Hydrogen sulfide generation from L-cysteine in the human glioblastoma-astrocytoma U-87 MG and neuroblastoma SHSY5Y cell lines*

Patrycja Bronowicka-Adamska, Anna Bentke and Maria Wróbel

Chair of Medical Biochemistry, Jagiellonian University, Collegium Medicum, Kraków, Poland

Hydrogen sulfide (H₂S) is endogenously synthesized from L-cysteine in reactions catalyzed by cystathionine beta-synthase (CBS, EC 4.2.1.22) and gamma-cystathionase (CSE, EC 4.4.1.1). The role of 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2) in H₂S generation is also considered; it could be important for tissues with low CTH activity, e.g. cells of the nervous system. The expression and activity of CBS, CTH, and MPST were detected in the human glioblastoma-astrocytoma (U-87 MG) and neuroblastoma (SHSY5Y) cell lines. In both cell lines, the expression and activity of MPST were the highest among the investigated enzymes, suggesting its possible role in the generation of H₂S. The RP-HPLC method was used to determine the concentration of cystathionine and alpha-ketobutyrate, products of the CBS- and CTH-catalyzed reactions. The difference in cystathionine levels between cell homogenates treated with totally CTH-inhibiting concentrations of DL-propargylglycine and without the inhibitor was used to evaluate the activity of CBS. The higher expression and activity of CBS, CTH and MPST in the neuroblastoma cells were associated with more intensive generation of H₂S in the presence of 2 mM cysteine. A threefold higher level of sulfane sulfur, a potential source of hydrogen sulfide, was detected in the astrocytoma cells in comparison to the neuroblastoma cells.

Key words: cystathionine, beta-synthase, gamma-cystathionase, glutathione, hydrogen sulfide, 3-mercaptopyruvate sulfurtransferase, sulfane sulfur

Received: 18 July, 2016; revised: 07 October, 2016; accepted: 05 November, 2016; available on-line: 14 March, 2017

INTRODUCTION

In mammalian tissues, H₂S is synthesized endogenously from L-cysteine in regulated enzymatic pathways catalyzed by pyridoxal phosphate-dependent enzymes: cystathionine beta-synthase (CBS, EC 4.2.1.22) and gamma-cystathionase (CSE, EC 4.4.1.1) and cysteine aminotransferase (CAT, EC 2.6.1.3) coupled with 3-mercaptopyruvate sulfurtransferase (MPST) (Wang, 2012) (Scheme 1A).

Enzymatic reactions involve L-cysteine hydrolysis by CBS to L-serine and H₂S, L-cysteine transformation by CTH into thiocysteine, pyruvate (PA) and ammonia and subsequent thiocysteine transformation into H₂S and CysSR (S-thiolane). Another pathway includes the transformation of L-cysteine into 3-mercaptopyruvate (3MP) by cysteine aminotransferase (CAT) and, subsequently, 3MP desulfuration catalyzed by MPST resulting in H₂S and pyruvate formation (Scheme 1A). H₂S is formed in a redox reaction between thiosulfate or RSSH (persulfides) and biological thios such as reduced glutathione (GSH) (Scheme 1B) (Libiad et al., 2014; Predmore et al., 2012). An additional pathway for the production of 3-MP and H₂S from D-cysteine by D-amino acid oxidase provides protection of cerebellar neurons from oxidative stress (Shibuya et al., 2013).

Astrocytes secrete and store antioxidative compounds, such as glutathione or ascorbate (Bartosz, 2006; Bélanger & Magistretti, 2009, Zablocka & Janusz, 2007). The cells play an important role in supplying precursors necessary for GSH synthesis in the neurons. GSH captured by astrocytes from the extracellular space is degraded in a reaction catalyzed by γ-glutamyl-transpeptidase (γ-GT) to free cysteine (Zablocka & Janusz, 2007) (Scheme 2).

