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Furthering the understanding of the redox control of soluble Epoxide Hydrolase and Protein Kinase G in the cardiovascular system

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Furthering the understanding of the redox control of soluble Epoxide Hydrolase and Protein Kinase G in the cardiovascular system

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Abstract

Redox regulation of proteins represents an important control mechanism that can finely tune cell homeostasis or responses to stress. Two proteins regulated in this way include soluble epoxide hydrolase (sEH) and protein kinase G (PKG). sEH hydrolyses epoxyeicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acids (DHETs), which are less potent in terms of their ability to dilate blood vessels and lower blood pressure (BP) or induce angiogenesis. Thus inhibitors of sEH, which include lipid electrophiles, that adduct to C521, increase EET levels. As sepsis is a time of oxidative stress when lipid electrophiles can be generated, the hypothesis that vasodilation and hypotension at this time is mediated by C521-dependent inhibition of sEH was explored. Wild-type (WT) or 'redox-dead' C521S sEH knock-in (KI) mice, that are resistant to lipid electrophile-induced inhibition, were subjected to sepsis. There was no difference in BP, heart rate (HR) or activity. Analysis of isolated mesenteries or aortae by myography following sepsis did not identify any differences between genotypes. Similarly, there were no differences in plasma markers of organ damage or metabolic acidosis. Comprehensive comparison of the plasma inflammatory responses of each genotype identified a significant increase solely in granulocyte-colony stimulating factor (G-CSF), together with a similar trend in IL-17A in WT compared to KI. Overall it was concluded that inhibition of sEH by electrophilic lipids is unlikely to mediate blood pressure lowering during sepsis.

The attenuated increase in G-CSF and IL-17A in the C521S sEH KI was notable as it is important in vasculogenesis and angiogenesis, in which increased EETs have been implicated. Postischaemic revascularisation in the model of hindlimb ischaemia failed to identify differences between genotypes. Furthermore, the capillary/myofibre ratio in the gastrocnemius muscle, as well as G-CSF levels therein, were also similar between genotypes.

Recombinant sEH was also studied with a library of nitro-alkene fatty acids with the position of the double bond or electron-withdrawing nitro moiety altered, potentially allowing for more potent inhibitors of the hydrolase to be identified than previous studies with 10-nitro-oleic acid. Notably it was determined that low concentration of many of the electrophiles actually stimulated sEH, before inhibiting at higher concentrations.

Accumulating evidence suggests that C117 and C195 in PKG can be oxidised to form an intraprotein disulfide bond within the high affinity cGMP binding pocket, and this directly mediates oxidant-induced activation. Moreover, recent study has shown that nitroxyl (HNO) can induce intradisulfide-mediated PKG activation. To determine the potential physiological impact of this modification, C195S PKG KI mice were generated and characterised for their haemodynamic function. Basal BP, HR and cardiac function were not different between each group. When each genotype was exposed to a sepsis protocol, no differences in indices of well-being or haemodynamic parameters were observed. Effect of HNO donors, NCA and CXL-1020, on BP and HR was investigated *in vivo*. While NCA administration resulted in BP lowering in both genotypes to the similar extent, CXL-1020 resulted in attenuated BP only in WT mice and not KI animals, although the differences were not statistically significant and more experiments are required. Although cGMP-dependent vasorelaxation is intact in KI mice, the impact of the C195S mutation on cGMP binding requires further examination. Notably, KI mice basally have enlarged caecum and increased whole gut transit time, which was also reported in mice deficient in the nitric oxide-cGMP pathway.

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List of abbreviations

14,15-EEZE – 14,15-epoxyeicosa-5(Z)-enoic acid

15d-PGJ2 – 15-deoxy- Δ 12,14-prostaglandin J2

20-HETE – 20-hydroxyeicosatetraenoic acid

3MNCA – 3-methyl-1-nitrosocyclohexyl acetate

4-AP – 4-aminopyridine

AA – arachidonic acid

AC – adenylate cyclase

Ach – acetylcholine

ADP – adenosinediphosphate

AI – autoinhibitory

ALA – α -linolenic acid

Ang II – angiotensin II

AnGap – anion gap

AS – Angeli's Salt

ATP- adenosinetriphosphate

BH₄ - tetrahydrobiopterin

BK_{Ca} – large-conductance calcium activated potassium channel

bpm – beats per minute

BSA – bovine serum albumin

BUN – blood urea nitrogen

CamKII – Ca^{2+} /calmodulin-dependent protein kinase II

cAMP – cyclic adenosine monophosphate

CBS – cystathionine β -synthase

cGMP – cyclic guanosine monophosphate

CGRP – calcitonin gene-related peptide

CLP – caecum ligation and puncture

Cm – calmodulin

Cn – calcineurin

CNB – cyclic nucleotide binding domain

CNP – C-type natriuretic peptide

CO –cardiac output

COPD – chronic obstructive pulmonary disease

COX-2 – cyclooxygenase 2

Cpm – count per minute

CSE – cystathionine gamma-lyase

cTnI – cardiac troponin I

CYP450 - cytochrome P450

Cys – cysteine

DHA – docosahexaenoic acid

DHDP – dihydroxydocosapentaenoic acid

DHEQ – dihydroxyeicosatetraenoic acid

DHET – dihydroxyeicosatrienoic acid

DiHODE – dihydroxyoctadecadienoic acid

DiHOME – dihydroxyoctadecamonoenoic acid

DMSO – dimethyl sulfoxide

EC – endothelial cell

EDP – epoxydocosapentaenoic acid

EDTA – ethylenediaminetetraacetic acid

EEQ – epoxyeicosatetraenoic acid

EET – epoxyeicosatrienoic acid

EH- epoxide hydrolase

eNOS – endothelial nitric oxide synthase

EPA – eicosapentaenoic acid

EpODE – epoxyoctadecadienoic acid

EpOME – epoxyoctadecamonoenoic acid

ERK – extracellular signal-regulated kinase

ES – embryonic stem cell

FIH – factor inhibiting hypoxia-inducible factor

FLP – flippase

FMD – flow-mediated dilation

FRET – Forster resonance energy transfer

FRT – flippase recognition target

G-CSF – granulocyte colony-stimulating factor

G-CSFR – granulocyte colony-stimulating factor receptor

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GC – gastrocnemius

GI – gastrointestinal

Gln - glutamine

GM-CSF – granulocyte macrophage colony-stimulating factor

GPCR – G protein-coupled receptor

Grad – gradient

GSH – glutathione

GSK3 – glycogen synthase kinase 3

GST – glutathione S-transferase

HA – hydroxylamine

HEK-293 – human embryonic kidney cells 293

HIF- hypoxia-inducible factor

HLI – hindlimb ischaemia

HNE – hydroxynonenal

hs-CRP – high sensitivity C-reactive protein

HSP – heat shock protein

HUVEC – human umbilical vein endothelial cell

i.p. – intraperitoneal

i.v. - intravenous

I/R – ischaemia/reperfusion

IBMX – 3-isobutyl-1-methylxanthine

ICAM1 – intercellular adhesion molecule 1

ICC – interstitial cells of Cajal

IFN γ – interferon gamma

IK_{Ca} – intermediate-conductance calcium activated potassium channel

IL – interleukin

iNOS – inducible form of nitric oxide synthase

IRAG – inositol triphosphate receptor associated cGMP kinase substrate

IVS – interventricular septum

K_{ATP} – ATP-sensitive potassium channel

KC – keratinocyte chemoattractant

K_{Ca} – calcium-sensitive potassium channel

Keap1 – Kelch-like ECH-associated protein 1

KI – knock-in

KO – knock-out

L-NAME – NG-nitro-L-arginine methyl ester

L-NMMA – NG-monomethyl-L-arginine

LA – linoleic acid

LOX – lipoxygenase

LPS – lipopolysaccharide

LV – left ventricle

LVAW – left ventricle anterior wall

LVID – left ventricle internal dimension

LVPW – left ventricle posterior wall

Lys – lysine

LZ – leucine zipper

MAPK – mitogen-activated protein kinase

MCP-1 – monocyte chemoattractant protein 1

MEF2 – myocytes enhancer factor 2

MIP-1 β – macrophage inflammatory protein 1 α

MLC – myosin light chain

MLCP – myosin light chain phosphatase

mmHg – millimeter of mercury

MMP- matrix metalloprotease

MYPT1 – myosin phosphatase target subunit 1

NADPH – nicotinamide adenine dinucleotide phosphate

NCA – nitrosocyclohexyl acetate

NCFA – nitrosocyclohexyl trifluoroacetate

NCP – nitrosocyclohexyl pivalate

NF- κ B – nuclear factor kappa-light-chain-enhancer of activated B cells

NFAT – nuclear factor of activated T cells

nNOS – neuronal nitric oxide synthase

NO₂-FA – nitro fatty acid

NO₂-OA – nitro-oleic acid

Nrf2 – nuclear factor (erythroid-derived 2)-like 2

ONE – 4-oxo-2-nonenal

PCR – polymerase chain reaction

PDE – phosphodiesterase

PDGF – platelet-derived growth factor

PE – phenylephrine

PGD2 – prostaglandin D2

PGE2 – prostaglandin E2

PGH2 – prostaglandin H2

PGI2 – prostaglandin I2

PHD – prolylhydroxylase domain protein

Phe – phenylalanine

PHOME – cyano-(6-methoxynaphthalen-2-yl)-methyl ester

PHP – pyridoxalated haemoglobin polyoxyethylene

PI3 – phosphatidylinositol-4,5-bisphosphate

PI3K – phosphatidylinositol-4,5-bisphosphate 3-kinase

PKA – cAMP-dependent protein kinase

PKG – cGMP-dependent protein kinase

PLB – phospholamban

PMSF – phenylmethylsulfonyl fluoride

PPAR γ – peroxisome proliferator-activated receptor γ

RANTES – regulated on activation normal T cell expressed and secreted

RGS – regulator of G protein signalling

ROS – reactive oxygen species

RyR – ryanodine receptor

s.c. - subcutaneous

sEH – soluble epoxide hydrolase

Ser – serine

SERCA – sarco/endoplasmic reticulum Ca^{2+} -ATPase

sGC- soluble guanylate cyclase

SK1 – sphingosine kinase 1

SK_{Ca} – small-conductance calcium activated potassium channel

SNAP – S-nitroso-N-acetylpenicillamine

SNP – sodium nitroprusside

SOD – superoxide dismutase

SR – sarcoplasmic reticulum

SV – stroke volume

TCEP – tris(2-carboxyethyl)phosphine

TNF – tumour necrosis factor

TRPC – transient receptor potential canonical cation channel

TRPV – transient receptor potential vanilloid cation channel

VASP – vasodilator-stimulated phosphoprotein

VCAM-1 – vascular cell adhesion molecule-1

VEGF- vascular endothelial growth factor

VEGFR – vascular endothelial growth factor receptor

Vel – velocity

VSMC – vascular smooth muscle cell

VTI – velocity time integral

WT – wild type

XO – xanthine oxidase

β -ME – β -mercaptoethanol

Chapter 1. General Introduction

1.1. Thiol-based redox regulation

Evolutionally, the emergence of oxygen that allowed the full oxidation of substrates was favourable and contributed to the variety and complexity of life we observe now, but one consequence of this is the ubiquitous presence of reactive oxygen species (ROS) [1]. ROS are endogenous metabolic by-products of cellular respiration and end-products of a number of metabolic reactions, however they can also be produced in the controlled manner by enzymes, such as NADPH oxidases. They were widely reported to be elevated in tissues during disease. Due to this, historically, ROS were strongly implicated in the pathogenesis of most major diseases, whereby they were thought to oxidise cellular materials including lipids, DNA and proteins [2], and these modifications were equated with damage. Over the past few decades, a new paradigm has arisen, where ROS are also regarded as targeted physiological regulators of intracellular signalling pathways. Examples of ROS signalling playing crucial physiological roles include responses to growth factors, hormones, hypoxia, as well as fundamental processes such as cell division, differentiation, migration, and immune responses [3, 4]. Thus, whilst ROS-dependent signalling is important for homeostasis and the maintenance during health, it can be dysregulated during disease and in some cases causatively contribute to the aetiology.

One way in which oxidants can signal in a tightly controlled manner is by inducing a variety of protein post-translational modification events. A variety of oxidative post-translational modifications, such as glutathiolation, palmitoylation, nitrosation, prenylation or adduction of lipid electrophiles allows fine control of numerous cell signalling events [5]. Perhaps the most susceptible target for reversible, oxidative modifications is the amino acid cysteine (Cys), which in some proteins can operate as a redox sensor because of their nucleophilic properties. In addition, methionine, tyrosine, and tryptophan are also relatively susceptible to oxidative modifications, but physiological role of it is less well established compared to redox state-dependent modifications of Cys. Oxidants post-translationally widely modify and regulate transcription factors, ion transporters, membrane receptors and metabolic enzymes, as

well as proteins that regulate proteins phosphorylation and calcium-dependent signalling pathways [6-8]. Thus, oxidants integrate extensively with major regulatory pathways, suggesting that oxidant signals are capable of fine-tuning cellular processes in response to alterations in cell redox state, and in this way, enable homeostatic or adaptive changes.

Cysteine thiols – principal players in redox-based signalling

The ability of Cys, at least in some cases, to act as a redox sensor is defined by its unique physical and chemical properties. The considerable number of sulfur oxidation states (from -2 to +6), along with the large atomic radius which results in the S-H bond having a very low dissociation energy, allows the cysteinyl thiol group in some proteins to function as major biological redox sensor and transducer. The reactivity of specific Cys is largely determined by the acid dissociation constant (pK_a) of the thiol and the structural microenvironment of the protein [9]. Most cytoplasmic protein thiols have pK_a values higher than the ambient pH, meaning they are mostly protonated and so unreactive at physiological conditions. The pK_a of reactive Cys thiols are generally much lower, as in glutaredoxin where it is 3.5, which allows the thiol group to be predominantly deprotonated at cellular pH and therefore highly reactive with oxidants [10, 11]. The precise position of a Cys residue within a protein generally defines whether it is redox active. This is because proximity to other residues influences the extent of protonation, as does solvent accessibility, which not only controls access of oxidants and reductants, but also that of acid or base present in the cellular milieu that can also modulate the pK_a . The protein environment can stabilise the negatively charged deprotonated and ionised thiol, known as a thiolate. Several types of interactions mediate stabilisation of the ionised state, such as electrostatic interactions between the thiolate and positively charged amino acids, or a positive dipole moment that can occur in the N-terminus of α -helix. Hydrogen bonds between thiolates and nearby amino acid residues can also stabilise a thiolate [9, 12]. Cys106 in DJ-1, a protein that protects cells against oxidative stress, provides an example where neighbouring amino acid side chain was shown to lower thiol pK_a from 8.3 to 5.4 by stabilising it [13].

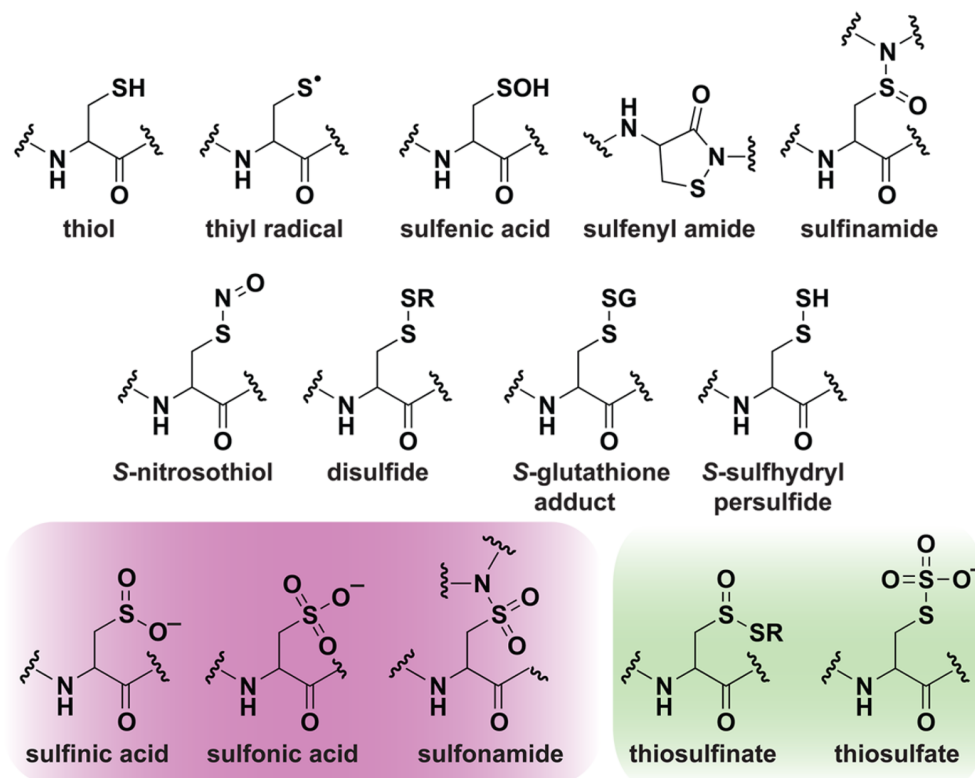
Currently, precise prediction of the thiolate status of a Cys is challenging. Assessing the

reactivity of a cysteine thiol only considering its pKa is an over-simplification. Additional properties that likely contribute to the reactivity include hydrophobicity and structural features of the protein, such as the presence of acid-base catalysts and special substrate pockets. For instance, in a hydrophobic pocket the interaction between oxygen and nitric oxide (NO) is augmented leading to facilitated formation of S-nitrosating species [14].

An interesting observation has been made which also implies an especially important role for Cys in redox regulation. With the evolution of organism complexity, the Cys content of proteins has increased from 0.41% in archaea to 2.26% for mammals [15]. Only a small proportion of this can be accounted for by an increase in zinc-binding structures or enzymes with catalytic thiols. This is consistent with most of the evolutionary changes perhaps relating to acquisition of redox regulation involving thiol oxidation. Moreover, numerous proteins contain Cys that are highly conserved between species and organisms, again suggesting regulatory functions beyond ion coordination or structural roles [16].

Cys residues can be modified through several different oxidative post-translational modifications as shown in Figure 1-1, each potentially inducing functionally different activities or graded responses, potentially allowing the sensor thiol to serve as a rheostat. That oxidative modification of protein thiols provides a vast number of different outcomes enabling fine “tuning” of cellular responses to perturbations in their redox state. Such redox state-dependent structural-functional alterations may mediate at the molecular level homeostatic or adaptive changes during health or disease.

The oxidation number of sulfur in the thiol group is 2, with up to nine different oxidation states possible in vitro, meaning there are many different post-translational modifications that can potentially regulate protein function [17].

**Figure 1-1**

Cysteine and some of its biologically-relevant oxidative modifications. Red, irreversible modifications; green, unique enzyme intermediates.

Oxidised forms of Cys thiols include sulfenic (SOH), sulfinic (SO₂H), sulfonic (SO₃H) acids, inter- and intraprotein disulfides, S-nitrosothiol, S-cysteinyl, glutathionyl, sulfenylamid, sulfhydryl (Figure 1-1). The complex chemistry of these reactions has been extensively discussed in reviews [18, 19]. All the Cys oxoforms differ in their stability, reactivity and reducibility. It is worth mentioning that the majority of Cys modifications mentioned and depicted in Figure 1-1 (white background) are reversible and can be removed by reduction reactions, which is an essential feature of a regulatory mechanism. The majority of oxidative thiols are reduced by the oxidoreductase enzymes, thioredoxin (Trx) or glutaredoxin [20]. Some modifications are considered to be terminal (Figure 1-1, red background). Sulfinic acids, which can be reduced by sulfiredoxin back to the thiols, form in 2-Cys peroxiredoxins [21], are predominantly irreversible when they form in most proteins. These terminal reactions also play important role in signal transduction; for example, sulfinylation of the active site Cys in protein tyrosine phosphatases leads to its inactivation [22]. It is important to note that

lipid-derived electrophiles can undergo redox reactions with reactive Cys thiols. Electrophilic lipids, such as 4-hydroxy-2-nonenal (HNE) or 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15d-PGJ₂), can form Michael adducts with nucleophilic Cys thiols.

Examples of thiol-based redox regulation

Many proteins have been reported to be directly regulated by cell redox state [22]. Oxidation of protein Cys may stimulate, modulate or inhibit catalytic activity, or promote conformational changes that change the propensity to interact with binding partners, and so potentially altering subcellular translocation. Many proteins have a catalytically-essential Cys, which often has enhanced reactivity with cellular biochemical by virtue of having a low pK_a thiol. As discussed above, such proteins are typically also reactive with oxidants, which would lead to enzyme inactivation as the essential catalytic thiol would no longer be available to perform biochemical reactions based on its nucleophilicity. Such oxidative inactivation mechanisms occur in protein tyrosine phosphatases and ribonucleotide reductases [23]. In the case of glyceraldehyde-3-phosphate dehydrogenase, oxidation of the catalytic thiol not only prevents its classical metabolic conversion of glyceraldehyde-3-phosphate to 3-phosphoglyceraldehyde, but switches its enzymatic activity [23, 24]. Thiol oxidation can also lead to alteration in protein structure, such as formation of intra- or intermolecular disulfide bonds. An activating intermolecular and intramolecular disulfide formation occurs in PKGI α in response to oxidants [25], which will be discussed in more detail in Chapter 3. Interdisulfide-induced activation of PKGI α lowers blood pressure and induces cardiac relaxation during diastole [26, 27]. Interestingly, although several oxidants can induce formation of the PKGI α interdisulfide, an intradisulfide in this kinase, that has only been recently identified, is selectively induced by nitroxyl (HNO) donors, consistent with the fine-tuning of cell signalling that can be achieved by redox mechanisms [28]. HNO-induced intradisulfide bond formation is a major focus of this work, and so is introduced in more detail below and is the subject of Chapter 3. Cys can also be modified by electrophilic lipids, as has been shown for many proteins, such as Kelch-like ECH-associated protein 1 (Keap1), heat shock protein 1 (HSP1), peroxisome proliferator-activated receptor gamma (PPAR γ) and soluble epoxide hydrolase (sEH). In a study relevant to this work, Charles

et al showed that soluble epoxide hydrolase activity can be inhibited by electrophilic lipids 15d-PGJ₂ or 10-nitro-oleic acid (NO₂-OA), via covalent adduction to Cys521 that is in proximal location to the hydrolase active centre [29]. Cys521 modification by NO₂-OA and subsequent sEH inhibition plays an important role in blood pressure regulation and was proposed as a new therapeutic strategy to combat hypertension [30]. Chapter 4 will focus on the redox regulation of sEH, extending previous studies to examine the potential role of electrophile-mediated inhibition of this hydrolase in pathology scenarios. Given the significant focus on lipid-electrophile signalling involving adduction to protein thiols, the chemistry and biology of such events is introduced in more detail below.

1.2. Electrophilic lipids

Post-translational modifications of proteins by electrophilic lipids were shown to contribute to oxidative stress-linked diseases, including atherosclerosis, rheumatoid arthritis, chronic inflammation, cancer, neurodegeneration, and Alzheimer's disease [31, 32]. However, as has been the case with other biochemical oxidant molecules, research continues to emerge demonstrating that oxidative protein modifications are also important for cell homeostasis during physiological processes. Moreover, electrophilic lipids may have therapeutic applications, for example protecting against neointimal hyperplasia, cardiac ischaemia-reperfusion injury, chronic inflammatory conditions, hypertension, as well as cancer in a number of animal models [30, 33-41]. A potential role of electrophilic lipids as therapeutic drugs is being evaluated in early-phase clinical trials assessing their efficacy against chronic inflammation and metabolic syndrome [42]. CXA-10 (10-nitro-oleic acid) is an anti-inflammatory and anti-fibrotic compound, which is produced by Complexa Inc. The company is initiating Phase 2 clinical trials in the nearest future. Nitro-oleic acid and other nitro-fatty acid compounds used in Chapter 3 were supplied by Complexa Inc. The therapeutic potential of electrophiles would be discussed in more detail in this chapter.

Generation of electrophilic lipids

Electrophiles can be ingested as they are present in food or exposure can occur through inhalation or skin contact [43]. Otherwise, electrophiles can be produced endogenously as a result of cellular metabolism, lipid peroxidation or nitration reactions [44, 45]. Endogenous electrophile formation can be either enzymatic or non-enzymatic. The enzymatic formation of electrophilic species is mediated by 3 families of heme- or non-heme- containing metallo-enzymes, such as cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450) [46]. As an example, 15d-PGJ₂ is generated via enzymatic pathway, where COX-2 catalyses arachidonic acid conversion into prostaglandin H₂ (PGH₂), which is further oxidised by prostaglandin D₂ (PGD₂) synthase into an unstable PGD₂ that undergoes dehydration to 15d-PGJ₂. Increased generation of 15d-PGJ₂ was shown in inflammatory conditions [47]. The enzymatic reactions are well controlled during normal metabolism and result in a plethora of biological responses (vasodilation, vasoconstriction, pro-inflammation or anti-inflammation), depending on the product array they generate [48]. Under oxidative conditions with high concentrations of reactive oxygen and nitrogen species, lipid electrophiles are predominantly produced via non-enzymatic free radical mechanisms, which represents an autocatalytic process, suggesting it is poorly regulated [42]. Lipid peroxidation by oxygen species occurs with both free and esterified fatty acids, leading to the formation of hydrophobic and hydrophilic electrophilic products. Membrane phospholipids with mono- and polyunsaturated fatty acids in S_n2 position provide the most relevant pool for lipid peroxidation. Lipid peroxidation by oxygen species results in the production of malondialdehyde, HNE, and 4-oxo-2-nonenal (ONE). Previously underappreciated lipid oxidation by NO-derived species has been gaining more attention lately. These reactions give rise to nitrosylated, nitrated, or nitro-containing fatty acids. Nitrated derivatives of oleic, linoleic, linolenic, arachidonic, and eicosapentaenoic acids have been identified in mammals [42]. Moreover, nitrated derivatives of unsaturated fatty acids are present in healthy human blood, plasma and lipoproteins [49-51]. Increased production of nitro-fatty acids (NO₂-FA) occurs in vivo in response to pathological stress. A recent study demonstrated that mice that have undergone cardiac ischaemia via coronary artery ligation generate increased levels of NO₂-FA [39]. Thus, unsaturated fatty acids, abundant in plasma and within cellular membranes, are highly

susceptible to oxidation mediated by free radical reactions, underlying relatively high levels of lipid electrophile generation during times of oxidative stress such as inflammation.

The low concentration of endogenously generated lipid electrophiles has led to a debate regarding their physiological significance. However, electrophiles readily react with other nucleophilic biomolecules, making their quantification complex and underestimating their abundance. Because of their reactivity, electrophiles are believed to react and signal in close proximity to the site of their generation. Although, as electrophiles can reversibly react with target thiols, potentially allow such conjugates with greater stability to diffuse more widely to allow post-translational modifications distant from where they are generated. Transport from the site of generation can involve binding to albumin or other carriers, or covalent adduction with glutathione (GSH) to be further degraded and excreted through urine and bile [42]. In addition to reaction with their targets such as protein thiolates, electrophilic lipids are susceptible to β -oxidation, and reduction of functionally critical double bonds.

Mechanisms of electrophile adduction

An electrophile is defined as an electron poor molecule, which can be attracted to an electron-rich nucleophilic molecule and undergo chemical reaction by accepting electrons (Figure 1-2).

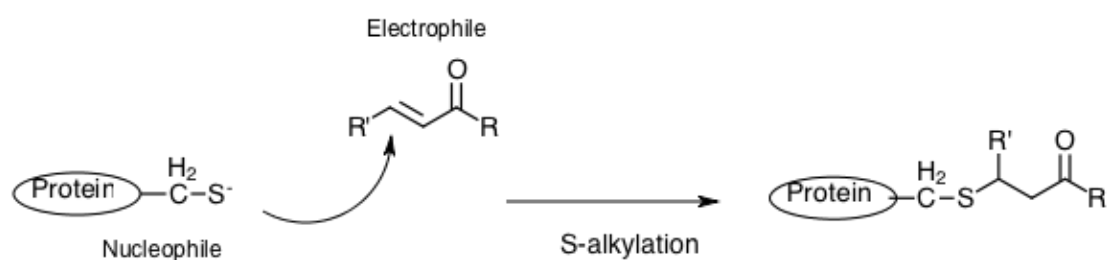


Figure 1-2

Post-translation modification by electrophilic adduction. Electrophile attacks cysteine thiol. The asterisk denotes for electrophilic carbon.

Noteworthy, there is selectivity or specificity in terms of which electrophiles and nucleophile will react together. This can be explained using the theory of hard/soft

acid–base [52]. Briefly, electrophiles and nucleophiles are classified as either hard or soft, based on the shape and energy of their outer electron orbitals. Hard electrophiles have much higher positive charge density at the electrophilic center, while partial positive charge density in soft electrophiles is diffused. The selectivity of electrophile–nucleophile reaction is mainly determined by the ability of the outer shell electrons to polarise. The hard electrophiles and nucleophiles are difficult to polarise, while soft electrophiles being formed by the electron-withdrawn substituents are more readily polarised. In general, hard electrophiles react more favourably with hard nucleophiles, whereas soft electrophiles react with soft nucleophiles [42]. The reaction occurs by electrons from the nucleophile being donated to the electrophile, resulting in the formation of a covalent bond.

Basic mechanisms of electrophilic lipid modification include Michael addition and Schiff base reactions. Electrophiles can react with nucleophilic nucleic acids, amino acid residues or other small molecules [32]. The formation of a Schiff's base adduct generally requires harder electrophiles such as aldehydes. In contrast, softer electrophiles preferentially form Michael adducts with thiolates (Figure 1-2). Cys is thought to be the softest biological nucleophile, and direct adduction of a lipid electrophile to Cys thiol is considered to be the major mechanism mediating the effects of lipid electrophiles. It is important to point out that the adduction rate depends on thiol reactivity, which is mainly defined by its pK_a , as discussed earlier. Despite the fact that lysine and histidine are weaker Michael donors than Cys, they can also undergo covalent adduction with lipid electrophiles [42].

Michael addition reactions are reversible while Schiff's base reactions are more stable and are not readily reversible. Reversible reactions generally display no cytotoxic effect at physiological concentrations, consistent with such reactions functioning as signalling events during normal cellular metabolism and redox status. Irreversible reactions, on the other hand, probably participate less in normal cell physiology, with evidence that they accumulate more during pathophysiological processes [53].

Physiological, pathophysiological roles of electrophiles and their therapeutic significance

Electrophilic lipids modulate numerous cell physiological and pathophysiological processes, including nuclear transcriptional factor function, heat-shock response, and kinase/phosphatase activities. More than 200 genes have been identified to have electrophile response elements in their regulatory regions, a feature that enables a response to oxidative stress [54]. Some electrophilic lipids, such as HNE or 15d-PGJ2, have been shown to trigger the induction of antioxidant response element-regulated genes through the modification of Keap1, which triggers the translocation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) into the nucleus [55]. Heat shock proteins (HSPs) are also well-known targets of cellular electrophiles and are key mediators of stress response, for example acting as molecular chaperones to refold damaged proteins. Rats exposed to a high fat or high ethanol diet showed accumulated HNE adducts on HSP72 and HSP90 proteins [56, 57]. Aldehyde dehydrogenase can be inactivated by electrophilic adduction during chronic alcohol consumption, as identified during a proteomic study of ethanol-induced hepatotoxicity [58]. Higher concentrations of electrophilic lipids can be pro-apoptotic, for example HNE can initiate binding of the death-associated protein death-associated protein 6 (Daxx) to the Fas receptor (FasR), modulating the apoptotic response through apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK) and caspase-3 [59]. Other proteins modified by electrophiles include AMP-activated serine-threonine kinase (AMPK) [60], protein serine-threonine phosphatase 2A (PP2A) [61], thioredoxin [62], sEH [29], and PPAR γ [63]. Numerous diseases have been linked with increased protein modification by electrophilic species. Glyoxal and methylglyoxal, electrophilic metabolites of glucose, have been associated with diabetes [64]. HNE has been suggested to contribute to cardiac hypertrophy, as well as being atherosclerogenic by adducting to low density lipoprotein [65, 66].

Electrophilic lipids and electrophile-sensitive proteins is an important area for therapeutic development. Electrophilic lipids have long been known to possess anti-inflammatory activities [67, 68]. Moreover, electrophilic PPAR γ agonists are being investigated for the treatment of cardiovascular diseases and type II diabetes [69]. An

interesting and rapidly growing area is the use of dietary electrophiles, such as sulforaphane and curcumin and 10-nitro-oleic acid. Administration of curcumin is neuroprotective in murine models of brain ischaemic injury and Alzheimer's disease [70, 71]. Similarly, carnosic acid protected mice from obesity and has been proposed as a therapy for metabolic syndrome [72]. Mice that consumed broccoli sprout extract, which contains sulforaphane, are protected against UV-induced cancer [33] and neurodegenerative diseases [73]. CXA-10 (10-nitro-oleic acid), developed by Complexa Inc for acute kidney injury completed 4 phase I clinical trials and is planned for a phase II clinical trials for focal segmental glomerulosclerosis in early 2018. Professor Bruce Freeman, one of the co-founders of Complexa Inc has provided us with novel library of nitro-fatty acids, their effect on sEH activity will be discussed in Chapter 4.

1.3.Nitroxyl

HNO or nitroxyl, which is explored in Chapter 3 in terms of its ability to oxidize PKGI, is a triatomic molecule that is a redox derivative of nitric oxide. However, nitroxyl possesses a very distinct chemical and biological properties, for example it is a weak acid with a pK_a of 11.4 [74, 75]. Deprotonation of HNO to NO^- is accompanied with a change of spin state, thus making deprotonation thermodynamically unfavourable [74]. This relatively high pK_a and slow spin-forbidden deprotonation suggests that HNO exists as a chemically reactive species at physiological pH. This high reactivity of HNO significantly complicates the study of its production, detection, and understanding of its chemistry, especially in a biological setting. HNO is a highly reactive electrophile that spontaneously dimerises to yield hyponitrous acid ($H_2N_2O_2$), which dehydrates to nitrous oxide (N_2O) [75]. This means that authentic HNO cannot be stored or concentrated and therefore more stable donor molecules are typically used to study its chemistry and biology [76]. It is important to note that the target molecule reactivity of HNO is much vaster than that of NO, potentially rapidly modifying many more targets which is anticipated to perhaps limit its spatial influence [77]. Being a potent electrophile, HNO readily reacts with nucleophiles, including thiols and amines to yield sulfinamides, disulfides and diazenes [78-81]. As HNO has lone pairs of electrons on both nitrogen and oxygen, it can react with metals, as demonstrated experimentally with

heme proteins [82]. This complex reaction chemistry of HNO underlies its biological effects and is also harnessed by the analytical methods developed for its detection.

Nitroxyl donors

HNO-releasing compounds can be divided into two general groups based on their chemistry [76, 83]. The first group, which includes Angeli's salt (AS), Pyloty's acid and CXL-1020 (Figure 1-3), represents hydroxylamine derivatives with a good leaving group attached to the nitrogen atom. Deprotonation of these compounds is accompanied by redox reactions that alter the oxidation state of the nitrogen atom [76, 84-86]. The second group includes nitroso compounds with a good leaving group. The nitrogen atom in these compounds is already in +1 oxidation state, thus they undergo non-redox reactions to release HNO. This group includes acyloxy nitroso compounds, such as the donor used in this work, namely 1-nitrosocyclohexyl acetate (NCA) (Figure 1-3) [87].

AS, which is also studied here, has been the most commonly used HNO donor due to its commercial availability and its well-studied chemistry. AS is highly unstable at physiological pH and readily decomposes to HNO with a half-life of approximately 3 minutes [84]. A disadvantage is that as an inorganic salt, AS cannot be modified to tune the release rate of HNO, with its short half-life making it difficult to study the impact of sustained levels which are perhaps more relevant to the cellular situation. AS is also oxidised by metals and oxygen to generate NO and nitrite, both of which possess their own biological properties [86, 88], making the interpretation of results with this donor complex.

Relatively recently, King's group developed new type of HNO donors - acyloxy nitroso compounds [87], which can be relatively easily synthesised and release HNO upon cleavage of an ester bond. By modifying the position of the acyl group it was possible to synthesise HNO donors with varying release properties. HNO is released through hydrolysis of acyloxy nitroso compounds, with the rate depending on the ease of ester hydrolysis and thus the structure of the ester group. A trifluoroacetate group makes nitrosocyclohexyl trifluoroacetate (NCFA) a very rapid HNO donor, whereas the half-

life of NCA is about 800 minutes, with that of nitrosocyclohexyl pivalate (NCP) being much longer at 37 hours. Lately, lots of attention has been focused on the Pyloty's acid derivative, CXL-1020, which is investigated in this study. This nitroxyl donor is currently being investigated in clinical trials as a potential therapy for patients with heart failure (clinical trials NCT01092325, NCT01096043). CXL-1020 is a longer-lasting than AS and more specific than both AS and NCA.

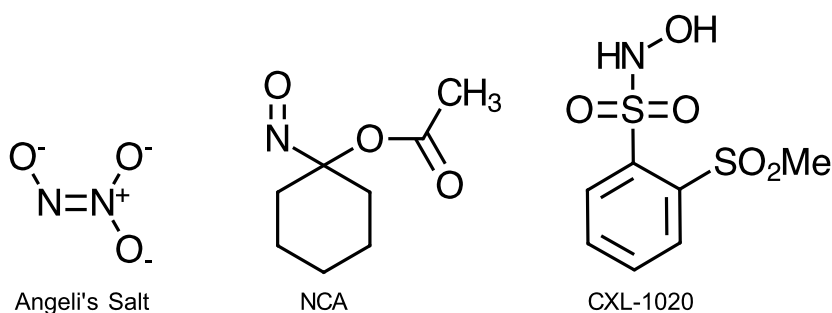


Figure 1-3

Nitroxyl donors, Angeli's salt, NCA and CXL-1020.

Endogenous HNO production

The biological properties of HNO and its selectivity towards certain biological targets suggest that it could be an endogenous signalling molecule. However whether HNO is indeed endogenously produced is still a matter of debate [89-93]. Several mechanisms as to how HNO may be produced endogenously have been proposed. For example, one of the first ways suggested involves nitric oxide synthase (NOS) uncoupling, due to reduced availability of substrates or cofactors such as tetrahydrobiopterin (BH_4), leading to production of nitroxyl instead of NO [94-96]. However, direct production of HNO via this mechanism has not been shown endogenously. Another potential cellular source of HNO involves heme-mediated oxidation of hydroxylamines, such as hydroxylamine itself or hydroxyurea. Such reactions occur at least in vitro with horse radish peroxidase, myoglobin or myeloperoxidase in the presence of H_2O_2 [97, 98].

Another way HNO may be generated in cells is via reduction of NO by superoxide dismutase (SOD) or xanthine oxidase (XO). Several studies suggested the chemistry

behind this reactions, however it is worth mentioning that all the evidence for HNO production in this way was acquired with in vitro experiments. [99, 100].

For a long time chemical reduction of NO in biological systems was thought to be thermodynamically unfavourable due to its negative reduction potential at physiological pH [75, 101]. Nonetheless, several studies provided indirect evidence for nitroxyl during the reaction of NO with physiological reductants. By far, the most physiologically relevant way that HNO is likely to be produced endogenously is by the reaction of nitrosothiols (RSNO) with other thiols (RSH). Almost two decades ago Wong and colleagues showed that in vitro reaction of nitrosothiols with excess thiols resulted in nitroxyl and disulfide [102]. Furthermore, NO treatment of albumin lead to N₂O production, strongly suggesting HNO as an intermediate [103]. Thioredoxin was also shown to decompose nitrosothiols [104], providing a plausible mechanism for HNO production in vivo. However, it is worth remembering that the S-N bond in nitrosothiol intermediates is relatively strong, thus complicating the release of HNO [105].

Recently, reaction of NO with H₂S was proposed to generate HNO [106]. In isolated rat ventricular myocytes exposed to both H₂S and NO donors, the associated increase in contraction was similar to that achieved by AS [107]. A study by Eberhardt et al perhaps provides the most compelling evidence supporting endogenous HNO generation [108]. Using an HNO-selective electrode they showed that HNO could indeed be produced in vitro by reaction of NO donor with H₂S donor. Next, they showed that overexpression of inducible NOS (iNOS) which produces NO, together with cystathionine β -lyase (CBS) which produces H₂S, in neurons resulted in HNO production, as measured using the fluorescent HNO detector CuBOT. It is important to note that they were able to detect basal levels of HNO in sensory neurons. Furthermore, depletion of cellular arginine, which is required for NO production, or Cys, which is required for H₂S production, resulted in attenuated HNO production. The authors also showed a physiological consequence of the nitroxyl production via this mechanism, namely transient receptor potential ankyrin 1 (TRPA1) channel activation in sensory neurons via the formation of a disulfide bond. This resulted in the release of calcitonin gene related peptide (CGRP), a known vasodilator. Although, the evidence for HNO

contributing to these physiological responses is convincing, it is worth considering that the reaction of NO with H₂S leads to several other products, such as polysulfides with very distinct biological properties [109]. Noteworthy, it can induce the interprotein disulfide formation in PKGI, the protein which regulation by HNO is extensively studied in this work [110].

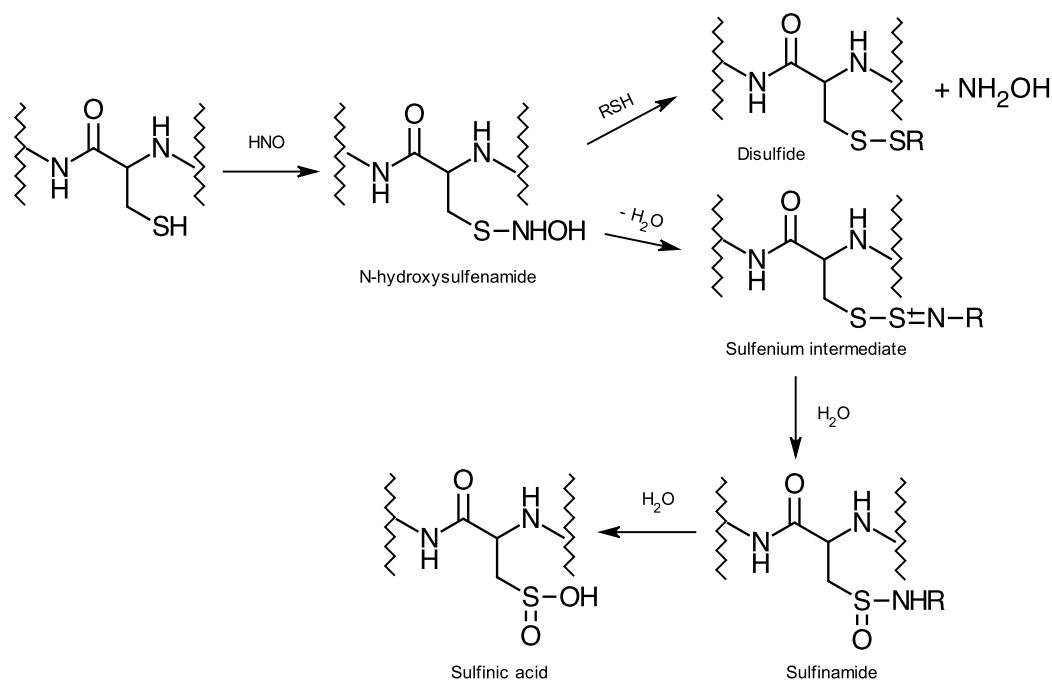
Another way that HNO may be generated is via the reduction of NO by aromatic or pseudoaromatic alcohols, such as hydroquinone or ascorbic acid – with these reactions occurring in vitro [111, 112]. Interestingly, treatment of bovine endothelial cells with ascorbic acid increased CuBOT-dependent fluorescence, suggesting generation of HNO [112]. Stimulation of RAW macrophages with iNOS-inducing agents and subsequent treatment with ascorbate resulted in HNO production as measured with an HNO-selective electrode [112].

To summarise, the proposed routes of HNO generation can be divided into two groups; the first dependent on amine oxidation, and the second on the reduction of NO. The first includes HNO production by BH₄-free NOS under oxidative conditions. The second mechanism requires thiols or aromatic alcohols which reduce NO to generate HNO. This could potentially happen in mitochondria during hypoxia, as stored nitrite can be reduced to NO by the action of globins or by reduced intermediates of the respiratory chain such as quinones [113]. As for the reaction of NO and H₂S it is clear that it can only happen in scenarios when their concentrations are high enough, perhaps when NO- and H₂S-producing enzymes function in close proximity.

The high reactivity of HNO, together with the numerous obstacles associated with selective detection of nitroxyl, makes it challenging to clearly establish whether this molecular species is truly generated endogenously. Although, several studies have provided evidence for production of HNO in vitro, it is perhaps fair to conclude that we cannot definitively say it is naturally formed in cells. Nevertheless, as HNO donors are in clinical development (clinical trials NCT01092325, NCT01096043) and they induce PKG intradisulfide formation, this provides a rationale for the studies Chapter 3 examining the novel C195S PKG KI mouse.

Nitroxyl - mechanisms of action

The biological actions of HNO are primarily based on its reactivity with thiols or metals in metallo-proteins. As mentioned before, HNO is a highly reactive electrophile that preferentially reacts with soft nucleophiles, such as Cys thiol [80, 91]. Doyle et al was one of the first to demonstrate nitroxyl reacts with thiols [79], showing HNO reacted with thiophenol to form a disulfide with N-hydroxysulfenamide supposedly as an intermediate. Subsequently, the reaction of nitroxyl with thiols was computationally calculated to be thermodynamically favourable [80]. It is important to note that the reaction rate of nitroxyl with thiols is very fast, being far greater than that for nitroxyl dimerization, consistent with its biological actions being significantly mediated by oxidation of thiols [114]. The reaction of HNO with thiols starts with a nucleophilic attack on the nitrogen atom to form N-hydroxysulfenamide (Figure 1-4) [79, 102, 114, 115]. In the presence of another proximal thiol, the reaction results in the formation of a disulfide bond and hydroxylamine. However, in the absence of thiols, N-hydroxysulfenamide isomerises to give N-hydroxysulfinamide [116-118]. The outcome of the reaction depends on the pK_a of the thiol, the hydrophobicity of the reaction environment and the availability of a chemical base [116, 118]. It is very important to emphasise that reaction of HNO with thiols can result in different protein modifications with distinct chemical and biological properties. While disulfides are known to be readily reduced in biological systems, the reduction of N-hydroxysulfinamide, although possible, is extremely slow [119]. N-hydroxysulfinamides can also be further hydrolysed to sulfinic acid (Figure 1-4).

**Figure 1-4****Reaction of HNO with thiols.**

Another primary target of HNO are metallo-proteins, reacting directly to form coordination complexes with transition metals [82]. Nitroxyl is effectively trapped by ferric heme proteins, as was observed when metmyoglobin or methaemoglobin was exposed to AS [120, 121]. The reaction results in generation of ferrous heme-nitrosyl complexes. Nitroxyl was also shown to react with copper and non-heme iron proteins [121]. An important biological outcome of HNO reacting with ferrous heme enzymes is its effect on soluble guanylate cyclase (sGC) [122]. Numerous studies have provided evidence that vasodilatory effects of HNO, which is investigated in Chapter 3, are mediated by its activation of sGC [123-126]. However, as mentioned before, HNO may be oxidised to NO, which might at least in part, be responsible for the attributed effects. Interestingly, one of the first studies investigating HNO effect on sGC activity reported that nitroxyl could not activate sGC [127]. However, the authors performed their studies in the presence of dithiothreitol (DTT), which can potentially scavenge nitroxyl before it was able to react with sGC. A later study showed that sGC cannot be activated by HNO in the absence of superoxide dismutase (SOD), suggesting that it is NO and not HNO that activates the enzyme [128]. Conversely, Miller and colleagues described sGC

activation in a thiol- and oxygen-free buffer upon treatment with several nitroxyl donors [122]. The HNO-induced activation of sGC was 2-3 times lower than for NO-induced activation at comparable concentrations. Experiments with heme-free sGC showed that heme is essential for the effect of nitroxyl on enzymatic activity. Interestingly, the interaction of HNO with thiols in sGC resulted in its inhibition [122].

Nitroxyl and the cardiovascular system

Vasodilatory effects of HNO

HNO is a potent vasodilator. Fukuto and colleagues were the first to show that AS relaxed rabbit aorta or bovine intrapulmonary artery via activation of sGC [129]. Since then numerous studies using different nitroxyl donors or varying vascular beds from diverse species provided robust evidence for the vasodilatory actions of nitroxyl [93, 125, 130-134]. Using pharmacological tools the signalling pathways that mediate HNO-induced vasorelaxation have been defined, at least in part. Inhibition of sGC with ODQ significantly impairs nitroxyl-induced vasodilation, strongly implicating this enzyme in the mechanism of vasodilation [125, 132-134]. The effect was shown to be independent of NO by experiments with NO scavengers or inhibitors of SOD, as the dismutase enzyme can oxidise HNO to NO [125, 132, 133]. Recent studies showed the voltage-dependent potassium channels (Kv) mediate nitroxyl-induced vasodilation, as 4-aminopyridine (4-AP) significantly reduced the relaxation it causes in human radial or rat mesenteric arteries [93, 132, 135]. Some studies reported iberiotoxin, which inhibits the large conductance Ca^{2+} -activated potassium channel (BK_{Ca}), to abolish responses to nitroxyl donors in rat mesenteric arteries. This is notable as interdisulfide-PKGI is thought to target BK_{Ca} channels, although nitroxyl signalling to these channels has been disputed [93, 132]. It is not clear whether HNO affects K channels directly or via some yet unidentified signalling pathways. Another signalling pathway implicated in nitroxyl-mediated vasorelaxation involves CGRP release [108, 124, 126]. Arterial plasma CGRP increased in dogs infused with AS, but not the NO donor diethylamine NONOate (DEA/NO) [92]. Additionally, a CGRP antagonist attenuated AS-induced vasorelaxation in the coronary vasculature of rat hearts [126]. Furthermore, a recent

study reported CGRP release from the cranial dura mater or sciatic nerves in mice exposed to AS [108].

HNO and cardiac function

In the early 2000s Paolocci et al suggested that nitroxyl induces positive inotropic and lusitropic effects on the myocardium [124]. Their studies showed that the nitroxyl donor AS increased cardiac contractility and reduced cardiac preload in dogs. This effect of HNO is thought to be independent of β -adrenergic signalling and rely on redox modifications of key proteins within the cardiomyocyte contractile machinery [92]. Tocchetti et al showed HNO enhances Ca^{2+} cycling by direct interaction with sarco/endoplasmic reticulum Ca^{2+} -ATPase 2a (SERCA2a) and the ryanodine receptor 2 (RyR_2) in a thiol-sensitive manner, which lead to increased Ca^{2+} uptake to, and release from the sarcoplasmic reticulum [136]. SERCA activation is mediated by S-glutathiolation at Cys674 as was shown in rat ventricular cardiomyocytes [137]. More recently oxidation of the important regulator of myocardial contractility, phospholamban (PLB), was demonstrated to contribute to the impact of nitroxyl on cardiomyocyte function. HNO promotes PLB oligomerisation to relieve the tonic inhibition of SERCA mediated by monomeric PLB, leading to enhanced sequestration of Ca^{2+} to the sarcoplasmic reticulum to enhance myocardial relaxation during diastole [138, 139]. Interestingly, nitroxyl not only improves Ca^{2+} cycling in cardiomyocytes, but it also increases Ca^{2+} sensitivity and force generation by directly modifying cardiac myofilament proteins [140, 141]. Using mass spectrometry together with a modified biotin switch assay, Gao et al demonstrated formation of disulfide bonds between actin-tropomyosin and myosin heavy or light chains, which increased myofilament Ca^{2+} responsiveness. It is noteworthy that while NCA donor enhanced both Ca^{2+} sensitivity and maximal force, AS only increased maximal force and had no effect on Ca^{2+} sensitivity - highlighting disparate actions of structurally-differing nitroxyl donors [141].

An overview of the biological chemistry of lipid electrophiles and nitroxyl donors has been provided in this introductory text. It is evident that both classes of electrophiles significantly mediate their biological responses by targeting reactive cysteine thiolates present in some proteins. Such proteins include sEH or PKG, which have also been

introduced here as they the focus of Chapters 3 and 4, are established targets of lipid electrophiles or nitroxyl donors respectively. Although sEH and PKG are structurally and functionally very distinct enzymes that are each regulated by their thiol-redox state, it is notable that that oxidation of these disparate enzymes induces similar responses within the cardiovascular system. Inhibition of sEH, which can occur when electrophiles adduct at Cys521, results in inhibition of the hydrolase and accumulation of EETs [30], which induce vasodilation and myocardial relaxation [29, 30], as well as cardioprotection [142]. In contrast oxidation of PKGI each associated with targeting or activation, which notably also induces vasodilation and myocardial relaxation [26-28], as well as cardioprotection [143, 144]. It is also notable that at the time of writing that the lipid electrophile nitro-oleic acid that inhibits sEH, and the nitroxyl donors CXL-1020 that activates PKGI, are each in clinical development by pharmaceutical companies. The studies presented in this thesis explore in more detail the regulation of sEH or PKG by these disparate thiol-oxidants and examine whether the redox-regulation of these enzymes also play important roles in the responses of the cardiovascular system to endogenous changes in the abundance of nitro-oxidants during times of stress, such as sepsis or ischaemia. The exploration of the role oxidative modification of sEH or PKG may play in vivo is viable through the use of transgenic knock-in mice in which the thiol-redox sensor in sEH or PKGI has been replaced by a Ser that is not oxidatively modified. This includes studies with a novel C195S PKG knock-in, which is anticipated to be resistant to the activation of this kinase mediated by oxidation of this residue [28, 145].

1.4. Protein kinase G

PKG, also known as cGMP-dependent protein kinase, belongs to the family of Ser/Thr protein kinases. In mammals, two genes encode PKG: *Prkg1* for PKGI and *Prkg2* for PKGII [146]. Both isoforms share similar structural characteristics and both are homodimers of two subunits [147]. PKG subunits have three functional domains, which will be discussed in more detail in this introduction. Despite PKGI and PKGII having similar structures, they differ widely in tissue localisation and substrate preference. Additionally, PKGI is mostly localised in the cytosol, while PKGII is anchored to the

plasma membrane by an N-terminal myristoyl residue [147]. From this point, only PKGI isoforms will be considered further, as they are more commonly involved in the cardiovascular system homeostasis and regulation. PKGII has so far been shown to regulate bone remodelling, intestinal secretion, renin secretion by the kidney and aldosterone production by the adrenal gland [148-150].

Two PKGI isoforms, PKGI α and PKGI β , are encoded by the *Prkg1* gene and are produced as a result of alternative splicing [146]. Both isoforms bind two molecules of cGMP and share similarities (in terms of k_{cat} , K_m , and V_{max}) in phosphorylating synthetic peptides, however they have differential protein substrates [151-153]. The two isoforms differ in their first 90-100 amino acids and this affects many of their features, including cGMP affinity, substrate specificity, and subcellular localisation [147, 151-157]. This N-terminus also mediates interaction with substrates, explaining differential substrate phosphorylation and targeting, which may involve binding to G-kinase anchoring proteins (GKAPs) targeting [158].

PKGI isoforms are highly expressed in all types of smooth muscle cells, platelets, and disparate parts of the brain, such as the cerebellum and hippocampus. In general, both PKGI α and PKGI β isoforms are co-expressed, however, are present in varying proportions in different tissues. In lungs, heart, and cerebellum, PKGI α predominates, while both isoforms are present at similar levels in smooth muscle cells in vessels, intestine, and uterus; only subtle amounts of PKGI α are detected in the hippocampus and platelets [159, 160]. In most cell types, both isoforms are abundant in the cytosol, but in platelets where PKGI β predominates, it is predominantly membrane bound [161]. It is now clear that PKGI isoforms can change subcellular localisation, however, the stimuli driving the process are not yet fully understood [147].

PKG knockout mice, both global and tissue-specific, have provided an invaluable tool in establishing the role of this kinase in health and disease [162-166]. These transgenic models, together with classical pharmacological tools allowed the development of drugs that enhance cGMP-dependent activation of PKG. Such pharmacotherapies efficiently dilate the vasculature of the penis and airways to provide effective treatments for erectile dysfunction or pulmonary hypertension, respectively [167, 168]. Whilst drugs that enhance cGMP-dependent activation of PKG have shown promise in limiting

cardiac ischaemia/reperfusion injury or progression to hypertrophy or heart failure [169-171], it is notable they have failed as anti-hypertensives or in the treatment of heart failure with preserved ejection fraction (HFpEF) [158].

What is known from studies with PKG knockout mice

As mentioned before, conventional and conditional PKG knockouts have been generated. Global, conventional PKG knockout mice have a limited life span, with more than half dying before 6 weeks of age. They have impaired relaxation of vascular and visceral smooth muscle, and show defects in axon guidance and neuronal plasticity [162, 172-176]. Although a lot of important information was acquired from conventional knockouts, these genetic models have limitations. Firstly, their short life span precludes investigation of adult mice, and the study of chronic disease models. Furthermore, it is very difficult to dissect whether the phenotype is primary due to the gene deletion in a certain cell type or whether it is secondary to changes in another. For these reasons, conditional PKG knockout mice were generated that allowed for the time and/or tissue-specific deletion of PKG. Conditional PKG knockout mice are fully viable as opposed to conventional knockouts [163-165, 177]. The cardiovascular phenotypes of, conventional or conditional PKGI knockouts are comprehensively documented in Table 1-1.

Table 1-1 Cardiovascular phenotype of PKG knockout mice

Type of knockout	Phenotype	References
Null mutation	Impaired NO/cGMP-dependent vasorelaxation	[162]
	Blunted cardiac negative inotropic effect in response to cGMP	[163]
	Systemic hypertension	[162]
	Pulmonary hypertension	[178]
	Impaired ischaemia-induced angiogenesis	[179]
	Increased platelet adhesion and aggregation	[173]
Cardiomyocyte-specific	Normal pressure overload- and iso- induced cardiac hypertrophy	[180]

	Blunted cardiac negative inotropic effect in response to cGMP	[163]
Smooth muscle-specific	Normal development of restenosis in response to ligation-induced injury	[181]
	Attenuated atherosclerosis on ApoE ^{-/-} background	[164]

Mice with mutations in the leucine zipper domain of PKGI α were also generated [182]. These mutations interfere with protein dimerisation and substrate recognition and result in mice being hypertensive and having impaired cGMP-induced vascular relaxation. They also show cardiac contractile dysfunction and age-dependent left ventricular hypertrophy [183]. As mentioned before, two PKGI isoforms exist and while having multiple similarities, they differ substantially in their endogenous substrates. Weber and colleagues created mouse lines on a PKG null background, which expressed either PKGI α or PKGI β under the SM22 α promoter, resulting in smooth-muscle specific expression [184]. Interestingly, these ‘rescue’ mice had normal blood pressure and smooth muscle cell responses, both vascular and non-vascular. There are several ways to explain these observations, however, there is still no definitive explanation for why rescue can be achieved with a sole isoform of the kinase. The substrate specificity of the isoforms might be not as prominent in vivo as in vitro experiments, suggesting that one isoform might be sufficient to maintain cardiovascular function. It is worth mentioning that smooth muscle-specific PKGI knockout mice have normal blood pressure [164]. Together these studies highlight that PKGI function is much more intricate than one might expect and future studies are required to unravel the complexities of PKG function.

PKG function in the cardiovascular system

PKGI and vasorelaxation

Regulation of vascular tone is essential for blood pressure homeostasis, and its dysregulation contributes to genesis of pathologies such as hypertension and atherosclerosis. Vascular smooth muscle cell (VSMC) tone is regulated by hormones and neurotransmitters, with contraction being initiated by an increase in cytosolic Ca²⁺.

This can happen either via inositol 1,4,5-trisphosphate (IP₃)-induced release of Ca²⁺ from intracellular stores or via extracellular Ca²⁺ influx through voltage-dependent and independent Ca²⁺ channels. Increased Ca²⁺ binds to calmodulin (CaM) and subsequently stimulates myosin light chain kinase (MLCK), which phosphorylates myosin light chain (MLC). This leads to increased myosin ATPase activity and induces vessel constriction via formation of actin-myosin cross bridges [185]. When intracellular Ca²⁺ decreases, MLCK is inactivated and MLC is dephosphorylated by myosin light chain phosphatase (MLCP), leading to relaxation. Additionally, the Rho/Rho kinase (ROCK) pathway modulates the sensitivity of the contractile system to Ca²⁺. ROCK inhibits MLCP, leading to sustained phosphorylation of MLC and tension at a given intracellular Ca²⁺ concentration. Therefore, VSMC tone is determined by the phosphorylation state of MLC and thus the signalling events that control MLCK and MLCP activity.

As mentioned above, cGMP-dependent vasorelaxation of small and large arteries is impaired in PKGI knockout mice. PKGI is shown to inhibit both, hormone-receptor-induced and depolarisation-induced constriction via modulating both Ca²⁺-dependent and/or independent mechanisms. There are two well-established Ca²⁺-dependent mechanisms that rely on two targets of PKGI, namely the IP₃-receptor-associated cGMP-dependent protein kinase substrate (IRAG) and the large conductance Ca²⁺-activated K⁺ channel (BK_{Ca}), which modulate intracellular Ca²⁺ release and extracellular Ca²⁺ entry, respectively. IRAG, a substrate of PKGIβ in VSMCs, is located at the ER membrane and is found in a complex with IP₃R and PKGIβ. PKGIβ phosphorylates IRAG to inhibit IP₃-induced Ca²⁺ release from the endoplasmic reticulum (ER). Thus, mice with IRAG mutated so that it cannot not bind inositol 1,4,5-trisphosphate receptor (IP₃R), lack cGMP-dependent inhibition of hormone-receptor-triggered intracellular Ca²⁺ release and VSMC contraction [186, 187]. This emphasises the importance of IRAG and PKGIβ localisation to their function. The advantage of colocalisation can be due to several factors, for example by increasing phosphate transfer efficiency due to enhanced proximity or perhaps inducing conformational changes that augment access to the residue that is phosphorylated. In addition to IRAG phosphorylation, PKGI is thought to activate regulator of G-protein signalling 2 (RGS2) and phospholipase Cβ3 (PLC) to inhibit its activity and reduce IP₃ synthesis [153, 188, 189]. Binding to RGS2

and its subsequent phosphorylation requires the leucine zipper domain in the N-terminus of PKGI α [190].

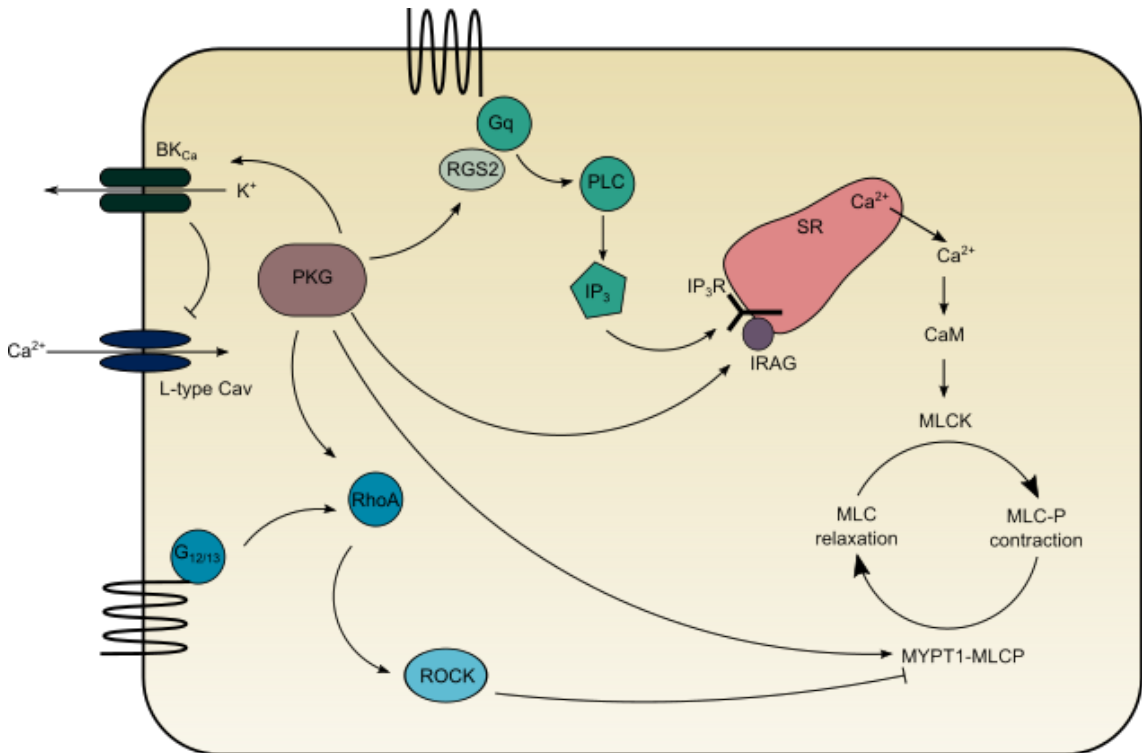


Figure 1-5

PKG-dependent mechanisms regulating vascular tone. Smooth muscle contraction is induced via the G-proteins G_q and G_{11} , which raise intracellular Ca^{2+} , or $G_{12/13}$, which activates a Ca^{2+} -sensitising mechanism. Increasing Ca^{2+} via receptor-activated or ion channel-activated mechanisms lead to myosin light chain (MLCK) activation. MLCK phosphorylates myosin light chain (MLC), activating myosin ATPase and actin-myosin cross bridging to promote VSMC contraction. Myosin light chain phosphatase (MLCP) dephosphorylates MLC, leading to VSMC relaxation. MLCP is activated by PKG. PKG also activates regulator of G-protein signalling 2 (RGS2), which attenuates G_q protein coupled receptor activation and increases intracellular Ca^{2+} . PKG-dependent phosphorylation of IP_3R -associated cGMP kinase substrate (IRAG) inhibits IP_3 -induced release of Ca^{2+} from the sarcoplasmic reticulum (SR). MLCP is inhibited by Rho-kinase (ROCK). PKG inhibits RhoA/ROCK signalling by phosphorylating RhoA. Ca^{2+} -activated K^+ channels, BK_{Ca} , are activated by PKG, resulting in cell hyperpolarisation and a decrease in Ca^{2+} entry.

PKGI can induce vasorelaxation independently of Ca^{2+} , which is achieved by phosphorylation of the myosin phosphatase target subunit 1 (MYPT1) at Ser695 and activation of MLCP [152, 191, 192]. MLCP dephosphorylates MLC to promote VSMC relaxation. Residues in the leucine zipper region of the PKGI α dimer form several

contacts with residues of MYPT1, interactions that are likely crucial for MYPT1 phosphorylation and MLCP activation [152, 193, 194].

RhoA is another well-studied target of PKGI, causing blockade of signalling through ROCK to inhibit contraction in VSMCs. In the absence of cGMP, RhoA is localised at the plasma membrane where it activates ROCK to phosphorylate MYPT1 at Thr696, thus inhibiting MLCP activity and causing VSMC constriction [195, 196]. PKGI phosphorylation of RhoA induces RhoA translocation to the cytosol, thereby inhibiting ROCK inactivation of MLCP. Additionally, MYPT1 phosphorylation at Ser695 blocks ROCK-dependent inactivation of the phosphatase [191, 197].

Additional targets of PKGI were reported, but their credibility have not been fully established. For example, PKGI can phosphorylate phospholamban (PLB), which is a negative regulator of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [198]. Phosphorylation of PLB activates SERCA to increase Ca^{2+} reuptake into SR thus reducing Ca^{2+} intracellular concentration [199]. It is not clear whether the PKGI-dependent phosphorylation of PLB is of physiological importance as cGMP-dependent relaxation was not affected in PLB knockout mice [200]. More than that, the concentration of PLB in VSMCs is marginal, when compared to cardiomyocytes.

Many studies show PKGI α and PKGI β interact with different proteins and induce vasorelaxation via distinct mechanisms, as discussed above. To reiterate, PKGI α interacts specifically with MYPT1 and RGS2, while PKGI β – with IRAG [151-153, 201]. Interestingly, some in vitro observations suggest that only PKGI α can restore cGMP-dependent inhibition of hormone receptor-induced increases in intracellular Ca^{2+} [202, 203]. On the other hand, there is strong evidence that PKGI β is essential for cGMP-dependent regulation of intracellular Ca^{2+} [187]. It is not clear what accounts for the discrepancy, but as mentioned above, Weber et al showed that both, PKGI α or PKGI β , can rescue the PKGI knockout phenotype independently of each other [184].

PKGI and cardiac function

NO and natriuretic peptides are known to modulate cardiac contractility and remodelling; several studies suggest that the NO-induced and natriuretic peptide-induced intracellular increase in cGMP in cardiac myocytes signals through PKG [204-

211]. Negative inotropic effects of NO in isolated cardiomyocytes, as well as in the myocardium of cardiomyocyte-specific PKG knockout mice were shown to be PKG-mediated [163, 207]. Additionally, cardiac-specific adenoviral overexpression of PKG1 β amplifies the anti-hypertrophic effects of NO donors [212]. Cardiomyocyte-specific overexpression of PKGI α enhances NO/cGMP-induced, but not muscarinic receptor-mediated inhibition of L-type Ca²⁺ channel activity, suggesting this mechanism to be responsible for negative inotropic effect of PKGI [213]. It is notable that C-type natriuretic peptide (CNP) increases cardiac contractility and exerts its effect via PKGI as well [214]. The dual effect of PKGI on cardiomyocyte contractility could be explained by spatial separation of NO/cGMP/PKG and CNP/cGMP/PKG signalling pathways. Unfortunately, the direct investigation of these beneficial effects in PKGI knockout mice is impossible because of their premature death. However, Lukowski et al tested the potential anti-hypertrophic effects of PKGI using the PKGI β rescue mice described above, but they did not show any differences in chronic β -adrenergic-induced or pressure-overload-induced cardiac hypertrophy as compared to WT mice [180]. Interestingly, Cys42-mediated redox regulation of PKG has been shown to play an important role in cardiac diastolic relaxation via phosphorylation of PLB [27]. PKG C42S KI mice have diastolic dysfunction and impaired tuning of the Frank-Starling response. The redox regulation of PKG will be discussed in more detail below.

