Role of Hydrogen Sulfide in the Cardioprotection Caused by Ischemic Preconditioning in the Rat Heart and Cardiac Myocytes

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ABSTRACT

Endogenous H$_2$S is synthesized mainly by cystathionine γ-lyase in the heart. The present study investigated the role of H$_2$S in cardioprotection induced by ischemic preconditioning. We have examined the effect of endogenous H$_2$S and exogenous application of NaHS (H$_2$S donor) on cardiac rhythm in the isolated rat heart subjected to low-flow ischemia insults as well as cell viability and function in isolated myocytes exposed to simulated ischemia solution. Preconditioning with NaHS (SP) or ischemia (IP) for three cycles (3 min each cycle separated by 5 min of recovery) significantly decreased the duration and severity of ischemia/reperfusion-induced arrhythmias in the isolated heart while increasing cell viability and the amplitude of electrically induced calcium transients after ischemia/reperfusion in cardiac myocytes. Both IP and SP also significantly attenuated the decreased H$_2$S production during ischemia. Moreover, decreasing endogenous H$_2$S production significantly attenuated the protective effect of IP in both the isolated heart and isolated cardiac myocytes. Blockade of protein kinase C with chelerythrine or bisindolylmaleimide I as well as ATP-sensitive K$^+$ (K$_{ATP}$) channel with glibenclamide (a non-selective K$_{ATP}$ blocker) and HMR-1098 (1-[[5-[2-(5-Chloro-o-anisamido)ethyl]-2-methoxyphenyl]sulfonyl]-3-methylthiourea) (a sarcolemmal K$_{ATP}$ Channel blocker) reversed the cardioprotection induced by SP or IP. However, blockade of mitochondrial K$_{ATP}$ Channels with 5-hydroxydecanoic acid had no effect on the cardioprotection of SP, suggesting that, unlike the mechanism involved in IP, mitochondrial K$_{ATP}$ channels most probably do not play a major role in the cardioprotection of SP. Our findings suggest that endogenous H$_2$S contributes to cardioprotection induced by IP, which effect may involve protein kinase C and sarcolemmal K$_{ATP}$ channels.

In 1986, Murry et al. (1986) first demonstrated that myocardial damage associated with ischemia was substantially reduced by several preceding short periods of ischemia, a phenomenon they termed ischemic preconditioning (IP). This finding has stimulated great interest in the mechanism underlying such endogenous protection. Although the precise molecular basis of IP is still unclear, several pathways have been implicated. These include activation of G protein-coupled receptors, protein kinase C (PKC), mitogen-activated protein kinases, ATP-sensitive K$^+$ (K$_{ATP}$) channels, heat shock protein, and nitric oxide synthase (Gross and Peart, 2003; Eisen et al., 2004). Among these various mechanisms, PKC and K$_{ATP}$ channels most probably play a central role in the immediate cardioprotection of IP (Eisen et al., 2004). PKC is believed to phosphorylate key proteins, which results in protection of the myocardium from the damaging effects of ischemia and/or reperfusion. The K$_{ATP}$ channel is considered to be one of the phosphorylated proteins that provides protection in this way. The K$_{ATP}$ channel subtype, which is involved in such cardioprotection, is still controversial. Initial evidence suggested that the surface or sarcolemmal K$_{ATP}$...
(sarcKATP) channel triggered or mediated cardioprotection induced by IP (Gross and Auchampach, 1992). More recent findings have suggested that the mitochondrial KATP (mitoKATP) channel may be involved (Liu et al., 1998), although the exact mechanism of the ensuing protection remains unclear.

Hydrogen sulfide (H2S) has been traditionally viewed as a toxic gas. Less recognized, however, is the fact that H2S is also an endogenously generated biological mediator. Indeed, it has recently been hypothesized that H2S is the “third endogenous signaling gasotransmitter,” alongside nitric oxide and carbon monoxide (Wang, 2002). Endogenous H2S is generated in mammalian tissues by two pyridoxal-5'-phosphate-dependent enzymes, cystathionine β-synthase and cystathionine γ-lyase (CSE). Both of these enzymes use L-cysteine as substrate. In the heart, there is little cystathionine β-synthase, whereas CSE is plentiful (Chen et al., 1999; Geng et al., 2004b). The H2S concentration in rat serum is approximately 46 μM (Zhao et al., 2001).

