J Pharmacol Sci **121**, 318 – 326 (2013)

Journal of Pharmacological Sciences

Full Paper

Acetylcholine- and Sodium Hydrosulfide–Induced Endothelium-Dependent Relaxation and Hyperpolarization in Cerebral Vessels of Global Cerebral Ischemia–Reperfusion Rat

Jun Han[1,2,3], Zhi-Wu Chen[1,*a], and Guo-Wei He[3,4,*b]

[1]*Department of Pharmacology, Anhui Medical University, Hefei, Anhui 230032, China*
[2]*Department of Pharmacology, Wannan Medical College, Wuhu, Anhui 241002, China*
[3]*TEDA International Cardiovascular Hospital, Medical College, Nankai University, Tianjin 300457, China*
[4]*Department of Surgery, Oregon Health and Science University, Portland, Oregon 97239-3098, USA*

Received December 12, 2012; Accepted February 19, 2013

Abstract. We investigated the effects of endothelium-derived hyperpolarizing factor (EDHF) and the role of hydrogen sulphide (H_2S) in the cerebral vasorelaxation induced by acetylcholine (ACh) in global cerebral ischemia–reperfusion (CIR) rats. CIR was induced by occlusion of bilateral carotid and vertebral arteries. Isolated arterial segments from the cerebral basilar (CBA) and middle artery (MCA) of CIR rats were studied in a pressurized chamber. Transmembrane potential was recorded using glass microelectrodes to evaluate hyperpolarization. In the CIR CBAs and MCAs preconstricted by 30 mM KCl, ACh induced concentration-dependent vasorelaxation and hyperpolarization that were partially attenuated by NG-nitro-L-arginine methyl ester (L-NAME, 30 μM) and L-NAME plus indomethacin (10 μM). The residual responses were abolished by the H_2S inhibitor DL-propargylglycine (PPG, 100 μM). The H_2S donor NaHS and L-Cys, the substrate of endogenous H_2S synthase, elicited similar responses to ACh and was inhibited by tetraethylamonine (1 mM) or PPG. ACh induces EDHF-mediated vasorelaxation and hyperpolarization in rat cerebral arteries. These responses are up-regulated by ischemia–reperfusion while NO-mediated responses are down-regulated. Further, the ACh-induced, EDHF-mediated relaxation, and hyperpolarization and the inhibition of these responses are similar to the H_2S-induced responses, suggesting that H_2S is a possible candidate for EDHF in rat cerebral vessels.

Keywords: endothelium, nitric oxide, endothelium-derived hyperpolarizing factor (EDHF), hydrogen sulphide, cerebral artery

Introduction

Endothelium-derived hyperpolarizing factor (EDHF), the third kind of relaxing factor and autacoids, is derived from vascular endothelium apart from nitric oxide (NO) and prostaglandin I_2 (PGI_2, prostacyclin) (1, 2). This factor is a potentially important modulator in the regulation of organ blood flow and vascular resistance during normal physiological states in animal (3, 4) and in the human circulation (5 – 8) and plays an even greater role following pathological conditions such as organ ischemia, hypoxia, and acidosis (9, 10).

Although EDHF has been proved to be present in various blood vessels, including mesenteric arteries (11), coronary arteries (8, 12), carotid artery (13), femoral artery (14), other somatic arteries (7, 8), and pulmonary arteries (6, 15), and so on. Yet, its chemical nature, particularly in cerebral arteries, still remains unclear. In this regard, multiple mechanisms of EDHF-mediated hyperpolarization and vasorelaxation have been proposed and the possible candidates include a number of substances such as epoxyeicosatrienoic acids (EETs) (16), potassium ion (17), H_2O_2 (18),and carbon monoxide (19). Recently, hydrogen sulphide (H_2S) has been demonstrated to relax smooth muscle cells through the

Corresponding authors.
*[a]ZW_Chen120@163.com, *[b]gwhezj@163.com
Published online in J-STAGE
doi: 10.1254/jphs.12277FP

release of EDHF and NO from the endothelium (20) and is defined as the third kind of novel gaseous transmitter besides NO and carbon monoxide (20). It has been shown that vascular endothelium can induce production of endogenous H_2S, leading to hyperpolarization and vasorelaxation responses (21) and that H_2S is involved in regulating a vast number of physiological and pathological processes in vitro (22, 23). Further, it has recently been reported that NaHS relaxes rat cerebral artery in vitro via inhibition of L-type voltage-sensitive Ca^{2+} channels (24).

In the cerebral circulation, it has been reported that both NO and EDHF played significant roles in controlling cerebrovascular tone (25). Further, EDHF-mediated dilations in the rat middle cerebral artery do not comprise the epoxidase pathway, lipoxygenase pathway, or reactive oxygen species including hydrogen peroxide (H_2O_2) (26). Therefore, other substances may be account for the EDHF-mediated responses in the cerebral artery.

In our previous studies (27, 28), we have found that in cerebral basilar arteries (CBAs) and middle cerebral arteries (MCAs) of healthy rats, H_2S had potential to induce EDHF responses.

The present study was designed to investigate the role of EDHF and the H_2S donor sodium hydrosulfide (NaHS) or the substrate of endogenous H_2S synthase, L-Cysteine (L-Cys) in acetylcholine (ACh)-induced vasodilation in the rat CBAs and MCAs with regard to the nature of EDHF in the cerebral vasculature and the role of EDHF and H_2S in the cerebral ischemia–reperfusion (CIR) rat model with implications in clinical therapy of brain injuries.

Materials and Methods

Drugs and solutions

ACh, *N*-nitro-L-arginine-methyl-ester (L-NAME), indomethacin (Indo), DL-propargylglycine (PPG), L-Cys, sodium hydrosulfide (NaHS), tetraethylammonium (TEA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of the highest purity and of analytical grade. Physiologic saline solution (PSS) (29) contained 118 mM NaCl, 3.4 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.1 mM glucose and was bubbled with 95% O_2 and 5% CO_2. The pH of the PSS solution was adjusted to 7.4 with NaOH and the solution was oxygenated during the incubation period.

