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Regulation of Heart Function by Endogenous Gaseous Mediators—Crosstalk Between Nitric Oxide and Hydrogen Sulfide

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Abstract

Both nitric oxide (NO) and hydrogen sulfide (H_2S) are two important gaseous mediators regulating heart function. The present study examined the interaction between these two biological gases and its role in the heart. We found that L-arginine, a substrate of NO synthase, decreased the amplitudes of myocyte contraction and electrically induced calcium transients. Sodium hydrogen sulfide (an H_2S donor), which alone had minor effect, reversed the negative inotropic effects of L-arginine. The effect of L-arginine + sodium hydrogen sulfide was abolished by three thiols (L-cysteine, *N*-acetyl-cysteine, and glutathione), suggesting that the effect of H_2S + NO is thiol sensitive. The stimulatory effect on heart contractility was also induced by GYY4137, a slow-releasing H_2S donor, when used together with sodium nitroprusside, an NO-releasing donor. More importantly, enzymatic generation of H_2S from recombinant cystathionine-γ-lyase protein also interacted with endogenous NO generated from L-arginine to stimulate heart contraction. In summary, our data suggest that endogenous NO may interact with H_2S to produce a new biological mediator that produces positive inotropic effect. The crosstalk between H_2S and NO also suggests an intriguing potential for the endogenous formation of a thiol-sensitive molecule, which may be of physiological significance in the heart. *Antioxid. Redox Signal.* 14, 2081–2091.

Introduction

THE LAST FEW YEARS have seen much interest in the biology of endogenous, physiologically and perhaps therapeutically important gasomediators (27). Of particular focus in the field of cardiovascular research is hydrogen sulfide (H_2S), the latest addition to the family of gasotransmitters together with its two counterparts, nitric oxide (NO) and carbon monoxide (27, 33, 44). H_2S has been long known for its toxic properties and as a pollutant, until recently it was found to be natively produced in mammalian tissues including the brain, vascular, and heart (7, 32, 35). As a biological gas, H_2S is synthesized by both endogenous enzymes and nonenzymatic pathways (by reduction of thiols and thiol-containing molecules) (27). Three enzymes that generate H_2S from L-cysteine (L-cys) have been elucidated, namely pyridoxal-5′-phosphate–dependent cystathionine β-synthase and cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (16, 36). To date, endogenously produced H_2S has been found to contribute significantly to the cardioprotective effects of ischemic preconditioning and postconditioning against ischemia/reperfusion injury in rat myocytes (33, 42, 47).

The discovery of NO as the first gasotransmitter was embraced by researchers in the field of life sciences (41). In the heart, NO is produced by neuronal and endothelial NO synthases (nNOS and eNOS) that are constitutively expressed. Physiological concentrations of NO *in vivo* range from 0.1 to 5 nM, although large variations in values have been reported (15). NO plays an important role in the modulation of heart contractility *via* guanylyl cyclase activation or *via* the direct nitrotyrosylation of various proteins involved in calcium handling and contractile machinery (31). The overexpression of eNOS (19, 21) or application of NO donors (43) can alleviate irreversible ischemia/reperfusion injury and other heart diseases (14). Further, NO is a well-known potent vasodilator of different blood vessels including the aorta and mesenteric and coronary arteries, hence reducing blood pressure (27).

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As both H_2S and NO are bioactive gaseous molecules, interaction between these two gasomediators has long been speculated (41, 46). In fact, there is growing evidence that the interaction between the gases in a number of ways can affect each other's biosynthesis and physiological response in a targeted tissue (27, 39). Several reports have demonstrated that H_2S and NO may influence the production of each other (4, 27, 33, 49). Julian *et al.* reported that sodium nitroprusside (SNP) potentiated H_2S-induced contractions in body wall muscle from a marine worm (22). In addition, Hosoki *et al.* reported that H_2S augmented the vasorelaxant effect of NO, probably by interaction with NO (16). On the other hand, Whiteman *et al.* (44) and Ali *et al.* (1) discovered that the inhibition of vasorelaxant effect of NO by H_2S was attributable to the possible "crosstalk" or biochemical reaction between the two gases. H_2S and NO may react together to form a yet unidentified nitrosothiol moiety that displays no vasorelaxant activity (1, 30). In view of this, the quenching of endogenous NO by H_2S and the formation of a novel nitrosothiol may be instrumental in the regulation or inactivation of NO and perhaps H_2S (9, 30, 44, 46). However, the exact physiological or pathophysiological functions in the heart have not been elucidated to date.

The accumulating data on the chemical interaction between the two gasotransmitters have stimulated mounting speculation that the interactions of gasotransmitters are of potential biological and therapeutic significance (30). Being a strong reducing agent, H_2S may potentially reduce endogenous NO to form a new substance. Therefore, in the present study, the objectives of the experiments contained herein are to evaluate the biological function of the interaction between H_2S and NO.

Materials and Methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

Drugs and chemicals

Type 1 collagenase, protease XIV, sodium hydrogen sulfide (NaHS), L-arginine, SNP, *N*-acetyl-L-cysteine (NAC), L-cys, pyridoxal-5'-phosphate (PLP), caffeine, NG-nitro-L-arginine methyl ester (L-NAME), and diethylamine NONOate sodium salt hydrate (DEA/NO) were purchased from Sigma Aldrich. The slow-releasing H_2S donor morpholin-4-ium 4 methoxyphenyl phosphinodithioate (GYY4137) was synthesised as previously described (28, 29, 45). Recombinant CSE was isolated as previously described (17). Fura-2 was purchased from Molecular Probes. All chemicals were dissolved in distilled water except Fura-2, which was dissolved in dimethyl sulfoxide.

In this study, NaHS was used as a soluble H_2S donor drug. NaHS is a preferred source of H_2S in comparison to bubbling H_2S gas in solutions as its use allows a better and more accurate determination of H_2S concentration in solution. When present in solutions, NaHS dissociates into Na^+ and HS^-. HS^- then combines with H^+ to give H_2S. At a physiological pH of 7.4 at 20°C, around one-third of H_2S in aqueous solution exists as the undissociated form and the remaining two-thirds exist as HS^- anions at equilibrium with H_2S (27). GYY4137, a slow-releasing H_2S donor, as well as CSE and its substrate L-cys and cofactor PLP were also employed as alternative sources of H_2S.