The main transport system for cysteine in the astrocytes and neurons is mediated by the Na⁺ dependent X₅M₉ and ASC (alanine-serine-cysteine) systems (Shanker et
al., 2001a; Shanker et al., 2001b). The degradation product returns to neuron cells, where it is used as a substrate for glutathione synthesis. When compared to the neurons, astrocytes have the higher level of GSH, both in vivo and in cell cultures (Dringen et al., 2000). Functional neuron-glial cell interrelations provide an important mechanism participating in brain functions control (Scheme 2).

The anaerobic conversion of cysteine can lead to the formation of hydrogen sulfide (H\textsubscript{2}S). It is known that endogenously formed H\textsubscript{2}S acts as a neuromodulator and neuroprotector in the brain (Panthi et al., 2016; Paul & Snyder, 2015). By means of activating NMDA receptors and increasing the response of peripheral neurons, H\textsubscript{2}S may play a significant role in processes associated with memorization and learning (Ishigami et al., 2009; Shibuya et al., 2009). An increased synthesis of H\textsubscript{2}S has been observed in patients with Down’s syndrome and septic shock, while its decreased generation has been noted in Alzheimer’s disease. H\textsubscript{2}S activates TRPA1 channels in the astrocytes in a similar way, but not as efficiently as polysulfides (Kimura, 2013; Moore & Whiteman, 2005). H\textsubscript{2}S has antioxidative properties and it increases the production of glutathione in neural cells (Kimura et al., 2010; Kimura & Kimura, 2004). The Scheme is based on the scheme presented by Kimura (2013). H\textsubscript{2}S activates TRPA1 channels in the astrocytes in a similar way, but not as efficiently as polysulfides (Kimura, 2013; Moore & Whiteman, 2005). H\textsubscript{2}S has antioxidative properties and it increases the production of glutathione in neural cells (Kimura et al., 2010; Kimura & Kimura, 2004).

The study was conducted to determine the activity and expression of the enzymes: CBS, CTH and MPST involved in the production of H\textsubscript{2}S in the human glioblastoma-astrocytoma (U-87 MG) and neuroblastoma (SHSY5Y) cell lines. The results of our previous studies (Jurkowska et al., 2011) showed the expression of CTH and MPST genes in the human neoplastic cell lines: astrocytoma U373 and neuroblastoma SH-SY5Y. The CTH and MPST enzymes, through an increase in sulfane sulfur levels, might increase H\textsubscript{2}S levels. The RP-HPLC method was used to detect and determine the amount of direct and indirect products of the CBS- and CTH-catalyzed reactions, such as cystathionine, cysteine, and glutathione. The difference in the cystathionine level between the cells incubated with totally CTH-inhibiting concentrations of DL-propargylglycine (PPG) and without the inhibitor was used to evaluate the activity of CBS. Differences in the expression and activity of CBS, CTH, and MPST point to a higher intensity of H\textsubscript{2}S generation in the neuroblastoma cells, which was confirmed by the higher level of H\textsubscript{2}S in SHS5Y cells determined using the H\textsubscript{2}S trapping method of Kartha et al. (2012).