Although it has been reported that H2S regulates rat heart contractility (Geng et al., 2004b), the effect of endogenous H2S on heart excitability and cell function remains unclear. Recent studies have shown that H2S activates KATP channels in both heart (Geng et al., 2004b) and vascular (Zhou et al., 2001) tissue. As such, we hypothesized that H2S may exert a cardioprotective effect via activation of KATP channels. Therefore, the present study was designed to define the role of H2S in the cardioprotective effect of ischemic preconditioning and to determine whether H2S preconditioning may protect the heart against ischemia/reperfusion-induced arrhythmias.

Materials and Methods

The study protocol was approved by the Institutional Animal Care and Use Committees of National University of Singapore.

Isolated Perfused Rat Heart Preparation. Sprague-Dawley rats (230–270 g, male) were anesthetized with 200 mg/kg pentobarbital by i.p. injection. Heparin (1000 IU) was administered i.p. to prevent coagulation during removal of the heart. The heart was removed, mounted in a Langendorff apparatus, and perfused retrogradely through the aorta with a Krebs solution containing 117 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.25 mM CaCl2, 25 mM NaHCO3, and 11 mM glucose and bubbled with 95% O2/5% CO2 (pH 7.4, 34°C) at a constant flow rate of 13 ml/min as described previously (Bian et al., 2000). The electrocardiogram was monitored continuously and recorded with two electrodes hooked to the apex and the aorta, respectively. Each heart was allowed to stabilize for 15 min before the experiment commenced. Any heart that exhibited arrhythmias during this period was discarded.

The experimental design for the NaHS preconditioning (SP) and IP protocols is shown in Fig. 1A. In the vehicle group (VP), hearts were superfused for 40 min with Krebs’ solution (13 ml/min) and then subjected to a low-flow ischemia insult (perfusion rate, 0.5 ml/min, 30 min) followed by 10 min of reperfusion. For the preconditioning group, hearts were subjected to three cycles of 3 min of perfusion with NaHS (100 μM, SP) or low-flow ischemia insults (perfusion rate 0.5 ml/min, IP), separated by 5 min of superfusion with normal Krebs’ solution at normal perfusion rate (13 ml/min). After SP or IP, hearts were subjected to low-flow ischemia insults for 30 min followed by 10 min of reperfusion as in the VP group. In an attempt to determine the involvement of H2S formation in the cardioprotection induced by IP, either DL-propargylglycine (PAG; 2 mM) or β-cyano-γ-l-alanine (BCA; 1 mM) (both CSE inhibitors) was given 15 min before as well as during IP (Fig. 1A). The concentration of such drug chosen was based on a previous study (Mok et al., 2004).

NaHS was used as a donor of H2S. NaHS was employed in these experiments because its use allows for a better definition of the concentration of H2S in solution than bubbling H2S gas, for example. NaHS dissociates to Na+ and HS− in solution. Thereafter, HS− associates with H+ and produces H2S. Approximately one-third of the H2S in aqueous solution exists in the undissociated form (H2S), whereas the remaining two-thirds exists as HS−, which is at equilibrium with H2S (Reifenstein et al., 1992).

Arrhythmia Scoring System. To quantify arrhythmias, the scoring system of Curtis and Walker (1988) was used with modifications. Since the arrhythmias induced by ischemia/reperfusion in the present study were mainly ventricular premature beats and ventricular tachycardia (VT), scoring emphasis was placed on ventricular arrhythmias. Therefore, the scoring system adopted was as follows: 0, no arrhythmia; 1, 1 to 30 premature ventricular contractions; 2, >30 premature ventricular contractions; 3, <three episodes of ventricular fibrillation (VF/VT; 4, three to five episodes of VF/VT; and 5, >five episodes of VF/VT. The score of a particular heart was the value of the most severe type of arrhythmias exhibited during 10 min of reperfusion.