Isolation of rat ventricular cardiomyocytes

Adult male Sprague–Dawley (SD) rats (230–270 g) were anesthetized by an intraperitoneal administration of ketamine/xylazine (75 mg/kg ketamine and 10 mg/kg xylazine) prior to cardiac myocyte isolation. Heparin (1000 international units) was subsequently injected intraperitoneally to prevent blood coagulation during removal of the heart. A central thoracotomy was performed, and the heart was rapidly excised, mounted onto a Langendorff apparatus *via* the aorta, and retrogradely perfused with calcium-free Tyrode's solution (in m*M*): 137 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 at 37°C). After 5 min, the perfusate was switched to the Ca^{2+}-free Tyrode's solution containing 1 mg/ml Type I collagenase and 0.28 mg/ml Type XIV protease and the heart was perfused with the enzyme solution for another 30 min. After the enzyme recirculation period, the rat heart was washed with Tyrode's solution containing 2×10^{-4} *M* $CaCl_2$ for an additional 5 min to stop enzymatic digestion. The ventricles of the heart were cut and finely minced in a Petri dish containing the prewarmed Ca^{2+}-Tyrode's solution. Then, the solution was shaken gently to ensure adequate dispersion of the dissociated cardiomyocytes. The myocytes were thereafter filtered through a 2.5×10^{-4} m mesh screen and washed three times in Ca^{2+}-Tyrode's solution to remove the digestive enzymes. Subsequently, the myocytes were spun in a centrifuge at 990 rpm and a temperature of 25°C for 1 min and resuspended and collected in Ca^{2+}-Tyrode's solution. The concentration of Ca^{2+} in the Tyrode's solution was then gradually increased to 1.25×10^{-3} *M* in 20 min, after which the cells were stabilized for 30 min at room temperature before experimentation.

Measurement of cardiomyocyte contractility

Twitch amplitudes of cells were measured and recorded. Before measurement, the cells were perfused with Krebs bicarbonate buffer containing (in m*M*) 118 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$ (pH 7.4). In general, rod-shaped cardiac myocytes with clear striation were chosen, and cells that do not respond to electrical stimulation (ES) were omitted from the experiments. Cell images were monitored through a 40× objective lens (Nikon) and transmitted to a charge-coupled device black and white video camera (NL-2332; National Electronic). The output from the charge-coupled device camera was displayed on a video monitor (National Electronic). Myocyte edge was measured using a video motion edge detector (VED-105; Crescent Electronics). Light–dark contrast of the edge of the myocytes provided a marker for measurement of the amplitude of motion. During contractility measurement, the amplitude of marker was directly proportional to the dark image of contraction and the action was in real time. The stability of the preparation was achieved when the amplitude of myocyte motion remained unchanged for at least 10 min. Data collection time point was fixed at 600 s after drug treatments, because maximum response was reached in less than 500 s after the administration of drugs.

Measurement of intracellular calcium

Ventricular cardiomyocytes were incubated with 4 μM fura-2 acetoxymethylester (fura-2/AM) for 30 min in Tyrode's

solution supplemented with 1.25×10^{-3} *M* $CaCl_2$. The cells were washed twice with fresh incubation solution to remove any unincorporated dye. Loaded cells were subsequently perfused at room temperature (25°C) for at least 20 min to allow the deesterification of fura-2/AM in the cytosol. Fura-2/AM-loaded ventricular myocytes were then transferred to the stage of an inverted microscope (Nikon) in a superfusion chamber at room temperature. The inverted microscope was coupled to a dual-wavelength excitation spectrofluorometer (Intracellular Imaging, Inc.). On the stage of the inverted microscope, the myocytes were perfused with Krebs bicarbonate buffer containing (in m*M*) 118 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$ (pH 7.4). Generally, rod-shaped myocytes with clear striations were selected prior to intracellular calcium ($[Ca^{2+}]_i$) measurement. To generate electrically induced (EI)-$[Ca^{2+}]_i$ transients, the cells were stimulated at suprathreshold (4 ms, 0.2 Hz) stimuli delivered by a stimulator (Grass S88) *via* two platinum field-stimulation electrodes immersed in the bathing fluid. In response, the myocytes exhibited simultaneous contraction. Cells that do not respond to ES were not chosen for experimentation. Fluorescent signals obtained at 340 nm (F_{340}) and 380 nm (F_{380}) excitation wavelengths were recorded and stored in a computer for data processing and analysis. The F_{340}/F_{380} ratio was used to represent $[Ca^{2+}]_i$ changes in the myocytes. F/F_o was also employed to assess the change in $[Ca^{2+}]_i$, where F_o represents the fluorescent signal before drug treatment and F represents the fluorescent signal after drug treatment. Response kinetics of various treatments was calculated using the software OriginPro 7.5. The maximum points of each amplitude of $[Ca^{2+}]_i$ transient were plotted, fitted, and analyzed with the function of Boltzmann sigmoidal function.

Intracellular NO measurement

Intracellular NO (NO_i) was measured by incubating myocytes with the NO-sensitive fluorescent dye 4,5-diaminofluorescein (DAF-2) (26), as essentially described in literature (6). Briefly, the cells were incubated with 5 μM DAF-2 at room temperature for 30 min in Kreb's solution containing (in m*M*) 117 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose and bubbled with 95% O_2/5% CO_2 (pH 7.4). The unincorporated dye was removed by washing the cells twice in fresh Kreb's solution. Ventricular myocytes were then transferred to the stage of an inverted microscope (Nikon) in a superfusion chamber filled with Kreb's solution additionally containing 1 m*M* L-arginine at room temperature. The inverted microscope was coupled to a dual excitation spectrofluorometer (PTI). These cells exhibited synchronous contraction in response to suprathreshold rectangular voltage pulses (4 ms, 1 Hz) delivered by a stimulator (Grass S88) *via* two platinum field-stimulation electrodes immersed in the bathing fluid. Fluorescent signal obtained at 488 nm (F) was normalized to the level of fluorescence recorded before stimulation (F_0) and the changes in NO_i were expressed in F/F_0.

Statistical analysis

Experimental values are presented throughout the Results section as the mean ± standard error of the mean, with the number of experimental observations indicated. Statistical analysis was performed on raw data by one-way analysis of variance, followed by *post hoc* Bonferroni test to determine the difference among groups. In general, a p-value of < 0.05 was considered to indicate statistical significance.