MATERIAL AND METHODS

Chemicals. l-Glutathione reduced (GSH), l-cysteine, cystathionine (CTN), DL-homoserine (HSer), 1-fluoro-2,4-dinitrobenzene (DNSFB), bathophenanthroline-disulfonic acid disodium salt (BPFD), acetoniitrile, pyridoxal phosphate (PLP), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), l-lactic dehydrogenase (LDH), 3-mercaptopropionate acid sodium salt, DL-dithiothreitol, (DTT), N-ethylmaleimide (NEM), DL-propargylglycine (PPG), sodium dihydrogen phosphate dihydrate pure, sodium sulfite, chloroform, isopropanol, agaroase, sodium hydroxide hydrate, sodium chloride, Folin-Ciocalteu’s phenol reagent, iron (III) nitrate nonahydrate, sodium thiosulfate pentahydrate, sodium carbonate and N,N-di-methyl-p-phenylenediamine sulfate salt, Coomassie Blue G250 were obtained from Sigma-Aldrich (Poznan, Poland). Trifluoroacetic acid (TFA), 2-mercaptoethanol were purchased from FlukaChemie GmbH (Buchs, Switzerland). Ethanol and 70% perchloric acid (PCA), 38% formaldehyde, 65% nitric acid, 38% hydrochloric acid, ammonia solution 25% pure, sodium potassium tartrate, copper sulphate pentahydrate, potassium dihydrogen phosphate, ferric chloride, zinc acetate dehydrate pure, sodium hydroxide were from Polskie Odezyniki Chemiczne S.A. (Gliwice, Poland). N-methyl-DL-histidine was obtained from Bachem (Bubendorf, Switzerland). DMEM/High glucose, trypsin 0.25%, fetal bovine serum and penicillin-streptomycin solution were purchased from Thermo Scientific (Waltham, MA, USA). Trizol, ethidium bromide and EDTA-disodium salt dihydrate were obtained from Lab-Emprise S.A. (Rzeszow, Poland). Potassium cyanide was from Merek Sp. z o.o. (Warszawa, Poland). Reverse Transcriptase M-MuLV was obtained from Roche Diagnostics Polska Sp. z o.o. and Promega Poland (Warszawa, Poland). Polymerase DNA Dream Taq™, Gene Ruler 100 bp DNA Ladder, Oligo(dT)18 primer and dNTP Mix were obtained from Abo Sp. z o.o. (Gdańsk, Poland). RIPA buffer was from TermoScientific (Rockford, USA). Antibodies: anti-CBS and -CTH were from Abino (Taiwan), anti-MPST was from GeneTex (Taiwan), anti-β-actin from Sigma-Aldrich (Poznan, Poland), anti-alpha-tubulin, alkaline phosphatase-conjugated goat anti-rabbit IgG antibody and anti-mouse IgG antibody were from Proteintech (Chicago, IL, USA). NBT/BCIP (nitroblue tetrazolium chloride/3-bromo-4-chloro-3-indolyl phosphate), toluidine was from Roche (Warszawa, Poland). All the chemicals and HPLC solvents were...
gradient grade. Water was deionized by passing through an EASY pure RF compact ultrapure water system.

**Cell lines. Cell culture.** Human glioblastoma-astrocytoma (U-87 MG) and neuroblastoma (SHSY5Y) cell lines were grown in a monolayer in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), in plastic culture dishes (100 mm in diameter), at 37°C in a humidified atmosphere containing 5% CO₂. Cell lines were purchased from the European Collection of Cell Cultures (ECACC-Sigma Aldrich, Poznań, Poland).

**Cytotoxicity of L-cysteine.** The cells were seeded in triplicates into 96-microwell plates at density of 20×10⁴ cells/well and incubated for 24 h with or without 2 mM L-cysteine in DMEM medium supplemented with 10% FBS. Colorimetric assay was performed according to the manufacturer instructions (Cytotoxicity Detection Kit, Roche, Thermo Fisher Scientific). Absorbance of the colored product – formazane – was measured at 490 nm by microreader (EPOCH, BioTEK).

**Expression of MPST, CTH, CBS in cell lines. RNA extraction.** The total RNA was extracted using TRIzol, according to the protocol provided by the manufacturer. The quality of RNA samples was determined by spectrophotometric analysis (A₂₆₀/A₂₈₀) and electrophoresis in 2.5% agarose gel followed by staining with ethidium bromide.

**Reverse Transcription of RNA.** Total RNA from the cell samples was reverse-transcribed using First-stand cDNA synthesis kit according to the manufacturer instructions (Promega, Company, Warszawa, Poland). For reverse transcription (RT) 3 µg of total RNA was mixed with 1 µl Oligo (dT)₁₅ (0.5 µg/µl reaction) and nuclease-free water and heated in a 70°C heat block for 5 minutes. After preincubation the reverse transcription reaction mix containing 4 µl of GoScript™ cDNA reaction buffer, 3 µl of MgCl₂ (final concentration 1.5–5.0 mM), 1 µl of dNTPs (final concentration 0.04 U/µl of DNA polymerase in 10 mM Tris-HCl buffer pH 8.8, 0.2 mM of each dNTPs, 10 mM), 1 µl of Recombinant RNases Ribonuclease Inhibitor (20 U/µl) and 1 µl of GoScript™ Reverse Transcriptase were prepared.

