The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener

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Hydrogen sulfide (H_2S) has been traditionally viewed as a toxic gas. It is also, however, endogenously generated from cysteine metabolism. We attempted to assess the physiological role of H_2S in the regulation of vascular contractility, the modulation of H_2S production in vascular tissues, and the underlying mechanisms. Intravenous bolus injection of H_2S transiently decreased blood pressure of rats by 12–30 mmHg, which was antagonized by prior blockade of K_{ATP} channels. H_2S relaxed rat aortic tissues *in vitro* in a K_{ATP} channel-dependent manner. In isolated vascular smooth muscle cells (SMCs), H_2S directly increased K_{ATP} channel currents and hyperpolarized membrane. The expression of H_2S-generating enzyme was identified in vascular SMCs, but not in endothelium. The endogenous production of H_2S from different vascular tissues was also directly measured with the abundant level in the order of tail artery, aorta and mesenteric artery. Most importantly, H_2S production from vascular tissues was enhanced by nitric oxide. Our results demonstrate that H_2S is an important endogenous vasoactive factor and the first identified gaseous opener of K_{ATP} channels in vascular SMCs.

Keywords: blood pressure/hydrogen sulfide/nitric oxide/ smooth muscle cells/vasorelaxation

Introduction

Hydrogen sulfide (H_2S) has been best known for decades as the toxic gas dubbed 'gas of rotten eggs' (Winder and Winder, 1933; Smith and Gosselin, 1979). Less recognized, however, is the fact that H_2S is also a biological gas endogenously generated from cysteine in a reaction catalysed by cystathionine β-synthase (CBS) and/or cystathionine γ-lyase (CSE) (Stipanuk and Beck, 1982; Hosoki *et al.*, 1997). By analogy to other endogenous gaseous molecules, such as nitric oxide (NO) and carbon monoxide (CO) (Wang *et al.*, 1997a,b), H_2S was hypothesized to fulfil a physiological role in regulating cardiovascular functions, distinctive from its toxicological effect. In line with this idea was the study showing that H_2S, at physiological concentrations, facilitated the induction of hippocampal long-term potentiation (Abe and Kimura, 1996). To date, the cardiovascular effects of both endogenous and exogenous H_2S have not been fully

understood. Hosoki *et al.* (1997) demonstrated that H_2S relaxed rat aortic tissues *in vitro*. However, the cellular mechanisms for this vascular effect of H_2S, as well as its physiological significance, remained to be examined. Does H_2S affect the cardiovascular function *in vivo* of whole animal or *in vitro* at the cellular level? Do the cardiovascular effects of H_2S have physiological significance? NO and CO mediate vasorelaxation by increasing the cellular cGMP activity and/or stimulating K_{Ca} channels in vascular smooth muscle cells (SMCs). Does H_2S also act on these cellular targets? NO and CO can be released from both SMCs and endothelial cells. Is H_2S synthesized in SMCs or endothelial cells or both? Once released, NO and CO act directly on SMCs independent of endothelium. Is the vascular effect of H_2S mediated by endothelium? The endogenous stimuli for the synthesis and release of NO and CO have been identified. What are the endogenous regulators of H_2S synthesis and release? Answers to these questions will not only help to establish the role of H_2S as another endogenous gaseous vasoactive factor, but also provide novel mechanisms for the fine regulation of vascular tone.

The purpose of the present study was to assess the physiological role of H_2S in the regulation of cardiovascular functions, the modulation of H_2S production in vascular tissues, and the underlying mechanisms. The vasoactive effects of H_2S were investigated by measuring the blood pressure change of rats *in vivo*, vascular tension development *in vitro* and K^+ channel currents in isolated vascular SMCs. Molecular biology and biochemistry techniques were used to detect the expression of H_2S-generating enzymes and the endogenous levels of H_2S. Our study indicates that H_2S is an endogenous vasorelaxant factor that activates K_{ATP} channels and hyperpolarizes membrane potential of vascular SMCs.

Results

The cardiovascular effects of H_2S in vivo

An intravenous bolus injection of H_2S at 2.8 and 14 μmol/ kg body weight provoked a transient (29.5 ± 3.6 s) decrease in mean arterial blood pressure of anaesthetized rats by 12.5 ± 2.1 and 29.8 ± 7.6 mm Hg, respectively ($n = 3$ for each group, $P < 0.05$) (Figure 1A). Heart rate was not altered by H_2S injection (Figure 1B). An 18 ± 3 mm Hg decrease of blood pressure was also observed after a bolus intravenous injection of a K_{ATP} channel opener pinacidil (2.8 μmol/kg) ($n = 3$, $P < 0.01$), mimicking the hypotensive effect of H_2S. Although a bolus intravenous or intraperitoneal injection of glibenclamide (a K_{ATP} channel blocker) at 2.8 μmol/kg did not alter mean blood pressure (106 ± 2.3 mm Hg compared with 109 ± 8.2 mm Hg, $P > 0.05$, $n = 3$), pretreatment of animals for 20 min with glibenclamide significantly reduced by
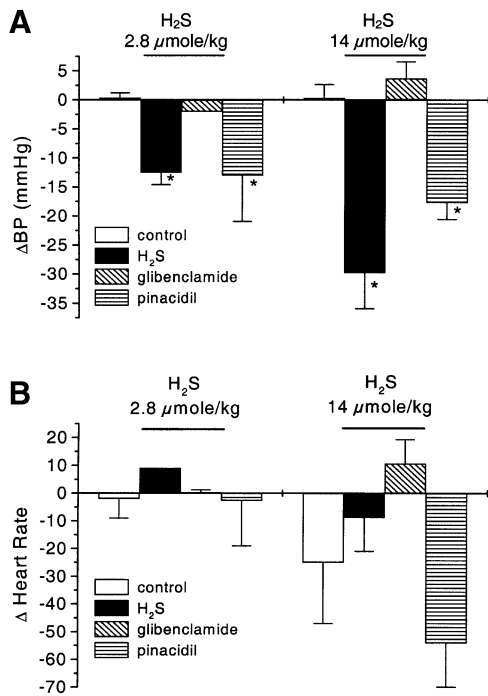
A

B

Fig. 1. The effect of H$_2$S *in vivo* on mean arterial blood pressure (BP) and heart rate of rats. (**A**) Intravenous injection of H$_2$S induced significant decrease in BP. This effect was mimicked by intravenous injection of pinacidil (2.8 μmol/kg) and antagonized by a prior intravenous injection of glibenclamide (2.8 μmol/kg). (**B**) Effect of H$_2$S on rat heart rate. Heart rate was recorded 30 s after intravenous injection of PBS (control), H$_2$S or pinacidil. *$P < 0.05$ compared with control.

83% the hypotensive effect of H$_2$S ($n = 3$, $P < 0.05$). Prior injection of vehicle used for preparing glibenclamide did not alter the hypotensive effect of H$_2$S ($P < 0.05$).