Methods

Male Sprague-Dawley rats (250 – 350 g body weight) were obtained from the Experimental Animal Centre of Anhui Medical University (Hefei, China) (Certificate No. SCXK 2005-001). All animal study protocols were approved by the Animal Care and Use Committee at Anhui Medical University, which conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication NO. 85-23, revised 1996).

Establishment of CIR rat model

Rats were anesthetized with a peritoneal injection (0.3 – 0.35 g/kg body weight) of 10% chloral hydrate (Shanghai, China), using the four-vessel occlusions methods of Pulsinelli et al. (4-VO) (30, 31). Briefly, skin incision was made above the spinous processes of the first cervical vertebral column to expose the vertebral arteries at the alar foramina of the atlas. The vertebral arteries were electrocoagulated before incision suturing. The bilateral common carotids were exposed and isolated with elastic surgery threads. Animals were kept in rearing cages for 24 h with free access to water and food. Bilateral carotid arteries had been simultaneously blocked using arteriole clamps for 0.5 h in the experiment with electroencephalogram (EEG; EB Neuro Corp., S.p.A, Firenze, Italy) monitoring and then the occluders were softly removed to restore the blood flow through bilateral carotid arteries. EEG was recorded for ischemia; ischemia 0.5 h; and reperfusion 5, 15, 30, 45, 60, and 120 min, respectively.

Sham-operation was used as controls in which the animals were free of electrocoagulation of vertebral arteries and occlusion of carotid arteries.

Rats with rigidity of the forepaws and loss of righting reflex during ischemia and survived after 2 h reperfusion, in which the wave amplitude of EEG was rapidly decreased to become a straight line during ischemia and gradually recovered when reperfused, were eligible for study (Fig. 1).

Experiments in all animals were performed under a specific environment by maintaining the core body temperature at 37°C through rectal temperature determination with a temperature controller coupled to a heating pad.

Isolated vessels experiments

After 2 h of reperfusion, the rats were anesthetized with 10% chloral hydrate and then killed by decapitation. Their brains were quickly removed from the cranium and placed in ice-cold PSS. CBA and MCA vessel were dissected carefully, made into a segment (0.6 – 0.8 mm in length) and kept in a vessel container. Each vessel was independently inserted into two glass micropipettes and tied in place within a vessel chamber, which was pressurized to a mean of 85 mmHg and established a flow discharge of 150 μl/min through the lumen. Each

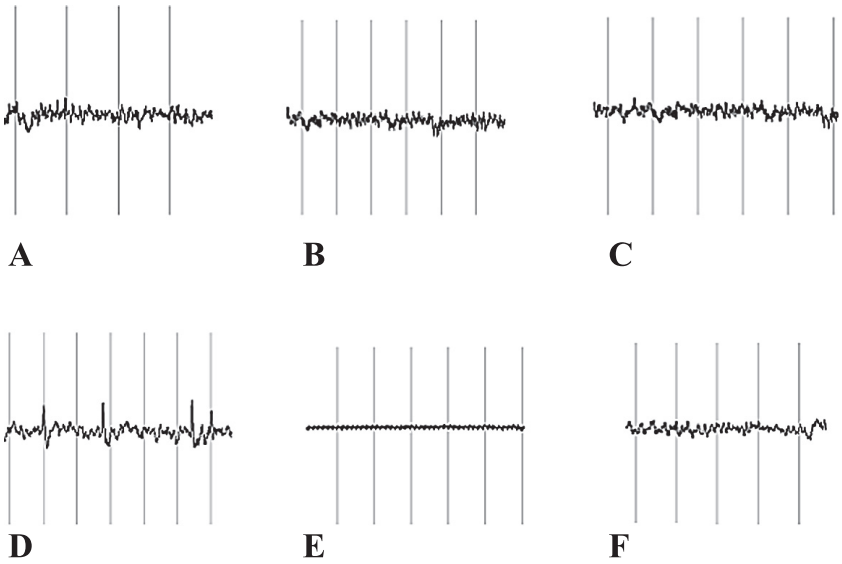

Fig. 1. The original record of electroencephalogram monitoring in sham and CIR rats. A: Sham, B: Sham (ischemia for 30 min), C: Sham (reperfusion for 2 h), D: CIR, E: CIR (ischemia for 30 min), F: CIR (reperfusion for 2 h).

CBA or MCA was immersed in the bathing chamber filled with PSS, which was aerated with 95% O_2–5% CO_2 (PH 7.3 – 7.5) and maintained at 37°C using a fixed heat-exchanger device. Reproducible constriction was obtained by adding 30 mM KCl to the luminal perfusate. Respectively, Indo (10 μM) and L-NAME (30 μM) were used respectively to block the effects of cyclooxygenase and NO synthase products in all experiments.

After CBA or MCA was mounted, the vessel container was placed on the stage of a stereoscopic microscope (Bengbu, China), which was equipped with a digital camera (Nikon, Tokyo) and a computer screen. Outer diameters were measured directly from the video screen (magnification of × 100). Vascular tissues were allowed to balance for 0.5 h before the study was implemented. During times of vasomotion, the average maximum and minimum diameters were recorded on CBAs and MCAs. The vessel diameter was measured continuously using image-analysis software (Optimas 6.0; Optimus Corp., Bothell, WA, USA) on a DELL Pentium computer.

Electrophysiology experiments

Isolated vessels for sham-operation and CIR vessels were perfused with warmed (37°C) PSS to achieve a mean pressure of 85 mmHg. Vascular smooth muscle cells (VSMC) of the CBA or MCA were pierced with glass microelectrodes and measured for intracellular membrane potential (E_m) as previously reported. In brief, each CBA or MCA was longitudinally dissected and fixed within the silica gel slot of 10-ml volume, which was perfused either with PSS kept at 37°C and oxygenated with 95% O_2–5% CO_2, or PPS with 30 μM L-NAME plus 10 μM Indo. Each VSMC was pierced with glass microelectrodes filled with KCl (30 mM, electrode resistances ranged from 40 to 80 MΩ). A successful impalement was shown by a sudden drop in the membrane voltage kept stable for at least 2 min before initiating the experiment. In order to avoid excessive vessel tissue lesion, the location was frequently displaced. A single E_m value for each condition in a specified CBA or MCA was obtained by averaging four to six different VSMC impalements. A traditional high-impedance amplifier (Intra 767; World Precision Instruments, Sarasota, FL, USA) was used to record the potential difference and interference (50 Hz) at the amplifier output that was selectively moved aside. The Powerlab/4sp system connected with Chart 5 software (AD Instruments, Castle Hill, NSW, Australia) was used to monitor and analyze the smooth muscle membrane potential.