**cDNA Synthesis and RT-PCR analysis.** Expression of MPST, CTH, CBS and β-actin was analyzed with RT-PCR as previously described by Jurkowska et al. (2004). No modifications. Amplification of cDNA samples was performed in a 12.5 µl reaction volume containing: 1 µl of synthesized cDNA, 0.2 µM of each gene-specific primer pair, 0.04 U/µl of DNA polymerase in 10 mM Tris-HCl buffer pH 8.8, 0.2 mM of each dNTPs and nuclease-free water. The temperature profile of RT-PCR amplification for the MPST consisted of activation of Taq polymerase at 94°C for 5 min, denaturation of cDNA at 94°C for 30 s, primer annealing at 56°C for 30 s, elongation at 72°C for 2 min for the following 28 cycles and was finished by the extension step for 8 min. For the CTH gene, after the initial denaturation for 5 min at 94°C, amplification was performed under the following conditions: 94°C for 30 s, 51°C for 1 min and 72°C for 8 min for 28 cycles, with the final incubation at 72°C for 10 min. For the CBS gene, after the initial denaturation for 5 min at 94°C, amplification was performed under the following conditions: 94°C for 30 s, 60°C for 30 s and 72°C for 2 min for 38 cycles, with the final incubation at 72°C for 8 min. For β-actin gene, after the initial denaturation for 5 min at 94°C, amplification was performed under the following conditions: 94°C for 30 s, 54°C for 30 s and 72°C for 2 min for 28 cycles, with the final incubation at 72°C for 8 min. The following specific primers (Oligo Company) were used:

**MPST – F:** 5'TCTTCGACATCGACAGCTGC' and **R:** 5'TGTGAAGGGGATGTTCAGCGG'  
**CTH – F:** 5'GCAAATGGCCATCTGAATTG' and **R:** 5'CCCATACCAACATCTAGTGG'  
**CBS – F:** 5'CGGTGCGTGTTGCTATTGC' and **R:** 5'TCCAGGTATCCGGCGCT'  
**β-actin – F:** 5'CTGTCGTTCACACCAT' and **R:** 5'GCAAATAGTCATAGTCG'  
β-actin was used as the internal standard to normalize all messenger RNA levels and the activity of MPST and CTH. For RP-HPLC analyses cells were suspended in 0.1 ml 0.9% saline and directly visualized under UV light and photographed.

**Western blotting analysis.** The cells were suspended in RIPA buffer, containing proteinase inhibitors cocktail, sonicated 3×5 s at 4°C (BandelinSonoplus GM 70) and centrifuged at 14000×g for 15 min – supernatants were used for further analysis. The relative amount of CBS, CTH, MPST was determined by Western blotting using the appropriate antibody: anti-CBS (1:1000), anti-CTH (1:1000), anti-MPST (1:1000). Anti-β-actin (1:5000) and anti-alpha-tubuline (1:5000) antibodies were used to check for equal loading. Proteins of interest were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:2000) or with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:2000). Proteins were visualized with NBT/BCIP staining solution.

**Detection of H₂S.** The H₂S produced during the incubation of the cell culture with H₂S-releasing compounds was trapped as zinc sulfide in the zinc agarose layer according to Kartha et al. (2012). The standard curve was linear at the concentration range of 0–250 µM with correlation coefficient of 0.994.

