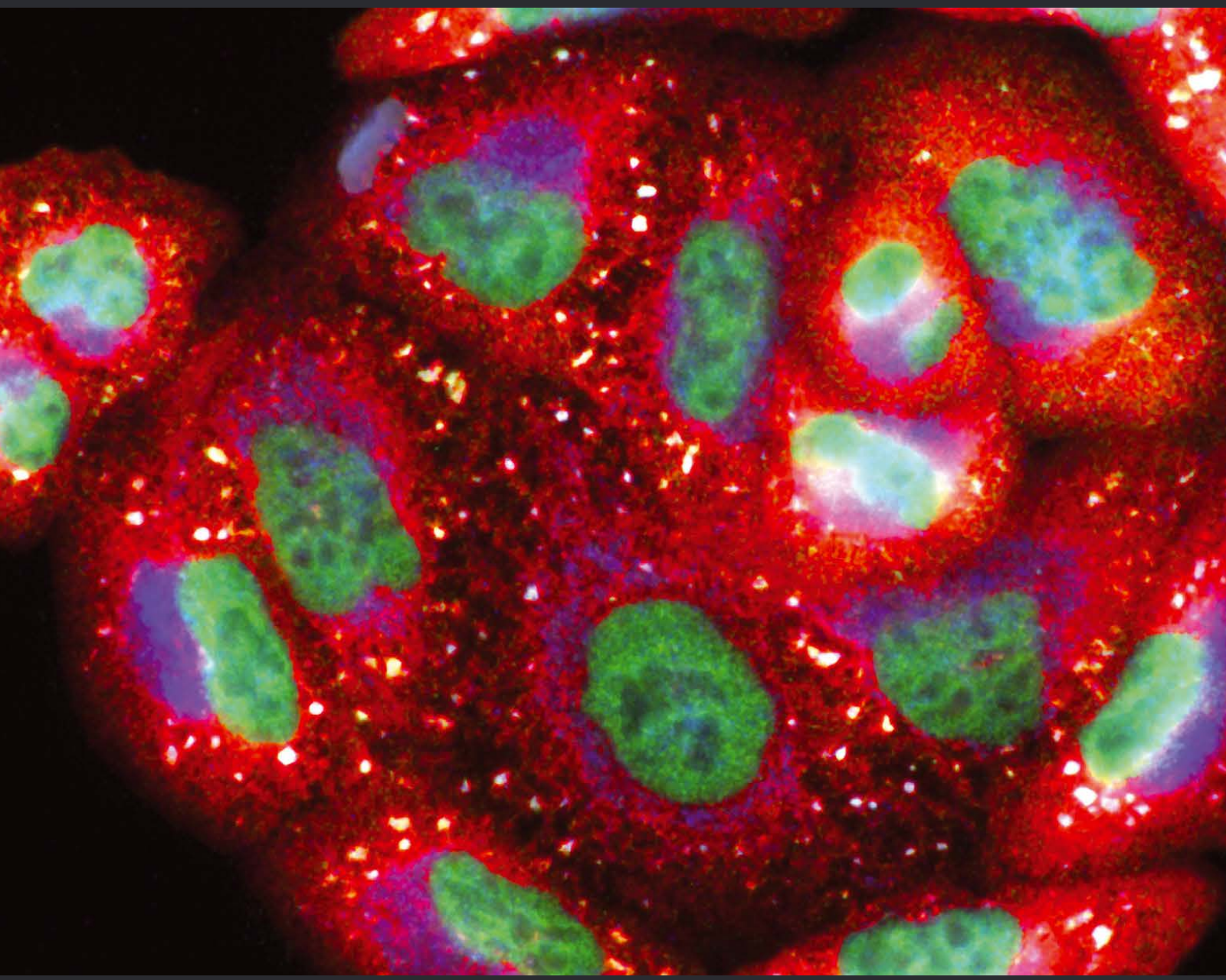
Oxidative Medicine and Cellular Longevity

Gasotransmitters in Biology and Medicine: Molecular Mechanisms and Drug Targets

Guest Editors: Guangdong Yang, Alp Sener, Yong Ji, Yanxi Pei, and Michael D. Pluth



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Editorial Board

Contents

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Editorial

Gasotransmitters in Biology and Medicine: Molecular Mechanisms and Drug Targets

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In the past two decades, an increasing number of reports have indicated the remarkable roles of gasotransmitters in biology and medicine. The term gasotransmitter was first coined by Wang in 2002 and further refined in 2012 and 2014 to encompass a group of small gaseous molecules, including nitric oxide (NO), hydrogen sulfide (H_2S), carbon monoxide (CO), and possibly other gases [1–3]. A gasotransmitter typically has high lipid solubility and can penetrate cell membranes without requiring a specific transporter or receptor. Gasotransmitters are generated endogenously by specific enzymes and can generate various functions at physiologically relevant concentrations by targeting specific cellular and molecular targets. Abnormal generation and metabolism of these gasotransmitters have been extensively demonstrated to be linked to diverse biological processes, such as vascular biology, immune functions, cellular survival, metabolism, longevity, and development and stress resistance.

This specific issue contains both review papers and original research articles that highlight novel discoveries and recent progress in relation to cellular function, molecular mechanisms, and drug targets of gasotransmitters in biology and in medicine as well as the involvement of gasotransmitters in response to environmental stressors in plants. Gasotransmitters have well-defined functions in the vascular system by regulating vascular contraction and dilation since both H_2S and NO act as endothelial derived hyperpolarizing factors or endothelium-derived relaxing factors [4]. In an original research article, L. Xiao et al. elucidated the protective effect of H_2S on the endothelium by using a rat two-kidney one-clip hypertensive model. Exogenous administration of H_2S-releasing donor NaHS lowered blood pressure and improved endothelium dependent contractions. These findings were attributed to the BMP4/ROS/p38 MAPK/COX-2 pathway in the H_2S-dependent endothelial function, further suggesting the potential therapeutic value of H_2S in clinical hypertension. In another study, W.-W. Zheng and colleagues demonstrated that NO is involved in high sodium-stimulated activation of epithelial sodium channel (ENaC) in human umbilical vein endothelial cells. High sodium concentrations inhibited endothelial nitric oxide synthase (eNOS) phosphorylation (Ser 1177) levels and NO production, while the specific ENaC blocker, amiloride, reversed this process; therefore NO may contribute to endothelial protection in response to high salt challenge. M. Forte et al. provided a thorough discussion of the possibility of targeting NO with natural derived compounds as a therapeutic strategy for vascular diseases.

In addition to its cardioprotective effects, H_2S provides potent protection in other systems, including neural system and gastrointestinal endocrine system. A review article by S. Panthi et al. summarized the pathophysiological roles of H_2S in the central nervous system as well as in peripheral nerve degeneration and regeneration. The authors concluded that a full understanding of H_2S and its complex interactions

with neural units could lead to potential therapeutic strategies that employ H_2S. D. Wu and colleagues explored the protective roles of H_2S in obesity-induced kidney injury in mice. They found that H_2S is able to reduce intrarenal lipid deposits, improve kidney function, and reduce the interstitial injury and fibrosis of the kidney through the reduction of kidney inflammation by downregulating NF-κB expression. These data suggested that H_2S or its releasing compounds may serve as a potential therapeutic molecule for obesity-induced kidney injury. A review paper by J. Pichette and J. Gagnon further discussed the regulation of H_2S on glucose metabolism and insulin secretion in both health and disease. Specially, they highlighted the potential roles of H_2S in the gastrointestinal endocrine system, possibly by direct interaction with the insulin-stimulating incretin hormones (insulinotropic polypeptide and/or glucagon peptide-1).

J. Nevoral et al. summarize recent knowledge on the action of gasotransmitters in maturing oocytes and early embryonic development in various animal species, including sea urchin, *Xenopus*, and mammalian models, pointing to the essential role of gasotransmitters in the beginning of life. They suggested that gasotransmitter regulation of gametogenesis may occur through cysteine residue modification of target proteins, including formation of nitrosothiols and persulfides. Further studies of gasotransmitters on gametogenesis are necessary to further establish the potential for advancement of human assisted reproductive technology and reproduction therapy.

In addition to NO, CO, and H_2S, sulfur dioxide (SO_2) has recently been suggested to be a potential gasotransmitter. J. Liu et al. reported investigations into the role of SO_2 in vascular structural remodeling. SO_2 may regulate vascular remolding by affecting smooth muscle proliferation and apoptosis, the balance between matrix metalloproteinase and tissue inhibitors of metalloproteinases, oxidative stress, the TGF-β1/Smad2/3 pathway, and so forth, all of which are closely related to the pathogenesis of hypertension. The authors also suggested that more clinical data are needed to demonstrate the potential therapeutic target for SO_2 in cardiovascular diseases.

Apart from the large volume of studies demonstrating their important roles in mammalian systems, the effects of gasotransmitters in plants are now recently being recognized, suggesting the gasotransmitters may act as universal signalling molecules. Z.-J. Ni et al. demonstrated that H_2S effectively alleviates postharvest senescence of grapes by preventing rachis browning and berry rotting, thus maintaining grape firmness, soluble solids, and titratable acidity during postharvest storage. The protective role of H_2S in grapes could be attributed to the induction of antioxidant enzymes and attenuation of lipid peroxidation, thereby maintaining the stability of cellular membrane structure.

In a special research paper, S. Brazier et al. reported the functional interactions between BKCa α-subunit and Annexin A5 in cell apoptosis. They found that the physical partnership of Annexin A5 and BKCa α-channels results in decreased Ca^{2+} sensitivity and removal of the Annexin A5 from the vicinity of the intracellular C-terminal of BKCa α-subunit, resulting in augmentation of K^+ efflux and subsequent apoptosis.

The research on gasotransmitters is quickly expanding and knowledge associated with the potential of gasotransmitters in biology and medicine is rapidly accumulating. It is clear that gasotransmitters play important roles in both health and diseases. Fully understanding the complex molecular mechanisms of gasotransmitters and developing gasotransmitter-related donors and/or inhibitors will be critical for boosting the progress from basic research to clinical or other commercial applications.

Acknowledgments

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Research Article

Functional Interactions between $BK_{Ca}\alpha$-Subunit and Annexin A5: Implications in Apoptosis

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Proteomic studies have suggested a biochemical interaction between α subunit of the large conductance, voltage- and Ca^{2+}-activated potassium channel ($BK_{Ca}\alpha$), and annexin A5 (ANXA5), which we verify here by coimmunoprecipitation and double labelling immunocytochemistry. The observation that annexin is flipped to the outer membrane leaflet of the plasma membrane during apoptosis, together with the knowledge that the intracellular C-terminal of $BK_{Ca}\alpha$ contains both Ca^{2+}-binding and a putative annexin-binding motif, prompted us to investigate the functional consequences of this protein partnership to cell death. Membrane biotinylation demonstrated that ANXA5 was flipped to the outer membrane leaflet of HEK 293 cells early in serum deprivation-evoked apoptosis. As expected, serum deprivation caused caspase-3/7 activation and this was accentuated in $BK_{Ca}\alpha$ expressing HEK 293 cells. The functional consequences of ANXA5 partnership with $BK_{Ca}\alpha$ were striking, with ANXA5 knockdown causing an increase and ANXA5 overexpression causing a decrease, in single BK_{Ca} channel Ca^{2+}-sensitivity, measured in inside-out membrane patches by patch-clamp. Taken together, these data suggest a novel model of the early stages of apoptosis where membrane flippage results in removal of the inhibitory effect of ANXA5 on K^+ channel activity with the consequent amplification of Ca^{2+} influx and augmented activation of caspases.

1. Introduction

Programmed cell death (apoptosis) is a series of controlled events which, when balanced with cell proliferation, is essential for the normal development of tissue and function of cells. Disturbing this equilibrium is a contributory factor in a number of disease states, such as neurodegenerative disorders, pulmonary hypertension, and cancer [1–3]. Apoptosis occurs in three main steps: (i) the initiation event when the signal for a cell to apoptosis is received and the cell begins to shrink, due to loss of cell volume; (ii) the effector phase when the mitochondrial membrane potential becomes depolarized, the mitochondrial transition pore opens, and the release of cytochrome C and other large molecules from the mitochondrial matrix results in activation of caspases; and (iii) the last phase in which DNA becomes degraded, apoptotic bodies excise from the plasma membrane, and the cytoskeleton is broken down (see [4] for review). As part of this process, phosphatidylserine is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet soon after the induction of apoptosis ("flippage"), where proteins such as the annexins are known to bind with high affinity [5]. This flippage phenomenon is utilized in diagnostic tests for apoptosis. However, little evidence exists as to how and when flippage occurs and what are the consequences that this process has for the apoptosis cascade.

K^+ fluxes have been shown to play an important role in both the early and late phases of apoptosis [4]. Once apoptosis is triggered, one of the earliest observed morphological changes is cell shrinkage or apoptotic volume decrease (AVD). This is due to the efflux of K^+ and Cl^- ions through their respective channels that leads to water exiting the cell through aquaporin water channels in order to maintain

the osmotic pressure balance between internal and external compartments. Identification of the specific K^+ channels, which are involved in apoptotic volume decrease, has employed pharmacological agents; to date, evidence suggests that all the major classes of K^+ channels (voltage-gated, Ca^{2+}-activated, ATP-sensitive, inwardly rectifying, and two pore domain K^+ channels) have some role to play in apoptosis [8–12]. Blockage of the large conductance and voltage- and calcium-activated potassium channel (BK_{Ca}) by iberiotoxin or tetraethylammonium (TEA) inhibits carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, a mitochondrial protonophore) induced apoptosis in both rat and human pulmonary smooth muscle cells [13]. Other studies indicate that the application of NS1619, a specific BK_{Ca} channel opener which can induce apoptosis in ovarian cancer cells [14] and exposure of erythrocytes to ionomycin (Ca^{2+} ionophore which raises internal Ca^{2+} concentrations ($[Ca^{2+}]_i$)) induces cell shrinkage and apoptosis via BK_{Ca} [15]. During the later stages of apoptosis, it has been demonstrated that only cells with a decreased cytosolic concentration of K^+ exhibit caspase activity [16, 17], suggesting that the efflux of K^+ during AVD is permissive for caspase activation during the later stages of cell death.

The annexins are a structurally related family of Ca^{2+}-sensitive proteins which participate in a wide range of cellular functions, including cellular signalling, cell migration, proliferation, and apoptosis [18–21]. These proteins have been shown to interact with a number of ion channels and are important in both membrane trafficking and modifying of ion channel function [1, 22, 23]. Using a proteomics-based approach, which previously identified heme oxygenase-2 (HO-2) as O_2-sensing protein partner of BK_{Ca}, we also identified ANXA5 as a potential interacting protein of BK_{Ca} [24]. A more recent study has shown that this particular protein partnership also occurs in native tissues, namely, the mouse cochlea [25]. Based on the knowledge that BK_{Ca} channel activation can induce apoptosis in a number of different cell types and the fact that annexins bind to externalized phosphatidylserine in response to apoptosis, we have begun to investigate how the partnership between ANXA5 and BK_{Ca} might influence this process. It has been proposed that annexins have a binding affinity for EF-hand Ca^{2+}-binding proteins and α-subunit of the human BK_{Ca} (KCNMA1) has a similar domain within its Ca^{2+} sensing region [26].

The aim of this study was to validate the interaction of ANXA5 and $BK_{Ca}\alpha$-subunit ($BK_{Ca}\alpha$), which was previously suggested by proteomics [24], to determine the biophysical consequences of the $BK_{Ca}\alpha$/ANXA5 partnership, in particular to elucidate the structural elements required, and to define the impact of this partnership on apoptosis.

2. Materials and Methods

2.1. Cell Culture. The studies were performed on both wild type HEK 293 cells and HEK 293 cell line stably expressing the human $BK_{Ca}\alpha$ subunit (see [24, 27–31]). HEK 293 cells were maintained in Earle's minimal essential medium (containing L-glutamine) supplemented with 10% fetal calf serum, 1% antibiotic/antimycotic, and 1% nonessential amino acids (Gibco BRL, Strathclyde, UK) in a humidified incubator gassed with 5% CO_2/95% air. Where indicated, apoptosis was induced by 48 h of serum deprivation (SD). Knockdown and overexpression of ANXA5 were achieved by transient transfection of cells with specific siRNA and ANXA5 plasmid DNA, respectively. Transfection was achieved using the Amaxa Nucleofector Kit V supplied with green fluorescent protein (GFP) for positive identification of transfected cells, following the manufacturer's protocols (Amaxa Biosystems, Germany). 24 h before performing the electrophysiological experiments, cells were passaged and plated onto glass coverslips, cultured as above, and then transferred into a continuously perfused (5 mL·min^{-1}) recording chamber (volume *ca.* 200 μL) mounted on the stage of an inverted microscope equipped with phase-contrast and fluorescent optics for (Olympus CK40).

2.2. Electrophysiological Recordings. $BK_{Ca}\alpha$ currents were recorded from inside-out membrane patches excised from the stably expressing the wild type HEK293 cells, transiently transfected with the ANXA5 siRNA or the ANXA5 plasmid. The pipette and bath solutions contained (in mM) 10 NaCl, 117 KCl, 2 $MgCl_2$, 11 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.2) with $[Ca^{2+}]_o$ at 1.2 mM and $[Ca^{2+}]_i$ adjusted to the quasiphysiological level of 300 nM using ethylene-glycol-tetra-acetic acid (EGTA), except when experiments were performed at the different range of Ca^{2+} concentration (10 nM–1 mM). The CO donor molecule, tricarbonyldichlororuthenium (II) dimer ($[Ru(CO_3)Cl_2]_2$, Sigma-Aldrich, Poole, Dorset, UK) (CORM-2), was employed to release free CO in the experimental solution [32].

All recordings were performed at the room temperature (22 ± 0.5°C) using an Axopatch 200 A amplifier and Digidata 1320 A/D interface (Axon Instruments, Forster City, CA, USA). $BK_{Ca}\alpha$ channel macroscopic currents were recorded using a protocol of 8 s voltage ramp from −30 mV to 120 mV, followed by three, 250 ms steps to +40, +60, and +120 mV, repeated at 0.1 Hz. All voltages are reported with the respect to the inner membrane leaflet (−Vp). All recordings were filtered with 8-pole Bessel filter at 5 kHz and digitized at 10 kHz. Half-activation voltages (Va_{50}) and half-activation concentrations (EC_{50}) were obtained from the current-voltage and concentration-response curves, respectively, and were fitted using Hill equation. Statistical comparisons were performed using one-way ANOVA and the differences were considered significant at the level of $p < 0.05$.

2.3. Caspase Assays. Apoptosis studies were performed using the Caspase-Glo 3/7 assay system (Promega). HEK 293 BK_{Ca} Cells and HEK 293 BK_{Ca} cells transfected with ANXA5 siRNA (see above) were plated into 96-well white walled plates, in triplicate for each experimental condition. The following day apoptosis was induced by replacing the media

with DMEM supplemented with 8% mannitol (serum-withdrawal media). Cells were then analysed for caspase-3/7 activity at 24-hour and 48-hour time points. Briefly, 100 μL of Caspase-Glo 3/7 reagent was added to each well and the plate was shaken at 300 rpm for 30 seconds; the plates were then incubated at room temperature for one hour before being assayed in a plate reading luminometer (Fluroskan Ascent FL, Thermo Labsystems). Duplicate plates were seeded at the start of all our apoptosis experiments; this allowed us to normalize our luminescence readings to cell count number. Cell count number was determined by using CyQUANT cell proliferation assay (Invitrogen), following the manufacturers' instructions.

2.4. Reciprocal Coimmunoprecipitations. Wild type HEK 293 cells were seeded into T75 flask and grown to about 80% confluency. The cells were then washed twice with ice cold PBS before adding 1 mL of ice cold RIPA buffer containing protease inhibitor cocktail (Sigma) to the cells. The cells were then scrapped from the flask and transferred to a precooled centrifuge tube and placed on a rocker for 30 minutes at 4°C. The lysate was then centrifuged at 14,000 ×g for 15 minutes and the supernatant transferred to a fresh tube. Protein G Dynabeads (Invitrogen) was prepared following the manufacturers' instructions, 50 μL of prepared Dynabeads was resuspended in 100 μL citrate phosphate buffer (pH 5), and either 5 μg of $BK_{Ca}\alpha$ (Alamone) or ANXA5 (Santa Cruz) antibody was added. The tubes were then incubated with rotation for 40 minutes at room temperature. Ig-Dynabeads complex was then washed 3 times in citrate phosphate buffer (pH 5) containing 0.01% Tween-20 using a magnetic stand to capture the beads. After the final wash precleared lysate (protein samples incubated with prepared Dynabeads to reduce nonspecific binding) was added to Ig-Dynabeads complex and incubated with rotation for a further hour at room temperature. The beads were then washed 3 times in 1 mL of PBS and the protein complex eluted from the beads by adding 20 μL 0.1 M citrate buffer (pH 2-3) and incubating for 2 minutes. Finally the pH of the eluate was adjusted by adding 1 M Tris pH 7.5. BK_{Ca} and ANXA5 were detected using standard western blotting techniques.

2.5. Immunocytochemistry. Recombinant HEK 293 $BK_{Ca}\alpha$ cells were plated onto coverslips for immunostaining. Media was aspirated from the dishes and cells were fixed with ice cold methanol for 10 minutes, followed by 3 washes with PBS. Cells were then blocked with 5% BSA for 1 hour at room temperature before applying the primary antibodies in 1% BSA. Both anti-$BK_{Ca}\alpha$ (Alamone) and anti-ANXA5 antibodies were used at 1 : 200 dilution. Antibodies were incubated overnight at 4°C with gently rocking followed by 3 washes with PBS. Following the final PBS wash appropriate TRITC and FITC conjugated secondary antibodies (Alexa) were added to the cells at a dilution of 1 : 500 and incubated at room temperature for 2 hours. The cells were washed 3 times with PBS and the coverslip was fixed onto a slide using VECTASHIELD (containing DAPI) and images taken with an Olympus BX61 camera (Olympus).

2.6. Surface Biotinylation and Western Blotting. HEK 293 $BK_{Ca}\alpha$ cells were seeded into T75 flasks and incubated for 48 hours in either normal or serum-withdrawal media. Pierces cell surface protein isolation kit (Thermo Scientific) was then used to biotinylate and extract cell surface membrane proteins. Cells were washed twice in ice cold PBS and 10 mL of biotin solution was added to each flask. The flasks were incubated at 4°C for 30 minutes on a rocking platform before terminating the reactions by adding 0.5 mL of quenching solution. Cells were gently scrapped into solution and pelleted by centrifugation before being resuspended in lysis solution. Cells were then incubated on ice for 30 minutes, vortexing every 5 minutes for 5 seconds. The cell lysate was then centrifuged at 10,000 ×g for 2 minutes and the biotin labelled proteins were isolated from the supernatant using a NeutrAvidin agarose column. Protein samples were separated by electrophoresis on 10% acrylamide gels and annexin A5 was detected by western blotting using an anti-ANXA5 antibody purchased from Santa Cruz antibodies.

3. Results

3.1. Interaction and Colocalization of $BK_{Ca}\alpha$ with ANXA5. Although binding of ANXA5 to phosphatidylserine that results in its exposure to the external leaflet of the plasma membrane is routinely used as an assay of cell death, little information is available on the potential role of annexins in the earlier stages of apoptosis. The effect that serum deprivation had on ANXA5 protein levels within the cell was elucidated using western blotting (Figure 1). Western blots of total lysates of cells incubated for 48 h in either serum-deprived (SD) or normal medium showed no difference in total ANXA5 expression levels (Figure 1(a)). However, western blot of extracts of cells, which had been surface biotinylated, demonstrated that serum deprivation resulted in a dramatic rise in the amount of ANXA5 at the outer membrane leaflet. These data evidently demonstrate that significantly increased proportion of ANXA5 becomes exposed to the outer side of the plasma membrane during the initial stages of apoptosis. To test directly the extent to which ANXA5 and $BK_{Ca}\alpha$ channels contribute to the early stages of apoptosis, caspase-3/7 assays were performed at 24 and 48 h following serum deprivation (Figure 2). Within 24 h serum deprivation resulted in a significantly larger increase of caspase-3/7 activation in $BK_{Ca}\alpha$ HEK 293 cells than it did in the wild type HEK 293 cells; this difference was maintained up to the 48 h time point. This initial observation provided a decent evidence that $BK_{Ca}\alpha$ contributed to the early apoptotic response of HEK 293 cells. In the absence of serum deprivation the effect of ANXA5 siRNA knockdown demonstrated by immunocytochemistry (Figure 2(c)) was to increase significantly the caspase-3/7 activity in $BK_{Ca}\alpha$ HEK 293 cells only (Figure 2(b)). Importantly, when cells were deprived of serum, ANXA5 knockdown did not affect further the caspase-3/7 activation of either cell type at 24 h but induced a dramatic amplification of this apoptotic response at 48 h in $BK_{Ca}\alpha$ HEK 293 cells only (Figure 2(d)).

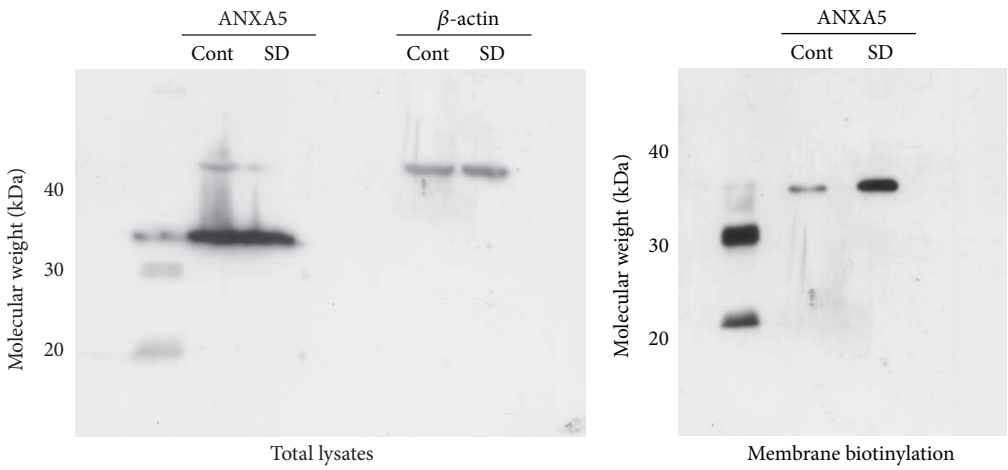

Figure 1: Serum deprivation-evoked apoptosis results in early localization of ANXA5 to plasma membrane outer leaflet. (a) The total cell lysates of $BK_{Ca}\alpha$ HEK239 cells cultured for 48 h in either normal (Cont) or serum-deprived (SD) media where prepared and western blotted to quantify the amounts of ANXA5 and β-actin. (b) HEK 293 cells stably expressing the human $BK_{Ca}\alpha$ subunit where incubated for 48 hours in before being incubated with biotin reagent. Extracts where prepared and normalized for protein content before the cell surface proteins were isolated with avidin-agarose beads. ANXA5 was detected on the blot using a specific antibody.

3.2. ANXA5 Modulates $BK_{Ca}\alpha$ Channel Function. A direct protein interaction between ANXA5 and $BK_{Ca}\alpha$ was validated using reciprocal coimmunoprecipitations of solubilized membrane proteins (Figure 3(a)) and immunocytochemistry (Figure 3(b)). The functional consequences of such physical interactions were then investigated using patch-clamp electrophysiology.

One of the most important physiological characteristics of BK_{Ca} channels is their ability to open in response to increase of $[Ca^{2+}]$. Knockdown of ANXA5 using siRNA (confirmed by the immunocytochemistry shown in Figure 2(c)) resulted in a significant leftward shift of Ca^{2+} concentration-response curve of $BK_{Ca}\alpha$ channel activity (Figure 4). This was reflected in a significant change in the mean Va_{50} values (Figure 4 and Table 1), indicating increased Ca^{2+} sensitivity of $BK_{Ca}\alpha$. Conversely, overexpression of ANXA5 (by transfection with a mammalian expression plasmid containing human ANXA5) evoked a significant rightward shift of Ca^{2+} concentration-response curve (Figure 4) and a change in Va_{50} values (Figure 4 and Table 1), indicating a decreased Ca^{2+} sensitivity. The alterations of Ca^{2+} sensitivities evoked by manipulation with ANXA5 expression occur within the physiological $[Ca^{2+}]_i$ window of between 100 nM and 1 μM. Ca^{2+} sensitivities of $BK_{Ca}\alpha$ channels in the cells transfected with a scrambled siRNA or empty vector were not significantly different from those of the wild type HEK 293 cells (data are not illustrated).

The fact that sensitivity of $BK_{Ca}\alpha$ to Ca^{2+} was effectively modulated by ANXA5 expression was also approved by the observation that the control $BK_{Ca}\alpha$ activity, at the beginning of each trace in the presence of 300 nM $[Ca^{2+}]_i$, was higher or lower than normal. Independently of Ca^{2+} sensitivity relative activation of $BK_{Ca}\alpha$ by the carbon monoxide donor CORM-2 (30 μM) was unaffected (Figure 4 and Table 2).

4. Discussion

During the course of the lifetimes many cells of our body undergo programmed death and replacement by the new ones; this process is finely tuned by a variety of mechanisms within the body. However when this strictly controlled cell death/regeneration is impaired it can lead to the serious conditions such as AIDS and cancer. The earliest observable sign that a cell will undergo programmed cell death is cell shrinkage. This is primarily caused by K^+ efflux through any of a steadily increasing number of K^+ channels [8–12]. One of such K^+ channels is BK_{Ca} of which activity is controlled by $[Ca^{2+}]_i$ that contributes to both maintenance of the resting membrane potential and repolarisation phase after action potential. Here we identify ANXA5 as a protein which physically communicates with $BK_{Ca}\alpha$. The main aim of the present research was to understand how these two proteins interact at the functional level and to determine whether this interaction might have consequences for cell death. Previously it was considered that annexins have only been implicated in the last stage of apoptosis, but our data show that ANXA5 has a functional role in the earlier stages of apoptosis when caspases are activated, and this process is significantly enhanced in the presence of $BK_{Ca}\alpha$. Based on this new information we propose that during resting conditions ANXA5 interacts with $BK_{Ca}\alpha$ on the intracellular side of the membrane; this protein partnership causes reduction of $BK_{Ca}\alpha$ channels sensitivity to $[Ca^{2+}]_i$; thus ANXA5 has an inhibitory effect on $BK_{Ca}\alpha$ channels' activity and keeps K^+ efflux to the minimum. In contrast,

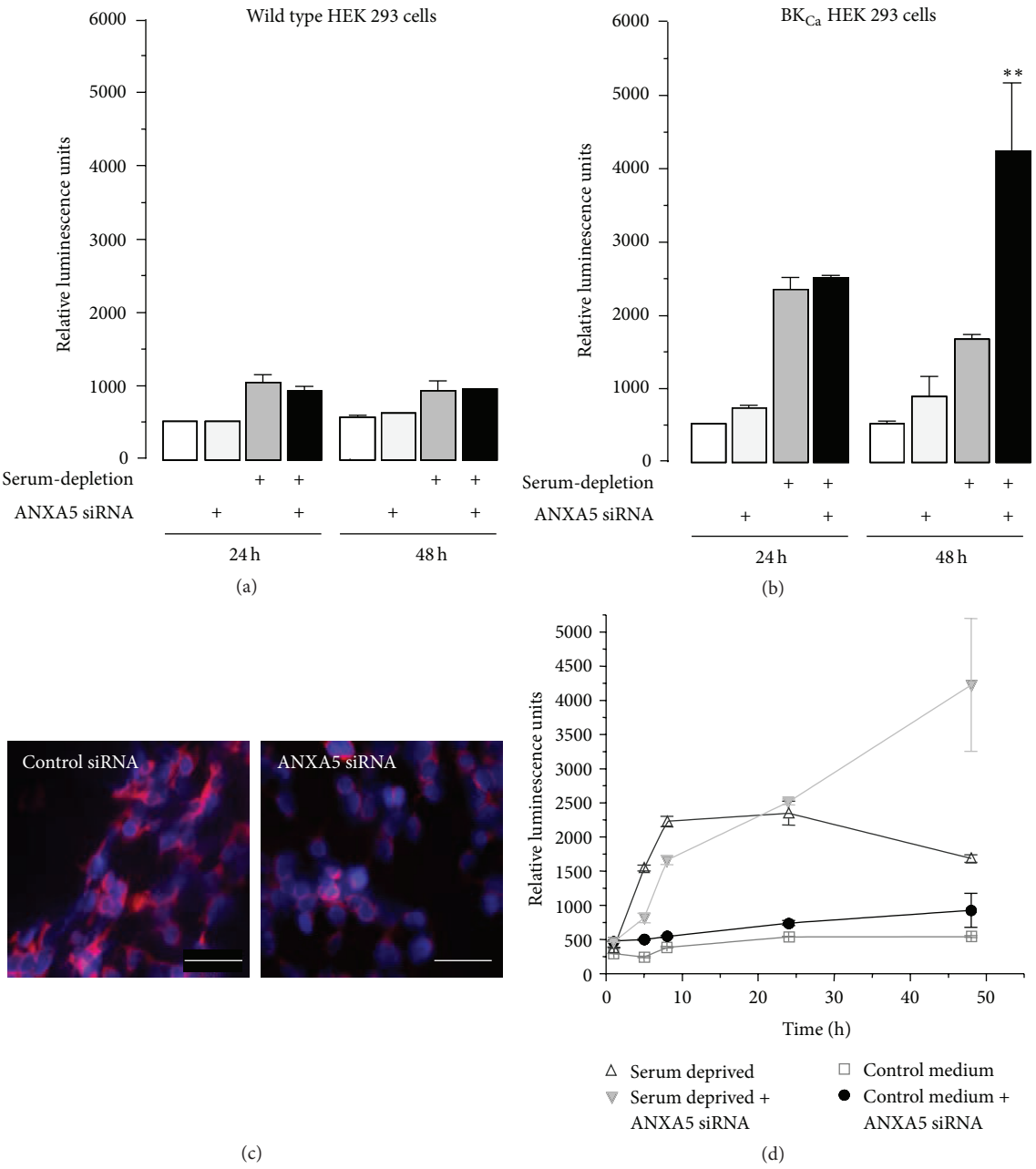

Figure 2: $BK_{Ca}\alpha$ augments the apoptotic response to serum deprivation: a response which is amplified by ANXA5 knockdown. (a) Caspase-3/7 activity in wild type HEK 293 cells was quantified as relative luminescence units at 24 h and 48 h. Apoptosis was induced by serum deprivation at time = 0 h, with and without ANXA5 knockdown using siRNA (shown beneath the bars). (b) As (a), but using $BK_{Ca}\alpha$ HEK239 cells. Asterisks indicate that, after 48 h, ANXA5 knockdown enhanced the serum-deprived-evoked caspase activation ($p < 0.05$, $n = 3$). (c) Immunocytochemistry using an antibody directed against ANXA5 in $BK_{Ca}\alpha$ expressing HEK 293 cells following treatment with control (left) and ANXA5 (right) siRNA. (d) Time course of caspase-3/7 activity, $BK_{Ca}\alpha$ HEK 293 cells (± ANXA5 knockdown) incubated in the presence of normal medium (control), or serum-deprived media (8% Mannitol, DMEM), over a 48-hour period.

Table 1: Ca^{2+} concentration-response EC_{50} values for $BK_{Ca}\alpha$ channel of the control, ANXA5 siRNA knockdown, and ANXA5 overexpression models.

	Control ($n = 8$)		ANXA5 siRNA knockdown ($n = 8$)		ANXA5 overexpression ($n = 8$)	
	I/I_{max} at +60 mV	Va_{50} (mV)	I/I_{max} at +60 mV	Va_{50} (mV)	I/I_{max} at +60 mV	Va_{50} (mV)
EC_{50} $[Ca^{2+}]$ (μM)	1.79 ± 0.57	2.07 ± 0.39	0.53 ± 0.09*	0.62 ± 0.07*	3.58 ± 0.48*	4.42 ± 0.65*

*The values were considered as significantly different from the respective values of the control ($p > 0.05$).

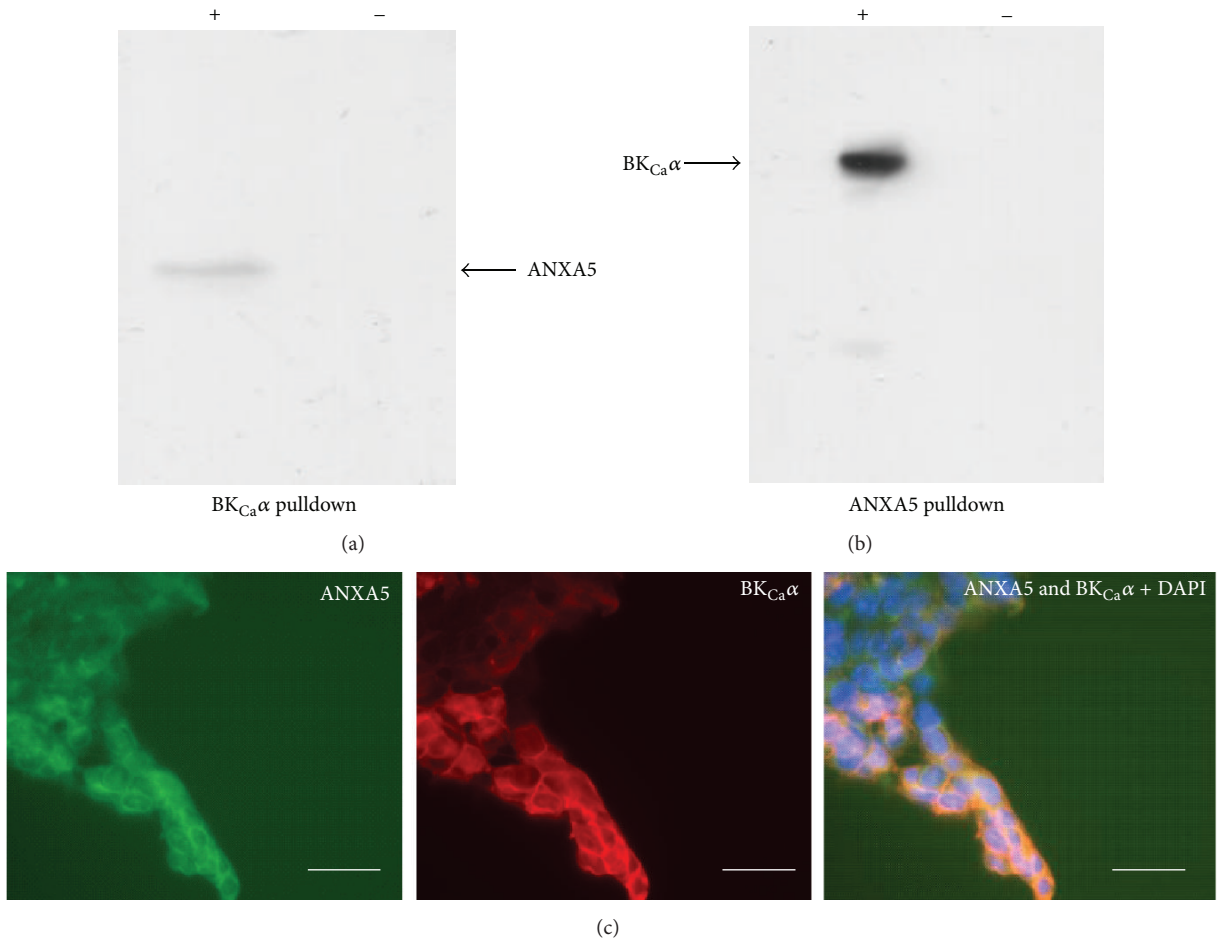

Figure 3: BK$_{Ca}\alpha$ subunit colocalizes with ANXA5. (a) Reciprocal coimmunoprecipitation from lysates of HEK 293 cells stably expressing BK$_{Ca}\alpha$ subunit with anti-BK$_{Ca}\alpha$ antibody (+). Bead controls (−) consisted of lysate mixed with DynaG beads lacking antibody. (b) Reciprocal coimmunoprecipitation from lysates of HEK 293 cells stably expressing BK$_{Ca}\alpha$ subunit with and anti-ANXA5 antibody (+) and bead control (−). (c) HEK 293 cells stably expressing BK$_{Ca}\alpha$ subunit were immunostained with anti-BK$_{Ca}\alpha$ subunit (1 : 250, red) and anti-ANXA5 (1 : 200 green) antibodies and cell nuclei (blue) where visualized with DAPI. Scale bars are 50 μM.

Table 2: Normalized current (I/I_{max}) at 60 mV and Va_{50} values for BK$_{Ca}\alpha$ channel of the control, ANXA5 siRNA knockdown, and ANXA5 overexpression models.

	Control ($n = 8$)		ANXA5 siRNA knockdown ($n = 8$)		ANXA5 overexpression ($n = 8$)	
	I/I_{max} at +60 mV	Va_{50} (mV)	I/I_{max} at +60 mV	Va_{50} (mV)	I/I_{max} at +60 mV	Va_{50} (mV)
[Ca^{2+}] 300 nM	0.25 ± 0.07	74.5 ± 4.6	0.38 ± 0.07	67.5 ± 2.8	0.19 ± 0.05*	79.5 ± 3.8*
CORM-2 30 μM	0.61 ± 0.1	55.9 ± 5.2	0.79 ± 0.06	44.1 ± 2.7	0.51 ± 0.06*	57.5 ± 2.5*
[Ca^{2+}] 1 μM	0.44 ± 0.08	60.0 ± 3.9	0.58 ± 0.10	54.8 ± 6.3	0.28 ± 0.03*	73.2 ± 2.7*

*The values were considered as significantly different from the respective values of ANXA5 siRNA knockdown ($p > 0.05$).

when cells are exposed to the serum deprivation, two important processes are initiated. Firstly, $[Ca^{2+}]_i$ runs up and activates BK$_{Ca}\alpha$ [6, 7]. Secondly, ANXA5, which is associated with the phosphatidylserine, flips to the outer membrane leaflet, resulting in uncoupling of BK$_{Ca}\alpha$ /ANXA5 physical interaction and a consequent progressive increase of Ca^{2+} sensitivity of BK$_{Ca}\alpha$. This augments BK$_{Ca}\alpha$ activity and results in an amplified K^+ efflux which accelerates AVD, caspase activation, and cell death. This scheme is represented pictorially in Figure 5.

Although these experiments have been conducted in a heterologous expression system, they provide conception for how we view the control of apoptosis in a variety of tissues which express BK$_{Ca}$ channels at a high level. For example,

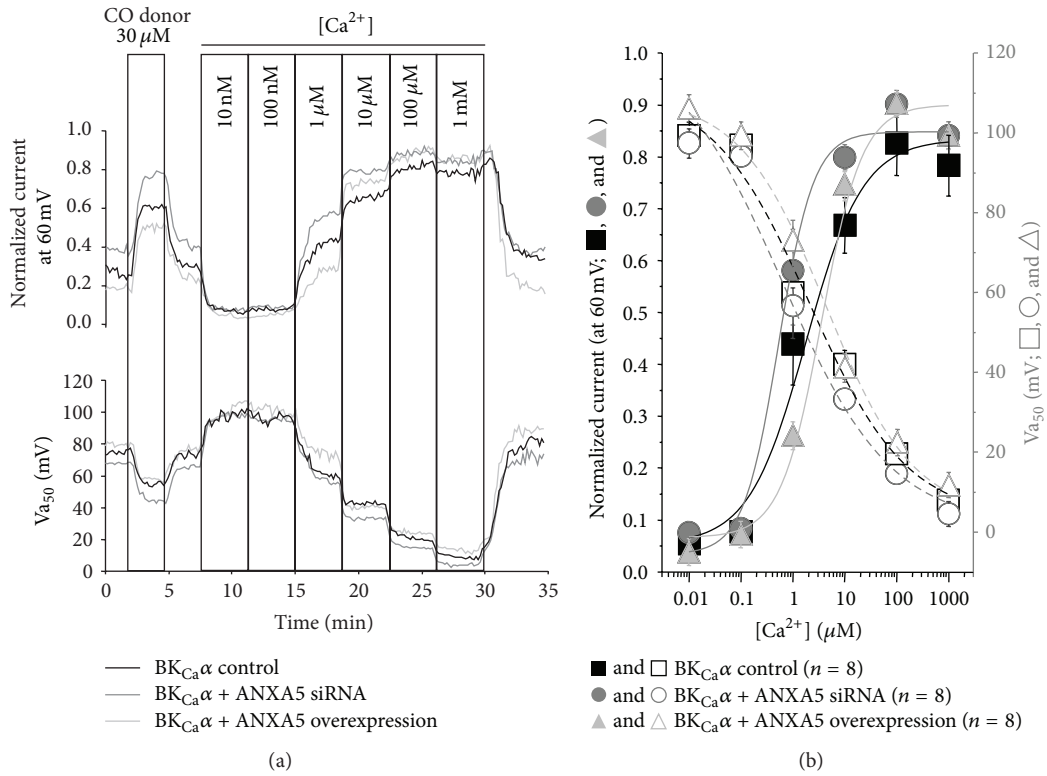

FIGURE 4: $BK_{Ca}\alpha$ channel function is modulated by its interaction with ANXA5 at different concentrations of Ca^{2+}. (a) Time course showing the stimulatory effect of 30 μM CORM-2 and the sequential increase of $[Ca^{2+}]$ in the concentration range 10 nM–1 mM on $BK_{Ca}\alpha$ subunit channel normalized current and Va_{50} in the control (black line), in the ANXA5 knockdown model achieved by transfection with siRNA (dark grey line), and in the ANXA5 overexpression model achieved by transfection with the DNA plasmid (light grey line). (b) Mean (± SEM) concentration-response plots displaying the stimulation effect of the sequential increase of $[Ca^{2+}]$ in the concentration range 10 nM–1 mM on $BK_{Ca}\alpha$ channel normalized current at the holding voltage of 60 mV in the wild type (black square and a solid black line, $n = 8$), in the ANXA5 knockdown model achieved by transfection with siRNA (grey circle and a solid grey line, $n = 8$), in the ANXA5 overexpression model achieved by transfection with ANXA5 DNA plasmid (light grey triangle and a solid light grey line, $n = 8$), on Va_{50} of $BK_{Ca}\alpha$ channels in the wild type (white square and a dashed black line, $n = 8$), in the ANXA5 knockdown model achieved by transfection with siRNA (white circle and a dashed grey line, $n = 8$), and in the ANXA5 overexpression model achieved by transfection with ANXA5 DNA plasmid (white triangle and a dashed light grey line, $n = 8$).

apoptosis is an important component of normal brain development and in the cerebellum such programmed cell death occurs in different cell layers at different points of pre- and postnatal development. Indeed, the level of apoptosis in the external granule cell layer gradually increases from week 26 until birth, whilst cells in the Purkinje layer only start to die postnatally [33, 34]. Such patterns of apoptosis can be modulated by extrinsic factors. For example, Purkinje cell apoptosis is enhanced *in utero* during fetal alcohol syndrome [35]. Since $BK_{Ca}\alpha$ channels are expressed in Purkinje neurones [36] and their activity is known to be augmented by ethanol [37], it seems likely that alcohol-evoked increases in $BK_{Ca}\alpha$ activity may contribute to the enhanced apoptosis observed in this neuronal cell layer.

Therefore, the data of this study show that the physical partnership of ANXA5 and $BK_{Ca}\alpha$ channels results in decreased Ca^{2+} sensitivity of the latter under conditions close to the physiological. The proposed mechanism may be particularly important for the programmed cell death mechanism, where membrane flippage removes the ANXA5 from the vicinity of the intracellular C-terminal of $BK_{Ca}\alpha$, resulting in augmentation of K^+ efflux and subsequent apoptosis.

Abbreviations

ANXA5:	Annexin A5
AVD:	Apoptotic volume decrease
$BK_{Ca}\alpha$:	Large conductance voltage-activated and calcium-sensitive potassium channel subunit
HEK 293 cells:	Human embryonic kidney cells
HO-2:	Heme oxygenase-2
TEA:	Tetraethylammonium
FCCP:	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
HEPES:	N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid
$[Ca^{2+}]_o$:	Extracellular calcium concentration

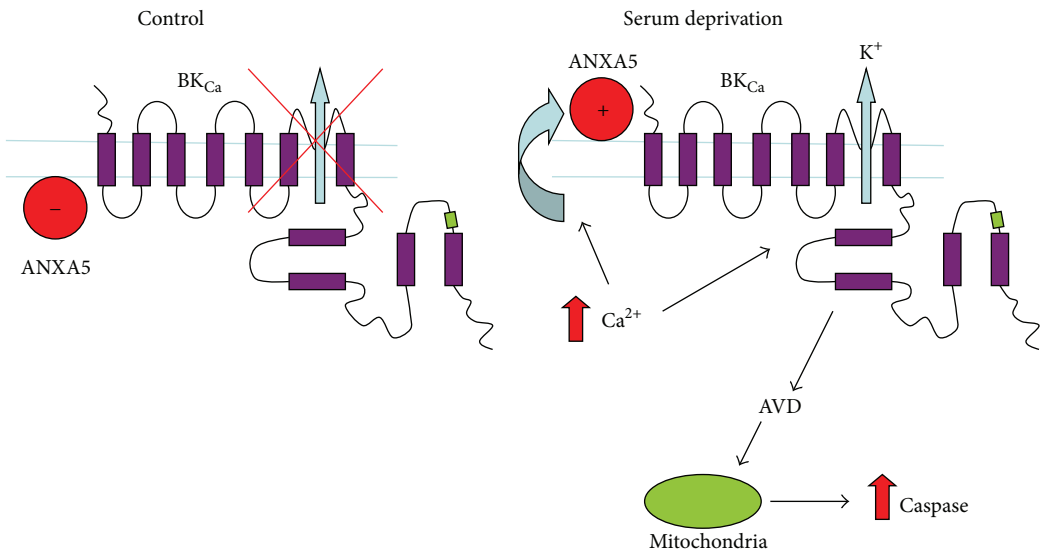

Figure 5: Proposed model to account for ANXA5/BK$_{Ca}\alpha$ interaction before and during apoptosis. During resting conditions, intracellular ANXA5 interacts with BK$_{Ca}\alpha$ and this partnership maintains the relatively low sensitivity to $[Ca^{2+}]_i$ of BK$_{Ca}\alpha$ channels. Thus, ANXA5 has an inhibitory effect on BK$_{Ca}\alpha$ channel activity and restricts K^+ efflux. During serum deprivation, $[Ca^{2+}]_i$ increases and the channel begins to activate [6, 7] and ANXA5, which is associated with the phosphatidylserine, flips to the outer membrane leaflet, resulting in the physical uncoupling of BK$_{Ca}\alpha$/ANXA5 interaction and a consequent increase in Ca^{2+} sensitivity. This augments channel activity further and results in an amplified K^+ efflux and accelerates AVD, caspase activation, and cell death.

$[Ca^{2+}]_i$: Intracellular calcium concentration
EGTA: Ethylene-glycol-tetra-acetic acid
CORM-2: Tricarbonyldichlororuthenium (II) dimer $([Ru(CO_3)Cl_2]_2$.

Competing Interests

The authors declare no competing interests.

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Review Article

Role of Endogenous Sulfur Dioxide in Regulating Vascular Structural Remodeling in Hypertension

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Sulfur dioxide (SO_2), an emerging gasotransmitter, was discovered to be endogenously generated in the cardiovascular system. Recently, the physiological effects of endogenous SO_2 were confirmed. Vascular structural remodeling (VSR), an important pathological change in many cardiovascular diseases, plays a crucial role in the pathogenesis of the diseases. Here, the authors reviewed the research progress of endogenous SO_2 in regulating VSR by searching the relevant data from PubMed and Medline. In spontaneously hypertensive rats (SHRs) and pulmonary hypertensive rats, SO_2/aspartate aminotransferase (AAT) pathway was significantly altered. SO_2 inhibited vascular smooth muscle cell (VSMC) proliferation, promoted apoptosis, inhibited the synthesis of extracellular collagen but promoted its degradation, and enhanced antioxidative capacity, thereby playing a significant role in attenuating VSR. However, the detailed mechanisms needed to be further explored. Further studies in this field would be important for the better understanding of the pathogenesis of systemic hypertension and pulmonary hypertension. Also, clinical trials are needed to demonstrate if SO_2 would be a potential therapeutic target in cardiovascular diseases.

