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

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Hydrogen sulfide (H_2S) 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 H_2S 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 H_2S. 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 H_2S 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

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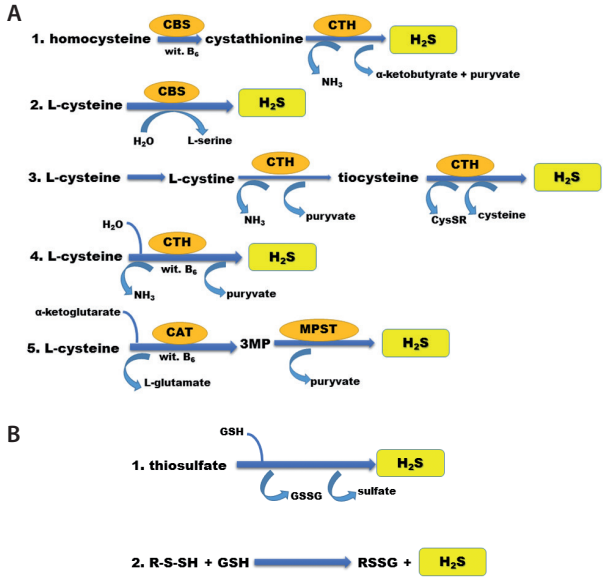
Abbreviations: CAT, cysteine aminotransferase; CBS, cystathionine β-lyase; CTH, γ-cystathionase; CTN, cystathionine; GSH, glutathione reduced form; MPST, 3-mercaptopyruvate sulfurtransferase; NBT/BCIP, nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine; PCA, perchloric acid

INTRODUCTION

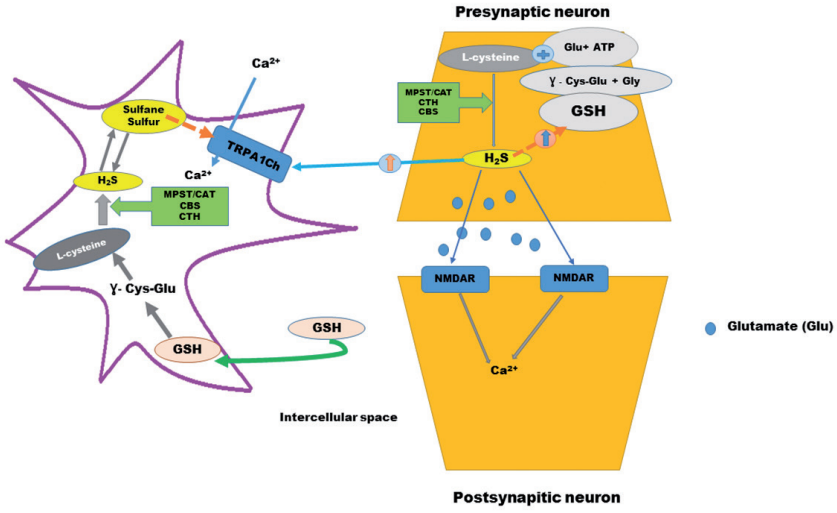
In mammalian tissues, H_2S is synthesized endogenously from L-cysteine in regulated enzymatic pathways catalyzed by pyridoxal phosphate-dependent enzymes: cystathionine beta-synthase (CBS, EC 4.2.1.22), gamma-cystathionase (CTH, EC 4.4.1.1) and cysteine aminotransferase (CAT, EC 2.6.1.3) coupled with 3-mercaptopyruvate sulfurtransferase (MPST) (Wang, 2012) (Scheme 1A).



Scheme 1. The enzymatic (A) and non-enzymatic (B) reactions generating hydrogen sulfide.