Results

Effect of endogenously produced NO on cardiomyocyte contractility in the presence and absence of NaHS

Freshly isolated rat myocytes were treated with L-arginine (500 μM; a substrate of NOS) in the presence or absence of the H_2S donor NaHS (10 μM). As shown in Figure 1A, administration of L-arginine for 10 min significantly decreased the twitch amplitudes of myocytes, whereas NaHS exerted a modest and insignificant effect on myocyte contraction. However, when NaHS was given right before L-arginine, L-arginine + NaHS elicited a positive inotropic effect on cardiomyocytes (+22.78% ± 5.30% when compared with the control group; $n = 5$, $p < 0.05$; Fig. 1A). We also tested the immediate effect of the premixture of NaHS + L-arginine. NaHS and L-arginine were mixed in Krebs solution at 10 min before addition to the myocytes. Treatment with the premixture of NaHS + L-arginine for 5 min did not produce any significant effect (Fig. 1A). These data suggest that the effect of NaHS + L-arginine was mediated by the interaction of H_2S + NO, but not by the other components in the mixture. Taken together, these data imply a putative interaction between NO and NaHS, forming a molecule that may be liable for the observed opposite effect. The ratio-dependent effect of NaHS and L-arginine is shown in Figure 1B. NaHS and L-arginine at a ratio of 50:100 produced the strongest effect.

Effect of endogenously produced NO on electrically and caffeine-induced $[Ca^{2+}]_i$ transients in cardiac myocytes in the presence and absence of NaHS

To find out the effect of NO and H_2S on calcium handling in cardiac myocytes, the amplitudes of EI-$[Ca^{2+}]_i$ transients were observed. As shown in Figure 2A and summarized in Figure 2B, NaHS alone elicited no substantial effect on $[Ca^{2+}]_i$ transients, whereas L-arginine alone induced a significant decrease in the amplitudes of EI-$[Ca^{2+}]_i$ transients. The co-application of L-arginine and NaHS significantly augmented the amplitudes of $[Ca^{2+}]_i$ transients. These data suggest that L-arginine + NaHS may also regulate calcium handling.

The Ca^{2+} decay time constant, τ, is also analyzed in Figure 3A. L-Arginine + NaHS significantly shortened the decay time, whereas L-arginine or NaHS alone caused no substantial changes in decay time. This suggests that L-arginine, in the presence of NaHS, may elicit a more rapid calcium removal from the cytosol by increasing the activity of either sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) or sarcolemmal Na^+/Ca^{2+} exchanger (NCX). Rapid application of 10 m*M* caffeine (Fig. 3B) abruptly releases all Ca^{2+} from the sarcoplasmic reticulum (SR) and the subsequent cytosolic Ca^{2+} removal is mainly mediated by SERCA and NCX (2, 35). As caffeine keeps the ryanodine receptor activated, SR Ca^{2+} sequestration would be substantially suppressed in the presence of caffeine (3). Therefore, the decline in $[Ca^{2+}]_i$ depends on the rate of Ca^{2+} extrusion *via* the NCX, which is correlated to the decay time of caffeine-induced $[Ca^{2+}]_i$ (32). As shown in Figure 3B, the mixture of L-arginine and NaHS failed to affect the amplitude and decay velocity of caffeine-induced $[Ca^{2+}]_i$

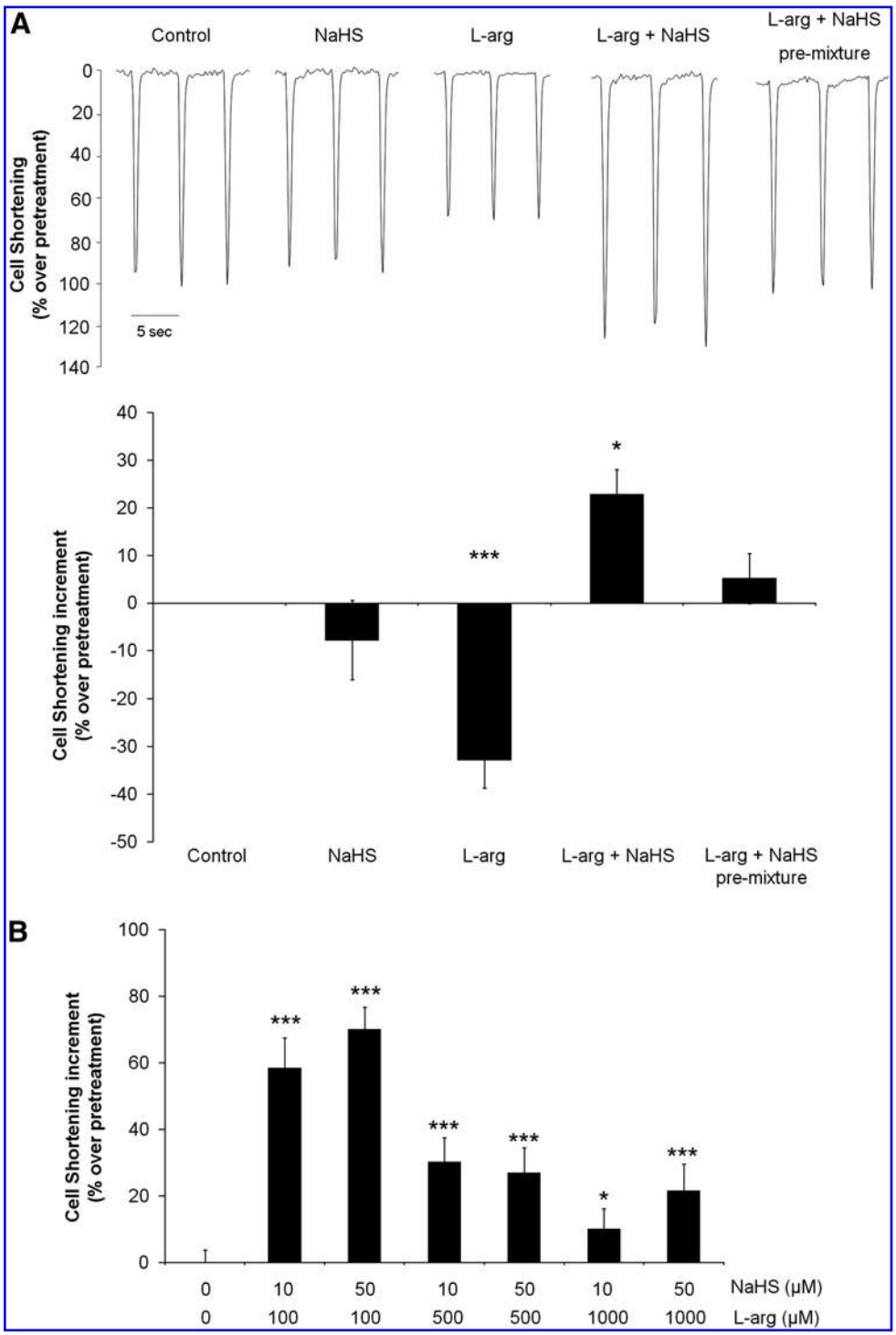

FIG. 1. Effect of endogenously produced NO on cardiomyocyte contractility in the presence and absence of NaHS. (A) Representative tracings (*upper panel*) and group data (*lower panel*) showing cell shortening in electrically stimulated rat ventricular myocytes treated with L-arg (substrate of NO synthase to produce NO, 500 μM) in the presence or absence of NaHS (10 μM), NaHS alone, or premixture of L-arg + NaHS. Treatment with L-arg + NaHS for 10 min significantly augmented the contractility in myocytes; however, treatment with premixture of L-arg + NaHS failed to produce any significant effect. $n = 5$–22. **(B)** Concentration-dependent effect of NaHS + L-arg on cardiomyocyte shortening. $n = 7$–10. Mean ± SEM; $^{*}p < 0.05$ and $^{***}p < 0.001$, *versus* control group. NO, nitric oxide; NaHS, sodium hydrogen sulphide; L-arg, L-arginine; SEM, standard error of the mean.

transients, as there were no substantial differences between groups. This indicates that the inotropic and lusitropic actions of L-arginine and NaHS do not occur by the alteration of NCX function or SR Ca^{2+} content.

