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Anti-inflammatory Effects of alpha1-Antitrypsin via Neutralization of Heme Toxicity in Human Endothelial Cells and Neutrophils

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ABSTRACT

Kukuh Madyaningrana

Anti-inflammatory Effects of alpha1-Antitrypsin via Neutralization of Heme Toxicity in Human Endothelial Cells and Neutrophils

Heme is a tetrapyrrole ring with a central iron ion that serves as a prosthetic group in proteins such as hemoglobin, myoglobin and cytochrome c, all of which play essential roles in oxygen transport and energy metabolism. In contrast, high concentrations of free heme, i.e. non-protein bound heme, that occur in clinical conditions with intravascular hemolysis or tissue damage, can cause prooxidant, pro-inflammatory and cytotoxic effects. The acute-phase reactant alpha1-antitrypsin (A1AT), a serum protein that is primarily known for neutralization of protease activity in neutrophils, has been demonstrated to exhibit high binding affinity for heme and can be considered a heme-binding protein (HBP). However, the potential functional roles of A1AT as a HBP are ill defined. Thus, it was the goal of the current thesis to investigate the potential protective effects of A1AT due to its interactions with heme in endothelial cells and neutrophils.

In the first paper of this thesis, the pro-inflammatory effects of heme and their potential neutralization by A1AT were investigated in endothelial cells. In particular, the protective function of A1AT for the endothelium against heme-mediated cytotoxicity was investigated in a cell culture model of primary human endothelial cells. Similar to other HBPs such hemopexin and albumin, A1AT counter-acted the cytotoxic effects of heme in this experimental model. However, in contrast to hemopexin and albumin, A1AT did not prevent the cellular up-take of heme by these cells. Furthermore, A1AT inhibited heme-mediated pro-inflammatory activation of endothelial cells and reduced the formation of reactive oxygen species in mitochondria. The mechanism of protection by A1AT appears to be fundamentally different to that of other known HBPs such as hemopexin and albumin.

The second paper of the thesis deals with the role of A1AT in human blood neutrophils. It is demonstrated in a cell culture model of primary human neutrophils that A1AT reduced heme-dependent cell spreading and surface expression of the filament protein vimentin in these cells. In addition, free radical production, expression of heme oxygenase-1, release of interleukin-8 and up-regulation of neutrophil adhesion with endothelial cells was markedly alleviated by A1AT. Heme-mediated activation of human neutrophils that involved the protein kinase C pathway was attenuated by A1AT. Finally, in a mouse model of acute kidney injury with injection of heme, administration of A1AT significantly reduced infiltration of neutrophils, and lowered levels of serum inflammatory markers.

To conclude, in the current thesis it is demonstrated that the prooxidant and pro-inflammatory effects of heme can be alleviated by A1AT in endothelial cells and neutrophils. These findings suggest that administration A1AT may be of clinical relevance in inflammatory settings with hemolysis and tissue damage.

ZUSAMMENFASSUNG

Kukuh Madyaningrana

Entzündungshemmende Wirkungen von alpha1-Antitrypsin durch Neutralisation der Toxizität von Häm in humanen Endothelzellen und Neutrophilen

Häm ist ein Tetrapyrrolring mit einem zentralen Eisenion, das als prosthetische Gruppe von Proteinen wie Hämoglobin, Myoglobin und Cytochrom c fungiert und dabei eine wichtige Rolle beim Sauerstofftransport und Energiestoffwechsel spielt. Im Gegensatz dazu kann ‚freies' oder nicht-proteingebundenes Häm, das bei Erkrankungen mit akuter Hämolyse oder Gewebeschädigung in hohen Konzentrationen auftreten kann, oxidative, pro-entzündliche und zytotoxische Wirkungen verursachen. Da für das Akute-Phase Serumprotein alpha1-Antitrypsin (A1AT), dessen wesentliche physiologische Funktion in der Neutralisierung von Proteasen aus neutrophilen Granulozyten besteht, eine hohe Bindungsaffinität für Häm festgestellt wurde, kann es auch als ein Häm-bindendes Protein (HBP) betrachtet werden. Die funktionelle Rolle von A1AT als ein mögliches HBP ist jedoch weitgehend unbekannt. Es war daher das Ziel der vorliegenden Arbeit, die möglichen Schutzwirkungen von A1AT aufgrund seiner Bindung mit Häm in Endothelzellen und neutrophile Granulozyten aufzuklären.

In der ersten Publikation der vorliegenden Dissertationsschrift wurden die pro-inflammatorischen Effekte von Häm und deren mögliche Neutralisierung durch A1AT in Endothelzellen untersucht. Insbesondere wurde dabei die endotheliale Schutzfunktion von A1AT gegen die Häm-vermittelte Zytotoxizität in einem Zellkulturmodell primärer humaner Endothelzellen untersucht. Vergleichbar zu anderen HBPs wie Hämopexin und Albumin neutralisierte A1AT die zytotoxischen Effekte von Häm in diesem experimentellen Modell. Im Gegensatz zu Hämopexin und Albumin verhinderte A1AT jedoch nicht die zelluläre Aufnahme von Häm durch diese Zellen. Darüber hinaus hemmte A1AT die durch Häm vermittelte entzündliche Aktivierung von Endothelzellen und verringerte die Bildung reaktiver Sauerstoffspezies in Mitochondrien. Der Schutzmechanismus durch A1AT scheint sich grundlegend von dem anderer bekannter HBPs wie Hämopexin und Albumin zu unterscheiden.

Die zweite Publikation der vorliegenden Dissertationsschrift beschäftigte sich mit der Rolle von A1AT in humanen neutrophilen Granulozyten. In einem Zellkulturmodell von primären humanen neutrophilen Granulozyten konnte gezeigt werden, dass A1AT das Häm-vermittelte *cell spreading* und die Expression des Filamentproteins Vimentin in diesen Zellen reduzierte. Darüber hinaus wurde die Produktion freier Radikale, die Expression des Häm-Oxygenase-1 Gens, die Freisetzung von Interleukin-8 und die Hochregulierung der Adhäsion von

neutrophilen Granulozyten an Endothelzellen durch A1AT deutlich verringert. Die Häm-vermittelte Aktivierung humaner neutrophiler Granulozyten, an der der Proteinkinase-C Signalweg beteiligt ist, wurde durch A1AT abgeschwächt. In einem Mausmodell von akutem Nierenversagen mit Injektion von Häm verminderte die Verabreichung von A1AT die Zahl der in die Niere infiltrierenden neutrophilen Granulozyten und verschiedene Entzündungsmarker im Serum signifikant.

Zusammenfassend wird in der vorliegenden Dissertationsschrift gezeigt, dass die pro-oxidativen und pro-inflammatorischen Wirkungen von Häm durch A1AT in Endothelzellen und neutrophilen Granulozyten abgeschwächt werden. Diese Ergebnisse lassen darauf schließen, dass die Gabe von A1AT bei entzündlichen Erkrankungen mit Hämolyse oder Gewebeschädigung klinisch bedeutsam werden könnte.

1. INTRODUCTION

Inflammation and the inflammatory response

Inflammation is a protective reaction of the innate immune system caused by infections or tissue damage (1, 2). The clinical symptoms of inflammatory reactions are pain, hyperthermia, redness, swelling and loss of function (3, 4). According to their time courses, inflammatory reactions are categorized into acute and chronic forms. Acute inflammation is often a severe immunological immune defense reaction that usually leads to the restoration of physiological homeostasis in affected organs and tissues. Chronic inflammation may be caused by dysregulated resolution of an acute inflammatory reaction after eliminating its stimulus and can play an important role in the development and progression of various diseases (5, 6).

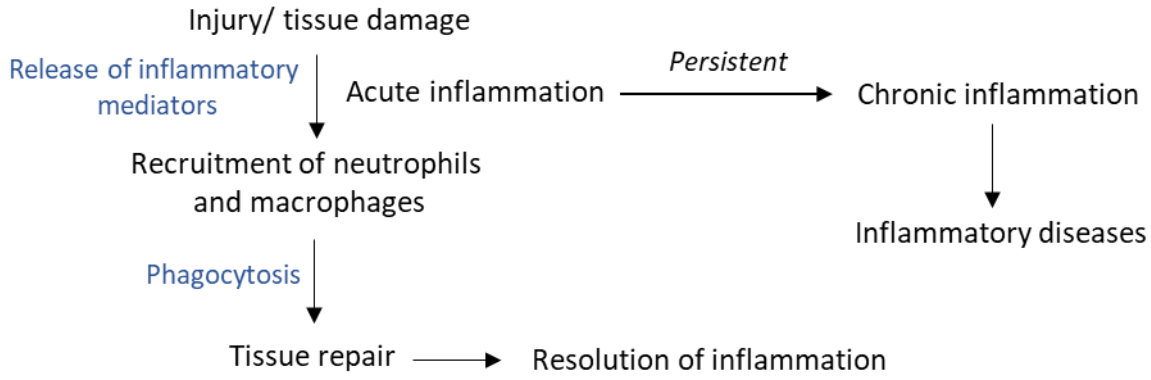

Figure 1 - Overview of the inflammatory response

In acute inflammation pathogens or damaged cells are recognized by macrophages, mast cells and dendritic cells via so-called pattern recognition receptors (PRRs) (7). PRRs interact with two types of ligands: exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs). PAMPs are immunogenic components of bacteria, viruses or other pathogens (8). A prototypical representative of the group of PAMPs is lipopolysaccharide (LPS), which is also known as endotoxin. LPS is a component of the outer cell membrane of gram-negative bacteria and is recognized by Toll-like receptor (TLR)-4, which is a prototypical member of TLRs. DAMPs, on the other hand, are endogenous compounds that are released when cells are destroyed (e.g. extracellular adenosine triphosphate (ATP) or uric acid).

After activation by PRRs, the respective tissue macrophages in various organs release pro-inflammatory mediators such as the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 or prostaglandins that induce inflammatory reactions (8, 9). This causes a local increase in blood flow, vascular permeability and activation of the endothelium that, in turn, leads to an increased accumulation of immunologically active cells at the site of inflammation. The first cells to reach the site of inflammation are neutrophils, followed by monocytes, lymphocytes (natural killer cells, T- and B-cells) and mast cells. Monocytes are recruited to the site of tissue damage via chemotaxis and then differentiate into macrophages (9). After halting of an inflammatory reaction, the infiltrating immune cells are removed from inflamed tissues. This process is referred to as resolution of inflammation and is regulated via anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (5, 10).

Endothelial cells and neutrophils in inflammation

Endothelial cells

Endothelial cells (ECs) make up the surface of blood vessels and form their inner cellular layer. The vascular endothelium plays an important role in regulating the homeostasis of arterial blood pressure, blood coagulation, exchange of fluids and macromolecules between blood vessels and tissues, as well as in the regulation of inflammatory reactions (11, 12). Acute inflammatory reactions induce activation of the endothelium that is mediated via activation of the histamine H1 receptor (type I activation) in the early phase and by pro-inflammatory mediators such as TNF-α and IL-1 (type II activation) in the late phase (11, 13). Furthermore, ECs can express PRRs and may therefore be activated by PAMPs and DAMPs such as LPS or heme (14, 15). Inflammatory activation of the endothelium leads to increased expression of the adhesion molecules vascular cell adhesion molecule (VCAM)-1 and intercellular cell adhesion molecule (ICAM)-1, as well as the secretion of von Willebrand factor and P-selectin, all of which cause further migration of leukocytes to the site of inflammation (4). This may be the result of an acute inflammatory response due to infection or may be caused by disorders such as sickle cell disease (SCD) or ischemia-reperfusion injury (IRI) associated with hemolysis and tissue damage. Activation of the endothelium can contribute to chronic inflammatory responses and plays a central role in inflammatory vascular diseases such as atherosclerosis or vascular graft rejection (16).

Neutrophils

Neutrophils are recruited in large numbers to the site of infection or tissue damage in the early phase of an inflammatory reaction. They are also known as polymorphonuclear neutrophilic

granulocytes and are the most abundant and predominant white blood cell population in human circulating blood (3). Moreover, neutrophils are generated by the bone marrow and their numbers can be markedly increased within a short time during acute inflammation in humans. Neutrophils are considered the first line of defense in infectious diseases caused by bacterial pathogens. In addition to their roles in killing and eliminating of bacteria, these cells have also been recognized to modulate the host response by interacting with other immunological cells such lymphocytes, dendritic cells and natural killer cells, the mechanisms of which are under current investigation (3, 17). Activation, mobilization and accumulation of neutrophils are modulated by a variety of regulatory mechanisms that are dependent on pathogen and host factors. Importantly, the various regulatory roles of neutrophils in the course innate immune response are subject of current investigations (17).

The tetrapyrrole heme

Physiological functions of heme

Heme is a molecular complex of iron and the tetrapyrrole protoporphyrin IX, that is also termed hemin (Fe^{3+}) or heme (Fe^{2+}) depending on the oxidation state of the central iron ion (18, 19). Heme is the prosthetic group of various heme proteins that are involved in a variety of physiological processes such as oxygen transport (hemoglobin (Hb), myoglobin), electron transport in the cellular respiratory chain (cytochrome c) or prostaglandin synthesis (cyclooxygenase-2 (COX-2)) (20).

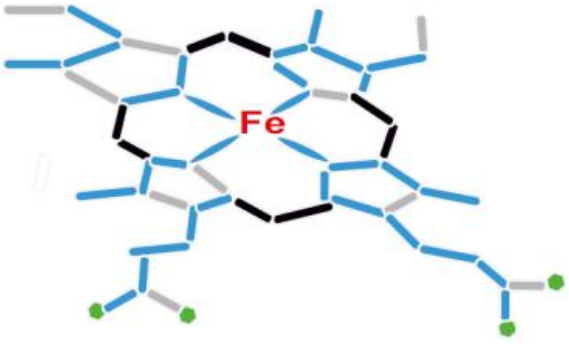

Figure 2 - Structure of heme (iron protoporphyrin IX) (adapted from Reference 47)

In hemoproteins, heme is either covalently, or non-covalently bound to the protein portion, in which it serves as a prosthetic group. In addition, a proportion of intracellular heme is also known as so-called free heme, which is weakly associated with heme-binding proteins (HBPs) and is also known as labile heme (21-24). The term 'free' heme is conflicting because heme is hydrophobic and highly toxic when not bound to proteins (25). It is therefore essential for the homeostasis of cells that the availability of free heme is limited or strictly controlled. It has recently been shown that the cellular availability of unbound free heme can be precisely controlled and limited, while the majority of so-called labile heme is protein-bound and can be released when needed (26). Labile heme may be a cellular heme reservoir, through which heme can be rapidly made available for processes such as *de novo* synthesis of hemoproteins and enzymes including COX-2, inducible nitric oxide synthase, catalases and peroxidases (24, 25, 27). Furthermore, it has been shown that labile heme inhibits the activity of transcription factors such as BTB-and-CNC homologue (BACH)1 and Rev-ERB (28, 29).

Toxicity of heme

Prooxidant effects of heme

High concentrations of free heme can have prooxidant, pro-inflammatory and cytotoxic effects effects that are critically involved in the pathogenesis of SCD, malaria and IRI (30-33). In various pathophysiological conditions such as hemolysis and tissue damage, large amounts of Hb and other hemoproteins can be released (19, 34, 35). Cell-free Hb and heme that is released from oxidized Hb mediate prooxidant effects via formation of reactive oxygen species (ROS) catalyzed by iron (Fenton reaction). Here, hydrogen peroxide reacts in the presence of the catalyst iron to form hydroxyl and hydroperoxyl radicals (36). In addition, due to its lipophilic character heme can intercalate with membranes of cells and cell organelles and cause damage in lipids, proteins and DNA through peroxidation (25, 37)

Pro-inflammatory effects of heme

Heme can also have pro-inflammatory effects that may be imvolved in various inflammatory processes. For example, it has been shown that neutrophils are activated by heme leading to increased production of ROS and formation of extracellular traps (38). In acute hemolysis, the vascular endothelium can be exposed to exceptionally high levels of heme (up to 100 µM) released from damaged red blood cells and cause activation of vascular ECs (see above) (39-43). Furthermore, it has also been demonstrated that heme promotes the inflammatory activation of murine macrophages via TLR4 (44) and enhance TLR4-mediated induction of IL-6 (33). Finally, heme has been shown to inhibit the nucleotide-binding domain and leucine-rich repeat pyrin 3 containing inflammasome in murine and human macrophages (45, 46).

Cytotoxic effects of heme

In addition to the prooxidant and pro-inflammatory effects, high concentrations of non-protein-bound heme can also be cytotoxic via the generation of ROS (25). It should be noted, however, that cell cultures of macrophages are significantly more resistant to the toxicity of heme compared to endothelial cells (47). In addition, heme appears to induce various types of cellular death in distinct cell types. Thus, heme has been shown to mediate cell death through necroptosis in peritoneal macrophages (37). The mechanism of necroptosis is independent of the activity of caspases and is mainly mediated by receptor-interacting serine/threonine-protein kinase (RIPK) 1 and RIPK3. Necroptosis is a regulated form of necrotic cell death, which leads to the release of DAMPs and cytokines thus activating immune cells (48). Moreover, it has been shown in ECs that heme leads to both apoptosis and necroptosis (14, 49). In contrast to necroptosis, apoptosis that is dependent on the activity of caspases does not induce the release of pro-inflammatory DAMPs (48).

Anti-inflammatory effects of heme

In addition to the prooxidant, pro-inflammatory and cytotoxic effects, anti-inflammatory effects have also been observed for heme. For example, heme has been shown to polarize primary human macrophages into an anti-inflammatory phenotype, which is referred to as *Mhem*. *Mhem* macrophages are characterized by increased levels of heme oxygenase (HO)-1 and IL-10 expression along with an anti-inflammatory phenotype and are protected against arteriosclerosis (50, 51). Similar findings were also found for liver macrophages in erythrophagocytosis in an *in vivo* disease model of sterile inflammation (52, 53). In a mouse model of myocardial infarction, it was also shown that liposomal encapsulated heme induces a switch of macrophages into an anti-inflammatory phenotype at the site of tissue damage (54). In summary, heme can exert pro-inflammatory, prooxidant and cytotoxic effects, but also anti-inflammatory effects. The major factor to determine the functional role of heme appears to be whether it is available in its free form or to which protein it is bound, respectively (55).

Protective mechanisms against the toxicity of heme

For protection against the toxic effects of free heme, several biological systems are known that control the concentration of heme and modulate or neutralize its cytotoxicity. These are divided into intra- and extracellular HBPs as well as HOs.

Intracellular and HBPs and HOs

Intracellular HBPs

The intracellular labile heme pool plays an important role for *de novo* synthesis of heme proteins and its concentration must be precisely regulated, because excessive intracellular heme may be cytotoxic (see above). Intracellular HBPs not only protect cells from prooxidant damage via binding of excess free heme, but appear to also have functions for the transport of free heme between different organelles and *de novo* synthesis of hemoproteins (24, 29, 56). One such example of an intracellular HBP is glyceraldehyde-3-phosphate dehydrogenase, which is carrier of heme independent of its main function as an enzyme of glycolysis (57). The HBPs p22 HBP and HBP23 have high binding affinities for heme and have been shown that to be up-regulated by heme (58, 59). The heme-degrading enzyme HO-2 also serves as an HBP that plays a role in regulating the concentrations of labile heme (60).

Heme oxygenases (HOs)

HOs have important functions in the protection against heme-induced toxicity and recycling of iron in aerobic organisms. HOs catalyze the reaction of heme to equimolar amounts of carbon monoxide, iron and billiverdin that is subsequently metabolized into billirubin by billiverdin reductase (61). Bilirubin is an antioxidant and protects cells and tissues from lipid peroxidation and biliverdin reductase also exerts antioxidant and anti-inflammatory functions (62, 63).

In mammals, two HO isoforms exist: the inducible isoform HO-1 and the constitutively expressed isoform HO-2. The most important enzyme for heme catabolism is the inducible HO-1 the expression of which is low in most cells and tissue types and only constitutively high in liver and spleen macrophages (64-66). HO-1 is induced by heme and other prooxidant stimuli, including heavy metals, ultraviolet light and hypoxia (67, 68). The expression of HO-1 is regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and the repressor BACH1. Nrf2 is a central antioxidant transcription factor that binds to the antioxidant regulatory element in the promoter region of numerous anti-oxidative genes, including HO-1 (69).

Extracellular HBPs

The non-covalent binding of free heme by HBPs can neutralize the toxic effects of free heme. Various extracellular serum serum proteins exhibit high binding affinity for heme or Hb and can neutralize free heme (25, 47, 70). Free Hb that is released from damaged red blood cells is bound and neutralized by the plasma protein haptoglobin and taken up via the CD163 receptor in tissue macrophages of liver and spleen (36, 71). In pathophysiological situations such as

massive hemolytic anemia that lead to the release of large amounts of Hb, the binding capacity of the serum protein haptoglobin may be saturated and Hb may be oxidized to met-Hb. The most important HBPs that control levels of heme in human circulating blood are hemopexin (Hx) (72, 73) and human serum albumin (HSA) (74, 75) (Table 1).

Table 1: Extracellular heme binding proteins (HBPs) in mammalians

Heme binding protein	Concentration	K_D	Ref.
Hemopexin	0.6 - 1.2 g/L	1×10^{-14}	72, 73
Albumin	35 - 53 g/L	1.2×10^{-8}	74, 75
α1-Microglobulin	0.03 g/L	1×10^{-6}	80
α1-Antitrypsin	1.3 - 2.5 g/L	2×10^{-8}	81

Hx binds heme non-covalently and has the highest binding affinity for heme of all known HBPs (Kd < 10-12 mol/l)(73, 76). Heme-Hx complexes are taken up by hepatocytes and macrophages via the CD91 receptor in the liver (77, 78). HSA is the most abundant protein in human blood with a concentration of 35-53 g/l. The heme-binding affinity of HSA is considerably lower than that of Hx. However, because HSA is present in markedly higher concentrations in human blood than Hx, free heme is initially mainly bound by HSA and then gradually transferred to Hx (76, 79) suggesting that under homeostatic conditions, HSA only temporarily binds heme. However, if the binding capacity of Hx is reduced or saturated (34), HSA can also function as a HBP (55). The mechanisms of cellular uptake of heme-HSA complexes are unclear and only incompletely understood. Further extracellular HBPs are alpha1-microglobulin (80) and alpha1-antitrypsin (A1AT) (81).

