Placental markers of folate-related metabolism in preeclampsia

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Abstract

The etiology and degree of clinical symptoms of preeclampsia depend on genotypic and phenotypic maternal and trophoblast factors, and elevated levels of plasma homocysteine (Hcy) are one of the pathogenetic factors of preeclampsia. To assess the impact of the folate-related metabolism, we characterized the indices of this metabolism in 40 samples from uncomplicated term placentas and 28 samples from preeclamptic pregnancies by quantifying the total content of folate, methionine (Met), Hcy and related cysteine, and glutathione (GSH) in compliance with the 677 C/T genotype of methylene tetrahydrofolate reductase (MTHFR). The prevalence of MTHFR genotypes was not significantly different between the two groups. The polymorphism of MTHFR was not unambiguously connected with the content of total placental Met, Hcy and related cysteine, and GSH either in uncomplicated or in complicated pregnancies. By contrast, the combination of the heterozygous MTHFR genotype with folate deficiency in the samples from preeclamptic pregnancies was characterized by a statistically significant decrease in the Met content, a trend toward increased Hcy levels and a tight association between metabolically directly and indirectly related compounds, e.g. positive relation between Hcy versus cysteine and folate versus GSH and negative relation between folate versus Hcy and both Hcy and cysteine versus GSH. We demonstrated the expression of cystathionine-β-synthase (CBS) in human placenta at term by RT-PCR and western blot analysis, for the first time, and confirmed its catalytic activity and the accumulation of cysteine and CBS in placental explants cultivated in the presence of elevated Hcy concentrations. We suggest that disturbance in placental folate-related metabolism may be one of the pathogenetic factors in preeclampsia.

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Introduction

The syndrome of preeclampsia is one of the major complications of pregnancy and the leading cause of maternal and fetal morbidity and mortality (Taylor 1988). According to the new hypothesis, preeclampsia results due to failure in placentation very early in gestation leading to the release of various products and factors from the impaired placenta and provoking the maternal syndrome (Huppertz 2008, Roberts & Hubel 2009). Characteristics of preeclampsia comprise oxidative stress, elevated inflammatory response, and generalized endothelial cell dysfunction, also affecting placental function. The most plausible genetic model to date postulates that the occurrence of the pathological phenotype in preeclampsia depends on the maternal genetic susceptibility, the genetic load from the trophoblast, and environmental factors (Lachmeijer et al. 2002).

Among different markers of preeclampsia, homocysteine (Hcy) concentrations in maternal blood have been of specific interest. Hcy concentrations are lower in pregnant women without complications than in nonpregnant women (Holmes et al. 2005) and are slightly increased in normotensive pregnancies that later develop preeclampsia and are considerably increased once preeclampsia is established (Poston & Rajmakers 2004, Ueland & Vollset 2004, Mignini et al. 2005). In established preeclampsia, Hcy reaches concentrations of 20–30 vs 5–11 μM in the control group and may even exceed 50 μM after methionine (Met) loading (D’Uva et al. 2007). A systematic review of the literature has revealed an overall association between higher Hcy concentrations in maternal blood and preeclampsia (Ueland & Vollset 2004, Mignini et al. 2005, Rajmakers et al. 2005).

Hcy, a thiol-containing amino acid, is the demethylated derivative of the essential amino acid Met and an intermediate in the Met cycle and transsulfuration pathway (Tarver & Schmidt 1939, Mudd & Levy 1989). It may be remethylated to Met or eliminated from the cycle by formation of cysteine in the transsulfuration pathway. The Met cycle takes place in all cell types, whereas transsulfuration occurs in a restricted number of tissues including liver, kidney, small intestine, and pancreas (Finkelstein 1990, 2000, 2006). Recently, this list was supplemented by evidence pointing to hydrogen...
sulfide (H$_2$S) synthesis in the human placenta and a potential transsulfuration activity (Patel et al. 2009, Solanky et al. 2010). The cystathionine-$\beta$-synthase (CBS; EC 4.2.1.22) catalyzes the first step of Hcy elimination from the system, the transsulfuration pathway, from Hcy to cystathionine, which is followed by cystathionine cleavage to yield cysteine. Cysteine undergoes desulfuration to H$_2$S or sulfane sulfur and oxidative catabolism by cysteine sulinate-dependent pathways, yielding either hypotaurine/taurine or sulfite/sulfate (Stipanuk & Ueki 2011). The expression and distribution of enzymes of the transsulfuration pathway and cysteine metabolism vary among tissues and so far were scarcely addressed in the human placenta in comparison with other enzymes of the folate metabolism (Christensen & MacKenzie 2006, Finkelstein 2006). The transit of Hcy either of maternal or of placental origin to the fetus will, to a great extent, depend on the efficiency of its catabolism.

