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SIRT3 mediates the anti-oxidant effect of hydrogen sulfide in endothelial cells

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Abstract

Aim Oxidative stress is a key contributor to endothelial dysfunction and associated cardiovascular pathogenesis. Hydrogen sulfide (H_2S) is an anti-oxidant gasotransmiter that protects endothelial cells against oxidative stress. Sirtuin3 (SIRT3), which belongs to silent information regulator 2 (SIR2) family, is an important deacetylase under oxidative stress. H_2S was able to regulate the activity of several sirtuins. The present study aims to investigate the role of SIRT3 in the antioxidant effect of H_2S in endothelial cells. **Results** Cultured EA.hy926 endothelial cells were exposed to hydrogen peroxide (H_2O_2) as a model of oxidative stress-induced cell injury. GYY4137, a slow-releasing H_2S donor, improved cell viability, reduced oxidative stress and apoptosis, and improved mitochondrial function following H_2O_2 treatment. H_2S reversed the H_2O_2-mediated inhibition of MAPKs phosphorylation, down-regulated SIRT3 mRNA and enhanced expression of superoxide dismutase 2 and isocitrate dehydrogenase 2. H_2S also increased activator protein-1 (AP-1) binding activity with SIRT3 promoter and this effect was absent in the presence of the specific AP-1 inhibitor SR11302 or curcumin. Paraquat administration to mice induced a defected endothelium-dependent aortic vasodilatation and increased oxidative stress in both mouse aorta and small mesenteric artery, which were alleviated by GYY4137 treatment. This vaso-protective effect of H_2S was absent in SIRT3 knockout mice. **Innovation** The present results highlight a novel role for SIRT3 in the protective effect of H_2S against oxidant damage in endothelium both *in vitro* and *in vivo.* **Conclusion** H_2S enhances AP-1 binding activity with the SIRT3 promoter, thereby up-regulating SIRT3 expression and ultimately reducing oxidant-provoked vascular endothelial dysfunction.

Keywords: hydrogen sulfide, oxidative stress, sirtuin3, endothelial cell

Introduction

Cardiovascular disease is one of the leading causes of death worldwide. A number of factors including hypertension, high blood sugar and/or cholesterol as well as smoking are known to impair vascular endothelial cell function leading to a plethora of deleterious cardiovascular pathogenesis including vasoconstriction, proliferation of vascular smooth muscle, vascular remodeling and changes in platelet function. These changes in turn contribute to the aetiology of hypertension, myocardial infarction and stroke (29). Accordingly, the protection of vascular endothelial cells from damage due, for example, to oxidative stress has long been considered as the key to the treatment and/or prevention of cardiovascular disease.

Silent information regulator 2 (SIR2) family is functionally important in endothelial cells under oxidative stress (45)**.** Sirtuin3 (SIRT3), one deacetylase belonging to SIR2, increases reactive oxygen species (ROS)-scavenging capacity by enhancing antioxidant enzyme (superoxide dismutase, SOD) activity (6). $SIRT3^{-/-}$ mice showed increased mitochondrial matrix oxidant stress without augmentation of intermembrane space or cytosolic oxidant signaling during sustained hypoxia (43).

Hydrogen sulfide (H_2S) is not only a potent anti-oxidant (19), vasodilator (52) and inhibitor of both vascular smooth muscle proliferation (49) and myocardial apoptosis (8), but also synthesized endogenously in a wide array of cell types either from L-cysteine by cystathionine γ-lyase (CSE) and/or cystathionine β-synthase (CBS) or from cysteine and 3-mercaptopyruvate by cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST) (19). Wen et al. reported that H_2S protected endothelial cells against oxidative stress by acting firstly as an anti-oxidant and secondly by maintaining mitochondrial structure and function (44). Several studies suggest that H_2S is able to regulate the activity of sirutuins family, such as upregulation of sirutuin1 (SIRT1) in human PC12 cells (18) and human umbilical vein endothelial cells (HUVECs) (36, 53), and increase of SIRT3 (4) and sirtuin 6 (SIRT6) (12), to

exert either physiological or patho-physiological effects. Nevertheless, the precise mechanisms of the anti-oxidant effect of H_2S in endothelial cells remain unclear.

In the present study, we used a slow-releasing H_2S donor drug, GYY4137 (17), to examine the anti-oxidant effect of H_2S in endothelial cells and to investigate the downstream signal mechanisms involved. We have identified a completely novel role for SIRT3 in regulating the endothelial response to H_2S, thereby raising the possibility that H_2S interfering with SIRT3 may be of value in the treatment of cardiovascular diseases which are underpinned by oxidative stress.

Results

The effect of GYY4137 on H_2S concentration, survival and apoptosis of endothelial cells exposed to H_2O_2. Assessment of H_2S release by amperometry showed that exposure of endothelial cells to H_2O_2 has no significant influence on H_2S concentration in the medium (1.56±0.13 μ*M* vs 1.37±0.09 μ*M*). Exposure of endothelial cells to GYY4137 (12.5, 25, 50 and 100 μ*M*) prior to H_2O_2 treatment enhanced H_2S concentration (2.15±0.11, 3.14±0.11, 5.46±2.56 and 6.96±0.38 μ*M,* respectively). Meanwhile, H_2O_2 caused 36±4% of cells to death, and GYY4137 (50 and 100 μ*M*) protected endothelial cells against the toxic effect of H_2O_2 (Figure 1A), which were confirmed by lactate dehydrogenase (LDH) assay (Figure 1B). Similar results were obtained when endothelial cells were visualized microscopically. Exposure to H_2O_2 caused cells to lose their typical fusiform shape as cobble-stones and a rounded appearance, which suggests that a number of cells were dead after H_2O_2 treatment, whilst H_2S pre-administration partially restored cell structure and decreased cell death (Figure 1C). Apoptosis was also measured by Hoechst 33342 staining and Annexin V/PI positive staining. H_2O_2 triggered apoptosis in endothelial cells as evidenced by the condensation and fragmentation of nuclei and that this apoptotic effect was significantly reduced by H_2S (representative photomicrographs are shown in Figure 1D). Likewise, Annexin V/PI positive staining, as assessed by flow cytometry, showed greater staining in the presence of H_2O_2, indicative of apoptosis, which effect was reduced in cells pre-treated with GYY4137 (Figure 1E-F). Collectively, the present results demonstrate that exogenous H_2S improves cell viability and decrease apoptosis in H_2O_2-treated endothelial cells.