A1AT as a HBP

Human A1AT is an acute-phase protein and an important inhibitor of neutrophil elastases that is also referred to as α_1-proteinase inhibitor or serpin A1 (82). It is the most abundant serine

proteinase inhibitor in human plasma encoded by the SERPINA1 gene. In structural analyses it has been demonstrated by various independent groups that A1AT is a single polypeptide chain glycoprotein with one free cysteine residue and three asparagine-linked carbohydrate side-chains (82, 83) (Figure 3). Notably, A1AT has been approved for clinical applications as pharmacological compound in patients with genetic A1AT deficiency and lung emphysema. More recently, it has been demonstrated that A1AT is a HBP with a heme binding affinity that is comparable to that of HSA. Thus, while the functions of A1AT in providing protection against protease activities in neutrophils have been well described in numerous previous studies, the functional roles of this serum protein as a HBP are only ill defined.

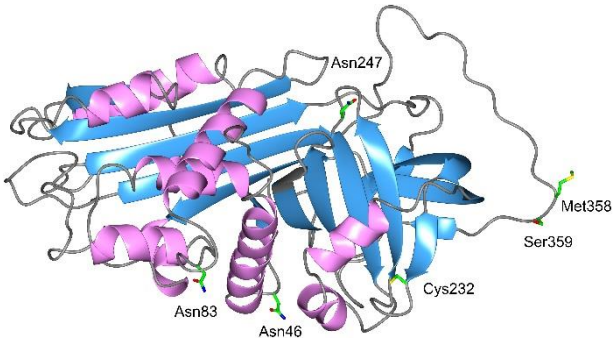

Figure 3 - Structure of A1AT (from Reference 82)

2. HYPOTHESES

- A1AT protects human endothelial cells against heme toxicity via a mechanism that is different to that mediated by the serum HBPs hemopexin and albumin.

- A1AT as a HBP may protect neutrophils from heme-induced oxidative damage and activation.

3. AIMS OF THE STUDY

The specific goals of this thesis were as follows:

- To investigate how A1AT controls heme toxicity in human ECs compared to the established serum HBPs hemopexin and albumin.

- To verify that heme binds A1AT and may neutralize heme-dependent neutrophil activation *in vitro* and *in vivo*.

4. RESEARCH ARTICLES

Research article 1

Alpha1-antitrypsin counteracts heme-induced endothelial cell inflammatory activation, autophagy dysfunction and death

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Author contribution statement

Kukuh Madyaningrana

- conducted all experiments
- analyzed data
- contributed to preparation of figures and writing of the manuscript

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Alpha1-antitrypsin counteracts heme-induced endothelial cell inflammatory activation, autophagy dysfunction and death

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ABSTRACT

Free heme toxicity in the vascular endothelium is critical for the pathogenesis of hemolytic disorders including sickle cell disease. In the current study, it is demonstrated that human alpha1-antitrypsin (A1AT), a serine protease inhibitor with high binding-affinity for heme, rescues endothelial cell (EC) injury caused by free heme. A1AT provided endothelial protection against free heme toxicity via a pathway that differs from human serum albumin and hemopexin, two prototypical heme-binding proteins. A1AT inhibited heme-mediated pro-inflammatory activation and death of ECs, but did not affect the increase in intracellular heme levels and up-regulation of the heme-inducible enzyme heme oxygenase-1. Moreover, A1AT reduced heme-mediated generation of mitochondrial reactive oxygen species. Extracellular free heme led to an increased up-take of A1AT by ECs, which was detected in lysosomes and was found to reduce heme-dependent alkalization of these organelles. Finally, A1AT was able to restore heme-dependent dysfunctional autophagy in ECs. Taken together, our findings show that A1AT rescues ECs from free heme-mediated pro-inflammatory activation, cell death and dysfunctional autophagy. Hence, A1AT therapy may be useful in the treatment of hemolytic disorders such as sickle cell disease.

1. Introduction

The vascular endothelium plays a major role in regulating the homeostasis of blood pressure, the plasma coagulation system, exchange of fluid and macromolecules between blood vessels and tissues as well as quiescence of inflammation [1–3]. In conditions associated with hemolysis and tissue damage such as sickle cell disease or ischemia-reperfusion injury (IRI) the endothelium encounters high levels of free heme released from damaged red blood cells or tissues [4–6]. Free heme can have pro-oxidant, pro-inflammatory and cytotoxic effects in the endothelium if its toxicity is not contained by covalent or non-covalent binding to hemoproteins or heme-binding proteins (HBPs) [7–12]. Therefore, intra- and extracellular levels of free heme are tightly controlled via a complex interplay of various protective mechanisms. For example, intracellular levels of heme are determined through a fine-tuned balance of enzymatic heme synthesis and degradation. Moreover, complex interactions of heme with intra- and extracellular HBPs are critically involved in controlling heme homeostasis [13–17].

A prototypical serum HBP is hemopexin, a glycoprotein with high binding affinity for free heme ($K_D < 10^{-12}$ in humans) that has been

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shown to counter-act free heme toxicity [18–20]. In conditions of major hemolysis serum levels of hemopexin are diminished [21] and, therefore, other serum HBPs such as albumin may become essential in neutralizing free heme toxicity [9]. Although albumin binds heme with lower affinity than hemopexin, its high serum concentrations may compensate a potential deficiency in hemopexin [22]. The latter may at least partially explain protective effects of albumin infusion to individuals with severe sepsis [23] or malaria [24,25].

Human alpha1-antitrypsin (A1AT), an acute-phase glycoprotein and important inhibitor of neutrophil proteases, has been shown to exhibit relatively high heme-binding affinity similar to that of albumin ($K_D \sim 10^{-7}$ in humans) [26]. We have previously reported in a human neutrophil model that A1AT markedly reduced free heme-activating effects such as reactive oxygen species (ROS) production, induction of heme oxygenase (HO)-1, release of interleukin (IL)-8, and inhibited neutrophil adhesion to ECs. In a mouse model of heme-induced acute kidney injury, A1AT was able to lower serum levels of heme and concomitant pro-inflammatory effects [27]. The aim of the current study was to investigate how A1AT controls heme toxicity in human ECs and to compare A1AT effects with two serum HBPs, hemopexin and albumin.

2. Results

2.1. A1AT blocks heme-induced activation of ECs

Since free heme causes inflammatory activation of ECs [28], we treated HUVECs with heme (2.5 μM) in the absence or presence of A1AT (0.5 mg/ml). For a comparison, albumin and hemopexin, two serum HBPs with high binding affinity for heme, as well as γ-globulin, a serum protein with no known binding affinity for heme, were applied under the same experimental conditions. As expected, free heme induced the EC surface expression of VCAM-1 and ICAM-1, and the secretion of cytokine IL-8 in the cell culture supernatant (Fig. 1A–C). The effects of heme were blocked by A1AT, albumin and hemopexin, but not by γ-globulin on both protein (Fig. 1A–C) and mRNA levels (Table 1). Because EC activation by heme is mediated via the NF-κB pathway [29], we hypothesized that treatment with the I-κB inhibitor, Bay11-7082, will block the effects of heme. As demonstrated in Table 2, BAY11-7082 and TAK-242, a small-molecule inhibitor of Toll-Like Receptor (TLR) 4, markedly inhibited heme-induced mRNA expression of VCAM-1, ICAM-1 and IL-8 (Table 2). Furthermore, Western blot analysis revealed that A1AT, albumin and hemopexin as well as BAY11-7082 and TAK-242, inhibited heme-induced phosphorylation of the NF-κB subunit p65 (Figs. S1A–B). These results support the notion that inhibition of heme-induced EC activation involves the NF-κB pathway.

2.2. A1AT prevents heme-caused EC death

Heme can cause EC death via apoptosis and necroptosis [30]. As determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays, prolonged exposure of ECs to heme (18 h) induced cytotoxicity, which was prevented by A1AT, albumin and hemopexin (Fig. 2A and B), but not by γ-globulin or BAY11-7082 or TAK242 (Fig. S1B). These latter findings implied that heme-induced NF-κB activation and/or TLR4 signaling are not involved in the observed cytotoxic effects of heme (Fig. S1C). The MTT assay, which measures cellular metabolic activity, was also applied for determining cell viability/cytotoxicity, but the results were in line with LDH assays (not shown). To further explore the mechanism/s of heme-induced cytotoxicity in ECs various small molecule inhibitors of cell death were employed. The apoptosis inhibitor Z-VAD-fmk, the necroptosis inhibitor necrostatin-1, but not the necrosis inhibitor IM-54 or the ferroptosis inhibitor ferrostatin-1, attenuated heme-induced cytotoxicity (Fig. 2C). Caspase activity assays further confirmed that treatment of ECs with heme alone, but not with heme plus A1AT, caused the activation of caspases 3 and 7 (Fig. 2D). The ability of A1AT to abolish free heme toxicity was also observed in aortic, pulmonary microvascular, and dermal microvascular ECs (Fig. S2). Altogether, the findings show that A1AT inhibits heme-mediated EC death.

2.3. Protective effects of A1AT are independent of HO-1

Albumin and hemopexin can prevent heme toxicity via limiting its entry into ECs [31]. To explore the mechanism of A1AT-mediated EC protection we treated cells with heme or heme-A1AT and intracellular levels of labile heme were determined with an apo-horseradish peroxidase (HRP)-based assay. In contrast to albumin and hemopexin, A1AT did not block the up-take of heme by ECs (Fig. 3A) and the up-regulation of mRNA and protein levels of the inducible heme-degrading enzyme, HO-1 (Fig. 3B and C). As expected, γ-globulin showed no effect on HO-1 induction (Fig. 3C). Because the up-regulation of HO-1 gene expression is considered a cytoprotective mechanism against free heme toxicity [32], we examined whether HO-1 expression and HO activity may contribute to heme-neutralizing effects of A1AT. Hence, before adding heme alone or heme with A1AT, ECs were pretreated with the HO inhibitor tin (IV)-mesoporphyrin IX (SnMPIX). As demonstrated in Fig. 3D, pretreatment with this compound did not affect A1AT-dependent cytoprotection against heme toxicity. Similarly, a transient knock-down of HO-1 by transfection with HO-1 small interfering RNA (siRNA)(Fig. 3E) did not alter A1AT-dependent protection of ECs (Fig. 3F). Thus, A1AT cytoprotection against heme-mediated EC injury is mediated via a mechanism that differs from that of albumin and hemopexin.

2.4. A1AT blocks heme-induced mitochondrial ROS (mROS) production

Mitochondrial dysfunction is associated with apoptosis and necroptosis [33]. Therefore, we evaluated the potential role of mitochondria in heme-mediated EC death. Staining with the mROS indicator MitoSOX [34] revealed that heme-mediated up-regulation of mROS is blocked by A1AT, as similarly observed for the mitochondria-specific superoxide scavenger mito-TEMPO [35] (Fig. 4A). Among the compounds applied as positive controls for mROS, the mitochondrial respiratory chain complex III inhibitor antimycin A, but not the complex I inhibitor rotenone, induced the production of mROS (Fig. 4A). To further determine the potential role of heme toxicity via mROS, ECs were treated with heme in the presence of mito-TEMPO. Whereas mito-TEMPO significantly blocked heme-induced cell death (Fig. 4B), rotenone, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and oligomycin A had no or only a minor effect on heme-induced cell death (Fig. 4B). Moreover, treatment with antimycin-A, which induced mROS in this model potentiated heme-induced toxicity. These findings indicate that generation of mROS, but not inhibition of the respiratory chain, contributes to heme-mediated cell death. These data indicate that heme-dependent cytotoxicity in ECs involves an increase of mROS that is blocked by A1AT.

2.5. Intracellular levels of A1AT are increased in the presence of heme

We next asked whether A1AT-mediated cytoprotection of ECs against heme toxicity might be related to the enhanced A1AT up-take by these cells. As determined by confocal microscopy, intracellular levels of A1AT were markedly higher in cells treated with A1AT plus heme in comparison to cells treated with A1AT alone. Notably, A1AT was not detectable in untreated or heme-treated ECs (Fig. 5A). Similarly, intracellular A1AT levels increased in ECs cultured with native human plasma in the presence of heme, further confirming that heme enhances the up-take of A1AT (Fig. 5B). A1AT is known to be taken up by ECs via endocytosis that is a lysosome delivery pathway [36]. Accordingly, A1AT was found to be co-stained with LysoTracker Red indicating lysosomal localization of this protein (Fig. 5C). Altogether, the data show that free heme increases the up-take of A1AT by ECs and localizes

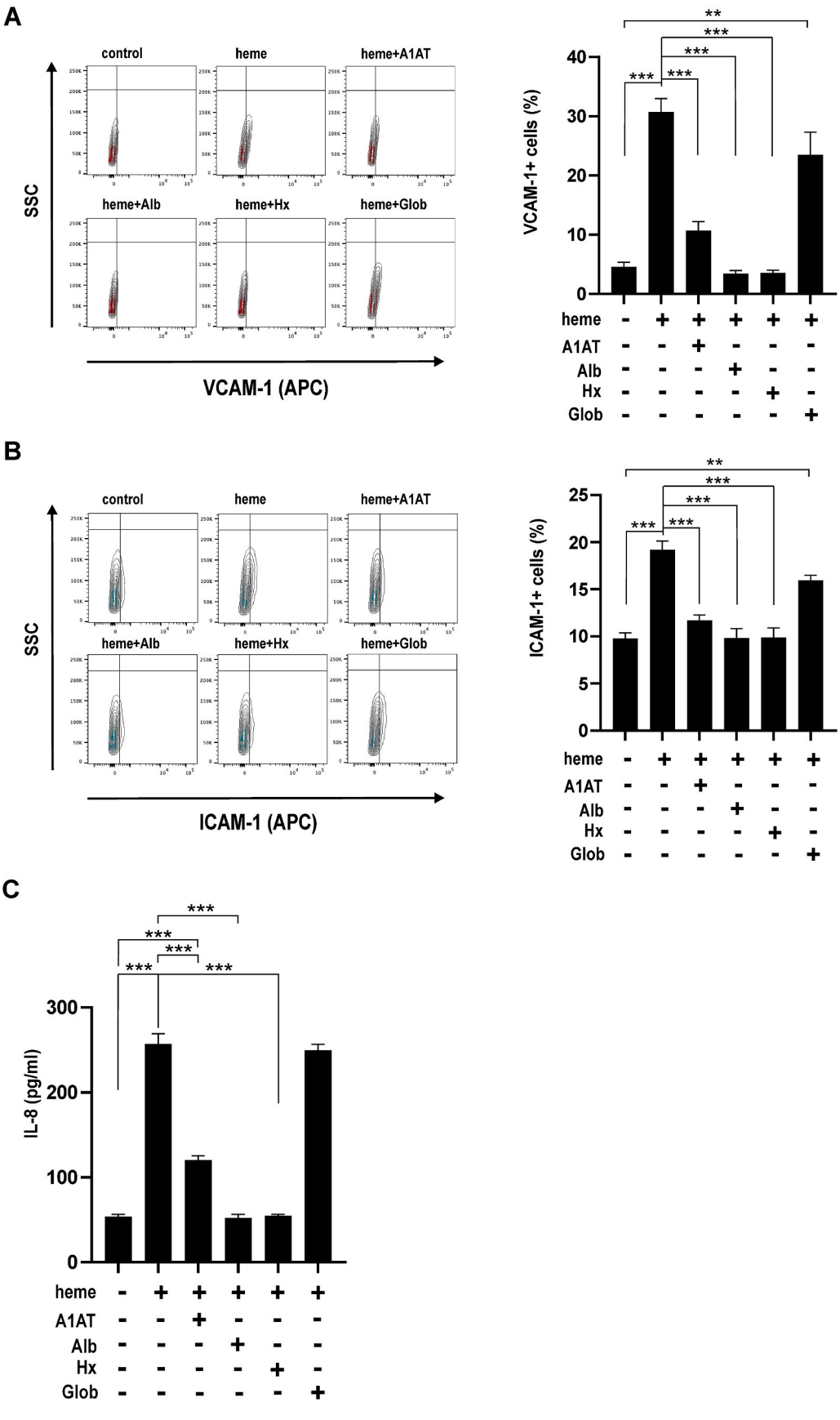

Fig. 1. A1AT attenuates heme-induced EC activation. (A–C) HUVECs were treated with heme (2.5 μM) in the presence of A1AT (0.5 mg/ml), albumin (0.5 mg/ml), Hx (0.5 mg/ml) or γ-globulin (0.5 mg/ml) for 12 h. (A–B) The cells were probed with antibodies against VCAM-1 and ICAM-1 and analyzed by flow cytometry. A representative flow cytometry contour plot is shown (left) and the mean % positive population of VCAM-1 and ICAM-1 is represented as a bar graph. (C) The cell culture supernatant was analyzed for the levels of secreted IL-8 by ELISA. Results shown are means ± SEM of at least three independent experiments. One-way ANOVA with Tukey's post-hoc analysis was performed for statistical analysis, $^{**}p < 0.01$, $^{***}p < 0.001$. Alb, albumin; Hx, hemopexin; Glob, γ-globulin.

Table 1
A1AT blocks heme-induced EC activation.

Treatment	VCAM-1/HPRT	ICAM-1/HPRT	IL-8/HPRT
Control	1	1	1
A1AT	1.032 ± 0.064	1.14 ± 0.21	0.83 ± 0.34
Hx	1.46 ± 0.46	1.34 ± 0.52	1.1 ± 0.07
HSA	0.69 ± 0.14	1.24 ± 0.67	0.99 ± 0.78
Heme	20.2 ± 7.21***	29.75 ± 21.35**	67.87 ± 40.38**
Heme + A1AT	0.58 ± 0.20	1.38 ± 0.91	1.87 ± 1.66
Heme + Hx	0.46 ± 0.10	0.99 ± 0.09	1.06 ± 0.10
Heme + HSA	0.78 ± 0.12	0.91 ± 0.50	0.82 ± 0.56

HUVECs were treated with heme (2.5 μM) in the presence or absence of the indicated serum proteins (0.5 mg/ml) for 9 h. Expression of VCAM1, ICAM1 and IL-8 was analyzed by real time RT-PCR and normalized to the expression of hypoxanthine phosphoribosyltransferase 1 (HPRT). Values are represented as mean ± SD fold induction in relation to control unstimulated cells from three independent experiments. One-way ANOVA with Tukey's post-hoc analysis was performed for statistical analyses; VCAM-1 (Heme vs others ***$p < 0.001$), ICAM-1(Heme vs others **$p < 0.01$) and IL-8 (Heme vs others **$p < 0.01$).

Table 2
EC activation is mediated via NF-κB and TLR4 signaling.

Treatment	VCAM-1/HPRT	ICAM-1/HPRT
Control	1	1
Bay-11082	0.59 ± 0.2	0.72 ± 0.02
TAK-242	0.87 ± 0.46	0.84 ± 0.52
Heme	15.58 ± 10.6**	49.88 ± 27.07**
Heme + Bay-11082	2.69 ± 1.05	8.76 ± 6.81
Heme + TAK-242	7.8 ± 0.20	26.53 ± 0.91

HUVECs were treated with heme (2.5 μM) in the presence or absence of the NF-κB signaling inhibitor, Bay-11082 (5 μM) or the TLR4 signaling inhibitor, TAK-242 (1 μM) for 9 h. Expression of VCAM1 and ICAM1 was analyzed by real time RT-PCR and normalized to the expression of HPRT. Values are represented as mean ± SD fold induction in relation to control unstimulated cells. One-way ANOVA with Tukey's post-hoc analysis was performed for statistical analyses; VCAM-1 (Heme vs others **$p < 0.01$) and ICAM-1 (Heme vs others **$p < 0.01$).

in lysosomes.

2.6. A1AT blocks heme-mediated lysosomal alkalization

To further investigate the role of A1AT in ECs, we performed additional experiments with acridine orange (AO), a lysomotropic dye. Treatment with heme alone caused a marked decrease in lysosomal AO staining, but this was not observed in the presence of A1AT (Fig. 6A). The decrease in AO fluorescence intensity by heme and its recovery by A1AT was also determined and quantified by flow cytometry (Fig. 6B). To corroborate that heme induces lysosomal alkalization ECs were stained with LysoSensor Green, a pH-dependent indicator probe. Stimulation of ECs with heme caused a marked decrease in the intensity of LysoSensor Green that was counter-acted by A1AT (Fig. 6C). In an attempt to re-acidify lysosomes, ECs were pretreated with forskolin, an inducer of cAMP [37]. Heme-mediated decrease in fluorescence intensity of LysoSensor Green was partially reversed by treatment with forskolin (Fig. 6D). Moreover, MTT assays revealed that forskolin significantly improves the viability of heme-treated ECs (Fig. 6E). Collectively, these findings indicate that heme causes lysosomal alkalization in ECs that is prevented by A1AT.

2.7. A1AT rescues heme-dependent dysfunction of autophagy in ECs

Lysosomes play a key role in autophagy, a process that is necessary for maintaining cellular homeostasis [38]. A significant increase in levels of the autophagy marker protein microtubule light chain (LC)3BII was observed in heme-treated ECs (Fig. 7A). In parallel, up-regulation of p62, also known as SQSTM1, was observed indicating an inhibition of autophagosome degradation (Fig. 7A). Heme-induced up-regulation of LC3BII and p62 was suppressed by A1AT (Fig. 7A). The addition of the lysosomal inhibitor chloroquine to non-treated or A1AT-treated ECs, and that of rapamycin, a positive control for autophagy, led to an increase in LC3BII indicating a normal autophagic flux (Fig. 7B). By contrast, chloroquine added to heme-treated cells failed to increase LC3BII levels indicating diminished autophagic flux (Fig. 7B). Moreover, the staining with cytoID revealed an accumulation of autophagosomes in heme-treated ECs, but not in heme plus A1AT-treated cells (Fig. 7C). Finally, we asked if distinct alterations of autophagy-associated pathways may affect cytoprotection against heme toxicity by A1AT. To this end, two lysosomal acidification blockers, concanamycin-A and bafilomycin-A1, as well as two autophagosome formation blockers, wortmannin (a phosphatidyl-inositol-3 kinase (PI3K) inhibitor) and MRT68921 (an Unc-51 like autophagy activating kinase (ULK) inhibitor) were applied. Inhibitors of lysosomal acidification, but not those of autophagosome formation, markedly enhanced cell death (Fig. 7D and E). When combined with lysosomal inhibitors, heme exerted an additive cytotoxic effect, which was not observed with two other inhibitors of autophagy (Fig. 7D and E). Notably, cytoprotective effects of A1AT against heme toxicity were preserved in the presence of autophagy inhibitors but partially lost in the presence of the lysosomal inhibitors (Fig. 7D and E) suggesting that reversal of lysosomal alkalization rather than direct correction of autophagy is responsible for inhibition of heme-induced cell death.