1. Introduction

Since the 1980s, studies have shown that the endogenous gaseous molecules nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) are endogenously produced and have a wide range of biological effects including vasodilation and inhibition of vascular smooth muscle cell (VSMC) proliferation and platelet aggregation. They have an important physiological and pathological significance. Gaseous signal molecules share the following characteristics [1]: (1) small gaseous molecules with low molecular weight; (2) freely passing through the cell membrane, independent of the specific cell receptor; (3) being endogenously generated under the enzyme catalysis and regulated by metabolic pathways; (4) having a clear specific function at physiological concentration; and (5) exerting biological effects, having specific cellular and molecular targets. Accordingly, gaseous signal molecule pathways and the physiologic and pathophysiologic significance have become hot topic in cardiovascular system and other systems [2].

In recent years, studies showed endogenous SO_2 pathways in tissues of rats including cardiovascular tissues [3, 4]. The physiological effects of SO_2 were also confirmed, including vasodilation [4–6], negative regulation of cardiac function [7], and regulation of lipid metabolism [8–10]. Also, the pathophysiologic role of SO_2 in the processes of vascular structural remodeling (VSR) [11, 12], inflammatory response [13], and oxidative response was indicated [12, 13]. In this review, the research progress of SO_2 in regulating VSR was summarized.

2. The Property of SO_2

2.1. General Physical and Chemical Properties of SO_2. SO_2 is a colorless gas accompanied by a pungent odor [14]. The relative molecular mass of SO_2 is 64 g/mol, and the ionic

charge of sulfur is 4+. Hence, it has both oxidative ability and reducibility. Reduced sulfur has a longer half-life in the body, and thereby it has a strong antioxidant effect [15]. In the atmosphere, however, SO_2 is oxidized to sulfuric acid or sulfate aerosols which severely pollute the atmosphere [16].

2.2. Generation and Metabolism of Endogenous SO_2. Endogenous SO_2 is produced during metabolism of sulfur-containing amino acids (Figure 1). Firstly, sulfur-containing amino acid is metabolized to L-cysteine that is then oxidized into L-cysteinesulfinate catalyzed by cysteine dioxygenase (CDO). L-Cysteinesulfinate, an analogue of L-asparaginic acid, can be transaminated into β-sulfinylpyruvate catalyzed by aspartate aminotransferase (AAT), which is then spontaneously decomposed into pyruvate and SO_2 [15, 17, 18]. *In vivo*, SO_2 produces HSO_3^-/SO_3^{2-} (molar ratio of 1 : 3) in water, which can be oxidated into SO_4^{2-} by sulfite oxidase and then excreted through the kidneys [19]. However, there are still some gaseous forms of SO_2 existing in the body. On the other hand, L-cysteinesulfinate can be also decarboxylated into CO_2 and hypotaurine by cysteinesulfinate decarboxylase (CSD). A large majority of hypotaurine can be further oxidized into taurine, which takes place during the metabolism of bile acids. Studies have found that taurine functions as an inhibitory neurotransmitter, membrane stabilizing factor, and broad spectrum cytoprotector, and so forth [20, 21].

In addition, endogenous SO_2 can be generated by intracellular H_2S. Firstly, H_2S is oxidized to thiosulfate by heme compounds, metal-protein complexes, and ferritin. Thiosulfate reacts with reduced glutathione under the catalysis of thiosulfate reductase (TSR) to form sulfite or SO_2 [19, 22]. Meanwhile, Mitsuhashi et al. [23] found that activated neutrophils in mammals could be catalyzed by NADPH to convert H_2S into sulfites through oxidative stress.

2.3. Detection Methods of SO_2. In 2003, Balazy et al. [24] detected SO_2 in the coronary artery and myocardium using gas chromatography/mass spectrometry. In 2008, Du et al. [4] first found that there was an endogenous SO_2/AAT pathway in cardiovascular system and other systems, with the highest SO_2 content in the arteries. In arteries, the highest SO_2 content was found in the aorta (5.55 ± 0.35 μmol/g protein), followed by the pulmonary artery (3.27 ± 0.21 μmol/g protein), mesenteric artery (2.67 ± 0.17 μmol/g protein), renal artery (2.50 ± 0.20 μmol/g protein), and the caudal artery (2.23 ± 0.19 μmol/g protein). The content in heart (1.78±0.12 μmol/g protein), liver (1.74 ± 0.16 μmol/g protein), lung (1.42 ± 0.11 μmol/g protein), and kidney (0.95±0.11 μmol/g protein) was relatively low. The content of SO_2 in plasma was 15.54 ± 1.68 μmol/L.

AAT, a key enzyme in the generation of endogenous SO_2, is distributed in mammals [25]. There are two kinds of isoenzymes AAT1 and AAT2. AAT1 is mainly located in the cytoplasm, and AAT2 is mainly in mitochondria [26]. However, the activity of AAT in tissues was not consistent with SO_2 tissue level. AAT activity was highest in heart tissue (4469 ± 278 U/g), followed by liver (1328 ± 198 U/g), kidney (381 ± 48 U/g), and lung tissue (175 ± 38 U/g). AAT activity value in the arteries was (188±30 U/g) in renal artery, (143±36 U/g) in caudal artery, (112±15 U/g) in mesenteric artery, (96±12 U/g) in pulmonary artery, and (88 ± 11 U/g) in the aorta. Serum AAT activity was (87 ± 18 U/g). The distribution of AAT mRNA levels was consistent with AAT activity in rats. In heart, liver, kidney, and other tissues, AAT mRNA levels were significantly higher than those found in the artery [3].

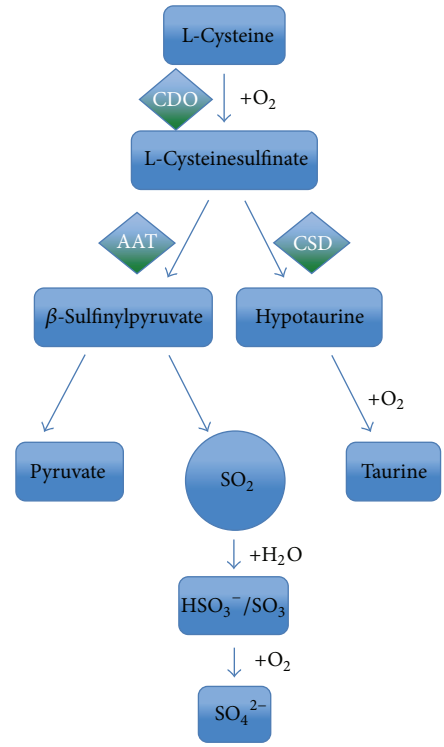

Figure 1: Endogenous generation of sulfur dioxide. Firstly, a sulfur-containing amino acid is metabolized to L-cysteine that is then oxidated into L-cysteinesulfinate under the role of CDO. L-Cysteinesulfinate, an analogue of L-asparaginic acid, can be transaminated into β-sulfinylpyruvate under the role of AAT, which is spontaneously decomposed into pyruvate and SO_2. *In vivo*, SO_2 can produce HSO_3^-/SO_3^{2-} (molar ratio of 1 : 3) after combining with water, which can be oxidated into SO_4^{2-} by sulfite oxidase and then excreted through the kidneys. On the other hand, L-cysteinesulfinate can be also decarboxylated into CO_2 and hypotaurine under the role of CSD. A large majority of hypotaurine can be further oxidized into taurine. SO_2: sulfur dioxide; CDO: cysteine dioxygenase; CSD: cysteinesulfinate decarboxylase; AAT: aspartate aminotransferase.

The synthesis of SO_2 is regulated by a variety of factors. Balazy et al. [24] showed that acetylcholine (Ach) could cause an increased synthesis of SO_2 in porcine coronary artery. However, Meng et al. [27] found that endogenous Ach could promote the generation of SO_2 in rat vascular tissue in cultured vascular endothelial and VSMCs in a dose-dependent manner, but noradrenaline (NE) suppressed the generation of SO_2. Factors regulating SO_2 generation need further studies.

3. Vascular Structural Remodeling

In 1989, Baumbach and Heistad [28] first proposed the concept of "vascular structural remodeling" when studying

the changes in cerebral artery of rats with chronic hypertension. With the wide use of pathological morphology, microstructure morphologic metrology, vascular perfusion *in vitro,* and other technologies, the study on VSR has been deepened. VSR is defined as any structural changes of blood vessels [29]. The most typical characteristics of VSR include intimal endothelial cell swelling, medial SMC hyperplasia, and adventitia extracellular matrix (ECM) deposition [29]. Gibbons and Dzau [29] pointed out that VSR was a dynamic process; it relied on the interaction among haemodynamics, other mechanical stimuli with locally produced growth factors, including signal perception, signal transduction, and synthesis and release of regulatory factors. This eventually leads to changes in the structure of the vessel wall, simultaneously accompanied by decreased vascular wall compliance, changes in the release of vasoactive substances, and other functional disorders.

4. SO_2 and Vascular Remodeling in Hypertension

4.1. The Role of SO_2 in the Development of Vascular Structural Remodeling in Systemic Hypertension. VSR is one of the important issues in pathogenesis of hypertension [30]. The characteristics of VSR in the process of hypertension include the hyperplastic medial SMCs, the swollen endothelial cells, and the increased ECM [31, 32]. Dzau et al. [33] summarized it up to four types: (1) both the media and intima are thickened; the inner diameter is shortened, resulting in an increased ratio of vessel wall thickness to inner diameter. This type of change is mainly due to the hypertrophy and proliferation of VSMCs. Or the proliferation may not be obvious; however, rearrangement of VSMCs and noncellular components may be significant; (2) both the inner and outer diameter are increased, suggesting that the hypertrophy of vessel wall is relatively mild. In this case, the ratio of vessel wall thickness to inner diameter is decreased. This change is due to the rearrangement of VSMCs and the proliferation is not obvious; (3) both the inner and outer diameter are decreased; and (4) the number of microcirculations is decreased.

SO_2 plays an important role in the development of VSR in hypertension. In spontaneously hypertensive rats (SHRs), Zhao et al. [34] found that SO_2 content and AAT activity in plasma and thoracic aorta were significantly decreased, but the ratio of vessel wall thickness and inner diameter was significantly increased, along with the accumulation of collagen I and collagen III in the aorta. However, intraperitoneal injection of SO_2 derivatives (Na_2SO_3/$NaHSO_3$, 0.54 mmol/kg: 0.18 mmol/kg, for five weeks) could significantly reduce the VSR and lower the blood pressure of SHRs. The results indicated that the downregulated endogenous SO_2 pathway was involved in the VSR in hypertension.

Excessive proliferation of VSMCs is one of the key mechanisms involved in hypertensive VSR. Under the hypertensive condition, VSMCs switch from a contractile to a synthetic phenotype, with a high rate of proliferation. The abnormal proliferation of VSMCs leads to a variety of cytokines secretion and a marked ECM production, which finally results in a decrease of lumen ratio and an increase of media-lumen ratio [35]. Zhao et al. [34] found that, in SHRs, aortic tunica media thickness and VSMCs proliferation index were significantly decreased after the exogenous supplement of SO_2 derivatives (0.54 mmol/kg: 0.18 mmol/kg, for five weeks). Liu et al. [36] found that SO_2 inhibited vascular proliferation by suppressing the progression of the cell cycle from G1 to S phase. Going further, AAT1 and AAT2 overexpression inhibited serum-induced VSMCs proliferation of rats, whereas AAT1 and AAT2 knockdown showed an opposite effect. These results showed that endogenous SO_2 had a negative effect on VSMCs proliferation. For the purpose of further exploring the potential mechanisms, a cell proliferation model stimulated by platelet derived growth factor-BB (PDGF-BB) was established. It was found that SO_2 dephosphorylated the active sites of ERK1/2, MAPK kinase 1/2, and c-Raf. Furthermore, SO_2 increased the AC activity of VSMCs of rats, thereby increasing the intracellular cAMP levels and activating PKA signaling molecules, which resulted in an increase in the Ser259 phosphorylation, an inhibitory site of Raf-1 molecule, thereby inhibiting the Raf-1 kinase activity and leading to the inactivation of the ERK/MAPK pathway [36]. The results suggested that SO_2 could attenuate VSR through suppressing proliferation of VSMCs. And cAMP/PKA/ERK/MAPK signaling was involved in the inhibitory effect of SO_2 on VSMC proliferation.

Numerous studies indicated that the VSMC apoptosis participated in the development of VSR. Zhao et al. [37, 38] found that, in SHRs, the exogenous supplement of SO_2 derivatives (0.54 mmol/kg: 0.18 mmol/kg, for five weeks) could promote apoptosis of VSMCs, and its mechanism might be related to inhibited Bcl-2 and activated Fas, ultimately inducing apoptosis through the molecule caspase-3. These data suggested that SO_2 inhibited VSR in hypertension in association with promoting VSMC apoptosis.

4.2. The Role of SO_2 in the Development of Vascular Structural Remodeling in Pulmonary Artery Hypertension. Pulmonary artery hypertension (PAH) is a pathophysiologic syndrome that leads to pulmonary vascular bed obstruction and progressively increased pulmonary vascular resistance, ultimately resulting in right heart failure. It is well known that pulmonary VSR contributes to all types of pulmonary hypertension. The main pathologic characteristics of pulmonary VSR in pulmonary artery hypertension include thickening of the adventitial, medial and/or intimal layer of the pulmonary arteries, elevated stiffening of the elastic pulmonary arteries, vascular bed occlusive lesions, proliferation of pulmonary fibroblasts, SMCs and endothelial cells, and excessive accumulation of ECM [39]. Interestingly, SO_2 plays an important role in the development of pulmonary artery hypertension, including hypoxic pulmonary hypertension, high pulmonary blood flow-induced pulmonary hypertension, and monocrotaline-induced pulmonary hypertension.

4.2.1. Downregulation of Endogenous SO_2 Pathway Is Involved in the Development of Pulmonary VSR in Hypoxic Pulmonary Hypertension. Hypoxic pulmonary vasoconstriction caused by acute hypoxia and hypoxic pulmonary vascular structural

remodeling caused by chronic hypoxia are the main pathophysiological processes of hypoxic pulmonary hypertension [40]. In comparison to the response of systemic blood vessels, the response of pulmonary vasculature to hypoxia has its specific characteristics; that is, pulmonary vascular reactive constriction in hypoxic condition can help maintain arterial oxygen saturation, which has an important physiological significance. However, if hypoxia persists, pulmonary arteriole will develop structural remodeling and pulmonary artery pressure will stay at a high level. And pulmonary hypertension will lead to aggravated hypoxia, and form a vicious circle. Interestingly, SO_2 content in the plasma and lung tissues from rats with hypoxic pulmonary hypertension was significantly decreased, as well as AAT1 mRNA expression and AAT activity [11]. In order to examine the significance of downregulated SO_2 pathway in the development of hypoxic pulmonary hypertension, investigators administered SO_2 derivatives to the hypoxic rats (0.54 mmol/kg: 0.18 mmol/kg, for three weeks). The results showed that it could significantly reduce mean pulmonary arterial pressure of the hypoxic rat [11]. Meanwhile, SO_2 derivatives significantly alleviated hypoxic pulmonary VSR, as demonstrated by a reduced percentage of muscular arteries and an increased percentage of nonmuscular arteries. Moreover, the relative medial thickness and relative medial areas of muscular arteries were decreased in hypoxic rats after SO_2 treatment. The above results implied that the reduction of endogenous SO_2 was involved in the development of hypoxic pulmonary VSR.

The studies have shown the advances in the mechanisms by which SO_2 plays a crucial role in the development of hypoxic pulmonary VSR. The deposition of ECM, including collagen, elastin, proteoglycans, and glycoproteins, participates in pulmonary VSR. Collagen deposition plays a crucial role in VSR. Exogenous supplementation of SO_2 derivatives could alleviate pulmonary VSR and attenuate pulmonary hypertension. Furthermore, SO_2 could significantly reduce the mRNA expressions of procollagens I and III in hypoxic pulmonary hypertension rats [11]. On the contrary, the mRNA levels of procollagens I and III were markedly increased in hypoxic pulmonary hypertensive rats administrated with hydroxamate (HDX), an inhibitor of AAT. The results indicated that SO_2 inhibited hypoxic hypertension-induced pulmonary VSR possibly by the suppression of collagen deposition. It is known that reduced collagen degradation has an important significance in pulmonary hypertensive VSR. It is regulated by the balance between matrix metalloproteinase (MMP) and the tissue inhibitor of metalloproteinase (TIMP). Zaidi et al. [41] found that MMPs activity was increased in the development of hypoxic pulmonary hypertension. Vieillard-Baron et al. [42] transferred the TIMP-1 gene into the lungs of rats by adenovirus, which successfully inhibited the expression and activity of MMPs, preventing from hypoxic pulmonary hypertension and pulmonary VSR. SO_2 was found to increase the mRNA ratio of MMP-13/TIMP-1 in pulmonary arteries of hypoxic pulmonary hypertensive rats by increasing MMP-13 mRNA level and decreasing TIMP-1 mRNA expression [11]. These data suggested that SO_2 attenuated hypoxic pulmonary VSR at least partly by reducing collagen degradation.

Additionally, SO_2 inhibited pulmonary VSMCs proliferation in association with the suppression of Raf-1 protein and the downstream ERK/MAPK pathway in hypoxic pulmonary hypertensive rats. Moreover, Bai and Meng [43] found that the exogenous inhalation of SO_2 downregulated mRNA levels of Bcl-2, increased protein expressions of p53 and Bax, and enhanced caspase-3 activity in rat lung, suggesting that SO_2 alleviated hypoxic pulmonary VSR by maintaining the balance between pulmonary vascular cell proliferation and apoptosis.

4.2.2. Downregulated Endogenous SO_2 Pathway Is Involved in the Development of Pulmonary VSR in High Pulmonary Blood Flow-Induced Pulmonary Hypertension. Pulmonary hypertension induced by high pulmonary blood flow is one of the most common complications of left-to-right shunt congenital heart disease. Increased pulmonary blood flow could result in pulmonary VSR and finally the development of pulmonary hypertension. Pronounced medial thickening and increased collagen content in pulmonary arteries were observed in cases of congenital heart disease accompanied with pulmonary hypertension [44]. Luo et al. [45] developed the rat model of high pulmonary blood flow-induced pulmonary hypertension by systemic-pulmonary shunting. They found that the content of SO_2, the mRNA, and protein expression of AAT2 as well as AAT activity in pulmonary vessels were decreased in pulmonary hypertensive rats. The administration of SO_2 derivatives (0.54 mmol/kg: 0.18 mmol/kg, for eight weeks) could significantly reduce the mean pulmonary artery pressure and improve the pulmonary vascular pathological changes of the rats, as demonstrated by the decreased percentage of muscularized pulmonary arteries. Furthermore, Liu et al. [46] found that SO_2 alleviated the protein expression of collagen I and collagen III.

However, the mechanisms by which SO_2 alleviates VSR are incompletely understood. It was suggested that endogenous SO_2 might alleviate pulmonary VSR via upregulating the reduced endogenous H_2S pathway [45, 47, 48]. To further investigate the signaling pathway by which SO_2 alleviated pulmonary VSR, Liu et al. used a Flexcell Fx-5000 Tension System to establish a cell model to mimic the mechanical stretching of high blood flow on vascular wall in high blood flow induced pulmonary hypertension. They found that mechanical stretching could downregulate endogenous SO_2/AAT1 pathway in pulmonary fibroblasts (PAFs) and then activate TGF-β1/Smad2/3 pathway, which ultimately resulted in an excessive collagen synthesis. Overexpression of AAT1, however, could antagonize the activation of TGF-β1/Smad2/3 pathway caused by mechanical stretching, which could be exaggerated by knockdown of AAT1. Furthermore, in the rat model of high pulmonary blood flow-induced pulmonary hypertension by surgical systemic-pulmonary shunting, the activated TGF-β1/Smad2/3 pathway could be inhibited by the administration of SO_2 derivatives [46]. The above results suggested that in high pulmonary blood flow-induced pulmonary hypertension the downregulated endogenous SO_2 activated TGF-β1/Smad2/3 pathway, which ultimately resulted in collagen remodeling.

4.2.3. Upregulated Endogenous SO_2 Pathway Is Involved in the Development of Pulmonary VSR in Monocrotaline-Induced Pulmonary Hypertension. Monocrotaline (MCT), a pyrrolizidine alkaloid, is metabolized into MCT pyrrole in the liver. The substance of MCT induces pulmonary hypertension in a rat model by causing pulmonary artery smooth muscle hypertrophy, inflammation, and endothelial cells injury. Mean pulmonary artery pressure (mPAP) and the ratio of right ventricle to left ventricle plus septum were increased markedly in the MCT-treated rats [12]. Meanwhile, pulmonary VSR developed accompanied with the increased SO_2 level, AAT activity, and mRNA expression. SO_2 administration significantly alleviated pulmonary VSR and reduced mPAP, while HDX aggravated them in the MCT-treated rats [12]. It suggested that endogenous SO_2 might play a protective role in the MCT-induced pulmonary VSR in pulmonary hypertensive rats.

Studies have suggested that enhanced oxidative stress by increasing superoxide anions and other reactive oxygen species production was involved in the pathophysiology of MCT-induced pulmonary hypertensive VSR [49, 50]. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione (GSH), and catalase (CAT) are antioxidant enzymes, and the malondialdehyde (MDA) is oxidation product. Although inhalation of SO_2 was considered to cause oxidative damage to mammals [51], the endogenous SO_2 at a low level was found to have an antioxidant effect in MCT-induced pulmonary hypertension. In MCT-induced pulmonary hypertensive rats [12], the activities of SOD, GSH-Px, GSH, CAT, and MDA were elevated in lung tissues in association with a protective upregulated SO_2 level. With supplement of SO_2 derivatives, the content of SOD, GSH-Px, and CAT went higher, while inhibition of endogenous SO_2 with HDX suppressed the activities of SOD and CAT [12]. These data indicate that upregulated SO_2 production plays a protective role in pulmonary VSR by promoting endogenous antioxidative capacity.

5. Conclusion

SO_2 was previously recognized as an industrial waste gas. Environmental SO_2 can cause oxidative damage to the cardiovascular system, respiratory system, and other systems [51]. SO_2 and sulphites can cause DNA damage in mammalian cells, such as chromatin breakage, sister chromatid exchange, micronucleus formation, DNA-protein cross-linking, and other stages [52, 53]. In recent years, however, SO_2 as discussed herein is found to have the characteristics of gaseous molecules such as endogenous continuous generation, fast transmission, extensive action, and low molecular weight and therefore play an important role in the physiology and pathophysiology of cardiovascular diseases.

VSR is an important pathogenic base of cardiovascular diseases, such as systemic hypertension and pulmonary hypertension. In the process of VSR in systemic hypertension or pulmonary hypertension, VSMC proliferation is excessive while apoptosis reduced, and extracellular collagen synthesis and degradation are imbalanced, leading to an excessive deposition of collagen in the vascular wall.

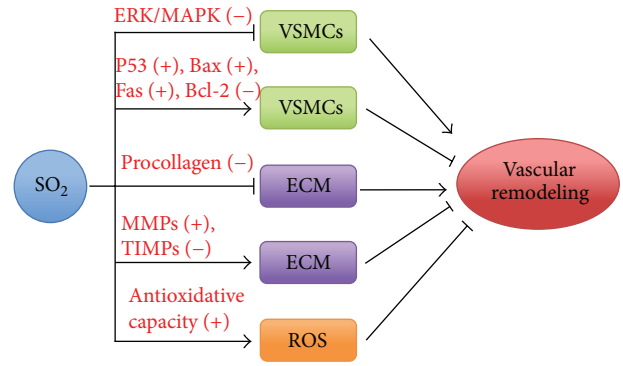

Figure 2: Mechanisms by which SO_2 attenuates VSR. SO_2 attenuates VSR by inhibiting proliferation of VSMCs, promoting apoptosis, inhibiting the synthesis of extracellular collagen, promoting the degradation of extracellular collagen, and increasing the antioxidative capacity. SO_2: sulfur dioxide; VSMCs: vascular smooth muscle cells; ECM: extracellular matrix; ROS: reactive oxygen species.

Although the increase in collagen content may help to resist the excessive stress in the vessel wall and maintain the integrity of blood vessels, the excessive collagen deposition can reduce the compliance and increase the resistance of the vascular wall, which has become an important factor in deterioration of systemic hypertension or pulmonary hypertension. Since VSR is a core pathological change of systemic hypertension and pulmonary hypertension, it is important to further explore its mechanisms in order to better understand its pathogenesis, which would ultimately provide scientific basis for potential therapeutic targets. *In vitro* and *in vivo* experiments demonstrated that downregulated SO_2/AAT pathway was involved in VSR of systemic hypertension and pulmonary hypertension. Furthermore, SO_2 has been shown to play an important regulatory role in VSR by inhibiting VSMC proliferation and the synthesis of extracellular collagen and promoting VSMC apoptosis and collagen degradation. In addition, SO_2 can also enhance antioxidative capacity, thereby playing a significant role in alleviating VSR (Figure 2). Hence, the regulatory role of endogenous SO_2 in VSR would help to further understand the pathogenesis and the potential therapeutic targets of VSR in systemic hypertension and pulmonary hypertension.

However, in the present studies, there are still some limitations. (1) In some studies, investigators used exogenous SO_2 derivatives to increase the level of SO_2 in rats or cells. Increasing endogenous SO_2 content by overexpressing AAT may be more in line with physiology. In addition, the AAT inhibitor HDX used to lower SO_2 content was not specific. Knocking down AAT to reduce endogenous SO_2 level together with a rescue experiment is likely a more convincing way to study the significance of endogenous SO_2. (2) As a gaseous molecule, the mechanisms by which SO_2 exerts its function need to be further explained. (3) Previous studies showed that gaseous molecules, such as NO, CO, H_2S, and SO_2, played an important role in the pathogenesis of VSR [54–57]. However, whether there are any interactions among these gaseous molecules when they function remains to be further studied.

(4) Effective treatment for hypertension and pulmonary hypertension is the clinical problem to be solved. Since the regulatory role of VSR in systemic hypertension and pulmonary hypertension is gaining more and more interest, the treatment of VSR has become a new area of the diseases. The regulation of endogenous SO_2 on VSR would likely become a new therapeutic target. Recently, benzothiazole sulfinate (BTS), a water-soluble SO_2 donor, was reported to exhibit slow and pH-dependent SO_2 release ability in aqueous solutions. And it also had SO_2-like vasorelaxant effect on rat aorta rings [58]. Although the biological activities and validity of BTS still need more exploration, the discovery of potentially better donors would undoubtedly provide a great help and potential for the future clinical application of SO_2. Meanwhile, although the *in vivo* experiments suggested that the exogenous supplement of SO_2 derivatives could play a protective role in hypertension and pulmonary hypertension, it still requires more validation in clinical trials to confirm its effects. Furthermore, there are still important things to note: (1) the suitable dosage. Although the current dosage of SO_2 derivatives (Na_2SO_3/$NaHSO_3$) used in animal model is 0.54 mmol/kg: 0.18 mmol/kg, we should still take into account the difference between rats and human beings; (2) the potential biomarker. Since the studies suggest that the development of hypertension and pulmonary hypertension is associated with alteration of SO_2/AAT pathway, it is speculated whether SO_2 or AAT would become a biomarker for the diseases. Of course, multiple clinical works need to be done to investigate the relationship between endogenous SO_2/AAT pathway and the severity or survival rate of the diseases.

In summary, SO_2 inhibited VSMC proliferation, promoted VSMC apoptosis, inhibited the synthesis of extracellular collagen but promoted its degradation, and enhanced antioxidative capacity, thereby playing a significant role in attenuating VSR, which ultimately alleviated systemic hypertension and pulmonary hypertension. Therefore, the understanding of the relationship between endogenous SO_2 and VSR offers important insight into the pathogenesis of VSR and provides a potential therapeutic target of systemic hypertension and pulmonary hypertension. In the future, with the depth of the study, we are confident to believe that greater progress would be made in the field of SO_2 biology and medicine to improve the prognosis of disease and enhance the life quality of patients.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Jia Liu and Yaqian Huang contributed equally to this work.

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Review Article

Targeting Nitric Oxide with Natural Derived Compounds as a Therapeutic Strategy in Vascular Diseases

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Within the family of endogenous gasotransmitters, nitric oxide (NO) is the smallest gaseous intercellular messenger involved in the modulation of several processes, such as blood flow and platelet aggregation control, essential to maintain vascular homeostasis. NO is produced by nitric oxide synthases (NOS) and its effects are mediated by cGMP-dependent or cGMP-independent mechanisms. Growing evidence suggests a crosstalk between the NO signaling and the occurrence of oxidative stress in the onset and progression of vascular diseases, such as hypertension, heart failure, ischemia, and stroke. For these reasons, NO is considered as an emerging molecular target for developing therapeutic strategies for cardio- and cerebrovascular pathologies. Several natural derived compounds, such as polyphenols, are now proposed as modulators of NO-mediated pathways. The aim of this review is to highlight the experimental evidence on the involvement of nitric oxide in vascular homeostasis focusing on the therapeutic potential of targeting NO with some natural compounds in patients with vascular diseases.

1. Introduction

Since 1992, when nitric oxide (NO) was nominated "molecule of the year" [1, 2], it continues to attract the interest of the scientific community. NO is the smallest gasotransmitter, recognized as an ubiquitous intercellular messenger; it is produced by three isoforms of NO synthases (NOS): endothelial NOS (eNOS) [3], neuronal NOS (nNOS) [4], and inducible NOS (iNOS) [5] and mitochondrial NOS (mtNOS) [6]. All NOS isozymes utilize L-arginine and oxygen and the reduced form of nicotinamide-adenine-dinucleotide phosphate (NADPH) as substrates and 6*R*-5,6,7,8-tetrahydro-L-biopterin (BH_4) as essential cofactor to generate NO and L-citrulline [7, 8]. Then, the main downstream signaling pathway carried out by the NO is the activation of soluble guanylyl cyclase (sGC), which in turn generates cyclic guanosine monophosphate (cGMP) [9] (Figure 1).

In the vascular system NO modulates blood flow [10], vascular tone [11], and platelet aggregation [12] exerting antihypertensive, antithrombotic, and atherosclerotic effects. It is also involved in the stimulation of the endothelial progenitor cells (EPCs) and proliferation of the smooth muscle cells (SMCs) [13]. Therefore, an impairment in the NO signaling is associated with the onset and perpetuation of the main clinical condition associated to cardiovascular diseases (CVDs) including endothelial dysfunction [14].

Given this premise, it is reasonable to consider NO as a therapeutic target for CVDs. Indeed, several approaches have been proposed to modulate NO pathways while preserving its physiological role [15]. From one side, the strategy consists in enhancing NO bioavailability, principally acting on NOS cofactors or avoiding NO breakdown; from the other side, different drugs act on the NO downstream signaling targets [16].

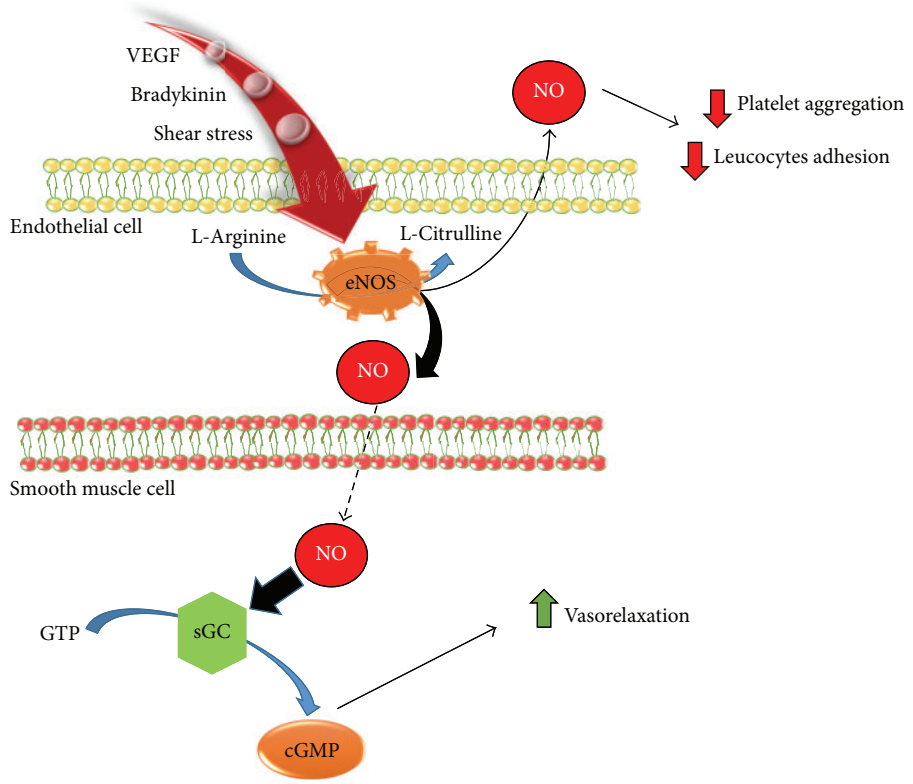

Figure 1: Nitric oxide generation: several stimuli induce eNOS activation and NO production in endothelial cells. NO diffusion in smooth muscle cells is responsible for cGMP generation and vasorelaxation.

Data from epidemiological studies have suggested the existence of a relationship between physical exercise and/or specific diets with a reduction of CVDs prevalence and incidence [17–20]. In addition, clinical trials and experiments in animal models have indicated NO as the main mediator of the beneficial effects of certain natural derived compounds, such as the polyphenols [17, 21].

In the present review, we discuss the biochemistry and pathophysiology of signaling pathways of NO focusing our attention on the experimental data showing that some natural derived compounds could be effective in the prevention and possibly treatment of CVDs.

2. Molecular Pathways of NO

Among the isoforms of NOS, eNOS represents the main source for the NO production in the vasculature. It is predominantly expressed in the endothelium but it has been also detected in kidney, human placenta, cardiomyocytes, platelets, and some neurons [22]. Several endogenous agonists, such as acetylcholine, bradykinin, and vascular endothelial growth factor (VEGF), as well as the shear stress induced by the blood flow, have been reported to activate eNOS [23]. Several studies have demonstrated that the phosphoinositide 3-kinase- (PI3K-) AKT pathway is mainly responsible for eNOS phosphorylation at Ser1177 especially in response to shear stress and VEGF [24–26]. Moreover, caveolin-1, the main component of the caveolae plasma membranes, has been reported as a negative regulator of eNOS [27, 28]. Another mechanism involved in the production of eNOS-derived NO is the activation of the β-adrenoreceptors [29] in response to the increase of catecholamines that are expressed at high levels in condition of oxidative stress associated with endothelial dysfunction [30, 31].

Neuronal NOS (nNOS) is expressed in specific neurons of the central nervous systems (CNS), as well as in the peripheral nervous systems (PNS) and in perivascular nerve fibers [32]. As in the case of eNOS, nNOS is responsible for the constitutively production of NO [33]. The inducible NOS (iNOS) is normally inactive in the vasculature [34], but its expression and activity can be induced in many cell types under oxidative and inflammatory stimuli; as a matter of fact several cytokines have been detected in the endothelium, in the media, and in the adventitia of blood vessels, as well as in neuronal cells and hepatocytes. Moreover, it is well known that NO produced by iNOS participates to the response of the immune system in killing bacteria and other exogenous compounds [35]. Several studies show the presence of a new isoform of eNOS enzyme in mitochondria (mtNOS) [36, 37]. This fourth isoform, the mtNOS, is responsible of the NO production in the mitochondria. It has been demonstrated that the NO-synthesizing capacity of mtNOS is higher than that derived from the combined activity of the all other NOS isoforms [38]. Moreover, recent findings suggest that

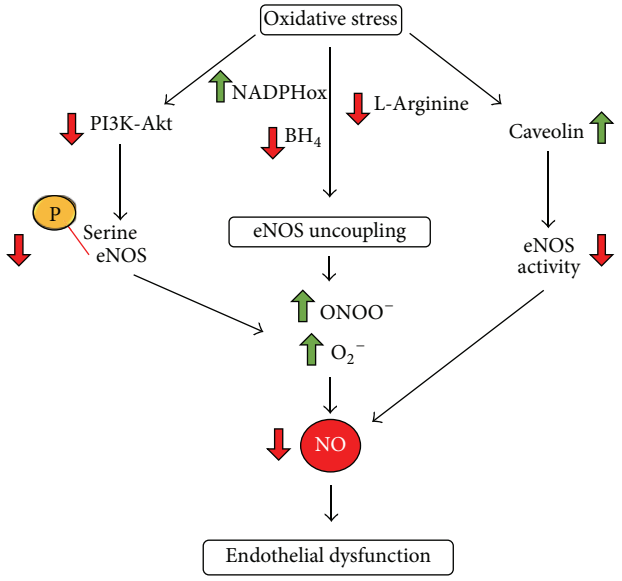

Figure 2: Decreased bioavailability of NO: oxidative stress is the cause of endothelial dysfunction, the common feature of CVDs; eNOS decreased activity due to different molecular pathways reduces NO production (see text for details).

an excessive stimulation of mtNOS leads to mitochondrial dysfunctions, which contribute to metabolic syndromes [39].

All NOS proteins are homodimers that transfer electrons from NADPH to the haem in the oxygenase domain where there are also binding sites for BH_4, oxygen, and L-arginine; at the haem site, the electrons are used to reduced O_2 and to oxidize L-arginine to L-citrulline and NO. Importantly, when oxidative stress increases, eNOS can lose its physiological properties in a process termed "eNOS uncoupling" [22, 40] (Figure 2). In such condition, NO reacts with superoxide O_2^-, leading to formation of peroxynitrite ($ONOO^-$), potent inducers of cell death, and eNOS produces reactive oxygen species (ROS), mainly O_2^-, rather than NO [41]. Therefore, eNOS uncoupling not only leads to decreasing NO bioavailability, but contributes to enhancing the preexisting oxidative stress [42]. Different mechanisms have been suggested to explain eNOS uncoupling; among these, the oxidation of BH_4 to the inactive form BH_3^- by O_2^- and $ONOO^-$ together with depletion of L-arginine plays a prominent role [4]. In particular, the decrease of L-arginine is caused by the upregulation of arginase isoforms (Arg I and Arg II) expression and activity. As we will discuss in the next sections, oxidative stress associated to eNOS uncoupling and the changing of the eNOS phosphorylation status (summarized in Figure 3) are characteristics of clinical conditions commonly associated to CVDs, such as diabetes mellitus, hypertension, atherosclerosis, and cerebral ischemia [22, 43].

2.1. Posttranslational Modifications of NOS. NOS enzymes are regulated by multiple interdependent mechanisms and signaling pathways, which can be calcium-dependent and/or calcium-independent. In particular, it has been demonstrated that the activity of eNOS is regulated by the increase of the cytosolic Ca^{2+} in endothelial cells, which leads to the activation of calmodulin that in turn binds eNOS, thus facilitating its function [23, 44]. Besides the increase of intracellular calcium, eNOS activity depends also on its phosphorylation status. In particular, It has been suggested that the phosphorylation of NOS isoforms at Tyr81 and Tyr657 represents a mechanism necessary to modulate the NO production above all during shear stress [45, 46].

Indeed, the phosphorylation is the major and most studied posttranslational modifications influencing the eNOS activity. Noteworthy, while the phosphorylation of serine at positions 617, 635, and 1179/1177 results in the activation of the eNOS, the same change at Ser116 and Thr497 reduces its function.

Also acetylation of the eNOS influences its activity and, in general, acetylation/deacetylation balance represents a crucial homeostatic mechanism mediating the response to metabolic changes in the cell [47].

Other important posttranslational changes are acylation, nitrosylation, glycosylation, and glutathionylation. All of them are necessary and often interconnected in controlling the subcellular localization and/or activity of the eNOS and thus the NO bioavailability in response to a variety of physiologic and pathophysiologic signals [48].

3. Physiopathological Role of NO in the Vascular System

The role of NO in the maintenance of vascular homeostasis is well defined and it depends on both eNOS distribution pattern and NO production rate. Perturbation of NO signaling pathways represents one of the major determinants of endothelial dysfunction, which is characterized by the reduction of the NO bioavailability and oxidative stress increase with the resulting impairment of the endothelium-dependent vasodilation [49, 50].

The NO synthesized by eNOS diffuses from endothelial cells into the underlying SMCs in which it stimulates sGC, thus generating cGMP, which in turn activates downstream protein kinases. Protein kinases predominantly act on myosin light chain phosphatase, the enzyme that dephosphorylates myosin light chains and leads to smooth muscle relaxation and vasodilatation. Moreover, NO may diffuse also in the blood flow where it inhibits several processes normally impaired during thrombotic and atherosclerotic events including platelet aggregation and leukocyte adhesion and migration into vascular wall [51].

Interestingly, over the well-known involvement of NO in the main cardio- and cerebrovascular diseases, other minor vascular forms of vascular diseases had been associated with impairment of the NO signaling. In this regard, it has been widely recognized that NO plays a key role in the physiology of penile erection eliciting its effect on guanylate cyclase leading to the production of cGMP. About this mechanism, the impairment of NO activity is similar to that observed in other forms of vascular diseases or in patients with cardiovascular risk factors (e.g., dyslipidemia, diabetes, and hypertension)

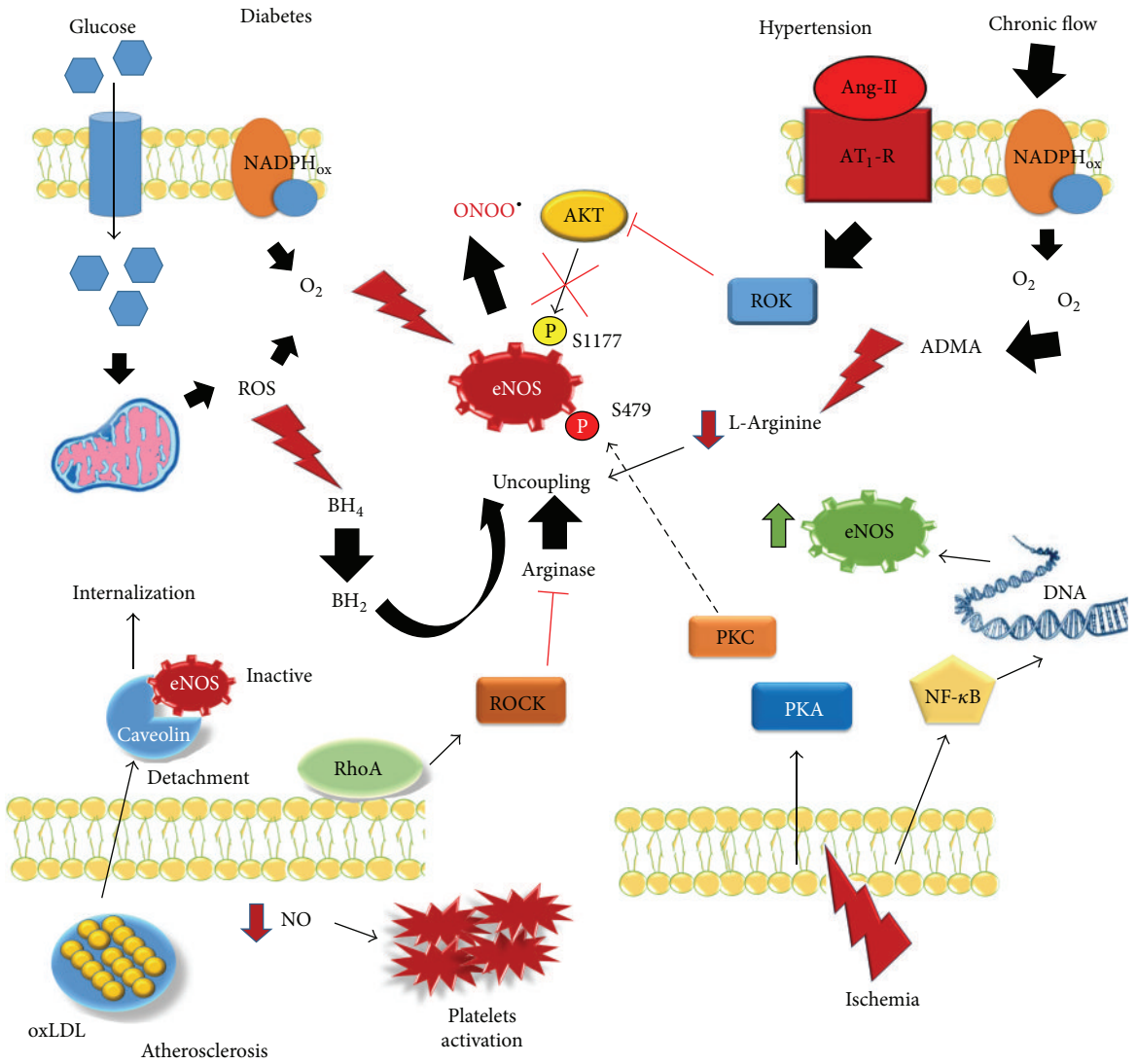

Figure 3: eNOS alteration, a common mechanism in different vascular diseases. The figure summarizes the main mechanisms of eNOS dysfunction promoted in the main cardiovascular diseases (see text for details).

[52]. Another form of vascular alteration in which changes in NO production and bioavailability have been reported is represented by varicose vein disease [53]. Furthermore, recent studies have found a link between endothelial dysfunction and NO alterations in venous valve dysfunction [53]. In particular, processes associated with varicose vein disease are increased destruction of collagen and matrix proteins triggered by endothelial dysfunction, which in turn is characterized by loss of NO bioavailability and increase of inflammation and ROS build-up [54]. Based on these findings, it is imperative to identify a new therapeutic strategy aiming at stimulating NO production and preventing the reduction of its bioavailability.

3.1. NO in Ischemia and Heart Failure. Many studies in animal models have documented the existence of a link between NO pathway impairment and CVDs. Kuhlencordt et al. showed that atherosclerosis, aortic aneurysm formation, and ischemic heart diseases can be accelerated as result of a chronic deficiency of eNOS [55]. The authors compared the atherosclerotic lesions occurring in two different knockout (KO) animal models, apolipoprotein E (apoE)/eNOS-double knockout (DKO) and apoE-KO, demonstrating that a genetic deficiency of eNOS significantly increased atherosclerosis in the apoE-KO mouse model. Of note, the location of the lesions, occurring mainly in the areas with disturbed flow, was similar in both KO models; therefore, the authors' conclusion was that the absence of eNOS did not determine the site of lesion formation in the aorta but appeared to accelerate its development. In addition, the ApoE/eNOS-DKO animals showed a more marked increase in blood pressure, comparable to that of eNOS-KO mice, indicating that eNOS deficiency could reflect different degrees of endothelial dysfunction. These findings are very important because they suggested eNOS deficiency/endothelial dysfunction as

a possible molecular mechanism linking hypertension to atherosclerosis [55].

Actually, Huang et al. have already demonstrated that in mice lacking the gene encoding eNOS the acetylcholine-induced relaxation was absent and the eNOS mutant mice had elevated blood pressure and developed hypertension [56].

Anti-ischemic actions of NO were also demonstrated by using of a transgenic (TG) mice model with cardiac specific overexpression of iNOS. After ischemia induced by coronary occlusion followed by 24 hours of reperfusion, the TG mice had a smaller infarct size compared to wild type. In addition, iNOS overexpression was able to attenuate the ROSs generation associated with reperfusion injury, in fact, the quantity of the ROSs trapped from reperfused hearts was lower in iNOS-TG than in wild type mice [57]. In another study performed in a model of eNOS-TG mice, it was demonstrated that a cardiomyocyte-specific overexpression of eNOS improved left ventricular performance and reduced compensatory hypertrophy after myocardial infarction (MI). Importantly, eNOS cardiac overexpression attenuated also a post-MI remodeling by reducing fibrosis in the noninfarcted area of the myocardium [58]. The beneficial role of the eNOS-derived NO has been demonstrated also in congestive heart failure (HF) in the study by Jones et al. in which the authors, by using a mouse model of infarct-induced HF, showed that eNOS overexpression enhanced animal survival, inhibited pulmonary edema, and improved cardiac function but did not attenuate the cardiac hypertrophy or improve cardiac contractility [59]. Moreover, it has been reported that the mitochondrial production of NO by mtNOS is reduced during ischemia because there is a lack of the O_2, necessary to generate the NO [36].

These findings demonstrated that strategies aimed at increasing NO bioavailability in the heart might be useful to counteract the structural and functional damage induced by myocardial ischemia.

3.2. NO in Diabetes and Atherosclerosis. NO production is reduced in diabetes mellitus and atherosclerosis, well-known risk factors for CVDs. In obese mouse model, eNOS activity was reported to be reduced by an enhanced phosphorylation at threonine 495 via PKC [60]. Similarly, Kashiwagi et al. showed that the lack of phosphorylation at serine 1176 residue was correlated with the development of obesity and insulin resistance in a mouse model [61].

Uncoupling eNOS also concurs to develop diabetes mellitus and, as mentioned above, both oxidation of BH_4 and depletion of L-arginine are the cause of such phenomenon. In addition, BH_4 was shown to be oxidized in diabetic mouse models, by a mechanism involving the activation of NADPH oxidases through PKC [62]. Similarly, in diabetic hypertensive rats, Alp et al. showed low levels of BH_4 and decreasing in NO production [63]. Heitzer and colleagues demonstrated that a supplementation of BH_4 improved endothelium-dependent vasodilation in patients with type II diabetes but not in control subjects. Of note, such beneficial effect was completely blocked by N(G)-monomethyl-L-arginine, a well-known inhibitor of NOS, suggesting that it was dependent on the NO production increase [64].

Also L-arginine deficiency has been reported in diabetic rats with a concomitant increase of the expression and activity of arginases, particularly, arginase, which has been recognized as the isoform responsible for eNOS uncoupling in diabetes [65]. In this regard, diabetic mice deficient of arginase I exhibited less endothelial dysfunction compared to wild type mice [66]. Notably, in the same way, in coronary arterioles of diabetic patients, arginase I was shown to contribute to the reduction of vasodilatation [67], and in plasma of patients with type II diabetes, arginase activity was reported to be elevated [68].

Interesting data in both humans and animal model have remarked the involvement of NO metabolism in the atherosclerosis. For example, depletion of BH_4 has been demonstrated in hypercholesterolemic patients [69] and high level of superoxide anions produced by uncoupled eNOS and increased formation of aortic atherosclerotic plaque with the concomitant deficiency of BH_4 were found in ApoE-KO mice where there were also observed an increased arginase II expression and activity [70]. Similarly, in human endothelial cells exposed to thrombin, Yang et al. found an enhancement of the arginase enzymatic activity [71]. In ApoE-KO mice, Ming et al. demonstrated that the small G protein RhoA and its effector ROCK play a role in the regulation of arginases activity involved in atherosclerotic process [72]. Moreover, posttranslational modifications of the eNOS have been shown to play a crucial role during atherosclerosis and diabetes [73]. Importantly, recent investigations have highlighted that phosphorylation and acetylation of the eNOS might concur to mediate the beneficial effects of some drugs. In this regard, Romero et al. have investigated the effects of BM-573, a compound that combines thromboxane synthase inhibition and thromboxane receptor antagonism, on endothelial dependent relaxation during early stage of atherosclerosis in apoE-KO mouse model. The authors demonstrated that BM-573 was able to ameliorate endothelial dysfunction by reducing oxidative stress and improving the NO bioavailability by increasing the eNOS phosphorylation [74]. Moreover, it has been demonstrated that lysine acetylation of the eNOS mainly contributes to the well-known atherothrombotic effects of low-dose acetylsalicylic acid [75].