**Enzymes assay. Cell homogenization.** U-87 MG and SHSY5Y cells (3.5–5×10⁴ cells) were suspended in 0.1 M phosphate buffer pH 7.5, in the proportion of 1 ml cells/0.07 ml of the buffer, sonicated 3×5 s at 4°C (BandelinSonoplus GM 70). After centrifugation at 1600×g for 10 min, the supernatant was used for the determination of protein concentration, sulfane sulfur levels and the activity of MPST and CTH. For RP-HPLC analyses cells were suspended in 0.1 ml 0.9% NaCl/1 70% PCA/1 mM BPDS. The sediment was separated by centrifugation at 14000×g for 10 min, and supernatant was stored at –80°C until analysis.

**MPST activity.** MPST activity was assayed according to the method of Valentine and Frankelfeld, (1974) following a procedure described in our earlier paper (Wrobel et al. 2004). The incubation mixture contained: 250 µl of 0.12 M sodium phosphate buffer, pH 8.0, 50 µl of 0.5M sodium sulphite, 50 µl of 0.15 M dithiothreitol, 50 µl of homogenates, 50 µl of H₂O and 50 µl of 0.1 M 3-mercaptopropionate acid sodium salt in a final volume of 500 µl. Mixture was incubated for 15 min. To stop the reaction 250 µl of 1.2 M PCA was added. Samples were centrifuged at 1600×g for 5 min, and 100 µl of supernatant was transferred to 1350 µl of mixture that contained 1200 µl of 0.12 M sodium phosphate buffer, pH 8.0, 100 µl of 0.1 M N-ethylmaleimide, 50 µl of NADH 5 mg/ml. After equilibration at 37°C, 2.5 µl of lactate dehydrogenase (7 IU) was added, and the decrease in absorbance was measured at 340 nm. The enzyme activity was expressed as nmol of pyruvate produced during 1 min incubation at 37°C per 1 mg of protein.

**CTH activity.** Cystathionase activity was determined using Matsuo and Greenberg’s method (1958) with modifications described by Czubak et al. (2002). The
incubation mixture contained: 25 µl of 1.3 mM PLP, 25 µl of 0.02 mM EDTA, 250 µl of 45 mM cystathionine solution in 0.1 M phosphate buffer, pH 7.5 (2.5 mg of cystathionine per sample) and 75 µl of homogenate and 0.1 M phosphate buffer, pH 7.5 containing 0.05 mM 2-mercaptoethanol in the final volume of 650 µl. The reaction was stopped after 15 min of incubation at 37°C by placing 125 µl of the incubation mixture in 25 µl of 10% PCA. Samples were centrifuged at 1600 × g for 10 min, and 25 µl of supernatant was transferred to 625 µl of 0.194 mM NADH solution and kept at 37°C. Control samples, without 45 mM cystathionine, were prepared in the same way as the examined samples. After 10 s of the measurement (absorbance at 340 nm), 25 µl (9.06 IU) of lactate dehydrogenase (LDH) was added and measurement was continued up to 180 s. The difference between the initial value of absorbance (before adding LDH) and the lowest value (after adding LDH) corresponded to the amount of alpha-ketobutyrate formed in the course of the cystathionase reaction. Cystathionase activity was expressed as nmols of α-ketobutyrate formed during 1 min incubation at 37°C per 1 mg of protein.

CBS activity. The activity of CBS was examined in cells homogenates in the presence of DL-homoserine as substrate after 15 minutes incubation at 37°C according to the description in Bronowicka-Adamska et al. (2011). PPG, in the concentration of 0.7 mM, was used to completely inhibit the activity of CTH in both cell lines. The level of cystathionine was determined using the HPLC method described by Bronowicka-Adamska et al. (2015). The CBS activity was expressed as pmols of cystathionine formed during 1 min incubation at 37°C per 1 mg of protein.

