Hydrogen Sulfide Is a Mediator of Cerebral Ischemic Damage

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Background and Purpose—We observed recently that elevated plasma cysteine levels are associated with poor clinical outcome in acute stroke patients. In a rat stroke model, cysteine administration increased the infarct volume apparently via its conversion to hydrogen sulfide (H₂S). We therefore investigated the effects of H₂S and the inhibition of its formation on stroke.

Methods—Cerebral ischemia was studied in a rat stroke model created by permanent occlusion of the middle cerebral artery (MCAO). The resultant infarct volume was measured 24 hours after occlusion.

Results—Administration of sodium hydrosulfide (NaHS, an H₂S donor) significantly increased the infarct volume after MCAO. The NaHS-induced increase in infarct volume was abolished by the administration of dizolcilpine maleate (an N-methyl-D-aspartate receptor channel blocker). MCAO caused an increase in H₂S level in the lesioned cortex as well as an increase in the H₂S synthesizing activity. Administration of 4 different inhibitors of H₂S synthesis reduced MCAO-induced infarct volume dose dependently. The potency of these inhibitors in effecting neuroprotection in vivo appeared to parallel their potency as inhibitors of H₂S synthesis in vitro. It also appeared that most of the H₂S synthesizing activity in the cortex results from the action of cystathionine β-synthase.

Conclusions—The present results strongly suggest that H₂S plays a part in cerebral ischemic damage after stroke. Inhibition of H₂S synthesis should be investigated for its potential as a novel neuroprotective stroke therapy. (Stroke. 2006;37:000-000.)

Key Words: hydrogen sulfide ■ N-methyl-d-aspartate ■ receptor ■ stroke

I n addition to being a risk factor of cardiovascular diseases, elevated plasma homocysteine (Hcy) is strongly linked with increased risk of acute stroke.12 Hcy may be converted by the action of cystathionine β-synthase (CBS; EC 4.2.1.22) to cystathionine, which, in turn, is acted on by cystathionine γ-lyase (CSE; EC 4.4.1.1) to form cysteine (Cys), thus suggesting a possible association of the actions of Cys and Hcy in stroke. Plasma Cys has been reported to be as important a risk factor as Hcy in coronary heart disease.3 However, it has also been reported that plasma Cys remained unchanged in stroke patients with hyperhomocysteinemia.4

Cys has been demonstrated to cause neuronal death when given orally to infant mice5 and is also an important mediator of the pathology of brain injury in immature animals subjected to hypoxic-ischemic brain injury.6 In rat hippocampal slices, Cys appears to be innocuous under normal conditions but causes toxicity to neurons deprived of glucose, oxygen, or both.7 Extracellular levels of Cys are also markedly elevated after ischemic brain injury caused by carotid artery ligation in Mongolian gerbils.8 Thus, elevation in extracellular Cys may occur during brain ischemia and contribute to the pathophysiology of ischemic brain injury. Cys is not an agonist at the N-methyl-D-aspartate (NMDA) receptor but is known to cause neuronal cell death that can be prevented by NMDA antagonists.9,10 In this respect, it has been suggested that Cys exerts an indirect action on the NMDA receptors via the excitatory amino acids or Cys derivatives such as S-nitrosocysteine or Cys sulfinate.9,11

However, it is now known that Cys is also the precursor of the novel gaseous mediator hydrogen sulfide (H₂S).12 It is interesting to note that the 2 enzymes involved in the conversion of Hcy to Cys are also the major enzymes involved in the production of H₂S from Cys, namely CBS and CSE.13,14 Both enzymes use Cys as substrate to form H₂S with the byproduct serine, in the case of CBS, or pyruvate and ammonia, in the case of CSE. The actions of H₂S include, in the cardiovascular system, vasodilation and thus lowering of blood pressure, and in the brain, the enhancement of NMDA receptor-mediated responses such as the facilitation of the induction of hippocampal long-term potentiation.15 The level

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of endogenous H$_2$S in the brain has been estimated at 50 to 160 μmol/L,\textsuperscript{16,17} concentrations high enough to suggest physiological functions. Most recently, we observed that high plasma Cys/cystine levels are associated with poor clinical outcome in acute stroke patients and that Cys neurotoxicity in cerebral ischemia in the rat is attenuated by inhibition of CBS.\textsuperscript{18} We therefore investigated the effects of H$_2$S and the inhibition of its formation in a rat model of stroke.