The eNOS posttranslational modifications are necessary also in mediating the antidiabetic effects of several therapeutic interventions. For example, a diet supplementation with l-arginine and sepiapterin along with salsalate has been proved to increase the eNOS phosphorylation and improved vasorelaxation of thoracic/abdominal aorta in type-1 diabetic mice [76]. Furthermore, Ding et al. showed that cardiac overexpression of SIRT1, a NAD+-dependent deacetylases, reduced diabetes-exacerbated myocardial ischemia reperfusion injury and oxidative stress in diabetic rats via eNOS activation and that such effect was mediated by increase of the eNOS phosphorylation and reduction of the eNOS acetylation [77]. It has been also showed that the eNOS phosphorylation might be also important in mediating the beneficial effects of metformin and thiazolidinediones into microvasculature. In this regard, Ghosh et al. demonstrated

that a brief 3 h exposure to metformin induced changes in eNOS signaling in mouse microvascular endothelial cells by reducing the ratio of phosphorylated (p-eNOS)/eNOS, but not the expression of total eNOS [78].

Xu et al. investigated the effects of ciglitazone in rat microvascular endothelial cells, finding that such antidiabetic drug was able to reverse the decrease of eNOS levels in the cells stressed with oxidized LDL thus improving the NO bioavailability [79].

3.3. NO and Hypertension. A decreased NO bioavailability is one of the mechanisms involved in the pathogenesis of hypertension. Indeed, the phosphorylation of eNOS at threonine 495 residue was shown to be enhanced in angiotensin II- (Ang II-) induced hypertensive rats [80]. Landmesser et al. showed an increase of BH_4 oxidation caused by the activation of the p47phox subunit of NADPH oxidase in a model of salt-induced hypertension rat [42]. A similar enhanced expression of NAPDH oxidase has been also shown in spontaneously [22] and in angiotensin II-induced hypertensive rats [81]. In addition, an oral administration of BH_4 was shown to suppress the hypertension in spontaneously hypertensive rats thanks to the reduction of $ONOO^-$ and O_2^- accumulation [82]. Similarly, a supplementation of BH_4 increased acetylcholine-dependent endothelium vasodilatation in hypertensive patients to the level of normal control subjects [83].

Besides the oxidation of BH_4, the depletion of L-arginine could contribute to hypertension causing eNOS/NO impairment. Indeed, in spontaneously hypertensive rats, as well as in the aorta of mineralocorticoid and salt-induced hypertensive rats, the expression/activity of arginases was found to be enhanced [84–86]. Moreover, angiotensin II, via stimulation of AT_1, receptor is reported to be a molecular pathway responsible for the increased expression/activity of arginases in hypertension. In particular, in arginases knockout mice Shatanawi et al. showed that the p38 MAPK is the downstream effectors of AT_1, leading to endothelial dysfunction [87]. Intravenous administration of L-arginine produces a vasodilatory effect by increasing the NO production in hypertensive individuals [88], as well as the arginase inhibitor *N*-(omega)-hydroxy-nor-l-arginine prevents the hypertension, lowering the blood pressure in a hypertensive rat model [89, 90].

3.4. NO and Cerebrovascular Diseases. Several experimental evidences have underlined the protective role of eNOS/NO pathways in neuronal injury after cerebral ischemia as well as in the prevention of stroke and severe subarachnoid hemorrhage (SAH) [91, 92].

In physiological conditions, eNOS-derived NO is the main molecule responsible for the control of the cerebral blood flow (CBF). In this regard, it has been shown that ischemic injury increases eNOS activity and NO availability, which in turn leads to the improvements of the CBF and to decreasing neuronal injury [93]. Osuka et al. [94], in rat cerebral models of ischemia, found increased level of phosphorylation at eNOS Ser1177 residue in microvessels, with temporary expression of VEGF. Similarly, in eNOS knockout mice, after middle cerebral artery (MCA) occlusion, Huang et al. demonstrated an enlargement of infarct size and showed that systemic administration of nitro-L-arginine prevented brain damage [43]. Moreover, thrombotic cerebral infarctions have been found in $eNOS^{+/-}$ mice after three–six months of age [95]. Other authors underlined the importance of NO in the angiogenesis and neurogenesis occurring after cerebral stroke; for example, neovascularization after stroke was found to be impaired in eNOS deficient mice, indicating that endothelial NO mediates this effect [96].

4. Main Modulators of NO Pathways

Several therapeutic strategies have been proposed to ameliorate the NO homeostasis. Currently, the best strategy is based on the drugs administration in order to activate downstream effectors of eNOS/NO from one side and to reduce eNOS uncoupling [16], improving BH_4 and L-arginine bioavailability and regulating post-translational modifications of eNOS, from the other side. Nevertheless, it is important to remark that a helpful strategy for the prevention and attenuation of CVDs is to make a good lifestyle and, in this context, physical exercise and specific diets such as diet rich in polyphenols have been suggested to improve the NO pathways.

The inhibition of the renin-angiotensin-aldosterone system is widely recognized as an effective therapy in CVDs [97]. In animal models, angiotensin-converting enzyme inhibitors (ACE-I) and AT1 receptor blockers (ARBs) are able to reduce eNOS uncoupling, while restoring BH_4 bioavailability [98], and to protect against cerebral ischemia via upregulation of the eNOS in middle cerebral artery [99] and cerebral infarct size via eNOS activation [100].

The renin-angiotensin system blockers exert also NO-dependent antithrombotic effects. In this regard, Kucharewicz et al. demonstrated that angiotensin 1–7, a component of the renin-angiotensin system, caused an increased production of NO, which contributes to reduction of thrombosis in rats [101].

Also the cholesterol-lowering drugs, the statins, improve endothelial functions by enhancing the NO bioavailability thanks to their antioxidant, anti-inflammatory, and antiatherosclerotic properties [102, 103]. For example, in hypercholesterolemic patients treated with fluvastatin, John et al. demonstrated an improvement of endothelial vasodilatation through increase of the NO production [104]. Moreover, in a rat experimental model of MI, statins were found to enhance NO bioavailability by restoring mobilization of EPCs, myocardial neovascularization, and, ultimately, increasing survival [42] and statins were also showed to decrease eNOS uncoupling through a reduction of vascular $O_2^{\bullet -}$ and BH_4 oxidation [105].

Another way to ameliorate endothelial homeostasis is the activation of the β-adrenoreceptor subtype 3 (β_3), which leads to eNOS activation and thus to the NO generation by increasing the levels of cAMP and Ca^{2+} [30, 31, 106]. Nebivolol, a third-generation β-adrenoreceptor blocker, is a promising drug able to improve NO pathways thanks to its

ability to antagonize β_1 and to activate β_3 receptors. Maffei et al. [107] showed that nebivolol induced endothelial NO production in both conductance and resistance rats arteries in a calcium-dependent manner. In another study, the same authors measured in mice the heart production of the NO consequent to the stimulation of β_3 receptor and iNOS increased activity, thus indicating nebivolol as therapeutic strategy for hypertension and heart failure [108].

Another aspect that deserves attention is the link between adrenergic pathway, NO bioavailability, and oxidative stress and, in this context, the beneficial effects of the nebivolol are attributable to its well-recognized antioxidant properties, which are considered an additional factor for increasing the NO bioavailability. For example, nebivolol and atenolol (a second-generation β-blockers) similarly reduced blood pressure values in hypertensive patients, but oxidative stress markers, such as LDL hydroperoxides, 8-isoprostanes, and ox-LDL were significantly improved only in patients treated with nebivolol [109, 110].

Moreover, in hypertensive patients, Okamoto et al. demonstrated that nebivolol lowered blood pressure [111], while in elderly patients with heart failure it was shown to reduce mortality and morbidity [112]. Interestingly, Falciani et al. highlighted also the role of nebivolol in inhibiting platelet aggregation by increasing L-arginine/NO, remarking also an antithrombotic effect of this β-blocker [113].

Nowadays, researchers are paying particular attention to the nonpharmacological strategies, including adoption of specific diet habits and exercise programs for the management of several chronic diseases.

In this context, several experimental and epidemiological findings have underlined the role of physical exercise (PE) in decreasing the oxidative stress associated with aging and in the prevention and attenuation of CVDs-associated risk factors [114–117]. It was suggested that the reduction of oxidative stress triggered by PE could be associated with the improvement of the NO function [118]. In this regard, in patients with chronic heart failure and coronary artery disease, Laurent et al. showed that water-based exercises increased NO metabolism by improving cardiorespiratory capacity and endothelial function [119]. Recently, a regular exercise was demonstrated to activate eNOS and nitrite production and to reduce oxidative stress in spontaneously hypertensive rats [120]. PE was also suggested to have a cardioprotective effects; in ischemic rats, high-intensity interval training increased NO metabolites levels and reduced myocardial infarction [121].

Different molecular mechanisms, such as phosphorylation status and transcription rate of eNOS, have been proposed to explain the effects of PE on the NO production. For example, in rats subjected to acute and chronic aerobic training eNOS mRNA levels were found to be upregulated [122]. Other authors underlined the role of β_3 adrenoreceptor in mediating the effects of PE on the NO production; in particular, Calvert et al. demonstrated that exercise could improve the cardiac function in ischemic rats via β_3 adrenoreceptor by increasing the eNOS phosphorylation [123].

Another molecular mechanism is represented by the NO-dependent changes in the vascular redox state and oxidative stress even if the beneficial role of the NO in this context could be complex to elucidate. In this regard, Farah et al. suggested that certain level of eNOS uncoupling could be required for exercise-induced myocardial cardioprotection during ischemia reperfusion. In particular, in such study, it was showed that eNOS uncoupling was associated with the improved myocardial antioxidant capacity that prevented excessive NO synthesis limiting the reaction between NO and $O_2^{\bullet-}$ to form peroxynitrites [124].

5. Crosstalk between NO and the Other Gasotransmitters

Besides the NO, other two gaseous molecules, hydrogen sulfide (H_2S) and carbon monoxide (CO), have been recognized as "gasotransmitters" [125]. Much like their predecessor NO, H_2S and CO have been historically considered as highly toxic and harmful agents; afterward, many investigations have showed that they not only play various physiological roles but could be effective against a number of diseases, including CVDs [126].

Indeed, also CO and H_2S mediate muscle relaxation and vasodilatation, the first, as well as the NO, through activation of GMP and consequent elevation of cGMP levels and the second through a cGMP-independent mechanism [127, 128].

Compelling evidence has demonstrated that each member of this triad of gasotransmitter can influence each other. For example, the inhibition of the NO synthesis might increase the CO production [129], while low-dose CO has been showed to decrease the eNOS mRNA expression [130].

Recently, particular attention is paid to the role of crosstalk existing among the gasotransmitters in determining cytoprotective effects in the heart and vessels. It was demonstrated that NO, CO, and H_2S act in concert to preserve the cardiovascular homeostasis thanks for instance to their antioxidant and anti-inflammatory properties [131].

Noteworthy, the gaseous nature of these compounds makes them attractive candidates for the treatment of several pathological conditions, especially ischemia reperfusion injury. In this regard, H_2S have been shown to stimulate vascular remodeling after ischemia in mice by enhancing the NO production [132]. Donnarumma et al. have recently investigated in murine and swine models of ischemia reperfusion the effects of an oral administration of zofenopril, an ACE-I containing a sulfhydrylic group. The authors found that zofenopril reduced myocardial infarct size in both animal models and preserved blood flow in swines and such effects were associated with an elevation of the H_2S and NO plasmatic levels [133].

Importantly, there are conflicting evidences on the anti-ischemic effects of the ACE-I and some studies have revealed such effects only for sulfhydryl-containing agents [134]. Moreover, it was demonstrated that an early treatment of the acute myocardial infarction with zofenopril is able to reduce morbidity and mortality any more than ramipril, dicarboxylate-containing ACE-I [135].

Therefore, the understanding of the mechanisms involved in the cytoprotective effects of all gasotransmitters either

individually or together is necessary to fully exploit their therapeutic potential.

6. Natural Derived Compounds and NO

Growing evidence leads to considering a healthy dieting regimen as an helpful strategy to reduce CVDs-associated risk factors acting, as well as the aforementioned drugs, via modulation of the NO pathways.

The Mediterranean diet, rich in fruits and vegetables and based on high consumption of red wine, was associated with a good prognosis in patients with CVDs [136–139]. In particular, in subjects who usually consume large amounts of fruits, vegetables, red wine, tea, chocolate, and nuts, a significant improvement of endothelial function has been reported, which in turn contributes to reduction of blood pressure, atherosclerosis, and cardiovascular mortality [140, 141]. Interestingly, the beneficial properties of the red wine were recognized as the solution of the "French paradox," a term used to describe the observation that the French population had a low incidence of CVDs, despite a diet predominantly characterized by a high consumption of wine and saturated fat food [142]. The protective effects against CVDs have been attributed, at least in part, to the high content in these specific foods of polyphenols, a class of chemicals characterized by the presence of phenol units in their chemical structure [143].

6.1. Classification and Source of Polyphenols

6.1.1. Flavonoids. Flavonoids represent a large group of polyphenols, characterized by two benzene rings linked via a heterocyclic pyran ring. The latter gives reason of the differences between the various classes of flavonoids. According to their chemical structure, the flavonoids can be subdivided in (i) flavones, such as apigenin (bilberry, raspberry, strawberry, plum, cherry, blackberry, red pepper, and tomato skin) [144], (ii) flavonols that include quercitin (red onions, tea, wine, apples, cranberries, buckwheat, and beans) [145], (iii) isoflavones, including genistein (soy, legumes) and coumestrol (soy, red clover), also known as phytoestrogens [146], and (iv) flavanols that include catechins and epicatechin (tea, apple juice, wine, and cocoa) [147]. Interestingly, these compounds have been found also in medical plants, such as *Aloe vera* [148] and *Cannabis sativa* [149]. Flavonoids have been demonstrated to exert a plethora of beneficial effects both *in vivo* and *in vitro* and to regulate specific molecular pathways and target several genes [150, 151]. In particular, the best characterized biological property for all flavonoids, as well as for polyphenols in general, is their ability to act as antioxidants, inhibiting ROS accumulation, acting either by scavenging ROS or inhibiting enzymes involved in the ROS production or by enhancing the natural antioxidant defenses [152]. Moreover, several studies have shown and are underlining anticancer activities of the flavonoids. For example, quercitin has been shown to inhibit cell proliferation in several human cells, such as lymphoid, colon, ovarian, and gastric cells, through modulation of several genes involved in cancer progression [153]. Moreover, genistein has been recently proposed as a chemopreventive agent especially against prostate cancer, thanks to several interesting results deriving from *in vitro* and epidemiological studies [154, 155].

6.1.2. Stilbenoids. Stilbenoids are a class of phenolic compounds synthesized as defense agents from the plants expressing stilbene synthase. Resveratrol is the most studied stilbenoid, but more than 400 compounds have been identified; most of them are currently used in Chinese traditional medicine [156]. Generally, stilbenoids are classified on the basis of the number of the C6-C2-C6 units (monomer, dimers, trimers, tetramers, and examers). They are present abundantly in berries (grape, blueberry, bilberry, cowberry, cranberry, and strawberries) and peanuts but are also detectable in cocoa powder, dark chocolate, and white tea [157, 158]. As mentioned for the flavonoids, several researches have suggested a role of stilbenoids as anticancer, antioxidant, and antiaging agents or as positive modulators of several human degenerative diseases [159, 160]. In this regard, resveratrol has been shown to induce apoptosis in breast cancer and prostate cells, by induction of caspases, Bax proteins, and p53 [161, 162]. Of note, a natural analog of resveratrol has been documented to inhibit growth of several cancers, such as pancreatic [163] and colon [164] cancer.

6.1.3. Curcuminoids. Cucrcuminoids are chemical compounds extracted from the rhizome of *Curcuma Longa* Linn. They are characterized by a linear structure (diarylheptanoid) with two phenolic groups (C6-C7-C6) and are widely used as colorants for vegetables. Curcumin and its derivates have been demonstrated to possess numerous pharmacological activities, including anti-inflammatory, antioxidant, and antitumorigenic effects. In particular, it has been reported that curcumin is able *in vitro* to downregulate the expression of cyclin D and E and to upregulate p53 and p21, which in turn contribute to arresting cell proliferation/migration and promoting apoptosis [165, 166]. Concerning its antioxidant properties, curcumin acts prevalently as superoxide radical scavenger [167].

6.1.4. Phenylethanoids. Phenylethanoids are polyphenols characterized by a phenethyl alcohol structure. Typical examples of phenylethanoids are tyrosol and its derivate oleuropein, present prevalently in olive oil and olive leaf. Oleuropein is the most abundant polyphenol in olives and thus it is receiving particular attention by the scientific community because extra virgin olive oil is an essential component of Mediterranean diet. Several studies have demonstrated that the oleuropein possesses a wide range of pharmacological properties such as antiatherogenic [168], hypotensive [169], and antidiabetic [170], as well as anticancer activity and antioxidant effects [171, 172]. Moreover, also hydroxytyrosol, a metabolite of oleuropein, has been shown to possess antioxidant properties [173] as well as anti-inflammatory, antiplatelet aggregation, antiatherogenic and cardioprotective, antimicrobial, antiviral, and anticancer activities [174].

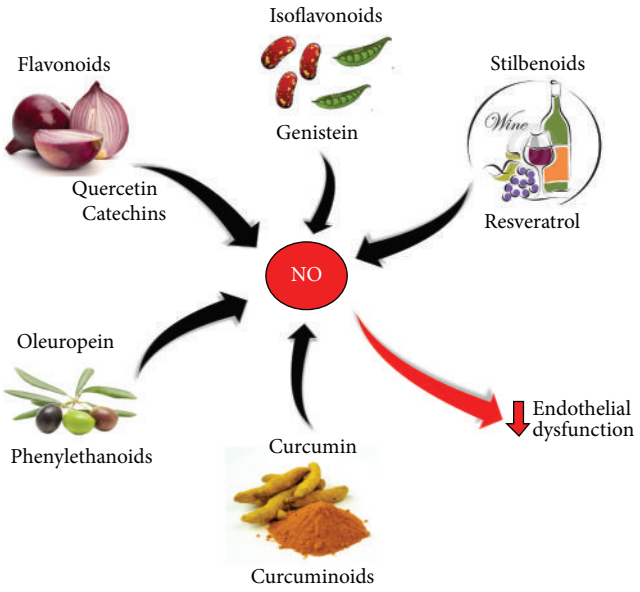

Figure 4: Natural derived compounds increase NO production: a diet rich in polyphenols, deriving from different sources, contributes to counteract oxidative stress and enhances NO generation, so improving the endothelial function.

7. Polyphenols and the NO Signaling

Concerning the effects on vascular physiology, several data suggest that polyphenols act on the NO signaling and metabolism, improving eNOS expression and activity, as well as reducing eNOS uncoupling. Nowadays, one of the limits during the characterization of the molecular pathways activated by polyphenols is that most of the experiments have been conducted with the total extracts of food, such as wine, cocoa powder, or olive leafs extracts; therefore often it is very difficult to identify the specific compound exerting protective effects. Nevertheless, some studies measured the effects of single compounds, such as resveratrol, quercitin, or curcumin [175]. Irrespective of their source, one of the main effects exerted by the polyphenols is the NO-dependent vasodilatation (Figure 4). For example, in isolated arteries of rabbits, Karim et al. demonstrated that cocoa extracts increased levels of intracellular Ca^{2+}, leading to L-arginine conversion in citrulline and to the eNOS activation [176]. Similarly, plant-derived polyphenols have been reported to induce vasodilatation of porcine coronary arteries through NO generation [177]. Moreover, in bovine endothelial cells, catechins of green tea activated eNOS by phosphorylation at Ser1179 and dephosphorylation at Thr495 in a PKA-Akt dependent manner [178, 179]. In addition, such compounds were also shown to exert protective effects in diabetic rats thanks to the reduction of oxidative stress obtained by downregulation of NADPH oxidase [180]. Interestingly, catechins were found to reduce platelet aggregation and to reverse endothelial dysfunction in patients with coronary artery disease, thus exerting antiatherosclerotic properties [181, 182]. Moreover, polyphenols of the black tea were found to enhance the activity of eNOS via p38 MAPK-dependent phosphorylation in porcine aortic endothelial cells. In fact, both pharmacological and genetic inhibition of p38 MAPK attenuated both eNOS activation and phosphorylation changes in response to these polyphenols [183].

Among plant-derived polyphenols, fruit extracts of *Camelia japonica* (CJF), a plant widely distributed in Asia and well known for its antioxidant properties [184], have been demonstrated to induce the NO production via Akt pathways in endothelial cells and to activate eNOS via phosphorylation at Ser1179. In the same study, CJF inhibited VSMCs proliferation and migration, suggesting its beneficial role in the prevention of atherosclerosis [185]. Similarly, polyphenols of the tropical plant *Terminalia* have been reported to induce a calcium-dependent activation of eNOS [186].

Interestingly, Appeldoorn et al. by using an *in vitro* screening to discover the potential effects of different polyphenols have found that quercitin, abundant in many vegetables and fruits, is one of the major stimulator of the NO production [187]. Indeed, the effects of quercitin have been extensively investigated in animal models of CVDs, especially with regard to its antihypertensive effects. For example, a reduction of blood pressure after administration of quercitin in spontaneously hypertensive rats has been showed [188], as well as in salt-hypertensive [189, 190] and NO deficient rats [191]. Recently, it has been reported that quercitin is able to ameliorate arterial erectile dysfunctions in rats via NOS regulation restoring, almost in part, the function of NO-cGMP pathway in the process of penis erection [192].

The molecular mechanism involved in the antihypertensive effect of the flavonoid quercitin was attributed to the inhibition/downregulation of NADPH oxidase. Concerning this, Perez-Vizcaino et al. demonstrated that quercitin was able to induce the lowering of blood pressure by diminishing superoxide-driven NO inactivation via downregulation of aortic p47phox, a regulatory subunit of NADPH oxidase, which is the main source of vascular superoxide [193]. These results are in accordance with others showing that quercitin decreased NADPH oxidase-mediated superoxide anion generation, as a consequence of inhibition of p47 protein subunit expression in [194].

In isolated rat aortic ring, Jin et al. found that apigenin, a polyphenol abundant in many plants, enhanced the NO bioavailability via reduction of oxidative stress. Apigenin evoked a concentration-dependent relaxation in aortas, which was specifically inhibited by L-NAME, a direct inhibitor of NOS. Of note, vasodilation occurred concomitantly with inhibition of superoxide anion and increasing of the NO levels [195]. In a similar way, curcumin has been reported to increase relaxation in porcine coronary arteries, probably thanks to mechanism involving NO, cGMP, and adrenergic β-receptor and, also in this case, such relaxant effect was specifically inhibited by L-NAME [196].

The involvement of caveolin-1 in polyphenols-mediated effects on the NO pathways has also been reported. Li et al. demonstrated in endothelial cells that green tea extracts downregulated the caveolin expression via activation of ERK and deactivation of p38 MAPK kinases [197]. Similarly, Vera et al. found in hypertensive rats that genistein, a soy isoflavone, was able to enhance eNOS activity via inhibition of caveolin-1 and NADPH oxidase and favoring

O_2^- reduction, thereby leading to decrease in blood pressure [198]. Moreover, soy isoflavones has also been demonstrated to improve the NO metabolism in carotid and cerebral rat arteries [199] as well as to enhance eNOS mRNA expression [200].

NO-mediated antihypertensive effects were also reported in rats after administration of other soy isoflavones, such as glucosyl hesperidin [201]. Yamamoto et al. found that the hypotensive effects of this natural compound were associated with reduction of oxidative stress and improvement of the NO metabolism [202]. In this regard, hesperidin was found to significantly prevent endothelial damage and leucocytes adhesion in animal models of ischemia reperfusion. Concomitantly, an increase of NO bioavailability and a reduction of inflammatory molecules which contribute to ameliorate edema and other symptoms of venous diseases have been reported [203].

Polyphenol-rich cocoa extracts have been demonstrated to reduce blood pressure in spontaneously hypertensive rats [204] and, similarly, in hypertensive patients, as well in healthy subjects, the intake of black cocoa extracts has been reported to reduce blood pressure and improve endothelial function through increase of the NO bioavailability [205–208]. Moreover, in patients with high cardiovascular risk it was showed that the administration of two different diets, one rich in polyphenols deriving from extra virgin olive oil and another rich in nuts, was shown to reduce systolic and diastolic pressure concomitantly with an increase of the NO plasma levels [209].

7.1. Red Wine Polyphenols and NO Pathways. Red wine is one of the main sources of the natural polyphenols. As mentioned above, epidemiological studies have suggested that the high consumption of red wine correlates with a reduction of the CVDs risk factors. The evidence corroborating vascular effects of red wine polyphenols (RWPs), as well as grape seed extracts (GSEs) and grape juice polyphenols (GJPs), is the induction of NO-dependent relaxation in isolate arteries and the activation of NO signaling pathways in endothelial cells [210–212]. Leikert et al. found that RWPs enhanced eNOS expression and release of NO in human endothelial cells [213]. In the same way, NO production and intracellular Ca^{2+} release have been shown in bovine endothelial cells treated with RWPs [214] and an increase of eNOS and Akt phosphorylation were also reported in endothelial cells exposed to GSEs [215]. Similar eNOS activation was also demonstrated in isolated arteries. For example, in porcine coronary arteries Madeira et al. showed endothelium relaxation induced by GSEs via Akt/eNOS phosphorylation [216], and also in isolated porcine coronary arteries, RWPs were found to enhance phosphorylation of eNOS at Ser1177, resulting in the increase of the NO production [217]. Interestingly, in rat femoral arteries, RWPs were shown to induce vasodilatation and to increase the NO levels in a concentration-dependent manner [218]. Moreover, RWPs were demonstrated in rat aorta to enhance NO bioavailability and to increase intracellular Ca^{2+} and cGMP concentrations [218, 219].

Several molecular mechanisms have been proposed to explain in both animal models and humans the beneficial effects of the RWPs in vascular physiology. In this regard, Bernátová et al. in hypertensive NO deficient rats showed that RWPs restored endothelial functions thanks to a reduction of blood pressure induced by increased eNOS activity in the left ventricle and aorta [220].

Similarly, in salt-induced hypertensive rats, RWPs were shown to improve vascular physiology by inhibiting NADPH oxidase [221]. The inhibition of NADPH oxidase was also reported in Ang II hypertensive rats treated with RWPs in which a reduction of superoxide anions level occurred concomitantly with restoration of the NO bioavailability [222]. RWPs have been demonstrated to exert protective effects also in animal models of ischemia and atherosclerosis. For example, in ischemic rats, RWPs were shown to reduce the angiogenic process [223], and, in hypercholesterolemic mice, Napoli et al. showed that low doses of RWPs reduced atherosclerosis by eNOS activation [224]. Interestingly, with an *in vitro* model of human atherosclerosis, Magrone et al. have reported enhanced production of the NO, after administration of red wine. The authors tested some red wines for their ability to trigger NO production from human healthy peripheral blood mononuclear cells, finding that flavonoids and resveratrol, abundant in the red wine, once absorbed at intestinal level and entered into circulation, induced monocytes to produce the NO [225].

Few clinical trials have planned with the aim to investigate the effects of a dietary regimen based on moderate consumption of wine about NO related improvement in vascular physiology in both healthy patients and patients with high risk of CVDs. For example, in healthy subjects, an oral supplementation of grape juice was found to inhibit platelet aggregation with decreased production of superoxide and enhanced NO levels [226, 227]. Moreover, besides its antithrombotic activity, red wine has also been suggested to exert cardiovascular protective effects by enhancing circulating endothelial progenitor cells thanks to a mechanism involving an increase of the NO bioavailability, as reported in studies performed in healthy individuals by Huang et al. [228]. In addition, red wine consumption has been shown to significantly decrease blood pressure and enhance plasma NO levels in hypertensive patients [229]. Interestingly, Karatzi et al. demonstrated that in smokers a consumption of red wine counterbalanced the endothelial dysfunction caused by oxidative stress induced by cigarettes smoke, in a pathway probably mediated by NO [230].

7.2. Resveratrol and NO Pathways. Among the RWPs, resveratrol (RSV) is one of the best characterized members. It has been used in the Indian medical herb named "Darakchasava" from about 4500 years ago and the clinical effects described in the past for "Darakchasava" are the same attributed to RSV today [231]. RSV was firstly described for its antitumorigenic properties [232]; it is present especially in grape skin and red wine, but also in peanuts, pistachios, and pine trees [233]. The interest of the scientific community for RSV derives from the observation that its administration mimics the effects of calorie restriction, a tool widely recognized to prevent the endothelial dysfunction, thereby attenuating atherosclerosis,

hypertension, diabetes, and CVDs risk factors and aging-associated diseases in general [234–236]. Thanks to some experiments conducted *in vitro* in endothelial cells, RSV has been shown to regulate several target molecules, such as the NAD^+-dependent deacetylases named sirtuins, acting at transcriptional and posttranscriptional levels [237–239].

Although the studies underlining the vascular protective effects exerted by RSV did not study the involvement of the NO signaling [157, 234], several findings, obtained in animal models of CVDs, have proposed the NO as the main downstream target mediating such effects. For example, Xia et al. demonstrated in ApoE deficient mice that RSV was able to modulate the oxidative stress responsible for atherosclerosis. From one side, NADPH oxidases were downregulated; from the other side superoxide dismutases (SOD) were upregulated. Moreover, oxidation of BH_4 was found to be reduced, attenuating the increase of eNOS uncoupling levels [240]. Other beneficial effects were shown in many different clinical settings reinforcing the idea that RSV could be considered an optimal therapeutic strategy against CVDs. For example, in hypercholesterolemic rabbits, RSV improved endothelial function in parallel with an increase of NO plasma levels [241]. In addition, RSV has been suggested to contrast the endothelial dysfunction correlated with metabolic syndromes. In this regard, in endothelial cells RSV was demonstrated to suppress superoxide generation and to activate eNOS through phosphorylation at Ser1177 thereby increasing the NO generation [242]. In aortas of diabetic mice, RSV restored vasodilatation by enhancing eNOS activity and inhibiting the tumor necrosis factor α-(TNFα-) induced activation of NADPH oxidase [243]. In the same way, a treatment in rats with RSV has been showed to increase muscle microvascular recruitment via an NO-dependent mechanism blocked by TNFα [244]. Also, RSV was shown to reduce blood pressure in obese rats and to enhance the expression of eNOS via AMPK and reduction of TNFα in adipose tissue [245]. Similarly, in rats fed with high fructose diet, RSV decreased blood pressure via AMPK-Akt-NOS pathway [246]. Interestingly, in the myocardium of diabetic mice, RSV reduced Cav-1 expression, which in turn contributes to enhance eNOS activity [247], and the same effects on Cav-1 expression were found in hypercholesterolemic rats [248].

Furthermore, RSV was shown to protect heart from ischemic reperfusion injury. Hattori et al. demonstrated that RSV reduced infarct size in rat hearts by enhancing iNOS expression [249]. The cardioprotective effects of the RSV has also been showed in spontaneously and angiotensin Ang II-induced hypertensive rats, in which RSV contributes to the upregulation of the eNOS activity and reduction of pressure and cardiac hypertrophy [250]. Moreover, the antihypertensive effect of the RSV was also shown to be mediated by the attenuation of eNOS uncoupling via reduction of L-arginine levels and oxidative stress [251].

The antithrombotic activity of the RSV has been also reported in human platelets. Gresele et al. showed that RSV stimulated platelet NO production through inhibition of p38 MAPK, NADPH oxidases, and superoxide formation, thus decreasing peroxynitrite accumulation [252].

RSV was also shown to mobilize endothelial progenitor cells in a NO-dependent manner, thus contributing to repairing the damage occurring in vessels after ischemic injuries [253].

In the arteries of patients with hypertension and dyslipidemia, Carrizzo et al. characterized many of the downstream effectors of the RSV-dependent NO generation. The authors found an enhanced vasodilatation of arteries due to the activation of AMPK and reduction of eNOS uncoupling via increasing levels of BH_4 and, in the same study, RSV was found to reduce vascular oxidative stress trough upregulation of manganese superoxide dismutase in a pathway mediate by nuclear factor erythroid-derived 2-like 2 [254].

Some authors have also suggested the potential therapeutic use of RSV for the prevention of stroke; for example, in rat models of stroke, RSV reduced brain damage in a NO-dependent manner [255]. Similarly, in rats subjected to focal cerebral ischemia Tsai et al. provided the evidence that RSV might enhance plasma levels of the NO and upregulate eNOS expression while it might downregulate iNOS expression and that these effects were abolished by the coadministration of selective NOS inhibitors [256].

8. Bioavailability of Polyphenols

Although the use of the polyphenols represents a promising tool for increasing the NO production and activity against CVDs, one of the biggest challenges for their employ in the clinical practice is to enhance their low bioavailability. In this regard, it has been shown that when orally administered, polyphenols concentration appears not to be sufficient to ensure therapeutic effects [257]. For example, the plasmatic levels of the resveratrol from dietary intake are often undetectable or very low when compared with the concentrations employed during *in vivo* and *in vitro* experiments [258]. Similarly, the pharmacological properties of curcumin are drastically restricted mainly because of its low water solubility and absorption from the gut, short half-life, and extremely poor bioavailability.

To overcome such problems, one of the best approach could be developing new pharmaceutical formulations, for example, polyphenols conjugated with cyclodextrins, or encapsulated in nanoparticles (NP), such as poly(lactic-co-glycolic acid) (PLGA) based NP or liposomes. In this regard, many of these formulations have been demonstrated to improve solubility, systemic half-life, resistance to metabolic degradation, and ultimately the bioavailability of the polyphenolic compounds in order to potentiate their biological activities [259, 260]. However, while the differences between polyphenols monoadministered or administered in encapsulated formulations have been extensively studied for what concerns the polyphenols antioxidant and anticancer properties, no experiments have been carried out on the effects of these formulations on the NO metabolism.

9. Conclusion

Targeting the gasotransmitter NO is becoming a new challenge in cardiovascular medicine. We here reviewed some

of the experimental evidences that have indicated several natural compounds as suitable activators of the NO signaling pathways.

It is necessary to remark that for most of them the molecular mechanism, as well as the precise concentration to obtain beneficial effects, especially because of their low bioavailability remains to be determined. Nevertheless, these agents, mainly the polyphenols, doubtless possess a great therapeutic potential above all when you consider that the available drugs, although effective, did not act exclusively on the NO pathways often causing deleterious side effects. Moreover, most of the investigations on the natural compounds have involved *in vitro* studies; thus it is difficult to draw definite conclusions about their therapeutic usefulness.

Although accumulating evidence suggests that the polyphenols exert beneficial effects against vascular diseases by restoring the impairment of the NO production and/or bioavailability, much remains to be clarified. Doubtless, many gaps must be filled in understanding the complex chemistry, biochemistry, and molecular biology of such natural agents in order to introduce such NO signaling modulators in the clinical practice.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Hydrogen Sulfide Improves Endothelial Dysfunction via Downregulating BMP4/COX-2 Pathway in Rats with Hypertension

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Aims. We object to elucidate that protective effect of H_2S on endothelium is mediated by downregulating BMP4 (bone morphogenetic protein 4)/cyclooxygenase- (COX-) 2 pathway in rats with hypertension. *Methods and Results*. The hypertensive rat model induced by two-kidney one-clip (2K1C) model was used. Exogenous NaHS administration (56 μmol/kg/day, intraperitoneally once a day) reduced mean arterial pressure (MAP) of 2K1C rats from 199.9 ± 3.312 mmHg to 159.4 ± 5.434 mmHg, while NaHS did not affect the blood pressure in the Sham rats and ameliorated endothelium-dependent contractions (EDCs) of renal artery in 2K1C rats. 2K1C reduced CSE level twofold, decreased plasma levels of H_2S about 6-fold, increased BMP4, Nox2, and Nox4 levels 2-fold and increased markers of oxidative stress MDA and nitrotyrosine 1.5-fold, upregulated the expression of phosphorylation-p38 MAPK 2-fold, and increased protein levels of COX-2 1.5-fold, which were abolished by NaHS treatment. *Conclusions*. Our results demonstrate that H_2S prevents activation of BMP4/COX-2 pathway in hypertension, which may be involved in the ameliorative effect of H_2S on endothelial impairment. These results throw light on endothelial protective effect of H_2S and provide new target for prevention and therapy of hypertension.

1. Introduction

Hydrogen sulfide (H_2S) has been proved to be the third endogenous gasotransmitter following nitric oxide (NO) and carbon oxide (CO). H_2S is endogenously produced from L-cysteine by two pyridoxal-5′-phosphate-dependent enzymes, that is, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in mammalian tissues [1]. Recently, it was found that another mitochondrial enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST) in conjunction with cysteine aminotransferase (CAT), contributes significantly in generating H_2S from L-cysteine in the presence of α-ketoglutarate [2]. The expression of those three enzymes is tissue-specific, and, in blood vessels, CSE is a major H_2S-producing enzyme expressed in both smooth muscle and endothelium [3–5]. H_2S is endowed with biological and physiological functions in cardiovascular system. H_2S has been well known as a vasodilator and plays an integral role in the homeostatic regulation of blood pressure [6].

Endothelial dysfunction is an initial factor in the pathogenesis of various vascular diseases such as atherosclerosis and hypertension. A number of studies have showed that H_2S played endothelium protection through decreasing the level of oxidative stress [7–10], strengthening endothelial NO production via activating eNOS Ser 1177 phosphorylation [11], and inhibiting inflammation of endothelium, which

resulted in ameliorating the development of hypertension [12]. However, the exact mechanism of H_2S remains to be fully clarified.

Bone morphogenetic protein 4 (BMP4) is one of the BMP family from BMP2 to BMP7, which belongs to TGF-β superfamily [13]. Original studies showed that BMP4 was bound to BMP receptors containing type I and type II [14] and then regulated physiological and pathological process of embryonic development, bone, and cartilage formation [15–17]. Several studies have further implied that BMP4 might be involved in exaggerating cardiac ischemia-reperfusion injury [18] and atherosclerotic calcification plaques [19]. Activating vascular BMP4 can promote vascular calcification in hyperglycemia and diabetes [20]. BMP4 infused chronically results in hypertension and is considered as a novel mediator of endothelial dysfunction and hypertension [21]. Endothelial dysfunction can be ameliorated via inhibiting BMP4 cascade [22].

As a proinflammatory gene, BMP4 induces endothelium dysfunction in systemic circulation and resulted in not only impairment of vascular relaxation [23] but also exaggeration of vascular contraction. BMP4 binds to BMP4 receptor which activates NADPH oxidase and then chronically increased the expression of cyclooxygenase- (COX-) 2 through p38 MAPK-dependent mechanism. COX-2 contributes to production of constrictive prostaglandins followed by impairing endothelial function and exacerbating endothelium-dependent contractions (EDCs) [24].

Our previous results found that H_2S lowered blood pressure and improved endothelial function [25]. However, the detailed mechanism of ameliorative effect of H_2S on impaired endothelial function in hypertension still remained underlying. Therefore, we hypothesize that H_2S can downregulate BMP4/COX-2 pathway, which may be involved in ameliorating endothelial dysfunction in hypertension. Here, the rat model of hypertension resulting from two-kidney one-clip (2K1C) model is used to verify the effect of H_2S on BMP4/COX-2 pathway and EDCs in renal artery.

2. Methods

2.1. Agents. NaHS, ACh, and *L*-NAME were purchased from Sigma-Aldrich Chemical (St. Louis, MO, United States). ACh and *L*-NAME were dissolved in distilled water.

2.2. Preparation of Hypertensive Model in Rats. 7-week-old male Sprague-Dawley rats were obtained from Animal Research Center of Hebei Medical University, which were kept in ordinary cages at room temperature of 25 ± 3°C with 12 h dark/light cycles (lights on 6:00) with food and water ad libitum. All animal procedures were complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (documentation number 55, 2001), and the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication number 85-23, revised in 1996) and approved by the Animal Care Committee of Hebei Medical University.

The rats were randomly divided into 4 groups ($n = 6$): Sham, Sham + NaHS, 2K1C, and 2K1C + NaHS. The rats in 2K1C and 2K1C + NaHS group were anesthetized with intraperitoneal injections of pentobarbital sodium (30 mg/kg) and then were subjected to unilateral clipping of the renal artery to establish 2K1C model. In brief, a left kidney was exposed via laparotomy and then the left renal artery was carefully separated from the left renal vein and connected tissues. In the 2K1C and 2K1C + NaHS groups, the left renal artery was clipped by a rigid U-shaped solid silver clip with an open slit of 0.25 mm, resulting in partial occlusion of renal perfusion. The contralateral kidney was left untouched. Sham and Sham + NaHS groups underwent the same procedure, but kidneys were only mobilized and renal vessels were only separated instead of being partially ligated. The rats were kept in cages after surgery for three weeks until blood pressure was stable; Sham + NaHS and 2K1C + NaHS groups received NaHS 56 μmol/kg/day intraperitoneally from the fourth week after the surgery which maintained for 20 weeks. Sham and 2K1C groups received saline as vehicle.

2.3. Mean Arterial Pressure Measurement. Mean arterial pressure (MAP) was measured noninvasively by tail-cuff plethysmography (BP-100A, Chengdu Taimeng Software CO. Ltd., Chengdu, China) after the rats were stabilized and remained quiescent. Briefly, MAP was measured before and further at every four weeks after surgery for 20 weeks. MAP measurement was always conducted between 9:00 and 12:00 AM and an average of 3 consecutive readings was taken as the systolic blood pressure of each rat.

2.4. Blood Vessel Preparation. Adult male rats were euthanized by CO_2 suffocation and rat intralobar renal arteries were dissected and placed in ice-cold Krebs solution (mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose, which gassed by 95% O_2/5% CO_2 at 37°C (pH~7.4). The arteries were carefully cleaned of adhering adipose tissue and cut into ring segments 2 mm in length for functional studies. Rings were suspended in a myograph (620 M, Danish MyoTechnology, Aarhus, Denmark) for recording of changes in isometric tension. Briefly, 2 stainless steel wires (40 μm in diameter) were put through the lumen of the vessel, and each wire was fixed to the jaws built in the myograph. The organ chamber was filled with 5 mL of Krebs solution. Each ring was stretched to an optimal tension of 2.5 mN and then allowed to stabilize for 60 minutes before the start of each experiment.

2.5. Endothelial Functional Studies. The series of experiments examined the alterations in EDCs. Firstly, renal artery rings were treated for 30 minutes with 100 μmol/L L-NAME to eliminate the interference of endothelium-derived nitric oxide (NO), a procedure commonly adopted to uncover ACh-induced EDCs [26, 27], and then contractions were elicited by Ach (0.03–100 μmol/L). Vasocontraction was determined in relative values as the percentage of 60 mmol/L KCl contraction.

2.6. Measurement of H_2S Content. H_2S levels in plasma were measured as described in previous experiment [28]. Briefly, 30 μL plasma was used to detect H_2S. H_2S concentrations were determined using a curve generated with sodium sulfide (0–40 μmol/L) standards, and the H_2S concentration in plasma was expressed as μmol/L.

2.7. Western Blot Analysis. Renal arteries from four groups were homogenized in ice-cold RIPA lysis buffer (1 μg/mL leupeptin, 5 μg/mL aprotinin, 100 μg/mL PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L NaF, and 2 mg/mL β-glycerophosphate). The homogenates were incubated on ice for 20 minutes and then centrifuged at 20 000 ×g for 20 minutes at 4°C. The supernatant was collected and the protein concentration was determined using the bicinchoninic acid (BCA) method (Generay biotechnology, Shanghai, China). Equal amounts of protein samples were electrophoresed through a 7.5% SDS-polyacrylamide gel and then transferred onto immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) using wet transfer at 100 V for 90 minutes at 4°C. Nonspecific binding sites were blocked by 5% nonfat milk or 1% BSA in 0.05% Tween-20 Tris-buffered saline (TBST) and then incubated overnight at 4°C with primary antibodies, anti-AT1R (1 : 1000, Abcam), anti-BMP4 (1 : 500, Sigma), anti-Nox2 (1 : 1000, Abcam), anti-Nox4 (1 : 1000, proteintech), anti-$p67^{phox}$ (1 : 1000, EPITOMICS), anti-Nitrotyrosine (1 : 1000, MILLIPORE), COX-2 (1 : 1000, Cayman), CSE (1 : 1000, Proteintech), and p38 MAPK, phospho-p38 MAPK (1 : 1000, Wanleibio). The blots were incubated with appropriate secondary antibodies with a horseradish peroxidase- (HRP-) conjugated goat anti-rabbit antibody (Proteintech, Chicago, United States) or HRP-conjugated rabbit anti-goat antibody (Proteintech, Chicago, United States) at 1 : 3000 dilution for 1 hour at room temperature. All blots washes were performed in TBST. Blots were developed with an enhanced chemiluminescence detection system (Sagecreation, Beijing, China). Densitometry was performed using lane-1 system (Sagecreation, Beijing, China).

2.8. Measurement of Malondialdehyde (MDA) Concentration. MDA concentration in plasma was measured by using thiobarbituric acid reactive substances (TBARS) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the instruction of manufacturer. The plasma was mixed with working solution, followed by 40-minute incubation in a boiling water bath. The mixed solution was centrifuged at 3500 rpm for 10 minutes. The absorbance of the supernatant (532 nm) was measured. The results were expressed as nmol/mL.

2.9. Statistical Analysis. Data are represented as means ± SEM. E_{max} denotes the maximal response produced by the constrictor or dilator. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests (GraphPad Software, San Diego, United States). P values less than 0.05 indicate statistical significance.

3. Result

3.1. Exogenous Administration of NaHS Lowered Blood Pressure and Ameliorated EDCs in Renal Artery in Hypertensive Rats. In 2K1C rats, NaHS (56 μmol/kg/day) treatment for 20 weeks significantly lowered MAP (159.4±5.434 mmHg versus 199.9 ± 3.312 mmHg, $P < 0.05$). MAP in Sham + NaHS rats had no changes compared with Sham rats (102.6 ± 2.687 versus 102.4 ± 3.721 mmHg) (Figure 1(a)). Rings in 2K1C rats displayed enhancing EDCs compared with Sham rings (E_{max}: 127.2 + 3.216% in 2K1C rings, $n = 7$ versus 34.68 + 10.34% in Sham, $n = 5$; $P < 0.05$). Renal arteries from 2K1C + NaHS rats reduced the enhanced EDCs to 72.66 + 6.007%. Chronically exogenously administrating NaHS had no effect on EDCs in Sham rats (Figure 1(b)).

3.2. Exogenous Administration of NaHS Improved the Level of Plasma H_2S and Renal Artery CSE Protein Expression in Hypertensive Rats. Western blot analysis showed that the protein level of CSE reduced in 2K1C rats compared with Sham rats. NaHS treatment in 2K1C rats increased the expression of CSE. There is no difference among the Sham, Sham + NaHS, and 2K1C + NaHS groups (Figure 2(a)). The plasma H_2S level in 2K1C rats was significantly lower than that in Sham rats (0.28 ± 0.04 versus 1.07 ± 0.08 μmol/L, $P < 0.05$). NaHS treatment elevated the plasma H_2S level to 0.67 ± 0.01 μmol/L ($P < 0.05$). There is no difference between Sham and Sham + NaHS group (1.07 ± 0.08 versus 1.24 ± 0.14 μmol/L, $P > 0.05$) (Figure 2(b)).

3.3. Exogenous Administration of NaHS Downregulated the Protein Expression of BMP4 in Hypertensive Rats. The Western blot results showed that the protein expression of BMP4 was elevated in hypertensive renal artery. Chronic treatment with NaHS decreased the level of BMP4 in hypertensive rats (Figure 3).

3.4. Exogenous Administration of NaHS Decreased the Level of Oxidative Stress in Hypertensive Rats. The level of oxidative stress as reflected by the expressions of NOX-2, NOX-4, and $p67^{phox}$ was augmented in 2K1C renal arteries. Exogenous treatment with NaHS rectified the overexpression of NOX-2, NOX-4, and $p67^{phox}$ (Figures 4(a)–4(c)). As stable markers of oxidative stress, the level of nitrotyrosine in 2K1C arteries was increased and the plasma MDA level in 2K1C rats was significantly elevated (6.779 ± 0.3518 versus 4.273 ± 0.1313 nmol/mL, $P < 0.05$). Treatment with NaHS decreased the arterial protein levels of nitrotyrosine and the plasma level of MDA (4.947 ± 0.1649 versus 6.779 ± 0.3518 nmol/mL, $P < 0.05$) (Figures 4(d)-4(e)).

3.5. Exogenous Administration of NaHS Decreased Phosphorylation-p38 MAPK Protein Expression in Hypertensive Rats. The Western blot results showed that the protein expression of the phosphorylation-p38 MAPK was upregulated in 2K1C renal arteries. Chronic treatment with NaHS could alleviate the increasing tendency of phosphorylation-p38 MAPK.

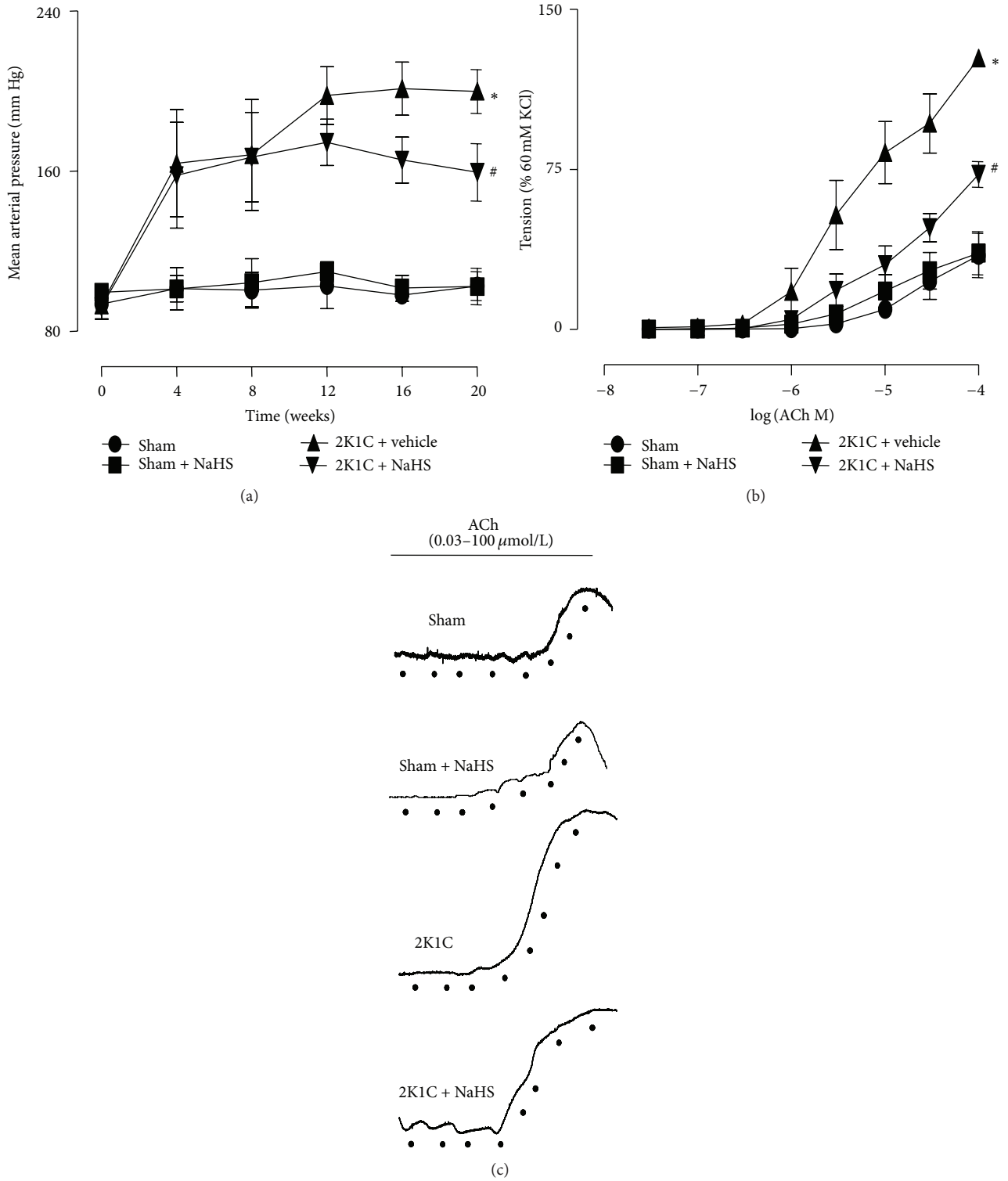

Figure 1: Effect of NaHS on blood pressure and endothelial-dependent renal arterial contraction. (a) Mean arterial pressure (MAP); (b) endothelium-dependent contractions (EDCs) in four groups; (c) original recording of EDCs in four groups. Data are means ± SEM. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus 2K1C vehicle. $n = 10$ in each group for MAP measurement; $n = 6$ in each group for EDCs measurement.