Enzymatic reactions involve L-cysteine hydrolysis by CBS to L-serine and H_2S, L-cystine transformation by CTH into thiocysteine, pyruvate (PA) and ammonia and subsequent thiocysteine transformation into H_2S 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 H_2S and pyruvate formation (Scheme 1A). H_2S is formed in a redox reaction between thiosulfate or RSSH (persulfides) and biological thiols 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 H_2S 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; Bèlanger & 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 X_{AG}^- and ASC (alanine-serine-cysteine) systems (Shanker *et*



Scheme 2. The possible cooperation between the astrocytes and neural cells.
The Scheme is based on the scheme presented by Kimura (2013). H_2S activates TRPA1 channels in the astrocytes in a similar way, but not as efficiently as polysulfides (Kimura, 2013; Moore & Whiteman, 2005). H_2S has antioxidative properties and it increases the production of glutathione in neural cells (Kimura *et al.*, 2010; Kimura & Kimura, 2004). TRPA1Ch, transient receptor potential cation channel; subfamily A, member 1; NMDAR, N-methyl-D-aspartate receptor; Gly, Glycine; GSH, reduced glutathione

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_2S). It is known that endogenously formed H_2S 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_2S 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_2S has been observed in patients with Down's syndrome and septic shock, while its decreased generation has been noted in Alzheimer's disease. H_2S activates TRPA1 channels in the astrocytes in a similar way, but not as efficiently as polysulfides (Kimura, 2013; Moore & Whiteman, 2005). H_2S 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_2S 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_2S 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_2S generation in the neuroblastoma cells, which was confirmed by the higher level of H_2S in SHSY5Y cells determined using the H_2S 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 (DNFB), bathophenanthroline-disulfonic acid disodium salt (BPDS), acetonitrile, pyridoxal phosphate (PLP), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), L-lactic dehydrogenase (LDH), 3-mercaptopyruvate acid sodium salt, D,L-dithiothreitol, (DTT), N-ethylmaleimide (NEM), DL-propargylglycine (PPG), sodium dihydrogen phosphate dihydrate pure, sodium sulfite, chloroform, isopropanol, agarose, sodium hydrosulfide hydrate, sodium chloride, Folin-Ciocalteu's phenol reagent, iron (III) nitrate nonahydrate, sodium thiosulfate pentahydrate, sodium carbonate and N,N-dimethyl-*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 tarate, copper sulphate pentahydrate, potassium dihydrogen phosphate, ferric chloride, zinc acetate dehydrate pure, sodium hydroxide were from Polskie Odczynniki Chemiczne S.A. (Gliwice, Poland). N^{ε}-methyllysine 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-Empire S.A. (Rzeszow, Poland). Potassium cyanide was from Merck 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 Abnova (Taiwan), anti-MPST was from GeneTex (Taiwan), anti-β-actin from Sigma-Aldrich (Poznan, Poland), anti-alpha-tubuline, alkaline phosphatase-conjugated goat anti-rabbit IgG antibody and anti-mouse IgG antibody were from Proteintech (Chicago, IL, USA). NBT/BCIP (nitroblue tetrazolium chloride/5-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 RFcompact 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% CO_2. 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 20×10^3 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 (A_{260}/A_{280}) 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/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™5X reaction buffer, 3 μl of $MgCl_2$ (final concentration 1.5–5.0 mM), 1 μl of deoxyribonucleotide triphosphates (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.* (2011) with 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 of 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'TCTTCGACATCGACCAGTGC' and R: 5'TGTGAAGGGGATGTTCACGG3'

CTH – F: 5'GCAAGTGGCATCTGAATTTG3' and R: 5'CCCATTACAACATCACTGTGG3'

CBS – F: 5'CGCTGCGTGGTCATTCTGCC3' and R: 5'TCCCAGGATTACCCCCGCCT3'

β-actin – F: 5'CTGTCTGTCACCACCAT3' and R: 5'GCAACTAAGTCATAGTCCGC3'

β-actin was used as the internal standard to normalize all samples for potential variations in mRNA content. PCR reaction products were separated electrophoretically in a 2.5% agarose gel, stained with ethidium bromide 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 $14\,000 \times g$ 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 (1:1 000), anti-CTH (1:1 000), anti-MPST (1:1000). Anti-β-actin (1:5 000) and anti-alpha-tubuline (1:5 000) antibodies were used to check for equal loading. Proteins of interest were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:2 000) or with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:2 000). Proteins were visualized with NBT/BCIP staining solution.