NO_i production induced by ES in the presence or absence of NaHS in the rat ventricular myocytes

If H_2S reacts with NO to form a new substance, NaHS may consume the production of NO. To confirm this, we measured NO_i production induced by ES in the presence of L-arginine. As shown in Figure 4A, ES significantly increased NO_i in the cardiomyocytes bathed in 1 mM L-arginine. This is consistent with previous reports (23). Administration of NaHS significantly attenuated NO_i production. These data suggest that H_2S may interact with the newly generated NO and therefore decrease its level.

To further confirm whether NaHS can interact with endogenous NO, we examined the effect of L-arginine + NaHS in the presence of the NOS inhibitor, L-NAME. As shown in

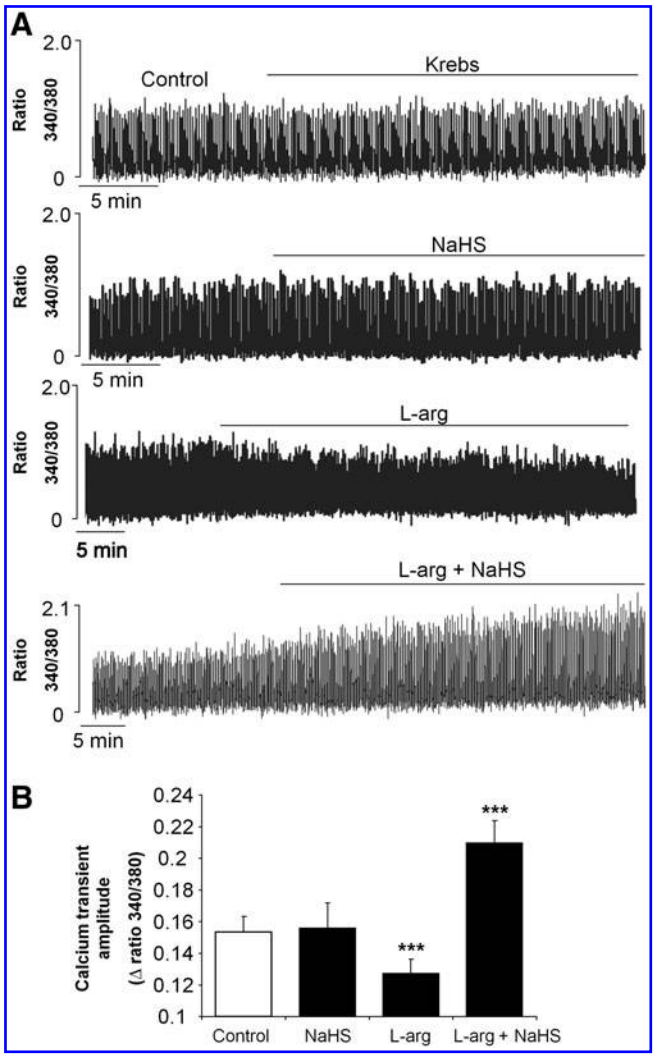

FIG. 2. Effect of endogenously produced NO on EI-$[Ca^{2+}]_i$ transients in cardiac myocytes in the presence and absence of NaHS. (A) Representative tracings of EI-$[Ca^{2+}]_i$ transients in rat ventricular myocytes in control, NaHS alone, L-arg alone, and L-arg + NaHS treatment group. **(B)** Group results showing that 500 μM L-arg + 10 μM NaHS increased, whereas L-arg alone decreased the amplitudes of EI-$[Ca^{2+}]_i$ transients. Mean ± SEM; $n = 5$–17; ***$p < 0.001$, *versus* control group. EI-$[Ca^{2+}]_i$, electrically induced intracellular calcium.

Figure 4B, pretreatment with L-NAME, which itself had no significant effect, abolished the positive inotropic effect of L-arginine + NaHS. These data clearly suggest that the effect was mediated by the interaction between H_2S and the endogenously generated NO.

Effect of NO donors on cardiomyocyte contractility in the presence and absence of NaHS

We continued to study the ratio-dependent effect of NO and H_2S. SNP and NaHS were given at different ratios. As shown in Figure 5A, only when the ratio of SNP:NaHS was at 1:1, it produced a positive inotropic effect on myocyte contraction significantly. These data suggest that H_2S may interact with NO at a special ratio to form a new substance.

We further studied whether the reaction product of H_2S + NO is stable. As shown in Figure 5B, the mixture of NaHS + DEA/NO produced positive inotropic effect at 5 min and 1 h. However, this effect did not last longer than an hour after being mixed (Fig. 5B). These data indicate that the reaction product of H_2S + NO is not stable at 2 h after being generated at room temperature.

Interaction of NO with H_2S generated from a slow-releasing H_2S donor or recombinant CSE protein

We also examined the interaction of NO and H_2S generated from a slow-releasing H_2S donor, GYY4137, which has been previously shown to generate H_2S at similar rates as enzymatic H_2S synthesis from CSE (45). As shown in Figure 6A, treatment with GYY4137 (1 mM), which alone had modest negative effect, and SNP (50 μM) significantly increased myocyte contractility.

We further investigated the interaction of endogenously produced NO with the endogenous generation of H_2S. Cardiomyocytes treated with recombinant CSE protein (10 μg) and its substrate L-cys (10 μM) and cofactor PLP (10 μM) showed no significant change in contractility in the absence of L-arginine. However, addition of 100 μM L-arginine significantly increased myocyte contractility. These data suggest that endogenous generation of NO also interacted with endogenously produced H_2S.