**Materials and Methods**

**Animals and Drugs**

Male Wistar rats (200 to 250 g) obtained from the University Laboratory Animal Center, National University of Singapore were housed singly under natural light/dark (≈12 to 12 hours) cycle with food and water available ad libitum. All procedures were performed in accordance with institutional guidelines, and all efforts were made to minimize suffering and the number of rats used.

Cys, di–propargylglycine (PAG), aminoxyacetic acid (AOAA), sodium hydrosulphide (NaHS), hydroxylamine (HA), β-cyanoalanine (β-CNA), and dizolcilpine maleate (MK-801) were obtained from Sigma. All drugs were dissolved in saline and administered by intraperitoneal injection. Control rats received saline only.

**Focal Cerebral Ischemic Stroke Model**

Cerebral ischemia was induced by permanent unilateral occlusion of the left middle cerebral artery (MCA) using a modified subtemporal approach described previously.\textsuperscript{19} Briefly, the MCA was occluded proximal to the origin of the lenticulo- striate branches extending to the point where it intersects the inferior cerebral vein by electrocauterization. Rectal temperature was maintained at 37±0.5°C until recovery from anesthesia. Control rats were sham operated by omitting only the occlusion.

**Measurement of Infarct Volume**

Rats were decapitated 24 hours after MCA occlusion (MCAO). Coronal sections (2 mm) of the cerebral cortex were then stained with 0.1% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37°C for 30 minutes and fixed using 4% formaldehyde in PBS. The size of the unstained infarcted area per section was analyzed (Olympus Micro Image Lite 4.0 system), and the true infarct volume was obtained by correcting for edema.\textsuperscript{20}

**H$_2$S Synthesizing Activity in Cortical Homogenate**

H$_2$S production in cortical homogenates was studied as described by Abe and Kimura\textsuperscript{12} with modification. Rat cerebral cortex was preincubated at 37°C with or without an inhibitor for 5 minutes in a 20-mL glass vial, was then cooled on ice for 10 minutes before l-Cys (10 mmol/L, final concentration) and pyridoxal 5′-phosphate (2 mmol/L) were added. The final volume was 1 mL. A 2-mL tube containing a piece of filter paper (0.5×1.5 cm) soaked with zinc acetate (1%; 0.3 mL) was put inside the vial. The vial was then flushed with a slow stream of nitrogen gas for 20 seconds and then capped with an airtight serum cap. The vials were then transferred to a 37°C shaking water bath. After 90 minutes, trichloroacetic acid (TCA; pH 6.0; 0.5 mL) was then added, centrifuged (14 000 g; 10 minutes; 4°C) and the clear supernatant (75 μL) was mixed with 0.25 mL Zn acetate (1%) and 0.45 mL water for 10 minutes at room temperature. TCA (10%; 0.25 mL) was then added, centrifuged (14 000 g; 10 minutes; 4°C), and the clear supernatant was collected and mixed with N,N-dimethyl-p-phenylenediamine sulfate (20 mmol/L; 133 μL) in 7.2 mol/L HCl and FeCl$_3$ (30 mmol/L; 133 μL) in 2 mL 50% HCl. After 20 minutes, absorbance was measured as described above.

**Reverse Transcriptase–Polymerase Chain Reaction**

Total mRNA from brain tissues were extracted using TriZol reagent (Invitrogen) as described.\textsuperscript{19} RNA (10 μg) was reverse transcribed into cDNA using Avian Myeloblastosis Virus reverse transcriptase (Promega) and oligo d(T)$_{12}$ as primers. The sequence of primers, annealing temperature/time, and PCR cycle are as follows: β-actin (internal standard; 870 bp; 60°C/2 minutes; 29 cycles); sense: 5′-ATCTGCGACCAACACCTTCTACAATGAGCTCG-3′; antisense: 5′-CGCTATCATCTGCTGTCCTTCAACTGATCGTGC-3′; CBS (559 bp; 58°C/2 minutes; 32 cycles); sense: 5′-ATGCTGCGGAAAAGCTTCAT-3′; antisense: 5′-GTTGAAACACAGTCGGTTCT-3′; CSE (579 bp; 58°C/2 minutes; 32 cycles); sense: 5′-CGCACAAAAATTGTCCAACAC-3′; antisense: 5′-GCTCTGCTCCTCTAGCCACAC-3′.