The effect of H_2S on oxidative stress, mitochondrial function, and mitochondrial permeability potential in endothelial cells exposed to H_2O_2. Oxidative stress plays an important role in the pathogenesis of cell death. To determine whether the protective role of H_2S against apoptosis is related to reduction of ROS, redox status was monitored by

dihydroethidium (DHE) oxidative fluorescence microtopography and dichloro-dihydro-fluorescein diacetate (DCFH-DA) method. Endothelial cells responded to H_2O_2 with a significant rise in ROS formation and this rise was reduced by pre-treatment of cells with GYY4137 (50 μ*M* and 100 μ*M*) (Figure 2A-2B). In addition, redox status is also related to the activity of antioxidant enzymes because H_2O_2 also reduced endothelial total superoxide dismutase (SOD) and Mn-SOD (SOD2) enzyme activity while these effects were again reversed by H_2S (Figure 2C-2D). In addition, H_2S also elevated nitric oxide (NO) content to improve endothelial function after H_2O_2 treatment (Figure 2E).

The decreased cellular ROS in H_2S-treated cells led us to postulate that mitochondrial function could be impaired by H_2O_2. To test this hypothesis, we measured oxygen consumption rate (OCR) in the presence and absence of H_2S using the Seahorse XF analyzer. Figure 2F-G showed that cellular respiration and the response to modifiers of mitochondria function were significantly decreased in H_2O_2-treated cells. We then performed the MitoStress test to quantify several bioenergetic parameters and we found that H_2O_2 significantly suppressed the basal respiration and oxygen consumption due to ATP turnover. Maximal respiration and respiratory reserve capacity were also significantly reduced by H_2O_2, indicating a generally depressed mitochondrial activity. Notably, H_2S prevented these effects of H_2O_2 (Figure 2F-G). In addition, mitochondrial permeability transition ($\Delta\psi m$) also was determined by JC-1 staining, as depicted in Figure 2H. Treatment of H_2O_2 resulted in the increased of green fluorescence intensity, but the decreased of red fluorescence intensity, indicating that the $\Delta\psi m$ of the cells was significantly decreased. Pretreatment with H_2S attenuated H_2O_2-induced collapse of $\Delta\psi m$ in endothelial cells (Figure 2H). These results suggest that H_2S can improve mitochondrial function to limit oxidative stress in H_2O_2-injured endothelial cells.

The effect of H_2S on MAPK signaling pathway and caspase-3. Mitochondrial ROS are known to activate mitogen-activated protein kinase (MAPKs) pathway, which in turn participates

in cell apoptosis. As shown in Figure 3A-C, H_2S pretreatment attenuated H_2O_2-induced phosphorylation of mitogen-activated protein kinase (MAPKs) family including p38 MAPK, ERK1/2 and Jun N-terminal kinase 1/2(JNK1/2). Caspases are crucial mediators of apoptosis. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins (26). As expected, the H_2O_2-induced expression of cleaved-caspase 3 was reversed by H_2S (Figure 3D).

SIRT3 is involved in the protective effect of H_2S in endothelial cells. SIR2 is a family of highly conserved NAD-dependent histone deacetylases that act as cellular sensors to detect energy availability and thus modulate metabolic processes, including mitochondrial function (45). SIR2 exists widely in mammalian and consists of 7 members (SIRT1-SIRT7), which vary in their cellular targeting and location, and play a significant role in metabolism, carcinomatosis, cell survival, and other physiological and pathological processes (1). To examine the involvement of SIR2 in the anti-oxidant effect of H_2S in endothelial cells, we examined mRNA expression of SIRT1-7 after exposure to H_2O_2. The mRNA expression of SIRT1, SIRT3 and SIRT4 was reduced in H_2O_2-treated cells whilst expression of SIRT2, SIRT5, SIRT6 and SIRT7 was unchanged. Expression of SIRT3 (but not SIRT1 or SIRT4) mRNA level was restored to near-baseline in cells incubated with GYY4137 (50 μ*M*, 4 h) (Figure 4A). Moreover, H_2S alone did not alter mRNA and protein expression of SIRT3 but it reversed H_2O_2-induced reduction in SIRT3 expression in endothelial cells exposed to H_2O_2 (Figure 4B-C). The positive role of SIRT3 in endothelial cells treated with H_2S was further supported by silencing experiments. SIRT3-specific siRNA was transfected into endothelial cells (Figure 4D) to reduce expression of SIRT3 (37% ± 10% *VS* 100% ± 11%, Figure 4E-F). SIRT3 silencing abolished the ability of H_2S to reverse the H_2O_2-induced oxidant stress (Figure 4G-H) and apoptosis in endothelial cells (Figure 4I-K).

The effect of H_2S on SIRT3-regulated signaling in endothelial cells. Two direct mechanism linking SIRT3 with reduced ROS have been proposed following the identification of isocitrate dehydrogenase 2 (IDH2) and SOD2 as direct targets for SIRT3. SIRT3, as a major mitochondrial NAD^+-dependent deacetylase, directly deacetylates and activates mitochondrial IDH2 and leads to increased NADPH levels and, thereby an increased ratio of reduced to oxidized glutathione in mitochondria (34). A pivotal mitochondrial ROS scavenging enzyme SOD2 can be activated by SIRT3-mediated deacetylation to reduce levels of superoxide anions (6, 28, 38). Therefore, the expression of IDH2 and SOD were measured. Exposure of endothelial cells to H_2O_2 reduced expression of both SOD2 and IDH2, and this effect was reversed by pretreatment with GYY4137 (50 μ*M*, 4 h). H_2S failed to reverse this effect of H_2O_2 in cells transfected with SIRT3 siRNA (Figure 5A-B). In addition, in order to test whether the effect of H_2S on MAPKs is mediated by SIRT3 in H_2O_2-injured endothelial cells, we determined the expression of p-JNK, p- p38 MAPK, p-ERK and total MAPK (p38 MAPK, ERK, JNK) in SIRT3 knockdown cells. As shown in Figure 5C-E, after transfection with SIRT3 siRNA, H_2S failed to decrease the phosphorylation of JNK, but it was still able to attenuate the levels of p-p38 MAPK and p-ERK. These results suggest that H_2S protects endothelial cells against oxidative damage not only by augmenting SIRT3-mediated IDH2, SOD2 and JNK pathways but also by impacting p38 MAPK and ERK pathways.