PKG activation in cardiomyocytes targets several mechanisms that modulate cardiac function or induce cardioprotection, including activation of RGS2, transient receptor potential channels, TRPC3 and TRPC6, glycogen synthase kinase 3 (GSK3 β) and mitochondrial K_{ATP} channels. A summary of PKG signalling in the heart is presented in Figure 1-6.

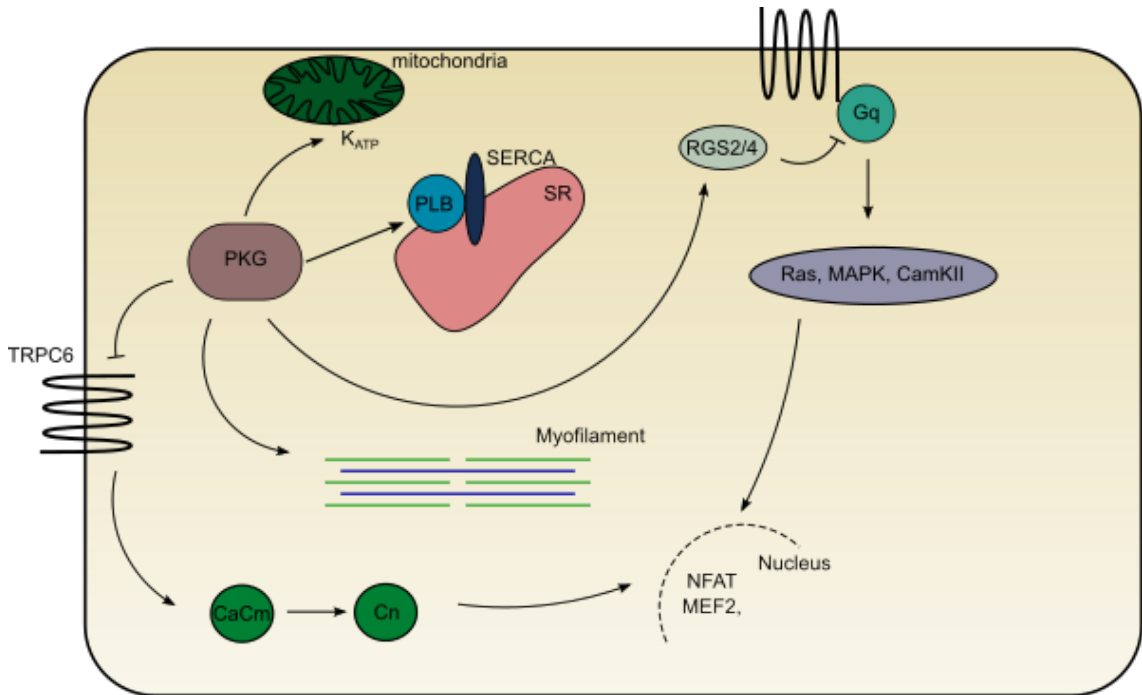


Figure 1-6

PKG regulation of cardiac myocytes. PKG phosphorylates phospholamban (PLB), enhancing Ca^{2+} uptake by SERCA in the sarcoplasmic reticulum (SR). PKG also binds and phosphorylates the regulator of G protein signalling 2 and 4 (RGS2/4), suppressing the Gq-coupled signalling cascades (Ras, MAPK and CamKII, and associated transcriptional factors, nuclear factor of activated T cells (NFAT) and myocyte enhancer factor 2 (MEF2)). PKG directly phosphorylates and suppresses transient receptor potential channel 6 (TRPC6), reducing Ca^{2+} -calmodulin (CaCm)-dependent calcineurin (Cn) activation. PKG can also provide cardioprotection via regulation of the open probability of mitochondrial ATP-sensitive K^{+} channels.

PKG structure and regulation

PKGI α and PKGI β are dimeric proteins with monomers arranged in a parallel configuration (Figure 1-7) [147]. Each monomer has regulatory and catalytic domains in a single polypeptide chain. The regulatory domain includes the N-terminal leucine zipper (LZ) domain, a flexible linker with autoinhibitory (AI) and autophosphorylation sequences, and two cyclic nucleotide-binding domains (CNB), CNB-A and CNB-B. The catalytic domain includes Mg^{2+} /ATP binding subdomain and substrate protein binding subdomain [215]. The LZ domain is responsible for protein dimerisation, interaction with protein substrates, and in certain cases subcellular localisation [154, 194, 215]. AI domain serves as a pseudo-substrate by binding to the catalytic domain and inhibiting PKGI activity in the absence of cGMP. The LZ and AI domains differ

between PKGI α and PKGI β isoform and define their specificity towards their substrates [147, 151-153]. Interestingly, PKGI α can be regulated by Cys oxidation in LZ domain that will be discussed later in more detail. The LZ and AI domain are followed by the CNB domains that bind cyclic nucleotides. Cyclic nucleotide binding induces a conformational change that activates PKG by releasing the catalytic domain from the AI domain [216, 217]. Both CNB domains have a preference towards cGMP over cAMP; however, they differ in their cGMP binding kinetics and cGMP analogue specificities [218, 219]. CNB-A is a high affinity (slow dissociation) site, while CNB-B is a low affinity (fast dissociation) site. Many studies have reported cGMP binding affinities for full-length PKGI α and PKGI β , as well as for the isolated binding domains. Unexpectedly, while full-length PKGI α has more than a 100 times lower affinity for cAMP than cGMP, isolated CNB-A affinity for cAMP is only twice weaker than for cGMP [218]. On the other hand, CNB-B is 240 times more selective for cGMP [220], suggesting that CNB-B is responsible for cGMP selectivity in PKG.

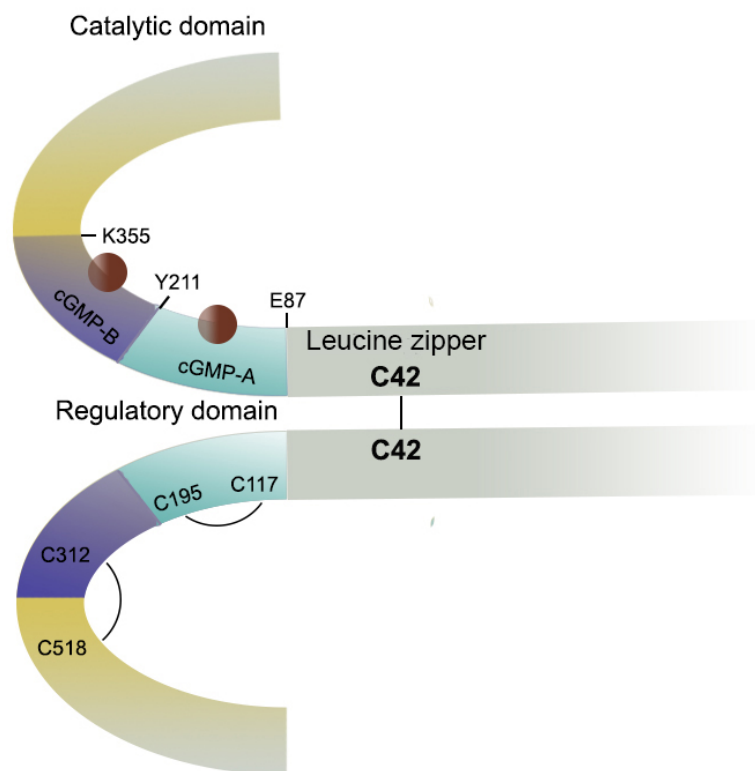


Figure 1-7

Domain organisation of PKG. PKG is a homodimer that is conjoined by virtue of an extended leucine zipper domain. cGMP binding sites (cGMP-A and cGMP-B) and catalytic domain are shown. Cysteine residues that have been shown or suggested to form disulfides are indicated.

Although the intracellular concentration of cAMP and cGMP is constantly changing, a maximum of 2-5 μM is likely [221, 222]. This supports the observations that there is a crosstalk between the cAMP and the cGMP pathways through binding of cAMP to PKG and cGMP to PKA [223-225]. The CNB-B domain is suggested to limit cAMP-mediated activation of PKG since the concentration of cAMP and cGMP nucleotides in cells is far below the EC_{50} for cAMP (52 μM) and far above the EC_{50} for cGMP (215 nM) [220]. To fully activate PKG, all four CNB sites have to be occupied, which strongly suggests that full activation of the kinase occurs only in response to cGMP and not cAMP [226]. This can limit the crosstalk and explain how cAMP and cGMP can mediate different, if not sometimes opposite, physiological responses [163, 227].

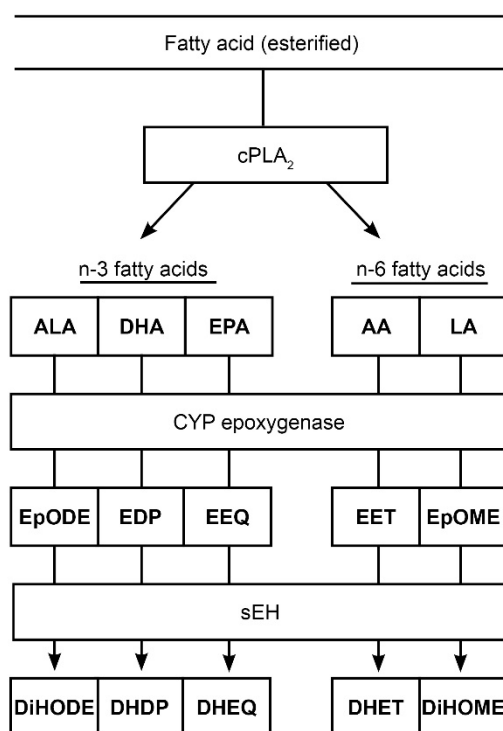
1.5.Soluble epoxide hydrolase

sEH in health and disease

Functional sEH in mammals is present as a homodimer of 62 kDa monomers arranged in an antiparallel mode [228]. Each monomer is comprised of 2 distinct structural domains, separated by a proline-rich linker. The C-terminal domain contains epoxide hydrolase activity, while the smaller N-terminal domain is a functional phosphatase that apparently acts on fatty acid diol phosphates [229].

sEH is expressed in multiple tissues and organs, with highest levels reported in the liver, followed by the kidney, heart, lung and brain [230]. sEH is broadly distributed in the liver, whereas its expression in other organs, such as kidney, lung or brain, is confined to the vascular tissue [231]. The enzyme is found mostly in the cytosol and peroxisomes [232]. For a long time, sEH activity was also thought to be present in a defined microsomal fraction, but this is now suggested to be the activity of membrane-bound epoxide hydrolases, EH3 and EH4 [233].

The most favoured substrates of the sEH hydrolase domain are epoxy fatty acids. Generally, sEH preferentially hydrolyses n-3 fatty acid epoxides and epoxides that are 14-16 atoms from the carboxyl group, while showing lower rates of hydrolysis for n-6 fatty acid epoxides and epoxides close to carboxyl termini [234]. sEH shows enantiomer and cis/trans-isomer selectivity, with trans-epoxides being preferable substrates [231]. sEH catalyses the hydrolysis by addition of water in a two-step mechanism via the formation of a covalent intermediate, as covered in a brilliant review [235]. The most studied endogenous substrates of sEH are epoxyeicosatrienoic acids (EETs), epoxides of arachidonic acid, but also to a lesser extent epoxides of linoleic acid (EpOMEs), α -linolenic acid (EpODEs), eicosapentaenoic acid (EEQs) and docosahexaenoic acid (EDPs) (Figure 1-8). This introduction will mostly focus on EETs as the most studied sEH substrates.

**Figure 1-8**

Cytochrome P450 epoxygenase metabolic pathway of n-3 and n-6 polyunsaturated fatty acids. n-3 fatty acids, α -linolenic acid (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and n-6 fatty acids, arachidonic acid (AA) and linoleic acid (LA), are metabolised by CYP450 epoxygenases to their corresponding epoxides, epoxyoctadecadienoic acid (EpODE) epoxydocosapentaenoic acid (EDP), epoxyeicosatetraenoic acid (EEQ), epoxyeicosatrienoic acid (EET) and epoxyoctadecamonoenoic acid (EpOME). Epoxide primary fate is conversion to corresponding diols by sEH, dihydroxyoctadecadienoic acid (DiHODE), dihydroxydocosapentaenoic acid (DHDP), dihydroxyeicosatetraenoic acid (DHEQ), dihydroxyeicosatrienoic acid (DHET), and dihydroxyoctadecamonoenoic acid (DiHOME).

Epoxygenated fatty acids are generated by cytochrome P450 epoxygenases (CYP450), which catalyse the epoxidation of unsaturated fatty acid double bonds. Most epoxygenated fatty acids have vasoactive, pro-angiogenic, anti-inflammatory and analgesic functions [236]. sEH regulates the biological effects of epoxy fatty acids by hydrolysing them to their corresponding vicinal diols and thus reducing the pool of active epoxides. Diols are mostly thought to be biologically inactive or at least less active than their corresponding epoxides. However, this is not always the case, as diols have been shown to be important in some systems. For example, 19,20-dihydroxydocosapentaenoic acid (19,20-DHDP) was found to be essential for retinal

angiogenesis, while some diols of EETs, dihydroxyeicosatrienoic acids (DHETs) were shown to be vasoactive and stimulate tissue plasminogen activator [237-240]. Through the metabolism of epoxide fatty acids, sEH is implicated in numerous diseases, including hypertension, cardiac hypertrophy, ischaemia-reperfusion injury, diabetes, pain, inflammation and cancer. The study of sEH function is facilitated by the availability of sEH-knockout (KO) mice and a great number of sEH inhibitors.

sEH and inflammation

sEH is implicated in the regulation of inflammation. This is consistent with EETs and n-3 polyunsaturated fatty acid epoxides having potent anti-inflammatory effects in the cardiovascular system, bronchi, kidney and nervous system [241, 242]. The first evidence for the role of EETs as anti-inflammatory mediators was that 11,12-EET attenuated the tumour necrosis factor (TNF- α)-induced activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) and the subsequent up-regulation of vascular cell adhesion molecule-1 (VCAM-1) expression in mice [243]. Notably, these effects were selectively induced by 11,12-EET, whereas 14,15-EET was ineffective. sEH-KO mice or mice overexpressing CYP450 epoxygenase in the endothelium (Tie2-CYP2J2-Tr) showed attenuated inflammatory response to endotoxin [244]. Moreover, the sEH inhibitor AUDA-BE reduced the production of pro-inflammatory lipid mediators and decreased lipopolysaccharide (LPS)-induced mortality in mice [245]. EETs attenuate inflammation via three mechanisms: blocking the NF- κ B pathway by inhibiting I κ B kinase [244], activating peroxisome proliferator-activated receptor (PPAR γ) transcriptional activity [246], and affecting levels of the inflammatory prostaglandin E2 (PGE2) [247].

Although several studies showed inhibition of sEH or overexpression of CYP450 epoxygenases is beneficial during inflammation, there are others that contradict this. LPS-induced expression of inflammatory genes or neutrophil liver infiltration was not diminished in sEH-KO mice or in WT mice administrated an sEH inhibitor [248]. Overexpression of CYP2C9 epoxygenase in endothelial cells resulted in the increased adhesion molecule expression [249]. Overexpression of CYP2C9 also increased COX-2 expression and PGE2 production in human vascular endothelial cells [250]. Moreover, mice overexpressing endothelial CYP2C8 are more susceptible to cardiac ischaemia-

reperfusion injury [251]. It should be noted, that CYP450 epoxygenases have differential preferences towards different polyunsaturated fatty acids. While arachidonic acid is a relatively poor substrate for CYP2J2, epoxygenases CYP2C8 and CYP2C9 more readily metabolise it. This could explain the observations that the overexpression of CYP2J2 attenuates adhesion molecule expression, whereas the overexpression of CYP2C8 and CYP2C9 results in exactly the opposite [243, 249]. This suggests that polyunsaturated acid mediators derived from lipids other than arachidonic acid may account for the anti-inflammatory effects observed. However, the pro-inflammatory effects of CYP2C8 and CYP2C9 mentioned above were proposed to be secondary to the epoxygenase-mediated production of reactive oxygen species (ROS) and not to the synthesis of EETs [249].

Although, the research has mainly focused on the anti-inflammatory actions of P450 epoxygenase-derived metabolites of arachidonic acid, it is now clear that bioactive lipids generated from other polyunsaturated fatty acids possess anti-inflammatory activities. Some of these actions are related to mobilisation of certain population of bone marrow-derived cells that give rise to neutrophils and monocytes [252]. sEH inhibition has been reported to reduce the infiltration of lymphocytes, neutrophils, and macrophages [244, 253-256]. However, elucidating which lipid mediators are responsible for which processes is extremely challenging, and the data available suggests a potential role of arachidonic acid and linoleic acid diols, rather than their epoxides [252].

sEH is involved not only in inflammatory signalling, but also in the regulation of inflammatory pain. Inhibitors of sEH and fatty acid epoxides reduce pain perception in rodent models of inflammatory pain, without influencing pain perception in healthy animals [257-259].

sEH and blood pressure regulation

After EETs were identified as vasodilatory compounds, the role of sEH in blood pressure regulation was suggested. In a simplified model, sEH hydrolyses vasodilatory EETs to less biologically active DHETs, which are also less lipophilic and are more easily removed from the site of action [232]. By doing this, it influences the ratio of

vasodilators to vasoconstrictors, leading to the change in vascular resistance, blood flow and blood pressure. sEH inhibition reduced hypertension in different animals models, including angiotensin II (Ang II), salt-sensitive or high-fat diet induced hypertension, while not lowering blood pressure in normotensive animals [260-262]. However, contradictory findings with sEH-KO mice have been reported. The first study with sEH-KO mice showed reduced systolic blood pressure compared to WT animals, with higher EET levels in their blood, liver and kidney. However, the reduction of blood pressure in sEH-KO mice was not corroborated when these mice were backcrossed into C57Bl/6 genetic background or in an independently generated sEH-KO mouse colony [263, 264]. Interestingly, mice from each of these sEH-KO colonies had higher levels of EETs.

EETs regulate blood pressure via their action on the vascular tone of small resistance vessels and enhanced sodium excretion in the kidney [234]. In resistance arteries EETs released by endothelial cells induce vasodilation by activating large-conductance calcium activated potassium channels (BK_{Ca}) on vascular smooth muscle cells (VSMC) and/or by transmitting endothelial cells hyperpolarisation to vascular smooth muscle cells via gap junctions [236] (Figure 1-9). Both 11,12-EET and 14,15-EET increase VSMC K_{Ca} channel opening [265]. Although, iberiotoxin-sensitive BK_{Ca} channels are believed to be the major mediators of EET vasodilatory effect, it is also suggested that intermediate-conductance (IK_{Ca}) and small-conductance (SK_{Ca}) calcium activated potassium channels are involved. The role of polyunsaturated fatty acid epoxides is most probably linked to their ability to increase the Ca^{2+} response of endothelial cells to agonists, so potentiating endothelial cell K_{Ca} activation and hyperpolarisation, an event that precedes the hyperpolarisation of VSMCs [266]. Another mechanism underlying the vasodilatory effects of EETs is believed to rely on transient receptor potential vanilloid (TRPV) channel activation. 5,6-EET has been reported to activate TRPV4 channel in endothelial cells and enhance flow-induced vasodilation of murine carotid artery [267-269]. EETs have also been suggested to contribute to vascular relaxation is via the facilitation of endothelial and possibly myoendothelial gap junction communication. In porcine coronary artery, 11,12-EET-induced hyperpolarisation was attenuated by gap junction blockers [270]. One characteristic of many EET-induced cellular responses is the dependence on protein kinase a (PKA) activation [266, 271,

272]. Several groups have speculated that a specific EET membrane receptor may exist, suggesting another way of EET-induced vascular relaxation [273].

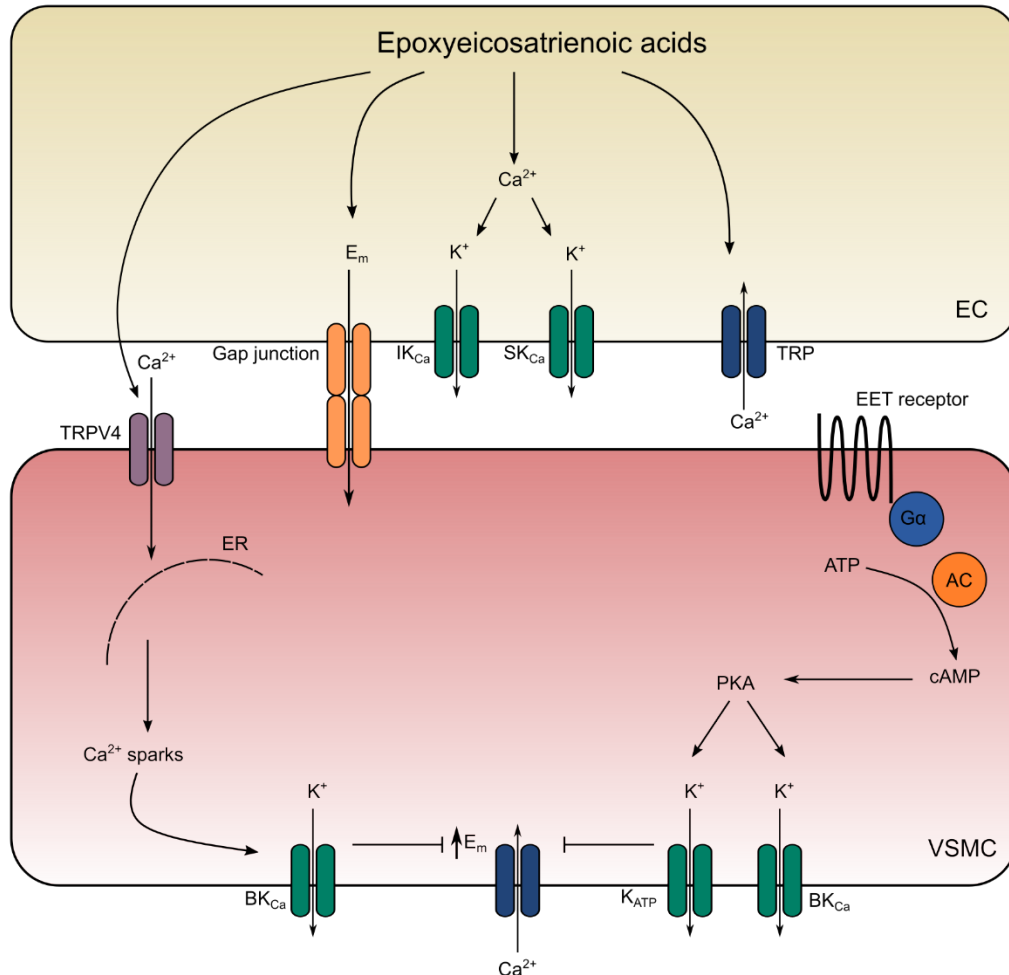


Figure 1-9

Signalling pathways mediating EET-induced vascular relaxation. EETs generated by endothelial cell (EC) activate transient receptor potential vanilloid channel 4 (TRPV4) on vascular smooth muscle cell (VSMC). Large-conductance calcium activated potassium channel (BK_{Ca}) activation is mediated by subsequent Ca^{2+} influx and Ca^{2+} sparks from the endoplasmic reticulum (ER). EETs activate TRP channel on EC, resulting in increased Ca^{2+} -induced intermediate-conductance (IK_{Ca}) and small-conductance (SK_{Ca}) potassium channels activation to cause cell hyperpolarisation, which spreads to VSMC via gap junctions. EETs released by EC are believed to activate a yet unidentified EET receptor on VSMC, leading to adenylate cyclase (AC) and protein kinase A (PKA) activation. This results in activation of BK_{Ca} and ATP-sensitive potassium channel (K_{ATP}) activation, potassium efflux and VSMC hyperpolarisation.

EETs also affect blood pressure by regulating renal sodium excretion. In the kidney, EETs induce natriuresis and increase renal blood flow [274]. Elevated sEH activity in

kidneys of spontaneously hypertensive rats was reported, further supporting the role of sEH in blood pressure regulation [275]. Using the two kidneys, one clip model or a transgenic rat model overexpressing renin to induce hypertension, sEH inhibition reduced blood pressure [276, 277]. Moreover, sEH was shown to be redox regulated by the electrophilic lipids nitro-oleic acid (NO₂-OA) and 15-deoxy-delta-12,14-prostaglandin J2 (15d-PGJ2), which inhibited sEH activity by adducting to Cys521 and lowered blood pressure in an Ang II-induced model of hypertension or dilated coronary vasculature, respectively [29, 30]. C521S sEH KI mice exhibit higher blood pressure than WT, which could be explained by a sustained level of hydrolase inhibition by electrophilic lipids in WT under basal conditions. Relying on such encouraging studies showing important role of sEH in blood pressure regulation, sEH has been pursued as a pharmacological target for treatment of hypertension. The sEH inhibitor, AR9281, was taken to a phase 2 clinical trials, but failed to show efficacy in early-stage hypertension [278].

Other roles of sEH

sEH is implicated in the pathogenesis of pulmonary hypertension [279, 280]. EETs act differently in different vascular beds; while they are vasodilators in the peripheral and coronary vasculature, they act as vasoconstrictors in the pulmonary vasculature. When sEH-KO mice are subjected to chronic hypoxia, they develop more pronounced right ventricular hypertrophy, pulmonary vascular remodelling and decreased exercise performance [281]. Interestingly, mice receiving chronic administration of sEH inhibitor do not replicate the phenotype observed in knockout animals [281]. This implies that the role of sEH in pulmonary hypertension is much more complicated than simple reduction of vasoconstrictory EET levels. sEH inhibition was shown to improve lung function and attenuate inflammation in a smoke-induced chronic obstructive pulmonary disease (COPD) rat model [282]. Moreover, the sEH inhibitor, GSK2256294 is currently being evaluated in clinical trials as a potential treatment for COPD.

sEH activity might be important in angiogenesis and the aetiology of cancer. The role of sEH in angiogenesis will be considered in more detail in this introduction. EETs promote cell proliferation, migration and angiogenesis, consistent with a potential role in the pathogenesis of cancer [283]. Indeed, EETs stimulated tumour growth and

metastasis in multiple mouse tumour models [284]. High levels of EETs in transgenic mice overexpressing CYP450 epoxygenases (Tie2-CYP2C8-Tr and Tie2-CYP2J2-Tr) or in sEH-KO mice are associated with a slightly potentiated primary tumour growth, but dramatically enhanced tumour metastasis. Although, these effects were not observed in the absence of spontaneous tumour models, these findings raise a serious concern about the use of sEH inhibitors as therapeutic drugs. Notably, mice lacking *Cyp2c44* gene were shown to have reduced tumour volumes and vascularisation [285]. CYP epoxygenase inhibitors also blunt brain tumour angiogenesis and growth [286].

Considering the numerous biological activities of sEH and its implication in various pathologies, enhancing our understanding of how sEH activity is regulated is of utmost importance. Although sEH was discovered more than four decades ago, the post-translational regulation of sEH activity in vivo is still poorly understood. Recent studies, reporting sEH redox regulation by adduction of electrophilic lipids, open new doors to understanding and manipulating the activity of this hydrolase to maintain health or combat disease. *Thus, the broad aims of the studies outlined in this chapter include: defining whether redox regulation of sEH contributes to the pathogenesis of sepsis or angiogenesis during hindlimb ischaemia.* Consequently, the rest of the introduction will focus on the background rationale for redox regulation of sEH mediating the progression of sepsis or angiogenesis during ischaemia.

Redox regulation of sEH and the pathogenesis of septic shock

Sepsis is a severe clinical condition manifested by a patient's response to infection. For a long time sepsis, historically described as "blood poisoning", was considered as a systemic infection. Recently, the International Sepsis Consensus Panel defined sepsis as a systemic inflammatory response to infection, rather than the infection itself [287]. The term severe sepsis was used to describe sepsis with organ dysfunction, with septic shock describing severe sepsis with hypotension despite adequate fluid resuscitation. Sepsis is a leading cause of mortality in critically ill patients, with more than 200,000 patients dying each year in United States alone, which is similar to the number of patients dying with acute myocardium infarction and far exceeds those who die from breast cancer or stroke [288].

Sepsis is associated with numerous cardiovascular derangements, leading to tissue hypoperfusion and subsequent multiple organ failure [289]. Several factors are known to contribute to impaired oxygen delivery in septic shock, such as hypotension, reduced red cell deformability and microvascular thrombosis [290] (Figure 1-10).

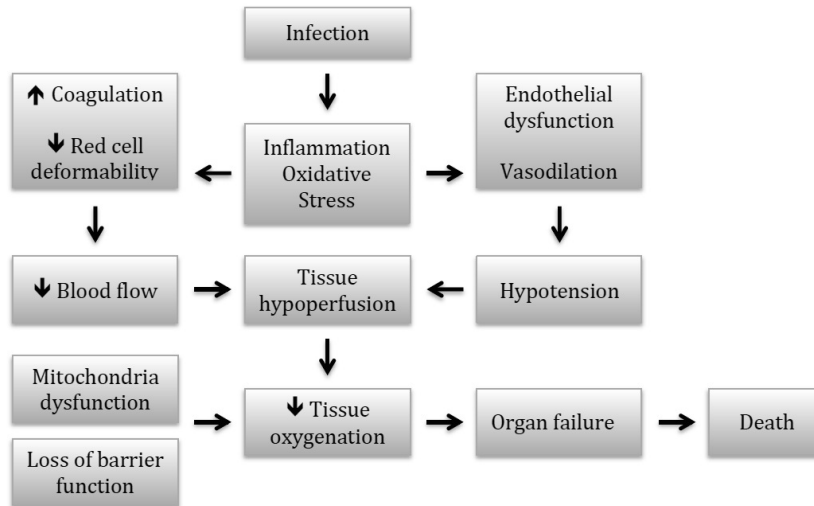


Figure 1-10

Pathogenesis of sepsis. Sepsis is triggered by an infection or inflammatory insult. Impairment of inflammatory response leads to endothelial dysfunction and microvascular thrombosis, resulting in tissue hypoperfusion, multi-organ failure and a high rate of mortality.

Hypotension is thought to be the main cause of mortality in septic shock and is considered to be the consequence of three major haemodynamic derangements, namely hypovolemia, vascular dysfunction and heart failure [291, 292]. One manifestation of vascular dysfunction is endothelial dysfunction, accompanied by the increased permeability, giving rise to subcutaneous and body-cavity oedema, and augmented endothelial cells apoptosis [293]. Another manifestation of this vascular dysfunction is a diminished ability of vascular smooth muscle cells to constrict and their resistance to vasopressors, promoting vasodilation. Septic shock patients showed a decreased response to α -adrenergic stimulation compared to healthy subjects [294]. It is worth noting, that septic shock stimulates the sympathetic system, producing the catecholamines norepinephrine and dopamine. This together with activation of the renin-angiotensin system is an adaptive mechanism to induce peripheral vasoconstriction to compensate for the vasodilation during septic shock [295].

Nevertheless, the vascular sensitivity and reactivity to vasopressors is altered during septic shock, making it unresponsive to system changes as stated above. One of the major mechanisms proposed to explain the vasodilation during septic shock include activation of ATP-sensitive potassium channels (K_{ATP}), activation of inducible form of nitric oxide synthase (iNOS), activation of cyclooxygenase 2 (COX-2) and deficiency of the hormone vasopressin (Figure 1-11).

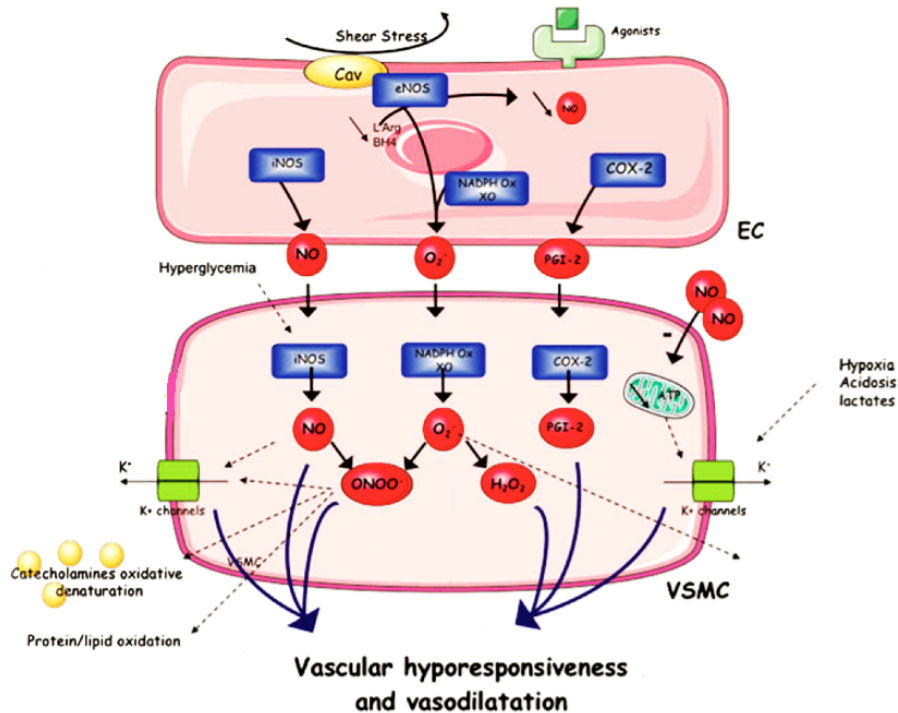


Figure 1-11

Proposed mechanism of vascular hyporesponsiveness during septic shock. Sepsis initiates multiple signalling cascades leading to vascular hyporesponsiveness and vasodilation. These include activation of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) and opening of K_{ATP} channels. Induction of iNOS results in increased levels of NO and subsequent vasodilation through the cGMP/PKG pathway. Increased levels of PGI₂ lead to VSMC relaxation via the cAMP/PKA pathway. Opening of K_{ATP} channels is followed by cell hyperpolarisation and vasorelaxation.

ATP-sensitive potassium channels

Opening of K_{ATP} channels allows efflux of potassium, thus leading to cell hyperpolarisation and preventing calcium entry. These channels contribute to resting

membrane potential in VSMCs and local blood flow, and can be activated by various stimuli, including tissue hypoxia and high cellular lactate [296]. Under physiological conditions K_{ATP} channels have low activity and only a minor effect on blood pressure regulation. However, during septic shock they are activated, leading to vasodilation and hypotension, also contributing to the hyporesponsiveness to catecholamines [92]. K_{ATP} channels were first demonstrated to be involved in sepsis when the injection of glibenclamide (a non-selective K_{ATP} inhibitor) restored arterial pressure in endotoxaemic dogs [297]. The result was attributed to an increase in systemic vascular resistance rather than enhanced cardiac output. Studies in endotoxaemic pigs or rats confirmed the observation, and showed increased vasopressor responses to α -adrenergic catecholamines [298-300]. Notably, some in vitro studies did not corroborate the effect of glibenclamide on vascular hyporeactivity, with some actually showing a potentiated vasodilation [298, 301, 302]. The involvement of K_{ATP} channels in vascular hyporesponsiveness during sepsis was challenged in two clinical trials studying the effect of glibenclamide during septic shock. Glibenclamide failed to restore arterial pressure or vascular sensitivity to vasoconstrictors in septic patients [303, 304].

The mechanism of K_{ATP} channel activation during septic shock is not fully established. Neurohormonal activation of K_{ATP} channels is thought to be involved in septic shock-induced vasodilation. Atrial natriuretic peptide, adenosine and calcitonin gene-related peptide (GCRP) were shown to activate K_{ATP} channels, with their plasma concentration increased during the later stages of septic shock [305]. Additional mechanisms leading to opening of K_{ATP} channels operate during sepsis, including a fall in the intracellular $[ATP]/[ADP]$ ratio, a rise in intracellular lactate and a decrease in pH.

Recent studies have shown the link between EETs, substrates of sEH and K_{ATP} channel, thus implying a possible role for sEH regulation of vascular tone during sepsis. EETs were shown to be potent activators of K_{ATP} channels in rat cardiac myocytes and vascular smooth muscle cells. However, the mechanism of activation differs; while cardiac K_{ATP} channels are directly activated by EETs, vascular K_{ATP} channels require the activity of protein kinase A (PKA) [306-310]. In various cardiac ischaemia/reperfusion (I/R) animal models, EETs exhibited protective effects against I/R injury through the activation of K_{ATP} channels. This EET protective effect was

abolished by K_{ATP} channel inhibitors [311-317]. In a rat model of cerebral I/R injury, EETs up-regulated the expression of K_{ATP} protein Kir6.1, consistent with the protective effect of EETs being mediated by the K_{ATP} channel [318]. Although, the role of EET-mediated K_{ATP} activation has not been established during septic shock, it is clearly a logical hypothesis that this mechanism might contribute to sepsis-induced hypotension.

Increased levels of prostacyclin

Prostacyclin (PGI_2) is produced in endothelial cells by the activity of COX enzymes. PGI_2 relaxes VSMCs through its action on G-protein coupled receptors, leading to increased cAMP levels and PKA activation, resulting in vasorelaxation [319]. During septic shock an inducible form of COX, namely COX-2, is responsible for the increase in PGI_2 . Inhibiting COX-2 in endotoxaemic mice restored systemic arterial pressure, but COX-2 knockout mice did not prevent LPS-induced hypotension, suggesting that PGI_2 may not be essential for vascular dysfunction during septic shock [320, 321]. Moreover, a clinical trial with ibuprofen, a non-specific inhibitor of prostaglandin formation, failed to show any beneficial effects during septic shock [322].

Increased synthesis of NO

In septic shock patients, plasma levels of NO metabolites are moderately increased. Elevated NO during septic shock is the result of activation of inducible form of NOS, (iNOS) and is believed to contribute to hypotension. iNOS was shown to be up-regulated in VSMC samples from septic shock patients [323, 324]. NO acts both directly and via cGMP to decrease intracellular calcium levels and activate K_{Ca} or K_{ATP} channels to promote dilation of blood vessels. Inhibitors of NOS, such as NG-nitro-L-arginine methyl ester (L-NAME), increased vascular resistance and arterial pressure in some animal models of sepsis, but failed to do so in others [325-328]. Moreover, iNOS knockout mice show attenuated hypotension in response to the administration of endotoxin [329], although the survival in iNOS knockout group was decreased after CLP induction [330]. Encouraged by the idea that excessive NO is an important cause of vasodilation and hypotension during septic shock, a clinical trial of the non-selective NOS inhibitor, NG-monomethyl-L-arginine (L-NMMA), was carried out. This trial was stopped because the mortality in treatment group exceeded the mortality in the placebo

group [331]. Another approach to target excessive NO was based on the findings that haemoglobin scavenges NO. A clinical trial examining pyridoxalated haemoglobin polyoxyethylene (PHP) showed that it ameliorated vascular hyporesponsiveness and hypotension in patients with distributive shock, characterised by hypotension due to reduced vascular resistance. However, this trial was also terminated because of excess mortality among the PHP cohort [332, 333]. The adverse effect of NOS inhibition or scavenging NO during septic shock could be explained, at least partly, by the fact that NO is an important factor in maintaining microcirculatory flow, has bactericidal activity and regulates blood coagulation. A number of studies showed beneficial effects of increased NO during endotoxic shock. Thus, in TNF α - or LPS-induced models of septic shock nitrite therapy prevented tissue damage, organ failure and decreased mortality [334]. Moreover, administration of nitroglycerin to septic patients increased microcirculatory flow [335]. Although, it should be emphasised that NO donors further lowered blood pressure. In addition, NO may have protective effects by attenuating apoptosis, by inducing anti-inflammatory gene expression or by kill pathogens. To some extent, the effects of NO during sepsis could be attributed in part to nitro fatty acids (NO₂-FAs) actions, an idea that is one focus of this work. NO free radical species can induce nitration of unsaturated fatty acids. NO₂-FAs display an electrophilic reactivity, facilitating adduction by nucleophilic targets, such as protein Cys residues. Current data reveal that NO₂-FAs serve as mediators of physiological and pathophysiological cell signalling processes. It was also demonstrated that NO₂-FAs exert anti-inflammatory actions. In a mouse model of LPS-induced sepsis NO₂-OA was reported to decrease the expression of TNF α , inflammatory cytokines, chemokines, iNOS and COX-2, as well as reduce lung and liver injury [336, 337]. Some of the anti-inflammatory actions of NO₂-OA were attributed to its activation of the PPAR γ receptor, but PPAR-independent mechanisms also contributed. These include inhibition of NF- κ B activity, activation of adenylyl cyclase and activation of the antioxidant transcription factor Nrf2 [338-340]. Some of these actions could potentially be mediated by anti-inflammatory EETs. Indeed, NO₂-OA was shown to inhibit sEH, leading to increased levels of EETs [29]. Thus, one can speculate that excessive formation of NO₂-FAs during septic shock could elevate EET levels and perhaps contribute to the vasodilation observed during septic shock.

sEH redox regulation and post-ischaemic revascularisation

Vascular homeostasis plays an important role in the remodelling of ischaemic tissues and potentially may constitute treatment targets for the restoration of function in ischaemic organs, such as the heart or limbs. Thus, it is of utmost importance to investigate the processes that regulate post-ischaemic vessel growth and remodelling. In general, the tissue healing process after ischaemia can be divided into several phases. After the initial hypoxia and tissue hypoperfusion, the damaged cells undergo apoptosis and necrosis. This initial phase is followed by the inflammatory phase, which features neutrophil, monocyte, and lymphocyte homing and migration into the necrotic tissue for the removal of dead cells. New vessel formation and remodelling of the pre-existing vasculature happens mainly under the control of local pro-angiogenic stimuli and local or circulating vascular progenitor cells. Arteriogenesis and remodelling of pre-existing vessels probably lead to a larger degree of blood flow recovery, while the development of new capillaries (vasculogenesis and angiogenesis) mitigates tissue loss in the ischaemic zone. While the initial stimuli for vasculogenesis, angiogenesis, arteriogenesis and collateral growth probably vary, all these processes involve the same growth factors, chemokines and proteases, whose activities are modulated and regulated differently and at distinct time points, but are broadly interdependent. It is worth noting that the complex tissue responses to ischaemia in hind limb are different in anatomically distinct regions. In areas of severe ischaemic injury (below the knee), tissue necrosis is accompanied by inflammation, angiogenesis, and muscle repair, while in areas proximal to the ischaemic region (the thigh), arteriogenesis predominates [341-343].

Endothelial cell proliferation and migration

Increasing evidence is consistent with EETs and other epoxygenated fatty acids exerting actions on endothelial and vascular smooth muscle cells to effect proliferation, migration, and angiogenesis [344-346]. In regards to endothelial cells, overexpression of the CYP2C family of proteins or exogenous application of EETs result in human or murine endothelial cell proliferation [347-350]. In vivo studies support these in vitro findings with EETs reported to induce vascularisation of Matrigel plugs implanted into mice or rats, as well as being angiogenic in the chick chorioallantoic membrane [349-351]. Further support that EETs are pro-angiogenic is shown by overexpression of

human CYP2J2 or CYP2C8 enzymes increasing skeletal muscle capillary density in the ischaemic rat hindlimb model [352]. Similarly, chronic sEH inhibition increased capillary density in stroke-prone spontaneously hypertensive rats, and insulin-resistant *Ephx2*^{-/-} mice had increased pancreatic vascular density [353, 354].

Several signalling pathways have been shown to mediate EET-induced proliferation and include the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, the p38 mitogen-activated protein kinase (MAPK) and the cAMP/PKA pathway. While all EET regioisomers have been reported to elicit an increase in Akt phosphorylation and murine endothelial cell proliferation, only 5,6-EET and 14,15-EET actions were shown to be sensitive to PI3K inhibitors, while 8,9-EET and 11,12-EET-induced proliferation seemed to be dependent on the p38 MAPK pathway. In bovine aortic endothelial cells, 8,9-EET-, 12,13-EET- or 14,15-EET-induced proliferation can be inhibited by MAPKK, extracellular signal-regulated kinase (ERK) and PI3K inhibitors. Other pathways contribute to EET-induced angiogenesis; for example, administration of 11,12-EET to human umbilical vein endothelial cells (HUVECs) induced proliferation through activation of sphingosine kinase 1 (SK1) [355]. Interestingly, EETs were reported to contribute to vascular endothelial growth factor (VEGF)-stimulated proliferation in lung endothelial cells [349, 356]. VEGF increased *Cyp2c44* expression and EET production, while silencing *Cyp2c44* reduced VEGF-induced endothelial proliferation [357].

Endothelial cell migration is essential for angiogenesis. Prior to migration, endothelial cells have to degrade the surrounding matrix, which is achieved by the action of matrix metalloproteases (MMPs) [358]. Both 11,12-EET and 14,15-EET activate one or more MMPs and enhance endothelial cell migration [250, 350, 359].

Actions of EETs on vascular smooth muscle cells are not defined to the same extent as those on endothelial cells. One of the first studies reported that 14,15-EET potentiated platelet-derived growth factor (PDGF)-induced vascular smooth muscle cell proliferation [247]. On the other hand, the majority of studies suggest that EETs have anti-migratory effects on cultured vascular smooth muscle cells, with all EET regioisomers inhibiting PDGF-induced rat aortic smooth muscle cell migration [360].

Hypoxia-inducible factor (HIF) and VEGF

In ischaemic tissue a principal response is the activation of the HIF transcriptional factor, which occurs due to the associated hypoxia. In oxygenated cells, HIF α is essentially non-functional. In such conditions prolylhydroxylase domain proteins (PHDs) and factor inhibiting HIF (FIH) hydroxylate certain amino acid residues in HIF α and addressing it to proteosomal degradation. In hypoxic conditions, PHDs and FIH are inactive, resulting in HIF α stabilisation and subsequent translocation to the nucleus. In the nucleus, HIF α interacts with HIF β to form a heterodimer that binds to the hypoxia response elements in the regulatory regions of many genes. In particular, HIF1 regulates the expression of the gene encoding VEGF-A, which is the principal master regulator of blood vessel sprouting and growth [361]. VEGF-A binds to its receptors VEGFR1 and VEGFR2 on the surface of endothelial and vascular smooth muscle cells. This results in activation of numerous signalling pathways, including MAPKs, and PI3K/Akt pathways [362-364]. As a result, VEGF-A signalling promotes endothelial cells proliferation, degradation of the extracellular matrix and chemotaxis. Heterozygous *Vegfa*^{+/-} mice show severe vascular defects, and mice null for *Vegfr2* show embryonic lethality and deficient vascular assembly [365-367]. PI3K γ knockdown abrogates endothelial cells angiogenic capacity, and mice deficient for PI3K γ show impaired reparative vascularisation [368].

Several studies support EETs significantly affecting the sequence of angiogenic events during hypoxia. For example, hypoxia stimulated CYP2C8 and CYP2C9 expression [369, 370], and the putative EET receptor antagonist, 14,15-EEZE, inhibited hypoxia-induced endothelial tube formation [371]. This latter study also showed the angiogenic effect of EETs is partially dependent on HIF1 α -mediated VEGF induction. *CYP2C11* gene expression is also directly regulated by HIF1 α . In hypoxic conditions in astrocytes HIF1 directly binds to *CYP2C11* promoter, enhancing gene expression and CYP2C11 mRNA and protein levels [372].

The effects of EETs and VEGF regulation are closely intertwined, with the former enhancing the effects of angiogenesis induced by the growth factor. EETs induce angiogenesis through activation of VEGF expression [373]. 14,15-EET-induced human dermal microvascular endothelial cell migration and tube formation and Matrigel plug

angiogenesis were completely blocked by anti-VEGF antibodies. On the other hand, VEGF increases CYP2C promoter activity in endothelial cells and induced the expression of CYP2C8, resulting in increased levels of EETs. 14,15-EEZE inhibits VEGF-induced endothelial cell tube formation. In cultured lung endothelial cells VEGF induced CYP2C44 epoxygenase, resulting in increased levels of 11,12-EET and 14,15-EET and angiogenesis [357, 374]. Recently sEH was shown to play an important role in VEGF-induced angiogenesis, as inhibition of the hydrolase enhanced endothelial cell tube formation and neovascularisation in Matrigel plugs [375]. The mechanism behind it being enhanced VEGF-induced phosphorylation of eNOS and increased NO production. eNOS plays a key role in post-ischaemic neovascularisation, with it being stimulated downstream of VEGF-A in a mouse of peripheral limb ischaemia [376]. Consistent with this, mice lacking eNOS show impaired vascular neogenesis in the ischaemic zone.

Haemodynamic forces and post-ischaemic revascularisation

Changes in mechanical forces within vessels as a result of arterial occlusion also contribute to post-ischaemic revascularisation. The difference in blood pressure between arterioles upstream of the occluded region and those downstream of it results in increased blood flow in collateral arteries [377]. The increase in shear stress leads to collateral vessel vasodilation, thickening, and endothelial cell activation and migration. The immediate response to the increase in shear stress is endothelial cells activation. Shear stress is recognised by extracellular “flow sensing” elements and is transmitted to cytoskeleton proteins to further trigger various adaptive mechanisms. In response to shear stress the expression of several integrins increased [378], which interact with growth factors to promote the development of collateral vessels. Interestingly, haemodynamic stimuli such as fluid shear stress and cyclic strain are suggested to regulate the expression of CYP epoxygenases [250, 379]. It is very tempting to make a direct link between the effects of shear stress and cyclic stretch on CYP expression and those on angiogenesis [380, 381]. Indeed, cyclic stretch-induced tube formation can be inhibited by N-methylsulfonyl-2-2-propynyloxy-benzenehexanamide (MS-PPOH), a CYP epoxygenase inhibitor, as well as by the EET antagonist 14,15-EEZE [250].

Shear stress potentiates communication between endothelial cells. This is achieved, at least partly, by the gap junction proteins connexin 37 and connexin 40. In mice, genetic ablation of connexin 40 resulted in reduced angiogenesis in femoral artery or mesenteric artery occlusion models [382]. Interestingly, EETs have also been shown to positively modulate gap junction communication [272].

Inflammation and post-ischaemic revascularisation

The effects of inflammation on vascular growth and remodelling are largely dependent on inflammatory cell adhesion to the endothelium and migration across the vessel wall, as well as their ability to release pro- and anti-angiogenic molecules. Interaction between the adhesion molecules expressed by myeloid cells, lymphocytes or endothelial cells are involved in the initial attachment, rolling, adhesion and migration of circulating cells. The initial adhesion of monocytes to the endothelium is mediated by adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). An early paper demonstrated that 14,15-EET administration enhanced U937 cell attachment to endothelial cells, indicating it likely plays a substantial role in angiogenesis [383]. Similarly, NF- κ B activity was enhanced after the induction or overexpression of CYP2C9, as was the expression of VCAM-1 [249]. However, subsequent studies have demonstrated that CYP epoxygenase-derived EETs, in particular 11,12-EET, significantly attenuate endothelial activation and leukocyte-endothelial interactions in various model systems [243, 249, 372, 384, 385]

Chapter 2. General Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich, unless states otherwise; where necessary catalogue numbers are given with number sign.

Mouse strains

Knock-in mice (KI) with a C521S mutation in sEH or those with a C195S mutation in PKG were studied and compared to their respective wild-type (WT) littermates. All the experiments were conducted according to the Animals (Scientific Procedures) Act 1986 (revised 1 January 2013).

Telemetric blood pressure measurements

Mean arterial pressure, heart rate, systolic, diastolic, pulse pressure and activity were assessed in conscious freely moving mice through implantation of radio-transmitters. Briefly, mice were anaesthetised as described earlier. Mice were placed in dorsal recumbency on a heating pad and then the neck was shaved and disinfected, after which a horizontal incision was made and the left carotid artery was exposed. This vessel was ligated with 7-0 silk-braided suture proximal to the bifurcation and punctured with 26-gauge needle. The catheter tip was advanced into the carotid artery and gently pushed into the aortic arch, while sutures were used to hold the catheter in place. A subcutaneous pocket was formed along the right flank by inserting scissors into the throat incision, and the transmitter was inserted. The skin incision was closed with a 5-0 Mersilk suture and mice were injected subcutaneously with 0.1 mg/kg Vetergesic (Alsoe Ltd).

Subcutaneous mini-pump implantation

Anesthesia was initiated and maintained as described above. Animals were then placed in the prone position on a heating pad, and the upper back area was shaved and

disinfected with Hydrex Pink (Ecolab). A 10 mm skin incision was made, and osmotic mini-pump implanted. The incision was closed with 5-0 Mersilk suture (Ethicon). All animals received subcutaneous injection of 0.1 mg/kg Vetergesic before the surgery.

Caecal Ligation and Puncture procedure

The Caecal Ligation and Puncture (CLP) procedure was used as a model of sepsis and was performed as described [386]. Anaesthesia was initiated using 5% isoflurane (Animal Care) with oxygen (flow rate of 0.5 L/min) in an induction chamber and subsequently maintained with 2% isoflurane with oxygen (flow rate of 0.5 L/min) via a nose cone. Mice were placed on a heating pad to maintain body temperature and positioned in dorsal recumbency. After the abdominal wall was shaved and disinfected with Hydrex Pink (Ecolab), mice were covered with adhesive film (Tegaderm). A 10 mm laparotomy was performed, and the cecum was exteriorised. The distal half of the cecum was subjected to ligation with a 7-0 silk-braided suture and subsequently punctured twice with a 19-gauge needle. The cecum was compressed to expose a small amount of faeces and replaced in the abdomen. The abdominal wall incision was closed in 2 layers using 5-0 Mersilk suture (Ethicon). After surgery, mice were injected with 0.1 mg/kg Vetergesic (Alsoe Ltd) intraperitoneally (i.p.) and resuscitated with 1ml per 30g body weight of pre-warmed physiological salt solution (Normasol, STERETS) subcutaneously (s.c.). Animals were left to recover for 30 minutes in an incubator at 26°C. Sham operated mice underwent the same procedure without caecal ligation and puncture and received 0.5 ml saline per 30g body weight subcutaneously instead of 1 ml. A validated sepsis score, reflecting the severity of the disease, was calculated 24 hours after CLP using as assessment protocol developed by Schrum et al [387]. The parameters evaluated included appearance, activity, respiration rate and response to stimulus and were given a score from 0 to 4, with 4 indicating the most severe state ().

Table 2-1. Sepsis Score to assess the severity of sepsis

Variable	Score and description
Appearance	0- Coat is smooth
	1- Patches of hair piloerected
	2- Majority of back is piloerected

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	3- Piloerection may or may not be present, mouse appears “puffy”
	4- Piloerection may or may not be present, mouse appears emaciated
Level of consciousness	0- Mouse is active
	1- Mouse is active but avoids standing upright
	2- Mouse activity is noticeably slowed. The mouse is still ambulant.
	3- Activity is impaired. Mouse only moves when provoked, movements have a tremor
	4- Activity severely impaired. Mouse remains stationary when provoked, with possible tremor
Activity	0- Normal amount of activity. Mouse is any of: eating, drinking, climbing, running, fighting
	1- Slightly suppressed activity. Mouse is moving around bottom of cage
	2- Suppressed activity. Mouse is stationary with occasional investigative movements
	3- No activity. Mouse is stationary
	4- No activity. Mouse experiencing tremors, particularly in the hind legs
Response to stimuli	0- Mouse responds immediately to auditory stimulus or touch
	1- Slow or no response to auditory stimulus; strong response to touch (moves to escape)
	2- No response to auditory stimulus; moderate response to touch (moves a few steps)
	3- No response to auditory stimulus; mild response to touch (no locomotion)
	4- No response to auditory stimulus. Little or no response to touch. Cannot right itself if pushed over
Eyes	0- Open
	1- Eyes not fully open, possibly with secretions
	2- Eyes at least half closed, possibly with secretions
	3- Eyes half closed or more, possibly with secretions
	4- Eyes closed or milky
Respiration rate	0- Normal, rapid mouse respiration
	1- Slightly decreased respiration (rate not quantifiable by eye)
	2- Moderately reduced respiration (rate at the upper range of quantifying

	by eye)
	3- Severely reduced respiration (rate easily countable by eye, 0.5 s between breaths)
	4- Extremely reduced respiration (>1 s between breaths)
Respiration quality	0- Normal
	1- Brief periods of laboured breathing
	2- Laboured, no gasping
	3- Laboured with intermittent gasps
	4- Gasping

Blood collection and Tissue Harvest

4 or 24 hours after CLP procedure, blood was collected for measurement of blood gases, electrolytes and haematological parameters. Anaesthesia was initiated and maintained in the same way as described above. The blood was collected from the left renal vein with the syringe containing 50 µl 100 IU/ml heparin sodium anticoagulant. Special care was taken to keep the same heparin-to-blood ratio in all the blood samples. Livers, hearts, kidneys, lungs and aortas were washed in cold physiological salt solution, snap-frozen in liquid nitrogen and stored at -80 °C before further analyses.

Wire Myography

Thoracic aortas and mesenteric arteries were rapidly isolated and placed in ice cold Ringer - Krebs solution (2.5 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 118 mM NaCl, 11 mM Glucose D (+), 25 mM NaHCO_3 , 4.7 mM KCl, 1.18 mM KH_2PO_4 , 1.16 mM MgSO_4). The adjacent tissue was gently removed from the vessels and 2 mm long segments of aortas or 2nd order mesenteric arteries were mounted on to two stainless steel 25 or 40 µm wires, respectively. The vessel segments were transferred to a 5 ml organ bath of a multi-wire myograph system (Danish Myo Technology 620M) with 37°C Krebs solution, oxygenated with 95% O_2 and 5% CO_2 . After the vessels were left to equilibrate under zero tension for 30 minutes, the normalisation process was performed. This involved stretching vessels stepwise, during which the wall tension/internal circumference ratio

was automatically determined by the system software. The normalised internal circumference of the vessel (IC_1) was set as 90% of the identified internal circumference (IC_{100}) that fully relaxed vessels would have at a transmural pressure of 100 mmHg. The normalised vessel internal diameter was calculated by the following equation $d=IC_1/\pi$, using Lab Chart software (ADInstruments). The vessels were equilibrated for 30 minutes, and exposed to 60 mM KCl during the wake-up protocol to reactivate the vessel signalling and functional properties, so to verify that vessel functionality was not compromised by the mounting process. Aortas that showed a constriction force less than 2 mN and mesenteric arteries that constricted less than 1 mN in response to 60 mM KCl were excluded from the studies. Precontracted vessels that relaxed more than 70% in response to 1 μ M Ach were considered suitable for experimentation.

Echocardiography

Anaesthesia was induced and maintained as described above. Mice were then placed in dorsal recumbence on a heated pad to maintain body temperature at 37°C. Heart rate was maintained at 500-550 beats per minute. Transthoracic echocardiography was performed using a Vevo-770 ultrasound (VisualSonics) and 707B linear transducer. A 2D long axis view and short axis view of the left ventricle (LV) were obtained at the level of the papillary muscles to record M-mode tracings.

LV systolic function was assessed by calculating fractional shortening (FS) and ejection fraction (EF) derived from the 2D M-mode long axis measurements using the following formulae:

$$FS(\%) = \left[\frac{LVID_d - LVID_s}{LVID_d} \right] * 100, EF(\%) = \left[\frac{LVID_d^3 - LVID_s^3}{LVID_d^3} \right] * 100,$$

where $LVID_d$ and $LVID_s$ are the diastolic and systolic LV internal dimensions, respectively.

Posterior wall (LVPW) and septal wall (IVS) thickness were obtained using 2D M-mode long axis measurements, and anterior wall (LVAW) thickness was obtained using

short-axis measurements. All the measurements were taken in systole and diastole. Stroke volume (SV) was calculated using Simpson's equation

$$SV = LVvol_d - LVvol_s,$$

where $LVvol_d$ and $LVvol_s$ are left ventricle volume in diastole and systole, respectively.

$$LVvol_d = \frac{7.0}{2.4 + LVID_d} * LVID_d^3, \quad LVvol_s = \frac{7.0}{2.4 + LVID_s} * LVID_s^3$$

The cardiac output (CO) was calculated as follows:

$$CO = SV * HR$$

From the apical view, with simultaneous visualisation of the aortic valve, aortic root and left ventricle outflow tract, the time-velocity integral (TVI) was generated. LV mass was calculated using the following formula:

$$LV\ mass\ (mg) = 1.053 * [(LVID_d + LVPW_d + LVAW_d)^3 - LVID_d^3],$$

where $1.053\ mg/mm^3$ is the density of myocardium. All the measurements were made from 3 sequential cardiac cycles.

Fluorescent magnetic bead-based immunoassay

The cytokine levels were assessed using a Bio-Plex MAGPIX fluorescent magnetic bead-based immunoassay reader (Bio-Rad) according to the manufacturer's instructions. The Bio-Plex MAGPIX system was validated and calibrated before the experiment using the Bio-Plex validation and calibration kits, respectively. Briefly, samples were diluted as indicated in materials and methods sections of Chapter 3 and 4 by adding Bio-Plex sample diluent and incubated with antibody-coupled beads at 800 rpm at room temperature. Biotinylated detection antibodies and streptavidin-phycoerythrin conjugates were added to the plate subsequently to measure cytokine levels. The plate was washed after each incubation step using the Bio-Plex Pro Wash station. After the plate was analysed, standard curves were generated for each analyte and cytokine concentrations were determined using BioManager 6.1 software (Bio-Rad). Each sample was measured in duplicate.

Cell culture, transfection and western blotting

HEK293T embryonic kidney cells were cultured in high glucose DMEM (Life Technologies) containing 10% foetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin (Life Technologies) at 37°C and 5% CO₂. Plasmid (0.1 µg) transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. 18 hours after transfection cells were lysed in 200 µl 2x sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate (SDS)). Some samples were reduced with 5% β-mercaptoethanol or 100 mM dithiothreitol (DTT) or 100 mM hydroxylamine before subjecting them to western blotting analysis. Samples were typically separated on 4-20% or 7.5% mini-protein TGX precast gels (Bio Rad) or 6% 'homemade' tris-glycine gels. Western blotting was performed according to the manufacturer's instructions with running buffer of the following composition: 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3. The samples were transferred on PVDF membranes using the following transfer buffer: 25 mM Tris-HCl, 192 mM glycine, 20% methanol. After blotting the PVDF membranes were blocked in 10% milk in PBS-Tween 20 (0.1%). All antibodies used were diluted in 5% milk in PBS-Tween 20 (0.1%). Western blots were developed using Pierce ECL western blotting substrate (Thermo Scientific).

Statistical analysis

All data sets are present as mean with standard error of the mean. Differences in means were analysed for significance using either t-test, one-way ANOVA or two-way ANOVA with a Tukey or Bonferroni post-hoc test where appropriate.

Chapter 3. Intradisulfide-mediated redox regulation of PKG

This chapter focuses on a novel mode of oxidative activation of cGMP-dependent protein kinase I (PKG). The physiological importance of PKG is demonstrated by its regulatory contribution to many processes, including smooth muscle relaxation and growth, neuronal adaptation, and platelet aggregation. PKG activity is regulated in a tightly controlled manner and is activated in several ways, including cGMP binding - which is known as classical activation, or by two oxidative mechanisms involving Cys42 disulfide-mediated protein dimerization or S-guanylation of Cys195. Recently, nitroxyl donors have been shown to activate PKG by a third oxidative mechanism in which an intradisulfide bond forms between Cys117 and Cys195. In this chapter, the physiological importance of this newly identified intradisulfide-mediated PKG activation is evaluated by using a novel knock-in mouse model. PKG C195S KI mice, which cannot form the intradisulfide due to the Cys to Ser substitution, were shown to have similar blood pressure and cardiac function as PKG WT mice. The heart rate of the PKG KI mice was slightly lower during day light hours. PKG expression in various tissues was not different between genotypes. The nitroxyl donors, 1-nitroso cyclohexyl acetate (NCA) or CXL-1020 had a blood pressure-lowering effect, but this was not different between WT and KI mice. This observation was consistent with observations made using in vitro myography studies, where each nitroxyl donor induced similar vasodilatory responses in U46619-precontracted mesenteric arteries from WT or C195S PKG KI mice. Unexpectedly, the PKG KI mice were lighter in weight, which could potentially be explained by impaired gastrointestinal (GI) tract function. The caecum of PKG KI animals was mostly enlarged and displaced, and their GI track transit time was slower. Further studies are needed to identify the mechanism behind this observation; however, this study suggests that Cys117 – Cys195 disulfide-mediated PKG activation might play an important role in GI tract function.

3.1. Introduction

The physiological and pathophysiological importance of PKG is extensively discussed in Chapter 1. In order to manifest such a broad and *significant array of function*, *PKG activity is regulated in a tightly controlled manner, with cGMP and oxidants identified so far as main regulators*. PKGI exists in two conformations, an inactive state with the catalytic domains bound with high affinity to the AI domains in the absence of cGMP, or an active state with the catalytic domain dissociated from the AI domain upon cGMP binding to the CNB domains [216, 217]. This increases PKG phosphotransferase rate 3-10 times [388, 389]. A recent paper has observed that cGMP binding resulted in more than a 50 fold increase in PKG activity, this will be discussed later and might be an aberrant finding explained by the presence of the N-terminal FLAG tag [390]. Small-angle X-ray scattering data suggest that cGMP binding induces a large conformational change and the activated PKG protein becomes extended by approximately 30% [217].

PKG is found in different cell compartments and is known to translocate in response to changes in cGMP concentration. Concentration of cGMP is thought to vary considerably between different cellular compartments, which can be regulated by multiple factors, such as cGMP production or degradation by specific phosphodiesterase isoforms [210, 391-393]. In addition to classical cGMP-induced activation of PKG, several reports indicate that PKGI can be activated in the absence of cGMP. PKGI α and PKGI β contain 11 or 10 Cys, respectively. Five out of them were suggested to contribute to cGMP-independent oxidative activation of PKG [25, 145, 394]. A decade ago Joseph Burgoyne described H₂O₂-induced formation of a disulfide bond between two PKGI α monomers at Cys42 [25]. Absence of cGMP resulted in a higher PKGI α affinity towards a synthetic peptide substrate and its physiological substrate RhoA, without a significant change in enzyme catalytic activity. In the presence of cGMP, although the K_m for the synthetic peptide significantly decreased, there was no effect on RhoA affinity. Interestingly, a recent paper reported that oxidation of Cys42 did not contribute to PKGI α substrate affinity [390]. Moreover, the authors suggested that C42S PKG binds cGMP with lower affinity, however, cGMP affinity was not measured in their studies, and the conclusion was made based on PKG activity studies. It is not clear how to explain the observed discrepancy, but it could be attributed to differences in

experimental procedures and the origin of the protein used. One of the most commonly used methods for PKGI protein preparation relies on cAMP bound resins, as with the study by Burgoyne, leading to the prospect that residual cAMP is present in the final preparation despite gel filtration or extensive dialysis. As mentioned before cAMP can bind to CNB pockets, and thus have an effect on PKG activity, or in this particular case it could potentially mask the effects of Cys42-mediated modification of PKG. C42S PKG KI mice, that have Cys substituted with Ser, are hypertensive and have an impaired resistance vessel reactivity in response to H_2O_2 and diastolic dysfunction [26, 27]. This mechanism was shown to be important for human coronary artery dilation [395].

A recent paper has suggested another way of cGMP-independent PKG activation [145]. By using in vitro kinase activity assays, the authors showed that Cys195 S-guanylation by 8-nitro-cGMP resulted in persistent and irreversible PKG activation. R_p -8-bromo-cGMP partially inhibited PKG S-guanylation, suggesting that classical, cGMP-dependent, and oxidative modes of PKG activation might negatively regulate each other. The authors also reported that PKG is basally S-guanylated in mouse hearts, with PKG S-guanylation significantly increasing during sepsis. However, the physiological importance of PKG S-guanylation has not been yet confirmed. Interestingly, Landgraf et al suggested that PKG can be reversibly activated by the action of divalent cations with a positive redox potential [394]. They suggested the formation of intramolecular disulfide bonds in PKGI, between Cys117 and Cys195, as well as between Cys312 and Cys518 upon treatment of native PKG purified from bovine lung tissue with Cu^+ and some other metal ions. The oxidation did not change the cGMP binding and kinetic parameters of PKG, however, it reduced the number of titratable $-SH$ groups, strongly suggesting that Cu^+ -induced oxidation resulted in PKGI activation via formation of at least one of the mentioned disulfide bonds. Although, these studies utilise cAMP-affinity purified PKGI, which may add complexity due to the likely presence of contaminant cAMP, as discussed above. Almost two decades after Landgraf et al published their results, a disulfide bond between Cys117 and Cys195 was shown in the PKG⁷⁸⁻³⁵⁵ crystal structure [396]. By identifying amino acids surrounding the disulfide bond, authors proposed a mechanism as to how PKGI can be activated in the absence of cGMP. They suggested that formation of a disulfide bond between Cys117 and Cys195

may disrupt the interaction between the AI and catalytic domains, thereby releasing the kinase from a state of autoinhibition. They also suggest that formation of a disulfide bond between Cys312 and Cys518 is highly unlikely to happen as the –SH groups face different directions.

Recently, Friederike Cuello's group in Hamburg, Germany has observed that formation of the intradisulfide bond between Cys117 and Cys195 results in PKG activation, as assessed by in vitro kinase activity assays [28]. Only nitroxyl donors, namely 1-nitroso cyclohexyl acetate (NCA) or Angeli's salt (AS) have been shown to induce intradisulfide formation, with H₂O₂ and various authentic nitric oxide donors failing to do so. The formation of the intradisulfide bond is visualised by western blotting. Nitroxyl-induced intradisulfide bond formation results in a PKG migrating faster on the gel. Thus, a shift down of PKG on a gel is indicative of Cys117 – Cys195 disulfide bond formation. Using Forster resonance energy transfer (FRET) analysis, they also showed that intradisulfide bond formation results in a conformational change in PKG structure similar to that found upon cGMP binding. In order to investigate the physiological or pathophysiological significance of this intradisulfide-mediated PKG activation, genetically modified mice were generated. These mice have Cys195 substituted with Ser, which prevents them forming the activating Cys117 – Cys195 intradisulfide. This chapter focuses on the basal characterisation and responses of these newly generated transgenic KI mice to nitroxyl donors.