Sulfane sulfur. Sulfane sulfur was determined by the method of Wood, (1987), based on cold cyanolysis and colorimetric detection of ferric thiocyanate complex ion. Incubation mixtures in a final volume 880 µl contained: 20 µl of 1 M ammonia solution, 20 µl of homogenate, 740 µl of H2O and 100 µl of 0.5 M sodium cyanide. Incubation was performed for 45 min at room temperature. After incubation, thiocyanate was estimated calorimetrically at 460 nm after the addition of 20 µl of 38% formaldehyde and 40 µl of ferric nitrate reagent. Sulfane sulfur level was expressed as nmols of SCN produced per 1 mg of protein.

Proteins. Protein concentration was determined with the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. Protein concentration measurement with Bradford assay was used for the determination of protein in Western blotting analysis (Bradford, 1976).

RP-HPLC (Reverse Phase High Performance Liquid Chromatography). The level of cystathionine, alpha-ketobutyrate and the reduced glutathione (GSH) in the incubation mixtures were determined using the RP-HPLC method of Dominick et al. (2001) with modifications (Bronowicka-Adamska et al., 2015; Bronowicka-Adamska et al., 2011; Wrobel et al., 2009). Table 1 shows the activity of MPST, CTH, CBS, and sulfane sulfur level in U-87 MG and SH-SY5Y cell.

RESULTS AND DISCUSSION

The studies showed U-87 MG and SH-SY5Y cells capacity of hydrogen sulfide formation from L-cysteine and an increased level of hydrogen sulfide in the neoroblastoma – SH-SY5Y cells (Fig. 1) by about 20%, as compared to the control cells without L-cysteine, and only by about 5% in the glioblastoma-astrocytoma (U-87 MG cells) after 24 h of incubation with 2 mM L-cysteine (Fig. 1). The cytotoxic effect on the SH-SY5Y and U-87 MG cell lines after 24 hours of incubation with 2 mM L-cysteine was lower than 10% for both cell lines.

Statistical analysis. All results were expressed as means ± S.D. The significance of the differences between controls and investigated samples were calculated using Student’s Test (P<0.05). Each experiment was repeated minimum three times.

The level of hydrogen sulfide in homogenates of U-87 MG and SH-SY5Y cells after 24 h incubation with 2 mM L-cysteine. The experiments were carried out for control homogenates of U-87 MG, SH-SY5Y cells with 2 mM L-cysteine as the main endogenous substrate for the hydrogen sulfide producing enzymes. The data represent the mean value from three independent experiments. Statistical analysis was performed using the Student’s t-test (P<0.05).

Figure 1. The level of hydrogen sulfide in homogenates of U-87 MG and SH-SY5Y cells after 24 h incubation with 2 mM L-cysteine.

Table 1. The mean value of MPST, CTH, CBS activity, and sulfane sulfur level in U-87 MG and SH-SY5Y cell.

| Cell line  | MPST nmol mg⁻¹ min⁻¹ | CTH pmol mg⁻¹ min⁻¹ | CBS pmol mg⁻¹ protein⁻¹ | Sulfane sulfur pmol mg⁻¹ protein⁻¹ |
|-----------|----------------------|---------------------|--------------------------|----------------------------------|
| SH-SY5Y   | 674±93               | 5.15±1.46           | 17.3±2.7                 | 41±15                            |
| U-87 MG   | 196±23               | 3.29±0.83           | 1.34±0.2                 | 139±47                           |

Values are the mean of four to five measurements from three independent experiments.
Changes in cystathionine levels were not observed in either of the cell lines in response to PPG after 15 min of incubation (Fig. 4).

CONCLUSIONS

In the glioblastoma-astrocytoma (U-87 MG) and neuroblastoma (SHSY5Y) cells, the pathway catalyzed by enzymatic tandem CAT/MPST can play a role in the generation of hydrogen sulfide from cysteine. In the neuroblastoma cells, the pathway from methionine to cysteine through the CBS and CTH reactions seems to play a more significant role as compared to the astrocytoma cells. The higher activity and expression of enzymes involved in H₂S generation from cysteine, in the neuroblastoma cells, provide an opportunity for more rapid response in H₂S production than in the astrocytoma cells. However, in the astrocytoma cells, the elevation of H₂S seems to be possible by releasing it from the pool of sulfane sulfur. Panthi et al. (2016) reviewed possible physiological roles of H₂S in neurons protection from oxidative stress or in the upregulation of the GABA β-receptors at pre- and postsynaptic sites along with astrocytes roles in the regulation of neurotransmitter levels or neuronal excitability.