Isolating Rat Cardiac Myocytes. Cardiac myocytes were isolated from the hearts of adult male rats using a collagenase perfusion method as described previously (Bian et al., 2000, 2004). In the present study, we examined the cardioprotective effect produced by simulated ischemia solution [i.e., glucose-free Krebs buffer containing 10 mM 2-deoxy-d-glucose (2-DOG), an inhibitor of glycolysis (Macianskiene et al., 2001), and 10 mM sodium dithionite (Na2S2O4), an oxygen scavenger (Otter and Austin, 2000); pH 6.6]. The use of simulated ischemia solution in this way produces a mixture of effects including metabolic inhibition, anoxia, and acidosis. The method used was adopted from previous publications (Ho et al., 2002), and the experimental procedures are detailed in figures. In brief, after separation of ventricular myocytes, cells were allowed to stabilize for 30 min before the experiment was commenced. Ventricular myocytes were subjected to three cycles of 3 min, each cycle of superfusion with 1 to 1000 μM NaHS (SP), ischemia solution (IP), or Dulbecco’s modified Eagle’s medium (DMEM) (VP), separated by 5 min of superfusion with DMEM solution. Cells were then subjected to ischemia solution for 9 min followed by reperfusion for 10 min with DMEM solution. In the control group, cells were treated with DMEM solution for 9 min (Figs. 2A and 6A). To probe the role of endogenous H2S, either PAG or BCA was administered 15 min before as well as during IP (Fig. 2A). To examine the mechanisms involved, a range of PKC inhibitors [chelerythrine (1 μM) and bisindolylmaleimide I (BSM, 100 nM)] and KATP channel blockers [glibenclamide (10 μM), 5-hydroxydecanoic acid (5-HD; 100 μM), and HMR-1098 (30 μM)] were given 5 min before and during SP or IP (Figs. 4A and 5A). The concentration of PKC inhibitors (Kawamura et al., 1998) and KATP channels blockers (Chen et al., 2003; Kristiansen et al., 2005) used in this study is based on previous reports in the literature.

Trypan blue exclusion was used as an index of myocyte viability (Zhou et al., 1996; Hiebert and Ping, 1997). After the live cells were incubated with 0.4% (w/v) trypan blue dye for 3 min, those that were unstained were termed to be nonblue cells. Nonblue cells/total cells were determined in a hemocytometer chamber using a light microscope (10× magnification).

Cell morphology was assessed by microscopic examination (Armstrong and Ganote, 1994; Zhou et al., 1996). Both rod-shaped (length/width ratio, >3:1) and square (length/width ratio, <3:1) cells were examined. Only results from rod-shaped cells are presented in this paper.

Measurement of [Ca2+]. To determine the functional status of the cells, the electrically induced [Ca2+]i transients before, during, and after ischemia/reperfusion were measured. Ventricular myocytes were incubated with 1 μM Fura-2/AM in DMEM solution for 45 min. The unincorporated dye was removed by washing the cells twice in fresh incubation solution. Loaded cells were maintained at room temperature...
for 30 min before measurement of [Ca\(^{2+}\)]

Ventricular myocytes loaded with fluo-3 were washed once and then transferred to the stage of an inverted microscope (Nikon, Tokyo, Japan) in a superfusion chamber at room temperature. The inverted microscope was coupled to a Digital Fluorescence Imaging System (Intracellular Imaging Inc., Cincinnati, OH). The myocytes selected for the study were red-shaped with clear striations. These cells exhibited a synchronous contraction (twitch) in response to suprathreshold 4-ms stimuli at 0.2 Hz delivered by a stimulator (Grass S88) through two platinum field stimulation electrodes in the bathing fluid. Fluorescent signals obtained at 488-nm excitation and 535-nm emission wavelengths were stored in a computer for data processing and analysis. [Ca\(^{2+}\)] changes were expressed as fluorescence measured over basal unstimulated fluorescence (F/F\(_{0}\)).

**H\(_2\)S Concentration Measurement.** Ventricular myocytes were divided into four groups including control (Con), VP, IP, and SP groups (Fig. 6A). The protocols for VP, IP, and SP are as described above. In the Con group, cardiac myocytes were subjected to sham ischemia with fresh DMEM solution for 9 min. At the end of 9-min ischemia or sham ischemia, 75 μl of culture media from each group was collected, diluted in deionized water (final volume, 500 μl), and added to an Eppendorf microtest tube containing zinc acetate (1% w/v, 250 μl) to trap H\(_2\)S. Subsequently, N,N-dimethyl-p-phenylenediamine sulfate (20 μM, 133 μl) in 7.2 M HCl was added followed by FeCl\(_3\) (30 μM, 133 μl) in 1.2 M HCl. Thereafter, trichloroacetic acid (10% w/v, 250 μl) was used to precipitate any protein that might be present in the culture media, and after centrifugation (10,000g), absorbance (670 nm) of aliquots from the resulting supernatant (300 μl) was determined using a 96-well microplate reader (Tecan, Durham, NC).