3.2. Materials and methods

Generation of PKG C195S KI mice

The generation of the C195S PKG1 KI mice was contracted to the well-established transgenesis company Cyagen Bioscience. Briefly, mouse *PRKG1* genomic DNA was isolated from the bacterial artificial chromosome library carrying the C57BL/6J mouse genome. The c.797A>T mutation was introduced into exon 4 of *Prkg1* (NM_001013833). A neo gene cassette with FRT sequence was inserted into intron 3 for positive selection. C57BL/6 embryonic stem cells were electroporated with a NotI-linearised targeting vector. Clones were selected based on a primary PCR screen followed by sequencing and Southern blot analysis. Southern blotting was performed using a neomycin probe, which was generated by polymerase chain reaction (PCR) using a primer pair: 5'-TCATCTCACCTTGCTCCTGC-3' and 5'-AAGGCGATAGAAGGCGATGC-3'. Selected clones were injected into C57BL/6 blastocysts, followed by chimera production. Chimeras were mated to C57BL/6N mice to obtain the F1 generation. The heterozygous mice were crossed with flippase (FLP) recombinase-expressing mice to remove flippase recognition target (FRT)-flanked neo cassette.

Genotyping mice

To genotype mice, genomic DNA was purified and amplified using a Rapid Extract PCR kit (PCR Biosystems) following the manufacturer's instructions. The mice were genotyped by PCR using the following primers: 5'-GAATACGTGTATGTCTGTGCAC-3' and 5'-GCAGGGCTTATTGAACGCA-3' and the following cycler program: 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds, and the final extension at 72°C for 10 minutes. Expected sizes for PCR products were 201 bp for WT and 312 bp for KI.

Blood pressure and heart rate measurements

Blood pressure and heart rate were measured in 8-12 weeks old mice by radio-telemetry as described in the general materials and methods provided in Chapter 2, the only difference being that measurements were recorded every minute.

Echocardiography

Echocardiography was performed in 8-12 weeks old weight-matched mice as described in Chapter 2.

Western blot analysis

Mouse organs, liver, heart, kidney, lung, brain, aorta or stomach, were crushed in liquid nitrogen using a mortar and pestle and homogenised using the following buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 mM maleimide, complete protease inhibitor tablet with EDTA (Roche) and phosphatase inhibitor cocktail 2 (Sigma). Samples for western blotting were prepared in 2X Laemmli sample buffer with or without 5% β -mercaptoethanol. Before loading on the gel, samples were boiled at 95°C for 5 minutes and centrifuged at 10000 g for 5 minutes. Samples were loaded onto 4-15 % mini-protein TGX precast gels (Bio-Rad) or for experiments indexing the intradisulfide downward shift in PKG, home-made 6% polyacrylamide Tris-glycine gels or 7.5 % mini-protein TGX precast gels from Bio-Rad were used. The western blotting was performed as described in Chapter 2. Membranes were probed with 1:1000 rabbit PKG antibody (#ADI-KAP-PK005, Enzo), rabbit p-VASP antibody (#3114, Cell Signalling), rabbit VASP antibody (#3112, Cell Signalling) and 1:3000 rabbit Vinculin antibody (#13901, Cell Signalling), rabbit GAPDH antibody (#2118, Cell Signalling).

Angiotensin II infusion

Ang II was delivered using osmotic mini-pumps (Alzet, ALZA Pharmaceuticals). Osmotic mini-pumps (#2002) delivering Ang II (1.1 mg/kg/day) were implanted subcutaneously. The subcutaneous mini-pump implantation is described in general material and methods section. Ang II (Sigma) was dissolved in saline.

CXL-1020 administration

8-12 weeks old mice were telemetered as described in the general materials and methods chapter. 10 days after implantation of the telemetry probes, CXL-1020 was delivered for 24 hours using osmotic mini-pumps as described above. Osmotic mini-pumps (2001D) delivered 9 mg/kg/day CXL-1020 dissolved in 30% (w/v) captisol (Ligand Pharmaceuticals) in sterile water, pH 4.5. Alternatively, before receiving CXL-1020, mice were implanted with osmotic mini-pumps delivering Ang II (1.1 mg/kg/day) for 10 days. At the end of the protocol mice were culled and their organs collected for western blot analysis.

NCA administration

8-12 weeks old mice were telemetered as described in the general material and methods chapter. 10 days after telemetry mice were implanted with osmotic mini-pumps delivering Ang II (1.1 mg/kg/day) as described above. 7 days after mini-pump implantation, NCA was administrated intravenously (i.v.). Anaesthesia was initiated and maintained as described above. Each mouse received 100 μ mol/kg of NCA in 2% DMSO/saline in a final volume of 50 μ l. Blood pressure and heart rate were recorded for 24 hours before and after the NCA treatment.

For western blot analysis NCA was administered intravenously as described above; 30 minutes after the injection mice were culled and their organs collected.

Sepsis induction

8-12 weeks old mice were telemetered as described in general materials and methods section. 10 days after telemetry mice were subjected to sham- or cecum ligation and puncture (CLP-) surgery as described in general materials and methods section with slight changes. Mice received 0.1 mg/kg vetergesic (Alsoe Ltd) subcutaneously before the surgery and did not receive resuscitation. 6 hours after surgery mice underwent echocardiography as described in Chapter 2. Body temperature of septic animals was increased to 37°C before the measurements were taken. After sepsis score evaluation, animals were culled and their organs collected.

PKG cGMP affinity in heart homogenates

Hearts were harvested as described in Chapter 2 and crushed in liquid nitrogen and homogenised in the following buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM IBMX, 100 mM bond-breaker TCEP solution (Sigma), protease inhibitor tablet with EDTA (Roche). Heart homogenates were centrifuged at 25000 g for 10 minutes and the supernatant was incubated with 8-AET-cGMP-immobilised agarose beads (BIOLOG) for 3 hours at 4°C on the rotating wheel. Agarose beads were blocked with 1% BSA and washed three times with washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Triton X-100, 1 mM bond-breaker TCEP) before mixing with supernatants prepared from heart homogenates. The elution was performed with 3',5'-cGMP-Na (Sigma) in a step-wise manner. At each step 1/10 of the mixture was collected and the same volume of cGMP solution (10 μ M – 10 mM final concentration) was added. The beads were incubated for 1 minute constantly shaking and centrifuged at 1000 g. This step was performed several times up to the point when all PKG was eluted from the beads. After addition of 2x sample buffer to samples, they were boiled at 95°C for 5 minutes and subjected to western blotting. Blots were probed for PKG with PKG polyclonal antibody (ADI-KAP-PK005, Enzo) and analysed using Image Studio Lite. The signal was normalised to input signal and corrected for the amount of eluted PKG sampled at each elution step.

Myography studies

The preparation, normalisation, and wake-up protocols for mesenteric arteries were performed as described in the general materials and methods chapter. The relaxation response curves to CPT-cGMP (300 nM – 300 μ M), NCA (100 nM – 100 μ M), and CXL-1020 (100 nM – 100 μ M) were evaluated in U46619-precontracted vessels (5nM). CPT-cGMP was dissolved in Krebs buffer, while NCA and CXL-1020 - in DMSO. The care was taken not to exceed 0.5% as final DMSO concentration in the myography bath.

Gut transit time

Whole gut transit time was assessed by using carmine red, a non-absorbable dye. Mice received 100 μ l of 6% carmine red (Sigma) in 0.5% methylcellulose (Sigma) by oral gavage and were placed in separate cages with white sheets of paper. The time taken to excrete red-coloured faeces was recorded and used to index whole gut transit time.

Treatment of mesenteric arteries with HNO donors

Mice were anaesthetised by inhalation of isoflurane as described above, and culled by cervical dislocation. The entire intestinal tract with mesenteric arteries was excised and placed in ice-cold Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.25 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11 mM glucose (+)). Mesenteric vessels, with or without adhesive fat tissue, were separated from the intestinal tract and treated with NCA, CXL-1020 or H₂O₂ at 100 μ M for 30 minutes at 37°C. NCA or CXL-1020 were prepared as a 500x stock solutions in DMSO. H₂O₂ was prepared as a 500x stock solution in water. After mesenteric vessels were treated, they were frozen in liquid nitrogen and stored at -80°C. For western blot analysis, mesenteric vessels were crushed in liquid nitrogen and homogenised in homogenisation buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl), containing protease inhibitor tablet with EDTA (Roche).

Cell treatment with NO and H₂S donors

HEK-293T cells overexpressing PKG were treated with 100 μ M NO donors, sodium nitroprusside (SNP) or S-nitroso-N-acetylpenicillamine (SNAP) and H₂S donor – NaSH. The NO and H₂S donors were applied either separately or in combination for 30 minutes. For some experiments, NO and H₂S donors were pre-incubated for 30 minutes before applying to cells for additional 30 minutes. Cell transfection is described in general materials and methods section.

3.3. Results

Generation of the C195S PKG knock-in mouse

Cys 117 and 195 in PKG are suggested to be involved in the formation of an intradisulfide bond, which could be an important regulator of PKG activity, with both residues being highly conserved among different species. The decision was made to mutate c.797 A>T to generate C195S PKG KI mouse. PKG C195S KI mice were generated by Cyagen Bioscience as described in Figure 3-1.

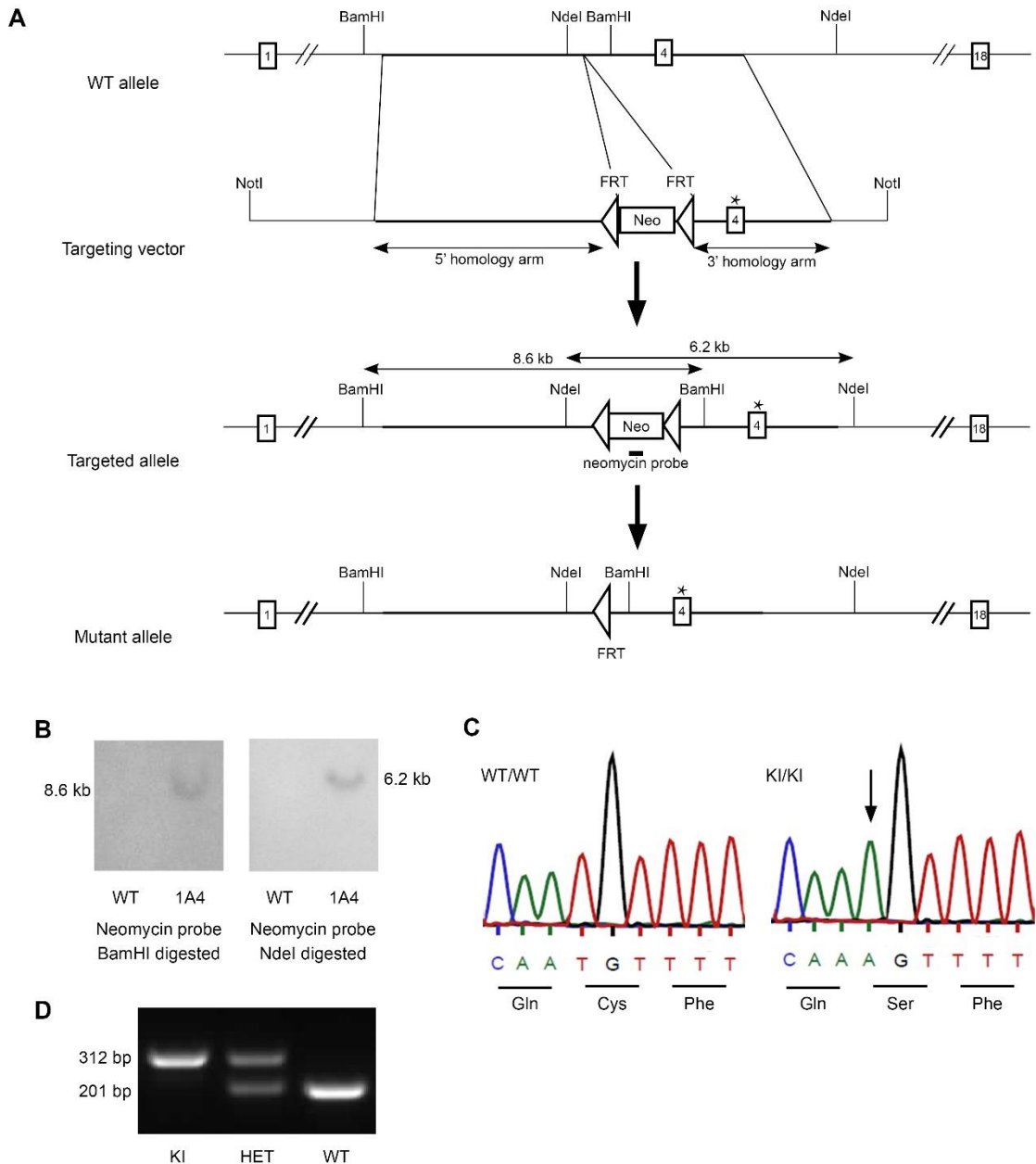


Figure 3-1

Generation of homozygous PKG C195S KI mice. A. PRKG1 wild-type allele, targeting vector and targeted allele. The mutation introduced to exon 4 is depicted by an asterisk. The position of the neomycin southern blot probe and restriction enzyme cleavage sites by BamHI and NdeI are indicated. Neo indicates the neomycin-resistance gene cassette, which is surrounded by the FRT sequence. B. Southern blot analysis for genomic DNA purified from the targeted embryonic stem cell clones and digested with either BamHI or NdeI and then hybridised with neomycin probe. C. Sequencing of the genomic DNA obtained from WT or C195S PKG KI mice. Changes in the genomic sequence and in the amino acid sequence are shown. D. Genotyping of WT or KI mice by PCR. The lower band is amplified from the WT allele and upper band – from the mutant allele.

The targeting vector designed to introduce the mutation in *Prkg1* is shown in Figure 3-1A. The homologous recombination was confirmed by PCR and southern blotting of the mutated region in embryonic stem (ES) cell clones. The DNA cleavage with BamHI or NdeI resulted in DNA fragments of 8.6 kDa and 6.2 kDa, respectively, which were hybridised with the neomycin probe (Figure 3-1B). Chimeric mice were generated by injecting ES cells into C57bl/6 blastocyst, with the offspring then mated with C57bl/6N mice to generate heterozygous mice. Founders were confirmed as germline-transmitted via crossbreeding with a FLP deleter. The mutation was confirmed by sequencing of the relevant regions in the WT and C195S PKG KI mice from DNA purified from ear punches (Figure 3-1C). The heterozygous and homozygous WT and KI mice were identified by a PCR genotyping strategy to detect any residual FRT site in intron 3 (Figure 3-1D).

Basal characterisation of the C195S PKG KI mice

To basally characterize cardiovascular function in PKG WT and KI mice, their blood pressure and heart rate were measured using radio-telemetry and cardiac function was assessed by echocardiography.

C195S PKG KI mice do not exhibit elevated blood pressure

PKG is an important regulator of blood pressure; global PKG knockouts are hypertensive, as are C42S PKG KI mice. Thus, we assessed blood pressure in 8-10 week old WT or C195S PKG KI mice using radio-telemetry. Figure 3-2A shows the WTs had a mean arterial pressure (MAP) of 101 ± 0.9 mmHg during the day and 116 ± 1.5 mmHg at night, with the KIs being essentially the same with 100 ± 1.3 mmHg during the day and 115 ± 1.3 mmHg at night.

Heart rate of the KI mice was significantly lower than WT during the day (554 ± 6.8 bpm vs 574 ± 6.9 bpm, (Figure 3-2B). At night the KI mice showed a trend towards a lower heart rate than WT (640 ± 6.9 bpm vs 653 ± 10.2 bpm), however, the difference was not statistically significant. WT and KI mice showed similar systolic, diastolic, and pulse pressure, both during day and night periods in accordance with the MAP results (Figure 3-2C-E).

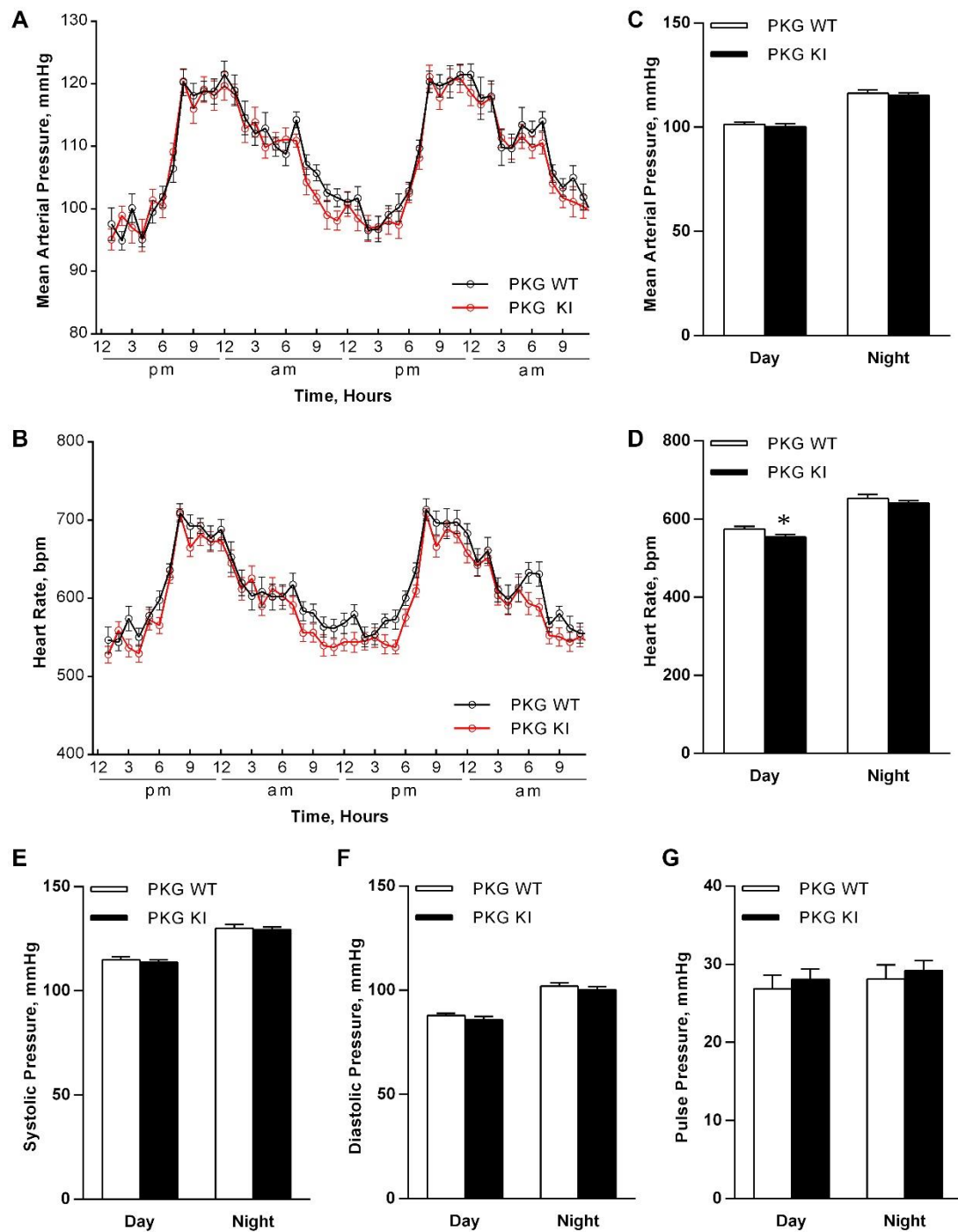


Figure 3-2

The effect of PKG C195S substitution on haemodynamic parameters. A-B. Time-course of mean arterial pressure and heart rate in PKG WT and KI mice. The data are given as hourly mean with SEM for the continuous period of 48 hours. C-D. Averaged mean arterial pressure, heart rate, systolic, diastolic, and pulse pressure. Data are present as mean with SEM for 12-hour day and night periods. The parameters were measured in conscious freely moving mice by radio-telemetry every

minute by radio-telemetry, n=15 mice per group. * p<0.05, statistical difference between genotypes was analysed by Student's t-test.

PKG C195S KI mice show normal cardiac function under basal conditions

The assessment of cardiac function was performed in body weight-matched 8-10 weeks old WT or C195S PKG KI mice using echocardiography. Figure 3-3 shows key left ventricle (LV) function indices, namely ejection fraction, fractional shortening, cardiac output, LV mass and stroke volume. All of them were found to be similar between genotypes. The velocity time integral, which reflects blood flow through aortic valve, was not different between PKG WT and KI mice.

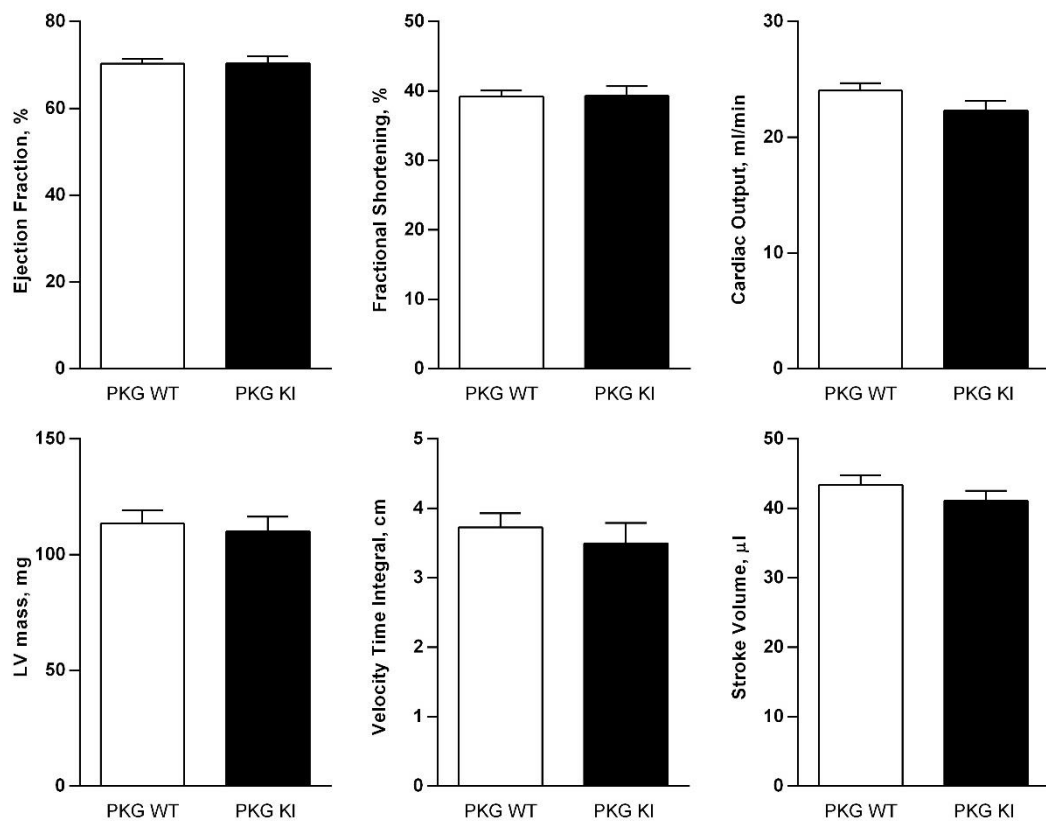


Figure 3-3

Cardiac function in WT and C195S PKG KI mice assessed by echocardiography. Data are presented as mean with SEM, n=10 mice per group. No statistical differences were detected between genotypes as analysed by a Student's t-test.

Ventricular wall thicknesses - interventricular septum (IVS), LV posterior wall (LVPW), and LV anterior wall (LVAW) were each found to be almost identical between WT and KI mice both during systole and diastole, as were LV dimensions and volumes as shown in Table 3-1.

Table 3-1 Echocardiographic characteristics of PKG WT and C195 KI mice

Parameter	PKG WT	PKG KI
Left Ventricle Measurements		
IVS;d (mm)	0.89 ± 0.034	0.88 ± 0.041
LVID;d (mm)	3.53 ± 0.059	3.58 ± 0.068
LVPW;d (mm)	0.80 ± 0.027	0.81 ± 0.042
Volume;d (μl)	61.85 ± 1.852	58.76 ± 2.620
LVAW;d (mm)	0.80 ± 0.047	0.83 ± 0.067
IVS;s (mm)	1.39 ± 0.037	1.38 ± 0.055
LVID;s (mm)	2.25 ± 0.029	2.23 ± 0.084
LVPW;s (mm)	1.18 ± 0.028	1.28 ± 0.043
Volume;s (μl)	18.43 ± 0.896	17.68 ± 1.623
LVAW;s (mm)	1.26 ± 0.075	1.32 ± 0.073
Aortic Valve Measurements		
Velocity Time Integral (cm)	3.95 ± 0.202	3.50 ± 0.296
Mean Velocity (cm/s)	827.16 ± 28.040	666.86 ± 82.425
Mean Gradient (mmHg)	2.90 ± 0.172	1.89 ± 0.508
Peak Velocity(cm/s)	1316.61 ± 29.401	1090.05 ± 99.479
Peak Gradient (mmHg)	7.10 ± 0.285	4.91 ± 0.949

Data are presented as mean with SEM, n = 10 mice per group. Abbreviations: IVS – interventricular septum, LVID – left ventricle internal dimension, LVPW – left ventricle posterior wall, LVAW – left ventricle anterior wall.

WT or C195S PKG KI mice express similar levels of PKG in their tissues

Sometimes in transgenic mice compensatory mechanisms may lead to protein levels being altered, potentially resulting in a lack of phenotype. Thus, PKG expression was assessed in gastric fundus, brain, aorta, liver, lung, heart, and kidney. Phosphorylation of VASP served as indirect indication of PKG activity in the mentioned tissues.

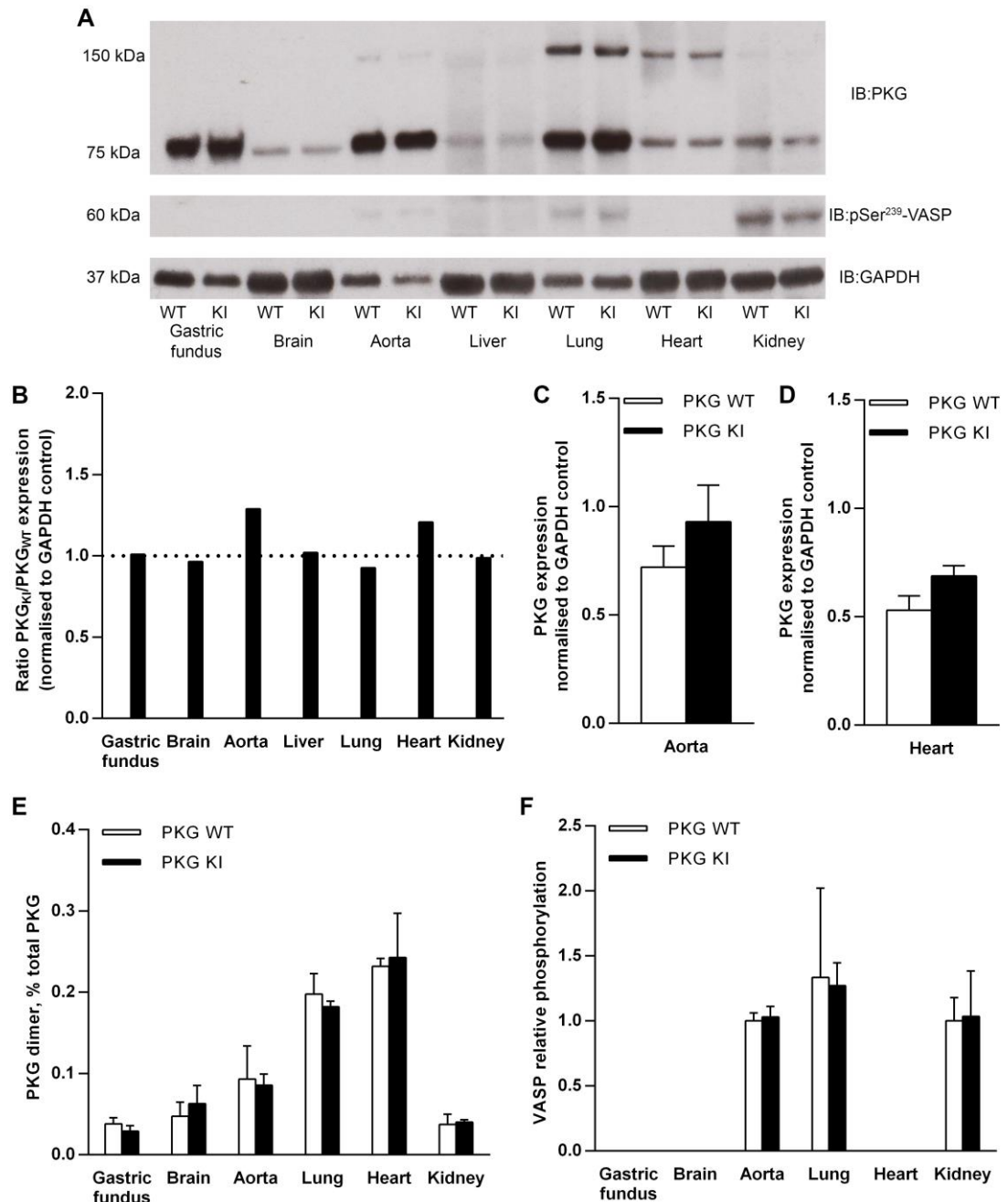


Figure 3-4

Comparison of PKG expression, PKG dimer, and VASP phosphorylation in various tissues of WT or C195S PKG KI mice. Expression of PKG, phosphorylation of VASP and expression of GAPDH were determined by western blotting. A. Representative non-reducing western blot. The membranes were probed with PKGI, pSer²³⁹-VASP and GAPDH-specific antibodies. B. Ratio of PKG expression in tissue homogenates from PKG KI and WT mice, calculated from reducing western blots, PKG signal was normalised to GAPDH signal prior to calculations. C. PKG expression in aorta homogenates, normalised to GAPDH signal. D. PKG expression in heart homogenates, normalised to GAPDH signal. E. Percentage of PKG dimer of total PKG. F. VASP Ser 239 phosphorylation, normalised to total VASP. Data are present either as ratio or as mean with

SEM, n=4 per group. No statistical differences were detected between genotypes as analysed by a Student's t-test.

Figure 3-4A shows the expression pattern of PKG in different mouse tissues. This western blot analysis revealed that PKG is predominantly expressed in tissues with smooth muscle, including stomach, aorta, lung, and heart. PKG expression was almost identical between genotypes in stomach, brain, liver, lung, and kidney (Figure 3-4B). In heart and aorta, PKG expression was slightly elevated in PKG KI mice, however, the differences were not statistically significant (Figure 3-4C and D).

Cys42-mediated PKG dimerisation is known to affect PKG targeting and subsequently modulates phosphorylation of PKG substrates. PKG C42S KI mice show elevated blood pressure, suggesting that PKG dimerisation is important in blood pressure regulation. Increased dimer formation in PKG C195S KI mice could represent another compensatory mechanism to account for the lack of blood pressure phenotype. Therefore, PKG disulfide dimer levels were assessed in different mouse tissues, being presented as a percentage of total PKG in Figure 3-4E. PKG dimerisation was found to be highest in heart, followed by lung, and aorta. Only very small amounts of dimer were detected in stomach, brain, and kidney. WT and C195S PKG KI mice showed similar levels of PKG dimerisation in all tissues examined.

Activation of PKG results in Ser239 VASP phosphorylation, which is often used to indirectly assess PKG activity. VASP phosphorylation was detected in aorta, lung, and kidney, and was similar between genotypes as shown in Figure 3-4F.

Ang II-induced hypertension is similar in WT and C195S PKG KI mice

Ang II is an important pathological determinant of hypertension and cardiac hypertrophy. Numerous studies have shown the beneficial effects of phosphodiesterase (PDE) inhibitors in the model of Ang II-induced cardiac hypertrophy, strongly suggesting that PKG mediates the beneficial effect. Additionally, HNO donors were shown to protect against Ang II-induced hypertrophy. Although, the role of PKG in Ang II-induced hypertension is not as well established as in cardiac hypertrophy, recent work has reported a new class of anti-hypertensive drugs that act via PKG disulfide

dimerisation [397]. Thus, the development of Ang II-induced hypertension was compared in WT or KI mice over 7 days. Ang II (1.1 mg/kg/day) was administered via osmotic mini-pumps and blood pressure was measured in mice using radio-telemetry. Basal blood pressure was similar between genotypes (Figure 3-5A), with Ang II treatment increasing blood pressure in both groups. Although the development of hypertension in the C195S PKG KI mice was initially slightly attenuated compared to WT animals, after 7 days of Ang II infusion the MAP was nearly identical between genotypes (Figure 3-5A).

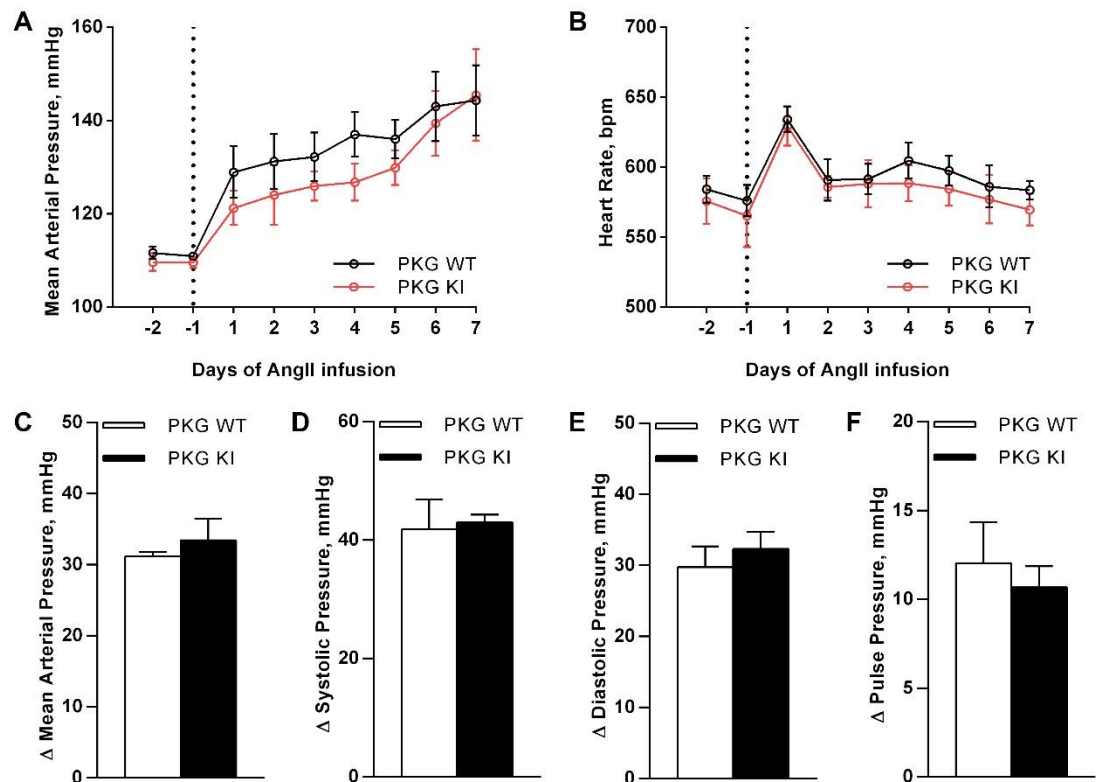


Figure 3-5

Angiotensin-induced hypertension development in WT versus C195S PKG KI mice. A-B. Time course of mean arterial pressure and heart rate measured in conscious mice via radio-telemetry before and after implantation of an osmotic mini-pump delivering Ang II (1.1mg/kg/day). Dotted line indicates mini-pump implantation. C-F. Increase in mean arterial pressure, systolic pressure, diastolic pressure, and pulse pressure over the average basal values, measured on day 7 of Ang II infusion. Data are present as mean with SEM, $n = 3-5$ mice per group. Statistical significance was determined by two-way ANOVA with Bonferroni post-hoc test or t-test.

Basally, heart rate was slightly lower in KI mice compared to WT mice. 1 day after implantation of osmotic mini-pumps delivering Ang II, the heart rate was significantly

increased, however it returned to basal by day two onwards and stayed relatively steady for the duration of this intervention, however remaining slightly lower in the KI mice (Figure 3-5B).

The increase over basal MAP on day 7 of Ang II treatment was 31 ± 0.6 mmHg and 33 ± 3.0 mmHg in WT and KI mice, respectively (Figure 3-5C). Ang II induced a greater increase in systolic pressure than in diastolic pressure, resulting in greater values for pulse pressure (Figure 3-5D-F). No statistical difference was detected between PKG WT and KI mice.

NCA lowers blood pressure in hypertensive WT or KI mice to a similar extent

The HNO donor NCA is an established vasodilator and blood pressure-lowering compound. The observation that NCA activates PKG, potentially via formation of the intradisulfide bond between Cys117 and Cys195, suggests that the blood pressure-lowering effect of this donor might be partly attributed to the oxidative activation of this kinase. The C195S PKG KI mice cannot form an intradisulfide and so should lack this mode of activation, meaning that we may anticipate attenuated blood pressure-lowering by NCA in KI compared to WT mice. WT or KI mice were rendered hypertensive by 1 week Ang II treatment (1.1 mg/kg/day) and their blood pressure and heart rate were measured before and after NCA administration by radio-telemetry. Basal mean arterial pressure and heart rate were similar between genotypes (Figure 3-6A,B). Intravenous injection of 10 μ mol/kg NCA had no effect on mean arterial pressure compared to vehicle control (data not shown). Administration of 100 μ mol/kg NCA had a profound effect on MAP, lowering it by 41 ± 12.8 mmHg and 39 ± 1.8 mmHg during first 10 minutes of administration in WT or KI, respectively (Figure 3-6A,C). Heart rate decreased in response to NCA administration in both genotypes, but this decrease was not different from the one observed in vehicle-treated control mice (Figure 3-6B,D). NCA lowered systolic and diastolic blood pressure to a similar extent in each genotype, however the decline in systolic pressure was slightly potentiated in WT mice compared to KI (48 ± 12.4 mmHg vs 40 ± 0.8 mmHg), which was not statistically different (Figure 3-6E) In contrast, the diastolic pressure was essentially the same in WT or KI (35 ± 12.7 vs 37 ± 4.0) (Figure 3-6F). This resulted in a potentiated pulse pressure drop

in WT mice compared to KI animals (13 ± 0.2 mmHg vs 4 ± 1.6 mmHg) (Figure 3-6G). The effect of NCA on blood pressure was maintained for up to 30 minutes after its administration.

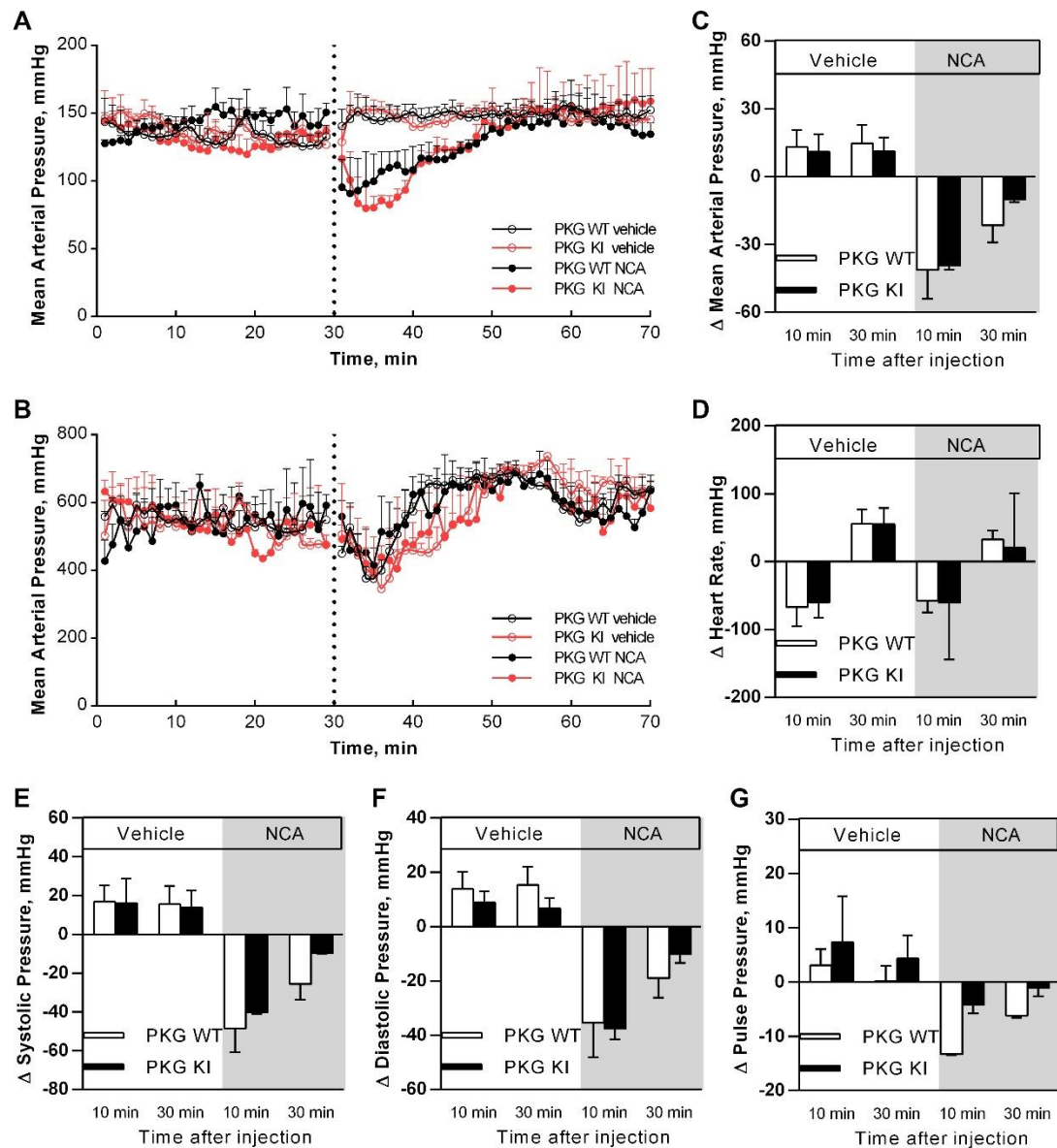


Figure 3-6

NCA lowers blood pressure comparably in Ang II-induced hypertensive WT or KI mice. A-B. Radio-telemetry recordings of MAP and heart rate in PKG WT and KI mice before and after administration of NCA (100 μ mol/kg i.v.) or vehicle (2% DMSO i.v.). The time of administration is indicated by dotted line. Values are plotted at 1 minute intervals. Prior to NCA administration, PKG WT and KI mice received Ang II treatment (1.1 mg/kg/day) for 7 days via subcutaneous osmotic mini-pumps. C-G. The difference of mean arterial pressure, heart rate, systolic pressure, diastolic pressure, and pulse pressure from the baseline for the duration of 10 minutes and 30 minutes after

vehicle or NCA injection. Baseline represents averaged 1-minute recordings for the duration of 30 minutes before the injection. Shaded regions represent NCA group. Data are present as mean with SEM, n = 2-3 mice per group.

The effect of *in vivo* NCA administration (100 $\mu\text{mol/kg}$) on PKG oxidation was assessed by western blotting. The mice were culled 30 minutes after the injection, and PKG oxidation was assessed in mesenteric arteries with or without perivascular fat (Figure 3-7). Compared to the positive control, *in vivo* administration of NCA did not induce PKG intradisulfide formation that was anticipated, which would be indicated by a downward gel shift. In contrast, a ‘positive control’ generated by exposing HEK cells to NCA showed the downward shift previously identified [28]. It is not clear why NCA did not induce the Cys117 - Cys195 intradisulfide formation in PKG present in the mesenteries, an unexpected issue which is explored further below.

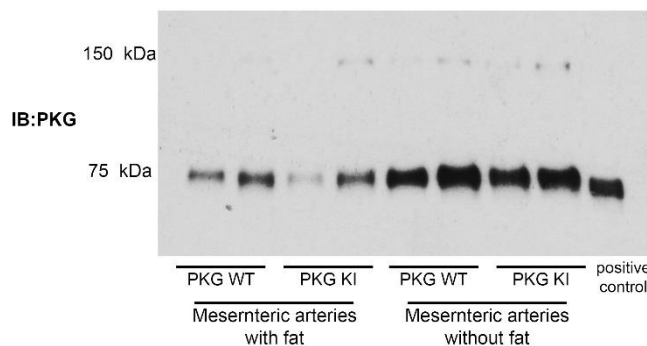


Figure 3-7

NCA does not induce PKG intradisulfide formation *in vivo*. WT or KI mice received 100 $\mu\text{mol/kg}$ of NCA via intravenous injection. 30 minutes after the injection the mice were culled and intestinal tract with mesenteric vessels collected. Mesenteric arteries were isolated with or without perivascular fat, homogenised, and subjected to western blotting using a 6% polyacrylamide gel, which has previously been used to successfully monitor PKG intradisulfide bond formation. Membranes were probed for PKG. Positive control represents lysate of HEK cells overexpressing Cys42S PKG and treated with 100 μM NCA, which causes a downward gel-shift.

CXL-1020 does not lower basal blood pressure in normotensive WT or KI mice

CXL-1020, an HNO donor currently being investigated in clinical trials, is considered superior to other HNO donors, as it only releases HNO and not NO. NCA and AS, on the other hand, release an amount of NO and other nitrogen species [398]. To assess the

impact of CXL-1020 on blood pressure and heart rate in WT or KI mice, 8-10 weeks old mice were telemetered and their haemodynamic parameters measured every minute for 24 hours before and after CXL-1020 administration. CXL-1020 (9 mg/kg/day) was administered via subcutaneous osmotic mini-pumps for the period of 24 hours. Basal mean arterial pressure was similar between PKG WT and KI mice, being 103 ± 0.1 mmHg and 107 ± 2.3 mmHg, respectively (Figure 3-8A). After implantation of the osmotic mini-pump delivering CXL-1020, MAP slightly increased, probably due to surgery-related stress, and stayed relatively stable for the 12-hour period without further increase during night time in both PKG WT and KI mice. 12 hours after mini-pump implantation, mean arterial pressure declined to basal levels (Figure 3-8A). Heart rate also decreased in WT mice, but not in KI, immediately after CXL-1020 administration (Figure 3-8B). Overall, there was no statistically significant difference in mean arterial pressure, heart rate, systolic pressure, diastolic pressure or pulse pressure over 24 hours before and after CXL-1020 administration in PKG WT or KI mice (Figure 3-8C-G).

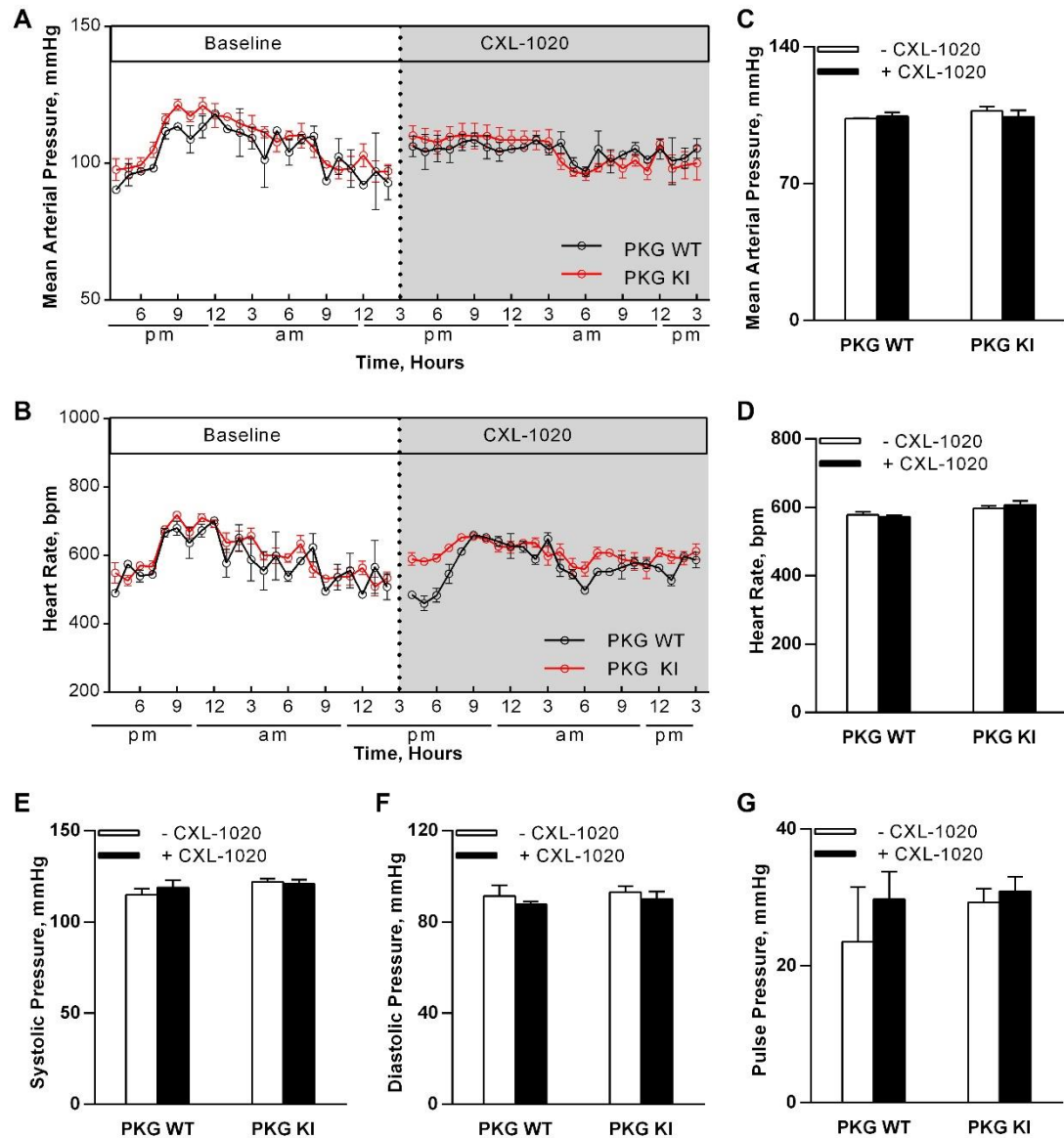


Figure 3-8

CXL-1020 does not lower basal blood pressure in WT or C195S PKG KI mice. A-B. Blood pressure and heart rate were measured by radio-telemetry 24 hours before and 24 hours after implantation of osmotic mini-pumps delivering CXL-1020 (9 mg/kg/day). The time of implantation is indicated by dotted line. Data are present as hourly mean with SEM, $n = 3-5$ mice per group. C-G. Mean arterial pressure, heart rate, systolic pressure, diastolic pressure, and pulse pressure are present as 24-hour average with SEM before and after osmotic mini-pump implantation, $n = 3-5$ mice per group. Statistical significance was determined as area under the curve calculation with subsequent one-way ANOVA and Tukey post-hoc test (A, B) or one-way ANOVA with Tukey post-hoc test (C-G).

A trend for CXL-1020 to lower blood pressure in Ang II-induced hypertensive mice

Because the effect of CXL-1020 on basal mean arterial pressure was negligible, its impact on blood pressure in a model of hypertension was studied. 8-10 weeks old PKG WT and KI mice were rendered hypertensive by Ang II treatment (1.1 mg/kg/day administered by subcutaneous osmotic mini-pumps for 10 days). CXL-1020 (9 mg/kg/day) was infused for 24 hours via osmotic mini-pumps. Blood pressure and heart rate were measured using radio-telemetry probes. Basal mean arterial pressure was similar between WT and KI mice and the Ang II treatment increased it to 142 ± 11.6 mmHg and 145 ± 9.4 mmHg in WT and KI mice, respectively as anticipated (Figure 3-9A). During the first 12 hours of administration, CXL-1020 lowered MAP by 12 ± 7.4 mmHg and 7 ± 4.4 mmHg in WT and KI mice, respectively. Subsequently, the mean arterial pressure was slightly elevated and stayed relatively stable.

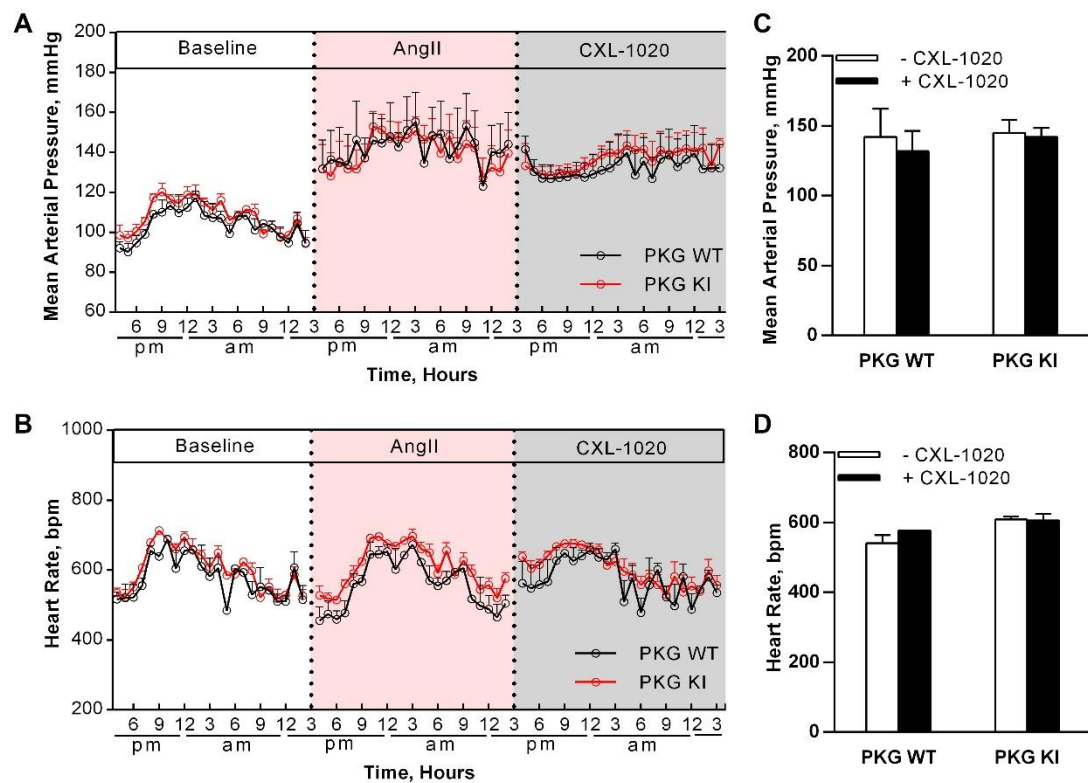


Figure 3-9

Effect of CXL-1020 on blood pressure and heart rate in hypertensive WT or KI mice. A-B. Blood pressure and heart rate were measured in PKG WT and KI mice by radio-telemetry before and after CXL-1020 administration (9 mg/kg/day, subcutaneous osmotic mini-pump). Prior to CXL-1020

administration, mice were rendered hypertensive (Ang II 1.1 mg/kg/day, subcutaneous osmotic mini-pump for 10 days). Implantation of osmotic mini-pump is indicated by the dotted line. C-D. Mean arterial pressure and heart rate 24 hours before and after CXL-1020 administration. Data are present as hourly mean with SEM (A-B) or as 24-hour mean with SEM (C-D), n = 3-5 mice per group. Statistical significance was determined as area under the curve calculations with subsequent one-way ANOVA and Tukey post-hoc test (A-B) or as one-way ANOVA with Tukey post-hoc test (C-D).

CXL-1020 abolished Ang II-induced increases in MAP variability in both genotypes, as highlighted by the decreased error bar size (Figure 3-9A). The heart rate responded similarly as before, increasing during the first 12 hours and then returning back to basal levels (Figure 3-9B). There was no difference in heart rate response to CXL-1020 administration between genotypes. The averaged 24-hour data for mean arterial pressure, systolic pressure, diastolic pressure, and pulse pressure before and after CXL-1020 administration are shown in Table 3-2. Mean arterial pressure decreased by 11 ± 5.6 mmHg and 3 ± 3.3 mmHg in WT and KI mice, respectively. The systolic pressure and diastolic pressure decreased similarly to that observed for the MAP in both genotypes. These differences were not statistically significant, probably due to the relatively low sample size.

Table 3-2. Effect of CXL-1020 on WT or C195S PKG haemodynamics

Genotype	MAP, mmHg		SP, mmHg		DP, mmHg		PP, mmHg	
	Ang II 10 days		Ang II 10 days		Ang II 10 days		Ang II 10 days	
	-	CXL-1020	-	CXL-1020	-	CXL-1020	-	CXL-1020
WT	142 $\pm$ 11.6	131 $\pm$ 8.4	163 $\pm$ 25.3	151 $\pm$ 17.0	122 $\pm$ 17.9	113 $\pm$ 14.3	41 $\pm$ 7.4	38 $\pm$ 2.7
KI	145 $\pm$ 9.3	142 $\pm$ 6.5	167 $\pm$ 7.7	162 $\pm$ 5.4	133 $\pm$ 7.3	128 $\pm$ 5.4	34 $\pm$ 1.5	32 $\pm$ 3.2

At the end of the protocol, tissues from hypertensive WT or KI mice exposed to CXL-1020 for 24 hours were collected. PKG oxidation state was analysed in thoracic aortas by western blotting. As shown in Figure 3-10, in vivo administration of CXL-1020 did not induce PKG shift down on the gel, suggesting the Cys117 – Cys195 intradisulfide was not formed.

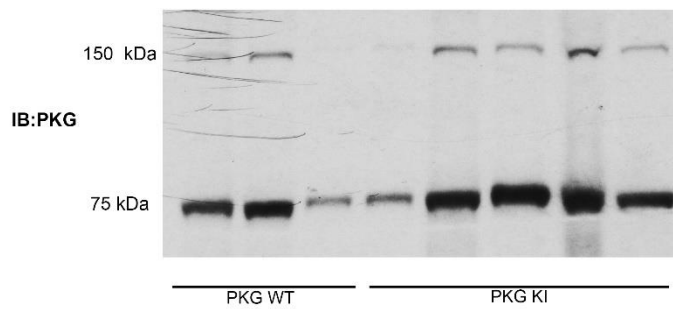


Figure 3-10

CXL-1020 does not induce PKG intradisulfide formation in vivo. WT or KI mice received CXL-1020 at 9 mg/kg/day via osmotic mini-pumps. 24 hours after mini-pump implantation mice were culled and thoracic aortas were collected. Aortas were homogenised and proteins were separated on a 6% gel. Membranes were probed for PKG.

Cys195 in PKG does not mediate NCA- or CXL-1020-induced vasorelaxation in vitro

Nitroxyl donors are known to be potent vasodilators. In order to identify the possible contribution of intradisulfide-mediated PKG activation in their vasodilatory action, the responses of mesenteric arteries from WT or KI mice to NCA or CXL-1020 were compared by using wire myography. Pre-constriction of mesenteric arteries with U46619 resulted in similar force development in vessels from WT or KI mice. NCA and CXL-1020 caused a concentration-dependent vasorelaxation in either genotype to a similar degree (Figure 3-11C,D). Interestingly, while NCA caused a persistent relaxation of mesenteric arteries, CXL-1020 resulted in relaxation that was reversed within two minutes of CXL-1020 administration (Figure 3-11A,B), which was expected due to the differences in kinetics of HNO release.

In vitro treatment of mesenteric arteries with nitroxyl donors at 100 μ M or 300 μ M did not result in Cys117 – Cys195- mediated PKG oxidation, as assessed by western blotting (Figure 3-11E,F). NCA and CXL-1020 failed to induce a downward gel-shift in PKG, which is observed when HEK293 cells are exposed to NCA.

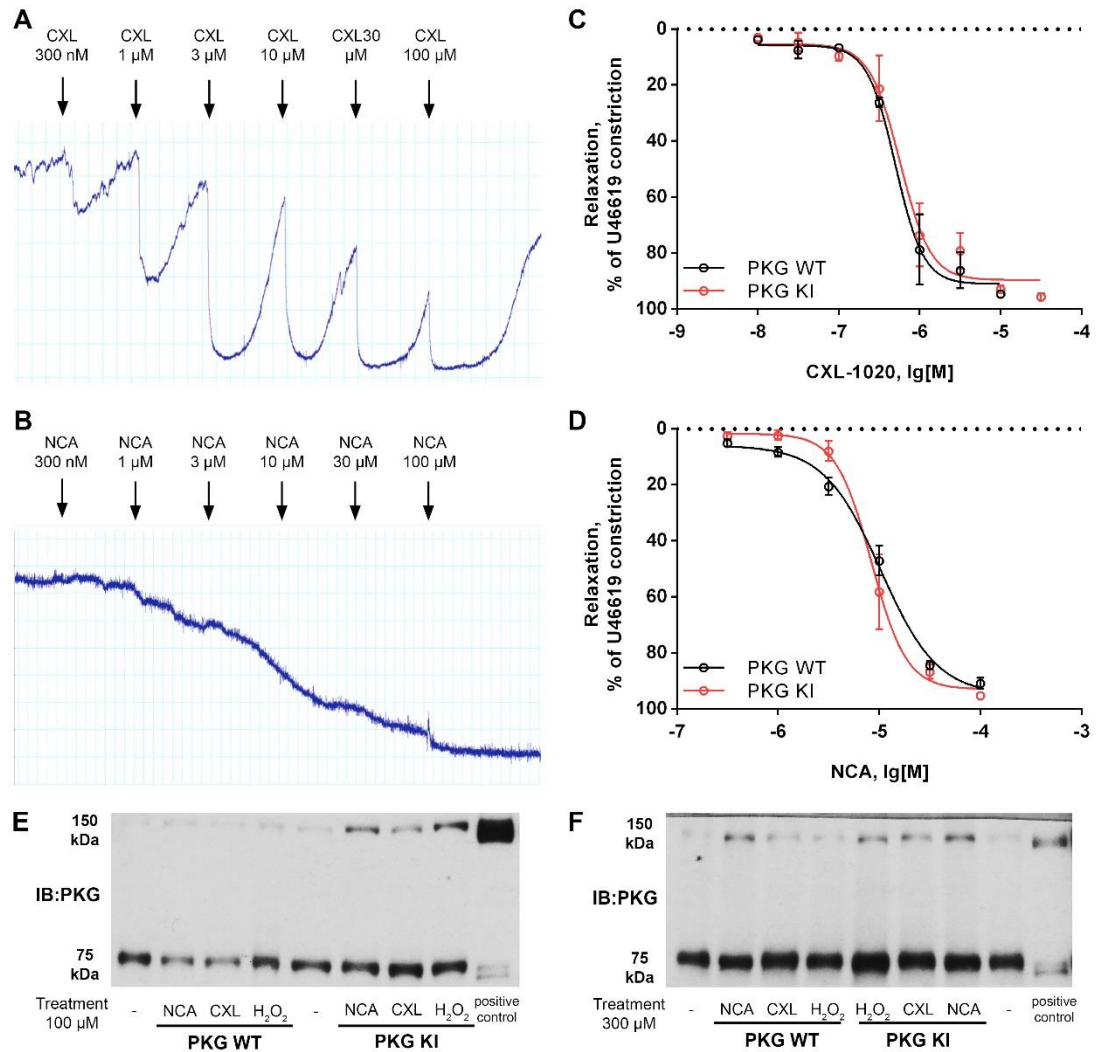


Figure 3-11

The nitroxyl donors, CXL-1020 and NCA, induce comparable arterial relaxation of mesenteries from PKG WT or KI mice. A-D. Representative traces and dose response curves showing relaxation responses of mesenteric arteries induced by CXL-1020 or NCA. Mesenteric arteries were precontracted with 5 nM U46619. Values are expressed as a percentage reversal of the pre-constriction value and presented as mean with SEM, $n = 8-12$ mesenteric segments from 3-4 mice. No statistical differences between genotypes were observed as determined by one-way ANOVA with Tukey post-hoc test. E-F. Western blot analysis of PKG oxidation state in WT and KI mice mesenteric arteries treated with NCA, CXL-1020 or H₂O₂ each at 100 μ M (E) and 300 μ M (F). Positive control was generated by exposing a lysate from HEK293 cells overexpressing PKG to 100 μ M NCA.

Cys195 in PKG does not mediate haemodynamic and cardiac changes in early sepsis

CLP in rodents is believed to resemble abdominal polymicrobial sepsis in humans more closely than any alternate models. 10-12 weeks old WT or C195S KI mice were subjected to CLP or sham surgery and blood pressure and heart rate were assessed by radio-telemetry and cardiac function by echocardiography.

Haemodynamic response to sepsis in PKG WT and KI mice

The blood pressure and heart rate were measured in freely moving conscious mice for 6 hours after surgery. There was no statistically significant difference between genotypes in haemodynamic responses to sepsis (Figure 3-12 and Figure 3-13).

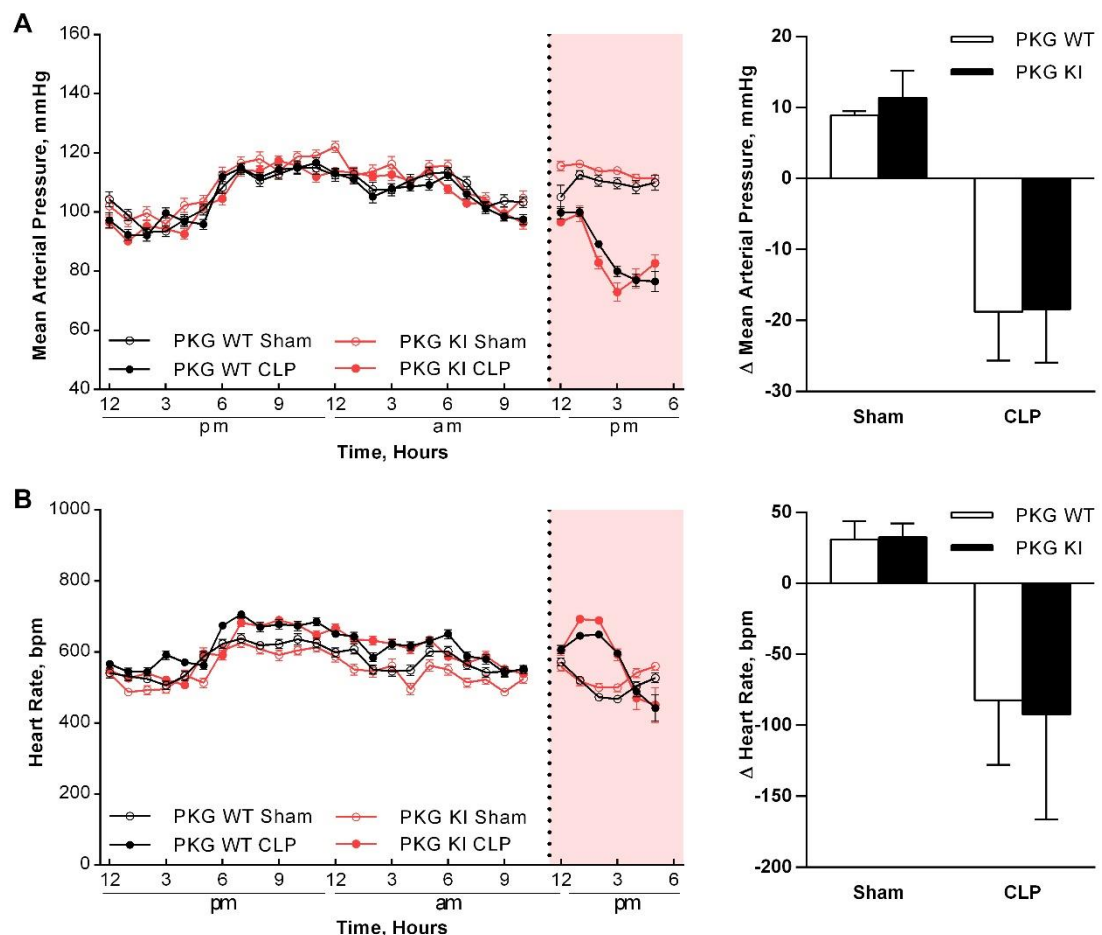


Figure 3-12

Systemic haemodynamic responses in PKG WT and PKG C195S KI mice subjected to CLP. A-B. Mean arterial pressure and heart rate were recorded in conscious sham- or CLP-operated mice using

radio-telemetry. Mean arterial pressure and heart rate were measured 24 hours before the surgery induction and for 6 hours after. The induction of the surgery is indicated by the dotted line. The data are present as an hourly mean with SEM (left) and as mean difference from the time-matched baseline with SEM (right), $n = 3$ mice per group. Statistical analysis was performed using one-way ANOVA with a Tukey post-hoc test, which failed to identify a difference between genotypes.

In WT or C195S PKG KI mice sepsis significantly reduced mean arterial pressure and heart rate, while both of these parameters slightly elevated in sham-operated controls. In sham animals mean arterial pressure stayed relatively stable after the surgery and was between 105 and 115 mmHg, which is about 10 mmHg higher than the time-matched baseline (Figure 3-12A). 2 hours after the surgery, MAP in septic animals started to decline to approximately 75 mmHg, reaching a plateau at 4-5-hour time point. Mimicking the trend observed for the MAP, the heart rate in sham-operated mice was slightly elevated 6 hours after the surgery, while both genotypes demonstrated tachycardia 2-3 hours after sepsis induction, which dropped further to statistically significant rate of 410 bpm by 6 hours (Figure 3-12B). This is around 100 mmHg lower than the time-matched baseline control. The decline in MAP in septic animals was attributed to a decline in both systolic and diastolic pressure, as seen in Figure 3-13. The changes in systolic pressure were more marked than those for diastolic function, resulting in a statistically significant decrease in pulse pressure. Statistical analysis of the results revealed no difference between genotypes in any of the parameters measured.