Acknowledgements

This work was supported by a grant from the National Committee for Scientific Research No. K/DSC/001372.

Conflicts of Interest

The authors declare no conflict of interest.

Contributions

P.B-A provided the experimental data. A.B performed and developed the Western blotting analysis. M.W. provided suggestions for the experiments. P.B-A and M.W. planned the experiments and wrote the paper.

REFERENCES

Abe K, Kimura H (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci 16: 1066–1071. doi: 10.1017/S1740925X09000864
Araque A (2008) Astrocytes process synaptic information. Neuron Glia Biol 4: 3–10.
Bartosz G (2006) Druga twarz tlenu. Bartosz G (2006) Druga twarz tlenu. Wyd. PWN (in Polish).
Bélanger M., Magistretti P.J. (2009) The role of astroglia in neuroprotection. Dialogues Clin Neurosci 11: 281–295.
Bonansco Ch, Court A, Perea G, Ferradas CA, Roncagliolo M, Fuenzalida M (2011) Glutamate released spontaneously from astrocytes sets the threshold for synaptic plasticity. Eur J Neurosci 33: 1483–1492. doi: 10.1111/j.1460-9580.2011.07631.x
Bradford MM (1976) A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.

Bronowicka-Adamska P, Wróbel M, Zagajewski J (2011) RP-HPLC method for quantitative determination of cystathionine cysteine and glutathione: An application for the study of the metabolism of cysteine in human brain. *J Chromatogr B* 879: 2005–2009. doi: 10.1016/j.jchromb.2011.05.026

Bronowicka-Adamska P, Wróbel M, Zagajewski J (2015) An application of RP-HPLC for determination of the activity of cystathionine beta-synthase and gamma-cystathionase in tissue homogenates. *Nitric Oxide* 46: 186–191. doi: 10.1016/j.niox.2014.09.159

Czubak J, Wróbel M, Jurkowska H (2002) Cystathionine γ-lyase (EC 4.4.1.1): an enzymatic assay of γ-koetobutyrate using lactate dehydrogenase. *Acta Biol Cracov Ser Zool* 44: 113–117.

Deitmer JW (2001) Strategies for metabolic exchange between gila cyprinids. *J Biol Chem* 276: 71–81. doi: 10.1016/S0021-9258(01)00283-3

Dominik PK, Cassidy PB, Roberts JC (2001) A new and versatile method for determination of thiolamines of biological importance. *J Chromatogr B* 761: 1–12.

Dringen R (2000) Metabolism and functions of glutathione in brain. *Prog Neurobiol* 62: 649–671.

Dringen R, Gutterer JM, Hirrlinger J (2000) Glutathione metabolism in brain. Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur J Biochem* 267: 4912–4916.

Ishigami M, Hiraki K, Umemura K, Ogasawara Y, Ishii K, Kimura H (2009) A source of Hydrogen sulfide and a mechanism of its release in the brain. *Antioxid Redox Signal* 11: 205–214. doi: 10.1089/ars.2008.2132

Jurkowska H, Placha W, Nagahara N, Wróbel M (2011) The expression and activity of cystathionine gamma-lyase and 3-mercaptoppyruvate sulfurtransferase in human neoplastic cell lines. *Amino Acids* 41: 151–158. doi: 10.1007/s00726-010-0606-3

Karthi RV, Zhou J, Hovde LB, Belinda WY, Cheung BWY, Schröder H (2012) Enhanced detection of hydrogen sulfide generated in cell culture using an agar trap method. *Anal Biochem* 423: 102–108. doi: 10.1016/j.ab.2012.01.001