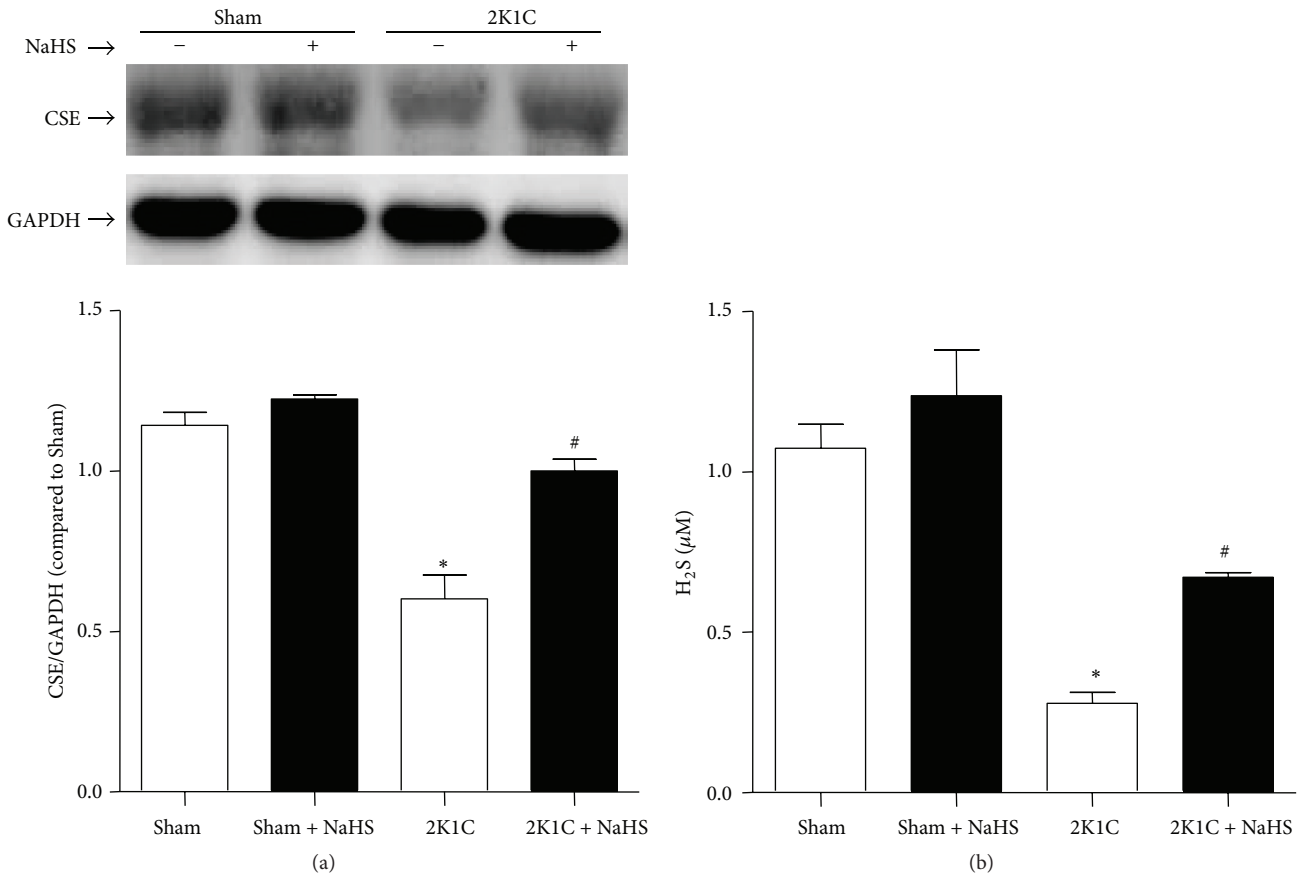

Figure 2: Effect of NaHS on protein levels of CSE in renal artery and plasma levels of H_2S. (a) Protein expression of CSE in renal arteries; (b) H_2S levels in plasma. Data are means ± SEM. $^*P < 0.05$ versus Sham; $^\#P < 0.05$ versus 2K1C vehicle. $n = 4$ in each group for Western blotting; $n = 10$ in each group for plasma H_2S level.

However, there is difference on the total expression of p38 MAPK among all groups (Figure 5).

3.6. Exogenous Administration of NaHS Decreased COX-2 Protein Expression in Hypertensive Rats. In renal arteries of 2K1C rats, protein expressions of COX-2 were increased compared with that of Sham rats. Chronic administration of NaHS inhibited the increased expression of COX-2 in 2K1C renal arteries (Figure 6).

4. Discussion

The present study demonstrates that NaHS reduces MAP and ameliorates EDCs which were both elevated in 2K1C hypertensive rats. Chronically administrating NaHS plays positive role in upregulating CSE protein expression and increasing the level of H_2S in plasma, in which both of them were also reduced in 2K1C hypertensive rats. Moreover, we found that in hypertensive rats the protein levels of BMP4 are increased, and then oxidative stress and p38 MAPK are activated, resulting in upregulation of COX-2, where the pathway contributes to augmentation of EDCs. Intraperitoneal injection of NaHS interestingly reverses the activation of the above pathway (Figure 7).

Among variety of experimental or genetic models of hypertension, the 2K1C hypertensive model is a classical one of renovascular angiotensin-II-dependent hypertension [29]. The 2K1C rats represent transient activation of renin-angiotensin system (RAS) and thereafter sustained rise in blood pressure [30]. This model is used widely to investigate hypertension. Previous study shows that endogenous cystathionine-γ-lyase (CSE)/H_2S pathway exited in vessels [31]. In hypoxia-induced pulmonary hypertension and maternal hypertension, endogenous CSE/H_2S pathway was downregulated [32, 33]. In our experiment, we also find that 2K1C rats exhibit increasing MAP, decreasing plasma level of H_2S, and downregulated protein expression of CSE. These results suggest that the hypertensive model has been established successfully, and endogenous CSE/H_2S system is downregulated in hypertensive artery. Moreover, NaHS supplement lowers MAP and restores the impairment of CSE/H_2S system. Our results demonstrate that endogenous CSE/H_2S system serves as a critical factor in the pathogenesis of hypertension, which is in accordance with the results of published article [4].

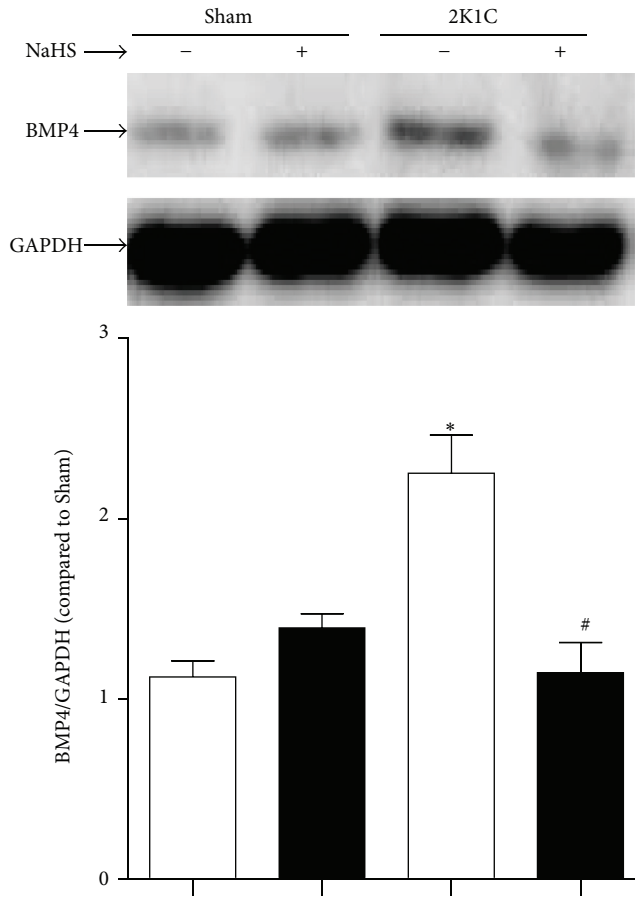

Figure 3: Effect of NaHS on protein levels of BMP4 in renal vascular hypertension rats. Data are means ± SEM. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus 2K1C vehicle. $n = 5$ in each group.

Previous studies have demonstrated that impairment of endothelial function is tightly associated with pathogenesis of hypertension [34, 35]. The damaged endothelium manifested not only the deterioration of endothelium-dependent relaxation, but also the enhancement of the EDCs [24]. Our results show that EDCs was strengthened in renal arteries of 2K1C rats, whereas exogenous administration of NaHS reverses the enhanced EDCs in hypertension. These results suggest that H_2S may ameliorate endothelial dysfunction and then reduces the elevated blood pressure.

Endothelial dysfunction induced by upregulation of NADPH oxidase and associated increasing oxidative stress has been found in spontaneously hypertensive rats [36, 37]. In experimental models of renovascular hypertension, the increased production of reactive oxygen species (ROS) mediates endothelial dysfunction resulting in progression of renovascular hypertension [38]. In an in vitro oscillatory shear stress (OS) model, it has firstly been confirmed that BMP4 coupled with oxidative stress [39]. A recent study also indicates that BMP4 can increase expression of NADPH oxidase and the level of ROS. These results suggest that BMP4 is a mediator and novel therapeutic target for cardiovascular diseases [40].

The endothelial dysfunction is due to the imbalance of the endothelium-derived relaxing and contracting factors [41, 42]. COX-2 is an inducible enzyme by inflammatory insult, and then its oxidative conversion of arachidonic acid in ECs results in the formation of an array of prostanoids that contributes to the occurrence of endothelium-dependent contractions [43, 44]. In renal arteries from hypertensive patients and SHR, BMP4 activates NADPH oxidase, leads to ROS overproduction and upregulation of COX-2 via p38 MAPK-dependent mechanism, which at last increases production of PGF2a, and then strengthens EDCs [24]. All of the above experiments indicated that BMP4/ROS/p38 MAPK/COX-2 pathway was involved in endothelium dysfunction of hypertension.

In our experiment, we find that the protein expressions of BMP4 are increased in hypertensive renal artery. The oxidative stress is accordingly strengthened in hypertension, verified by the increased expression of NADPH oxidase subtype, including NOX2, NOX4, and $p67^{phox}$. Nitrotyrosine is considered as a biomarker for endogenous level of peroxynitrite [45] and has been correlated with elevated levels of other indices of oxidative stress [46]. Malondialdehyde (MDA) is formed in the lipid peroxidation caused by ROS and is also used as a biomarker to measure the level of oxidative stress.

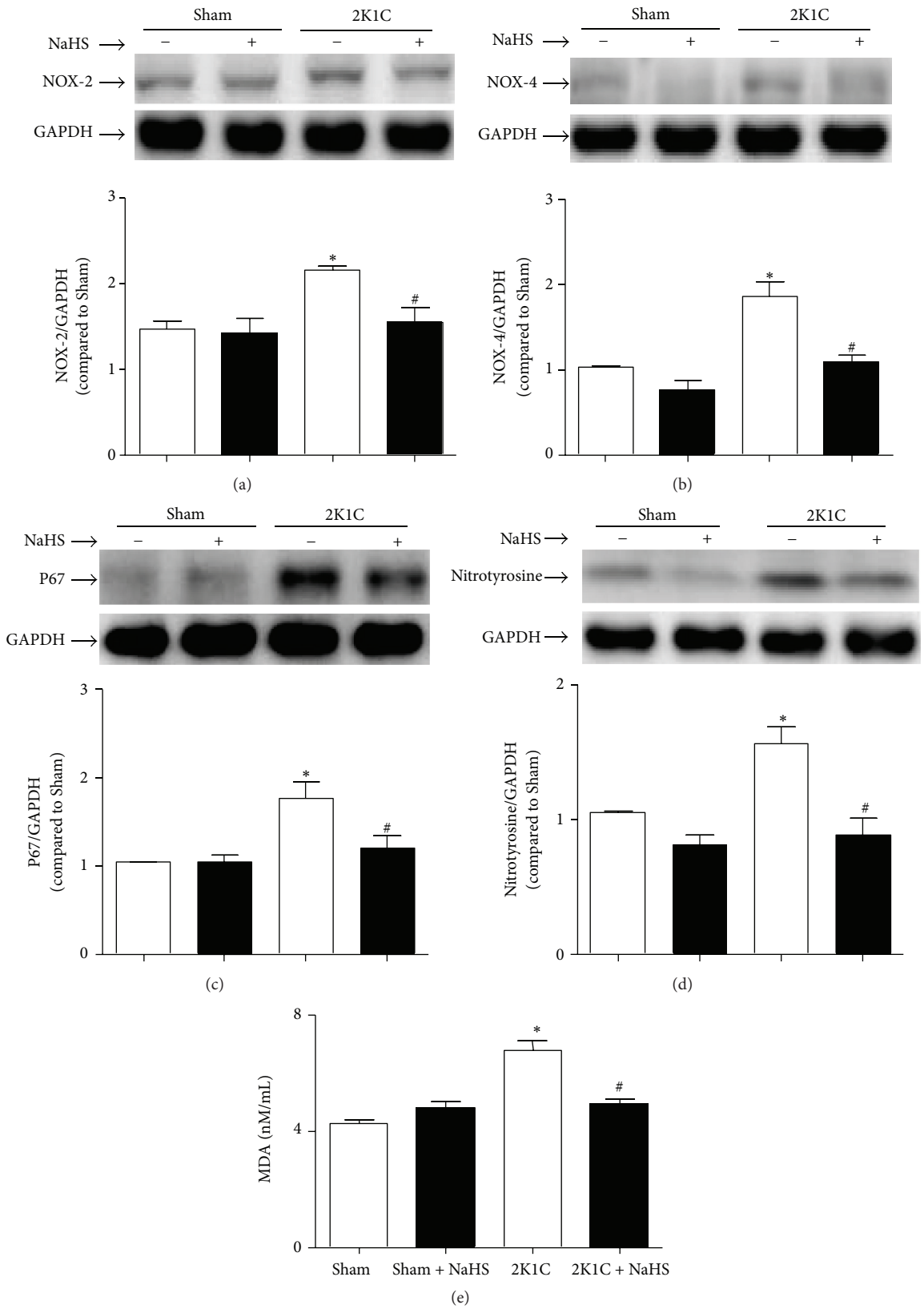

Figure 4: Effect of NaHS on oxidative stress. The oxidative stress was determined by the protein expression of NOX-2 (a), NOX4 (b), P67phox (c), and nitrotyrosine (d) in renal artery and the plasma level of MDA (e). Data are means ± SEM. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus 2K1C vehicle. $n = 5$ in each group for Western blotting; $n = 20$ for MDA measurement.

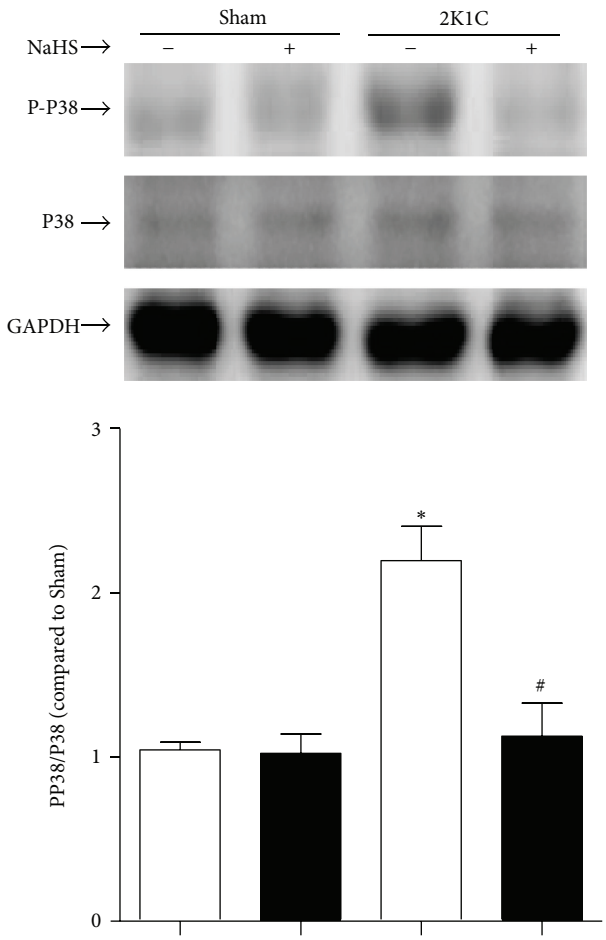

Figure 5: Effect of NaHS on the activation of p38 MAPK. Data are means ± SEM. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus 2K1C vehicle. $n = 5$ in each group.

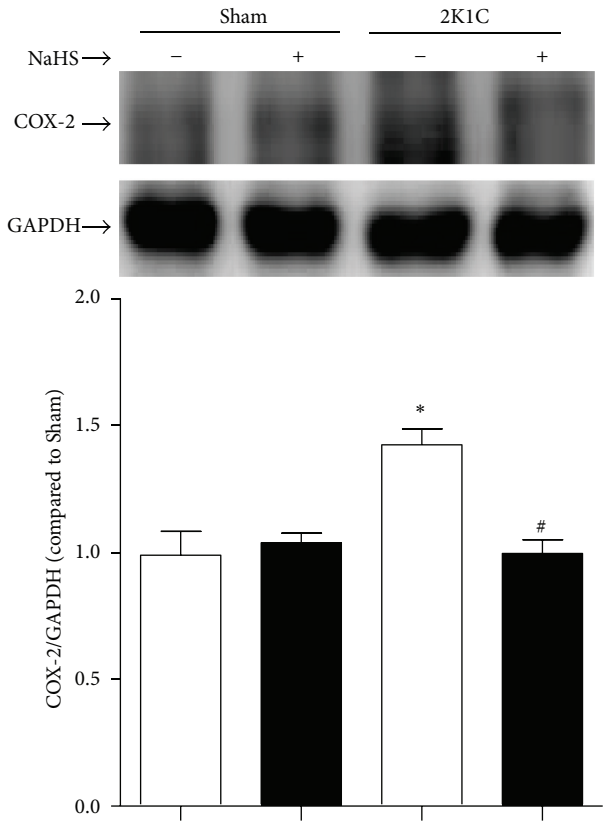

Figure 6: Effect of NaHS on protein levels of COX-2. Data are means ± SEM. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus 2K1C vehicle. $n = 5$ in each group.

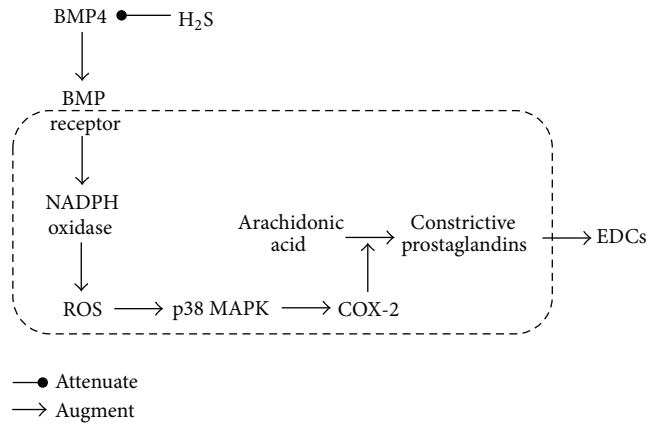

Figure 7: Schematic representation of the ameliorative effect of H_2S on EDCs.

As stable marker of oxidative/nitrative stress [47], the expression of nitrotyrosine and the level of MDA elevated in 2K1C rats. Otherwise, the phosphorylation level of p38 MAPK is also increased in hypertensive renal artery. At last, protein expression of COX-2 is elevated in hypertensive renal artery. These results demonstrate that the activation of BMP4/ROS/p38 MAPK/COX-2 pathway involves the pathogenesis of EDCs and hypertension, in accordance with that in other published articles. Moreover, exogenous treatment of NaHS interestingly prevents the activation of the above pathways. These results suggest that protective effect of H_2S on endothelium may be mediated by BMP4/ROS/p38 MAPK/COX-2 pathway.

Of course, there are several limits in our study. In our experiment, hypertension was induced by 2K1C animal model as previously described [48]. This model was used to mimic hypertension which is characterized by renin-angiotensin system (RAS) being excessively activated [49]. The results derived from the present study should be verified in other hypertensive animal models, such as spontaneous hypertension rats. Moreover, we only found the association between H_2S and the BMP4/ROS/p38 MAPK/COX-2 pathway. The exact mechanism of how H_2S regulates the above pathways needs to be further investigated.

5. Conclusion

Taken together, our present results demonstrate the inhibitory effect of H_2S on BMP4 mediated cellular signaling cascade in hypertension, which may be involved in the

ameliorative effect of H_2S on endothelial dysfunction. Our findings further suggest the potential therapeutic value of H_2S for hypertension.

Competing Interests

The authors declare that there are no competing interests.

Acknowledgments

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Research Article

AMP-Activated Protein Kinase Attenuates High Salt-Induced Activation of Epithelial Sodium Channels (ENaC) in Human Umbilical Vein Endothelial Cells

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Recent studies suggest that the epithelial sodium channel (ENaC) is expressed in the endothelial cells. To test whether high salt affects the NO production via regulation of endothelial ENaC, human umbilical vein endothelial cells (HUVECs) were incubated in solutions containing either normal or high sodium (additional 20 mM NaCl). Our data showed that high sodium treatment significantly increased α-, β-, and γ-ENaC expression levels in HUVECs. Using the cell-attached patch-clamp technique, we demonstrated that high sodium treatment significantly increased ENaC open probability (P_O). Moreover, nitric oxide synthase (eNOS) phosphorylation (Ser 1177) levels and NO production were significantly decreased by high sodium in HUVECs; the effects of high sodium on eNOS phosphorylation and NO production were inhibited by a specific ENaC blocker, amiloride. Our results showed that high sodium decreased AMP-activated kinase (AMPK) phosphorylation in endothelial cells. On the other hand, metformin, an AMPK activator, prevented high sodium-induced upregulation of ENaC expression and P_O. Moreover, metformin prevented high salt-induced decrease in NO production and eNOS phosphorylation. These results suggest that high sodium stimulates ENaC activation by negatively modulating AMPK activity, thereby leading to reduction in eNOS activity and NO production in endothelial cells.

1. Introduction

Previous investigations have shown that dietary high salt intake impairs relaxation of blood vessels in response to vasodilator stimuli [1, 2]. A possible contributor to this impairment of vasodilator-stimulated vascular relaxation in animals on a high salt diet could be an impaired function of the endothelium, which normally plays a critical role in regulating vascular tone by generating vasodilator and vasoconstrictor chemicals [2, 3]. Nitric oxide (NO) is an important endothelium-derived relaxation factor, which is produced by the action of endothelial nitric oxide synthase (eNOS). Reduced NO release impairs the vasodilation of blood vessels, which promotes endothelial dysfunction [4]. A moderate increase in sodium concentration has been shown to stiffen cultured endothelial cells within minutes, thereby reducing NO release [5]. However, the mechanism by which changes in sodium concentration induce these cellular responses in the endothelium is currently unknown.

Epithelial sodium channel (ENaC) mediates sodium transport across the apical membrane of epithelia and is considered the predominant site for regulating sodium reabsorption in kidney, lung, colon, and sweat glands [6]. ENaC consists of three different subunits (α, β, and γ) that are

expressed in a tissue specific manner [7, 8] and can be blocked by amiloride [9]. The regulation of ENaC is tissue specific and mediated by the mineralocorticoid hormone aldosterone and aldosterone-induced proteins, for example, the serum- and glucocorticoid-regulated kinase 1 [10, 11]. Thus, various proteins and extracellular factors interact directly or indirectly with ENaC [9, 12]. ENaC is the typical sodium channel found in a variety of epithelial cells of kidney, colon, and lung. However, recent studies indicate that ENaC is also expressed in vascular endothelial cell, where its roles are similar to that in the epithelia [13–15]. In endothelial cells, an acute application of aldosterone leads to amiloride-sensitive cell swelling and a reduction in NO release, which is probably caused by sodium and water uptake mediated by the endothelial ENaC [16]. Moreover, inhibiting endothelial ENaC activates eNOS and increases NO production in mesenteric arteries [17]. However, the role of ENaC in high salt-induced endothelium dysfunction is unknown.

The metabolic sensor AMP-activated kinase (AMPK) is ubiquitous metabolite-sensing Ser/Thr kinase that is a heterotrimer comprising catalytic α-subunit and regulatory β- and γ-subunits. AMPK activity increases under the conditions of metabolic stress in response to elevated intracellular AMP : ATP ratios [18]. It has been demonstrated *in vitro* that stimulation of AMPK by metformin, phenformin, and 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) inhibits ENaC conductance in *Xenopus* oocytes, HEK293 cells, and polarized renal epithelial cells [19, 20].

In this study, we aim to test whether high sodium treatment may affect eNOS activity and NO production levels by altering the expression profile and activity of ENaC via AMPK-dependent signaling.

2. Materials and Methods

2.1. Endothelial Cell Culture. HUVECs were cultured in endothelial cell growth medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) plus 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Confluent cells were used for experiments between passages 3 and 6. When HUVECs had grown to 85–90% confluence in 6-well plates, they were incubated with or without different concentrations (10, 20, and 30 mM) of additional NaCl and maintained in 95% air and 5% CO_2 at 37°C for 24 h.

2.2. Cell Viability Assay. Cell viability was estimated by measuring mitochondrial dehydrogenase activity, using the colorimetric MTT assay, based on the fact that viable cells (but not dead cells) can reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as previously described in our previous works [21]. Briefly, cells were cultured in 96-well plates and treated with either 10, 20, or 30 mM NaCl in RPMI 1640 medium supplemented with 10% FBS for 24 or 48 h. The cells were then incubated with MTT solution (5 mg/mL) for 4 h. The formazan crystals, thus, formed were dissolved in dimethyl sulfoxide (DMSO) (150 μL/well). The absorbance was recorded at a wavelength of 490 nm using a microplate reader (Tecan, Switzerland). All experiments were performed at least 3 times.

2.3. Patch-Clamp Studies. ENaC single-channel currents were recorded using cell-attached patch-clamp configuration using an Axon Multiclamp 200B amplifier (Axon Instruments, Foster City, CA, USA) at room temperature (22–25°C). HUVECs were thoroughly washed with a NaCl solution containing (in mM) 115 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, and 5 Na-HEPES, adjusted to pH 7.2 with NaOH. This NaCl solution was used as the bath solution for recordings. Patch pipettes were pulled from borosilicate glass with a Sutter P-97 horizontal puller, and resistance of the pipettes was ranged between 6 and 10 MΩ when filled with the NaCl solution. The data were acquired by application of 0 mV pipette potential and were sampled at 5 kHz and low-pass filtered at 1 kHz with Clampex 10.2 Software (Molecular Devices, Sunnyvale, CA, USA). Prior to analysis, the single-channel traces were further filtered at 30 Hz. ENaC activity was recorded for 2 min after the formation of the cell-attached mode and stabilization of ENaC activity. A single patch was typically recorded for at least 30 min and P_O was analyzed using at least 30 min recordings. The open probability (P_O) of ENaC was calculated as follows: $P_O = NP_O/N$, where N (N was estimated by the current amplitude histogram) represents the apparent number of active channels in the patch.

2.4. Western Blot Analysis for ENaC, eNOS, and AMPK. For western blot analysis, protein samples were extracted from HUVECs, separated by 10% SDS-PAGE, and transferred to nitrocellulose membrane using a Trans-Blot unit for 1.5 h at 250 mA. Membranes were blocked with 5% (wt/vol) nonfat milk in TBS (pH 7.4) containing 0.1% (vol/vol) Tween 20 (TBS-T) for 1 h at room temperature (25°C). Then, the membranes were incubated with primary antibodies against α-ENaC (StressMarq, Victoria, BC, Canada), phospho-eNOS (Ser1177; Thermoscientific, Waltham, MA, USA), eNOS (Abcam, NJ, USA), AMPKα and phospho-AMPKα (Cell Signaling Technology, Boston, MA, USA), and β-actin (Santa Cruz Biotechnology, USA) overnight at 4°C, followed by washing in TBS-T and incubation with the corresponding secondary antibodies (1 : 10,000) for another 1 h at 22–25°C. Membranes were finally washed with TBS-T and the protein bands were detected by ECL kit (Invitrogen, Carlsbad, CA, USA) and scanned densitometry (Bio-Rad, CA,USA).

2.5. Measurement of NO Production by Laser Confocal Fluorescence Microscopy. Fluorimetric measurements were performed on HUVECs using the Olympus Fluoview FV1000 laser scanning confocal system. 4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA; Life Technology, Rockford, IL, USA) was used as the NO indicator. Briefly, DAF-FM DA (10 μM) was added to the HUVECs for 1 h. Next, the labeled cells were washed twice in modified PBS before analysis using confocal microscopy. The amount of NO in response to high salt incubation was evaluated by measuring the fluorescence intensity at 515 nm upon excitation at 495 nm.

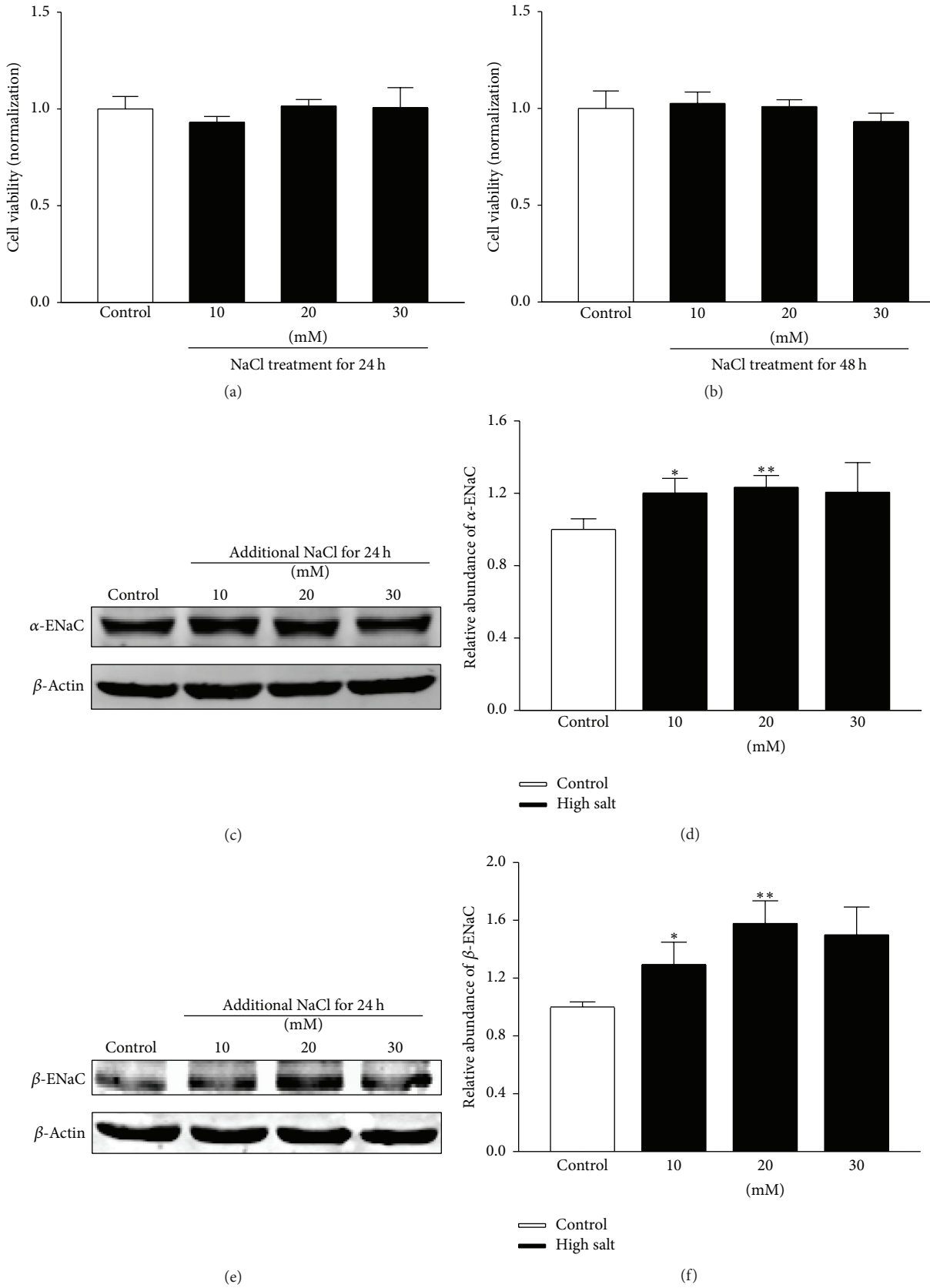

Figure 1: Continued.

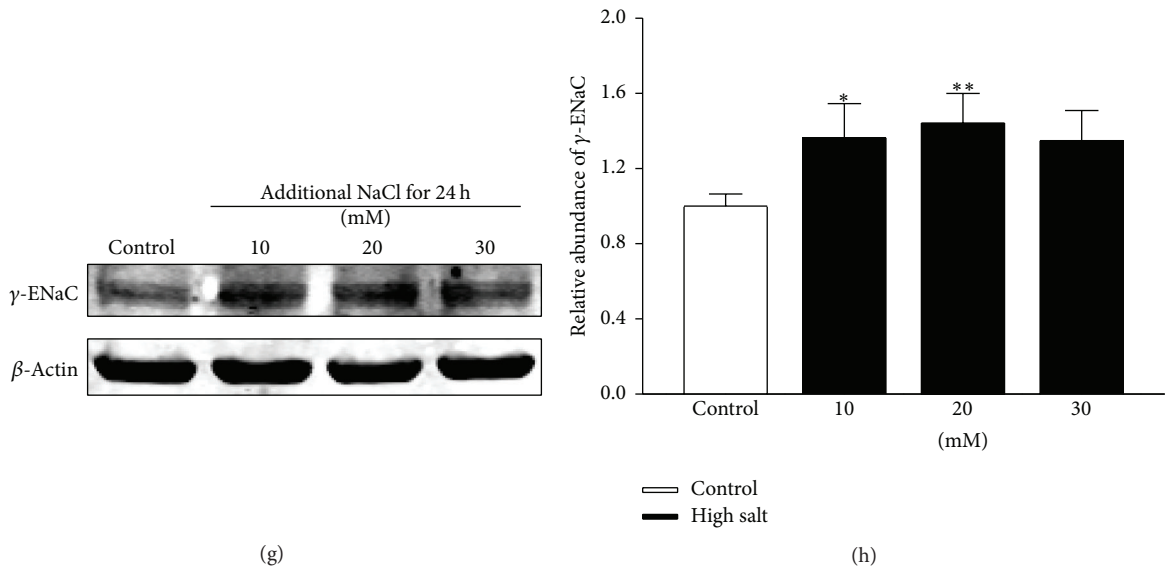

Figure 1: Effect of high sodium treatment on ENaC expression in HUVECs. ((a) and (b)) MTT assay was performed to measure the effect of high sodium concentration on cell viability. NaCl at concentrations of 10, 20, and 30 mM was, respectively, applied to the cells for (a) 24 h and (b) 48 h ($n = 6$ in each group). ((c)–(h)) Effects of high sodium application (additional 10, 20, and 30 mM NaCl treated for 24 h) on α-, β-, and γ-subunit levels of ENaC in HUVECs. Levels of ENaC subunits and β-actin were evaluated using western blot analysis. The densitometry values were normalized to β-actin ($n = 5$ in each group). $*$ indicates $p < 0.05$; $**$ represents $p < 0.01$ versus control.

2.6. Statistical Analysis. All data are represented as mean ± SEM. Statistical analysis was performed using SigmaPlot and SigmaStat Software (Jandel Scientific, CA, USA). One-way ANOVA, ANOVA for repeated measurements (followed by Student-Newman-Keuls *post hoc* test), or Student's t-test was used for statistical analysis. Differences were considered statistically significant for $p < 0.05$.

3. Results

3.1. High Salt Treatment Increases ENaC Expression in HUVECs. We first determined whether incubation of HUVECs with high sodium could affect cell viability. Our data showed that treatment of the HUVECs with additional 10, 20, and 30 mM NaCl for up to 48 h did not affect cell viability (Figures 1(a) and 1(b)).

Our data show that α-, β-, and γ-subunits of ENaC are expressed in HUVECs. To test whether high salt alters the expression profile of ENaC, we incubated HUVECs with an aldosterone-free medium containing additional 10, 20, or 30 mM NaCl for 24 h. Our data show that addition of 10 mM and 20 mM NaCl but not 30 mM NaCl significantly enhanced the abundance of all the three subunits of ENaC compared with that in the normal-sodium condition (Figures 1(c)–1(h)). We then examined whether the effect of high salt on ENaC expression of HUVECs was due to the change in osmolarity. As the osmolarity of 20 mM of mannitol equals the osmolarity of 10 mM of NaCl, 20, 40, and 60 mM mannitol were, respectively, used to examine whether osmolarity can alter ENaC expression. We found that mannitol did not affect ENaC expression at any concentration we used (data not shown).

3.2. ENaC Activity in HUVECs Was Increased by High Salt. Since additional 20 mM NaCl treatment had the most impact on the expression levels of all three subunits of ENaC, we therefore chose additional 20 mM NaCl to treat HUVECs for 24 h followed by cell-attached patch-clamp analysis. Under control conditions, we detected a single-channel current with small amplitude in HUVECs (Figure 2(a)). This current was blocked by 0.5 μM amiloride (Figure 2(b)). Furthermore, this amiloride-sensitive current was significantly upregulated by additional 20 mM NaCl, but not by additional 40 mM mannitol (Figures 2(c) and 2(d)). The high sodium-induced activation of amiloride-sensitive currents was significantly blocked by 0.5 μM amiloride (Figure 2(e)). These results together suggest that the regulatory effect of high sodium on ENaC activity in HUVECs was not due to osmotic stress (Figure 2(f)).

3.3. ENaC Activity Contributes to High Salt-Induced Downregulation of eNOS Phosphorylation and NO Production. Although accumulated evidence suggests that excess salt can stiffen the vascular endothelium and reduce NO release, it is presently not known whether the endothelial ENaC is involved in high sodium stimulated cellular responses. Western blotting experiments with total protein homogenates obtained from HUVECs showed that treatment with high sodium (additional 20 mM NaCl) for 24 h significantly reduced eNOS phospho-Ser 1177 levels. In contrast, ENaC blockade after using treatment with 0.5 μM amiloride for 1 h significantly prevented high sodium-induced downregulation of eNOS phospho- Ser 1177 (Figures 3(a) and 3(b)).

To determine whether high sodium can decrease NO production, HUVECs were loaded with a NO-sensitive probe,

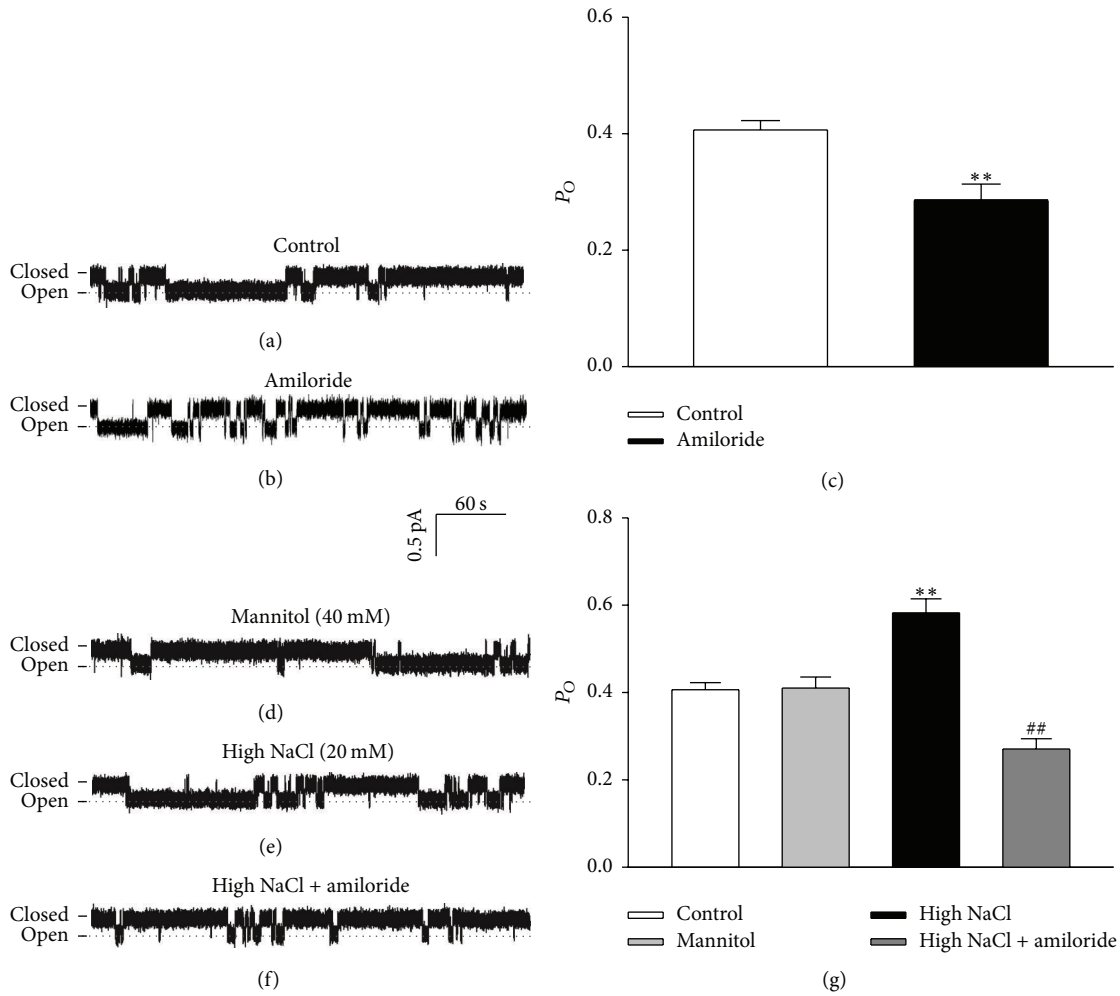

Figure 2: Effect of high sodium on ENaC activity in HUVECs. ((a) and (b)) Representative traces of ENaC single-channel current recorded from HUVECs with and without amiloride treatment. (c) Summarized P_O obtained from the single-channel recordings as shown in (a) and (b). The data show that P_O calculated from amiloride group significantly decreased compared to that from control group ($n = 5$; ** indicates $p < 0.01$ versus control group). ((d)–(f)) Representative traces of ENaC single-channel current recorded from HUVECs under indicated conditions. (g) Summarized P_O obtained from the single-channel recordings as shown in (d)–(f). The data show that P_O calculated from high NaCl group significantly increased compared to that from control group ($n = 5$ in each group). ** indicates $p < 0.01$ versus control group; ## represents $p < 0.01$ versus high NaCl group.

DAF-FM DA. The fluorescent intensity was significantly reduced after addition of 20 mM NaCl, suggesting a reduction of NO production under this condition. Interestingly, the inhibitory effect of high salt on NO production was significantly restored by amiloride in HUVECs (Figures 3(c) and 3(d)). These results support the notion that ENaC activity may contribute to high sodium treatment-induced reduction of eNOS activity and NO production.

3.4. AMPK Attenuates High Salt-Induced Increase in ENaC Expression. It has been demonstrated *in vitro* that AMPK inhibits ENaC [19, 20, 22]. Therefore, we reasoned that manipulation of AMPK activity may affect ENaC expression profile and/or ENaC activity. The data shown in Figure 4(a) suggest that AMPK activity was blunted by high sodium (Figure 4(a)). However, blocking ENaC by amiloride had no obvious effects on AMPK activity (Figure 4(a)). We speculated that the inhibition of AMPK activity might be a reason for enhancement of ENaC expression and activity. We then examined whether metformin, an AMPK activator, could reverse the high sodium treatment-induced increase in ENaC expression. Consistent with the results described above, the expression levels of α-, β-, and γ-ENaC were significantly upregulated by high sodium; however, the effect of high sodium on ENaC expression in HUVECs was almost completely diminished in HUVECs treated with 2 mM metformin for 24 h (Figures 4(b), 4(c), and 4(d)).

3.5. AMPK Activation Reduces High Salt-Induced Elevation of ENaC Activity. We next determined whether AMPK activity contributes to the regulatory effect of high salt on ENaC P_O. The data show that application of 2 mM metformin to HUVECs under control conditions had no effect on ENaC P_O (0.41 ± 0.02 to 0.37 ± 0.03), suggesting that metformin does not

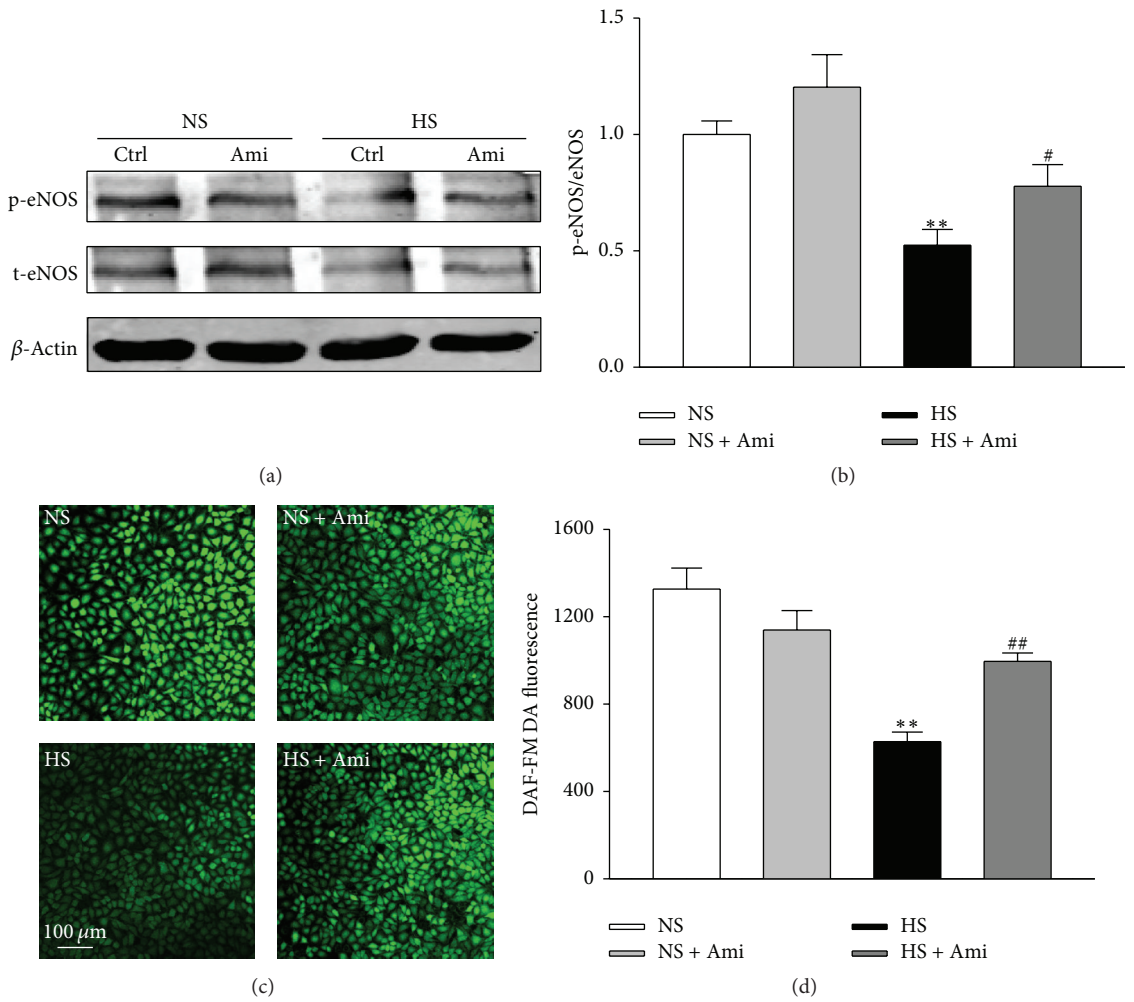

Figure 3: Effect of ENaC blockade on the phosphorylation of eNOS Ser 1177 and production of NO. (a) Representative western blots demonstrating the expression levels of total eNOS, p-eNOS, and β-actin in HUVECs cultured with normal sodium (NS), NS plus 0.5 μM of amiloride (Ami), high sodium (additional 20 mM NaCl treated for 24 h; HS), and HS plus 0.5 μM of amiloride (Ami). (b) Summaries of eNOS activity in response to amiloride in NS or HS group. (c) The images represent the levels of intracellular NO detected using membrane-permeable fluorescent probe, DAF-FM DA, under indicated conditions in HUVECs. (d) Summary of fluorescence results from (c) ($n = 5$ in each group). ** indicates $p < 0.01$ versus NS group; # represents $p < 0.05$; ## indicates $p < 0.01$ versus HS group.

affect ENaC activity under control conditions (Figures 5(a), 5(b), and 5(e)). However, high sodium-induced increase in ENaC activity was significantly attenuated by application of 2 mM metformin (Figures 5(c), 5(d), and 5(e)). These results suggest that metformin exerts a protective effect on high sodium-induced enhancement of ENaC activity in HUVECs.

3.6. AMPK Activation Prevents High Salt-Induced Downregulation of eNOS Phosphorylation and NO Production. High salt concentration stimulates ENaC and leads to the reduction of eNOS activity and NO production. The stimulated ENaC expression and activity could be inhibited by an AMPK activator, metformin. Therefore, we tested whether AMPK activation could prevent high salt-induced downregulation of eNOS activity and NO production. We found that metformin administration significantly increased high salt-induced inhibition of eNOS phosphorylation (Figures 6(a) and 6(b)). Furthermore, high salt-induced inhibition of NO production could also be prevented by metformin (Figures 6(c) and 6(d)).