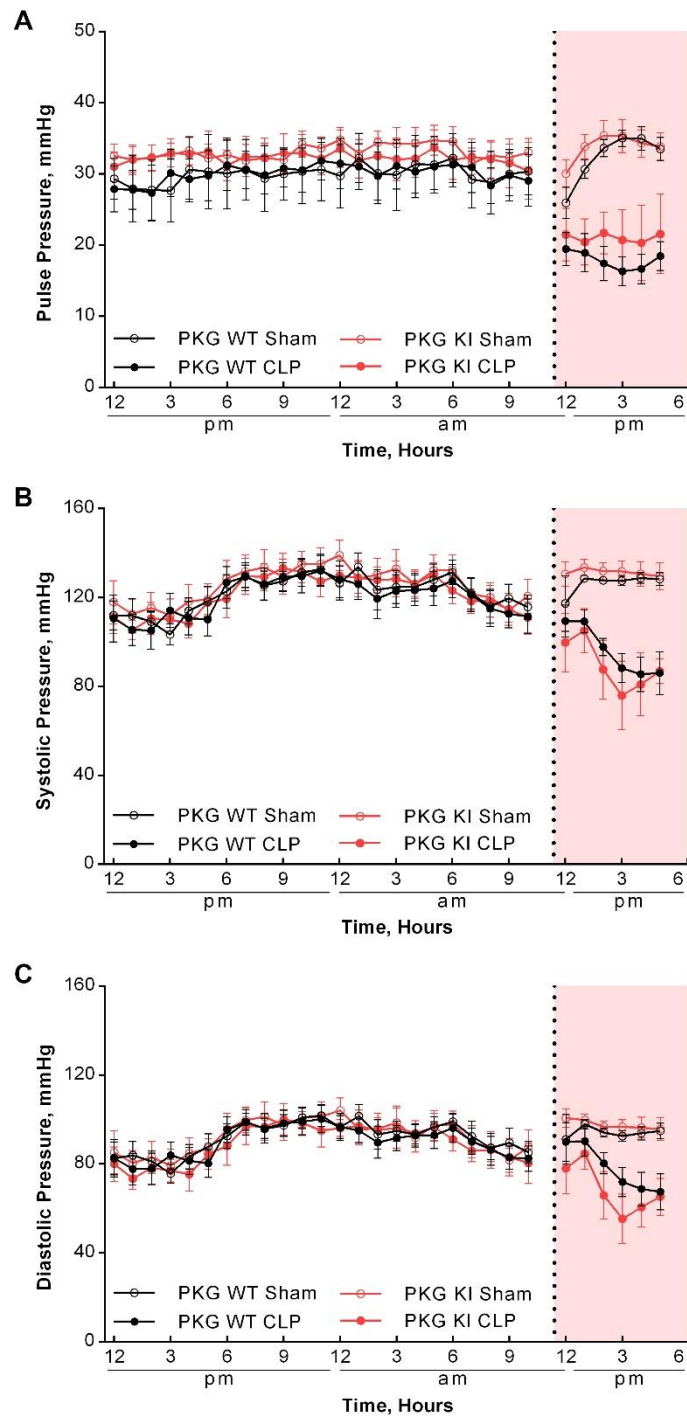


Figure 3-13

Haemodynamics of WT or C195S PKG KI mice basally and in response to CLP. A-C. Pulse pressure, systolic pressure and diastolic pressure were measured in freely moving conscious mice by radio-telemetry 24 hours before sham or CLP surgery and for 6 hours after. The induction of the surgery is indicated by dotted line. Data are present as hourly mean with SEM, $n = 3$ per group. Statistical significance was analysed by determining the area under the curve calculation for

individual mice with a subsequent one-way ANOVA and Tukey post-hoc test, no statistical difference between genotypes.

PKG cysteine 195 does not mediate cardiac dysfunction in early sepsis

To investigate the role of Cys195 in cardiac dysfunction caused by sepsis, 6 hours after sham or CLP surgery LV function was assessed in WT or KI mice by echocardiography. Figure 3-14B shows sepsis markedly attenuated cardiac function; FS and EF decreased approximately two-fold as compared with sham-operated controls. In both WT and KI animals these effects were attributable to changes in diastolic, but not systolic LVID. LVID during diastole was significantly decreased from approximately 4 mm to 3 mm. The decrease is illustrated in Figure 3-14A showing M-mode echocardiograms of sham or septic animals from each genotype. The diminished chamber dimension also resulted in a profound decrease in SV, and corresponding the CO was markedly reduced in the septic mice, however, there was no statistically significant difference between genotypes (Figure 3-14B).

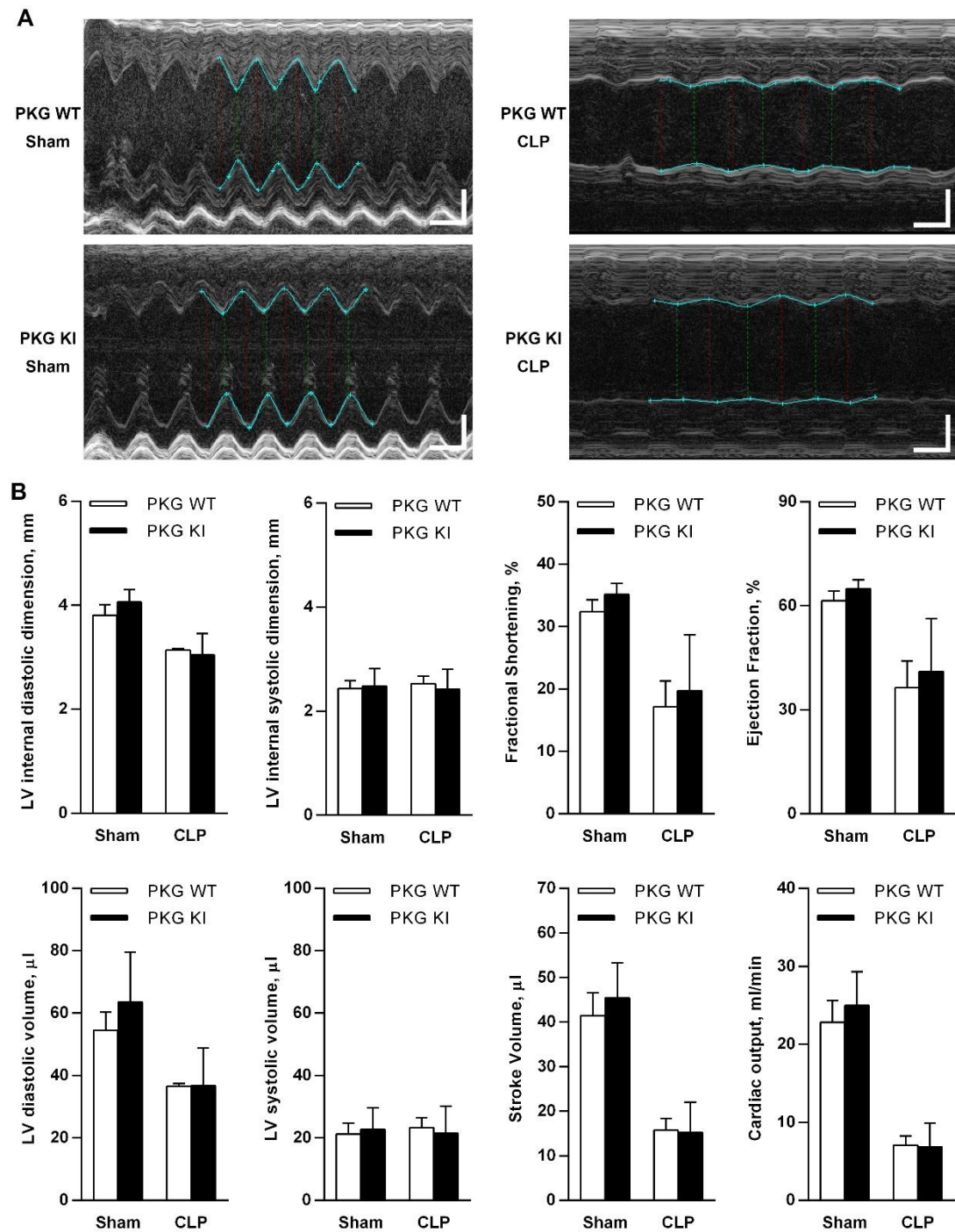


Figure 3-14

Cardiac function in WT or C195S PKG KI mice subjected to CLP. A. Representative long axis M-mode echocardiograms from sham- or CLP-operated mice 6 hours after surgery induction. B. Cardiac parameters calculated from echocardiography data. Data are present as mean with SEM, $n = 3$ mice per group. Statistical significance was analysed by one-way ANOVA with Turkey post-hoc test and revealed no difference between genotypes.

Sepsis did not alter LVPW or LVAW thickness during systole or diastole (Table 3-3), but significantly increased IVS thickness. Aortic blood flow was assessed by pulsed Doppler imaging. Sepsis significantly decreased aortic flow, as was indicated by the velocity time integral in WT and KI mice (Table 3-3), but there was no statistical difference between genotypes. Blood flow was undetectable in two mice throughout the study, one from WT septic group and another from the C195S PKG KI septic group.

Table 3-3. Echocardiographic measurements of left ventricle and aortic valve function in PKG or KI mice subjected to sepsis

	Sham		CLP	
	PKG WT	PKG KI	PKG WT	PKG KI
Left Ventricle measurements				
IVS;d (mm)	0.76 ± 0.022	0.76 ± 0.055	0.99 ± 0.071	0.93 ± 0.123
LVPW;d (mm)	0.88 ± 0.010	0.98 ± 0.189	0.97 ± 0.041	1.04 ± 0.161
IVS;s (mm)	1.29 ± 0.015	1.33 ± 0.075	1.20 ± 0.078	1.18 ± 0.096
LVPW;s (mm)	1.17 ± 0.047	1.32 ± 0.116	1.20 ± 0.075	1.24 ± 0.117
LVAW;d (mm)	0.9 ± 0.030	0.81 ± 0.009	0.98 ± 0.097	0.92 ± 0.115
LVAW;s (mm)	1.22 ± 0.010	1.33 ± 0.052	1.18 ± 0.084	1.30 ± 0.022
LV Mass (mg)	101.73 ± 6.401	108.45 ± 2.750	105.33 ± 7.910	104.81 ± 14.939
Aortic Valve Measurements				
VTI (cm)	4.73 ± 0.448	4.41 ± 0.519	2.87 ± 0.335	2.01 ± 0.170
Mean Vel (cm/s)	811.91 ± 78.692	728.34 ± 71.455	399.50 ± 90.930	375.23 ± 29.955
Mean Grad (mmHg)	2.68 ± 0.523	2.22 ± 0.418	0.67 ± 0.290	0.57 ± 0.090
Peak Vel (cm/s)	1300.74 ± 199.284	1238.90 ± 123.622	717.78 ± 114.100	729.66 ± 78.660
Peak Grad (mmHg)	7.08 ± 1.931	6.35 ± 1.225	2.12 ± 0.655	2.16 ± 0.455

Data are present as mean with SEM, n = 3 mice per group. Abbreviations: IVS – interventricular septum, LVPW – left ventricular posterior wall, LVAW – left ventricular anterior wall, VTI – velocity time integral, Vel – velocity, Grad – gradient.

The severity of sepsis is similar between PKG WT and KI mice

The severity of sepsis can be evaluated by using a scoring system, which takes into account several parameters, such as the appearance of the mouse, its responses to stimuli, and respiration rate. A score of 4 represents the most severe state, while a score of 0 means that no noticeable symptoms are present. 6 hours after sham or CLP surgery, sepsis scoring was performed in WT or KI mice. The sepsis score was similar between genotypes, being 2 ± 0.6 for septic mice and 0 for sham-operated mice (Figure 3-15A). Significant hypothermia was noted in the septic animals. While body temperature was

36 ± 0.1 °C in sham-operated mice, it dropped to 31 ± 1.9 °C in WT mice and 31 ± 2.9 °C in KI mice (Figure 3-15B). Locomotor activity in sham and septic animals was assessed by radio-telemetry. Figure 3-15C shows locomotor activity increased in all operated animals during the first two hours after surgery, rapidly declining afterwards - more so in those exposed to sepsis, but with no differences between the genotypes.

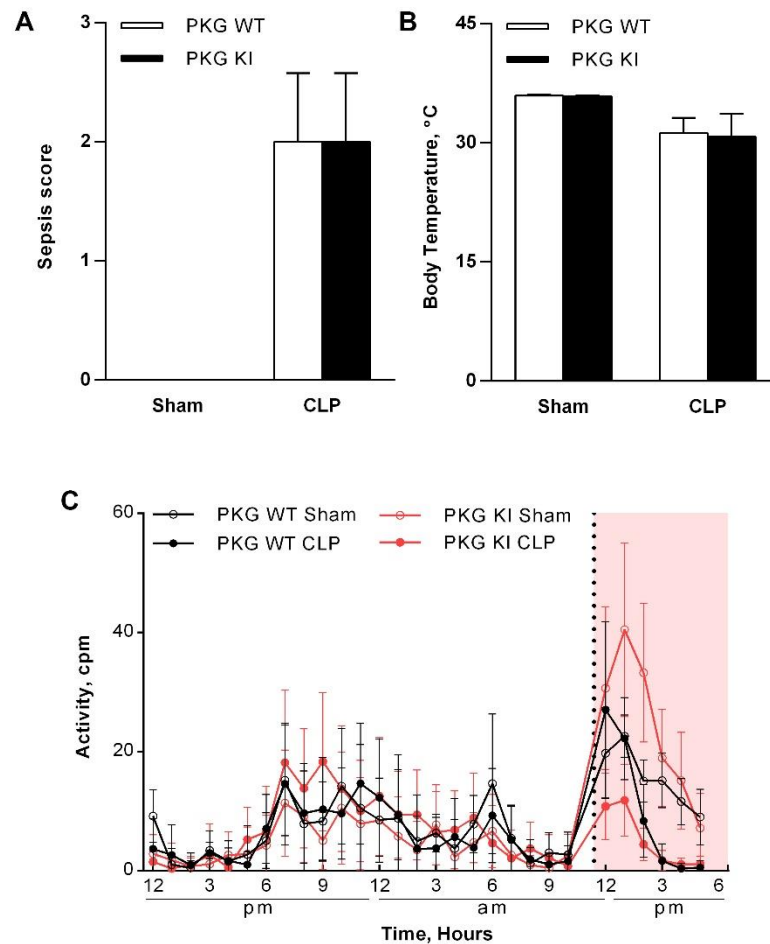


Figure 3-15

Severity of sepsis in WT and C195S PKG KI mice. A-B. Sepsis score and body temperature were assessed in WT or KI mice 6 hours after sham- or CLP surgery. C. The locomotor activity was measured by radio-telemetry 24 hours before the surgery and 6 hours after surgery, which occurred as indicated by the dotted line. Data are presented as mean with SEM, $n = 3$ mice per group. Statistical significance was analysed by one-way ANOVA with Turkey post-hoc test.

In order to assess whether sepsis induced intradisulfide formation in PKG, hearts from sham or septic mice were homogenised and subjected to western blotting. Figure 3-16A reveals that while sepsis resulted in PKG dimerisation in hearts from septic mice, both the WT and the KI, it did not induce a downward gel shift that would be indicative of

intradisulfide formation. PKG KI mice had higher percentage of dimerised PKG after sham surgery, however, the percentage of PKG dimerisation was similar in hearts from septic animals of different genotypes (Figure 3-16B).

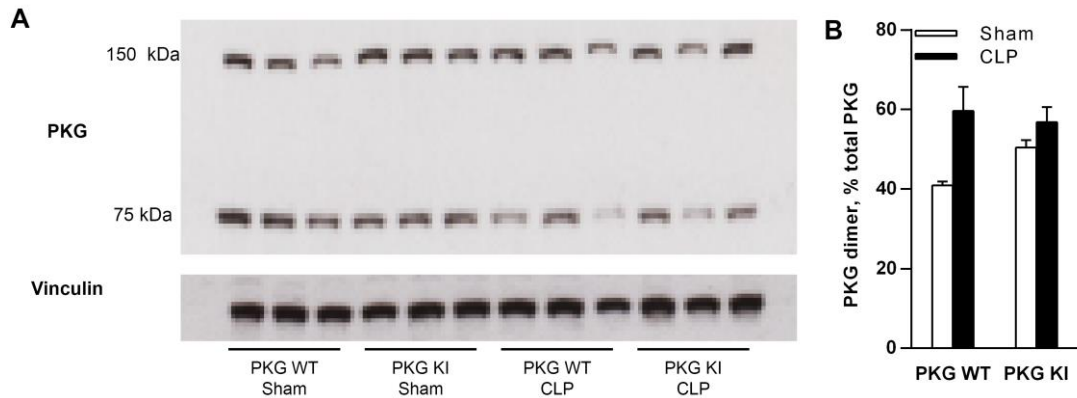


Figure 3-16

Sepsis does not induce intradisulfide-mediated PKG oxidation. A. PKG oxidation was assessed by western blotting-based gel shift assays, comparing hearts from WT or KI mice subjected to sham or CLP surgery. Representative non-reducing western blot of heart homogenates separated on a 6% gel, with western blots subsequently probed for PKG and vinculin (loading control). B. Percentage PKG disulfide dimerisation between experimental groups. Data are presented as mean with SEM, n=3. Statistical difference was determined by one-way ANOVA with Tukey post-hoc test.

C195S PKG KI mice have an enlarged caecum and increased whole gut transit time

C195S PKG KI mice have lower body weight compared to their age-matched WT siblings (Figure 3-17A), also exhibiting a higher mortality rate (Figure 3-17B). While the reasons behind these observations remain unclear, the growth retardation could be explained by malnutrition. The majority of PKG KI mice had a grossly enlarged caecum (Figure 3-17D,E), which in most cases was displaced from its normal position. To assess the functional relevance, whole gut transit time was used as a marker of GI motility. Whole gut transit time was measured as time until excretion of carmine red dye, administered by oral gavage. Whole gut transit time in PKG KI mice was significantly increased compared with WT mice (Figure 3-17F), suggesting impaired gut motility. No difference in organ weights, apart from the caecum, was observed in the KI mice compared with WT animals (Figure 3-17C).

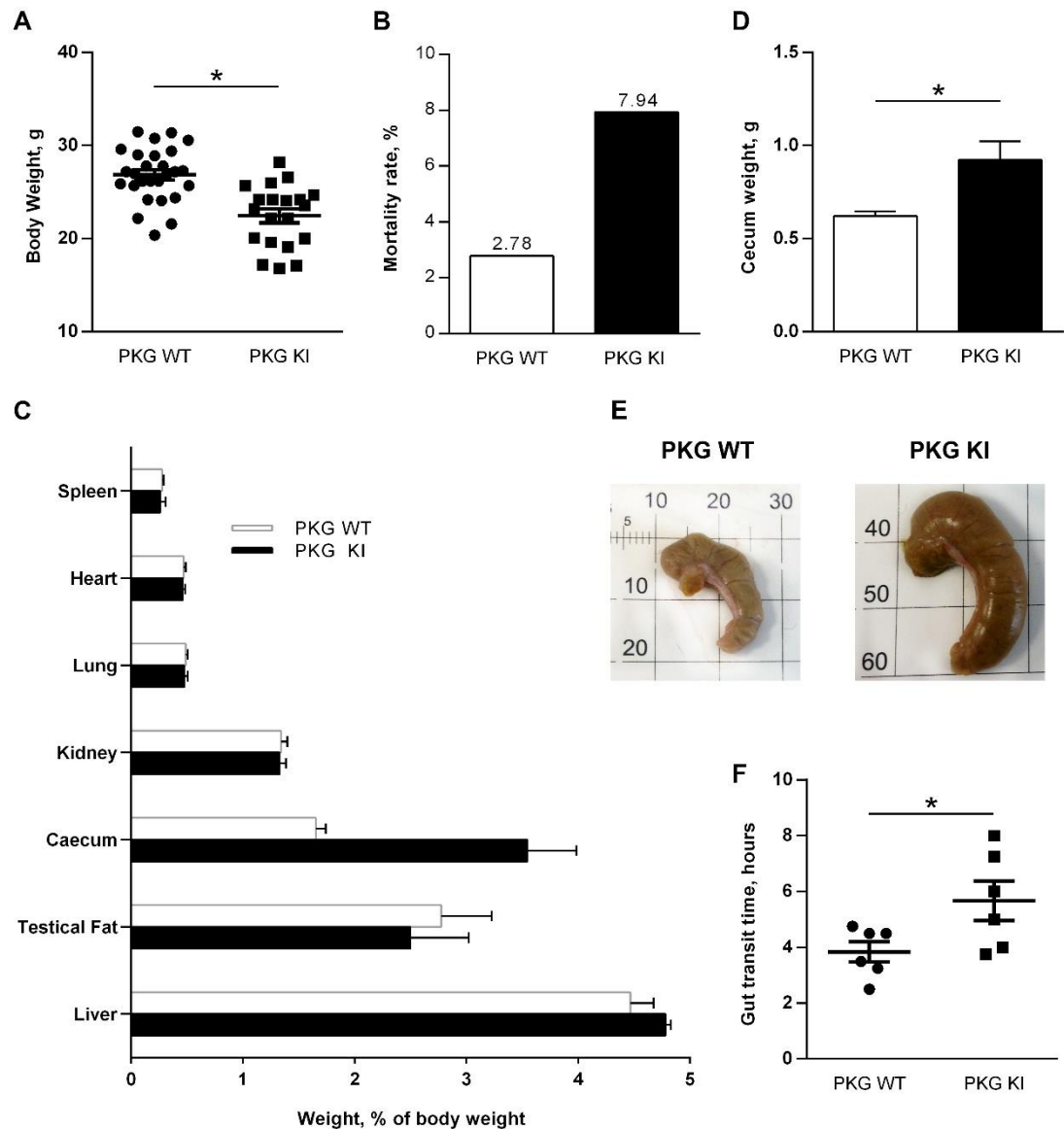


Figure 3-17

C195S PKG KI mice have lower body weight, an enlarged caecum and increased total gut transit time. A. Body weight was measured in 9-11 weeks old PKG WT and KI animals. B. Mortality rate of WT or KI mice is presented as a ratio of unexpected deaths to total number of WT or KI animals in the colony, n = 54-63 mice per group. C. Weight of organs from WT and KI mice, n = 10 mice per group. D. Caecum weight in WT or KI mice, n = 20 mice per group. E. Representative images of a caecum from WT or KI mice. F. Total gut transit time in each genotype was measured by administering carmine red by oral gavage. The gut transit time is presented as time between carmine red administration and first appearance of the dye the faeces. Data are present as scatter dot plot with horizontal line at the mean (A,F) or as mean with SEM (B,C,D). * statistically significant difference ($p < 0.05$), determined by Student's t-test.

cGMP binding affinity and vascular responses to cGMP are similar between PKG C195S KI and WT mice

Cys195 is located in the cGMP-binding domain, and its substitution for Ser in PKG C195S mice might result in lower affinity of cGMP, potentially contributing to the development of some phenotype. The affinity of cGMP to WT and C195S PKG was indirectly assessed by competitive affinity chromatography. Heart homogenates from PKG WT and KI mice were incubated with 8-AET-cGMP-coated agarose beads and bound PKG was subsequently eluted with increasing concentration of cGMP. A representative western blot is shown in Figure 3-18A. Competitive studies demonstrated similar amounts of PKG being eluted in response to different cGMP concentrations, suggesting both WT and C195S PKG bind cGMP with similar affinity (Figure 3-18B).

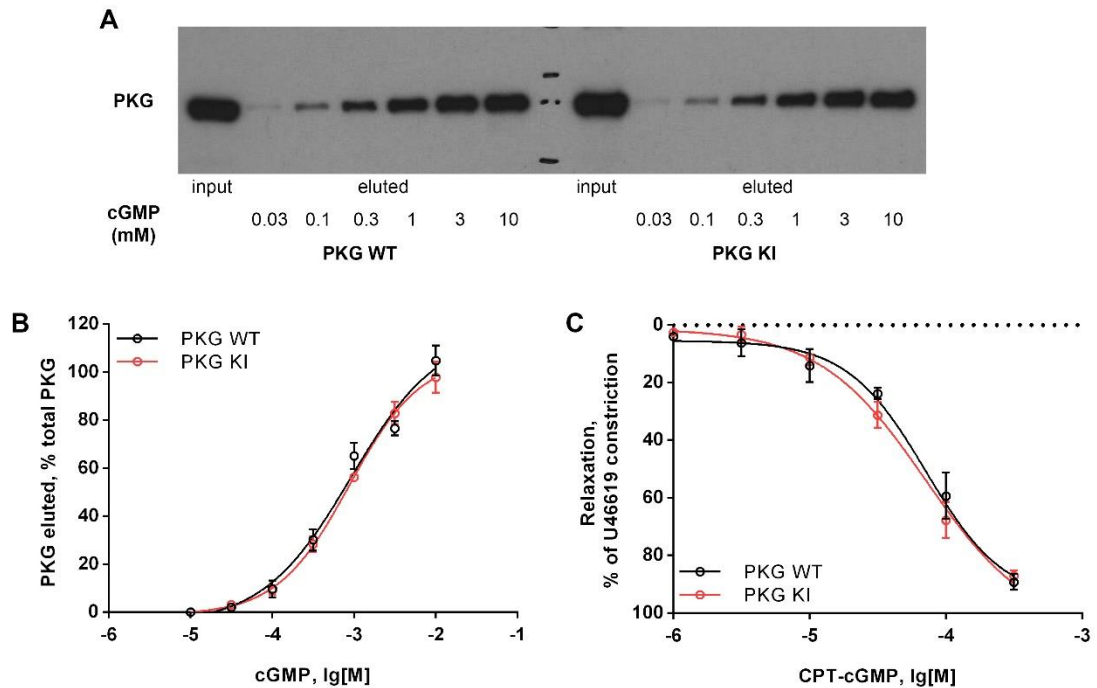


Figure 3-18

Affinity for cGMP and cGMP-dependent vasorelaxation are not different between genotypes.

A-B. Heart homogenates from WT or KI mice were incubated with 8-AET-cGMP agarose beads and subsequently eluted in a step-wise manner with a cumulatively-increasing concentration of cGMP. Representative western blot for eluted fractions and PKG elution curve calculated from western blots for eluted fractions are presented. C. Relaxation curve in response to CPT-cGMP in mesenteric vessels precontracted with 5 nM U46619, as assessed using a DMT wire myograph. Data are presented as mean with SEM, $n = 3-4$ mice per group. Statistical comparison was made using two-way ANOVA with Bonferroni post-hoc test.

The effect of a cell-permeable derivative of cGMP on the relaxation of mesenteric arteries from WT or KI mice was assessed by using wire myography. Figure 3-18C shows this compound, namely CPT-cGMP, induced a concentration-dependent relaxation in U46619-precontracted mesenteric arteries. The responses of each genotype to CPT-cGMP were almost identical.

The NO donor SNP in combination with a salt of H_2S induce intradisulfide-mediated PKG oxidation

HNO donors have been shown to induce Cys117 – Cys195 intradisulfide formation. Several studies suggest that interaction of NO with H_2S may generate nitroxyl, and so the effect of NO donors in combination with Na_2S , a salt of H_2S , on PKG oxidation was

assessed in HEK293 cells overexpressing PKG. Figure 3-19 shows that the only treatment, apart from NCA, that induced intradisulfide-mediated PKG oxidation involved mixing sodium nitroprusside (SNP) and Na_2S for 30 minutes before application to the cells. Interestingly, simultaneous application of SNP/ Na_2S failed to do so, as did SNAP/ Na_2S , pre-incubated or not.

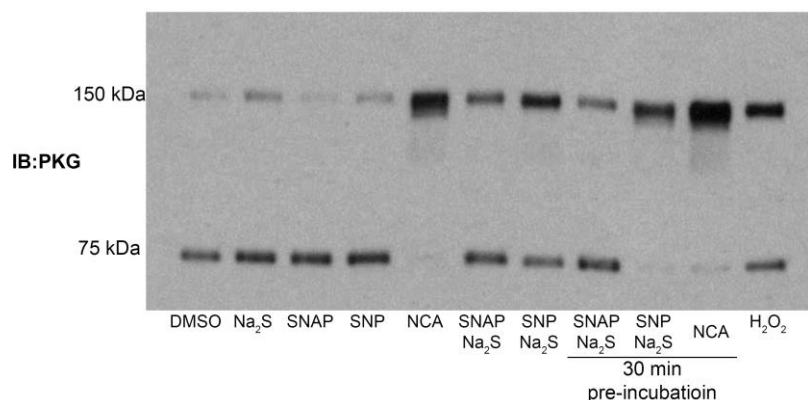


Figure 3-19

PKG oxidation in response to various treatments. HEK-293 cells transfected to express PKG were exposed to H_2O_2 , the nitroxyl donor NCA, the H_2S salt Na_2S or the NO donors SNAP or SNP. Each compound was assessed at 100 μM for 30 minutes. In some cases, NCA or mixtures of NO donors and Na_2S were pre-incubated for 30 minutes before adding the mixture to the cells. HEK-293 cells were lysed and subjected to western blotting and the membranes were probed for PKG.

3.4. Discussion

PKG is an important regulator of vascular tone and blood pressure [147]. Activation of PKG results in phosphorylation of a variety of substrates, leading to VSMC relaxation and blood pressure lowering. cGMP binding to PKG leads to a conformational change that releases the catalytic domain, resulting in increased substrate phosphorylation. Alternatively, in the absence of cGMP, thiol oxidation has been reported as another mechanism for PKG activation [25]. Oxidation of Cys42 by H₂O₂ and formation of an interdisulfide between two identical subunits of PKG is associated with vasodilation and contributes to blood pressure regulation [26]. S-guanylation of PKG at Cys195 results in the persistent activation of the enzyme and has been reported to take place in LPS-induced sepsis [145]. More recently, Donzelli et al reported that PKG could be activated by nitroxyl, the mechanism behind it being the oxidation of Cys117 and Cys195 and the formation of an intradisulfide bond between them [28]. The current study explores the physiological role of HNO-induced PKG activation by utilising genetically modified mouse model in which Cys195 in PKG is substituted for Ser. This conservative C195S mutation renders PKG mice incapable of forming an intradisulfide bond between Cys117 and Cys195 and subsequently incapable of intradisulfide bond-mediated PKG activation.

In this study C195S PKG KI mice were shown to have similar blood pressure to PKG WT control mice, suggesting that oxidation of Cys195 and formation of intradisulfide bond does not contribute to basal blood pressure homeostasis. Blood pressure control is a tightly regulated process, and is determined by a range of physiological factors such as cardiac output and vascular resistance [399]. PKG, which is important in maintaining vascular tone and blood pressure, directly activates MLCP to cause MLC dephosphorylation and vascular relaxation [152]. Mice with mutations in PKGI that prevent interaction with myosin phosphatase are hypertensive [182]. PKG can also relax smooth muscle by interacting with RGS2, which terminates signalling by G_q-coupled contractile agonist receptors [153]. Mice lacking RGS2 have impaired vascular relaxation and are hypertensive [153, 400]. By interacting with IRAG, PKG modulates release of Ca²⁺ from intracellular stores and induces vasodilation [201]. Consistent with this, IRAG-deficient mice have impaired vascular relaxation [187]. PKG knockout mice

are hypertensive, as are C42S PKG KI mice that cannot form the interprotein disulfide [26, 162]. Thus, numerous studies strongly suggest that the observed hypertension in aforementioned transgenic mouse models is primarily due to altered vascular resistance. While vascular contraction and relaxation in response to endogenously relevant stimuli were not assessed in the C195S PKG KI mice, the absence of basal hypertension does not necessarily mean that Cys195 oxidation in PKG does not participate in the control of vascular tone basally. Blood pressure is a tightly regulated process and represents a function of vascular resistance and cardiac output. Thus, changes in cardiac output could be responsible for the lack of blood pressure phenotype, or perhaps there are other adaptive, compensatory changes that normalise the blood pressure to match that of WT.

Cardiac output is dependent on three regulated variables, including end-diastolic volume, myocardial contractility and heart rate. End-diastolic volume itself is determined by blood volume and venous smooth muscle tone. The direct measurement of end-diastolic volume is usually achieved by pressure volume loop analysis using a conductance catheter [401]. This is technically challenging and was not available during this study; however, stroke volume was used as an indirect indicator of end-diastolic volume and myocardial contractility. In accordance with Frank – Starling law, changes in preload directly affect how much blood is ejected at each cardiac cycle [402]. LV volumes during systole and diastole and stroke volume in C195S PKG KI mice were not different from WT mice as assessed by echocardiography. However, it should be noted that echocardiography has limitations. For example, values for LV volumes derive from one-dimensional measurements that are raised to the power of 3, thus a relatively small difference in M-mode plane could result in over- or under-estimated values [403, 404]. Interestingly, the relationship between end-diastolic volume and cardiac output is impaired in C42S PKG mice, illustrating the complexity of PKG regulation in cardiovascular homeostasis [27]. PKG is a well-known negative modulator of myocardial contractility [405-407], by reducing L-type Ca^{2+} current and increasing Ca^{2+} uptake in sarcoplasmic reticulum, as well as directly phosphorylating cTnI. Cardiomyocytes from global and cardiomyocyte-specific PKG knockout mice showed that activation of this kinase is essential for cGMP-mediated effect on cardiac contractility [163]. Noteworthy, there is a recent work demonstrating that nitroxyl

affects the venous capacitance probably more so than the arterial compartment basally in dogs [124, 408]. Although, the physiological significance of this observation and the mechanism behind it remain to be elucidated, one cannot discard the possibility of PKG in nitroxyl-induced venodilation, so potentially influencing end-diastolic pressure. Venous tone in PKG C195S mice was not assessed in the current study. However, if nitroxyl-induced venodilation was mediated via Cys195 oxidation and PKG activation, the C195S KI mice would perhaps have higher venous tone, and consequently a higher end-diastolic volume and blood pressure, which is not what was observed in this study.

Another parameter that affects cardiac output and blood pressure is heart rate. Heart rate was significantly decreased in C195S PKG KI mice during day-time and tended to be lower over a 24-hour cycle, however, this failed to reach statistical significance. This bradycardia could potentially be a result of reflex changes due to blood pressure elevation and so could account for the lack of blood pressure phenotype. One possible explanation could relate to the baroreceptor reflex, which responds to alterations in blood pressure. An acute increase in blood pressure decreases heart rate via the baroreflex mechanism. However, during long-term increases in blood pressure, the baroreceptor resets to the new pressure and the heart rate returns to normal. A decrease in heart rate is not normally observed in either hypertensive patients or spontaneously hypertensive rats or mice lacking atrial natriuretic factor [409, 410]. Although, a satisfactory explanation for the decreased heart rate in the KI mice is currently lacking, it is possible that PKG could affect the baroreflex resetting or be involved in establishing the baroreceptor set point. The role of PKG in baroreflex regulation has not been extensively studied, although it has been suggested that adrenomedullin-induced baroreflex enhancement is mediated via activation of nitric oxide synthase (nNOS) and PKG [411]. Several studies have shown a role of NO in baroreflex function; however, whether this is definitively mediated by PKG remains undetermined [412-414]. Interestingly, blood pressure elevation in RGS2 knockout mice was suggested to be attributed not only to increase in peripheral vascular resistance, but also to increased sympathetic tone, leading to baroreflex resetting [415]. Another possibility for the reduction of heart rate is that PKG could play a direct role in the modulation of heart rate, such as via its action on cholinergic receptors.

Blood pressure in conscious mice has been shown to be strongly affected by locomotor activity [416]. However, in PKG C195S KI mice the locomotor activity was not different from WT mice, suggesting that it could not be a compensatory mechanism for blood pressure control.

Indices of cardiac function measured *in vivo* by echocardiography appear to be normal in the KI and comparable to WT controls. The similar values for cardiac output do not necessarily mean that cardiac output in conscious mice of either genotype is similar, as anaesthesia can have a profound effect on heart rate [417], which was not different in anaesthetised mice. LV mass and posterior wall thickness were similar between genotypes, indicative of the absence of cardiac hypertrophy in the KI mice.

Another possibility for the lack of a blood pressure phenotype is enhanced PKG expression in the C195S PKG KI mice, as a compensatory mechanism. PKG expression was assessed in aorta, heart, lung, kidney, liver, brain and stomach. While levels of PKG in lung, kidney, liver, brain and stomach were very similar between genotypes, PKG expression in the heart and aortas of the KI tended to be around 25 - 30 % higher than in WT, although the differences were not statistically significant. More replicates are required to clarify whether PKG protein levels are indeed higher in the heart and the aorta of PKG C195S KI mice. As mentioned above, Cys42 oxidation and formation of an interdisulfide results in enhanced phosphorylation of specific PKG substrates, consistent with enhanced activity in certain situations [25]. Potentiated PKG disulfide dimerisation in the KI could potentially account for PKG activity being similar to WT mice, even if intradisulfide-mediated activation of PKG is impaired. Thus, the proportion of the kinase in the interdisulfide state was assessed in different tissues. Only PKGI α can form an interdisulfide and the interprotein disulfide is most abundant in tissues with relatively high levels of the kinase. The highest levels of PKG dimer were detected in heart, lung, and aorta, with no difference in PKG disulfide dimerisation between genotypes. The actual values of PKG dimer percentage should be taken with caution, as the PKG antibody likely has different affinities for monomeric and dimeric PKG. No methods were available at the time to directly measure PKG activity in PKG WT and KI mice, thus phosphorylation of VASP, a well-recognised PKG substrate, was used as indirect measure of PKG activity. Phosphorylation of VASP was measured in

aorta, lung and kidney and was not different between genotypes, suggesting that basal activities of PKG are similar in these tissues. However, different PKG isoforms phosphorylate different substrates. Furthermore, different modes of PKG activation, for example cGMP- versus oxidant-induced, might result not only in changes in activity, but also in changes in PKG substrate targeting.

Overall, basal characterisation of cardiovascular function in the C195S PKG KI mice did not reveal any differences compared to WT, apart from a decreased heart rate in the transgenic during the day, with a similar trend at night. The mechanism behind the decreased heart rate is unclear, but the fact that mice have similar blood pressure might simply mean that intradisulfide-induced activation of PKG is not involved in basal blood pressure regulation. However, the difference between WT and KI mice might be more apparent in the presence of a stimulus address potential compensatory occurring basally, and so Ang II was assessed. There is substantial amount of evidence regarding the functional interplay between NO/cGMP/PKG pathway and Ang II in vascular and cardiac tissues [418]. Ang II acts as a vasoconstrictor, while activation of PKG induces vasodilation. Ang II stimulates VSMC and cardiomyocyte growth, while PKG inhibits it. Interestingly, a recent work showed a novel type of anti-hypertensive compounds, that acts via Cys42– mediated PKG targeting and activation [397]. While there is ample evidence for a protective role of PKG in Ang II-induced cardiac hypertrophy, several observations suggest that the kinase does not have a role in Ang II-induced hypertension and hypertrophy. Mice lacking PKG in cardiomyocytes did not exhibit aggravated blood pressure or cardiac hypertrophy in response to Ang II [419]. The development of Ang II-induced hypertension was assessed in WT and KI mice, but no statistically significant differences were observed, however, the C195S PKG KI mice trended to have a delayed development of Ang II-induced hypertension, albeit the number of mice studied was quite low and the errors in the measurements were large. However, even if the interdisulfide-induced activation of PKG played a role in attenuating Ang II-induced hypertension, C195S PKG KI mice would be expected to have a potentiated development of hypertension, not the other way around.

Although the C195S PKG KI mice did not show significant differences in blood pressure from WT mice basally or during Ang II treatment, an important remaining

question related to whether they have differential responses to compounds that induce PKG intradisulfide formation. So far only nitroxyl-releasing compounds have been shown to induce intradisulfide-mediated activation of PKG, and so two HNO donors were assessed *in vivo*, namely NCA and CXL-1020.

Administration of NCA intravenously induced a significant drop in blood pressure in Ang II-induced hypertensive WT or KI mice. The blood pressure decreased rapidly by approximately 40 mmHg in both genotypes after NCA was administered and returned to basal values 30 minutes after the injection. Such rapid and profound drop in blood pressure was not anticipated, because NCA has been reported to be a slow releasing nitroxyl donor. The half-life for NCA in physiological solutions was estimated to be over 8 hours [87, 420]. However, it should be noted that the experiments were performed *in vitro* and decomposition rate of NCA and its bioavailability *in vivo* might differ substantially. Importantly, NCA is a potent electrophile itself and rapidly reacts with soft nucleophiles, such as free Cys. Incubation of NCA or 3-methyl 1-nitrosocyclohexyl acetate (3MNCA), which has a similar structure to NCA, but does not release HNO, with GSH both result in the formation of disulfides [87]. This together with a relatively short lasting effect of NCA on blood pressure suggests that the observed effect might be not due to nitroxyl, but instead to the electrophilic properties of NCA modulating an alternate target. *In vitro*, NCA was shown to induce vasodilation mainly via activation of sGC, and to a lesser extent voltage-dependent K⁺ channels, as demonstrated using ODQ or 4-aminopyridine [87, 135]. In this study, the targets of NCA were not identified, however, oxidation of Cys195 in PKG does not seem to be involved in the physiological response as C195S PKG KI mice showed similar responses to WT. However, the number of mice used for the study was low, so definitive conclusions cannot be drawn. Myography studies showed that NCA-induced relaxation in U44619-precontracted mesenteric arteries was not impaired in C195S KI mice compared to WT, however it might be masked by sGC activation, as the donor can also release NO. Future studies with the sGC inhibitor ODQ would be rational to test any direct effects of NCA on classical activation of PKG. Although, numerous studies have shown the vasodilatory effect of NCA on vasculature, its effect on blood pressure has not been extensively studied. *In vivo* administration of NCA has only been assessed in one study, which focuses on diabetes-induced cardiac hypertrophy [421]. The authors

chronically administrated NCA (500 $\mu\text{mol/kg/day}$ i.p.) for four weeks to diabetic or control mice. Systolic blood pressure measured in control mice treated with NCA, although being slightly lower than in vehicle-treated animals (94 ± 16 mmHg vs 103 ± 23 mmHg), was not statistically lower than control. Although, the authors suggest NCA has no impact on blood pressure, their data do not contradict the effect on NCA observed in this study. The authors measured blood pressure using a micromanometer-tipped catheter and while they do not state how long after NCA injection the blood pressure was measured, it is likely that the measurements were done more than 30 minutes after the injection, when blood pressure may have returned back to basal. Interestingly, a profound drop in heart rate was noticed in NCA- and vehicle-injected mice, which potentially relates to the use of 2% DMSO, which was used as a vehicle, or perhaps most likely to anaesthetic isoflurane. However, this drop did not result in lower blood pressure in vehicle-treated mice, suggesting that the effect on blood pressure in NCA-treated mice was NCA-specific.

As experiments comparing NCA in WT or KI mice were in some ways inconclusive, CXL-1020 was chosen to further assess the role of nitroxyl-induced activation of PKG on blood pressure. CXL-1020 decomposes to produce HNO and an inactive by-product. Unlike NCA, CXL-1020 is thought to be a pure HNO donor that does not release other endogenously produced species such as NO. Human clinical trials with CXL-1020 are currently in progress, as a potential therapy for heart failure. Administration of CXL-1020 (9 mg/kg/day) did not change basal blood pressure or heart rate in WT or KI mice. Previously, CXL-1020 has been shown to reduce systemic vascular resistance in mice or dogs as measured by pressure-volume loop analysis [422, 423]. These results from these investigations do not contradict published studies, as the doses used here were significantly lower than those of others. Previously CXL-1020 was delivered to mice at 200 – 500 $\mu\text{g/kg/min}$ via intravenous infusion [422], which is the mode of administration being investigated in the current human clinical trials. The effect of CXL-1020 on systemic vascular resistance was observed only from 300 $\mu\text{g/kg/min}$ (432 mg/kg/day). The concentration of CXL-1020 used in this study was determined by the solubility of the drug and its method of administration. Unfortunately, intravenous infusion did not seem possible at the time, and the drug was delivered via subcutaneous osmotic mini-pumps with 9 mg/kg/day being the highest concentration possible. CXL-

1020 at the chosen concentration did not exert a blood pressure-lowering effect basally, and so the drug was tested in mice rendered hypertensive by Ang II treatment for ten days. CXL-1020 (9 mg/kg/day) tended to lower blood pressure in both hypertensive WT and KI mice, but the difference was not statistically significant. Further investigations to increase the sample size are required to provide greater confidence in these preliminary studies. Overall blood pressure decreased by 11 ± 5.6 mmHg in WT mice and 3 ± 3.3 mmHg in the KI mice. The blood pressure drop is consistent with the published data, where MAP fell by 9 mmHg in dogs receiving 3 μ g/kg/min of CXL-1020 [423]. Although, decreases in blood pressure observed in this study are not statistically significant, there was a marked trend for CXL-1020 to lower blood pressure, which was more clearly evident during the first 12 hours after implantation of mini-pumps delivering CXL-1020. The difference between WT and KI mice, on the other hand, is not that convincing, and it would be careless to speculate on something, which is not yet substantiated. Repeating these experiments to increase sample size would help clearly define whether blood pressure lowering in response to CXL-1020 is truly deficient in the KI mice. If this were found to be true, it would be a direct indication of Cys195 involvement in nitroxyl-induced haemodynamic responses. However, it should be noted that vascular responses to CXL-1020 were not impaired in PKG C195S KI mice. Interestingly, administration of CXL-1020 decreased blood pressure variability in PKG WT and KI mice, as illustrated by the size of error bars. The impact of CXL-1020 on blood pressure variability is unlikely to be only the consequence of blood pressure normalisation, because variability was lowered to levels below those observed in control animals. Enhanced blood pressure variability is known to be a risk factor for cardiovascular injury and end-organ damage [424]. Two major mechanisms are known to buffer blood pressure fluctuations, namely the arterial baroreceptor reflex and the NO pathway. Blood pressure variability is increased in baroreceptor-denervated mice and eNOS knockout mice [425, 426], with CXL-1020 potentially affecting one of these mechanisms. Interestingly, although the baroreflex blockade does not impair the positive inotropic response to nitroxyl, it balances nitroxyl-induced arterial and venous dilation, perhaps suggesting a role in preferential venodilation by nitroxyl [124]. The mechanism behind blood pressure lowering by CXL-1020 is not clear, but there is some evidence that activation of sGC might be involved. Zhu et al showed that while CXL-

1020 lowered systemic vascular resistance in WT mice, it failed to do so in sGC knockout mice [422]. While it seems clear cut, it should be noted that sGC knockout mice are hypertensive and have elevated arterial stiffness [427, 428]. The authors also showed that CXL-1020-induced vasorelaxation was absent in aortic rings from mice lacking sGC. From this they concluded that the blood pressure lowering effect of CXL-1020 is primarily based on activation of sGC. However, one should not forget that while the vasodilatory NO/sGC/PKG signalling pathway is predominant in large conduit vessels, it is not so in smaller resistance vessels [429]. And it is resistance vessels that regulate blood pressure. Moreover, downregulation of NO/sGC/PKG signalling has been shown to take place during several pathologic conditions, suggesting that while basally CXL-1020 might lower blood pressure predominantly via activation of sGC, it might do so via different mechanisms in disease scenarios [430].

Large increases in NO species are frequently observed in rodent models of sepsis and in septic patients with hypotension [431, 432]. iNOS protein expression is significantly increased in response to sepsis and can be observed 2 hours and onwards after sepsis induction [433], but declines by 12 hours. NO is implicated in sepsis-induced vasodilatory shock by both cGMP/PKG-dependent and independent mechanisms, however, a major effect of NO is believed to be mediated by sustained activation of sGC [434]. Interestingly, not only cGMP-dependent, but also redox-dependent regulation of PKG has been shown to contribute to the pathogenesis of sepsis [435]. During sepsis large amounts of NO may result in formation of S-nitrosothiols, and as mentioned in the introduction, reaction of S-nitrosothiols with thiols lead to disulfide and HNO formation [102, 103]. Thus, it is logical to speculate that increased production of nitroxyl may occur during sepsis. Interestingly, NO donors have been shown to induce Cys42 oxidation with S-nitrosothiol as an intermediate, with formation of HNO likely to occur during this process. This could potentially offer a mechanism for PKG-induced formation of nitroxyl and subsequent PKG activation via nitroxyl-induced formation of the Cys117 – Cys195 intradisulfide bond. In this study WT or KI mice were rendered septic by CLP and the role of Cys195 oxidation in the pathogenesis of sepsis was assessed by measuring blood pressure and heart rate of the animals, as well as cardiac function. The 6-hour time point was chosen because, as mentioned before, NO levels increase early in sepsis. If HNO is produced via S-nitrosothiol reaction with

thiols, HNO-mediated regulation could potentially take place in early sepsis. Moreover, the CLP is a severe model of sepsis with some mice occasionally dying before the 24-hour time point, and assessment of cardiac function would be extremely challenging at later time points when myocardial contractility is substantively impaired. There was no difference between in the haemodynamic responses to sepsis between genotypes. Sepsis induced blood pressure lowering in both genotypes to a similar extent. The cardiac function was impaired in both genotypes 6 hours after sepsis induction, as evidenced by a significantly reduced ejection fraction and cardiac output – but there were no differences between genotypes. These results suggest that Cys195 oxidation does not contribute to cardiac or haemodynamic changes during early sepsis. It is worth mentioning that PKG Cys195 S-guanylation by 8-nitro-cGMP has been shown to take place during sepsis, and this modification results in sustained activation of the kinase [145]. The authors have observed basal PKG S-guanylation and its significant increase in heart samples from LPS-treated mice by western blotting. The physiological and pathophysiological significance of the observation is not fully elucidated.

During the characterisation, the KI mice they were found to have a lower body weight than age-matched WT control mice. A similar reduced weight reported for smooth muscle cell-specific PKG knockout mice [436]. The difference in body weight could be potentially explained by impaired function of the GI tract. Indeed, the majority of PKG KI mice showed a profoundly enlarged caecum, which was commonly out of place, suggesting a possible impairment in gut motility and dysfunctional peristaltic. The gut motility studies using carmine red showed significantly increased whole-gut transit time, suggesting a role of Cys195 oxidation in the normal function of the GI tract. It is widely known that PKGI is ubiquitously expressed in the smooth muscle cells of the GI tract, including stomach, intestine and caecum. It is also found in the intrinsic nervous system that regulates the GI tract function - in a population of pacemaker cells - interstitial cells of Cajal (ICC) [159, 162, 437]. ICC orchestrate motor activity of the gut by generating electric waves and smooth muscle constriction [438, 439]. PKGI is a principal mediator of nonadrenergic, noncholinergic neurotransmitters, NO and CO, which are generated in intrinsic neurons by the action of neuronal nNOS and heme oxygenase, respectively [440]. Deletion of nNOS disrupts GI motility and mice show gastric stasis and pylorus stenosis [441, 442]. PKG knockout mice exhibit severe

impairment of intestinal motility [162]. The stomach, duodenum, and caecum of these mice is largely dilated, they also show symptoms of pyloric stenosis and contraction of the ileocecal region. A similar phenotype is found in sGC knockout mice, as well as in mice with the mutated IRAG (IRAG^{Δ12/Δ12}) that cannot bind IP₃R [187, 428]. The cGMP-dependent relaxation of the gastric fundus or colon strips precontracted with carbachol is impaired in PKGI knockout and IRAG^{Δ12/Δ12} mice [174, 187]. It is worth noting, that inducible smooth muscle cell-specific PKGI knockout mice do not mirror global PKG knockout GI phenotype, suggesting there might be an important role for other cell types in neuronal-dependent smooth muscle relaxation [438]. Interestingly, conditional ICC-specific PKGI knockout mice, have severe GI tract dysfunction, which suggests a role of PKGI in pacemaker current generation and signal integration between interstitial neurons and smooth musculature in the gut. Intestinal smooth muscle cells express several PKGI targets, including MYPT1, RhoA and telokin, which mediate both Ca²⁺-dependent and independent contraction [185, 191, 443, 444]. Thus, it is logical that PKGI deletion affects MLCP activity and smooth muscle contraction at constant intracellular Ca²⁺, while IRAG mutation affects only IP₃-dependent intracellular Ca²⁺ release [187, 445]. It is worth noting that cGMP-dependent relaxation of gastric fundus or colon strips precontracted with submaximal concentration of K⁺ was impaired in PKGI knockout mice, but not in IRAG^{Δ12/Δ12} mice. This suggests that PKGI can relax gastrointestinal smooth muscle cells via additional signalling pathways that do not require IRAG.

While it is logical to speculate that Cys195 modification in PKG is important for GI tract function, a lower affinity of the mutant kinase for cGMP might potentially account for the observation. Mutating Cys for Ser might have an effect on cGMP affinity, as Cys195 is located in cGMP-binding domain. Unfortunately, methods for direct measurement of cGMP affinity were not readily available, and so cGMP affinity with WT or C195S PKG were measured by eluting kinase from heart homogenates pre-bound to a cGMP-affinity matrix with increasing concentrations of cGMP. The eluted fractions were analysed by western blotting and the cGMP affinity was represented as a percentage of eluted PKG at a given concentrations of cGMP. There was no difference between the amounts of WT and C195S PKG eluted at any given concentration of cGMP, suggesting that they bind cGMP with similar affinities. However, the method

has got several limitations, and cannot be used without further complementary analyses to make definitive conclusions. Because PKG was pre-bound to cGMP-ligated beads, the concentrations of cGMP required for elution was substantially higher than previously observed K_d values for cGMP. Myography studies assessing mesenteric vessels from WT or C195S PKG KI mice were used to further indirectly assess the potential effect of Cys195 substitution for Ser on PKG activity. Mesenteric vessels relax in response to cGMP; if the affinity for cGMP was severely impaired, it is anticipated to result in attenuated cGMP-dependent vasorelaxation. However, no differences were observed between genotypes, with relaxation curves in response to the membrane-permeable cGMP analogue CPT-cGMP being almost identical. This suggests that the cGMP-dependent vascular smooth muscle cell relaxation is not impaired in the KI mice. It does not necessarily mean that cGMP-dependent GI smooth muscle cell relaxation is not impaired in PKG C195S mice. Studies with precontracted gastric strips are required to shed light on this question. As mentioned in the introduction, nitroxyl has been suggested to form upon reaction of NO with H_2S . Numerous studies have shown the importance of both mediators in GI tract function [446-449]. Endogenous H_2S production depends upon two enzymes, cystathionine β -synthase and cystathionine γ -lyase, and occurs in the GI tract [450-452]. In the GI tract, exogenously applied NaSH inhibits gastric and intestinal motility by inducing smooth muscle cell relaxation [451, 453]. Importantly, H_2S has an inhibitory role on spontaneous rhythmic contractile activity, which is dependent on ICC, which generate intrinsic pacemaker potentials [454]. Recent work has shown that H_2S interacts with NO to regulate functional pacemaker activity [448]. The profound effect on pacemaker currents in ICC was noticed in the presence of NO and H_2S donors together, but was only marginal when the donors were administered separately [448, 454, 455]. The effect was attenuated by preincubating ICC with ODQ or glibenclamide, suggesting a role for cGMP and K_{ATP} channels, although the exact mechanism remains unclear. It is reasonable to speculate that PKG activation via nitroxyl-induced Cys117 – Cys195 intradisulfide formation might be partly responsible for the observed cooperative effect of NO and H_2S in ICC, especially as PKG C195S KI mice have impaired intestinal motility. The impaired GI smooth muscle relaxation in C195S PKG KI mice cannot be fully ruled out, however, there is no data supporting it.

It should be noted that endogenous formation of the intradisulfide bond between Cys117 and Cys195 has not been confirmed in this study. In vitro treatment of mesenteric arteries with nitroxyl donors, NCA or CXL-1020, did not induce the intradisulfide bond formation as evidenced by a lack of a downward gel-shift on non-reducing western blots, the shift has been observed before by Donzelli et al [28]. In vivo administration of CXL-1020 via osmotic mini-pumps or intravenous injection of NCA also failed to induce this shift. It is not clear as what the reasons are, but thiol redox state in mesenteric arteries is believed to be predominantly reduced. Redox state has been shown to greatly impact on vascular function. Thioredoxin-deficient mice have impaired vascular responses, and this is thought to be due to the change in their redox state [456]. The redox state of mesenteric arteries in thioredoxin-deficient mice has been shown to be predominantly oxidised as assessed by the ratio of oxidised to reduced thioredoxin. As shown by western blotting, only about 20% of PKG in vessels is in the interdisulfide state, as opposed to approximately 40% in the heart. Thus, it is perhaps possible that the intradisulfide could have been formed, but was resolved before there was a chance to visualise it by western blotting. It is also not clear how much of the PKG intradisulfide is formed endogenously and would be required to induce its activity and impact on vasomotor function. The method used for to monitor PKGI redox state does not allow detection of subtle changes. Also, structural modification of PKG could have affected binding of antibodies to the protein, complicating the detection of changes in oxidation state. Moreover, it is suggested that different modes of PKG activation negatively affect each other. Thus, dimerisation of PKG negatively affects cGMP-dependent activation of the kinase. Because Cys195 is located in the cGMP binding domain, cGMP bound to at this location will probably hinder the formation of intradisulfide. Basally, the concentration of cGMP in vascular smooth muscle cells is approximately 0.1 μ M, suggesting that the high affinity cGMP binding site in PKG is occupied with cGMP and formation of intradisulfide is obstructed [147]. Although, there is likely to be a turnover in occupancy, which is supported by a study showing 8-nitro-cGMP that can adduct to the Cys in this site in vivo. Interestingly, in vitro treatment of mesenteric arteries with HNO donors resulted in more dimer formation in vessels from PKG KI mice compared to WT mice. It is not clear what the reasons are, but this could be attributed to the crosstalk discussed above. Also it should be noted that

most of the *in vitro* experiment were performed with mesenteric arteries with intact perivascular fat. This could potentially attenuate the impact of the HNO donors on the mesenteric arteries. Moreover, perivascular fat contains a lot of the PKGI β isoform, thus hindering assessment of PKG oxidation by homodimer formation (only PKGI α can form it). Future experiments using mesenteric arteries with their perivascular fat removed would be a logical way forward.

The visualisation of the shift is a challenging task and requires the use of low percentage polyacrylamide gels in an attempt to separate the PKG in different redox states. The gels are prepared in advance and are left to polymerase for at least 16 hours to make sure that no residual unpolymerised acrylamide monomer in the gel, as it can react with amino, sulfhydryl, and phenolic hydroxyl groups [457, 458]. While the PKG shift down in most cases is well-resolved in cell samples, in tissue samples it is quite often observed as a smear, and sometimes it is hard to tell whether there is actually a shift or not. Different protein gel systems, namely Tris-HCl, bis-Tris, and Tris-acetate, were assessed for their ability to separate PKG in its intradisulfide state. They have different leading and trailing ions. The pH of the gels also differs. While Tris-HCl system is the most commonly used and works well in most cases, bis-Tris and Tris-acetate systems have been suggested to provide better resolution. Unfortunately, all systems showed similar results and Tris-HCl system was used throughout the study.

Overall, this study shows that Cys195 in PKG is not involved in basal blood pressure regulation or left ventricle function. C195S PKG KI mice were shown to have lower heart rate, however, the mechanism behind it is unclear. Nitroxyl donors, NCA or CXL-1020 were shown to have a blood pressure-lowering effect in Ang II-induced hypertensive animals, but WT or C195S KI responded similarly. This suggested that oxidation of Cys195 and formation of the intradisulfide bond between Cys117 and Cys195 are not involved in blood pressure-lowering effect of these molecules. This was further corroborated by myography studies. WT and C195S PKG KI mice also had similar responses to sepsis. Unexpectedly, the C195S PKG KI mice had significantly enlarged cecum and impaired gut motility, as assessed by whole gut transit time, suggesting that intradisulfide-mediated activation of PKG might play a role in GI smooth muscle cell relaxation, its regulation by intestinal neurons or integration of the

signal between them. Further studies are required to definitively confirm this suggestion, as well as to define the signalling mechanisms responsible for the differential responses between genotypes.

Chapter 4. sEH redox regulation in sepsis

This chapter focuses on the potential role for alterations in the redox state of soluble epoxide hydrolase (sEH) to contribute to physiological dysfunction in sepsis. sEH regulates numerous physiological processes, including control of blood pressure and inflammation. However, these processes can become dysregulated and contribute to disease pathogenesis, potentially mediated by redox alterations of the hydrolase. The post-translational mechanisms regulating sEH activity *in vivo* are still not completely understood, however recent studies have shown that adduction of electrophilic lipids to Cys521 in sEH results in its inhibition. This chapter documents a series of experiments performed to compare the responses of WT or C521S sEH KI mice to septic shock. C521S sEH KI mice are resistant to inhibition by electrophiles and showed aggravated clinical symptoms of sepsis compared to WT, as assessed by their appearance, activity, respiration rate and response to stimuli. Both genotypes became highly hypotensive in response to cecum ligation and puncture (CLP) induction, with C521S sEH KI mice showing the trend towards an even lower blood pressure and lower heart rate. There was no difference in blood biochemistry and cytokine levels between genotypes, except for granulocyte colony-stimulating factor, which was lower in KI mice comparing to WT. Wire myography analysis was performed to compare the responses of small mesenteric vessels from C521S KI to those from WT animals subjected to CLP, and showed impaired contraction in response to U44619 in septic animals. However, no difference was observed between different genotypes, suggesting that sEH inhibition by electrophilic lipids does not mediate the impaired vessel contractility in response to sepsis.

4.1. Introduction

Sepsis, which is introduced in significant depth in Chapter 1, is an inflammatory response to infection, characterised by a range of cardiovascular derangements, including vascular dysfunction, hypotension, and impairment of cardiac function [290]. Cytochrome P450 epoxygenases oxidise polyunsaturated fatty acids to corresponding epoxides, including those derived from arachidonic acid, namely the epoxyeicosatrienoic acids (EETs). EETs have been shown to possess potent vasodilatory, proangiogenic, and antiapoptotic effects in the vasculature [344]. Soluble epoxide hydrolase (sEH) rapidly hydrolyses EETs to their corresponding dihydroxyeicosatrienoic acids (DHETs), which are believed to lack EETs' physiological effects, or at least to be less active. Pharmacological inhibition of sEH or *Ephx2* gene deletion resulted in increased levels of cellular and circulating EETs, potentiating their biological effects.

Hypotension accompanies sepsis, and is considered to be a major contribution to sepsis-associated mortality in critically ill patients [288]. A large number of studies have tried to untangle the mechanism underlying sepsis-induced hypotension. So far the ability of iNOS and K_{ATP} channel inhibitors, as well as genetic knockout of iNOS, to reduce vascular hyporesponsiveness and hypotension in septic shock animal models have demonstrated a pivotal role for NO and its derivatives and K_{ATP} channels in the development of hypotension during sepsis [303, 304, 331-333]. However, numerous pre-clinical studies, as well as clinical trials have failed to demonstrate increased survival in animal models and septic shock patients following K_{ATP} channel, COX or NOS inhibition. The mechanisms extensively discussed in Chapter 1 do not fully explain the hypotension during septic shock. Thus, it is reasonable to suggest that other factors might be important in causing the systemic hypotension. Oxidative inhibition of sEH by lipid electrophiles could be one of these factors. Sepsis is accompanied by excessive production of reactive oxygen and nitrogen species both in the circulation and in the organs, leading to lipid peroxidation and formation of electrophilic lipids [459]. sEH is inhibited by adduction of electrophilic lipids [29, 30], leading to increased levels of the hydrolase's substrates, namely EETs and other epoxygenated fatty acids. EETs are potent vasodilators with established roles in the regulation of vascular tone and

vascular homeostasis [236]. Notably, gene expression of several epoxygenases is altered during sepsis, with some genes being up-regulated and others down-regulated, suggesting they could potentially impact the regulation of vascular reactivity during sepsis [460]. Thus, one can speculate that during sepsis excessive formation of electrophilic lipids could inhibit sEH, leading to higher levels of epoxide fatty acids and subsequent vasodilation and hypotension. Here the redox regulation of sEH in the pathogenesis of polymicrobial sepsis is investigated by comparing the responses of WT mice to those of the C521S sEH KI to CLP.

4.2. Materials and Methods

Mouse strains

Knock-in mice (KI) with a C521S mutation in sEH were generated by homologous recombination, as described in detail before [30]. Briefly, the nucleotide changes were introduced in exon 18 of EPHX2 gene (NM_007940.4) (c.1649C>G, c.1650C>T), leading to amino acid change from cysteine to serine. WT (C57bl/6J) and the KI mice were bred in-house, with those aged between 8-28 weeks used for experiments. All the experiments were conducted according to the Animals (Scientific Procedures) Act 1986 (revised 1 January 2013).

Genotyping

Genomic DNA was extracted from mice ear tissue samples using a DNeasy blood and tissue kit (QIAGEN) according to the manufacturer's instructions. The KI allele and WT allele were amplified with the polymerase chain reaction (PCR) using the following primers: Forward – (5' – CCT CAG AGG CCA GAA TAG GG – 3'), Reverse – (5' – TAG AAG GGT GGG AGG AGC TG – 3'); expected sizes for PCR products were 297 bp for WT and 389 bp for KI. The amplification reaction was performed using GoTaq Flexi DNA Polymerase (Promega) according to the manufacturer's instructions in a reaction volume of 25 µl. Thermocycling conditions were as follows: 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, elongation at 72°C for 30 seconds, with the final extension at 72°C for 10 minutes.

Induction of sepsis

8-12 weeks old mice or 24-28 weeks old mice were subjected to sham- or cecum ligation and puncture (CLP) surgery as described in the general materials and methods section.

Blood pressure and heart rate measurements in septic mice

Blood pressure and heart rate were measured in conscious freely moving mice by using radio-telemetry as described in the general materials and methods section. After implantation of telemetry probes, mice were left to recover for at least 10 days before sepsis induction.

Assessment of sepsis severity in mice

Sepsis scoring, body temperature and locomotor activity were used to assess the severity of sepsis 4 and 24 hours after induction of sepsis. The sepsis score assessment is described in the general materials and methods section. The locomotor activity was measured using radio-telemetry as also described in the general materials and methods section.

Blood collection and tissue harvest from septic mice

4 or 24 hours after the CLP procedure blood was collected for measurement of their gas content, electrolytes and haematological parameters. Anaesthesia was initiated and maintained the same way as described in the general materials and methods section. The blood was collected from the left renal vein with a syringe containing 50 µl 100 IU/ml heparin sodium anticoagulant. Special care was taken to keep the same heparin-to-blood ratio in all the blood samples. Livers, hearts, kidneys, lungs and aortas were washed in cold physiological salt solution, snap-frozen in liquid nitrogen and stored at -80°C before further analyses. Blood was analysed using VetScan i-STAT 1 handheld analyser (Abaxis) and i-STAT EC8+ and i-STAT cardiac troponin I cartridges (Abaxis).

Vessel reactivity studies in septic mice

Vessel reactivity was measured in conduit and resistance vessels. The preparation, normalisation and wake-up protocols were performed as described in the general materials and methods section. Vascular constriction was assessed by the contractile response to KCl (15 mM – 90 mM), phenylephrine (PE) (10 nM – 10 µM) or U44619 compound (1 nM – 1 µM). Relaxation of PE (1 µM) precontracted aortas or U44619

(15 nM) precontracted mesenteric arteries was evaluated by measuring the dilatory response to acetylcholine (Ach) (1nM - 10 μ M). Precontracted vessels that relaxed more than 70% in response to 1 μ M Ach were considered suitable for experimentation.

Measurement of plasma cytokine levels in septic mice

Plasma was separated from the whole blood by centrifugation at 2500 g for 5 min at room temperature, aliquoted and stored at -80°C until further analyses. The cytokine levels were assessed using the Bio-Plex Pro Mouse Cytokine 23-plex assay (Bio-Rad) together with a Bio-Plex MAGPIX fluorescence magnetic bead-based immunoassay reader (Bio-Rad) according to the manufacturer's instructions. Bio-Plex MAGPIX was validated and calibrated before the experiment using the Bio-Plex validation and calibration kits, respectively. Briefly, plasma samples were diluted 1:4 by adding Bio-Plex sample diluent and incubated with antibody-coupled beads at 800 rpm at room temperature. Biotinylated detection antibodies and streptavidin-phycoerythrin conjugates were added to the plate subsequently to measure cytokine levels. The plate was washed after each incubation step using the Bio-Plex Pro Wash station. Subsequently the plate, which also contained standard curves generated for each analyte, was analysed. Each sample was measured in duplicate and cytokine concentrations were determined using BioManager 6.1 software (Bio-Rad).

4.3. Results

C521S sEH KI mice are not protected from sepsis-induced haemodynamic derangements

To measure the systemic haemodynamic changes during sepsis, 24 – 28 weeks old sEH WT or KI mice were subjected to CLP and mean arterial pressure and heart rate were recorded in conscious mice for 24 hours following the procedure using radio-telemetry (Figure 4-1). No statistically significant alterations in haemodynamic parameters were observed between genotypes in the septic animals, although KI mice showed a trend, albeit statistically insignificant, towards a greater decline in heart rate compared to WT. In sham-operated mice mean arterial pressure remained stable between 95 and 125 mmHg before and after the surgery (Figure 4-1A). In contrast, mean arterial pressure in septic mice was significantly lower compared with sham mice after the surgery. Mean arterial pressure progressively declined during the first 4 hours to approximately 75 mmHg in WT mice and 60 mmHg in KI mice, after which it remained relatively stable for the next 20 hours. As with mean arterial pressure, heart rate did not change in sham operated animals, while it was significantly elevated in septic mice during the first hour after the procedure and subsequently declined to approximately 450 bpm and 330 bpm for WT and KI mice, respectively (Figure 4-1B). The decrease in heart rate was delayed relative to the decrease in mean arterial pressure and reached its lowest point 16 hours after the surgery. The heart rate drop in C521S sEH KI mice after the CLP procedure and had a trend towards being more marked than that in WT mice, although these differences were not statistically significant. The averaged decreases in mean arterial, systolic or diastolic pressure in response to sepsis were similar between genotypes (Figure 4-1C-F). Sepsis induced a decline in systolic pressure, which was greater than the corresponding alteration to diastolic pressure, resulting in a pulse pressure drop (Figure 4-1G). However, there was no difference between sepsis-induced alterations in pulse pressure genotypes.

