Hydrogen Sulphide and Nitric Oxide Cooperate in Cardioprotection Against Ischemia/Reperfusion Injury in Isolated Rat Heart

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**Abstract.** Background/Aim: This study was designed to provide further evidence for the interactions between hydrogen sulfide (H2S) and nitric oxide (NO) in ischemia/reperfusion (I/R) injury. Materials and Methods: Rat hearts were studied with the Langendorff technique using the H2S donor sodium hydrosulfide (NaHS, 40 μM) and the cystathionine gamma-lyase (CTH or CSE) inhibitor DL-propargylglycine (PAG, 1 mM). NO synthase inhibitor L-NG-nitroarginine methyl ester (L-NAME, 30 mg/kg, 7 days) was administered before the isolation. The hearts were homogenized for biochemical and molecular analysis. Results: NaHS reversed I/R-induced cardiac performance impairment, increased tissue nitric oxide production and decreased tissue markers for cardiac injury, while L-NAME inhibited these effects. The expression of CTH was increased with PAG, which was suppressed by L-NAME. Conclusion: H2S and NO increase each other’s production suggesting their interaction and cooperation in cardioprotection against I/R injury.

Nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H2S), in the order of their discovery, are gasotransmitters, a term that refers to a gaseous transmitter, and was first coined by Wang (1). All are endogenously produced small signaling molecules with low molecular weight (NO, 30 Da; CO, 28 Da; H2S, 34 Da). Because they are small gaseous molecules, they reach easily their intracellular targets to activate them, by diffusing freely across the plasma membrane. They play a pivotal role in the control of many physiological functions, including regulation of cardiovascular, nervous, gastrointestinal, excretory, immune, and reproductive systems (2-5). Of these three gaseous transmitters, H2S that was first introduced as a metabolic product in mammals by the American biochemist Vincent Du Vigneaud, has gained much attention in recent years due to its involvement in the above-mentioned physiological functions (2, 6, 7). It is endogenously synthesized in most mammalian tissues from L-cysteine and/or L-homocysteine by cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CTH or CSE), and cysteine aminotransferase together with 3-mercaptopropionate sulfurtransferase (2, 4, 8).

Heart failure, the major health issue in the world and the leading cause of deaths, is a complicated disease caused by
a variety of common stresses to the heart, such as hypertension, diabetes, and myocardial infarction that is the result of ischemic heart disease (9, 10). Therefore, novel complementary compounds that are safe and effective alternatives to conventional pharmacotherapy of heart failure are needed. In recent years, a considerable number of studies have revealed that H2S plays important roles in alleviating ischemia/reperfusion (I/R) injury (11), and that plasma sulfur concentration is inversely proportional to the severity of congestive heart failure (12). In addition, exogenous administration of H2S or cardiac-specific CTH overexpression provides protection against acute myocardial I/R injury and heart failure (10, 12, 13).

Since 1997, when the first experimental study by Hosoki, et al. (14) revealed that endogenous H2S may regulate smooth muscle tone in synergy with NO, many studies have provided strong and growing evidence that these two molecules could modulate each other’s activities by altering the functions of the related proteins (15-17). Kondo, et al. (18) has shown that H2S protected against heart failure via up-regulation of endothelial nitric oxide synthase (eNOS) activity, while a new thiol sensitive molecule resulted from the reaction of H2S with NO was found to regulate heart function (19). However, the precise mechanisms of interactions between NO and H2S that affect heart failure, and whether H2S modulates the biological effects of NO are not entirely clear (8). Therefore, it is urgently needed to deeply understand the underlying mechanisms, so that novel strategies can be developed to provide protection against heart failure (10, 17).

In view of these facts, the present study aimed to provide further evidence for the effects of H2S and NO, and their interactions in I/R injury by employing the Langendorff technique of isolated rat heart perfusion.

Materials and Methods

Animals. Forty-eight male Wistar albino rats weighing 250-300 g were used. They were housed under 12/12 h day/night cycle and controlled room temperature (22±2°C) and were allowed free access to food and water, and received humane care according to the criteria outlined in the ‘Guide for the Care and Use of Laboratory Animals (2011)’ prepared by the National Academy of Science and published by the National Institutes of Health. Animal experiments were reviewed and approved by the Animal Care and Use Committee of Istanbul University.

