Reduced Production of Hydrogen Sulfide and Sulfane Sulfur Due to Low Cystathionine $\beta$-Synthase Levels in Brain Astrocytes of Stroke-Prone Spontaneously Hypertensive Rats

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**MATERIALS**

**Materials** Acutase, Dulbeco’s modified Eagle’s medium (DMEM), penicillin-streptomycin mixed solution, 0.25% Trypsin solution and Alamar Blue (TM) were obtained from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was kindly provided by Dojindo. SulfoBiotics Sulfane Sulfur Probe 4 (SSP4) was kindly provided by Dojindo. Sulfide fluor-7 acetoxymethyl ester (SF7-AM) was kindly gift from Dr. Alexander R. Lippert (Southern Methodist University, TX, U.S.A.).

**METHODS**

**Cell Culture** SHRSP and SHR were supplied by the Disease Model Cooperative Study Association (Kyoto, Japan).
Astrocyte preparation was carried out as described by McCarthy and de Vellis and Yamagata et al. Astrocytes from the cerebrum in 1- or 2-d-old SHR and SHRS rats were cultured in DMEM supplemented with 10% FBS, 100 unit/mL penicillin and 100 µg/mL streptomycin, at 37°C in a CO₂ incubator (95% air and 5% CO₂). Cultures with a homogeneous cell population (consisting of >95% astrocytes as determined by glial fibrillary acidic protein staining) were used for the experiments.

**Measurement of Cell Viability by GOD Using Alamar Blue (TM)**

Astrocytes were plated on a 96-well plate and incubated at 37°C until sub-confluency. The cell culture medium was changed to high glucose DMEM with 1% FBS with or without GOD, a reagent that sustains release of H₂O₂ from glucose followed by incubation at 37°C for 3h. After 3h, the cell culture medium was changed to phenol red free DMEM with 1% FBS and 10% Alamar Blue (TM) and incubated at 37°C for 3h. Finally, the fluorescence intensity was measured (λex = 560nm, λem = 595nm) using the fluorescence plate reader and the viability was calculated.

**Measurement of Total Thiols with ThiolTracker Violet**

A 5 mM stock solution of ThiolTracker violet (fluorescent probes for the thiol group including GSH and thiol group of protein) was prepared with dimethyl sulfoxide (DMSO). Cells were seeded on a 6-well plate and cultured at 37°C for 24h. They were incubated with 5 µM ThiolTracker violet at 37°C for 30min and washed with phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS(−)). Fluorescence was observed using a fluorescence microscope (Keyence, Osaka, Japan) (λex = 404nm, λem = 526nm) with randomly taken photographs. The fluorescence intensity was quantified using BZ-X Analyzer (Keyence) for 885–1712 cells per view.

**Quantification of GSH**

Cells were seeded on 6-well plates and cultured at 37°C for 24h and washed with PBS(−), collected using 0.25% trypsin, centrifuged and collected again. To destroy the cell membrane, 1mM HCl was added and freeze-thawing was repeated twice. For deproteinization, 5% sulfosalicylic acid was added to the cell lysate, followed by centrifugation. The sulfosalicylic acid concentration was diluted to 0.5% with MilliQ water. Total GSH, reduced form of GSH and glutathione disulfide (GSSG) were measured according to the protocol of GSSG/GSH Quantification Kit by Dojindo. The values were corrected by the protein concentration of the cell lysates. In this study, protein quantification was done using Bio-Rad Protein Assay, based on the method of Bradford.

**Quantification of H2S in Astrocytes**

Sulfidefluor-7 acetoxymethyl ester (SF7-AM) is a dye that enters cells and fluoresces when it combines with hydrogen sulfide. A 5 mM stock solution of SF7-AM was prepared with DMSO. Astrocytes were seeded on a 96-well plate and cultured until subconfluent. They were then incubated with 5 µM SF7-AM at 37°C for 30min and washed with PBS(−) according to a previously described method. Fluorescence was measured using a fluorescence plate reader (λex = 485 nm, λem = 535 nm). The fluorescence was corrected with protein concentration of the cell lysates.

