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ORIGINAL ARTICLE

Homocysteine metabolism in peripheral blood mononuclear cells: evidence for cystathionine beta-synthase activity in resting state

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Abstract Activated peripheral blood mononuclear cells (PBMC) release homocysteine and possess cystathionine β-synthase (CBS) activity; however, it was thought that there is no CBS in resting state. Previously, we found that nickel decreased intracellular homocysteine concentration in un-stimulated (e.g. resting) PBMC, suggesting that resting PBMC might also have active homocysteine metabolism. Here, we demonstrated that un-stimulated PBMC synthesize (incorporate L-[methyl-^{14}C]methionine to DNA, lipids and proteins), release (increase extracellular homocysteine), and metabolize homocysteine. Intracellular homocysteine concentration varied with incubation time, depending on extracellular concentrations of methionine, homocysteine, and glutathione. Methionine synthase activity was constant and independent of thiol concentrations. In Western blot, CBS protein was clearly identified in freshly isolated PBMC. CBS protein level and activity increased with incubation time, upon stimulation, and similar to intracellular homocysteine, depending on intra- and extracellular homocysteine and glutathione concentrations. According to our knowledge, this is the first evidence that certifies homocysteine metabolism and regulatory role of CBS activity to keep balanced intracellular homocysteine level in resting PBMC. Homocysteine, released by PBMC, in turn can modulate its functions contributing to the development of hyperhomocysteinemia-induced diseases.

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Introduction

Homocysteine is a non-essential, sulfur-containing amino acid. It is synthesized from methionine via multi step process (Fig. 1). First, methionine receives an adenosine group from ATP, a reaction catalyzed by S-adenosylmethionine synthase (MAT), to give S-adenosylmethionine (SAM). SAM then transfers the methyl group to acceptor molecules by methyltransferases (MT)—involving DNA methyltransferase—and forms S-adenosylhomocysteine (SAH). SAH is then hydrolyzed by S-adenosylhomocysteine hydrolase (SAHH) to yield homocysteine. Homocysteine is metabolized by two primary fates; it can convert via Vitamin B_{12} dependent methionine synthase (MS) in folate cycle to methionine, or it can be irreversibly removed in transsulfuration pathway. One key enzyme in the transsulfuration pathway is the cystathionine β-synthase (CBS), which catalyses condensation of homocysteine and serine to cystathionine. This reaction uses Vitamin B_6 as cofactor. Cystathionine γ-lyase then converts this amino acid to cysteine, ammonia, and α-ketobutyrate. Cysteine is a substrate of glutathione (GSH) synthesis whose availability is the main determinant of cellular GSH synthesis. The third remethylation pathway using betaine as methylation agent for homocysteine is restricted to liver and kidney (Delgado-Reyes et al. 2001) (Fig. 1). The main organ of homocysteine formation is the liver.

Deficiencies of vitamins, e.g., folate, Vitamin B_6, or B_{12} can lead to elevated homocysteine levels (Chanarin et al. 1985; Morrow and Barness 1972). Hyperhomocysteinemia

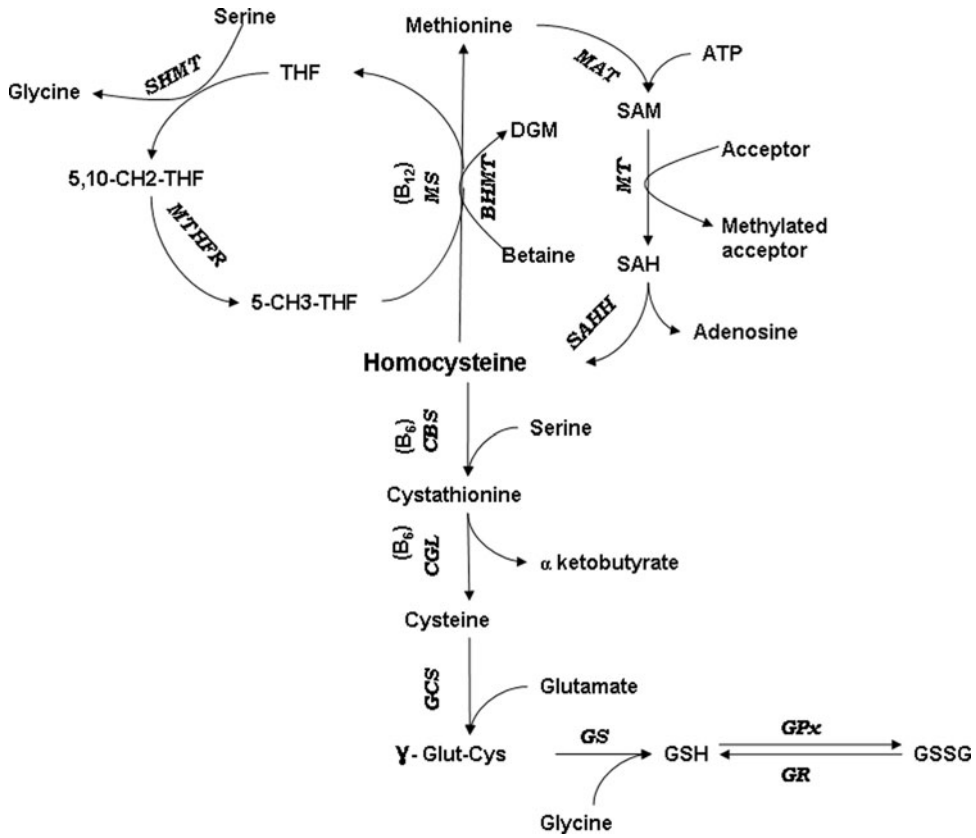

Fig. 1 Homocysteine formation and metabolism [*MAT* methionine-adenosyltransferase, *MT* methyltransferase, *SAHH* SAH hydrolase, *MS* methionine synthase, *MTHFR* 5,10 methylenetetrahydrofolate reductase, *SHMT* serinehydroxymethyltransferase, *BHMT* betainehomocysteine methyltransferase, *CBS* cystathionine-*β*-synthase, *CGL* cystathionine *γ*-lyase, *GCS* *γ*-glutamyl-cysteine synthetase (or glutamate-cysteine ligase), *GS* glutathione synthetase, *GR* glutathione reductase, *GPx* glutathione peroxidase, *SAM* S-adenosylmethionine, *SAH* S-adenosylhomocysteine, *THF* tetrahydrofolate, *γ-Glut-Cys* *γ*-glutamyl-cysteine, *GSH* reduced glutathion, *GSSG* oxidized glutathione]

is known as a risk factor for cardiovascular disease, such as myocardial infarction, stroke etc. (McCully and Wilson 1975; van Guldener and Stehouver 2000; Selhub 1999). Immune activation appears to be involved in atherogenesis as well as in other diseases that are associated with mild–moderate hyperhomocysteinemia such as rheumatoid arthritis, or neurodegenerative diseases (Wallberg-Jonsson et al. 2002; Schroecksnadel et al. 2003a; Van Dam and Van Gool 2009; Minagawa et al. 2010).

Peripheral blood mononuclear cells (PBMC) play important role in immune activation. Connection between PBMC activation and methionine cycle was also demonstrated; PBMC utilize methionine and release homocysteine in activated state (Schroecksnadel et al. 2003b; Crott et al. 2001a). However, very little is known of homocysteine metabolism, if any, in un-stimulated PBMC, especially of transsulfuration pathway. It is mainly due to the fact that in an early work, Goldstein et al. could not measure CBS activity in freshly isolated PBMC using radioactive labeled substrate (Goldstein et al. 1972). Based on this observation until now, CBS activity is studied in stimulated PBMC (Fensom et al. 1983; Tsai et al. 1996). However, CBS mRNA was isolated from resting PBMC (Devi et al. 1998).

Previously, we found that intracellular homocysteine (i-Hcy) concentration in un-stimulated 24-h incubated PBMC can be decreased by nickel (Katko et al. 2008). We supposed that decreased i-Hcy might be a consequence of enhanced extracellular release or its enhanced metabolism in remethylation or probably transsulfuration pathways. To confirm this hypothesis, intra- and extracellular concentrations of homocysteine, and enzyme activities involved in homocysteine metabolism such as MS responsible for remethylation and CBS in transsulfuration pathway was determined in freshly isolated, un-stimulated and phytohemagglutinin (PHA) stimulated PBMC incubated for various time. CBS protein was identified by Western blot analysis.

Materials and methods

Separation of peripheral blood mononuclear cells (PBMC) was performed from the fasting blood of healthy

volunteers by density gradient centrifugation (Boyum 1968) using Histopaque 1077 (Sigma, St. Louis, MO, USA). Cells were suspended in RPMI 1640 (Sigma, St. Louis, MO, USA). 1 ml of 10^6 cells/ml was placed to a 24-well plate (Falcon, Becton–Dickinson Labware, New Jersey, USA) and incubated for indicated time in a humidified CO_2 incubator at 37°C. Intra- and extracellular thiols were determined in fetal bovine serum-free (FBS, Sigma, St. Louis, MO, USA) conditions because of its homocystein content. At the end of incubation, supernatant was collected for the determination of extracellular homocysteine (e-Hcy) concentration. Remaining supernatant was removed totally, and cells without washing, which can modify intracellular thiol content (Korendyaseva et al. 2010), were used for homocysteine determination. PBMC were disrupted by three cycles of freezing (5 min) and thawing in 100 µl 0.1 M sodium acetate (pH = 6.0) lysis buffer and used for HPLC analysis and protein determination. Healthy volunteers gave their informed consent, which met with the rules of the Ethics Committee of the University of Debrecen.

Homocysteine was determined by the method of Ubbink et al. (1991) without modification by HPLC (Hitachi-Merck L 6200A) using fluorescence detection (Hitachi-Merck, F 1050). Reduction of thiols was performed with tri-n-butylphosphine, and derivatization with 7-fluoro-2,1,3-benzoxazole-4-sulfonamide (SBDF, 1 mg/ml). Mobile phase was 0.1 M KH_2PO_4 buffer (pH = 2.1) containing 4% acetonitrile. Flow rate was 1.5 ml/min, excitation wavelength was 358 nm, and emission wavelength was 515 nm. Calibration curve was prepared from analytical grade homocysteine (Sigma, St. Luis, MO, USA). Measured thiol concentration was normalized to protein content of cell lysate, and change was expressed as percentage of control. PBMC incubated for 24 h in normal RPMI were considered as control. All determinations were performed in duplicate. Results are expressed as mean ± SD of indicated independent experiments.

Protein content of cell suspension was determined by the method of Lowry (Lowry et al. 1951) or Bradford assay.

Methionine consumption by PBMC was assessed by determining incorporation of L-[methyl-^{14}C]methionine (specific activity 54 mCi/mmol, Sigma, St. Louis, MO, USA) into cells. Un-stimulated- and PHA-stimulated PBMC (1×10^6 cells/ml) were incubated in methionine, cystine- and glutamate-free modified RPMI (Sigma, St. Louis, MO, USA), that was supplemented with 50 µM L-[methyl-^{14}C]methionine, 50 µM DL-methionine, and 2 mM L-glutamine, 0.2 mM L-cysteine, and 10% FBS. Cysteine at this concentration does not affect cell viability. After 72-h incubation, cells were washed with phosphate balanced salt solution (PBS, Sigma St. Louis, MO, USA) to remove excess of radioactivity and then were scrapped by a rubber policeman. Cell suspension was transferred into an Eppendorf tube, centrifuged at 400 g, for 5 min at 4°C, and re-suspended in 600 µL of fresh PBS. Re-suspended cells were divided into three fractions: From one portion, proteins were precipitated with trichloroacetic acid (at 6% final concentration). After wash with TCA, precipitate was dissolved in scintillation fluid. From another portion of cell suspension, lipids were extracted two times with chloroform/methanol (2:1 v/v). Lower organic phase was collected and evaporated until dryness under a nitrogen stream. From the third portion, Genomic DNA was isolated by GenElute^{TM} Blood Genomic DNA Kit (Sigma St. Louis, MO, USA) according to manufacturer instructions. The samples were transferred into counting vials, and the incorporated radioactivity was measured in a liquid scintillation counter (Packard 2200CA). Measured radioactivity was normalized to protein content of samples. Data show results of five independent experiments. All determinations were performed in duplicate.

Experimental conditions to study concentration-dependent effects of methionine and thiols

Methionine dependency was determined in PBMC incubated for 24 h in normal RPMI supplemented with indicated concentrations of DL-methionine (Sigma, St. Louis, MO, USA). In separated experiments, to assess effect of absence of methionine, cystine and glutamate on intracellular homocysteine, PBMC were incubated for 24 h in methionine, cystine and glutamate-free modified RPMI (Sigma, St. Louis, MO, USA). Modified RPMI was used without any supplementation (met−) or was supplemented with 0.1 mM L-methionine but not with cystine (or cysteine) and glutamate (met+). To determine effects of excess of extracellular thiols, normal RPMI was used containing 0.1 mM homocysteine (Hcy+) or 0.1 mM GSH (GSH+). Cells of the same subject, incubated in normal RPMI served as control (n-RPMI). At the end of incubation, cells were treated for HPLC determination as described above. Results are expressed as mean ± SD of five independent experiments. All determinations were performed in duplicate.

Incubation conditions to study enzyme activities

Freshly isolated or incubated (1, 4, 24, 48, 72 h) PBMC ($1x10^6$cells/ml), before disruption were scrapped by a rubber policeman, and transferred into an Eppendorf tube, and centrifuged at 400 g, for 5 min, at 4°C. Cells were disrupted in 200 µL of lysis buffer that was 0.1 M phosphate containing 2.5 mM DTT (pH = 7.2) in the case of MS, and without DTT in the case of CBS, by a Teflon

homogenizer in ice. Cell homogenate was centrifuged at 14000 g for 10 min at 4°C, and protein content was determined in the supernatant. Mean protein content in samples was 0.3 ± 0.04 mg/ml. In some experiments, PBMC were cultured in presence of 0.1 mM GSH (GSH+), or 0.1 mM Hcy (e-Hcy+) in normal RPMI, or methionine-free RPMI (met−) for 24 h. For stimulation, Phytohemagglutinin (PHA) was used at 10 μg/ml concentration and cells were incubated for 48, or 72 h. In these experiments, to provide optimal conditions for proliferation, RPMI was supplemented with 10% FBS in both un-stimulated and stimulated cases. Results are expressed as mean ± SD of indicated experiments. All determinations were performed in duplicate.

Methionine synthase activity was determined by method of Drummond et al. (1995). The incubation mixture consisted of 100 mM phosphate buffer (pH = 7.2), 25 mM dithiothreitol (DTT), 19 μM S-adenosylmethionine (SAM), 500 μM L-homocysteine, 50 μM hydroxocobalamin, 50 μl homogenate and 210 μM 5-methyl-tetrahydrofolate in total volume of 0.8 ml. L-homocysteine was liberated from L-homocysteine-thiolacton (Sigma, St. Louis, MO, USA) with preincubation of NaOH (0.1 M) for 5 min at 60°C to allow cleavage of the thiolacton ring. After incubation at 37°C, the reaction was stopped by adding 200 μl of 5 N HCl/60% formic acid. The product (tetrahydrofolate) was detected spectrofotometrically at 350 nm following its conversion to 5,10-methenyl-tetrahydrofolate by heating at 80°C for 10 min. Activity in mU/mg protein (one unit is defined as 1 μmol product formed/min) is calculated from the extinction coefficient of 5,10-methenyl-tetrahydrofolate in acid (26,500 $M^{-1}cm^{-1}$). Results are expressed as mean ± SD of five independent experiments. All determinations were performed in duplicate.

Cystathionine β-synthase activity was measured according to Hamelet and Zou (Hamelet et al. 2007; Zou and Banerjee 2003). The supernatant of disrupted cells was mixed with D,L-propargylglycin (250 μM), an inhibitor of cystathionine γ-lyase, and piridoxal phosphate (40 μM) in 100 mM Tris, pH = 8.3. To determine SAM dependency of enzyme 380 μM SAM was added. After pre-incubation for 15 min at 37°C, reaction was initiated by addition of L-serine and L-homocysteine at final concentrations of 10 mM and reaction mixture was incubated for 60 min at 37°C. Enzyme activity was determined by measuring homocysteine consumption by Ellman's reagent (Hamelet et al. 2007) or cystathionine formation by acidified nynhidrin (Zou and Banerjee 2003), and was expressed as mU/mg protein. One unit is defined as μmol substrate conversion/h. Data show results of at least five independent experiments. All determinations were performed in duplicate.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from whole blood of ten healthy individuals with QIAamp® RNA Blood Mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription were carried out using SuperScript III Reverse Transcriptase (Invitrogen). PCRs were performed using iQ SYBR Green Supermix (Bio-Rad) and the following primers: CBS forward: 5′-AGG ATA AAG CCA AGG AG-3′ and reverse: 5′-TGT TGA TTC TGA CCA TAG GG-3′. PCRs were carried out using the iCycler iQ Real Time PCR System (Bio-Rad). Results were normalized to GAPDH mRNA levels. Results are expressed as mean ± SD of ten independent experiments.

Western blot analysis

Freshly isolated, 24- and 72-h incubated (1×10^6 cells/ml) un-stimulated and PHA-stimulated PBMC were disrupted in 0.1 M phosphate buffer containing 0.1% TritonX 100, and a mixture of protease inhibitors (Sigmafast™ Protease Inhibitor Tablets containing 2 mM AEBSF, 1 mM EDTA, 130 μM bestain, 14 μM E64, 0.3 μM aprotinin, 1 μM leupeptin) was added. Cell lysate was kept at −70°C until use. To evaluate CBS protein expression, cell lysate (25 μg total protein) was denaturated for 5 min at 100°C in Laemmli buffer, electrophoresed in 12.5% SDS–PAGE, and blotted onto nitrocellulose membrane (Amersham™ Hybond™-ECL, GE Healthcare, UK, Buckinghamshire). Membrane was blocked in 6% Non-Fat Dry Milk (NFDM) in TBS containing 0.1% Tween-20 for 1 h. Polyclonal anti-CBS antibody from Sigma (AV45746) was added at concentration of 1.25 μg/ml in TBS-Tween-20 with 1% NFDM incubated for 16 h followed by the incubation with peroxidase-linked anti-rabbit IgG from donkey (Amersham™ECL™ NA934, GE Healthcare) at dilution 1:15000. Antigen–antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (Amersham™ECL™ Western blotting detection reagents, GE Healthcare). Signals were visualized by exposure of the blot to Amersham Hyperfilm™ ECL X-ray film (GE Healthcare) for 10 min. After detection membrane was stripped and re-probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

The data were expressed as the mean and the SD of indicated independent experiments. Statistical analyses were conducted by ANOVA test followed by post hoc Newmann–Keuls test for comparisons. The differences were considered significant at $P < 0.05$.

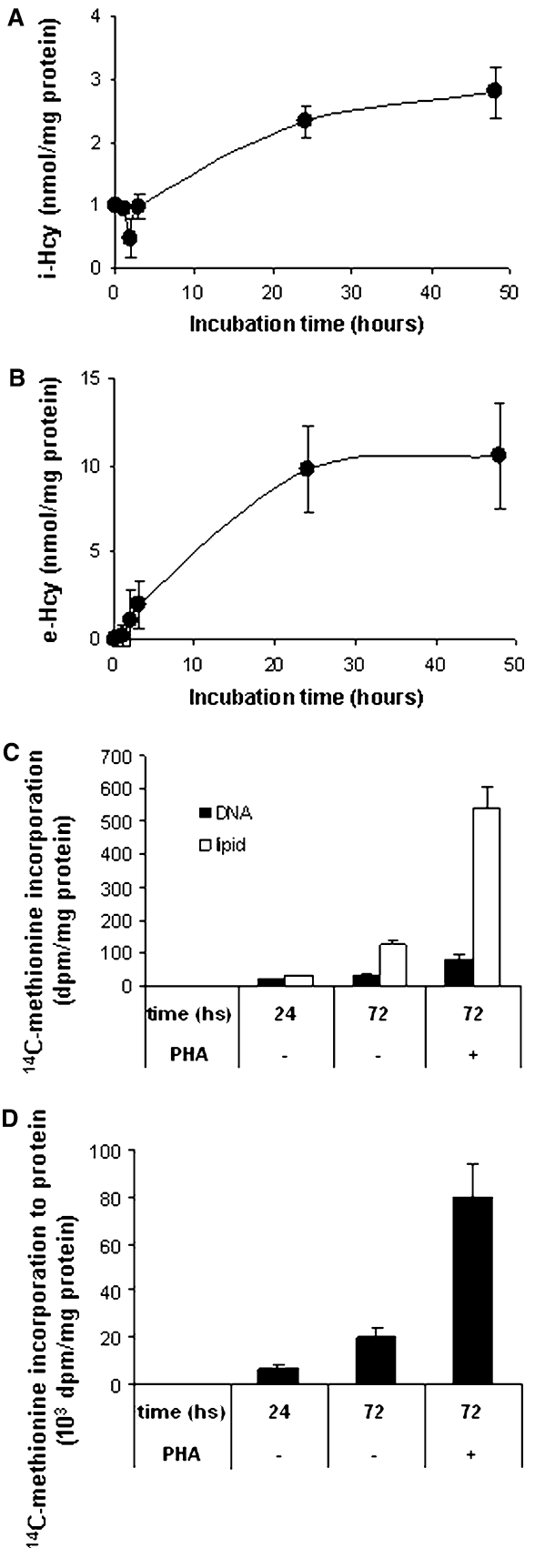

Fig. 2 Intra- and extracellular homocysteine concentrations and methionine utilization by un-stimulated (resting) PBMC. **a** Time dependent change in intracellular (i-Hcy), and **b** extracellular homocysteine (e-Hcy) concentrations. Results are expressed as mean ± SD of five independent experiments. **c** L-[methyl-^{14}C]methionine incorporation to DNA and lipids, and **d** proteins in 24- and 72-h incubated resting and 72-h incubated PHA-stimulated states. Results are expressed as mean ± SD of three independent experiments. All determination was performed in duplicate

Results and discussion

Incubation conditions affect intra- and extracellular homocysteine concentration in un-stimulated (e.g. resting) PBMC

Stimulated PBMC release homocysteine (Schroecksnadel et al. 2003b) and homocysteine at high concentrations (>100 μM) affects PBMC, B and T lymphocyte functions in vitro (Hadzic et al. 2005; Zhang et al. 2001; Crott et al. 2001b). However, i-Hcy concentration in neither resting nor stimulated PBMC is well studied. To get inside into methionine utilization in resting PBMC, we first assessed i-Hcy as well as e-Hcy concentrations as a function of incubation time. During the first 1–2 h, i-Hcy decreased at about 50% in PBMC compared to freshly isolated state; and then i-Hcy tended to reach its original concentration at 4–5 h. After that, i-Hcy remained near constant during entire observation period (Fig. 2a). At the same time, PBMC released homocysteine continuously (Fig. 2b), and this process might be responsible for i-Hcy depletion observed in early phase of incubation. At the same time, total intracellular GSH concentration increased slightly (about 10%). Reduced to total GSH ratio was stable, suggesting that no significant change in intracellular redox state developed during incubation in un-stimulated PBMC.

To confirm that time-dependent change in i-Hcy in resting PBMC can be due to de novo synthesis of homocysteine, incorporation of L-[methyl-^{14}C]methionine into lipids, DNA (Fig. 2c) and proteins (Fig. 2d) in 24- and 72-h incubated resting PBMC compared to PHA-stimulated case were determined. As shown in Fig. 2c, L-[methyl-^{14}C]methionine incorporated to lipids and DNA in both un-stimulated and PHA-stimulated states. Incorporated radioactivity in lipids and DNA can be due to methyltransferase reactions using SAM as methyl donor (Fig. 1). However, as expected, incorporation of L-[methyl-^{14}C]methionine into DNA, lipids and proteins was significantly lower in un-stimulated than in PHA-stimulated cells (Fig. 2c, d, respectively).

Homocysteine is coming exclusively from methionine and methionine availability by providing SAM—the main regulator of intracellular homocysteine metabolism—determines homocysteine intracellular concentration. Methionine supplementation significantly increased e-Hcy

concentration in Concanavalin A, pokeweed mitogen and PHA stimulated PBMC, suggesting that during immune response, proliferating immunocompetent cells could contribute to the development of hyperhomocysteinemia (Schroecksnadel et al. 2003b). In contrast to stimulated case, methionine supplementation did not modify e-Hcy (Fig. 3a, open circles) and had only minor increasing effect on i-Hcy (Fig. 3a, black circles) in 24-h incubated un-stimulated PBMC. When extracellular methionine exceeded 0.3 mM, no further elevation in i-Hcy was observed (Fig. 3a, black circles). Steady state distribution of methionine between intra- and extracellular space was found to be developed within few minutes in hepatocytes providing possibility for increased metabolism to remove its excess (Korendyaseva et al. 2010).

Absence of methionine resulted in total depletion of i-Hcy (Fig. 3b, Met−) and supplementation with methionine (0.1 mM) in absence of cysteine or cysteine and glutamate resulted in decreased i-Hcy (Fig. 3b, Met+) as well compared to control cells incubated in normal RPMI (Fig. 3b, nRPMI) for 24 h. Furthermore, in met− and

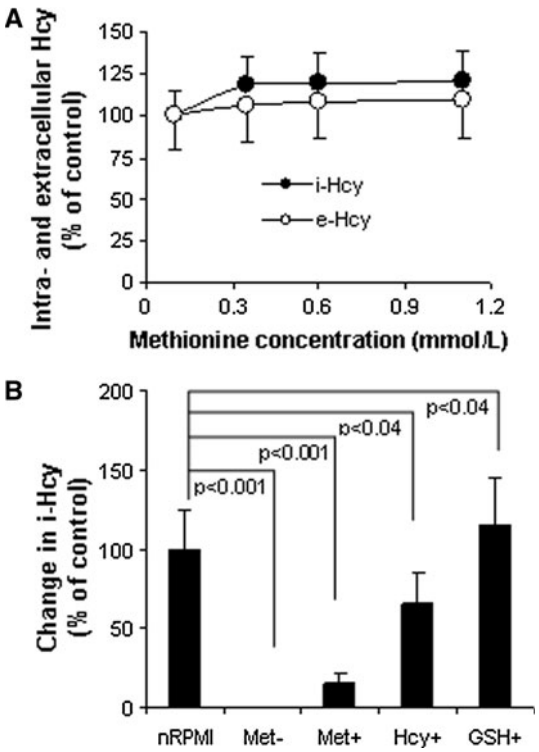

Fig. 3 Effects of extracellular methionine, homocysteine and glutathione concentrations on i-Hcy in resting PBMC. **a** Concentration dependent effect of methionine on i-Hcy and e-Hcy. **b** Effects of methionine absence (Met−), glutamate and cystine absence in presence of 0.1 mM methionine (Met+), and supplementation with homocysteine (Hcy+, 0.1 mM), and glutathione (GSH+, 0.1 mM) on i-Hcy compared to normal RPMI (nRPMI) after 24-h incubation. Results are expressed as mean ± SD of five independent experiments. All determination was performed in duplicate

met+ conditions e-Hcy decreases by 30% compared also to 24-h incubated cells in normal RPMI at which time point both i-Hcy and e-Hcy tended to reach a plateau (Fig. 2a, b, respectively). In PHA stimulated lymphocytes—they have more intense methionine utilization as resting ones—incubated in methionine and homocysteine-free RPMI total absence of e-Hcy have been found (Crott et al. 2001b). These results suggest that independently from the fact that homocysteine is synthesized exclusively from methionine, presence of sufficient methionine itself in absence of cystine and glutamate does not provide normal homocysteine synthesis and/or metabolism in PBMC. Moreover, without extracellular cystine/cysteine cells try to produce cysteine from methionine via transsulfuration pathway that can lead to decreased i-Hcy.

Several experimental data support that not only methionine but also extracellular concentration of GSH and homocysteine modifies lymphocyte functions and IL-2 secretion, and in turn, intracellular thiol availability regulates selective signalling pathways and are essential for IL-2 production and cell proliferation in T lymphocytes (Zhang et al. 2001; Crott et al. 2001b). Buthionine-sulfoximine (BSO), an inhibitor of GSH synthesis, blocked T cell proliferation (Hamilos et al. 1989) that was reversed by co-incubation with N-acetylcysteine (Hadzic et al. 2005) or GSH (Suthanthiran et al. 1990). Moreover, homocysteine (≥1 mM) induced B lymphocyte proliferation and ROS production and antioxidant enzymes and p38 MAPK inhibitors could reverse homocysteine-induced processes in B cells (Zhang et al. 2001). In this context, we demonstrated here that extracellular concentration of these thiols modify i-Hcy in resting state. Excess of extracellular homocysteine (0.1 mM) significantly decreased (Fig. 3b, Hcy+), while GSH (100 μM) increased i-Hcy (Fig. 3b, GSH+). It has to be noted that PBMC incorporated GSH that caused 1.5 ± 0.4-fold increase in i-GSH level. These observations might be explained by the fact that high extracellular concentration of homocysteine stimulates intracellular homocysteine metabolism, leading to decreased i-Hcy. In contrast, GSH supplementation by its incorporation into cells probably inhibits homocysteine metabolism that can lead to elevated i-Hcy.

On the basis that un-stimulated PBMC incorporate methionine, and extracellular concentration of homocysteine and GSH modify i-Hcy, we concluded that PBMC synthesize and metabolise homocysteine. Initiation of these processes could partially be due to the modification of extracellular environment e.g. thiol concentrations. Change in extracellular thiols during incubation might provide continuous "stimulation" for homocysteine formation that lead to its metabolism to keep i-Hcy in PBMC balanced in vitro. Biological relevance of these observations in vivo might be underlined by the facts that plasma concentrations

of homocysteine (Svatikova et al. 2004), methionine (Velez-Carrasco et al. 2008), cystathionine (Guttormsen et al. 2004) and GSH (Blanco et al. 2007) exhibit diurnal variation. Diurnal variation of thiol concentrations could cause activation of methionine–homocysteine cycle in circulating PBMC, and probably other tissues, that lead not only release of homocysteine but also its metabolism.

Homocysteine metabolism in un-stimulated (e.g. resting) human PBMC

Homocysteine can metabolize in folate cycle by MS to form methionine. MS works in two steps in a ping-pong reaction. First, methylcobalamin (MeB_{12}) is formed by a methyl group transfer from 5-methyl-tetrahydrofolate with the formation of MeB_{12} and tetrahydrofolate. Next, MeB_{12} transfers this methyl group to homocysteine, regenerating the cofactor cobalamin and releasing the product methionine (Banerjee and Matthews 1990). MS activity increases when SAM concentration in cells decreases. We found that neither change in concentration of thiols e.g. homocysteine depletion by 1-h incubation (Fig. 4, i-Hcy−) and incubation for 24 h with GSH (Fig. 4, GSH+) nor incubation time (Fig. 4, 0-, 24- and 48-, respectively), and PHA stimulation (Fig. 4, 48 PHA) modified protein normalized MS activity. Methionine depletion (Fig. 4, met−) was the only parameter that has modification effect. Therefore, the role of the transsulfuration pathway in i-Hcy regulation in resting PBMC emerged.

The first enzyme in the transsulfuration pathway is CBS (Fig. 1). The physiological significance of transsulfuration pathway in production of thiols, such as cysteine and GSH, results from the fact that approximately 30–50% of intracellular cysteine is synthesized in situ by most cells. Cysteine is necessary to GSH synthesis (Reed et al. 2008; Mosharov et al. 2000). On the basis that MS is stable, we supposed that PBMC at resting state might also possess CBS activity. However, as we mentioned previously in an early work, Goldstein et al. could not measure CBS activity in un-stimulated PBMC using radioactive labeled substrate (Goldstein et al. 1972).

Therefore, to assess whether freshly isolated PBMC express any CBS protein we performed Western blot analysis. As shown in Fig. 5, CBS protein could be detected in freshly isolated PBMC. Cultured un-stimulated and PHA-stimulated PBMC and HepG2 cells serve as positive controls for CBS expression. Double-line on Western blot in the case of HepG2 cells is due to trypsin treatment inducing partial truncation of the enzyme (Kery et al. 1998). Therefore, it can also be concluded that freshly isolated PBMC express full-length CBS protein.

Then, we determined CBS mRNA and protein content in PBMC derived from eight volunteers. Cells were disrupted immediately after separation followed by RT-PCR and Western blot analysis. Relative CBS/GAPDH mRNA level was found to be 0.964 ± 0.28. In Western blot, CBS protein was also identified in all samples (Fig. 6a, b respectively) with mean levels of 0.105 ± 0.037 CBS/GAPDH range between 0.075 and 0.175 CBS/GAPDH. All samples possessed measurable CBS activity (Fig. 6c) with mean activity of 0.99 ± 0.32 mU/mg protein range between 0.6 and 1.59 mU/mg protein. CBS protein level and activity correlated positively $r = 0.526$, $P < 0.001$. CBS protein level (Fig. 6d, e, respectively) as well as activity (Fig. 6f) significantly increased with incubation time and upon stimulation with PHA.

CBS is a modular protein in which the N-terminal stretch constitutes to the heme domain. The heme domain

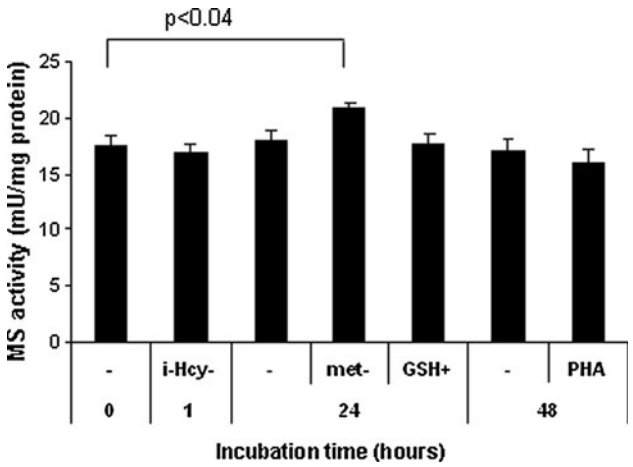

Fig. 4 Methionine synthase activity in resting PBMC. Cells were disrupted after separation (0) or incubated for 1 h to induce i-Hcy depletion (i-Hcy−), and 24 h in absence (met−) and presence of methionine, and GSH (GSH+, 0.1 mM), and for 48 h in absence and presence of PHA. Results are expressed as mean ± SD of five independent experiments. All determination was performed in duplicate

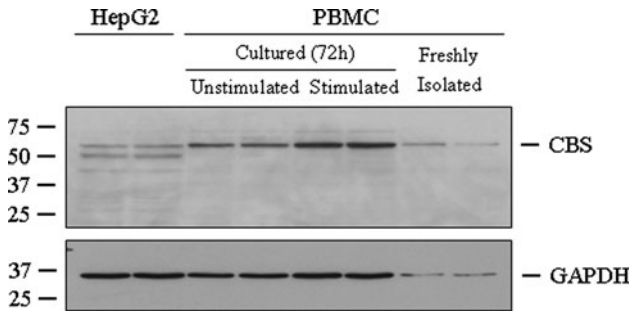

Fig. 5 Identification of CBS protein in freshly isolated PBMC using 72-h incubated un-stimulated and PHA stimulated PBMC and confluent HepG2 cells as positive controls. HepG2 cells were scrapped by trypsinization that caused partial truncation of CBS. Membrane was re-probed for GAPDH and shown on the lower panel

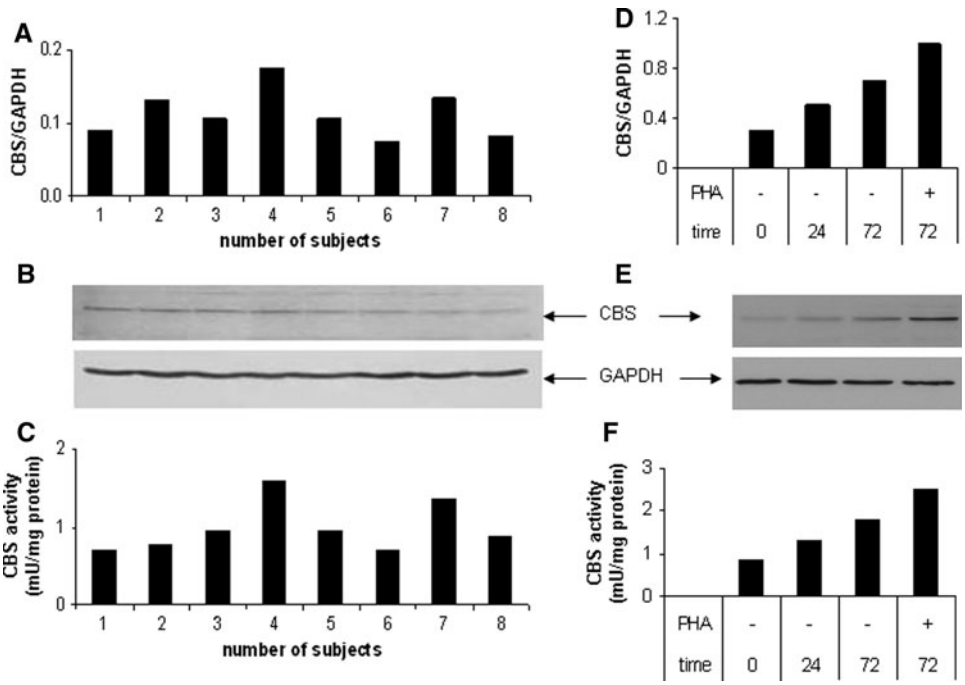

Fig. 6 Identification of CBS protein and activity in human PBMC. **a** Densitometry results, **b** of Western blot analysis that confirmed presence of CBS protein, and **c** CBS activity in PBMC disrupted immediately after separation from blood of eight different donors. **d** Densitometry results, **e** of Western blot analysis, and **f** CBS activity in freshly disrupted (0), 24- and 72-h incubated un-stimulated (−) and 72-h PHA-stimulated PBMC. Typical results in one subject. Enzyme activity determination was performed in duplicate

is followed by a core domain in which pyridoxal phosphate is covalently linked via a Schiff-base in the active site and leads to the C-terminal regulatory domain. C-terminal domain exerts intrasteric inhibition that is reversed by binding of SAM that increases activity of the enzyme (Banerjee and Zou 2005; Ignoul and Eggermont 2005; Prudova et al. 2006). C-terminal truncation causes deletion of regulatory domain. The truncated enzyme is highly active but unresponsive to activation with SAM (Zou and Banerjee 2003; Prudova et al. 2006).

To prove that CBS in resting PBMC is a fully active enzyme, the effect of SAM on its activity was determined. As shown in Fig. 7a, addition of SAM nearly doubled CBS activity. SAM responsiveness of CBS from un-stimulated PBMC together with results of Western blot analysis confirms that CBS is a full length and an active enzyme in resting PBMC. Moreover, taking into consideration that cystathionine γ-lyase, second enzyme in transsulfuration pathway (Fig. 1), is also active in resting human lymphocytes (Barathi et al. 2007), it can be concluded that they possess the full transsulfuration pathway.

CBS activity apart from SAM concentration is regulated by a heme cofactor that functions as a redox sensor, and enzyme activity is doubled when the Fe(II) is oxidized to Fe(III) (Banerjee and Zou 2005; Prudova et al. 2006). The physiological relevance of the redox sensitivity of CBS is that it would enhance conversion of methionine to cysteine, the limiting reagent in the synthesis of GSH, when oxidative stress occurs, and this switching would serve as an auto-regulatory response to an oxidative insult by increasing GSH synthesis (Prudova et al. 2006). High concentration of GSH is known as a competitive inhibitor against glutamate of γ-glutamyl-cysteine synthetase (GCS), the enzyme that catalyzes synthesis of glutamyl-cysteine (Fig. 1). The primary effect of the product (GSH) inhibition on GCS is to prevent excess accumulation of GSH (Reed et al. 2008). On the other hand, antioxidants such as superoxide dismutase, catalase or water soluble variant of Vitamin E (Trolox) inhibit CBS activity (Vitvitsky et al. 2003). In this respect, our findings, that extracellular GSH induced a decrease in CBS activity (Fig. 7b, e-GSH+) that caused elevation in i-Hcy (Fig. 3b) is in good agreement, and confirm that increased extracellular GSH concentration leading to elevated i-GSH as well can also modulate redox-state of CBS that in turn decreases its activity.

Finally, CBS activity is known to be regulated by homocysteine availability and increase in SAM and homocysteine concentrations enhances CBS activity providing possibility to cell to maintain its optimal homocysteine concentration in methionine overflow cases (Banerjee and Zou 2005; Reed et al. 2008). Our results, that excess of extracellular homocysteine decreased significantly i-Hcy (Fig. 3b, Hcy+), is in good agreement with homocysteine-induced elevation of CBS (Fig. 7b, e-Hcy+). Finally, we also showed that CBS activity is sensitive to decrease of i-Hcy since in 1-h incubated un-stimulated PBMC—that leads to i-Hcy depletion (Fig. 2a)—CBS activity decreased (Fig. 7b, i-Hcy−). Altogether, intra- and extracellular thiol availability regulate CBS activity that seems to be responsible for regulation of i-Hcy, at least in resting sate.

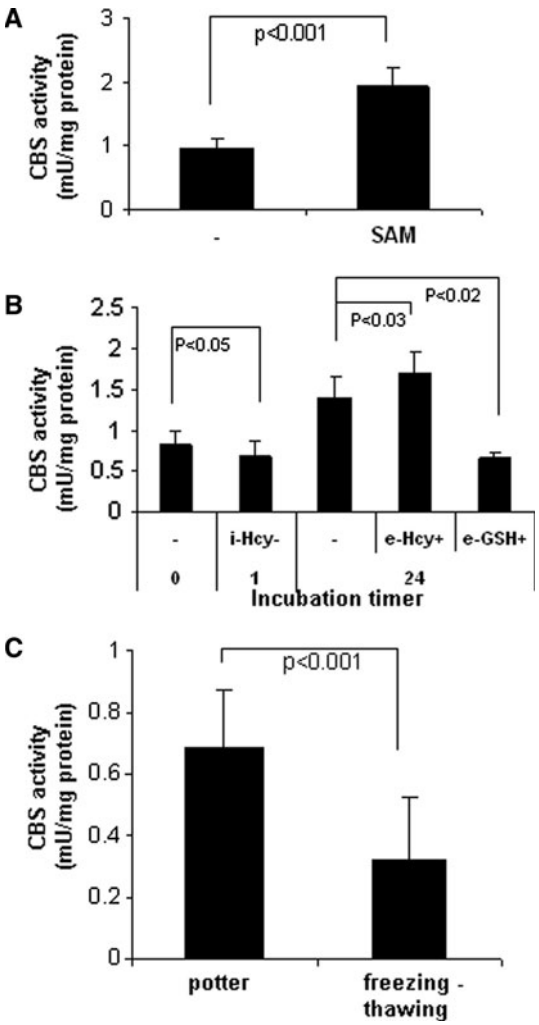

Fig. 7 Identification of some parameters that affect CBS activity in resting PBMC. **a** SAM responsiveness of CBS in PBMC disrupted immediately after separation. **b** CBS activity in homocysteine depleted PBMC (i-Hcy−) induced by 1-h incubation (1) compared to freshly isolated state (0), and in 24 h incubated control (−) and homocysteine (e-Hcy+, 0.1 mM) and GSH (GSH+, 0.1 mM) supplemented resting PBMC. **c** Effect of cell disruption method on CBS activity in PBMC. Cells were disrupted by three-cycle of freezing/thawing or Teflon homogenizer (potter). Results are expressed as mean ± SD of five independent experiments. All determination was performed in duplicate

These results are in contradiction with Goldstein et al. findings (Goldstein et al. 1972) who used freezing and thawing for cell disruption. First, we also used freezing and thawing to disrupt PBMC and, as seen in Fig. 7c, we found no or low CBS activity in PBMC. However, when Teflon homogenizer (potter)—that is widely used for disruption of PBMC—was used we were able to measure CBS activity in all PBMC lysate; therefore, it can be supposed that the different disruption method could be an explanation for contradiction between our and Goldstein et al. findings. Furthermore, Goldstein et al. (Goldstein et al. 1972) and other researchers who could not measure CBS activity in resting PBMC used ^{14}[C]-serine for CBS determination (Gartler et al. 1981; Fensom et al. 1983; Tsai et al. 1996). We supposed that the other explanation could be for the contradictory observations that ^{14}[C]-labeled substrate provides high specificity for CBS activity determination but probably less sensitive than spectrophotometric methods measuring substrate consumption by Ellmans' or product formation by acidified nynhidrin reagents used widely for the determination of CBS activity.

Summary

According to our knowledge, this is the first evidence that proves existence of CBS protein and activity in resting PBMC. CBS protein level as well as activity increase with incubation time and upon stimulation. Its activity is modified by changes in intra- and extracellular homocysteine and GSH concentrations that occur in vitro and more importantly in vivo. Continuous release of homocysteine in resting PBMC results in increase of its extracellular concentration that in turn can modulate its functions, and might contribute to the development of hyperhomocysteinemia-induced diseases. These observations might open new possibilities in the study of hyperhomocysteinemia-related diseases.

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Conflict of interest None of the authors shares any conflict of interest or is consultants for companies.

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