The Met cycle is tightly associated with the tetrahydrofolate (THF) cycle. 5-Methyltetrahydrofolate (5-CH$_3$THF) donates its CH$_3$ group to Hcy remethylation and 5,10-methylenetetrahydrofolate (5,10-CH$_2$THF) is irreversibly reduced to 5-CH$_3$THF by the key enzyme of this cycle – methylene THF reductase (MTHFR; EC 1.5.1.20; Fig. 1). MTHFR is a polymorphic enzyme. Its alleles with C677→T677 or alanine 222 to valine substitutions are highly represented in the Caucasian population (Frost et al. 1995). This mutation is associated with thermolability and lower catalytic activity of MTHFR, elevated Hcy, and increased risk of obstetrical pathologies (De Franchis et al. 1995, Shields et al. 1999, Van der Put et al. 2001, Dodds et al. 2008).

Regarding Hcy in pregnancy, numerous questions and problems remain unanswered such as the specific input of each organ or different stages of the folate-related metabolism into the integral value of Hcy concentrations in plasma and the particular role of the placenta in the overall folate-related metabolism in mother and fetus.

Taking into account a pivotal role of the placenta in the development of preeclampsia, metabolizing activity, and the genetic load from the trophoblast in disease development, we have focused our attention on folate and folate-related markers in this organ. To this aim, we a) measured the concentration of total folate; b) measured the concentration of Hcy, Met, Cys, and glutathione (GSH); c) characterized the MTHFR genotype in human placental samples at term from uncomplicated and preeclamptic pregnancies; d) investigated expression and catalytic activity of the key enzyme of the transsulfuration pathway, CBS; and e) the effect of elevated concentrations of Hcy in the medium on Cys and CBS content in placental explants.

**Results**

**Social, economic, demographic, and clinical characteristics of the cohorts**

According to the questionnaires, the women from Ukraine whose specimens were taken for investigations were employees with average earnings, similar lifestyles, and traditional national diets. They denied alcohol and drug dependencies and did not smoke during pregnancy. All women had no professional contact with xenobiots.

All the samples were subdivided into two groups – those from uncomplicated and preeclamptic pregnancies. These two groups did not significantly differ in terms of maternal age (25.9±4.9 vs 27.6±5.7 years), gestational age (279±11 vs 271±10 days of gestation), and newborn weight (3.3±0.32 vs 3.2±0.51 kg), but Apgar index was significantly higher in the group with uncomplicated pregnancies (8.7±1.4) compared with preeclampsia (7.6±0.9; t-test; P<0.001).

**The frequency of C677T MTHFR gene mutation in the cohorts**

The prevalence of MTHFR gene polymorphism was estimated in the Ukrainian population for the first time. The frequency of the C677C genotype composed 48.5% (33/68), C677T, 41.1% (28/68), and T677T, 10.3% (7/68) of the total cohort and was in the range of that reported for Caucasians (Gasparovic et al. 2004). The samples from uncomplicated and complicated pregnancies revealed no statistically significant difference in the prevalence of MTHFR genotypes, although the percentage of C/C and T/T genotypes was correspondingly lower and higher in the group with preeclampsia than in the group of normotensive pregnancies (Table 1).