**Total RNA Extraction and Reverse Transcription (RT)–Real Time PCR**

Total RNA was isolated from SHR and SHRS astrocytes. Cultured cells were washed with ice cold PBS(−) and total RNA was extracted using NucleoSpin RNA II. Next, 2 µg of total RNA was transcribed into cDNA using High-Capacity cDNA Reverse Transcription kits, according to the manufacturer's protocols. Real-time PCR was performed using LightCycler TaqMan Master (Applied Biosystems Assay ID: ribosomal protein L13A (RPL13a); Rn00821946_g1, CBS; Rn00560948_m1). The amplification reaction was monitored using a LightCycler nano (Roche Diagnostics, Tokyo, Japan). Expression values were determined using the ∆∆Ct equation and corrected with expression levels of Ribosomal Protein L13a as a housekeeping gene.

**Western Blot Analysis for Quantification of CBS Protein**

Astrocyte samples were prepared in RIPA buffer (50mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet®P 40 and 0.5% Sodium Deoxy Cholate) with protease and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan). Protein samples (30 µg/lane) were separated in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (ATTO, Osaka, Japan). The membranes were blocked with EZ-block Chemi (ATTO) and washed with Tris-buffered saline with 1% Tween. After blockage and probing with primary antibodies against CBS at 1:1000, followed by secondary rabbit immunoglobulin G (IgG) antibodies (CST, Tokyo, Japan) the signals were developed with ECL reagent (Nacalai Tesque) and then recorded using CCD camera system; Ez-CaptureII (ATTO). The results

![Fig. 1. The Astrocyte Cell Viability Was Measured as Fluorescence Intensity (Ex 560 nm/Em 595 nm) Using Alamar Blue (TM)](image-url)

Values are indicated as the mean±S.E. (n=4–5). The cell viability is shown as a percentage of 0 unit/mL GOD group of SHR (a) and SHRS (b). *p<0.05 and compared with the 0unit/mL GOD group using William’s test.
were quantifiably assayed with a CS Analyzer 3.0 (ATTO). The expressions of CBS protein were corrected using the expression of \( \beta \)-actin.

**CBS Enzyme Activity** CBS activity was measured as an *in vitro* enzymatic reaction using SF7-AM. The astrocytes were lysed with 50 mM phosphate buffer (pH 8.2). One mg of protein was put in a 96-well plate and the fluorescent probe (5 \( \mu \)M SF7-AM) was preloaded at 37°C for 20 min. After 20 min, the fluorescence was measured (\( \lambda_{ex} = 485 \) nm, \( \lambda_{em} = 535 \) nm) using a fluorescence plate reader and used as a blank. Next, 1 mM L-cysteine, 1 mM Dl-homocysteine and 0.2 mM pyridoxal-5'-phosphate were added to the cells to start the enzyme reaction, followed by treatment with or without 1 mM AOAA as a CBS inhibitor. The plate was incubated at 37°C for the enzyme reaction. The fluorescence was measured every 30 min and the CBS activity was calculated. The details of calculation are listed in Results.

**Effect of GYY4137 on Cell Death by \( \text{H}_2\text{O}_2 \) Oxidative Stress** GYY4137, a reagent that sustainably releases \( \text{H}_2\text{S} \), was developed by Moore and colleagues \( \text{GYY4137} \) (Dojindo) when added at 100 \( \mu \)M, led to the release of 20 \( \mu \)M \( \text{H}_2\text{S} \). Astrocytes were plated on a 96-well plate and cultured until subconfluent. Cells were incubated with 0.1 unit/mL GOD with or without 20, 50 and 100 \( \mu \)M GYY4137 in DMEM with 1% FBS at 37°C for 3 h. After 3 h, the medium was changed to 1% FBS in colorless DMEM supplemented with Alamar Blue (TM) and incubated at 37°C for 3 h. Finally, the fluorescence was measured (\( \lambda_{ex} = 560 \) nm, \( \lambda_{em} = 595 \) nm) using a fluorescence plate reader.

**Quantification of Sulfane Sulfur in Astrocytes Using SSP4** SSP4 is a dye that enters cells and fluoresces when it combines with sulfane sulfur. Cells were seeded on a 96-well plate and cultured until subconfluent. They were incubated with 10 \( \mu \)M SSP4 and 0.5 \( \mu \)M hexadecyltrimethylammonium bromide into DMEM without FBS at 37°C for 15 min according to a protocol by Dojindo. After 15 min, cells were washed...
with PBS(−) and fluorescence were measured using fluorescence plate reader ($\lambda_{ex}=485\text{nm}$, $\lambda_{em}=535\text{nm}$). To investigate the participation of CBS, the cells were treated with 1 and 2 mM AOAA as a CBS inhibitor and pre-incubated at 37°C for 1 h. The fluorescence was corrected by the protein concentration of the cell lysates.