4. Discussion

This study provides evidence that endothelial ENaC is regulated by AMPK and that this regulation may play an important role in dietary salt-induced endothelial dysfunction. The major findings include the following: (1) high salt significantly elevated ENaC expression and activity in endothelial cells; (2) high salt-induced reduction of eNOS activity and NO level were prevented by the specific ENaC blocker, amiloride; (3) AMPK activity was reduced in high salt-treated endothelial cells and metformin, an AMPK activator, significantly reversed high salt-induced elevation of ENaC expression and

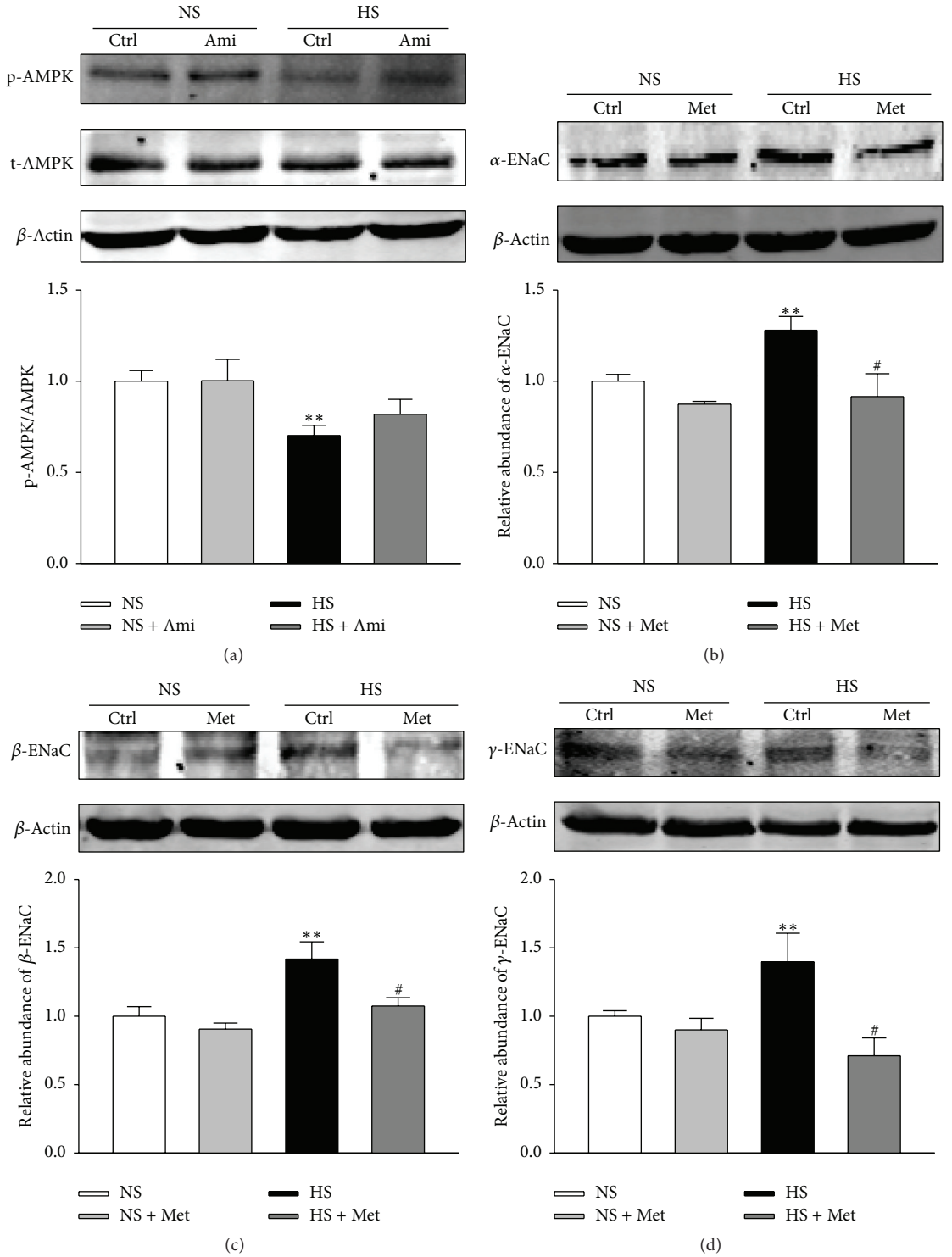

Figure 4: High salt-induced increase in expression of ENaC protein was attenuated by metformin (AMPK activator). (a) Representative western blots demonstrating the expression levels of total AMPK, p-AMPK, and β-actin in HUVECs cultured with normal sodium (NS), NS plus 0.5 μM of amiloride (Ami), high sodium (additional 20 mM NaCl treated for 24 h; HS), and HS plus 0.5 μM of amiloride (Ami). ((b)–(d)) Representative western blots demonstrating the expression levels of α-, β-, and γ- ENaC subunits in HUVECs cultured with NS, NS plus 2 mM metformin (Met), HS, and HS plus 2 mM metformin (Met) for 24 h. The densitometry values were normalized to β-actin ($n = 5$ in each group). ** indicates $p < 0.01$ versus NS group; # represents $p < 0.05$ versus HS group.

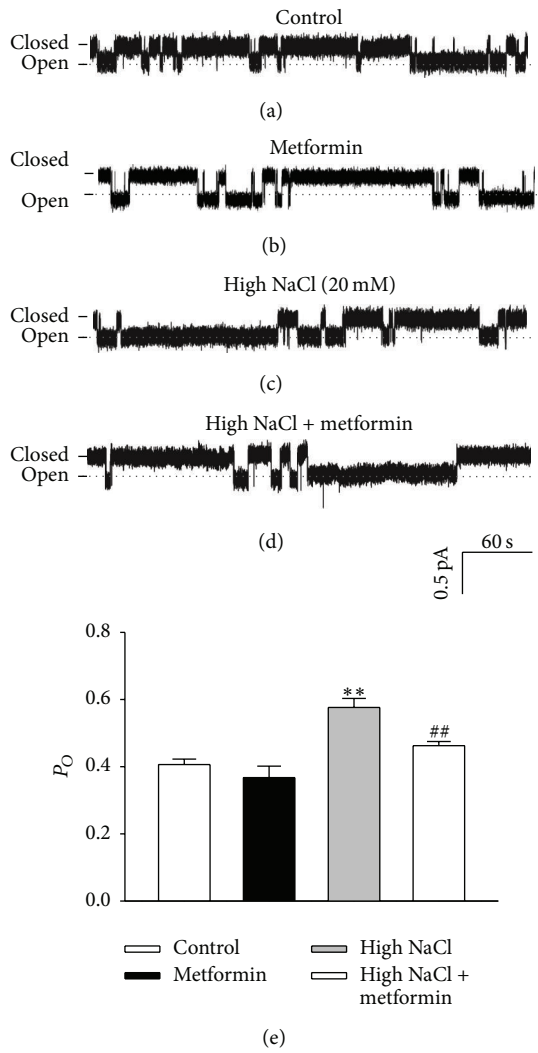

Figure 5: High salt-induced increase in ENaC activity was blunted by metformin. ((a)–(d)) Representative traces of ENaC single-channel current recorded from HUVECs under indicated experimental conditions. (e) Summarized P_O obtained from the single-channel recordings as shown in ((a)–(d)). The data showed that metformin significantly reduced ENaC P_O compared to that from high NaCl group ($n = 5$ in each group). ** indicates $p < 0.01$ versus control group; ## represents $p < 0.01$ versus high NaCl group.

activity; and (4) activation of AMPK also prevented high salt-induced reduction of eNOS activity and NO level in endothelial cells.

Dietary salt loading in rats is known to result in increase of arterial blood pressure and impairment of endothelium-dependent vascular relaxation. Sodium in the plasma has been suggested to play a primary role in controlling blood pressure because a small increase in plasma sodium level (1–3 mM) was found in individuals with hypertension [23, 24]. Moreover, an acute increase in plasma sodium concentration observed in people on high salt diet has been proposed to alter the mechanical properties of the vascular endothelium [5, 24]. The sodium-selective ion channel, ENaC, is expressed on the surface of endothelial cells; therefore, it could act as a functional link between the plasma and the endothelial cells.

It has been reported that the elevation of plasma Na^+ concentration stimulates the membrane insertion of α-subunit of ENaC in human endothelial cells [16, 25]. Pérez et al. [17] found that the inhibition of endothelial ENaC activates eNOS and increases NO production in mesenteric arteries. In this study, our results showed that high sodium concentration significantly elevates ENaC abundance and activity and reduces eNOS activity and NO level. Here, we showed, for the first time, that α-, β-, and γ-subunits of ENaC are expressed in cultured HUVECs, and high sodium concentration upregulated both the protein level and the channel activity of ENaC. Moreover, the protein abundance of α-, β-, and γ-ENaC was significantly increased by high sodium treatment. Whereas, Wang et al. [15] reported that α-subunit of ENaC, but not β- and γ-subunits, was expressed in cultured endothelial cells by PCR. We speculate that the reason for this discrepancy might be due to the experimental conditions or the difference in antibody preparation. Moreover, our earlier results have also shown that high salt diet inhibits ENaC and leads to the enhancement of acetylcholine-induced relaxation of the vasculature in SD rats, which might be a feedback inhibition of the development of salt-sensitive hypertension [26]. However, high salt diet significantly increased the expression and activity of ENaC and induced hypertension in salt-sensitive rats (our unpublished data). Together, we suggest that high salt challenge upregulates ENaC and leads to endothelial dysfunction, which might play an important role in the development of salt-sensitive hypertension.

AMPK regulates ENaC activity in oocytes, polarized kidney cells, and lung epithelial cells [19, 20, 22]. In this study, we examined the effects of AMPK activation by metformin on ENaC abundance and activity in HUVECs. We first examined the effect of high sodium treatment on activation of AMPK, measured by phosphorylated AMPKα appearance. Our data show that phosphorylated AMPK levels in HUVECs decreased 24 h after exposure to additional 20 mM NaCl and that ENaC blockade had no effect on high sodium-induced inhibition of AMPK activation in these cells. The results obtained in kidney regarding the effects of high salt on AMPK activity are controversial. It appeared that, in rat kidney, high salt diet activated AMPK, whereas low salt diet led to inhibition of AMPK activity. Interestingly, both low and high salt media transiently activated AMPK in the cultured macula densa cell line MMDD1, an effect due to changes in osmolarity [27]. In contrast, another study suggests that renal expression of activated AMPK was dramatically decreased in rat fed with high salt intake [28]. These conflicting results suggest that the effects of high salt on AMPK activity may depend upon experimental model and cell types. Nevertheless, we suggest that high salt attenuates AMPK activity in HUVECs.

Accordingly, we examined the effects of metformin on ENaC abundance and activity and found that metformin markedly inhibited the all three subunits of ENaC protein expression and reduced ENaC P_O in HUVECs. These results demonstrate that activation of AMPK abrogates the activated effect of high sodium treatment on ENaC current and expression of α-, β-, and γ-subunits. Since AMPK is a sensor of

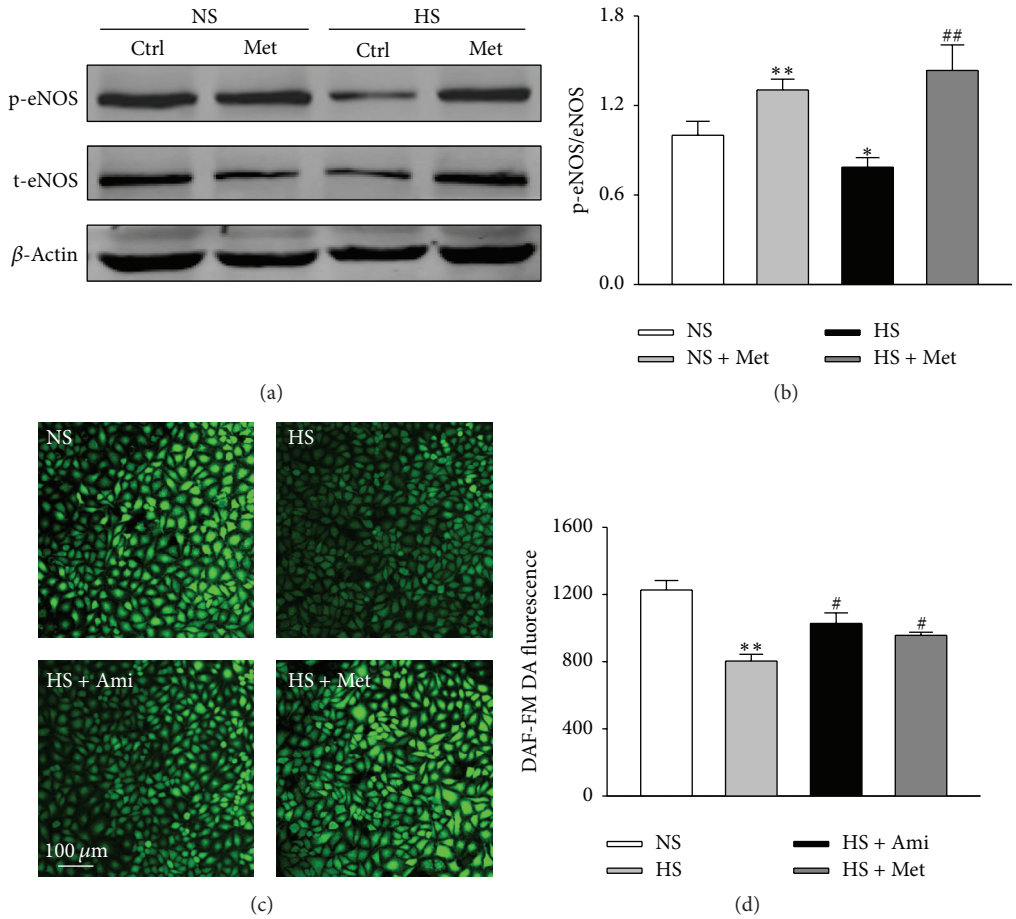

Figure 6: High salt-induced reductions in the levels of eNOS phosphorylation and NO production were partially reversed by metformin. (a) Representative western blots demonstrating the expression levels of total eNOS, p-eNOS, and β-actin in HUVECs cultured with NS, NS plus 2 mM metformin (Met), HS, and HS plus 2 mM metformin (Met) for 24 h. (b) Summaries of eNOS activity in response to metformin in NS or HS group. (c) The images represent the levels of intracellular NO detected by a membrane-permeable fluorescent probe, DAF-FM DA, under indicated conditions in HUVECs. (d) Summary of fluorescent intensity analyzed from the experiments shown in (c) ($n = 5$ in each group). $*$ and $**$, respectively, indicate $p < 0.05$ and $p < 0.01$ versus NS group; # and ##, respectively, represent $p < 0.05$ and $p < 0.01$ versus HS group.

the "cellular fuel," which responds to changes in cellular ATP, therefore, AMPK regulation of ENaC might provide a mechanism to adapt to high sodium concentration and/or metabolic stress. There are several lines of evidence to suggest that AMPK inhibits ENaC through functional regulation of the ubiquitin ligase Nedd4-2 [19, 29]. Nedd4-2 interacts with β- and γ-subunits of ENaC at their C-terminal tails, thereby contributing to the reduction of ENaC cell surface expression [30, 31]. Recent work has also suggested that Nedd4-2 activation may affect opening probability in addition to an effect on cell surface expression of ENaC [32, 33]. Therefore, we speculated that the protective effects of AMPK activation by metformin on ENaC could be mediated by Nedd4-2. However, there are numerous possible mechanisms by which ENaC regulation may be linked to AMPK. Investigating intermediate pathways and underlying mechanisms involved are important goals for future studies.

5. Conclusions

Our study suggests that endothelial ENaC is stimulated by high concentration of salt and negatively modulates eNOS in response to high salt treatment. Blocking ENaC in endothelial cells increases eNOS activity and NO production. High salt stimuli-induced enhancement of ENaC expression and activity in HUVECs was downregulated by AMPK. Therefore, AMPK might act directly in the endothelium by inhibiting ENaC expression and activity, thereby contributing to endothelial protection in response to high salt challenge.

Abbreviations

ENaC: Epithelial sodium channel
AMPK: AMP-activated protein kinase
eNOS: Endothelial nitric oxide synthase

NO: Nitric oxide
HUVEC: Human umbilical vein endothelial cells
P_O: Open probability.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Wei-Wan Zheng and Xin-Yuan Li contributed equally to this work.

Acknowledgments

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Research Article

Hydrogen Sulfide Alleviates Postharvest Senescence of Grape by Modulating the Antioxidant Defenses

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Hydrogen sulfide (H_2S) has been identified as an important gaseous signal in plants. Here, we investigated the mechanism of H_2S in alleviating postharvest senescence and rotting of Kyoho grape. Exogenous application of H_2S released from 1.0 mM NaHS remarkably decreased the rotting and threshing rate of grape berries. H_2S application also prevented the weight loss in grape clusters and inhibited the decreases in firmness, soluble solids, and titratable acidity in grape pulp during postharvest storage. The data of chlorophyll and carotenoid content suggested the role of H_2S in preventing chlorophyll breakdown and carotenoid accumulation in both grape rachis and pulp. In comparison to water control, exogenous H_2S application maintained significantly higher levels of ascorbic acid and flavonoid and total phenolics and reducing sugar and soluble protein in grape pulp. Meanwhile, H_2S significantly reduced the accumulation of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and superoxide anion ($O_2^{\bullet-}$) in grape pulp. Further investigations showed that H_2S enhanced the activities of antioxidant enzymes ascorbate peroxidase (APX) and catalase (CAT) and decreased those of lipoxygenase (LOX) in both grape peels and pulp. In all, we provided strong evidence that H_2S effectively alleviated postharvest senescence and rotting of Kyoho grape by modulating antioxidant enzymes and attenuating lipid peroxidation.

1. Introduction

Grapes are subject to postharvest senescence during storage, in the syndromes of serious water loss, berry softening, off-flavour occurrence, and decay caused mainly by *Botrytis cinerea*, which reduces the commodity and consumption of grapes [1]. Besides, rachis browning is also an important storage problem of grapes, which greatly affects consumer preference and fruit price [2]. The universal practice to control postharvest decay is to fumigate grapes with SO_2. Despite the obvious effect of SO_2 in controlling fungal spreading and postharvest rotting, SO_2 treatment causes tissue damage to grape berry such as cracks and bleaching and also leads to excessive sulfite residue which may induce allergenic effects [3, 4]. Thus, developing novel technologies to prolong the shelf life of grapes is of great importance to both grape growers and consumers.

Hydrogen sulfide (H_2S) has been identified as a third multifunctional endogenous gaseous signal after nitric oxide (NO) and carbon monoxide (CO) in animal system [5]. In plants, H_2S emission has been found in many plant species such as cucumber, squash, pumpkin, soya bean, and cotton [6, 7]. More recently, the metabolism and function of endogenous H_2S have been clarified through H_2S-generation defect mutants, revealing its role in plant growth and development [8–10]. Accumulating evidence indicates that H_2S functions in various processes in plants, including seed germination, root organogenesis, abiotic stress tolerance, photosynthesis, guard cell movement, and postharvest senescence, suggesting that H_2S acts as an important signaling molecule in plants, as do NO and CO [11–17].

Fruit senescence is usually accompanied by physiological and biochemical changes among which oxidative damage

caused by reactive oxygen species (ROS) such as $O_2^{\bullet-}$ and H_2O_2 are universally observed [18]. Several recent studies found that H_2S could attenuate oxidative stress by modulating antioxidant enzymes in some postharvest fruits and vegetables including strawberry, mulberry, kiwifruit, and broccoli [19–22]. However, there is no study on whether H_2S plays a role in delaying the senescence of postharvest grape. In the present research, H_2S donor sodium hydrosulfide (NaHS) solution was applied to fumigate grapes and the effects of H_2S signal on grape senescence, the metabolism of natural antioxidants, and ROS and on the activities of antioxidant enzymes were investigated.

2. Materials and Methods

2.1. Plant Material and Treatments. Clusters of fresh Kyoho grape (*Vitis vinifera* L. × *V. labrusca* L. cv. Kyoho) were kindly supplied by the orchard of Anhui Academy of Agricultural Sciences, Anhui, China, and grape samples of commercial ripeness, similar bunch size, and no disease and injury were used in this study. Solution of sodium hydrosulfide ($NaHS{\cdot}3H_2O$, Sigma) was used as H_2S donor. Aqueous solutions of NaHS at different concentrations (150 mL) of 0, 0.20, 0.40, 0.60, 0.80, 1.00, 1.20, 1.40, 1.60, 1.80, 2.00, or 2.20 mM were prepared in sealed containers (volume 3 L) and the solutions were renewed daily. Twelve groups of grape clusters in three replicates (approx. 150 g per replicate) were fumigated with H_2S in the sealed containers at 25°C and a relative humidity of 85–90%. Grape clusters exposed to H_2S fumigation were photographed daily, and the rotten and threshing berries of three replicate grapes were recorded. Rotten fruit rate (%) = (the number of rotten berries (berries with mildew or rot) + the number of threshing berries)/total number of berries in a replicate.

2.2. Quality Evaluation of Grapes. Grape clusters were fumigated with water or H_2S released from 1.0 mM NaHS in the sealed containers at 25°C and a relative humidity of 85–90% for 7 days and relative data were analyzed. Browning index of grape rachis was evaluated according to the browning scales as follows: 0, no browning; 1, browning scale less than a quarter of total area of rachis; 2, browning of scales less than 1/2 of total area of rachis; 3, browning of scales less than three-quarters of total area of rachis; and 4, more than 3/4 of total area of rachis. Browning index (BI) was calculated daily by the following formula: $BI = \sum(df)/ND$, where d is the browning of scales on the grape rachis and f is its respective quantity; N is the total number of grape rachis examined; and D is the highest browning of scales.

For weight loss percentage, the weight of grape clusters was measured before treatment (a) and after storage (b). The weight loss was calculated as $(a - b)/a$.

Grape firmness was measured at the equatorial part of individual grape by a 5-mm diameter flat probe with a texture analyzer (Model TA XT plus, SMS). The penetration depth was 5 mm and the crosshead speed was $5\,mm{\cdot}s^{-1}$. Fruit firmness values were an average of 8 grape berries ± SD (standard deviation).

The total soluble solids (TSS) were determined by measuring the refractive index of the fruit with a hand refractometer (Tongfang Inc., Shanghai, China) according to the method of Jiang et al. [23]. The values were an average of 10 replicates of grapes ± SD.

The titratable acidity of the grape (pooled juice of 15 berries, three replicates per treatment) was measured by titration with 0.1 mM NaOH to pH 8.3. The results were expressed as $g{\cdot}L^{-1}$ [24].

2.3. Determination of Chlorophyll and Carotenoid Contents in Grape Rachis and Pulp. Chlorophyll content of grape was determined using the colorimetric method according to Lichtenthaler and Wellburn [25] with minor modifications. About 5.0 ± 0.05 g of finely chopped grape flesh samples or 2.5 ± 0.02 g of finely chopped grape rachis samples was homogenized using a pestle and mortar on ice and incubated in an Erlenmeyer flask containing 10 mL of 80% acetone as extraction solvent. After extraction in darkness for 24 h at 4°C, the supernatant was measured at 663 and 645 nm, respectively. Chlorophyll and carotenoid contents were calculated with the following equations: Carotenoid = $A_{440}V/W$; Chla = $(12.7A_{663} - 2.69A_{645})V/W$; Chlb = $(22.9A_{663} - 4.68A_{645})V/W$; and Chl = Chla + Chlb. Chlorophyll and carotenoid contents were expressed as $mg{\cdot}g^{-1}$ FW.

2.4. Determination of Ascorbic Acid, Flavonoid, Total Phenolics, Reducing Sugar, and Soluble Protein in Grape Pulp. Ascorbic acid was determined by the method described by Nath et al. [26]. Grape pulp samples 5.0 ± 0.05 g were ground with 15 mL of 4% oxalic acid. After centrifugation at 5,000 rpm for 15 min at 4°C, the supernatant was adjusted to 25 mL with 2% oxalic acid and titrated with 2,6-dichlorophenol-indophenol to a pink color.

Determination of total phenolics and flavonoid in grape pulp was performed according to the methods of Pirie and Mullins [27] and Zhishen et al. [28], respectively.

Soluble protein and reducing sugar contents in grape pulp were measured according to Bradford [29] and Miller [30], respectively. Grape pulp samples at 5.0 ± 0.05 g were ground with 3 mL of sodium phosphate buffer (pH 7.0, 200 mM), and the homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. Then, the supernatant was collected for the determination of soluble protein and reducing sugar content. For soluble protein, 0.1 mL of supernatant was mixed with 0.9 mL of dH_2O and 5 mL of Coomassie Brilliant Blue. Absorbance was recorded at 595 nm after 5 min. The results were expressed as $\mu g{\cdot}g^{-1}$ FW.

Reducing sugar was measured by the dinitrosalicylic acid method. The supernatant (0.2 mL) was mixed with 1.5 mL of 3,5-dinitrosalicylic acid and 1.8 mL of dH_2O, and then the mixture was heated at 100°C for 5 min, cooled, and added to 25 mL distilled water. Reducing sugar was determined at 540 nm by a spectrophotometer, and the results were expressed as $mg{\cdot}g^{-1}$ FW.

2.5. Determination of Malondialdehyde (MDA), Hydrogen Peroxide (H_2O_2), and Superoxide Anion ($O_2^{\bullet-}$) in Grape Pulp. Contents of MDA and H_2O_2 and generation of $O_2^{\bullet-}$ were

determined according to the methods described by Hu et al. [22] with minor modifications. For MDA analysis, grape pulp samples (5.00 ± 0.05 g) were ground in liquid nitrogen and extracted in 3 mL 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 20 min and 1.8 mL of the supernatant fraction was mixed with 1.8 mL of 20% TCA containing 0.5% thiobarbituric acid. The mixture was incubated at 100°C for 30 min, cooled, and centrifuged at 12,000 rpm for 10 min. Absorbance was recorded at 532 nm, and the value for nonspecific absorption at 600 nm was subtracted. An extinction coefficient of 155 $mM^{-1}\cdot cm^{-1}$ was used to calculate MDA content which was expressed as $\mu mol\cdot g^{-1}$.

For determination of H_2O_2, grape pulp samples (5.00 ± 0.05 g) were ground and extracted in 3 mL cold acetone. The homogenate was centrifuged at 12,000 rpm at 4°C for 30 min and 0.5 mL of the supernatant fraction was mixed with 1.5 mL of $CHCl_3$ and CCl_4 (1 : 3, V/V) mixture. Subsequently, 2.5 mL of distilled water was added and the mixture centrifuged at 12,000 rpm for 1 min and the aqueous phase collected for determination. The reaction system included 1 mL sample, 0.5 mL of buffer (phosphate-buffered saline, 200 mM, pH 7.8), and 20 μL (0.5 unit) of catalase as control or inactive catalase protein (catalase inactivated by heating in boiling water for 5 min). After the mixture was incubated at 37°C for 10 min, 0.5 mL of 200 mM titanium 4-(2-pyridylazo) resorcinol (Ti-PAR) was added. The reaction mixture was incubated at 45°C for another 20 min. Absorbance at 508 nm was measured and H_2O_2 content was indicated as $\mu g\cdot g^{-1}$ FW.

The generation rate of $O_2^{\bullet-}$ was determined using hydroxylamine method. Grape pulp samples (5.00 ± 0.05 g) were ground with 3 mL of 50 mM Tris-HCl buffer (pH 7.8) and the homogenate was centrifuged at 12,000 rpm at 4°C for 30 min. The reaction mixture (0.5 mL) contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM XTT [sodium, 3-1-(phenylamino-carbonyl)-3, 4-tetrazolium-bis(4-methoxy-6-nitro), and benzenesulfonic acid hydrate], and 50 μL of sample extracts. Corrections were made for the background absorbance in the presence of 50 U of superoxide dismutase (SOD).

2.6. Activity Assays of APX, CAT, and LOX in Grape Peels and Pulp. Activities of ascorbate peroxidase (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6) were determined by the procedures described by García-Limones et al. [31]. Grape pulp samples (5.00±0.05 g) or grape peel samples (2.0±0.02 g) were homogenized with 5 mL of ice-cold sodium phosphate buffer (50 mM, pH 7.5, containing 5 mM beta mercaptoethanol and 1% polyvinyl pyrrolidone). The homogenate was centrifuged at 10,000 rpm (4°C, 20 min), and the supernatant was used for activity measurement.

APX activity was determined by the decrease of ascorbate which was measured in absorbance at 290 nm. The reaction mixture contained 2.5 mL 50 mM K-phosphate buffer (pH 7.0), 0.2 mL 10 mM ascorbic acid, 0.1 mL 3% H_2O_2, and 0.2 mL crude enzyme extract.

CAT activity was determined spectrophotometrically by monitoring the decrease in absorbance at 240 nm. The reaction mixture contained 2.8 mL of sodium phosphate buffer (50 mM, pH 7.0), 100 μL of 3% H_2O_2, and 100 μL enzyme extract.

Activity of LOX (EC 1.13.11.12) was detected by the procedure described by Surrey [32]. One unit of LOX was defined as a decrease of 0.01 OD value in absorbance per minute. The results of APX, CAT, and LOX were expressed as $U\cdot g^{-1}$ FW.

2.7. Statistical Analysis. The data in the paper are based on three or more replicates in each experiment, and the experiments were repeated independently for three times and similar change pattern was observed. Statistical significance was tested by one-way analysis of variance (ANOVA) using IBM SPSS Statistics (SPSS version 20.0, Armonk, NY), and the results were expressed as the means ± SD. Least significant difference test was performed on all data following ANOVA tests to test for significant ($P < 0.05$ or $P < 0.01$) differences between treatments.

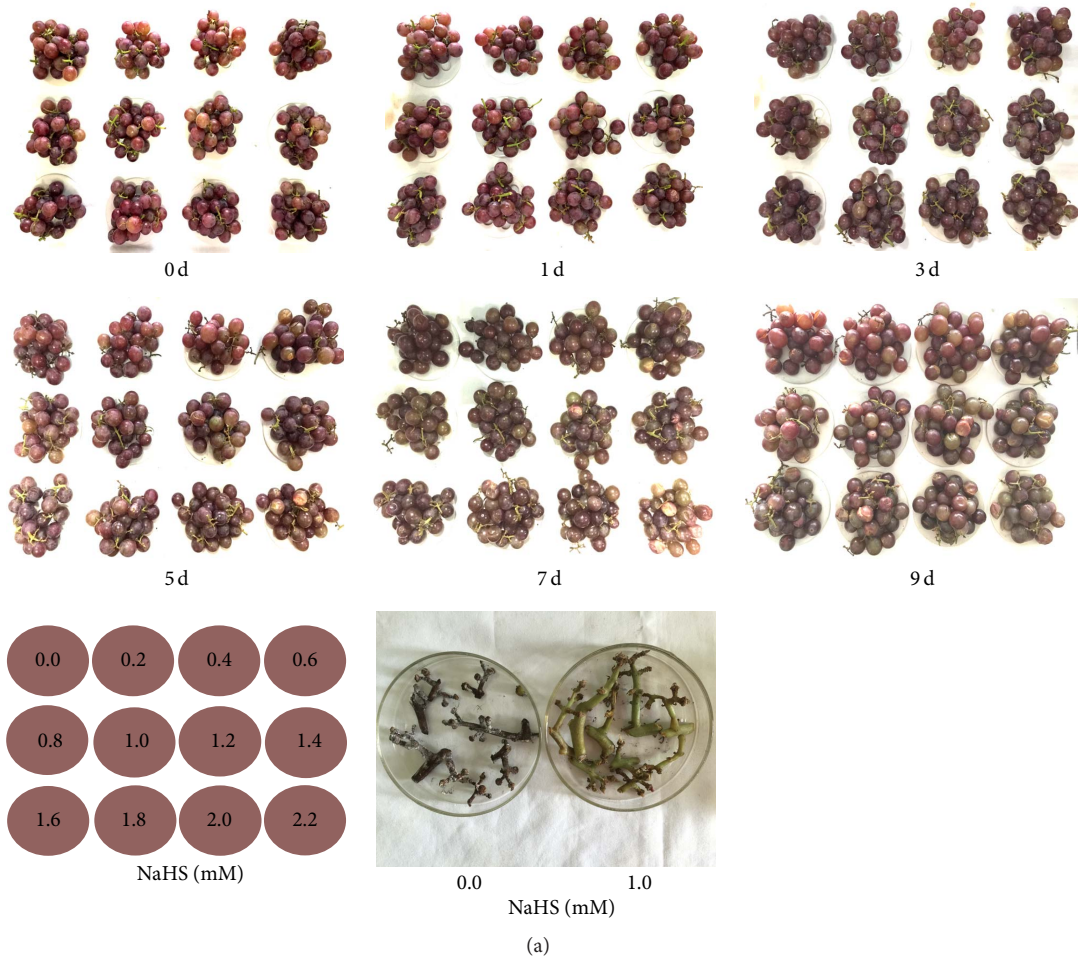
3. Results

3.1. H_2S Alleviates Postharvest Senescence and Rotting of Kyoho Grape. Grape clusters were fumigated with H_2S released from aqueous solutions of NaHS ranging from 0.2 mM to 2.2 mM with water treatment as controls. The visual effects of H_2S on delaying grape senescence, berry cracking, rotting, and threshing are shown in Figure 1(a). As for control berries and 2.2 mM NaHS treated ones, rotten fruit rate increased steadily with storage time, whereas 1.0 mM NaHS could remarkably decrease the rotten and threshing rate of grape berries and was used for subsequent experiments (Figure 1(b)).

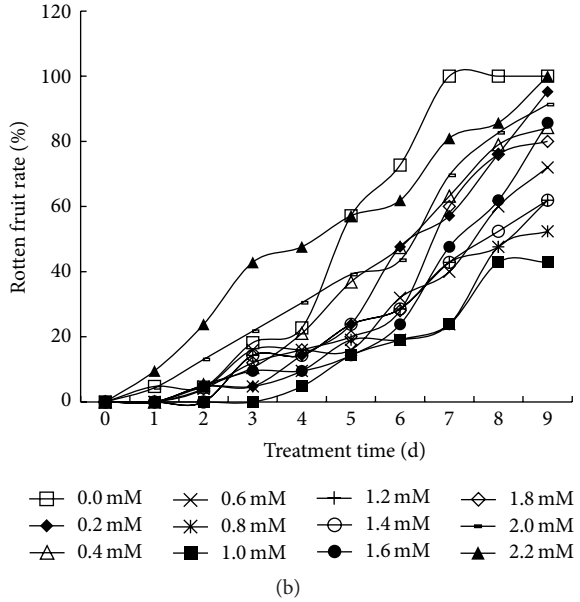
3.2. Effect of H_2S on the Browning Index of Grape Rachis, Weight Loss, Firmness, Total Soluble Solids, and Titratable Acidity of Berries. Rachis browning is a common problem that affects grape quality and consumer preference. As shown in the lower right part of Figure 1(a), grape rachis in 1.0 mM NaHS treated grape clusters still retained a green appearance on day 5 of storage, while the control rachis developed serious browning. Also illustrated in Figure 2(a), browning index of water control rachis increased steadily to 100% on day 5 compared with 30% of NaHS treated ones.

Whole-cluster weight loss of H_2S treatment and water control is presented in Figure 2(b). The weight loss percentage of control grape clusters went up steadily to about 4.3 on day 8 of storage, while the weight loss was effectively alleviated in H_2S treatment. Berry firmness of water control declined gradually during storage, whereas only slight decrease in firmness was observed in berries treated with H_2S (Figure 2(c)).

As shown in Figure 2(d), the content of soluble solids of control grape berries decreased sharply along with storage. However, H_2S application maintained significantly higher levels of soluble solids compared with that of control except on day 2 of storage. Titratable acidity (Figure 2(e)) in control berries dropped sharply until day 4 and thereafter maintained a stable level, which is a symbol of an enhanced ripening. In contrast, titratable acidity in NaHS treatment showed a



(a)



(b)

Figure 1: Hydrogen sulfide (H_2S) treatment delays the senescence and rotting of Kyoho grapes in a dose-dependent manner. Grape clusters were fumigated with H_2S released from different concentrations of aqueous NaHS (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, and 2.2 mM) and the photographs of grapes were taken every two days (a). Grape rachis of control and 1.0 mM NaHS treatment on day 5 of storage were presented in third panel of (a). Meanwhile, rotten fruit rates were recorded daily as shown in (b). The experiments and the following ones were carried out at 25°C and 85–90% relative humidity.

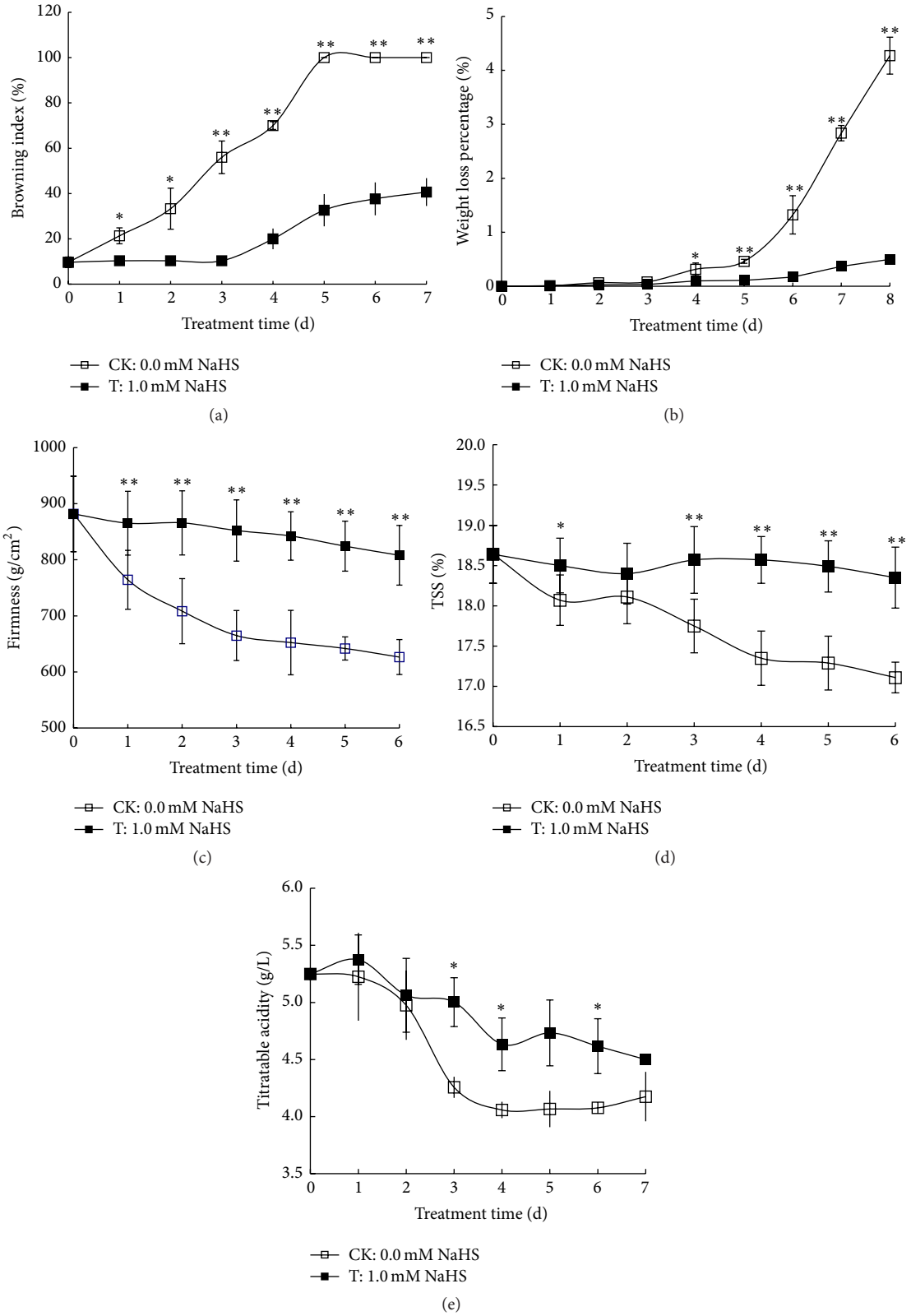


FIGURE 2: Effects of H_2S on browning index of grape rachis (a), weight loss percentage of grape berries (b), grape berry firmness (c), total soluble solids (TSS) (d), and titratable acidity (e) in grape flesh. Grape clusters were fumigated with 1.0 mM H_2S donor NaHS aqueous solution with water as the control groups for 0–8 d. Data are presented as means ± SD (standard deviation) ($n = 3$ rachis for (a), $n = 3$ grape clusters for (b), $n = 8$ grape berries for (c), $n = 10$ replicates for (d), and $n = 3$ replicates for (e)). The symbols $*$ and $**$ in this figure and the following ones stand for a significant difference between water control and 1.0 mM NaHS treatment at $P < 0.05$ and $P < 0.01$, respectively. FW = fresh weight.

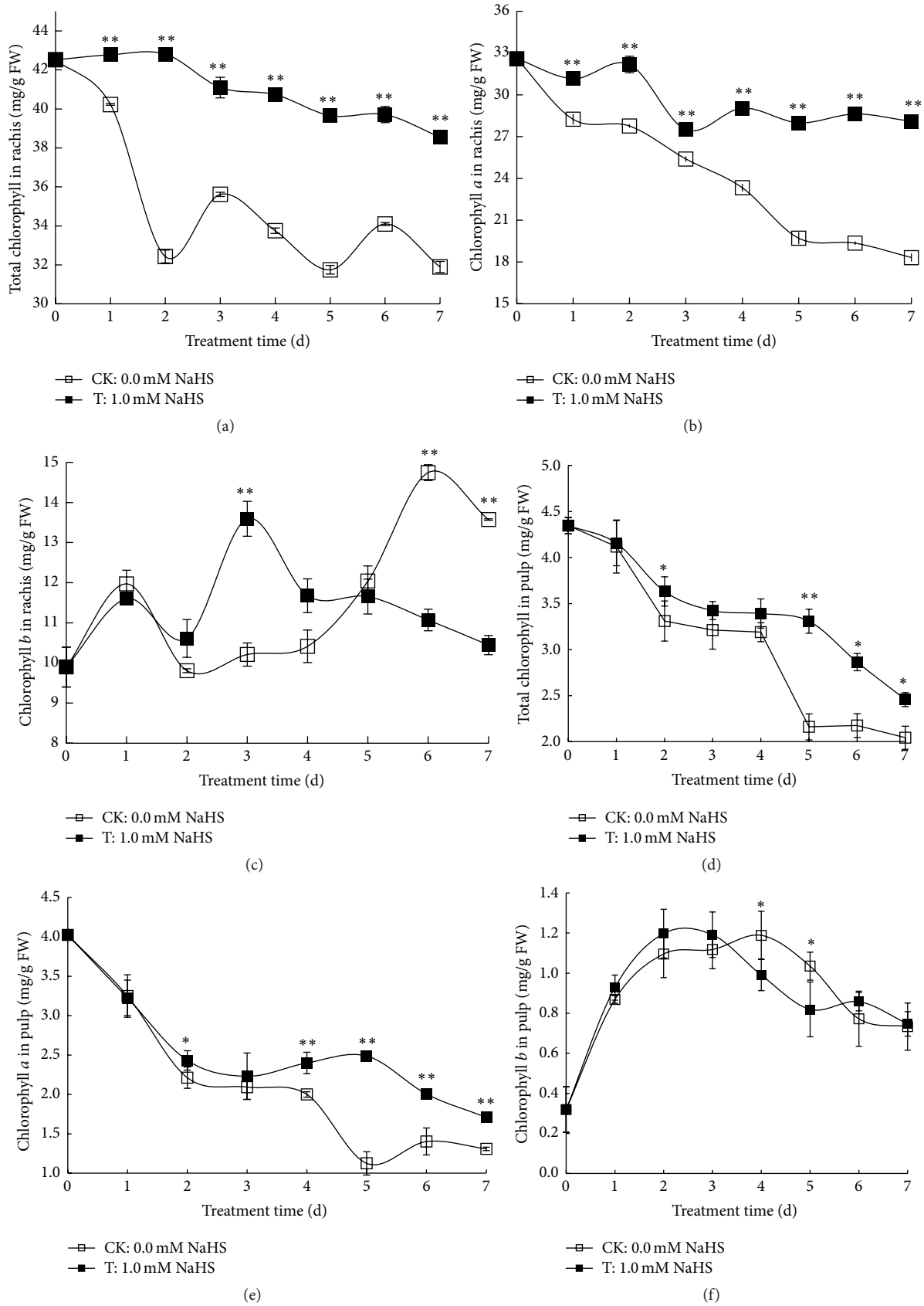


Figure 3: Continued.

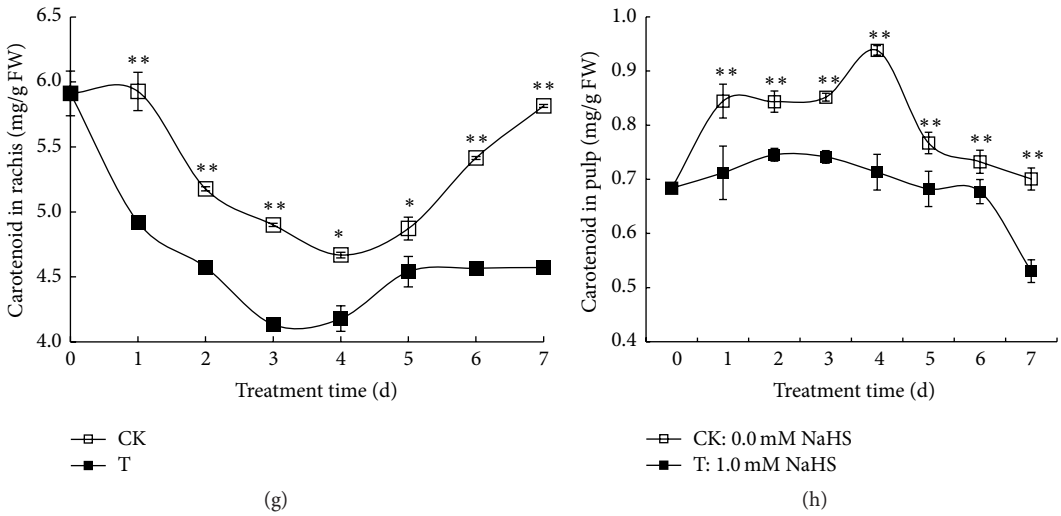


Figure 3: Effects of H_2S on the contents of total chlorophyll (a) and chlorophyll *a* (b) and chlorophyll *b* (c) in rachis and total chlorophyll (d) and chlorophyll *a* (e) and chlorophyll *b* (f) in grape pulp and on the content of carotenoid in rachis (g) and grape pulp (h). Grape clusters were fumigated with 1.0 mM H_2S donor NaHS aqueous solution with water as the control groups for 0–7 d. Data are presented as means ± SD ($n = 3$ for (a), (b), (c), and (g), $n = 6$ for (d), (e), (f), and (h)). FW = fresh weight.

slower downward trend and was significantly higher than that of water control on days 3, 4, and 6 (Figure 2(e)).

3.3. Effect of H_2S on the Contents of Chlorophyll and Carotenoid in Postharvest Grape Rachis and Pulp. Chlorophyll breakdown is shown to be associated with the first steps of the senescence process [33]. Besides, fruit ripening is often accompanied with the destruction of the green chlorophyll pigments and accumulation of yellow carotenoids in the flesh [34]. Thus, to understand how H_2S alleviated rachis browning and berry senescence, we determined the contents of chlorophyll and carotenoid in rachis and pulp. Chlorophyll contents (Figures 3(a) and 3(d)) were expressed as the sum of chlorophyll *a* (Figures 3(b) and 3(e)) and chlorophyll *b* (Figures 3(c) and 3(f)). In rachis, total chlorophyll content as well as the amounts of chlorophyll *a* showed a decline trend during storage in both water controls and H_2S treatment, while H_2S fumigation maintained a relatively stable level of total chlorophyll and chlorophyll *a* during the storage (Figures 3(a) and 3(b)). Similarly, a decrease in total chlorophyll content in pulp was also observed in water controls and H_2S treatment, whereas H_2S helped to maintain significantly higher level of total chlorophyll on days 2, 5, 6, and 7 compared with water control (Figure 3(d)). The content of chlorophyll *a* in both control and H_2S treatment showed a decreasing trend along with time, while the content in H_2S treatment was significantly higher than that of control on days 2, 4, 5, 6, and 7 (Figure 3(e)). However, a slightly higher level of chlorophyll *b* was found in control grape compared with H_2S-treated ones on days 4 and 5 (Figure 3(f)).

Changes of carotenoid content in rachis and pulp are shown in Figures 3(g) and 3(h). During the whole storage period, carotenoid content in control group was always higher than that of H_2S treatment in both the rachis and pulp. In grape rachis, carotenoid content decreased and bottomed on day 4 for water control and day 3 for NaHS treatment followed by an increase (Figure 3(g)). Carotenoid content in grape pulp of water control increased steadily and peaked on day 4 followed by a decline, while only slight change was observed in H_2S-treated berries except a drop on day 7. The data of chlorophyll and carotenoid content suggested the role of H_2S in preventing chlorophyll breakdown and carotenoid accumulation in both grape rachis and pulp.

3.4. Effect of H_2S on the Contents of Ascorbic Acid, Flavonoid, Total Phenolics, Reducing Sugar, and Soluble Protein in Grape Pulp. Ascorbic acid, flavonoid, and phenols are natural antioxidants and important nutrient traits of fruit. As shown in Figure 4(a), the content of ascorbic acid decreased to a bottom on day 2 for control and on day 3 for H_2S treatment followed by a gradual increase. However, H_2S treatment sustained significantly higher content of ascorbic acid on days 1, 2, 6, and 7 in comparison to water control. Figure 4(b) illustrated a decreasing trend of flavonoid content in grape berries treated with H_2S or not, whereas H_2S treatment sustained significantly higher level of flavonoid compared with water control. Similar decreasing trend was also observed in the changes of phenolics content (Figure 4(c)). However, in comparison to water control, H_2S fumigation significantly alleviated the decrease and maintained higher content of phenolics during the whole storage.

The contents of reducing sugar and soluble protein in grape berries are shown in Figures 4(d) and 4(e). Reducing sugar, as a primary energy substance, is a key energy source in postharvest fruit and vegetables. Reducing sugar in water control declined sharply and bottomed on day 4 followed by a surge till day 6. However, there was only slight fluctuation of reducing sugar in H_2S-treated berries and significantly higher level of reducing sugar was observed on days 2 to 5 relative to that of water control. Soluble protein content

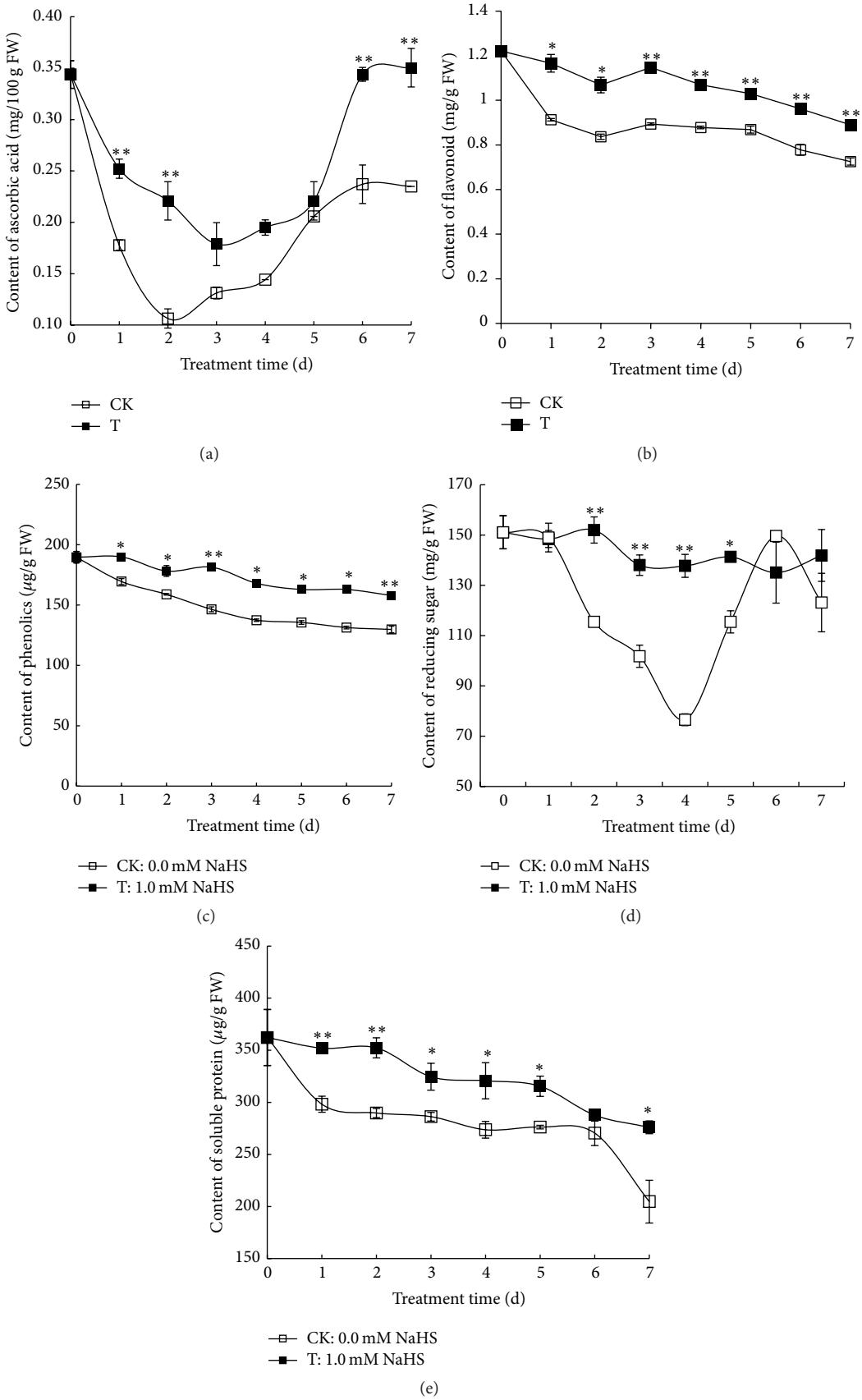


FIGURE 4: Effects of H_2S on the contents of ascorbic acid (a), flavonoid (b), total phenolics (c), reducing sugar (d), and soluble protein (e) in grape pulp. Grape clusters were fumigated with 1.0 mM H_2S donor NaHS aqueous solution with water as the control groups for 0–7 d. Data are presented as means ± SD (n = 3). FW = fresh weight.