Characterization of the H$_2$S-induced vasorelaxation

Unless otherwise stated, each experiment shown in this section was composed of eight aortic rings. H$_2$S induced a concentration-dependent relaxation of the phenylephrine (PHE)-precontracted rat aortic tissues (IC$_{50}$, 125 ± 14 μM) (Figure 2A). For instance, at a concentration of 180 μM, H$_2$S relaxed the tissue by 63 ± 2.2% ($P < 0.05$). A positive cooperativity for H$_2$S in operating its acting sites on vascular smooth muscles was evidenced by the calculated Hill coefficient of 4.9 (Ruiz *et al.*, 1999). Pretreatment of the endothelium-intact tissues with L-NAME (N^G-nitro-L-arginine methyl ester, 100 μM) to block endogenous NO production from endothelium shifted the H$_2$S concentration-dependent relaxation curve to the right with IC$_{50}$ changed to 220 ± 12 μM ($P < 0.05$) (Figure 2B). Similar results were obtained by just removing endothelium from the aortic tissues (Figure 2C). Moreover, co-application of charybdotoxin (50 nM) and apamin (50 nM) to the endothelium-intact tissues reduced the H$_2$S-induced vasorelaxation (Figure 2C).

Further studies were carried out to identify the involvement of various signal transduction pathways in the vascular effect of H$_2$S. Treatment of tissues with indomethacin (10 μM, not shown) (Rodriguez-Martinez *et al.*, 1998) or staurosporine (30 nM, not shown) (Hattori *et al.*, 1995; Huang, 1996) or SQ22536 (100 μM) (Talpain
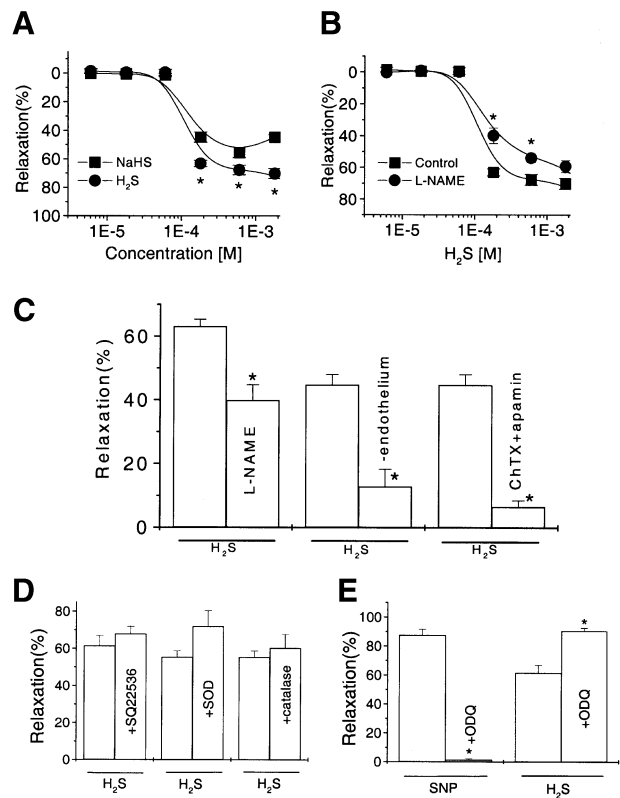
A

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Fig. 2. The H$_2$S-induced relaxation of rat aortic rings and the underlying mechanisms. (**A**) Relaxation of the PHE-precontracted tissues by H$_2$S in the form of either standard NaHS solution (square) or H$_2$S gas-saturated solution (circle). (**B**) Inhibitory effect of L-NAME (100 μM, 20 min, circle) on the H$_2$S-induced relaxation (control, square). (**C**) The effects of H$_2$S (180 μM) on the endothelium-free or endothelium-intact aortic tissues pretreated with L-NAME or charybdotoxin (ChTX)/apamin. (**D**) The relaxant effect of H$_2$S was not affected by pretreating the tissues with SQ22536, SOD or catalase, respectively. (**E**) The effect of ODQ treatment (10 μM for 10 min) on the relaxant effects of SNP (0.1 μM) or H$_2$S (600 μM). $n = 8$ for each data point. *$P < 0.05$ compared with control.

et al., 1995) did not change the effect of H$_2$S (Figure 2D), disapproving the involvement of prostaglandin, protein kinase C or cAMP pathways, respectively. The generation of superoxide anion or hydrogen peroxide by H$_2$S (Nicholls, 1961) was unlikely responsible for the H$_2$S-induced vasorelaxation since the inclusion of superoxide dismutase (SOD, 160 U/ml) and catalase (1000 U/ml) (Rodriguez-Martinez *et al.*, 1998) failed to alter the effect of H$_2$S (Figure 2D). The vasorelaxation induced by sodium nitroprusside (SNP), a NO donor, was virtually abolished by the specific inhibitor of the soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ). However, the vasorelaxant effect of H$_2$S was not blocked by ODQ (Figure 2E). Clearly, the vasorelaxant effect of H$_2$S was not mediated by the cGMP pathway.

Involvement of K$^+$ channel activities in the H$_2$S-induced vasorelaxation

Different relaxation potencies of H$_2$S on vascular tissues precontracted by high or low concentration of KCl. To examine whether the H$_2$S-induced vasorelaxation was mediated by the increased potassium conductance, aortic rings were precontracted with either 20 or 100 mM KCl and the vasorelaxant effect of H$_2$S was then examined. The
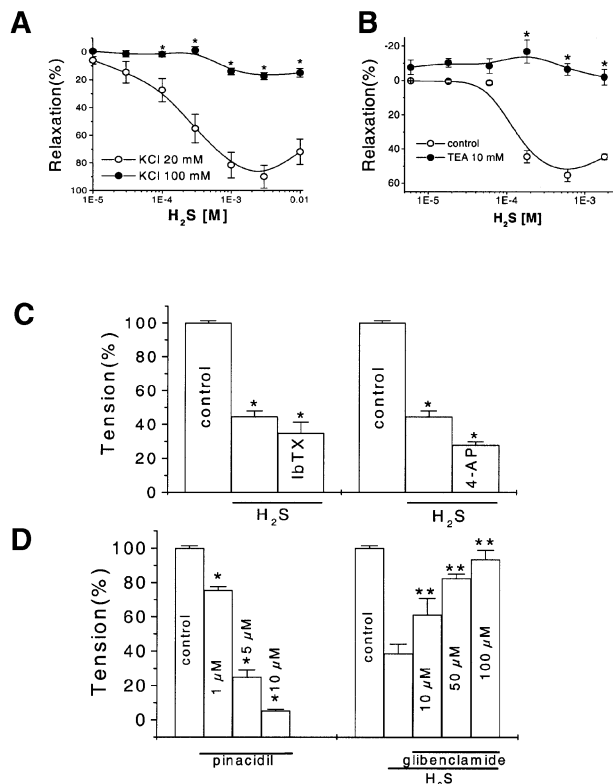
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Fig. 3. The K^{+} channel-mediated vascular effects of H$_2$S. (**A**) The relaxant effect of H$_2$S on the aortic tissues precontracted with 20 or 100 mM KCl. (**B**) Inhibitory effect of TEA on the H$_2$S-induced vasorelaxation. The concentration-dependent vasorelaxant effects of H$_2$S with or without TEA (10 mM) pretreatment of aortic rings were determined. (**C**) The H$_2$S (600 µM)-induced vasorelaxation was not affected by pretreatment of aortic tissues with either 10 µM iberiotoxin (IbTX) or 2.5 mM 4-aminopyridine (4-AP). *$P < 0.05$ compared with control, $n = 8$. (**D**) The vasoactive effects of pinacidil (left) and H$_2$S (right) on the precontracted aortic tissues. The vasorelaxant effect of H$_2$S (600 µM) was examined in the presence of glibenclamide at different concentrations. *$P < 0.05$ compared with control, **$P < 0.05$ compared with the H$_2$S group in the absence of glibenclamide, $n = 8$.