The effect of H_2S on SIRT3 gene transcription. In order to investigate the mechanism by which H_2S regulated the transcription of SIRT3 gene in response to oxidative stress triggered by H_2O_2, a number of luciferase reporter plasmids containing a series SIRT3 promoter constructs with various length were constructed. EA.hy926 endothelial cells were transiently transfected with luciferase reporter plasmids containing the SIRT3 promoter (-491/+146). The reporter assays revealed a diminished SIRT3 promoter activity in endothelial cells exposed to H_2O_2, which was reversed by H_2S (Figure 6A). With a series of deletion constructs，the stimulatory

effects of H_2S on SIRT3 promoter activity was observed in -491 Luc and -242 Luc, of which the 5'-ends correspond to 491bp and 242bp from the transcription start site, respectively. However, H_2S-induced enhancement of SIRT3 promoter activity was abolished in -161 Luc (Figure 6B), suggesting that the presence of a critical site between 242bp and 161bp on the upstream of the SIRT3 promoter was responsible for the effect of H_2S on SIRT3 transcription. The putative activator protein 1 (AP1) binding site is present in this region of the SIRT3 promoter and the ChIP assay showed that H_2S increased AP-1 binding activity with the SIRT3 promoter which had been decreased in endothelial cells treated with H_2O_2 (Figure 6C). The enhanced effect on SIRT3 promoter activity in the presence of H_2S was absent when specific AP-1 inhibitors SR11302 (1 μ*M*) or curcumin (20 μ*M*) was present (Figure 6D-E). Collectively, these results suggest that H_2S upregulated SIRT3 gene expression via increasing the AP-1 binding activity with the SIRT3 promoter.

The protective effect of H_2S in SIRT3 KO mice. Our results suggest that H_2S-regulated SIRT3 is a critical endogenous inhibitor of oxidative damage induced by H_2O_2 in endothelial cells *in vitro*. To further explore the pathophysiological significance of H_2S-induced SIRT3 expression *in vivo,* a state of oxidative stress was induced in mice by administration of paraquat (21). Mice treated with paraquat showed impaired endothelium-dependent relaxations and increased oxidative stress in their aortas (Figure 7A-B). H_2S administration improved endothelium-dependent relaxations accompanied by reducing oxidative stress in paraquat-treated mice. More importantly, H_2S treatment lost its ability to augment endothelium-dependent relaxations and to lower oxidative stress in the aortas of SIRT3 KO mice (Figure 7A-B). Although vascular oxidative stress was eliminated by tempol, H_2S produced a greater improvement of endothelium-dependent relaxations in tempol pre-incubated aortas in WT mice followed by paraquat and GYY4137 administration than in SIRT3 KO mice ($P<0.05$, Figure 7C-D). And there was no difference in aortic relaxation in response to NO donor (SNP) among all groups,

suggesting that the sensitivity of vascular smooth muscle to NO was not altered (Figure 7E-F). Moreover, H_2S treatment augmented endothelium-dependent dilatations in small mesenteric arteries from WT mice but not SIRT3 KO mice (Figure 7G-H). These results demonstrate that SIRT3 is most likely required for H_2S to inhibit endothelial oxidative damage *in vivo*, leading to improved endothelial function.

Discussion

Oxidative stress can be broadly defined as an imbalance of ROS production over antioxidant defenses. The mitochondrial respiratory chain is a major source of intracellular ROS generation but also an important target for the damaging effects of ROS (31). Endothelial cells are crucial both for vascular homeostasis and for protecting the vasculature against oxidant species. Endothelial cells are replete with CSE and are endogenous sources of H_2S. The latter is a powerful antioxidant and it may defend the endothelium against oxidative stressors.

In the present study, oxidative injury was induced by H_2O_2 and paraquat, both *in vitro* and *in vivo*, and the protective effect of H_2S was assessed using the donor GYY4137. In consistence with previous studies (13, 44)，the present study shows that H_2S protects endothelial cells from cytotoxicity and improves mitochondrial function against H_2O_2 insult.

The seven mammalian sirtuin orthologs (Sirt1-7) have been studied in diverse disease models, including insulin resistance and diabetes, inflammation, neurodegenerative disease, cancer, and more recently, in cardiovascular pathologies such as cardiac hypertrophy, heart failure, and atherosclerosis (45). mRNA expressions of all SIRT1–7 genes were reduced with advanced passages in several kinds of endothelial cells and such reduction was exaggerated in high glucose-treated cells (22). Our study shows that mRNA expression of SIRT1, SIRT3 and SIRT4 was reduced in H_2O_2-treated cells whilst the expression of SIRT2, SIRT5, SIRT6 and SIRT7 was unaffected. Other studies show that the expression and activity of SIRT1 can be increased by H_2S (18, 36, 53). Zheng et al. found that H_2S delayed nicotinamide-induced premature senescence of HUVECs via up-regulation of SIRT1 (53). H_2S also prevented H_2O_2-induced senescence of HUVECs through SIRT1 activation (36). The present results indicate that SIRT1 mRNA levels decreased in cultured endothelial cells following H_2O_2 treatment; however, this effect was not restored by H_2S co-treatment. As such, our observation contrasted with previous report that NaHS and GYY4137 were able to reverse H_2O_2-induced reduction of

SIRT1 mRNA in HUVECs and in hyperoxic lungs (36, 41). This discrepancy may be attributed to different regulatory mechanisms after H_2O_2 exposure in different cell types, the use of different H_2S treatment regiments giving rise to different kinetics of H_2S release. The observation that reduced SIRT3 mRNA by H_2S in H_2O_2-exposed endothelial cells drove us to focus on SIRT3 in the present study.