Isolated heart perfusion. All Langendorff isolated heart studies were performed as previously described (20). Briefly, animals were anesthetized by intraperitoneal injection of 75 mg/kg pentobarbital sodium (Pental Sodyum, IE Ulaga, Istanbul, Turkey). Tracheotomy was performed, and mechanical ventilation (Small Animal Ventilator Model 683, Harvard Apparatus, Holliston, MA, USA) was initiated soon after surgical opening of the thorax. Heparin (150 IU) was administered from the abdominal vein, and before excision of the heart the aorta was cannulated in situ. The hearts were then Langendorff-perfused at 37°C with Krebs-Henseleit buffer containing (mM) 118 NaCl, 0.5 EDTA, 4.7 KCl, 1.25 CaCl2, 1.2 MgSO4, 25 NaHCO3, 1.2 KH2PO4, and 11 glucose, 1 lactate, 0.5 glutamine, and 0.1 pyruvate, gassed with 95% O2 - 5% CO2. End-diastolic pressure was adjusted at 5-10 mmHg. The hearts were perfused by a mini pulse peristaltic pump (ML172B, ADInstruments, Sydney, Australia) at a constant flow with initial perfusion pressure of approximately 80 mmHg. After stabilization of pressure development during the first 20 min of Langendorff-perfusion, 6 groups of hearts, each composed of 8 animals, were studied (Figure 1). All hearts were subjected to 30 min ischemia and 60 min reperfusion by switching the peristaltic pump off and on. The ischemia/reperfusion (IR) group was just perfused with Krebs-Henseleit solution for 20 min, while the sodium hydrosulphide (NaHS) group was perfused with 40 μM NaHS (as H2S donor), and the DL-propargylglycine (PAG) group with 1 mM PAG (as CTH inhibitor) prior to ischemia. L-NG-Nitroarginine methyl ester (L-NAME), L-NAME+NaHS, and L-NAME+PAG groups additionally received 30 mg/kg L-NAME (as NOS inhibitor) intraperitoneally for 7 days before Langendorff studies.

Cardiodynamic analysis. Left ventricular pressure was recorded by means of a balloon catheter placed inside the left ventricle and connected to a physiological pressure transducer (MLT844, ADInstruments) for assessment of contractile performance, while a second physiological pressure transducer was connected to the system in order to record the perfusion pressure via the data acquisition unit (PowerLab ML870B2, ADInstruments). The obtained data were analyzed with an appropriate software (LabChart 7; ADInstruments), and the records at certain time points [0th min: end of stabilization (baseline), 20th min: end of drug administration before ischemia; 55th min: 5th min of reperfusion, 60th min: 10th min of reperfusion, 110th min: end of experiment] were used for further analyses of cardiac parameters, including end diastolic pressure (EDP), left ventricular developed pressure (LVEDP), Max dP/dt (a specific index used to determine the ability of the heart to contract), and rate pressure product (RPP, an indirect index of myocardial oxygen consumption and cardiac function).

Biochemical analysis. At the end of the experiment, the hearts were homogenized with a teflon piston homogenizer (Sartorius Potter S, Goettingen, Germany) in ice-cold PBS (ph 7.4) in a borosilicate
glassware of 15 ml. The homogenate was centrifuged at 15,000g at 4°C for 20 min.

As tissue markers for cardiac injury, the levels of creatine kinase-MB (CK-MB) (Uscn Life Science Inc., Wuhan, PR China), a marker of myocardial injury, lactate dehydrogenase (LDH) (Elabsience, Wuhan, PR China), a marker of necrosis, and glutathione peroxidase (GPx) (Elabsience), an endogenous antioxidant enzyme, were measured in supernatant by ELISA. The production of NO and H2S was measured with a nitrate/nitrite assay kit (Cayman Chemical, Ann Arbor, MI, USA), and a H2S ELISA kit (Elabsience, respectively).

Gene expression analysis
Homogenization and RNA isolation. Total RNA was isolated from cardiac tissue via TRIzol® Reagent (Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) as described in the manufacturer’s protocol. Briefly, 0.1 g of tissue was thawed, and homogenized in 1 ml TRIzol® Reagent with a benchtop homogenizer (MP Biomedicals LLC, CA, USA). Then, the resulting homogenate was incubated for 5 min at room temperature. After the addition of 200 μl chloroform to each sample, they were incubated for 2-3 min at room temperature, and centrifuged for 15 min 12,000g at 4°C, forming a lower red phenol-chloroform interphase and a colorless upper aqueous phase. The latter containing RNA was transferred to a new tube, and the former containing protein was stored at −20°C for RNA isolation. Precipitation of RNA was performed with the addition of 70% ethanol and incubation for 10 min at room temperature. The quantity and quality of RNA was measured by a spectrophotometer (Nanodrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA).