**Statistics** Data were analyzed by one-way ANOVA or $t$-test. In all cases, $p<0.05$ or $p<0.01$ was taken to indicate a significant difference.

**RESULTS**

**Astrocyte Cells Viability in SHR and SHRSP by GOD Released H$_2$O$_2$** Alamar Blue (TM) is a reagent for evaluating cells viability. Astrocytes in SHRSP were more vulnerable than SHR. Cell death of astrocytes was observed more at 0.02 unit/mL of GOD for SHRSP, but SHR astrocytes did not die at 0.1 unit/mL (Figs. 1a, b). The astrocytes from SHRSP are more vulnerable to H$_2$O$_2$ oxidative stress, possibly due to the impaired of scavenging ROS.

**Measurement of Total Thiols and GSH to Maintain the Reducing Condition in Astrocytes from SHR and SHRSP** Intracellular thiols play an important role in maintaining reduced conditions in the cells. ThioTracker violet, a fluorescent probe for intracellular thiols, reacts actively with reduced thiols including GSH in live cells. The total thiol levels in the SHRSP astrocytes were significantly lower than those of the SHR (Fig. 2). We therefore measured GSH. The total amount of GSH and the reduced form of GSH in SHRSP were not significantly different. However, the oxidized form of GSSG in SHRSP was significantly higher than in SHR. Therefore, GSH/GSSG in SHRSP was significantly lower than in SHR (Fig. 3). These results show that the cause of the decreased total thiols in SHRSP was not due to a decrease in GSH.

**Comparison of H$_2$S in Astrocyte Cells from SHR and SHRSP** We measured H$_2$S, known to have a reducing effect in cells, in astrocytes from SHR and SHRSP. SF7-AM is a fluorogenic reagent designed and synthesized to detect H$_2$S. It has been engineered specifically for use in live cell imaging of H$_2$S. In quantifying H$_2$S using SF7-AM, the value per protein was found to be significantly decreased in SHRSP astrocytes compared with those of SHR (Fig. 4). As H$_2$S is produced by CBS in astrocytes, we next investigated the reduction of H$_2$S in SHRSP caused by the expression of CBS or reduction of CBS enzyme activity.

**Expression of CBS mRNA and Protein in Astrocytes from SHR and SHRSP** We investigated the expression of CBS known to be specifically expressed in astrocytes of the brain cortex and to synthesize the H$_2$S. CBS mRNA expression levels in SHRSP astrocytes were significantly lower than those from SHR using real time PCR (Fig. 5a). Expressions of CBS protein in astrocytes were also significantly reduced in SHRSP compared with SHR (Fig. 5b).

**Enzyme Activity of CBS in Astrocytes from SHR and SHRSP** The intensity of the fluorescence of SF7-AM bound with H$_2$S increased linearly up to the measurement period of 1 h. Thus, CBS activity was calculated using the values at 1 h.
CBS enzyme activity was calculated by subtracting the portion due to the AOAA CBS inhibitor. The result was that the CBS activity in SHRSP was significantly lower than in SHR (Fig. 6).

**Effect of GYY4137 on Cell Death by H₂O₂ Oxidative Stress** SHRSP astrocytes were vulnerable to H₂O₂ oxidative stress. The CBS activity in cells is indicated as the mean ± S.E. (n=5). *Significant at p<0.05 versus SHR using t-test.

**Fig. 6.** The CBS Activity in Astrocytes of SHR and SHRSP Was Measured for 1 h Using SF7-AM

The values of CBS activity are shown as the fluorescence intensity without AOAA group—-with AOAA group. White column is SHR, black column is SHRSP. The CBS activity in cells are indicated as the mean ± S.E. (n=5). *Significant at p<0.05 versus SHR using t-test.