**Drugs and Chemicals.** Type 1 collagenase, protease XIV, 2-DOG, PAG, BCA, NaHS, 5-HD, N,N-dimethyl-p-phenylenediamine sulfate, FeCl\(_3\), and trypan blue dye were purchased from Sigma-Aldrich (St. Louis, MO). Glibenclamide was obtained from Tocris Cookson Inc. (Bristol, UK). HMR-1098 was a generous gift from Aventis Pharma Deutschland GmbH (Frankfurt, Germany). Fluo-3 was purchased from Invitrogen (Carlsbad, CA). Chelerythrine and BSM were from Calbiochem (San Diego, CA). All chemicals were dissolved in distilled water except Fluo-3/AM, chelerythrine, and BSM, which were dissolved in dimethyl sulfoxide at a final concentration of 0.1% (w/v).

**Statistical Analysis.** Values presented are mean ± S.E.M. One-way analysis of variance was used with a post hoc (Bonferroni) test to determine the difference between groups. The significance level was set at p < 0.05.

**Results**

SP Attenuated Ischemia/Reperfusion-Induced Arrhythmias. To determine whether SP is able to produce a cardioprotective effect on cardiac rhythm, we measured elec-
trocardiogram in isolated rat hearts. Like numerous previous researchers, we utilized NaHS as a soluble H₂S donor drug. Previous studies have shown that the actual amount of H₂S generated in such solutions is approximately 33% of the amount of NaHS (Reiffenstein et al., 1992). As such, NaHS (100 μM) is likely to produce approximately 33 μM H₂S, which is well within the physiological concentration range in, for example, rat plasma (Zhao et al., 2001). Figure 1, B and C, show that low-flow ischemia/reperfusion induced severe arrhythmias in the VP group. Both the duration of arrhythmias (VP, 78.2 ± 19.6 s versus SP, 8.8 ± 4.7 s; n = 6, p < 0.01) and the arrhythmia scores (VP, 3.6 ± 0.2 versus SP, 0.5 ± 0.4, n = 6, p < 0.001) during the 10-min reperfusion period were significantly decreased in the SP group. These data suggest that SP protects the heart against ischemia/reperfusion induced arrhythmias.

**Effect of SP on Cell Viability and Morphology Subjected to Ischemia Solution.** To further substantiate the cardioprotective effect of H₂S, we also assessed the concentration-dependent effect of NaHS on cell viability and morphology in isolated rat ventricular myocytes that were exposed to ischemia solution. As shown in Fig. 2B, preconditioned with three cycles of different concentrations of NaHS (1, 10, and 100 μM and 1 mM), the percentage of nonblue cells following ischemia increased in a concentration-dependent manner. This effect was significantly greater than that of the VP group at an NaHS concentration of 10 μM, and the maximum protective response was observed at a concentration of 100 μM NaHS (VP, 32.6 ± 2.1%; 10 μM NaHS, 45.9 ± 2.3%; 100 μM NaHS, 47.9 ± 2.2%; all n = 7; Fig. 2B).

To compare the responses in terms of myocyte viability and cell shape, the rod-shaped cells were counted 10 min into the reperfusion period. As shown in Fig. 2C, treatment with NaHS at 10 and 100 μM resulted in a greater percentage of rod-shaped cells per total number of cells than that detected in the VP group (VP, 28.9 ± 3.3%; 10 μM NaHS, 41.3 ± 2.8%; 100 μM NaHS, 43.4 ± 3.1%; all n = 7, Fig. 2C).