Detection of H_2S. The H_2S produced during the incubation of the cell culture with H_2S-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 ($3.5–5 \times 10^6$ cells) were suspended in 0.1 M phosphate buffer pH 7.5, in the proportion of 1 mln cells/0.07 ml of the buffer, sonicated 3×5 s at 4°C (BandelinSonoplus GM 70). After centrifugation at $1600 \times g$ 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% NaC/l 70% PCA/1 mM BPDS. The sediment was separated by centrifugation at $1400 \times g$ 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 (Wróbel *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 sulfite, 50 μl of 0.15 M dithiothreitol, 50 μl of homogenates, 50 μl of H_2O and 50 μl of 0.1 M 3-mercaptopyruvate 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 $1600 \times g$ 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 nmoles 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-mercaptoethanolin 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 nmoles of α-ketobutyrate formed during 1min 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 pmoles of cystathionine formed during 1min 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 H_2O 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 nmoles 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; Wróbel *et al.*, 2009).

Table 1. The mean value of MPST, CTH, CBS activity, and sulfane sulfur level in U-87 MG and SHSY5Y cell.

Cell line	MPST	CTH	CBS	Sulfane Sulfur
	nmol·mg^{-1} min^{-1}		pmol·mg^{-1}·min^{-1}	nmol·mg protein^{-1}
SHSY5Y	674±93	5.15±1.46	17.3±2.7	41±15
U87MG	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.

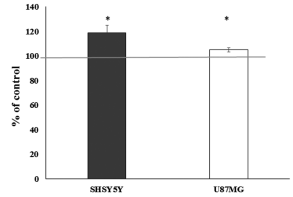


Figure 1. The level of hydrogen sulfide in homogenates of U-87 MG and SHSY5Y cells after 24 h incubation with 2 mM L-cysteine.
The experiments were carried out for control homogenates of U-87 MG, SHSY5Y cells with 2 mM L-cysteine as the main endogenous substrate for the hydrogen sulfide producing enzymes. The data represent the mean value from three independent experiments. Statistical analysis was performed using the Student's *t*-test (*P<0.05).

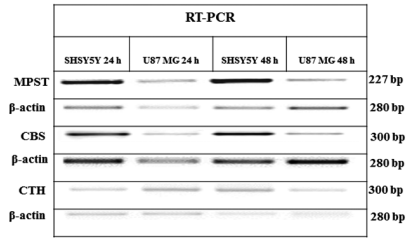


Figure 2. RT-PCR analysis of MPST, CBS and CTH expression in SHSY5Y and U-87 MG cell lines.
One set of representative results is shown. β-actin was used as the internal control. The length of the products: MPST, 227 bp; CBS, 300 bp; CTH, 300 bp; β-actin, 280 bp. Specific primers and temperature profiles for particular proteins are given in Material and Methods.

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.

RESULTS AND DISCUSSION

The studies showed U-87 MG and SHSY5Y cells capacity of hydrogen sulfide formation from L-cysteine and an increased level of hydrogen sulfide in the neuroblastoma – SHSY5Y 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 SHSY5Y and U-87 MG cell lines after 24 hours of incubation with 2 mM L-cysteine was lower than 10% for both cell lines.