cDNA synthesis and real-time PCR. Random hexamers (pdN6) (Roche Applied Science, Mannheim, Germany) and M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA, USA) were employed to synthesize cDNA from 1 μg of total RNA. The obtained cDNA samples were kept at −20°C. cNOS, inducible nitric oxide synthase (iNOS), and CTH gene expression analysis was performed using a LightCycler 480 instrument (Roche Applied Science, Penzberg, Upper Bavaria, Germany). Gene-specific primers and probes were designed using Universal ProbeLibrary reference gene assays for mouse or rat (Universal Probe Library Rat-ACTB Gene Assay; Cat. No. 05 046 203 001, Roche Applied Science, Penzberg, Upper Bavaria, Germany) (Table I). The housekeeping gene ACTB (β-actin) was used to standardize quantification in gene expression, by performing two-color real-time PCR. Based on the mathematical model described by Livak et al. (21), double delta Ct analysis was used to calculate relative gene expressions.

Statistical analysis. All data were statistically analyzed with GraphPad Prism 6.0 (GraphPad Prism Software, San Diego, CA, USA), and presented as mean±standard error of mean (mean±SEM). A value of p<0.05 was considered as statistically significant.

Cardiodynamic results were evaluated by two-way analysis of variance (ANOVA), while gene expression and biochemical results were analyzed by one-way ANOVA and followed by post-hoc Bonferroni’s multiple comparison test.

Results
Cardiodynamic results. The end diastolic pressure values were close to each other in all groups before ischemia, which increased them significantly (p<0.001), implying diminished cardiac contractility that is observed in heart failure. Administration of L-NAME, NaHS or PAG induced a statistically significant (p<0.01) decrease in EDP values. Following ischemia, the highest EDP value was determined at the 5th min of reperfusion in the L-NAME+NaHS group, while the lowest value was in the groups of L-NAME and NaHS. At the end of reperfusion, values were approximated to those of the initial point in the groups of L-NAME and NaHS. It was interesting that perfusion with PAG alone reversed this effect and caused an increase in EDP values when compared to perfusion with NaHS alone (Figure 2A). As to values of LVDP, there was a significant decrease only in the NaHS group compared to the IR (p<0.001) and L-NAME (p<0.001) groups. The decrease observed due to ischemia at the 5th min of reperfusion was found to be increased in the later stages of reperfusion in all groups. The highest value was observed in the L-NAME group, whereas the lowest value was in the IR group (Figure 2B). Max dp/dt values were also similar with the LVDP results (Figure 3A). Before ischemia, the initial RPP values of all groups were in parallel with each other except for the NaHS group. Following ischemia, the values in all groups decreased until the first 5th min of reperfusion, and then increased with the progression of reperfusion. The best recovery occurred in L-NAME (p<0.001), NaHS (p<0.01), and L-NAME+PAG (p<0.05) groups in descending order, whereas the lowest recovery was in the IR (p<0.001) and L-NAME+NaHS (p<0.001) groups (Figure 3B).

Biochemical results. CK-MB levels showed a significant increase (p<0.01) in the L-NAME+PAG group compared to the
IR group, while a significant decrease was observed in all other groups \((p<0.001)\) (Figure 4A). Apart from the L-NAME+PAG group \((p<0.001)\), all groups had decreased levels of LDH in comparison with both the IR and L-NAME groups (Figure 4B). NaHS and PAG administration increased the levels of GPx compared to both the IR and L-NAME groups \((p<0.001)\).

In the L-NAME+NaHS group, the levels of this enzyme also increased compared to the IR group, while they significantly decreased in the L-NAME+PAG group (Figure 4C).

The levels of \(\text{H}_2\text{S}\) were increased by NaHS administration in the tissue compared to both the IR and L-NAME groups \((p<0.001)\), but PAG administration caused a decrease \((p<0.001)\). However, \(\text{H}_2\text{S}\) increased in the L-NAME and L-NAME+NaHS groups \((p<0.001\text{ for both})\), whereas it decreased in the L-NAME+PAG group as in the PAG group \((p<0.001)\) (Figure 4D). NaHS administration led to an increase in NO levels \((p<0.001)\), but PAG administration did not cause a significant change. Nitrate/nitrite levels significantly decreased \((p<0.001)\) in all L-NAME-administered groups compared to the IR group. L-NAME+NaHS combination increased nitrate/nitrite levels \((p<0.05)\) compared to the L-NAME group, whereas PAG had no effect (Figure 4E).
Gene expression results

eNOS, iNOS, and CTH mRNA expression. Relative mRNA expressions of genes were evaluated by RT-PCR analysis. The expression levels of eNOS mRNA were similar in all groups except for the NaHS-administered groups, in that NaHS administration caused a decrease in eNOS mRNA expression, while a further decrease was detected in the L-NAME+NaHS group. However, this decrease was not statistically significant (Figure 5A). L-NAME administration alone caused a prominent increase in the expression of iNOS mRNA, although it was not statistically significant. In contrast to the increment with L-NAME alone, the administration of NaHS and PAG with L-NAME resulted in a decrease (Figure 5B).