**Effects of IP on Electrically Induced [Ca²⁺]ᵢ Transients of the Ventricular Myocytes Subjected to Ischemia Solution in the Single Surviving Cells.** To determine the functional status of the cells, electrically induced [Ca²⁺]ᵢ transients before, during, and after ischemia were determined. As shown in Fig. 3, A and B, electrically induced [Ca²⁺]ᵢ transients were significantly (p < 0.001) decreased after ischemia/reperfusion (25.8 ± 3.0%, n = 25, Fig. 3, A and B). These results are in agreement with the effects of metabolic inhibition or hypoxia as reported in a previous study (Seki and MacLeod, 1995). Similar to the change in cell viability, the decrease in [Ca²⁺]ᵢ transients during reperfusion was also significantly attenuated by SP (90.4 ± 4.6%; n = 25; Fig. 3, A and B). These results suggest that cell function was significantly improved by SP.

**Effects of IP on Cardiac Rhythm, Cell Viability, and Electrically Induced [Ca²⁺]ᵢ Transients in the Presence and Absence of H₂S Synthase Inhibitors.** This series of experiments was designed to determine whether en-
PAG or 1 mM BCA (Teague et al., 2002) 15 min before and during IP increased the duration of arrhythmias (PAG, 82.4 ± 0.2, n = 6; BCA, 107.4 ± 7.8, n = 5; p < 0.05; Fig. 1D) and the arrhythmia scores (PAG, 2.75 ± 0.2, n = 6; BCA, 3.2 ± 0.4, n = 5; p < 0.05; Fig. 1E) in the IP group. Co-administration of 100 μM NaHS abolished these effects, suggesting that the effects of PAG and BCA were most probably secondary to a decrease in endogenous H₂S. Taken together, these data suggest that myocyte endogenous H₂S production may be decreased during ischemia and that IP may attenuate this effect.

To further confirm whether the cardioprotection associated with IP is mediated by endogenous H₂S, cell viability and electrically induced [Ca²⁺] transients were used as markers. Neither PAG nor BCA alone affected cell viability (control, 69.2 ± 3.2%; PAG, 63.4 ± 2.4%; BCA, 65.1 ± 1.7%, all n = 5) or morphology (control, 62.8 ± 1.2%; PAG, 60.3 ± 1.8%; BCA, 59.2 ± 1.2%, all n = 5). The percentage of nonblue cells and rod-shaped cells at 10 min into reperfusion in the IP group was significantly higher than that of the VP group (Fig. 2D and E). Both PAG and BCA reversed the cardioprotection due to IP on cell viability and morphology. We also tested whether inhibition of endogenous H₂S formation with PAG or BCA reversed the cardioprotection of IP on cell function by observing the amplitude of electrically induced [Ca²⁺] transients during 10 min of reperfusion. Figure 3, A and C, shows that the amplitudes of electrically induced [Ca²⁺] transients induced by IP were significantly higher than those in the VP group (VP, 20.2 ± 3.8%; IP, 70.0 ± 3.5%; n = 26; p < 0.001). Both PAG and BCA reversed the increased amplitudes in the IP group (PAG, 34.8 ± 3.6%, n = 19; BCA, 37.8 ± 4.6%; n = 10, p < 0.001; Fig. 3C) but by themselves did not affect electrically induced [Ca²⁺] transients in the VP group. Therefore, taken together, these data suggest that endogenous H₂S production is likely to contribute to the cardioprotection caused by IP.

Effects of IP and SP on Cell Viability and Electrically Induced [Ca²⁺], Transients in the Presence and Absence of PKC Inhibitors. The goal of this series of experiments was to probe the mechanism(s) involved in the cardioprotection of IP and SP. To determine whether PKC is involved in cardioprotection induced by IP and SP, two specific PKC inhibitors, chelerythrine (1 μM) and BSM (100 nM) (Kawamura et al., 1998), were used. Either chelerythrine or BSM was given 5 min before as well as during preconditioning. As shown in Fig. 4, B and C, chelerythrine or BSM alone had no significant effect on cell viability or function in the VP group. However, both drugs significantly reversed the cardioprotective effect of IP and SP on cell viability (IP, 40.5 ± 3.0%; chelerythrine, 30.1 ± 3.6; BSM, 30.6 ± 1.7; all n = 7; p < 0.05). Likewise, the improved cell functions in the IP and SP groups were also attenuated by chelerythrine and BSM (Fig. 4C). The amplitudes of electrically induced [Ca²⁺] transients were decreased from 74.2 ± 4.2% (n = 26) in the IP group to 37.2 ± 3.6% in the chelerythrine group (n = 28, p < 0.001) and 35.6 ± 5.7% (n = 13) in the BSM group and decreased from 97.3 ± 5.8% (n = 38) in the SP group to 38.0 ± 3.3% in the chelerythrine group (n = 25, p < 0.001) and 58.2 ± 4.7% in the BSM group (n = 18, p < 0.001).