We found that the effect of H_2S was attenuated when SIRT3 was knocked down, suggesting that the protective effect of H_2S against oxidative stress and apoptosis depends, at least in part, on SIRT3. SIRT3 plays a role in the maintenance and regulation of normal physiological function of mitochondria (25), which is achieved via its ability to deacetylate acetylated proteins in mitochondria (9, 10, 14). SIRT3 exists as a soluble protein in mitochondria to regulate acetylation reaction including those of acetyl coenzyme A synthase, glutamate dehydrogenase, Ku70, IDH2, a fork head protein FOXO3a and SOD2 (28, 38, 40). Over-expression of SIRT3 can enhance the binding between FoxO3a and the promoter of SOD2 to strengthen the activity of SOD2 transcription (23, 35). SIRT3 may increase mitochondrial ROS scavenging capacity by enhancing antioxidant enzyme activity, including SOD2 (6). SIRT3 also deacetylates IDH2, which mediates intermediary metabolism and energy production (34), (39). Mitochondrial oxidative respiration is thus enhanced, maintaining mitochondrial energy metabolism and reducing ROS generation. It has been reported that H_2S reduces atherosclerotic plaques and endoplasmic reticulum stress by SOD2 activation (7). Likewise, we found that both SOD2 and IDH2, downstream of SIRT3, were activated by H_2S in endothelial cells. Therefore, we demonstrated that the SIRT3-SOD2 and SIRT3-IDH2 pathways were also involved in the protective effect of H_2S against endothelial dysfunction.

Little is currently known about the molecular mechanism of the regulation of SIRT3 gene expression. A previous study showed that peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) activated the mouse SIRT3 promoter, which was mediated by an

estrogen-related receptor binding element mapped to the promoter region (15). Our study suggests a potential role of H_2S in modulating SIRT3 promoter activity in response to H_2O_2. This observation agrees with previous report, indicating that mitochondria, especially during cellular stress or damage, are able to regulate a series of nuclear targeted genes by transcriptional factors acting as mediators of the well-known nucleus-mitochondrion cross-talk (3). We also found that H_2S could increase AP-1 binding activity with SIRT3 promoter which was decreased after H_2O_2 treatment in endothelial cells. The enhanced effect of H_2S on SIRT3 promoter activity was absent when specific AP-1 inhibitors SR11302 or curcumin were used. This result suggests that H_2S can enhance the ability of AP-1 binding into SIRT3 promoter. AP-1 is a multi-functional transcription factor, which regulates gene expression, either positively or negatively, in different types of cells under various physiological and pathological conditions. It is noted that H_2S donor inhibits the activation of AP-1, which is bound to COX-2 gene promoter, in 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion in mouse skin (33). The factors such as AP1 and GATA2, have several motif binding sites located within the SIRT3 promoter region (2). Our study is probably the first one to show that H_2S increases AP-1 binding activity with the SIRT3 promoter and thus enhances SIRT3 transcription to attenuate endothelial oxidative stress.

MAPKs family is one of the most important downstream signal pathways of oxidative stress. Previous study found that exposure of endothelial cells to H_2S increased the phosphorylation of p38 MAPK (24). H_2S also activated ERK1/2 to inhibit angiogenic features of human endothelial cells (46). However, others reported that H_2S scavenged particulate matter-induced endothelial cells ROS and inhibited the oxidative activation of p38 MAPK (42). Inhibiting ERK decreased the H_2S-induced rise in cell migration rate (50). H_2S also decreased the expression of caspase-3 to protect human endothelial cells under a hypoxic condition (32). MAPK, as a double-edged sword, plays a vital role in regulating oxidative stress and other pathophysiological processes. Therefore, H_2S plays diverse roles in MAPK signaling pathway in endothelial cells in response

to different stimulations. In the present study, prior treatment of endothelial cells with H_2S not only conferred protection against death but also reduced activation of intracellular MAPKs signaling and apoptosis. Similar results have been reported by other researchers albeit using different types of cells (11, 30, 48). In addition, the present results show that the SIRT3 mediates the effect of H_2S on JNK but not p38 MAPK and ERK in H_2O_2-treated endothelial cells. The further study is required to establish whether H_2S, SIRT3, MAPK, and oxidative stress will form a complicated regulatory circuit. Nevertheless, this study demonstrates a significant role of SIRT3 activation in the protective effects of H_2S against H_2O_2- and paraquat- induced injury of endothelial cells.

In summary, we provide new evidence that SIRT3 plays an important role in the anti-oxidant effect of H_2S in vascular endothelial cells. In addition to inhibition of p38 MAPK and ERK signaling, H_2S also enhances AP-1binding activity with the SIRT3 promoter which up-regulates SIRT3 and subsequent elevation of SOD2 and IDH2 expression and down-regulation of JNK activity. We therefore propose that H_2S protects endothelial cells against oxidative damage not only by inhibiting p38 MAPK and ERK pathways but also by increasing the expression and activity of SIRT3 as a new mechanism by which H_2S protects endothelial cells. Whether H_2S donors or other drugs which can modify SIRT3 signaling are of value to diminish endothelial dysfunction, a key early event in the development of cardiovascular and metabolic diseases, warrants further investigation.

Innovation

H_2S is known to protect endothelial cells against oxidative stress although the underlying molecular mechanism is only partially understood. The present study demonstrates that H_2S protects endothelial cells against oxidative damage by enhancing activator protein 1 (AP-1) binding activity with the sirtuin3 (SIRT3) promoter thereby up-regulating the expression of SIRT3 and its downstream genes SOD2 and IDH2 as well as improving vascular endothelial function. The present study sheds new light on the molecular mechanism responsible for the cyto-protective effect of H_2S through SIRT3 activation in endothelial biology.