3. Discussion

Vascular ECs control blood vessel homeostasis and play a critical role in the pathogenesis of inflammatory disorders [1]. In various clinical conditions such as sickle cell disease, sepsis or IRI, high levels of extracellular free heme can arise from hemolysis or tissue damage and cause endothelial injury, dysfunction or death via pro-oxidant, pro-inflammatory and cytotoxic effects [4,5,9]. In the current study, we show that human A1AT, an acute phase protein with high binding affinity for heme ($K_D \sim 10^{-7}$ M) [26], protects against free heme toxicity in ECs via a mechanism different from that of albumin and hemopexin, two major serum HBPs. In contrast to these latter proteins, A1AT has protective effects without blocking the cellular up-take of heme. Furthermore, we provide experimental evidence that A1AT-dependent protection against heme in ECs is mediated via pathways that block EC activation, death and dysfunction of autophagy.

Exposure to low concentrations of heme can cause activation of ECs with increased expression of adhesion molecules and cytokine production [28] whereas higher concentrations of heme can cause EC death [30,39]. We show that A1AT counteracts heme-induced EC activation and death (Figs. 1 and 2). According to previous reports, heme scavenger proteins such as albumin and hemopexin protect the endothelium via blocking the cellular uptake of heme [31]. By contrast, A1AT does not inhibit the up-take of heme by ECs and heme-mediated up-regulation of HO-1, an inducible heme-degrading enzyme (Fig. 3). Because HO-1 overexpression is known to be protective in the vascular endothelium [40], we assumed that the up-regulation of HO-1 by heme and A1AT may be critical for A1AT-dependent protection against heme toxicity. However, blockage of HO enzyme activity or silencing of HO-1 gene expression, did not affect A1AT's ability to inhibit the toxicity of heme (Fig. 3). Therefore, the protective effects of A1AT appear to be independent of heme degradation by HO-1.

Laser confocal microscopy assays revealed that extracellular free heme strongly enhanced A1AT uptake by ECs (Fig. 5A). It is also important to note that heme increased endothelial up-take of purified A1AT (Fig. 5A) and also that of A1AT from whole human plasma (Fig. 5B). ECs lack the ability to synthesize their own pool of intracellular A1AT and are entirely dependent on circulating levels of A1AT. Previous studies have shown that A1AT is internalized by ECs in a time-, dose- and conformation-dependent manner. Moreover, internalization

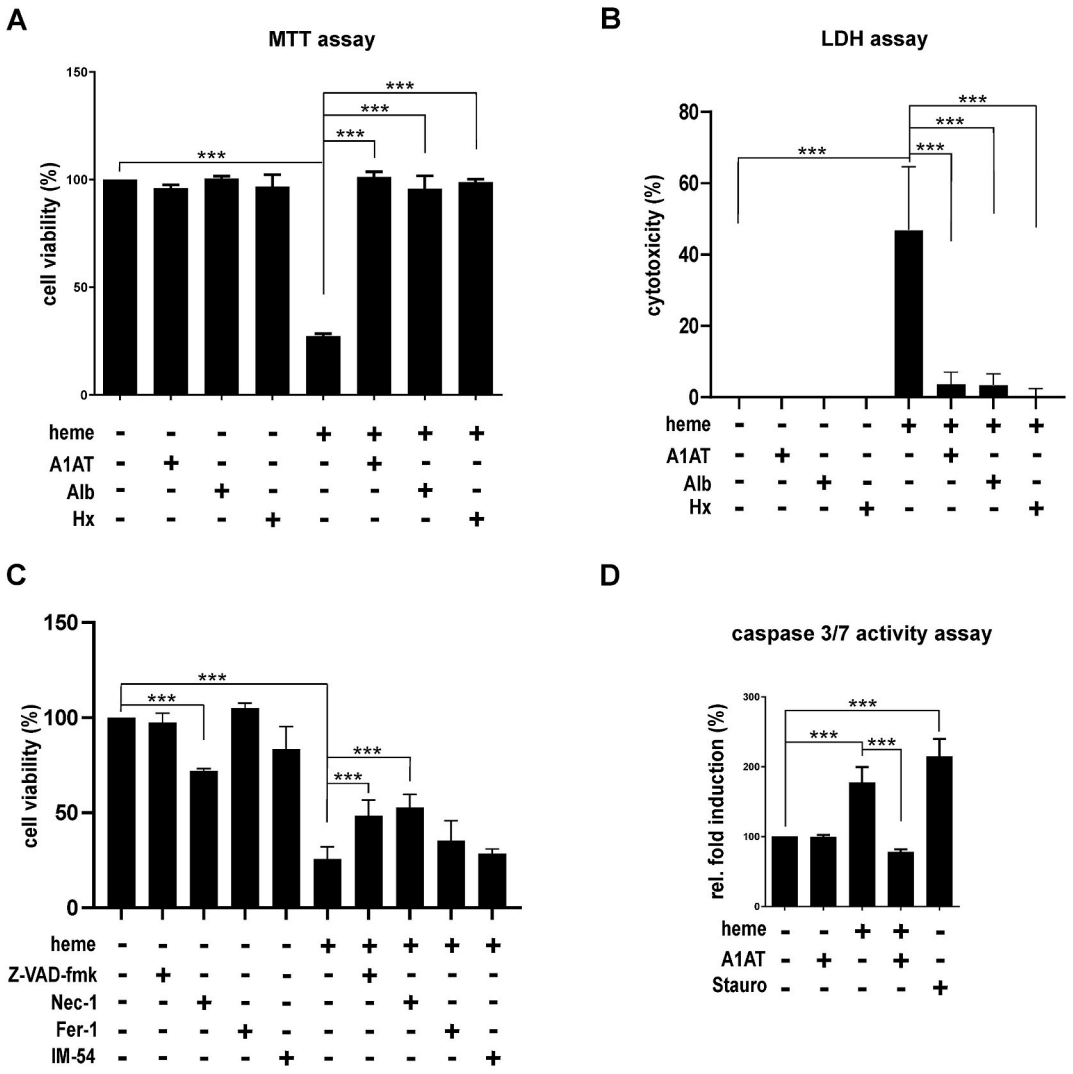

Fig. 2. A1AT blocks heme-induced EC death. The viability of HUVECs treated with heme (2.5 μM) in the presence of A1AT (0.5 mg/ml), albumin (Alb) (0.5 mg/ml) or hemopexin (Hx) (0.5 mg/ml) for 18 h was assessed by (A) MTT assay and LDH assay (B). (C) The viablility of HUVECs stimulated with heme (2.5 μM) in the presence of z-VAD-fmk (50 μM), necrostatin-1 (nec-1) (50 μM), ferrostatin-1 (fer-1) (10 μM) or IM-54 (10 μM) was assessed by MTT assay (D) HUVECs treated for 12 h were subjected to caspase 3/7 activity measurements as detailed in Materials and Methods. Staurosporine (0.75 μM), an inducer of apoptosis was used as a positive control. Results shown are means ± SEM of at least three independent experiments. One-way ANOVA with Tukey's post-hoc analysis was performed for statistical analysis, ***$p < 0.001$. Alb, albumin; Hx, hemopexin.

of A1AT has been demonstrated to be mediated via both clathrin- and caveolae-mediated endocytosis [41,42]. The increased up-take of A1AT by ECs in the presence of extracellular free heme (Fig. 5) supports a protective nature of this event and the mechanisms of how heme mediates the intracellular up-take of A1AT in ECs warrant further investigation.

More detailed microscopy studies indicated that A1AT is localized in lysosomes (Fig. 5). In addition, heme caused lysosomal alkalization via a yet unknown mechanism, which was markedly inhibited in the presence of A1AT (Figs. 5 and 6). Previously, apolipoprotein D has been demonstrated to reduce lysosomal permeabilization and to stabilize the pH of these organelles resulting in a significant improvement of cell viability [43]. Therefore, it is tempting to speculate that the effect of A1AT might be similar to that of apolipoprotein D. Further studies are required to find out if A1AT's property to maintain lysosomal alkalization is associated with binding of free heme or direct lysosomal interactions.

Lysosomal alkalization can lead to alterations of the autophagy pathway [44,45] that is important for cell homeostasis [46]. Therefore, diminution or blockage of autophagy can result in intracellular accumulation of misfolded proteins and dysfunctional organelles [47]. Indeed, heme-induced lysosomal alkalization appeared to coincide with impaired autophagy in ECs (Fig. 7) as observed by the decrease in autophagy flux (Fig. 7) [48], which was rescued in the presence of A1AT. It should be noted that earlier findings on the effects of free heme and autophagy are contradictory. Heme has been shown to block autophagy flux in cardiomyocytes and to induce cytotoxicity [49]. On the other hand, heme has been shown to induce autophagy, which was then considered a protective mechanism in bovine aortic ECs [50].

Free heme concentrations in human plasma may range between 2 and 5 μM, as determined with an antibody-based assay [51]. Thus, heme concentrations used in our study are relatively low (2.5 μM) as compared to those in previous reports (10 μM and higher). These latter differences may, at least in part, explain discrepancies between studies on the heme effects in autophagy. Similar to heme, A1AT has also been reported to play contrasting roles in the regulation of autophagy. Endogenous A1AT has previously been shown to act as a negative regulator of autophagy in cultured breast cancer cells [52]. By contrast, in human macrophages A1AT has been reported to enhance autophagy upon mycobacterial infection [53]. The mechanistic details on how

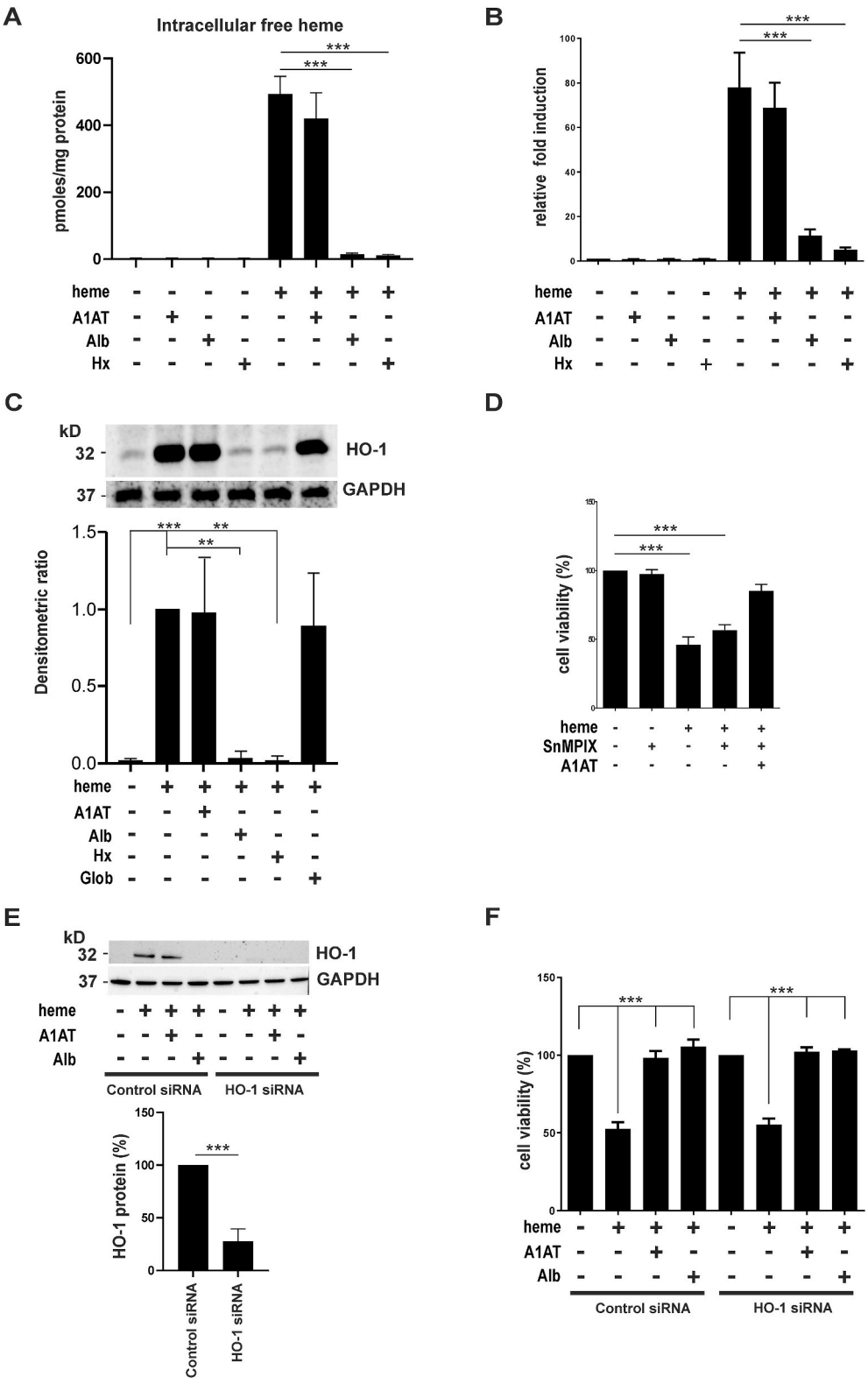

Fig. 3. A1AT mediated protection against heme toxicity is independent of HO-1. (A–C) HUVECs were treated as indicated for 4 h. (A) Intracellular free heme was measured using an apo-HRP assay as detailed in Materials and Methods. (B) RNA isolated was analyzed by real-time RT-PCR for the expression of HO-1 and normalized to the expression of HPRT. (C) A representative Western blot of total cell lysates probed with antibodies against HO-1 and GAPDH (Top) and a bar graph that represents the mean of densitometric quantification normalized to GAPDH from three independent experiments is shown as fold change in relation to heme-induced HO-1 expression (bottom). (D) HUVECs were pretreated with SnMPIX (10 μM) for 1 h before adding heme (2.5 μM) and A1AT (0.5 mg/ml), as indicated, for another 18 h. Cell viability was assessed by MTT assay. (E) A representative Western blot of total cell lysates probed with the antibodies against HO-1 and GAPDH isolated from cells transfected with the indicated siRNA for 48 h followed by 4 h of treatment with heme (2.5 μM) (Top) and a comparison of HO-1 densitometric quantification in heme-treated control and HO-1 siRNA transfected lanes is shown as a bar graph (Bottom). The values are normalized to GAPDH and represents the mean of at least three independent experiments (F) HUVECs transfected with the indicated siRNAs were treated with heme (2.5 μM) and A1AT (0.5 mg/ml) and subjected to MTT assays as described in Materials and Methods. Results shown are mean of at least three independent experiments. Student's t-test or one-way ANOVA with Tukey's post-hoc analysis was performed for statistical analyses, $^{**}p < 0.01$, $^{***}p < 0.001$. Alb, albumin; Hx, hemopexin; Glob, γ-globulin.

A1AT corrects heme-induced autophagy needs to be explored further.

The current results allow to conclude that A1AT interacts with various heme-dependent pro-oxidant and pro-inflammatory pathways that cause EC activation, death and impairment of autophagy all of which may not necessarily be linked with each other. It is conceivable that A1AT can prevent EC activation via inhibiting NF-κB signaling whereas EC death is blocked by reduction of mROS production, caspase activation and lysosomal alkalization. Although most recognized for its inhibitory role on neutrophil elastase and proteinase 3, A1AT also exhibits a number of other anti-inflammatory and immunomodulatory cell-type specific functions [54]. For example, A1AT has been shown to inhibit caspase activity and to prevent EC death [55] which is in line with findings of the current report. Increased intracellular levels of A1AT in the presence of heme (Fig. 5) could be a protective mechanism

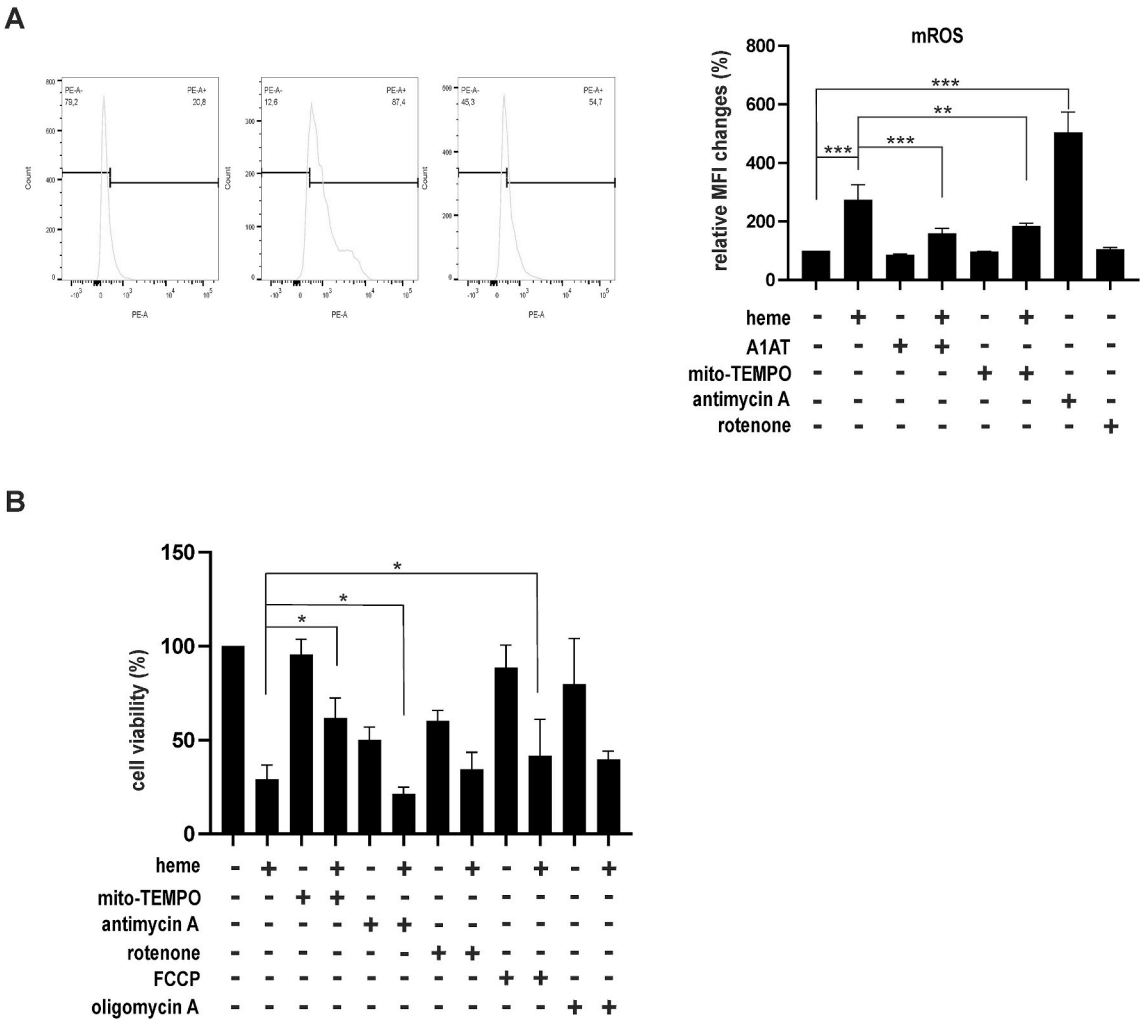

Fig. 4. A1AT blocks heme-induced mitochondrial ROS production. (A–B) HUVECs were treated with heme (2.5 μM) in the presence of A1AT (0.5 mg/ml), mito-TEMPO (75 μM), antimycin-A (4 μM), rotenone (1 μM), FCCP (1 μM) and oligomycin A (2 μM) for 3 h (A) or 18 h (B), as indicated. (A) A representative histogram of mitochondrial ROS levels assessed by flow cytometry using MitoSox (left) and the percentage changes in relative mean fluorescence intensity (MFI) shown as a bar graph. (B) Cell viability was assessed by MTT assays. Values represent mean ± SEM of at least three independent experiments. One-way ANOVA with Tukey's post-hoc analysis was performed for statistical analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

that might be of particular relevance in pathophysiological situations, in which the heme-binding capacities of albumin and hemopexin are exhausted or high levels of free heme occur as a consequence of tissue damage by IRI or other pathophysiological conditions [9]. Recent studies have shown that A1AT has protective effects against renal IRI by inhibiting pathways of inflammation and apoptosis [56]. It is conceivable that these effects of A1AT may be related to its free heme-neutralizing properties.

Hence, we provide novel aspects of how the acute-phase protein A1AT, a recently identified HBP, can exert protection of ECs against the toxicity of extracellular free heme. Our findings expand knowledge on some as yet unknown functions of A1AT in pathophysiological settings, in which large amounts of free heme are released. The ability of A1AT to counter-act clinically relevant toxicity of free heme in the endothelium may help to develop novel therapeutic applications of A1AT for the treatment of disorders associated with hemolysis or tissue damage.

4. Materials and methods

4.1. Reagents

A1AT was purchased from CSL Behring (Kankakee, IL, USA), human-serum albumin (HSA) from Octapharma (Lachen, Switzerland) and human hemopexin from Athens research and technology (Athens, GA, USA). Hemin and SnMPIX were obtained from Frontiers Scientific (Logan, UT, USA). IM-54 and Z-VAD-fmk were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All other materials and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Tocris Bioscience (Bristol, UK), unless otherwise indicated.