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**Figure 1** The general scheme of folate-mediated one-carbon unit metabolism. 5-CH$_3$THF, 5-methyltetrahydrofolate; 5,10-CH$_2$THF, 5,10-methylenetetrahydrofolate; THF, tetrahydrofolate; 5,10-CH$_2$THF, 5,10-methylenetetrahydrofolate; 10-CHOTHF, 10-formyl tetrahydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.
Folate content in placental samples

The folate content in placental samples was variable with values in the range 2.0–32.0 μg/g tissue or 0.2–0.8 μg/mg protein (Table 1). As the approximate placental weight is about 400 g, the total content of placental folates comprises about 0.8–12.8 mg. This value is in the same order of folate content in the liver with 3.8–11.3 μg/g tissue during the first year of life (Hoppner & Lampi 1980) and about 5.2 μg/g or about 11 mg in the whole liver in adulthood (Suh et al. 2001).

Categorization of folate content according to genotype and uncomplicated/complicated pregnancies revealed differences between these categories. The folate content was significantly lower in the samples from complicated pregnancies with a C/T genotype (Mann–Whitney U test, \( P<0.05 \)) and higher in the samples with a T/T genotype. The samples from other categories revealed no statistically significant differences (Table 1).

Met and aminothiols content in placenta samples

The concentration of Met and total (reduced and oxidized free and protein-bound) Hcy, cysteine, and GSH in placental samples was detected with HPLC combined with coulochemical detection. Cysteine, GSH, Hcy, and Met were eluted with retention times of 5.0, 10.0, 12.5, and 17.0 min respectively. Mean recoveries for Met, cysteine, Hcy, and GSH were 98.6, 98.4, 98.3, and 98.2% respectively. Within-run coefficients of variation in placental samples were 0.78–5.79% for cysteine, 0.92–6.89% for Hcy, 0.98–6.84% for Met, and 0.78–5.89% for GSH. Day-to-day coefficients of variation were 1.29–7.85% for Hcy, and 1.28–7.85% for Met.

Among the investigated compounds, cysteine had the highest concentration, whereas Hcy had the lowest (Table 1). Overall, placental thiols were composed of 63% cysteine, 19.7% GSH, 17% Met, and 0.4% Hcy. The ratio Cys:GSH was about three to one, which is in the range of ratios for embryonic (Cys:GSH=15:1) and fetal (Cys:GSH=1.5:1) liver but reverse to other embryonic and fetal organs including placenta at 13 weeks of gestation (Raijmakers et al. 2001).

Categorization of data according to MTHFR genotype and uncomplicated/complicated pregnancies revealed differences of the values between the categories (Table 1). Met level was lower in the samples from complicated pregnancies with C/T genotype in comparison with C/C genotype and with uncomplicated pregnancies with C/T genotype (Mann–Whitney U test, \( P<0.05 \)). The level of Hcy in the samples from complicated pregnancies with C/T genotype was the highest among all the categories though statistically non-significant. The carriers of T/T genotype from complicated pregnancies possessed the lowest value of Hcy at the background of the highest value of folate (Mann–Whitney U test, \( P<0.05 \)).

A pairwise correlation analysis revealed strong positive and negative correlations between all the indices but two in the samples with a C/T genotype from complicated pregnancies (Fig. 2A). A tight negative correlation was detected between folate and Hcy, which are the cosubstrates in the remethylation reaction. A positive association \( r_s=0.73, P=0.006 \) existed between Hcy and cysteine. This value was similar to that reported by Raijmakers \( r_s=0.74, P<0.01 \) for the Hcy and cysteine association in plasma of pregnant normotensive and hypertensive women (Raijmakers et al. 2000). A positive but non-significant association \( r_s=0.71, P=0.07 \) existed between Hcy and Met; one compound is the precursor of the other.

There was strong negative correlation between cysteine and GSH in the samples with C/T genotype from preeclamptic women \( r_s=−0.69, P=0.012 \). It corresponded to \( r_s=−0.74 (P=0.04) \) reported by Raijmakers et al. (2001) for the embryo. The metabolism of both compounds is tightly connected. Cysteine is a limiting factor in the synthesis of γ-glutamylcysteine, the first product in the two-step synthesis of GSH, and part of cysteine is produced in result of GSH breakdown via cysteinylglycine (Meister 1988). All other strong associations in this category of samples notably folate versus
Expression and catalytic activity of CBS in term placenta