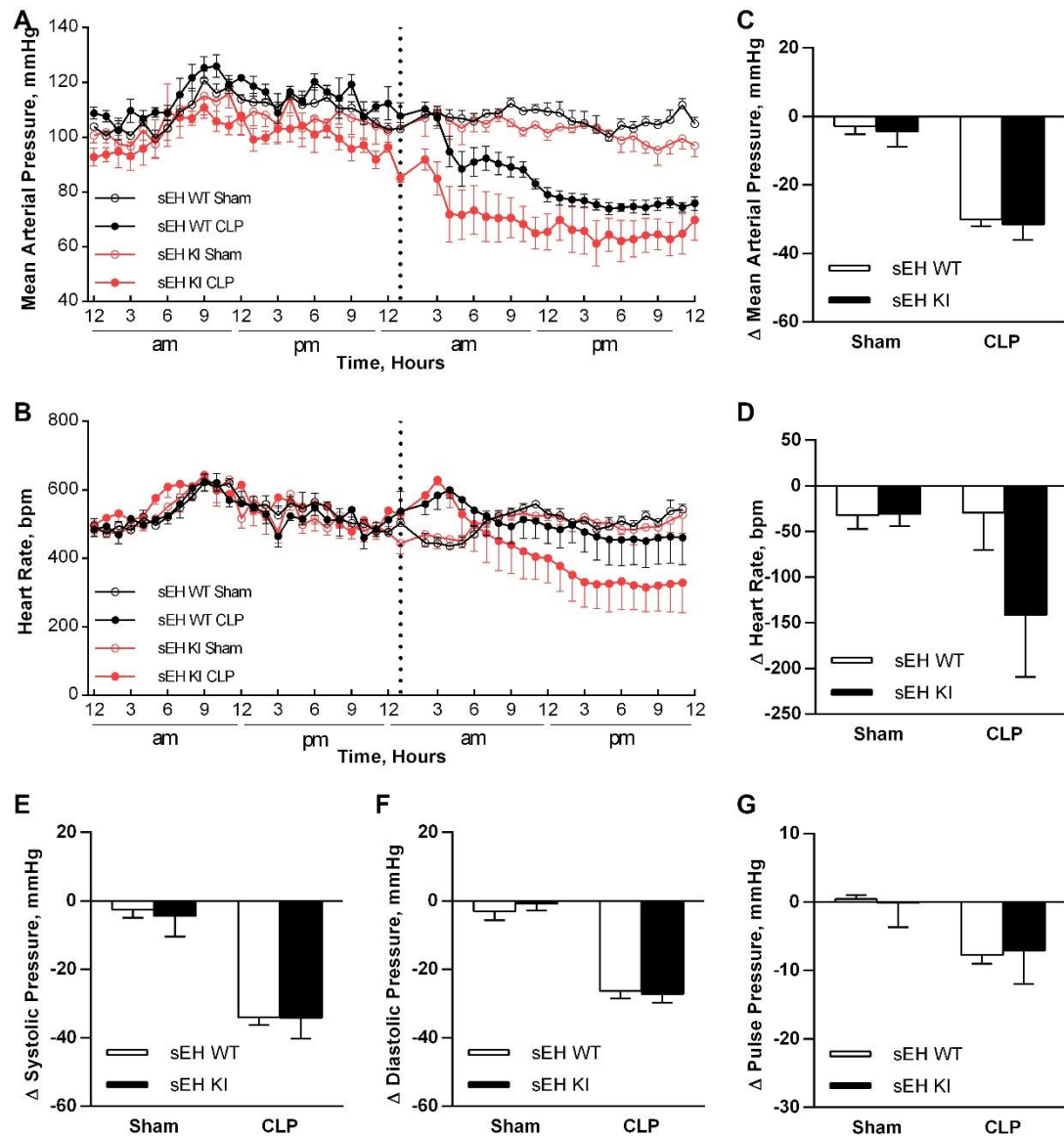


Figure 4-1

Haemodynamic parameters in WT versus KI mice subjected to sepsis. A-B. Time course of mean arterial pressure and heart rate 24 hours before and after CLP/Sham procedure. The induction of surgery is indicated by the dotted line. Variables were measured in conscious WT or KI mice every 5 minutes by radio-telemetry and are present as hourly mean with SEM, $n = 4$ mice per group. C-G. Sham- or CLP-induced changes in mean arterial pressure, heart rate, systolic, diastolic and pulse pressure relative to the basal values. The data are present as mean difference from the time-matched baseline with SEM, $n = 4$ mice per group. Statistical analysis was performed using one-way ANOVA with a Tukey post-hoc test, which failed to identify difference between genotypes.

C521S sEH KI mice demonstrate aggravated sepsis-related disease symptoms

To determine the severity of sepsis in WT compared to KI mice, a behaviour score, body temperature and locomotor activity of the animals were evaluated at 4 and 24 hours after the sham or CLP procedure (Figure 4-2).

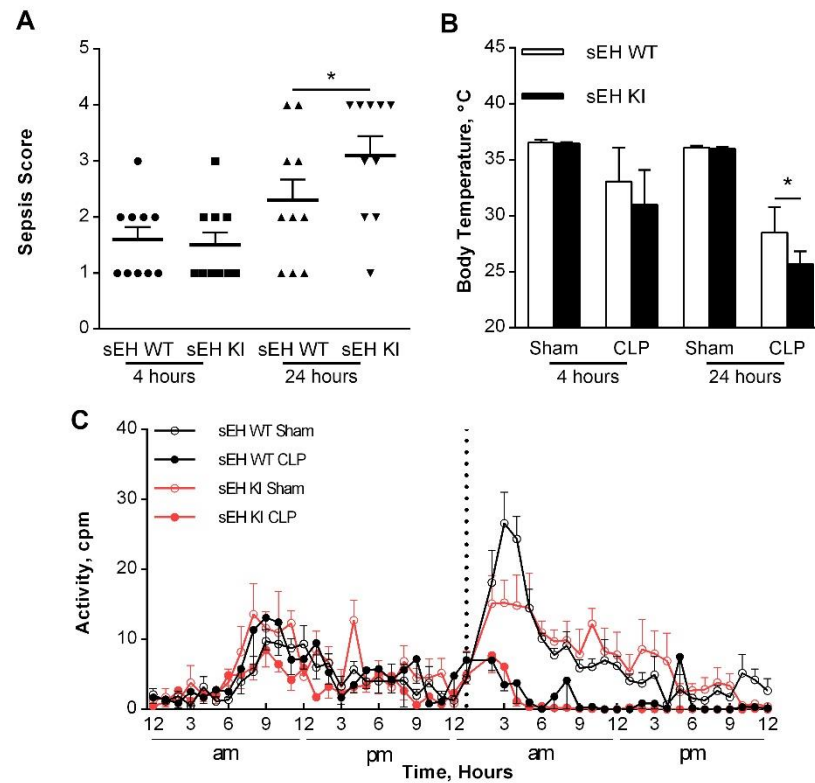


Figure 4-2

The severity of sepsis in WT versus KI mice subjected to sepsis. A. Sepsis scoring was performed in WT or KI mice 4 and 24 hours after sham or CLP surgery. B. Body temperature was measured by rectal probe in WT or KI mice 4 and 24 hours after sham or CLP surgery. C. Locomotor activity was measured in WT or KI mice by radio-telemetry 24 hours before and after surgery, which is indicated by the dotted line. Data are present as mean with SEM, n = 10 mice per group (A-B) and n = 4 mice per group (C). * significantly different as analysed by one-way ANOVA with Tukey post-hoc test.

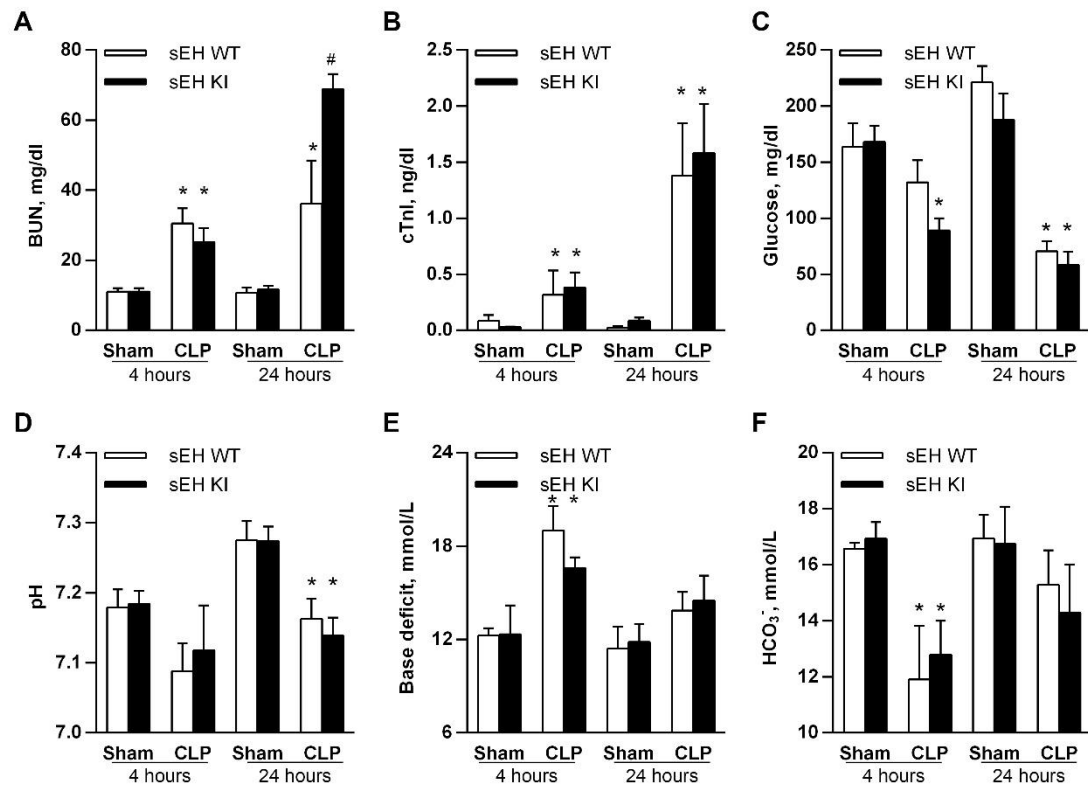
A sepsis score representing the clinical disease symptoms was used to assess the severity of sepsis induced by the CLP model (Figure 4-2A). It takes into consideration several alterations in the appearance and behaviour of mice as described fully in the methods section. The score for sham-operated animals was always 0, meaning there was no discernible difference in these groups at 4 or 24 hours following the surgical procedure. 4 hours after CLP induction mice showed relatively mild clinical signs of

sepsis, with no significant differences in the sepsis scores of 1.6 ± 0.22 and 1.5 ± 0.22 for WT or KI mice, respectively. The sepsis score, indexed 24 hours after the surgery, was significantly higher in KI mice than WT mice (3.1 ± 0.35 vs 2.3 ± 0.36), which correlated with the augmented difference in heart rate between septic and sham KI mice compared to WT animals. Mice subjected to the CLP procedure became progressively more hypothermic, dropping to 31-33°C by 4 hours in each genotype. 24 hours after the surgery KI mice showed a significantly augmented drop in body temperature compared to WT animals ($26 \pm 1.1^{\circ}\text{C}$ vs $29 \pm 2.3^{\circ}\text{C}$) (Figure 4-2B). Locomotor activity was markedly attenuated 4 hours after the CLP procedure in each genotype, and remained low for the ensuing 20 hours in both groups (Figure 4-2C).

Septic mice show symptoms of organ damage and metabolic acidosis

Sepsis is associated with systemic acid-base imbalance and organ damage. Assessment of blood biomarkers of organ injury 4 or 24 hours after CLP procedure showed significant organ damage in both WT and KI mice, as evidenced by increased levels of blood urea nitrogen (BUN) and cardiac troponin I (cTnI) (Figure 4-3A,B).

BUN, a marker of acute kidney injury, was elevated in a time-dependent manner showing a 3-fold increase in WT and 6-fold increase in KI mice 24 hours after CLP, which was consistent with the septic score and body temperature drop (Figure 4-3A). Sepsis produced a significant escalation in the blood concentration of cTnI, a specific marker of myocardial damage, reaching ~ 1.5 ng/dl 24 hours after sepsis induction (Figure 4-3B). There was no difference between KI or WT mice in terms of sepsis-induced blood cTnI levels. Mice also became severely hypoglycaemic over the 24 hours after CLP, which is consistent with organ damage and metabolic disturbance (Figure 4-3C).

**Figure 4-3**

Sepsis-induced multi organ injury and acid-base imbalance in WT or KI mice. A,B. Markers of organ injury, blood urea nitrogen (BUN) and cardiac troponin I (cTnI) in WT and KI mice subjected to Sham or CLP surgery. C. Glucose levels in WT or KI mice subjected to Sham or CLP surgery. D-F. Acid-base balance in WT or KI mice subjected to Sham or CLP surgery. The markers were measured in venous blood 4 and 24 hours after surgery induction using an iSTAT analyser. The data are presented as mean with SEM, $n = 10$ mice per group. *significantly different from sham-operated controls as analysed by one-way ANOVA with Tukey post-hoc test.

Assessment of venous blood gases showed metabolic acidosis in WT or KI mice. Venous blood pH was significantly reduced in septic mice compared to sham-operated controls, but was not significantly different between genotypes (Figure 4-3D). Results for blood base deficit paralleled the findings for blood pH. Specifically, the base deficit concentrations were higher in CLP-operated animals, significantly at 4 hours, with a trend towards this difference remaining at 24 hours (Figure 4-3E). Although, blood bicarbonate (HCO₃⁻) concentration, another indicator of acid-base physiology, was not significantly different in septic animals compared to sham-operated control, the trend was towards being slightly lower in septic mice (Figure 4-3F).

Table 4-1. Blood biochemistry in septic sEH WT and KI mice

Parameter	4h Sham		4h CLP		24h Sham		24h CLP	
	WT	KI	WT	KI	WT	KI	WT	KI
Na⁺	147.5	146.3	145.3	147.6	145.4	145.7	148.1	144.8
(mmol/L)	±0.5	±0.9	±0.5	±0.8	±1.2	±0.7	±1.0	±1.3
K⁺	3.4	3.5	4.6	3.8	3.4	3.4	3.7	4.3
(mmol/L)	±0.1	±0.1	±0.1*	±0.2	±0.2	±0.1	±0.1	±0.4
Cl⁻	120.8	118.3	122.8	123.2	121.4	118.5	119.0	122.3
(mmol/L)	±0.6	±1.9	±1.3	±0.7	±1.9	±0.7	±1.0	±1.2
TCO₂	17.5	19.0	13.3	14.0	18.2	17.7	19.4	17.3
(mmol/L)	±0.3	±1.2	±2.3	±1.5	±1.0	±1.4	±1.3	±2.9
Haematocrit	29.0	29.7	33.7	30.4	24.6	26.7	29.0	31.8
(%PCV)	±1.4	±0.9	±2.0	±3.2	±2.4	±2.5	±1.2	±1.6
pCO₂	44.1	53.4	48.1	43.0	32.1	35.6	44.0	47.3
(mmHg)	±2.5	±3.2	±12.2	±8.7	±4.4	±3.5	±1.7	±8.0
AnGap	13.5	14.3	15.5	15.0	12.0	13.8	14.7	11.3
(mmol/L)	±0.6	±1.2	±0.9	±0.7	±1.5	±0.9	±0.6	±1.7
Haemoglobin	9.9	10.1	11.5	10.4	8.3	9.1	9.9	10.8
(g/dl)	±0.5	±0.3	±0.7	±1.1	±0.8	±0.8	±0.4	±0.5

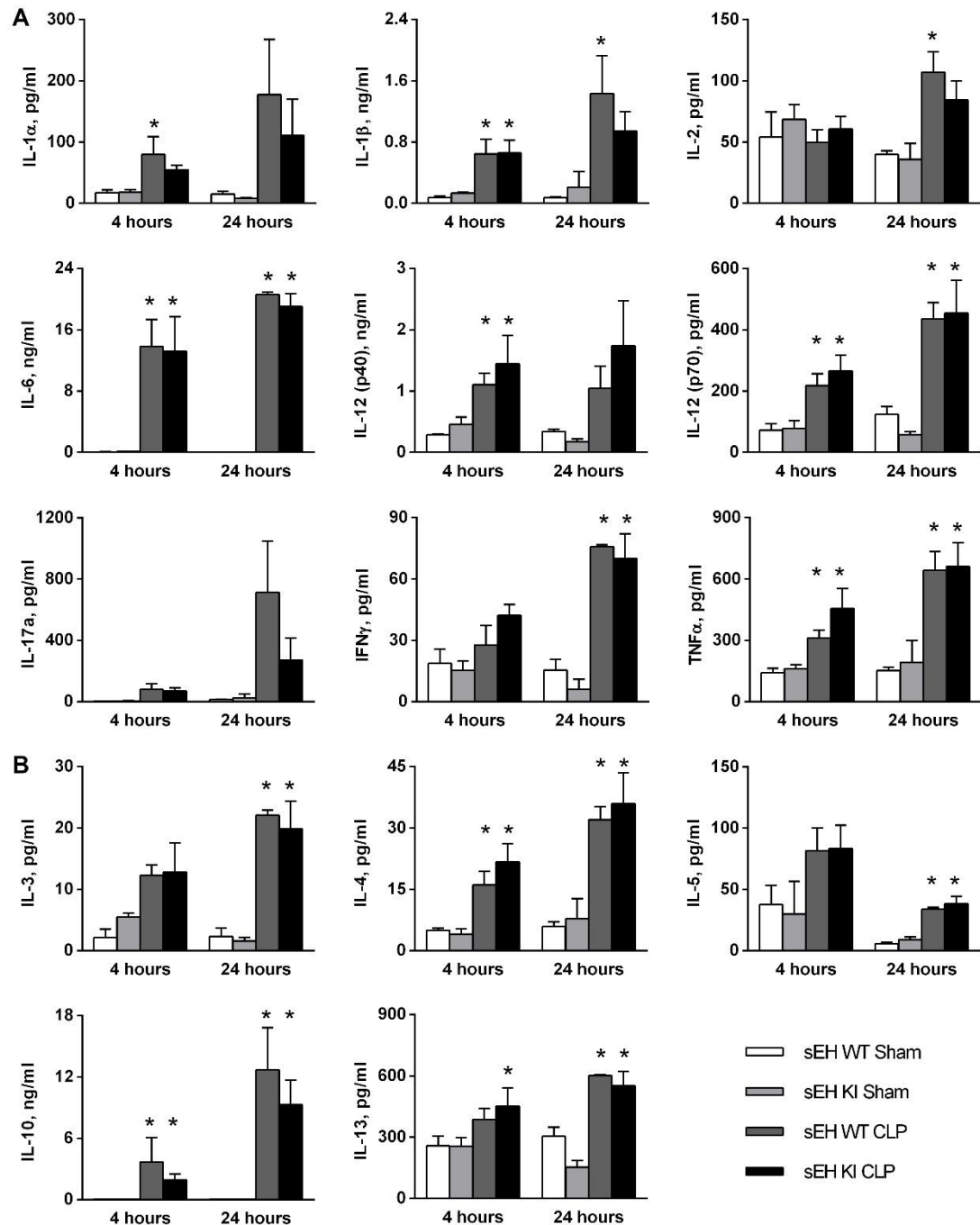
Blood biochemistry was measured 4 or 24 hours after CLP with an iSTAT analyser. Sham-operated animals served as a control. Data are present as mean with SEM, n = 10 mice per group. * significantly (p < 0.05) different from the control, one-way ANOVA and Tukey post-hoc test.

Blood Na⁺, Cl⁻, and total CO₂ levels were similar between WT or KI animals and did not change in response to CLP (Table 4-1). WT mice exhibited significantly higher blood potassium concentration compared to sham-operated control (4.6 ± 0.1 mmol/L vs 3.4 ± 0.1 mmol/L) 4 hours after CLP, probably due to deficiency in renal potassium excretion. In contrast, the KI mice did not exhibit this elevation in blood potassium at the 4-hour point, but had a trend towards it at 24 hours. Haematocrit, partial pressure of carbon dioxide, anion gap and haemoglobin were similar among the groups (Table 4-1).

Cytokine secretion in peripheral blood was similar between sEH WT and KI animals

As sepsis is characterised by altered cytokine and chemokine expression, their levels were measured in plasma of WT or KI mice 4 and 24 hours after sepsis induction using a multiplexed magnetic bead-based array.

Septic mice showed a time-dependent increase in pro-inflammatory cytokines Il-1 α , Il-1 β , Il-6, Il-12 (p70), Il-17A, IFN γ and TNF α (Figure 4-4A). Il-2 levels did not show any changes 4 hours after CLP, but were significantly higher by 24 hours. Il-12 (p40) was elevated 4 hours after the induction of sepsis, and remained at the same level 24 hours post-CLP. The most profound increase was for Il-6, which occurred in both genotypes. No significant difference was detected between septic WT or KI mice for pro-inflammatory cytokine levels, although, there was a trend towards decreased levels of Il-17A in septic KIs compared to WTs. Th2 cytokines (Il-3, Il-4, Il-5, Il-13) exhibit mostly anti-inflammatory actions and were found to increase in a time-dependent manner, as was observed for pro-inflammatory cytokines, except for Il-5, which was significantly reduced at 24 hours compared to the 4-hour point (Figure 4-4B). Il-10 expression was augmented more than 200-fold 24 hours after induction of CLP, but this increase was similar in both genotypes.

**Figure 4-4**

Pro-inflammatory and anti-inflammatory cytokine levels in septic WT versus KI mice. A. Pro-inflammatory cytokines in WT or KI mice subjected to the sham or CLP procedure. B. Anti-inflammatory cytokines in WT or KI mice subjected to sham or CLP procedure. Cytokine concentration was measured in plasma of WT or KI mice 4 and 24 hours after surgery induction using the Bio-Plex mouse cytokines 23-plex panel assay. Data are present as mean with SEM, $n = 4 - 5$ mice per group. *significantly different from sham-operated controls as based on one-way ANOVA with Tukey post-hoc test.

Chemokines mediate tissue infiltration and play crucial roles in the mechanisms of organ damage during sepsis. Most chemokines showed time-dependent increases in response to CLP (Figure 4-5). Eotaxin, G-CSF, GM-CSF and KC exhibited a significant increase 4 hours after CLP induction, and were further elevated 24 hours after the surgery. Levels of MCP-1 or MIP-1 β were not significantly different at 4 hours, but were significantly increased at 24 hours. RANTES was not different at the 4-hour time point compared to sham-operated controls, but increased more than 10-fold 24 hours post-CLP. 24 hours after induction of sepsis, KI mice exhibited significantly lower levels of G-CSF than WT mice (141.5 ± 25.4 ng/ml vs 254.6 ± 22.1 ng/ml).

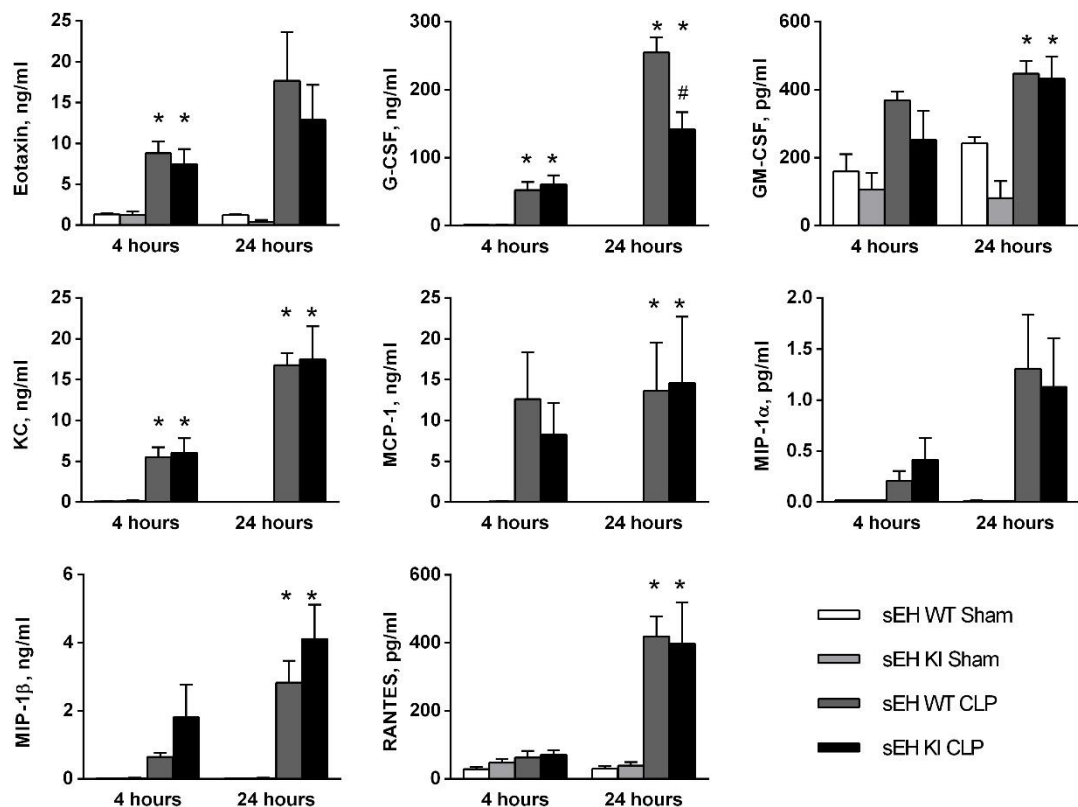


Figure 4-5

Chemokine levels in septic WT versus KI mice. WT or KI mice were subjected to Sham or CLP surgery. The concentration of eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), keratinocyte chemoattractant (KC), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 α (MIP-1 α), macrophage inflammatory protein 1 β (MIP-1 β) and regulated on activation normal T cell expressed and secreted (RANTES) were measured in plasma 4 and 24 hours after surgery using the Bio-Plex mouse cytokines 23-plex panel assay. Data are present as mean with SEM, $n = 4 - 5$ mice per group.

*significantly different from sham-operated control based on one-way ANOVA with Tukey post-hoc test. #significantly different as based on one-way ANOVA with Tukey post-hoc test.

Vascular reactivity of conduit and resistance arteries of septic WT and KI mice

To determine vascular reactivity during sepsis, the responses to increasing concentration of potassium chloride or the thromboxane A2 mimetic, U46619, were measured using wire myograph in resistance or conduit vessels 24 hours after sham operations or induction of CLP.

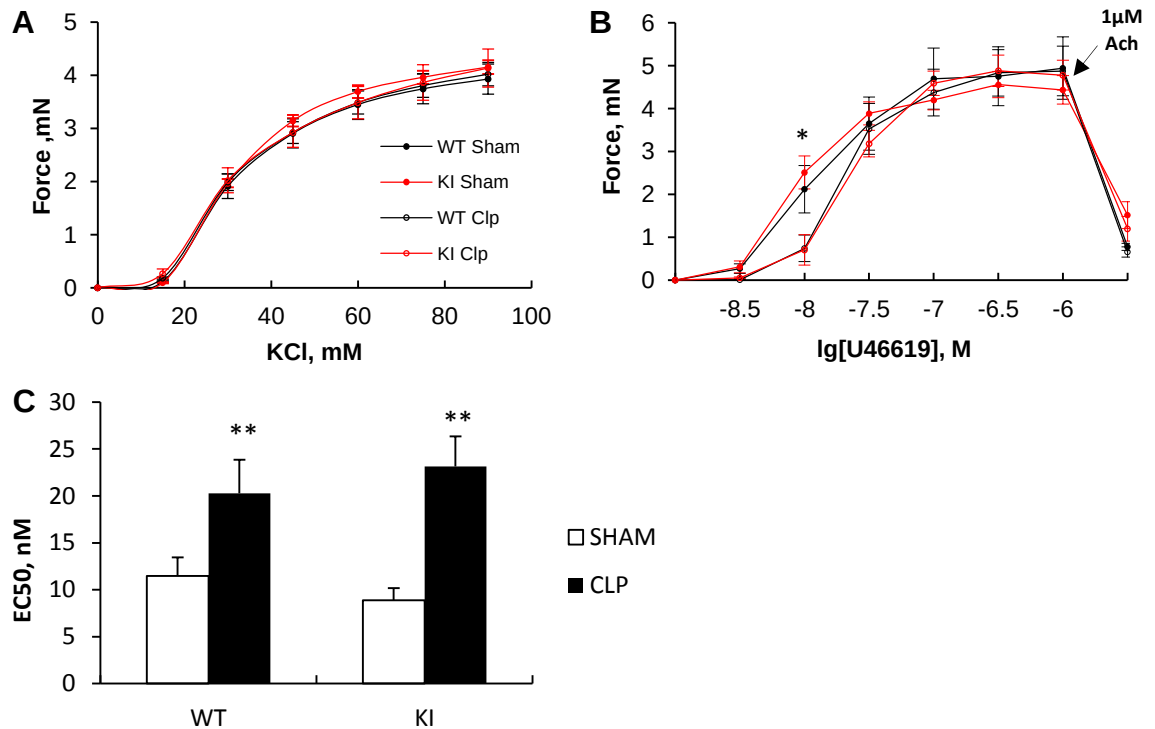


Figure 4-6

Vascular contraction of small mesenteric arteries from WT or KI mice subjected to CLP. A-B. Mice were sacrificed 24 hours after inducing sepsis by CLP. The constriction and relaxation were assessed using a DMT Wire Myograph. Concentration response curves to KCl and U46619 in 3rd order mesenteric arteries. C. Half maximum effective concentration (EC50), expressed as the concentration of U46619 required to produce 50% of the maximal tension. Data are present as mean with standard errors. * significantly ($p < 0.0001$) different from sham-operated controls as based on 2-

way ANOVA with Bonferroni post-hoc test; ** significantly ($p < 0.001$) different from sham-operated controls as based on 1-way ANOVA with Tukey post-hoc test. $N = 4-5$ mice per group.

KCl caused a similar contraction response of resistance vessels in septic and sham-operated mice, with no difference between WT or KI animals (Figure 4-6A). In small mesenteric arteries from CLP-induced mice the concentration response curve to the thromboxane A₂ mimetic, U46619, was shifted rightward, without change in the maximal response, compared to sham-operated control mice. Although, there was no significant difference between the two genotypes (Figure 4-6B). The half maximal effective concentration (EC_{50}) of U46619 was increased in septic mice compared to sham controls in both WT and KI mice during sepsis (Figure 4-6C). As shown in Figure 4-6B, mesenteric vessels relaxed to the same extent in response to 1 μ M acetylcholine (Ach), confirming that the endothelium was intact.

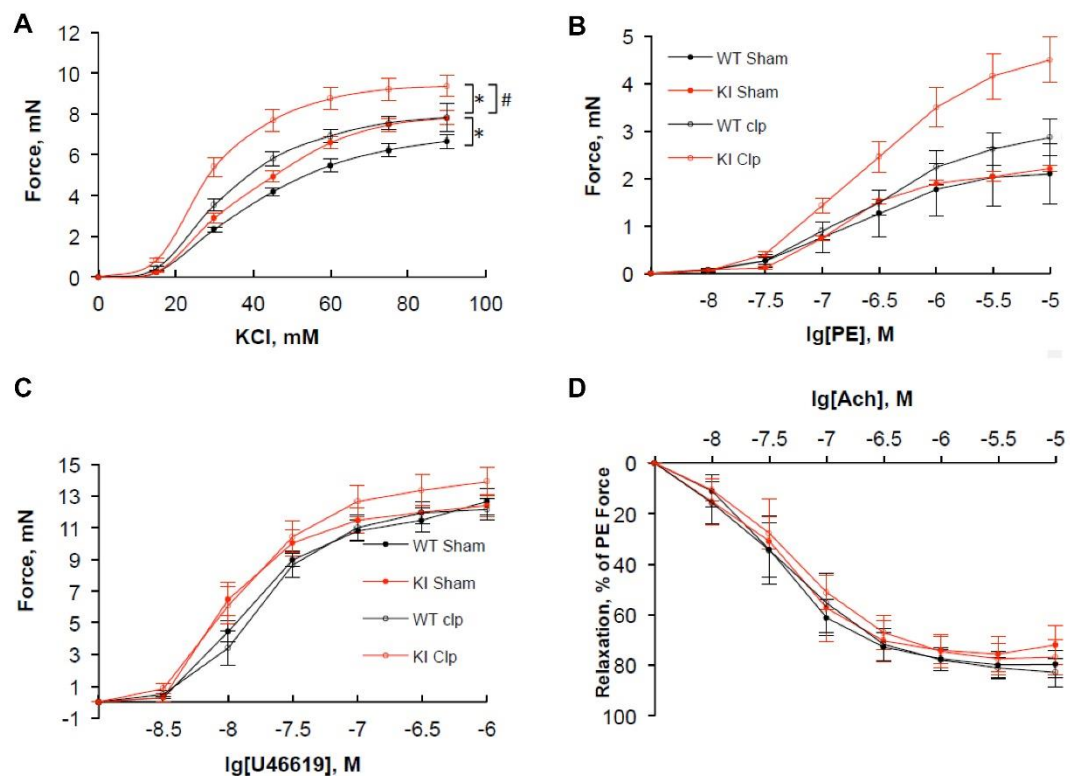


Figure 4-7

Vascular contraction and relaxation of aortic rings from WT or KI animals subjected to CLP.

A-D. Mice were subjected to CLP and sacrificed 24 hours later. Aortic rings constriction or relaxation was measured using DMT Wire Myograph. Concentration response curves to KCl, PE, U46619, and Ach in isolated aortic rings from WT and KI mice. Data are present as mean with

standard errors. * significantly ($p < 0.001$) different from sham-operated controls, # significantly ($p < 0.001$) different between WT CLP or KI CLP mice. The statistical analysis was based on 2-way ANOVA with Bonferroni post-hoc test. $N = 4 - 5$ mice per group.

CLP surgery induced a significant increase in the maximal response to potassium as well as a significant decrease in EC_{50} in aortae of WT or KI mice (Figure 4-7A). Moreover, sham-operated animals of different genotypes reacted differently to increasing concentrations of potassium, with vessels from KI mice exhibiting a potentiated response. Similarly, responses to the α_1 -adrenergic receptor agonist, phenylephrine (PE), were potentiated in aortae of KI mice subjected to CLP compared to those from septic WT animals or sham-operated controls from either genotype (Figure 4-7B). U46619 produced a concentration-dependent constriction in aortic rings, however, it was neither different between CLP- or sham-operated mice, nor between KI or WT mice (Figure 4-7C). The endothelium-dependent vascular relaxation in response to Ach was measured in aortic rings precontracted with PE. As shown in Figure 4-7D, the genotype had no significant effect on Ach-induced dilation, and sepsis did not affect the relaxation induced by this compound.

Identification of issues in mouse colony used in the sepsis studies outlined above

As the studies outlined above evolved, it became apparent that both the WT and KI mice (which were from the same colony) appeared visibly bigger than C57bl6/J mice of the same age from other colonies. The body weight of 8 weeks old mice was 33 ± 0.7 g and 30 ± 0.4 g for WT and KI mice respectively, and reached values of 43 ± 1.9 g and 40 ± 1.2 g by 30 weeks of age (Figure 4-8A). Based on literature review the body weight of C57bl/6 mice should be around 22 g at 8 weeks of age and 32 g at 30 weeks of age [461]. The mean arterial pressure of WT mice was significantly higher than that of KI animals (Figure 4-8B), which was surprising as sEH WT mice were originally reported to have lower blood pressure compared to KI littermates. Although, the reasons for the unexpected observations about the large mass of colony mice and lower blood pressure in the KI, were not clear, the decision was made to backcross mice to their original background.

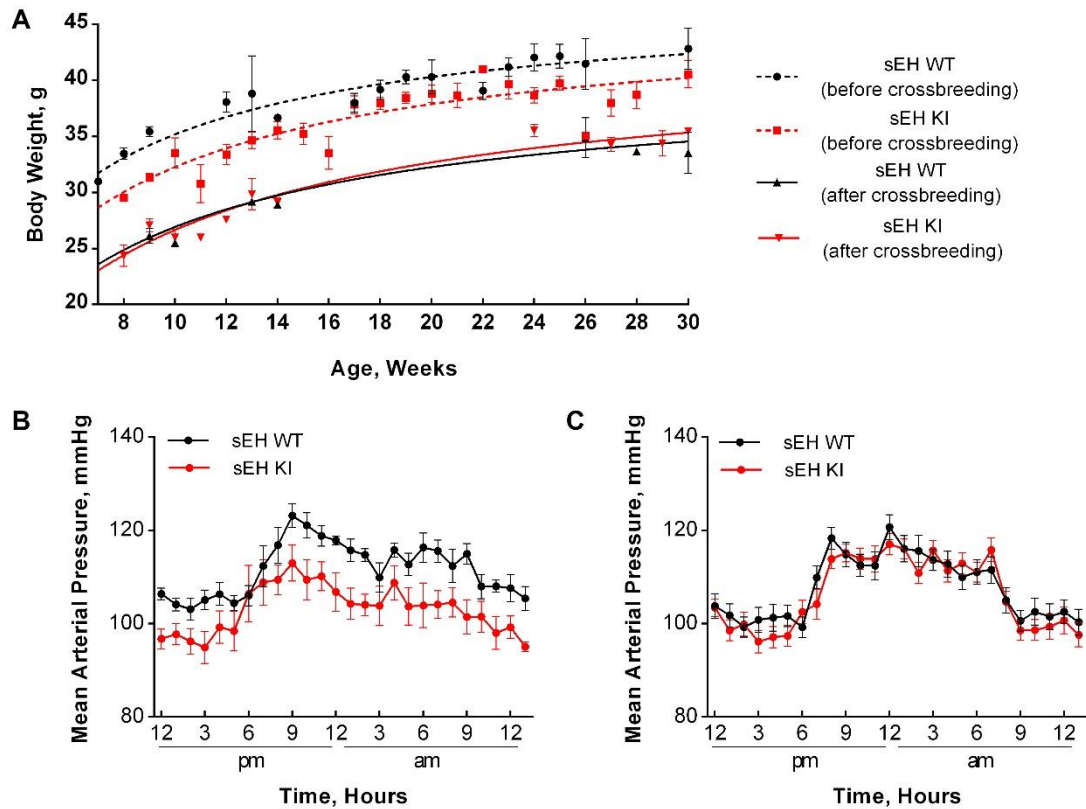


Figure 4-8

Effect of crossbreeding on body weight and blood pressure of WT or KI mice. A. Body weight curves of WT or KI mice before and after backcrossing. Data are present as mean with SEM, $n = 54 - 98$ mice per group. B. Mean arterial pressure in 24 – 28 weeks old sEH WT and KI mice before crossbreeding. Data are present as mean with SEM, $n = 8$ mice per group. C. Mean arterial pressure in 8-12 weeks old sEH WT and KI mice after crossbreeding. Data are present as mean with SEM, $n = 12$ mice per group.

After mice were backcrossed into C57bl6/J for 8 generations, the body weight and blood pressure of WT and KI mice were measured. Body weight curves for WT or KI mice returned to values typical for C57bl6/J mice (Figure 4-8A), and mean arterial blood pressure radio-telemetry measurements showed no difference between genotypes (Figure 4-8C).

Fatty acid content in chow diet does not have a short-term effect on mean arterial pressure

The difference in mean arterial pressure observed between WT and KI mice (Figure 4-8B) could potentially be attributed to the chow diet that mice were on, especially as

they differ considerably in their unsaturated fatty acid content and so their propensity to form lipid electrophiles. Consequently, the effects of diet on mean arterial pressure was assessed in C57bl/6 mice. Mice were fed either a normal chow diet (RM1) or a chow diet with higher fatty acid content (RM5 and Transbreed) for 10 days and blood pressure and heart rate were measured using radio-telemetry. Mean arterial pressure and heart rate were similar between mice fed either diet as shown in Figure 4-9.

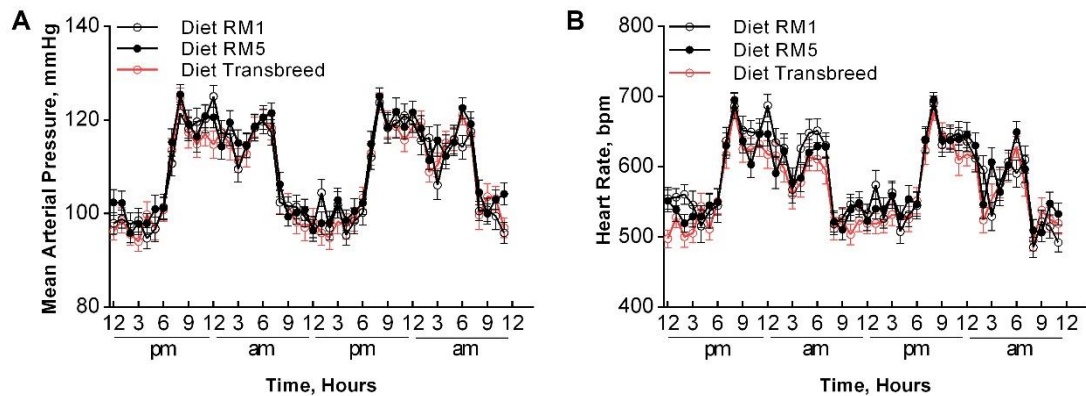


Figure 4-9

Effect of diets with different fatty acid content on blood pressure and heart rate. Mean arterial pressure and heart rate were measured in conscious 8-12 weeks old C57bl/6/J mice by radio-telemetry. Mice were kept on the stated diets for 10 days before the haemodynamic data was acquired. Variables were recorded every five minutes. Data are present as mean with SEM, $n = 9$ mice per group.

Young WT or KI mice have similar haemodynamic responses to sepsis

While sepsis resulted in a similar blood pressure drop in older WT or KI mice, the body weight and basal blood pressure of the mice used for those experiments were somewhat abnormal as reported above. Thus, the effect of sepsis on haemodynamic parameters was assessed in a younger cohort of mice with normal body weight after crossbreeding. 8-12 weeks old WT or KI mice were subjected to sham or CLP surgery and their blood pressure and heart rate were measured using radio-telemetry. Sepsis induced a drop in mean arterial pressure both genotypes (Figure 4-10A). The mean arterial pressure started steadily declining two hours after sepsis induction, reaching its lowest point 5-8 hours after the surgery. Over the 24-hour period mean arterial pressure dropped by 25 ± 5.7 mmHg or 34 ± 7.2 mmHg in WT or KI mice, respectively, which was not statistically different (Figure 4-10C). As expected, sepsis increased heart rate, which

was similar between genotype and peaked two hours after the surgery. Subsequently, heart rate steadily declined and fell below basal 5-6 hours after induction of sepsis (Figure 4-10B). The heart rate decreased by 140 ± 49.0 bpm or 183 ± 26.9 bpm in WT or KI mice respectively (Figure 4-10D). Although, there was no statistical difference between genotypes, KI mice showed a trend towards having more of a decline in mean arterial pressure and heart rate compared to the WT.

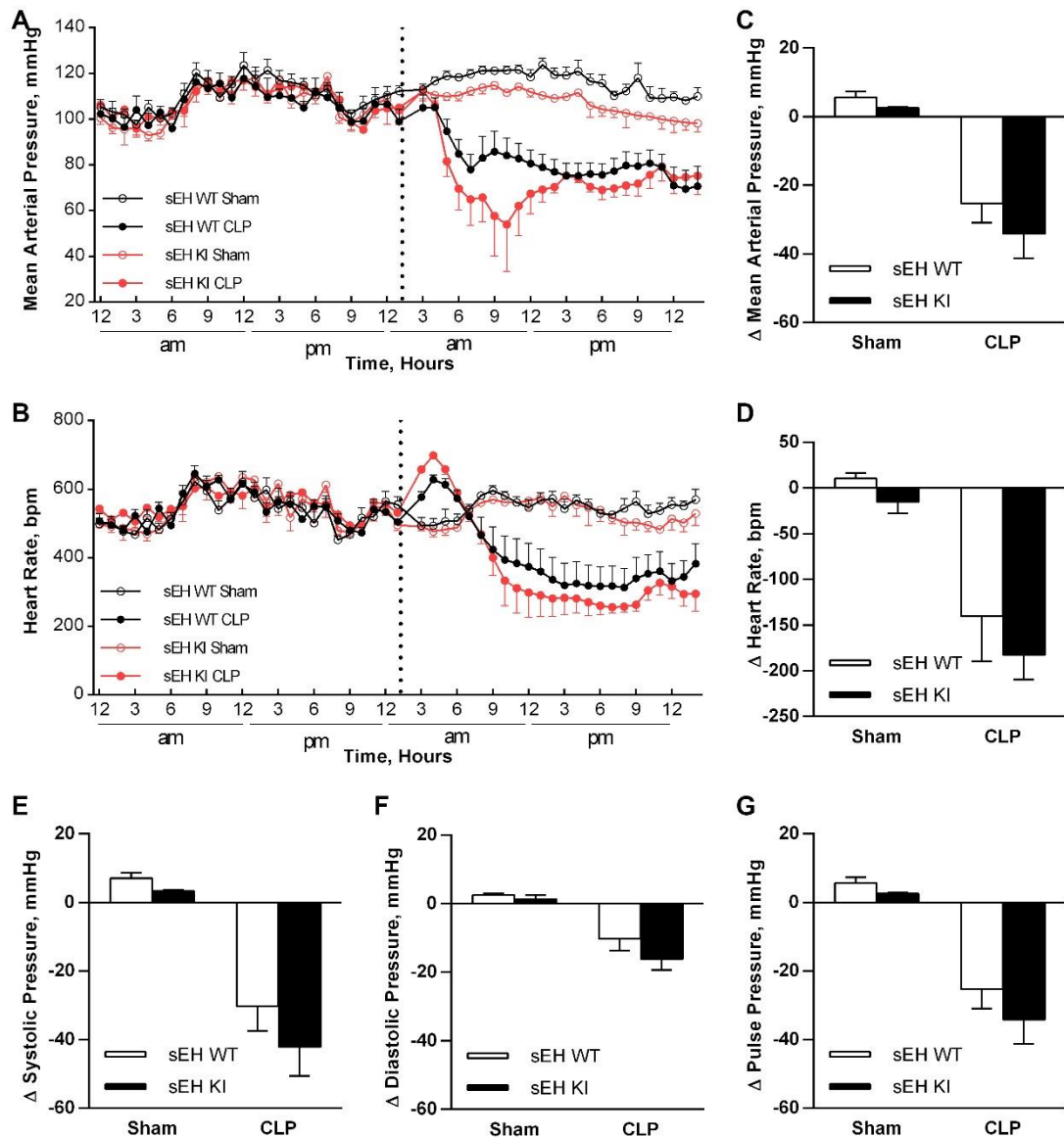


Figure 4-10

Haemodynamic parameters in young WT or KI mice subjected to sepsis. A-B. Time course of mean arterial pressure and heart rate in WT and KI mice 24 hours before and after Sham or CLP surgery. The induction of surgery is represented by the dotted line. Data are present as mean with

SEM, n = 6 mice per group. C-G. Averaged difference in mean arterial pressure, heart rate, systolic, diastolic and pulse pressure before and after Sham or CLP surgery. Data are present as mean differences from the time-matched baseline with SEM, n = 6 mice per group.

Changes in systolic and diastolic pressure reflected those reported for mean arterial pressure data (Figure 4-10E,F). Systolic pressure decreased more than diastolic pressure, resulting in a decreased pulse pressure (Figure 4-10G), with this change being the same between genotypes.

The severity of sepsis was similar between young sEH WT and KI mice

Sepsis score, body temperature and locomotor activity were used to assess the severity of sepsis. Both WT and KI mice showed similar sepsis scores as measured after induction surgery (Figure 4-11A), their body temperature dropped to $26 \pm 2.3^{\circ}\text{C}$ and $27 \pm 2.4^{\circ}\text{C}$, respectively, which was not statistically significant (Figure 4-11B). Locomotor activity was measured using radio-telemetry. Compared to time-matched basal values, septic mice of either genotype were more active the first 4 hours post-CLP, after which they remained relatively inactive until they were culled (Figure 4-11C).

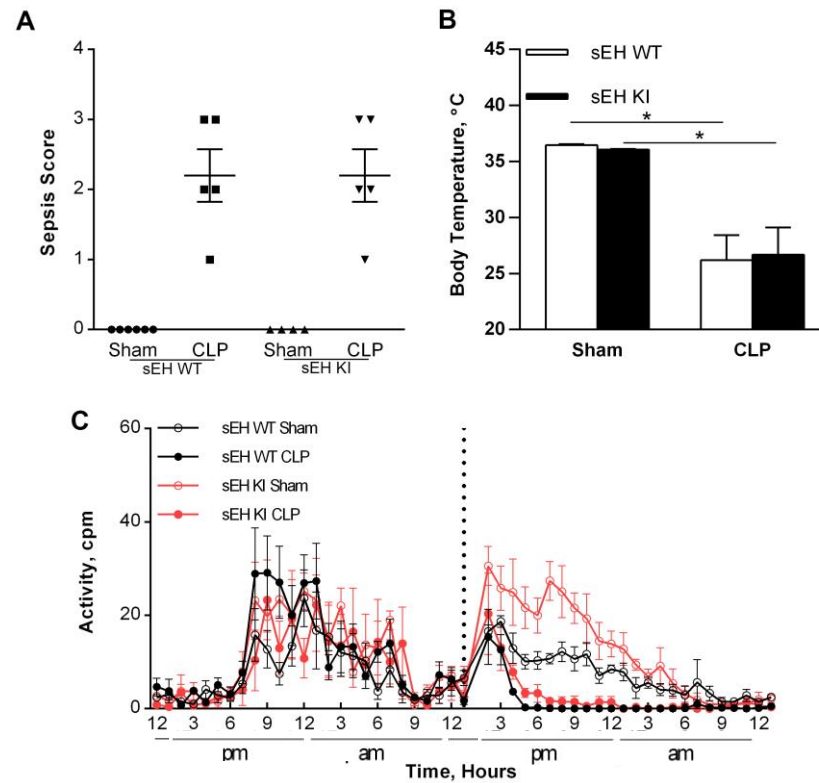


Figure 4-11

Severity of sepsis in young WT or KI mice. A. Sepsis score was indexed 24 hours after Sham or CLP surgery induction. B. Body temperature was measured in WT or KI mice 24 hours after Sham or CLP surgery induction using a rectal probe. C. Locomotor activity in WT or KI mice subjected to Sham or CLP surgery was measured 24 hours before and after surgery induction by radio-telemetry. Data are present as mean with SEM, n = 6 mice per group.

sEH WT and KI mice show similar levels of organs injury and metabolic acidosis in response to sepsis

Concentrations of BUN and cTnI, markers of kidney and cardiac injury, respectively, were measured in venous blood 24 hours after Sham or CLP surgery. Both of the markers significantly increased in response to sepsis, however, there was no difference between genotypes (Figure 4-12A,B). Septic WT and KI mice became hypoglycaemic 24 hours after sepsis induction to the similar extent (Figure 4-12C).

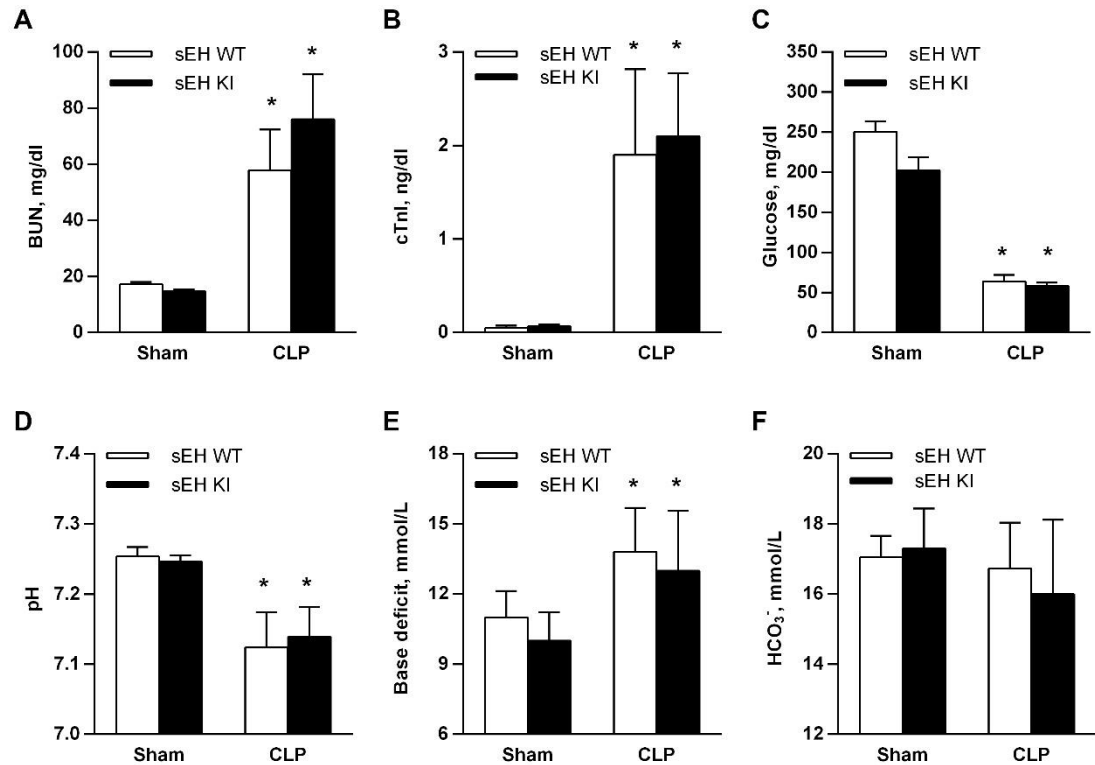


Figure 4-12

Sepsis induced multiorgan injury and acid-base imbalance in young WT or KI mice. A-B. Markers of organ injury, namely blood urea nitrogen (BUN) and cardiac troponin I (cTnI), in WT or KI mice subjected to Sham or CLP surgery. C. Glucose levels in WT and KI mice subjected to Sham or CLP surgery. D-F. Acid-base balance in WT and KI mice subjected to Sham or CLP surgery. The markers were measured in venous blood 4 hours and 24 hours after surgery induction by iSTAT analyser. The data are present as mean with SEM, n = 10 mice per group. *significantly different from sham-operated controls as analysed by one-way ANOVA with Tukey post-hoc test.

Signs of metabolic acidosis were noted in septic mice of either genotypes 24 hours after CLP surgery. The pH of venous blood significantly decreased, and base deficit significantly increased, however, the concentration of HCO₃⁻ was not statistically different. No statistical difference was observed between genotypes (Figure 4-12D-F).

Venous blood concentration of Na⁺, K⁺ or Cl⁻ were similar between WT and KI mice and did not change in response to sepsis (Table 4-2).

Table 4-2. Blood biochemistry in young septic sEH WT and KI mice

Parameter	24h Sham		24h CLP	
	WT	KI	WT	KI
Na⁺ (mmol/L)	145.0 ±0.89	144.8 ±0.85	144.6 ±1.0	145.0 ±0.45
K⁺ (mmol/L)	3.7 ±0.2	3.7 ±0.1	3.7 ±0.2	4.2 ±0.5
Cl⁻ (mmol/L)	118.4 ±0.7	118.5 ±1.3	120.8 ±0.8	121.2 ±1.9
TCO₂ (mmol/L)	18.0 ±0.7	18.5 ±1.3	17.0 ±1.7	17.4 ±2.3
Haematocrit (%PCV)	30.5 ±0.7	30.5 ±1.0	37.0 ±2.0*	31.2 ±0.7
pCO₂ (mmHg)	38.78 ±2.2	39.73 ±2.5	48.0 ±6.8	46.9 ±5.7
AnGap (mmol/L)	13.2 ±0.7	13.0 ±1.1	12.0 ±0.95	11.8 ±1.2
Haemoglobin (g/dl)	10.4 ±0.2	10.4 ±0.3	12.0 ±0.5*	10.6 ±0.2

Blood biochemistry was measured 4 or 24 hours after CLP with an iSTAT analyser. Sham-operated animals served as a control. Data are present as mean with SEM, n = 6 mice per group. * significantly ($p < 0.05$) different from the control, one-way ANOVA and Tukey post-hoc test.

Interestingly, haematocrit levels significantly increased in septic WT mice (37 ± 2.0 % vs 31 ± 0.7 %), but not sEH KI mice (31 ± 1.0 % in sham animals and 31 ± 0.7 in septic mice). Haemoglobin mimicked the pattern for haematocrit, being significantly elevated in WT mice in response to sepsis (12.0 ± 0.5 g/dl vs 10.4 ± 0.2 g/dl), but not in septic KI animals (10.6 ± 0.2 g/dl vs 10.4 ± 0.3 g/dl). TCO₂ and pCO₂ were similar between all groups of animals.

4.4. Discussion

sEH hydrolyses EETs, endogenously generated epoxy lipids which modulate numerous vascular effects, such as angiogenesis or dilation of peripheral and coronary blood vessels. sEH is redox regulated by adduction of electrophilic lipids to Cys521 [29, 30].

Although the mechanisms contributing to vasodilation and hypotension during septic shock or those mediating revascularisation after ischaemia have been extensively studied, a potential role for sEH C521-dependent redox regulation in each of these scenarios has not been reported. This study aimed to characterise the role of sEH redox regulation in each of these biomedically important processes by utilising a C521S sEH KI redox-dead mice that express a form of the hydrolase that is resistant to lipid electrophile-dependent inhibition [30].

sEH redox regulation and sepsis

The CLP model of sepsis, which is considered to be a “gold standard” because it recreates the aetiology of human sepsis more closely than those administering endotoxins, was utilised. CLP induces similar haemodynamic and metabolic changes to those that occur in human sepsis, which is characterised by the presence of both hyper- and hypoinflammatory phases [462]. Due to the fact that sepsis affects mostly the elderly population, with 80% of septic deaths occur in individuals over 65, the decision was made to use 24 – 28 weeks old mice [463]. The blood pressure and vascular reactivity were attenuated during septic shock, which is in accordance with previous studies [290]. CLP produced a profound fall in mean arterial pressure in both WT as well as C521S sEH KI mice, which reached its lowest point approximately 4 hours after the induction and was sustained for the next 20 hours till animals were culled. During the first 4 hours of septic shock mice exhibited tachycardia which is consistent with reflex neurohumoral responses attempting to sustain perfusion by maintaining cardiac output and blood pressure. These include baroreceptor reflexes, release of catecholamines, activation of renin-angiotensin system, release of antidiuretic hormones and generalised sympathetic stimulation. KI mice had a lower heart rate compared to WT after CLP induction, although this difference was not statistically significant. EETs do not seem to regulate heart rate [464], although they were shown to be cardioprotective in different models [314, 465]. However, there was no difference between genotypes in levels of cTnI, a biomarker of heart injury, measured in plasma of sEH KI or WT animals after sepsis induction. Interestingly, 24 after sepsis induction KI mice had aggravated severity of sepsis as assessed by sepsis score and body

temperature. Hypothermia was evident 4 hours after the induction of CLP and the body temperature of septic mice was even lower 24 hours after.

Several studies have assessed the role of sEH during inflammation and sepsis, however these data are contradictory. Some studies reported that inhibition of sEH or deletion of EPHX2 gene are beneficial during inflammation. Schmelzer et al showed that pharmacological inhibition of sEH with AUDA-BE, which increased levels of EETs, reduced LPS-induced mortality and tissue injury, as well as increased inflammatory cytokines in an LPS-induced sepsis model in mice [245]. Furthermore, application of sEH inhibitors reduced thermal hyperalgesia and inflammatory pain in an LPS-induced sepsis model in rats [258]. sEH knock-out mice, as well as mice overexpressing endothelial CYP2J2 and CYP2C8 epoxygenases showed reduced levels of inflammatory gene expression and neutrophil recruitment in the lung following LPS injection [244]. Inhibition of sEH also reduced tobacco smoke-induced lung inflammation in spontaneously hypertensive rats [253]. Treatment with sEH inhibitors decreased total bronchoalveolar lavage cell number with significant reductions noted in neutrophils, alveolar macrophages and lymphocytes in tobacco smoke-exposed rats. Co-administration of sEH inhibitors with EETs further enhanced the anti-inflammatory effect of sEH inhibition [253]. Deletion of sEH reduced the inflammatory cell infiltration, pro-inflammatory cytokine expression and NF- κ B signalling activation caused by IL-10 deficiency in IL-10 KO mice [466, 467].

As EETs have anti-inflammatory roles, a logical suggestion is that sEH inhibition during sepsis may be protective. However, the evidence for this is contradictory. Fife et al reported that sEH knockout mice did not have attenuated inflammatory gene expression or neutrophil accumulation in the liver in the LPS-induced model of sepsis [248]. Another study demonstrated that sEH inhibition did not reduce neutrophil recruitment in the lung, utilising a rat model of tobacco smoke-induced lung inflammation. sEH inhibition also failed to reduce tobacco smoke-induced adhesion molecule expression in the lung vasculature [468]. Moreover, this study was unable to replicate the previously reported reduction of TNF α -induced expression of the adhesion molecules following EET application to human lung microvascular endothelial cells or human umbilical vein endothelial cells. Additionally, 5,6-EET and 14,15-EET were

reported to increase endothelial permeability in isolated rat lungs via activation of the TRPV4, suggesting their participation in the pathogenesis of acute lung injury [469, 470]. Unfortunately, the EET/DHET ratio was not measured in this study as the method is not available in the facilities.

Brachial artery flow-mediated dilation (FMD) is a common assessment tool of endothelial function in humans [471]. As endothelial function is disturbed during inflammation, whether changes in FMD correlated with EET/DHET ratios in patients with stable atherosclerotic disease was investigated, but no association was observed [472]. Moreover, Schuck et al did not observe any association between biomarkers of CYP-mediated eicosanoid metabolism and high sensitivity C-reactive protein (hs-CRP), which is a well-known marker of systemic inflammation in patients [473]. This suggests that EETs might not be as important as they have been depicted in the literature in terms of attenuating inflammation in humans [472]. This discrepancy in the data regarding a role for sEH during inflammation may be due to methodological differences between the studies. The methods to induce inflammation vary considerably, with some studies utilising CLP and other LPS. CLP-induced and LPS-induced inflammation vary significantly in the severity, with the exact composition of the lipopolysaccharide varying a lot as it often originates from different bacteria strains. Moreover, pre-clinical studies often utilise different species, are inconsistent in the organs in which inflammation is assessed, with measurements made at varying time points.

Due to the wealth of data suggesting EETs have anti-inflammatory properties, sEH KI mice were speculated to have higher pro-inflammatory markers. Thus, the cytokine and chemokine levels were measured in plasma of WT or KI mice, but no substantial difference between genotypes was observed, neither 4 hours after septic shock induction, nor at 24 hours. G-CSF was the only cytokine that significantly differed between genotypes. Although, the mechanisms behind it are unclear, it is important to point out that this highlights a difference in the biological responses of each genotype. Inflammatory biomarkers were significantly elevated in response to CLP induction with increasing magnitude overtime. These results are in agreement with previous studies [474-477].

One consideration is that the lack of difference in BP between genotypes during septic shock be explained by the changes in sEH mRNA and protein expression. Notably, several studies have shown that sepsis causes an increase in sEH mRNA and protein expression in the kidney and aorta, with a correlative decrease in the EETs/DHETs ratio [478, 479]. However, it should be highlighted that this is not uniformly the case, as other studies found sepsis down-regulated sEH mRNA in the liver, kidney, and lung [248, 479]. Thus, one could speculate that CLP-induced septic shock down-regulates sEH mRNA levels and protein expression, potentially explaining why the sEH C521S KI mice were not protected from hypotension during sepsis. The sEH mRNA levels have not been assessed in the current study, however the protein levels in heart and aorta tissue samples were not different between sham and septic animals. Changes in the CYP450 epoxygenases protein expression pattern during sepsis adds additional complexity to the current picture of EET/DHET levels during septic shock [479].

Another important consideration is that EETs can undergo a number of other metabolic conversions besides the major hydrolysis by sEH. These include partial β -oxidation, chain elongation or oxidation by COX, LOX or CYP ω -oxidase enzymes, as well as conjugation with glutathione [480]. During pathological processes, including septic shock, alternative pathways for EET catabolism could predominate, potentially minimising the role of sEH in EET degradation. Moreover, catabolism of EETs by enzymes other than sEH generate vasoactive species that may impact on blood pressure, including during sepsis. For example, 10,11-Epoxy-16:2, the 14,15-EET β -oxidation product, relaxed porcine or human coronary arterioles contracted with endothelin with the same potency as 14,15-EET [481, 482]. A 22-carbon chain-elongated EET product, 16,17-epoxydocosatrienoic acid, was able to dilate constricted canine and porcine coronary arterioles with a potency comparable to that of EETs [481, 483]. COX was reported to metabolise 5,6-EET and 8,9-EET. The 5,6-epoxy-PGE1 is equipotent to 5,6-EET as a vasodilator, and the 5,6-EET vasodilator activity in the isolated perfused rabbit kidney was blocked by a COX inhibitor [484, 485].

Vascular function was assessed in WT or sEH C521S KI mice subjected to a CLP or a sham procedure. Numerous *in vivo* or *in vitro* studies have shown vascular hyporesponsiveness to different vasoconstrictors, including α -adrenergic agonists, Ang

II, KCL, endothelin or thromboxane A₂, following induction of sepsis. Similarly in the studies here, sepsis impaired constriction in response to the thromboxane A₂ mimetic U46619, in mesenteric arteries of WT or sEH C521S KI mice with no difference between genotypes. Interestingly, in contrast to a pronounced impairment of contraction in mesenteric arteries, sepsis did not impair the contractile responses to KCl or PE in aortae of mice subjected to CLP. Moreover, KCl-induced constriction was potentiated in aortae from septic animals compared to sham controls. Aortae from sEH C521S KI mice also showed an elevated contractile response to KCl compared to those from WT animals. There are conflicting data concerning the effects of sepsis on vascular constriction. Farmer et al showed that constriction induced by KCl was unaffected by LPS infusion in rats at 24 hours and showed a trend towards an increase instead of the decreased observed by others [486]. Similarly, in the study by Parker and Adams, LPS did not modify the KCl contraction in aortae from LPS-injected guinea pigs [487]. It should be emphasised that resistance arteries are more significant in the distribution of blood flow and determining peripheral resistance than the conduit arteries.

The sEH KI mice were previously reported to have elevated blood pressure compared to WT controls [30]. In marked contrast, the KI were not found to be hypertensive in this study, and actually showed a significantly lower blood pressure. Ejection fraction and cardiac output, assessed by echocardiography, were similar in sEH KI mice and WT mice (data not shown). Another surprising characteristic of the mice was that their weight was significantly higher for a given age than is typical, although the weight was elevated in mice of either genotype. There is a possibility that the initial phenotype of a newly generated transgenic line can change over time. For instance, sEH knockout mice when generated were reported to have lower blood pressure than WT animals, however, the difference disappeared over time, as later studies could not confirm it in two independently derived colonies [263]. The authors suggested this to be due to a phenotypic adaptive response in renal lipid metabolism. Another possibility is that a modifying mutation can arise over the generations that give a selective advantage toward reducing a phenotype. As an example, transgenic mice expressing activated calcineurin in the heart originally died from 3 to 5 months of age, however, sometime after they were able to survive up to 1.5 years, despite strong overexpression as originally characterised with massive cardiac hypertrophy [488]. It should be noted that

mice used in this study were double the age of the mice characterised in the study by Charles et al. Backcrossing mice into C57bl6/J background normalised their body weight and, although, blood pressure of sEH KI mice did not return to values described in the published work, it was not significantly different from WT mice, as was reported for KI mice before backcrossing. In backcrossed WT or KI mice the severity of sepsis, sepsis-induced haemodynamic changes, organ damage, and markers of metabolic acidosis were similar to the ones reported for mice before backcrossing.

The current study used complementary genetic and chemical approaches to study sEH redox regulation involvement in septic shock. The findings overall support the conclusion that severe hypotension and inflammation during sepsis cannot be rescued by preventing sEH redox regulation via Cys521. It is not clear from the study whether sEH is not redox regulated during septic shock, whether unknown products of sEH hydrolysis are detrimental or whether beneficial anti-inflammatory properties of EETs outweigh the possible detrimental vasodilatory properties. Further studies are required to identify the role of sEH during septic shock. Using LC-MS/MS to quantifying the lipid profile in the WT or KI mice before or after sepsis would potentially be illuminating.

Chapter 5. sEH redox regulation in post-ischaemic revascularisation

Chapter 5 focuses on soluble epoxide hydrolase (sEH) redox regulation in settings of post-ischaemic revascularisation. sEH substrates are well-known regulators of angiogenesis, suggesting a role for sEH in revascularisation. Numerous proteins were shown to be redox regulated during ischaemia, which could also be the case for sEH. The aim of the current study was to investigate a role of sEH redox regulation in angiogenesis by comparing responses in sEH WT and C521S KI mice. Following induction of hindlimb ischaemia, blood flow recovery in sEH KI mice was similar to WT littermates as assessed by laser Doppler flowmetry. Likewise, angiogenesis, as measured by CD31-stained capillary density in the gastrocnemius muscle, did not differ between each genotype. Granulocyte colony-stimulating factor levels increased in the muscle in response to ischaemia, however, this was equivalent in both genotypes, as were the G-CSFR levels. Notably, in hindlimb ischaemic WT and KI sEH formed high molecular weight complexes, which could be reduced by β -mercaptoethanol. These data suggest that regulation of sEH activity by the adduction of electrophiles to cysteine 521 probably is not important for the regulation of angiogenesis during post-ischaemic revascularisation.