Statistical analysis

All data are expressed as the mean ± S.E.M. Analyses of percent diameter changes in CBAs and MCAs were calculated by using the following formula: % Relaxation = $[(D - D_{min}) / (D_{max} - D_{min})] \cdot 100$, where D expresses the vessel diameter after the addition of the reagents, either ACh or L-NAME etc., D_{min} stands for the minimal diameter after drug addition of 30 mM KCl, and D_{max} is the maximum diameter. The maximum diameter was obtained at 85 mmHg luminal pressure after 1-h equilibrium.

The differences between Sham-operated and CIR vessels and between different treatments were tested using the unpaired Student's *t*-test. *P*-value < 0.05 was regarded as statistically significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software.

Results

Effect of L-NAME on ACh-mediated dilatation in CIR vessels

ACh ($10^{-7} - 10^{-4.5}$ M) evoked concentration-dependent relaxation in CIR CBAs and MCAs, which were precontracted by 30 mM KCl. The percentage of maximal relaxation (E_{max}) was 85.2% ± 6.2% and 70.8% ± 4.7% ($P = 0.000$) in sham-operated and CIR CBAs and 78.7% ± 5.4% and 69.3% ± 7.5% ($P = 0.012$) in sham-operated and CIR MCAs, respectively. ACh-mediated dilatations were inhibited by 30 μM L-NAME added in the lumen perfusate, E_{max} being reduced to 50.3% ± 6.1% and 61.8% ± 5.3% ($P = 0.001$) in sham and CIR CBAs (Fig. 2A) and 53.0% ± 4.0% and 61.4% ± 5.9% ($P = 0.005$) in sham and CIR MCAs, respectively (Fig. 2B).

These results revealed that NO was involved in vasorelaxation in 30 mM KCl–preconstricted rings for sham and CIR vessels; as compared with NO-mediated relaxation in sham vessels, the relaxation was attenuated in CIR vessels. Interestingly, after blockade of NO production by L-NAME, the residual relaxation in CIR vessels was remarkably greater than that in sham vessels (E_{max}: 61.8% ± 5.3% vs. 50.3% ± 6.1%, $P = 0.001$), suggesting that non-NO–mediated relaxation may be augmented in CIR vessels.

Effect of L-NAME + Indo on ACh-induced vasorelaxation in CIR vessels

Co-application of L-NAME (30 μM) and Indo (10 μM) did not completely block ACh-induced vasorelaxation, leading to attenuation of E_{max} by 58.1% ± 4.1% and 40.8% ± 3.5% ($P = 0.001$) in the CIR and sham CBAs

