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Vascular Endothelium Expresses 3-Mercaptopyruvate Sulfurtransferase and Produces Hydrogen Sulfide

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Hydrogen sulfide (H_2S) has been recognized as a smooth muscle relaxant. Cystathionine γ-lyase, which is localized to smooth muscle, is thought to be the major H_2S-producing enzyme in the thoracic aorta. Here we show that 3-mercaptopyruvate sulfurtransferase (3MST) and cysteine aminotransferase (CAT) are localized to vascular endothelium in the thoracic aorta and produce H_2S. Both 3MST and CAT were localized to endothelium. Lysates of vascular endothelial cells produced H_2S from cysteine and α-ketoglutarate. The present study provides a new insight into the production and release of H_2S as a smooth muscle relaxant from vascular endothelium.

Key words: 3-MST, EDRF, endothelium, H_2S, relaxation, smooth muscle.

Abbreviations: CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; EDRF, endothelium-derived relaxing factor; H_2S, hydrogen sulfide; 3MP, 3-mercaptopyruvate; 3MST, 3-mercaptopyruvate sulfurtransferase; SDS, sodium dodecyl sulfate.

Hydrogen sulfide (H_2S) has been recognized as a physiological mediator in many tissues. It facilitates the induction of hippocampal long-term potentiation (LTP), a synaptic model of memory in neurons, and induces calcium waves in astrocytes (*1*, *2*). H_2S relaxes smooth muscle of the thoracic aorta, portal vein and ileum (*3–5*). In addition to its functions as a signalling molecule, H_2S protects neurons from oxidative stress by enhancing the production of glutathione (*6*). It also protects cardiac muscle from ischaemia reperfusion injury by preserving mitochondrial function (*7*). Finally, H_2S is involved in the regulation of insulin release from pancreatic β-cells (*8*, *9*) and in inflammatory reactions (*10*, *11*).

The release of vasodilators from endothelial cells regulates vascular tone and maintains local blood flow and systemic blood pressure. The endothelial cells release endothelium-derived relaxation factor (EDRF), which was identified as nitric oxide (NO), as well as other dilators including endothelium-derived hyperpolarizing factor (EDHF) (*12*, *13*). In addition to dilators released from endothelial cells, it has also been demonstrated that smooth muscle releases relaxing factors (*14*, *15*).

Since cystathionine γ-lyase (CSE), an H_2S-producing enzyme, is expressed in smooth muscle of rat thoracic aorta, H_2S has been thought to be a smooth muscle-derived relaxing factor (*3*, *5*). Although species were different, it was recently reported that CSE could be detected in mouse endothelial cells (*16*). These observations along with our present data suggest that CSE is an enzyme that produces H_2S in rat smooth muscle, but a potential source of H_2S in rat endothelial cells has not been determined.

We recently demonstrated that 3-mercaptopyruvate sulfurtransferase (3MST) along with cysteine aminotransferase (CAT), which is identical with aspartate aminotransferase (*17*, *18*), produces H_2S in the brain (*19*). 3MST produces H_2S from 3-mercaptopyruvate (3MP), which is produced by CAT from cysteine and α-ketoglutarate (α-KG) (*19*, *20*). The present study shows that 3MST and cytosolic and mitochondrial CATs (cCAT and mCAT) are localized to endothelial cells of the thoracic aorta and that these enzymes produce H_2S in this cell type. These results indicate that vascular endothelium produces H_2S through the metabolism by 3MST and CAT and that H_2S is a smooth muscle relaxant released from endothelium.

All animal procedures were approved by the National Institute of Neuroscience Animal Care and Use Committee. Male Sprague-Dawley rats (Clea Japan, Tokyo, Japan) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and transcardially perfused with 200 ml phosphate-buffered saline (PBS). The descending thoracic aorta was excised and immersed into PBS. After incision of the aorta, endothelial cells on the luminal side were collected with a cotton swab and transferred into a tube containing sodium dodecyl sulfate (SDS) sample buffer [125 mM Tris–HCl (pH 6.8), 50 mM dithiothreitol, 4% SDS, 10% sucrose, 0.004% bromophenol blue]. The remaining aorta was chopped with razor blades in nine volumes of SDS sample buffer, and homogenized using a Potter-type glass homogenizer with a Teflon pestle and used as a sample of smooth muscle.