Materials and Methods

Cell culture and treatment. The EA.hy926 endothelial cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were seeded into 6-well plates and cultured until they reached about 80% confluence. Dulbecco's Modified Eagle Midium (DMEM; Gibco BRL, Gaithersburg, MD, USA) with 10% (v/v) fetal bovine serum (FBS, Gibco) was replaced immediately prior to addition of different concentration of H_2S donor GYY4137 (12.5-100 μ*M*). After 4 h treatment, cells were incubated in freshly prepared medium containing H_2O_2 (250 μ*M*; Sigma-Aldrich, MO, USA;) for a further 4 h. Microscopic observation of cells to monitor the differentiation status of cultures and to record any cell changes during treatment were conducted using a converted phase-contrast microscope (Olympus, Tokyo, Japan).

Measurement of H_2S in culture medium. H_2S in culture medium was measured using a H_2S-specific microelectrode (ISO-H_2S-2; World Precision Instruments, USA) connected to a free radical analyzer (TBR4100; World Precision Instruments) as described previously (51). The sensor was set to the 10-nA range and the poise voltage to +150 mV. Prior to initiation of the experiments, the sensor was polarized and calibrated by adding 4 aliquots of the Na_2S stock solution at final concentrations of 0.5、1、2、4μM. Concentrations of H_2S in the samples were calculated using a standard curve of Na_2S.

Cell viability assay. Cell proliferation was measured by Cell Counting Kit-8 (CCK-8, Beyotime, Jiangsu, PR China) according to the manufacturer's directions. Briefly, EA.hy926 cells (1×10^4) were seeded in a 96-well plate and cultured overnight, then exposed to H_2O_2 (250 μ*M*, 4 h) after pretreatment with GYY4137 or vehicle (medium, DMEM) for 4 h, followed by addition of 10 μl of the WST-8 mixture to each well. The cells were then incubated for 1 h at 37 °C in the incubator. The absorbance was measured in a microplate reader (Biotek Instruments, Winooski, VT, USA) at a wavelength of 450 nm.

Cell death was evaluated by the quantification of plasma membrane damage which resulted in the release of LDH. The level of LDH released in the cell culture medium was detected by LDH cytotoxicity assay detection kit (Beyotime, Jiangsu, PR China) following the manufacturer's instructions. The optical density was measured spectrophotometrically at 490 nm on a microplate reader (Biotek Instruments).

Quantification of apoptosis. EA.hy926 endothelial cells were seeded in 6-well plates and incubated overnight. Cells were then treated with H_2O_2 (250 μ*M*, 4 h) after pretreatment with GYY4137 (25-100 μ*M*) or vehicle 4h. To visualize nuclear morphology, EA.hy926 endothelial cells were fixed in 4% v/v paraformaldehyde and stained with Hoechst 33342 DNA dye (2.5 μg/mL). Uniformly stained nuclei were scored as healthy and viable cells. Condensed or fragmented nuclei were scored as apoptotic. The percentage of cells undergoing apoptosis was also determined by Annexin V staining using the BU-ANNEXIN V-FITC apoptosis detection kit (Biouniquer Technology CO., Ltd, Nanjing, China) following the manufacturer's instructions. Annexin V-FITC binding was detected by flow cytometry (BD Biosciences, Franklin Lakes, USA, FL1 filter for Annexin-V-FITC and FL3 filter for PI). The data was analyzed by Cellquest Pro software.

Measurement of reactive oxygen species in endothelial cells. Cells were exposed to H_2O_2 (250 μ*M*, 4 h) after pretreatment with GYY4137 or vehicle (DMEM medium) for 4 h and washed with phosphate-buffered saline (PBS) twice, then switched to serum-free DMEM medium containing dihydroethidium (DHE, 5 μ*M*, Vigorous Biotechnology Beijing Co.,Ltd. Beijing, PR China) or dichlorodihydrofluorescein diacetate (DCFH-DA, 10 μ*M*, Beyotime, Jiangsu, PR China) as described previously (37, 47). After that, cells were washed with PBS again, and the red or green fluorescence was measured with a Zeiss Inverted Microscope (Carl Zeiss, Jena, Germany). DHE enters the cells and is oxidized by $O_2^{\bullet-}$ to yield ethidium, which binds to DNA to produce bright red fluorescence. DCFH-DA is a lipophilic cell permeable compound that is

deacetylated in the cytoplasm to DCF by cellular esterases. DCF is then oxidized by radicals such as hydroxyl, peroxyl, alkoxyl, nitrate and carbonate to a fluorescent molecule (excitation 530 nm, emission 485 nm).

Measurement of total SOD and SOD2 activity in endothelial cells. EA.hy926 endothelial cells were treated with H_2O_2 (250 μ*M*, 4 h) after pretreatment with GYY4137 (25-100 μ*M*) or vehicle (DMEM medium) 4h as described above. Then, cells were washed using PBS and lysed in ice-cold 0.1 M Tris/HCl (pH 7.4) containing 0.5% Triton, 5 m*M* β-mercaptoethanol and 0.1 mg/ml phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 14000g at 4°C for 5 min and cell debris was discarded. SOD activity was detected using a commercial 'SOD Assay Kit-WST' according to the manufacturer's protocol (Dojindo Molecular Technologies, Tokoyo, Japan). SOD2 activity was measured in the presence of a CuZnSOD inhibitor (3mM NaCN) and normalized to total protein content. The highly water-soluble tetrazolium salt WST-1, which produces a water-soluble formazan dye upon reduction by the superoxide anion, was used to measure SOD2 activity. Absorbance values at 450 nm were measured using microplate reader (Biotek Instruments).

Measurement of NO. The production of NO in cultured supernatants was measured using an Apollo 1000 single-channel, free radical detector employing an amperometric-type NO probe (ISO-NOPF100H, World Precision Instruments, USA) as described previously (27).

Analysis of mitochondrial respiration. EA.hy926 endothelial cells were plated (1.2 x 10^3 cells/well) in an XF-96-well plate (Seahorse Bioscience, Massachusetts, USA) and 18 h thereafter cells were treated with GYY4137 (25-100 μ*M*, 4 h) followed by addition of medium or H_2O_2 (250 μ*M*, 4 h) immediately before the medium was changed to unbuffered DMEM containing glucose (10 m*M*), pyruvate (10 m*M*), and GlutaMAX (2 m*M*, Invitrogen, Carlsbad, CA, USA). Mitochondrial respiration oxygen consumption rate (OCR) was measured by a Seahorse Extracellular Flux Analyzer XF96 (Seahorse Bioscience, Massachusetts, USA) (5).