On the basis of a strong association between Hcy and Cys in two categories of samples, we decided to check whether the key enzyme of the transsulfuration pathway, CBS, is expressed in human term placenta. Moreover, we aimed to clarify whether it is expressed in a catalytically active form. The expression of the CBS gene was confirmed by RT-PCR and western blot analyses that detected specific mRNA (amplicon of 151 bp) in total RNA and CBS-specific bands of 63 kDa in the protein assay, both isolated from term placenta (Fig. 3A and B). The sizes of both bands corresponded to those detected in the liver samples where expression of catalytically active CBS was previously ascertained (Kraus et al. 1978). Recently, Solanky et al. (2010) have also detected CBS-specific mRNA by semiquantitative RT-PCR in term placenta.

The catalytic activity of CBS was 3.2 mU/h per mg of total cytosolic protein from term placenta and 0.1 mU/h per mg of protein in preheated samples. In contrast, the catalytic activity of CBS in human liver comprises 53 mU/h per mg of total cytosolic protein, i.e. 16.5 times higher than in placenta (Kraus et al. 1978). According to our knowledge, evidence of functional activity of the transsulfuration pathway in human placenta was obtained for the first time.

**The impact of Hcy on cysteine synthesis by placental explants**

Cultivation of placental explants for 48 h in the presence of increasing concentrations of Hcy resulted in a substantial increase in cysteine content in the tissue. While after cultivation in the absence of Hcy the cysteine content was in the range 35.8–39.2 nmol/g of explant, in the presence of 20, 40, and 80 μM Hcy it comprised 168.0–182.0, 575.6–652.4, and 452.5–480.3 nmol/g tissue respectively (Fig. 4A). The involvement of CBS in this process was confirmed by western blot analysis. The protein accumulates at 20 and 40 μM Hcy and decreases at a concentration of 80 μM (Fig. 4B and C).

**Discussion**

Unlike frequent analyses of correlation between maternal and fetal levels of amino acids, GSH, and folate in plasma (Milman et al. 2006), we have estimated the level of Met, Hcy and related cysteine, and GSH in compliance with folate content and MTHFR polymorphism in placental tissues using samples from uncomplicated and complicated pregnancies. So far, the role of the placental folate-related metabolism had scarcely been addressed in the study of pathogenesis of preeclampsia, although placental genotype and phenotype define the final set of metabolites reaching the fetus and from the fetus to the maternal circulation.
Along with a mutated MTHFR genotype, folate deficiency can occur due to different factors – inadequate dietary intake, malabsorption, altered hepatic metabolism, or increased elimination of folate. The placenta concentrates folate in the intervillous space (Guigliani et al. 1985a, 1985b, Cetin 2001, Camelo et al. 2004). Folate receptors accumulate 5-CH\textsubscript{3}THF, the principal circulating form of folate, at the brush border of the syncytiotrophoblast and mediate the transport of folate compounds into the syncytiotrophoblast by endocytosis (Suh et al. 2001, Van der Put et al. 2001, Solanky et al. 2010). The further fate of folate inside the placenta is not clear so far. Active synthetic processes in the human placenta like the synthesis of nucleic acids in which THF derivatives are donators of one-carbon units indicate that the THF cycle and the conjugated Met cycle are active in this organ, which, therefore, is sensitive to folate deficiency.

Whatever is the cause of elevated folate content in the samples from complicated pregnancies with T/T genotype, they reveal the lowest Hcy content and once more point to the importance of the combination of several factors, particularly folate deficiency with MTHFR SNP, to provoke preeclampsia. Recently, it has been shown that folate deficiency in HepG2 cells produces time-dependent transcriptional changes that involve clusters of genes related to the remethylation and transmethylation pathways and transport of folates (Chango et al. 2009).