Effect of L-arginine + NaHS is sensitive to thiol

Cardiomyocytes were preincubated with thiols for 10 min prior to the administration of L-arginine and NaHS. Figure 7 shows that a high concentration (1 mM) of thiols (L-cys, NAC, and glutathione [GSH]) substantially blunted the contractility response elicited by L-arginine and NaHS (L-cys: −3.00% ± 5.70%; NAC: 4.90% ± 6.86%; GSH: 4.25% ± 5.90% compared with L-arginine + NaHS treatment group), suggesting that a thiol-sensitive molecule may act as a mediator in the positive inotropic action of NO and H_2S in cardiomyocytes.

Discussion

The complex interaction between H_2S and NO in the regulation of cardiovascular functions in health and diseases may be of great significance (46). The "crosstalk" between H_2S and NO and the hypothesized endogenous formation of thiol-sensitive molecule may offer potential strategies in the management of heart failure. In instances of heart inflammation wherein H_2S and NO levels are elevated, thiols could have a novel utility as prophylactic and therapeutic agents in the treatment of inflammation-induced arrhythmias (5, 46).

It has been previously reported by us and other groups that NaHS produced negative inotropic effect in the cardiac myocytes by suppression of opening of ATP-sensitive K^+ channels (12, 13), blockade of L-type calcium channels (37), and suppression of cAMP/PKA pathway (48). In the present study, we found that NaHS at a low concentration (10 μM) only produced marginal negative effect on cardiomyocyte contractility and EI-$[Ca^{2+}]_i$ transients, whereas L-arginine alone exerted a negative inotropic effect in myocytes. The effect of L-arginine is consistent with the findings in various past studies (10, 38). Interestingly, the co-application of NaHS

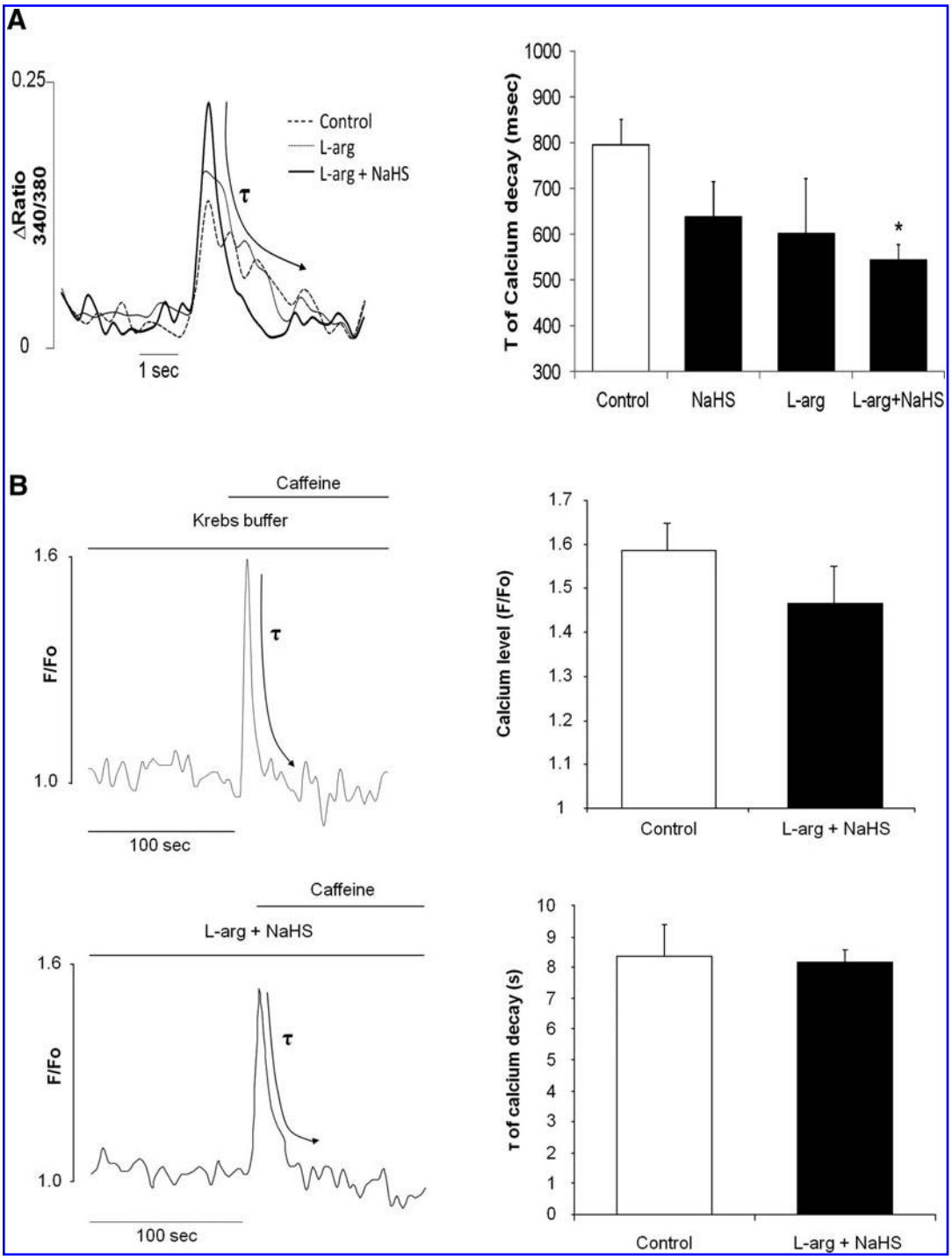

FIG. 3. Effect of L-arg and NaHS on the decay time (τ) of electrically stimulated (A) and caffeine-induced $[Ca^{2+}]_i$ (B) transients in rat ventricular myocytes. (A) Representative tracings and group data showing the effect of L-arg and L-arg + NaHS on the decay time (τ) of EI-$[Ca^{2+}]_i$ transients in rat ventricular myocytes. L-arg + NaHS significantly shortened the decay time, indicating that L-arg + NaHS increased the velocity of calcium decay. Mean ± SEM; $n = 5–9$; $^*p < 0.05$, *versus* control group. **(B)** Representative tracings of caffeine (10 m*M*)-induced $[Ca^{2+}]_i$ transient in the presence or absence of L-arg and NaHS. Group data showed that L-arg + NaHS had no significant effect on caffeine-induced $[Ca^{2+}]_i$ transient amplitudes and calcium decay time. Mean ± SEM; $n = 4–5$; p = not significant.

and L-arginine elicited a substantial increase in the amplitudes of EI-$[Ca^{2+}]_i$ transients and myocyte contractility accordingly. The opposite responses elicited by the combination of L-arginine and NaHS in contrast to that by L-arginine or NaHS alone led to the proposal that a new molecule may be produced *via* interaction between H_2S and NO.