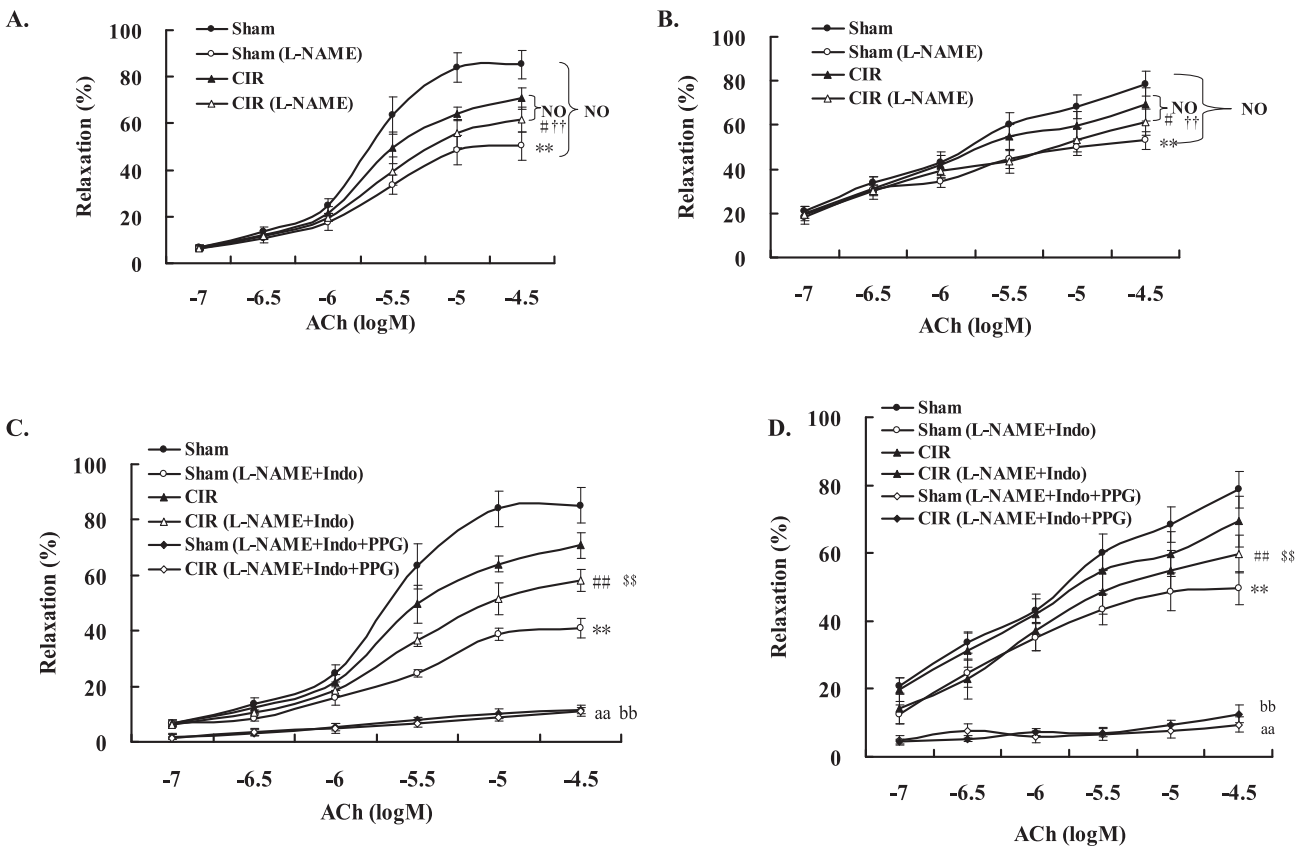

Fig. 2. The relaxation to acetylcholine (ACh) by 30 mM KCl–precontracted cerebral arteries in CIR and sham rat. Effects of 30 μM L-NAME (an inhibitor of nitric oxide synthase) alone in cerebral basilar arteries (CBAs) (n = 8) (A) and middle cerebral arteries (MCAs) (n = 8) (B); Effects of 30 μM L-NAME and 10 μM Indo (an inhibitor of cyclooxygenase) or plus 100 μM PPG (an inhibitor of cystathionine-γ–lyase) in CBAs (n = 8) (C) and MCAs (n = 8) (D). $^{**}P < 0.01$, compared with the Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with CIR group; $^{\dagger\dagger}P < 0.01$, CIR compared with the Sham group for reduction in the percentage maximal relaxation; $^{\$\$}P < 0.01$, compared with the Sham group in the presence of 30 μM L-NAME + 10 μM Indo; $^{aa}P < 0.01$, compared with the Sham group after treatment with 30 μM L-NAME plus 10 μM Indo. $^{bb}P < 0.01$, compared with the CIR group after treatment with 30 μM L-NAME + 10 μM Indo. All values are presented as mean percentage relaxation ± S.E.M. All comparisons were assessed by the unpaired Student's *t*-test.

(Fig. 2C) and 59.6% ± 5.5% and 49.6% ± 4.8% (P = 0.002) in the CIR and sham MCAs (Fig. 2D), respectively. These results showed that in the presence of the inhibitors, ACh was still capable of producing concentration-dependent relaxation in CIR segments. Further, compared to the dilations to ACh in sham vessels, those of the CIR vessels were obviously potentiated ($P < 0.01$). Figure 2, C and D revealed that the non-NO, non-PGI_2 vasorelaxation response may be upregulated in CIR segments.

Compositive effect of L-NAME + Indo + PPG on ACh-induced dilation in CIR vessels

PPG, an inhibitor of cystathionine-γ–lyase (CSE) that is a synthase of the endogenous production of H_2S, is frequently used to inhibit the biosynthesis of CSE (32 – 36). Figure 2, C and D demonstrate that PPG (100 μM) markedly restrained ACh-induced dilation in the presence of 30 μM L-NAME plus 10 μM Indo. In CIR CBAs, the E_{max} was reduced from 58.1% ± 4.1% to 10.9% ± 1.6% (P = 0.000, Fig. 2C). Similarly, the E_{max} of CIR MCAs was decreased from 59.6% ± 5.5% to 12.7% ± 2.5% (P = 0.000, Fig. 2D). These findings suggested that ACh-induced non-NO, non-PGI_2 mediated vasorelaxation was potentially associated with the endogenic release of H_2S.

Effect of Indo + L-NAME on ACh-mediated hyperpolarization in CIR vessels

The resting membrane potential (E_m) of VSMCs was measured in rat CBAs and MCAs under similar conditions and the original records of E_m in rat MCAs are summarized in Fig. 3. For 1 h of equilibration, the average value of E_m was determined at −49.2 ± 3.5 mV in CIR CBAs and −44.3 ± 2.1 mV in CIR MCAs (P = 0.052). ACh ($10^{-7} - 10^{-4.5}$ M) produced notable concentration-dependent hyperpolarization in both sham and CIR vessels. Figure 4A (CBA) and 4B (MCA) show that in the presence of Indo plus L-NAME, ACh-mediated hyperpolarization was partially suppressed in both CIR and sham groups although there was a considerable residual response. The maximal change in E_m was significantly different between the groups with more depression in the Sham than in the CIR ($P < 0.05$). Furthermore, the absolute value of the maximal changes in E_m for CIR segments was significantly greater than that of sham segments ($P < 0.05$).

Combined effect of Indo + L-NAME + PPG on ACh-induced hyperpolarization in CIR vessels

After treatment with L-NAME and Indo, the hyperpolarization to ACh was further attenuated by PPG in CIR CBAs (−10.1 ± 1.3 vs. −4.9 ± 0.4 mV, $P < 0.01$; Fig. 4A) and MCAs (12.5 ± 1.2 vs. −3.9 ± 0.3 mV, $P < 0.01$; Fig. 4B), and the maximal change in E_m was −4.9 ± 0.4 mV and −3.9 ± 0.3 mV, respectively.

H_2S donor–elicited relaxation and hyperpolarization

After blockade of NO and PGI_2 synthase with 30 μM L-NAME plus 10 μM Indo, L-Cys ($10^{-5} - 10^{-2.5}$ M), the substrate of endogenous H_2S synthesis, induced dose-dependent relaxation (E_{max}: 57.0% ± 4.3%, Fig. 5A) and