Effects of IP and SP on Cell Viability and Electrically Induced [Ca²⁺], Transients in the Presence and Absence of Kₐᵢₜ Channel Blockers. To determine the involvement of Kₐᵢₜ channels in the cardioprotection in-
duced by SP and IP, glibenclamide (10 μM), a nonselective K\textsubscript{ATP} channel blocker, 5-HD (100 μM), a selective mitoK\textsubscript{ATP} channel blocker, or HMR-1098 (30 μM), a selective sarcK\textsubscript{ATP} blocker, was administered 5 min before and during preconditioning (Chen et al., 2003). The experimental procedures are shown in Fig. 5A. As shown in Fig. 5, B and C, all three drugs alone did not affect cell viability and function in the VP group. Figure 5B shows that glibenclamide significantly reduced cell viability in both the IP and SP groups (IP, 44.9 ± 3.1%, n = 15; versus glibenclamide + IP, 32.2 ± 3.7%, n = 9; p < 0.05; SP, 50.8 ± 2.7%, n = 18; versus glibenclamide + SP, 34.1 ± 2.7%; n = 8; p < 0.05). Similar results were also obtained in cell function using electrically induced \([Ca^{2+}]_i\) transients as the endpoint (Fig. 5C). Glibenclamide significantly attenuated the increased amplitudes of electrically induced \([Ca^{2+}]_i\) transients in the IP (IP, 67.9 ± 3.5%, n = 37; versus glibenclamide + IP, 37.4 ± 3.1%; n = 40; p < 0.001) and SP (SP, 81.9 ± 3.7, n = 12; versus glibenclamide + SP, 39.4 ± 4.1; n = 32; p < 0.001) groups. These data suggest that K\textsubscript{ATP} channel is involved in the cardioprotection of both IP and SP.

5-HD and HMR-1098 were further employed to determine the involvement of mitoK\textsubscript{ATP} or sarcK\textsubscript{ATP} channels in the cardioprotection of IP and SP. HMR-1098 significantly decreased the cell viability (IP + HMR, 30.9 ± 4.6%; n = 8; SP ± HMR, 35.2 ± 4.7%; n = 8, p < 0.05; Fig. 5B) and the amplitudes of electrically induced calcium transients (IP + HMR, 23.1 ± 2.2%, n = 21; SP + HMR, 23.0 ± 3.3%; n = 12; Fig. 5C) in both IP and SP groups. However, 5-HD only significantly attenuated the cardioprotection of IP (cell viability, 29.6 ± 2.8%, n = 8; [Ca\textsuperscript{2+}]\textsubscript{i}, transients, 30.1 ± 4.2%, n = 29) but had no significant effect on these parameters in the SP group (cell viability, 49.7 ± 1.6%, n = 8; [Ca\textsuperscript{2+}]\textsubscript{i}, transients, 75.9 ± 5.4, n = 12; Fig. 5, A and B). These data suggest that unlike the mechanism of IP, sarcK\textsubscript{ATP} channel, but not the mitoK\textsubscript{ATP} channel, mediates the cardioprotection of SP.

Effects of H\textsubscript{2}S Synthesis Inhibitors, IP and SP, on H\textsubscript{2}S Production in the Culture Medium of Cardiac Myocytes. We first observed whether PAG and BCA treatment for 40 min inhibited endogenous H\textsubscript{2}S production in these experiments. As shown in Fig. 6B, both PAG and BCA significantly decreased H\textsubscript{2}S production by 78.8 ± 7.1% (n = 5) and 60.4 ± 7.6% (n = 5), respectively.