The effects of H_2S generated from a slow-releasing H_2S donor, GYY4137, and enzymatic H_2S from recombinant CSE

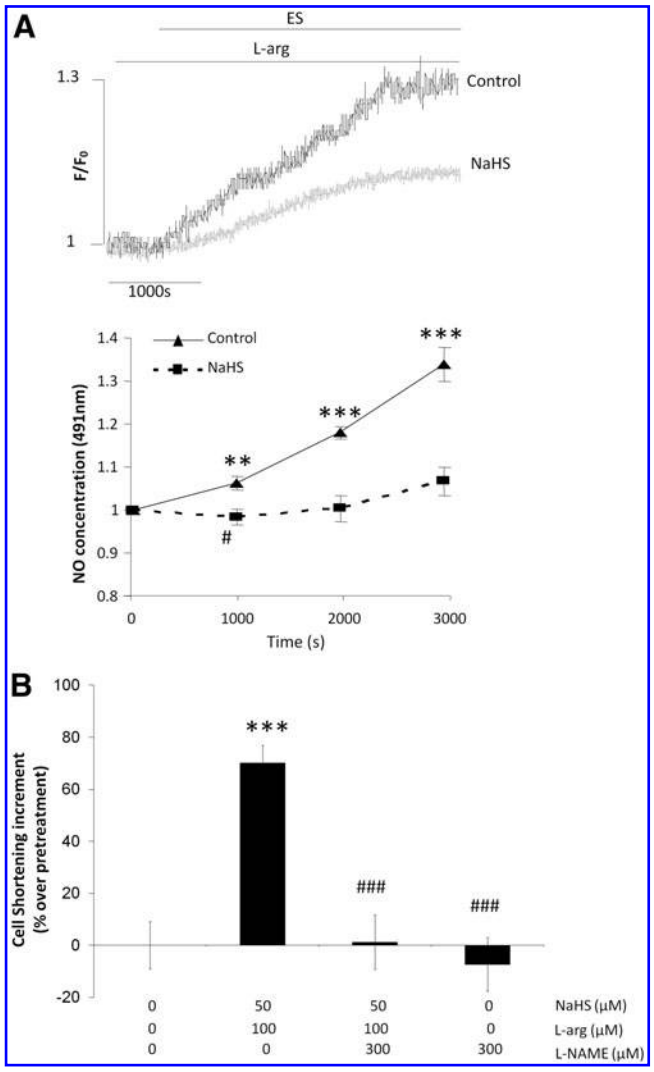

FIG. 4. NO production and its role in the effect of L-arg + NaHS. (A) The effect of H_2S on NO_i generation induced by ES in the cardiomyocyte bathed in L-arg–containing solution. $^{**}p < 0.01$, and $^{***}p < 0.001$, *versus* the NO_i concentration before L-arg treatment; $n = 4$–7; $^{\#}p < 0.05$, *versus* starting NO_i concentration in control group. **(B)** Pretreatment with L-NAME (300 μM, 30 min) abolished the positive inotropic effect of L-arg (100 μM) + NaHS (50 μM). Mean ± SEM; $n = 7$–10; $^{***}p < 0.001$, *versus* control group; $^{\#\#\#}p < 0.001$, *versus* group treated with NaHS + L-arg. H_2S, hydrogen sulfide; NO_i, intracellular NO; ES, electrical stimulation; L-NAME, NG-nitro-L-arginine methyl ester.

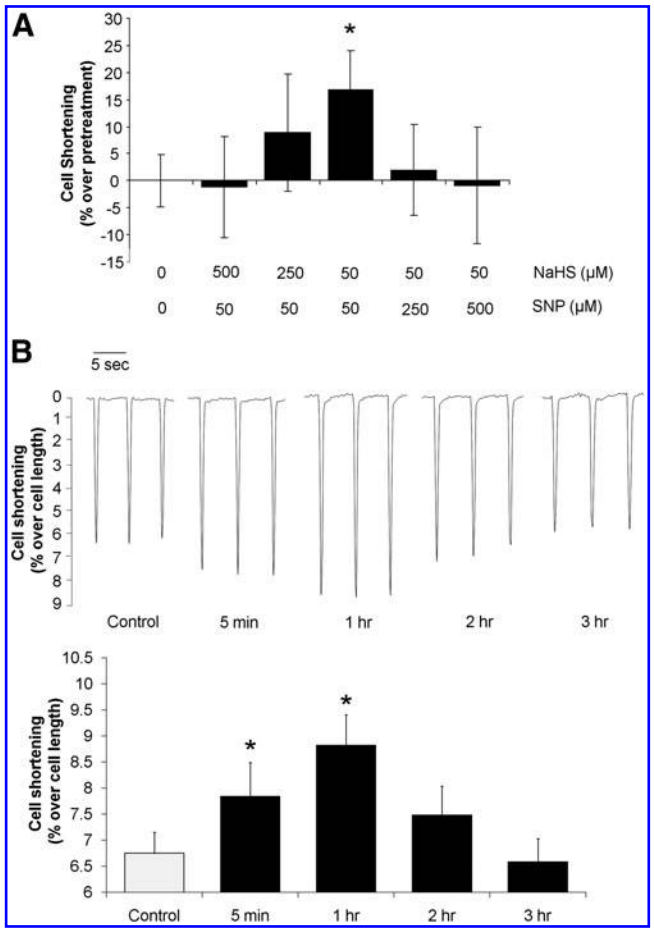

FIG. 5. Concentration- and time-dependent effects of NO + H_2S. (A) Effect of different concentration ratios of NaHS and SNP on cardiomyocyte shortening. $n = 6$–9. **(B)** The time course for the inotropic effect of NaHS + DEA/NO. Upper panel: Representative tracings of cell shortening in the presence of NaHS + DEA/NO after being mixed for 5 min, 1, 2, and 3 h, respectively. Lower panel: The inotropic effect of NaHS + DEA/NO only lasted for 1 h after being mixed. $n = 10$. Mean ± SEM; $^{*}p < 0.05$, *versus* control group. DEA/NO, diethylamine NONOate sodium salt hydrate; SNP, sodium nitroprusside.

were also examined. Different from NaHS, GYY4137 and CSE appear to release H_2S very slowly (29, 45). We found in the present study that GYY4137 also interacted with SNP to produce positive inotropic effects. The interaction between endogenously generated H_2S and NO was also studied. Endogenous H_2S was produced by addition of recombinant CSE proteins and its substrate L-cys and cofactor PLP, whereas endogenous NO was generated by application of L-arginine. This treatment induced a similar positive inotropic effect as that caused by exogenous application of NaHS and DEA/NO. Our data suggest that endogenously generated H_2S and NO may also interact with each other and produce a thiol-sensitive new compound.