4.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) (3 independent donors), human aortic endothelial cells (HAoECs), human pulmonary microvascular endothelial cells (HPMVECs) and human dermal microvascular endothelial cells (HDMVECs) (2 independent donors, respectively) were purchased from PromoCell (Heidelberg, Germany). HUVEC and HAoEC were cultivated in endothelial cell growth medium (PromoCell) with supplements including 2%/5% heat-inactivated fetal calf serum, respectively and used in passages 4 to 7. HPMVEC and HDMVEC were cultivated in endothelial cell growth medium MV (PromoCell) with supplements and 5% heat-inactivated fetal calf serum and used in passages 4 to 7. Cells were maintained at 37 °C, 5% CO_2 and 100% humidity until confluence. For the experiments, ECs were plated in 12 well plates at a seeding density of 1.5×10^5 cells/well or in 24 well plates at a seeding density of 8×10^4 cells/well. Except for immunofluorescence

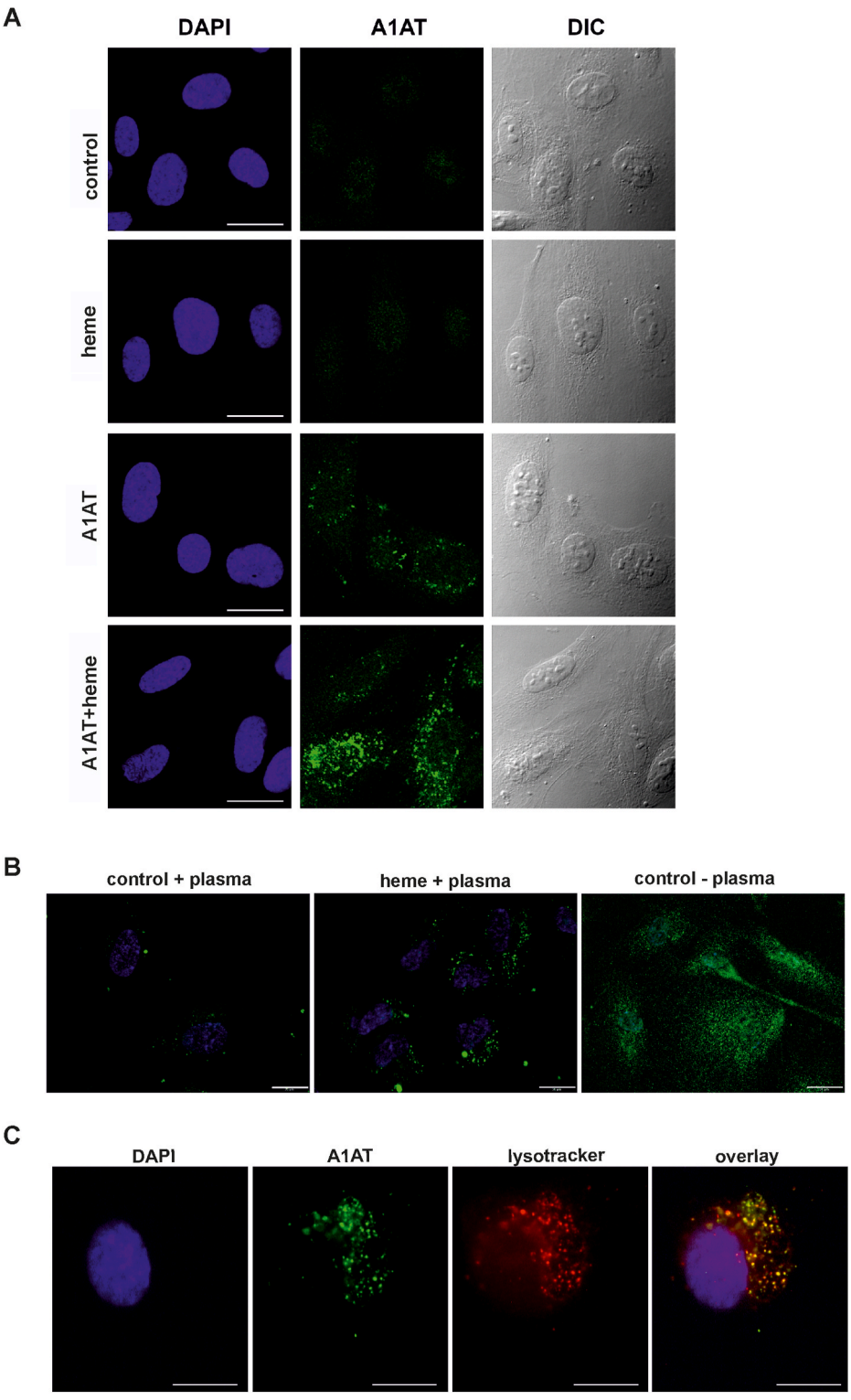

Fig. 5. Heme-mediated up-take of extracellular A1AT in ECs. (A–B) HUVECs were treated for 4 h with heme (2.5 μM) and A1AT (0.5 mg/ml) as indicated. (A) A representative confocal microscopy image of cells stained with an antibody against A1AT is shown. Nuclei were stained with 4′,6-diamidin-2-phenylindol (DAPI, 1 μg/ml). (B) A representative fluorescence microscopy image of HUVECs treated with heme (2.5 μM) in medium supplemented with native human plasma (10%) for 4 h and stained with DAPI and an antibody against A1AT (Bar = 20 μm) is shown. (C) A representative fluorescence microscopy image of cells co-stained with LysoTracker Red, DAPI and an antibody against A1AT (Bar = 20 μm) is shown. Co-localization of A1AT and lysosomes were calculated using Image J (Pearson's r value = 0.79 ± 0.075). All images are representatives of at least three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

studies, cells were used for the experiment when it reached a desired confluency of 75–80%. The final molar concentration of serum proteins A1AT, hemopexin and albumin used for experiments were 9.62 μM, 8.47 μM and 7.52 μM respectively, corresponding to a concentration of 0.5 mg/ml in either 1 ml of medium/well for 12 well plates or 0.5 ml/well for 24 well plates.

4.3. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was performed as described previously [57]. Briefly, the medium was replaced with fresh serum free medium supplemented with MTT (0.5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C for 2 h. The resulting formazan crystals were dissolved in DMSO and absorbance read at 570 nm using a spectrophotometer (Biotek). The cell viability was calculated by the following formula: A570 of treated

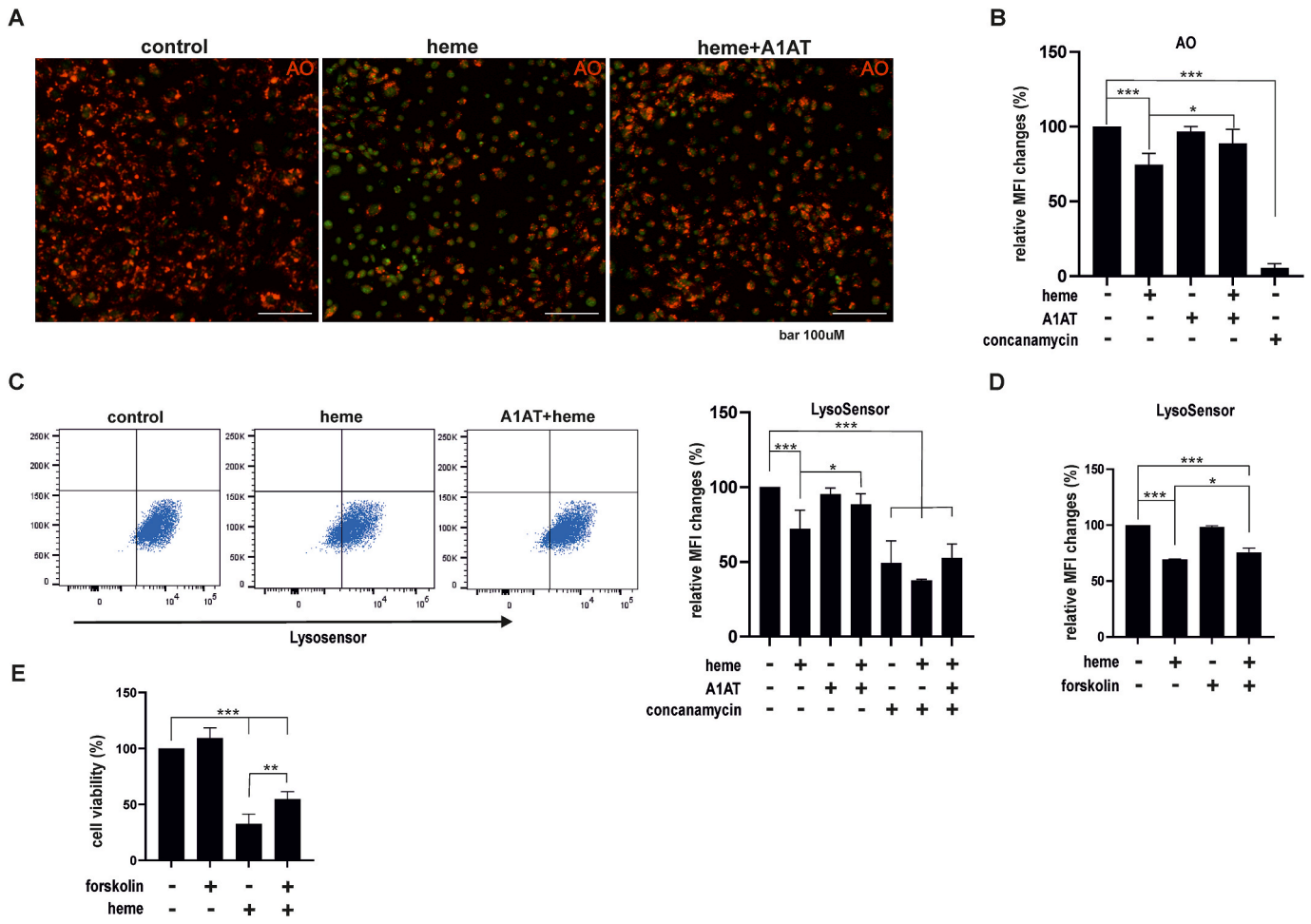

Fig. 6. Heme-induced lysosomal alkalization is reduced by A1AT. (A–E) HUVECs were treated with heme (2.5 μM) in the presence A1AT (0.5 mg/ml) or concanamycin (0.1 μM) as indicated for 4 h. (A) A representative fluorescence image of acridine orange (AO) staining as mentioned in Materials and Methods (Bar = 100 μm) is shown. (B) Relative changes in AO staining of ECs as determined by flow cytometry are shown in % of MFI. (C) A representative dot plot of ECs stained with LysoSensor Green assessed by flow cytometry (left) and relative changes (% of MFI) (right) are shown. (D) HUVECs were pretreated with forskolin (10 μM) for 1 h before addition of heme for another 4 h (2.5 μM), as indicated. Cells were stained with lysosensor and analyzed by flow cytometry. (E) HUVECs were pre-treated with forskolin (10 μM) for 1 h before stimulation with heme (2.5 μM) for an additional 18 h. Cell viability was assessed by MTT assays. Values represent mean ± SEM of at least three independent experiments. One-way ANOVA with Tukey's post-hoc analysis was performed for statistical analyses, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cells/A570 of non-treated cells x 100.

4.4. Lactate dehydrogenase (LDH) assay

The amount of LDH released in the cell culture supernatant was determined using Cytotoxicity Detection Kit Plus (Roche, Basel, Switzerland) according to the manufacturer's protocol.

4.5. Western blot

Western blotting was performed with primary antibodies against HO-1 (1:1,000, Enzo Life Sciences, Farmingdale, NY, USA), phospho-p65 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), LC3B (1:1,000, Sigma-Aldrich, St. Louis, MO, USA), β-actin (1:5,000, Sigma-Aldrich) and secondary horse radish-conjugated goat anti-rabbit or rabbit anti-mouse (1:4,000, Agilent Dako, Santa Clara, CA, USA), as previously described. Signals were visualized by Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and quantified with a ChemiDoc MP Imaging System (Bio-Rad). Images were processed using Corel Draw Graphic Suite ×5 Software (Corel Corporation, Ottawa, Canada).

4.6. Analysis of mRNA expression

RNA isolation was performed using an RNeasy mini kit (Qiagen GmbH, Hilden, Germany). Synthesis of cDNA was performed by employing High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Inventoried primers for quantification of mRNA levels of VCAM-1, ICAM-1, IL-8, HO-1 and hypoxanthine phosphoribosyltransferase 1 (HPRT) were purchased from Applied Biosystems. Amplification was performed with TaqMan Gene Expression Master Mix on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Thermal cycling was performed at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. HPRT was used as a control for normalization of cDNA values. The ΔΔCT method was used to semi-quantify mRNA levels.

4.7. Immunofluorescence

Cells plated on coverslips in 24-well plates were subjected to an indirect immunofluorescence staining protocol as previously described [58]. Briefly, at the end of the experiment cells were washed with PBS and fixed using 4% paraformaldehyde and 2% saccharose for 20 min, following permeabilization using 1% glycine containing 0.02% Triton

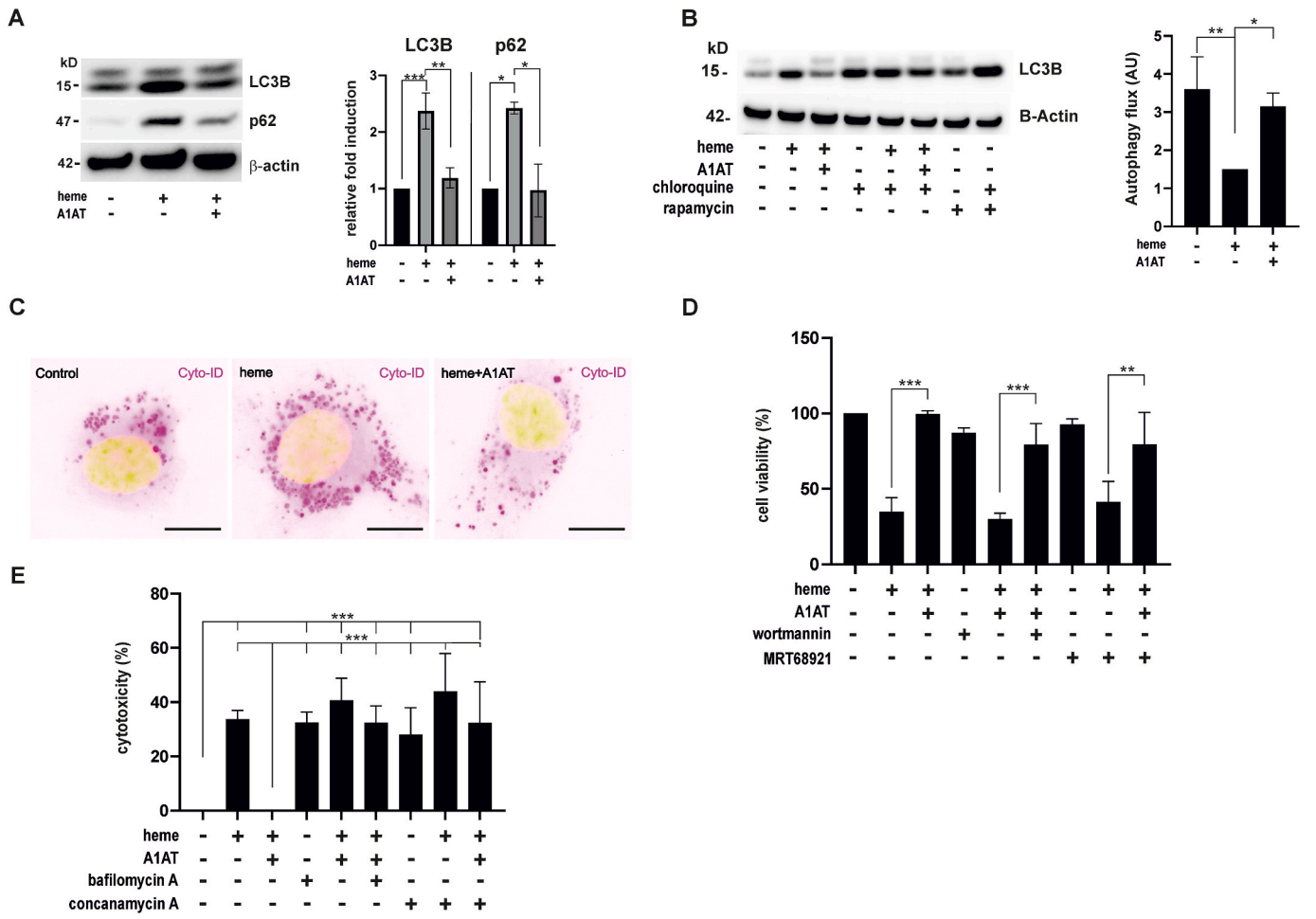

Fig. 7. A1AT corrects heme-induced autophagy dysfunction. (A–C) HUVECs were treated with heme (2.5 μM) in the presence of A1AT (0.5 mg/ml) and chloroquine (0.1 mM), rapamycin (500 nM) for 8 h, as indicated. (A–B) Representative Western blots of total cell lysates using antibodies against LC3BI/II, p62 and β-actin. (C) A representative image of cyto-ID staining (bar = 20 μm) is shown. (D–E) HUVECs pretreated with the lysosomal inhibitors bafilomycin A (25 nM), concanamycin A (0.1 μM) or the autophagy inhibitors wortmannin (1 μM), MRT68921 (1 μM) for 1 h before heme treatment for another 18 h. Cell viability was determined with MTT and LDH assays, respectively. Values represent mean ± SEM of at least three independent experiment). One-way ANOVA with Tukey's post-hoc analysis was performed for statistical analyses, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

X-100 for an additional 10 min. Next, cells were washed using PBS containing 0.05% Tween. Non-specific binding sites were blocked with 1% bovine serum albumin prepared in washing buffer for 30 min at room temperature. The primary antibody against A1AT was incubated overnight at 4 °C followed by incubation with an Alexa Fluor 488-conjugated secondary antibody at 1:500 dilution (Invitrogen). Nuclei were visualized with 1 μM Hoechst 33,342 for 5 min at RT embedded in Mowiol 4–88. All samples were inspected with an Olympus IX81 microscope equipped with a DR4 camera. Digital pictures were processed with image J software. For confocal microscopy images were acquired using confocal laser microscope Olympus FluorView 1000 equipped with a 60× oil immersion objective in sequential mode.

4.8. Apo-horseradish peroxidase (HRP) assay to determine intracellular heme levels

Intracellular levels of free heme were determined with a method based on the reconstitution of apo-HRP as described previously [59]. The assay works on the principle that amount of free transferable heme is directly proportional to the amount of active form of HRP (holo-HRP) formed when an inactive form (non-heme containing or apo-HRP) is supplied. A heme standard was used to determine the concentration of heme in the cell lysates based on the holo-HRP activity. 2–40 μL of protein cell lysates from each sample were added to the required amount of HBSS to get an initial volume of 50 μL. From a 10 nM heme stock solution, a heme standard curve with concentrations of 0.25, 0.5, 1, 1.5, 2.0 and 2.5 nM for a reaction volume of 100 μL was calculated and prepared initially in 50 μL. Next, 50 μL of 750 nM apo-HRP (APO-HRP4C; BBI Solution, Gwent, UK), was added to all the samples and standards. The reconstitution reaction was carried out in a 96-well plate for 10 min at 4 °C. Following incubation, 5 μL of each reaction was transferred to a new 96-well plate. The holo-HRP activity of the samples and standards were recorded by adding 200 μl of TMB substrate (KEM-EN-TEC Diagnostics, Copenhagen, Denmark) and measuring the absorbance at 652 nm kinetically on a BioTek Synergy 2 (Agilent BioTek, Winooski, VT, USA) plate reader until the absorbance of the highest standard was between 1.5 and 1.8. Unknown labile heme concentrations in the cell lysate were calculated from the linear regression analysis of the heme standard curve and normalized to protein concentrations in cell lysates. The final free heme values are expressed as pmoles/mg protein.

4.9. Caspase 3/7 activity assay

Caspase 3/7 activity assay kit (Promega, Madison, WI, USA) was used according to the manufacturer's protocol.

4.10. Mitochondrial ROS (mROS) production

HUVECs (0.75×10^5 cells/well) were plated in 24-well plates and allowed to rest overnight. The following day medium was replaced with serum free medium and cells were stimulated for 3 h with different reagents as detailed in the respective Figure Legends. The medium was replaced with a fresh serum free medium containing 5 μM of Mitosox (Thermo Fisher Scientific, Inc. Waltham, MA, USA) and incubated for 10 min followed by FACS analysis.

4.11. Autophagy flux measurement

To calculate the autophagy flux the densitometry values of LC3BII was determined and respective fold induction in relation to the band in control unstimulated cells were calculated. The autophagy flux was determined by subtracting the fold induction values of chloroquine treatment and the respective treatment without chloroquine.

4.12. CytoID staining

HUVECs (1×10^5 cells/well) plated on coverslips in a 24 well plate were treated with heme in the presence or absence of A1AT for 6 h followed by CytoID staining (CYTO-ID® Autophagy detection kit, Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's protocol. Pictures were taken using an Olympus IX81 microscope equipped with a DR4 camera. Digital pictures were processed with image J software.

4.13. Lysosomal parameters (acridine orange (AO), LysoTracker red and LysoSensor green)

HUVECs (0.75×10^5 cells/well) were cultured in 24-well plates and allowed to rest overnight. At the end of the experiment, the medium was replaced with fresh serum free medium containing AO (2 μM), LysoTracker Red (50 nM, Life Technologies, Carlsbad, CA, USA) or LysoSensor Green (1 μM, Life Technologies) and incubated for further 20 min. Cells were analyzed by flow cytometry or by fluorescence microscopy as described previously [60]. For co-localization experiments, LysoTracker Red staining was performed before fixing the cells.

4.14. Knockdown experiments

HUVECs were transfected in 12-well plates with 75 nM of HO-1 small interfering RNA (siRNA) (ID:s194530) from Thermo Fischer Scientific, and control siRNA (Pre-designed validated AllStars Negative Control siRNA, Qiagen, Venlo, Netherlands) using ScreenFect A-plus transfection reagent (Incella, Eggenstein-Leopoldshafen, Germany) according to the manufacturer's instructions. The knockdown was verified by Western blot analyses after 48 h of transfection.

4.15. Flow cytometry analysis

Flow cytometry analysis was performed on a FACS Canto II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and quantification was performed using FACS Diva software.

4.16. Statistical analyses

All statistical data analysis was performed using One-way ANOVA with Post Tukey's test or Student's *t*-test as indicated in the figure legends using GraphPad Prism Version 8 (GraphPad Prism Software Inc.).

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Author contributions

VV, SJ and SI designed the outline and experiments of the study. KM, VV, CN, AA, ST, EK, HS, HF and MS conducted experiments. KM, VV, SJ, and SI wrote the manuscript.

Declaration of competing interest

The author's state no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102060.