contraction forces induced by 20 and 100 mM KCl were 0.86 ± 0.08 and 1.58 ± 0.08 *g*, respectively. In general, H$_2$S induced a greater relaxation of the vascular tissues precontracted by low concentration of KCl (20 mM) than by PHE. For instance, the threshold concentrations of H$_2$S to initiate relaxation were 18 and 60 µM for 20 mM KCl- and PHE-precontracted tissues, respectively. The maximum vascular relaxation induced by H$_2$S was 90 ± 8.2% or 19 ± 3.9% when the tissues were precontracted with 20 or 100 mM KCl, respectively (Figure 3A). This difference in the relaxation potency of H$_2$S represents the portion of relaxation possibly mediated by potassium conductance.

Involvement of K$_{Ca}$ channels in the vascular effects of H$_2$S. In order to identify the role of specific types of K^{+} channels in the H$_2$S-induced relaxation, aortic rings were incubated with either 10 mM tetraethylammonium (TEA), 100 nM charybdotoxin or 100 nM iberiotoxin (IbTX) (two specific K$_{Ca}$ channel inhibitors) for 20 min prior to the application of H$_2$S. In the presence of TEA, the H$_2$S-induced vasorelaxation was completely inhibited (Figure 3B). At this high concentration, TEA is known to block many different types of K^{+} channels (Nelson and Quayle, 1995).

The H$_2$S-induced vasorelaxation was not affected by iberiotoxin (Figure 3C) or charybdotoxin (not shown), suggesting that big-conductance K$_{Ca}$ channels might not be responsible for the H$_2$S-induced vasorelaxation.

Involvement of voltage-dependent K^{+} (Kv) channels in the vascular effects of H$_2$S. A previous study has shown that 4-AP specifically inhibited the Kv channel with an IC$_{50}$ of 1.4 mM (Remillard and Leblanc, 1996). In the present study, 2.5 mM 4-AP was used to treat vascular tissues 20 min before the application of H$_2$S. The relaxant effect of H$_2$S was not affected by 4-AP, eliminating the involvement of Kv channels (Figure 3C).

Involvement of K$_{ATP}$ channels in the vascular effects of H$_2$S. To elucidate whether an ATP-sensitive K^{+} channel (K$_{ATP}$) was the target of H$_2$S, the interaction of H$_2$S with known K$_{ATP}$ channel modulators was examined. The H$_2$S-induced vasorelaxation was inhibited in a concentration-dependent manner by glibenclamide (IC$_{50}$, 140 µM) (Figure 3D). The H$_2$S effect was also mimicked by pinacidil that relaxed the PHE-precontracted vascular tissues in a concentration-dependent manner (IC$_{50}$, 2.4 µM) (Figure 3D).

The direct effect of H$_2$S on K$_{ATP}$ channel currents and membrane potential in single vascular SMCs

After exposure to 300 µM H$_2$S, K$_{ATP}$ channel currents in rat aortic SMCs were significantly increased in amplitude (Figure 4A). This excitatory effect of H$_2$S was fully manifested 3 min after the application and nullified immediately after washing out H$_2$S from the bath solution. Pinacidil (5 µM) also increased K$_{ATP}$ channel currents, similar to the effect of H$_2$S (Figure 4B). The effects of H$_2$S and pinacidil on K$_{ATP}$ channel currents were observed in a wide test potential range (–40 to +60 mV). The reversal potential of K$_{ATP}$ channels in these cells was not altered by either H$_2$S or pinacidil (Figure 4A and B, right panels).

On average, H$_2$S (300 µM) and pinacidil (5 µM) increased K$_{ATP}$ channel currents from 85.8 ± 12.4 to 149.0 ± 21.4 pA and from 99.1 ± 5.1 to 191.8 ± 28.1 pA (test potential, +40 mV), respectively (Figure 4C). Increased K$_{ATP}$ channel currents by H$_2$S would lead to membrane hyperpolarization, resulting in smooth muscle relaxation. This hypothesis was tested by directly measuring membrane potential change using the conventional whole-cell patch–clamp technique and the results are shown in Figure 4D. After exposing SMCs to H$_2$S (300 µM) or pinacidil (5 µM), the cell membrane was hyperpolarized from –35.7 ± 2.0 to –53.3 ± 2.5 mV or from –36.6 ± 4.5 to –56.7 ± 5.0 mV, respectively. The hyperpolarization developed within 3 min of the application of H$_2$S or pinacidil. Glibenclamide (5 µM) alone had no effect on resting membrane potential ($n = 6$). The hyperpolarization induced by H$_2$S or pinacidil was reversed to the control level 3–5 min after the subsequently applied glibenclamide to the same cells ($n = 5$). Using the perforated whole-cell recording technique, we also found that H$_2$S (300 µM) significantly hyperpolarized the resting membrane potential from –51.2 ± 6.5 to –69.2 ± 7.6 mV ($n = 6$, $P < 0.05$). It may be argued that the increase in K$_{ATP}$ channel currents in the presence of H$_2$S resulted from the interfered ATP metabolism by
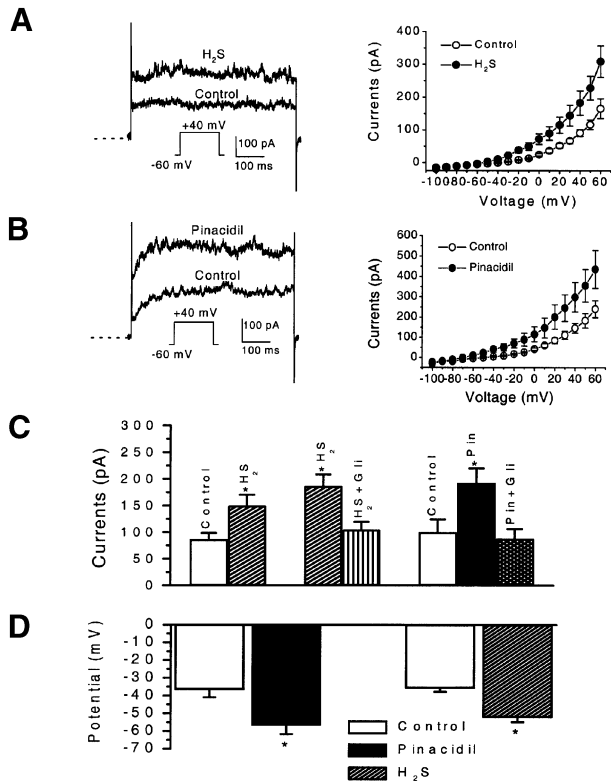
Fig. 4. The effect of H$_2$S and pinacidil on K$_{ATP}$ channel currents in rat aortic SMCs. (**A**) The effect of H$_2$S on K$_{ATP}$ channel currents. The original records from one cell (left) and the mean current–voltage relationship of six cells (right) in the absence and then presence of H$_2$S (300 μM) are shown. (**B**) The effect of pinacidil (5 μM) on K$_{ATP}$ channel currents. The original records from one cell (left) and the mean current–voltage relationship of five cells (right) in the absence and then presence of pinacidil are shown. Dashed line indicates zero current level. (**C**) The antagonistic effect of glibenclamide (Gli, 5 μM) on the effect of H$_2$S or pinacidil (Pin) on K$_{ATP}$ channel currents (holding potential, –60 mV; test potential, +40 mV). (**D**) The membrane hyperpolarization induced by H$_2$S (300 μM) or pinacidil (5 μM), respectively. *P < 0.05 compared with control; n = 3–7 for each group.
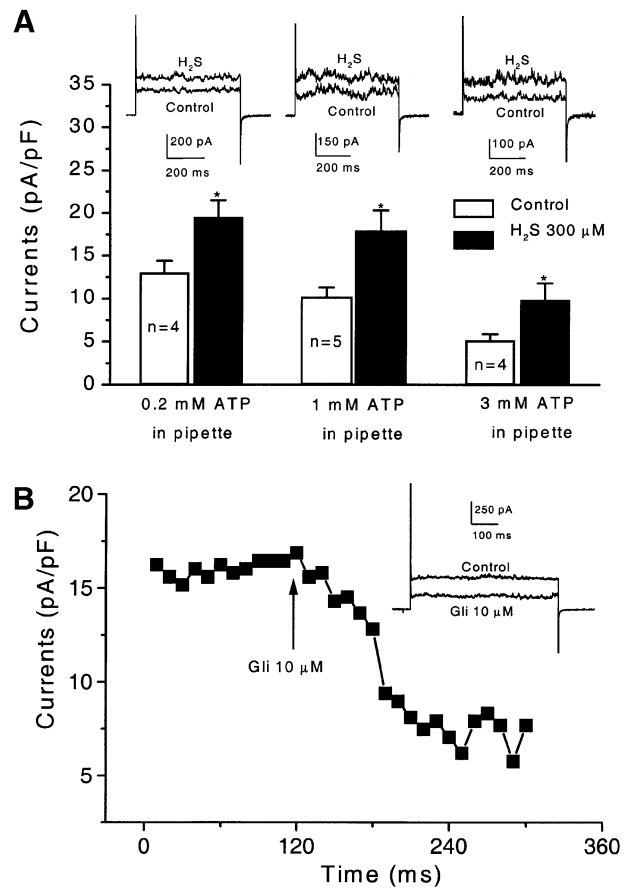
Fig. 5. The modulation of K$_{ATP}$ channel currents by H$_2$S with different intracellular ATP concentrations and by glibenclamide (Gli) in rat aortic SMCs. (**A**) The ATP-dependence of the H$_2$S (300 μM)-induced increases in K$_{ATP}$ channel currents. Holding potential, –60 mV; test potential, +40 mV. *P < 0.05 compared with control. (**B**) The time course of the inhibition of K$_{ATP}$ currents by glibenclamide. Aortic SMCs were dialysed with 0.2 mM ATP and 1 mM GDP. The voltage pulses of +30 mV were applied from a holding potential of –60 mV. Inset shows the representative of original current traces before and after the application of 10 μM glibenclamide.