The production of a new compound by the interaction between H_2S and NO may further be supported by the fact that H_2S is a strong reducing agent. It is highly possible that H_2S can reduce NO to form a thiol-sensitive molecule, thereby producing positive inotropic and lusitropic effects in the heart. Myocytes were pretreated with specific thiols prior to L-arginine + NaHS exposure. Sources of thiols utilized in the experiments were different types of thiol-donating agents (L-cys, NAC, and GSH) (11, 18, 34). All of them at 1 mM abolished the effects of L-arginine + NaHS, clearly relating L-arginine + NaHS to their reactivity to thiols. Although millimolar concentrations of L-cys have been reported to enhance the potency and prolong the actions of NO (8), NO does not react directly with thiols and must be first converted to a reactive nitrogen species (*e.g.*, N_2O_3) before reacting with thiols to form *S*-nitrosothiols (11). This suggests that NO itself is unlikely to be scavenged by the thiols, hence preserving the effect of NO. With regard to H_2S, numerous studies have

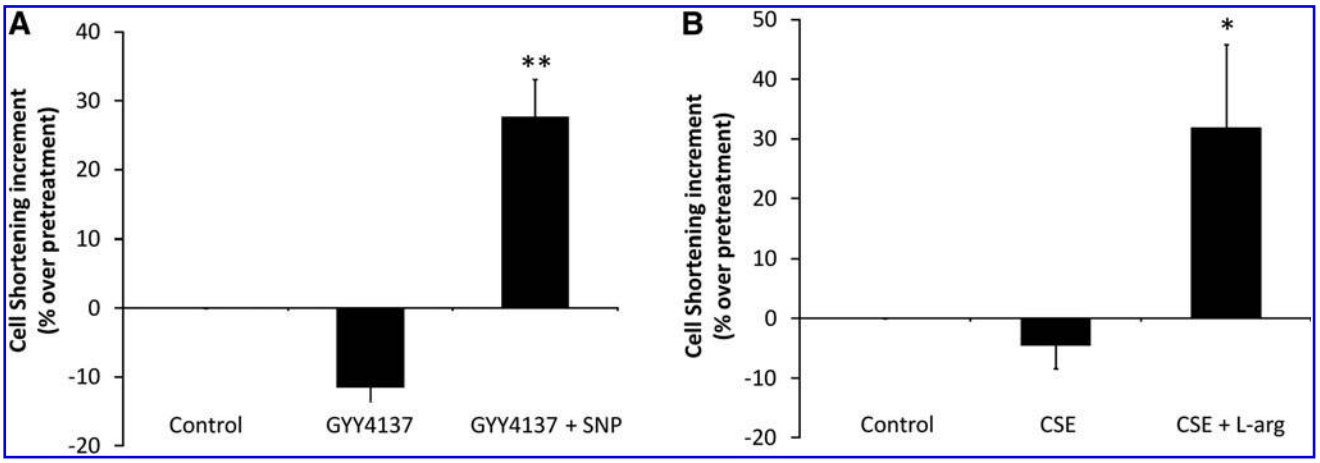

FIG. 6. Interaction between NO and H_2S generated from an H_2S slow-releasing donor or by CSE protein. (A) Treatment with GYY4137 (1 m*M*, an H_2S slow-releasing agent) and SNP (50 μM) significantly increased myocyte contractility. GYY4137 was given 15 min before addition of SNP. Data were collected 10 min after GYY4137 + SNP. **(B)** Effect of endogenous H_2S produced by CSE proteins on cardiomyocytes contractility. Cardiomyocytes treated with CSE (10 μg) and its substrate L-cys (10 μM) and cofactor pyridoxal-5′-phosphate (10 μM) for 30 min showed no significant change in contractility in the absence of L-arg. In CSE + L-arg group, 100 μM L-arg was added at 15 min after CSE + L-cys + pyridoxal-5′-phosphate being administered. CSE + L-arg significantly increased myocyte contractility. Data were collected at 10 min after addition of L-arg. Mean ± SEM; $n = 6$–7; *$p < 0.05$ and **$p < 0.01$, *versus* control group. CSE, cystathionine-γ-lyase; L-cys, L-cysteine.

shown that H_2S can boost GSH and cysteine levels (24) and that cysteine can increase H_2S production conversely (9, 24). This again suggests that H_2S is unlikely to be scavenged by thiols, hence preserving the effect of H_2S. As the thiols can markedly abolish the inotropic actions of L-arginine + NaHS, it may be conclusive that the effect of NO + H_2S may involve a thiol-sensitive molecule.

The speculation that H_2S (a strong reducing agent) can directly reduce NO to form an endogenous thiol-sensitive compound should not be overlooked (18). As mentioned earlier, reports have suggested that the interaction of H_2S and NO may react together to form a yet unidentified nitrosothiol moiety (44). Perhaps the hypothesis of the generation of a thiol-sensitive compound by the biochemical reaction between the gasotransmitters H_2S and NO may additionally address the controversy on the conflicting roles of NO in modulating cardiac contractile function (31). Several studies have indicated that low concentrations of NO (submicromolar) may exert a positive inotropic effect on cardiac contractile function in the absence of agonist stimulation (25). Conversely, high concentrations of NO (above micromolar concentrations) have been reported to induce a negative