5.1. Introduction

Soluble epoxide hydrolase in post-ischaemic revascularisation

A role for cytochrome P450 epoxygenases and their products, epoxygenated fatty acids, in angiogenesis has been demonstrated in several studies using in vitro and in vivo models [344-346]. However, the majority of studies investigating the role of epoxygenated fatty acids in angiogenesis have so far mostly focused on the effects of EETs and largely ignored other epoxygenated fatty acids and the products of their hydrolysis, namely diols. Moreover, it should be noted that the majority of studies were performed using cultured cells and utilised either the exogenous application of EETs, CYP450 inhibitors or the overexpression of epoxygenases of interest. Therefore, so far very little evidence has been gained linking *endogenous* production of EETs with proliferation and angiogenesis. To elucidate the lipid mediators responsible for revascularisation after ischaemia, studies investigating the inhibition of sEH activity or decreasing its expression are anticipated to be illuminating. Such an approach was used to study the role of epoxygenated fatty acids and corresponding diols in postnatal retinal angiogenesis [237]. Genetic deletion of sEH, as well as its inhibition, attenuated sprouting angiogenesis in mouse retina [237]. These effects were attributed to the lack of the sEH product 19,20-DHDP. Attenuated fatty acid diol levels have also been linked to defects in the formation of the caudal vein plexus in zebrafish. In the mouse model of hindlimb ischaemia, deletion of sEH results in significantly impaired blood flow recovery and vascularisation, which can be rescued by treatment with 12,13-DiHOME.

While EETs, CYP450 epoxygenase metabolites derived from ω -6 fatty acid, promote endothelial cell proliferation and have pro-angiogenic effects, metabolites derived from ω -3 fatty acids have been reported to inhibit angiogenesis and attenuate neovascularisation. Dietary ω -3 polyunsaturated fatty acids protect against pathological retinal angiogenesis, both in mice and humans [489-491]. CYP epoxygenases metabolise ω -3 acids, DHA and EPA, resulting in accumulation of 19,20-EDP and 17,18-EEQ, respectively. Both of these epoxides act as mediators of vascular regression in a mouse model of neovascular age-related macular degeneration

The studies considered above together are consistent with an important role for sEH in angiogenesis as shown in different animal models. The implication is that sEH could be a potential therapeutic target in angiogenic diseases. The inhibition of sEH has opposing effects on angiogenesis in different tissues or disease models, consistent with sEH hydrolysing both pro-angiogenic EETs or anti-angiogenic EDPs and EEQs. Thus, it is essential to further elucidate the mechanisms that regulate sEH endogenously, which is a key aim of the studies described in this chapter. Oxidative stress is a well-known mediator of angiogenesis [492]. One of the major consequences of ROS presence is peroxidation of lipids. Polyunsaturated fatty acid components of phospholipids in membranes are particularly prone to oxidation [45, 493]. As discussed in the general introduction, sEH activity can be regulated by adduction of electrophilic lipids to Cys521 [29, 30]. Numerous electrophilic lipids are formed via membrane peroxidation processes, that are known to occur during hypoxia, and thus might contribute to post-ischaemic revascularisation [494]. The endogenous redox regulation of sEH by electrophilic lipids might be one of the factors regulating such revascularisation. This chapter investigates this possibility by utilising C521S sEH KI mice, which are insensitive to sEH inhibition by electrophilic lipid adduction - comparing their responses to WT in a hindlimb ischaemia model.

5.2. Materials and methods

Mouse model of hindlimb ischaemia (HLI)

8-10-week old WT or KI mice were anaesthetised with isoflurane (Animal Care). The anaesthesia was induced in the induction chamber with 5% isoflurane and 0.5 L/min oxygen flow, and subsequently maintained via inhalation of 2% isoflurane via a nose cone. Throughout the surgery, the mice were kept on a 37°C heated pad in the dorsal recumbency position. The right lower abdominal quadrant of the limb was shaved and disinfected with Hydrex Pink (Ecolab) and hindlimb ischaemia was induced as described previously [495]. Briefly, the left femoral artery was exposed via a 10 mm incision in the skin. It was separated from the femoral vein and femoral nerve, with extra care taken to avoid injury to the nerve or vein. The femoral artery was ligated above and below the proximal caudal femoral artery branch for induction of mild ischaemia, or the lateral circumflex femoral artery for induction of moderate ischaemia. After the ligation with 8-0 nylon suture (Ethicon) the intervening artery segment was excised. The skin was closed with 6-0 Mersilk suture (Ethicon) and a single dose of buprenorphine (Vetergesic) (0.1 mg/kg) was administered subcutaneously to reduce pain.

Administration of sEH inhibitor, t-AUCB

t-AUCB at 3 mg/kg/day was delivered for 28 days subcutaneously by osmotic mini-pumps (Alzet, Alza Pharmaceuticals). Mini-pumps were implanted as discussed in general materials and methods section. t-AUCB was dissolved in 1:1 DMSO and polyethylene glycol 600.

Measurement of blood flow recovery

Immediately after the femoral artery ligation surgery, as well as at 7 day intervals, the blood flow was assessed in the operated or control limbs using a Laser Doppler Imager LDI2-HIR (Moor Instruments). The anaesthesia was initiated and maintained as described above. For the duration of the scanning the body temperature was kept at

37°C. The data was presented as the mean ratio of blood flow in the ischaemic to that in the non-ischaemic hindlimb.

Tissue collection for G-CSF measurements and western blotting

24 hours after the HLI surgery induction, the gastrocnemius (GC) muscle of the operated and control limbs were excised and washed twice with ice-cold PBS buffer. The muscle was cut into small pieces and homogenised in 1 ml of Bio-Plex cell lysis buffer (Bio-Rad) with a shaft homogeniser at 19000 rpm for 1 minute on ice. 2 mM phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, was added to the cell lysis buffer prior homogenisation. The homogenates were frozen in liquid nitrogen, thawed on ice and subsequently sonicated using an ultrasonic processor (Jencons, 3 mm microtip) with 10 second cycles repeated 10 times at a 40% amplitude. The samples were aliquoted and stored at -80°C until further analyses.

Measurement of protein concentration

The protein concentration in muscle lysate samples was measured using the bicinchoninic method (Pierce BCA protein assay kit, Thermo Scientific) according to the manufacturer's instruction. Briefly, the muscle homogenate samples were thawed on ice and centrifuged at 4500g for 5 min. The reaction was performed in clear polystyrene 96 well plates by adding 5 µl of supernatant to 200 µl working solution (50:1, reagent A:B). The plate was incubated for 30 min at 37°C and subsequently the absorbance was measured with an EL808 absorbance reader (Biotek) at 560 nm.

Measurement of G-CSF in muscle lysates

The samples were thawed on ice and centrifuged at 4500 g for 5 minutes. Supernatants were diluted in Bio-Plex sample diluent (Bio-Rad) with 0.5% BSA added to a final protein concentration of 500 µg/ml. The G-CSF concentration in muscle lysates was measured using Bio-Plex G-CSF coupled beads (Bio-Rad) together with a Bio-Plex MAGPIX fluorescence magnetic bead-based immunoassay reader (Bio-Rad) as described above.

Immunohistochemistry

28 days after the HLI surgery, the gastrocnemius (GC) muscle was collected for immunohistochemical analysis. The anaesthesia was initiated and maintained as described as above. The skin and fasciae were removed from the lower limb and GC muscle was excised. After it was washed with ice-cold PBS, it was covered with cryo-embedding media (O.C.T. compound, VWR) and snap-frozen in 2-methylbutane (Sigma Aldrich) suspended in liquid nitrogen. The frozen muscles were cut into 5 μ m sections with a cryotome and placed onto positively charged glass slides (Microscope Slide Superfrost, VWR Collection). After rehydration sections were fixed in ice-cold acetone and blocked with 5% foetal bovine serum (Thermo Fisher Scientific) in phosphate buffer saline containing 1% BSA and 0.1% Triton X-100. For staining, CD31 rat anti-mouse antibodies (#550274, BD Biosciences) or laminin conjugated with DyLight 488 (Novus NB300-144G) were used. Donkey anti-rat DyLight 594 (#SA510028, Thermo Fisher Scientific) was used as secondary antibody. All antibodies were diluted in blocking buffer without Triton X-100.

Western blotting analysis of muscle lysates

The muscle homogenate samples kept at -80°C were thawed on ice and samples were diluted to 2 mg/ml protein concentration in 100 mM Tris-HCl (pH7.4). After equal volume of sample buffer was added, samples were incubated at 95°C for 5 minutes and centrifuged at 4500 g for 5 minutes. Some samples were reduced with either 5% β -mercaptoethanol or 100 mM hydroxylamine. Samples were separated using 4-20% mini-protein TGX precast gels (Bio-Rad) and membranes were probed for sEH (#sc-25797, Santa Cruz Biotechnology) and G-CSFR (#sc-9173, Santa Cruz Biotechnology). Goat anti-rabbit secondary antibodies were used (#sc-2004, Santa Cruz Biotechnology).

Measurement of soluble epoxide hydrolase activity

To measure sEH activity a fluorescent substrate, cyano-(6-methoxynaphthalen-2-yl)-methyl ester (PHOME) (Cayman Chemical), was used according to the method developed by Jones and colleagues [496]. The reaction was performed in 100 mM Tris-HCl, pH 7.4 with 50 mM NaCl and 100 μ M bond breaker TCEP solution (Thermo

Fisher Scientific). The recombinant sEH was incubated with vehicle or nitro-lipid compounds (0.1 nM – 100 μ M) for 15 minutes at room temperature. The reaction was initiated by adding PHOME, diluted in DMSO, to the final concentration of 0.5 μ M (final DMSO content/well was 2.5% vol/vol) and final volume of 100 μ l. The reaction was performed at room temperature in black polystyrene 96-well plates. Fluorescent measurements were taken 15 minutes after reaction initiation. Fluorescence was measured using SpectraMax Geminin XS with the following settings: excitation wavelength, 330 nm (bandwidth, 9 nm); emission wavelength, 465 nm (bandwidth, 9 nm); number of flashes, 6; temperature, 23°C. All the determinations were conducted in triplicate. sEH activation potency of nitro-fatty acids was assessed by using an activation integral. This integral represents the peak area under the concentration-dependent curve of nitro-fatty acid effect on sEH activity, and provides a measure of the ability of different test compounds to activate the hydrolase. The nitro lipid compounds were provided by Professor Bruce Freeman, University of Pittsburgh.

5.3. Results

WT or KI mice have similar blood flow recovery in mild or moderate models of hindlimb ischaemia

To investigate the role of sEH redox regulation in angiogenesis, WT or KI mice were subjected to hindlimb ischaemia and blood flow recovery was evaluated at 7, 14, 21 and 28 days after ischaemia induction by laser Doppler flowmetry. The severity of experimental ischaemia induced depends on the location and length of artery occlusion (Figure 5-1B). Blood flow recovery was assessed in models of mild or moderate hindlimb ischaemia. Figure 5-1C shows representative laser Doppler blood flow images of hindlimb blood flow before and at different time points after surgical induction of mild or moderate ischaemia. Immediately after surgery, hindlimb blood flow was severely reduced in both genotypes. In the model of mild ischaemia, blood flow rapidly recovered to $51 \pm 2.8 \%$ or $48 \pm 2.8 \%$ 7 days after surgery in WT or KI mice respectively, reaching $67 \pm 2.8 \%$ and $65 \pm 4.0 \%$ 28 days post-surgery. There was no difference in blood flow recovery between two genotypes (Figure 5-1D). In the model of moderate ischaemia, blood flow recovery in hindlimb was more gradual compared to the mild ischaemia model (Figure 5-1C,E). 7 days after surgery blood flow in ischaemic hind limb reached $39 \pm 3.5 \%$ or $33 \pm 2.5 \%$ in WT or KI mice respectively (Figure 5-1E). The maximum blood flow recovery at day 28 post surgery was $61 \pm 3.6 \%$ in WT mice or $54 \pm 2.6 \%$ in KI littermates. Although, the KI mice showed a trend towards attenuated blood flow recovery after induction of moderate hindlimb ischaemia, the difference was not statistically significant.

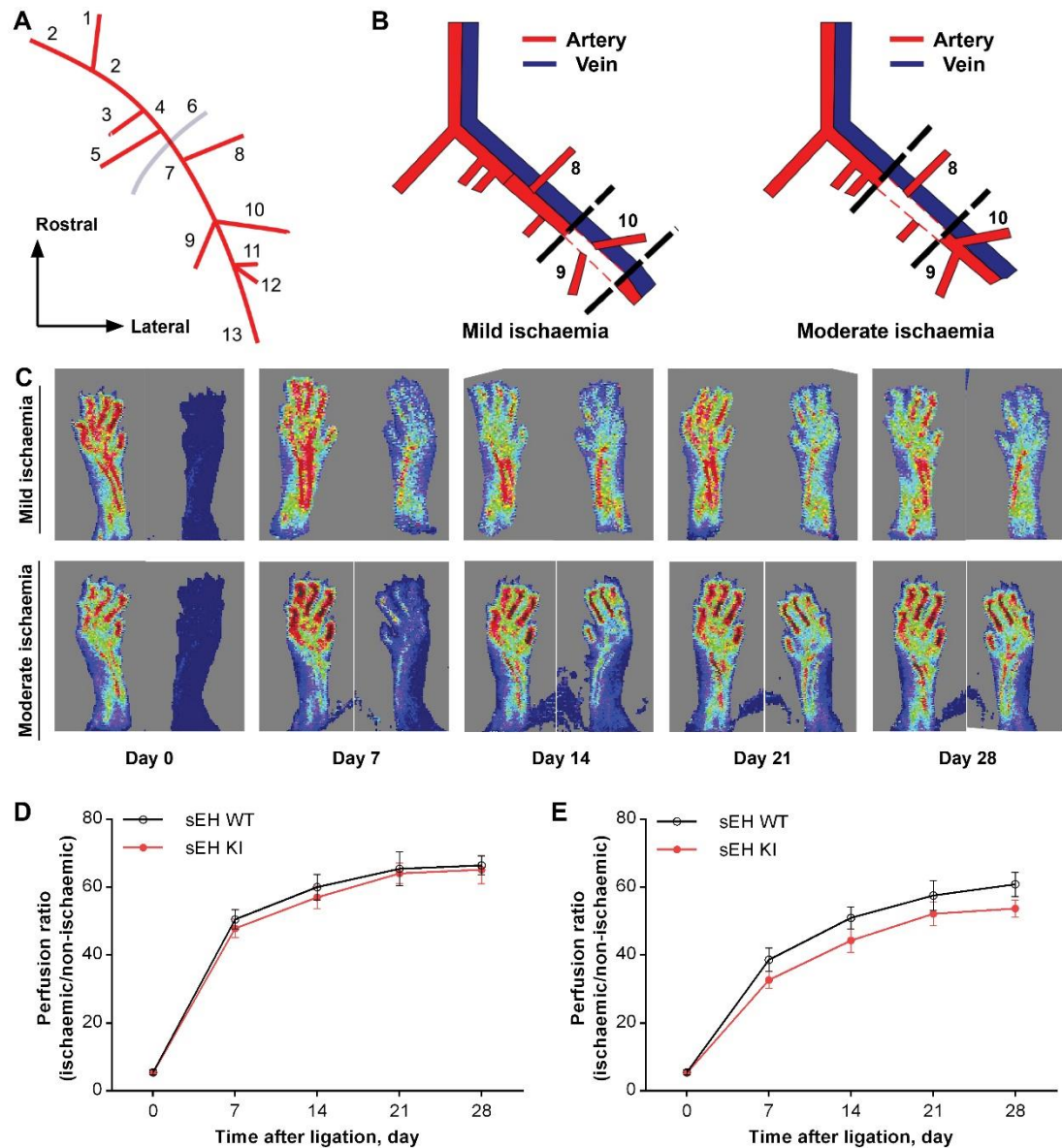


Figure 5-1

Blood flow recovery in WT or KI mice after mild or moderate hindlimb ischaemia induction.

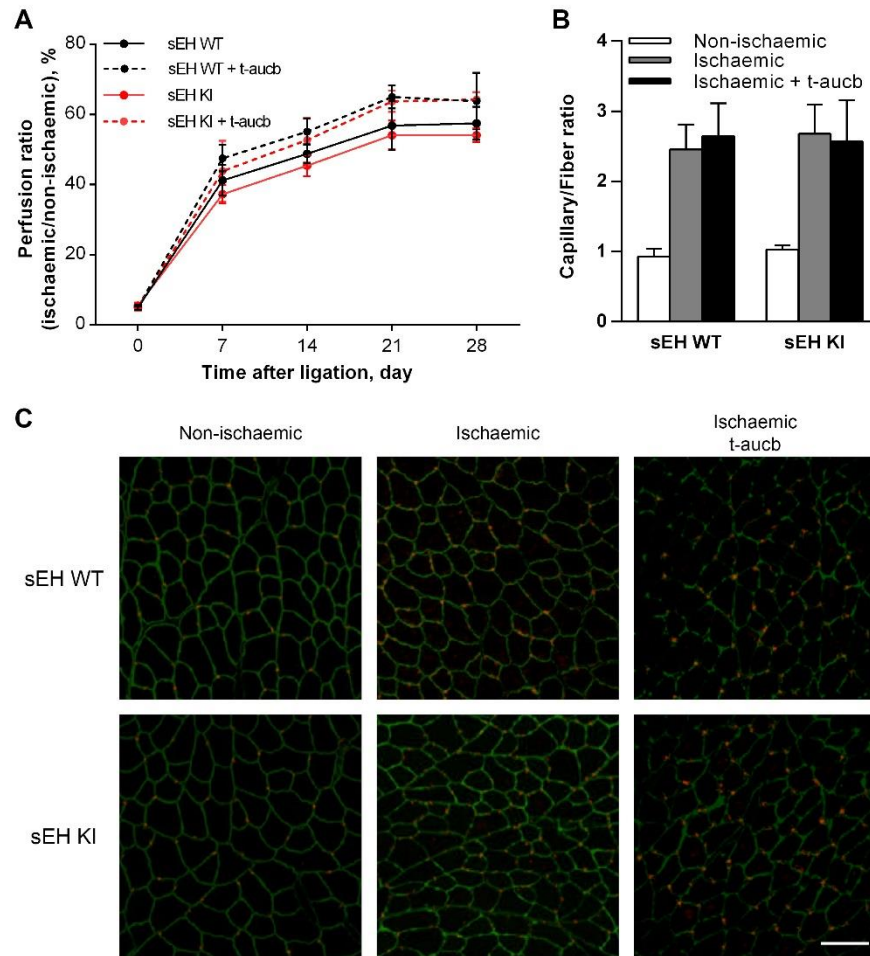
A. Schematic diagram of the anatomy of the major lower limb arteries in mice. Major branches are labelled with numbers; (1) aorta, (2) common iliac artery, (3) internal iliac artery, (4) external iliac artery, (5) epigastric artery, (6) inguinal ligament, (7) femoral artery, (8) lateral circumflex femoral artery, (9) proximal caudal femoral artery, (10) superficial caudal epigastric artery, (11) medial proximal genicular artery, (12) popliteal artery, (13) saphenous artery. B. Simplified diagram showing surgical techniques employed to induce mild or moderate hindlimb ischaemia. The positions of ligature are indicated by dashed lines. C. Representative laser Doppler flowmetry images recorded immediately after and every 7 days after surgical induction of mild and moderate hindlimb ischaemia. D. Time course of blood flow recovery in WT or KI mice after surgical induction of mild ischaemia. Mean perfusion ratio is expressed as a ratio of perfusion in the ischaemic paw to the non-ischaemic paw. Data are present as mean with SEM, n = 9 mice per group.

E. Time course of blood flow recovery in WT or KI mice after surgical induction of moderate ischaemia. Mean perfusion ratio is expressed as a ratio of perfusion in the ischaemic paw to the non-ischaemic paw. Data are present as mean with SEM, $n = 17$ mice per group. Statistical analysis was performed using two-way ANOVA with Bonferroni post-hoc test, which failed to identify difference between genotypes.

The impact of the sEH inhibitor t-AUCB on post-ischaemic blood flow recovery

The effect of sEH inhibition was assessed in WT or KI mice subjected to hindlimb ischaemia surgery. t-AUCB was delivered at 3 mg/kg/day for the duration of 28 days via osmotic mini-pumps implanted subcutaneously. The blood flow recovery in control WT or KI animals was similar to the results obtained previously, with no statistical difference between genotypes (Figure 5-2A). Administration of t-AUCB slightly improved blood flow recovery in both WT and KI animals, although it was not statistically significant. At 7 days post-ischaemia in animals treated with t-AUCB, blood flow recovered to 48 ± 3.8 % in WT mice or 44 ± 8.7 % in KI mice, as opposed to 41 ± 4.4 % and 37 ± 2.6 % in control WT or KI mice respectively (Figure 5-2A). A similar trend was seen 28 days after ischaemia induction. t-AUCB increased blood flow in the ischaemic limb to 67 ± 4.7 % in WT mice and 64 ± 2.1 % in KI mice, while in control animals it was 58 ± 4.7 % and 54 ± 2.0 %, respectively. No statistical difference was detected between genotypes.

Histological analysis of the ischaemic hindlimbs was performed 28 days after surgery. Ischaemia resulted in the enhancement of capillary density in both genotypes, as analysed by CD31 staining (Figure 5-2B,C). The number of capillaries per muscle fibre increased to similar levels in WT or KI mice after hindlimb ischaemia, being 2.5 ± 0.4 or 2.7 ± 0.4 capillaries per fibre respectively (Figure 5-2B). t-AUCB did not change the alteration in capillary density occurring in the muscle as a result of ischaemia.

**Figure 5-2**

Effect of sEH inhibitor t-AUCB on blood flow recovery and capillary density in WT or KI mice subjected to moderate hindlimb ischaemia. A. Time course of blood flow recovery in WT or KI mice. Two groups of mice received t-AUCB treatment (3 mg/kg/day) delivered via subcutaneous mini-pumps for 4 weeks. Blood flow recovery is expressed as mean ratio of perfusion in ischaemic paw to non-ischaemic paw. Data are present as mean with SEM, $n = 5$ mice per group. Statistical analysis was performed using two-way ANOVA with a Bonferroni post-hoc test, which failed to identify differences between genotypes. B. Number of capillaries per muscle fibre in WT or KI mice 28 days after hindlimb ischaemia induction. Capillary density was assessed by CD31 and laminin staining in the gastrocnemius muscle of the non-ischaemic limb and the ischaemic limb of each genotype, some of which were treated with t-AUCB (3 mg/kg/day s.c. for 4 weeks). Data are presented as mean with SEM, $n = 5$ mice per group. C. Representative immunohistochemical staining of gastrocnemius muscle sections from non-ischaemic and ischaemic WT and KI mice for CD31 (red) and laminin (green).

Granulocyte colony-stimulating factor is increased in early stages of hindlimb ischaemia and correlates with increased muscle oedema

The cytokine G-CSF is known to be induced in several ischaemic settings. The levels of G-CSF were measured in muscle tissue of WT or KI mice 24 hours after hindlimb ischaemia induction using a Magpix multiplex reader. The concentration of G-CSF significantly increased in response to ischaemia in WT and KI mice to similar levels (Figure 5-3A). Additionally, muscle oedema was noticed in ischaemic hindlimbs of WT and KI mice. The oedema index, representing the weight ratio of ischaemic to non-ischaemic hindlimb, was similar between genotypes (Figure 5-3B).

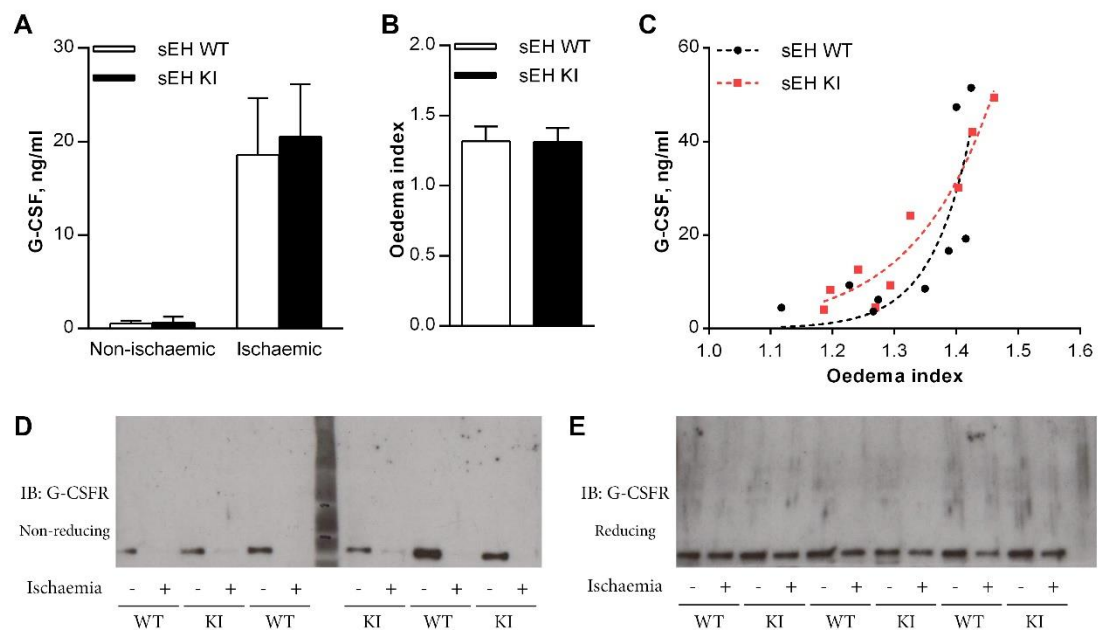


Figure 5-3

Levels of granulocyte colony-stimulating factor and granulocyte colony-stimulating factor receptor in WT or KI mice 24 hours after induction of hindlimb ischaemia. A. Concentration of granulocyte colony-stimulating factor (G-CSF) in gastrocnemius muscle. WT or KI mice were subjected to hindlimb ischaemia and the concentration of G-CSF was measured using a Bio-Plex Magpix system. Data are present as mean with SEM, $n = 9$ mice per group. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test. B. Oedema index in WT or KI mice 24 hours after hindlimb ischaemia induction. The oedema index represents the ratio of ischaemic gastrocnemius muscle weight to non-ischaemic gastrocnemius muscle weight. Data are present as mean with SEM, $n = 9$ mice per group. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test. C. Correlation between oedema index and G-CSF levels in gastrocnemius muscle of WT or KI mice 24 hours after ischaemia induction. Non-linear regression analysis was performed for G-CSF concentration vs oedema index. Non-linear regression curves were compared

between genotypes using Akaike's information criteria, which indicated there were no statistical differences. D-E. Levels of granulocyte colony-stimulating factor receptor (G-CSFR) in gastrocnemius muscle of WT or KI mice 24 hours after hindlimb ischaemia analysed by non-reducing or reducing western blot.

Interestingly, the levels of G-CSF correlated with the oedema index, although the correlation curves were not different between genotypes (Figure 5-3C). The levels of G-CSF receptor (G-CSFR) were also assessed using western blotting. While no G-CSFR could be visualised in non-reduced ischaemic muscle samples from WT or KI mice, reducing the samples with β -ME resulted in G-CSFR detection in both non-ischaemic and ischaemic samples (Figure 5-3D,E).

sEH forms high molecular weight complexes in the early stages of hindlimb ischaemia

sEH expression was assessed by western blotting in muscle lysates of WT and KI mice subjected to hindlimb ischaemia for 24 hours (Figure 5-4).

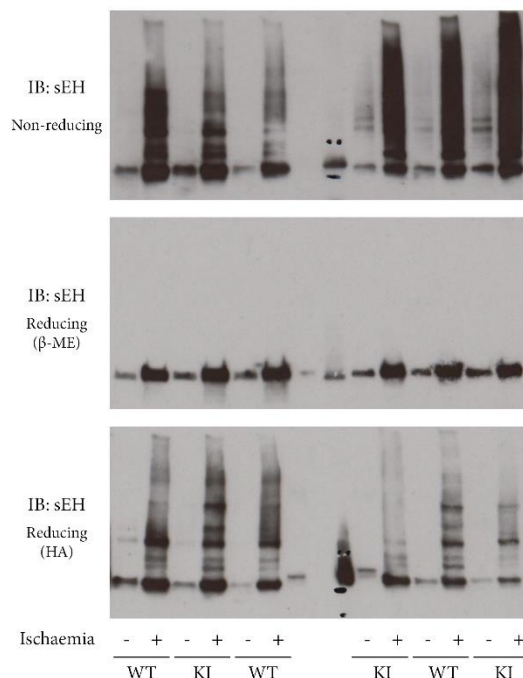


Figure 5-4

sEH expression in gastrocnemius muscle of WT or KI mice subjected to hindlimb ischaemia surgery. 24 hours after hindlimb ischaemia induction gastrocnemius muscle was excised and analysed for sEH expression by western blot under non-reducing conditions or after samples were reduced with β -mercaptoethanol (β -ME) or hydroxylamine (HA).

In ischaemic samples from WT or KI mice, numerous high-molecular bands were detected, all of which could be reduced by disulphide bond reducing agent β -ME, but not by the thioester bond breaker hydroxylamine (HA) (Figure 5-4). This analysis by western blotting showed levels of sEH increased in ischaemic muscle compared to control, to a similar extent in each genotype.

sEH activity is inhibited by nitro-fatty acids

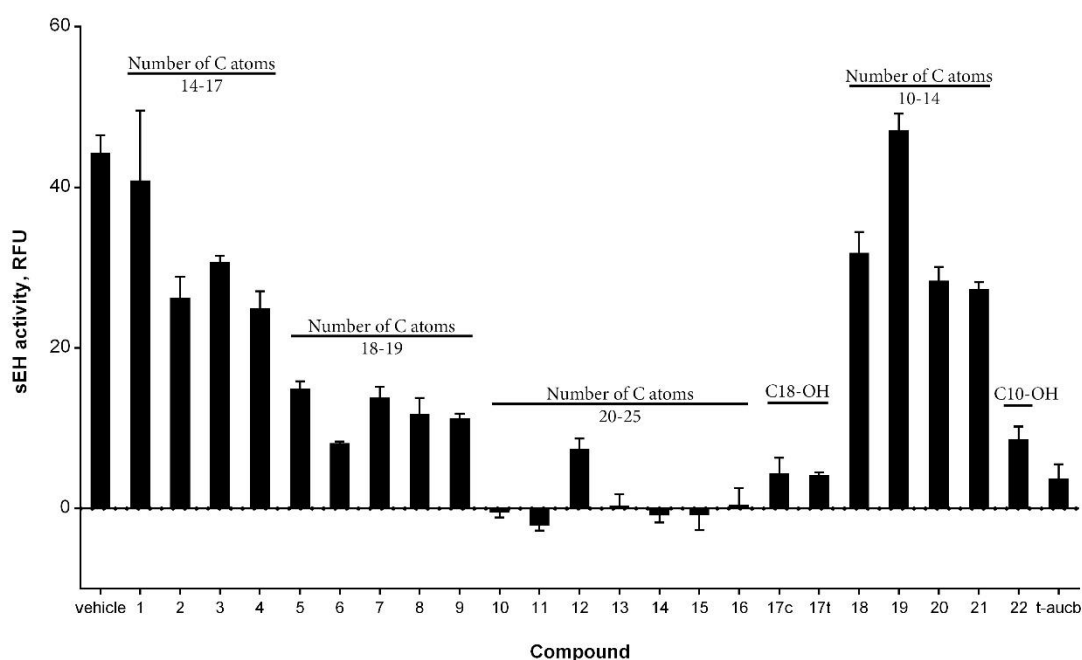
In order to assess the effect of electrophilic nitro-fatty acids on sEH activity, a fluorescence-based in vitro activity assays utilising recombinant sEH were performed. 23 compounds, the structures of which are summarised in Table 5-1, were tested.

Table 5-1. Structural features of compounds 1-22

C1 9/10:14	C2 10/11:15	C3 9/10:16	C4 10/11:17	C5 6/7:18	C6 9/10:18	C7 11/12:18	C8 7/8:19
C9 10/11:19	C10 8/9:20	C11 11/12:20	C12 5/6:20	C13 12/13:21	C14 13/14:22	C15 14/15:23	C16 15/16:24
C17c 9/10cis:18- OH	C17t 9/10tr:18- OH	C18 10:10	C19 11:11	C20 12:12	C21 14:14	C22 10:10- OH	

The position of nitro group and total number of carbon atoms are indicated

All compound, apart from C1 or C19, at a concentration of 20 μ M resulted in inhibition of sEH activity (Figure 5-5). The ability of compounds to inhibit sEH activity was dependent on the number of carbon atoms. C10-C16, having 20-25 carbon atoms, were the most potent inhibitors, and fully inhibited the hydrolase. The potency with which compounds inhibited sEH declined as the number of carbon atoms decreased. Compounds C5–C9, which have 18 or 19 carbon atoms, when used at 20 μ M inhibited sEH activity more than 50 %. In notable contrast, compounds C1-C4 or C18-C21, which have 14 or less carbons in their structure, were not as effective.

**Figure 5-5**

sEH inhibitory activities of 23 compounds. sEH activity was measured using the PHOME fluorimetric activity assay. Each compound was assessed at a concentration of 20 μ M. Data are present as mean with SEM, $n = 3$.

Nitro-fatty acids potentiate sEH activity at low concentrations

The concentration-dependence of sEH inhibition by each of the nitro-fatty acids was next studied. Figure 5-6 and Figure 5-7 summarise the effect of 23 compounds used at 0.1 nM – 100 μ M on sEH activity. The vast majority of the compounds induced dose-dependent inhibition of sEH, however, notably some of them activated the hydrolase when used at low concentrations (10 nM – 1 μ M).

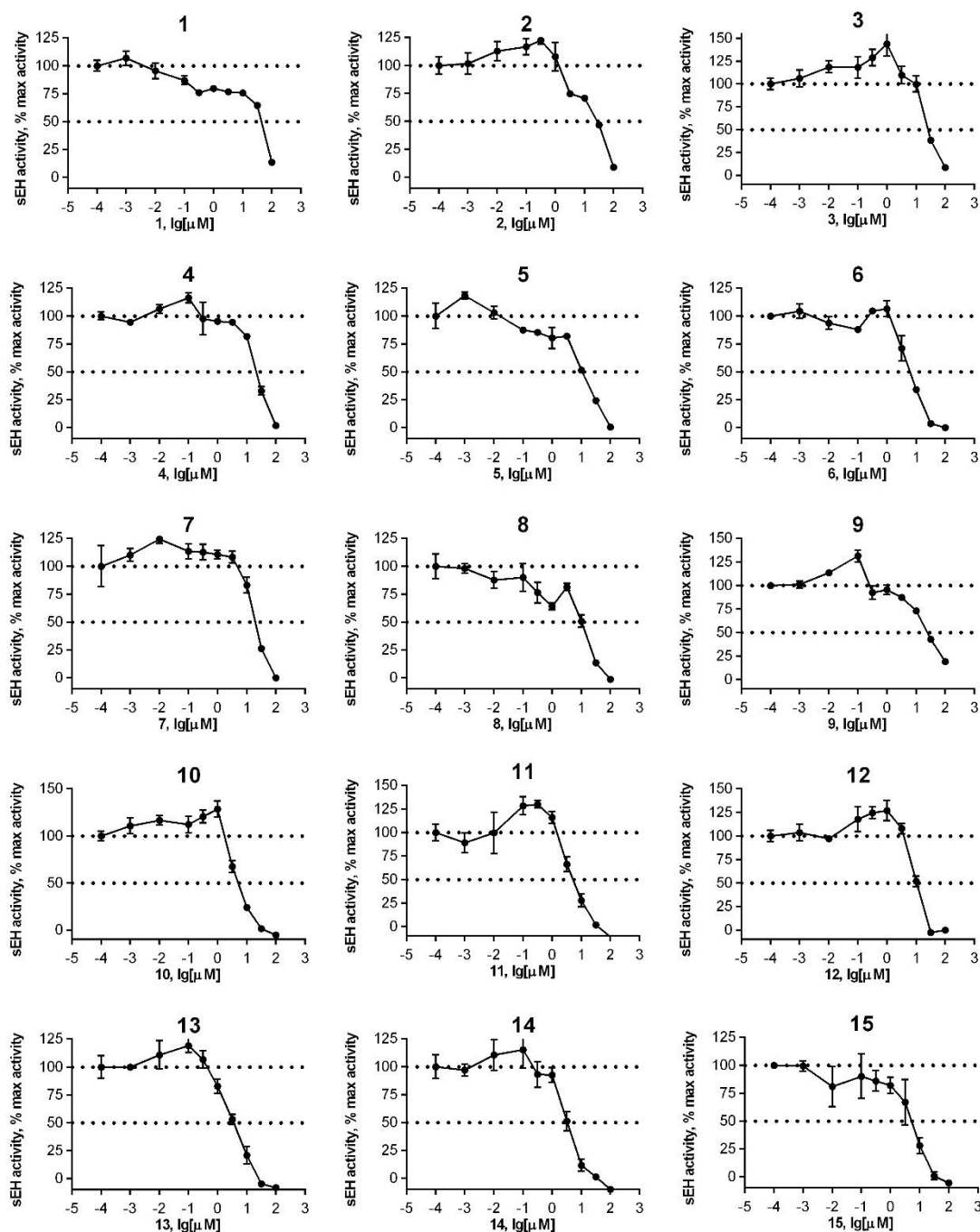


Figure 5-6

Effect of compounds C1 – C15 on sEH activity. sEH activity was measured using recombinant sEH and PHOME fluorescence activity assay. The data are present as mean with SEM, n = 3.

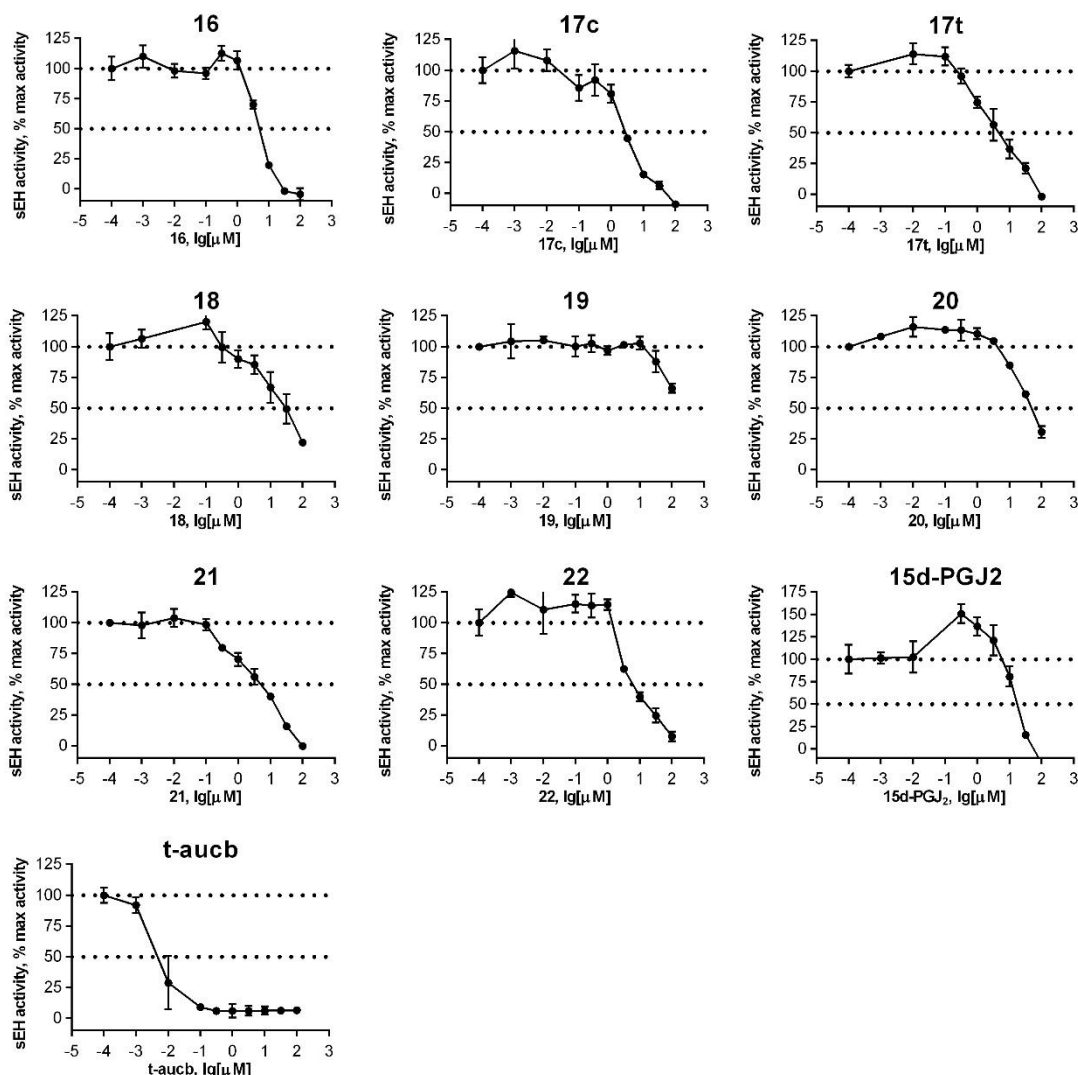
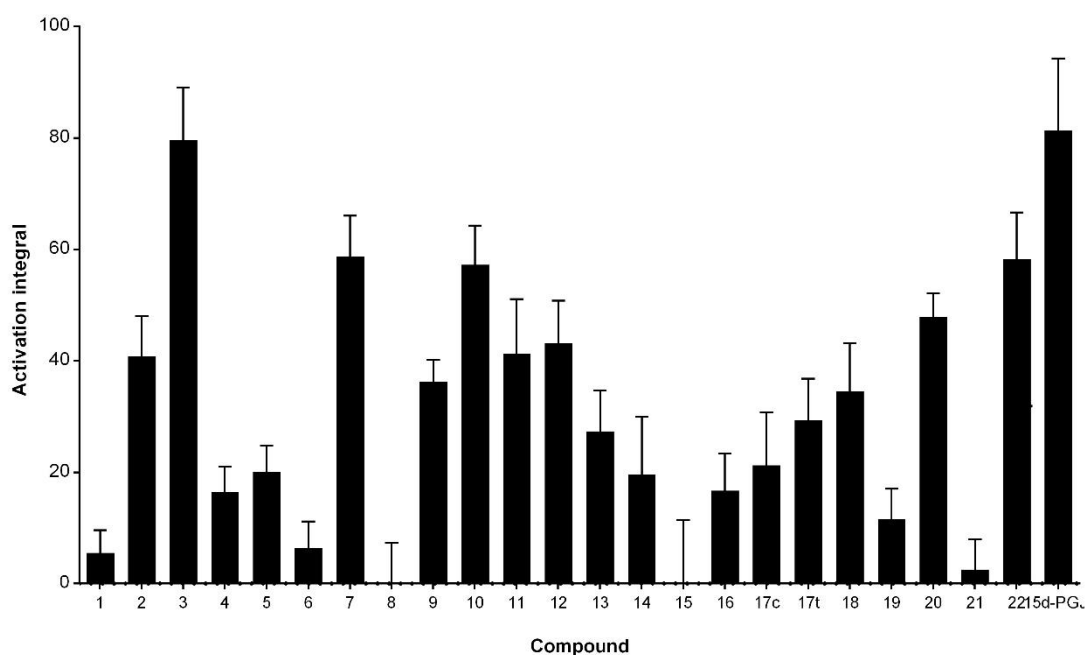


Figure 5-7

Effect of compounds C16 – C22 on sEH activity. sEH activity was measured using recombinant sEH and PHOME fluorescence activity assay, sEH inhibitor, t-AUCB was used as a negative control. The data are present as mean with SEM, n = 3.

In order to quantify the sEH activation potency of nitro-fatty acids, the activation integral for each compound was calculated. The activation integral represents the total peak area under the curve with 100% sEH activity used as a baseline.

**Figure 5-8**

The sEH activation potency of compounds C1 – C23. sEH activity was measured using recombinant sEH and PHOME fluorescence activity assay. The activation integral was calculated from concentration-dependent curves as peak area under the curve with 100% sEH activity as a baseline. Data are present as mean with SEM, n=3.

Compounds 3, 7, 10, and 22 had the most profound effect on sEH activity in terms of activation, while compounds 1, 6, 8, 15, 19, and 21 failed to activate sEH (Figure 5-8). Unlike their inhibitory activity, no dependence on the number of carbon atoms or position of nitro group in nitro-fatty acids was found for their ability to induce activation.

5.4. Discussion

sEH redox regulation and responses to hindlimb ischaemia

As mentioned before and extensively considered in the introduction, epoxygenated fatty acids, substrates of sEH, and their corresponding diols are mostly pro-angiogenic and regulate post-ischaemic revascularisation. Numerous processes contribute to post-ischaemic revascularisation, including vasculogenesis, angiogenesis and arteriogenesis. These multiple, complex processes require coordination and precise regulation of many activating and inhibitory factors that interact with many types of vascular and extravascular cells. These regulatory mechanisms probably differ considerably between organs and contribute to the constitution of vascular networks. The vast diversity of epoxygenated fatty acids and their diols could provide means for such regulation. Interestingly, the expression of the epoxygenated fatty acids producing enzymes, CYP 450 epoxygenases, which is an important controller of major steps in post-ischaemic revascularisation is regulated by hypoxia, [497, 498]. The role of endogenous sEH regulation in post-ischaemic revascularisation has not been excessively studied. Consequently, the current studies investigated the redox control of sEH as an endogenous mechanism of regulating post-ischaemic revascularisation. These studies utilised C521S sEH KI mice, which are resistant to inhibition induced by electrophilic lipids. It is worth noting, that electrophilic nitro-lipids were reported to promote angiogenesis [499]. Nitro-fatty acids are known endogenous by-products of nitrooxidative stress, which takes place during inflammation, which is well established as an important contributor to post-ischaemic vascularisation [500, 501]. This supports the possibility that sEH activity could be modulated during ischaemia, and could potentially contribute to post-ischaemic revascularisation.

C521S sEH KI mice had similar blood flow recovery to sEH WT mice in the model of mild hindlimb ischaemia. Many studies have used animal hindlimb ischaemia model to delineate which of multiple factors influence tissue responses to ischaemia. Numerous hindlimb ischaemia models exist, ranging from simple femoral artery ligation to extensive excision of the femoral artery, vein and nerve [502]. The degree of ischaemic damage varies markedly depending on the site of artery ligation. The location of ligature

influences the degree of collateral vessel growth involvement in blood flow recovery during hindlimb ischaemia [503]. It should be noted, that collateral vessel growth and arteriogenesis, despite the considerable confusion with terminology, are different processes that are regulated by different factors. Thus, if the recovery after hindlimb ischaemia is mostly attributed to collateral growth, and not arteriogenesis or angiogenesis, the factors influencing collateral growth would be more central to the recovery process and vice versa. As no difference in blood flow recovery between WT or KI mice was observed in the mild model of hindlimb ischaemia, moderate hindlimb ischaemia model was used throughout the study. Although, KI mice showed a trend towards attenuated blood flow recovery in the moderate hindlimb ischaemia model, the difference was marginal and was not statistically different from WT animals. It is important to recognise major limitations of the technique. There is a great degree of anatomical variations in the origins of femoral artery branches and in arteries responsible for collaterals. These variations may result in unexpected circulatory states even if the surgeries performed are the same. Moreover, laser Doppler flowmetry can vary widely depending on the animal's body temperature and position. Thus, large numbers of mice per experimental group are required for reliable results to be obtained.

Ischaemia-induced angiogenesis was determined in gastrocnemius muscle of the lower limb. After 4 weeks of ischaemia, there was an increase in the number of CD31-positive capillaries, surrounding the skeletal muscle fibres, but this was similar between genotypes. Increased number of CD31-positive cells was shown during neovascularisation in the mouse ear wound model in response to treatment with t-AUCB or exogenous application of EETs [504]. Moreover, the sEH inhibition improved vascularisation of engineered skin grafts after transplantation in mice [505]. Genetic deletion of sEH or endothelial cell-specific overexpression of CYP450 epoxygenases, CYP2J2 or CYP2C8, also increased vascularisation in healing wounds [506]. Thus, inhibiting sEH activity in WT or KI mice was suggested to result in increased number of CD31-positive capillaries. However, t-AUCB administration (3 mg/kg/day) via osmotic mini-pump implanted subcutaneously did not increase the number of CD31-positive blood vessels in gastrocnemius muscle. Although, this observation was unexpected, it should be noted that different tissues have different unsaturated fatty acid content, varying CYP450 epoxygenases, and disparate epoxide/diol ratios. While most

observations suggest that sEH inhibition is beneficial for revascularisation in wound healing models in mice, as well as in spinal cord injury model in rats [507], this is not always the case. Numerous other studies suggested that sEH activity is essential for retinal neovascularisation, as genetic deletion or inhibition of sEH attenuated physiological sprouting angiogenesis in murine retina [237]. Moreover, genetic deletion of sEH or its inhibition were shown to reduce G-CSF-induced vascularisation of Matrigel plugs [252].

Interestingly, sEH KI mice were shown to have significantly reduced levels of G-CSF in response to sepsis compared to WT animals. Moreover, levels of cytokine IL-17, which is known to induce the release of G-CSF, showed a similar trend (Figure 4-4). In response to infection, serum G-CSF is often increased. Levels of G-CSF were measured in gastrocnemius muscle of WT or KI mice 24 hours after ischaemia induction and showed a significant increase compared to non-ischaemic control muscle, although, without any difference between genotypes. G-CSF is the principal haematopoietic cytokine regulating the granulopoiesis and is widely used to treat or prevent neutropenia in a variety of clinical settings. In addition to its well-characterised ability to induce neutrophil production, G-CSF is a potent stimulus for inducing neutrophil release from bone marrow. Notably, sEH activity is essential for progenitor cell proliferation and their mobilisation from bone marrow, and vascular repair in a model of murine hindlimb ischaemia [252]. Although, it is not clear whether sEH potentially mediates G-CSF release in inflammatory settings, its involvement in G-CSF-induced mobilisation of progenitor cells seems to be highly plausible. G-CSF treatment induced changes in levels of epoxygenated fatty acids and their diols in bone marrow and plasma of WT mice, although not in sEH^{-/-} mice, suggesting that it might manifest its actions via altering the activity of CYP450 and/or the hydrolase [252]. G-CSF-induced mobilisation of progenitor cells was attenuated in sEH^{-/-} mice, as well as in WT mice treated with the sEH inhibitor t-AUCB. After G-CSF treatment, bone marrow cells release a wide number of proteases such as MMP-9, cathepsin G and neutrophil elastase [508]. Some studies suggest that G-CSF-mediated mobilisation is inhibited by systemic administration of inhibitors to MMP-9 [509]. Interestingly, a link between sEH activity and MMP-9 expression has been reported by several groups [243, 510-512].

Angiogenesis encompasses cell proliferation, migration and differentiation. Loosening of cell-to-cell contacts is the major initial event that causes normally quiescent endothelial cells to initiate angiogenesis. Moreover, in certain circumstances, a transient increase in vascular permeability allows the transport of macromolecules and inflammatory cells, which is essential for repair of damaged tissue and resolution of pathological conditions. Thus, vascular permeability is a common hallmark of normal angiogenesis. VEGF, a well-known inducer of angiogenesis, is also known by its potent ability to induce permeability of blood vessels through destabilisation of the adherent junctions and induction of actomyosin cytoskeleton contraction [513, 514]. Increased vascular permeability results in oedema, which is a current problem in therapeutic angiogenesis [515, 516]. Oedema severity was assessed in gastrocnemius muscles of sEH WT and KI mice by measuring an ‘oedema index’, which represents ratio of ischaemic muscle to non-ischaemic muscle weight. The oedema index was similar between genotypes. Interestingly, G-CSF levels correlated with oedema index, however, analysis of correlation curves showed them to be similar between WT and KI mice.

G-CSF mediates its actions by binding to the G-CSF receptor (G-CSFR). The levels of G-CSFR were analysed in muscle lysates of WT or KI mice by western blotting, and interestingly, were completely undetectable under non-reducing conditions in ischaemic muscle. A variety of homo- or heterodimer, as well as higher order complexes, are formed upon the interaction of different cytokines with their cognate receptors. Binding of G-CSF to its receptor, induces its dimerisation, however, the complex stoichiometry of G-CSF and the receptor remains controversial [517]. This could potentially explain why G-CSFR could not be visualised in non-reducing conditions. Interestingly, several conserved Cys were found in G-CSFR and were shown to form disulfide bonds [518, 519]. G-CSFR was detected in ischaemic muscle lysate samples, when the latest were reduced with β -mercaptoethanol, suggesting that disulfide bond formation might be involved in G-CSFR dimerisation. The levels of G-CSFR in ischaemic reduced samples were similar between genotypes.

sEH protein levels have been shown to be significantly reduced during several pathological conditions, one of them being hypoxia [281]. As mentioned before, hindlimb ischaemia model is characterised by hypoxia. The levels of sEH were analysed

in muscle samples from WT or KI mice subjected to hindlimb ischaemia. It was notable that sEH was detected as part of high-molecular weight complexes in ischaemic samples, in both genotypes. Sample incubation with β -mercaptoethanol reduced complexes. Ischaemic conditions were reported to induce degradation of various proteins [520, 521]. These sEH-specific high-molecular bands were considered to possibly represent poly-ubiquitinated forms, so accounting for the multiple bands observed. However, as this banding pattern was reduced by β -mercaptoethanol to generate a single band of the weight of sEH, any ubiquitination would have to be non-canonical, and likely mediated by conjugated to Cys instead of Lys. In this case, ubiquitin may be conjugated to a Cys residue on the enzyme via thioester bond [522]. However, hydroxylamine, a thioester bond breaker, failed to reduce high-molecular bands, strongly suggesting that they are not formed via thioester bonds and thus the altered banding pattern of sEH is not explained by cysteine ubiquitination. Further studies are required to identify the nature of these complexes.

Overall, the results of the current study suggest that redox regulation of sEH via Cys521 does not mediate post-ischaemic revascularisation. Blood flow recovery was similar between genotypes in response to either mild or moderate ischaemia. Moreover, angiogenesis as assessed by the density of CD31-stained capillaries in the lower limb, was also not different between sEH WT and KI mice. In early ischaemia, the concentration of G-CSF in gastrocnemius muscle increased in WT and KI mice to the similar level. Interestingly, sEH formed high-molecular complexes during ischaemia, but these were not dependent on C521 and their molecular basis remains to be elucidated.

sEH is a ubiquitously expressed enzyme that is found in disparate tissues, regulating numerous physiological or pathophysiological processes. However, little is known about endogenous sEH regulation, although electrophilic lipids are known to inhibit the enzyme's hydrolase activity [30]. As mentioned, lipid electrophiles are present endogenously, however, the concentration they accumulate at in cells is anticipated to limit their ability to inhibit hydrolase effectively. The effect of various different nitro-fatty acids on sEH activity was assessed in the current study. Although these electrophiles inhibited sEH activity at higher concentrations, intriguingly many of these

nitro-fatty acids in fact stimulated the hydrolase when used at lower concentrations. It was notable that some nitro-fatty acids showed a profound effect on sEH activity, while the others failed to activate it. This observation suggests that sEH regulation is extremely complex, and while sEH was hypothesised to be inhibited during sepsis and ischaemia, it might not be the case, as endogenously generated electrophilic lipids are mostly present at low concentrations. It is important to highlight the significance of the aforementioned observation as this might represent an endogenous regulatory mechanism. Moreover, it might possibly have a therapeutic potential, by inhibiting tumour angiogenesis as an example.

Chapter 6. General conclusion and future directions

The work presented in this thesis explored the emerging and rapidly expanding role of redox signalling in biology. Numerous studies have demonstrated the unique chemistry of reactive Cys residues within certain target proteins, allowing them to be covalently modified. Such oxidative post-translational modifications of protein Cys residues are akin to the well-established phosphorylation of Ser, Thr or Tyr residues that is integral to the regulation of the function of such proteins. The current work investigated two proteins that can be redox regulated, namely soluble epoxide hydrolase (sEH) and cGMP-dependent protein kinase (PKG). Although being structurally and functionally different, it is notable that both of these proteins are each involved in a number of similar physiological processes, such as vascular relaxation, platelet aggregation and vessel growth. It is apparent that signalling pathways involving PKG or sEH, are intertwined [523-525]. Some studies have suggested that EETs have a potentiated ability to decrease vascular resistance when endothelial nitric oxide levels are decreased. Reactive oxygen species have been shown to modulate sEH and PKG activity in opposite ways, with oxidants typically inhibiting the hydrolase [29, 30], but activating the kinase [25, 394]. However, despite oxidants inducing such differential activity responses in these two enzymes, it is interesting that the functional outcomes of their oxidative modification from the physiological perspective can be similar. Thus, sEH inhibition increases EETs levels that are vasodilatory [480], whereas oxidative activation of PKG similarly promotes vascular relaxation [26]. Elevated levels of EETs can promote elevations in cAMP, which can also induce vasodilation by activating PKA [272, 344]. Interestingly, PKA can also be activated by oxidants [526], a mechanism that can couple to vasodilation [527], as well as angiogenesis [528, 529] - which can also be promoted by EETs [236, 273].

The current study examined the role of sEH redox regulation during two pathological scenarios that are accompanied by inflammatory processes and increased levels of electrophilic lipids. C521S sEH KI mice, that harbour an engineered mutation that renders the hydrolase resistant to inhibition by electrophilic lipids, were hypothesised to be protected from sepsis-induced hypotension. However, the responses of the KI mice

to sepsis were similar to that of WT littermates. Similarly, the responses of the C521S sEH KI mice to hindlimb ischaemia were not any difference to WT controls, suggesting that sEH redox regulation by electrophilic lipid adduction is not involved in post-ischaemic revascularisation. The potential role of intradisulfide-induced activation of PKG on cardiovascular function during health or with sepsis was also assessed in the current studies using a newly generated C195S KI PKG mice, which lack this mode of oxidative activation. While C195S PKG KI mice showed similar basal haemodynamic and cardiac parameters to WT mice, as were their responses to sepsis, an impaired gastrointestinal tract function was observed in the mutant mice. The KI mice were shown to have smaller weight, a bigger caecum and an increase in whole gut transit time. This strongly suggesting that intradisulfide-induced PKG activation might play an important role in GI tract function, however further studies are required to elucidate the exact mechanism behind this phenotype. The rest of the discussion will focus on possible future directions involving sEH and PKG redox regulation.

Redox regulation of PKG and gastrointestinal tract function

Gastrointestinal (GI) motility is based on coordinated contraction and relaxation of smooth muscle [530]. Nitrergic (mediated by NO) neurotransmission is known to inhibit smooth muscle cell constriction and is suggested to act via PKG in different GI cell types including smooth muscle cells (SMC) and interstitial cells of Cajal (ICC). Notably, mice lacking nNOS, sGC or PKGI have been reported to have impaired GI tract function [166, 428, 441, 442]. It is not fully understood at the moment, but several studies have suggested that PKG activation in ICC cells is essential for transduction of nitrergic signals and regulation of colonic contractions [437, 438]. Interestingly, NO and H₂S are important gaseous molecules regulating pacemaker activity of ICC, and act cooperatively [448]. Moreover, NO and H₂S have been shown to react with the formation of nitroxyl, which represents one of the pathways suggested for endogenous HNO generation. As shown by Donzelli et al, nitroxyl induces PKG oxidative activation via formation of an intradisulfide bond between Cys117 and Cys195, and could potentially represent a mechanism regulating GI motility. Notably, as shown in this study C195S PKG KI mice that cannot form the disulfide, have an enlarged caecum and

impaired GI motility as determined by whole gut transit time studies. Thus, any future work might rationally focus on unravelling the mechanisms behind this ‘gut phenotype’. After the observation has been further corroborated using additional cohorts of mice, studies aimed at identify which cell types are responsible for phenotype, and which signalling pathways contribute to it, would be a logical way to proceed.

sEH redox regulation and myogenic tone

Apart from neurohormonal inputs, vascular myogenic response is a major mechanism that maintains basal tone to regulate blood pressure and organ perfusion [531]. Myogenic tone is characterised by the ability of arteries and arterioles to react to a change in transmural pressure, thus allowing constant blood flow despite changes in arterial pressure. Myogenic response requires a mechanism that transforms physical stimuli into biological reactions, leading to a change in vessel diameter. The mechanisms underlying myogenic response are not fully understood, but a role for CYP450 hydroxylase product 20-hydroxyeicosatetraenoic acid (20-HETE) is well established. 20-HETE depolarises VSMCs by blocking K_{Ca} channels and increasing the conductance of VDCC channels [532, 533]. EETs, produced by CYP450 epoxygenases, are potent vasodilators that hyperpolarise VSMCs, mainly by activating K_{Ca} channels. The role of EETs in regulation of myogenic tone has not been extensively studied [534]. Alterations in the ratio of 20-HETE/EETs could be an important regulator of myogenic tone. sEH rapidly hydrolyses EETs to their vicinal diols, which diminishes the pool of vasodilatory EETs and might consequently participate in myogenic response regulation. The inhibition of sEH by adduction of electrophiles to Cys521 has been shown to be important in blood pressure regulation [30]. With the considerations above in mind, it would be rational to test the hypothesis that inhibition of sEH by lipid adduction to Cys521 might be a component mechanism in the regulation of myogenic tone. To test this hypothesis resistance arteries from C521S sEH KI mice or WT mice will be studied using pressure myography. Further investigation of the molecular mechanism would subsequently be performed if small arteries from each genotype showed differential myogenic tone responses.

sEH redox regulation and hypoxic pulmonary vasoconstriction?

Hypoxic pulmonary vasoconstriction is an important physiological mechanism whereby pulmonary arteries constrict in oxygen-deficient areas of the lung in order to redirect blood flow from poor to better ventilated areas. This incompletely understood mechanism allows the efficient oxygenation of blood, but in some disease scenarios this mechanism can be over-recruited throughout the lung and so actually limit oxygenation. EETs have been shown to contribute to pulmonary vascular tone and hypoxic pulmonary vasoconstriction [535]. Although EETs are vasodilators in the peripheral and coronary vasculature, they are potent vasoconstrictors in the pulmonary vasculature. Selective CYP450 epoxygenase inhibitors significantly reduce acute hypoxic pulmonary vasoconstriction and chronic hypoxia-induced vascular remodelling [280, 536]. Moreover, preventing the breakdown of EETs by inhibiting sEH strongly augments the response to hypoxia [281]. Although it might seem paradoxical, numerous observations with a variety of methods and in many cells and tissue types suggest that hypoxia induces ROS production [537]. Increased ROS generally lead to lipid peroxidation and formation of lipid electrophiles, which can regulate numerous proteins via post-translational modifications. sEH is known to be inhibited by several electrophilic lipids by their adduction to Cys521 [29]. Thus, it is reasonable to speculate that redox regulation of sEH might play an important role in modulating pulmonary vasoconstriction. C521S sEH KI mice are therefore hypothesised to have lower EET levels in pulmonary vasculature and be protected from pulmonary vascular remodelling and pulmonary hypertension associated with hypoxia. WT and C521S sEH KI mice would be subjected to hypoxia, and right ventricle wall thickness and pulmonary artery blood flow will be analysed using echocardiography. The right ventricle hypertrophy would also be measured as the ratio of right ventricle weight and the weight of left ventricle plus septum (Fulton index). Lungs would be isolated and lung histology performed for further assessment of small pulmonary arteries muscularisation, which together with the other investigations would allow a potential role for sEH regulation by lipid electrophile in pulmonary hypertension to be defined.

As has been discussed in this thesis, understanding redox control of proteins is important, and studies in this area continue to emerge. Such new work may help in the

development of new pharmacotherapies, for example drugs with electrophilic properties. Indeed, drugs with oxidant or electrophilic features have shown promise in preclinical studies [67, 68, 70, 71, 73] and some such compounds are in clinical development [44, 69]. Indeed, 10-nitro-oleic acid which was used in this work is in clinical development (clinical trials number NCT02248051 and NCT02313064), and may, at least in part, exert its therapeutic actions by targeting sEH. The nitroxyl donor CXL-1020, which was also used in this work, is also in clinical development, and part of its action is likely to be through inducing intradisulfide bond formation in PKG [28].

List of references

1. Stadtman ER. *Protein oxidation in aging and age-related diseases*. Ann N Y Acad Sci, 2001; **928**: p. 22-38.
2. Cross CE, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL, McCord JM, Harman D. *Oxygen radicals and human disease*. Ann Intern Med, 1987; **107**(4): p. 526-45.
3. Holmstrom KM, Finkel T. *Cellular mechanisms and physiological consequences of redox-dependent signalling*. Nat Rev Mol Cell Biol, 2014; **15**(6): p. 411-21.
4. Schieber M, Chandel NS. *ROS function in redox signaling and oxidative stress*. Curr Biol, 2014; **24**(10): p. R453-62.
5. Couvertier SM, Zhou Y, Weerapana E. *Chemical-proteomic strategies to investigate cysteine posttranslational modifications*. Biochim Biophys Acta, 2014.
6. Antelmann H, Hellmann JD. *Thiol-based redox switches and gene regulation*. Antioxid Redox Signal, 2011; **14**(6): p. 1049-63.
7. Pace NJ, Weerapana E. *Diverse functional roles of reactive cysteines*. ACS Chem Biol, 2013; **8**(2): p. 283-96.
8. Truong TH, Carroll KS. *Redox regulation of protein kinases*. Crit Rev Biochem Mol Biol, 2013; **48**(4): p. 332-56.
9. Roos G, Foloppe N, Messens J. *Understanding the pK(a) of redox cysteines: the key role of hydrogen bonding*. Antioxid Redox Signal, 2013; **18**(1): p. 94-127.
10. Giles GI, Tasker KM, Jacob C. *Hypothesis: the role of reactive sulfur species in oxidative stress*. Free Radic Biol Med, 2001; **31**(10): p. 1279-83.
11. Lohse DL, Denu JM, Santoro N, Dixon JE. *Roles of aspartic acid-181 and serine-222 in intermediate formation and hydrolysis of the mammalian protein-tyrosine-phosphatase PTP1*. Biochemistry, 1997; **36**(15): p. 4568-75.
12. Madzalan P, Labunska T, Wilson MA. *Influence of peptide dipoles and hydrogen bonds on reactive cysteine pKa values in fission yeast DJ-1*. FEBS J, 2012; **279**(22): p. 4111-20.
13. Witt AC, Lakshminarasimhan M, Remington BC, Hasim S, Pozharski E, Wilson MA. *Cysteine pKa depression by a protonated glutamic acid in human DJ-1*. Biochemistry, 2008; **47**(28): p. 7430-40.
14. Nedospasov A, Rafikov R, Beda N, Nudler E. *An autocatalytic mechanism of protein nitrosylation*. Proc Natl Acad Sci U S A, 2000; **97**(25): p. 13543-8.
15. Miseta A, Csutora P. *Relationship between the occurrence of cysteine in proteins and the complexity of organisms*. Mol Biol Evol, 2000; **17**(8): p. 1232-9.
16. Go YM, Jones DP. *Thiol/disulfide redox states in signaling and sensing*. Crit Rev Biochem Mol Biol, 2013; **48**(2): p. 173-81.
17. Giles NM, Giles GI, Jacob C. *Multiple roles of cysteine in biocatalysis*. Biochem Biophys Res Commun, 2003; **300**(1): p. 1-4.
18. Paulsen CE, Carroll KS. *Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery*. Chem Rev, 2013; **113**(7): p. 4633-79.