The very special result in the group of samples from complicated pregnancies and C/T genotype concerns the tight interrelation between compounds of the folate-related metabolism. The negative association of folate with Hcy is in agreement with the biochemistry of remethylation where Hcy and 5-methylTHF are co-substrates and Met is one of the products. Another strong positive correlation in this category of samples exists between Hcy and cysteine and corresponds to that reported for plasma of pregnant women (Raijmakers et al. 2000). In the majority of tissues, cysteine content depends on several factors including cysteine supply in the diet, by cysteine synthesizing organs, mainly the liver, membrane transport of extracellular cysteine converting intracellularly to cysteine, protein breakdown, and cysteine catabolism to taurine and inorganic sulfur (Lu 1999, Stipanuk & Ueki 2011). Cysteine is also connected with Hcy by the transsulfuration pathway (Tarver & Schmidt 1939) restricted to several organs (Finkelstein 1990, 2000), but to our knowledge, there was no comprehensive evidence showing that this reaction is active in the human placenta.

For the first time, we have shown the expression of the enzyme CBS in term placenta at the RNA and protein levels and confirmed its catalytic activity. CBS ‘opens’ the transsulfuration pathway catalyzing the \(\beta\)-replacement of the hydroxyl group of serine with Hcy and forming the thioether cystathionine with release.

We could not detect statistically significant prevalence of mutated forms of MTHFR (i.e. C677T and T677T) in preeclamptic placentas nor could we show statistically significant differences between the levels of folate, Met, and aminothiols in the samples with different genotypes from uncomplicated pregnancies. In contrast, the lower catalytic (reducing) activity of the C/T MTHFR genotype (60% in comparison with 100% of C/C genotype (Frostell et al. 1995)) may promote the accumulation of Hcy, depletion of Met, and imbalance between reduced methylTHF and oxidative methylene- and formylTHF, with the shift to oxidative forms in cases suffering from preeclampsia.

If the higher susceptibility of oxidative forms of THF to catabolism (Suh et al. 2001) may partly explain the lower folate content in the samples with C/T genotype, then it cannot explain the higher content of folate and the lowest content of Hcy in the samples with T/T genotype that retain only 30% of MTHFR activity (Frost et al. 1995). Thus, our data can be interpreted in such a way that deviant incidences of placental MTHFR genotypes alone do not seem to be significant for the placental folate-mediated metabolism. The causative role of maternal MTHFR polymorphism in preeclampsia is also still under debate (Lachmeijer et al. 2002, GOPEC Consortium 2005).

Figure 4 Content of cysteine and cystathionine-\(\beta\)-synthase in placental explants cultivated with elevated concentrations of homocysteine. (A) Changes of cysteine content in placental explants cultivated without homocysteine (1) and at 20 (2), 40 (3), and 80 (4) \(\mu\)M homocysteine. (B) Western blot analysis. The lanes: L – protein ladder; other designations of X axis are the same as in A. (C) The results of densitometry in relative units. Equal amounts of total protein (20 \(\mu\)g) were loaded per lane.
of water. Further cleavage of cystathionine by cystathionine γ-lyase (CSE; EC 4.4.1.1) yields cysteine. Our recent experiments with the samples from term placentas have revealed CSE expression at the mRNA level. Therefore, CSE may accomplish the cleavage of cystathionine yielding cysteine (data not shown).

The incubation of placental explants with elevated concentrations of Hcy in the range 20–40 μM induced the synchronous accumulation of cysteine and CBS, whereas higher concentrations seem to slow down these processes pointing to potential exhaustion of probable adaptive reactions.

We speculate that the positive correlation between Hcy and Cys in the samples with C/T genotype from complicated pregnancies, the accumulation of cysteine, and CBS protein in explants cultivated at elevated concentrations of Hcy reflect the activation of CBS expression and the whole transsulfuration pathway as adaptive reaction for elimination of Hcy. So far, the detailed mechanisms of this activation are not clear. Also, it is not clear what the fate of increased cysteine production is. Cysteine is used for multiple cellular functions. It must be sufficiently high to meet the prior needs of protein synthesis and the production of other essential molecules like GSH, taurine, and sulfate. At the same time, however, cysteine levels must be kept below the threshold of toxicity due to feasibility of its oxidation. Therefore, elevated levels of Hcy and Cys may provoke oxidative stress in the placenta with noxious consequences at the molecular and cellular levels. The negative association between both Hcy and cysteine versus GSH may enhance the oxidative stress by reduction of the antioxidant capacity of GSH under hyperhomocysteinemia.