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**Fig. 7.** Measurement of Fluorescence (Ex 560 nm/Em 595 nm) Using Alamar Blue (TM)

The graph shows the effect of GYY4137 for astrocyte cell death by GOD. Values are indicated as the mean ± S.E. (n=4–5). White column is non-treatment group, black column is treatment of GOD 0.1 unit/mL group. The gray columns are GOD 0.1 unit/mL + GYY4137 each. **Significant at p<0.01 versus GOD 0.1 unit/mL group using Tukey test.

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**Fig. 8.** The Amount of Sulfane Sulfur Using SSP4

Values of fluorescence intensity/protein shows the mean ± S.E. (n=5). *Significant at p<0.05 versus SHR using t-test. The white column is SHR, black column is SHRSP. The amount of sulfane sulfur in astrocytes in (a) and with AOAA CBS inhibitor for 1h in (b–c). Values for levels of fluorescence intensity of SSP4 bound sulfane sulfur in cells are indicated the mean ± S.E. (n=5). **Significant at p<0.01 versus control using Dunnet test.
stress. Treatment with 0.1 unit/mL GOD injured the astrocytes, but 20 nM GYY4137 led to rescue from cell death at a significant level. High concentration groups of GYY4137 did not result in rescue from cell death (Fig. 7).

Quantification of Sulfane Sulfur with or without CBS Inhibitor SSP4 is a fluorescent dye which is bound with sulfane sulfur without H$_2$S. The amount of sulfane sulfur was significantly lower in SHRSPr than SHR (Fig. 8a). Treatment with AOAA dose-dependently and significantly decreased the fluorescence intensity in SHR and SHRSPr astrocytes (Figs. 8b, c).

DISCUSSION

We showed the vulnerability of astrocytes in SHRSPr to H$_2$O$_2$ oxidative stress. Astrocytes were exposed to the continuously released H$_2$O$_2$ from GOD with glucose in the culture medium. GOD catalyzes the oxidation of glucose to produce H$_2$O$_2$. SHRSPr astrocytes died at lower concentrations of GOD than SHR.

Cellular redox balance is maintained by cellular thiols mainly GSH. The amount of total thiols in SHRSPr was significantly lower than SHR. Total and reduced GSSG significantly decreased in SHRSPr astrocytes compared with those from SHR. The decreased tendency of total GSH in SHRSPr astrocytes may be related to CBS expression, because CBS is partly related with GSH synthesis. The ratio of GSH to GSSG is critical to cellular redox balance, and the significant decreased ratio of GSH to GSSG cannot rule out of vulnerability in SHRSPr astrocytes for oxidative stress.

Recent studies suggest that H$_2$S has many physiological roles in processes, such as cytoprotection through an antioxidant effect or anti-inflammation and vasodilatation. In SHRSPr astrocytes, production of H$_2$S was significantly decreased compared with that in SHR.

The reduction in SHRSPr astrocytes was caused by decreased expression of mRNA, protein and enzyme activity of CBS. CBS has been found in regions of the brain responsible for localized in astrocytes. In the brain, CBS contributes mainly to the production of H$_2$S using cysteine and homocysteine as a substrate. It is found in SHRSP astrocytes, with increased expression of mRNA, protein and enzyme activity of CBS. CBS in Dahl salt-sensitive rats is regulated by hypoxia. CBS has been found in regions of the brain responsible for producing H$_2$S, and it induces vasodilatation. The reduction of H$_2$S in SHRSPr may cause not enough vasodilation in condition of hypoxia. This decreased CBS expression may contribute to stroke-proneness in SHRSPr.

H$_2$S has been reported to modify specific cysteine residues in proteins through the formation of a polysulfide and persulphide bond and to be involved in the regulation of such activity by sulfhydration of the enzyme. H$_2$S also regulates the cerebral microvascular response to hypoxia. Some reports showed that under normal O$_2$ condition, CO binds to the heme in CBS and inhibits the activity of producing H$_2$S. Hypoxia induces production of H$_2$S through decline of the generation of CO and vasodilatation. As explained above, the cerebral blood flow reduces in SHRSPr when blood pressure elevates. Ischemia induces hypoxia subsequently hypoxia causes production of ROS. In condition of hypoxia, CBS is activated and enhanced production of H$_2$S, and it induces vasodilatation. The reduction of H$_2$S in SHRSPr may cause not enough vasodilation in condition of hypoxia. This decreased CBS expression may contribute to stroke-proneness in SHRSPr.

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Conflict of Interest The authors declare no conflict of interest.

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