19. Ullrich V, Kissner R. *Redox signaling: bioinorganic chemistry at its best*. J Inorg Biochem, 2006; **100**(12): p. 2079-86.
20. Berndt C, Lillig CH, Holmgren A. *Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system*. Am J Physiol Heart Circ Physiol, 2007; **292**(3): p. H1227-36.
21. Lowther WT, Haynes AC. *Reduction of cysteine sulfinic acid in eukaryotic, typical 2-Cys peroxiredoxins by sulfiredoxin*. Antioxid Redox Signal, 2011; **15**(1): p. 99-109.
22. Tanner JJ, Parsons ZD, Cummings AH, Zhou H, Gates KS. *Redox regulation of protein tyrosine phosphatases: structural and chemical aspects*. Antioxid Redox Signal, 2011; **15**(1): p. 77-97.
23. Meng TC, Fukada T, Tonks NK. *Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo*. Mol Cell, 2002; **9**(2): p. 387-99.
24. Schmalhausen EV, Nagradova NK, Boschi-Muller S, Branlant G, Mironetz VI. *Mildly oxidized GAPDH: the coupling of the dehydrogenase and acyl phosphatase activities*. FEBS Lett, 1999; **452**(3): p. 219-22.
25. Burgoyne JR, Madhani M, Cuello F, Charles RL, Brennan JP, Schroder E, Browning DD, Eaton P. *Cysteine redox sensor in PKGI α enables oxidant-induced activation*. Science, 2007; **317**(5843): p. 1393-7.
26. Prysyazhna O, Rudyk O, Eaton P. *Single atom substitution in mouse protein kinase G eliminates oxidant sensing to cause hypertension*. Nat Med, 2012; **18**(2): p. 286-90.
27. Scotcher J, Prysyazhna O, Boguslavskiy A, Kistamas K, Hadgraft N, Martin ED, Worthington J, Rudyk O, Rodriguez Cutillas P, Cuello F, Shattock MJ, Marber MS, Conte MR, Greenstein A, Greensmith DJ, Venetucci L, Timms JF, Eaton P. *Disulfide-activated protein kinase G I α regulates cardiac diastolic relaxation and fine-tunes the Frank-Starling response*. Nat Commun, 2016; **7**: p. 13187.
28. Donzelli S, Goetz M, Schmidt K, Wolters M, Stathopoulou K, Diering S, Prysyazhna O, Polat V, Scotcher J, Dees C, Subramanian H, Butt E, Kamynina A, Schobesberger S, King SB, Nikolaev VO, de Wit C, Leichert LI, Feil R, Eaton P, Cuello F. *Oxidant sensor in the cGMP-binding pocket of PKGI α regulates nitroxyl-mediated kinase activity*. Sci Rep, 2017; **7**(1): p. 9938.
29. Charles RL, Burgoyne JR, Mayr M, Weldon SM, Hubner N, Dong H, Morisseau C, Hammock BD, Landar A, Eaton P. *Redox regulation of soluble epoxide hydrolase by 15-deoxy-delta-prostaglandin J2 controls coronary hypoxic vasodilation*. Circ Res, 2011; **108**(3): p. 324-34.
30. Charles RL, Rudyk O, Prysyazhna O, Kamynina A, Yang J, Morisseau C, Hammock BD, Freeman BA, Eaton P. *Protection from hypertension in mice by the Mediterranean diet is mediated by nitro fatty acid inhibition of soluble epoxide hydrolase*. Proc Natl Acad Sci U S A, 2014; **111**(22): p. 8167-72.
31. LoPachin RM, Barber DS, Gavin T. *Molecular mechanisms of the conjugated α,β -unsaturated carbonyl derivatives: relevance to neurotoxicity and neurodegenerative diseases*. Toxicol Sci, 2008; **104**(2): p. 235-49.
32. Groeger AL, Freeman BA. *Signaling actions of electrophiles: anti-inflammatory therapeutic candidates*. Mol Interv, 2010; **10**(1): p. 39-50.
33. Dinkova-Kostova AT, Jenkins SN, Fahey JW, Ye L, Wehage SL, Liby KT, Stephenson KK, Wade KL, Talalay P. *Protection against UV-light-induced skin*

- carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts.* Cancer Lett, 2006; **240**(2): p. 243-52.
34. Satoh T, Kosaka K, Itoh K, Kobayashi A, Yamamoto M, Shimojo Y, Kitajima C, Cui J, Kamins J, Okamoto S, Izumi M, Shirasawa T, Lipton SA. *Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1.* J Neurochem, 2008; **104**(4): p. 1116-31.
 35. Cornblatt BS, Ye L, Dinkova-Kostova AT, Erb M, Fahey JW, Singh NK, Chen MS, Stierer T, Garrett-Mayer E, Argani P, Davidson NE, Talalay P, Kensler TW, Visvanathan K. *Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast.* Carcinogenesis, 2007; **28**(7): p. 1485-90.
 36. Johnson JJ, Mukhtar H. *Curcumin for chemoprevention of colon cancer.* Cancer Lett, 2007; **255**(2): p. 170-81.
 37. Satoh T, Lipton SA. *Redox regulation of neuronal survival mediated by electrophilic compounds.* Trends Neurosci, 2007; **30**(1): p. 37-45.
 38. Cole MP, Rudolph TK, Khoo NK, Motanya UN, Golin-Bisello F, Wertz JW, Schopfer FJ, Rudolph V, Woodcock SR, Bolisetty S, Ali MS, Zhang J, Chen YE, Agarwal A, Freeman BA, Bauer PM. *Nitro-fatty acid inhibition of neointima formation after endoluminal vessel injury.* Circ Res, 2009; **105**(10): p. 965-72.
 39. Rudolph V, Rudolph TK, Schopfer FJ, Bonacci G, Woodcock SR, Cole MP, Baker PR, Ramani R, Freeman BA. *Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion.* Cardiovasc Res, 2010; **85**(1): p. 155-66.
 40. Zhang J, Villacorta L, Chang L, Fan Z, Hamblin M, Zhu T, Chen CS, Cole MP, Schopfer FJ, Deng CX, Garcia-Barrio MT, Feng YH, Freeman BA, Chen YE. *Nitro-oleic acid inhibits angiotensin II-induced hypertension.* Circ Res, 2010; **107**(4): p. 540-8.
 41. Rudolph TK, Rudolph V, Edreira MM, Cole MP, Bonacci G, Schopfer FJ, Woodcock SR, Franek A, Pekarova M, Khoo NK, Hasty AH, Baldus S, Freeman BA. *Nitro-fatty acids reduce atherosclerosis in apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2010; **30**(5): p. 938-45.
 42. Schopfer FJ, Cipollina C, Freeman BA. *Formation and signaling actions of electrophilic lipids.* Chem Rev, 2011; **111**(10): p. 5997-6021.
 43. Rappaport SM, Li H, Grigoryan H, Funk WE, Williams ER. *Adductomics: characterizing exposures to reactive electrophiles.* Toxicol Lett, 2012; **213**(1): p. 83-90.
 44. Freeman BA, Baker PR, Schopfer FJ, Woodcock SR, Napolitano A, d'Ischia M. *Nitro-fatty acid formation and signaling.* J Biol Chem, 2008; **283**(23): p. 15515-9.
 45. Gueraud F, Atalay M, Bresgen N, Cipak A, Eckl PM, Huc L, Jouanin I, Siems W, Uchida K. *Chemistry and biochemistry of lipid peroxidation products.* Free Radic Res, 2010; **44**(10): p. 1098-124.
 46. Fritz KS, Petersen DR. *An overview of the chemistry and biology of reactive aldehydes.* Free Radic Biol Med, 2013; **59**: p. 85-91.
 47. Gilroy DW, Colville-Nash PR, Willis D, Chivers J, Paul-Clark MJ, Willoughby DA. *Inducible cyclooxygenase may have anti-inflammatory properties.* Nat Med, 1999; **5**(6): p. 698-701.

48. Brown HA, Marnett LJ. *Introduction to lipid biochemistry, metabolism, and signaling*. Chem Rev, 2011; **111**(10): p. 5817-20.
49. Lima ES, Di Mascio P, Abdalla DS. *Cholesteryl nitrolinoleate, a nitrated lipid present in human blood plasma and lipoproteins*. J Lipid Res, 2003; **44**(9): p. 1660-6.
50. Tsikas D, Zoerner A, Mitschke A, Homsy Y, Gutzki FM, Jordan J. *Specific GC-MS/MS stable-isotope dilution methodology for free 9- and 10-nitro-oleic acid in human plasma challenges previous LC-MS/MS reports*. J Chromatogr B Analyt Technol Biomed Life Sci, 2009; **877**(26): p. 2895-908.
51. Balazy M, Iesaki T, Park JL, Jiang H, Kaminski PM, Wolin MS. *Vicinal nitrohydroxyeicosatrienoic acids: vasodilator lipids formed by reaction of nitrogen dioxide with arachidonic acid*. J Pharmacol Exp Ther, 2001; **299**(2): p. 611-9.
52. Lopachin RM, Gavin T, Decaprio A, Barber DS. *Application of the Hard and Soft, Acids and Bases (HSAB) theory to toxicant--target interactions*. Chem Res Toxicol, 2012; **25**(2): p. 239-51.
53. Lin D, Saleh S, Liebler DC. *Reversibility of covalent electrophile-protein adducts and chemical toxicity*. Chem Res Toxicol, 2008; **21**(12): p. 2361-9.
54. Nerland DE. *The antioxidant/electrophile response element motif*. Drug Metab Rev, 2007; **39**(1): p. 235-48.
55. Itoh K, Tong KI, Yamamoto M. *Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles*. Free Radic Biol Med, 2004; **36**(10): p. 1208-13.
56. Carbone DL, Doorn JA, Kiebler Z, Ickes BR, Petersen DR. *Modification of heat shock protein 90 by 4-hydroxynonenal in a rat model of chronic alcoholic liver disease*. J Pharmacol Exp Ther, 2005; **315**(1): p. 8-15.
57. Carbone DL, Doorn JA, Kiebler Z, Sampey BP, Petersen DR. *Inhibition of Hsp72-mediated protein refolding by 4-hydroxy-2-nonenal*. Chem Res Toxicol, 2004; **17**(11): p. 1459-67.
58. Venkatraman A, Landar A, Davis AJ, Ulasova E, Page G, Murphy MP, Darley-Usmar V, Bailey SM. *Oxidative modification of hepatic mitochondria protein thiols: effect of chronic alcohol consumption*. Am J Physiol Gastrointest Liver Physiol, 2004; **286**(4): p. G521-7.
59. Sharma R, Sharma A, Dwivedi S, Zimniak P, Awasthi S, Awasthi YC. *4-Hydroxynonenal self-limits fas-mediated DISC-independent apoptosis by promoting export of Daxx from the nucleus to the cytosol and its binding to Fas*. Biochemistry, 2008; **47**(1): p. 143-56.
60. Wagner TM, Mullally JE, Fitzpatrick FA. *Reactive lipid species from cyclooxygenase-2 inactivate tumor suppressor LKB1/STK11: cyclopentenone prostaglandins and 4-hydroxy-2-nonenal covalently modify and inhibit the AMP-kinase kinase that modulates cellular energy homeostasis and protein translation*. J Biol Chem, 2006; **281**(5): p. 2598-604.
61. Codreanu SG, Adams DG, Dawson ES, Wadzinski BE, Liebler DC. *Inhibition of protein phosphatase 2A activity by selective electrophile alkylation damage*. Biochemistry, 2006; **45**(33): p. 10020-9.
62. Shibata T, Yamada T, Ishii T, Kumazawa S, Nakamura H, Masutani H, Yodoi J, Uchida K. *Thioredoxin as a molecular target of cyclopentenone prostaglandins*. J Biol Chem, 2003; **278**(28): p. 26046-54.

63. Shiraki T, Kamiya N, Shiki S, Kodama TS, Kakizuka A, Jingami H. *Alpha,beta-unsaturated ketone is a core moiety of natural ligands for covalent binding to peroxisome proliferator-activated receptor gamma*. J Biol Chem, 2005; **280**(14): p. 14145-53.
64. Rabbani N, Thornalley PJ. *Glyoxalase in diabetes, obesity and related disorders*. Semin Cell Dev Biol, 2011; **22**(3): p. 309-17.
65. Benderdour M, Charron G, DeBlois D, Comte B, Des Rosiers C. *Cardiac mitochondrial NADP⁺-isocitrate dehydrogenase is inactivated through 4-hydroxynonenal adduct formation: an event that precedes hypertrophy development*. J Biol Chem, 2003; **278**(46): p. 45154-9.
66. Wall SB, Smith MR, Ricart K, Zhou F, Vayalil PK, Oh JY, Landar A. *Detection of electrophile-sensitive proteins*. Biochim Biophys Acta, 2014; **1840**(2): p. 913-22.
67. Surh YJ, Na HK, Park JM, Lee HN, Kim W, Yoon IS, Kim DD. *15-Deoxy-Delta(1)(2),(1)(4)-prostaglandin J(2), an electrophilic lipid mediator of anti-inflammatory and pro-resolving signaling*. Biochem Pharmacol, 2011; **82**(10): p. 1335-51.
68. Villacorta L, Chang L, Salvatore SR, Ichikawa T, Zhang J, Petrovic-Djergovic D, Jia L, Carlsen H, Schopfer FJ, Freeman BA, Chen YE. *Electrophilic nitro-fatty acids inhibit vascular inflammation by disrupting LPS-dependent TLR4 signalling in lipid rafts*. Cardiovasc Res, 2013; **98**(1): p. 116-24.
69. Schopfer FJ, Cole MP, Groeger AL, Chen CS, Khoo NK, Woodcock SR, Golin-Bisello F, Motanya UN, Li Y, Zhang J, Garcia-Barrio MT, Rudolph TK, Rudolph V, Bonacci G, Baker PR, Xu HE, Batthyany CI, Chen YE, Hallis TM, Freeman BA. *Covalent peroxisome proliferator-activated receptor gamma adduction by nitro-fatty acids: selective ligand activity and anti-diabetic signaling actions*. J Biol Chem, 2010; **285**(16): p. 12321-33.
70. Wu J, Li Q, Wang X, Yu S, Li L, Wu X, Chen Y, Zhao J, Zhao Y. *Neuroprotection by curcumin in ischemic brain injury involves the Akt/Nrf2 pathway*. PLoS One, 2013; **8**(3): p. e59843.
71. Mishra S, Palanivelu K. *The effect of curcumin (turmeric) on Alzheimer's disease: An overview*. Ann Indian Acad Neurol, 2008; **11**(1): p. 13-9.
72. Wang T, Takikawa Y, Satoh T, Yoshioka Y, Kosaka K, Tatemichi Y, Suzuki K. *Carnosic acid prevents obesity and hepatic steatosis in ob/ob mice*. Hepatol Res, 2011; **41**(1): p. 87-92.
73. Tarozzi A, Angeloni C, Malaguti M, Morroni F, Hrelia S, Hrelia P. *Sulforaphane as a potential protective phytochemical against neurodegenerative diseases*. Oxid Med Cell Longev, 2013; **2013**: p. 415078.
74. Bartberger MD, Liu W, Ford E, Miranda KM, Switzer C, Fukuto JM, Farmer PJ, Wink DA, Houk KN. *The reduction potential of nitric oxide (NO) and its importance to NO biochemistry*. Proc Natl Acad Sci U S A, 2002; **99**(17): p. 10958-63.
75. Shafirovich V, Lyman SV. *Nitroxyl and its anion in aqueous solutions: spin states, protic equilibria, and reactivities toward oxygen and nitric oxide*. Proc Natl Acad Sci U S A, 2002; **99**(11): p. 7340-5.
76. DuMond JF, King SB. *The chemistry of nitroxyl-releasing compounds*. Antioxid Redox Signal, 2011; **14**(9): p. 1637-48.

77. Miranda KM, Paolocci N, Katori T, Thomas DD, Ford E, Bartberger MD, Espey MG, Kass DA, Feelisch M, Fukuto JM, Wink DA. *A biochemical rationale for the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system.* Proc Natl Acad Sci U S A, 2003; **100**(16): p. 9196-201.
78. Lemal DM, Rave TW. *Diazenes from Angelis Salt.* Journal of the American Chemical Society, 1965; **87**(2): p. 393-&.
79. Doyle MP, Mahapatro SN, Broene RD, Guy JK. *Oxidation and Reduction of Hemoproteins by Trioxodinitrate(Ii) - the Role of Nitrosyl Hydride and Nitrite.* Journal of the American Chemical Society, 1988; **110**(2): p. 593-599.
80. Bartberger MD, Fukuto JM, Houk KN. *On the acidity and reactivity of HNO in aqueous solution and biological systems.* Proceedings of the National Academy of Sciences of the United States of America, 2001; **98**(5): p. 2194-2198.
81. Reisz JA, Klorig EB, Wright MW, King SB. *Reductive Phosphine-Mediated Ligation of Nitroxyl (HNO).* Organic Letters, 2009; **11**(13): p. 2719-2721.
82. Farmer PJ, Sulc F. *Coordination chemistry of the HNO ligand with hemes and synthetic coordination complexes.* Journal of Inorganic Biochemistry, 2005; **99**(1): p. 166-184.
83. King SB, Nagasawa HT. *Chemical approaches toward generation of nitroxyl.* Nitric Oxide, Pt C, 1999; **301**: p. 211-220.
84. Hughes MN, Wimbledon PE. *Chemistry of Trioxodinitrates .1. Decomposition of Sodium Trioxodinitrate (Angelis Salt) in Aqueous-Solution.* Journal of the Chemical Society-Dalton Transactions, 1976(8): p. 703-707.
85. Dutton AS, Fukuto JM, Houk KN. *Mechanisms of HNO and NO production from Angeli's salt: Density functional and CBS-QB3 theory predictions.* Journal of the American Chemical Society, 2004; **126**(12): p. 3795-3800.
86. Miranda KM, Dutton AS, Ridnour LA, Foreman CA, Ford E, Paolocci N, Katori T, Tocchetti CG, Mancardi D, Thomas DD, Espey MG, Houk KN, Fukuto JM, Wink DA. *Mechanism of aerobic decomposition of Angeli's salt (sodium trioxodinitrate) at physiological pH.* Journal of the American Chemical Society, 2005; **127**(2): p. 722-731.
87. Shoman ME, DuMond JF, Isbell TS, Crawford JH, Brandon A, Honovar J, Vitturi DA, White CR, Patel RP, King SB. *Acyloxy Nitroso Compounds as Nitroxyl (HNO) Donors: Kinetics, Reactions with Thiols, and Vasodilation Properties.* Journal of Medicinal Chemistry, 2011; **54**(4): p. 1059-1070.
88. Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, Freeman BA, Frenneaux M, Friedman J, Kelm M, Kevil CG, Kim-Shapiro DB, Kozlov AV, Lancaster JR, Lefer DJ, McColl K, McCurry K, Patel RP, Petersson J, Rassaf T, Reutov VP, Richter-Addo GB, Schechter A, Shiva S, Tsuchiya K, van Faassen EE, Webb AJ, Zuckerbraun BS, Zweier JL, Weitzberg E. *Nitrate and nitrite in biology, nutrition and therapeutics.* Nature Chemical Biology, 2009; **5**(12): p. 865-869.
89. Kemp-Harper BK. *Nitroxyl (HNO): A Novel Redox Signaling Molecule.* Antioxidants & Redox Signaling, 2011; **14**(9): p. 1609-1613.
90. Martin W. *Nitroxyl anion - the universal signalling partner of endogenously produced nitric oxide?* British Journal of Pharmacology, 2009; **157**(4): p. 537-539.
91. Miranda KM, Paolocci N, Katori T, Thomas DD, Ford E, Bartberger MD, Espey MG, Kass DA, Feelisch M, Fukuto JM, Wink DA. *A biochemical rationale for*

- the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system.* Proceedings of the National Academy of Sciences of the United States of America, 2003; **100**(16): p. 9196-9201.
92. Paolocci N, Katori T, Champion HC, St John ME, Miranda KM, Fukuto JM, Wink DA, Kass DA. *Positive inotropic and lusitropic effects of HNO/NO- in failing hearts: Independence from beta-adrenergic signaling.* Proceedings of the National Academy of Sciences of the United States of America, 2003; **100**(9): p. 5537-5542.
 93. Yuill KH, Yarova P, Kemp-Harper BK, Garland CJ, Dora KA. *A Novel Role for HNO in Local and Spreading Vasodilatation in Rat Mesenteric Resistance Arteries.* Antioxidants & Redox Signaling, 2011; **14**(9): p. 1625-1635.
 94. Clague MJ, Wishnok JS, Marletta MA. *Formation of N delta-cyanoornithine from NG-hydroxy-L-arginine and hydrogen peroxide by neuronal nitric oxide synthase: implications for mechanism.* Biochemistry, 1997; **36**(47): p. 14465-73.
 95. Pufahl RA, Wishnok JS, Marletta MA. *Hydrogen peroxide-supported oxidation of NG-hydroxy-L-arginine by nitric oxide synthase.* Biochemistry, 1995; **34**(6): p. 1930-41.
 96. Rusche KM, Spiering MM, Marletta MA. *Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase.* Biochemistry, 1998; **37**(44): p. 15503-12.
 97. Donzelli S, Espey MG, Flores-Santana W, Switzer CH, Yeh GC, Huang J, Stuehr DJ, King SB, Miranda KM, Wink DA. *Generation of nitroxyl by heme protein-mediated peroxidation of hydroxylamine but not N-hydroxy-L-arginine.* Free Radic Biol Med, 2008; **45**(5): p. 578-84.
 98. Huang J, Sommers EM, Kim-Shapiro DB, King SB. *Horseradish peroxidase catalyzed nitric oxide formation from hydroxyurea.* J Am Chem Soc, 2002; **124**(13): p. 3473-80.
 99. Niketic V, Stojanovic S, Nikolic A, Spasic M, Michelson AM. *Exposure of Mn and FeSODs, but not Cu/ZnSOD, to NO leads to nitrosonium and nitroxyl ions generation which cause enzyme modification and inactivation: an in vitro study.* Free Radic Biol Med, 1999; **27**(9-10): p. 992-6.
 100. Saleem M, Ohshima H. *Xanthine oxidase converts nitric oxide to nitroxyl that inactivates the enzyme.* Biochem Biophys Res Commun, 2004; **315**(2): p. 455-62.
 101. Lyman SV, Shafirovich V, Poskrebyshev GA. *One-electron reduction of aqueous nitric oxide: a mechanistic revision.* Inorg Chem, 2005; **44**(15): p. 5212-21.
 102. Wong PS, Hyun J, Fukuto JM, Shirota FN, DeMaster EG, Shoeman DW, Nagasawa HT. *Reaction between S-nitrosothiols and thiols: generation of nitroxyl (HNO) and subsequent chemistry.* Biochemistry, 1998; **37**(16): p. 5362-71.
 103. DeMaster EG, Quast BJ, Redfern B, Nagasawa HT. *Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide.* Biochemistry, 1995; **34**(36): p. 11494-9.
 104. Stoyanovsky DA, Tyurina YY, Tyurin VA, Anand D, Mandavia DN, Gius D, Ivanova J, Pitt B, Billiar TR, Kagan VE. *Thioredoxin and lipoic acid catalyze the denitrosation of low molecular weight and protein S-nitrosothiols.* J Am Chem Soc, 2005; **127**(45): p. 15815-23.

105. Bartberger MD, Mannion JD, Powell SC, Stamler JS, Houk KN, Toone EJ. *S-N dissociation energies of S-nitrosothiols: on the origins of nitrosothiol decomposition rates*. J Am Chem Soc, 2001; **123**(36): p. 8868-9.
106. Filipovic MR, Eberhardt M, Prokopovic V, Mijuskovic A, Orescanin-Dusic Z, Reeh P, Ivanovic-Burmazovic I. *Beyond H₂S and NO interplay: hydrogen sulfide and nitroprusside react directly to give nitroxyl (HNO). A new pharmacological source of HNO*. J Med Chem, 2013; **56**(4): p. 1499-508.
107. Yong QC, Hu LF, Wang S, Huang D, Bian JS. *Hydrogen sulfide interacts with nitric oxide in the heart: possible involvement of nitroxyl*. Cardiovasc Res, 2010; **88**(3): p. 482-91.
108. Eberhardt M, Dux M, Namer B, Miljkovic J, Cordasic N, Will C, Kichko TI, de la Roche J, Fischer M, Suarez SA, Bikiel D, Dorsch K, Leffler A, Babes A, Lampert A, Lennerz JK, Jacobi J, Marti MA, Doctorovich F, Hogestatt ED, Zygmunt PM, Ivanovic-Burmazovic I, Messlinger K, Reeh P, Filipovic MR. *H₂S and NO cooperatively regulate vascular tone by activating a neuroendocrine HNO-TRPA1-CGRP signalling pathway*. Nat Commun, 2014; **5**: p. 4381.
109. Cortese-Krott MM, Kuhnle GG, Dyson A, Fernandez BO, Grman M, DuMond JF, Barrow MP, McLeod G, Nakagawa H, Ondrias K, Nagy P, King SB, Saavedra JE, Keefer LK, Singer M, Kelm M, Butler AR, Feelisch M. *Key bioactive reaction products of the NO/H₂S interaction are S/N-hybrid species, polysulfides, and nitroxyl*. Proc Natl Acad Sci U S A, 2015; **112**(34): p. E4651-60.
110. Stubbert D, Prysyazhna O, Rudyk O, Scotcher J, Burgoyne JR, Eaton P. *Protein kinase G Ialpha oxidation paradoxically underlies blood pressure lowering by the reductant hydrogen sulfide*. Hypertension, 2014; **64**(6): p. 1344-51.
111. Hamer M, Suarez SA, Neuman NI, Alvarez L, Munoz M, Marti MA, Doctorovich F. *Discussing endogenous NO(*)/HNO interconversion aided by phenolic drugs and vitamins*. Inorg Chem, 2015; **54**(19): p. 9342-50.
112. Suarez SA, Neuman NI, Munoz M, Alvarez L, Bikiel DE, Brondino CD, Ivanovic-Burmazovic I, Miljkovic J, Filipovic MR, Marti MA, Doctorovich F. *Nitric oxide is reduced to HNO by proton-coupled nucleophilic attack by ascorbate, tyrosine, and other alcohols. A new route to HNO in biological media?* J Am Chem Soc, 2015; **137**(14): p. 4720-7.
113. Shiva S. *Nitrite: A Physiological Store of Nitric Oxide and Modulator of Mitochondrial Function*. Redox Biol, 2013; **1**(1): p. 40-44.
114. Miranda KM. *The chemistry of nitroxyl (HNO) and implications in biology*. Coordination Chemistry Reviews, 2005; **249**(3-4): p. 433-455.
115. Fukuto JM, Carrington SJ. *HNO signaling mechanisms*. Antioxid Redox Signal, 2011; **14**(9): p. 1649-57.
116. Sherman MP, Grither WR, McCulla RD. *Computational investigation of the reaction mechanisms of nitroxyl and thiols*. J Org Chem, 2010; **75**(12): p. 4014-24.
117. Jackson SG, Zhang F, Chindemi P, Junop MS, Berti PJ. *Evidence of kinetic control of ligand binding and staged product release in MurA (enolpyruvyl UDP-GlcNAc synthase)-catalyzed reactions*. Biochemistry, 2009; **48**(49): p. 11715-23.

118. Shen B, English AM. *Mass spectrometric analysis of nitroxyl-mediated protein modification: comparison of products formed with free and protein-based cysteines*. Biochemistry, 2005; **44**(42): p. 14030-44.
119. Keceli G, Toscano JP. *Reactivity of nitroxyl-derived sulfinamides*. Biochemistry, 2012; **51**(20): p. 4206-16.
120. Miranda KM, Nims RW, Thomas DD, Espey MG, Citrin D, Bartberger MD, Paolucci N, Fukuto JM, Feelisch M, Wink DA. *Comparison of the reactivity of nitric oxide and nitroxyl with heme proteins. A chemical discussion of the differential biological effects of these redox related products of NOS*. J Inorg Biochem, 2003; **93**(1-2): p. 52-60.
121. Doctorovich F, Bikiel DE, Pellegrino J, Suarez SA, Marti MA. *Reactions of HNO with metal porphyrins: underscoring the biological relevance of HNO*. Acc Chem Res, 2014; **47**(10): p. 2907-16.
122. Miller TW, Cherney MM, Lee AJ, Francoleon NE, Farmer PJ, King SB, Hobbs AJ, Miranda KM, Burstyn JN, Fukuto JM. *The effects of nitroxyl (HNO) on soluble guanylate cyclase activity: interactions at ferrous heme and cysteine thiols*. J Biol Chem, 2009; **284**(33): p. 21788-96.
123. Kimura H, Mittal CK, Murad F. *Activation of guanylate cyclase from rat liver and other tissues by sodium azide*. J Biol Chem, 1975; **250**(20): p. 8016-22.
124. Paolucci N, Saavedra WF, Miranda KM, Martignani C, Isoda T, Hare JM, Espey MG, Fukuto JM, Feelisch M, Wink DA, Kass DA. *Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling*. Proc Natl Acad Sci U S A, 2001; **98**(18): p. 10463-8.
125. Ellis A, Li CG, Rand MJ. *Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta*. Br J Pharmacol, 2000; **129**(2): p. 315-22.
126. Favalaro JL, Kemp-Harper BK. *The nitroxyl anion (HNO) is a potent dilator of rat coronary vasculature*. Cardiovasc Res, 2007; **73**(3): p. 587-96.
127. Dierks EA, Burstyn JN. *Nitric oxide (NO), the only nitrogen monoxide redox form capable of activating soluble guanylyl cyclase*. Biochem Pharmacol, 1996; **51**(12): p. 1593-600.
128. Zeller A, Wenzl MV, Beretta M, Stessel H, Russwurm M, Koesling D, Schmidt K, Mayer B. *Mechanisms underlying activation of soluble guanylate cyclase by the nitroxyl donor Angeli's salt*. Mol Pharmacol, 2009; **76**(5): p. 1115-22.
129. Fukuto JM, Chiang K, Hszieh R, Wong P, Chaudhuri G. *The pharmacological activity of nitroxyl: a potent vasodilator with activity similar to nitric oxide and/or endothelium-derived relaxing factor*. J Pharmacol Exp Ther, 1992; **263**(2): p. 546-51.
130. Andrews KL, Irvine JC, Tare M, Apostolopoulos J, Favalaro JL, Triggle CR, Kemp-Harper BK. *A role for nitroxyl (HNO) as an endothelium-derived relaxing and hyperpolarizing factor in resistance arteries*. Br J Pharmacol, 2009; **157**(4): p. 540-50.
131. Botden IP, Batenburg WW, de Vries R, Langendonk JG, Sijbrands EJ, Danser AH. *Nitrite- and nitroxyl-induced relaxation in porcine coronary (micro-) arteries: underlying mechanisms and role as endothelium-derived hyperpolarizing factor(s)*. Pharmacol Res, 2012; **66**(5): p. 409-18.

132. Irvine JC, Favalaro JL, Kemp-Harper BK. *NO- activates soluble guanylate cyclase and Kv channels to vasodilate resistance arteries*. Hypertension, 2003; **41**(6): p. 1301-7.
133. Wanstall JC, Jeffery TK, Gambino A, Lovren F, Triggle CR. *Vascular smooth muscle relaxation mediated by nitric oxide donors: a comparison with acetylcholine, nitric oxide and nitroxyl ion*. Br J Pharmacol, 2001; **134**(3): p. 463-72.
134. Andrews KL, Lumsden NG, Farry J, Jefferis AM, Kemp-Harper BK, Chindusting JP. *Nitroxyl: a vasodilator of human vessels that is not susceptible to tolerance*. Clin Sci (Lond), 2015; **129**(2): p. 179-87.
135. Donzelli S, Fischer G, King BS, Niemann C, DuMond JF, Heeren J, Wieboldt H, Baldus S, Gerloff C, Eschenhagen T, Carrier L, Boger RH, Espey MG. *Pharmacological characterization of 1-nitrosocyclohexyl acetate, a long-acting nitroxyl donor that shows vasorelaxant and antiaggregatory effects*. J Pharmacol Exp Ther, 2013; **344**(2): p. 339-47.
136. Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di Benedetto G, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, Paolocci N. *Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca²⁺ cycling*. Circ Res, 2007; **100**(1): p. 96-104.
137. Lancel S, Zhang J, Evangelista A, Trucillo MP, Tong X, Siwik DA, Cohen RA, Colucci WS. *Nitroxyl activates SERCA in cardiac myocytes via glutathiolation of cysteine 674*. Circ Res, 2009; **104**(6): p. 720-3.
138. Froehlich JP, Mahaney JE, Keceli G, Pavlos CM, Goldstein R, Redwood AJ, Sumbilla C, Lee DI, Tocchetti CG, Kass DA, Paolocci N, Toscano JP. *Phospholamban thiols play a central role in activation of the cardiac muscle sarcoplasmic reticulum calcium pump by nitroxyl*. Biochemistry, 2008; **47**(50): p. 13150-2.
139. Sivakumaran V, Stanley BA, Tocchetti CG, Ballin JD, Caceres V, Zhou L, Keceli G, Rainer PP, Lee DI, Huke S, Ziolo MT, Kranias EG, Toscano JP, Wilson GM, O'Rourke B, Kass DA, Mahaney JE, Paolocci N. *HNO enhances SERCA2a activity and cardiomyocyte function by promoting redox-dependent phospholamban oligomerization*. Antioxid Redox Signal, 2013; **19**(11): p. 1185-97.
140. Dai T, Tian Y, Tocchetti CG, Katori T, Murphy AM, Kass DA, Paolocci N, Gao WD. *Nitroxyl increases force development in rat cardiac muscle*. J Physiol, 2007; **580**(Pt.3): p. 951-60.
141. Gao Y, Marshall P. *An experimental and computational study of the reaction of ground-state sulfur atoms with carbon disulfide*. J Chem Phys, 2011; **135**(14): p. 144306.
142. Oni-Orisan A, Alsaleh N, Lee CR, Seubert JM. *Epoxyeicosatrienoic acids and cardioprotection: the road to translation*. J Mol Cell Cardiol, 2014; **74**: p. 199-208.
143. Prysyazhna O, Burgoyne JR, Scotcher J, Grover S, Kass D, Eaton P. *Phosphodiesterase 5 Inhibition Limits Doxorubicin-induced Heart Failure by Attenuating Protein Kinase G Ialpha Oxidation*. J Biol Chem, 2016; **291**(33): p. 17427-36.

144. Nakamura T, Ranek MJ, Lee DI, Shalkey Hahn V, Kim C, Eaton P, Kass DA. *Prevention of PKG1 α oxidation augments cardioprotection in the stressed heart.* J Clin Invest, 2015; **125**(6): p. 2468-72.
145. Akashi S, Ahmed KA, Sawa T, Ono K, Tsutsuki H, Burgoyne JR, Ida T, Horio E, Prysyazhna O, Oike Y, Rahaman MM, Eaton P, Fujii S, Akaike T. *Persistent Activation of cGMP-Dependent Protein Kinase by a Nitrated Cyclic Nucleotide via Site Specific Protein S-Guanylation.* Biochemistry, 2016; **55**(5): p. 751-61.
146. Hofmann F, Feil R, Kleppisch T, Schlossmann J. *Function of cGMP-dependent protein kinases as revealed by gene deletion.* Physiol Rev, 2006; **86**(1): p. 1-23.
147. Francis SH, Busch JL, Corbin JD, Sibley D. *cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action.* Pharmacol Rev, 2010; **62**(3): p. 525-63.
148. Gambaryan S, Butt E, Marcus K, Glazova M, Palmethofer A, Guillon G, Smolenski A. *cGMP-dependent protein kinase type II regulates basal level of aldosterone production by zona glomerulosa cells without increasing expression of the steroidogenic acute regulatory protein gene.* J Biol Chem, 2003; **278**(32): p. 29640-8.
149. Rangaswami H, Marathe N, Zhuang S, Chen Y, Yeh JC, Frangos JA, Boss GR, Pilz RB. *Type II cGMP-dependent protein kinase mediates osteoblast mechanotransduction.* J Biol Chem, 2009; **284**(22): p. 14796-808.
150. Wagner C, Pfeifer A, Ruth P, Hofmann F, Kurtz A. *Role of cGMP-kinase II in the control of renin secretion and renin expression.* J Clin Invest, 1998; **102**(8): p. 1576-82.
151. Ammendola A, Geiselhoringer A, Hofmann F, Schlossmann J. *Molecular determinants of the interaction between the inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) and cGMP kinase Ib β .* J Biol Chem, 2001; **276**(26): p. 24153-9.
152. Surks HK, Mochizuki N, Kasai Y, Georgescu SP, Tang KM, Ito M, Lincoln TM, Mendelsohn ME. *Regulation of myosin phosphatase by a specific interaction with cGMP-dependent protein kinase I α .* Science, 1999; **286**(5444): p. 1583-7.
153. Tang KM, Wang GR, Lu P, Karas RH, Aronovitz M, Heximer SP, Kaltenbronn KM, Blumer KJ, Siderovski DP, Zhu Y, Mendelsohn ME. *Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure.* Nat Med, 2003; **9**(12): p. 1506-12.
154. Richie-Jannetta R, Francis SH, Corbin JD. *Dimerization of cGMP-dependent protein kinase Ib β is mediated by an extensive amino-terminal leucine zipper motif, and dimerization modulates enzyme function.* J Biol Chem, 2003; **278**(50): p. 50070-9.
155. Ruth P, Pfeifer A, Kamm S, Klatt P, Dostmann WR, Hofmann F. *Identification of the amino acid sequences responsible for high affinity activation of cGMP kinase I α .* J Biol Chem, 1997; **272**(16): p. 10522-8.
156. Sekhar KR, Hatchett RJ, Shabb JB, Wolfe L, Francis SH, Wells JN, Jastorff B, Butt E, Chakinala MM, Corbin JD. *Relaxation of pig coronary arteries by new and potent cGMP analogs that selectively activate type I α , compared with type I β , cGMP-dependent protein kinase.* Mol Pharmacol, 1992; **42**(1): p. 103-8.

157. Wolfe L, Francis SH, Corbin JD. *Properties of a cGMP-dependent monomeric protein kinase from bovine aorta*. J Biol Chem, 1989; **264**(7): p. 4157-62.
158. Redfield MM, Chen HH, Borlaug BA, Semigran MJ, Lee KL, Lewis G, LeWinter MM, Rouleau JL, Bull DA, Mann DL, Deswal A, Stevenson LW, Givertz MM, Ofili EO, O'Connor CM, Felker GM, Goldsmith SR, Bart BA, McNulty SE, Ibarra JC, Lin G, Oh JK, Patel MR, Kim RJ, Tracy RP, Velazquez EJ, Anstrom KJ, Hernandez AF, Mascette AM, Braunwald E, Trial R. *Effect of phosphodiesterase-5 inhibition on exercise capacity and clinical status in heart failure with preserved ejection fraction: a randomized clinical trial*. JAMA, 2013; **309**(12): p. 1268-77.
159. Geiselhöringer A, Gaisa M, Hofmann F, Schlossmann J. *Distribution of IRAG and cGKI-isoforms in murine tissues*. FEBS Lett, 2004; **575**(1-3): p. 19-22.
160. Keilbach A, Ruth P, Hofmann F. *Detection of cGMP dependent protein kinase isozymes by specific antibodies*. Eur J Biochem, 1992; **208**(2): p. 467-73.
161. Eigenthaler M, Nolte C, Halbrugge M, Walter U. *Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets. Estimating the rate of cAMP-regulated and cGMP-regulated protein phosphorylation in intact cells*. Eur J Biochem, 1992; **205**(2): p. 471-81.
162. Pfeifer A, Klatt P, Massberg S, Ny L, Sausbier M, Hirneiss C, Wang GX, Korth M, Aszodi A, Andersson KE, Krombach F, Mayerhofer A, Ruth P, Fassler R, Hofmann F. *Defective smooth muscle regulation in cGMP kinase I-deficient mice*. EMBO J, 1998; **17**(11): p. 3045-51.
163. Wegener JW, Nawrath H, Wolfsgrubner W, Kuhbandner S, Werner C, Hofmann F, Feil R. *cGMP-dependent protein kinase I mediates the negative inotropic effect of cGMP in the murine myocardium*. Circ Res, 2002; **90**(1): p. 18-20.
164. Wolfsgrubner W, Feil S, Brummer S, Kuppinge O, Hofmann F, Feil R. *A proatherogenic role for cGMP-dependent protein kinase in vascular smooth muscle cells*. Proc Natl Acad Sci U S A, 2003; **100**(23): p. 13519-24.
165. Kleppisch T, Wolfsgrubner W, Feil S, Allmann R, Wotjak CT, Goebbels S, Nave KA, Hofmann F, Feil R. *Hippocampal cGMP-dependent protein kinase I supports an age- and protein synthesis-dependent component of long-term potentiation but is not essential for spatial reference and contextual memory*. J Neurosci, 2003; **23**(14): p. 6005-12.
166. Pfeifer A, Aszodi A, Seidler U, Ruth P, Hofmann F, Fassler R. *Intestinal secretory defects and dwarfism in mice lacking cGMP-dependent protein kinase II*. Science, 1996; **274**(5295): p. 2082-6.
167. Steers WD. *Pharmacologic treatment of erectile dysfunction*. Rev Urol, 2002; **4 Suppl 3**: p. S17-25.
168. Duarte JD, Hanson RL, Machado RF. *Pharmacologic treatments for pulmonary hypertension: exploring pharmacogenomics*. Future Cardiol, 2013; **9**(3): p. 335-49.
169. Bryan NS, Bian K, Murad F. *Discovery of the nitric oxide signaling pathway and targets for drug development*. Front Biosci (Landmark Ed), 2009; **14**: p. 1-18.
170. Egemnazarov B, Sydykov A, Schermuly RT, Weissmann N, Stasch JP, Sarybaev AS, Seeger W, Grimminger F, Ghofrani HA. *Novel soluble guanylyl*

- cyclase stimulator BAY 41-2272 attenuates ischemia-reperfusion-induced lung injury.* Am J Physiol Lung Cell Mol Physiol, 2009; **296**(3): p. L462-9.
171. Krieg T, Liu Y, Rutz T, Methner C, Yang XM, Dost T, Felix SB, Stasch JP, Cohen MV, Downey JM. *BAY 58-2667, a nitric oxide-independent guanylyl cyclase activator, pharmacologically post-conditions rabbit and rat hearts.* Eur Heart J, 2009; **30**(13): p. 1607-13.
 172. Hedlund P, Aszodi A, Pfeifer A, Alm P, Hofmann F, Ahmad M, Fassler R, Andersson KE. *Erectile dysfunction in cyclic GMP-dependent kinase I-deficient mice.* Proc Natl Acad Sci U S A, 2000; **97**(5): p. 2349-54.
 173. Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W, Fassler R, Ruth P, Krombach F, Hofmann F. *Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I.* J Exp Med, 1999; **189**(8): p. 1255-64.
 174. Ny L, Pfeifer A, Aszodi A, Ahmad M, Alm P, Hedlund P, Fassler R, Andersson KE. *Impaired relaxation of stomach smooth muscle in mice lacking cyclic GMP-dependent protein kinase I.* Br J Pharmacol, 2000; **129**(2): p. 395-401.
 175. Persson K, Pandita RK, Aszodi A, Ahmad M, Pfeifer A, Fassler R, Andersson KE. *Functional characteristics of urinary tract smooth muscles in mice lacking cGMP protein kinase type I.* Am J Physiol Regul Integr Comp Physiol, 2000; **279**(3): p. R1112-20.
 176. Schmidt H, Werner M, Heppenstall PA, Henning M, More MI, Kuhbandner S, Lewin GR, Hofmann F, Feil R, Rathjen FG. *cGMP-mediated signaling via cGKIalpha is required for the guidance and connectivity of sensory axons.* J Cell Biol, 2002; **159**(3): p. 489-98.
 177. Feil R, Hartmann J, Luo C, Wolfsgruber W, Schilling K, Feil S, Barski JJ, Meyer M, Konnerth A, De Zeeuw CI, Hofmann F. *Impairment of LTD and cerebellar learning by Purkinje cell-specific ablation of cGMP-dependent protein kinase I.* J Cell Biol, 2003; **163**(2): p. 295-302.
 178. Zhao YD, Cai L, Mirza MK, Huang X, Geenen DL, Hofmann F, Yuan JX, Zhao YY. *Protein kinase G-I deficiency induces pulmonary hypertension through Rho A/Rho kinase activation.* Am J Pathol, 2012; **180**(6): p. 2268-75.
 179. Yamahara K, Itoh H, Chun TH, Ogawa Y, Yamashita J, Sawada N, Fukunaga Y, Sone M, Yurugi-Kobayashi T, Miyashita K, Tsujimoto H, Kook H, Feil R, Garbers DL, Hofmann F, Nakao K. *Significance and therapeutic potential of the natriuretic peptides/cGMP/cGMP-dependent protein kinase pathway in vascular regeneration.* Proc Natl Acad Sci U S A, 2003; **100**(6): p. 3404-9.
 180. Lukowski R, Rybalkin SD, Loga F, Leiss V, Beavo JA, Hofmann F. *Cardiac hypertrophy is not amplified by deletion of cGMP-dependent protein kinase I in cardiomyocytes.* Proc Natl Acad Sci U S A, 2010; **107**(12): p. 5646-51.
 181. Lukowski R, Weinmeister P, Bernhard D, Feil S, Gotthardt M, Herz J, Massberg S, Zernecke A, Weber C, Hofmann F, Feil R. *Role of smooth muscle cGMP/cGKI signaling in murine vascular restenosis.* Arterioscler Thromb Vasc Biol, 2008; **28**(7): p. 1244-50.
 182. Michael SK, Surks HK, Wang Y, Zhu Y, Blanton R, Jamnongjit M, Aronovitz M, Baur W, Ohtani K, Wilkerson MK, Bonev AD, Nelson MT, Karas RH, Mendelsohn ME. *High blood pressure arising from a defect in vascular function.* Proc Natl Acad Sci U S A, 2008; **105**(18): p. 6702-7.

183. Blanton RM, Takimoto E, Aronovitz M, Thoonen R, Kass DA, Karas RH, Mendelsohn ME. *Mutation of the protein kinase I alpha leucine zipper domain produces hypertension and progressive left ventricular hypertrophy: a novel mouse model of age-dependent hypertensive heart disease.* J Gerontol A Biol Sci Med Sci, 2013; **68**(11): p. 1351-5.
184. Weber S, Bernhard D, Lukowski R, Weinmeister P, Worner R, Wegener JW, Valtcheva N, Feil S, Schlossmann J, Hofmann F, Feil R. *Rescue of cGMP kinase I knockout mice by smooth muscle specific expression of either isozyme.* Circ Res, 2007; **101**(11): p. 1096-103.
185. Somlyo AP, Somlyo AV. *Ca²⁺ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase.* Physiol Rev, 2003; **83**(4): p. 1325-58.
186. Desch M, Sigl K, Hieke B, Salb K, Kees F, Bernhard D, Jochim A, Spiessberger B, Hoherl K, Feil R, Feil S, Lukowski R, Wegener JW, Hofmann F, Schlossmann J. *IRAG determines nitric oxide- and atrial natriuretic peptide-mediated smooth muscle relaxation.* Cardiovasc Res, 2010; **86**(3): p. 496-505.
187. Geiselhoring A, Werner M, Sigl K, Smital P, Worner R, Acheo L, Stieber J, Weinmeister P, Feil R, Feil S, Wegener J, Hofmann F, Schlossmann J. *IRAG is essential for relaxation of receptor-triggered smooth muscle contraction by cGMP kinase.* EMBO J, 2004; **23**(21): p. 4222-31.
188. Sun X, Kaltenbronn KM, Steinberg TH, Blumer KJ. *RGS2 is a mediator of nitric oxide action on blood pressure and vasoconstrictor signaling.* Mol Pharmacol, 2005; **67**(3): p. 631-9.
189. Xia C, Bao Z, Yue C, Sanborn BM, Liu M. *Phosphorylation and regulation of G-protein-activated phospholipase C-beta 3 by cGMP-dependent protein kinases.* J Biol Chem, 2001; **276**(23): p. 19770-7.
190. Osei-Owusu P, Sun X, Drenan RM, Steinberg TH, Blumer KJ. *Regulation of RGS2 and second messenger signaling in vascular smooth muscle cells by cGMP-dependent protein kinase.* J Biol Chem, 2007; **282**(43): p. 31656-65.
191. Wooldridge AA, MacDonald JA, Erdodi F, Ma C, Borman MA, Hartshorne DJ, Haystead TA. *Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of Serine 695 in response to cyclic nucleotides.* J Biol Chem, 2004; **279**(33): p. 34496-504.
192. Lee MR, Li L, Kitazawa T. *Cyclic GMP causes Ca²⁺ desensitization in vascular smooth muscle by activating the myosin light chain phosphatase.* J Biol Chem, 1997; **272**(8): p. 5063-8.
193. Lee E, Hayes DB, Langsetmo K, Sundberg EJ, Tao TC. *Interactions between the leucine-zipper motif of cGMP-dependent protein kinase and the C-terminal region of the targeting subunit of myosin light chain phosphatase.* J Mol Biol, 2007; **373**(5): p. 1198-212.
194. Sharma AK, Zhou GP, Kupferman J, Surks HK, Christensen EN, Chou JJ, Mendelsohn ME, Rigby AC. *Probing the interaction between the coiled coil leucine zipper of cGMP-dependent protein kinase Ialpha and the C terminus of the myosin binding subunit of the myosin light chain phosphatase.* J Biol Chem, 2008; **283**(47): p. 32860-9.
195. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. *Regulation of myosin*

- phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science*, 1996; **273**(5272): p. 245-8.
196. Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. *Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation*. *J Biol Chem*, 1997; **272**(19): p. 12257-60.
 197. Nakamura K, Koga Y, Sakai H, Homma K, Ikebe M. *cGMP-dependent relaxation of smooth muscle is coupled with the change in the phosphorylation of myosin phosphatase*. *Circ Res*, 2007; **101**(7): p. 712-22.
 198. Cornwell TL, Pryzwansky KB, Wyatt TA, Lincoln TM. *Regulation of sarcoplasmic reticulum protein phosphorylation by localized cyclic GMP-dependent protein kinase in vascular smooth muscle cells*. *Mol Pharmacol*, 1991; **40**(6): p. 923-31.
 199. Wray S, Burdyga T. *Sarcoplasmic reticulum function in smooth muscle*. *Physiol Rev*, 2010; **90**(1): p. 113-78.
 200. Lalli MJ, Shimizu S, Sutliff RL, Kranias EG, Paul RJ. *[Ca²⁺]_i homeostasis and cyclic nucleotide relaxation in aorta of phospholamban-deficient mice*. *Am J Physiol*, 1999; **277**(3 Pt 2): p. H963-70.
 201. Schlossmann J, Ammendola A, Ashman K, Zong X, Huber A, Neubauer G, Wang GX, Allescher HD, Korth M, Wilm M, Hofmann F, Ruth P. *Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta*. *Nature*, 2000; **404**(6774): p. 197-201.
 202. Christensen EN, Mendelsohn ME. *Cyclic GMP-dependent protein kinase Ialpha inhibits thrombin receptor-mediated calcium mobilization in vascular smooth muscle cells*. *J Biol Chem*, 2006; **281**(13): p. 8409-16.
 203. Feil R, Gappa N, Rutz M, Schlossmann J, Rose CR, Konnerth A, Brummer S, Kuhbandner S, Hofmann F. *Functional reconstitution of vascular smooth muscle cells with cGMP-dependent protein kinase I isoforms*. *Circ Res*, 2002; **90**(10): p. 1080-6.
 204. Kinoshita H, Kuwahara K, Nishida M, Jian Z, Rong X, Kiyonaka S, Kuwabara Y, Kurose H, Inoue R, Mori Y, Li Y, Nakagawa Y, Usami S, Fujiwara M, Yamada Y, Minami T, Ueshima K, Nakao K. *Inhibition of TRPC6 channel activity contributes to the antihypertrophic effects of natriuretic peptides-guanylyl cyclase-A signaling in the heart*. *Circ Res*, 2010; **106**(12): p. 1849-60.
 205. Koitabashi N, Aiba T, Hesketh GG, Rowell J, Zhang M, Takimoto E, Tomaselli GF, Kass DA. *Cyclic GMP/PKG-dependent inhibition of TRPC6 channel activity and expression negatively regulates cardiomyocyte NFAT activation Novel mechanism of cardiac stress modulation by PDE5 inhibition*. *J Mol Cell Cardiol*, 2010; **48**(4): p. 713-24.
 206. Kruger M, Kotter S, Grutzner A, Lang P, Andresen C, Redfield MM, Butt E, dos Remedios CG, Linke WA. *Protein kinase G modulates human myocardial passive stiffness by phosphorylation of the titin springs*. *Circ Res*, 2009; **104**(1): p. 87-94.
 207. Layland J, Li JM, Shah AM. *Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes*. *J Physiol*, 2002; **540**(Pt 2): p. 457-67.
 208. Lee DI, Vahebi S, Tocchetti CG, Barouch LA, Solaro RJ, Takimoto E, Kass DA. *PDE5A suppression of acute beta-adrenergic activation requires*

- modulation of myocyte beta-3 signaling coupled to PKG-mediated troponin I phosphorylation.* Basic Res Cardiol, 2010; **105**(3): p. 337-47.
209. Li P, Wang D, Lucas J, Oparil S, Xing D, Cao X, Novak L, Renfrow MB, Chen YF. *Atrial natriuretic peptide inhibits transforming growth factor beta-induced Smad signaling and myofibroblast transformation in mouse cardiac fibroblasts.* Circ Res, 2008; **102**(2): p. 185-92.
 210. Takimoto E, Koitabashi N, Hsu S, Ketner EA, Zhang M, Nagayama T, Bedja D, Gabrielson KL, Blanton R, Siderovski DP, Mendelsohn ME, Kass DA. *Regulator of G protein signaling 2 mediates cardiac compensation to pressure overload and antihypertrophic effects of PDE5 inhibition in mice.* J Clin Invest, 2009; **119**(2): p. 408-20.
 211. Tokudome T, Kishimoto I, Horio T, Arai Y, Schwenke DO, Hino J, Okano I, Kawano Y, Kohno M, Miyazato M, Nakao K, Kangawa K. *Regulator of G-protein signaling subtype 4 mediates antihypertrophic effect of locally secreted natriuretic peptides in the heart.* Circulation, 2008; **117**(18): p. 2329-39.
 212. Wollert KC, Fiedler B, Gambaryan S, Smolenski A, Heineke J, Butt E, Trautwein C, Lohmann SM, Drexler H. *Gene transfer of cGMP-dependent protein kinase I enhances the antihypertrophic effects of nitric oxide in cardiomyocytes.* Hypertension, 2002; **39**(1): p. 87-92.
 213. Schroder F, Klein G, Fiedler B, Bastein M, Schnasse N, Hillmer A, Ames S, Gambaryan S, Drexler H, Walter U, Lohmann SM, Wollert KC. *Single L-type Ca(2+) channel regulation by cGMP-dependent protein kinase type I in adult cardiomyocytes from PKG I transgenic mice.* Cardiovasc Res, 2003; **60**(2): p. 268-77.
 214. Wollert KC, Yurukova S, Kilic A, Begrow F, Fiedler B, Gambaryan S, Walter U, Lohmann SM, Kuhn M. *Increased effects of C-type natriuretic peptide on contractility and calcium regulation in murine hearts overexpressing cyclic GMP-dependent protein kinase I.* Br J Pharmacol, 2003; **140**(7): p. 1227-36.
 215. Francis SH, Blount MA, Zoraghi R, Corbin JD. *Molecular properties of mammalian proteins that interact with cGMP: protein kinases, cation channels, phosphodiesterases, and multi-drug anion transporters.* Front Biosci, 2005; **10**: p. 2097-117.
 216. Alverdi V, Mazon H, Versluis C, Hemrika W, Esposito G, van den Heuvel R, Scholten A, Heck AJ. *cGMP-binding prepares PKG for substrate binding by disclosing the C-terminal domain.* J Mol Biol, 2008; **375**(5): p. 1380-93.
 217. Wall ME, Francis SH, Corbin JD, Grimes K, Richie-Jannetta R, Kotera J, Macdonald BA, Gibson RR, Trewella J. *Mechanisms associated with cGMP binding and activation of cGMP-dependent protein kinase.* Proc Natl Acad Sci U S A, 2003; **100**(5): p. 2380-5.
 218. Kim JJ, Casteel DE, Huang G, Kwon TH, Ren RK, Zwart P, Headd JJ, Brown NG, Chow DC, Palzkill T, Kim C. *Co-crystal structures of PKG Ibeta (92-227) with cGMP and cAMP reveal the molecular details of cyclic-nucleotide binding.* PLoS One, 2011; **6**(4): p. e18413.
 219. Reed RB, Sandberg M, Jahnsen T, Lohmann SM, Francis SH, Corbin JD. *Fast and slow cyclic nucleotide-dissociation sites in cAMP-dependent protein kinase are transposed in type Ibeta cGMP-dependent protein kinase.* J Biol Chem, 1996; **271**(29): p. 17570-5.

220. Huang GY, Kim JJ, Reger AS, Lorenz R, Moon EW, Zhao C, Casteel DE, Bertinetti D, Vanschouwen B, Selvaratnam R, Pflugrath JW, Sankaran B, Melacini G, Herberg FW, Kim C. *Structural basis for cyclic-nucleotide selectivity and cGMP-selective activation of PKG I*. *Structure*, 2014; **22**(1): p. 116-24.
221. Ponsioen B, Zhao J, Riedl J, Zwartkruis F, van der Krogt G, Zaccolo M, Moolenaar WH, Bos JL, Jalink K. *Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator*. *EMBO Rep*, 2004; **5**(12): p. 1176-80.
222. Trivedi B, Kramer RH. *Real-time patch-clamp detection of intracellular cGMP reveals long-term suppression of responses to NO and muscarinic agonists*. *Neuron*, 1998; **21**(4): p. 895-906.
223. Cornwell PA, Barry BW, Stoddart CP, Bouwstra JA. *Wide-angle X-ray diffraction of human stratum corneum: effects of hydration and terpene enhancer treatment*. *J Pharm Pharmacol*, 1994; **46**(12): p. 938-50.
224. Jiang H, Colbran JL, Francis SH, Corbin JD. *Direct evidence for cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary arteries*. *J Biol Chem*, 1992; **267**(2): p. 1015-9.
225. Komalavilas P, Lincoln TM. *Phosphorylation of the inositol 1,4,5-trisphosphate receptor. Cyclic GMP-dependent protein kinase mediates cAMP and cGMP dependent phosphorylation in the intact rat aorta*. *J Biol Chem*, 1996; **271**(36): p. 21933-8.
226. Corbin JD, Doskeland SO. *Studies of two different intrachain cGMP-binding sites of cGMP-dependent protein kinase*. *J Biol Chem*, 1983; **258**(18): p. 11391-7.
227. Vila-Petroff MG, Younes A, Egan J, Lakatta EG, Sollott SJ. *Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes*. *Circulation Research*, 1999; **84**(9): p. 1020-1031.
228. Beetham JK, Tian T, Hammock BD. *cDNA cloning and expression of a soluble epoxide hydrolase from human liver*. *Arch Biochem Biophys*, 1993; **305**(1): p. 197-201.
229. Newman JW, Morisseau C, Harris TR, Hammock BD. *The soluble epoxide hydrolase encoded by EPXH2 is a bifunctional enzyme with novel lipid phosphate phosphatase activity*. *Proc Natl Acad Sci U S A*, 2003; **100**(4): p. 1558-63.
230. Gill SS, Hammock BD. *Distribution and properties of a mammalian soluble epoxide hydrolase*. *Biochem Pharmacol*, 1980; **29**(3): p. 389-95.
231. Newman JW, Morisseau C, Hammock BD. *Epoxide hydrolases: their roles and interactions with lipid metabolism*. *Prog Lipid Res*, 2005; **44**(1): p. 1-51.
232. Harris TR, Hammock BD. *Soluble epoxide hydrolase: gene structure, expression and deletion*. *Gene*, 2013; **526**(2): p. 61-74.
233. Decker M, Adamska M, Cronin A, Di Giallonardo F, Burgener J, Marowsky A, Falck JR, Morisseau C, Hammock BD, Gruzdev A, Zeldin DC, Arand M. *EH3 (ABHD9): the first member of a new epoxide hydrolase family with high activity for fatty acid epoxides*. *J Lipid Res*, 2012; **53**(10): p. 2038-45.
234. Morisseau C, Hammock BD. *Impact of soluble epoxide hydrolase and epoxyeicosanoids on human health*. *Annu Rev Pharmacol Toxicol*, 2013; **53**: p. 37-58.

235. Morisseau C, Hammock BD. *Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles*. Annu Rev Pharmacol Toxicol, 2005; **45**: p. 311-33.
236. Imig JD. *Epoxides and soluble epoxide hydrolase in cardiovascular physiology*. Physiol Rev, 2012; **92**(1): p. 101-30.
237. Hu J, Popp R, Fromel T, Ehling M, Awwad K, Adams RH, Hammes HP, Fleming I. *Muller glia cells regulate Notch signaling and retinal angiogenesis via the generation of 19,20-dihydroxydocosapentaenoic acid*. J Exp Med, 2014; **211**(2): p. 281-95.
238. Lu T, Katakam PV, VanRollins M, Weintraub NL, Spector AA, Lee HC. *Dihydroxyeicosatrienoic acids are potent activators of Ca(2+)-activated K(+) channels in isolated rat coronary arterial myocytes*. J Physiol, 2001; **534**(Pt 3): p. 651-67.
239. Node K, Ruan XL, Dai J, Yang SX, Graham L, Zeldin DC, Liao JK. *Activation of Galpha s mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids*. J Biol Chem, 2001; **276**(19): p. 15983-9.
240. Oltman CL, Weintraub NL, VanRollins M, Dellsperger KC. *Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation*. Circ Res, 1998; **83**(9): p. 932-9.
241. Shahabi P, Siest G, Meyer UA, Visvikis-Siest S. *Human cytochrome P450 epoxygenases: variability in expression and role in inflammation-related disorders*. Pharmacol Ther, 2014; **144**(2): p. 134-61.
242. Thomson SJ, Askari A, Bishop-Bailey D. *Anti-inflammatory effects of epoxyeicosatrienoic acids*. Int J Vasc Med, 2012; **2012**: p. 605101.
243. Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK. *Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids*. Science, 1999; **285**(5431): p. 1276-9.
244. Deng Y, Edin ML, Theken KN, Schuck RN, Flake GP, Kannon MA, DeGraff LM, Lih FB, Foley J, Bradbury JA, Graves JP, Tomer KB, Falck JR, Zeldin DC, Lee CR. *Endothelial CYP epoxygenase overexpression and soluble epoxide hydrolase disruption attenuate acute vascular inflammatory responses in mice*. FASEB J, 2011; **25**(2): p. 703-13.
245. Schmelzer KR, Kubala L, Newman JW, Kim IH, Eiserich JP, Hammock BD. *Soluble epoxide hydrolase is a therapeutic target for acute inflammation*. Proc Natl Acad Sci U S A, 2005; **102**(28): p. 9772-7.
246. Wray J, Bishop-Bailey D. *Epoxygenases and peroxisome proliferator-activated receptors in mammalian vascular biology*. Exp Physiol, 2008; **93**(1): p. 148-54.
247. Fang X, Moore SA, Stoll LL, Rich G, Kaduce TL, Weintraub NL, Spector AA. *14,15-Epoxyeicosatrienoic acid inhibits prostaglandin E2 production in vascular smooth muscle cells*. Am J Physiol, 1998; **275**(6 Pt 2): p. H2113-21.
248. Fife KL, Liu Y, Schmelzer KR, Tsai HJ, Kim IH, Morisseau C, Hammock BD, Kroetz DL. *Inhibition of soluble epoxide hydrolase does not protect against endotoxin-mediated hepatic inflammation*. J Pharmacol Exp Ther, 2008; **327**(3): p. 707-15.
249. Fleming I, Michaelis UR, Bredenkotter D, Fisslthaler B, Dehghani F, Brandes RP, Busse R. *Endothelium-derived hyperpolarizing factor synthase (Cytochrome*

- P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. Circ Res, 2001; 88(1): p. 44-51.*
250. Michaelis UR, Falck JR, Schmidt R, Busse R, Fleming I. *Cytochrome P4502C9-derived epoxyeicosatrienoic acids induce the expression of cyclooxygenase-2 in endothelial cells. Arterioscler Thromb Vasc Biol, 2005; 25(2): p. 321-6.*
 251. Edin ML, Wang Z, Bradbury JA, Graves JP, Lih FB, DeGraff LM, Foley JF, Torphy R, Ronnekleiv OK, Tomer KB, Lee CR, Zeldin DC. *Endothelial expression of human cytochrome P450 epoxygenase CYP2C8 increases susceptibility to ischemia-reperfusion injury in isolated mouse heart. FASEB J, 2011; 25(10): p. 3436-47.*
 252. Fromel T, Jungblut B, Hu J, Trouvain C, Barbosa-Sicard E, Popp R, Liebner S, Dimmeler S, Hammock BD, Fleming I. *Soluble epoxide hydrolase regulates hematopoietic progenitor cell function via generation of fatty acid diols. Proc Natl Acad Sci U S A, 2012; 109(25): p. 9995-10000.*
 253. Smith KR, Pinkerton KE, Watanabe T, Pedersen TL, Ma SJ, Hammock BD. *Attenuation of tobacco smoke-induced lung inflammation by treatment with a soluble epoxide hydrolase inhibitor. Proc Natl Acad Sci U S A, 2005; 102(6): p. 2186-91.*
 254. Manhiani M, Quigley JE, Knight SF, Tasoobshirazi S, Moore T, Brands MW, Hammock BD, Imig JD. *Soluble epoxide hydrolase gene deletion attenuates renal injury and inflammation with DOCA-salt hypertension. Am J Physiol Renal Physiol, 2009; 297(3): p. F740-8.*
 255. Zhang LN, Vincelette J, Cheng Y, Mehra U, Chen D, Anandan SK, Gless R, Webb HK, Wang YX. *Inhibition of soluble epoxide hydrolase attenuated atherosclerosis, abdominal aortic aneurysm formation, and dyslipidemia. Arterioscler Thromb Vasc Biol, 2009; 29(9): p. 1265-70.*
 256. Revermann M, Schloss M, Barbosa-Sicard E, Mieth A, Liebner S, Morisseau C, Geisslinger G, Schermuly RT, Fleming I, Hammock BD, Brandes RP. *Soluble epoxide hydrolase deficiency attenuates neointima formation in the femoral cuff model of hyperlipidemic mice. Arterioscler Thromb Vasc Biol, 2010; 30(5): p. 909-14.*
 257. Wagner K, Inceoglu B, Hammock BD. *Soluble epoxide hydrolase inhibition, epoxygenated fatty acids and nociception. Prostaglandins Other Lipid Mediat, 2011; 96(1-4): p. 76-83.*
 258. Inceoglu B, Jinks SL, Schmelzer KR, Waite T, Kim IH, Hammock BD. *Inhibition of soluble epoxide hydrolase reduces LPS-induced thermal hyperalgesia and mechanical allodynia in a rat model of inflammatory pain. Life Sci, 2006; 79(24): p. 2311-9.*
 259. Inceoglu B, Jinks SL, Ulu A, Hegedus CM, Georgi K, Schmelzer KR, Wagner K, Jones PD, Morisseau C, Hammock BD. *Soluble epoxide hydrolase and epoxyeicosatrienoic acids modulate two distinct analgesic pathways. Proc Natl Acad Sci U S A, 2008; 105(48): p. 18901-6.*
 260. Huang H, Morisseau C, Wang J, Yang T, Falck JR, Hammock BD, Wang MH. *Increasing or stabilizing renal epoxyeicosatrienoic acid production attenuates abnormal renal function and hypertension in obese rats. Am J Physiol Renal Physiol, 2007; 293(1): p. F342-9.*

261. Imig JD, Zhao X, Capdevila JH, Morisseau C, Hammock BD. *Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension*. Hypertension, 2002; **39**(2 Pt 2): p. 690-4.
262. Loch D, Hoey A, Morisseau C, Hammock BO, Brown L. *Prevention of hypertension in DOCA-salt rats by an inhibitor of soluble epoxide hydrolase*. Cell Biochem Biophys, 2007; **47**(1): p. 87-98.
263. Luria A, Weldon SM, Kabcenell AK, Ingraham RH, Matera D, Jiang H, Gill R, Morisseau C, Newman JW, Hammock BD. *Compensatory mechanism for homeostatic blood pressure regulation in Ephx2 gene-disrupted mice*. J Biol Chem, 2007; **282**(5): p. 2891-8.
264. Sinal CJ, Miyata M, Tohkin M, Nagata K, Bend JR, Gonzalez FJ. *Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation*. J Biol Chem, 2000; **275**(51): p. 40504-10.
265. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. *Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors*. Circ Res, 1996; **78**(3): p. 415-23.
266. Fleming I, Rueben A, Popp R, Fisslthaler B, Schrodtt S, Sander A, Haendeler J, Falck JR, Morisseau C, Hammock BD, Busse R. *Epoxyeicosatrienoic acids regulate Trp channel dependent Ca²⁺ signaling and hyperpolarization in endothelial cells*. Arterioscler Thromb Vasc Biol, 2007; **27**(12): p. 2612-8.
267. Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. *Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels*. Nature, 2003; **424**(6947): p. 434-8.
268. Vriens J, Owsianik G, Fisslthaler B, Suzuki M, Janssens A, Voets T, Morisseau C, Hammock BD, Fleming I, Busse R, Nilius B. *Modulation of the Ca²⁺ permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium*. Circ Res, 2005; **97**(9): p. 908-15.
269. Loot AE, Popp R, Fisslthaler B, Vriens J, Nilius B, Fleming I. *Role of cytochrome P450-dependent transient receptor potential V4 activation in flow-induced vasodilatation*. Cardiovasc Res, 2008; **80**(3): p. 445-52.
270. Edwards G, Thollon C, Gardener MJ, Feletou M, Vilaine J, Vanhoutte PM, Weston AH. *Role of gap junctions and EETs in endothelium-dependent hyperpolarization of porcine coronary artery*. Br J Pharmacol, 2000; **129**(6): p. 1145-54.
271. Fukao M, Mason HS, Kenyon JL, Horowitz B, Keef KD. *Regulation of BK(Ca) channels expressed in human embryonic kidney 293 cells by epoxyeicosatrienoic acid*. Mol Pharmacol, 2001; **59**(1): p. 16-23.
272. Popp R, Brandes RP, Ott G, Busse R, Fleming I. *Dynamic modulation of interendothelial gap junctional communication by 11,12-epoxyeicosatrienoic acid*. Circ Res, 2002; **90**(7): p. 800-6.
273. Pfister SL, Gauthier KM, Campbell WB. *Vascular pharmacology of epoxyeicosatrienoic acids*. Adv Pharmacol, 2010; **60**: p. 27-59.
274. Imig JD. *20-HETE or EETs: which arachidonic acid metabolite regulates proximal tubule transporters and contributes to pressure natriuresis?* Am J Physiol Regul Integr Comp Physiol, 2004; **287**(1): p. R3-5.
275. Yu Z, Xu F, Huse LM, Morisseau C, Draper AJ, Newman JW, Parker C, Graham L, Engler MM, Hammock BD, Zeldin DC, Kroetz DL. *Soluble epoxide*

- hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids.* Circ Res, 2000; **87**(11): p. 992-8.
276. Honetschlagerova Z, Sporkova A, Kopkan L, Huskova Z, Hwang SH, Hammock BD, Imig JD, Kramer HJ, Kujal P, Vernerova Z, Chabova VC, Tesar V, Cervenka L. *Inhibition of soluble epoxide hydrolase improves the impaired pressure-natriuresis relationship and attenuates the development of hypertension and hypertension-associated end-organ damage in Cyp1a1-Ren-2 transgenic rats.* J Hypertens, 2011; **29**(8): p. 1590-601.
 277. Sporkova A, Kopkan L, Varcabova S, Huskova Z, Hwang SH, Hammock BD, Imig JD, Kramer HJ, Cervenka L. *Role of cytochrome P-450 metabolites in the regulation of renal function and blood pressure in 2-kidney 1-clip hypertensive rats.* Am J Physiol Regul Integr Comp Physiol, 2011; **300**(6): p. R1468-75.
 278. Chen D, Whitcomb R, MacIntyre E, Tran V, Do ZN, Sabry J, Patel DV, Anandan SK, Gless R, Webb HK. *Pharmacokinetics and pharmacodynamics of AR9281, an inhibitor of soluble epoxide hydrolase, in single- and multiple-dose studies in healthy human subjects.* J Clin Pharmacol, 2012; **52**(3): p. 319-28.
 279. Loot AE, Fleming I. *Cytochrome P450-derived epoxyeicosatrienoic acids and pulmonary hypertension: central role of transient receptor potential C6 channels.* J Cardiovasc Pharmacol, 2011; **57**(2): p. 140-7.
 280. Keseru B, Barbosa-Sicard E, Popp R, Fisslthaler B, Dietrich A, Gudermann T, Hammock BD, Falck JR, Weissmann N, Busse R, Fleming I. *Epoxyeicosatrienoic acids and the soluble epoxide hydrolase are determinants of pulmonary artery pressure and the acute hypoxic pulmonary vasoconstrictor response.* FASEB J, 2008; **22**(12): p. 4306-15.
 281. Keseru B, Barbosa-Sicard E, Schermuly RT, Tanaka H, Hammock BD, Weissmann N, Fisslthaler B, Fleming I. *Hypoxia-induced pulmonary hypertension: comparison of soluble epoxide hydrolase deletion vs. inhibition.* Cardiovasc Res, 2010; **85**(1): p. 232-40.
 282. Wang L, Yang J, Guo L, Uyeminami D, Dong H, Hammock BD, Pinkerton KE. *Use of a soluble epoxide hydrolase inhibitor in smoke-induced chronic obstructive pulmonary disease.* Am J Respir Cell Mol Biol, 2012; **46**(5): p. 614-22.
 283. Panigrahy D, Greene ER, Pozzi A, Wang DW, Zeldin DC. *EET signaling in cancer.* Cancer Metastasis Rev, 2011; **30**(3-4): p. 525-40.
 284. Panigrahy D, Edin ML, Lee CR, Huang S, Bielenberg DR, Butterfield CE, Barnes CM, Mammoto A, Mammoto T, Luria A, Benny O, Chaponis DM, Dudley AC, Greene ER, Vergilio JA, Pietramaggiori G, Scherer-Pietramaggiori SS, Short SM, Seth M, Lih FB, Tomer KB, Yang J, Schwendener RA, Hammock BD, Falck JR, Manthathi VL, Ingber DE, Kaipainen A, D'Amore PA, Kieran MW, Zeldin DC. *Epoxyeicosanoids stimulate multiorgan metastasis and tumor dormancy escape in mice.* J Clin Invest, 2012; **122**(1): p. 178-91.
 285. Pozzi A, Popescu V, Yang S, Mei S, Shi M, Puolitaival SM, Caprioli RM, Capdevila JH. *The anti-tumorigenic properties of peroxisomal proliferator-activated receptor alpha are arachidonic acid epoxxygenase-mediated.* J Biol Chem, 2010; **285**(17): p. 12840-50.
 286. Zagorac D, Jakovcevic D, Gebremedhin D, Harder DR. *Antiangiogenic effect of inhibitors of cytochrome P450 on rats with glioblastoma multiforme.* J Cereb Blood Flow Metab, 2008; **28**(8): p. 1431-9.