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Research article 2

Alpha1-antitrypsin binds heme and prevents oxidative activation of human neutrophils: putative pathophysiological significance

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Author contribution statement

Kukuh Madyaningrana

- conducted experiments on human endothelial cells and heme measurement
- analyzed data
- contributed to preparation of figures and writing of the manuscript

JLB

Article

Alpha1-antitrypsin binds hemin and prevents oxidative activation of human neutrophils: putative pathophysiological significance

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ABSTRACT

Heme is a ubiquitous compound of human tissues, and it is involved in cellular physiology and metabolism. Once released from the cell, free heme oxidizes to the ferric state (hemin). High levels of hemin can cause oxidative stress and inflammation if not neutralized immediately by specialized scavenger proteins. Human alpha1-antitrypsin (A1AT), an acute-phase glycoprotein and important inhibitor of neutrophil proteases, is also a hemin-binding protein. A short-term exposure of freshly isolated human blood neutrophils to 4 μM hemin results in cell spreading, surface expression of filament protein, vimentin, free radical production, expression of heme oxygenase-1 (HO-1), release of IL-8, and enhanced neutrophil adhesion to human endothelial cells. Consequently, the phosphorylation of protein kinase C (PKC) occurs after 25 min. Under the same experimental conditions, addition of 1 mg/ml A1AT markedly reduces or abolishes neutrophil-activating effects of hemin and prevents PKC phosphorylation. In a mouse model of acute kidney injury (AKI) plus injection of hemin, monotherapy with 4 mg/mouse A1AT significantly lowered serum levels of free hemin at 2 h after surgery. Moreover, a tendency toward lower AKI scores, reduced infiltration of neutrophils, and lower levels of serum chemokine [CXCL1/keratinocyte-derived chemokine (KC)] was observed. Our findings highlight A1AT as a potential serum scavenger of hemin and suggest that the commercial preparations of human plasma A1AT might prove to be useful therapeutics in conditions associated with hemolysis.

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Introduction

Plasma hemoglobin, released after the rupture of RBCs, is strongly associated with an adverse clinical prognosis in gastrointestinal, cardiovascular, pulmonary, urogenital, hematologic, and renal pathologies [1]. Hemoglobin is a source of free heme, and high plasma levels of heme (up to 20 μM) have been reported in patients with bacterial infections and hemolytic diseases, such as sickle-cell anemia, β-thalassemia, I/R injury, and malaria [2–4]. The free heme rapidly oxidizes to the ferric state—hemin—which catalyzes the formation of ROS, induces cell damage, and amplifies inflammatory responses. For example, injection of hemin in experimental mice leads to vascular permeability, leukocyte migration, and increased synthesis of acute-phase proteins [5, 6]. Hemin induces expression of the adhesion molecules on endothelial cells [7, 8] and enables firm neutrophil attachment to the endothelium and initiation of an inflammatory response [9, 10].

Previous studies revealed that neutrophil activation by hemin depends on ROS generation and activation of PKC [9]. With the triggering of the oxidative burst and modification of actin cytoskeleton dynamics, hemin also induces neutrophil migration [10]. Hemin-activated neutrophils can promote vascular injury [11]. Although human neutrophils are considered short-lived cells, with a half-life in the circulation of ~8–12 h [12], according to more recent studies, the lifespan of activated neutrophils can

Abbreviations: 7-AAD = 7-amino-actinomycin, A1AT = alpha1-antitrypsin, AKI = acute kidney injury, BUN = blood urea nitrogen, CER = cytoplasmic extraction reagent, Ct = threshold cycle, DHR-123 = dihydrorhodamine-123, GR = glutathione reductase, GSH = glutathione, HO-1/*HMOX1* = heme oxygenase-1, HPRT = hypoxanthine phosphoribosyltransferase, Hpx = hemopexin, I/R = ischemia-reperfusion, KC = keratinocyte-derived chemokine,

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The online version of this paper, found at www.jleukbio.org, contains supplemental information.

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increase by several-fold [13]. Some reports imply that hemin increases neutrophil longevity to enhance their harmful effects [14, 15].

Through evolution, mammals acquired specialized proteins that sequester free hemin. Hpx is considered as a main scavenger that binds hemin with high affinity at the molar ratio of 1:1 ($K_d < 10^{-12}$ M/l) [16]. Subsequently, the Hpx–hemin complexes are primarily cleared by hepatocytes via the LRP1 receptor pathway [17]. When Hpx becomes saturated, HSA is proposed to play a pivotal role as a hemin scavenger [18, 19]. Alpha1-microglobulin, a glycoprotein (~26 kDa) that belongs to the lipocalin protein family, is another hemin and free-radical scavenger primarily acting in the extravascular compartments [20–24].

Previously, Karnaukhova and coauthors [25] demonstrated in vitro that human plasma A1AT is a hemin-binding protein with an affinity to hemin comparable with that of albumin. Although A1AT is mostly viewed as an inhibitor of neutrophil elastase, A1AT is also an acute-phase glycoprotein with broad anti-inflammatory and immunomodulatory properties [26, 27]. For example, A1AT has been reported to inhibit neutrophil adhesion, chemotaxis, and superoxide production [26, 28]; reduce IL-1β and induce an IL-1R antagonist expression [29]; and regulate HO-1 activity in Alzheimer's brain [30]. The mechanisms involved in mediating these effects are not fully identified or understood. Therefore, we hypothesized that A1AT, as a putative hemin-binding protein, might protect neutrophils from hemin-induced oxidative activation. First, we verified that A1AT binds free hemin in vitro, and secondly, we provide novel evidence that A1AT significantly neutralizes hemin to induce neutrophil activation and adhesion to endothelial cells. In addition, in a Balb/c mice model of AKI, aggravated by free hemin, we demonstrate that monotherapy with human A1AT significantly lowers systemic release of cytotoxic-free hemin at 2 h after AKI induction. Our results highlight A1AT as a physiologically relevant scavenger of hemin.

MATERIALS AND METHODS

Preparation of hemin and A1AT protein

Hemin was always prepared freshly by dissolving 6–8 mg hemin chloride (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 N NaOH. The 1 M HCl was added to bring the pH to 8. The concentration of hemin was determined spectrophotometrically at 400 nm in 40% DMSO using the molar extinction coefficient of 180. The absorbance was measured in a Synergy 2 Multi-Mode plate reader (BioTek Instruments, Winooski, VT, USA). Plasma-purified human A1AT (Zemaira; CSL Behring, Kankakee, IL, USA) was reconstituted in sterile water, and the commercial buffer was exchanged to sterile PBS by using Amicon Ultra-2 10K centrifugal filter units (Millipore Sigma, Billerica, MA, USA). oxA1AT was prepared as described previously [31]. In brief, *N*-chlorosuccinimide was added to A1AT solution at a molar ratio of 25:1 and incubated for 30 min at room temperature. The excess of *N*-chlorosuccinimide was removed by the 3× wash with PBS using 30K centrifugal units Vivaspin 20 (Sartorius Stedim Biotech, Göttingen, Germany). Native and oxA1AT preparations were used immediately or were kept no longer than 1 wk at −20°C.

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LDH = lactate dehydrogenase, LRP1 = LDL receptor-related protein 1, NGAL = neutrophil gelatinase-associated lipocalin, oxA1AT = oxidized alpha1-antitrypsin, PAS = periodic acid–Schiff, PKC = protein kinase C, qPCR = quantitative PCR, R-123 = rhodamine 123, ROS = reactive oxygen species, SERPINA1 = serpin family A member 1, TNB = thiobenzoate, VF = view field

Hemin–A1AT binding assay

Before use, hemin–agarose beads (Sigma-Aldrich) were washed 3 times with sterile PBS. Washed beads, containing 40 nM hemin, were added to a solution containing 2 nM A1AT and incubated for 30 min at room temperature. Aminohexyl–agarose beads (Sigma-Aldrich) were used in parallel, as a control for nonspecific binding. After incubation, beads were washed 3 times with PBS, suspended in 30 μl, 2-fold SDS-PAGE sample buffer. Samples were boiled and spun down at 14,000 *g* for 4 min. Equal amounts of supernatant were applied to 7.5% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250.

Human blood neutrophil isolation and culture

Human neutrophils were isolated from the peripheral blood of healthy volunteers using Polymorphprep (Axis Shield, Oslo, Norway), as described elsewhere [32]. Neutrophil counts were determined by using the Cellometer automatic cell counter (Nexcelom Bioscience, Lawrence, MA, USA). The neutrophil purity was typically ≥96%, as judged by the examination of cytospin. Cell viability exceeded 97%, according to staining with 0.4% Trypan blue solution (Sigma-Aldrich). Freshly isolated neutrophils were suspended in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and plated into FCS-precoated plates (5×10^6/well) at 37°C, 5% CO_2. After 30 min, neutrophils were incubated with 4 μM hemin, 1 mg/ml A1AT, or combinations of hemin with A1AT for a determined time point (no longer than 5 h). For controls, cells were incubated with appropriate buffer. In some experiments, neutrophils were incubated in suspension we placed into sterile cryovials (or glass tubes) and treated directly. For PKC activity assays, neutrophils were preincubated for 30 min with 50 nM calphostin C, a specific PKC inhibitor (Sigma-Aldrich). Following incubation, supernatants and cells were collected and used for further examinations. In some experiments, neutrophils were photographed after 1 h of incubation using a CKX41 microscope, equipped with a 40× objective and U-CMAD3 phototube and SC30 camera (Olympus, Tokyo, Japan).

LDH cytotoxicity assay

Treatment-associated cytotoxicity was determined based on LDH release from ruptured cells into the cell supernatant using the Cytotoxicity Detection Kit (LDH; Roche Diagnostics and Red Cross, Basel, Switzerland). Neutrophils we incubated according to the experimental settings. For 100% control, total cell lysates were used, and for the low and background controls, supernatants from untreated cells and assay medium alone were used, respectively. Absorbance of the colorimetric product of LDH reaction was measured at 490 nm using an Infinite M200 microplate reader (Tecan, Wien, Austria). Measurements were carried out in triplicates.

Apoptosis assay

PE–Annexin V Apoptosis Detection Kit I (#559763; BD PharMingen, San Diego, CA, USA) was used to quantify the percentage of apoptotic neutrophils. The 7-AAD, a standard flow cytometric viability probe, was used to distinguish viable from nonviable neutrophils. Neutrophils that stained positive for Annexin V and negative for 7-AAD were considered as undergoing apoptosis, whereas cells negative for both PE–Annexin V and 7-AAD were considered as not undergoing measurable apoptosis. Cells were analyzed in BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometry acquisition and analysis were performed on at least 20,000 acquired events. The cytometric data were analyzed using FlowJo version 7.6.1 software (Tree Star, Ashland, OR, USA).

Neutrophil cytosol isolation

Cytoplasmic extracts were prepared using the NE PER Nuclear and CERs (Thermo Fisher Scientific), according to the manufacturer's recommendations. In brief, neutrophils (1×10^7/cryovial) were suspended in media and treated with hemin, A1AT, or A1AT in combination with hemin for 2 h. Cells were then pelleted, and cell membranes were disrupted by adding ice-cold CER I and CER II. Cytoplasmic content was collected postcentrifugation at 17,000 *g* for 10 min at 4°C and analyzed by electrophoresis and Western blotting.

Neutrophil adhesion to endothelial cells

The adherence of calcein-labeled neutrophils to HUVECs (American Type Culture Collection, Manassas, VA, USA; Lonza, Basel, Switzerland; PromoCell, Heidelberg, Germany) was analyzed, as described earlier [33]. In brief, freshly isolated neutrophils (5×10^6 cells/ml) were suspended in RPMI-1640 medium, supplemented with BSA (RPMI + 0.1% BSA), and labeled at 37°C with calcein-acetoxymethyl ester (Thermo Fisher Scientific). Labeled neutrophils were washed with RPMI + 0.1% BSA, resuspended in the same media (2.5×10^6 cells/ml), and added to the confluent HUVEC (125,000 cells/well in a 96-well plate), alone or together with the test compounds, i.e., A1AT (1 mg/ml), hemin (4 μM), or A1AT plus hemin. The fMLP (100 nM)-treated cells were used as a positive control. Cell cultures were incubated for 25 min at 37°C 5%, CO_2. Nonadherent neutrophils were aspirated, and the wells were washed with warm medium. The adherent cells were analyzed with a fluorescence spectrophotometer (Infinite M200; Tecan) using an excitation λ of 485 nm and emission λ of 520 nm. The fluorescence of the total number of neutrophils added per well was considered as 100% and was used to calculate the percentage of adherent cells. Triplicate determinations were performed for each condition, in 5 independent experiments.

Gene-expression analysis

Total RNA was prepared using the RNeasy Mini kit (Qiagen Sample and Assay Technologies; Qiagen, Valencia, CA, USA.). The RNA amounts were determined with the NanoDrop spectrophotometer (Thermo Scientific, Bremen, Germany). For cDNA synthesis, 1 μg total RNA was transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). mRNA levels of selected genes (**Table 1**) were analyzed using TaqMan Gene Expression Assays (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR Systems machine (Thermo Fisher Scientific). The Ct value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit. GAPDH was used as a reference gene in the same run. Basal expression of genes was calculated according to the method $2^{\Delta Ct}$ (Ct value of target gene − Ct value of reference gene). All measurements were performed in triplicates, with samples from 3 to 4 independent experiments.

ROS production assay

Isolated neutrophils, 5×10^6/condition, were suspended in RPMI-1640 medium and incubated in glass tubes alone, with 4 μM hemin, 1 mg/ml A1AT separately, or hemin plus A1AT for 1 h at 37°C, 5% CO_2. For the high positive control, cells were treated with PMA (5 μM), and for the low positive control, cells were treated with fMLP (5 μM). The percentage of ROS-producing cells was determined with the PhagoBurst kit (Glycotype Biotechnology, Heidelberg, Germany), according to the protocol of the manufacturer. In brief, cells were resuspended in wash solution provided with the kit and incubated with DHR-123-containing substrate solution for 10 min at 37°C. The reaction was stopped at room temperature by partial fixation with Fixation Buffer (BD Biosciences, San Jose, CA, USA) for 25 min (reaction was protected from the light). The percentage of R-123-positive cells (producing ROS) was analyzed by an FC 500 flow cytometry analyzer (Beckman Coulter, Krefeld, Germany).

TABLE 1. TaqMan primers for gene-expression analysis

Primer	Code
GAPDH	Hs02758991_g1
Bcl2	Hs00608023_m1
HMOX1	Hs01110250_m1
IL1B	Hs00174097_m1
CXCL8	Hs00174103_m1
TLR2	Hs01872448_s1
TLR4	Hs00152939_m1
LRP1	Hs00233856_m1
SERPINA-1	Hs00165475-m1

All primers are from Thermo Fisher Scientific.

GR assay

Following incubation, neutrophils were washed once with PBS and lysed in ice-cold GR assay buffer provided with a GR Assay Kit (Abcam, Cambridge, United Kingdom). Sample preparation and assay were performed, as suggested by the manufacturer. In brief, lysates were first pretreated with H_2O_2 and thereafter, with catalase to destroy endogenous GSH. Pretreated samples were mixed with an oxidized GSH dimer and NADPH and 5,5′-dithiobis (2-nitrobenzoic acid)-containing reaction mix and incubated at 25°C. Color development was followed at 405 nm in an Infinite M200 plate reader (Tecan). Measurements were carried out in duplicate. GR activity (milliunits per 10^6 cells) was calculated from a 2-nitro-5-TNB standard curve. One unit is defined as the amount of enzyme that generates 1.0 μmol TNB/min at 25°C.

Total hemin assay

Hemin concentrations in neutrophil culture supernatants and lysates were examined directly after 5 h of incubation with 4 μM hemin or hemin plus 1 mg/ml A1AT by using a Hemin Colorimetric Assay Kit (BioVision, Milpitas, CA, USA), according to the manufacturer's instructions. In brief, supernatants and lysates were diluted by 1:250 and 1:500, respectively, and applied to 96-well plates. The reaction mix (supplied with the kit) was added, and the plates were incubated for 60 min at room temperature in the dark. The absorption was determined at 570 nm using an Infinite M200 plate reader (Tecan). Measurements were carried out in duplicate or triplicate.

Western blot analyses

Cells were lysed in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Dallas, TX, USA) or PKC lysis buffer (20 mM MOPS, 5 mM EGTA, 2 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1 mM Na-orthovanadate β, 50 mM glycerophosphate disodium salt hydrate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The protein concentration in the lysates was determined by the Bradford assay (Thermo Fisher Scientific). Equal amounts of protein were loaded onto 12.5, 10 or 7.5% SDS-PAGE. After separation, proteins were transferred onto polyvinylidene fluoride membranes (Millipore Sigma) using semi-dry blot transfer. Blots were blocked with 5% BSA (Calbiochem, Darmstadt, Germany) or milk (5%; Carl Roth, Karlsruhe, Germany) in TBS with 0.1% Tween 20 for at least 1 h at room temperature and then probed with the following primary antibodies: mouse monoclonal anti-HO-1 (Clone 23; Santa Cruz Biotechnology), rabbit polyclonal anti-A1AT (Dako; Agilent Technologies, Waldbronn, Germany), rabbit monoclonal anti-PKC β2 (phospho-S660, Clone EP1902Y; Abcam), and mouse monoclonal anti-β-actin (Clone AC-15; Sigma-Aldrich). The immune complexes were visualized with HRP-conjugated secondary antibodies (Dako) and the ECL Western blot analysis kit (Bio-Rad Laboratories, München, Germany) using ChemiDoc Touch Imaging System (Bio-Rad Laboratories). The density of the specific bands was quantified using Bio-Rad's Image Lab software.

PKC activity assay

Neutrophils (5×10^6/condition) were incubated alone, with 1 mg/ml A1AT and 4 μM hemin, separately or in combination, for 25 min at 37°C, 5% CO_2. The activity of PKC was determined using a PKC Kinase Activity

Assay Kit (ab139437; Abcam) that is based on a solid-phase ELISA, using a specific synthetic peptide as a PKC substrate and a polyclonal antibody specific for the phosphorylated form of the substrate. The assay was performed according to the manufacturer's instructions. Cells were washed with ice-cold PBS and lysed in ice-cold PKC lysis buffer by 2 sonication steps each for 1 s. Homogenates were centrifuged for 15 min at 16,000 *g* to obtain cytosolic fractions. Samples, kinase assay dilution buffer (negative control), and 100 ng PKC (for the reference value) were applied to precoated, prewetted microtiter wells. The reaction was started by adding ATP. For quantification of phosphorylated substrate, reaction mixtures were removed, and plates were incubated with phosphor-specific antibody, HRP-conjugated anti-rabbit IgG, and 3,3′,5,5′-tetramethylbenzidine substrate solution. Absorbance at 450 nm was measured with Infinite M200 (Tecan). Measurements were carried out in duplicate. For analysis absorbance, values were normalized to the absorbance of 1 μg PKC, calculated from the PKC reference value.

IL-8 ELISA

Neutrophils were resuspended in RPMI-1640 medium at a concentration of 5×10^6 cells/ml and stimulated with 4 μM hemin and 1 mg/ml A1AT, separately or in combination, for 5 h at 37°C, 5% CO_2. Cell culture supernatants and cell lysates were analyzed with IL-8 DuoSet ELISA Sets (detection limit: 31.25 pg/ml; R&D Systems, Minneapolis, MN, USA).

Vimentin FACS assay

Neutrophils (1×10^6/well) were plated in 12-well plates and incubated with test compounds for 2.5 h at 37°C, 5% CO_2. Afterward, cells were harvested for flow cytometry and incubated with anti-vimentin mAb (Clone 3E9; Abnova, Taipei City, Taiwan) at a final concentration of 0.2 μg/ml for 30 min at 4°C in the dark, followed by incubation with polyclonal Alexa Fluor 488-conjugated goat anti mouse IgG (Fc) antibody (Dianova, Hamburg, Germany) for 25 min at 4°C in the dark. Cells were analyzed by using a flow cytometer, FACSCanto II (BD Biosciences, Heidelberg, Germany).

Studies in vivo

Male Balb/c mice (20–22 wk of age; body weight 28–30 g) were purchased from the Institute of Laboratory Animals, Hannover Medical School (Hannover, Germany). Mice were housed under conventional conditions in a 14/10 h light/dark cycle and had free access to tap water and food (Altromin 1324 standard mice diet; Altromin, Lage, Germany). The local Animal Protection Committee (33.12-42502-04-14/1657) approved the study. The German guidelines are in accordance with the NIH guidelines for animal welfare.

Surgical model for AKI

For general anesthesia, isoflurane (3% for induction and 1.5% for maintenance) was used. For analgesia, butorphanol (1 mg/kg s.c.) was given before surgery. AKI was induced by the clamping of the renal pedicle for 15 min, and after releasing the clamps, 20 mg/kg body weight hemin was injected i.v. Aqueous hemin solution was prepared as described previously [8]. Fifteen minutes after the surgery, A1AT (4 mg/mouse, in a total volume of 200 μl) was injected i.p. Another group of mice were injected with the same volume of sterile PBS and used as controls. After surgery, mice were monitored until they were fully awake. Blood samples were drawn 2 h (for free heme analysis) and 24 h after the start of surgery before euthanasia. Mice were euthanized in deep anesthesia by total body perfusion with ice-cold PBS via the left ventricle, causing circulatory arrest. Clinical chemistry for renal function (i.e., serum-creatinine and BUN) was measured by an automated method using an AU400 Olympus analyzer.

Histology and immunohistochemistry

Kidney tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Two micrometers sections were stained with PAS. Sections were scored for AKI by using a semiquantitative grading system: 0 = focal AKI with <5% of tubuli of the cortex affected, 1 = mild AKI with 5–25% of tubuli affected, 2 = moderate AKI with 26–50% of tubuli affected, 3 = severe AKI with 51–75% of the tubuli affected, 4 = very severe AKI with >75% of tubuli affected. To detect neutrophils, immunohistochemistry was performed with anti-CXCR4 antibody, which is a receptor for the CXC chemokine CXCL12/stromal cell-derived factor 1 motive (CXCR4, bs-1011R; Bioss Antibodies, Woburn, MA, USA; Biozol, Eching, Germany). Cell quantification was performed according to the semiquantitative score: 0 = <5 cells/VF, 1 = mild infiltrates of up to 10 cells/VF, 2 = moderate infiltration of 11–20 cells/VF, 3 = 21–50 cells/VF, 4 = >50 cells/VF. Anti-NGAL (antibody shop; ABS 043-10) was used to detect stressed tubuli. For quantification, the proportion of NGAL-positive tubuli/VF was determined in the cortical area. Ten VFs were quantified/section.

qPCR

Total RNA was purified from fixed mouse kidney using the RNeasy Mini Kit system (Qiagen, Hilden, Germany). For qPCR, 1 μg DNase-treated total RNA was reverse transcribed using the PrimeScript Reverse Transcriptase Reagent Kit (Takara, Kyoto, Japan), and qPCR was performed with StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using Applied Biosystems TaqMan probes (Thermo Fisher Scientific). Ready-to-use mouse primers from Qiagen were used for the PCR reactions (QuantiTech, Hofheim, Germany): TNF-α (Mm_Tnf_1_SG), IL-6 (Mm_Il6_1_SG), KC (QT249900; Qiagen), and HPRT (Mm_Hprt_1_SG), and PCR amplification was 96°C (10 min) and then 40 cycles with 10 s 95°C and 1 min at 60°C. For normalization, HPRT (QT00166768; Qiagen) was used as a housekeeper. Quantitation was carried out by QGene software using the QGene Excel add-on (5–6 mice/group were analyzed).