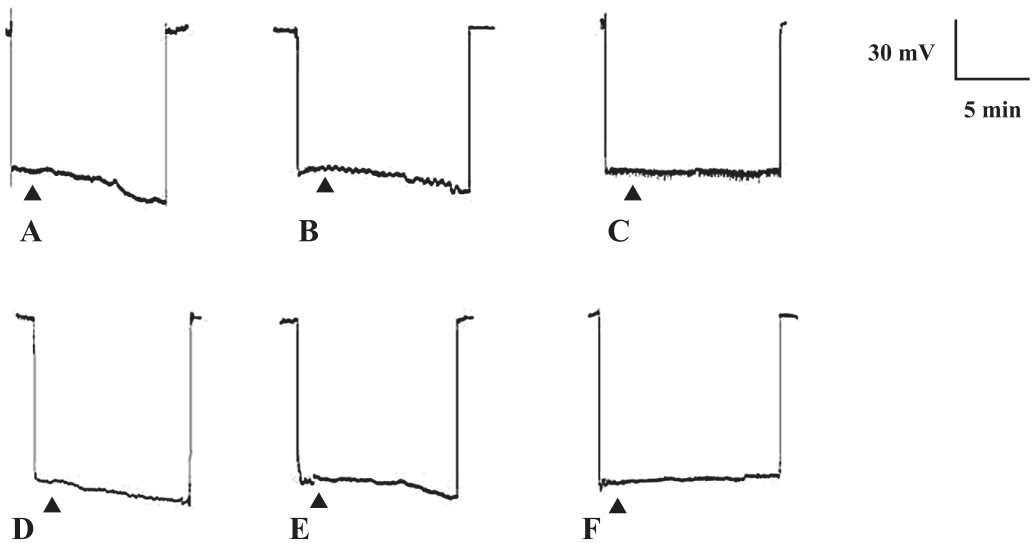

Fig. 3. The original trace record of membrane potential measurement in the smooth muscle cell in sham and CIR rat middle cerebral arteries in various protocols. A: Sham (ACh), B: Sham (ACh + L-NAME + Indo), C: Sham (ACh + L-NAME + Indo + PPG), D: CIR (ACh), E: CIR (ACh + L-NAME + Indo), F: CIR (ACh + L-NAME + Indo + PPG).

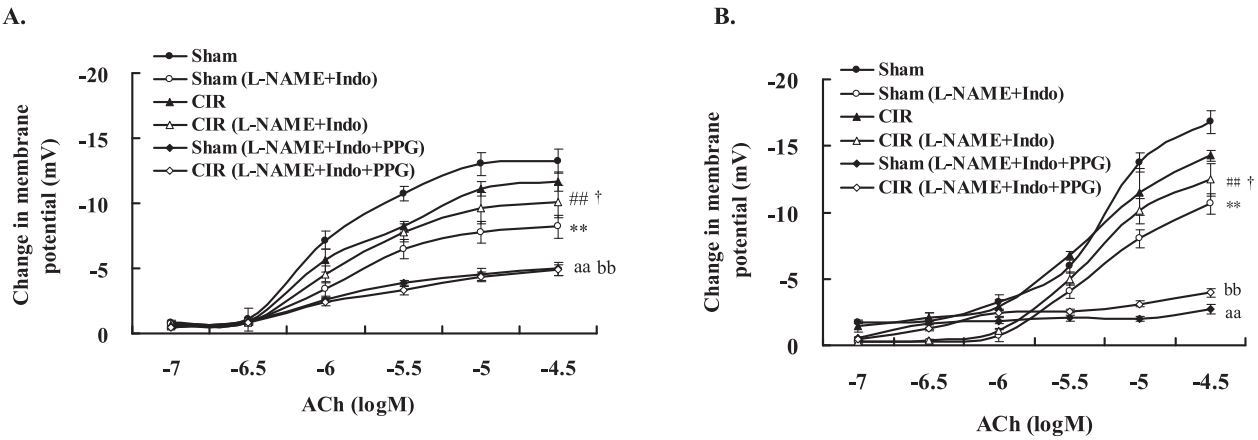

Fig. 4. Effects of 30 μM L-NAME and 10 μM Indo or plus 100 μM PPG on the hyperpolorization to ACh by 30 mM KCl–precontracted in CIR and sham rat cerebral basilar arteries (CBAs) (n = 6) (A) and middle cerebral arteries (MCAs) (n = 6) (B). Values are presented as means ± S.E.M. $^{**}P < 0.01$, compared with the Sham group; $^{\#\#}P < 0.01$, compared with the CIR group; $^{\dagger}P < 0.05$, compared with the Sham group in the presence of 30 μM L-NAME + 10 μM Indo; $^{aa}P < 0.01$, compared with the Sham group after treatment with 30 μM L-NAME plus 10 μM Indo; $^{bb}P < 0.01$, compared with the CIR group after treatment with 30 μM L-NAME + 10 μM Indo. The unpaired Student's *t*-test was used for comparison.

hyperpolarization (maximal change in E_m: -10.2 ± 1.1 mV, Fig. 5C) in MCA of rat subjected to CIR, and these were almost abolished by PPG (100 μM), an inhibitor of the endogenous H_2S synthase-CSE.

Similarly, NaHS ($10^{-5} - 10^{-2.5}$ M), a donor of exogenous H_2S, significantly elicited concentration-dependent relaxation and hyperpolarization of VSMC in CIR MCA that were partially inhibited by 30 μM L-NAME plus 10 μM Indo (Fig. 5: B and D). The residual relaxation and hyperpolarization were abolished by 1 mM TEA, an inhibitor of potassium channels ($P < 0.01$, Fig. 5: B and D).

Discussion

In this study, we have found that 1) in both Sham and CIR rats, ACh induced EDHF-mediated vasorelaxation and hyperpolarization of the VSMCs of cerebral arteries in a concentration-dependent manner; 2) in cerebral arteries of rat subjected to ischemia-reperfusion, the EDHF-mediated responses were upregulated while NO was downregulated; and 3) in rat CIR vessels, H_2S was likely to be the chemical identity of EDHF.

ACh induced NO- and EDHF-mediated relaxation and hyperpolarization of the VSMCs of cerebral arteries in both Sham and CIR rats

The present study has demonstrated that EDHF-mediated responses exist in normal rats (Sham). This was shown by the residual relaxation and hyperpolarization in the presence of inhibitors of eNOS and PGI_2 (Figs. 2A and 4A). The effect of ischemia–reperfusion attenuated both relaxation (Fig. 2) and hyperpolarization by ACh (Fig. 4). In fact, the NO-mediated responses were significantly attenuated by ischemia–reperfusion (compare Sham and CIR in Figs. 2 and 4). In contrast, the EDHF-mediated relaxation and hyperpolarization induced by ACh were upregulated by ischemia–reperfusion [compare CIR (L-NAME) to Sham (L-NAME) and CIR (L-NAME + Indo) to Sham (L-Name + Indo)]. The upregulation of the EDHF-pathway under ischemia–reperfusion has been previously reported by others and in our own observations. The present study is in accordance with these resorts.