H$_2$S. However, the reversibility of H$_2$S effect was not in favour of this argument. Moreover, in the aforementioned experiments, the cells were dialysed with the pipette solution that contained a pre-determined ATP concentration, i.e. 0.5 mM. In another set of experiments, the concentrations of ATP in the pipette solution were intentionally altered (Figure 5A). Similar K$_{ATP}$ current densities were observed in the cells with 0.2 or 1 mM ATP in the pipette solution. The excitatory effect of H$_2$S on K$_{ATP}$ currents was not altered by changing intracellular ATP concentrations within this range (P > 0.05). In the presence of 3 mM ATP in the pipette solution, however, the K$_{ATP}$ current density was significantly smaller (5.1 ± 0.7 pA/pF) than that with 0.2–1 mM ATP (P < 0.05). The increase in K$_{ATP}$ channel currents induced by H$_2$S was also significantly decreased in the presence of 3 mM ATP (49.7 ± 2.7% increase) as compared with the effect of H$_2$S in the presence of 0.2 mM ATP (87.3 ± 12.2% increase) (P < 0.05) (Figure 5A). Furthermore, pinacidil (5 μM) increased the K$_{ATP}$ currents in aortic SMCs by 72.4 ± 10.5% (n = 5, P < 0.05 compared with control) or 107.6 ± 32.2% (n = 5, P < 0.05 compared with control)

in the presence of 0.2 or 1 mM ATP in the pipette solution, respectively (P > 0.05 between the effects of pinacidil at the two ATP concentrations). In the presence of 3 mM ATP in the pipette solution, pinacidil (5 μM) increased K$_{ATP}$ currents by 44.7 ± 4.9% (n = 4, P < 0.05), which was significantly smaller than the effect of pinacidil at 0.2 or 1 mM ATP (P < 0.05). These results demonstrated a low ATP sensitivity of the K$_{ATP}$ channels in the examined vascular SMCs and revealed that the effect of K$_{ATP}$ channel openers was reduced in the presence of high intracellular ATP concentrations (Quayle *et al.*, 1995).

To further characterize the K$_{ATP}$ channels in these cells, the effect of glibenclamide was studied. The H$_2$S-stimulated or pinacidil-stimulated K$_{ATP}$ channel currents were significantly reduced by glibenclamide (5 μM, n = 6 for each group) to the control level (Figure 4C). Glibenclamide *per se* did not change the basal K$_{ATP}$ channel current under our recording conditions (5.4 mM KCl in the bath solution and 0.5 mM ATP in the pipette solution) (n = 5, P > 0.05). In the presence of 1 mM GDP in the pipette solution containing 0.2 mM ATP, the basal K$_{ATP}$ current density was significantly increased to
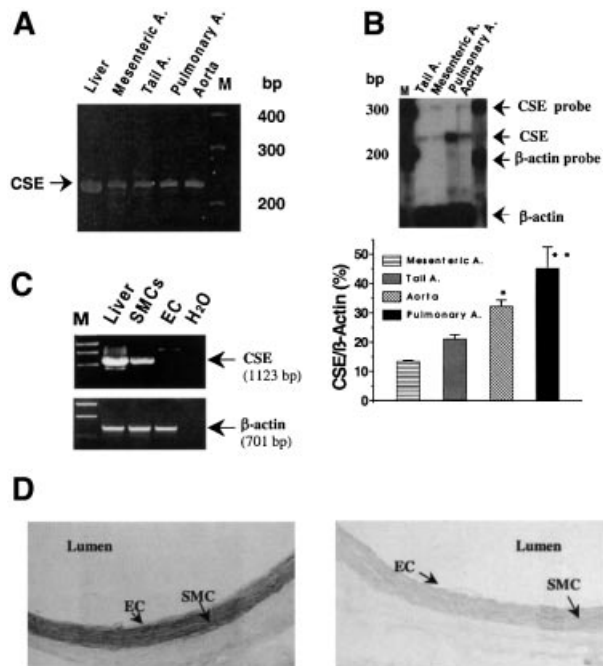
Fig. 6. Differential expression of CSE in rat vascular tissues.
(**A**) RT–PCR analysis of the expression of CSE (234 bp) in rat
liver, mesenteric artery, tail artery, pulmonary artery and aorta.
(**B**) Quantitative comparison of CSE mRNA levels in rat tail artery,
mesenteric artery, pulmonary artery and aorta with RPA. This is
representative of three experiments. *$P < 0.05$ compared with
mesenteric artery; **$P < 0.01$ compared with tail artery, mesenteric
artery and aorta. A, artery. (**C**) The transcriptional expressions of CSE
and β-actin in cultured SMCs and EC (endothelial cell) detected by
RT–PCR. (**D**) *In situ* hybridization showing the location of CSE
mRNA in rat aorta wall by antisense probe on the left and sense
probe as control on the right.