Table 1 shows the activity of CTH, MPST, CBS and the level of sulfane sulfur in the SHSY5Y and U-87 MG cells. One can summarize that all the enzymatic pathways (Scheme 1) generating H_2S can occur in the investigated cell lines. CBS is regarded as the principal enzyme responsible for H_2S synthesis in the brain, while CTH plays a similar role in the circulatory system (Abe & Kimura, 1996). More than tenfold higher CBS activity and three fold higher MPST activity were estimated in the neuroblastoma cells, as compared to the astrocytoma cells. Therefore, the neuroblastoma cells have potentially higher capacity of H_2S generation from cysteine than astrocytoma. In the neuroblastoma cells, specific activity of MPST being the highest among the investigated enzymes and the highest expression of MPST (Fig. 2 and

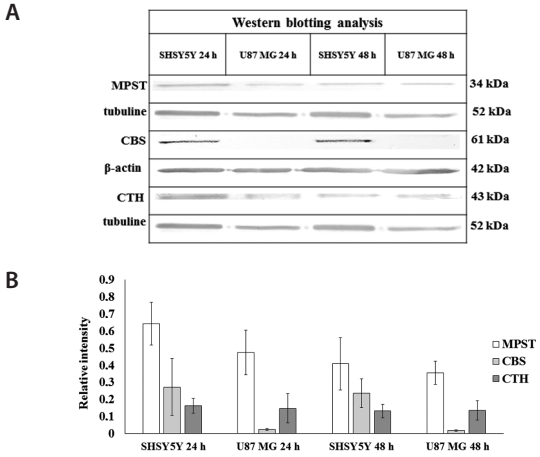


Figure 3. Western blotting analysis of MPST, CBS, CTH proteins in SHSY5Y and U-87 MG cell lines.
(**A**) Western blotting analysis. Western blot analysis of MPST, CBS and CTH was performed for the cell lines using a mixture of rabbit- (MPST) and mouse-derived (CBS, CTH, β-actin and alpha-tubuline) primary antibodies with the appropriate mixture of alkaline phosphatase-conjugated secondary antibodies. One set of representative results is shown. β-Actin and alpha-tubuline were used as the internal control of protein loading; 20 µg of protein was added to each lane. For details see Material and Methods. (**B**) The relative intensity normalized to β-actin and alpha-tubulin. Analysis of protein bands imaged with the ChemiDoc™ MP. The relative intensity value is the average from three independent experiments. The relative intensity was normalized using β-actin and alpha-tubulin signals, the average of which was taken as one.

3) can both suggest that this enzyme plays a role in the generation of H_2S.

On the other hand, the sulfane sulfur level was more than threefold higher in the astrocytoma cells, which can suggest that a nonenzymatic release of H_2S from sulfane sulfur-containing compounds (Scheme 2) is possible. It seems probable that in the neuroblastoma cells, H_2S, functioning as a neurotransmitter, is synthesized in response to the signal-to-date. The astrocytoma cells, in turn, can release H_2S from sulfane sulfur reserves to transmit a signal within the astrocyte network (Perea & Araque, 2003; Zablocka & Janusz, 2007). The conditions under which physiological signals mobilize H_2S from sulfane sulfur stores have not been elucidated so far (Paul & Snyder, 2015).

The U-87 MG astrocytoma cells had the higher level of GSH in comparison to the neuroblastoma cells, which confirms the differences in glutathione content between the astrocytes and neurons (Dringen *et al.* 2000) (Fig. 4).The high levels of glutathione in the astrocytes seem to be essential for neurons protection e.g.: against the toxicity of reactive oxygen species. In the control cultures and cultures with PPG (inhibitor of CTH) added in the concentration of 0.7 mM, both the level of α-ketobutyrate and the level of cysteine were undetectable. The inhibition of CTH with PPG in the astrocytoma cells resulted in a diminished level of GSH after 15 min of incubation, but no such effect was seen in the neuroblastoma cells (Fig. 4). This may suggest that the pathway of cysteine generation through CTH-catalyzed reaction is important in the U-87 MG cells, while the SHSY5Y cells depend to a great extent on an exogenous source of cysteine. The importance of the transsulfuration pathway in astrocytes and glioblastoma/astrocytoma cells as a reserve pathway when the demand for glutathione is high was recently discussed by McBean (2012). Changes in cystathionine levels were not