H_2S is the possible chemical identity of EDHF in rat cerebral vessels

Since EDHF has been proposed (1, 37), there have been great efforts to identify the chemical nature of EDHF as mentioned above. In comparison to other peripheral vessels, little is known about the identity of EDHF in cerebral vessels. You et al. reported that the identity and/or process of EDHF in cerebral vessels was possibly distinct with that in peripheral vessels. Interestingly, neither EETs nor H_2O_2 in the rat MCA was proven to have a relationship with EDHF-mediated dilations (31).

H_2S, a conventional toxic gas, is recently recognized as an important gasotransmitter and capable of producing dilation by activating ATP-sensitive K^+ channel (K_{ATP}) in VSMC, such as in rat aorta, tail artery, and mesenteric

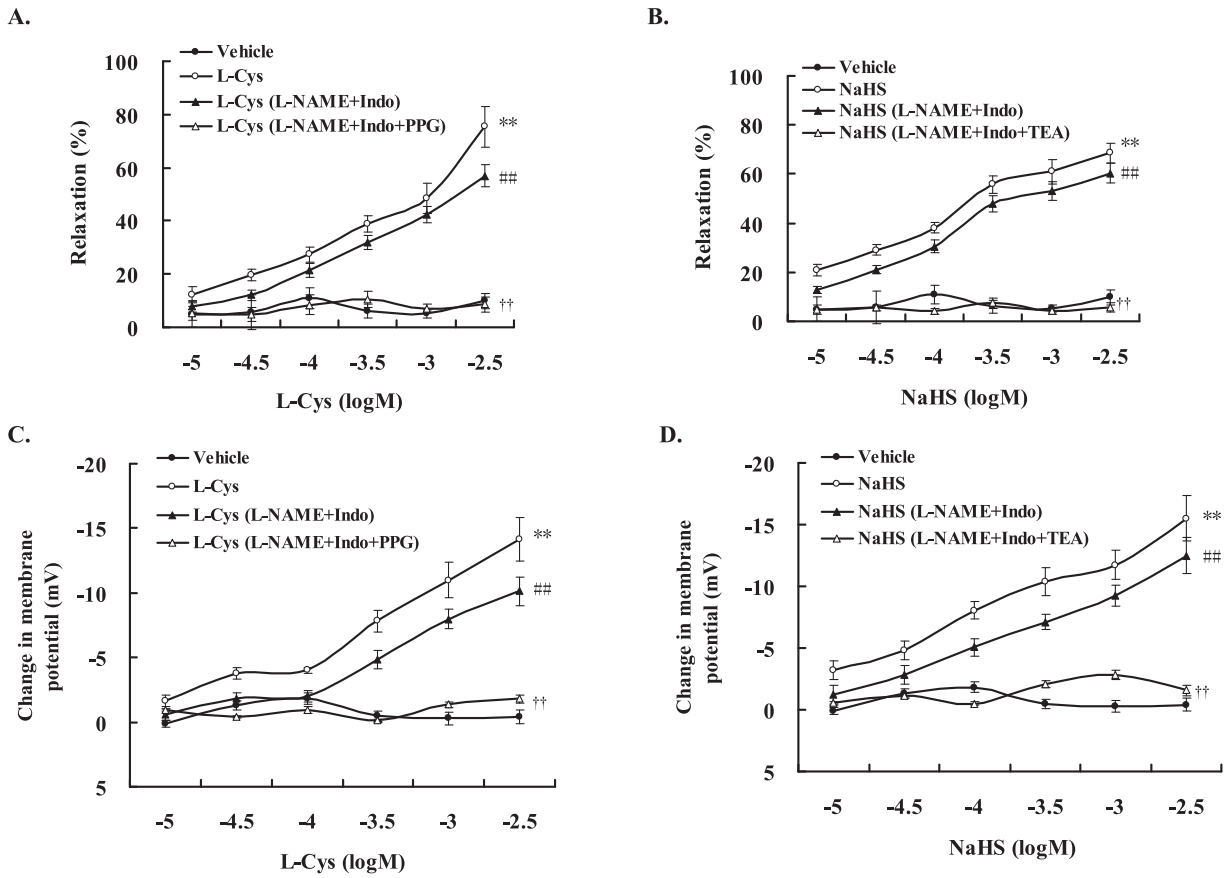

Fig. 5. L-Cys–elicited relaxation of 30 mM KCl–preconstricted middle cerebral artery in CIR rat and the effect of PPG on the relaxation in the presence of 30 μM L-NAME and 10 μM Indo (n = 8) (A); NaHS-mediated relaxation for the same treatment and the effect of TEA, an inhibitor of calcium-activated potassium channels (n = 8) (B); L-Cys–induced hyperpolarization of membrane potential of VSMC from the middle cerebral artery of rat subjected to CIR in the presence of L-NAME plus Indo and the effect of PPG on the hyperpolarizing action (C); NaHS-produced hyperpolarization for the same treatment and the effect of TEA (n = 8) (D). Values are presented as means ± S.E.M. $^{**}P < 0.01$ vs. Vehicle, $^{\#\#}P < 0.01$ vs. L-Cys or NaHS group. $^{\dagger\dagger}P < 0.01$, compared with L-Cys or NaHS group in the presence of 30 μM L-NAME + 10 μM Indo. Comparisons were performed by the unpaired Student's *t*-test.

artery. It can be generated endogenously from L-Cys by catalysis of two pyridoxal-5′-phosphate-dependent enzymes, cystathionine-b-synthase (CBS) and CSE, in mammalian cells (38). Expression of these two enzymes possesses tissue-type specificity. In pancreatic β-islets, CSE is the capital H_2S-generating enzyme with different vascular tissues (39). The expression of CSE proteins has been found in vascular endothelial cells (40). PPG, an inhibitor of CSE could prevent H_2S production and H_2S-induced vasodilation by restraining L-Cys–dependent increase in vascular tissue. We have detected the expression of CSE proteins in rat CBAs (28).