PCR amplification products were analyzed on a 1% agarose gel. Bands were visualized with an ultraviolet transilluminator and band intensities were quantified by a SynGene Multi Genius Bio Imaging System.

**Statistical Analysis**

All comparisons were performed by 1-way ANOVA followed by post hoc analysis with Bonferroni correction using SPSS for Windows (v13). Data are expressed as mean±SEM. The critical P level set for significance is 0.05.

**Figure 1.** Dose-dependent effect of NaHS on infarct volume 24 hours after MCAO. NaHS (0.18 mmol/kg or otherwise stated in parentheses) was injected intraperitoneally 10 minutes before MCAO. MK-801 (3 μmol/kg), injected intraperitoneally 10 minutes before NaHS administration, completely abolished the effect of NaHS. The mean infarct volume was 179±10 mm$^3$ (100%) for control rats receiving saline only. n=4. One-way ANOVA: F$_{3,10}$=7.388; P<0.01. *P<0.01 against control group by post hoc analysis with Bonferroni correction.
Results

MCAO causes widespread tissue infarction predominantly in the cortex of the occluded side of the brain. Administration of NaHS at 0.09 mmol/kg (IP) before MCAO had no significant effect on the infarct volume, but at 0.18 mmol/kg, the infarct volume was increased to 110% of control. NaHS was administered at a sublethal dose, and no increase in mortality was observed in this group of rats. Coadministration of the NMDA receptor channel blocker MK-801 (3 mol/kg) completely abolished the proinfarct effect of NaHS (Figure 1).

The endogenous level of H$_2$S in the cerebral cortex almost doubled in the lesioned cortex after MCAO when compared with sham-operated controls. As expected, H$_2$S levels increased further after previous Cys loading (Figure 2). In addition, the H$_2$S synthesizing activity, at maximal Cys concentration, in cortical homogenates also increased after MCAO (Figure 3). However, when the cortical expression of CBS and CSE were investigated using RT-PCR, no significant difference in the expression of either enzyme was observed between sham-operated and MCAO rats (Figure 4). It is noted that the expression of CSE was observed to be higher than that of CBS in both groups of animals.

To gain further insight into the role played by H$_2$S in this stroke model, the inhibitory effects of 2 CBS inhibitors (AOAA and HA) and 2 CSE inhibitors (β-CNA and PAG) on the H$_2$S synthesizing activity in cortical homogenate was studied. All 4 inhibitors inhibited H$_2$S production in vitro in a dose-dependent manner (Figure 5). AOAA exhibited the greatest potency with an IC$_{50}$ value of 12.6 μmol/L, reaching 98% inhibition at a concentration of 0.5 mmol/L (data not shown in Figure 5). The IC$_{50}$ values for the other inhibitors are 0.5 mmol/L (HA), 2.5 mmol/L (β-CNA), and 7.1 mmol/L (PAG). In addition, the CSE inhibitors achieved only 70% (β-CNA) and 55% (PAG) inhibition at the highest concentration used (10 mmol/L).

Administration of each of the 4 inhibitors to rats before MCAO revealed that all 4 compounds were able to reduce infarct volume in a dose-dependent manner (Figure 6). Notably, their effectiveness in this respect paralleled their ability to inhibit H$_2$S synthesis in vitro, thus AOAA was the most effective at a dose of 0.05 mmol/kg followed by HA,
which was effective at 0.5 to 1.0 mmol/kg. It is interesting to note that AOAA was not effective at higher doses (ie, up to 0.5 mmol/kg). In contrast, β-CNA and PAG were effective at much higher doses of 1 or 2 mmol/kg. However, relative to β-CNA, PAG appeared to be more effective in reducing infarct volume than its potency in inhibiting H2S production suggested (Figure 5).

Discussion

Cys has been shown previously to increase infarct volume after MCAO in a dose-dependent manner. This effect of Cys was abolished by AOAA, a CBS inhibitor, suggesting that Cys exerts its effect after conversion to H2S via the action of CBS in the brain. We show here that NaHS, an H2S donor, is also able to enhance the destructive effects of cerebral ischemia, leading to a marked increase in the extent of tissue damage. At a dose of 0.18 mmol/kg of NaHS, the effective dose of H2S is ≈0.06 mmol/kg based on a yield of ≈30%; this is equivalent to only 0.6% of the effective dose of Cys at 10 mmol/kg. This is therefore consistent with the possibility that Cys increased the cerebral infarct by production of H2S. Moreover, the effects of both Cys (K. Qu and P.T.H. Wong, unpublished data, 2005) and NaHS (Figure 1) were abolished by MK-801 pretreatment, confirming that H2S acts most likely by an effect via NMDA receptors.