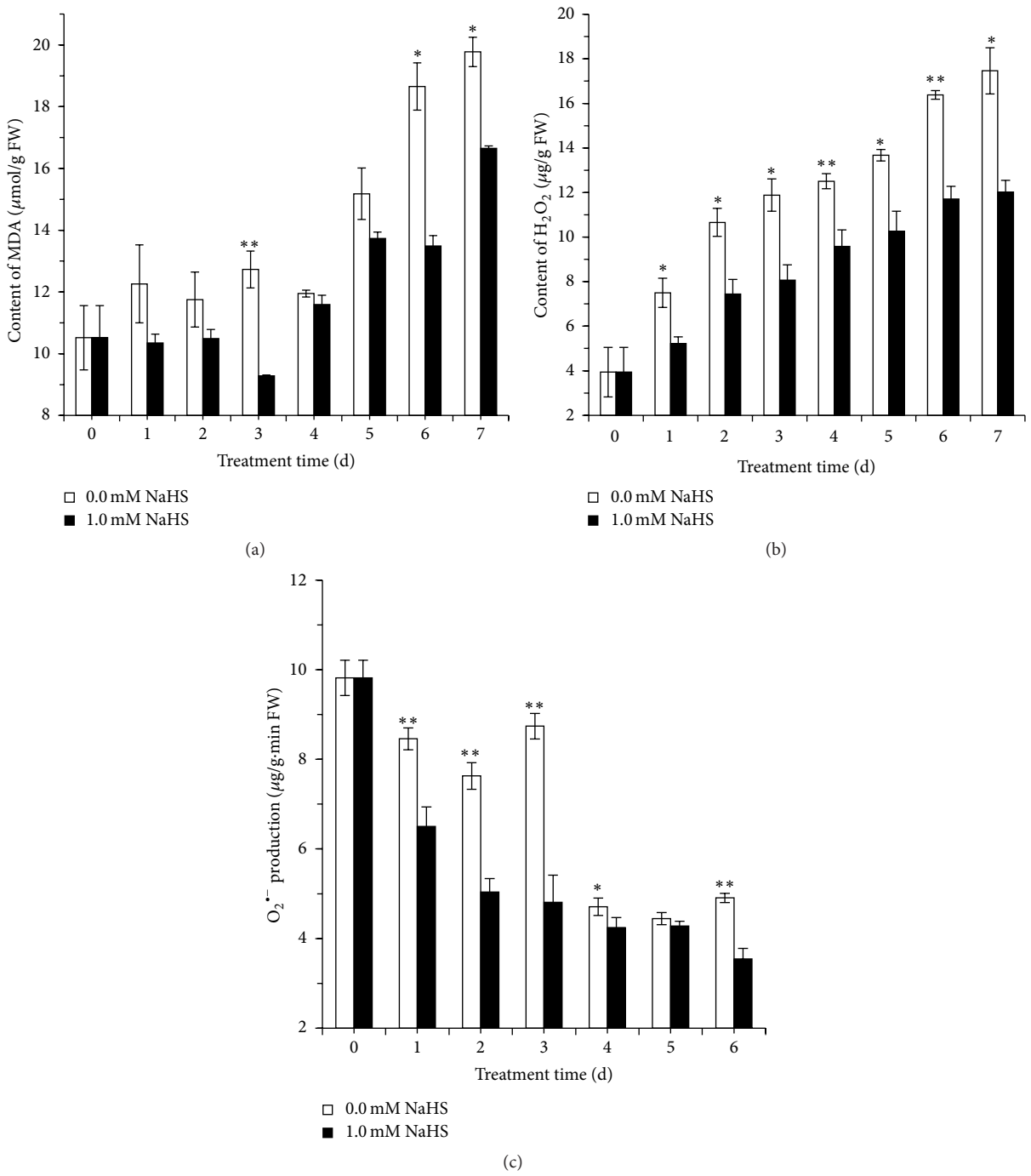


FIGURE 5: Effects of H_2S on the contents of malondialdehyde (MDA) (a), hydrogen peroxide (H_2O_2) (b), and superoxide anion ($O_2^{\bullet-}$) (c) production rate in grape pulp. Grape clusters were fumigated with 1.0 mM H_2S donor NaHS aqueous solution with water as the control groups for 0–7 d. Data are presented as means ± SD ($n = 3$). FW = fresh weight.

in both water control and H_2S decreased continually during the storage, but H_2S treatment significantly alleviated the decrease, suggesting the role of H_2S in preventing protein degradation.

3.5. Effect of H_2S on the Contents of MDA, H_2O_2, and $O_2^{\bullet-}$ in Grape Pulp. The contents of MDA and H_2O_2 and the generation of $O_2^{\bullet-}$ in grape fumigated with H_2S or water are shown in Figure 5. MDA is determined as an index of lipid peroxidation. As shown in Figure 5(a), MDA content in water control pulp fluctuated during the first four days of storage followed by a surge. An increase of MDA content was also observed in H_2S-treated berries on day 4, but H_2S treatment significantly reduced MDA accumulation on days 3, 6, and

7, implicating the role of H_2S in alleviating lipid peroxidation.

The overproduction of reactive oxygen species (ROS) is universally occurring during fruit senescence [21, 22]. Figure 5(b) shows that H_2O_2 content in grape pulp increased steadily in both H_2S treatment and water control, while H_2S treatment significantly reduced H_2O_2 accumulation. However, the content of $O_2^{\bullet-}$ in control pulp fluctuated during the first 3 days of storage followed by a decrease on day 4. In contrast, the content of $O_2^{\bullet-}$ in H_2S treatment declined continuously in pulp and was significantly lower compared to water control except on day 5.

3.6. Effect of H_2S on the Activities of APX, CAT, and LOX in Grape Peels and Pulp. To further understand the role of H_2S in ROS metabolism in grape, we determined the activities of enzymes involved in oxidative metabolism in plants, such as APX, CAT, and LOX. As showed in Figure 6(a), the activity of APX in grape peels of both control and H_2S treatment increased steadily and peaked on day 3 of storage followed by a gradual decline. However, APX activity in H_2S-treated peels was significantly enhanced on days 1 to 3 compared with that of water control. The changes in APX activity in grape pulp are shown in Figure 6(b). APX activity in control peels increased during the first 2 days of storage and then fluctuated and peaked on day 4 followed by a drop on day 5 and then a plateau. Similar increase in APX activity was observed in H_2S-treated peels till day 2 followed by a slight decrease till day 5. However, H_2S treatment induced an about 3-fold increase in APX activity on day 6 in grape peels compared with control. As shown in Figure 6(c), H_2S treatment induced a swift increase of CAT activity on day 1 in grape peels followed by a gradual decrease till day 4. Then, an increase was observed in CAT activity in peels treated with H_2S followed by a drop. Similar trend of the changes in CAT activity was seen in control peels, except that there was no activity increase on day 2 compared with that of H_2S treatment (Figure 6(c)). However, H_2S significantly promoted CAT activity in peels during the whole storage in comparison to water control except on day 7. Figure 6(d) illustrates the effect of H_2S on CAT activity in grape pulp. CAT activity in control pulp increased on day 1 followed by a slight decrease thereafter, whereas H_2S was found to induce an increase during the first two days of storage followed by a gradual increase. CAT activity in H_2S-treated pulp was significantly higher than that of control on days 2 and 3 but was lower than that of control on days 6 and 7.

LOXs are enzymes that catalyze the hydroperoxidation of polyunsaturated fatty acids. As shown in Figure 6(e), LOX activity in control peels increased steadily and reached a maximum value on day 3 followed by a slight decline. Similar trend of changes of LOX activity was observed in H_2S-treated peels, while H_2S significantly reduced LOX activity on days 2, 4, 6, and 7 compared with water control. Figure 6(f) showed that H_2S also attenuated LOX activity in pulp during the first 4 days of storage. LOX activity in control pulp rose significantly till day 3 followed by a drop on day 5. However, an attenuated increase in LOX activity was observed in H_2S-treated pulp during the first 4 days of storage (Figure 6(f)).

4. Discussion

Table grapes are highly perishable and their quality deteriorates quickly after harvest because of water loss and fungal spoilage especially in developing countries where cold chain transportation is not always available [1]. Besides, rachis browning also has a great impact on consumer preference. Here, we provide an alternative strategy other than SO_2 fumigation to maintain the freshness of grape berries and green color of the rachis. Water loss is responsible for large and significant changes in the composition and metabolism of detached fruit, which induces changes in color and palatability and loss of nutritional quality [35, 36]. We found that H_2S treatment effectively reduced weight loss in grape clusters and maintained higher berry firmness compared to water control (Figures 2(b) and 2(c)). Higher titratable acidity (TA) indicates a marked delay in process of maturation and ripening and loss of acidity can cause the fruits to taste insipid during storage. H_2S fumigation alleviated the decrease in TA during grape storage, further suggesting the role of H_2S in delaying fruit maturation and ripening (Figure 2(e)).

During fruit ripening and senescence, green chlorophyll pigments were decomposed and yellow carotenoids accumulated in the flesh [33, 34]. In the present research, chlorophyll degradation was observed in both grape rachis and pulp, which was consistent with previous findings that during storage there was upregulation of chlorophyll breakdown-related genes in rachis [37]. However, H_2S significantly prevented chlorophyll degradation and carotenoid accumulation in both rachis and pulp, further confirming the antisenescence role of H_2S in plants (Figure 3). Although grapes are nonclimacteric, the effect of ethylene on ripening at veraison is well established [38]. Rachis browning was believed to be associated mainly with dehydration, but there is evidence showing that ethylene acts as a major factor in rachis browning [1, 39]. Recent study shows that 1-methylcyclopropane (1-MCP), which is a potent inhibitor of ethylene action, delays rachis browning in three table-grape varieties whereas ethylene tends to enhance it [39]. Besides, treatment with cytokinin or abscisic acid (ABA) improves rachis quality during storage, further suggesting the involvement of senescence during rachis browning because cytokinin and ABA are known to have antisenescent effect in plant [40, 41]. Thus, our finding of the role of H_2S in alleviating grape senescence and rachis browning highlights the possibility that H_2S might act as an antagonist to counteract ethylene-induced fruit senescence.

Plant senescence is usually accompanied with the accumulation of ROS which can potentially cause oxidative damage to cellular components, including lipid, protein, and nucleic acid [42]. The metabolism of ROS is controlled by a series of antioxidant enzymes including CAT and APX. We found that H_2O_2 was accumulated in control grape pulp while H_2S effectively reduced H_2O_2 accumulation and $O_2^{\bullet-}$ content (Figures 5(b) and 5(c)). APX and CAT are the two enzymes responsible for H_2O_2 breakdown. In the present study, H_2S treatment significantly enhanced the activities of APX and CAT in both grape peels and pulp during storage, which helped to scavenge excessive ROS and reduced

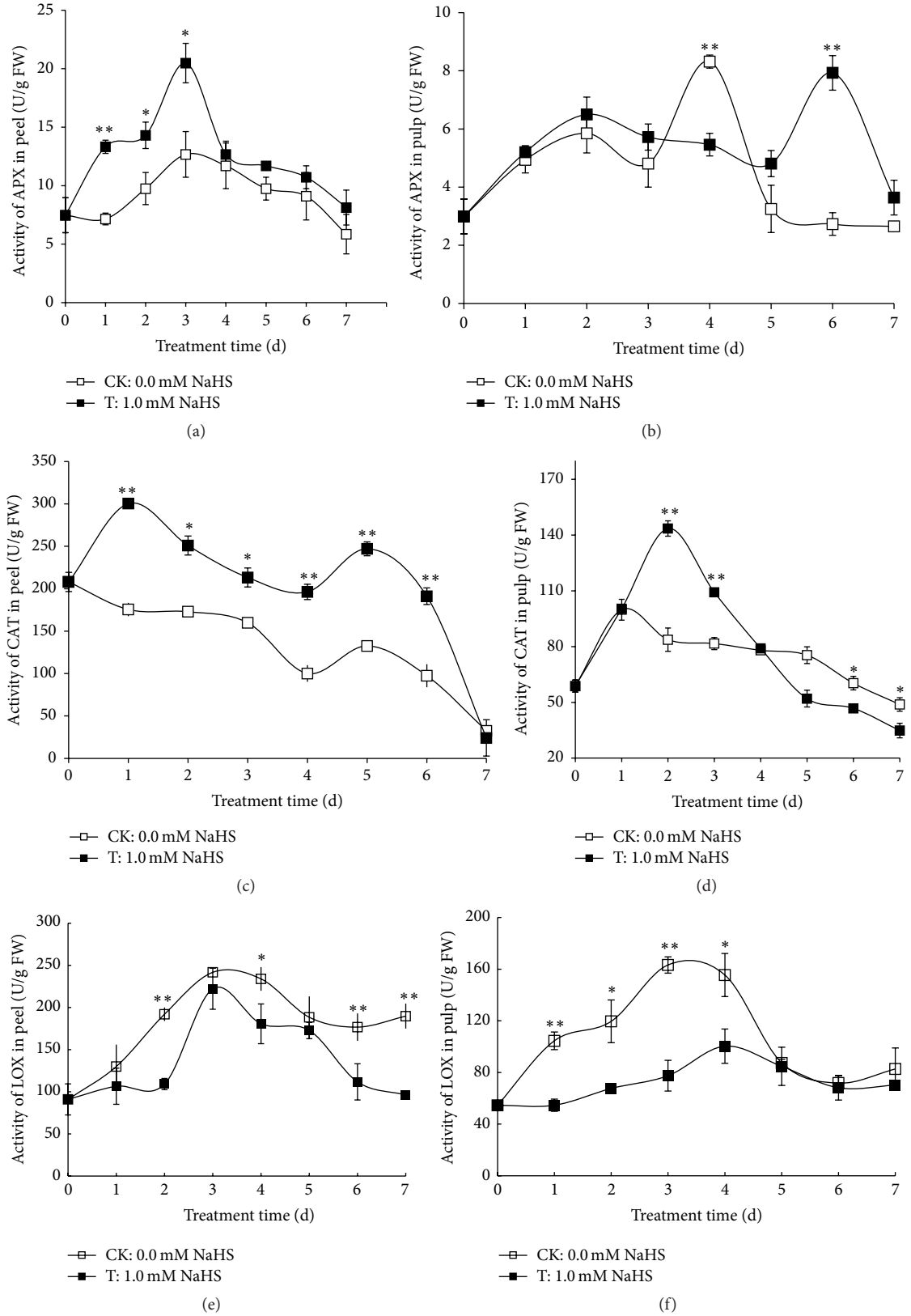


FIGURE 6: Effects of H_2S on the activities of ascorbate peroxidase (APX) in grape peels (a) and pulp (b), catalase (CAT) in peels (c) and pulp (d), and lipoxygenase (LOX) in peels (e) and pulp (f). Grape clusters were fumigated with 1.0 mM H_2S donor NaHS aqueous solution with water as the control groups for 0–7 d. Data are presented as means ± SD (n = 3). FW = fresh weight.

ROS-caused damage to tissues (Figures 6(a), 6(b), 6(c), and 6(d)). In addition to the antioxidative effect of antioxidant enzymes, nonenzymatic antioxidants or nutritional components such as ascorbic acid, flavonoid, and phenolics, which are important quality parameters used to evaluate the storage effect on table grapes, also help to maintain a balanced ROS metabolism by quenching ROS [43]. In the present research, H_2S was found to maintain higher levels of ascorbic acid, flavonoid, and phenolics in grape pulp compared with water control (Figures 4(a), 4(b), and 4(c)), highlighting the positive role of H_2S in grape storage. Other compounds such as chitosan-glucose complex, which has superior antioxidant activity in grape, were also found to delay the declines of ascorbic acid and titratable acidity and to induce antioxidant enzymes, thereby extending the postharvest life of grape [44]. Further, preharvest polyamines application which maintained higher value of antioxidant activity during grape storage also improved grape quality as indicated by the higher levels of phenolics and anthocyanins and alleviated weight loss and softening [45]. All the above publications highlighted the central role of oxidative stress during grape senescence and the effectiveness of antioxidant compounds (including H_2S, polyamines, and chitosan-glucose complex) in delaying grape senescence.

LOX, as one of the key enzymes in membrane lipid peroxidation, is capable of catalyzing the peroxidation of unsaturated fatty acids to form a series of reactive oxygen species and thereby causing disorders in the normal physiological metabolic activity of cells [46]. We found that H_2S significantly inhibited the increase in LOX activity and meanwhile reduced the accumulation of MDA, which is a product of lipid peroxidation and a marker of oxidation of the plasma membrane [46] (Figures 5(a), 6(e), and 6(f)).

In all, our results indicated that H_2S could alleviate postharvest senescence of grape and maintain high fruit quality by decreasing ROS accumulation, improving antioxidant enzyme activities, and reducing lipid peroxidation, thereby maintaining the stability of the membrane structure.

5. Conclusion

In summary, we demonstrated that exogenous application of H_2S effectively alleviated postharvest senescence of grapes by preventing rachis browning and berry rotting and maintaining grape firmness, soluble solids, titratable acidity, and natural antioxidants during postharvest storage. The protective role of H_2S in grapes could be attributed to the increased activities of ROS-scavenging enzymes which bring about a repression on the production of ROS such as H_2O_2 and $O_2^{\bullet-}$ and to the decreased level of LOX activity. In all, we provided strong evidence that H_2S effectively alleviated postharvest senescence and rotting of Kyoho grape by modulating antioxidant enzymes and attenuating lipid peroxidation. Considering the critical role of ethylene in postharvest senescence of grape berries and rachis, it will be interesting to know whether H_2S is antagonistically involved in ethylene pathway.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Zhi-Jing Ni and Kang-Di Hu contributed equally to this work.

Acknowledgments

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Review Article

Gasotransmitters in Gametogenesis and Early Development: Holy Trinity for Assisted Reproductive Technology—A Review

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Creation of both gametes, sperm and oocyte, and their fusion during fertilization are essential step for beginning of life. Although molecular mechanisms regulating gametogenesis, fertilization, and early embryonic development are still subjected to intensive study, a lot of phenomena remain unclear. Based on our best knowledge and own results, we consider gasotransmitters to be essential for various signalisation in oocytes and embryos. In accordance with nitric oxide (NO) and hydrogen sulfide (H_2S) physiological necessity, their involvement during oocyte maturation and regulative role in fertilization followed by embryonic development have been described. During these processes, NO- and H_2S-derived posttranslational modifications represent the main mode of their regulative effect. While NO represent the most understood gasotransmitter and H_2S is still intensively studied gasotransmitter, appreciation of carbon monoxide (CO) role in reproduction is still missing. Overall understanding of gasotransmitters including their interaction is promising for reproductive medicine and assisted reproductive technologies (ART), because these approaches contend with failure of *in vitro* assisted reproduction.

1. Introduction

Human reproductive medicine and assisted reproductive technologies (ART) have been gaining increasing significance, dealing with human reproduction failure. Doubtlessly, the oocyte and sperm are crucial cells for assisted reproduction because these haploid gametes are required to build a diploid zygote, capable of further development. Female and male gametes exhibit different morphological features and, excluding brought genome, they differently contribute to embryo formation. While centrosomes, small noncoding RNAs, and posttranslationally modified residual histones are sperm-inherited, oocytes provide mitochondria, mRNAs (distributed according to a specific pattern), histones, metabolic enzymes, and cytoplasmic factors to sustain development, as summarized elsewhere [1–5]. Hence, one has to consider the oocyte as a microenvironment filled with a precisely balanced cocktail of the numerous factors that are essential for embryonic development. Also, oocytes offer physical environments favourable to self-organizing process like division spindle assembly. Upon fertilization, a succession of mitotic divisions is triggered; the transition from maternal to zygotic mRNAs transcription and transformation of a low organized cellular mass into a blastocyst will occur prior to implantation. Together with gametogenesis and DNA integrity maintenance, these events are of high interest for ART. Indeed, any failure in these processes will impact severely the embryo's fate. Untangling the processes at the molecular and cellular levels is crucial for ART and we should underline that the effects of many contributors, besides the main regulators of gametogenesis and early embryogenesis, remain uncovered.

Oocyte maturation, which can be simulated in *in vitro* conditions, deserves particular attention because meiotic division and achievement of developmental competence are finalized during this short and extremely important period (summarized in [5]). The quality of matured oocytes is decisive for the fertilization rate, as a result of sperm penetration and complex oocyte changes including cortical granule exocytosis-prevented polyspermy and oocyte activation for embryonic development [6–8]. In fact, the early embryonic development, where high-quality blastocyst is optimal for embryo transfer into the recipient body, is decisive for the success of ART [9, 10]. Numerous factors have been identified to play different roles in chromosome segregation and developmental competence achievement, regulating kinases, structural cytoskeletal proteins, enough histones, and second messengers (cAMP, cGMP, and Ca^{2+} ions) [11–14]. In addition to these known key factors, gaseous molecules with signal transduction ability, hence named gasotransmitters [15–17], have been involved in the oogenesis as well [18, 19]. Their impact is acknowledged along with a better understanding of gasotransmitters' signalling pathways. Moreover, recent observations point out imperfect *in vitro* imitation [20] and some gasotransmitter signalisation seems to be lacking in complete gametes' maturation and early embryogenesis.

Only matured oocytes are able to go through *in vitro* fertilization, a key technique of assisted reproduction [21]. Fertilization consists in the interactions of male and female gametes leading to embryonic development. The high cell division rate, typical of this period, is highly sensitive to well-orchestrated cell cycle regulation [2, 22, 23]. Oocyte maturation and early embryonic development persist as delicate steps for *in vitro* approaches, calling for ART improvement. Nevertheless, gasotransmitters rise expectations due to their broad physiological effect and promising results of gasotransmitters supplementation.

The aim of this review is to compare the biological necessity of all three gasotransmitters in the oocyte and embryo, observing their *in vitro* culture in ART, as a key factor for creating a new individual. This comparison highlights protein posttranslational modifications as crucial molecular action of gasotransmitters during oogenesis and preimplantation embryonic development.

2. Gasotransmission in Female Reproductive Processes

2.1. NO as a "Yes Signal" for Fertilization and Early Development. Only matured gametes, which underwent adequate changes, are capable of fertilization. These changes involve especially oocyte maturation, sperm capacitation, and acrosome reaction and are an essential prerequisite for both successful fertilization and further embryonic development. Biochemical changes regulate gametes' changes and their interactions during fertilization process. Originally, these changes were believed to be exclusively regulated *via* kinase signalling, such as protein kinase A- (PKA-) M-phase/maturation promoting factor- (MPF) mitogen-activated protein kinase (MAPK) and calmodulin-dependent protein kinase II (CaMKII), either directly dependent or indirectly regulated by molecules of second messengers, Ca^{2+} and cAMP. In addition to these two messengers, the involvement of NO, a small gaseous molecule, in cell signalling of physiological processes has been described [24, 25]. Along with NO, gasotransmitters H_2S and CO were suggested to participate in the above-mentioned processes as well [17, 26–28] (see Figure 1).

NO represents the most read-up gasotransmitter, with ability to regulate molecular processes in gametes and embryo [29–31]. All NO synthases, that is, endothelial (eNOS), neuronal (nNOS), and inducible (iNOS), are present in mammalian oocyte with various subcellular localization, where they are essential for endogenous production of NO and its cell signalisation [32, 33]. NO action leads to ovulation of matured and fertilizable oocytes [31, 32] as a result of reinitiation of oocyte meiosis and correct oocyte maturation [18, 31, 32]. Accordingly, NO level in oocytes of young mice is significantly higher than old animals and NO antiaging effect is obvious [34]. On the contrary, increased eNOS expression accompanies improved mouse oocyte quality after estrogen administration [35]. One of NO action modes, S-nitrosylation of proteins, has been observed in oocytes during meiotic maturation [36]. However, NO is able to stimulate soluble guanylate cyclase (sGC), which is a NO-specific receptor, in cGMP production and thus NO increases protein kinase G (PKG) activity [37–40]. On the other hand, S-nitrosylation of sGC affects the decreasing responsiveness to NO in somatic cells and molecular mechanism-dependent dual effect of NO is obvious [41]. In contrast to oocyte maturation [38], NO-sGC-cGMP-PKG signal pathway is capable of inducing spontaneous oocyte activation and subsequent parthenogenetic development [42]. NO-induced oocyte activation indicates a pulsation pattern of NO action in porcine oocytes [43]. Based on an observation of *Xenopus* oocytes, the parthenogenetic NO effect is Ca^{2+}-dependent and occurs due to MAPK inactivation [44].

While NO might promote but is dispensable for *Xenopus* and mammals oocyte activation, NO is essential for oocyte activation event during the fertilization process in sea urchin oocytes [45]. In accordance with this variable effect, cortical granules exocytosis has been reported in *Xenopus* oocytes [44] but not in porcine oocytes [46]. The interspecies differences of NO action during fertilization are obvious and NO seems to be even nonessential during mammalian fertilization (Figure 2). The ambiguous NO necessity could be a result of a more diverse NO effect when NO is associated with inflammation and/or oxidative stress [47, 48]. Accordingly, the role of NO during subsequent embryonic development after fertilization remains controversial [49–51] for inflammation (endometriosis), accompanying NO [52, 53] and protein nitration [54]. However, creation of secondary products of NO interactions seems to be one of possible mechanisms of NO negative action [55]. The physiological role of NO in embryogenesis is still unexceptionable when NO is involvement in embryonic stem cell differentiation through transcriptional factors [56]. Therefore, NO is able to be considered as trigger for oocyte maturation and fertilization as well as subsequent embryonic development.

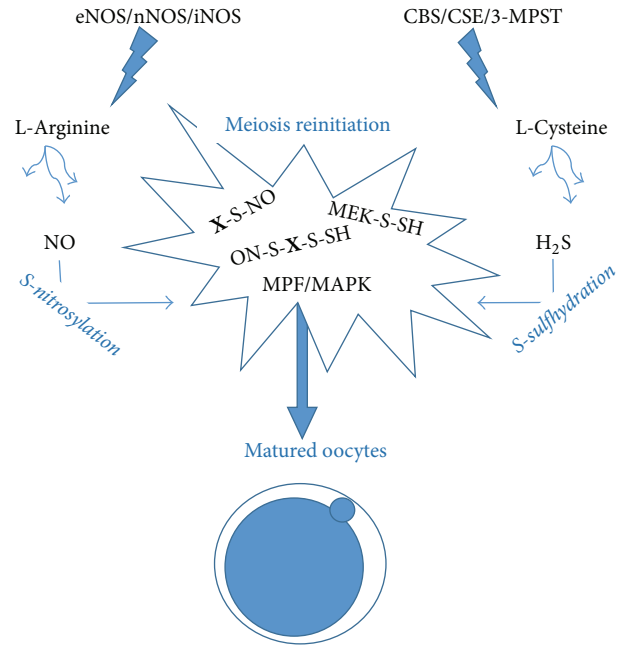

FIGURE 1: Gasotransmission in oocytes, resulting in S-sulfhydration and nitrosylation of various factors. Both gasotransmitters NO and H_2S are enzymatically released, respectively, from L-arginine and L-cysteine. Subsequently, NO- and/or H_2S-posttranslationally modified proteins lead to MPF/MAPK-orchestrated meiotic maturation reinitiation (equal to GVBD, germinal vesicle breakdown) and completion (with extruded polar body and small particles visible in perivitelline space). S-sulfhydration of MEK, upstream MAPK kinase, is known [61] and more S-sulfhydrated factors are considered. In addition to S-sulfhydration, S-nitrosylation seems to be exclusive mechanism of NO-regulated oocyte maturation [34]. Disclosure of complete "S-sulfhydration" and "S-nitrosylation" is still lacking (**X**-S-SH, **X**-S-NO) and we can assume wide protein index underwent this posttranslational modifications as well as NO-H_2S intraprotein cross-talking (HS-S-**X**-S-NO).

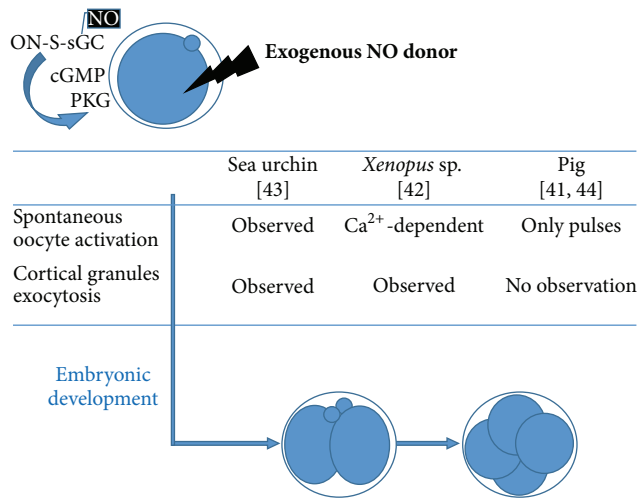

FIGURE 2: NO action in oocyte activation is evolutionary inconsistent. The NO/sCG/cGMP/PKG signal pathways are presumed, where dual NO effect on sCG, resulting in its S-nitrosylation and NO binding, is expectable. Obviously, dependency of fertilization and oocyte activation, followed by cleavage and the second polar bod extrusion, is shaded in evolutionary more developed organisms, where fulfilment of certain conditions (Ca^{2+} presence, pulsative character of NO) is necessary.

2.2. H_2S in Gametogenesis and Embryo Development. S-sulfhydration, another gasotransmitter-derived posttranslational modification, is supposed to be a prime way of H_2S molecular action [57, 58] without known H_2S-specific receptors. In contrast to NO, little is known about H_2S and S-sulfhydration involvement in gametogenesis and embryonic development. Nevertheless, all three H_2S-releasing enzymes, CBS, CSE, and 3-MPST, were observed in porcine oocyte and surrounding cumulus cells [59]. This observation is in accordance with earlier finding of H_2S involvement in folliculogenesis and oocyte maturation [19, 20]. The necessity of H_2S in matured oocytes interferes with the contribution to developmental competence acquirement and subsequent embryonic development [20]. In addition, there is the observation of a protective effect of H_2S against oocyte aging and H_2S-positively affected further embryonic development [60]. Physiological action of endogenously released H_2S immediately in oocyte has been described and modified kinase activity of MPF and MAPK has been observed [20, 59, 60]. S-sulfhydration of these kinases and their upregulated factors are presumable. Activating S-sulfhydration of MEK, leading to MAPK signalling [61], confirms this assumption and the findings mean that S-sulfhydration is crucial for enzyme activity and shift its significance to protein phosphorylation.

However, in contrast to the essential and protective effect of H_2S in mammalian oocytes, our own observation of oxidative stress-like effect of H_2S in *Xenopus* oocytes indicates less conservative evolutionary mechanism through species. Moreover, some findings support that H_2S action is at least comparable to reactive oxygen species (ROS) throughout reactive sulfide species (RSS) creation [62–64].

Although the role of the third gasotransmitter, CO, remains uncovered, the necessity of gasotransmitters for male and female reproduction including fertilization and embryonic development is unquestionable. Accordingly, S-nitrosylation and sulfhydration of sulphur amino acid cysteine seem to be crucial protein posttranslational modifications for reproductive processes and their understanding brings relevant possibilities for ART.

3. An Increasing Attractiveness of S-Nitrosylation and S-Sulfhydration

Decades of research have established a high potential for NO and S-nitrosylation in controlling cellular mechanisms. Indeed, both NO and H_2S might engage in protein short-lived covalent reactions, which modulate proteins structure and functions. NO builds its signalling activity by binding to sulfhydryl groups of cysteine residues in target proteins. The latter process is called S-nitrosylation. In a similar manner, S-sulfhydration is a posttranslational modification of specific

residues, through the formation of persulfide (-SSH) bonds. Both sulfhydration and S-nitrosylation are reversible.

There is a broad spectrum of S-nitrosylated proteins. An exhaustive list would be beyond the scope of this review. Nevertheless, it is to note that nitrosylated proteins include cytoskeleton, cell migration, cell cycle, and antiapoptotic proteins, as well as proteins involved in transcription and protein synthesis [65–69]. In a similar way, protein-SSH formation is now admitted to mediate in a fundamental manner the cellular signalling by H_2S, based on the detection of S-sulfhydrated proteins and on the demonstration of their perturbed functions [70]. Spatial environments of the modified residues drive the impact of S-sulfhydration on protein function. For example, it may protect residues from oxidation under oxidative stress and therefore may sustain protein activities.

3.1. From Cell Cycle to Implantation, Potential Roles for S-Nitrosylation. Therefore, S-nitrosylation is a well-established posttranslation modification, whose potential involvements at physiological level in oocytes and embryos go from cell cycle regulation (meiotic transition, segmentation) to embryo survival and implantation.

Indeed, S-nitrosylation targets can be found within main modulators of meiosis progression or cell cycle progression and their regulators. Though the M-phase promoting factor, made up with cyclin B and cyclin-dependent kinase 1 (CDK1), was not reported to be itself S-nitrosylated, the S-nitrosylation of CDKs was observed for CDK2, CDK5, and CDK6 [71–73]. While CDK2-nitrosylation increases its activity independently of any effects on protein levels expression, the effect of S-nitrosylation on CDK5 and CDK6 remains elusive. S-nitrosylation of cyclin B was sought in HL-60 cells, but not observed [72]. No S-nitrosylation was reported for polo-like kinases (PLKs), anaphase promoting factor/cyclosome (APC/C), WEE1, and MYT1, which are among the close regulators of MPF. Nevertheless, the dual specificity cell division cycle 25 phosphatase (CDC25), which is the main activator of MPF, is clearly impacted since its S-nitrosylation annihilates its phosphatase activity ([71, 74]; Gelaude and Bodart: personal observations).

Beyond the cell cycle regulators, S-nitrosylation has been called to play a role in preimplantation embryos and implantation. Microenvironmental presence of NO was reported to contribute to the pathologic effects of endometriosis on the development potential of embryos. In this context, NO effects on embryo survival could either rely upon S-nitrosylation, NO/GC/cGMP or peroxynitrite formation. Lee et al. [50] suggested that the apoptotic effects of excessive NO on embryos were related to S-nitrosylation rather than to any other mechanisms. These effects were closely associated with lipid-rich organelles (mitochondria and endoplasmic reticulum) [50, 75]. Regarding implantation, NO was shown to influence trophoblasts motility [76, 77]. It was further suggested that the effects of NO on trophoblast migration and invasion, which are critical processes for the successful embryonic development, were mediated by nitrosylation of the matrix metalloprotease MMP9 [78]. Indeed, while MMP9 has been reported to be nitrosylated [79], it was colocalized with iNOS and S-nitrosylated proteins at the leading edge in trophoblast [78]. Finally, the trophoblast also appeared to be protected from apoptosis *via* S-nitrosylation of caspase 3 [80].

Thus, S-nitrosylation of proteins might play pivotal roles throughout the early development, modulating cell cycle, trophoblast motility, and embryo survival (Table 1).

3.2. S-Sulfhydration as Another Modulator of Enzymatic Activities. The impacts of H_2S and S-sulfhydration have been addressed and considered to a lesser extent, mainly due to the lack of methodologies [81]. Since the specification of protein S-sulfhydration sites has been enabled, increasing evidence has come to underline the ability of S-sulfhydration to enhance or impair an enzymatic activity. S-sulfhydration was reported to impair the activity of KEAP1 [82], while it increases the activity of K_{ATP} and Ca^{2+} channels, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), nuclear factor κB (NF-κB), and MAPK/ERK kinase 1 (MEK1) [57, 61, 82–84]. In addition to the above-mentioned S-sulfhydrated proteins, S-sulfhydration of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), H_2S-releasing enzymes, has been observed [57] and existence of feedback in H_2S production is supported.

Protein phosphatase serves as points of flexibility and crucial regulation in network signalling. Evidence had raised the fact that it might be particularly subject to S-sulfhydration. Among the phosphatase types involved in early embryogenesis and/or signalling pathways and whose activity might be modulated by S-sulfhydration are phosphatase and tensin homolog (PTEN), protein-tyrosine phosphatase 1B (PTP1B), and aforementioned CDC25. Protein phosphatase PTEN is requested at early steps for proper embryonic development [85]. In the case of PTEN, S-sulfhydration was reported to maintain the activity of the phosphatase [86], by preventing its S-nitrosylation, which would result in protein degradation [87]. PTP1B belongs to the family of ErbB, involved in numerous signalling pathways modulating proliferation, adherence, migration, or survival. PTP1B was shown to be inactivated by S-sulfhydration of cysteine C215, located in its catalytic site [88].

Also, CDC25 might be sulhydrated and inactivated presumably by modification of the cysteine in its active site [89]. There is no direct evidence for CDC25 sulfhydration, but since organosulphur compounds inhibit CDC25A and promote G2/M arrest [90] and CDC25 are targeted by ROS and S-nitrosylation, CDC25 are likely to be S-sulfhydrated [91]. Further studies are obviously needed to gather an exhaustive list of S-sulfhydrated proteins, and one might first focus on proteins, which have been already reported as being S-nitrosylated. MKP1, ERK1, CDK2 and CDK5, CDC25, and MMP9 appear as appealing candidate (Table 1). Indeed, evidences have been raised for cross-talk between S-sulfhydration and S-nitrosylation for many proteins.

3.3. A Cross-Talk of S-Sulfhydration and S-Nitrosylation? Many protein sites have been reported to undergo either S-nitrosylation or S-sulfhydration. As an example, the residue cysteine C150 in GAPDH had been found either S-nitrosylated or S-sulfhydrated [54, 92–94]. Susceptibility

Table 1: Examples of S-nitrosylated and/or S-sulfhydrated proteins.

Protein	Sulfhydration site	Sulfhydration effect on function	Nitrosylation site	Nitrosylation effect on function	References
MKP1	n.d.	n.d.	C258	Stability of protein	Guan et al., 2012 [130]
ERK1	n.d.	n.d.	C183 (potential)	Prevention of phosphorylation	Feng et al., 2013 [131]
CDK2	n.d.	n.d.	n.d.	Increase of kinase activity	Kumar et al., 2010 [72]
CDK5	n.d.	n.d.	n.d.	n.d.	Foster et al., 2009 [71]
CDC25	n.d.	n.d.	n.d.	Loss of phosphatase activity	Foster et al., 2009 [71]; Majumdar et al., 2012 [74]
MMP9	n.d.	n.d.	n.d.	Increase of activity	Harris et al., 2008 [78]
PTP1B	C215	Reduction of phosphatase activity	n.d.	n.d.	Krishnan et al., 2011 [88]
PTEN	C71, C124	Maintenance of enzyme activity and prevention of further oxidation by NO	C83	Promotion of survival signal and protein degradation	Kwak et al., 2010 [87]; Ohno et al., 2015 [86]
Actin	n.d.	Increase of polymerization activity	Cys 374	Decrease in polymerization activity and network formation	Dalle-Donne et al., 2000 [100]; Mustafa et al. 2009 [57]; Thom et al., 2008 [101]
MEK1	C341	Facilitation of Parp activation	n.d.	Loss of kinase activity	Ben-Lulu et al., 2014 [73]; Zhao et al., 2014 [61]
Parkin	n.d.	Increase of activity	n.d.	Decrease of activity	Chung et al., 2004 [95]; Vandiver et al., 2013 [96]
GAPDH	C150	Increase of the activity sevenfold	C150	Inhibition of glycolytic activity	Greco et al., 2006 [93]; Hao et al., 2006 [94]; Hara et al., 2005 [92]; Mustafa et al., 2009 [57]

for both modifications may strike root in the chemical properties of the involved thiols by S-nitrosylation and S-sulfhydration [81]. If S-sulfhydration and nitrosylation can occur on reactive cysteine residues, they frequently involve the same residue, generally by promoting different and opposing effects. Indeed, S-nitrosylation typically reduces cysteine thiols reactivity while S-sulfhydration increases cysteine thiols reactivity, thereby making them more nucleophilic. For instance, S-sulfhydration and nitrosylation on the same sites have been reported for GAPDH, Parkin, and the p65 subunit of NF-κB (nuclear factor-κB) (Table 1). The increase of GAPDH activity stimulated by S-sulfhydration is antagonized by nitrosylation, which impairs the glycolytic activity of the enzyme [54, 92–94]. Similarly for Parkin, the S-nitrosylation impairs the enzyme activity whereas sulfhydration stimulates it [95, 96].

S-nitrosylation and sulfhydration both regulate the p65 subunit of the antiapoptotic transcription factor NF-κB, which provided quite a school-case for the interplay of S-nitrosylation and sulfhydration [82]. S-sulfhydration of NF-κB has been reported to inhibit apoptosis. Persulfidation of cysteine 38 of p65 unit of NF-κB promotes binding of NF-κB to the coactivator ribosomal S3, thereby increasing its binding to promoters of antiapoptotic genes. Also, cysteine 38 persulfidation might function as the molecular "key" by which hydrogen sulfide prevents NF-κB pathway activation in ox-LDL-induced macrophage inflammation by impairing NF-κB p65 phosphorylation, nuclear translocation, and, therefore, DNA binding activity [97]. One has to note that NF-κB S-sulfhydration may not account for all the protective effects of H_2S towards inflammation. Subsequent to sulfhydration, nitrosylation of p65 reversed the activation of NF-κB targets [98, 99].

Similarly, actin, whose modifications of properties are requested for the rapid cadence of cytokinesis during early embryogenesis, is nitrosylated or sulfhydrated. While S-sulfhydration of actin resulted in an increase of filament polymerization [57], S-nitrosylated actin exhibited a decrease in polymerization activity and thus an impairment in actin network formation [100, 101]. Actin-binding proteins such as profilin [101] and cofilin [102] are also subject to S-nitrosylation and may contribute through the latter modifications to modulate the remodelling of the actin network. Thus, if we are to compare S-sulfhydration and nitrosylation, we should mainly outline that (1) proteins are rather S-sulfhydrated than S-nitrosylated and (2) nitrosylation is more likely to inhibit and impair protein functions (Table 1).

One may also hypothesize that the sequence of S-nitrosylation and sulfhydration could provide a way for a fine tuning of signalling pathways and cellular functions regulation. Because protein S-nitrosylation can foster intramolecular disulfide bond formation, a protein S-nitrosylation event might promote the formation of a more enduring S-sulfhydration reaction. Moreover, S-sulfhydration of eNOS and its increased activity have been described [103]. Supervision of Ca^{2+} influx and availability of eNOS, Ca^{2+}-dependent,

is another mechanism of H_2S-controlled NO creation [104]. Likewise, reverse NO modulation effect on H_2S releasing is assumed; however, it has not been uncovered so far.

4. Perspectives of Gasotransmitters for Assisted Reproductive Technologies

4.1. About Recent Reproductive Medicine. With respect to the above-described posttranslational modifications, the causality of some of the phenomena is explained. Assisted reproductive technologies (ART), as a medicinal approach to the solution of human infertility, are a field where the posttranslational modifications and their consequences could be utilized.

Embryos by produced *in vitro* by ART show differences compared to the *in vivo* grown embryos. Routinely used ART techniques, such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), may affect embryonic development differentially on cellular and molecular levels. Moreover, individual approaches are not equal where a slight delay of early embryonic development of IVF-produced human embryos compared to those fertilized by ICSI has been described [105, 106]. An alteration of embryonic chromatin modifications, including posttranslational modifications of nucleosomal proteins, essential for genome reprogramming and successful development into blastocyst, is a possible explanation of this phenomenon [107–109]. To this respect, gasotransmitter involvement could be justly considered.

Supplementation of culture media with donors of NO or H_2S improves embryonic development *in vitro* [20, 58, 110]. The necessity of physiological NO production has been demonstrated for *in vitro* fertilization and embryo culture [111–113]. Moreover, NO plays a role in the second meiotic block release and oocyte activation [42, 43, 114]. The suppression of H_2S physiological production even leads to oocyte maturation failure [59]. Therefore, precise supplementation of NO and H_2S allows optimizing fertilization conditions [112] as well as relieving embryonic development defects. However, the molecular mechanisms are not known and target system is questionable.

4.2. Epigenetic Dimension of Gasotransmitters. S-nitrosylation, one of the above-mentioned NO-derived protein posttranslational modifications, affects direct chromatin modification, namely, tyrosine nitration of nucleosomal core histones [115]. Histone modification *via* NO is supposed to be decisive for gene activity [116]; however, final effect of histone nitration remains unclear [117]. In addition to S-nitrosylation, core histone is affected by acetylation and methylation [118, 119] and some evidence (mentioned below) indicates that NO and/or H_2S could be indirectly required in upstream signalling of these modifications. Accordingly, influencing histone modifying upstream enzymes is another NO/H_2S-modulated chromatin gene activity. Hereby, NO interacts with histone deacetylases (HDACs) when their activity is inhibited by NO in neurons [120]. On the other hand, NO is capable of activating Sirtuin 1 (SIRT1), one of NAD^+-dependent HDACs [121]. Similarly, H_2S has been described as a potent activator of SIRT1 [122]. Presumably, S-nitrosylation and S-sulfhydration are responsible for NO and H_2S effect, respectively. On the other hand, NO/H_2S-derived modifications do not seem to be strictly upstream, because H_2S releasing stimulated by resveratrol, a strong activator of SIRT1, has been recently observed [123].

The above-mentioned SIRT1 is responsible for modifications of both histone and nonhistone targets [124–126] and through modulation of its activity it brings a broad spectrum of S-nitrosylation and sulfhydration effect. In addition to histone deacetylation, complex SIRT1 signalling leads to histone methylation and thus chromatin stabilization, which is, however, accompanied by gene silencing (summarized in [127]). Apparently, gasotransmitters are involved in wide epigenetic regulations, affecting gene expression without changes in gene sequences themselves. Some evidence indicates targeted chromatin modulation and transcriptional activation of certain genes [128, 129], due to a molecular mechanism which is yet unknown.

4.3. Delicacy of Gasotransmitter Involvement in Epigenetic Regulation. In contrast to the lifespan beneficial genome stability, embryonic genome reprogramming requires transcriptional activity, nevertheless, followed by DNA damage-prone euchromatin creation, marked by histone acetylation [132–135]. Therefore the equilibrium between chromatin stability and transcriptional activity is obviously the compromise for successful embryonic development. The dual effect of NO and H_2S on HDACs and NAD^+-dependent HDACs [136, 137] and the delicate balance between them, obvious in somatic cells [138], could be the key to embryonic genome activation and impeccable further embryogenesis.

In accordance with presumption of H_2S-epigenetically affected embryogenesis, cell cycle and proliferation are affected by H_2S as well [139, 140]. The involvement of H_2S in regulation of specific promoters has been described in vascular smooth muscle cells [141]. Interestingly, ten-eleven translocation (Tet) proteins, factors playing a role in epigenetics of early embryo [142], are included in described H_2S-modulated genes [143]. Although there are evidences of H_2S-derived epigenetic regulation of cell cycle, the characterization of H_2S-caused chromatin modifications remains clean.

In general, presence of NO and H_2S has been reported in mammalian oocytes and embryos as well [32, 59, 144] and their cross-talk due to S-nitrosylation and sulfhydration was reported, where their necessity is assumed. Meanwhile, there is poor knowledge of all gasotransmitters' potentiality, for example, (a) direct H_2S-derived S-sulfhydration of core histones, (b) NO/H_2S/SIRT1 axis, leading to chromatin equilibrium between an adequate transcriptional gene activity and genome stability, and (c) absent knowledge of CO involvement in epigenetics-driven embryogenesis. Regarding CO, its molecular action remains fully unidentified and CO-derived modifications have not yet been completely explained.

Obviously, understanding the molecular mechanism of NO/H_2S interaction and HDACs-modified embryonic chromatin offers a possibility for improvement of *in vitro*

embryo production *via* a gasotransmitter tool. A complete understanding of the cross-talk between all gasotransmitters, including CO, is necessary and a holistic approach should be emphasized.

5. Conclusion

This review summarizes the recent knowledge of gasotransmitters' action in maturing oocytes and early embryonic development, in various animal species, including sea urchin, *Xenopus,* and mammalian models. Current observations point out the necessity of NO and H_2S in these processes; however, the role of CO remains unexplained.

Based on our best knowledge, the observations, performed on amphibian and mammalian female reproduction, enlightened various species-specific biological action of both NO and H_2S. Nevertheless, the gasotransmitter-derived posttranslational modifications are shared throughout the studied animal models. Both S-nitrosylation and S-sulfhydration may be required for adequate protein activities/functions and therefore, patterns of posttranslational modifications create NO- and H_2S-modulated proteome in oocytes and embryos. Importantly, most of gasotransmitter-modified proteins may not have been yet described. Although the understanding is limited, S-nitrosylation and sulfhydration seem to be equal to other posttranslational modifications' impact. In contrast to the wide spectrum of kinases mediating phosphorylation and regulation of various proteins, NO and H_2S decide on the activity of a comparable spread of proteins. However, other alternative molecular mechanisms could be considered, often epoch-making, such as possible ROS-generating H_2S due to RSS creation [62, 63].

In addition to above-mentioned absence of insight, CO, the third known gasotransmitter, is still unexplored and its molecular involvement in gametogenesis and embryogenesis waits for verification. The principle of CO molecular action is unknown and NO and H_2S like posttranslational modifications can be presumed. The evolutionary permanence of CO biological effect is questionable, with respect to the existing recognition traits of NO and H_2S. In addition to the single CO action, the interaction of all gasotransmitters offers infinite consequences resulting in various effects in gametes and embryos. Three gasotransmitters have been described so far and some other small molecules, such as sulphur dioxide [145, 146] or hydrogen [147, 148], exhibit possible gasotransmitter features as well.

Obviously, the understanding and further study of gasotransmitters are necessary for the advancement of human ART. The *in vitro* technologies are based on a simulation of *in vivo* conditions, still lacking undefined factors. Gasotransmitters are among the essential molecules, missing in *in vitro* protocols where their failure is appreciable. However, their volatility makes them difficult to supplement into culture media and the development of an applicable gasotransmitter treatment is subject to research. A serious consideration of gasotransmitters as signal molecules, respecting their evolutionary consequences, represents an expectation for current therapy of human reproduction.

Abbreviations

APC/C: Anaphase promoting factor/cyclosome
ART: Assisted reproductive technologies
CaMKII: Calmodulin-dependent protein kinase II
CBS: Cystathionine β-synthase
CDC25: Cell division cycle 25 phosphatase
CDKs: Cyclin-dependent kinases
CSE: Cystathionine γ-lyase
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HDACs: Histone deacetylases
ICSI: Intracytoplasmic sperm injection
IVF: *In vitro* fertilization
MAPK: Mitogen-activated protein kinase
MEK1: MAPK/ERK kinase 1
MPF: M-phase/maturation promoting factor
3-MPST: 3-Mercaptopyruvate sulphurtransferase
NF-κB: Nuclear factor κB
eNOS: Endothelial nitric oxide (NO) synthase
nNOS: Neuronal nitric oxide (NO) synthase
iNOS: Inducible nitric oxide (NO) synthase
PKA: Protein kinase A
PKG: Protein kinase G
PLKs: Polo-like kinases
PTEN: Phosphatase and tensin homolog
PTP1B: Protein-tyrosine phosphatase 1B
ROS: Reactive oxygen species
RSS: Reactive sulfide species
sGC: Soluble guanylate cyclase
SIRT1: Sirtuin 1, SirT1, NAD^+-dependent HDAC 1.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Implications of Hydrogen Sulfide in Glucose Regulation: How H_2S Can Alter Glucose Homeostasis through Metabolic Hormones

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Diabetes and its comorbidities continue to be a major health problem worldwide. Understanding the precise mechanisms that control glucose homeostasis and their dysregulation during diabetes are a major research focus. Hydrogen sulfide (H_2S) has emerged as an important regulator of glucose homeostasis. This is achieved through its production and action in several metabolic and hormone producing organs including the pancreas, liver, and adipose. Of importance, H_2S production and signaling in these tissues are altered during both type 1 and type 2 diabetes mellitus. This review first examines how H_2S is produced both endogenously and by gastrointestinal microbes, with a particular focus on the altered production that occurs during obesity and diabetes. Next, the action of H_2S on the metabolic organs with key roles in glucose homeostasis, with a particular focus on insulin, is described. Recent work has also suggested that the effects of H_2S on glucose homeostasis goes beyond its role in insulin secretion. Several studies have demonstrated important roles for H_2S in hepatic glucose output and adipose glucose uptake. The mechanism of H_2S action on these metabolic organs is described. In the final part of this review, future directions examining the roles of H_2S in other metabolic and glucoregulatory hormone secreting tissues are proposed.