Kimura H (2013) Physiological role of hydrogen sulfide and polysulfide in the central nervous system. *Neuromol Med* 16: 492–497. doi: 10.1007/j.neunet.2013.09.003

Kimura Y, Goto Y, Kimura H (2010) Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxid Redox Signal* 12: 1–13. doi: 10.1089/ars.2008.2282

Kimura Y, Kimura H (2004) Hydrogen sulfide protects neurons from oxidative stress. *FASEB J* 18: 1168–1167. doi: 10.1096/fj.04-1815fj

Lhtiad M, Yadav Pk, Vrielynck V, Martinov M, Banerjee R (2014) Organization of the human mitochondrial Hydrogen sulfide oxidation pathway. *J Biol Chem* 289: 30901–30910. doi: 10.1074/jbc.M114.602664

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275.

Matsuo Y, Greenberg DM (1958) A crystalline enzyme that cleaves homoserine and cystathionine. *J Biol Chem* 230: 545–560.

McBean GJ (2012) The transulfuration pathway: a source of cysteine for glutathione in astrocytes. *Amino Acids* 42: 190–205. doi: 10.1007/s00726-011-0964-8

Moore PK, Whiteman M (2005) Chemistry Biochemistry and Pharmacology of Hydrogen Sulfide. Springer-Verlag GmbH.

Pantri S, Chung HJ, Jung J, Jeong NY (2016) Physiological importance of hydrogen sulfide: emerging potent neuroprotector and neuromodulator. *Oxid Med Cell Longe* 2016: 1–11. doi: 10.1155/2016/9049782

Paul BD, Snyder SH (2015) Modes of physiology H2S signaling in the brain and peripheral tissues. *Antioxid Redox Sign* 22: 411–425. doi: 10.1089/ars.2014.5917

Perea G, Araque A (2003) New information pathways in the nervous system: communication between astrocytes and neurons. *Rev Neurol* 36: 137–144.

Predmore BL, Lefer DJ, Gojon G (2012) Hydrogen sulfide in biochemistry and medicine. *Antioxid Redox Signal* 17: 119–140. doi: 10.1089/ars.2012.4612

Shanker G, Allen JW, Mutkus LA, Aschner M (2001) Methylmercury inhibits the in vitro uptake of the glutathione precursor cysteine in astrocytes but not in neurons. *Brain Res* 894: 131–140. doi: 10.1016/S0006-8993(01)02342-3

Shanker G, Allen JW, Mutkus LA, Aschner M (2001) The uptake of cysteine in cultured primary astrocytes and neurons. *Brain Res* 902: 156–163.

Shibuya N, Koike S, Tanaka M, Ishigami-Yuasa M, Kimura Y, Ogasawara Y, Fukui K, Nagahara N, Kimura H (2013) A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. *Nat Commun* 4: 1366–1373. doi: 10.1038/ncomms2571

Shibuya N, Tanaka M, Yoshida M, Ogasawara Y, Togawa T, Ishii K, Kimura H (2009) 3-Mercaptoppyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxid Redox Signal* 11: 703–714. doi: 10.1089/ars.2008.2253

Valentine WN, Frankelid JK (1974) 3-Mercaptoppyruvate sulfurtransferase is a physiological hydrogen sulfide producing enzyme. *J Biol Chem* 249: 113–117.

Wróbel M, Jurkowska H, Slowa L, Srebzro Z (2004) Sulfurtransferases and cyanide detoxification in mouse liver kidney and brain. *Toxicol Mech Methods* 14: 331–337. doi: 10.1080/15376520400343683

Wróbel M, Lewandowska I, Bronowicka-Adamska P, Paszewski A (2009) The level of sulfane sulfur in the fungus *Aspergillus niger* wild type and mutant strains. *Amino Acids* 37: 565–571. doi: 10.1007/s00726-008-0175-x

Zabłocka A, Janusz M (2007) Structure and function of the central nervous system. *Postepy Hig Med Dosw* 61: 454–460.