Briefly, a classical MitoStress test was performed according to the following procedure: (1) basal respiration was measured in unbuffered medium; (2) oligomycin (2 μg/ml final concentration), an inhibitor of ATP synthesis, was injected to determine respiration linked to ATP production; (3) the uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhy-drazone (FCCP, 2 μ*M*) was added to measure maximal respiration and (4) antimycin A (4 μ*M*) plus rotenone(4 μ*M*) were applied in combination to block respiration due to simultaneous inhibition of complexes III and I, respectively.

Assessment of mitochondrial membrane potential (Δ*ψ*m). Mitochondrial membrane potential measured by a commercial JC-1 kit (Beyotime, Jiangsu, PR China) following the manufacturer's instructions. Briefly, EA.hy926 were grown on 6-well plate, and were treated with GYY4137 (25-100 μ*M*, 4 h) followed by addition of medium or H_2O_2 (250 μ*M*, 4 h), then confluent cells were rinsed with PBS and incubated in 1mL JC-1 staining solution at 37°C for 20 min. Cells were then rinsed twice with JC-1 washing solution and Digital pictures for JC monomers (Green fluorescence; 535 nm) and JC aggregates (Red fluorescence; 570 nm) were captured with a Zeiss Inverted Microscope (Carl Zeiss, Jena, Germany), The ratio of red (J-aggregate)/green (monomeric JC-1) emission is directly proportional to the Δ*ψ*m.

RNA interference. The following double-strand RNA oligos specific for SIRT3 (sense, 5'-GCCCAACGTCACTCACTACTT-3', antisense, 5'-GUAGUGAGUGACGUUGGGCTT-3') were synthesized by Shanghai GenePharma (Shanghai, PR China). Commercially available siRNA to random noncoding sequences were used for control transductions (Shanghai GenePharma). To obtain SIRT3 knock-down cells with transient transfection, cells were transfected with siRNA duplexes at the final concentration of 100 n*M* using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The cells were used for experiments in 24 h after transfection and gene silencing was detected by analysis of SIRT3 protein expression with Western blot. Transfected with control siRNA or SIRT3 siRNA for 24h, EA.hy926 cells were exposed to H_2O_2 (250 μ*M*, 4 h)

after pretreatment with GYY4137 or vehicle (DMEM medium) for 4 h, ROS and apoptosis were measured as described earlier.

Western blot analysis. Cytoplasmic protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and then immunoblotted with primary anti-IDH2 (1:500, Santa Cruz Biotechnology, CA, USA), anti-SIRT-3, anti-p38 MAPK, anti-p-p38 MAPK, anti-ERK, anti-p-ERK, anti-JNK, anti-p-JNK, anti-cleaved-caspase 3 (1:1000, Cell Signaling Technology, MA, USA), anti-SOD2 (1:500, Abcam, Cambridge, UK), anti-GAPDH (1:6000, Sigma, MO, USA). Proteins were visualized by enhanced chemiluminescence substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted using Trizol reagent (Takara, Osaka, Japan). RNA (500ng) was added as a template to reverse-transcriptase reactions carried out using PrimeScript^{TM} RT Master Mix Kit (Takara). Quantitative real-time PCR (qRT-PCR) was carried out with the resulting cDNAs in triplicate using SYBR Green Rremix (Takara,) and ABI 7500 Real Time PCR System (Appliedbiosystems ABI, IL, USA). Experimental Ct values were normalized to 18s and relative mRNA expression was calculated versus a reference sample. Each sample was run and analyzed in triplicate (Structures of all primers used are listed in Table 1).

Plasmids and luciferase reporter assays. The mSIRT3 promoter (-491 to +146) and pGL3-Basic were provided Professor Yongsheng Chang of the Chinese Academy of Medical Sciences and Peking Union Medical College. Various fragments of the 5' flanking promoter region of mSIRT3 were generated by PCR amplification of Luc-491 followed by cloning into pGL3-Basic using the Acc65 I and Xho I sites as described previously (15).

EA.hy926 endothelial cells were cultured in 24-well plates and cotransfected with luciferase reporter construct (0.2 mg/well) and pRL-TK reporter plasmid (control reporter, 0.006 mg/well)

using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). After transfection (24 h), cells were treated with GYY4137 or DMEM medium as vehicle for 4 h and then exposed to H_2O_2 (250 μ*M*) for a further 4 h. Cells were harvested in lysis buffer and luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to that of control reporter.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed using the Pierce Agarose ChIP Kit (Thermo Fisher Scientific Inc. IL, USA) according to the manufacturer's recommendations. In brief, EA.hy926 endothelial cells were treated with H_2S (GYY4137 50μ*M*) for 4 h before H_2O_2 (250 μ*M*) treatment for 4 h. Protein samples were then pre-cleared with protein A-agarose/salmon sperm DNA (30 min, 4°C) followed by overnight incubation at 4°C with antibodies specific for AP-1 or normal rabbit IgG (as a negative control). The immune complexes were precipitated with protein A-agarose for 1 h. Precipitated genomic DNA was amplified by real-time PCR with primers. Potential AP-1-binding sites on SIRT3 promoter region was amplified with the following primer pairs: 5'-AATCTCCCGGTTTGGCTTCC-3' (sense) and 5'-CCCGCACGATAACCCGAAGT-3' (antisense).

Animals and treatment. $SIRT3^{+/-}$ mouse were the gift of Professor Hongliang Li (Wuhan University, China). These mice were intercrossed to produce homozygous $SIRT3^{-/-}$ and wild-type 129S1/SvImJ animals. Male $SIRT3^{-/-}$ mouse and wild-type (WT) control animals (8-10 weeks) were randomly treated with either GYY4137 (133 μM/kg, i.p.) or vehicle (saline, i.p.) 1 h after paraquat (50 mg/kg, i.p.) or vehicle (saline, i.p.) injection. The dose of both GYY4137 and paraquat used were based upon prior reports in the literature (5, 16). Endothelium-dependent vasorelaxation of mouse aorta and small mesenteric artery were assessed after paraquat injected for 24 h. All animal experiments were conformed to the Guide for the Care and Use of

Laboratory Animals published by NIH and was also approved by the Committee on Animal Care of Nanjing Medical University (NJMU-ERLAUA-20100112).