The intriguing question is which mechanisms support the tight pairwise correlation that is predominantly observed in the samples with C677T MTHFR genotype, low level of folate, and originating from preeclamptic women. We suggest that intracellular folate deficiency plays an important role. Whatever the reason of folate deficiency in the organ was, the subsequent consequences may develop by more than one way and define the tight correlation between the indices. Usually, a lower concentration of folate in a tissue is accompanied by higher enzymatic activity of folypolyglutamate synthetase and consequently longer glutamate chain length of folates in contrast to the monoglutamate form in serum. It increases retention of folates once transported and makes the folate molecule a more efficient carrier of one-carbon units. Significant amounts of folypolyglutamate bind to a number of specific folate-binding proteins, regulate their activity, and prone to enhance channeling of substrates through sequential enzyme reactions (Schirch & Strong 1989, Suh et al. 2001, Van der Put et al. 2001).

A mutated C/T MTHFR genotype and oxidative stress due to preeclampsia and potential cysteine oxidation (Hogg 1999) may exert the additive effect on inter-relation between the amino acids, folate, and GSH. Numerous independent biomarkers of oxidative stress indicate its presence in placentas of women with preeclampsia (Raijmakers et al. 2005) and some enzymes of folate-related metabolism are redox sensitive (Zou & Banerjee 2005). The activity of Met synthase is diminished under oxidizing conditions possibly due to oxidative lability of the cofactor intermediate, cobalamin (Chen et al. 1995), and/or as result of cysteine oxidation in the essential Zn-binding site of the enzyme (Goulding & Mathews 1997). In contrast, CBS is activated under oxidizing conditions because heme groups in the ferric state are more favorable for the binding of Hcy (Banerjee & Zou 2005). Therefore, various molecular mechanisms may stipulate the changes in the folate-related metabolism in human placenta under preeclampsia and hyperhomocysteinemia. They include deviant gene expression, functional activity of proteins, and perturbed interrelations between metabolites of the system. This area merits further investigations, especially at the systems level when polymorphisms of folate-related enzymes, content of external and internal metabolites, redox status of the tissue, and gene expression would be taken into account.

Conclusion

Altogether, these results point to changes in the folate-related one-carbon metabolism in human placenta in women with preeclampsia, whereas the significance of this event for fetal development remains to be elucidated. We suggest that disturbances in the placental folate-related metabolism may contribute to the development of preeclampsia.

Materials and Methods

The object of investigation

This study was carried out according to the Declaration of Helsinki. The ethics committee of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine approved the study protocol and the use of human tissues. Preeclampsia was defined according to the standards of the International Society for the Study of Hypertension in Pregnancy as a multi-system disorder with hypertension (diastolic pressure above 90 mmHg on two or more consecutive occasions each more than 4 h apart) and proteinuria (ratio above 0.3 g protein to 10 mM creatinine). After written informed consent was obtained, placental samples of women with normotensive pregnancies \((n = 40; 279 \pm 11\) days of gestation) and late-onset preeclampsia \((n = 28; 271 \pm 10\) days of gestation; diastolic blood pressure in the range 90–110 mmHg and without HELLP syndrome) were collected in the state regional maternity hospitals in Ukraine. Immediately after delivery, placental tissue (50 g) was excised from the central part of the organ through all layers, frozen in liquid
nitrogen, and stored at −70 °C before use. Each sample was accompanied by a personal questionnaire that included data such as lifestyle factors, consumption of alcohol, cigarette smoking, diet, and occupational contamination risk. Maternal clinical background data and newborn health status were taken from medical histories. Placental villous tissue from normal term placentas (n = 3) was collected in the maternity hospital of the Medical University of Graz (Austria) after written informed consent was obtained. The experimental protocol was approved by the ethics committee of the Medical University of Graz (Austria). The samples of human liver were obtained from the tissue adjacent to the removed tumor in the National Institute of Surgery and Transplantology n.a. A Shalimov (Kiev, Ukraine) after written consent given by the patient.