Discussion

A considerable number of studies have been performed to examine the cardioprotective effects of H₂S and NO, and the interactions between them in I/R injury, by employing several in vitro and in vivo experimental models of cardiac injury, including cultured cardiomyocytes, isolated perfused...
hearts, and rodent and large animal (rabbit, dog, pig) models (17, 22-26). However, to the best of our knowledge, no study has examined the effect of NaHS, PAG and L-NAME on isolated rat heart administered, and the present study is the first to establish the role of these donors and inhibitors in cardioprotection against I/R injury in rat heart.

The studies that aim to reveal the role of H$_2$S in physiological functions are generally based on two strategies: (i) inhibition of endogenous H$_2$S, and (ii) administration of exogenous H$_2$S by employing NaHS as donor. Although the latter strategy is found inconvenient because the large and quick release of H$_2$S from NaHS may have detrimental effects on the experimental animals, this may be negligible since the resulting effects will be fairly short lasting (27). Similarly, the NO synthase inhibitor L-NAME is exogenously administered to the experimental animals in studies investigating the biological functions of NO. Accordingly, the experimental setup of our study is based on these strategies.

In our study, the EDP values that were decreased by ischemia, suggesting diminished cardiac contractility in heart failure, were restored to those of initial levels at the end of

Figure 4. Biochemical analysis in heart tissue samples. A) CK-MB, B) LDH, C) GPx, D) H$_2$S and E) Nitrate/nitrite levels of all groups. **p<0.01, ***p<0.001, statistical significance compared to the IR group; *p<0.05, **p<0.01, ***p<0.001, statistical significance compared to the L-NAME group.
reperfusion in the L-NAME and NaHS groups, while a significant decrease in Max dP/dt and LVDP values was observed in the NaHS group. Concerning the RPP values, the best recovery was observed with the L-NAME, NaHS, and L-NAME+PAG. These results obviously show the role of both H$_2$S and NO in cardioprotection against I/R injury in rat heart, and are concordant with many studies. Johansen et al. (28) have shown that exogenous H$_2$S decreased left ventricular Max dP/dt in a concentration- and time-dependent manner in rat heart. In a study performed in isolated rat hearts, NaHS administration led to a significant reduction in heart rate (HR) and LVDP, and this effect was ascribed to the muscle relaxant role of H$_2$S, suggesting that it has a similar effect on myocardium (29). Moreover, postconditioning with H$_2$S improved the contractile and diastolic functions of the heart subjected to I/R, as revealed with improved HR, Max dP/dt, Min dP/dt, and LVDP, and reduced left ventricular EDP in the left atrium after reperfusion in the study in isolated rat hearts by Luan et al. (24). We obtained similar results in our isolated heart study, which suggests that a decrease in H$_2$S and NO levels due to the inhibition of synthesizing enzymes might be responsible for the impaired cardiac parameters in ischemia. It has been reported that cardioprotection by H$_2$S occurs in I/R injury through the inhibition of oxidation, increase in mitochondrial biogenesis, restoration of mitochondrial dysfunction, inhibition of heart cell apoptosis, reduction in the expression of proinflammatory cytokines and iNOS, up-regulation of eNOS, modulation of autophagy, and increase in angiogenesis (30).

There are numerous studies demonstrating the role of NO in protecting the heart against I/R injury, although some results highlight a controversial role (31). NO plays a positive role by being involved in the mechanisms of protection triggered by cardiac adaptation and ischemic preconditioning, on the other hand it has deleterious effects on the normal heart subjected to I/R alone (32). It is accepted that H$_2$S and NO cooperatively provide a cardioprotection, although there are fewer studies on the interaction between H$_2$S and NO in the cardiovascular system. In our study, exogenous NaHS resulted in increased NO levels, and combined L-NAME and NaHS caused an increase in nitrate/nitrite levels, indicating that H$_2$S participates in NO production. As it is known, NO and H$_2$S interact with each other in various ways, including the formation of S-nitrosothiols, protein S-nitrosylation, and enzyme activation or inhibition (33).