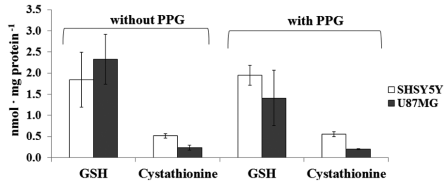


Figure 4. The level of GSH and cystathionine in U-87 MG and SHSY5Y cells.
The experiments were carried out for control homogenates of U-87 MG, SHSY5Y cells with or without PPG as the inhibitor of CTH in concentration of 0.7 mM for both cells lines. The homogenates were incubated with PPG for 15 minutes. The data represent the mean value from three independent experiments.

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 H_2S generation from cysteine, in the neuroblastoma cells, provide an opportunity for more rapid response in H_2S production than in the astrocytoma cells. However, in the astrocytoma cells, the elevation of H_2S seems to be possible by releasing it from the pool of sulfane sulfur. Panthi *et al.* (2016) reviewed possible physiological roles of H_2S 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.

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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/S1740925X09000064

Araque A (2008) Astrocytes process synaptic information. *Neuron Glia Biol* **4**: 3–10.

Bartosz G (2006) Druga twarz tlenu. Wyd. PWN (in Polish).

Bélanger M, Magistretti PJ (2009) The role of astroglia in neuroprotection. *Dialogues Clin Neurosc* **11**: 281–295.

Bonansco Ch, Couve 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.1460-9568.2011.07631.x

Bradford MM (1976) A rapid and sensitive for the quantitation of microgram quantitites 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 Chromatography 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 α-ketobutyrate using lactate dehydrogenase. *Acta Biol Cracov Ser Zool* **44**: 113–117.

Deitmer JW (2001) Strategies for metabolic exchange between gila cells and neurons. *Respir Physiol* **129**: 71–81. doi: 10.1016/S0034-5687(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 cystahionine gamma-lyase and 3-mercaptoppyruvate sulfurtransferase in human neoplastic cell lines. *Amino Acids* **41**: 151–158. doi: 10.1007/s00726-010-0606-3

Kartha 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. *Neurochem Int* **63**: 492–497. doi: 10.1016/j.neuint.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**: 1165–1167. doi: 10.1096/fj.04-1815fje

Libiad M, Yadav PK, Vitvitsky 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 RI (1951) Protein measurement with the Folinphenol 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 transsulfuration pathway: a source of cysteine for glutathione in astrocytes. *Amino Acids* **42**: 190–205. doi: 10.1007/s00726-011-0864-8

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

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

Paul BD, Snyder SH (2015) Modes of physiologic H_2S signaling in the brain and peripheral tissues. *Antioxid Redox Sign* **22**: 411–423. 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 cystine 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/ncomms2371

Shibuya N, Tanaka M, Yoshida M, Ogasawara Y, Togawa T, Ishii K, Kimura H (2009) 3-Mercaptopyruvate 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, Frankelfeld JK (1974) 3-Mercaptopyuruvate sulfurtransferase (EC 2.8.1.2): A simple assay adapted to human blood cells. *Clin Chim Acta* **14**: 205–210. doi: 10.1016/0009-8981(74)90031-X

Wang R (2012) Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Pysiol Rev* **92**: 791–896. doi: 10.1152/physrev.00017.2011

Wood L (1987) Sulfane sulfur. *Methods Enzymol* **143**: 25–29. doi: 10.1016/0076-6879(87)43009-7

Wróbel M, Jurkowska H, Śliwa L, Srebro Z (2004) Sulfurtransferases and cyanide detoxification in mouse liver kidney and brain. *Toxicol Mech Methods* **14**: 331–337. doi: 10.1080/15376520490434683

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

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