22.3 $\pm$ 4.9 pA/pF ($n = 8$) in comparison with the K_{ATP}
current density of 13.0 $\pm$ 1.4 pA/pF recorded in the
absence of GDP and presence of 0.2 mM ATP in the
pipette solution ($n = 4$) (test potential, +40 mV;
$P < 0.05$). Under this modified condition, glibenclamide
reduced the basal K_{ATP} current density by 47.3 $\pm$ 9.2%
($n = 8$, test potential at +40 mV, $P < 0.01$) (Figure 5B).

The identification, differential expression and cloning of the H_2S-generating enzymes in vascular tissues

To corroborate the physiological importance of the
vasorelaxant effect of H_2S, the endogenous sources of
H_2S were determined in vascular tissues. Using RT–PCR,
a PCR-amplified 234 bp fragment of CSE, but not CBS
(not shown), was detected in endothelium-free rat
pulmonary artery, mesenteric artery, tail artery and aorta
as well as rat liver (Figure 6A). To ascertain that the
amplified PCR product was free of genomic DNA
contamination, every RNA sample was simultaneously
amplified by PCR without reverse transcriptase treatment.
Under these conditions, no PCR product was detected. The
gel-purified PCR products of CSE from rat liver and
vascular tissues were ligated with a synthesized de-
oxyooligonucleotide (Amersham Pharmacia Biotech)
that contains the phage T7 promoter sequence and then
sequenced manually using T7 primer. The sequences of

our PCR-amplified CSE fragments from rat arteries
matched the corresponding CSE sequence cloned from
rat liver (DDBJ/EMBL/GenBank accession No. X53460),
demonstrating that the same isoform of CSE gene was
expressed in rat vascular tissues and liver. RNase protec-
tion assay revealed the abundant levels of CSE mRNA in
different vascular tissues with an intensity rank of
pulmonary artery, aorta, tail artery and mesenteric artery
(Figure 6B). Due to the lack of commercially available
antibodies against CSE, the protein levels of CSE in
vascular tissues were not examined.

The differential expression of CSE between vascular
SMCs and endothelial cells was studied further. Using
RT–PCR, a 1123 bp fragment of CSE was detected in
cultured rat aortic SMCs, but not in cultured vascular
endothelial cells (Figure 6C). *In situ* hybridization was
applied to further locate CSE mRNA in the aortic wall.
The expression of CSE mRNA was clearly identified in the
smooth muscle layer of the artery wall, but not in the
endothelial layer (Figure 6D, left panel). To exclude the
non-specific staining, the sense probe for CSE was used to
hybridize rat aortic tissues under the same condition as for
the antisense probe. Figure 6D (right panel) shows that
no positive staining could be spotted using the sense probe
for CSE.

Finally, a PCR-based cloning technique was used with a
pair of primers covering the translation initiation and
termination codons of CSE to obtain a cDNA clone
encoding the whole open reading frame (ORF) region of
CSE. The PCR-amplified product of ~1300 bp was
detected in rat aorta, tail artery and mesenteric artery.
We have cloned and sequenced two isoforms of CSE from
rat mesenteric arteries, which contained an ORF of
1197 bp, encoding a 398 amino acid peptide. We further
cloned and sequenced CSE from rat liver and found no
differences among all the clones from artery and liver. The
sequence data of our cloned rat vascular CSE and liver
CSE have been submitted to GenBank (AB052882 and
AY032875).

The endogenous level of H_2S and its regulation

The physiological role of H_2S could not be established
before the endogenous level of this gas in vascular tissues
or in circulation had been determined. Thus, the
endogenous production of H_2S in vascular tissues and in
circulation was assayed. Figure 7A showed that various
vascular tissues produced different levels of H_2S. When
the specific inhibitor of CSE, DL-propargylglycine, was
added to the reaction medium at the final concentration
of 20 mM (Hosoki *et al.*, 1997), H_2S production was
completely abolished in all tested arteries ($n = 3$), indi-
cating that the generation of H_2S from vascular tissues was
due to the specific catalytic activity of CSE. Using a
modified sulfide electrode method, the H_2S concentration
of rat serum was determined to be 45.6 $\pm$ 14.2 μM
($n = 4$).

The effect of NO on the endogenous production of H_2S
was examined by incubating homogenized rat vascular
tissues with different concentrations of SNP, a NO donor,
for 90 min. An accumulated H_2S production was upregu-
lated by SNP in a concentration-dependent manner
(1–100 μM) (Figure 7B). Nitric oxide has been shown
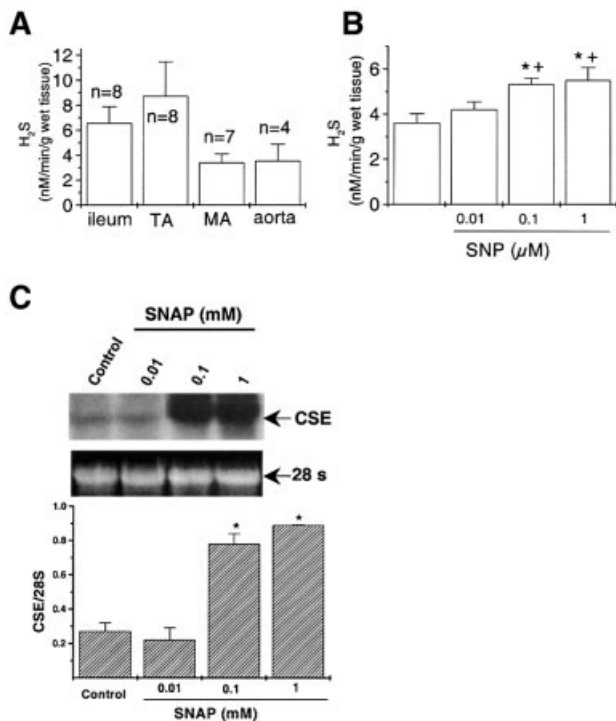to regulate protein expression and synthesis, including

Fig. 7. Regulation of the endogenous H$_2$S production in different rat tissues. (**A**) Accumulated endogenous H$_2$S levels in rat tail artery (TA), mesenteric artery (MA), aorta and ileum. (**B**) H$_2$S production rate of aorta tissues was stimulated by SNP in a concentration-dependent manner. *$P < 0.05$ compared with control, $n = 3$. (**C**) The SNAP-induced concentration-dependent upregulation of CSE transcriptional expression in cultured aortic SMCs, determined using northern blotting. The 28S ribosome RNA was assayed as the housekeeping control. $n = 3$, *$P < 0.05$ compared with control.

growth factors, leukocyte adhesive proteins and extracellular matrix proteins (Kourembanas *et al.*, 1993; Kolpakov *et al.*, 1995; Zeiher *et al.*, 1995). In our study, incubating the cultured vascular SMCs with SNAP (0.1 or 1 mM), another NO donor, for 6 h significantly increased the transcriptional level of CSE (Figure 7C).