1. Introduction

Hydrogen sulfide (H_2S) is a colorless and odorless gas that is produced both endogenously by a variety of mammalian cells and by the sulfate reducing bacteria in the lower gastrointestinal (GI) tract. H_2S has emerged as an important gasotransmitter that regulates several systems including the cardiovascular, GI, immune, endocrine, and nervous systems (reviewed in detail in [1]). One area of recent interest is the potential role that H_2S may play in glucose regulation and metabolic health. Indeed, several groups have demonstrated that obese and diabetic individuals have altered H_2S levels in their circulation [2, 3] and tissues [4, 5]. The precise mechanisms of how H_2S can drive metabolic changes are beginning to be understood. A major factor in the regulation of glucose metabolism is the secretion and action of metabolic hormones. These hormones include insulin, glucagon, leptin, and glucagon like peptide-1. Several groups have already described the action of H_2S on insulin secretion [6–8]. Furthermore, recent work has demonstrated the effects of H_2S on downstream hormone signaling [9]. These studies and others suggest that H_2S may be a potential target in the treatment of metabolic diseases through modulating metabolic hormone secretion and signaling. The goal of this review is to describe the roles of H_2S in the regulation of metabolic hormone secretion, with a particular focus on insulin, and the downstream signaling of these hormones in the regulation of energy homeostasis.

2. H_2S Production

Although the presence of H_2S in the body has been known for some time, the precise locations of its production remain an active area of research. H_2S is produced by a large variety of cell types in the body (here named endogenous) and by host microbes including the sulfate reducing

bacteria in the GI tract. The main enzymatic machineries in the endogenous production of H_2S are the cystathionine metabolizing cystathionine-β-synthase (CBS) [10] and cystathionine γ-lyase (CSE) [11]. Other enzymes such as 3-mercaptopyruvate sulfurtransferase (MST) and cysteine aminotransferase (CAT) are also important in specific tissue types [12]. CSE activity is much higher than CBS in peripheral tissues, while CBS mainly predominates in the brain [13, 14]. The precise mechanisms involving the production of endogenous H_2S are thoroughly reviewed by Wang in [1]. Once H_2S is produced in the cell, it can act on different cellular pathways or be stored for later release. H_2S can store its sulfur group with iron (acid labile sulfur) [15] or in sulfane sulfur (a persulfide) [16] in mammalian tissues. When required and under the appropriate conditions, this bound sulfur can be released as S^{2-}, HS^-, or H_2S [17].

In addition to endogenous generation, H_2S can be produced from microorganisms in the GI tract. The gut microbiota aids in the decomposition and harvest of nutrients from food, a crucial step in energy production. Primary fermenters break down protein and complex carbohydrates into short-chain fatty acids (e.g., acetate, propionate, and butyrate) that are an important energy source, and gases (e.g., hydrogen, carbon dioxide) that are released or absorbed by the system. Hydrogenotrophs, or H_2-consuming bacteria, are essential in keeping luminal hydrogen levels low and stabilizing the environment for these primary fermenters. Among the groups of hydrogenotrophs are methanogens (producing methane), acetogens (producing acetate), and sulfate reducing bacteria (producing H_2S). Sulfate reducing bacteria use hydrogen or organic compounds as electron donors and use sulfate as their terminal electron acceptor leading to a large production of H_2S. This process is known as dissimilatory sulfate reduction and can lead to mM concentrations of H_2S in the lumen [18]. Sulfur sources from diet can originate from amino acids, preservatives, and food additives (carrageenan) or as dietary supplements (chondroitin sulfate) [18]. Microbial produced H_2S is a significant contributor to the bodies H_2S pool, as germ free mice have between 50 and 80% less H_2S in their tissues and circulation [19]. Microbial H_2S has been associated with both maintaining gastric health and being implicated in disease. Several groups have shown that H_2S regulates various physiological functions including maintenance of GI barrier function and injury repair [20]. Some earlier studies have suggested that H_2S may be involved in the etiology of ulcerative colitis [21]. However, more recent work points towards a protective role [22]. Regardless of its source, H_2S has emerged as a regulator of glucose metabolism. The mechanisms of this action are described below.

3. Importance of H_2S in Diabetes and Insulin Regulation

Insulin is one of the most researched and clinically important metabolic hormones. Strategies that seek to enhance insulin secretion and sensitivity are the cornerstone of diabetes treatment. Insulin biosynthesis is regulated by many physiological events; however the main driver of its secretion is circulating glucose, such that, after a meal is consumed, the levels of insulin spike in circulation. Insulin then acts on a variety of tissues in the body, including, but not limited to, adipose, liver, and muscle. The target cells are activated through the insulin receptor which then leads to increased translocation of glucose transporters to the membrane and glucose uptake. During the development of type 2 diabetes mellitus (T2DM), insulin signaling in the target tissues is impaired, and in order to overcome this resistance, the β cells of the pancreas begin to proliferate and produce more insulin. In cases where the pancreas is unable to produce sufficient insulin to regulate the rising glucose levels, T2DM develops. In this scenario, a variety of treatments that act to increase insulin levels or enhance insulin signaling are employed. Nevertheless, additional strategies to enhance insulin levels and signaling are of great interest in the treatment of diabetes and metabolic disease.

The investigation of hydrogen sulfide's potential involvement in glucose metabolism began in 1990 when Hayden and colleagues showed that H_2S exposure (2.2 mM) increased circulating glucose in postpartum rats [23]. Later on, several groups began to investigate how H_2S levels fluctuate in metabolic disease. Human studies that have examined circulating H_2S in T2DM have found them to be reduced. Jain and colleagues found that T2DM individuals had significantly lower H_2S compared to age matched nondiabetics [2]. Whiteman and colleagues confirmed these findings and further demonstrated that adiposity was negatively correlated with H_2S [3]. This is of particular interest since obesity is one of the principal causes of T2DM. Unfortunately, the mechanisms driving these changes in circulating H_2S, or their effects on glucose metabolism, were not investigated. As such, it is unclear whether the altered circulating H_2S observed in obese individuals is a driving force in their metabolic disease. A more mechanistic understanding of how H_2S can alter glucose metabolism has come to light through the examination of glucoregulatory hormones such as insulin and its target tissues. These pathways and their role in glucose homeostasis are described below.

4. H_2S Production and Function in the Pancreas

The first evidence that H_2S was produced in the pancreas and that it played a role in the regulation of insulin secretion came from Yang and colleagues. Using the INS-1 cell line, they demonstrated that β cells express the enzymatic machinery required to produce H_2S, including CSE, and can produce high levels of H_2S which blocks glucose-stimulated insulin secretion [8]. This was later confirmed in another β cell model, Min6 [24]. Yang and colleagues also demonstrated that treating INS-1 cells with H_2S, or overexpressing CSE, stimulated apoptosis [7]. This latter effect appeared to be caused by increased endoplasmic reticulum stress and may be a driving factor in the reduced insulin secretion observed [7]. In addition, other groups have demonstrated the mRNA expression of both CSE and CBS in the rat pancreas and that streptozocin-induced diabetes (a model of type 1 diabetes) causes increased mRNA expression of CBS and increased H_2S

production [4]. Using a rodent model of obese diabetes (the Zucker diabetic fatty rat), Wu and colleagues demonstrated that the animals impaired glucose metabolism was due to an overproduction of pancreatic H_2S and impaired insulin secretion [6]. Together, these studies suggest that increases in H_2S may be responsible for a reduction in insulin secretion and ultimately the impaired glucose clearance that occurs in diabetes. However, other groups have suggested that the elevated H_2S production from the β cell is occurring as a result of elevated circulating glucose and that H_2S is acting as a pancreatic brake, which may protect these insulin producing cells from being overstimulated by chronic hyperglycemia [25]. Indeed, it was later demonstrated that mice on a high fat diet lacking CSE have significantly worse islet glucotoxicity compared to WT animals [26]. This protective role for H_2S in β cell apoptosis occurs through H_2S mediated activation of thioredoxin, a system responsible for controlling redox homeostasis that protects β cells from glucotoxicity. The difference in reports of the protective versus toxic effect of H_2S in the pancreas may be due to the cell/animal model being used (whole animal versus cell studies and type 1 versus type 2 diabetes models). The differences in H_2S concentrations used would warrant further research into what concentration threshold is protective or detrimental to cellular function. Nevertheless, H_2S is produced in the pancreas and this appears to have important implications in insulin secretion and glucose homeostasis. How this gasotransmitter can elicit its effects on the cell is discussed below.

5. Mechanism of H_2S Action in the Pancreas

The earliest reports on the intracellular target of H_2S in insulin regulation were found to be an opening of the K_{ATP} channel [8]. When glucose enters the β cell, it generates ATP, causing the closure of ATP sensitive K_{ATP} channels and opening of calcium channels leading to depolarization and thus insulin secretion [27]. When K_{ATP} channels are kept open by H_2S, the β cell is hyperpolarized and insulin secretion is suppressed. Based on this, several groups have demonstrated that compounds that suppress the production of H_2S can increase the secretion of insulin from β cells [8, 24]. The precise mechanisms that cause the opening of this channel remain an active area of research. It has been suggested that direct binding of H_2S to cysteine residues in proteins (sulfhydration) may be a potential mechanism [28]. Using the patch clamp method coupled with channel subunit mutagenesis, Jiang and colleagues demonstrated the importance of the rvKir6.1/rvSUR1 subunits in mediating K_{ATP} channel opening [29]. It should be noted however that the above studies on the precise mechanisms of H_2S on the K_{ATP} have not been done in the β cell.

Voltage-dependent calcium channels (VDCCs) in the β cells control the movement of calcium, a crucial step in glucose-stimulated insulin release. One of the early studies examining the effect of H_2S in β cells found that NaHS (an H_2S donor) caused a decrease in the calcium oscillations caused by glucose, which ultimately led to reduced insulin secretion [24]. Using whole mouse islets, Tang and colleagues demonstrated (via patch clamp) that L-type VDCC current density is inhibited by the H_2S donor NaHS and that islets from mice lacking CSE had reduced L-type VDCC activity [29]. Of interest, these reports of decreased VDCC activity in β cells and islets are in contrast to the increased calcium concentrations that result from H_2S in cerebellar granule neurons [30]. This difference suggests that H_2S may regulate similar intracellular pathways in distinct manners depending on the cell type.

In addition to ion channel activities, H_2S may also regulate insulin secretion through the modulation of intracellular kinases. Several of these kinases are known to be modulated during the secretion of insulin including PI3K, ERK, AKT, and MAPK. Indeed, both endogenous and exogenous H_2S have been shown to directly activate the p38 MAPK [7]. Importantly, activation of the MAPK/JNK pathway is a known mechanism in impaired insulin release from the β cell [31]. More studies are required to determine if additional cell signaling pathways are altered through the activity of H_2S.

6. H_2S Effects on Metabolic Tissues

The description thus far focused on the production and effects of H_2S in the insulin secreting β cell. A vital part of glucose homeostasis is the function of the insulin sensitive metabolic organs, including adipose tissue, liver, and muscle.

One of the principle targets of insulin is the adipocyte. Insulin promotes the storage of excess glucose and its conversion to fat, leading to increased adiposity, a major risk factor for the development of metabolic disease. Several groups have demonstrated that adipose tissue produces H_2S, and that gasotransmitter production and signaling in the adipocyte are altered during obesity. Feng and colleagues were the first group to describe the expression of CBS and CSE and production of H_2S from rat adipocytes [32]. In this report they demonstrated that H_2S impairs insulin mediated glucose uptake and that high fructose-induced diabetes led to increased production of H_2S in epididymal adipose tissue, an effect that could be blocked by inhibiting CSE. This result points towards a negative effect of H_2S on glucose uptake in the adipocyte. Interestingly, circulating levels of H_2S are lower in obese humans [3], suggesting a disconnection in the increased production observed in the rodent adipose tissue. Some groups have demonstrated a positive role for H_2S in glucose metabolism in the adipocyte. One study in 3T3L1 adipocytes found that H_2S is required for vitamin D induced GLUT4 translocation and glucose uptake [33]. Another positive role for H_2S in adipose tissue metabolism appears to be its role in reducing inflammatory cytokine production from resident adipose macrophages. These cytokines are a known causal factor in the development of insulin resistance in adipose and other metabolic tissues [34]. In one study, macrophages isolated from mice with diet-induced obesity produced less H_2S and more cytokines than macrophages from lean mice [5]. Based on these reports, it may be important that future work in adipose tissue (from obese subjects) separates the adipocytes from the stromal vascular fraction. Several studies have also shown a role for the H_2S/CSE system in perivascular adipose tissue, although

most of this work has described its importance in vascular tone (reviewed in [35]) rather than glucose homeostasis.

Another key organ in the regulation of glucose metabolism is the liver. During an elevated circulating glucose scenario, insulin acts on the liver to stimulate glucose uptake and its conversion to glycogen and fatty acids for storage. In a low glucose scenario, pancreatic glucagon acts on the liver to promote the production or liberation of glucose through gluconeogenesis or glycogenolysis, respectively. Dysregulation of insulin signaling in the liver (hepatic insulin resistance) is a common phenomenon in T2DM (reviewed in [36]). The mRNA expression of both CSE and CBS was demonstrated in the liver of rats and was found to increase after inducing type 1 diabetes with STZ [4]. Later on it was demonstrated that overexpressing CSE in hepatocytes leads to reduced glycogen content. In this study, it was also shown that CSE KO animals (lower H_2S) have a reduction in endogenous glucose production [37]. A recent study by Ju and colleagues demonstrated a mechanism by which H_2S may directly stimulate gluconeogenesis. They found that pyruvate carboxylase (a key enzyme in gluconeogenesis) is sulfhydrated by H_2S, which leads to increased activity and glucose production [9]. These findings seem to indicate that H_2S production in the liver causes enhanced glucose release, an effect that could aggravate the hyperglycemia observed in diabetes. However, since type 2 diabetics are known to have lower rather than higher circulating H_2S, further studies investigating the liver production of H_2S during T2DM are required.

Surprisingly, there is a paucity of studies that have examined the role of H_2S in skeletal muscle, let alone skeletal muscle glucose uptake. This may be due in part to the low or nondetectable levels of the H_2S producing enzymes in rodent models (in contrast to the higher levels found in human muscle, reviewed in [38]). Nevertheless, future work should, at the very least, examine the effects of H_2S donors since H_2S may act on muscle tissue via its circulating stores.

7. Other Hormones and Future Work

While H_2S plays important roles in the metabolism of hormones like insulin and glucagon, a variety of other metabolic hormones remain to be examined. One emerging area holding potential for this is the gastrointestinal endocrine system. Here, a variety of enteroendocrine cells secrete numerous peptide hormones that play important roles in glucose homeostasis and energy metabolism. Some important candidates are the insulin-stimulating incretin hormones: glucose-dependent insulinotropic polypeptide (GIP) and glucagon peptide-1 (GLP-1). Recently, Bala and colleagues examined the role of endogenous H_2S in a GI endocrine cell line, STC-1 [39]. This cell line secretes GLP-1 and the anorexic hormone peptide YY (PYY). They found that H_2S donors and l-cysteine impaired oleic acid-stimulated GLP-1 and PYY secretion. While their primary focus was on the modulatory effect of H_2S on oleic acid-stimulated hormone secretion, their results support further investigation of H_2S on GI hormone secretion and signaling. Indeed, the question remains: can GI endocrine cells produce their own H_2S, and is the altered H_2S level observed in obesity responsible for the dysregulation in GI hormone secretion [40]? Of importance, GLP-1 therapies have become a major tool in the treatment of type 2 diabetes [41] and recently obesity [42]. Therefore, the role H_2S has in GLP-1 and other endocrine cells may be an additional mechanism by which this gasotransmitter can regulate glucose homeostasis.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Physiological Importance of Hydrogen Sulfide: Emerging Potent Neuroprotector and Neuromodulator

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Hydrogen sulfide (H_2S) is an emerging neuromodulator that is considered to be a gasotransmitter similar to nitrogen oxide (NO) and carbon monoxide (CO). H_2S exerts universal cytoprotective effects and acts as a defense mechanism in organisms ranging from bacteria to mammals. It is produced by the enzymes cystathionine β-synthase (CBS), cystathionine ϒ-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (MST), and D-amino acid oxidase (DAO), which are also involved in tissue-specific biochemical pathways for H_2S production in the human body. H_2S exerts a wide range of pathological and physiological functions in the human body, from endocrine system and cellular longevity to hepatic protection and kidney function. Previous studies have shown that H_2S plays important roles in peripheral nerve regeneration and degeneration and has significant value during Schwann cell dedifferentiation and proliferation but it is also associated with axonal degradation and the remyelination of Schwann cells. To date, physiological and toxic levels of H_2S in the human body remain unclear and most of the mechanisms of action underlying the effects of H_2S have yet to be fully elucidated. The primary purpose of this review was to provide an overview of the role of H_2S in the human body and to describe its beneficial effects.

1. Introduction

Hydrogen sulfide (H_2S) is a poisonous gas that is a toxicant in most organs in the human body. It acts as a gaseous signaling molecule and chemical reagent involved in many physiological processes, including the pathogeneses of various diseases such as neurodegenerative disease, heart failure, and diabetes [1, 2]. As a result, the beneficial roles of this compound were neglected for many years due to its toxic nature. However, in recent years, the benefits of gasotransmitters such as nitric oxide (NO) and carbon monoxide (CO) have acted as a fillip to investigating the benefits of H_2S [3]. Furthermore, a reevaluation of the endogenous levels of H_2S confirmed its existence and advantages in mammalian tissues [4].

H_2S is a sulfur analog of water and, due to its weak intermolecular force, exists in a gaseous form that is colorless but has an offensive odor [5]. At a pH of 7.4 in the mammalian body, one-fifth of the total H_2S subsists in an undissociated form, with the remaining content existing as hydrosulfide anions (HS^-) and sulfide (S^{2-}). The high lipid solubility of H_2S allows it to easily penetrate the plasma membrane of cells in its undissociated form [6] but it remains unclear whether this undissociated form is physiologically pertinent [7]. Various studies performed on rat, human, and bovine brain tissues have determined that H_2S is present at levels of up to 50–160 μmol/L in tissues and that sodium hydrogen sulfide (NaHS) is one of the physiological donors of H_2S [8].

Although it is known that H_2S protects nerves from oxidative stress, saves photoreceptor cells in the retina from light-induced degeneration, regulates endoplasmic reticulum stress, and defends the kidneys from ischemic reperfusion injury [9], the role of H_2S in the central nervous system (CNS) has attracted a lot of attention over the past few decades. The neuromodulation and neuroprotection of nerve cells are a common feature of H_2S [9] and its enzymes exert effects in a variety of diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Down's syndrome, cerebral ischemia, and Huntington's disease [10, 11]. Additionally, following injury in the peripheral nervous system (PNS), peripheral nerves undergo Wallerian degeneration and macrophages are recruited into the distal nerve pump [12]. The regeneration of these injured peripheral nerves is associated with the demyelination, dedifferentiation, and proliferation of Schwann cells, which takes place serially. Recently, the possible involvement of H_2S in demyelinating disorders and nerve degenerative disorders has also been suggested [13, 14].

Thus, the present review focuses on the physiological roles of H_2S in different systems and organs, including the CNS, and in peripheral nerve degeneration and regeneration. A consensus regarding findings in these areas will aid in the determination of appropriate avenues for H_2S in research investigating neural regeneration and the treatment of neurodegenerative diseases and may lead to potential therapeutic strategies that employ H_2S.

2. Biosynthesis of H_2S

Two pyridoxal-5′-phosphate- (PLP-) dependent enzymes present in mammalian tissues, cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine Υ-lyase (CSE; EC 4.4.1.1), are primarily responsible for the biosynthesis of H_2S from L-cysteine (L-Cys) [15, 16]. H_2S can be produced by other pathways as well. Cysteine first reacts with ketoacids to form 3-mercaptopyruvate via the catalytic action of cysteine aminotransferase (CAT; EC 2.6.1.3) and then 3-mercaptopyruvate is desulfurated by 3-mercaptopyruvate sulfurtransferase (MST; EC 2.8.1.2) to form H_2S [17]. H_2S is mostly synthesized by cystathionase in the liver, kidneys, enterocytes, and vascular smooth muscle cells, by CBS in the brain, and by MST in cardiac tissues [18]. Recently, it was reported that dihydrolipoic acid (DHLA) and thioredoxin are the endogenous reducing substances that cause MST to release H_2S [16] and that CBS is the catalytic agent responsible for the condensations of cysteine and homocysteine, which produces H_2S and cystathionine via a β-replacement reaction [19, 20]. The distributions of CBS and CSE in mammalian tissue are different. A cardiovascular study determined that CSE plays a major role in producing H_2S under normal physiological conditions [21] while another study found that the MST pathway mainly contributes to its production in the brain and that this pathway is regulated by intracellular calcium (Ca^{2+}) in a concentration-dependent manner [16, 22, 23].

A recent study used Western blot and immunohistochemistry analyses to investigate DAO expression and methylene blue assays to assess H_2S biosynthesis and revealed that H_2S is also produced from D-cysteine via the enzyme D-amino acid oxidase (DAO) [24]. The H_2S production pathway that uses D-cysteine primarily operates in the cerebellum and kidney and, thus, was termed the DAO/MST pathway [25, 26]. Although DAO is confined to peroxisomes and MST is located in the mitochondria, these factors exchange numerous enzymes and metabolites [16]; these findings provide strong support for calling this pathway the DAO/MST pathway. Interestingly, the production of H_2S via DAO is subdued by indole-2-carboxylic acid but this effect was not seen with MST [26]. A nonenzymatic pathway for the production of H_2S also exists and involves the reduction of elemental sulfur to H_2S by reducing equivalents procured via the oxidation of glucose [27]. Either hyperglycemia or the escalation of oxidative stress conditions can nurture the production of H_2S via this nonenzymatic route in mammalian erythrocytes [8]. The endogenous production and metabolism of H_2S and its aforementioned biosynthetic and transformation pathways are illustrated in Figure 1.

It is believed that two possible mechanisms can explain the release of H_2S. First, after its production, H_2S may be liberated by the enzymes involved in its synthesis and, second, H_2S may be stored and released in response to physiological needs in mammalian tissue [28]. H_2S is released from acid-liable sulfur (sulfur atom in iron-sulfur complex) under acidic conditions, while under alkaline conditions bound sulfane-sulfur comes into play; the favorable pH range for its release under these conditions is approximately 5.4 and 8.4, respectively [29]. The discovery of the DAO/MST pathway suggests that additional H_2S biosynthetic pathways may be revealed in the future.

3. Interrelationships with Other Gasotransmitters

Although H_2S, NO, and CO have similar molecular targets and cellular actions, these compounds also have a tendency to compete with each other. For example, the gasotransmitters CO and NO have particular relationships with CBS [8]. After the initial demonstration of the physiological roles of H_2S in 1996 [31], it took 5 more years to determine that NO can bind to CBS and can impede enzymatic activity and that CBS has a high affinity for CO [32]. However, the exact mechanisms underlying these relationships remain unknown. NO also modulates the endogenous production of H_2S in smooth muscle cells and vessels [33]. Previously, it was thought that the signaling pathways and functional mechanisms associated with H_2S and NO were autonomous, but their combined effects on angiogenesis and vasorelaxation have complicated this issue for researchers. If endothelial NO synthase (eNOS) is blocked, then it fully rescinds the angiogenic effects of H_2S whereas the silencing of H_2S significantly attenuates the angiogenic effects of NO [34]. Additionally, both H_2S and NO activate protein kinase G, as well as its cellular signaling, and 1-H-[1,2,4]oxadiazolo[4-3,-a]quinoxalin-1-one (ODQ), which is a soluble guanylyl cyclase inhibitor that decreases both H_2S- and NO-induced angiogenesis [35].

Under severe conditions such as heart inflammation and heart failure events, H_2S and NO cooperate and communicate

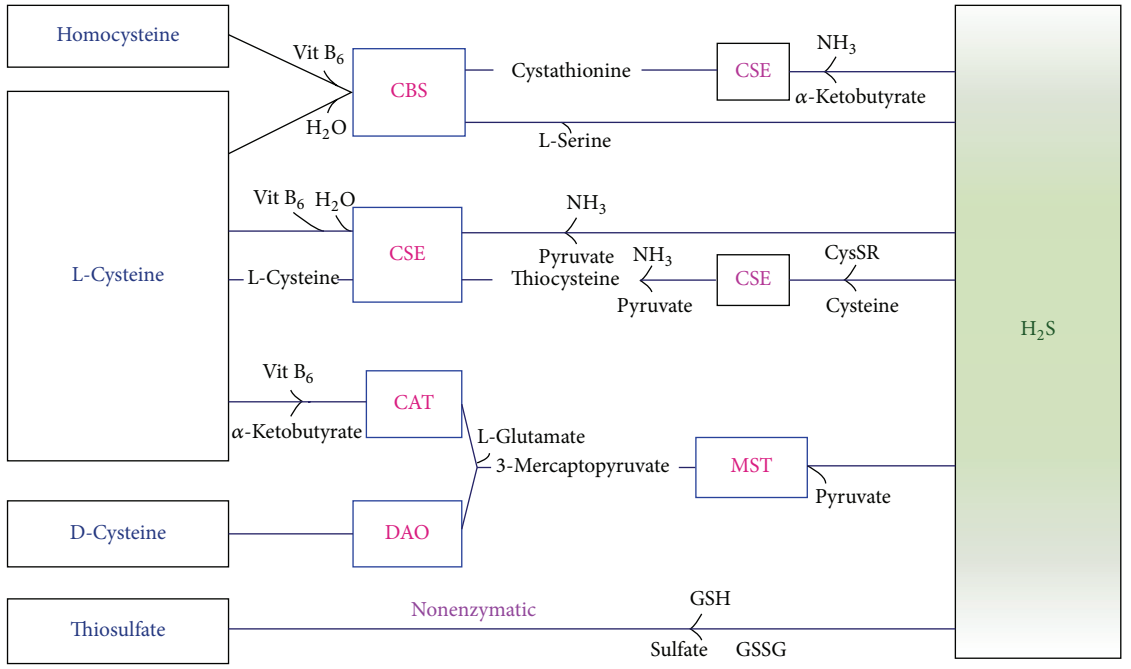

FIGURE 1: Biosynthetic pathways and the transformation of H_2S in the mammalian body. H_2S is synthesized in the mammalian body via both enzymatic and nonenzymatic pathways but the nonenzymatic pathway accounts for only a small portion of its production. CBS and CSE both use PLP and vitamin B_6 as cofactors. In the presence of cysteine and more so in the presence of homocysteine, CBS catalyzes the production of H_2S, and the condensations of homocysteine and serine are the most recognized reactions catalyzed by CBS. CSE uses L-cysteine as the substrate to form two gases, H_2S and NH_3, as well as pyruvate. MST and CAT produce H_2S and pyruvate from 3-mercaptopyruvate, which is formed from L- or D-cysteine, and α-ketoglutarate, which is produced by CAT. Thiosulfate nonenzymatically produces H_2S and all essential components of this nonenzymatic path are present in vivo. Thiosulfate can be converted into sulfite in the liver, kidney, or brain tissues via thiosulfate reductase or by thiosulfate sulfurtransferase in the liver. H_2S is also released from thiosulfate and persulfides [8, 30].

with each other to stimulate thiol-sensitive compounds that produce unambiguous positive outcomes for inotropic and lusitropic heart abnormalities [36]. Ex vivo aortic explants of wild-type and CSE-knockout (CSE-KO) mice were used to explore the possible interaction between H_2S and NO and revealed that CSE-KO mice exhibit a significant decrease in vascular neogenesis [37]. The authors also proposed that the interaction of these gasotransmitters stimulates endothelial cell proliferation. The administration of H_2S to rats with clinical symptoms of hypoxic pulmonary hypertension has also been shown to increase plasma CO concentrations [38]. CO and H_2S act on the same target but have opposite patterns of outcome. For example, coevoked channel activation is completely suppressed by potassium cyanide (KCN; 1 mM) but there are no consequences during H_2S-induced channel activation [39]. Thus, the precise nature of the relationship between these gasotransmitters in various physiological pathways remains unclear and requires further investigation. However, even if H_2S, NO, and CO compete with each other, their interactions are likely to result in beneficial effects on mammalian physiology.

4. Effect of H_2S on the CNS

H_2S has a proven neuromodulatory role in the protection of neurons from oxidative stress, as evidenced by its inhibition of hypochlorous acid-mediated oxidative damage [40] and $ONOO^-$-mediated protein nitration and cytotoxicity [41] in neuroblastoma cells. Recent studies have also established that H_2S plays an important role in the upregulation of the GABA β-receptor at both pre- and postsynaptic sites [42]. Astrocytes and microglial cells play important roles in the regulation of brain pH levels, neurotransmitter levels, and neuronal excitability [43] while microglia are also associated with the progression of neuronal diseases such as AD [44] and PD [45]. H_2S evokes Ca^{2+} waves in astrocytes that trigger a Ca^{2+} influx via its channels in the plasma membrane [7] and also reversibly increases Ca^{2+} levels in microglia in a dose-dependent manner [46].

In addition to regulating Ca^{2+} homeostasis, H_2S may also be involved in long-term potentiation (LTP) and the modulation of various neurotransmitters [5]. H_2S facilitates hippocampal LTP via the activation of N-methyl-D-aspartate (NMDA) receptors as well as the phosphorylation of these receptors by protein kinase A (PKA) [47] and regulates intracellular Ca^{2+} level in astrocytes and hippocampal slices [10]. H_2S also safeguards neurons by controlling endoplasmic stress via the balancing of membrane potentials and the activation of K_{ATP} and cystic fibrosis transmembrane conductance regulator chloride (CFTR Cl^-) channels [9]. Most neurons in the nucleus solitarius, which are believed to play a role in the cardiovascular system, are depolarized by H_2S [48]. Additionally, a donor of H_2S, NaHS, is linked to

the inhibition of apoptosis, decreases of edema in the brain, and the amelioration of cognitive dysfunction, which could attenuate early brain injury development due to subarachnoid hemorrhage via several mechanisms [49]. The major roles and possible therapeutic targets of H_2S are illustrated in Figure 2.

Thus, it can be concluded that H_2S exerts protective and modulatory effects on nerve cells, either cooperatively or independently. Although the physical and chemical properties of H_2S remain elusive, its roles in these processes have become more clearly defined and most of its mechanisms of action are understood.

4.1. H_2S and PD. PD is primarily characterized by cognitive deficiencies resulting from changes in the nucleus basalis of Meynert and the cerebral cortex and the continuous loss of dopaminergic neurons in the mesencephalon [50]. H_2S inhibits oxygen consumption and 6-OHDA-evoked nicotinamide adenine dinucleotide phosphate (NADPH) oxidation and activates microglial cells in the midbrain which, in turn, lead to the accumulation of proinflammatory factors in the subcortical part of the forebrain [51]. This is the primary mechanism by which H_2S decreases the chances of further neuronal injury and degeneration [52]. The neuroprotective role of H_2S has also been demonstrated in experimental rat models of neurotoxin-induced PD [50]. The advantageous effects of H_2S are due to its activation or suppression of different protein kinases, such as PKC, PI3K/Akt, p38, JNK, and the ERK-MAPKs [53], which decrease oxidative stress and inflammation and exert antiapoptotic actions.

Although L-Dopa is the most commonly used drug for the treatment of PD due to its ability to maintain dopamine levels, it cannot block or reverse the progression of PD. Additionally, long-term L-Dopa therapy may lead to neurodegeneration [54] and dyskinesia [55] in and of itself. H_2S stimulates glutamate transporter functioning and leads to direct sulfa hydration via the ERK/MAPK pathway, which attenuates the production of reactive oxygen species and decreases oxidative stress [56, 57]. Thus, it can be concluded that L-Dopa and H_2S may be more effective for the treatment of PD when used in combination. A mouse model of PD constructed using l-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) plus probenecid injections results in the destruction of dopaminergic neurons but the administration of H_2S increases the survival rate of neurons and is protective against MPTP-induced toxicity [58]. Thus, H_2S not only protects peripheral tissues but also effectively treats neurological damage related to PD. Recently, it was shown that drinking coffee and inhaling cigarettes can inhibit monoamine oxidase (MAO) [59], which suggests that there is a lower risk of PD in coffee drinkers and smokers. Cakmak found that coffee contains *Prevotella*-derived H_2S and that H_2S is a well-documented constituent of cigarette smoke.

4.2. H_2S and AD. AD, which is one of the most familiar types of dementia, is caused by activated microglia and increases in neuritic plaques carrying the β-amyloid protein [60]. This neurodegenerative disease has been exhaustively researched because it affects the cortex and hippocampus and leads to severe cognitive dysfunction [61]. The etiology of AD is multifactorial and presumably includes a number of distinctive etiopathogenic mechanisms [62]. CBS is thought to be the main source of H_2S in the brain. In 1996, it was first shown that S-adenosylmethionine, which is a CBS activator, is significantly reduced in subjects with AD [63]. Moreover, the severity of AD is related to altered levels of H_2S [64] because the pathological state of AD in the human body has been associated with lower levels of endogenous H_2S and the accumulation of homocysteine in the brain [65, 66]. Several series of in vitro and in vivo experiments demonstrated the role of H_2S in the promotion of cell growth and preservation of mitochondrial function [67] as well as in the retardation of oxidative stress factors such as amyloid beta peptides (Aβ), malondialdehyde (MDA), hypochlorite (HOCI), and 4-hydroxy-2-nonenal (4-HNE) [68, 69]. In adult male Wistar rats without serious signs of H_2S toxicity, spa water with excessive amounts of H_2S has the ability to improve cognitive processes by decreasing Aβ deposits and targeting the APP, PST, and ON/4R-tau isoforms [70]. Additionally, NaHS, which is a donor of H_2S, retards protein oxidation and lipid peroxidation in the neuroblastoma cells of AD patients [71].

Cerebral atrophy, seizures, and intellectual disabilities can be caused by the autooxidation of homocysteine [72, 73] and hyperhomocysteinemia has been identified in brains of AD patients [74]. H_2S protects against and reduces homocysteine-induced toxicity and oxidative stress through its antioxidant properties in the adrenal medulla (PC12 cells) and vascular smooth muscle cells of rats [75, 76]. Synaptic dysfunction and vascular inflammation are also believed to play crucial roles in the pathogenesis of AD [77]. Recent analyses of the expressions of mRNA and synaptic proteins in C57BL/6J wild-type male mice clearly demonstrated that plasma homocysteine-induced alterations in learning and memory processes were associated with synaptic remodeling in the hippocampus [78]. Thus, H_2S can influence synaptic remodeling. Vascular dementia (VD) is another common neurodegenerative disorder that, much like AD, is caused by cerebral ischemia. H_2S modulates oscillatory coupling in the hippocampus and may represent a possible molecular mechanism underlying the changes in VD patients [79].

Although neurodegenerative pathologies like AD and PD do not initially involve inflammation, various experimental findings suggest that the inflammatory responses of macrophages, microglia, and astrocytes contribute to the progressions of both diseases [14]. The relevance of the CBS, CSE, MST, and CAT enzymes in the development of AD and PD is still unexplained and direct evidence supporting the potential advantages of H_2S as a therapeutic strategy for these diseases is unavailable.

4.3. Other CNS Diseases. Various experimental studies have correlated the effects of H_2S in different pathological states of the human body. Ischemic stroke increases tissue levels of H_2S in the cerebral cortex [80] while H_2S has been shown to protect the embryonic brain against ischemia-reperfusion injury [81]. A rat model of febrile seizure is associated with elevated plasma levels of H_2S, and Down's syndrome is known to cause the overaccumulation of H_2S

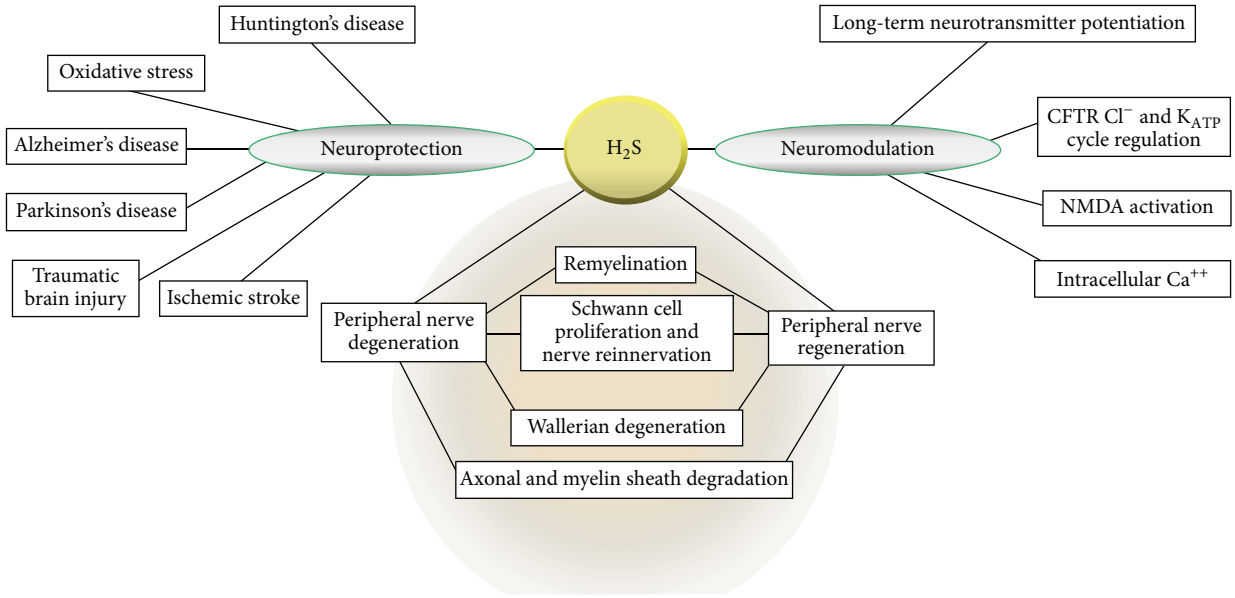

Figure 2: Therapeutic targets and possible physiological functions of H_2S. Enough experimental evidence has been collected to prove the prominent role of H_2S in normal pathophysiology. Therefore, many therapeutic targets exist for H_2S in mammalian body; its roles in neuroprotection, neuromodulation, and antiproliferation, as well as its functions during peripheral nerve degeneration and regeneration, are widely appreciated.

in the brain [42]. Similarly, there are increased total plasma homocysteine levels in patients with Huntington's disease and CBS deficiencies lead to homocystinuria [50]. Additionally, H_2S reverses learning and memory problems caused by damage to the hippocampus [82, 83].

5. H_2S and the PNS

Even if the most important roles that glial cells play involve the physical and metabolic support of neurons via the maintenance of the extracellular environment, these support cells are often referred to as "glial culprits" because the CNS lacks the ability to regenerate itself, even after injury [84]. On the other hand, Schwann cells in the PNS are best known for their roles in supporting nerve regeneration, conducting nerve impulses along axons, and modulating neuromuscular synaptic activity and nerve development [85]. The possible roles of H_2S in peripheral nerve degeneration and regeneration are discussed below and supported with experimental evidence.

5.1. H_2S in Peripheral Nerve Degeneration. Based on the degrees of damage in the nerve and surrounding connective tissue, peripheral nerve damage may be classified as neurapraxia, axonotmesis, and neurotmesis, with the latter being a severe type of peripheral nerve injury [86]. As stated above, nerve regeneration after injury is possible in the PNS and involves major events such as Wallerian degeneration, axonal degeneration, remyelination, axonal regeneration, and nerve reinnervation. H_2S plays vital roles throughout this process. Axonal regeneration and remyelination begin in the distal pump of injured peripheral nerves and involve axonal degeneration and the degradation of the myelin sheath of Schwann cells, which is termed Wallerian degeneration [87].

The effects of H_2S on peripheral nerve degeneration and regeneration may be best explained by recent ex vivo experiments using the sciatic nerves of mice. In these experiments, Park et al. [12] utilized N-ethylmaleimide (NEM), which is an inhibitor of all cysteine peptidases, to inhibit the production of H_2S during Wallerian degeneration and found that NEM inhibits not only CSE but also the basal expression of MST. Based on analyses of several markers of Schwann cell dedifferentiation and proliferation, these authors concluded that H_2S signaling has great value after peripheral nerve injury regarding myelin fragmentation, axonal degradation, and Schwann cell dedifferentiation and proliferation. Additionally, these findings demonstrated that H_2S production influences transcriptional regulation in Schwann cells during Wallerian degeneration. A similar study inhibited these processes via NEM or through several Schwan cell dedifferentiation or immaturity markers including lysosomal associated membrane protein 1 (LAMP1), neurotrophin receptor p75 ($p75^{NTR}$), the protein coded by JUN gene (c-jun), and phospho-ERK 1/2 (p-ERK1/2) [15]. Jung and Jeong found that these types of inhibition decreased myelin ovoid fragmentation, axonal degeneration, and Schwann cell dedifferentiation, demyelination, and proliferation through neurotrophin receptors, the MAPK pathway, lysosomal protein degradation, and transcriptional regulation.

The use of these markers in current research has garnered much attention. For example, the LAMP1 marker is related to the degeneration of peripheral nerves, including the nonuniform morphology of myelinated axons, in aged individuals [88]. The function of $p75^{NTR}$ is associated not only

with cell death and survival but also with the maintenance of axonal elongation and the release of neurotransmitters between sympathetic neurons and cardiac myocytes [89]. Furthermore, the reexpression of $p75^{NTR}$ has been identified in various pathological scenarios, including neurodegenerative disorders [90]. Due to its roles as a protein and a component of a transcription factor, the inactivation of c-jun in mice resulted in delays and/or the failure to properly complete the regeneration procedure and an enhancement of neuronal death [91]. Although the molecular pathways are not yet fully understood, the absence of c-jun causes the complete downregulation of neurotrophic genes such as artemin (Artn), brain-derived neurotrophic factor 1 (BDNF-1), glial cell-derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), and nerve growth factor (NGF). These findings suggest that neurotrophins may be a potential target for the treatment of various neuropathies. Studies of the molecular mechanisms involved in those processes explained above have revealed the capability of Schwann cells to regenerate axons and to rescue motor neurons following nerve injury [92].

However, many issues relevant to this process remain unexplained, including the relationships between neurotrophic factors and H_2S, H_2S production, and remyelination that is associated with the extracellular matrix (ECM) protein, as well as intracellular regulators, hormones, and transcriptional regulators that involve H_2S [15]. Other than H_2S, the ubiquitin proteasome system (UPS) is another factor that is essential for the regenerative functions of peripheral nerves after injury [93, 94].

5.2. H_2S in Peripheral Nerve Regeneration. Other issues associated with the role of H_2S in peripheral nerve regeneration involve the responses of Schwann cells in H_2S production. Schwann cells are believed to be an important factor in the regeneration process because peripheral axons have no function as H_2S producers during Wallerian degeneration [12]. Nerve injury leads to the dedifferentiation of Schwann cells from myelinated cells to immature undeveloped cells [15] and, as discussed in Section 5.1, decreases in H_2S production attenuate changes in markers of dedifferentiation. Thus, it is important that reductions in H_2S production nurture both the regeneration of axons and the remyelination during the last stage of Wallerian degeneration. Immature undeveloped Schwann cells undergo multiplication and reach the target, injured organ, which is when the possible beneficial effects of H_2S come into play. Remyelination is another important process involved in nerve regeneration whereby Schwann cells are able to flourish due to myelination and the inhibition of H_2S production (via transcriptional regulation through krox20 and c-jun), which is fundamental to this stage of regeneration [15].

Despite the above findings, very few studies have attempted to analyze the roles that H_2S plays in peripheral nerve degeneration and regeneration. Although our research group is currently conducting studies to identify the core effects of H_2S, the existing literature strongly supports the importance of H_2S in nerve degeneration and regeneration.

6. Other Functions of H_2S

The exciting evolution of H_2S in the field of neuroscience has revealed its importance not only in the PNS but also in various physiological and pathological conditions. For example, endogenous levels of H_2S have been shown to influence exogenous H_2S during cell apoptosis [95]. Additionally, the antiproliferative and proapoptotic effects of H_2S have a significant influence on various disorders such as vascular graft occlusion, atherosclerosis, and neointimal hyperplasia [96]. H_2S also regulates glutathione levels by enhancing the hustle of the cysteine/glutamate antiporter at the cellular level, which directly neutralizes free radicals and reactive oxygen compounds and balances the levels of vitamins C and E in their reduced form [97]. The acute inhalation of H_2S protects lungs from injuries induced by ventilators and relaxes pulmonary vascular tissue but epidemiological data suggest that the long-term exposure to even low levels of H_2S can cause bronchial hyperresponsiveness [8].

Relative to other parts of the body, the gastrointestinal tract (GIT) contains the largest amount of H_2S, where it subsidizes the homeostatic control of GIT mucosal defenses and repairs damage [31]. However, the signaling pathways of H_2S in the GIT remain unclear. Other beneficial effects of H_2S on translation and transcription include the control of endoplasmic reticulum stress and activating the unfolding protein response. During these processes, CSE increases H_2S production and ultimately restores endoplasmic reticulum homeostasis via the sulfa hydration reaction [98]. Additionally, type 1 diabetes is believed to involve the overproduction of H_2S [18], and H_2S may be helpful for patients with erectile dysfunction due to its involvement in the relaxation of the smooth muscle that causes the erection of the penis [99]. Recently, six fatal cases of H_2S poisoning were reported during an attempt to unblock a wastewater cistern in which the primary reason for death was H_2S aspiration and because it is a mediator and regulator of various physiological conditions, H_2S can have serious toxic effects [100].

The roles of H_2S in insulin resistance syndrome and regional ischemic damage, as well as its dose-dependent relationship with methylglyoxal (MG) in vascular smooth muscle cells, also require explanation [3]. Because it is an antioxidant bulwark, H_2S has the capacity to sense chromaffin cells and chemoreceptors [101] but it has yet to be determined how H_2S increases local blood perfusion and/or ventilation [102]. Further justification is needed for the production of H_2S via MST under physiological conditions because this process requires full alkaline conditions [103].

7. Therapeutic Prospect and Potential of H_2S

Almost two decades of research on H_2S unfastened series of positive outcomes and its potential is expanding every day. Molecular mechanisms of H_2S are being uncovered and various types of molecular and sulfa hydration targets are on the phase of identification which may lead us to reveal its biological activities [10]. Several in vivo and in vitro studies on AD and PD model have already proved their therapeutic effectiveness for treatment [40, 68, 104, 105].

Surprisingly, inhaled H_2S has found to have protective effect against neuropathic pain and brain edema which has also made researchers think seriously in this topic because we can develop our opinion and research arena on hydrogen sulfide donor compounds against neuropathic pain and brain edema [106, 107]. Even though H_2S has to deal with its own double face (toxic and protective) attitude which has pushed it into several controversies, it is recently found that various ion channels in multiple systems and organs advocate the protective role of H_2S [108]. Because of the overall effect, H_2S-releasing drugs are now under clinical trial after their verified effectiveness in animal model. This trend of clinical trial on finding H_2S donors or H_2S-releasing drugs can be a very big breakthrough for the treatment of several diseases which are almost incurable till date [109]. H_2S-releasing derivatives of mesalamine [110] and diclofenac [111] have already been shown to decrease inflammatory disease and gastric hemorrhagic lesions compared to original drugs and some possibility for the treatment of injured brain after subarachnoid hemorrhage (SAH) via H_2S is also a great progress [112] because another study demonstrated that it reduces the level of reactive oxygen species and lipid peroxides malondialdehyde following SAH [113]. Recent studies on the effect of H_2S on brain synaptic remodeling and its role in GABA-mediated and glutamate neurotransmission have even increased its prospective potential in physiological standpoint [114]. Even with the difference between community and industry based results, the effect of environmental exposure to H_2S on CNS has exposed its novel and diverse role [115]. Neuroprotective role of H_2S after traumatic brain injury has also proven its potentiality and efficacy for other CNS related diseases [116]. Thus, it can be summarized that future prospect of H_2S in development of new therapeutic strategy is wide and bright.

8. Concluding Remarks

An adequate amount of evidence has been gathered in support of H_2S as a gasotransmitter and modulator in mammalian tissue, particularly in the nervous system. H_2S is principally produced in the liver, kidney, enterocytes, and vascular smooth muscle cells via cystathionase and partially produced in cardiac tissue by MST. It is catabolized in mitochondria by thiosulfate reductase, and thiosulfate in the urine may be used as a marker of H_2S biosynthesis. H_2S is capable of both suppressing and promoting inflammation but it remains unclear how these proinflammatory and anti-inflammatory activities can be enhanced or attenuated. The role of H_2S in the Schwann cell response to peripheral nerve injury has been well established by experimental evidence, and the importance of H_2S signaling during Wallerian degeneration, where it broadly affects Schwann cell dedifferentiation and proliferation, has been repeatedly demonstrated.

The bidirectional relationship between H_2S and NO is another area of particular research interest and therapeutic potential. Experimental studies using inhibitors or donors of endogenous H_2S have produced clear evidence of its effects but clinical studies that can define its role in the treatment of diseases are necessary. Pharmaceutical companies should accelerate their attempts to formulate, design, and produce H_2S-releasing drugs with sustained/controlled release functions as they are likely to be highly innovative for the treatment of neurodegenerative diseases. Thus, therapeutic prospects of H_2S signaling for patients with neurodegenerative disease and demyelination disorders are imminent. However, the uncontrollable release, unidentified byproducts, and unclear mechanisms of release and action represent major issues related to the research and clinical use of H_2S. Although it is currently a time of great interest and excitement regarding the potential of H_2S in medicine and biology, many hypotheses regarding H_2S have yet to be supported and many issues remain unanswered. Even though various novel molecular targets have been identified and research is hastily expanding, biomedical research into this gasotransmitter is still in its initial stages.

Competing Interests

The authors reported no potential conflict of interests.

Authors' Contributions

Sandesh Panthi and Hyung-Joo Chung contributed equally to this work.

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Research Article

Hydrogen Sulfide Mitigates Kidney Injury in High Fat Diet-Induced Obese Mice

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Obesity is prevalent worldwide and is a major risk factor for the development and progression of kidney disease. Hydrogen sulfide (H_2S) plays an important role in renal physiological and pathophysiological processes. However, whether H_2S is able to mitigate kidney injury induced by obesity in mice remains unclear. In this study, we demonstrated that H_2S significantly reduced the accumulation of lipids in the kidneys of high fat diet- (HFD-) induced obese mice. The results of hematoxylin and eosin, periodic acid-Schiff, and Masson's trichrome staining showed that H_2S ameliorated the kidney structure, decreased the extent of interstitial injury, and reduced the degree of kidney fibrosis in HFD-induced obese mice. We found that H_2S decreased the expression levels of tumor necrosis factor-α, interleukin- (IL-) 6, and monocyte chemoattractant protein-1 but increased the expression level of IL-10. Furthermore, H_2S treatment decreased the protein expression of p50, p65, and p-p65 in the kidney of HFD-induced obese mice. In conclusion, H_2S is able to mitigate renal injury in HFD-induced obese mice through the reduction of kidney inflammation by downregulating the expression of nuclear factor-kappa B. H_2S or its releasing compounds may serve as a potential therapeutic molecule for obesity-induced kidney injury.