To further investigate the hypothesis that endogenous H\textsubscript{2}S may mediate the cardioprotection associated with IP, H\textsubscript{2}S concentration in culture medium after 9 min of ischemia was determined. The experimental procedures are shown in Fig. 6A and described under Materials and Methods. As shown in Fig. 6B, ischemia for 9 min (VP group) significantly decreased (23.7 ± 6.9%, n = 10, p < 0.001) H\textsubscript{2}S level in the VP group, suggesting that endogenous H\textsubscript{2}S production is markedly decreased during ischemia. Interestingly, preconditioning with three cycles of ischemia or NaHS (100 μM) significantly attenuated the
Inhibitory effect of ischemia on H₂S production (IP, 49.6 ± 9.5%; SP, 59.5 ± 8.6%; n = 5, p < 0.05 versus ischemia group). These data suggest that both IP and SP may be able to reverse the inhibitory effect of ischemia on H₂S production.

Discussion

In the present study, we have observed a cardioprotective effect of exogenous application of NaHS. We used NaHS (an H₂S donor) to produce H₂S. Previous study has shown that
the actual amount of H$_2$S is about 33% of the amount of NaHS (Reifenstein et al., 1992). The concentration of NaHS we used is 100 $\mu$M, which may produce about 33 $\mu$M H$_2$S. This is within the physiological range of H$_2$S in the serum (~46 $\mu$M) (Zhao et al., 2001). We observed that preconditioning with 100 $\mu$M NaHS attenuated arrhythmias in the isolated Langendorf-perfused heart (subjected to low-flow ischemia insults), increased cell viability, and improved cell function in cardiac myocytes during ischemia/reperfusion. Our data clearly suggest that, at physiological concentrations, H$_2$S produces a cardioprotective effect.

Subsequently, we examined the potential role of endogenous H$_2$S in cardioprotection due to IP. Treatment of cardiac myocytes with either PAG or BCA markedly decreased endogenous H$_2$S production and significantly attenuated the protective effect of IP in the isolated rat heart and cardiac myocytes. Moreover, we also observed that H$_2$S production was decreased when ventricular myocytes were subjected to ischemia. Both IP and SP significantly attenuated the inhibitory effect of ischemia on H$_2$S production. Taken together, our data provide the first evidence that endogenous H$_2$S plays an important role in protecting heart function.

Ischemia/reperfusion-induced arrhythmias originate from a series of complex cellular and humoral reactions. The primary causes are considered to be the endogenous metabolites produced and accumulated in the myocardium during reperfusion. These various metabolites include, for example, reactive oxygen species (ROS), calcium, thrombin, and platelet-activating factor. H$_2$S may protect the heart against arrhythmias by scavenging ROS (Geng et al., 2004a) and opening K$_{ATP}$ channel (Zhao et al., 2001), which reduce calcium influx and shorten action potential duration (APD). During ischemia, H$_2$S production was markedly decreased. Therefore, this effect may increase harmful chemical substances such as ROS, which may, in turn, modulate cellular electrophysiology causing the complex changes at the level of ion channels and induce the arrhythmias. After preconditioning, both IP and SP could stimulate the heart to produce more endogenous H$_2$S and therefore protect the hearts. Blockade of endogenous H$_2$S synthesis increased both the duration of ischemia/reperfusion-induced arrhythmias and the severity of the arrhythmias. Thus, these data suggest that the endogenous H$_2$S system may mediate the cardioprotection induced by ischemic preconditioning.

In the present study, we also investigated the signaling mechanism underlying the cardioprotection of SP and IP. Both chelerythrine and BSM (two specific PKC inhibitors), attenuated the protective effect of SP and IP, thereby suggesting that PKC may mediate the cardioprotection caused by both SP and IP. This is consistent with a previous observation that PKC plays an important role in mediating IP (Gross and Peart, 2003). During IP, PKC stimulation is secondary to activation of G$_q$ or G$_{i/o}$ protein-coupled receptor (Eisen et al., 2004). The mechanism(s) by which PKC is activated during SP is unclear. However, an effect to open of K$_{ATP}$ channels may be involved. This is supported by previous findings that showed that H$_2$S opens K$_{ATP}$ channels in vascular smooth muscle cells (Zhao et al., 2001). Indeed, activation of PKC and K$_{ATP}$ channel may be codependent (Baxter et al., 1995; Gross and Peart, 2003). Protection provided by direct K$_{ATP}$ channel openers may be abolished by PKC antagonists and vice versa, which implies that activation of PKC and K$_{ATP}$ channels are both codependent and necessary for cardioprotection (Gaudette et al., 2000). Additional experiments are needed to determine whether opening K$_{ATP}$ channels is an event upstream of PKC activation.