Discussion

In the present study, the cardiovascular effects of H$_2$S were demonstrated *in vivo* and *in vitro*. Intravenous injection of H$_2$S provoked a transient but significant decrease in mean arterial blood pressure. Similar to our *in vitro* vascular contractility assay and patch–clamp studies, the H$_2$S-induced decrease in blood pressure was antagonized by glibenclamide and mimicked by pinacidil. Glibenclamide and pinacidil are specific K$_{ATP}$ channel blocker and opener, respectively (Beech *et al.*, 1993; Quayle *et al.*, 1994). Thus, these *in vivo* results indicated that the hypotensive effect of H$_2$S was likely provoked by the relaxation of resistance blood vessels through the opening of K$_{ATP}$ channels. The short duration of the hypotensive effect of H$_2$S could be attributed to the scavenging of H$_2$S by metalloproteins, disulfide-containing proteins, thio-S-methyl-transferase and haem compounds. The administration of H$_2$S as a bolus injection also partially explains the transient effect. Similarly, the hypotensive

effect of pinacidil was also transient. Furthermore, our data showed that the *in vivo* hypotensive effect of H$_2$S was due to a specific action on vascular smooth muscles, since heart rate was not significantly affected. Our *in vitro* study showed that H$_2$S relaxed the isolated aortic tissues at concentrations as low as 18 and 60 μM for the aortic tissues precontracted with either 20 mM KCl or PHE, respectively. It has been reported that the normal blood level of H$_2$S in Wistar rats was ~10 μM (Mason *et al.*, 1978). Our study demonstrated that the plasma level of H$_2$S in SD rats was ~50 μM. The tissue level of H$_2$S is known to be higher than the circulating level. For instance, the physiological concentration of H$_2$S in brain tissue has been reported to be ~50–160 μM (Hosoki *et al.*, 1997). Taken together, H$_2$S is believed to induce vasorelaxation within a physiologically relevant concentration range. These findings strongly suggest that, similar to NO and CO, H$_2$S is another intrinsic vasoactive gas factor.

The physiological role of H$_2$S in the cardiovascular system was further substantiated by its endogenous sources and regulated production process. Using RT–PCR, RPA and northern blotting, we detected the transcriptional expression of the H$_2$S-generating enzyme CSE in all rat arteries tested. The whole sequence of the ORF of CSE had been cloned in this study from rat vascular tissues, which had not been done in any vascular tissues from any species until the present study. CSE has the capability to cleave L-cysteine to produce H$_2$S, ammonium and pyruvate. This enzyme has a unique tissue distribution and was not detectable in brain and lungs (Smith and Gosselin, 1979; Abe and Kimura, 1996).

Akin to the release of NO from endothelium by acetylcholine, the regulation of H$_2$S production by other endogenous substances is an essential piece of evidence for establishing the physiological role of H$_2$S. We showed for the first time that NO regulates the endogenous levels of H$_2$S in vascular tissues via two mechanisms. First, NO increases CSE activity in vascular tissues. Incubating aortic tissue homogenate with a NO donor for 90 min significantly increased H$_2$S generation in a concentration-dependent manner (Figure 7B). NO may increase the activity of cGMP-dependent protein kinases, which in turn stimulates CSE. It is also possible that NO directly acts on CSE protein. Rat mesenteric artery CSE protein contains 12 cysteines that are the potential substrate of S-nitrosylation. Currently, the three-dimensional structure of CSE is unknown and which cysteine contains free -SH group cannot yet be assured. However, the nitrosylation of certain free -SH group of CSE in the presence of NO does represent a possibility (Stamler *et al.*, 1997; Broillet, 1999). Secondly, NO upregulates the expression of CSE. Incubating cultured vascular SMCs with a NO donor for 6 h significantly increased the expression level of CSE (Figure 7C). The mechanism by which NO increased CSE transcription is not yet clear.

Unlike NO, which can be produced from both endothelial cells and SMCs, H$_2$S is only generated from SMCs since no H$_2$S-generating enzyme was expressed in vascular endothelium according to our *in situ* hybridization and RT–PCR studies. Although a previous study claimed that the relaxation of aortic tissues by H$_2$S was not endothelium dependent, the data to support this claim and the procedure to remove endothelium were not presented

in that report (Hosoki *et al*., 1997). Our study demonstrated that a small portion of the vasorelaxant effect of H_2S was indeed potentiated by endothelium (Figure 2B and C). This indicated that H_2S might act as a hyperpolarizing factor, of which the effect was amplified by endothelium. Alternatively, endothelium-derived vasorelaxant factors might have been released by H_2S as both L-NAME and the co-application of charybdotoxin and apamin to the endothelium-intact tissues, a treatment reported to inhibit the effect of endothelium-derived hyperpolarizing factor (Doughty *et al*., 1999), reduced the H_2S-induced vasorelaxation. Nevertheless, our results demonstrate that H_2S acts on both vascular SMCs and endothelial cells with distinctive mechanisms.

Our data did not support the mediation of the H_2S-induced vasorelaxation by prostaglandin, protein kinase C, cAMP or cGMP pathways. Meanwhile, the modulation of K^+ channels in general, and the K_{ATP} channel in particular, in vascular SMCs by H_2S gained ground from several lines of evidence. (i) H_2S effectively relaxed aortic tissues precontracted by 20 mM KCl, but not so when tissues were precontracted by 100 mM KCl. (ii) The H_2S-induced vasorelaxation was inhibited by high concentrations of TEA that blocked many K^+ channels in vascular SMCs, including K_{Ca}, Kv and K_{ATP} channels. Interestingly, different blockers for K_{Ca} or Kv channels failed to affect the vascular effects of H_2S (Nelson and Quayle, 1995), leaving the K_{ATP} channel as the most likely candidate target of H_2S. (iii) Pinacidil relaxed aortic tissues, showing the active role of K_{ATP} channels in regulating vascular tone. (iv) Glibenclamide inhibited the H_2S-induced vasorelaxation, showing indirectly the interaction of H_2S and K_{ATP} channels. (v) Both pinacidil and H_2S effectively increased the whole-cell K_{ATP} channel currents in single SMCs isolated from rat aortae. These effects of H_2S and pinacidil were antagonized by glibenclamide. (vi) H_2S induced a significant membrane hyperpolarization of SMCs. (vii) Finally, we demonstrated that the hypotensive effect of H_2S *in vivo* was suppressed by the K_{ATP} channel blocker. The modulatory effect of H_2S on K_{ATP} channels can be explained by a direct interaction of H_2S and K_{ATP} channel proteins. Potentially, H_2S may induce the reduction of disulfide bonds of the K_{ATP} channel protein (Warenycia *et al*., 1989).