Apo-peroxidase-based assay to measure free hemin

Determination of free hemin was performed as described previously [34]. Compared with this assay, HBSS, instead of PBS, was applied as an assay diluent, and apo-peroxidase was used at a concentration of 0.75 μM for each reaction. The absorbance at 652 nm was determined with the plate reader BioTek Synergy 2 (BioTek Instruments) and was plotted against the femtomole of hemin in each well to establish the standard curve.

ELISA assay for mice CXCL1/KC

Serum levels of mouse CXCL1/KC were determined using the DuoSet ELISA kit (R&D Systems). Samples were diluted with PBS containing 10% FCS, and assay was performed, according to the manufacturer's recommendations. Detection range was 15.60–1000 pg/ml.

Statistical analysis

A statistical package (SPSS for Windows, release 21.0; IBM, Armonk, NY, USA) was used for the statistical calculations. The differences in the means of experimental results were analyzed for their statistical significance using *t* test and 1-way ANOVA, combined with a multiple-comparison procedure (Scheffé multiple range test), with an overall significance level of $\alpha = 0.05$.

RESULTS

An interaction between A1AT and free hemin, in vitro

A previous study used various techniques to investigate an in vitro interaction between A1AT and hemin [25] and revealed that A1AT is a hemin-binding protein. To investigate further the binding of the A1AT to hemin, we used hemin-immobilized on agarose beads. As shown in **Fig. 1**, A1AT binds hemin, as after 30 min of incubation with hemin beads, a marked amount of protein retained associated with heme–agarose but not with the negative-control ω-aminohexyl-agarose beads. To investigate if hemin binding requires anti-elastase activity of A1AT, we

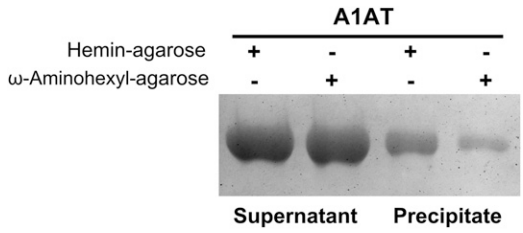

Figure 1. Precipitation of A1AT with hemin-agarose. A1AT (2 nM) was mixed with hemin–agarose slurry corresponding to 40 nM hemin or with a similar amount of aminohexyl-agarose and incubated for 30 min at room temperature. Supernatants were collected. Beads were cleared from unbound A1AT by 3 washes with PBS. Precipitated protein was released by boiling with SDS-PAGE sample buffer. Samples were analyzed using 7.5% SDS-PAGE, and gels were stained with Coomassie Brilliant Blue R-250. One representative gel from 3 independent experiments, performed with different A1AT preparations, is shown.

performed similar experiments with a noninhibitory oxA1AT. Results revealed that oxA1AT interacts equally well with hemin-immobilized agarose beads as native A1AT (Supplemental Fig. 1).

Hemin does not affect A1AT complex formation with elastase

Western blot analysis of cytosol fractions isolated after neutrophil incubation for 2 h with A1AT or A1AT/hemin showed that hemin does not influence the level and molecular profiles of A1AT (**Fig. 2**). Next, native A1AT alone or A1AT/hemin was incubated in vitro for 30 min with elastase and analyzed by SDS-PAGE electrophoresis. Figure 2A shows that A1AT/elastase mixtures exhibit 3 typical major protein bands, corresponding to a complex between A1AT and elastase (~78 kDa), unreacted A1AT (52 kDa), and the cleaved A1AT (45 kDa). The A1AT–hemin/elastase mixtures exhibited a similar profile to that of A1AT/elastase. The oxA1AT was a substrate for the elastase, and similar low molecular-size fragments of oxA1AT were detected after incubation with elastase, independent of the presence of hemin (Fig. 2B). We also examined if the A1AT–hemin interaction affects levels of total hemin in cell supernatants and lysates prepared from neutrophils treated for 5 h with hemin in the absence or presence of A1AT. As shown in Fig. 2C, the levels and distribution of total soluble hemin were not affected by the presence of A1AT.

A1AT prevents hemin-induced neutrophil spreading and adhesion to the culture plates

As illustrated in **Fig. 3A**, nontreated control neutrophils exhibit a rounded shape. In contrast, most of the neutrophils exposed to 4 μM hemin for 5 h were spreading and adhered to the cell-culture plates. Under the same experimental conditions, addition of 1 mg/ml A1AT significantly prevented hemin-induced neutrophil spreading and adhesion (Fig. 3A). With the use of the Cellometer cell counter with preoptimized settings for neutrophil counting, comparable cell counts were found in controls, A1AT, or A1AT/hemin-treated samples, whereas significantly fewer cells were detected in hemin-treated samples (Fig. 3B). Notably, neutrophils incubated for 5 h alone, with hemin or hemin/A1AT, remained viable, as confirmed using LDH toxicity assay (Fig. 3C).

A1AT inhibits hemin-induced neutrophil adhesion to human endothelial cells

Based on the previous findings that hemin induces neutrophil adhesion to endothelial cells [8] and that A1AT protects

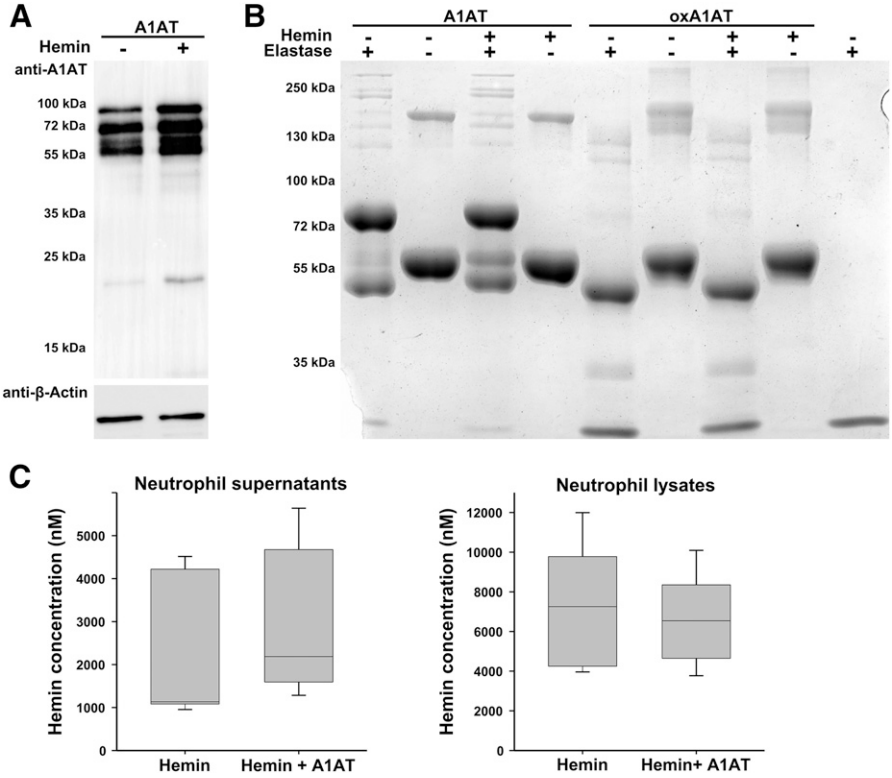

Figure 2. Hemin does not change inhibitory properties of A1AT. (A) Neutrophils were incubated for 2 h with native A1AT (1 mg/ml) or A1AT–hemin mixture, which was prepared by the incubation of 1 mg/ml A1AT with 4 μM hemin for 30 min at 37°C, 5% CO_2. Afterward, a cytosol fraction was isolated and applied to SDS-PAGE 12.5% gels. Electrophoretically separated samples were immunoblotted by using rabbit polyclonal antibody against A1AT. For loading control, blots were reprobed with antibodies to β-actin. A representative blot from 4 independent experiments is shown. (B) SDS-PAGE analysis (7.5%) of native, oxidized, or complexed with hemin A1AT alone and in complex with elastase at molar ratios 1.2:1. All samples, including elastase alone, were incubated for 30 min at room temperature. Electrophoretically separated samples were stained with Coomassie Brilliant Blue-R-250. Representative gel from 3 independent experiments is shown. (C) Hemin distribution in neutrophil supernatants and lysates after 5 h incubation with 4 μM hemin alone or 4 μM hemin plus 1 mg/ml A1AT. The concentration of hemin was determined by using a colorimetric assay. Box plots represent data from 4 individual blood donors; 2 replicates for each experiment.

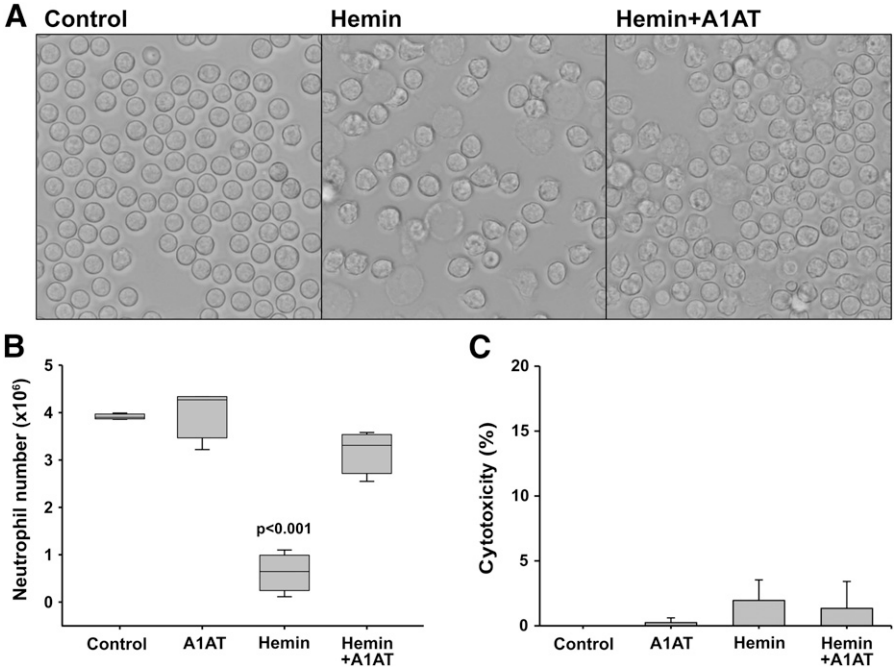

Figure 3. Effects of hemin, hemin + A1AT, and A1AT alone on spreading, adhesion, and viability of neutrophils. Freshly prepared neutrophils were incubated for 4 h at 37°C, 5% CO_2, in FCS-precoated cell culture plates alone and with 4 μM hemin or 4 μM hemin plus 1 mg/ml A1AT. (A) Cells were photographed under the Olympus SC30 digital camera at an original magnification, ×400. Representative pictures from 3 independent experiments are shown. (B) Nonadherent neutrophils were quantified with the Cellometer cell counter. Box plots represent data from 3 individual blood donors; 3 replicates for each experiment. *P* value indicates significant differences compared with the values seen in control neutrophils and those incubated with A1AT and hemin plus A1AT. (C) Cytotoxicity was determined by measuring release of LDH by using the LDH cytotoxicity assay. Each bar represents means ± SD of 3 individual donors.

endothelial cells from neutrophil adhesion induced by fMLP [27], we investigated whether A1AT, as a scavenger of hemin, can prevent hemin-induced neutrophil adhesion to HUVECs. As shown in **Fig. 4**, neutrophils treated with hemin or fMLP (used as a positive control) exhibited a 3-fold higher adhesion to HUVECs compared with controls. However, the adherence of neutrophils treated with hemin/A1AT did not differ from controls (Fig. 4).

A1AT prevents hemin to induce surface expression of vimentin

Vimentin is cytoskeletal protein responsible for maintaining neutrophil shape and integrity and is expressed on the surfaces of activated neutrophils [35]. To investigate cell-surface expression of vimentin, neutrophils were incubated for 2.5 h with A1AT or hemin alone or in combination and analyzed by the flow cytometry (**Fig. 5A**). When compared with control cells, A1AT had no effect on the percentage of neutrophils expressing vimentin [mean (SD) 2.5% (1.4) vs. 2.5% (0.8), $n = 5$; not significant]. In contrast, hemin strongly increased the percentage of neutrophils positive for surface expression of vimentin [mean (SD) 48.8% (20) vs. 2.5% (0.8), $n = 5$; $P < 0.001$]. In the presence of A1AT, this latter effect of hemin was significantly inhibited and did not differ from controls (Fig. 5B).

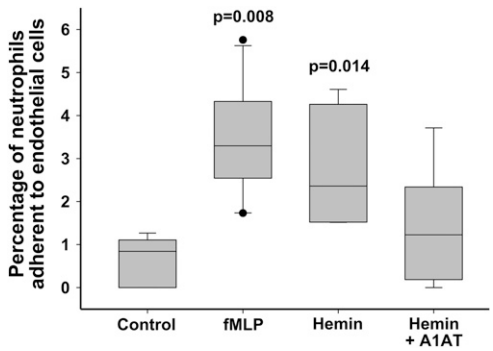

Figure 4. Neutrophil adhesion to human endothelial cells. Calcein-labeled neutrophils were added for 25 min alone or together with 100 nM fMLP, 4 μM hemin, or 4 μM hemin plus 1 mg/ml A1AT to confluent HUVEC cultures. Adherent neutrophils were analyzed with a fluorescence spectrophotometer using an excitation λ of 485 nm and emission λ of 520 nm. The results are expressed in percentage of neutrophil adhesion [(adherent cells/total cells) × 100%]. Box plots represent data from 5 individual blood donors; 3 replicates for each experiment. *P* values indicate significant differences compared with the values seen in control neutrophils. The solid circles on the box plots are outliers.

A1AT and hemin did not significantly influence neutrophil apoptosis

Vimentin has been shown to be expressed on the surface of apoptotic neutrophils [35]. The putative proapoptotic effects of hemin and hemin/A1AT were assessed by using an Annexin V binding assay (**Fig. 6A**). We have observed that incubation of human neutrophils for 5 h with 4 μM hemin only slightly increased the number of apoptotic cells relative to controls or cells incubated with A1AT. Nevertheless, in the presence of A1AT, even a minor proapoptotic effect of hemin was abolished (Fig. 6B). After 5 h of culture, neutrophils were expressing low levels of *bcl-2* and showed no significant differences between controls and hemin or hemin/A1AT treatments (Fig. 6C).

A1AT inhibits hemin-induced IL-8 release but does not affect intracellular IL-8 levels

We evaluated the mRNA and protein levels of IL-8 in neutrophils exposed to hemin or hemin/A1AT. Incubation of neutrophils with 4 μM hemin resulted in an ~2-fold increase in *CXCL8* mRNA expression and in a significant increase in released and cell-associated levels of IL-8 protein compared with the non-treated controls or A1AT-treated cells (**Fig. 7A–C**). In the presence of A1AT, hemin-induced release of IL-8 protein was

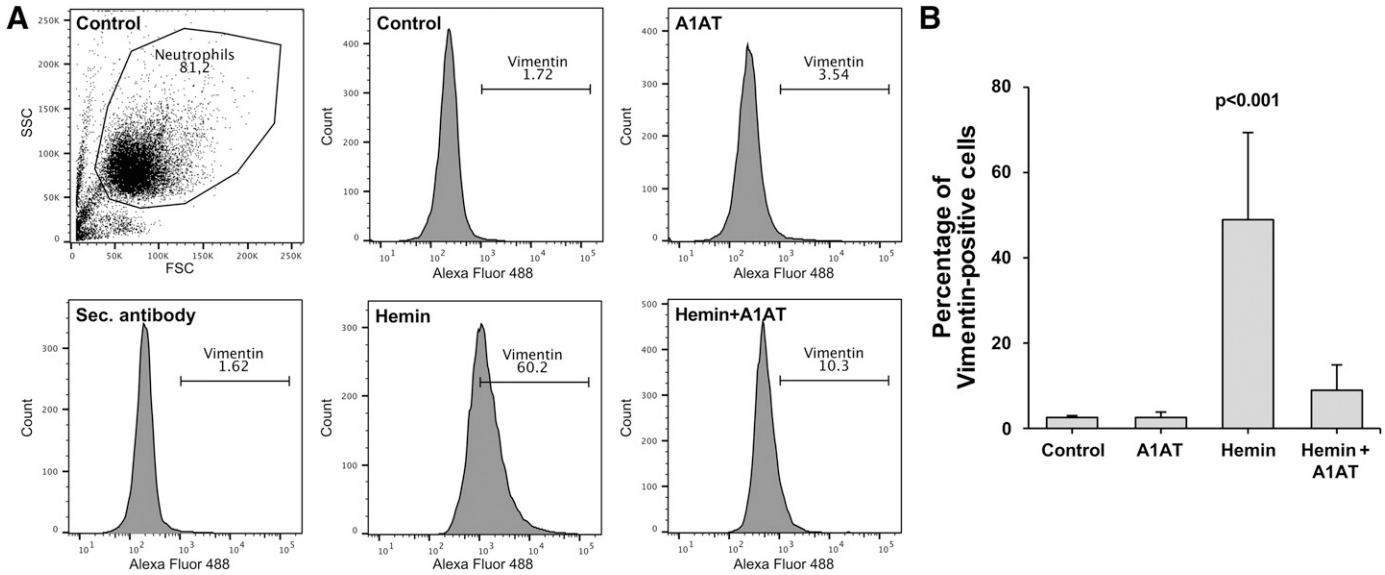

Figure 5. Reduced hemin-induced vimentin surface expression in the presence of A1AT. Human neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing 1 mg/ml A1AT, 4 μM hemin, or 4 μM hemin plus 1 mg/ml A1AT. Afterward, cells were incubated with anti-vimentin mAb for 30 min, followed by incubation with polyclonal Alexa Fluor 488-conjugated goat anti -mouse IgG (Fc) antibody. Surface staining of vimentin was analyzed by using the flow cytometer, FACSCanto II (BD Biosciences, San Jose, CA, USA). (A) Gating strategy and percentage of vimentin-positive cells relative to secondary antibody (Sec. antibody) control. SSC, Side-scatter; FSC, forward-scatter. (B) Bars represent quantitative data of flow cytometric analysis as means ± SD of 5 individual donors. *P* values indicate significant differences compared with the values seen in controls and A1AT or hemin plus A1AT-treated neutrophils.

inhibited significantly (Fig. 7B). However, hemin-induced *CXCL8* mRNA and intracellular IL-8 protein levels were only slightly reduced by the addition of A1AT (Fig. 7A and C).

A1AT prevents hemin induction of ROS production by human neutrophils

Following 5 h treatments with 1 mg/ml A1AT, 4 μM hemin, or hemin/A1AT, neutrophils were incubated with the fluorogenic molecule DHR-123, which oxidizes into fluorescent R-123 in the presence of ROS. As expected, incubation of neutrophils with hemin resulted in a significant number of cells producing ROS (**Fig. 8A** and **B**). The ability of hemin to trigger ROS production in neutrophils was abrogated significantly in the presence of A1AT (Fig. 8).

A1AT inhibits hemin-induced HO-1 expression

HO-1 is an essential enzyme neutralizing hemin-induced ROS, and neutrophils are among the main HO-1-expressing cells in peripheral blood [36]. As predicted, *HMOX1* expression levels (means ± SD) in hemin-treated cells were much higher (7.25 ± 5.02, $n = 12$; $P < 0.001$) than in nontreated controls (0.14 ± 0.18, $n = 9$) or A1AT-treated cells (0.09 ± 0.1, $n = 9$). In the presence of A1AT, hemin effect on *HMOX1* expression was diminished significantly (**Fig. 9A**). Western blot analysis performed on total cell lysates confirmed that exposure of neutrophils to hemin increases protein levels of HO-1, whereas this increase did not occur in the presence of A1AT (Fig. 9B).

A1AT prevents decrease in GR activity

GR is an enzyme that catalyzes the reduction of oxidized GSH to GSH that serves as an antioxidant by reacting with ROS [37]. As shown in **Fig. 10**, when neutrophils were incubated for 2 h with hemin, GR activity decreased by 50% ($P < 0.05$). Under the same experimental conditions, A1AT blocked the ability of hemin to reduce GR activity (Fig. 10).

A1AT inhibits hemin-induced PKC activation

The hemin-induced ROS and expression of HO-1 are suggested to be modulated via the PKC pathway [38]. Therefore, we examined effects of hemin on PKC phosphorylation. We exposed neutrophils for 25 min to hemin (4 μM) and A1AT (1 mg/ml) separately or in combination. As shown in **Fig. 11A**, in hemin-treated neutrophils, PKC activity increased by ~2.6-fold ($P < 0.001$) relative to controls. However, in the presence of A1AT, hemin did not change PKC activity significantly. The reduction of hemin-induced PKC activity was also achieved by using calphostin C, a specific PKC inhibitor. Data from Western blot analysis confirmed the ability of hemin to induce phospho-PKC. This induction did not occur in the presence of A1AT or calphostin C, used as a positive control (Fig. 11B).

Levels of LRP1 and TLR2/4 receptors unaffected by hemin or hemin plus A1AT

Hemin signaling via TLR4 [8] and clearance via LRP1 [39] have been demonstrated. In general, expression of TLR2/4 and especially LRP1 was very low in neutrophils compared with the housekeeping gene *GAPDH*. Exposure of cells to hemin or hemin plus A1AT did not change *TLR2/4* mRNA levels (**Fig. 12**). Of note, hemin significantly lowered *LRP1* mRNA, whereas hemin/A1AT had no significant effect on *LRP1* expression relative to controls (Supplemental Fig. 2).