287. Goldstein B, Giroir B, Randolph A, International Consensus Conference on Pediatric S. *International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics*. *Pediatr Crit Care Med*, 2005; **6**(1): p. 2-8.
288. Martin GS. *Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes*. *Expert Rev Anti Infect Ther*, 2012; **10**(6): p. 701-6.
289. Zanotti Cavazzoni SL, Dellinger RP. *Hemodynamic optimization of sepsis-induced tissue hypoperfusion*. *Crit Care*, 2006; **10 Suppl 3**: p. S2.
290. Angus DC, van der Poll T. *Severe sepsis and septic shock*. *N Engl J Med*, 2013; **369**(9): p. 840-51.
291. Levy B, Collin S, Sennoun N, Ducrocq N, Kimmoun A, Asfar P, Perez P, Meziani F. *Vascular hyporesponsiveness to vasopressors in septic shock: from bench to bedside*. *Intensive Care Med*, 2010; **36**(12): p. 2019-29.
292. Bernardin G, Pradier C, Tiger F, Deloffre P, Mattei M. *Blood pressure and arterial lactate level are early indicators of short-term survival in human septic shock*. *Intensive Care Med*, 1996; **22**(1): p. 17-25.
293. Goldenberg NM, Steinberg BE, Slutsky AS, Lee WL. *Broken barriers: a new take on sepsis pathogenesis*. *Sci Transl Med*, 2011; **3**(88): p. 88ps25.
294. Benedict CR, Rose JA. *Arterial norepinephrine changes in patients with septic shock*. *Circ Shock*, 1992; **38**(3): p. 165-72.
295. Annane D, Bellissant E, Cavaillon JM. *Septic shock*. *Lancet*, 2005; **365**(9453): p. 63-78.
296. Buckley JF, Singer M, Clapp LH. *Role of KATP channels in sepsis*. *Cardiovasc Res*, 2006; **72**(2): p. 220-30.
297. Landry DW, Oliver JA. *The ATP-sensitive K⁺ channel mediates hypotension in endotoxemia and hypoxic lactic acidosis in dog*. *J Clin Invest*, 1992; **89**(6): p. 2071-4.
298. Sorrentino R, d'Emmanuele di Villa Bianca R, Lippolis L, Sorrentino L, Autore G, Pinto A. *Involvement of ATP-sensitive potassium channels in a model of a delayed vascular hyporeactivity induced by lipopolysaccharide in rats*. *Br J Pharmacol*, 1999; **127**(6): p. 1447-53.
299. Vanelli G, Hussain SN, Aguggini G. *Glibenclamide, a blocker of ATP-sensitive potassium channels, reverses endotoxin-induced hypotension in pig*. *Exp Physiol*, 1995; **80**(1): p. 167-70.
300. Vanelli G, Hussain SN, Dimori M, Aguggini G. *Cardiovascular responses to glibenclamide during endotoxaemia in the pig*. *Vet Res Commun*, 1997; **21**(3): p. 187-200.
301. O'Brien AJ, Thakur G, Buckley JF, Singer M, Clapp LH. *The pore-forming subunit of the K(ATP) channel is an important molecular target for LPS-induced vascular hyporeactivity in vitro*. *Br J Pharmacol*, 2005; **144**(3): p. 367-75.
302. Preiser JC, Zhang H, Debelle F, Fesler P, Kafi SA, Naeije R, Vincent JL. *Hemodynamic effects of glibenclamide during endotoxemia: contrasting findings in vitro versus in vivo*. *Shock*, 2003; **19**(3): p. 223-8.
303. Morelli A, Lange M, Ertmer C, Broeking K, Van Aken H, Orecchioni A, Rocco M, Bachetoni A, Traber DL, Landoni G, Pietropaoli P, Westphal M. *Glibenclamide dose response in patients with septic shock: effects on norepinephrine requirements, cardiopulmonary performance, and global oxygen transport*. *Shock*, 2007; **28**(5): p. 530-5.

304. Warrillow S, Egi M, Bellomo R. *Randomized, double-blind, placebo-controlled crossover pilot study of a potassium channel blocker in patients with septic shock*. Crit Care Med, 2006; **34**(4): p. 980-5.
305. Landry DW, Oliver JA. *The pathogenesis of vasodilatory shock*. N Engl J Med, 2001; **345**(8): p. 588-95.
306. Lu T, Hoshi T, Weintraub NL, Spector AA, Lee HC. *Activation of ATP-sensitive K(+) channels by epoxyeicosatrienoic acids in rat cardiac ventricular myocytes*. J Physiol, 2001; **537**(Pt 3): p. 811-27.
307. Ye D, Zhou W, Lee HC. *Activation of rat mesenteric arterial KATP channels by 11,12-epoxyeicosatrienoic acid*. Am J Physiol Heart Circ Physiol, 2005; **288**(1): p. H358-64.
308. Lu T, Hong MP, Lee HC. *Molecular determinants of cardiac K(ATP) channel activation by epoxyeicosatrienoic acids*. J Biol Chem, 2005; **280**(19): p. 19097-104.
309. Ye D, Zhou W, Lu T, Jagadeesh SG, Falck JR, Lee HC. *Mechanism of rat mesenteric arterial KATP channel activation by 14,15-epoxyeicosatrienoic acid*. Am J Physiol Heart Circ Physiol, 2006; **290**(4): p. H1326-36.
310. Lu T, Ye D, Wang X, Seubert JM, Graves JP, Bradbury JA, Zeldin DC, Lee HC. *Cardiac and vascular KATP channels in rats are activated by endogenous epoxyeicosatrienoic acids through different mechanisms*. J Physiol, 2006; **575**(Pt 2): p. 627-44.
311. Batchu SN, Chaudhary KR, El-Sikhry H, Yang W, Light PE, Oudit GY, Seubert JM. *Role of PI3Kalpha and sarcolemmal ATP-sensitive potassium channels in epoxyeicosatrienoic acid mediated cardioprotection*. J Mol Cell Cardiol, 2012; **53**(1): p. 43-52.
312. Batchu SN, Law E, Brocks DR, Falck JR, Seubert JM. *Epoxyeicosatrienoic acid prevents postischemic electrocardiogram abnormalities in an isolated heart model*. J Mol Cell Cardiol, 2009; **46**(1): p. 67-74.
313. Bodiga S, Zhang R, Jacobs DE, Larsen BT, Tampo A, Manthathi VL, Kwok WM, Zeldin DC, Falck JR, Gutterman DD, Jacobs ER, Medhora MM. *Protective actions of epoxyeicosatrienoic acid: dual targeting of cardiovascular PI3K and KATP channels*. J Mol Cell Cardiol, 2009; **46**(6): p. 978-88.
314. Gross GJ, Hsu A, Falck JR, Nithipatikom K. *Mechanisms by which epoxyeicosatrienoic acids (EETs) elicit cardioprotection in rat hearts*. J Mol Cell Cardiol, 2007; **42**(3): p. 687-91.
315. Nithipatikom K, Moore JM, Isbell MA, Falck JR, Gross GJ. *Epoxyeicosatrienoic acids in cardioprotection: ischemic versus reperfusion injury*. Am J Physiol Heart Circ Physiol, 2006; **291**(2): p. H537-42.
316. Seubert J, Yang B, Bradbury JA, Graves J, Degraff LM, Gabel S, Gooch R, Foley J, Newman J, Mao L, Rockman HA, Hammock BD, Murphy E, Zeldin DC. *Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K⁺ channels and p42/p44 MAPK pathway*. Circ Res, 2004; **95**(5): p. 506-14.
317. Seubert JM, Sinal CJ, Graves J, DeGraff LM, Bradbury JA, Lee CR, Goralski K, Carey MA, Luria A, Newman JW, Hammock BD, Falck JR, Roberts H, Rockman HA, Murphy E, Zeldin DC. *Role of soluble epoxide hydrolase in postischemic recovery of heart contractile function*. Circ Res, 2006; **99**(4): p. 442-50.

318. Qu YY, Yuan MY, Liu Y, Xiao XJ, Zhu YL. *The Protective Effect of Epoxyeicosatrienoic Acids on Cerebral Ischemia/Reperfusion Injury is Associated with PI3K/Akt Pathway and ATP-Sensitive Potassium Channels*. Neurochem Res, 2014.
319. Vane J, Corin RE. *Prostacyclin: a vascular mediator*. Eur J Vasc Endovasc Surg, 2003; **26**(6): p. 571-8.
320. Hocherl K, Dreher F, Kurtz A, Bucher M. *Cyclooxygenase-2 inhibition attenuates lipopolysaccharide-induced cardiovascular failure*. Hypertension, 2002; **40**(6): p. 947-53.
321. Staehr M, Madsen K, Vanhoutte PM, Hansen PB, Jensen BL. *Disruption of COX-2 and eNOS does not confer protection from cardiovascular failure in lipopolysaccharide-treated conscious mice and isolated vascular rings*. Am J Physiol Regul Integr Comp Physiol, 2011; **301**(2): p. R412-20.
322. Bernard GR, Wheeler AP, Russell JA, Schein R, Summer WR, Steinberg KP, Fulkerson WJ, Wright PE, Christman BW, Dupont WD, Higgins SB, Swindell BB. *The effects of ibuprofen on the physiology and survival of patients with sepsis. The Ibuprofen in Sepsis Study Group*. N Engl J Med, 1997; **336**(13): p. 912-8.
323. Annane D, Sanquer S, Sebille V, Faye A, Djuranovic D, Raphael JC, Gajdos P, Bellissant E. *Compartmentalised inducible nitric-oxide synthase activity in septic shock*. Lancet, 2000; **355**(9210): p. 1143-8.
324. Stoclet JC, Martinez MC, Ohlmann P, Chasserot S, Schott C, Kleschyov AL, Schneider F, Andriantsitohaina R. *Induction of nitric oxide synthase and dual effects of nitric oxide and cyclooxygenase products in regulation of arterial contraction in human septic shock*. Circulation, 1999; **100**(2): p. 107-12.
325. Kilbourn RG, Gross SS, Jubran A, Adams J, Griffith OW, Levi R, Lodato RF. *NG-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide*. Proc Natl Acad Sci U S A, 1990; **87**(9): p. 3629-32.
326. Kilbourn RG, Jubran A, Gross SS, Griffith OW, Levi R, Adams J, Lodato RF. *Reversal of endotoxin-mediated shock by NG-methyl-L-arginine, an inhibitor of nitric oxide synthesis*. Biochem Biophys Res Commun, 1990; **172**(3): p. 1132-8.
327. Thiernemann C, Szabo C, Mitchell JA, Vane JR. *Vascular hyporeactivity to vasoconstrictor agents and hemodynamic decompensation in hemorrhagic shock is mediated by nitric oxide*. Proc Natl Acad Sci U S A, 1993; **90**(1): p. 267-71.
328. Thiernemann C, Vane J. *Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo*. Eur J Pharmacol, 1990; **182**(3): p. 591-5.
329. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N, et al. *Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase*. Cell, 1995; **81**(4): p. 641-50.
330. Cobb JP, Hotchkiss RS, Swanson PE, Chang K, Qiu Y, Laubach VE, Karl IE, Buchman TG. *Inducible nitric oxide synthase (iNOS) gene deficiency increases the mortality of sepsis in mice*. Surgery, 1999; **126**(2): p. 438-42.
331. Lopez A, Lorente JA, Steingrub J, Bakker J, McLuckie A, Willatts S, Brockway M, Anzueto A, Holzapfel L, Breen D, Silverman MS, Takala J, Donaldson J, Arneson C, Grove G, Grossman S, Grover R. *Multiple-center, randomized,*

- placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: effect on survival in patients with septic shock.* Crit Care Med, 2004; **32**(1): p. 21-30.
332. Kinasewitz GT, Privalle CT, Imm A, Steingrub JS, Malcynski JT, Balk RA, DeAngelo J. *Multicenter, randomized, placebo-controlled study of the nitric oxide scavenger pyridoxalated hemoglobin polyoxyethylene in distributive shock.* Crit Care Med, 2008; **36**(7): p. 1999-2007.
 333. Vincent JL, Privalle CT, Singer M, Lorente JA, Boehm E, Meier-Hellmann A, Darius H, Ferrer R, Sirvent JM, Marx G, DeAngelo J. *Multicenter, Randomized, Placebo-Controlled Phase III Study of Pyridoxalated Hemoglobin Polyoxyethylene in Distributive Shock (PHOENIX)*.* Crit Care Med, 2015; **43**(1): p. 57-64.
 334. Cauwels A, Buys ES, Thoonen R, Geary L, Delanghe J, Shiva S, Brouckaert P. *Nitrite protects against morbidity and mortality associated with TNF- or LPS-induced shock in a soluble guanylate cyclase-dependent manner.* J Exp Med, 2009; **206**(13): p. 2915-24.
 335. Spronk PE, Ince C, Gardien MJ, Mathura KR, Oudemans-van Straaten HM, Zandstra DF. *Nitroglycerin in septic shock after intravascular volume resuscitation.* Lancet, 2002; **360**(9343): p. 1395-6.
 336. Reddy AT, Lakshmi SP, Reddy RC. *The Nitrated Fatty Acid 10-Nitro-oleate Diminishes Severity of LPS-Induced Acute Lung Injury in Mice.* PPAR Res, 2012; **2012**: p. 617063.
 337. Wang H, Liu H, Jia Z, Olsen C, Litwin S, Guan G, Yang T. *Nitro-oleic acid protects against endotoxin-induced endotoxemia and multiorgan injury in mice.* Am J Physiol Renal Physiol, 2010; **298**(3): p. F754-62.
 338. Coles B, Bloodsworth A, Clark SR, Lewis MJ, Cross AR, Freeman BA, O'Donnell VB. *Nitrolinoleate inhibits superoxide generation, degranulation, and integrin expression by human neutrophils: novel antiinflammatory properties of nitric oxide-derived reactive species in vascular cells.* Circ Res, 2002; **91**(5): p. 375-81.
 339. Cui T, Schopfer FJ, Zhang J, Chen K, Ichikawa T, Baker PR, Batthyany C, Chacko BK, Feng X, Patel RP, Agarwal A, Freeman BA, Chen YE. *Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators.* J Biol Chem, 2006; **281**(47): p. 35686-98.
 340. Kansanen E, Bonacci G, Schopfer FJ, Kuosmanen SM, Tong KI, Leinonen H, Woodcock SR, Yamamoto M, Carlberg C, Yla-Herttuala S, Freeman BA, Levonen AL. *Electrophilic nitro-fatty acids activate NRF2 by a KEAP1 cysteine 151-independent mechanism.* J Biol Chem, 2011; **286**(16): p. 14019-27.
 341. Shireman PK, Contreras-Shannon V, Reyes-Reyna SM, Robinson SC, McManus LM. *MCP-1 parallels inflammatory and regenerative responses in ischemic muscle.* J Surg Res, 2006; **134**(1): p. 145-57.
 342. Scholz D, Thomas S, Sass S, Podzuweit T. *Angiogenesis and myogenesis as two facets of inflammatory post-ischemic tissue regeneration.* Mol Cell Biochem, 2003; **246**(1-2): p. 57-67.
 343. Scholz D, Ziegelhoeffer T, Helisch A, Wagner S, Friedrich C, Podzuweit T, Schaper W. *Contribution of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice.* J Mol Cell Cardiol, 2002; **34**(7): p. 775-87.

344. Fleming I. *DiscrEET regulators of homeostasis: epoxyeicosatrienoic acids, cytochrome P450 epoxygenases and vascular inflammation*. Trends Pharmacol Sci, 2007; **28**(9): p. 448-52.
345. Zhang G, Kodani S, Hammock BD. *Stabilized epoxygenated fatty acids regulate inflammation, pain, angiogenesis and cancer*. Prog Lipid Res, 2014; **53**: p. 108-23.
346. Michaelis UR, Fleming I. *From endothelium-derived hyperpolarizing factor (EDHF) to angiogenesis: Epoxyeicosatrienoic acids (EETs) and cell signaling*. Pharmacol Ther, 2006; **111**(3): p. 584-95.
347. Munzenmaier DH, Harder DR. *Cerebral microvascular endothelial cell tube formation: role of astrocytic epoxyeicosatrienoic acid release*. Am J Physiol Heart Circ Physiol, 2000; **278**(4): p. H1163-7.
348. Zhang C, Harder DR. *Cerebral capillary endothelial cell mitogenesis and morphogenesis induced by astrocytic epoxyeicosatrienoic Acid*. Stroke, 2002; **33**(12): p. 2957-64.
349. Medhora M, Daniels J, Munday K, Fisslthaler B, Busse R, Jacobs ER, Harder DR. *Epoxygenase-driven angiogenesis in human lung microvascular endothelial cells*. Am J Physiol Heart Circ Physiol, 2003; **284**(1): p. H215-24.
350. Michaelis UR, Fisslthaler B, Medhora M, Harder D, Fleming I, Busse R. *Cytochrome P450 2C9-derived epoxyeicosatrienoic acids induce angiogenesis via cross-talk with the epidermal growth factor receptor (EGFR)*. FASEB J, 2003; **17**(6): p. 770-2.
351. Webley AC, Popp R, Korff T, Michaelis UR, Urbich C, Busse R, Fleming I. *Cytochrome P450 2C9-induced angiogenesis is dependent on EphB4*. Arterioscler Thromb Vasc Biol, 2008; **28**(6): p. 1123-9.
352. Wang Y, Wei X, Xiao X, Hui R, Card JW, Carey MA, Wang DW, Zeldin DC. *Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways*. J Pharmacol Exp Ther, 2005; **314**(2): p. 522-32.
353. Luria A, Bettaieb A, Xi Y, Shieh GJ, Liu HC, Inoue H, Tsai HJ, Imig JD, Haj FG, Hammock BD. *Soluble epoxide hydrolase deficiency alters pancreatic islet size and improves glucose homeostasis in a model of insulin resistance*. Proc Natl Acad Sci U S A, 2011; **108**(22): p. 9038-43.
354. Simpkins AN, Rudic RD, Schreihof DA, Roy S, Manhiani M, Tsai HJ, Hammock BD, Imig JD. *Soluble epoxide inhibition is protective against cerebral ischemia via vascular and neural protection*. Am J Pathol, 2009; **174**(6): p. 2086-95.
355. Yan G, Chen S, You B, Sun J. *Activation of sphingosine kinase-1 mediates induction of endothelial cell proliferation and angiogenesis by epoxyeicosatrienoic acids*. Cardiovasc Res, 2008; **78**(2): p. 308-14.
356. Dhanasekaran A, Al-Saghir R, Lopez B, Zhu D, Gutterman DD, Jacobs ER, Medhora M. *Protective effects of epoxyeicosatrienoic acids on human endothelial cells from the pulmonary and coronary vasculature*. Am J Physiol Heart Circ Physiol, 2006; **291**(2): p. H517-31.
357. Yang S, Wei S, Pozzi A, Capdevila JH. *The arachidonic acid epoxygenase is a component of the signaling mechanisms responsible for VEGF-stimulated angiogenesis*. Arch Biochem Biophys, 2009; **489**(1-2): p. 82-91.

358. Heissig B, Hattori K, Friedrich M, Rafii S, Werb Z. *Angiogenesis: vascular remodeling of the extracellular matrix involves metalloproteinases*. *Curr Opin Hematol*, 2003; **10**(2): p. 136-41.
359. Chen JK, Capdevila J, Harris RC. *Heparin-binding EGF-like growth factor mediates the biological effects of P450 arachidonate epoxygenase metabolites in epithelial cells*. *Proc Natl Acad Sci U S A*, 2002; **99**(9): p. 6029-34.
360. Sun J, Sui X, Bradbury JA, Zeldin DC, Conte MS, Liao JK. *Inhibition of vascular smooth muscle cell migration by cytochrome p450 epoxygenase-derived eicosanoids*. *Circ Res*, 2002; **90**(9): p. 1020-7.
361. Shweiki D, Itin A, Soffer D, Keshet E. *Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis*. *Nature*, 1992; **359**(6398): p. 843-5.
362. Lohela M, Bry M, Tammela T, Alitalo K. *VEGFs and receptors involved in angiogenesis versus lymphangiogenesis*. *Curr Opin Cell Biol*, 2009; **21**(2): p. 154-65.
363. Coultas L, Chawengsaksophak K, Rossant J. *Endothelial cells and VEGF in vascular development*. *Nature*, 2005; **438**(7070): p. 937-45.
364. Shibuya M. *Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis*. *J Biochem Mol Biol*, 2006; **39**(5): p. 469-78.
365. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. *Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele*. *Nature*, 1996; **380**(6573): p. 435-9.
366. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. *Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene*. *Nature*, 1996; **380**(6573): p. 439-42.
367. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. *Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice*. *Nature*, 1995; **376**(6535): p. 62-6.
368. Siragusa M, Katare R, Meloni M, Damilano F, Hirsch E, Emanuelli C, Madeddu P. *Involvement of phosphoinositide 3-kinase gamma in angiogenesis and healing of experimental myocardial infarction in mice*. *Circ Res*, 2010; **106**(4): p. 757-68.
369. Earley S, Pastuszyn A, Walker BR. *Cytochrome p-450 epoxygenase products contribute to attenuated vasoconstriction after chronic hypoxia*. *Am J Physiol Heart Circ Physiol*, 2003; **285**(1): p. H127-36.
370. Fleming I. *Epoxyeicosatrienoic acids, cell signaling and angiogenesis*. *Prostaglandins Other Lipid Mediat*, 2007; **82**(1-4): p. 60-7.
371. Suzuki S, Oguro A, Osada-Oka M, Funae Y, Imaoka S. *Epoxyeicosatrienoic acids and/or their metabolites promote hypoxic response of cells*. *J Pharmacol Sci*, 2008; **108**(1): p. 79-88.
372. Liu M, Alkayed NJ. *Hypoxic preconditioning and tolerance via hypoxia inducible factor (HIF) 1alpha-linked induction of P450 2C11 epoxygenase in astrocytes*. *J Cereb Blood Flow Metab*, 2005; **25**(8): p. 939-48.
373. Cheranov SY, Karpurapu M, Wang D, Zhang B, Venema RC, Rao GN. *An essential role for SRC-activated STAT-3 in 14,15-EET-induced VEGF expression and angiogenesis*. *Blood*, 2008; **111**(12): p. 5581-91.

374. Webler AC, Michaelis UR, Popp R, Barbosa-Sicard E, Murugan A, Falck JR, Fisslthaler B, Fleming I. *Epoxyeicosatrienoic acids are part of the VEGF-activated signaling cascade leading to angiogenesis*. Am J Physiol Cell Physiol, 2008; **295**(5): p. C1292-301.
375. Hou HH, Hammock BD, Su KH, Morisseau C, Kou YR, Imaoka S, Oguro A, Shyue SK, Zhao JF, Lee TS. *N-terminal domain of soluble epoxide hydrolase negatively regulates the VEGF-mediated activation of endothelial nitric oxide synthase*. Cardiovasc Res, 2012; **93**(1): p. 120-9.
376. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM. *Nitric oxide synthase modulates angiogenesis in response to tissue ischemia*. J Clin Invest, 1998; **101**(11): p. 2567-78.
377. Eitenmuller I, Volger O, Kluge A, Troidl K, Barancik M, Cai WJ, Heil M, Pipp F, Fischer S, Horrevoets AJ, Schmitz-Rixen T, Schaper W. *The range of adaptation by collateral vessels after femoral artery occlusion*. Circ Res, 2006; **99**(6): p. 656-62.
378. Tzima E, del Pozo MA, Shattil SJ, Chien S, Schwartz MA. *Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment*. EMBO J, 2001; **20**(17): p. 4639-47.
379. Fisslthaler B, Popp R, Michaelis UR, Kiss L, Fleming I, Busse R. *Cyclic stretch enhances the expression and activity of coronary endothelium-derived hyperpolarizing factor synthase*. Hypertension, 2001; **38**(6): p. 1427-32.
380. Rivilis I, Milkiewicz M, Boyd P, Goldstein J, Brown MD, Egginton S, Hansen FM, Hudlicka O, Haas TL. *Differential involvement of MMP-2 and VEGF during muscle stretch- versus shear stress-induced angiogenesis*. Am J Physiol Heart Circ Physiol, 2002; **283**(4): p. H1430-8.
381. Cullen JP, Sayeed S, Sawai RS, Theodorakis NG, Cahill PA, Sitzmann JV, Redmond EM. *Pulsatile flow-induced angiogenesis: role of G(i) subunits*. Arterioscler Thromb Vasc Biol, 2002; **22**(10): p. 1610-6.
382. Buschmann I, Pries A, Styp-Rekowska B, Hillmeister P, Loufrani L, Henrion D, Shi Y, Duelsner A, Hoefer I, Gatzke N, Wang H, Lehmann K, Ulm L, Ritter Z, Hauff P, Hlushchuk R, Djonov V, van Veen T, le Noble F. *Pulsatile shear and Gja5 modulate arterial identity and remodeling events during flow-driven arteriogenesis*. Development, 2010; **137**(13): p. 2187-96.
383. Pritchard KA, Jr., Tota RR, Stemerman MB, Wong PY. *14, 15-Epoxyeicosatrienoic acid promotes endothelial cell dependent adhesion of human monocytic tumor U937 cells*. Biochem Biophys Res Commun, 1990; **167**(1): p. 137-42.
384. Falck JR, Reddy LM, Reddy YK, Bondlela M, Krishna UM, Ji Y, Sun J, Liao JK. *11,12-epoxyeicosatrienoic acid (11,12-EET): structural determinants for inhibition of TNF-alpha-induced VCAM-1 expression*. Bioorg Med Chem Lett, 2003; **13**(22): p. 4011-4.
385. Moshal KS, Zeldin DC, Sithu SD, Sen U, Tyagi N, Kumar M, Hughes WM, Jr., Metreveli N, Rosenberger DS, Singh M, Vacek TP, Rodriguez WE, Ayotunde A, Tyagi SC. *Cytochrome P450 (CYP) 2J2 gene transfection attenuates MMP-9 via inhibition of NF-kappaB in hyperhomocysteinemia*. J Cell Physiol, 2008; **215**(3): p. 771-81.

386. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. *Immunodesign of experimental sepsis by cecal ligation and puncture*. Nat Protoc, 2009; **4**(1): p. 31-6.
387. Shrum B, Anantha RV, Xu SX, Donnelly M, Haeryfar SM, McCormick JK, Mele T. *A robust scoring system to evaluate sepsis severity in an animal model*. BMC Res Notes, 2014; **7**: p. 233.
388. Hofmann F, Bernhard D, Lukowski R, Weinmeister P. *cGMP regulated protein kinases (cGK)*. Handb Exp Pharmacol, 2009(191): p. 137-62.
389. Schlossmann J, Desch M. *cGK substrates*. Handb Exp Pharmacol, 2009(191): p. 163-93.
390. Kalyanaraman H, Zhuang S, Pilz RB, Casteel DE. *The activity of cGMP-dependent protein kinase Ialpha is not directly regulated by oxidation-induced disulfide formation at cysteine 43*. J Biol Chem, 2017; **292**(20): p. 8262-8268.
391. Castro LR, Verde I, Cooper DM, Fischmeister R. *Cyclic guanosine monophosphate compartmentation in rat cardiac myocytes*. Circulation, 2006; **113**(18): p. 2221-8.
392. Fischmeister R, Castro LR, Abi-Gerges A, Rochais F, Jurevicius J, Leroy J, Vandecasteele G. *Compartmentation of cyclic nucleotide signaling in the heart: the role of cyclic nucleotide phosphodiesterases*. Circ Res, 2006; **99**(8): p. 816-28.
393. Piggott LA, Hassell KA, Berkova Z, Morris AP, Silberbach M, Rich TC. *Natriuretic peptides and nitric oxide stimulate cGMP synthesis in different cellular compartments*. J Gen Physiol, 2006; **128**(1): p. 3-14.
394. Landgraf W, Regulla S, Meyer HE, Hofmann F. *Oxidation of cysteines activates cGMP-dependent protein kinase*. J Biol Chem, 1991; **266**(25): p. 16305-11.
395. Zhang DX, Borbouse L, Gebremedhin D, Mendoza SA, Zinkevich NS, Li R, Gutterman DD. *H2O2-induced dilation in human coronary arterioles: role of protein kinase G dimerization and large-conductance Ca2+-activated K+ channel activation*. Circ Res, 2012; **110**(3): p. 471-80.
396. Osborne BW, Wu J, McFarland CJ, Nickl CK, Sankaran B, Casteel DE, Woods VL, Jr., Kornev AP, Taylor SS, Dostmann WR. *Crystal structure of cGMP-dependent protein kinase reveals novel site of interchain communication*. Structure, 2011; **19**(9): p. 1317-27.
397. Burgoyne JR, Prysyazhna O, Richards DA, Eaton P. *Proof of Principle for a Novel Class of Antihypertensives That Target the Oxidative Activation of Protein Kinase G Ialpha*. Hypertension, 2017.
398. Switzer CH, Flores-Santana W, Mancardi D, Donzelli S, Basudhar D, Ridnour LA, Miranda KM, Fukuto JM, Paolocci N, Wink DA. *The emergence of nitroxyl (HNO) as a pharmacological agent*. Biochim Biophys Acta, 2009; **1787**(7): p. 835-40.
399. Mendelsohn ME. *In hypertension, the kidney is not always the heart of the matter*. J Clin Invest, 2005; **115**(4): p. 840-4.
400. Le TH, Coffman TM. *RGS2: a "turn-off" in hypertension*. J Clin Invest, 2003; **111**(4): p. 441-3.
401. Cingolani OH, Kass DA. *Pressure-volume relation analysis of mouse ventricular function*. Am J Physiol Heart Circ Physiol, 2011; **301**(6): p. H2198-206.

402. Katz AM. *Ernest Henry Starling, his predecessors, and the "Law of the Heart"*. Circulation, 2002; **106**(23): p. 2986-92.
403. Stypmann J, Engelen MA, Troatz C, Rothenburger M, Eckardt L, Tiemann K. *Echocardiographic assessment of global left ventricular function in mice*. Lab Anim, 2009; **43**(2): p. 127-37.
404. Tournoux F, Petersen B, Thibault H, Zou L, Raher MJ, Kurtz B, Halpern EF, Chaput M, Chao W, Picard MH, Scherrer-Crosbie M. *Validation of noninvasive measurements of cardiac output in mice using echocardiography*. J Am Soc Echocardiogr, 2011; **24**(4): p. 465-70.
405. Shah AM, Spurgeon HA, Sollott SJ, Talo A, Lakatta EG. *8-bromo-cGMP reduces the myofilament response to Ca²⁺ in intact cardiac myocytes*. Circ Res, 1994; **74**(5): p. 970-8.
406. Lohmann SM, Fischmeister R, Walter U. *Signal transduction by cGMP in heart*. Basic Res Cardiol, 1991; **86**(6): p. 503-14.
407. Kinugawa KI, Kohmoto O, Yao A, Serizawa T, Takahashi T. *Cardiac inducible nitric oxide synthase negatively modulates myocardial function in cultured rat myocytes*. Am J Physiol, 1997; **272**(1 Pt 2): p. H35-47.
408. Miranda KM, Katori T, Torres de Holding CL, Thomas L, Ridnour LA, McLendon WJ, Cologna SM, Dutton AS, Champion HC, Mancardi D, Tocchetti CG, Saavedra JE, Keefer LK, Houk KN, Fukuto JM, Kass DA, Paolocci N, Wink DA. *Comparison of the NO and HNO donating properties of diazeniumdiolates: primary amine adducts release HNO in Vivo*. J Med Chem, 2005; **48**(26): p. 8220-8.
409. Susic D, Nunez E, Hosoya K, Frohlich ED. *Coronary hemodynamics in aging spontaneously hypertensive and normotensive Wistar-Kyoto rats*. J Hypertens, 1998; **16**(2): p. 231-7.
410. John SW, Kregge JH, Oliver PM, Hagaman JR, Hodgin JB, Pang SC, Flynn TG, Smithies O. *Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension*. Science, 1995; **267**(5198): p. 679-81.
411. Yen DH, Chen LC, Shen YC, Chiu YC, Ho IC, Lou YJ, Chen IC, Yen JC. *Protein kinase A-dependent neuronal nitric oxide synthase activation mediates the enhancement of baroreflex response by adrenomedullin in the nucleus tractus solitarii of rats*. J Biomed Sci, 2011; **18**: p. 32.
412. Hogan N, Kardos A, Paterson DJ, Casadei B. *Effect of exogenous nitric oxide on baroreflex function in humans*. Am J Physiol, 1999; **277**(1 Pt 2): p. H221-7.
413. Li Z, Chapleau MW, Bates JN, Bielefeldt K, Lee HC, Abboud FM. *Nitric oxide as an autocrine regulator of sodium currents in baroreceptor neurons*. Neuron, 1998; **20**(5): p. 1039-49.
414. Spieker LE, Corti R, Binggeli C, Luscher TF, Noll G. *Baroreceptor dysfunction induced by nitric oxide synthase inhibition in humans*. J Am Coll Cardiol, 2000; **36**(1): p. 213-8.
415. Gross V, Tank J, Obst M, Plehm R, Blumer KJ, Diedrich A, Jordan J, Luft FC. *Autonomic nervous system and blood pressure regulation in RGS2-deficient mice*. Am J Physiol Regul Integr Comp Physiol, 2005; **288**(5): p. R1134-42.
416. Van Vliet BN, Chafe LL, Montani JP. *Characteristics of 24 h telemetered blood pressure in eNOS-knockout and C57Bl/6J control mice*. J Physiol, 2003; **549**(Pt 1): p. 313-25.

417. Constantinides C, Mean R, Janssen BJ. *Effects of isoflurane anesthesia on the cardiovascular function of the C57BL/6 mouse*. ILAR J, 2011; **52**(3): p. e21-31.
418. Yan C, Kim D, Aizawa T, Berk BC. *Functional interplay between angiotensin II and nitric oxide: cyclic GMP as a key mediator*. Arterioscler Thromb Vasc Biol, 2003; **23**(1): p. 26-36.
419. Patrucco E, Domes K, Sbroggio M, Blaich A, Schlossmann J, Desch M, Rybalkin SD, Beavo JA, Lukowski R, Hofmann F. *Roles of cGMP-dependent protein kinase I (cGKI) and PDE5 in the regulation of Ang II-induced cardiac hypertrophy and fibrosis*. Proc Natl Acad Sci U S A, 2014; **111**(35): p. 12925-9.
420. Sha X, Isbell TS, Patel RP, Day CS, King SB. *Hydrolysis of acyloxy nitroso compounds yields nitroxyl (HNO)*. J Am Chem Soc, 2006; **128**(30): p. 9687-92.
421. Cao N, Wong YG, Rosli S, Kiriazis H, Huynh K, Qin C, Du XJ, Kemp-Harper BK, Ritchie RH. *Chronic administration of the nitroxyl donor 1-nitrosocyclohexyl acetate limits left ventricular diastolic dysfunction in a mouse model of diabetes mellitus in vivo*. Circ Heart Fail, 2015; **8**(3): p. 572-81.
422. Zhu G, Groneberg D, Sikka G, Hori D, Ranek MJ, Nakamura T, Takimoto E, Paolucci N, Berkowitz DE, Friebe A, Kass DA. *Soluble guanylate cyclase is required for systemic vasodilation but not positive inotropy induced by nitroxyl in the mouse*. Hypertension, 2015; **65**(2): p. 385-92.
423. Sabbah HN, Tocchetti CG, Wang M, Daya S, Gupta RC, Tunin RS, Mazhari R, Takimoto E, Paolucci N, Cowart D, Colucci WS, Kass DA. *Nitroxyl (HNO): A novel approach for the acute treatment of heart failure*. Circ Heart Fail, 2013; **6**(6): p. 1250-8.
424. Su DF, Miao CY. *Blood pressure variability and organ damage*. Clin Exp Pharmacol Physiol, 2001; **28**(9): p. 709-15.
425. Martinka P, Fielitz J, Patzak A, Regitz-Zagrosek V, Persson PB, Stauss HM. *Mechanisms of blood pressure variability-induced cardiac hypertrophy and dysfunction in mice with impaired baroreflex*. Am J Physiol Regul Integr Comp Physiol, 2005; **288**(3): p. R767-76.
426. Stauss HM, Godecke A, Mrowka R, Schrader J, Persson PB. *Enhanced blood pressure variability in eNOS knockout mice*. Hypertension, 1999; **33**(6): p. 1359-63.
427. Dunnes SG, D.; Herold, V.; Jakob, P.; Friebe, S., *Arterial stiffness and collagen expression in mice lacking NO-sensitive guanylyl cyclase*, in 7th International conference on cGMP Generators, Effectors and Therapeutic Implications. 2015, BMC Pharmacology and Toxicology. p. A46.
428. Friebe A, Mergia E, Dangel O, Lange A, Koesling D. *Fatal gastrointestinal obstruction and hypertension in mice lacking nitric oxide-sensitive guanylyl cyclase*. Proc Natl Acad Sci U S A, 2007; **104**(18): p. 7699-704.
429. Kang KT. *Endothelium-derived Relaxing Factors of Small Resistance Arteries in Hypertension*. Toxicol Res, 2014; **30**(3): p. 141-8.
430. Kovacs A, Alogna A, Post H, Hamdani N. *Is enhancing cGMP-PKG signalling a promising therapeutic target for heart failure with preserved ejection fraction?* Neth Heart J, 2016; **24**(4): p. 268-74.
431. Evans T, Carpenter A, Kinderman H, Cohen J. *Evidence of increased nitric oxide production in patients with the sepsis syndrome*. Circ Shock, 1993; **41**(2): p. 77-81.

432. Strand OA, Leone A, Giercksky KE, Kirkeboen KA. *Nitric oxide indices in human septic shock*. Crit Care Med, 2000; **28**(8): p. 2779-85.
433. Lange M, Connolly R, Traber DL, Hamahata A, Nakano Y, Esechie A, Jonkam C, von Borzyskowski S, Traber LD, Schmalstieg FC, Herndon DN, Enkhbaatar P. *Time course of nitric oxide synthases, nitrosative stress, and poly(ADP ribosylation) in an ovine sepsis model*. Crit Care, 2010; **14**(4): p. R129.
434. Abraham E, Singer M. *Mechanisms of sepsis-induced organ dysfunction*. Crit Care Med, 2007; **35**(10): p. 2408-16.
435. Rudyk O, Phinikaridou A, Prysyazhna O, Burgoyne JR, Botnar RM, Eaton P. *Protein kinase G oxidation is a major cause of injury during sepsis*. Proc Natl Acad Sci U S A, 2013; **110**(24): p. 9909-13.
436. Lutz SZ, Hennige AM, Feil S, Peter A, Gerling A, Machann J, Krober SM, Rath M, Schurmann A, Weigert C, Haring HU, Feil R. *Genetic ablation of cGMP-dependent protein kinase type I causes liver inflammation and fasting hyperglycemia*. Diabetes, 2011; **60**(5): p. 1566-76.
437. Salmhofer H, Neuhuber WL, Ruth P, Huber A, Russwurm M, Allescher HD. *Pivotal role of the interstitial cells of Cajal in the nitric oxide signaling pathway of rat small intestine. Morphological evidence*. Cell Tissue Res, 2001; **305**(3): p. 331-40.
438. Klein S, Seidler B, Kettenberger A, Sibaev A, Rohn M, Feil R, Allescher HD, Vanderwinden JM, Hofmann F, Schemann M, Rad R, Storr MA, Schmid RM, Schneider G, Saur D. *Interstitial cells of Cajal integrate excitatory and inhibitory neurotransmission with intestinal slow-wave activity*. Nat Commun, 2013; **4**: p. 1630.
439. Huber A, Neuhuber WL, Klugbauer N, Ruth P, Allescher HD. *Cysteine-rich protein 2, a novel substrate for cGMP kinase I in enteric neurons and intestinal smooth muscle*. J Biol Chem, 2000; **275**(8): p. 5504-11.
440. Xue L, Farrugia G, Miller SM, Ferris CD, Snyder SH, Szurszewski JH. *Carbon monoxide and nitric oxide as coneurotransmitters in the enteric nervous system: evidence from genomic deletion of biosynthetic enzymes*. Proc Natl Acad Sci U S A, 2000; **97**(4): p. 1851-5.
441. Sivarao DV, Mashimo H, Goyal RK. *Pyloric sphincter dysfunction in nNOS-/- and W/Wv mutant mice: animal models of gastroparesis and duodenogastric reflux*. Gastroenterology, 2008; **135**(4): p. 1258-66.
442. Sivarao DV, Mashimo HL, Thatte HS, Goyal RK. *Lower esophageal sphincter is achalasic in nNOS(-/-) and hypotensive in W/W(v) mutant mice*. Gastroenterology, 2001; **121**(1): p. 34-42.
443. Choudhury N, Khromov AS, Somlyo AP, Somlyo AV. *Telokin mediates Ca²⁺-desensitization through activation of myosin phosphatase in phasic and tonic smooth muscle*. J Muscle Res Cell Motil, 2004; **25**(8): p. 657-65.
444. Ellerbroek SM, Wennerberg K, Burridge K. *Serine phosphorylation negatively regulates RhoA in vivo*. J Biol Chem, 2003; **278**(21): p. 19023-31.
445. Bonnevier J, Fassler R, Somlyo AP, Somlyo AV, Arner A. *Modulation of Ca²⁺ sensitivity by cyclic nucleotides in smooth muscle from protein kinase G-deficient mice*. J Biol Chem, 2004; **279**(7): p. 5146-51.
446. Linden DR, Levitt MD, Farrugia G, Szurszewski JH. *Endogenous production of H₂S in the gastrointestinal tract: still in search of a physiologic function*. Antioxid Redox Signal, 2010; **12**(9): p. 1135-46.

447. Linden DR. *Hydrogen sulfide signaling in the gastrointestinal tract*. Antioxid Redox Signal, 2014; **20**(5): p. 818-30.
448. Yoon PJ, Parajuli SP, Zuo DC, Shahi PK, Oh HJ, Shin HR, Lee MJ, Yeum CH, Choi S, Jun JY. *Interplay of hydrogen sulfide and nitric oxide on the pacemaker activity of interstitial cells of cajal from mouse small intestine*. Chonnam Med J, 2011; **47**(2): p. 72-9.
449. Magierowski M, Magierowska K, Kwiecien S, Brzozowski T. *Gaseous mediators nitric oxide and hydrogen sulfide in the mechanism of gastrointestinal integrity, protection and ulcer healing*. Molecules, 2015; **20**(5): p. 9099-123.
450. Martin GR, McKnight GW, Dickey MS, Coffin CS, Ferraz JG, Wallace JL. *Hydrogen sulphide synthesis in the rat and mouse gastrointestinal tract*. Dig Liver Dis, 2010; **42**(2): p. 103-9.
451. Hosoki R, Matsuki N, Kimura H. *The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide*. Biochem Biophys Res Commun, 1997; **237**(3): p. 527-31.
452. Linden DR, Sha L, Mazzone A, Stoltz GJ, Bernard CE, Furne JK, Levitt MD, Farrugia G, Szurszewski JH. *Production of the gaseous signal molecule hydrogen sulfide in mouse tissues*. J Neurochem, 2008; **106**(4): p. 1577-85.
453. Gallego D, Clave P, Donovan J, Rahmati R, Grundy D, Jimenez M, Beyak MJ. *The gaseous mediator, hydrogen sulphide, inhibits in vitro motor patterns in the human, rat and mouse colon and jejunum*. Neurogastroenterol Motil, 2008; **20**(12): p. 1306-16.
454. Parajuli SP, Choi S, Lee J, Kim YD, Park CG, Kim MY, Kim HI, Yeum CH, Jun JY. *The inhibitory effects of hydrogen sulfide on pacemaker activity of interstitial cells of cajal from mouse small intestine*. Korean J Physiol Pharmacol, 2010; **14**(2): p. 83-9.
455. Park CG, Kim YD, Kim MY, Kim JS, Choi S, Yeum CH, Parajuli SP, Park JS, Jeong HS, So I, Kim KW, Jun JY. *Inhibition of pacemaker currents by nitric oxide via activation of ATP-sensitive K⁺ channels in cultured interstitial cells of Cajal from the mouse small intestine*. Naunyn Schmiedeberg's Arch Pharmacol, 2007; **376**(3): p. 175-84.
456. Hilgers RH, Das KC. *Role of in vivo vascular redox in resistance arteries*. Hypertension, 2015; **65**(1): p. 130-9.
457. Allison JH, Agrawal HC, Moore BW. *Effect of N,N,N',N'-tetramethylethylenediamine on the migration of proteins in SDS polyacrylamide gels*. Anal Biochem, 1974; **58**(2): p. 592-601.
458. Dirksen ML, Chrambach A. *Studies on the Redox State in Polyacrylamide Gels*. Separation Science, 1972; **7**(6): p. 747-772.
459. Andrades ME, Morina A, Spasic S, Spasojevic I. *Bench-to-bedside review: sepsis - from the redox point of view*. Crit Care, 2011; **15**(5): p. 230.
460. Cui X, Wu R, Zhou M, Simms HH, Wang P. *Differential expression of cytochrome P450 isoforms in the lungs of septic animals*. Crit Care Med, 2004; **32**(5): p. 1186-91.
461. Guo J, Jou W, Gavrilova O, Hall KD. *Persistent diet-induced obesity in male C57BL/6 mice resulting from temporary obesigenic diets*. PLoS One, 2009; **4**(4): p. e5370.

462. DeJager L, Pinheiro I, Dejonckheere E, Libert C. *Cecal ligation and puncture: the gold standard model for polymicrobial sepsis?* Trends Microbiol, 2011; **19**(4): p. 198-208.
463. Pinheiro da Silva F, Zampieri FG, Barbeiro DF, Barbeiro HV, Goulart AC, Torggler Filho F, Velasco IT, da Cruz Neto LM, de Souza HP, Machado MC. *Septic shock in older people: a prospective cohort study.* Immun Ageing, 2013; **10**(1): p. 21.
464. Moffat MP, Ward CA, Bend JR, Mock T, Farhangkhoe P, Karmazyn M. *Effects of epoxyeicosatrienoic acids on isolated hearts and ventricular myocytes.* Am J Physiol, 1993; **264**(4 Pt 2): p. H1154-60.
465. Gross GJ, Gauthier KM, Moore J, Falck JR, Hammock BD, Campbell WB, Nithipatikom K. *Effects of the selective EET antagonist, 14,15-EEZE, on cardioprotection produced by exogenous or endogenous EETs in the canine heart.* Am J Physiol Heart Circ Physiol, 2008; **294**(6): p. H2838-44.
466. Zhang W, Yang AL, Liao J, Li H, Dong H, Chung YT, Bai H, Matkowskyj KA, Hammock BD, Yang GY. *Soluble epoxide hydrolase gene deficiency or inhibition attenuates chronic active inflammatory bowel disease in IL-10(-/-) mice.* Dig Dis Sci, 2012; **57**(10): p. 2580-91.
467. Zhang W, Liao J, Li H, Dong H, Bai H, Yang A, Hammock BD, Yang GY. *Reduction of inflammatory bowel disease-induced tumor development in IL-10 knockout mice with soluble epoxide hydrolase gene deficiency.* Mol Carcinog, 2013; **52**(9): p. 726-38.
468. Davis BB, Liu JY, Tancredi DJ, Wang L, Simon SI, Hammock BD, Pinkerton KE. *The anti-inflammatory effects of soluble epoxide hydrolase inhibitors are independent of leukocyte recruitment.* Biochem Biophys Res Commun, 2011; **410**(3): p. 494-500.
469. Alvarez DF, Gjerde EA, Townsley MI. *Role of EETs in regulation of endothelial permeability in rat lung.* Am J Physiol Lung Cell Mol Physiol, 2004; **286**(2): p. L445-51.
470. Alvarez DF, King JA, Weber D, Addison E, Liedtke W, Townsley MI. *Transient receptor potential vanilloid 4-mediated disruption of the alveolar septal barrier: a novel mechanism of acute lung injury.* Circ Res, 2006; **99**(9): p. 988-95.
471. Anderson TJ. *Prognostic significance of brachial flow-mediated vasodilation.* Circulation, 2007; **115**(18): p. 2373-5.
472. Schuck RN, Theken KN, Edin ML, Caughey M, Bass A, Ellis K, Tran B, Steele S, Simmons BP, Lih FB, Tomer KB, Wu MC, Hinderliter AL, Stouffer GA, Zeldin DC, Lee CR. *Cytochrome P450-derived eicosanoids and vascular dysfunction in coronary artery disease patients.* Atherosclerosis, 2013; **227**(2): p. 442-8.
473. Ridker PM. *High-sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease.* Circulation, 2001; **103**(13): p. 1813-8.
474. Yiu HH, Graham AL, Stengel RF. *Dynamics of a cytokine storm.* PLoS One, 2012; **7**(10): p. e45027.
475. Williams DL, Li C, Ha T, Ozment-Skelton T, Kalbfleisch JH, Preiszner J, Brooks L, Breuel K, Schweitzer JB. *Modulation of the phosphoinositide 3-*

- kinase pathway alters innate resistance to polymicrobial sepsis. *J Immunol*, 2004; **172**(1): p. 449-56.
476. Bozza FA, Salluh JI, Japiassu AM, Soares M, Assis EF, Gomes RN, Bozza MT, Castro-Faria-Neto HC, Bozza PT. *Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis*. *Crit Care*, 2007; **11**(2): p. R49.
 477. Huber-Lang M, Barratt-Due A, Pischke SE, Sandanger O, Nilsson PH, Nunn MA, Denk S, Gaus W, Espevik T, Mollnes TE. *Double blockade of CD14 and complement C5 abolishes the cytokine storm and improves morbidity and survival in polymicrobial sepsis in mice*. *J Immunol*, 2014; **192**(11): p. 5324-31.
 478. Tunctan B, Korkmaz B, Sari AN, Kacan M, Unsal D, Serin MS, Buharalioglu CK, Sahan-Firat S, Cuez T, Schunck WH, Falck JR, Malik KU. *5,14-HEDGE, a 20-HETE mimetic, reverses hypotension and improves survival in a rodent model of septic shock: contribution of soluble epoxide hydrolase, CYP2C23, MEK1/ERK1/2/IKKbeta/IkappaB-alpha/NF-kappaB pathway, and proinflammatory cytokine formation*. *Prostaglandins Other Lipid Mediat*, 2013; **102-103**: p. 31-41.
 479. Theken KN, Deng Y, Kannon MA, Miller TM, Poloyac SM, Lee CR. *Activation of the acute inflammatory response alters cytochrome P450 expression and eicosanoid metabolism*. *Drug Metab Dispos*, 2011; **39**(1): p. 22-9.
 480. Spector AA, Fang X, Snyder GD, Weintraub NL. *Epoxyeicosatrienoic acids (EETs): metabolism and biochemical function*. *Prog Lipid Res*, 2004; **43**(1): p. 55-90.
 481. Fang X, Kaduce TL, Weintraub NL, Harmon S, Teesch LM, Morisseau C, Thompson DA, Hammock BD, Spector AA. *Pathways of epoxyeicosatrienoic acid metabolism in endothelial cells. Implications for the vascular effects of soluble epoxide hydrolase inhibition*. *J Biol Chem*, 2001; **276**(18): p. 14867-74.
 482. Fang X, Weintraub NL, Oltman CL, Stoll LL, Kaduce TL, Harmon S, Dellsperger KC, Morisseau C, Hammock BD, Spector AA. *Human coronary endothelial cells convert 14,15-EET to a biologically active chain-shortened epoxide*. *Am J Physiol Heart Circ Physiol*, 2002; **283**(6): p. H2306-14.
 483. Zhang Y, Oltman CL, Lu T, Lee HC, Dellsperger KC, VanRollins M. *EET homologs potently dilate coronary microvessels and activate BK(Ca) channels*. *Am J Physiol Heart Circ Physiol*, 2001; **280**(6): p. H2430-40.
 484. Carroll MA, Balazy M, Margiotta P, Falck JR, McGiff JC. *Renal vasodilator activity of 5,6-epoxyeicosatrienoic acid depends upon conversion by cyclooxygenase and release of prostaglandins*. *J Biol Chem*, 1993; **268**(17): p. 12260-6.
 485. Carroll MA, Garcia MP, Falck JR, McGiff JC. *Cyclooxygenase dependency of the renovascular actions of cytochrome P450-derived arachidonate metabolites*. *J Pharmacol Exp Ther*, 1992; **260**(1): p. 104-9.
 486. Farmer MR, Roberts RE, Gardiner SM, Ralevic V. *Effects of in vivo lipopolysaccharide infusion on vasoconstrictor function of rat isolated mesentery, kidney, and aorta*. *J Pharmacol Exp Ther*, 2003; **306**(2): p. 538-45.
 487. Parker JL, Adams HR. *Selective inhibition of endothelium-dependent vasodilator capacity by Escherichia coli endotoxemia*. *Circ Res*, 1993; **72**(3): p. 539-51.

488. Davis J, Maillet M, Miano JM, Molkentin JD. *Lost in transgenesis: a user's guide for genetically manipulating the mouse in cardiac research*. Circ Res, 2012; **111**(6): p. 761-77.
489. Connor KM, SanGiovanni JP, Lofqvist C, Aderman CM, Chen J, Higuchi A, Hong S, Pravda EA, Majchrzak S, Carper D, Hellstrom A, Kang JX, Chew EY, Salem N, Jr., Serhan CN, Smith LEH. *Increased dietary intake of omega-3-polyunsaturated fatty acids reduces pathological retinal angiogenesis*. Nat Med, 2007; **13**(7): p. 868-873.
490. SanGiovanni JP, Chew EY, Clemons TE, Davis MD, Ferris FL, 3rd, Gensler GR, Kurinij N, Lindblad AS, Milton RC, Seddon JM, Sperduto RD, Age-Related Eye Disease Study Research G. *The relationship of dietary lipid intake and age-related macular degeneration in a case-control study: AREDS Report No. 20*. Arch Ophthalmol, 2007; **125**(5): p. 671-9.
491. Yanai R, Mulki L, Hasegawa E, Takeuchi K, Sweigard H, Suzuki J, Gaissert P, Vavvas DG, Sonoda KH, Rothe M, Schunck WH, Miller JW, Connor KM. *Cytochrome P450-generated metabolites derived from omega-3 fatty acids attenuate neovascularization*. Proc Natl Acad Sci U S A, 2014; **111**(26): p. 9603-8.
492. Kim YW, Byzova TV. *Oxidative stress in angiogenesis and vascular disease*. Blood, 2014; **123**(5): p. 625-31.
493. Negre-Salvayre A, Auge N, Ayala V, Basaga H, Boada J, Brenke R, Chapple S, Cohen G, Feher J, Grune T, Lengyel G, Mann GE, Pamplona R, Poli G, Portero-Otin M, Riahi Y, Salvayre R, Sasson S, Serrano J, Shamni O, Siems W, Siow RC, Wiswedel I, Zarkovic K, Zarkovic N. *Pathological aspects of lipid peroxidation*. Free Radic Res, 2010; **44**(10): p. 1125-71.
494. Behn C, Araneda OF, Llanos AJ, Celedon G, Gonzalez G. *Hypoxia-related lipid peroxidation: evidences, implications and approaches*. Respir Physiol Neurobiol, 2007; **158**(2-3): p. 143-50.
495. Limbourg A, Korff T, Napp LC, Schaper W, Drexler H, Limbourg FP. *Evaluation of postnatal arteriogenesis and angiogenesis in a mouse model of hind-limb ischemia*. Nat Protoc, 2009; **4**(12): p. 1737-46.
496. Jones PD, Wolf NM, Morisseau C, Whetstone P, Hock B, Hammock BD. *Fluorescent substrates for soluble epoxide hydrolase and application to inhibition studies*. Anal Biochem, 2005; **343**(1): p. 66-75.
497. Fradette C, Du Souich P. *Effect of hypoxia on cytochrome P450 activity and expression*. Curr Drug Metab, 2004; **5**(3): p. 257-71.
498. Michaelis UR, Fisslthaler B, Barbosa-Sicard E, Falck JR, Fleming I, Busse R. *Cytochrome P450 epoxigenases 2C8 and 2C9 are implicated in hypoxia-induced endothelial cell migration and angiogenesis*. J Cell Sci, 2005; **118**(Pt 23): p. 5489-98.
499. Rudnicki M, Faine LA, Dehne N, Namgaladze D, Ferderbar S, Weinlich R, Amarante-Mendes GP, Yan CY, Krieger JE, Brune B, Abdalla DS. *Hypoxia inducible factor-dependent regulation of angiogenesis by nitro-fatty acids*. Arterioscler Thromb Vasc Biol, 2011; **31**(6): p. 1360-7.
500. O'Donnell VB, Eiserich JP, Chumley PH, Jablonsky MJ, Krishna NR, Kirk M, Barnes S, Darley-Usmar VM, Freeman BA. *Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion*. Chem Res Toxicol, 1999; **12**(1): p. 83-92.

501. Baker PR, Schopfer FJ, O'Donnell VB, Freeman BA. *Convergence of nitric oxide and lipid signaling: anti-inflammatory nitro-fatty acids*. Free Radic Biol Med, 2009; **46**(8): p. 989-1003.
502. Goto T, Fukuyama N, Aki A, Kanabuchi K, Kimura K, Taira H, Tanaka E, Wakana N, Mori H, Inoue H. *Search for appropriate experimental methods to create stable hind-limb ischemia in mouse*. Tokai J Exp Clin Med, 2006; **31**(3): p. 128-32.
503. Westvik TS, Fitzgerald TN, Muto A, Maloney SP, Pimiento JM, Fancher TT, Magri D, Westvik HH, Nishibe T, Velazquez OC, Dardik A. *Limb ischemia after iliac ligation in aged mice stimulates angiogenesis without arteriogenesis*. J Vasc Surg, 2009; **49**(2): p. 464-73.
504. Sander AL, Jakob H, Sommer K, Sadler C, Fleming I, Marzi I, Frank J. *Cytochrome P450-derived epoxyeicosatrienoic acids accelerate wound epithelialization and neovascularization in the hairless mouse ear wound model*. Langenbecks Arch Surg, 2011; **396**(8): p. 1245-53.
505. Supp DM, Hahn JM, McFarland KL, Combs KA, Lee KS, Inceoglu B, Wan D, Boyce ST, Hammock BD. *Soluble Epoxide Hydrolase Inhibition and Epoxyeicosatrienoic Acid Treatment Improve Vascularization of Engineered Skin Substitutes*. Plast Reconstr Surg Glob Open, 2016; **4**(12): p. e1151.
506. Panigrahy D, Kalish BT, Huang S, Bielenberg DR, Le HD, Yang J, Edin ML, Lee CR, Benny O, Mudge DK, Butterfield CE, Mammoto A, Mammoto T, Inceoglu B, Jenkins RL, Simpson MA, Akino T, Lih FB, Tomer KB, Ingber DE, Hammock BD, Falck JR, Manthati VL, Kaipainen A, D'Amore PA, Puder M, Zeldin DC, Kieran MW. *Epoxyeicosanoids promote organ and tissue regeneration*. Proc Natl Acad Sci U S A, 2013; **110**(33): p. 13528-33.
507. Chen X, Chen X, Huang X, Qin C, Fang Y, Liu Y, Zhang G, Pan D, Wang W, Xie M. *Soluble epoxide hydrolase inhibition provides multi-target therapeutic effects in rats after spinal cord injury*. Mol Neurobiol, 2016; **53**(3): p. 1565-1578.
508. Levesque JP, Hendy J, Takamatsu Y, Williams B, Winkler IG, Simmons PJ. *Mobilization by either cyclophosphamide or granulocyte colony-stimulating factor transforms the bone marrow into a highly proteolytic environment*. Exp Hematol, 2002; **30**(5): p. 440-9.
509. Pelus LM, Bian H, King AG, Fukuda S. *Neutrophil-derived MMP-9 mediates synergistic mobilization of hematopoietic stem and progenitor cells by the combination of G-CSF and the chemokines GRObeta/CXCL2 and GRObetaT/CXCL2delta4*. Blood, 2004; **103**(1): p. 110-9.
510. Sander AL, Sommer K, Neumayer T, Fleming I, Marzi I, Barker JH, Frank J, Jakob H. *Soluble epoxide hydrolase disruption as therapeutic target for wound healing*. J Surg Res, 2013; **182**(2): p. 362-7.
511. Bettaieb A, Chahed S, Bachaalany S, Griffey S, Hammock BD, Haj FG. *Soluble Epoxide Hydrolase Pharmacological Inhibition Ameliorates Experimental Acute Pancreatitis in Mice*. Mol Pharmacol, 2015; **88**(2): p. 281-90.
512. Dong XW, Jia YL, Ge LT, Jiang B, Jiang JX, Shen J, Jin YC, Guan Y, Sun Y, Xie QM. *Soluble epoxide hydrolase inhibitor AUDA decreases bleomycin-induced pulmonary toxicity in mice by inhibiting the p38/Smad3 pathways*. Toxicology, 2017; **389**: p. 31-41.

513. Dvorak HF. *Discovery of vascular permeability factor (VPF)*. Exp Cell Res, 2006; **312**(5): p. 522-6.
514. Vandenbroucke E, Mehta D, Minshall R, Malik AB. *Regulation of endothelial junctional permeability*. Ann N Y Acad Sci, 2008; **1123**: p. 134-45.
515. Baumgartner I, Rauh G, Pieczek A, Wuensch D, Magner M, Kearney M, Schainfeld R, Isner JM. *Lower-extremity edema associated with gene transfer of naked DNA encoding vascular endothelial growth factor*. Ann Intern Med, 2000; **132**(11): p. 880-4.
516. Isner JM, Vale PR, Symes JF, Losordo DW. *Assessment of risks associated with cardiovascular gene therapy in human subjects*. Circ Res, 2001; **89**(5): p. 389-400.
517. Aritomi M, Kunishima N, Okamoto T, Kuroki R, Ota Y, Morikawa K. *Atomic structure of the GCSF-receptor complex showing a new cytokine-receptor recognition scheme*. Nature, 1999; **401**(6754): p. 713-7.
518. Hiraoka O, Anaguchi H, Ota Y. *Evidence for the ligand-induced conversion from a dimer to a tetramer of the granulocyte colony-stimulating factor receptor*. FEBS Lett, 1994; **356**(2-3): p. 255-60.
519. Hiraoka O, Anaguchi H, Yamasaki K, Fukunaga R, Nagata S, Ota Y. *Ligand binding domain of granulocyte colony-stimulating factor receptor*. J Biol Chem, 1994; **269**(35): p. 22412-9.
520. Martins-Marques T, Catarino S, Zuzarte M, Marques C, Matafome P, Pereira P, Girao H. *Ischaemia-induced autophagy leads to degradation of gap junction protein connexin43 in cardiomyocytes*. Biochem J, 2015; **467**(2): p. 231-45.
521. Hwang JY, Gertner M, Pontarelli F, Court-Vazquez B, Bennett MV, Ofengeim D, Zukin RS. *Global ischemia induces lysosomal-mediated degradation of mTOR and activation of autophagy in hippocampal neurons destined to die*. Cell Death Differ, 2017; **24**(2): p. 317-329.
522. McDowell GS, Philpott A. *Non-canonical ubiquitylation: mechanisms and consequences*. Int J Biochem Cell Biol, 2013; **45**(8): p. 1833-42.
523. Huang A, Sun D, Smith CJ, Connetta JA, Shesely EG, Koller A, Kaley G. *In eNOS knockout mice skeletal muscle arteriolar dilation to acetylcholine is mediated by EDHF*. Am J Physiol Heart Circ Physiol, 2000; **278**(3): p. H762-8.
524. Larsen BT, Gutterman DD, Sato A, Toyama K, Campbell WB, Zeldin DC, Manthati VL, Falck JR, Miura H. *Hydrogen peroxide inhibits cytochrome p450 epoxigenases: interaction between two endothelium-derived hyperpolarizing factors*. Circ Res, 2008; **102**(1): p. 59-67.
525. Fleming I. *Cytochrome p450 and vascular homeostasis*. Circ Res, 2001; **89**(9): p. 753-62.
526. Brennan JP, Bardswell SC, Burgoyne JR, Fuller W, Schroder E, Wait R, Begum S, Kentish JC, Eaton P. *Oxidant-induced activation of type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation*. J Biol Chem, 2006; **281**(31): p. 21827-36.
527. Burgoyne JR, Eaton P. *Transnitrosylating nitric oxide species directly activate type I protein kinase A, providing a novel adenylate cyclase-independent cross-talk to beta-adrenergic-like signaling*. J Biol Chem, 2009; **284**(43): p. 29260-8.
528. Nedvetsky PI, Zhao X, Mathivet T, Aspalter IM, Stanchi F, Metzger RJ, Mostov KE, Gerhardt H. *cAMP-dependent protein kinase A (PKA) regulates*

- angiogenesis by modulating tip cell behavior in a Notch-independent manner. Development*, 2016; **143**(19): p. 3582-3590.
529. Burgoyne JR, Rudyk O, Cho HJ, Prysyazhna O, Hathaway N, Weeks A, Evans R, Ng T, Schroder K, Brandes RP, Shah AM, Eaton P. *Deficient angiogenesis in redox-dead Cys17Ser PKARIAalpha knock-in mice*. *Nat Commun*, 2015; **6**: p. 7920.
 530. Sanders KM, Koh SD, Ro S, Ward SM. *Regulation of gastrointestinal motility--insights from smooth muscle biology*. *Nat Rev Gastroenterol Hepatol*, 2012; **9**(11): p. 633-45.
 531. Henrion D. *Pressure and flow-dependent tone in resistance arteries. Role of myogenic tone*. *Arch Mal Coeur Vaiss*, 2005; **98**(9): p. 913-21.
 532. Huang A, Sun D, Yan C, Falck JR, Kaley G. *Contribution of 20-HETE to augmented myogenic constriction in coronary arteries of endothelial NO synthase knockout mice*. *Hypertension*, 2005; **46**(3): p. 607-13.
 533. Roman RJ, Maier KG, Sun CW, Harder DR, Alonso-Galicia M. *Renal and cardiovascular actions of 20-hydroxyecosatetraenoic acid and epoxyecosatrienoic acids*. *Clin Exp Pharmacol Physiol*, 2000; **27**(11): p. 855-65.
 534. Sun D, Cuevas AJ, Gotlinger K, Hwang SH, Hammock BD, Schwartzman ML, Huang A. *Soluble epoxide hydrolase-dependent regulation of myogenic response and blood pressure*. *Am J Physiol Heart Circ Physiol*, 2014; **306**(8): p. H1146-53.
 535. Han Y, Yan G, Wang Q, Ma G, Tang C, Gu Y, Li L, Zhao J. *Predominant role of vasoconstrictors over dilators derived from arachidonic acid in hypoxic pulmonary vasoconstriction*. *Mol Med Rep*, 2013; **8**(4): p. 1263-71.
 536. Pokreisz P, Fleming I, Kiss L, Barbosa-Sicard E, Fisslthaler B, Falck JR, Hammock BD, Kim IH, Szelid Z, Vermeersch P, Gillijns H, Pellens M, Grimminger F, van Zonneveld AJ, Collen D, Busse R, Janssens S. *Cytochrome P450 epoxigenase gene function in hypoxic pulmonary vasoconstriction and pulmonary vascular remodeling*. *Hypertension*, 2006; **47**(4): p. 762-70.
 537. Clanton TL. *Hypoxia-induced reactive oxygen species formation in skeletal muscle*. *J Appl Physiol (1985)*, 2007; **102**(6): p. 2379-88.