In conclusion, we demonstrate that H_2S is an important endogenous vasorelaxant factor. This finding enlarges the family of gaseous vasorelaxant factors by having H_2S to join another two established members, i.e. NO and CO (Wang, 1998). The vasorelaxation induced by H_2S comprises a minor endothelium-dependent effect and a major direct effect on smooth muscles, which differs from the effects of NO and CO that act only on smooth muscles. While activation of the cGMP pathway is an important mechanism for NO- and CO-induced vasorelaxation, the H_2S-induced vasorelaxation is mediated mainly by the opening of K_{ATP} channels in vascular SMCs and partially through a K^+ conductance in endothelial cells. Thus, this study reveals novel mechanisms underlying the vascular effects of different endogenous molecules of gas. To our knowledge, H_2S is the only endogenous gaseous K_{ATP} channel opener in vascular SMCs ever reported. Some endogenous substances, such as calcitonin gene-related peptide, have been shown to act on K_{ATP} channels

(Reslerova and Loutzenhiser, 1998; Szilvassy *et al*., 1999). However, the effect of these endogenous substances is mediated by membrane receptor- or second messenger-coupled mechanisms. Being a gas, H_2S directly acts on K_{ATP} channels independent of membrane receptors. Importantly, for the first time we showed that NO appears to be a physiological modulator of the endogenous production of H_2S by increasing the CSE expression and stimulating CSE activity. Conceivably, the interaction between two gases, NO and H_2S, may function as a molecular switch for regulating vascular tone.

Materials and methods

Tissue contractility study
A total of 50 male Sprague–Dawley (SD) rats (10–12 weeks old) were used, following an approved protocol by the Committee on Animal Care and Supply of the University of Saskatchewan. The rings of isolated thoracic aortae were prepared as described in our previous publication (Wang *et al*., 1989). When the concentration of KCl in Krebs' solution was increased to 20 or 100 mM in some experiments, the osmolality of solution was adjusted by equimolarly decreasing NaCl concentration. Tissues were contracted with submaximal dose of PHE (0.3 µM). At the plateau phase of contraction, the cumulative concentration-response curves of H_2S were built and fitted with a Hill equation (Weiss, 1997). Endothelium was kept functionally undamaged in most experiments unless otherwise specified, which was confirmed by the acetylcholine (1 µM)-induced vascular tissue relaxation.

Blood pressure and heart rate measurement
Animals were anaesthetized by intraperitoneal injection of pentobarbital (52 mg/kg). Left carotid artery was cannulated and connected to a pressure transducer (AH 51-4844 Harvard, Holliston, USA). One femoral vein was cannulated for delivering chemicals. A heating pad was used to keep the rat's body temperature stable at 37°C. Data acquisition and analysis were accomplished with a Biopac System (Biopac Systems, Inc., Golata).

After 60 min of equilibration, H_2S or other agent was injected in bolus intravenously. Same dose was repeated once with the intervals between injections of at least 10 min.

K_{ATP} channel activity in freshly isolated aortic SMCs
Single aortic SMCs were isolated according to our previously published method with modification (Wang *et al*., 1989). Briefly, thoracic section of aorta was dissected from male SD rats and kept in ice-cold physiological salt solution (PSS). The dissected aorta was incubated at 37°C in low-Ca^{2+} (0.1 µM) PSS containing 1 mg/ml albumin, 0.75 mg/ml papain and 1 mg/ml dithioerythritol for 30 min. After removing the adventitia, aorta was cut into small pieces and transferred to calcium-free PSS in which 1 mg/ml collagenase and 1 mg/ml hyaluronidase were added. Single cells were released by gentle triturating through a Pasteur pipette, stored in the calcium-free PSS at 4°C and used on the same day of preparation.

The whole-cell patch–clamp technique was used to record K_{ATP} channel currents (Tang *et al*., 1999). Briefly, cells were placed in a Petri dish mounted on the stage of an inverted phase contrast microscope (Olympus IX70). Currents were recorded with an Axopath 1-D amplifier (Axon Instruments Inc.), controlled by a Digidata 1200 interface and pClamp software (version 6.02, Axon Instruments Inc.). Membrane currents were filtered at 1 kHz with a four-pole Bessel filter and sampled at 3.3 kHz, digitized and stored. At the beginning of each experiment, junctional potential between pipette solution and bath solution was electronically adjusted to zero (Wang *et al*., 1997b). No leakage subtraction was performed to the original recordings and all cells with visible changes in leakage currents during the course of study were excluded from further analysis. Test pulses were made with a 10 mV increment from –100 to +60 mV. The holding potential was set at –60 mV. *I–V* relationships were constructed using the stable current amplitude at the end of 600 ms test pulses. Membrane potential was recorded with the conventional whole-cell or the perforated whole-cell recording techniques at the current-clamp mode while holding the current at 0 pA. A stable recording of membrane potential was achieved at least 2 min after penetration of cell membrane. The bath solution contained (mM): NaCl 140, KCl 5.4, $MgCl_2$ 1.2, HEPES 10, EGTA 1, glucose 10

(pH adjusted to 7.3 with NaOH). The pipette solution used in the conventional whole-cell recording was composed of (in mM): KCl 140, MgCl$_2$ 1, EGTA 10, HEPES 10, glucose 5, Na$_2$–ATP 0.5 (pH adjusted to 7.3 with KOH). The pipette solution used in the perforated whole-cell recording comprised (mM): KCl 140, MgCl$_2$ 1, EGTA 10, HEPES 10, nystatin 250 µg/ml. Cells were continuously superfused with the bath solution containing tested chemicals.

Measurement of endogenous H$_2$S production

Tissue H$_2$S production rate was measured as described previously (Stipanuk and Beck, 1982) with modifications. Briefly, vascular tissues were isolated from rats and homogenized in 50 mM ice-cold potassium phosphate buffer pH 6.8. The reaction mixture contained (mM): 100 potassium phosphate buffer pH 7.4, 10 L-cysteine, 2 pyridoxal 5′-phosphate and 10% (w/v) tissue homogenate. Cryovial test tubes (2 ml) were used as the centre wells each contained 0.5 ml 1% zinc acetate as trapping solution and a filter paper of 2 × 2.5 cm^2 to increase the air/liquid contacting surface. The reaction was performed in a 25 ml Erlenmeyer flask (Pyrex, USA). The flasks containing reaction mixture and centre wells were flushed with N$_2$ before being sealed with a double layer of Parafilm. Reaction was initiated by transferring the flasks from ice to a 37°C shaking water bath. After incubating at 37°C for 90 min, 0.5 ml of 50% trichloroacetic acid was added into the reaction mixture to stop the reaction. The flasks were sealed again and incubated at 37°C for another 60 min to ensure a complete trapping of the H$_2$S released from the mixture. The contents of the centre wells were then transferred to test tubes each containing 3.5 ml of water. Subsequently, 0.5 ml of 20 mM N,N-dimethyl-p-phenylenediamine sulphate in 7.2 M HCl was added immediately followed by addition of 0.4 ml 30 mM FeCl$_3$ in 1.2 M HCl. The absorbance of the resulting solution at 670 nm was measured 20 min later with a spectrophotometer (Siegel, 1965). The H$_2$S concentration was calculated against the calibration curve of the standard H$_2$S solutions.