Effects of A1AT therapy in AKI mice model, in vivo

AKI was induced by renal pedicle clamping for 15 min and aggravated by i.v. injection of hemin. AKI was evident by an

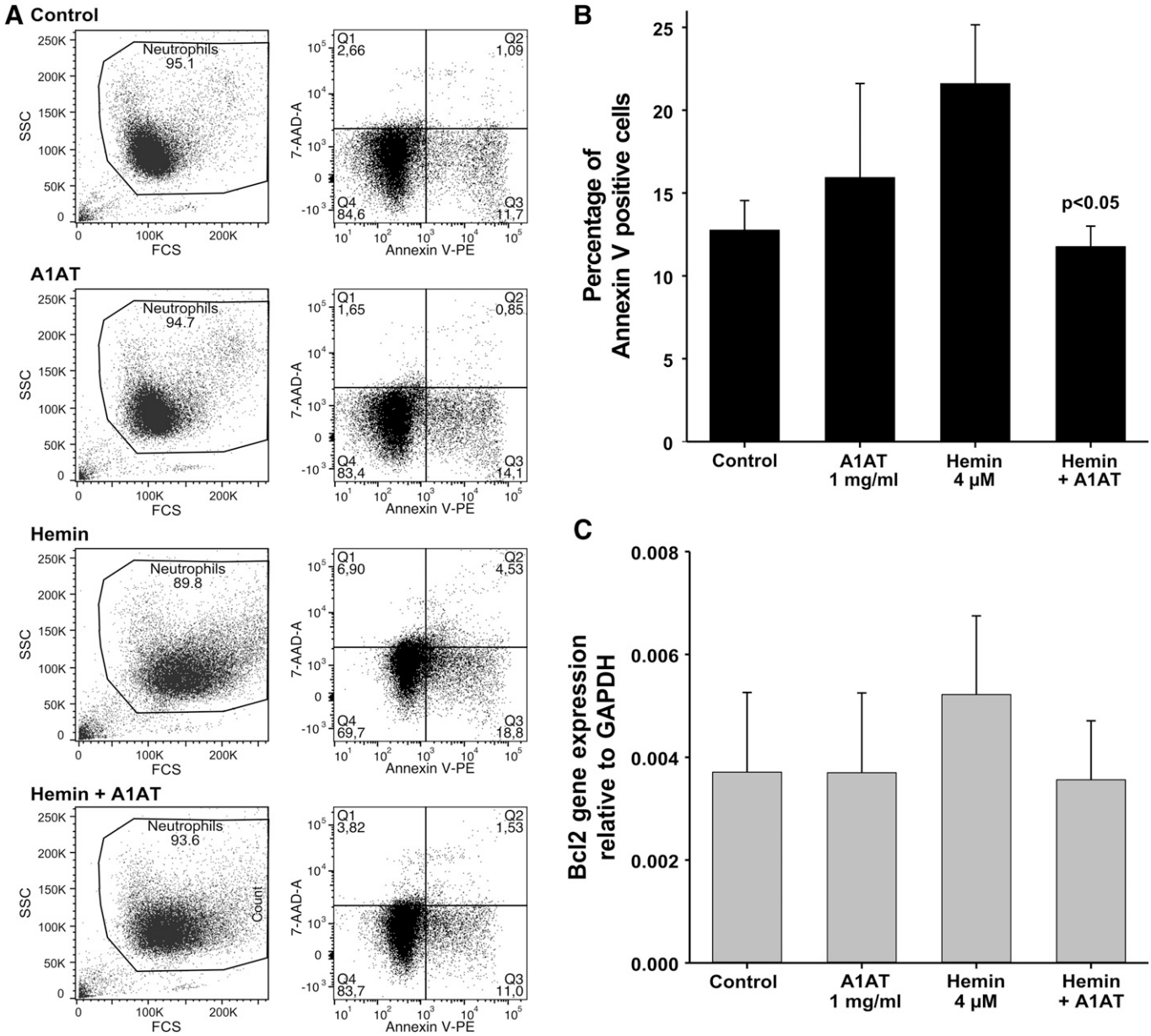

Figure 6. Effects of hemin and hemin plus A1AT on neutrophil apoptosis. Neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing 1 mg/ml A1AT, 4 μM hemin, or 4 μM hemin plus 1 mg/ml A1AT. (A) Gating strategy to get flow cytometry data for all conditions (control, A1AT, hemin and Hemin+A1AT). The 7-AAD was used to distinguish viable from nonviable neutrophils. Neutrophils that stained positive for Annexin V and negative for 7-AAD were considered as undergoing apoptosis, whereas cells negative for both PE-Annexin V and 7-AAD were considered as not undergoing measurable apoptosis. Cells were analyzed in the BD FACSCanto II flow cytometer, and results were calculated by using FlowJo version 7.6.1 software (Tree Star). Q1–Q4, Quadrants 1–4. (B) Bars represent means ± SD of 3 independent experiments. *P* value indicates significant differences compared with the values seen in hemin-treated neutrophils. (C) Expression of the *Bcl2* gene was analyzed by RT-PCR and normalized to *GAPDH*, a housekeeping gene. Each bar represents means ± SD of 4 individual donors, 3 replicates for each experiment.

increase in S-creatinine and BUN. This approach mimics the clinical situation of major surgery when hemin is released from hemoproteins (e.g., myoglobin) as a result of cell damage. Two hours after surgery, A1AT-treated mice exhibited significantly lower serum hemin concentrations compared with the control animals (**Fig. 13**, but after 24 h, hemin levels were similar between both groups (data not shown).

Within 24 h after surgery, histologically renal damage was obvious in the outer medulla in both groups. There was a tendency toward lower AKI scores and the lower infiltration of neutrophils to the outer medulla in the A1AT-treated mice relative to controls, but this did not reach statistical significance (**Fig. 14**). In both, vehicle- and A1AT-treated mice, tubular NGAL expression, as a sign of oxidative stress, increased to a similar extent (Fig. 14). As illustrated in **Fig. 15**, both groups of mice showed similar up-regulation of IL-6, TNF-α, and KC mRNA, although in general, the expression levels of these cytokines were very low. Therapy with A1AT slightly reduced serum CXCL1/KC levels (Fig. 15).

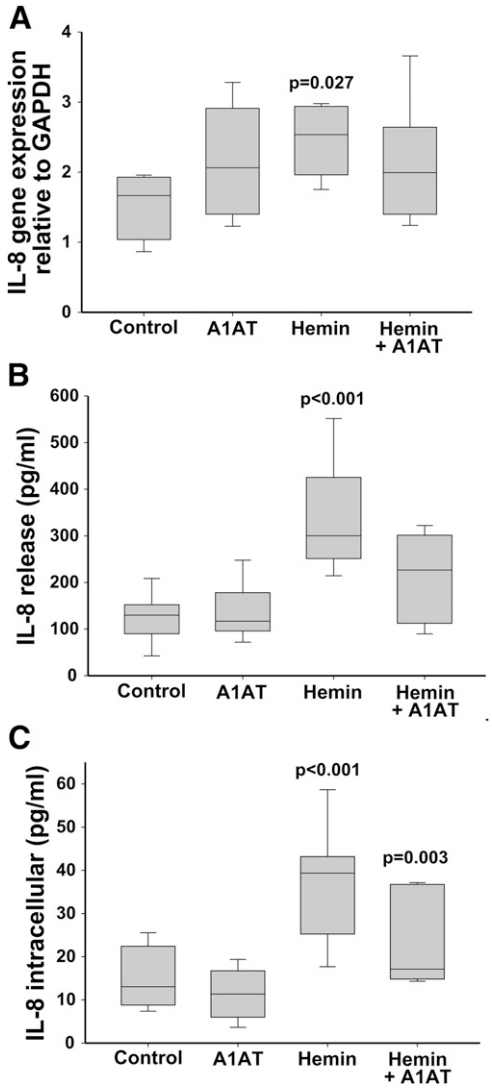

Figure 7. Effects of A1AT in hemin-induced IL-8 expression and release. Neutrophils (5×10^6) were incubated for 5 h in either medium alone or medium containing 1 mg/ml A1AT, 4 μM hemin, or 4 μM hemin plus 1 mg/ml A1AT. (A) Expression levels of the *CXCL8* gene were analyzed by RT-PCR and normalized to *GAPDH,* a housekeeping gene. Box plots represent data from 3 individual blood donors; 3 replicates for each experiment. *P* values indicate significant difference in hemin-treated neutrophils compared with the values seen in controls; measurements of IL-8 protein by ELISA in cell culture supernatants (B) and lysates (C). Box plots represent data from 6 individual blood donors; 2 replicates for each experiment. *P* values indicate significant difference compared with the values seen in controls.

DISCUSSION

Free hemin is a cytotoxic molecule that mediates oxidative stress, endothelial activation, and inflammation, and it is implicated in malaria pathogenesis [40] and AKI, among others [41]. Toxic concentrations of hemin (3.8–38.4 μM) are achievable under pathologic conditions [42]. For example, plasma levels of free hemin can reach 25 μM in symptomatic sickle-cell patients, whereas in stable patients, basal plasma hemin levels can be 5–10 μM [43]. Hence, the scavenging of free hemin by plasma proteins is one of the mechanisms controlling this toxicity.

Hpx is a high binding-affinity, hemin-scavenging protein [44]; however, its levels become depleted under hemolytic conditions, suggesting that other scavenger proteins are important in clearance and/or detoxification of extracellular hemin [45]. Although it has been known for some time that A1AT interacts with hemin, here, we demonstrate for the first time that this interaction neutralizes the ability of hemin to induce neutrophil activation and adhesiveness to the endothelial cell. Moreover, in vivo experiments using the ARI (acute renal injury) mice model provide evidence of the short-term free hemin lowering and protective (albeit modest) effect of A1AT therapy.

Previous studies reported that human A1AT binds hemin and suggested that this interaction be relatively stable [25, 46]. With the use of a hemin–agarose pull-down assay, we further confirm that A1AT interacts with hemin. Besides, samples processed by SDS/PAGE analysis showed that hemin did not affect complex formation between A1AT and its target enzyme, elastase. In pull-down assays, a noninhibitory (at least as an elastase inhibitor) oxA1AT was capable of binding free hemin, as well as the native form of A1AT. Therefore, it appears that an interaction between A1AT and hemin does not require anti-elastase activity of A1AT. Human plasma A1AT has been found in complexes with free fatty acids, LDLs, and HDLs, in which A1AT preserves its anti-elastase activity but also gains additional anti-inflammatory properties [26]. This propensity of A1AT to interact with hydrophobic substances probably also makes it a hemin-binding protein. However, the goal of this study was not to gain insight to the A1AT hemin-binding mechanisms in detail. Instead, the ability of A1AT to bind hemin had stimulated our efforts to investigate the putative pathophysiological importance of such an interaction.

The average concentration of A1AT in plasma is ~1.3–2 g/L, with a half-life of 3–5 d. As A1AT is an acute-phase protein, its concentration may rise up to 3- to 5-fold above normal during inflammation and infections. The main function of A1AT is to inhibit neutrophil elastase and proteinase 3. However A1AT has broader functions [26, 27], abrogating inflammation via both enzyme-inhibitory and noninhibitory mechanisms [47]. For example, A1AT has been found to suppress superoxide production by activated neutrophils [28] and thereby, to reduce oxidant-driven amplification of neutrophilic responses. Neutrophils account for 50–70% of all circulating leukocytes. Under the normal physiologic conditions, neutrophils have a round shape and exist in a resting state to ensure no release of their toxic intracellular contents. Upon exposure to proinflammatory stimuli, such as free hemin, neutrophils mobilize to the site of inflammation [48] and release ROS and cytokines, which initiate and amplify inflammation [49]. For instance, earlier studies have demonstrated that neutrophil elastase degrades the hemoglobin-liberating free hemin that induces ROS production. The generated ROS may cause formation of met-hemoglobin and a subsequent release of more hemin [50]. This vicious cycle well illustrates how neutrophils can amplify oxidative stress and inflammation.

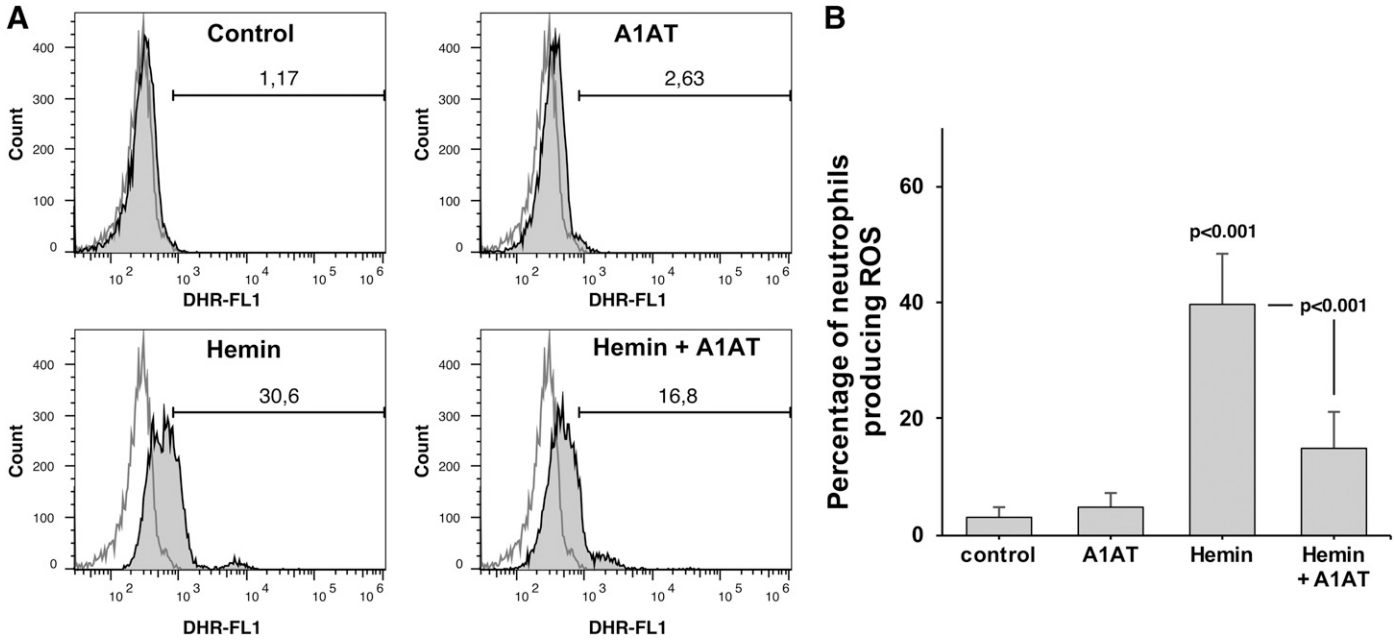

Figure 8. A1AT reduces hemin-induced ROS production. Neutrophils 5×10^6/condition were incubated in glass tubes alone, with 4 μM hemin, 1 mg/ml A1AT separately, or hemin plus A1AT for 1 h at 37°C, 5% CO_2. Afterward, cells were incubated with DHR-123-containing substrate solution for 10 min at 37°C (PhagoBurst kit, Glycotype Biotechnology). The reaction was stopped at room temperature using Fixation Buffer. The percentage of R-123-positive cells (producing ROS) was analyzed by a Beckman Coulter FC 500 flow cytometry. (A) In each condition (filled histograms), the percentage of ROS-producing cells was determined compared with unstained cells (open histograms). One representative experiment is shown. FL1, Fluorescence 1. (B) Bars represent means ± SD of 2 independent experiments, each carried out in duplicates. *P* value for hemin indicates significant differences compared with the values seen in control, A1AT, and hemin plus A1AT-treated cells. *P* value for hemin plus A1AT indicates a significant difference compared with the values seen in hemin-treated cells.

We hypothesized that the anti-oxidant effects of A1AT can be explained, not only by its ability to inhibit elastase but also by the property to bind hemin. To test this, we conducted an in vitro study, in which freshly isolated blood neutrophils from healthy donors were incubated with a fixed amount of hemin (4 μM), alone or with hemin plus a physiologic concentration of A1AT (1 mg/ml), for no longer than 5 h. We focused our studies on the oxidative activation and adherence of neutrophils.

As expected, neutrophils exposed to pathologic amounts of hemin (4 μM) showed shape changes, increased cell-surface vimentin expression and high ROS production, decreased activity of GR, and increased levels of HO-1. Up-regulation of HO-1 represents one of the most critical cytoprotective mechanisms during hemin-induced oxidative stress. HO-1 is a stress-responsive enzyme that catabolizes hemin into biliverdin, carbon monoxide, and free iron (Fe^{2+}) [51, 52]. The GSH is another critical molecule in resisting oxidative stress, and its depletion may trigger HO-1 induction as well [53]. An inverse correlation has been shown between the levels of GSH and HO-1 activity [54]. We also noted that up-regulation of HO-1 in hemin-activated neutrophils was accompanied by reduced activity of GR.

However, we found significantly lower number of ROS generating neutrophils when A1AT and hemin were added to the cells simultaneously. Consequently, the expression of HO-1 did not increase significantly, and the GR activity did not differ from control cells. Along with this, A1AT inhibited hemin to induce PKC phosphorylation, which is an essential step for the production of ROS [9]. In this way, A1AT ameliorated the propensity of hemin to induce oxidative activation of neutrophils. At the same time, when compared with hemin alone, addition of A1AT markedly prevented neutrophil flattening and adhesiveness to the plastic plates. Neutrophil flattening not only increases the size of the cell [55] but also leads to increased adhesion to vascular endothelial cells [56]. Concomitantly, a significantly lower percentage of vimentin-positive neutrophils was found in hemin/A1AT than hemin-treated neutrophils. It is known that surface-exposed vimentin is a mediator of neutrophil-endothelial interactions [57]. It is also important to point out that IL-8 is a well-known chemokine, which triggers the neutrophil respiratory burst, degranulation, chemotaxis, and adhesion to endothelial cells [58]. Resting neutrophils contain IL-8, which is stored in the specific vesicles [59] and rapidly released upon cell activation. In our experiments, A1AT significantly inhibited hemin to induce IL-8 release but only slightly reversed hemin-induced *CXCL8* expression and intracellular levels of IL-8 protein. The induction of IL-8 expression by hemin is suggested to be a PKC-independent event [54]. Thus, our data support the notion that the capability of A1AT to neutralize hemin-induced neutrophil spreading, cell-surface vimentin exposure, IL-8 secretion, and ROS production depends on its interaction with hemin and the prevention to induce the PKC signaling pathway. Further detailed studies in vivo are

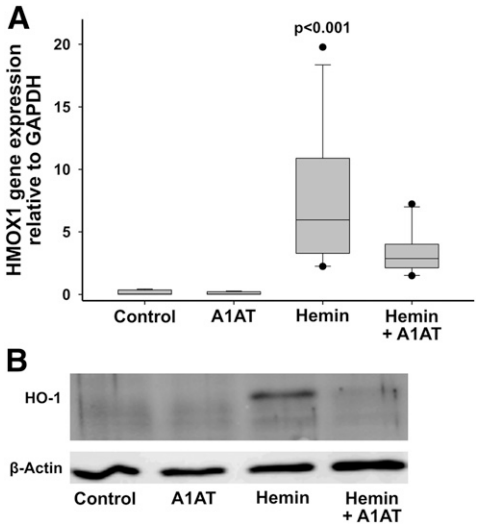

Figure 9. A1AT suppresses hemin-induced HO-1 gene and protein expression. Neutrophils were incubated for 5 h with medium alone, 1 mg/ml A1AT, 4 μM hemin, or 4 μM hemin plus 1 mg/ml A1AT. (A) Expression levels of *HMOX1* were analyzed by RT-PCR and normalized to GAPDH. Box plots represent data from 4 individual blood donors; 3 replicates for each experiment. *P* values indicate significant differences compared with the values seen in control neutrophils. The solid circles on the box plots are outliers. (B) Total cell lysates were analyzed for HO-1 protein levels by Western blotting. For loading control, blots were reprobed with antibodies to β-actin. Representative blot from 3 independent donors with similar results is shown.

required to understand the mechanism(s) by which A1AT, in complex with liberated hemin, regulates neutrophil and endothelial interactions during health and pathologic conditions.

Motivated by the findings that A1AT has the capacity to prevent hemin to induce neutrophil activation and adhesion

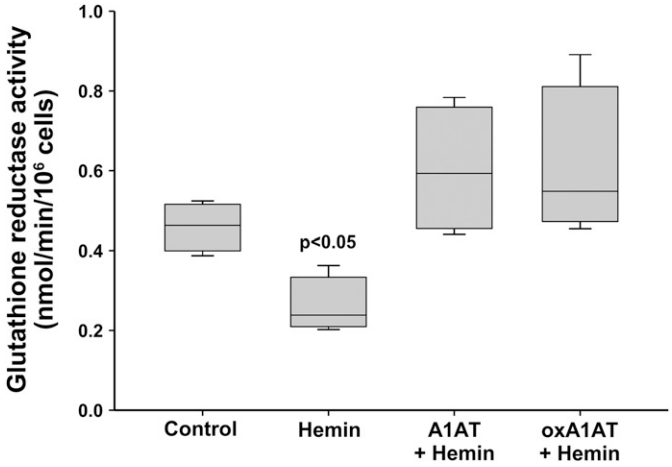

Figure 10. A1AT blocks hemin-induced down-regulation of GR activity. Neutrophils were incubated for 2 h with medium alone, 1 mg/ml A1AT, 4 μM hemin, or 4 μM hemin plus 1 mg/ml A1AT. Afterward, cell lysates were prepared and analyzed for GR activity using the GR Assay Kit (Abcam; as described in Materials and Methods). Box plots represent data from 3 individual blood donors; 2 replicates for each experiment. *P* value indicates significant differences compared with the values seen in control neutrophils.