Measurement of superoxide formation in mouse aorta. Superoxide production in tissue sections of mouse aorta was detected by fluorescence microtopography using the fluorescent probe DHE as previously described (20).

Assessment of endothelium-dependent relaxations. Vasorelaxation of isolated aortic ring segments was determined in organ baths containing oxygenated Krebs' solution. After an equilibration period of 60 min, aortic rings were precontracted with norepinephrine or phenylephrine (Phe, 0.1 μ*M*). Endothelium dependent or independent relaxation was then assessed in response to cumulative addition of acetylcholine (Ach, 10^{-9} to 10^{-5} *M*) or sodium nitroprusside (SNP, 10^{-10} to 10^{-6} *M*) with or without superoxide scavenger tempol (1 m*M*) pre-incubation for 60 min. Relaxation at each concentration was measured and expressed as the percentage of force generated in response to norepinephrine.

Statistical analysis. All data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test as appropriate (Stata13.0 software). $P<0.05$ was considered to be statistically significant.

Materials. Unless otherwise stated, all reagents used were obtained from Sigma-Aldrich (St.Louis, MO, USA).

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Author Disclosure Statement

None.

Abbreviations

AP1: activator protein 1

CBS: cystathionine β-synthase

ChIP: Chromatin immunoprecipitation

CSE: cystathionine γ-lyase

DMEM: Dulbecco's Modified Eagle Midium

DHE: dihydroethidium

DCFH-DA: dichlorodihydrofluorescein diacetate

H_2O_2: hydrogen peroxide

H_2S: hydrogen sulfide

IDH: isocitrate dehydrogenase

LDH: lactate dehydrogenase

MAPK: mitogen-activated protein kinase

MST: mercaptopyruvate sulfurtransferase

NO: nitric oxide

ROS: reactive oxygen species

SIRT3: sirtuin3

SOD: superoxide dismutase

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Table1 Sequences of primers

Human Gene	Sequences(5'-3')
SIRT1-Reverse	CTTCATCTTTGTCATACTTCATGGCTCTATG
SIRT1-Forward	TGCGGGAATCCAAAGGATAATTCAGTGTC
SIRT2-Reverse	AAGGTCCTCCAGCTCCTTCTTC
SIRT2-Forward	CAGAACATAGATACCCTGGAGCGAA
SIRT3-Reverse	CAGCGGCTCCCCAAAGAACAC
SIRT3-Forward	CGGCTCTACACGCAGAACATC
SIRT4-Reverse	TTCCCCACAATCCAAGCAC
SIRT4-Forward	ACCCTGAGAAGGTCAAAGAGTTAC
SIRT5-Reverse	ACTCTTGTAATTCTCAGCCACAACTCCAC
SIRT5-Forward	CGAGTCGTGGTCATCACCCAGAACATC
SIRT6-Reverse	GGCCAGACCTCGCTCCTCCATGG
SIRT6-Forward	GAGGAGCTGGAGCGGAAGGTGTG
SIRT7-Reverse	CACAGTTCTGAGACACCACATGCT
SIRT7-Forward	TGTGGACACTGCTTCAGAAAGGGA

Figure Legends

Figure 1 Protective effect of H_2S on H_2O_2-induced cell injury in endothelial cells. EA.hy926 endothelial cells were pre-treated with GYY4137 (12.5, 25, 50 and 100 μ*M*) for 4 h prior to H_2O_2 (250 μ*M*, 4 h). (A) Cell viability in EA.hy926 endothelial cells was measured by CCK-8 kit. (B) Lactate dehydrogenase (LDH) release in cell culture medium was assayed by a commercial LDH kit. (C) Shape of EA.hy926 cells was detected with light microscope. (D) Cells were stained with Hoechst 33342 and taken the images under a fluorescence microscope. (E-F) Cells were stained with Annexin V/PI and apoptotic rates were analyzed by flow cytometry. $^{**}P<0.01$ *vs* control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* H_2O_2 treated group, n=5-7. H_2O_2, hydrogen peroxide; LDH, Lactate dehydrogenase. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars)

Figure 2 Effect of H_2S on ROS accumulation, SOD activity, mitochondrial function and NO synthesis in endothelial cells with H_2O_2 treatment. EA.hy926 endothelial cells were pre-treated with GYY4137 (25-100 μ*M*) for 4 h prior to H_2O_2 (250 μ*M*, 4 h). (A-B) Cellular ROS production was detected by DHE and DCFH-DA staining. (C-D) Total SOD and Mn-SOD (SOD2) enzyme activity were assessed using a SOD kit. (E) NO production was measured with an NO sensor. (F) The bioenergetic profiles of EA.hy926 cells were detected by a Seahorse Extracellular Flux Analyzer, oxygen consumption rate (OCR) in cells treated with oligomycin (2 μg/ml), FCCP (2 μ*M*) and antimycin A and rotenone (rot and AA, respectively, 4 μ*M*), (G) basal respiration, ATP genenration, maximal respiratory and respiratory reserve capacity are shown. (H) Mitochondrial permeability transition (ΔΨm) was determined by JC-1 staining. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* H_2O_2 treated group, n=3-8. ROS, reactive oxygen species; SOD, superoxide dismutase; NO: nitric oxide; DHE, dihydroethidium; DCFH-DA: dichlorodihydrofluorescein diacetate. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars)

Figure 3 Effect of H_2S on MAPK signal pathway and caspase-3. EA.hy926 endothelial cells were pre-treated with GYY4137 (50 μ*M*) for 4 h prior to H_2O_2 (250 μ*M*, 4 h). (A-C) Representative western blots and quantification of p-p38 MAPK, p-ERK and p-JNK protein expression. (D) Representative examples of western blots and quantification of cleaved-capase3 protein expression. $^{*}P<0.05$, $^{**}P<0.01$ *vs* control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* H_2O_2 treated group, n=4-6. MAPK, mitogen-activated protein kinase.