The present study revealed that ACh-mediated relaxation and membrane potential hyperpolarization were markedly decreased by PPG after L-NAME and Indo administration in both Sham and CIR rats, suggesting that H_2S plays a role in the EDHF-mediated responses. Further evidence of the role of H_2S in EDHF-mediated responses is that the upregulation of EDHF in CIR rats was abolished with PPG.

In the present study, we also studied the role of exogenous H_2S donor NaHS and the substrate of endogenous H_2S synthesis CSE L-Cys. The results showed that NaHS and L-Cys generated a similar extent of hyperpolarization and relaxation in MCA of rat subjected to ischemia–reperfusion. The similar responses elicited by ACh and H_2S in the rat cerebral vessels and the blockage of the ACh-induced EDHF-mediated responses by H_2S synthase inhibitor have provided solid evidence that the ACh-induced EDHF-mediated responses in this vasculature might be through the H_2S pathway. These experiments therefore suggest that H_2S is a possible candidate for EDHF in the cerebral vessels. The significant relaxation and hyperpolarization elicited by

L-Cys in the present study probably imply that H_2S is a back-up for NO in the rat cerebral artery subjected to ischemia–reperfusion while NO-mediated responses are down-regulated.

Further, TEA, an inhibitor of Ca^{2+}-activated potassium channel, abolished the NaHS-induced effects, suggesting that the NaHS-induced, non-NO and non-PGI_2 effect may be relevant to Ca^{2+}-activated potassium channels. However, further studies would be warranted to identify the subtypes of the Ca^{2+}-activated potassium channels.

An interesting aspect of the gaseous messengers is their multiple interaction with substances such as O_2, NO, H_2S, and carbon monoxide (41). The complex interaction of H_2S with NO in regulating cardiovascular function in health and disease has been highlighted (35). It has been shown that there is 'crosstalk' between NO and H_2S in mice and rats with endotoxic shock (42). The present study, although it was not focused on this aspect, also shows some evidence of the interaction between NO and H_2S. Indeed, our data showed that in cerebral arteries of rats subjected to ischemia–reperfusion, the responses mediated by EDHF, likely related to H_2S as discussed above, were upregulated while NO was down-regulated. This may suggest that H_2S may act as a back-up for NO under pathological conditions such as ischemia–reperfusion. This interesting topic should be further explored.

In summary, the present study has found that ACh induces EDHF-mediated vasorelaxation and hyperpolarization in rat cerebral arteries. These responses are up-regulated by ischemia–reperfusion while NO-mediated responses are down-regulated. Further, the ACh-induced, EDHF-mediated relaxation and hyperpolarization and the inhibition of these responses in rats subjected to cerebral ischemia–reperfusion are similar to the H_2S-induced responses, suggesting that H_2S is a possible candidate of EDHF in rat cerebral vessels.

Acknowledgments

The experimental work aforementioned was supported by the National Natural Science Foundation of China under Grant No. 30840104, No. 81173596, and No. 81170148; the Doctor Subject Fund of Education of Ministry of China under Grant No. 20070366005; the Natural Science Foundation of Anhui province under Grant No. 11040606M196; and the Natural Science Foundation of the Department of Education of Anhui province under Grant No. kj2011B200. The authors appreciated the contributions from the other members of our laboratory: YF Fan, B Song, YF Chen, and QH Wang.

References

1 Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of canine coronary smooth muscle. Br J Pharmacol. 1988;93:515–524.
2 Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of vascular smooth muscle cells. Acta Pharm Sin. 2000; 21:1–18.
3 Golding EM, Marrelli SP, You J, Bryan RM Jr. Endothelium-derived hyperpolarizing factor in the brain: a new regulator of cerebral blood flow? Stroke. 2002;33:661–663.
4 Ge ZD, Zhang XH, Fung PC, He GW. Endothelium-dependent hyperpolarization and relaxation resistance to N(G)-nitro-L-arginine and indomethacin in coronary circulation. Cardiovasc Res. 2000;46:547–556.
5 Yang Q, Yim AP, He GW. The significance of endothelium-derived hyperpolarizing factor in the human circulation. Curr Vasc Pharmacol. 2007;5:85–92.
6 Zhang RZ, Yang Q, Yim AP, Huang Y, He GW. Role of NO and EDHF-mediated endothelial function in the porcine pulmonary circulation: comparison between pulmonary artery and vein. Vascul Pharmacol. 2006;44:183–191.
7 He GW, Liu ZG. Comparison of nitric oxide release and endothelium-derived hyperpolarizing factor-mediated hyperpolarization between human radial and internal mammary arteries. Circulation. 2001;104:I344–I349.
8 Liu ZG, Ge ZD, He GW. Difference in endothelium-derived hyperpolarizing factor-mediated hyperpolarization and nitric oxide release between human internal mammary artery and saphenous vein. Circulation. 2000;102:III296–III301.
9 Dong YY, Wu M, Yim AP, He GW. Hypoxia-reoxygenation, St. Thomas cardioplegic solution, and nicorandil on endothelium-derived hyperpolarizing factor in coronary microarteries. Ann Thorac Surg. 2005;80:1803–1811.
10 Schildmeyer LA, Bryan RM Jr. Effect of NO on EDHF response in rat middle cerebral arteries. Am J Physiol Heart Circ Physiol. 2002;282:H734–H738.
11 Liu MY, Hattori Y, Fukao M, Sato A, Sakuma I, Kanno M. Alterations in EDHF-mediated hyperpolarization and relaxation in mesenteric arteries of female rats in long-term deficiency of oestrogen and during oestrus cycle. Br J Pharmacol. 2001;132: 1035–1046.
12 Zhang R, Bai N, So J, Laher I, MacLeod KM, Rodrigues B. The ischemic metabolite lysophosphatidylcholine increases rat coronary arterial tone by endothelium-dependent mechanisms. J Mol Cell Cardiol. 2009;47:112–120.
13 Corriu C, Félétou M, Canet E, Vanhoutte PM. Vanhoutte. Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig. Br J Pharmacol. 1996;119:959–964.
14 Leung HS, Leung FP, Yao X, Ho WH, Chen ZY, Vanhoutte PM, et al. Endothelia mediators of the acetylcholine-induced relaxation of the rat femoral artery. Vascul Pharmacol. 2006; 44:299–308.
15 Yang Q, Shigemura N, Underwood MJ, Hsin M, Xue HM, Huang Y, et al. NO and EDHF pathways in pulmonary arteries and veins are impaired in COPD patients. Vascul Pharmacol. 2012;57:113–118.
16 Gauthier KM, Deeter C, Krishna UM, Reddy YK, Bondlela M, Falck JR, et al. 14, 15-Epoxyeicosa-5(Z)-enoicacid: a selective epoxyeicosatrienoic acid antagonist that inhibits endothelium-dependent hyperpolarization and relaxation in coronary arteries. Circ Res. 2002;90:1028–1036.
17 Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH. K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. Nature. 1998;396:269–272.