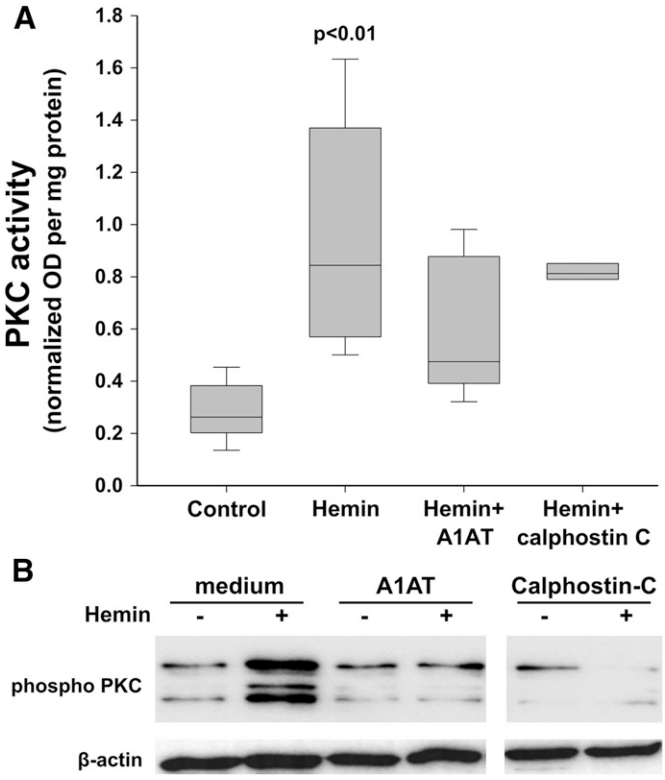

Figure 11. A1AT reduces hemin-induced PKC activity and phosphorylation. Neutrophils (5×10^6/condition) were incubated for 25 min alone, with 1 mg/ml A1AT and 4 μM hemin separately or in combination at 37°C, 5% CO_2. For positive controls, neutrophils were preincubated for 30 min with 50 nM calphostin C, a specific PKC inhibitor. (A) Activity of PKC was determined using the PKC Kinase Activity Assay Kit (Abcam). Box plots represent data from 4 individual blood donors. *P* value indicates significant differences compared with the values seen in control neutrophils. (B) Cell lysates prepared in PKC lysis buffer were analyzed by Western blotting using rabbit monoclonal anti-PKC β2 (phospho-S660, Clone EP1902Y). Representative blot from 3 independent experiments is shown.

to endothelial cells, we investigated whether hemin-neutralizing effects of A1AT are associated with the changes in intra- and extracellular distribution of hemin. In this regard, we analyzed total hemin concentration in neutrophil supernatants and cell lysates. In neutrophils incubated with A1AT alone or hemin/A1AT, we found no differences in intra- or extracellular levels of total hemin, as well as no difference in cytosol levels and molecular profiles of the A1AT protein. As a matter of fact, hemin has been suggested to enhance the expression of positive acute-phase proteins [5]. A1AT is a positive acute-phase protein, and the *SERPINA1* gene encoding the A1AT protein increases in LPS-activated neutrophils [60]. However, in our experiments, neutrophil exposure to hemin did not affect SERPINA1 mRNA expression (data not shown). Hence, although A1AT does not affect hemin distribution, it neutralizes effects of hemin. Free hemin is lipophilic and toxic to cells. Previous studies have found that cytosolic fatty acid-binding proteins are able to bind hemin and interact with phospholipid-rich membranes and other proteins required for hemin transfer

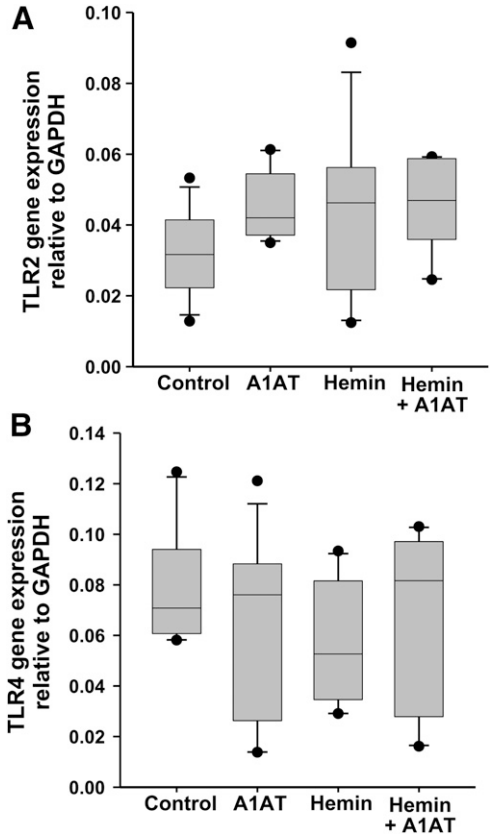

Figure 12. Effects on TLR2 and TLR4 gene expression. Neutrophils (5×10^6/condition) were incubated for 5 h with medium alone, 1 mg/ml A1AT, 4 μM hemin, or 4 μM hemin plus 1 mg/ml A1AT. (A) TLR2 and (B) TLR4 gene-expression levels were analyzed by RT-PCR. GAPDH was used as a housekeeping gene. Box plots represent data from 4 individual donors; 3 replicates for each experiment. The solid circles on the box plots are outliers.

and signaling [61]. We hypothesize that hemin, in complex with A1AT does not interact with the same intracellular carriers and/or compartments as hemin alone. Therefore, hemin toxicity is not necessarily directly linked to the total hemin levels but rather, to the function of the hemin-binding proteins, which redirects hemin metabolism and/or signaling.

Hemin is suggested to bind specifically to TLR4 [8]. However, other studies reported that hemin-induced oxidative burst, neutrophil recruitment, and HO-1 expression are independent of TLR4 [62]. We found that hemin and A1AT/hemin do not affect surface levels and expression of TLR2/4. It is most probable that A1AT neutralizes the effects of hemin without involving the TLR2/4 pathway. LRP1 (CD91) has been identified as a receptor for the Hxp–heme complexes and hemin recycling [17]. LRP1 is also suggested as a receptor for A1AT neutrophil-elastase complexes [63]. The expression level of neutrophil LRP1 was very low, and we think this receptor is unlikely to be involved in A1AT/hemin complex elimination in neutrophils.

Altogether, our data provide novel evidence that A1AT, as a hemin scavenger, prevents hemin-induced activation of neutrophils in vitro. It is important to point out that oxA1AT, without anti-elastase activity, still binds hemin. It means that even if a large fraction of A1AT would lose anti-elastase activity during neutrophilic inflammation, it still would be able to bind hemin. Additional studies are needed to investigate further in detail the pathophysiological role of the oxA1AT/hemin interaction.

Human A1AT is found to modulate and/or prevent tissue injury in experimental animal models of various human diseases, including graft-versus-host disease, inflammatory bowel disease, rheumatoid arthritis, acute liver failure, autoimmune diabetes, gouty arthritis, and I/R injuries [26]. In a recent study, Maicas and coauthors [64] have used a mild renal I/R injury in C57Bl/6 mice and demonstrated that therapy with human A1AT partially preserved renal function and tubular integrity, which was accompanied with a significant decrease of NGAL protein levels in urine and plasma. Here, we used a more severe AKI mouse model, in which we combined 15 min bilateral renal clamping with systemic hemin injection. Within 2 h, monotherapy with 4 mg i.p. A1AT significantly lowered systemic levels of free hemin. This in vivo finding supports the hemin-binding role of A1AT. However, the beneficial, early effect of A1AT in this model was not strong enough to overcome renal inflammation. After 24 h, only slight benefits in reducing serum KC levels and tissue neutrophil infiltration were observed in AKI mice treated with A1AT compared with vehicle controls. Neutrophil accumulation in the ischemic kidney was observed in animal models and in biopsies from patients with AKI [65, 66]. Neutrophil recruitment typically starts as early as 30 min after reperfusion [67], and the exact role of neutrophils in renal I/R is not well understood. One could speculate that a shorter time, causing more pronounced neutrophil infiltration, and/or multiple doses of A1AT would have given a more marked result. Therefore, the clinical relevance of our findings still needs further evaluation. The short-term protection against hemin injury provided by human A1AT could positively affect graft outcome after organ transplantation. Novel therapies free of side effects are needed to minimize the consequences of I/R

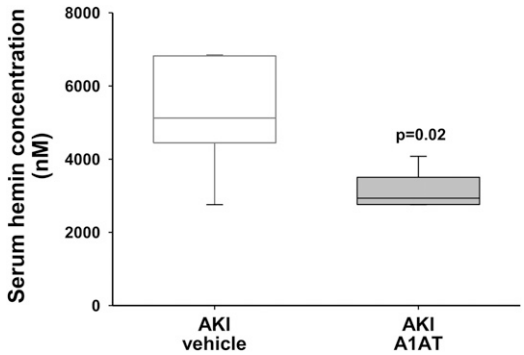

Figure 13. Levels of systemic-free hemin, 2 h after AKI. Serum concentration of free hemin was measured 2 h after AKI by an apoperoxidase assay. A1AT-treated mice displayed significantly less hemin compared with the control, vehicle-treated animals. Box plots represent data from $n = 6$ (vehicle) and $n = 5$ (A1AT) mice. P values indicate significant differences compared with the values seen in control animals.

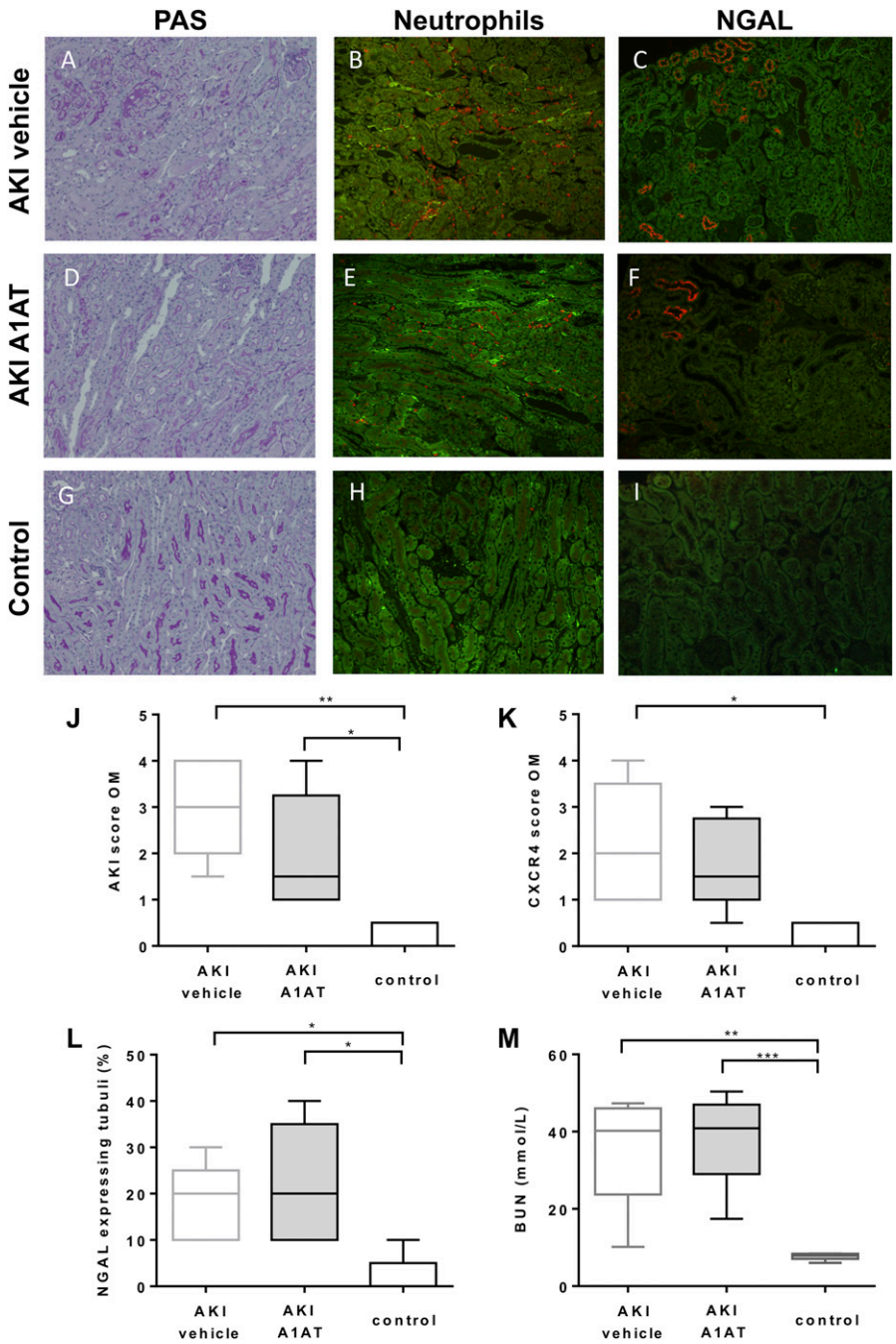

Figure 14. Histology 24 h after AKI. (A, D, G, and J) Renal morphology was assessed after PAS stain and revealed a loss of the brush-border membrane and tubular damage mainly in the outer medulla (OM; B, E, H, and K). The infiltration of granulocyte receptor 1-positive (CXCR4) neutrophils was prominent in the outer medulla (red cells, green autofluorescence of the tubuli). The expression of NGAL, a biomarker of AKI, was similar in both groups (C, F, I, and L). Original magnification, ×200-fold, $n = 5$–6 mice in each group. (M) Deterioration of renal function in the AKI model was detected by BUN increase in both groups at 24 h after injury. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

injury of allografts. Human A1AT is a natural, acute-phase protein with proven safety in the treatment of emphysema patients with severe inherited A1AT deficiency. Therefore, further studies will be designed to investigate the beneficial effects of A1AT therapy under hemolytic conditions.

Although the blood concentration of A1AT (up to 44 μM) is lower than that of albumin (~500 μM), it is much higher than that of α1-microglobulin (1–2 μM), another well-recognized hemin-binding protein [44]. Human albumin is a carrier for many endogenous and exogenous compounds, including drugs, which may affect the albumin–hemin interaction. Therefore, therapy with A1AT might prove to be useful in hemin detoxification under hemolytic conditions, especially after solid organ transplantation.

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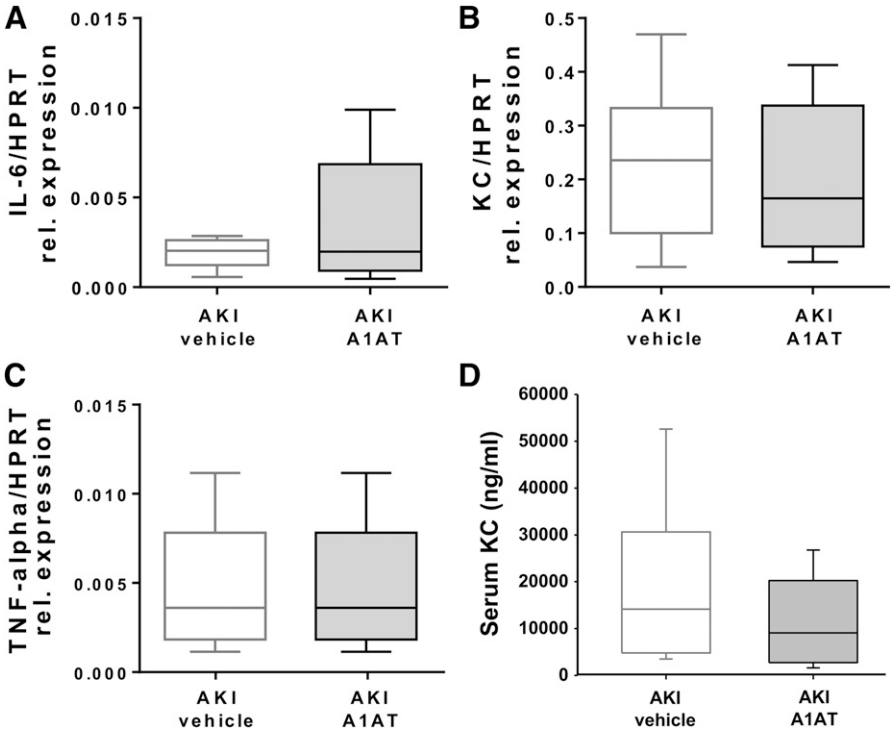

Figure 15. Gene expression of proinflammatory cytokines and serum levels of KC, 24 h after AKI. (A–C) Proinflammatory IL-6, TNF-α, and KC mRNA were elevated in both AKI groups at 24 h after AKI. Systemic release of the chemoattractant KC was slightly lower in the A1AT-treated group relative to vehicle controls. Box plots represent data from 6 mice; 2 replicates for each experiment. (D) Box plot represents KC serum levels at 24 h after AKI from $n = 6$ mice treated with vehicle compared with $n = 5$ mice treated with A1AT (4 mg/mouse).

DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
free radicals · heme oxygenase-1 · IL-8 · protein kinase C · endothelial cells · adhesion

1. RESULTS AND DISCUSSION

Research article 1

Alpha1-antitrypsin counteracts heme-induced endothelial cell inflammatory activation, autophagy dysfunction and death

The endothelium plays a crucial regulatory role for the physiological homeostasis of blood vessels and is critically involved in the pathogenesis of major vascular disorders including atherosclerosis. Free heme mediates inflammatory responses activation of the endothelium via TLR4 signaling. Because A1AT has been demonstrated to exhibit high binding affinity for heme the goal of the current study was to investigate whether and how this serum acute-phase phase protein may protect the endothelium against heme toxicity in direct comparison to the serum HBPs Hx and albumin. To this end, the effects of heme-A1AT interactions were determined in cell cultures of various primary human ECs.

It is shown that A1AT provides protection against the toxicity of extracellular free heme in cell cultures of various types of primary human ECs. A1AT-mediated protection against heme toxicity was distinct to that mediated via Hx and albumin, two serum HBPs that have previously been shown to neutralize heme toxicity via its scavenging. In contrast to Hx and albumin, both of which blocked the cellular up-take of heme in human ECs, heme was taken up by these cells in the presence of A1AT. This was revealed by increased gene expression of HO-1 and high levels of free heme in ECs. A remarkable observation was that intracellular levels of A1AT in ECs were higher when ECs were simultaneously treated with heme and A1AT suggesting that the up-take of A1AT may be mediated via clathrin- and caveolae-dependent pathways. The underlying mechanisms, however, remained partially elusive. Morphological studies with fluorescence microscopy indicated that A1AT was localized in lysosomes. Because heme led to alkalization of these organelles that was markedly reduced by the presence of A1AT, regulation of the cellular acid-base homoeostasis of lysosomes may be involved in the A1AT-dependent protection of the endothelium against heme toxicity. A1AT-dependent protection against endothelial damage by heme was mediated via an autophagy-dependent pathway. Specifically, the heme-induced decrease of autophagy flux that was associated with lysosomal

alkalization could be rescued by A1AT. The findings of this study indicated that A1AT may be important to provide anti-inflammatory protective effects in clinical settings of free heme toxicity. This could be relevant for clinical applications, because administration of A1AT may have beneficial effects in conditions of hemolysis or tissue damage such as in SCD or IRI in which large amounts of free heme can cause harmful effects. Because A1AT has previously been approved for treatment of lung diseases with genetic A1AT deficiency, it is a candidate compound for drug repurposing.

Research article 2

Alpha1-antitrypsin binds heme and prevents oxidative activation of human neutrophils: putative pathophysiological significance

Neutrophils, also known as polymorphonuclear neutrophilic granulocytes, are circulating phagocytes that are the most abundant population of immune blood cells. They are among the first immune cells recruited to sites of infection or tissue damage, and recognize and ingest microbes or dead cells. Heme can not only activate neutrophils, but can also induce migration of these cells to the site of inflammation. The major goal of the current study was to investigate whether and how the high binding affinity of A1AT for heme can affect activation of blood neutrophils in a cell culture model of primary human neutrophils and in an experimental *in vivo* mouse model of IRI-dependent acute kidney injury.

In cell cultures of freshly isolated neutrophils from healthy blood donors, exposure to heme caused spreading of neutrophils with an increased expression of vimentin, a major filament protein associated with inflammatory activation of these cells. It is demonstrated that the heme-dependent effect was blocked by treatment with A1AT. Moreover, a marked increase of ROS production, HO-1 expression, IL-8 release and enhanced adhesion to the endothelium was observed in the presence of heme, all of which were reduced by simultaneous exposure to A1AT. A protein kinase C-dependent pathway mediated activation of human neutrophils by heme. These findings suggested that A1AT-dependent binding of heme by A1AT counter-

acted the activation of neutrophils. Finally, in an *in vivo* mouse model of bilateral IRI, heme levels in circulating blood were markedly increased as determined with an apo-horseradish peroxidase-based assay, which is a dedicated method for measuring free heme. However, the reduced levels of free heme in circulating blood of IRI mice did not appreciably alleviate renal failure in ischemic mouse kidneys. Findings of the current study suggest that A1AT adds to other serum HBPs such as Hx and albumin that are known to counter-act the toxicity of free heme in circulating blood via scavenging of this compound. Thus, A1AT may be a potential therapeutic application in conditions of hemolysis or tissue damage such as in SCD or IRI, in which large amounts of free heme can induce major pathological effects.

In conclusion, A1AT does not only provide protective effects via neutralizing protease activity of from neutrophils, but also via blocking pro-inflammatory activation of neutrophils by heme.

2. Conclusion and Outlook

From the experimental studies of this thesis, we can draw the following conclusions:

- A1AT provided protective functions against the pro-inflammatory effects of heme in human endothelial cells.

- A1AT alleviated pro-inflammatory activation of human blood neutrophils induced by the prooxidant heme.

Outlook

The findings of the current thesis suggest that the beneficial role of A1AT remains to be investigated in preclinical mouse models, in which levels of free heme are increased. As A1AT is a clinically approved drug it appears conceivable that this compound may become applicable for treating disorders with acute hemolysis and/ or IRI.

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4. APPENDIX

Abbreviations

A1AT	alpha1-antitrypsin
ATP	Adenosine triphosphate
BACH1	BTB-and-CNC-homology 1
COX-2	Cyclooxgenase-2
DAMP	Damage-associated molecular pattern
EC	Endothelial cell
Hb	Hemoglobin
HBP	Heme-binding protein
HO	Heme oxygenase
HSA	Human serum albumin
Hx	Hemopexin
IL	Interleukin
IRI	Ischemia-reperfusion injury
LPS	Lipopolysaccharide
Nrf2	Nuclear factor erythroid 2-related factor-2
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
SCD	Sickle cell disease
TLR	Toll-like receptor
TNF	Tumor necrosis factor

List of figures and tables

Animal experiments

All experiments were carried out are in accordance with the version of the German Animal Welfare Act in force at the time of implementation, the German Animal Welfare Ordinance on Experimental Animals and the Directive of the European Parliament and Council of Protection of animals used for scientific purposes (2010/63/EU).

Animal experiments in research article 2 were performed by Prof. Dr. Faikah Güler and were approved by the Local Institutional Animal Care and Research Advisory Committee and the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Lower Saxony, Germany; license No. 33.12-42502-04-14/1657). Kukuh Madyaningrana was not directly involved in animal experimentation and analyzed tissue samples from these experiments. All procedures were carried out according to the German animal protection law and the European Directive 2010/63/EU.

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Declaration

I hereby declare that the above information is true to my knowledge.

Yogyakarta, January 31, 2024

Kukuh Madyaningrana

Declaration

Hiermit erkläre ich, dass ich die Dissertation *Anti-inflammatory Effects of alpha1-Antitrypsin via Neutralization of Heme Toxicity in Human Endothelial Cells and Neutrophils* selbstständig verfasst habe. Bei der Anfertigung wurden keine Hilfen Dritter in Anspruch genommen.

Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Ich habe die Dissertation am Institut für Transfusionsmedizin und Transplantat Engineering angefertigt. Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht. Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Hiermit erkläre ich außerdem, dass die promotionsrelevanten Originaldaten und -aufzeichnungen einschließlich der elektronischen Daten verfügbar sind.

Yogyakarta, January 31 2024

Kukuh Madyaningrana

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