Figure 4 Role of SIRT3 in H_2S-mediated protection of endothelial cell injury by H_2O_2. (A-B) EA.hy926 endothelial cells were pre-treated with GYY4137 (50 μ*M*) for 4 h prior to H_2O_2 (250 μ*M*, 4 h). Quantification of SIRT family (SIRT1 to SIRT7) mRNA expression was determined by real-time PCR. $^{*}P<0.05$ *vs* control, $^{\#}P<0.05$ *vs* H_2O_2 treated group, n=6-7. (C) Representative examples of western blots and quantification of SIRT3 protein. $^{*}P<0.05$ *vs* control, $^{\#\#}P<0.01$ *vs* H_2O_2 treated group, n=5. (D) EA.hy926 endothelial cells were transfected with SIRT3-specific siRNA (SIRT3siRNA) or a nonspecific control siRNA (CTLsiRNA). Transfection efficiency was assessed by immunofluorescence. (E-F) Representative examples of western blots and quantification of SIRT3 after transfection. $^{**}P<0.01$ *vs* CTLsiRNA, n=6. Transfected with CTLsiRNA or SIRT3siRNA for 24 h, EA.hy926 endothelial cells were exposed to H_2O_2 (250 μ*M*, 4 h) after pretreatment with GYY4137 (50 μ*M*) for 4 h, (G-H) ROS in endothelial cells was examined by DHE and DCFH-DA staining, and (I) apoptosis in endothelial cells was detected with Hochest 33342 staining. (J-K) Cells were stained with Annexin V/PI and apoptotic rates were analyzed by flow cytometry. $^{**}P<0.01$ *vs* CTLsiRNA transfection, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* H_2O_2 treated group with CTLsiRNA transfection, $^{\&\&}P<0.01$ *vs* SIRT3siRNA transfection, n=5. **(**To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars**)**

Figure 5 Effect of H_2S on the SIRT3-regulated SOD2, IDH2 and MAPKs signaling pathways in endothelial cells. Transfected with CTLsiRNA or SIRT3siRNA for 24 h, EA.hy926 endothelial cells were exposed to H_2O_2 (250 μ*M*, 4 h) after pretreatment with GYY4137 (50 μ*M*) for 4 h, (A-B) Representative western blots and quantification of SOD2 and IDH2 protein expression. (C-F) Representative western blots and quantification of p-p38 MAPK, p-ERK and p-JNK protein expression. *P<0.05, ***P<0.001 *vs* CTLsiRNA transfection, $^{\#}$P<0.05, $^{\#\#}$P<0.01 *vs* H_2O_2 treated group with CTLsiRNA transfection, $^{\&}$*P*<0.05, $^{\&\&}$*P*<0.01 *vs* SIRT3siRNA transfection, n=3-4. IDH, isocitrate dehydrogenase.

Figure 6 Effect of H_2S on SIRT3 gene transcript activity. (A) EA.hy926 endothelial cells were transfected with SIRT3 promoter (-491/+146)-luciferase fusion plasmid and pRL-TK plasmids. 24 h later, cells were pre-treated with GYY4137 (50 μ*M*) for 4 h prior to H_2O_2 (250 μ*M*, 4 h). SIRT3 promoter luciferase activity was determined using a dual-luciferase reporter assay system. (B) Cells were transfected with plasmids contain the SIRT3 promoter region up to -491, -242 and -161 respectively. The 3' end of the promoter of all of these constructs corresponds to +146. Transfected cells were treated and promoter luciferase activities were measured as described above. ***P*<0.01 *vs* control, $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01 *vs* H_2O_2 treated group, n=5-7. (C) Cells were pre-treated with GYY4137 (50 μ*M*) for 4 h prior to H_2O_2 (250 μ*M*, 4 h). Chromatin fragments for ChIP assays were immunoprecipitated with anti-AP-1 antibody. Precipitated DNA was amplified by real-time PCR with primers spanning the SIRT3 promoter region. IgG as a negative control. ***P*<0.01 *vs* control, $^{\#\#}$*P*<0.01 *vs* H_2O_2 treated group, n=4. (D-E) Cells were transfected with SIRT3 promoter (-491/+146)-luciferase fusion plasmid. 24h later, cells were treated with specific AP-1 inhibitor SR11302 (1 μ*M*) or curcumin (20 μ*M*) for 4 h. Then GYY4137 (50 μ*M*) were pre-treated for 4 h prior to H_2O_2 and then luciferase activities were determined. $^{\#\#}$*P*<0.01 *vs* H_2O_2 treated group, n=4. AP-1, activator protein 1.

Figure 7 Effects of H_2S on vasorelaxation in SIRT3 KO mice. (A) WT or SIRT3 KO mice were injected with either paraquat (50 mg/kg, i.p) or saline 1 h before either GYY4137 (133 μ*M*/kg, ip) or saline. Mice were killed and aortic segments were removed 24 h thereafter. Endothelium-dependent vasorelaxation to acetylcholine of pre-contracted aortic sections was assessed. $^{**}P<0.01$ *vs* saline treated group of the same genotype, $^{\#\#}P<0.01$ *vs* paraquat treated group of the same genotype, n=6. (B) DHE staining of aorta for superoxide production (n=4). (C-D) Above aortic segments were pre-incubated with tempol (1 m*M*) for 30 min, then endothelium-dependent vasorelaxation to acetylcholine of pre-contracted aortic sections was assessed. $^{**}P<0.01$ *vs* saline treated group of the same genotype, $^{\#\#}P<0.01$ *vs* paraquat treated group of the same genotype, $^{\&}P<0.05$ *vs* paraquat and GYY4137 treated group of the same genotype, n=6. (E-F) Vasorelaxation to SNP of pre-contracted aortic sections was assessed. (G-H) Endothelium-dependent vasorelaxation in small mesenteric artery to acetylcholine of pre-contracted aortic sections was assessed. $^{**}P<0.01$ *vs* saline treated group of the same genotype, $^{\#\#}P<0.01$ *vs* only paraquat treated group of the same genotype, n=4-6. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars)