Expression and activity of hydrogen sulfide generating enzymes in murine macrophages stimulated with lipopolysaccharide and interferon-γ

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Abstract
Murine macrophages of the J774A.1 line are hydrogen sulphide-producing cells with the primary role of γ-cystathionase (CTH) and secondary role of 3-mercaptopyruvate sulfurtransferase (limited by cysteine availability) and with a negligible role of cystathionine β-synthase (CBS) in H2S generation. J774A.1 cells stimulation with lipopolysaccharide (LPS) or interferon-gamma (IFNγ) resulted in decreased H2S levels after 24 h of incubation; however, they were restored to the control level after 48 h. Negligible CBS expression and activity in J774A.1 cells can result in homocysteine availability for CTH-catalyzed, H2S-generating reactions. This was supported by an increased CTH expression (IFNγ, 24 h and 48 h, and LPS, 48 h) and activity (24 h, LPS) in the stimulated cells. The results confirm the suggested feedback regulation between CBS and CTH.

Keywords γ-Cystathionase · Hydrogen sulfide · Interferon · Lipopolysaccharide · Mouse macrophages

Abbreviations
CBS Cystathionine β-synthase
CTH γ-Cystathionase
HCys Homocysteine
IFNγ Interferon γ
LPS Lipopolysaccharide
MPST 3-Mercaptopyruvate sulfurtransferase
TST Rhodanese

Introduction
Methionine is metabolized to homocysteine (HCys) to produce cysteine, which is a substrate of two enzymes—cystathionine β-synthase (CBS) and γ-cystathionase (CTH)—involved in endogenous production of H2S (Scheme 1) [1]. In cells with low CBS expression, H2S can be alternatively generated by CTH-catalyzed condensation reaction between cysteine and HCys or between two molecules of HCys (Scheme 1) [2].

Another endogenous source of H2S is cysteine transamination by cysteine aminotransferase (CAT) and subsequent 3-mercaptopyruvate (3MP) conversion by 3-mercaptopyruvate sulfurtransferase (MPST) [3]. 3MP provides sulfur to the active-site cysteine residue of MPST to produce persulfide, which releases H2S in the presence of thioredoxin (Trx), abundant in cells. Another physiological reducing disulfide is dihydrolipoic acid (DHLA), cofactor for mitochondrial α-ketacid dehydrogenases. Trx and DHLA release H2S from persulfide provided by 3MP at the active site of MPST [4]. The expression of CBS, CTH and MPST are tissue-specific.

Hydrogen sulfide (H2S) plays an important role in inflammation [5] but there is no clear consensus as to its precise role in inflammatory signaling. Macrophages in general play important roles in the initiation and progression of many chronic inflammatory diseases. Li et al. [6] observed biphasic effects of H2S on inflammatory signals in murine lipopolysaccharide (LPS)-treated macrophages, where low H2S levels inhibited LPS-induced synthesis of PGE2, NO, IL-1β, IL-6 and NF-κB activity, but higher NaHS concentrations promoted synthesis of pro-inflammatory mediators. LPS—a potent activator of macrophages, and interferon-gamma (IFNγ)—the principal macrophage-activating factor,
was used to stimulate murine macrophages of the J774A.1 cell line. H$_2$S measured by the zinc acetate-trapping method [7] was used to compare the H$_2$S level in the control, and LPS- and IFNγ-stimulated cells after 24 and 48 h of incubation. The expression and the activity of hydrogen sulfide-generating enzymes, i.e. CBS, MPST, CTH, and TST, were investigated in J774A.1 cells. The results indicate an important role of CTH in the generation of hydrogen sulfide in J774A.1 cells with low activity of the transsulfuration pathway catalyzed by the CBS/CTH enzymatic system. They also confirm a negative feedback regulation between CBS and CSE [8].

The studies presented in this paper were designed to determine the activity and expression of hydrogen sulfide generating enzymes, i.e.: cystathionine β-synthase (CBS), γ-cystathionase (CTH) and MPST in murine macrophages stimulated with LPS and IFN-γ.

**Materials and methods**

**Chemicals**

1-Fluoro-2,4-dinitrobenzene, bathophenanthrolinedisulfonic acid disodium salt, 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), D,L-propargylglycine (PPG), Na$_2$HPO$_4$·2H$_2$O Na$_2$SO$_3$, chloroform, isopropanol, agarose, sodium hydrosulfide hydrate, NaCl, Folin–Ciocalteu’s phenol reagent, Fe(NO$_3$)$_3$·9H$_2$O, Na$_2$S$_2$O$_3$·5H$_2$O, Na$_2$CO$_3$ and N,N-dimethyl-p-phenylenediamine sulfate salt (DMPPDA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid and 2-mercaptoethanol were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Ethanol and 70% perchloric acid 38% formaldehyde, 65% HNO$_3$, 38% HCl, ammonia solution 25%, sodium potassium tartrate, copper sulphate pentahydrate (CuSO$_4$·5H$_2$O) potassium dihydrogen phosphate (KH$_2$PO$_4$), ferric chloride (FeCl$_3$), (Zn(CH$_3$COO)$_2$·2H$_2$O), and sodium hydroxide (NaOH) were from Polskie Odczynniki Chemiczne S.A. (Gliwice, Poland). N'-methyllysine was obtained from Bachem (Bubendorf, Switzerland). DMEM/high glucose (Lanza, Basel, Switzerland), fetal bovine serum and penicillin–streptomycin solution were purchased from Gibco Invitrogen, Life Technologies, (Grand Island, NY, USA). Trizol, ethidium bromide and EDTA–disodium salt dihydrate were obtained from Lab-Empire (Rzeszow, Poland). KCN was obtained from Merck (Darmstadt, Germany). Reverse transcriptase M-MuLV was obtained from Promega (Madison, WI, USA). Polymerase DNA Dream Taq™, Gene Ruler 100 bp DNA Ladder, Oligo(dT)18 primer and dNTP mix were obtained from Abo (Gdańsk, Poland). All the chemicals were analytical grade and HPLC solvents were gradient grade. Water was deionized by passing through an EASY pure RF compact ultrapure water system.
Cell culture

Mouse BALB/c monocyte macrophage of the J774A.1 cell line (ATCC, Manassas, VA, USA) were grown in DMEM medium supplemented with 10% FCS, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), in sterile, plastic culture flasks (BD Falcon, Franklin Lakes, NJ, USA), at 37 °C, in a humidified atmosphere containing 5% CO₂. For experiments, the cells were cultured in DMEM medium supplemented with 5% FCS and antibiotics (as above) at 37 °C, in a humidified atmosphere containing 5% CO₂, either on sterile, plastic Petri dishes (100 mm in diameter) or on sterile, plastic, 24-well, flat-bottom plates. J774A.1 cells were stimulated for 24 h and 48 h with LPS (100 ng/5 × 10⁵ cells/ml; Sigma Aldrich, St. Louis, MO, USA) or with recombinant IFNγ (100 U/5 × 10⁵ cells/ml; PeproTech, London, UK). When necessary, after a certain time of stimulation, dry cell pellets were frozen and stored at − 80 °C prior to RNA isolation.

The release of TNFα and IL-6 by J774A.1 cells stimulated with IFNγ and LPS

J774A.1 cells (5 × 10⁵ cells/ml/well), cultured on sterile, plastic, 24-well, flat-bottom plates at 37 °C and 5% CO₂, were stimulated with either LPS (100 ng/ml) or IFNγ (100 U/ml), as described above. After 24 h, cell-free culture supernatants were collected, frozen and stored at − 20 °C for further detection of cytokine concentrations. TNFα and IL-6 concentrations were measured in ELISA according to procedures delivered by manufacturers with ELISA kits, i.e. mouse TNFα ELISA Ready-SET-Go!® (Bioscience, Inc., San Diego, CA, USA) and mouse IL-6 BD OptEIA™ Set (BD Biosciences, San Diego, CA, USA).

Expression of MPST, CTH, CBS, and rhodanese (TST) in J774A.1 cells

Isolation of total RNA

Total RNA was extracted with Trizol reagent (Lab-Empire, Poland) following the manufacturer’s instructions and RNA quantity and integrity were verified by agarose gel electrophoresis. Isolated RNA was stored at − 80 °C and was used for RT-PCR.

Reverse transcription of RNA

Total RNA (3 µg) was reversely transcribed with 1 µl of GoScript™ Reverse Transcriptase (160 U/µl; Promega Corporation), 4 µl of GoScript™ 5 x reaction buffer (Promega Corporation), 3 µl of MgCl₂, 1 µl of RNase Inhibitor (20 U/µl; Thermo Scientific), 1 µl of dNTP mix (10 mM; Thermo Scientific), and 1 µl of GoScript™ Reverse Transcriptase (160 U/µl; Promega Corporation) in 20 µl of final volume of reaction mixture. RNA was mixed with Oligo d(T) primer and was heated for 5 min at 70 °C. Then, samples were incubated in the reaction mixture for 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C.

Polymerase chain reaction (PCR)

PCR was performed using 2 µl of cDNA, 0.2 µM of each primer, 0.04 U/µl of DNA polymerase (Thermo Scientific) in 10 mM buffer Tris–HCl pH 8.8 (supplemented with 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100), 0.2 mM of dNTP mix (Thermo Scientific) and H₂O–DEPC in total reaction volume of 25 µl. The primer sequences are described in Table 1.

For CTH, after an initial 5 min denaturation at 94 °C, amplification was performed under the following conditions: 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min for 35 cycles, with a final incubation at 72 °C for 8 min.

For CBS, after an initial 5 min denaturation at 94 °C, amplification was performed under the following conditions:

### Table 1 Primer sequences used for RT-PCR

| Genes | Forward (F) and reverse (R) primers (5′–3′) | RT-PCR product size (bp) |
|-------|------------------------------------------|-------------------------|
| CTH   | F: CATGGATGAAGTGTATGGGAGGC R: CGGCAGCGAGTAAACATCG | 445 [10] |
| CB    | F: TGGGACACTACATGTCACAAG R: TTGCAGACTTCGTCGATGG | 307 [11] |
| MPST  | F: GATCCTTCTTCTTATCAAG R: CATGACCACCTACCCA | ~ 400 [12] |
| TST   | F: AGGCACGCCCAAGAGTACCAG R: GTCGTATGCGCCAGCTTGC | 149 (NCBI data base) |
| β-Actin | F: ATGGTGGGAAATGGGTCAGAAAGGAC R: TCTTGTATGTCAGCCAGTTTC | 513 [9] |
94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min for 35 cycles, with a final incubation at 72 °C for 8 min.

For MPST, after an initial 5 min denaturation 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 30 cycles, with a final incubation at 72 °C for 8 min [9].

For TST, after an initial 5 min denaturation at 95 °C, amplification was performed under the following conditions: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min for 34 cycles, with a final incubation at 72 °C for 8 min.

For β-actin, after an initial 5 min denaturation at 94 °C, amplification was performed under the following conditions: 94 °C for 30 s, 55 °C for 35 s, and 72 °C for 35 s for 32 cycles, with a final incubation at 72 °C for 8 min [10].

β-actin was used as an internal standard to normalize mRNA abundance. PCR products were analyzed by 2.0% agarose gel electrophoresis and imaged by UVI-KS 4000i/ImagePC (Syngen Biotech, Poland).

Detection of H$_2$S

H$_2$S produced during the incubation of the cell culture with H$_2$S-releasing compounds was trapped as zinc sulfide in the zinc agarose layer according to [7]. The standard curve was linear at the concentration range of 0–250 µM with a correlation coefficient of 0.994.

Enzymes assay

Cell homogenization

Murine macrophage cells (line J774A.1) (3.5–5 × 10$^6$ cells/pellet) were resuspended in 0.1 M phosphate buffer pH 7.5, in the proportion 1 × 10$^6$ cells/0.07 ml of the buffer, sonicated 3 × 5 s at 4 °C (Bandelin Sonoplus 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, CTH and rhodanese. For reverse phase high performance liquid chromatography (RP-HPLC) analyses, the cells were resuspended in 0.1 ml 10% PCA/1 mM BPDS. The sediment was separated by centrifugation at 1400 × g for 10 min, and the supernatant was stored at −80 °C until analysis.

MPST activity

The MPST activity was assayed according to the method of Valentine and Frankelfeld 1974 [13] with some modification as described by Wróbel et al. [14] and Bronowicka-Adamska et al. [15]. The enzyme activity was expressed as nmols of pyruvate produced during 1 min incubation at 37 °C/1 mg of protein.

CTH activity

The cystathionase activity was determined by Matsuo and Greenberg [16] with modifications described by Czubak et al. [17]. 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. The cystathionase activity was expressed as nmols of α-ketobutyrate formed during 1 min incubation at 37 °C/1 mg of protein.

CBS activity

The activity of CBS was examined in cell homogenates in the presence of δL-homoserine as a substrate after 15 min incubation at 37 °C according to the description by Bronowicka-Adamska et al. [18]. The level of cystathionine was determined using the HPLC method described by Bronowicka-Adamska et al. [19]. The CBS activity was expressed as pmols of cystathionine formed during 1 min incubation at 37 °C/1 mg of protein.

Sulfane sulfur level

Sulfane sulfur was determined by the method of Wood [20] following the procedure described by Bronowicka-Adamska et al. [15]. The method is based on cold cyanolysis and colorimetric detection of ferric thiocyanate complex ion. The sulfane sulfur level was expressed as nmols of SCN$^−$ produced/1 mg of protein.

Protein level

Protein concentration was determined by the method described by Lowry et al. [21] using crystalline bovine serum albumin as a standard.

RP-HPLC (reverse phase high performance liquid chromatography)

The levels of cystathionine in the incubation mixtures were determined using the RP-HPLC method Dominik et al. [22] with modifications [19].

Statistical analysis

All the results from at least three independent experiments, 15–25 individual determinations, were expressed as mean ± SD. The significance of the differences between the controls and investigated samples were calculated using a nonparametric Mann–Whitney U test (p < 0.05).
Results

The release of TNFα and IL-6 by J774A.1 cells stimulated with IFNγ or LPS

After stimulation with both factors, macrophages released proinflammatory mediators. The effects of LPS stimulation was weaker than IFNγ (Table 2).

| Cytokines | TNFα (pg/ml) | IL-6 (pg/ml) |
|-----------|--------------|--------------|
| IFNγ      | 209.3 ± 8.5  | 296.6 ± 20.6 |
| LPS       | 10.46 ± 2.11 | 22.01 ± 3.72 |

The release of TNFα and IL-6 by J774A.1 cells stimulated with IFNγ or LPS

Table 2

The results represent three independent experiments. The macrophages of the J774A.1 cell line (5 × 10^5 cells/ml/well) were stimulated with IFNγ (100 U/ml) or LPS (100 ng/ml) for 24 h. TNFα and IL-6 concentrations in culture supernatants were measured using the ELISA test.

H₂S levels in control, and LPS and IFNγ-stimulated J774A.1 cells

The level of hydrogen sulphide was significantly lower after 24 h in case of both factors (Fig. 1). It was restored to the control levels after 48 h of incubation.

The expression of MPST, CTH, CBS and TST in murine macrophage cells (J774A.1 cells)

After 24 h incubation the control cells, not stimulated (Fig. 2a, line 1), express γ-cystathionase (CTH) and MPST, two H₂S-forming enzymes. Low TST expression and only trace expression of cystathionine β-synthase (CBS) was found. Similarly after 48 h (Fig. 2a, line 4) with the exception of higher expression of TST, as compare to 24 h, and lower expression of CTH.

CBS expression did not change significantly in the LPS-stimulated cells and it seemed even to drop in the IFNγ-stimulated cells (Fig. 2a, b). The expression of MPST significantly decreased during incubation with 100 U IFNγ and 100 ng/ml LPS. IFNγ, in turn, up-regulated CTH expression in J774A.1 cells after both 24 h and 48 h stimulation, in comparison to the control cells (Fig. 2a, b).

The activity of MPST, CTH, TST and the level of sulfane sulfur in J774A.1 cells after stimulation

CBS activity in both the control and stimulated cells was undetectable. The activity of MPST (24 h) and rhodanese (24 h and 48 h) was significantly decreased after incubation with IFNγ and LPS after 48 h, in comparison to the control cells (Table 3A, B). Significantly increased CTH activity was determined after 24 h and 48 h stimulation with LPS (Table 3A, B) but in case of IFNγ it was below the detection limit after 24 h or significantly decreased after 48 h, as compare to control values. A homeostasis of the level of sulfane sulfur was observed—it remains unchanged independently of the compound used and time of incubation (Table 3A, B).

Discussion

Hydrogen sulfide (H₂S) has been identified as a regulator of inflammatory responses [5].

The results have confirmed the expression of CTH and MPST in J774A.1 cells suggesting that macrophages are hydrogen sulphide producing cells. CBS seems to be of a low importance for H₂S formation in J774A.1 cells.

Low CBS expression can result in Hcys availability for CTH-catalyzed H₂S generating reaction [2, 23] (Scheme 1). The IFNγ-stimulated production of NO was detected to result in a decrease in CBS activity [24] and it is attributed to a reduced rate of the transsulfuration pathway, CBS/CTH dependent, converting majority of Hcys (Scheme 1). In the IFNγ-stimulated cells, an increased expression of CTH (Fig. 2a, b) after 24 h is correlated with a decreased - as compared to control—level of H₂S. This can result from significantly decreased activity of CTH and TST observed after both 24 h and 48 h of incubation.
with IFNγ and MPST activity after 24 h (Table 3A, B), the activity of which is regulated through a redox-switch regulatory mechanism [25]. However, higher expression of CTH after 48 h, in comparison to 24 h, resulted in an increased level of hydrogen sulfide (Figs. 1, 2a, b).

Table 3  The activity of MPST, CTH, TST and the level of sulfane sulfur in J774A.1 cells after 24 h (A) and 48 h (B) of stimulation with LPS and IFNγ

| J774A cells | MPST (nmol/mg/min) | CTH (nmol/mg/min) | TST (nmol/mg/min) | Sulfane sulfur (nmol/mg) |
|------------|-------------------|-------------------|-------------------|-------------------------|
| (A)        |                   |                   |                   |                         |
| Control    | 617 ± 98          | 1.46 ± 0.67       | 101 ± 28          | 158 ± 36                |
| IFNγ 100 U | 489 ± 88*         | ND                | 66 ± 10*          | 183 ± 34                |
| LPS 100 ng/ml | 456 ± 92*     | 4.55 ± 2.04*      | 112 ± 25          | 124 ± 30                |
| (B)        |                   |                   |                   |                         |
| Control    | 355 ± 116         | 2.51 ± 0.57       | 161 ± 22          | 172 ± 41                |
| IFNγ 100 U | 302 ± 109         | 1.94 ± 0.63       | 114 ± 32*         | 191 ± 55                |
| LPS 100 ng/ml | 418 ± 111       | 2.97 ± 0.63       | 77 ± 13*          | 182 ± 50                |

ND not detectable
*p < 0.05 LPS, IFNγ versus control (Mann–Whitney U test)

In case of LPS, a higher production of H₂S after 48 h, as compare to 24 h, is also correlated with higher increase in CTH expression after both IFNγ and LPS stimulation. An increased expression of CTH and H₂S production in the macrophages stimulated with LPS was earlier reported.
A decreased expression and activity of MPST after 24 h (Fig. 2a, b; Table 3A) limits the amount of cysteine converted by this enzyme and make it more available for H$_2$S-generating reactions catalyzed by CTH (Scheme 1). Interestingly, the expected increased level of HCs in J774A.1 cells with low CBS expression and activity, is associated with a high CTH expression. A decreased level of H$_2$S after 24 h stimulation with both IFN$\gamma$ and LPS was also associated with an increased CTH expression. These observations may confirm the suggested effect of HCs (increased due to low CBS level) and H$_2$S on CTH expression, which is upregulated by high levels of HCs and low H$_2$S levels and a negative feedback regulation between CBS and CTH [8]. Increased HCys level together with the elevated level of CTH (IFN$\gamma$, 48 h) can result in cysteine sucking into reactions with HCs and its lower availability for GSH synthesis. Decreased GSH levels result in an increased level of reactive oxygen species which are, in turn, involved in the redox regulation of immune cells [27].

H$_2$S can be regarded an O$_2^-$ scavenger—it reacts with superoxide radical anion (Scheme 1). H$_2$S is a reducing agent and a weak acid with approximately 4:1 HS$^-$/$H_2$S ratio at physiological pH [28]. HS$^-$, as opposed to H$_2$S, can reduce the metal center of cytochrome c and lead to production of O$_2^-$ from molecular oxygen [29]. On the other hand, HS$^-$/$H_2$S can reduce Cu$^{2+}$ to Cu$^{+}$ and enhance O$_2^-$ scavenging activity of the copper–zinc SOD (CuZnSOD) [29, 30]. Sulfide is a potent reversible inhibitor of myeloperoxidase activity [31]. It has also been shown that HCys can induce Trx-1 expression in human monocytes [32] and in this way potentiate the antioxidant protection of cells (Scheme 1). Thus, in cells, H$_2$S can act as a pro- or antioxidant, depending on the H$_2$S/HS$^-$ ratio.

In stimulated cells, hydrogen sulphide can react with nitric oxide [33], what can result in decreased H$_2$S levels, especially pronounced after 24 h, and particularly in the IFN$\gamma$-stimulated cells with a massive amount of pro-inflammatory mediators released (Table 1). On the other hand, H$_2$S might also exert anti-inflammatory effects by inhibiting NO production [11]. H$_2$S oxidation results in persulfides and thiosulfate formation [34], substrates of mitochondrial rhodanese. It catalyzes the transfer of sulfane sulfur atom from thiosulfate to various acceptors (e.g. GSH), leading to H$_2$S, and allows for recycling H$_2$S without consuming additional cysteine. However, it seems this does not occur due to its significantly decreased activity after 48 h stimulation with both IFN$\gamma$ and LPS (Table 3A, B). On the other hand, a homeostasis of sulfane sulfur level was observed (Table 3A, B).

**Conclusions**

J774A.1 macrophage cells are hydrogen sulphide-producing cells with CTH as the main enzyme and MPST playing a supporting role. A low CBS expression and non-detectable CBS activity in the IFN$\gamma$-stimulated cells can result in HCys availability for CTH-catalyzed, H$_2$S-generating reactions (Scheme 1). A decreased level of H$_2$S after 24 h stimulation with both IFN$\gamma$ and LPS associated with an increased CTH expression can confirm CTH upregulation by a high level of HCys (due to low CBS expression) and low H$_2$S and a negative feedback regulation between CBS and CTH.

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**Author contributions** P.B.-A. provided the experimental data, analyzed the data, wrote the manuscript in consultation with M.W., P.S., A.G. provided the experimental data. H.J. performed and developed the RT-PCR analysis, contributed to the interpretation of the results. K.N. supervised and performed cell cultures. J. M. and M.W. discussed the results and contributed to the final manuscript.

**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

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