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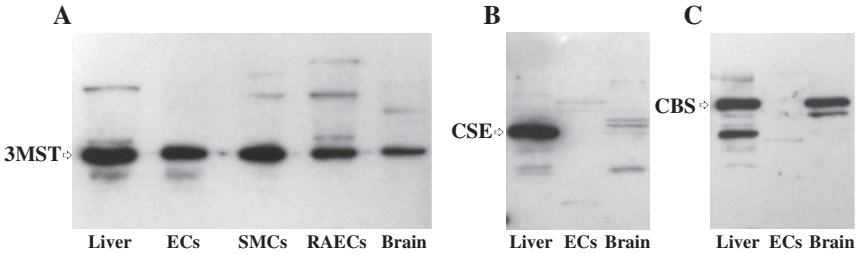

Fig. 1. **Western blot analysis of the localization of 3MST, CSE and CBS in the thoracic aorta.** (A) 3MST is localized to endothelium and smooth muscle in rat thoracic aorta. Western blot analysis showed that 3MST is localized to endothelium (ECs) and smooth muscle (SMCs) in thoracic aorta, rat aortic endothelial cells (RAECs, Toyobo), rat liver and brain. (B and C) Neither CSE nor CBS is localized to thoracic endothelium. Note that CSE is localized to the liver but not to the brain (B), and CBS is localized both to the liver and the brain (C).

For western bolt analysis, one microgram of protein samples were separated by 15% SDS–polyacrylamide gel electrophoresis (DRC, Tokyo, Japan) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked by 2% non-fat milk in PBS/0.1% Tween-20 overnight at 4°C and incubated with either anti-3MST antibody [1 : 3,000; Atlas antibodies AB, Stockholm, Sweden), anti-CSE antibody (1 : 3,000; established in our laboratory (*21*)], or anti-cystathionine β-synthase (CBS) antibody [1 : 3,000; established in our laboratory (*22*)] for 4 h at 4°C. After incubation with secondary antibodies conjugated with horseradish peroxidase, the binding of antibodies was detected by chemiluminescence.

For immunohistochemistry, the rat thoracic aorta was fixed with 4% paraformaldehyde/PBS overnight at 4°C. After rinsing with PBS, tissues were submerged sequentially into 5, 15 and 30% sucrose/PBS. Twelve micrometer thick sections were prepared after being embedded and frozen in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo). After blocking with 10% normal goat serum for an antibody against 3MST (*23*) or with blocking solution (Roche Diagnostics, Mannheim, Germany) for antibodies against cCAT (1 : 3,000; Rockland, Gilbertsville, PA, USA) or mCAT (1 : 1,000; Lifespan Biosciences, Seattle, WA, USA) at room temperature for 1 h, sections were incubated overnight with the antibodies at 4°C. The slides were washed three times with PBS for 5 min. For detection of 3MST, the slides were incubated at room temperature with biotinylated anti-rabbit immunoglobulin G for 1 h, then with peroxidase-conjugated streptavidin for 30 min using a Histfine SAB-PO® kit (Nichirei, Tokyo). For detection of cCAT and mCAT, slides were incubated with peroxidase conjugated anti-sheep immunoglobulin G. Immunoreaction was visualized with 3,3′-diaminobenzidine.

For immunofluorescence, after blocking with 10% normal goat serum, sections were incubated with antibodies against 3MST, CD31 (a marker for endothelial cells) or ASM-1 (a marker for smooth muscle) at 4°C overnight and incubated with second antibodies (Alexa Fluor 488 labelled for 3MST, Cy3 labelled for CD31 and ASM-1) for 1 h at room temperature. Sections were imaged by an epifluorescence microscopy (Axiophot, Zeiss, Germany) using a Plan-NEOFLUAR 40× objective lens (Axiophot).

For the measurement of H_2S production, subconfluent monolayers of rat aortic endothelial cells (CAR304K05, Toyobo, Osaka, Japan) were washed and scraped into PBS, then precipitated by centrifugation at 1,000*g* for 5 min. The cell precipitates were resuspended with the buffer solution consisting of 100 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol and protease inhibitor cocktail 'complete, EDTA free' (Roche Diagnostics) and sonicated for 10 s using a sonifier (Branson Model 450; Branson Ultrasonics, Danbury, CT). For enzyme reactions, 11 μl of substrate solution was added to 0.1 ml of cell lysates in a 15 ml centrifugation tube to a final concentration as indicated in the figure legends. After incubation of the reaction mixture at 37°C for 50 min, H_2S was detected by gas chromatography as previously described (*19*).

H_2S relaxes smooth muscle in the thoracic aorta, and CSE is thought to be a major H_2S-producing enzyme in this tissue (*3*, *5*, *16*). We recently identified 3MST as another H_2S-producing enzyme (*19*, *20*). 3MST produces H_2S from 3MP, which is synthesized by CAT from cysteine and α-KG (*19*, *20*). This finding led us to investigate whether or not 3MST is expressed in the thoracic aorta. Western blot analysis showed that 3MST is localized to both endothelium and smooth muscle (Fig. 1A). This observation was confirmed by immunohistochemistry. 3MST co-localized with markers for endothelial cells, CD31 (Fig. 2A, D, E and F) and for smooth muscle, α-smooth muscle isoform of actin, ASM-1 (Fig. 2A, G, H and I). Although CAT mRNA was reported to be expressed in both endothelial cells and smooth muscle (*24*), our immunohistochemistry showed that both cCAT and mCAT were localized only to endothelial cells (Fig. 2B and C). These observations suggest that 3MST along with CATs produces H_2S in endothelial cells.

Although Yang *et al*. (*16*) recently reported that CSE was found in mouse endothelial cells, the same group previously reported that CSE mRNA was expressed only in smooth muscle in rats (*5*). The present study shows that neither CSE nor CBS was found in rat endothelial cells (Fig. 1B and C), in agreement with their previous finding.

To examine the possibility that 3MST in endothelial cells produces H_2S, production of H_2S by these cells was examined. Lysates of endothelial cells produced H_2S using 3MP as a substrate (Fig. 3A). Because cCAT and mCAT co-localize with 3MST to endothelium, endothelial cells can also produce H_2S from cysteine

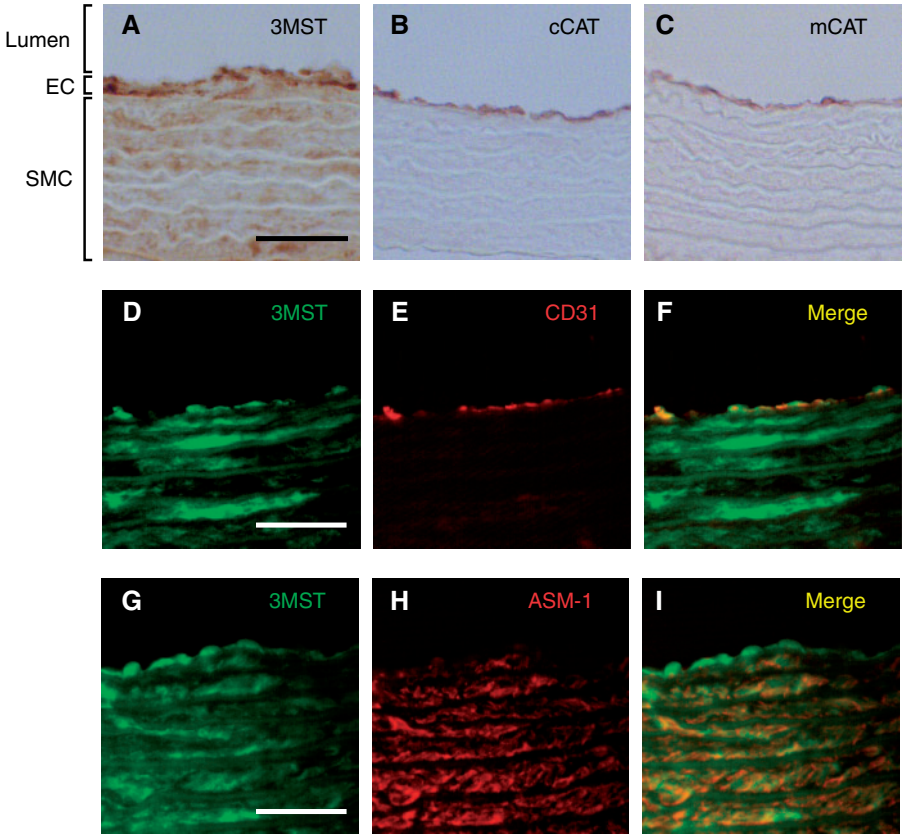

Fig. 2. **Immunohistochemistry of 3MST in thoracic aorta.** (A–C) Localization of 3MST (A), cCAT (B) and mCAT (C). 3MST is localized to both endothelium and smooth muscle (A). EC: endothelial cells, SMC: smooth muscle cells. cCAT and mCAT are localized to endothelium but not to smooth muscle (B and C). (D–I) Co-localization of 3MST with a marker for endothelium (CD31) and a marker for smooth muscle (ASM-1). The merge (F) shows that 3MST (D) is co-localized with an endothelium marker, CD31 (E). The merge (I) shows that 3MST (G) is also co-localized with a smooth muscle marker, ASM-1 (H). Scale bars indicate 50 μm.

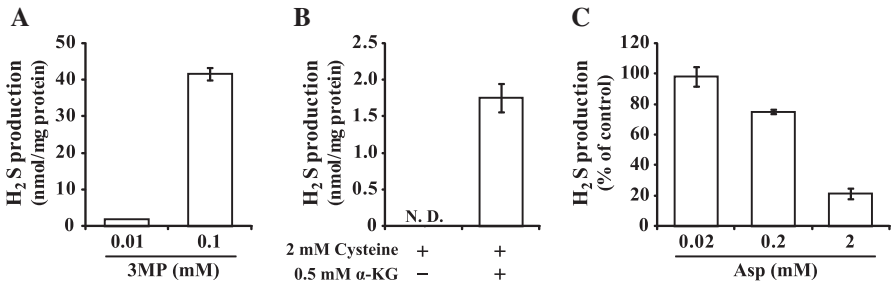

Fig. 3. **Aortic endothelial cells produce H_2S.** (A) Endothelial cells produce H_2S from 3MP as a substrate. Lysates of rat aortic endothelial cells (RAECs) were incubated with 3MP, and released H_2S was measured. (B) Requirement of α-KG for H_2S production from cysteine in the aortic endothelial cells. Lysates of RAECs were incubated with combinations of cysteine, α-KG. Note that H_2S is not produced in the absence of α-KG. (C) H_2S production is suppressed by aspartate, a CAT inhibitor. H_2S production from lysates of RAECs incubated with 2 mM cysteine, 0.5 mM α-KG and 0.05 mM pyridoxal-5′-phosphate was suppressed by aspartate (Asp), a CAT inhibitor in a dose-dependent manner. All data are represented as the mean ± SEM of three experiments.

and α-KG. This possibility was examined. Lysates of endothelial cells produced H_2S from cysteine in the presence of α-KG (Fig. 3B). In the absence of α-KG no H_2S was produced from cysteine, suggesting that H_2S production in endothelium is highly dependent on the activity of CATs. This conclusion is also supported by the observation that the production of H_2S from cysteine and α-KG was suppressed by aspartate, a preferred substrate of CATs, in a dose-dependent manner (Fig. 3C).

The endothelial cells release dilators such as NO, prostacycline and EDHF, of which the candidates include K^+

ion, cyclic AMP, cytochrome P450 2C products, H_2O_2 and C-type natriuretic peptide (*25*). The present study shows that 3MST with CAT produces H_2S in endothelial cells and suggests that H_2S is a smooth muscle relaxant released from endothelium. In addition to the dilators released from endothelial cells, smooth muscle-derived relaxing factors have also been demonstrated (*14*, *15*). In the cerebral artery, flow-induced relaxation is composed of both endothelium-dependent and independent components (*15*). A muscle-derived relaxing factor possesses pharmacological and chemical properties similar to those of NO, suggesting that NO is produced in both endothelial cells and smooth muscle (*14*).

In conclusion, 3MST and CATs are localized to endothelium of the thoracic aorta and produce H_2S from cysteine and α-KG. The present study provides a new insight into the production and release of H_2S as a smooth muscle relaxant from vascular endothelium.

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CONFLICT OF INTEREST

None declared.

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