1. Introduction

Obesity, well known as a major public health issue worldwide, has been reported to be associated with many diseases, such as hypertension and type 2 diabetes mellitus [1–3]. Accumulating evidence suggests that obesity is also a major risk factor for the development and progression of chronic kidney disease (CKD) [3–5]. Along with the development of obesity, fast body weight gain increases tubular sodium reabsorption in the kidney, which in turn leads to renal vasodilation and glomerular hyperfiltration and eventually results in an increased glomerular filtration rate. Excess body weight gain may raise blood pressure, which can lead to increased renal blood flow. Consequently, these hemodynamic changes cause an increase of glomerular size accompanied by mesangial matrix expansion and podocyte injury [6–8]. Both hemodynamic and morphological changes, together with other factors, such as renal inflammation [9, 10], oxidative stress [9, 11], lipotoxicity [12, 13], insulin resistance [14], and fibrosis [15, 16], may result in renal dysfunction and ultimately lead to end-stage renal disease (ESRD).

Hydrogen sulfide (H_2S) is an endogenous gaseous signaling molecule in mammalian tissues, which is enzymatically synthesized by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) [17–19]. All three enzymes are abundantly expressed in the kidney and are involved in renal H_2S production [18, 20]. Accumulating evidence supports the roles of H_2S in renal physiology and pathology [18–21]. Under physiological conditions, basal renal H_2S regulates tubular functions and renal hemodynamics, including changes of renal blood flow, natriuresis, kaliuresis, glomerular filtration rate, and urinary excretion [18, 19, 22]. H_2S can also act as an oxygen sensor in the renal medulla, where its accumulation in hypoxic conditions could restore oxygen balance by increasing medullary blood flow, reducing energy requirements for tubular transport, and directly inhibiting mitochondrial respiration [23, 24]. Because medullary hypoxia is a common pathologic

feature of CKD, H_2S deficiency may contribute to progression of CKD by limiting the important adaptive mechanism [23, 25].

Under pathological conditions, H_2S plays a renoprotective role in the kidney. For instance, treatment with NaHS (a donor of H_2S) could effectively attenuate ischemia/reperfusion-induced acute kidney injury by accelerating tubular cell proliferation and reducing superoxide formation and lipid peroxidation [26]. Importantly, the significant reduction of plasma H_2S concentration and the downregulation of H_2S-producing enzymes have been found in a rodent model of kidney injury and ESRD patients [23, 27]. H_2S supplementation has been shown to reduce macrophage infiltration, glomerulosclerosis, and interstitial fibrosis as well as inhibit the blunt upregulation of adhesion molecules and inflammatory mediators [28]. Plasma H_2S levels are also reduced in overweight subjects and patients with type 2 diabetes [29]. In obese mice, the biosynthesis of H_2S was significantly reduced in the kidney after 16 weeks of feeding with a high fat diet [30]. Given the potent antioxidative, cytoprotective, and anti-inflammatory properties of H_2S, we hypothesize that the application of exogenous H_2S may protect against obesity-related kidney damage.

To test our hypothesis, a diet-induced obesity mouse model was employed in the present study. We found that the administration of NaHS could significantly improve kidney structure and function and suppress inflammation in the kidney of HFD-induced obese mice. Thus, H_2S or its releasing compounds may possess therapeutic potential in treating obesity-related kidney disease.

2. Materials and Methods

2.1. Animals. The protocols for animal experiments were reviewed and approved by the Committee of Medical Ethics and Welfare for Experimental Animals of Henan University School of Medicine. Eight-week-old male C57BL/6JNju mice were purchased from the Nanjing Biomedical Research Institute of Nanjing University (Jiangsu, China). Mice were housed in individual ventilated cages in a temperature- and humidity-controlled environment on a 12-hour light/dark cycle with food and water *ad libitum*. The mice were fed either a low fat diet (LFD, 10% kcal as fat, Medicience Ltd., Jiangsu, China) or a high fat diet (HFD, 45% kcal as fat, Medicience Ltd., Jiangsu, China) for a total of 16 weeks. After 12 weeks of feeding, the LFD-fed mice were divided into the LFD group (6 mice) and the LFD + H_2S group (6 mice); the HFD-fed mice were divided into the HFD group (6 mice) and the HFD + H_2S group (6 mice). The mice from LFD and HFD groups received an intraperitoneal (i.p.) injection of saline; the mice from the LFD + H_2S group and HFD + H_2S group received an i.p. injection of NaHS (50 μmol/kg/day, dissolved in saline) for 4 weeks. At the end of experiments, the mice were killed and the plasma was collected. Tissues were rapidly removed from the mice. The tissues were frozen in liquid nitrogen or embedded in FSC 22 frozen section compound (Leica, Buffalo Grove, IL, USA) or immersed in 4% neutral buffered formalin. Frozen tissues and plasma samples were stored at −80°C.

2.2. Histological Analysis. Kidney tissues were fixed in formalin, embedded in paraffin, and cut into 5 μm thick sections. The sections were stained with a hematoxylin and eosin (HE) staining kit (Baibo Biotechnology Co., Ltd., Shandong, China), periodic acid-Schiff (PAS) staining kit (Baso Diagnostics Inc., Guangdong, China), and Masson's trichrome (MT) staining kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's protocols. The PAS score is defined as follows: 0 = no deposits of PAS-positive material, 1 = up to one-third, 2 = one-third to two-thirds, and 3 = more than two-thirds of the glomerular cross section stain positive for PAS. Tubulointerstitial injury scores are defined as follows: 0 = no injury, 1 = less than 25%, 2 = 25–50%, 3 = 50–75%, and 4 = more than 75% [31]. Renal interstitial fibrosis (RIF) was scored from 0 to 3 as follows: 0 = absent (n_0), 1 = less than 25% of the area (n_1), 2 = 25–50% of the area (n_2), and 3 = more than 50% of the area (n_3). The RIF index was calculated according to the following equation: RIF index = $(0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3)/(n_0 + n_1 + n_2 + n_3) \times 100\%$ [32]. All sections were scanned using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.3. Biochemical Analysis. Kidney triglyceride (TG) was measured by enzymatic colorimetric method using a commercial kit according to the manufacturer's protocols (Applygen Technologies Inc., Beijing, China). Serum TG, cystatin C (Cys-C), creatinine (Cre), blood urea nitrogen (BUN), and kidney injury molecule- (KIM-) 1 were measured using a Roche Cobas 8000 automatic biochemical analyzer (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocols. Tumor necrosis factor- (TNF-) α, interleukin- (IL-) 6, monocyte chemoattractant protein- (MCP-) 1, and IL-10 were determined using ELISA kits (Wuhan Elabscience Biotechnology Co., Ltd., Hubei, China) according to the manufacturer's protocols.

2.4. RNA Extraction and RT-PCR. Total RNA was isolated from kidney tissues using TRIzol reagent (Life Technologies, Rockville, MD, USA), treated with DNase I (Roche Applied Science, Mannheim, Germany), and purified using RNA clean-up kit (Cwbiotech, Beijing, China). One microgram of total RNA was applied for cDNA synthesis using a cDNA reverse transcription kit (Cwbiotech, Beijing, China). Primers were designed according to the primer design principles with Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA): TNF-α, forward 5′-GACGTGGAACTGGCAGAAGAG-3′ and reverse 5′-TTGGTGGTTTGTGAGTGTGAG-3′; IL-6, forward 5′-TAGTCCTTCCTACCCCAATTTCC-3′ and reverse 5′-TAAGACAATGGATCGGTCTAC-3′; MCP-1, forward 5′-CAGCCAGATGCAGTTAACGC-3′ and reverse 5′-GCCTACTCATTGGGATCATCTTG-3′; IL-10, forward 5′-GCTCTTACTGACTGGCATGAG-3′ and reverse 5′-CGCAGCTCTAGGAGCATGTG-3′; and 18S rRNA, forward 5′-AGAGTCGGCATCGTTTATGGTC-3′ and reverse 5′-CGAAAGCATTTGCCAAGAAT-3′. The PCR reactions were performed in a total volume of 20 μL using the following thermal cycling parameters: 95°C for

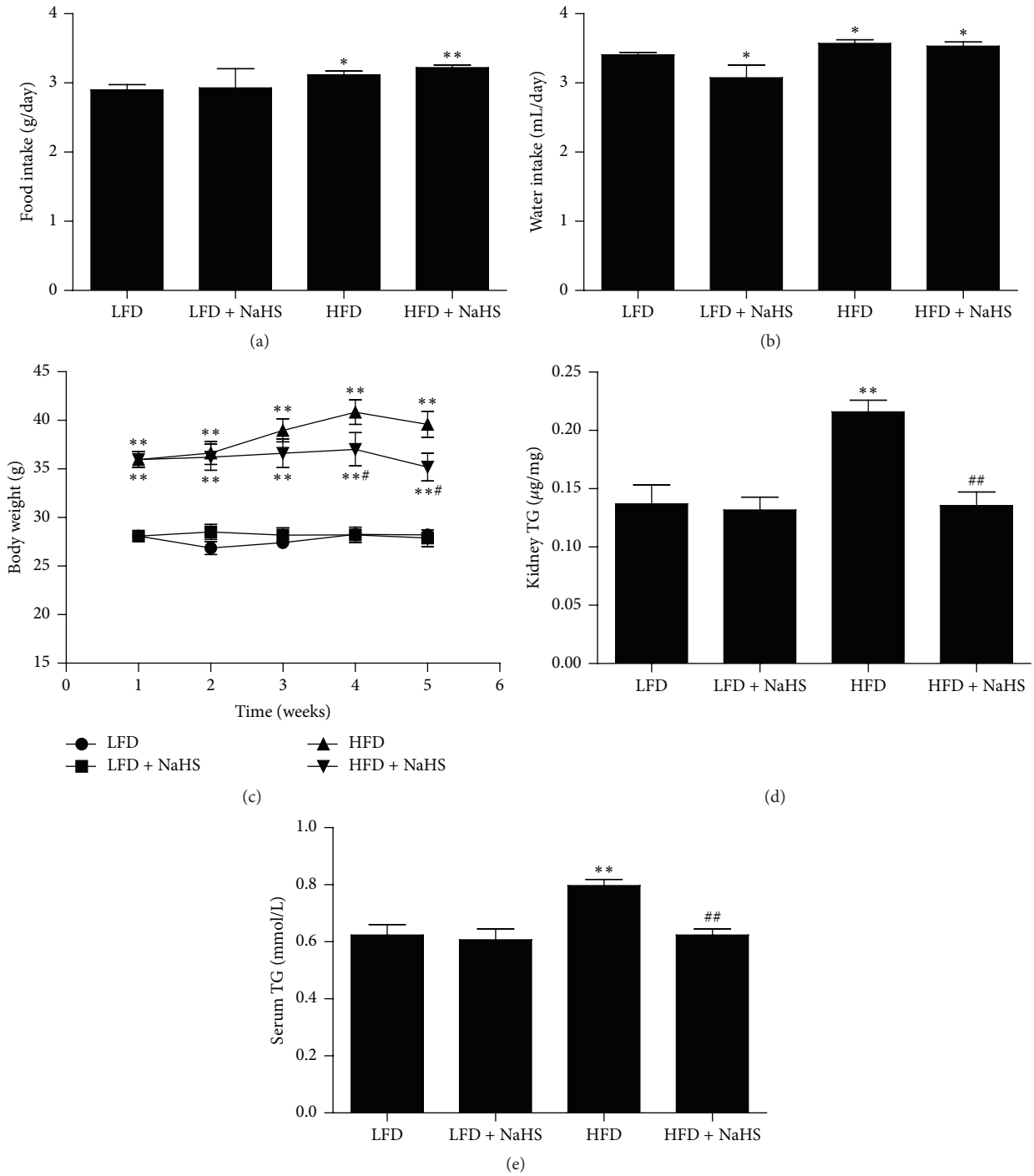

FIGURE 1: Effect of H_2S on the body weight and TG levels of mice. ((a), (b)) Food intake and water intake were calculated. (c) The body weight of mice. (d) Kidney TG levels of mice. (e) Serum TG levels of mice. Values were presented as mean ± SEM ($n = 6$); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the LFD group; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with the HFD group.

1 min, 40 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min. The mRNA expression levels of the test genes were normalized to 18S rRNA levels.

2.5. Western Blot Analysis. Kidney tissues were homogenized in RIPA buffer (Sigma Chemical, St. Louis, MO, USA). Protein concentrations of kidney homogenates were measured with the BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The extracted proteins (40 μg) were separated on SDS-PAGE gel and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated with anti-TNF-α antibody (Beyotime Institute of Biotechnology, Shanghai, China), antinuclear factor-kappa B (NF-κB), p65 antibody (Wuhan Boster Biotech Co., Ltd.,

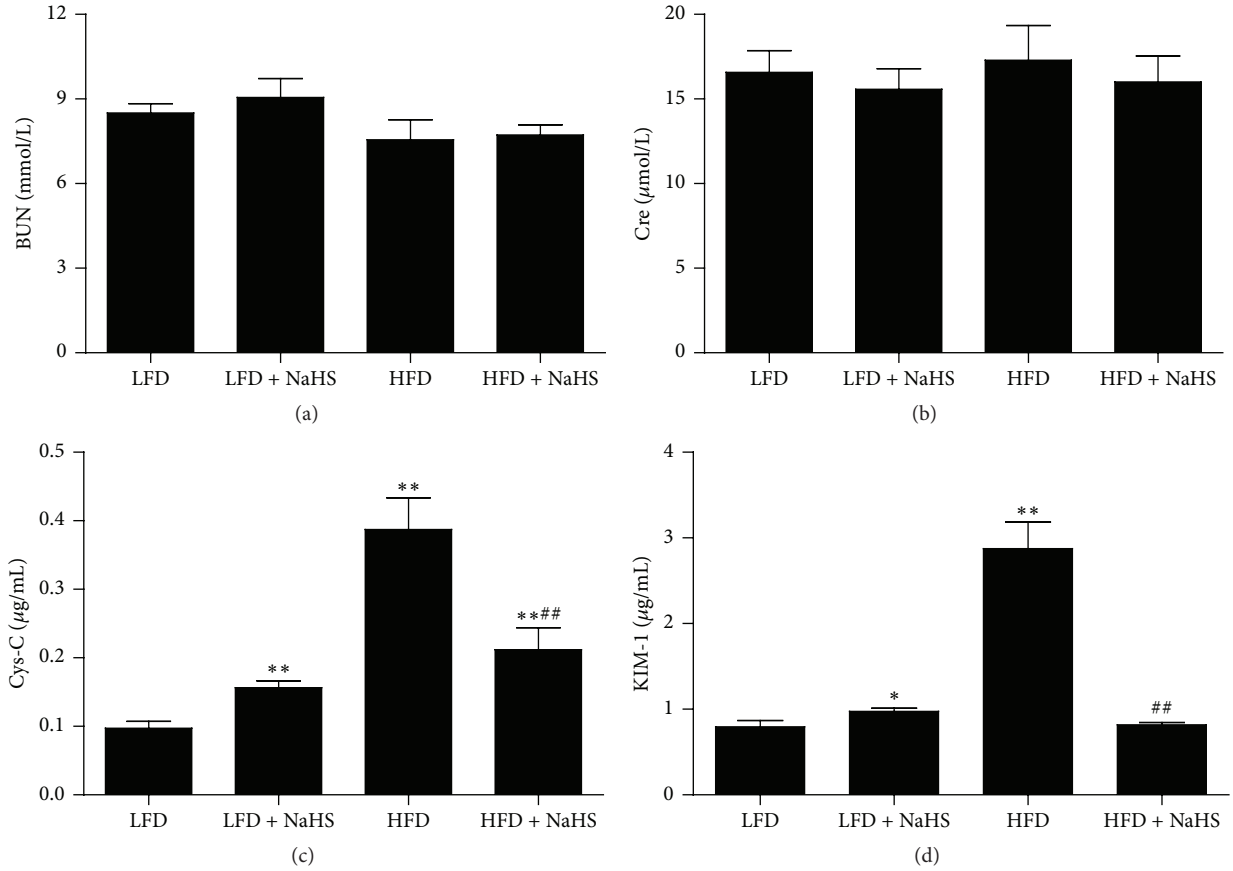

FIGURE 2: Effect of H_2S on the kidney function of mice. The serum levels of BUN (a), Cre (b), Cys-C (c), and KIM-1 (d) were analyzed in each group of mice. Values were presented as mean ± SEM ($n = 6$); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the LFD group; $^{\#\#}P < 0.01$ compared with the HFD group.

Hubei, China), anti-NF-κB p50 antibody (Wuhan Boster Biotech Co., Ltd., Hubei, China), anti-phospho-NF-κB p65 (Ser536) antibody (Beyotime Institute of Biotechnology, Shanghai, China), and anti-β-actin antibody (Proteintech, Hubei, China). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Beyotime Institute of Biotechnology, Shanghai, China). The reaction was visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific, Rockford, IL, USA). Immunoblots were quantified by densitometry using Quantity One software (Bio-Rad, CA, USA).

2.6. Statistical Analysis. Data were presented as the means ± standard error of the mean (SEM). Differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 17.0 software, followed by Tukey's test. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. H_2S Reduces Body Weight and TG Levels in HFD-Induced Obese Mice. As shown in Figures 1(a)–1(c), in comparison with mice fed with LFD, HFD-fed mice exhibited increased food intake, water intake, and body weight. H_2S treatment significantly decreased the body weight and TG levels of mice fed HFD, whereas H_2S treatment did not change the body weight and TG levels of mice fed LFD (Figures 1(c)–1(e)).

3.2. H_2S Treatment Improves the Kidney Function in HFD-Induced Obese Mice. In clinical practice, serum BUN and Cre are the most frequently used markers of kidney function [33, 34]. Cys-C and KIM-1 are considered sensitive and specific biomarkers in early kidney injury [33]. To investigate the effects of HFD and NaHS treatment on kidney function, the plasma levels of BUN, Cre, Cys-C, and KIM-1 in these mice were analyzed using biochemical methods. The results showed that there were no significant differences in plasma BUN and Cre levels between the LFD and LFD + NaHS group (Figures 2(a) and 2(b)). After 12 weeks of HFD feeding followed by 4 weeks of H_2S treatment, the levels of plasma BUN and Cre were not changed, suggesting that HFD did not cause end-stage kidney injury. However, HFD induced 3- to 4-fold increases in plasma Cys-C and KIM-1 levels (Figures 2(c) and 2(d)), indicating that the kidney injury induced by HFD was in the early stage. After 4 weeks of H_2S treatment, the plasma Cys-C and KIM-1 levels were significantly reduced, indicating that H_2S has a therapeutic effect on early kidney injury induced by HFD.

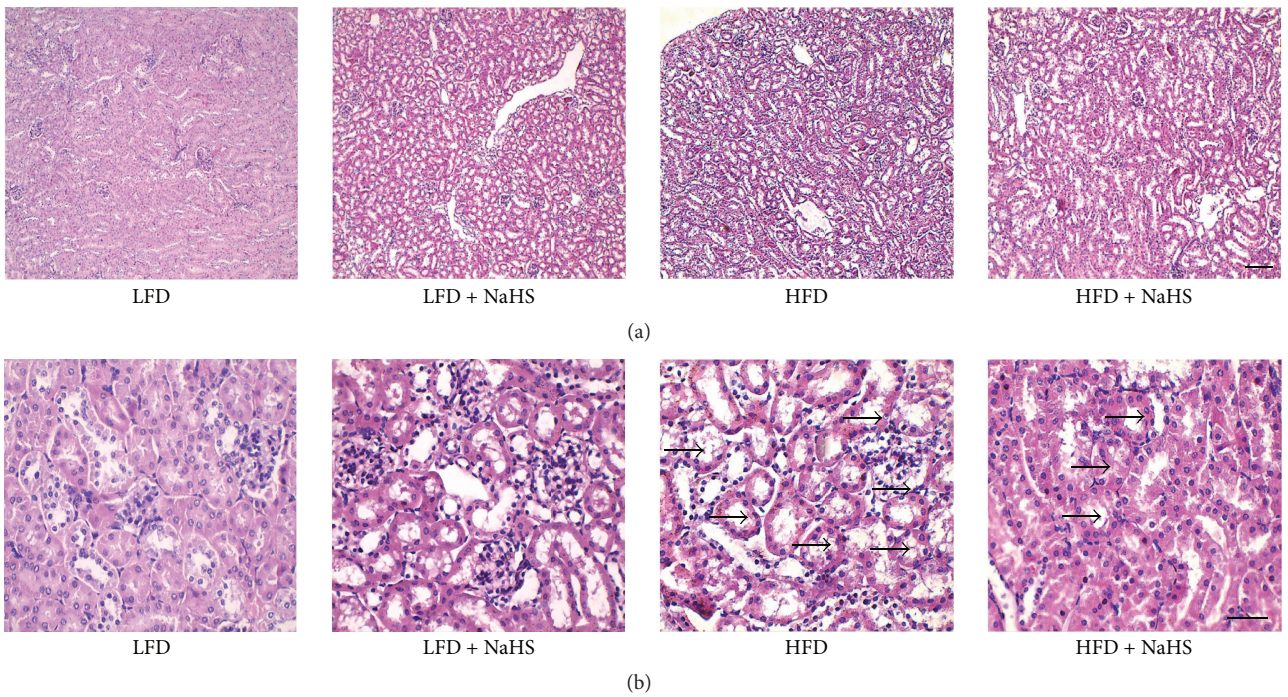

Figure 3: Evaluation of the kidney structure of mice using HE staining. Representative photographs of HE-stained kidney sections in each group of mice (n = 6/group). (a) Original magnification, ×100; scale bar = 200 μm. (b) Original magnification, ×400; scale bar = 20 μm.

3.3. H_2S Ameliorates Kidney Structure in HFD-Induced Obese Mice. To investigate the effect of H_2S on kidney structure in HFD-induced obese mice, HE, PAS, and MT staining were performed. HE staining demonstrated that the kidney of the LFD control group showed clear tubular and glomerular structures and the tubular endothelial cells presented a good arrangement (Figures 3(a) and 3(b)). Upon HFD, the glomerular volume was increased, glomerular structure was unclear, Bowman's capsule was reduced, and sclerosis was observed in the glomerulus. The tubular endothelial cells swelled and became vacuoles, part of the tubular cavity expanded (Figures 3(a) and 3(b)). After NaHS treatment, the structure of the kidney was significantly improved, the glomerular volume was reduced, Bowman's cavity and the structure of the glomerulus became clear, and the tubular endothelial cells were arranged favorably (Figures 3(a) and 3(b)).

3.4. H_2S Decreases the Carbohydrate Content and Interstitial Injury in HFD-Induced Obese Mice. PAS staining can be used to stain structures containing a high proportion of carbohydrate macromolecules, such as glycoprotein, glycogen, and proteoglycans [35]. Compared with the LFD, HFD increased the amount of PAS-positive staining in both glomerular and tubular structures (Figures 4(a)–4(c)), suggesting that the carbohydrate content was increased in the kidney of mice fed HFD. After NaHS treatment, the PAS-positive staining in both glomerular and tubular structures was reduced, demonstrating that NaHS treatment could effectively decrease the carbohydrate content in HFD-induced obese mice. Furthermore, as shown in Figure 4(d), HFD increased the interstitial injury, while NaHS treatment significantly decreased the interstitial injury.

3.5. H_2S Ameliorates the Fibrosis of the Kidney in HFD-Induced Obese Mice. Masson's trichrome is a three-color staining protocol used in histology, which produces red fibers, blue collagen, pink cytoplasm, and dark brown nuclei [32]. In the LFD control group, kidney tubular endothelial cells presented fullness of pink cytoplasm. By HFD, the amounts of blue collagen staining and red fiber staining were increased in kidney glomerular basement membrane and tubular interstitial area (Figure 5(a)), suggesting the accumulation of collagen and connective tissue fibers in the kidney. After NaHS treatment, the amounts of both blue collagen staining and red fiber staining were reduced (Figure 5(a)), and the RIF index was decreased (Figure 5(b)), indicating that H_2S could play a role in ameliorating the fibrosis of kidney.

3.6. Treatment of NaHS Reduces Kidney Inflammation in HFD-Induced Obese Mice. Our results demonstrated that HFD induced injuries in kidney structure and function, and the injuries could be ameliorated by the administration of NaHS. To investigate the underlying mechanism, we examined kidney inflammation by analyzing the cytokine levels in the kidney using ELISA techniques. Compared with the LFD, HFD increased the expression levels of TNF-α, IL-6, and MCP-1 in the kidney (Figures 6(a)–6(c)). Treatment with NaHS significantly decreased the levels of TNF-α, IL-6, and MCP-1 (Figures 6(a)–6(c)), whereas it increased the IL-10 level (Figure 6(d)). The expressions of cytokine genes were further detected by RT-PCR. As shown in Figures 7(a)–7(d),

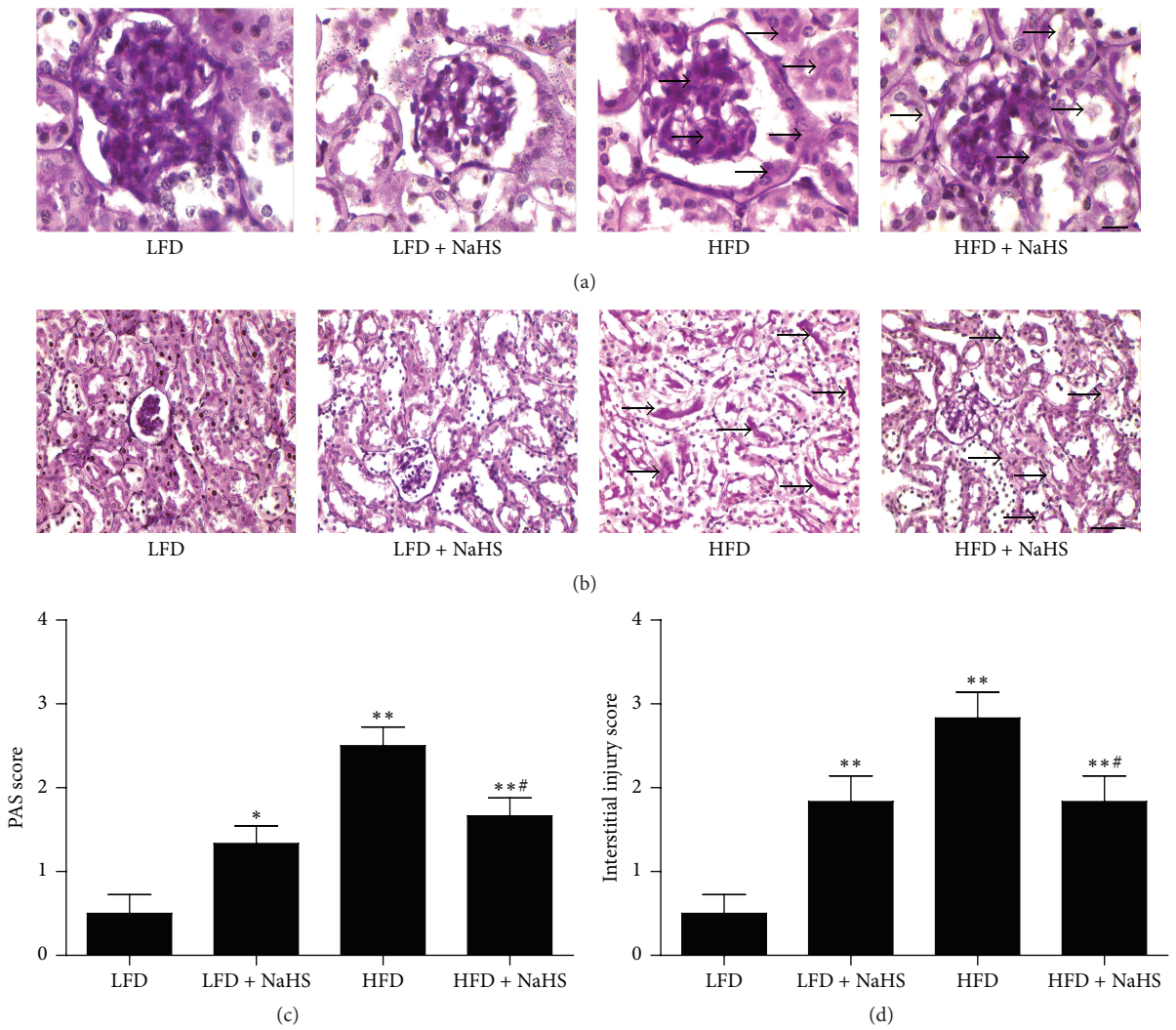

Figure 4: Evaluation of carbohydrate content in the kidney of mice using PAS staining. Representative photographs of PAS-stained glomerulus ((a) original magnification, ×1000; scale bar = 5 μm) and renal tubular ((b) original magnification, ×400; scale bar = 20 μm). Quantitative analysis of PAS-positive deposits (c) and interstitial injury (d) in each group of mice. Values were presented as mean ± SEM ($n = 6$); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the LFD group; $^{\#}P < 0.05$ compared with the HFD group.

compared with the LFD, HFD increased the gene expressions of TNF-α, IL-6, and MCP-1 in the kidney. Treatment of NaHS significantly decreased kidney TNF-α, IL-6, and MCP-1 gene expressions (Figures 7(a)–7(d)), whereas it increased kidney IL-10 expression (Figure 7(e)). These results indicated that H_2S could reduce the kidney inflammation in HFD-induced obese mice.

3.7. H_2S Reduces the Expression of NF-κB in the Kidney of Obese Mice. To investigate the mechanisms of NaHS on cytokine regulation, the expression level of NF-κB was detected. NF-κB is a transcription factor that plays a key role in the regulation of the expression of multiple cytokine genes involved in a variety of physiological processes [36]. The p50/p65 heterodimer is the most abundant and well understood of the NF-κB dimers in most cells [37, 38]. We examined the protein expressions of p50, p65, and p-p65 in the kidneys of mice. As shown in Figures 8(a)–8(e), compared with the LFD, HFD increased kidney p50, p65, and p-p65 protein expressions and the p-p65/p65 ratio. Treatment with NaHS significantly decreased the protein expressions of p50, p65, and p-p65, as well as the ratio of p-p65/p65 in the kidney (Figures 8(a)–8(e)). The results indicated that HFD increased kidney inflammation through the upregulation of NF-κB expression, and treatment with NaHS effectively reduced kidney inflammation by downregulating NF-κB expression.

4. Discussion

H_2S has been widely considered to be an endogenous gaseous signaling molecule, along with carbon monoxide and nitric oxide [17, 20, 21, 39]. Recently, an increasing number of studies suggest that H_2S plays important and complex roles in renal physiology and pathophysiology, including the regulation of baseline hemodynamics and tubular properties [18, 19, 22], action as an oxygen sensor/transducer [28, 40],

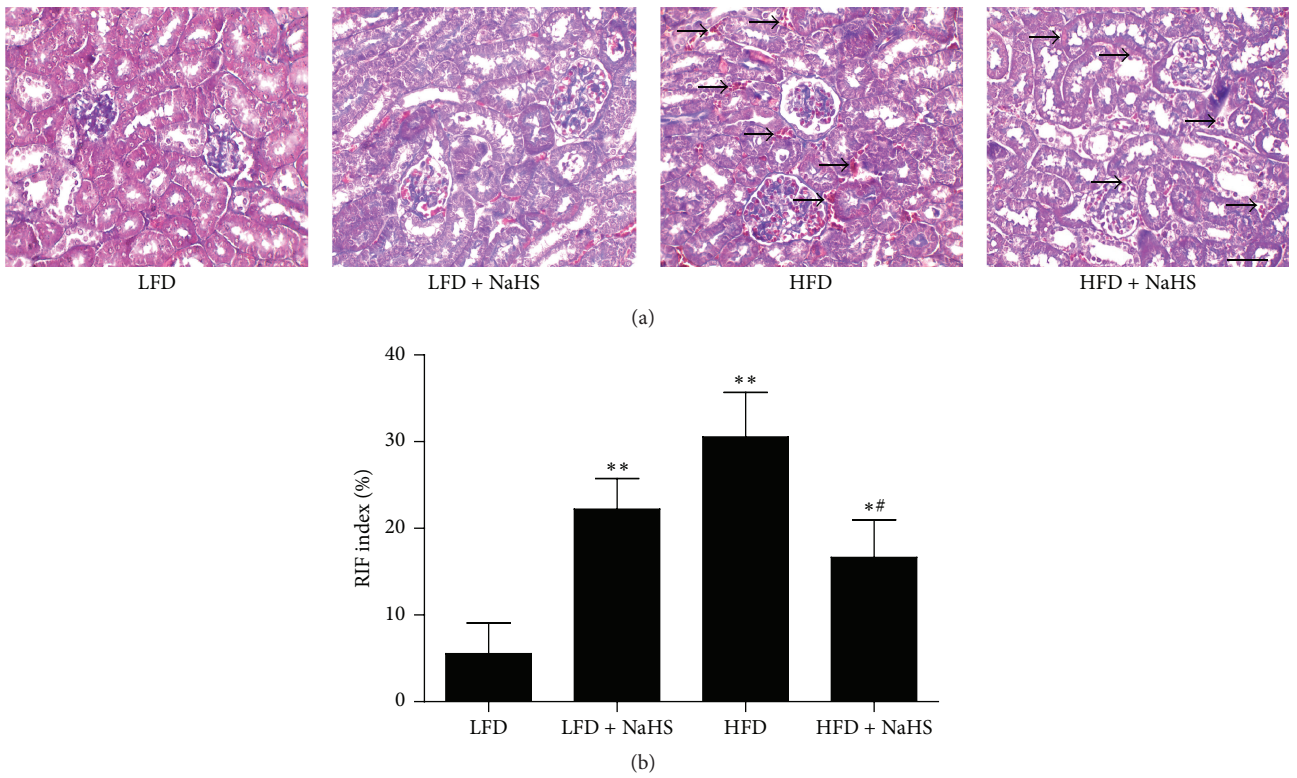

Figure 5: Evaluation of renal collagen deposition using MT staining (blue: collagen, pink: cytoplasm, and brown-black: nuclei). (a) Representative samples of MT staining (original magnification, ×400; scale bar = 20 μm) in each group of mice. (b) The extent of the renal lesions is represented by the RIF index. Values were presented as mean ± SEM ($n = 6$); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the LFD group; $^{\#}P < 0.05$ compared with the HFD group.

protection against renal I/R injury [21, 26, 41], attenuation of renal fibrosis [18, 42], and protection against glomerulosclerosis [28, 43]. Recent studies indicate that the H_2S level was significantly reduced in overweight patients with type 2 diabetes and HFD-induced obese mice [29, 30]. In addition, there is increasing evidence that the obesity epidemic has coincided with an increased incidence of CKD [3, 8]. Therefore, we speculate that H_2S deficiency may potentially contribute to the progression of CKD and the administration of exogenous H_2S could mitigate CKD induced by obesity.

In the current study, we demonstrated that H_2S significantly reduced the body weight and kidney TG levels of mice fed with HFD, suggesting that H_2S plays an important role in lipid metabolism. However, the precise molecular mechanisms behind the role of H_2S in the regulation of lipid metabolism need to be further investigated. A recent study showed that intrarenal arterial infusion of NaHS increased glomerular filtration rate, renal blood flow, urinary sodium, and potassium excretion, indicating that H_2S is involved in the control of renal function [22]. Our data indicated that the kidney injury induced by HFD was in the early stage and H_2S could maintain normal renal function by reducing the plasma levels of specific biomarkers in early kidney injury, such as Cys-C and KIM-1.

HE staining is the most common method used in the anatomic pathology diagnosis [44]. Our results showed that HFD increased the glomerular volume, reduced the Bowman's capsule, and resulted in glomerulosclerosis, suggesting that HFD could successfully induce renal injury. After treatment with H_2S, the structure of the kidney was significantly improved in HFD-induced obese mice. It has been reported that the glomerular tuft area and the mesangial matrix area in mice fed with HFD tended to be larger than that in mice fed with LFD [45]. Similarly, we found that HFD increased the amount of PAS-positive staining in both glomerular and tubular structures, while H_2S significantly reduced the PAS-positive staining in the kidney. Furthermore, H_2S could decrease the interstitial injury in HFD-induced obese mice. A recent study revealed that H_2S exhibited potent antifibrotic effects on obstructed nephropathy and inhibited the proliferation and differentiation of renal fibroblasts both *in vitro* and *in vivo* [18]. Our results indicated that HFD increased the red fiber staining in the glomerular basement membrane and tubular interstitial area and H_2S effectively reduced the degree of renal fibrosis.

Obesity-related nephropathy is associated with regenerative cell proliferation, monocyte infiltration, and increased renal expressions of systemic proinflammatory TNF-α, IL-6, and MCP-1 [1, 9]. Similarly, our data indicated that HFD increased the expression levels of TNF-α, IL-6, and MCP-1 in the kidney. The administration of NaHS significantly decreased the expression levels of these

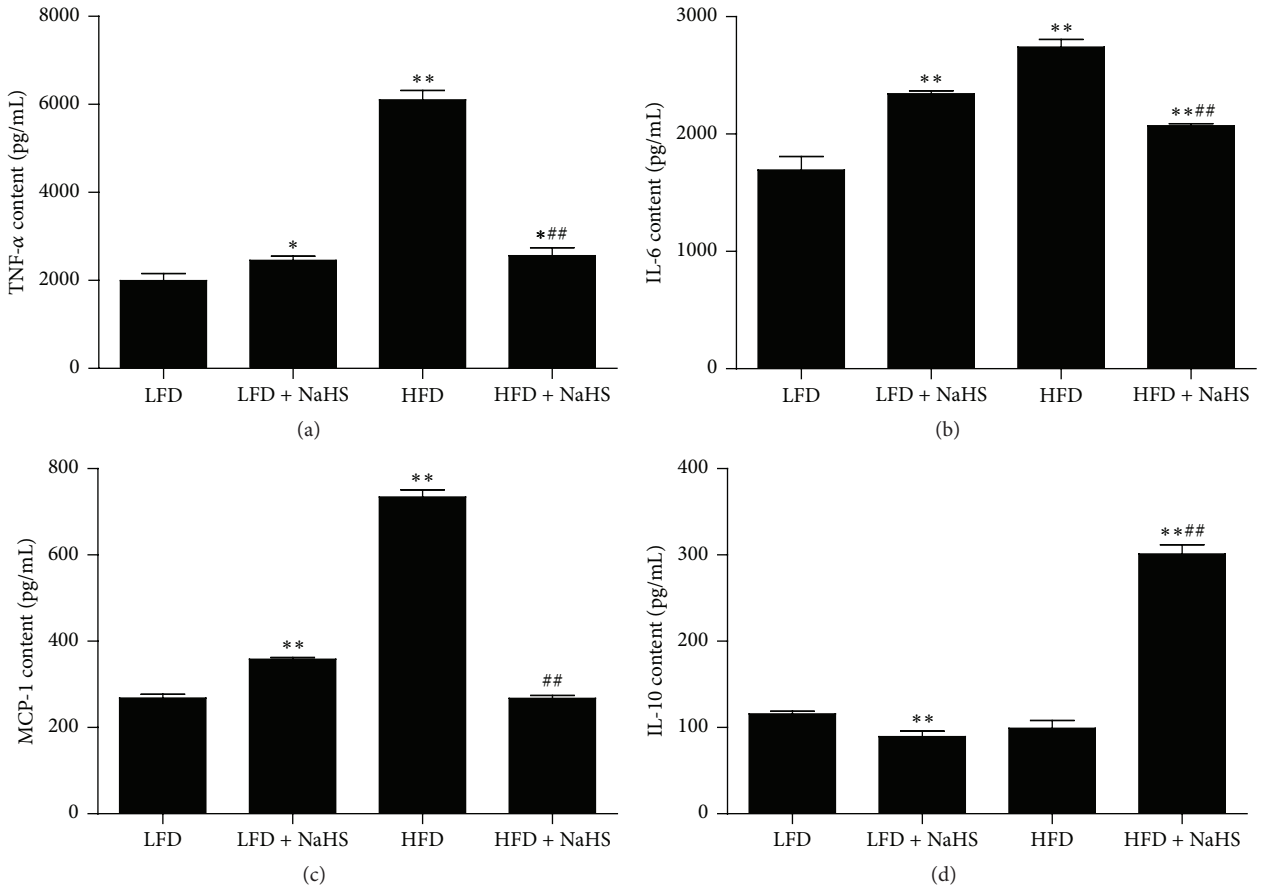

FIGURE 6: Effect of H_2S on the cytokine levels in the kidney of mice was assayed using ELISA techniques. The expression levels of TNF-α (a), IL-6 (b), MCP-1 (c), and IL-10 (d) were analyzed. Values were presented as mean ± SEM ($n = 6$); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the LFD group; $^{\#\#}P < 0.01$ compared with the HFD group.

proinflammatory cytokines. IL-10 is a Th_2-type cytokine that is produced by a number of immunological cell types, including macrophages/monocytes, and it is a potent inhibitor of proinflammatory cytokines and chemokines [46, 47]. Notably, we found increased expression levels of IL-10. Furthermore, the gene expression of TNF-α, IL-6, MCP-1, and IL-10 exhibited similar changes. Thus, when the TNF-α/IL-6/MCP-1 results were contrasted with the IL-10 results, it appeared that the relative balance between proinflammatory and anti-inflammatory was "tipped" toward a proinflammatory state. The proinflammatory state played a mechanistic role in the progression of kidney injury. In contrast, treatment with H_2S resulted in an anti-inflammatory state, which played an inhibitory role in the progression of kidney injury. However, it should also be noted that the administration of H_2S increased the expression levels of TNF-α, IL-6, and MCP-1 and decreased the expression levels of IL-10 in LFD-fed mice. Furthermore, H_2S increased the plasma levels of Cys-C and KIM-1 and enhanced the interstitial injury and fibrosis of the kidney in LFD-fed mice, which may be attributed to the opposite effect of H_2S on inflammation [20, 48, 49]. Based on these studies, we hypothesize that H_2S is able to exhibit an anti-inflammatory effect in HFD-fed mice and exert a proinflammatory effect in LFD-fed mice.

The NF-κB network plays a crucial role in human health, and aberrant NF-κB activation contributes to the development of a wide range of autoimmune, inflammatory, and malignant disorders, including atherosclerosis, multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, and malignant tumors [36, 38, 50]. The NF-κB family comprises five major members: c-Rel, p50, p52, p65 (Rel A), and Rel B [36, 38]. The p50/p65 heterodimer is the most important transcription factor of the canonical NF-κB pathway and is commonly referred to as NF-κB [38, 51]. A recent study demonstrated that high body adiposity could induce an inflammatory and proliferative microenvironment in the rat kidney [1]. Similarly, our data showed that HFD increased kidney p50, p65, and p-p65 protein expressions and the p-p65/p65 ratio, suggesting that HFD induced an inflammatory microenvironment in the kidneys of mice. However, the administration of NaHS significantly decreased the protein expressions of p50, p65, and p-p65, as well as the ratio of p-p65/p65 in the kidney. These results indicate that H_2S could reduce kidney inflammation by downregulating NF-κB expression.

In conclusion, our results suggest that H_2S is able to reduce kidney lipids, improve kidney function, and reduce the interstitial injury and fibrosis of the kidney through the

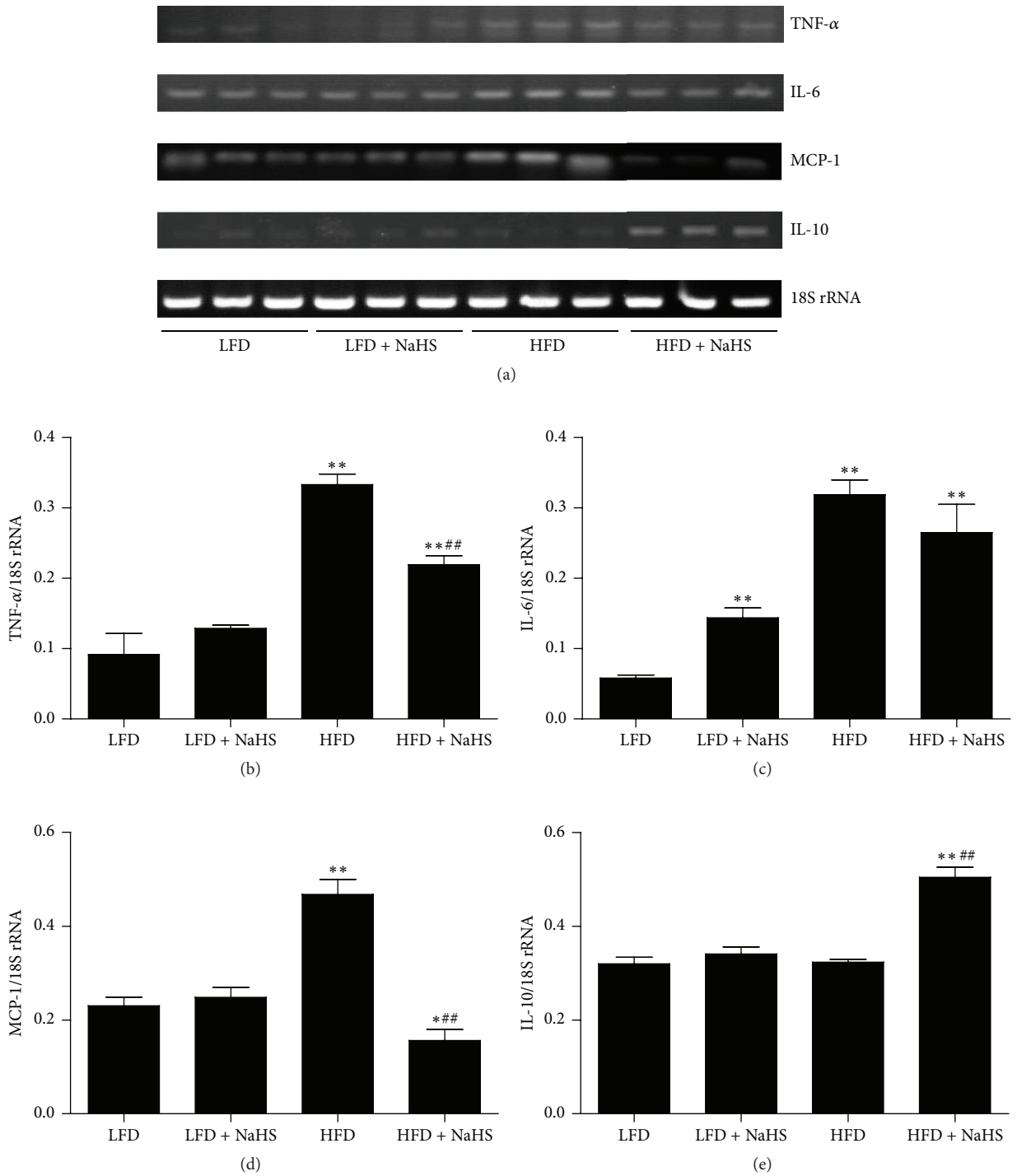

Figure 7: Effect of H_2S on the cytokine levels in the kidney of mice was assayed using RT-PCR techniques. (a) The expression levels of TNF-α, IL-6, MCP-1, and IL-10 were measured by RT-PCR. 18S rRNA was used as an internal control. Bar graphs showed the quantification of TNF-α (b), IL-6 (c), MCP-1 (d), and IL-10 (e). Values were presented as mean ± SEM ($n = 3$); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the LFD group; $^{\#\#}P < 0.01$ compared with the HFD group.

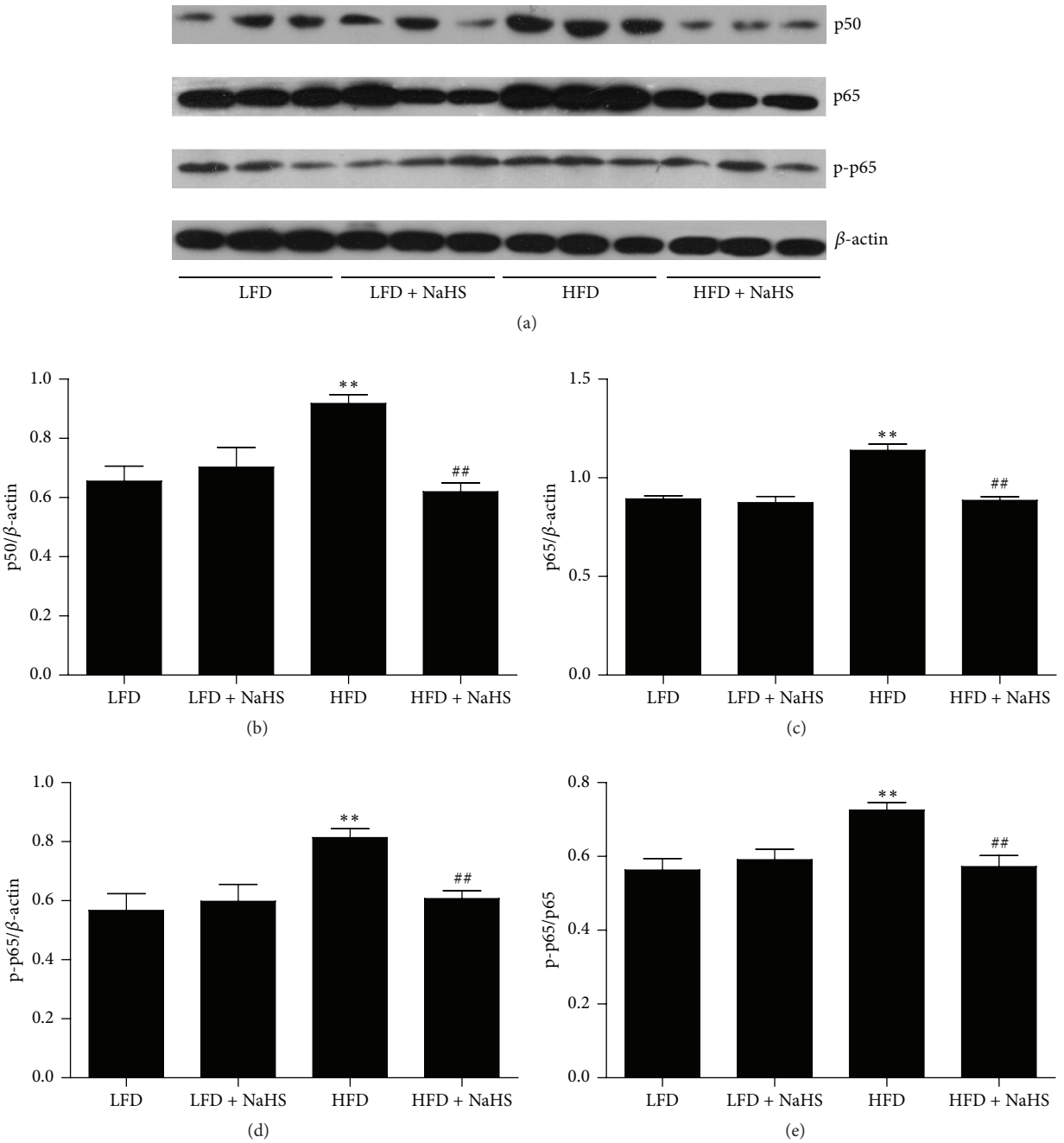

Figure 8: Effect of H_2S on the protein level of NF-κB in the kidney of mice. (a) The expression levels of p50, p65, and p-p65 were measured by Western blot. β-actin was used as an internal control. Bar graphs showed the quantification of p50 (b), p65 (c), p-p65 (d), and p-p65/p65 (e). Values were presented as mean ± SEM ($n = 3$); $^{**}P < 0.01$ compared with the LFD group; $^{\#\#}P < 0.01$ compared with the HFD group.

reduction of kidney inflammation by downregulating NF-κB expression. Therefore, H_2S or its releasing compounds may serve as a potential therapeutic molecule for obesity-induced kidney injury.

Competing Interests

The authors declare that they have no competing interests related to this work.

Authors' Contributions

Dongdong Wu and Biao Gao contributed equally to this work.

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