It has been reported previously that physiological concentrations of H2S enhances NMDA receptor function through activation of adenyl cyclase. Increased production of cAMP, observed in primary cultures of both neuronal and glial cells, may lead to phosphorylation of the NMDA receptor subunits at specific sites by protein kinase A, resulting in the activation of NMDA receptor-mediated excitatory postsynaptic current. Thus, in cerebral ischemia, H2S may enhance the NMDA receptor mediated excitotoxicity of glutamate. Together with the observed increase in the endogenous level of both H2S and H2S synthesizing activity in the MCAO lesioned cortex (Figures 2 and 3), these various observations strongly suggest that H2S plays an important role in tissue damage in the ischemic brain, possibly through enhancement of NMDA receptor-mediated calcium overload.

Another possibility is that H2S influences the ischemic infarction by altering cerebral blood flow. However, because H2S causes vasodilation and vasodilators are generally cerebroprotective, leading to reduced infarct size, this possible mechanism is much less likely.

The ability of cortical tissue to increase production of H2S very quickly after MCAO or Cys loading suggests that the enzyme responsible for this conversion is not saturated by its substrate in vivo. Abe and Kimura noted that CBS inhibitors including AOAA completely inhibited the production of H2S in rat whole brain homogenates, whereas CSE inhibitors, including PAG, were ineffective at a concentration of 2 mmol/L. Abe and Kimura concluded that CBS is the predominant enzyme responsible for H2S production in the brain. It has also been reported that CBS is localized in most areas of the adult mouse brain but predominantly in the cell bodies and neuronal processes of Purkinje cells and Ammon’s horn neurons.

Consistently, our present results (Figure 5) also suggest a predominantly CBS-catalyzed production of H2S in the cerebral cortex. AOAA inhibited H2S production effectively with an IC50 value of 12.6 μmol/L and caused almost complete inhibition of H2S production at 0.5 mmol/L. In contrast, PAG, a potent CSE inhibitor, inhibited cortical H2S production with an IC50 value of 7.1 mmol/L, suggesting that PAG may be acting as a low-affinity inhibitor of CBS in this instance rather than as an inhibitor of CSE. It has been reported that CSE is not expressed at detectable levels or expressed at a barely detectable level in the rat and mouse brain by Northern or Western blot analysis. However, it has to be noted that the expression of CSE mRNA (by RT-PCR) is apparently higher than that of CBS (Figure 4), which is in stark contrast to the data obtained in the in vitro assay (Figure 6). It is possible that CSE is expressed at the mRNA level but not at the protein level. More conclusive studies can be made only when antibodies to both CBS and CSE become available.

All 4 inhibitors used reduced the MCAO-induced infarct volume in a dose-dependent manner. The rank order of potency was AOAA > HA > PAG > β-CNA (Figure 6). Significantly, the observed potencies of the compounds as H2S synthesis inhibitors in vitro paralleled their effectiveness in reducing MCAO infarct size in vivo. AOAA, as the most potent inhibitor, significantly reduced infarct volume at a dose of 0.05 mmol/kg. Interestingly, at higher doses, AOAA no longer exhibited any protective effects, probably indicating overinhibition of H2S formation, leading to detrimental effects, supporting an important neuromodulator role for H2S in the brain. It was further noted that at doses >0.5 mmol/kg, rats showed an unacceptably high mortality rate (data not shown).

Summary

The present results clearly demonstrate that H2S, produced from Cys in the cerebral cortex most probably by CBS, is an important mediator of ischemic damage. H2S acts via the
NMDA receptor, which has become a prime target for stroke research over the past decade. Indeed, some NMDA antagonist and glycine antagonists have shown promise in clinical trials. Current evidence suggests that H2S promotes ischemic damage by a direct degenerative effect on cerebral neurons, although effect on cerebral blood flow may not be, as yet, excluded. Whatever the mechanism of action, these results suggest, for the first time, that inhibition of H2S production using a CBS inhibitor may represent a novel therapeutic approach to the treatment of stroke.

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