18 Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. J Clin Invest. 2000;106:1521–1530.
19 Félétou M, Vanhoutte PM. Endothelium-dependent hyperpolarizations: past beliefs and present facts. Ann Med. 2007;39: 495–516.
20 Skovgaard N, Gouliaev A, Aalling M, Simonsen U. The role of endogenous H_2S in cardiovascular physiology. Curr Pharm Biotechnol. 2011;12:1385–1393.
21 Mustafa AK, Sikka G, Gazi SK, Steppan J, Jung SM, Bhunia AK, et al. Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. Circ Res. 2011;109: 1259–1268.
22 Li XH, Du JB, Bu DF, Tang XY, Tang CS. Sodium hydrosulfide alleviated pulmonary vascular structural remodeling induced by high pulmonary blood flow in rats. Acta Pharmacol Sin. 2006; 27:971–980.
23 Geng B, Yang J, Qi Y, Zhao J, Pang Y, Du J, et al. H_2S generated by heart in rat and its effects on cardiac function. Biochem Biophys Res Commun. 2004;313:362–368.
24 Tian XY, Wong WT, Sayed N, Luo J, Tsang SY, Bian ZX, et al. NaHS relaxes rat cerebral artery in vitro via inhibition of l-type voltage-sensitive Ca^{2+} channel. Pharmacol Res. 2012;65:239–246.
25 Watanabe Y, Kusama1 N, Itoh T. Effects of chronic in vivo administration of nitroglycerine on ACh-induced endotheliumdependent relaxation in rabbit cerebral arteries. Br J Pharmacol. 2008;153:132–139.
26 You J, Golding EM, Bryan RM Jr. Arachidonic acid metabolites, hydrogen peroxide, and EDHF in cerebral arteries. Am J Physiol Heart Circ Physiol. 2005;289: H1077–H1083.
27 Fan YF, Chen ZW, Guo Y, Wang QH, Song B. Cellular mechanisms underlying Hyperin-induced relaxation of rat basilar artery. Fitoterapia. 2011;82:626–631.
28 Cai SN, Fan YF, Chen ZW. The hyperpolorization produced by H_2S in VSMC from middle cerebral artery of rat. Chin J Clin Pharmacol Ther. 2011;16:155–159.
29 Bryan RM Jr, Eichler MY, Swafford MW, Johnson TD, Suresh MS, Childres WF. Stimulation of 2 adrenoceptors dilates the rat middle cerebral artery. Anesthesiology. 1996;85:82–90.
30 Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke. 1979;10:267–272.
31 Marrelli SP, Childres WF, Goddard-Finegold J, Bryan RM Jr. Potentiated EDHF-mediated dilations in the rat middle cerebral artery following ischemia/reperfusion. In: Vanhoutte PM, editor. EDHF 2000. London: Taylor & Francis; 2001.
32 Leffler CW, Parfenova H, Basuroy S, Jaggar JH, Umstot ES, Fedinec AL. Hydrogen sulfide and cerebral microvascular tone in newborn pigs. Am J Physiol Heart Circ Physiol. 2011; 300:H440–H447.
33 Schleifenbaum J, Köhn C, Voblova N, Dubrovska G, Zavarirskaya O, Gloe T, et al. Systemic peripheral artery relaxation by KCNQ channel openers and hydrogen sulfide. J Hypertens. 2010;28:1875–1882.
34 Lee AT, Shah JJ, Li L, Cheng Y, Moore PK, Khanna S. A nociceptive-intensity-dependent role for hydrogen sulphide in the formalin model of persistent inflammatory pain. Neuroscience. 2008;152:89–96.
35 Whiteman M, Moore PK. Hydrogen sulfide and the vasculature: a novel vasculoprotective entity and regulator of nitric oxide bioavailability? J Cell Mol Med. 2009;13:488–507.
36 Köhn C, Schleifenbaum J, Szijártó IA, Markó L, Dubrovska G, Huang Y, et al. Differential effects of cystathionine-γ-lyase-dependent vasodilatory H_2S in periadventitial vasoregulation of rat and mouse aortas. PLoS One. 2012;7:e41951.
37 Chen G, Suzuki H, Weston AH. Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. Br J Pharmacol. 1988;95:1165–1174.
38 Levonen AL, Lapatto R, Saksela M, Raivio KO. Human cystathionine gamma-lyase: developmental and in vitro expression of two isoforms. Biochem J. 2000;347:291–295.
39 Wang R. Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? FASEB J. 2002;16:1792–1798.
40 Wang R. Hydrogen sulfide: a new EDRF. Kidney Int. 2009; 76:700–704.
41 Kajimura M, Fukuda R, Bateman RM, Yamamoto T, Suematsu M. Interactions of multiple gas-transducing systems: hallmarks and uncertainties of CO, NO, and H_2S gas biology. Antioxid Redox Signal. 2010;13:157–192.
42 Anuar F, Whiteman M, Siau JL, Kwong SE, Bhatia M, Moore PK. Nitric oxide-releasing flurbiprofen reduces formation of proinflammatory hydrogen sulfide in lipopolysaccharide-treated rat. Br J Pharmacol. 2006;147:966–974.