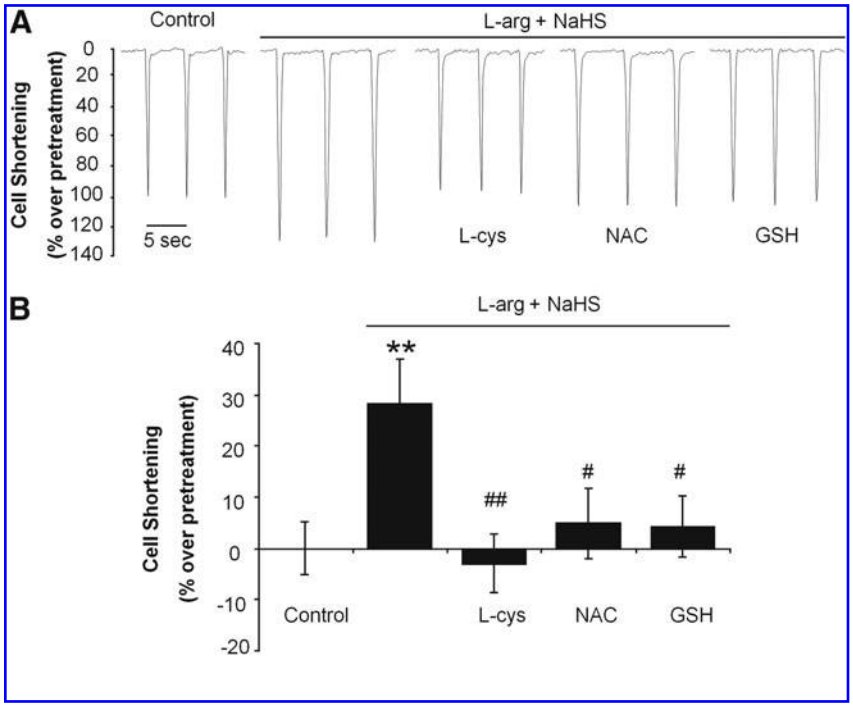

FIG. 7. Effect of NO + H_2S is sensitive to thiol. Representative tracings **(A)** and group data **(B)** showing the effect of L-arg + NaHS on cell contractility in electrically induced myocytes in the presence and absence of thiols (1 m*M*). The positive inotropic action of L-arg + NaHS on myocytes was significantly attenuated when myocytes were preincubated with thiols L-cys, NAC, and GSH. Mean ± SEM; $n = 8$–40; **$p < 0.01$, *versus* control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, *versus* L-arg + NaHS treatment group. NAC, *N*-acetyl-L-cysteine; GSH, glutathione.

inotropism on basal contractile function in hearts (31). The bimodal effect of NO may be alluded to the ability of H_2S to interact with NO and the formation of a thiol-sensitive substance may, at least partly, exert the positive inotropic effect correspondingly, in the light of the data that have been presented so far. Although speculative at this time, the study has raised a putative explanation accounting for this effect and also highlighted the importance of examining the effects and functions of the gases as a whole rather than "stand-alone" individuals.

Given that H_2S and NO would potentially interact in biological systems, it is crucial to examine the possibility of this reaction and its implications under physiological conditions. If ample concentrations of H_2S and NO are present, there may be generation of a new compound with different chemical properties under physiological conditions in biological systems and there is speculation that a species with chemical properties resembling an *S*-nitrosothiol is formed (44), although the precise molecular identity of this intermediate is not known. However, the absolute levels of "free" H_2S gas in blood and tissues are controversial and it is plausible that H_2S circulates as part of an as yet unidentified carrier molecule(s) analogous to hemoglobin and *S*-nitrosothiols for NO. Similarly, the absolute physiological concentrations of NO are also controversial and *in vivo* levels range from 0.1 to 5 nM, but large variations in values are also reported (15). In view of this, low concentrations (nM) of the new compound may be presumably generated under physiological conditions in biological systems. It would therefore be appropriate to ascertain the physiological relevance of H_2S + NO by knocking out CSE together with eNOS and nNOS in the hearts of experimental mice, to understand the potential physiological effect of H_2S + NO in biological systems, especially on cardiac functions.

Another consideration is the possible pathology implicated by H_2S + NO. In instances of heart inflammation, elevated levels of NO and H_2S may lead to increased production of the thiol-sensitive compound, which may augment Ca^{2+} levels in the heart. During inflammation, inducible NOS becomes highly expressed when stimulated by inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β (46). Consequently, the production of NO increases greatly (27). Similarly, during inflammation, H_2S biosynthesis is markedly increased and CSE expression is upregulated (44). Moreover, further interaction between H_2S and NO in inflammation may additionally add on to the production of the new compound, thus potentially exacerbating Ca^{2+} levels in the heart. Jeong *et al.* found that H_2S enhanced interleukin-1β–induced inducible NOS expression and NO production (20). In a similar fashion, NO could increase H_2S production by influencing CSE expression and activity (49). This is also supported by Zhong's findings that inhibition of NO production by L-NAME also suppresses CSE expression in thoracic aorta (50). Collectively, the large amounts of H_2S and NO generated during inflammation may translate into the overproduction of the thiol-sensitive compound, leading to unregulated Ca^{2+} overloading, which may potentially induce arrhythmias. As such, this study may be helpful in suggesting a new therapeutic strategy to treat cardiovascular diseases such as inflammation-induced arrhythmias.

As described earlier, H_2S and NO can increase cardiac Ca^{2+} cycling by possibly activating ryanodine receptor and SERCA in the heart, hence enhancing heart contractility. Both H_2S and NO donors may represent an opportunity to supersede the current pharmacological therapeutic agents in treating heart failure in the light of the present findings. However, further assessments of the therapeutic utility of H_2S and NO donors must be undertaken. Henceforth, the present findings may provide a valuable starting point for future studies on the therapeutic implications of the interaction between H_2S and NO.

We also found in the present study that ES significantly increased NO_i in cardiomyocyte in the presence of L-arginine. This is consistent with a previous report that basal calcium influx elicited by ES is sufficient to increase nitrite levels in rat ventricular myocytes (23). Interestingly, preincubation with NaHS significantly reduced NO_i augmentation, suggesting that H_2S may either decrease the production of NO_i by inhibiting NO synthase (46) or simply interact with NO and form another substance, which cannot be recognized by the NO-specific probe, DAF-2.

In conclusion, the present data suggest that H_2S and NO may interact together to form a thiol-sensitive compound, which produces inotropic and lusitropic effects in the heart. The crosstalk between H_2S and NO also suggests an intriguing potential for the endogenous formation of thiol-sensitive molecule, which may be of physiological significance in the heart. In addition, the findings may offer a new perspective in the study of gasotransmitters, in which cell function is modulated by the concerted activities of these gases together. Besides, the study also offers new therapeutic strategies for the treatment of cardiovascular diseases. Much work remains to be done to fully establish the identity and formation of the thiol-sensitive molecule from H_2S and NO, and assuming the hypothesis is valid, it may be expected that many more interesting studies on the significance of the interaction between gasotransmitters await.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

$[Ca^{2+}]_i$ = intracellular calcium
CSE = cystathionine-γ-lyase
DAF-2 = 4,5-diaminofluorescein
DEA/NO = diethylamine NONOate sodium salt hydrate
EI = electrically induced
eNOS = endothelial NO synthases
ES = electrical stimulation
fura-2/AM = fura-2 acetoxymethylester
GSH = glutathione
H_2S = hydrogen sulfide
L-Arg = L-arginine
L-Cys = L-cysteine
L-NAME = NG-nitro-L-arginine methyl ester
NAC = *N*-acetyl-L-cysteine
NaHS = sodium hydrogen sulphide
NCX = Na^+/Ca^{2+} exchanger
nNOS = neuronal NO synthases
NO = nitric oxide
NO_i = intracellular nitric oxide
PLP = pyridoxal-5'-phosphate
SEM = standard error of the mean
SERCA = sarcoplasmic reticulum Ca^{2+}-ATPase
SNP = sodium nitroprusside
SR = sarcoplasmic reticulum

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