K$_{ATP}$ channels are well known to play an important role in the cardioprotection induced by IP. However, the subtype of K$_{ATP}$ channel that confers cardioprotective activity is still controversial. Since the first evidence of a role of the K$_{ATP}$ channels in acute IP is presented (Gross and Auchampach, 1992) in the canine heart, results obtained in a number of studies using a variety of different models and species supported that the possibility sarcK$_{ATP}$ channels triggered or mediated the cardioprotective effects of IP. Thus, IP and K$_{ATP}$ channel openers shorten APD (Noma, 1983; Tan et al., 1993), whereas K$_{ATP}$ channel blockers attenuate the effect of IP on APD shortening (Cole et al., 1991; Yao and Gross, 1994). More evidence for the involvement of sarcK$_{ATP}$ channel was provided by Suzuki et al. (2002). They demonstrated that cardioprotection due to IP was blocked by HMR-1098 (a putative sarcK$_{ATP}$ channel blocker) but not by 5-HD. However, Sasaki et al. (2001) found MCC-134, a novel pharmacological agent that opens sarcK$_{ATP}$ channel and blocks mitoK$_{ATP}$ channels, attenuated the effects of IP. These data suggest that the sarcK$_{ATP}$ channel may not be totally accountable for the protective effects afforded by IP. In the present study, we found that the subtypes of K$_{ATP}$ channels involved in the cardioprotection of IP and SP may differ. We observed that various K$_{ATP}$ channel blockers, including glibenclamide, 5-HD, and HMR-1098, attenuated the cardioprotection of IP, which suggests that both sarcK$_{ATP}$ and mitoK$_{ATP}$ are involved. However, selective blockade of mitoK$_{ATP}$ channels with 5-HD had no effect on the protective effect of SP on cell viability and cell function, whereas glibenclamide and HMR-1098 significantly attenuated this effect. Thus, these data suggested that sarcK$_{ATP}$ but not mitoK$_{ATP}$ may mediate the cardioprotection of SP.

Opening of the sarcK$_{ATP}$ channel induced by SP would be expected to enhance shortening of the cardiac APD by accelerating phase 3 repolarization, thereby inhibiting Ca$^{2+}$ entry into the cell via L-type channels and preventing Ca$^{2+}$ overload. Furthermore, the slowing of depolarization would also be expected to reduce Ca$^{2+}$ entry and slow or prevent the reversal of the Na$^-$/Ca$^{2+}$ exchanger. All of these actions may increase cell viability via a reduction in Ca$^{2+}$ overload during ischemia and early reperfusion.

Both PAG and BCA have been widely used to inhibit CSE activity and endogenous H$_2$S production. PAG causes an irreversible inhibition of CSE activity in vitro (Johnston et al., 1979) and in vivo (Uren et al., 1978; Porter et al., 1996; Mok et al., 2004), whereas BCA is a reversible inhibitor of CSE (Pfeffer and Ressler, 1967; Uren et al., 1978). Despite the widespread use of both PAG and BCA to inhibit H$_2$S formation, there is a possibility that one or both of these compounds may produce effects by mechanism(s) that are unrelated to inhibition of CSE. However, this possibility seems unlikely since neither PAG nor BCA alone significantly affected cell viability or heart rhythm. Furthermore, coadministration of NaHS reversed the effect of both CSE inhibitors that both drugs attenuated the cardioprotection of IP on cardiac rhythm, implying that the effects of PAG and BCA most likely result from a decrease in endogenous H$_2$S formation.
In conclusion, the present study has demonstrated, for the first time, that endogenous H$_2$S contributes to the cardioprotection of IP and that pharmacological preconditioning with the H$_2$S donor NaHS confers cardioprotection. SarK$_{ATP}$ channel and PKC may be involved in the cardioprotective effect of H$_2$S. In additions, mitoK$_{ATP}$ may also be involved in the cardioprotective effect induced by IP.

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References


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