Figure 5. RT-PCR analysis in heart tissue samples. A) eNOS B) iNOS and C) Cth mRNA expressions. All expression levels were measured relative to β-actin by RT-PCR. **p<0.01, statistical significance compared to the PAG group.
other’s synthesizing enzymes, and affect their generation, but
the precise mechanism remains unclear (17).

It has been shown that H₂S provides cardioprotection
against I/R injury by augmenting NO bioavailability via
activation of eNOS (33). Congestive heart failure in mice
was attenuated by eNOS overexpression, while eNOS
deficiency resulted in heart failure and congenital septal
defects during cardiac development due to increased
apoptotic cardiomyocyte death (34, 35). Based on the results
of the studies with L-NAME-induced hypertensive rats, Ji et
al. (36) and Zhong et al. (37) have suggested that the
eNOS/NO pathway was involved in the antihypertensive
effects of H₂S. As confirmed by the significantly increased
expression of iNOS mRNA and protein, iNOS is often induced
to produce higher NO in certain pathological conditions
involving I/R injury (38, 39). However, exogenous NaHS
administration suppressed iNOS activity and reduced NO content in the plasma and myocardial tissue
to improve heart function in a metabolic syndrome model of
rats (38). In a mice study by Hua et al. (40), exogenous H₂S
provided protection against virus-induced myocardial injury
through the inhibition of myocardial iNOS mRNA and
protein expression. However, studies reporting conflicting
results are available in the current literature. For instance,
H₂S inhibited the activity of eNOS in rat and mouse aortic
rings (41), and both exogenous and endogenous H₂S reduced
NO generation and prevented eNOS activity and transcription (42). Additionally, in the present study, a slight
non-significant increase of iNOS expression was observed
with L-NAME administration, while the administration of
NaHS and PAG in combination with L-NAME resulted in
decreased expression. Moreover, NaHS administration
caus ed a decrease in eNOS mRNA expression.

Our biochemical results also indicate that H₂S and NO are
required for cardioprotection since the levels of CK-MB and
LDH were increased in the L-NAME+PAG group in which
NO and H₂S were inhibited, while their levels were decreased
in the other groups. Our results are in agreement with those of
Yang et al. (38) who have found that exogenous NaHS
ameliorated cardiac hypertrophy and myocardial injury in
diabetic cardiomyopathy and reduced LDH and CK-MB
activities in rats. In addition, another study has shown that
CK-MB and LDH levels decreased following NaHS administration
(4). Reactive oxygen species (ROS) production is accelerated
and cellular antioxidants become depleted during myocardial
ischemia. H₂S is a cytochrome C oxidase inhibitor and
therefore inhibits respiration and thus can decrease the
production of ROS and preserve mitochondrial function at low
concentrations. In addition, it has been reported that,
glutathione peroxidase, an antioxidant enzyme, was increased
by NaHS application (4). The GPx, which functions in the
detoxification of hydrogen peroxide, increased in the NaHS
group of our study, as well as in other studies (4, 38). Thus,
H₂S may be involved in the activation of endogenous
antioxidant mechanism by elevating enzyme levels.

We also found that PAG increased expression of CTH,
whereas L-NAME suppressed it. In the PAG group, NO
levels were not changed despite increased CTH expression,
and this result is in conflict with studies stating that CTH
overexpression promotes NO production (10), and that mice
lacking CTH exhibit reduced NO levels (42). It is possible
that different H₂S/NO donors/inhibitors and amounts, and
different experimental models and parameters may result in
conflicting results. Therefore, there is need to examine further
the interactions between H₂S and NO in cardioprotection.

In conclusion the results of our study strengthen the
evidence that NaHS and L-NAME alone reverse I/R injury
induced cardiac performance impairments, while co-
administration adversely affected cardiodynamic values as
reflected by the biochemical results of tissue markers of
cardiac injury. It was also demonstrated that both H₂S and
NO increased each other’s production. We suggest that H₂S
and NO cooperated in cardioprotection against I/R injury in
isolated rat heart. However, there is no doubt that the precise
mechanisms underlying these interactions require further
studies.

Conflicts of Interest

The Authors declare that there are no conflicts of interest associated
with this work.

Authors’ Contributions

S.U. conceived the work, designed and performed the experiments,
analysed the data, and wrote the manuscript. S.T., H.B., D.A.,
O.H.N., C.D.T., S.U.D., U.O. and E.I.A. designed and performed
the experiments and analysed the data. N.Y. and E.G.G. conceived
the work, designed and performed the experiments, and critically
reviewed and supervised the study.

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