Serum H$_2$S concentration was measured with a sulfide sensitive electrode (Model 9616, Orion Research, Beverly) (Khan et al., 1980).

Screening the expression of CSE and CBS in various arteries by RT–PCR

Total RNA was extracted from vascular tissues of animals using a RNeasy Total RNA Kit (Qiagen) and treated with RNase-free DNase I (Ambion). cDNA was synthesized using random hexamers and MULV reverse transcriptase (Perkin-Elmer). The reverse transcription reaction was carried out at room temperature for 15 min followed by incubation at 42°C for 1 h. PCR was performed using Advanced PCR II mixer (Clontech) with gene-specific primers designed based on the reported sequences of the CSE (DDBJ/EMBL/GenBank accession No. D17370) (Nishi et al., 1994) and CBS (DDBJ/EMBL/GenBank accession No. M88344) (Sun et al., 1992), including CSE-forward (5′-aagcagtggctg-cactgg-3′), CSE-reverse (5′-tctgtggtgtgatcgctg-3′), CBS-forward (5′-gccaacttctggcaacac-3′) and CBS-reverse (5′-caccagcatgtccaccttc-3′). cDNA obtained from rat liver was used as the positive expression control for both CSE and CBS. The amplified fragments by PCR were gel-purified and sequenced by T7 sequencing kit (Amersham Pharmacia Biotech). RT–PCR was also used to detect the CSE transcription in cultured SMCs and endothelial cells.

Cloning and sequencing of CSE from rat vascular tissues

A set of primers was synthesized to cover the ORF of CSE: 5′-cgtcccagcatgcagaagaa-3′ and 5′-cagttattcagaaggtctggcccc-3′. PCR was used to amplify the ORF from rat vascular tissues with 32 thermal cycles (denaturing at 95°C for 20 s and annealing/extending at 68°C for 90 s). The amplified ORF of CSE was subcloned into a TOPO TA cloning vector (PCR4-TOPO). Positive clones containing CSE ORF insert were sequenced by automatic sequencing (ABI 373A) and named PCR4/CSE.

Quantitative determination of CSE mRNA level by RNase protection assay (RPA)

DNA templates for the transcription of CSE were obtained by linearizing PCR4/CSE with HindIII (New England Biolabs) digestion. In vitro transcription using T7 polymerase (Ambion) was performed to generate anti-sense [α-^{32}P]UTP (Amersham Pharmacia Biotech) labelled CSE probes, according to the company's protocol (RPA II^{TM}, Ambion). β-actin was used as the housekeeping gene and tRNA (Sigma) was applied as negative control. The signals of the protected fragments were scanned and quantified using an imaging analyser (UN-SCAN-IT). The level of CSE transcript was normalized to that of β-actin in each sample.

In situ hybridization study

To generate sense and antisense probes, the CSE-ORF plasmids were linearized by NotI or PmeI and in vitro transcribed by T3 or T7 RNA polymerase, respectively, in the presence of digoxigenin-labelled UTP (Roche Molecular Biochemicals). Probes were fragmented to ~150 bp in length by alkaline treatment before use. Rat aorta was isolated and immediately snap-frozen by liquid nitrogen and stored at –80°C until use. Cryostat sections of 8 µm were cut on a Micron cryostat at –20°C and thaw-mounted onto ethanol-cleaned slides coated with 1% geltin and 0.1% poly-L-lysine. The sections were vacuum dried and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. After permeablized by incubating with 10 µg/ml proteinase K (Promega) for 30 min at 37°C and post-fixed in 4% paraformaldehyde for 10 min, the sections were dehydrated and prehybridized for 1 h at 50°C in a hybridization buffer consisting of 50% deionized formamide, 2× SSC (1× SSC: 0.15 M NaCl, 0.017 M sodium citrate), 10% dextran sulfate, 1× Denhardt's solution, 0.1 mM EDTA, 0.05 mM Tris–HCl pH 7.5 and 250 µg/ml denatured sonicated salmon sperm DNA (Invitrogen). The prepared slides were then incubated with an anti-sense riboprobe (or a sense probe as a negative control) at 50°C overnight in a humidified box followed by successive incubations in 50% formamide, 2× SSC at 50°C for 30 min and digestion of the single-stranded RNA probe with 20 mg/ml RNase A (Ambion) at 37°C for 30 min. The slides were consecutively washed in 2× SSC at 37°C for 20 min, 1× SSC at 50°C for 20 min, and 0.1× SSC at 50°C for 20 min twice in the shaking water bath. Positive signals were detected by immunoassay with anti-digoxigenin-alkaline phosphatase conjugate and the color substrates NBT/X-phosphate (Roche Molecular Biochemicals).

SMC culture and northern blotting

Single SMCs were isolated and identified from rat aortic tissue as reported previously (Wang et al., 1989; Wu and de Champlain, 1996). Cells were maintained in DMEM containing 10% fetal calf serum in a CO$_2$ incubator at 37°C. The cells at passages 9–13 were seeded into 100 mm dishes for 3–4 days until they became confluent. The confluent monolayers were first incubated in the serum-free DMEM for 24 h and then different concentrations of S-nitroso-N-acetyl-D,L-penicillamine (SNAP) were added to the calcium-free DMEM for another 6 h. At the end of the incubation period, total RNA was extracted by RNeasy Total RNA Kit (Qiagen). Total RNA (15 µg/lane) denatured by glyoxal/dimethylsulf-oxide (Ambion) was subjected to electrophoresis in 1% agarose gels, transferred to positive-charged nylon membranes (Hybond, Amersham Pharmacia Biotech) and UV cross-linked. Hybridization was carried out with the [α-^{32}P]dCTP (Amersham Pharmacia Biotech) labelled DNA probes by Random Primed DNA Labelling Kit (Boehringer Mannheim) in an ULTRAhyb hybridization buffer (Ambion) at 42°C overnight. cDNA fragments cut from PCR4/CSE were used as template for probe labelling. After one low stringency (2× SSC for 20 min at room temperature) and two high stringency (0.1× SSC for 20 min at 42°C) washings, the membranes were exposed to Kodak film for 24–48 h. Levels of CSE mRNA were normalized by the intensity of the corresponding 28S ribosomal RNA band that was visualized with ethidium bromide staining under UV light.

Chemicals and data analysis

H$_2$S solution was prepared daily either by directly bubbling the saline with pure H$_2$S gas (Praxair, Mississauga, Ontario) to make the saturated H$_2$S solution (0.09 M at 30°C) or by using the stock solution of NaHS (1 M) that has been widely used as the in vitro precursor of H$_2$S. Glibenclamide was purchased from RBI (Natick, MA). All other chemicals were from Sigma (St Louis, MO).

The results were expressed as mean ± SE. All concentration–response curves were fitted with a Hill equation, from which IC$_{50}$ was calculated. Student's t-test for unpaired samples was used to compare the mean values between control and tested groups. The changes in blood pressure or heart rate of the same rats were analysed using paired Student's t-test. Statistical significance was set at $P < 0.05$. For comparison of the differences between control and experimental groups and among experimental groups, ANOVA followed by a post-hoc analysis (Newman–Keuls test) was used.

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