**MTHFR genotyping**

Genomic DNA from placental samples was assessed for the presence of the C677T mutation by PCR coamplification of the MTHFR gene fragment (+510...+707) with the exon III of the gene, coding for fibrinogen Aa (+1723...+2252) and subsequent restriction analysis by HinfI restrictase according to an adapted method described by van Amerongen et al. (1998). DNA was extracted from 50 mg placental samples by proteinase K digestion with consequent salting out procedure (Miller et al. 1988). The primers used were as follows: MTHFR (forward) 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'; MTHFR (reverse) 5'-AGG ACG CTG CGG TGA GAG TG-3'; fibrinogen Aa (forward) 5'-CTC CCT TCA TCT GAA GCT CA-3'; fibrinogen Aa (reverse) 5'-GAC CTC TCA TCT TGC ACC TTT A-3'. To ensure primer specificity, the PCR products were sequenced by standard procedure on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit according to the instructions of manufacturer. The results were analyzed with the software ‘Sequencing Analysis’ (Applied Biosystems) and Chromas 1.55 (Technelysium Ltd, Helensvale, Australia) and verified using BLAST (http://blast.ncbi.nlm.nih.gov/BLAST.cgi). The sequences of amplicons were identical with those of the gene, coding for fibrinogen Aa (forward) 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'; MTHFR (reverse) 5'-AGG ACG CTG CGG TGA GAG TG-3'; fibrinogen Aa (forward) 5'-CTC CCT TCA TCT GAA GCT CA-3'; fibrinogen Aa (reverse) 5'-GAC CTC TCA TCT TGC ACC TTT A-3'. To ensure primer specificity, the PCR products were sequenced by standard procedure on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit according to the instructions of manufacturer. The results were analyzed with the software ‘Sequencing Analysis’ (Applied Biosystems) and Chromas 1.55 (Technelysium Ltd, Helensvale, Australia) and verified using BLAST (http://blast.ncbi.nlm.nih.gov/BLAST.cgi). The sequences of amplicons were identical with those of the expected products. Coamplification was performed in a reaction volume of 50 μl containing 1 μg placental DNA, 10 pmoles of each primer (Syntol Ltd, Moscow, Russia), 200 nM dNTP (MBI Fermentas, Vilnius, Lithuania), 10 mM Tris–HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, and 2.5 units of Taq polymerase (AmpliSence Ltd, Moscow, Russia). Amplification consisted of an initial denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 58.5 °C for 30 s, 72 °C for 50 s, and final cycle of 72 °C for 7 min. The PCR products were subjected to electrophoresis in a 2% agarose gel with 0.5 μg/ml ethidium bromide to confirm the correct amplicon size. Restriction analysis was performed in a volume of 25 μl containing 14 μl ampiclon, 10 mM Tris–HCl, pH 8.5, 10 mM MgCl₂, 100 mM KCl, 0.1 mg/ml BSA, and 10 units of HinfI restrictase (MBI Fermentas) for 4 h at 37 °C. After digestion, all fragments were resolved in a 3% TopVision agarose gel (MBI Fermentas) in 1×TBE buffer with 0.5 μg/ml ethidium bromide. The homozygous MTHFR 677C/C genotype resulted in a single fragment of 198 bp; the heterozygous 677C/T genotype produced 198, 175, and 23 bp fragments; and the homozygous 677T/T resulted in two fragments, 175 and 23 bp. The amplified fibrinogen Aa resulted in three fragments, 56, 136, and 350 bp, indicative of proper amplification and complete digestion.

**Determination of the folate content by a microbiological test with Lactobacillus casei**

To estimate the folate content in placental samples, we chose a classical microbiological test with auxotrophic Lactobacillus casei, which shows equivalent response to various folate derivatives (Grossowicz et al. 1962, 1981, Wilson & Horne 1982).

Lyophilized culture of L. casei ATCC 7469 was obtained from the Russian collection of productive microorganisms, No. 3464 (Institute of Genetics, RAS, Moscow, Russia). Lactobacilli Broth MRS (Institute of Microbiology, Kiev, Ukraine), 1.0 ml, was added to the vial with lyophilized culture, left overnight at 37 °C, inoculated into Lactobacilli Agar OADC (Difco Laboratories, Detroit, MI, USA), and transferred every 2 weeks to the fresh maintenance Lactobacilli Agar. Prior to each experiment, 2.0 ml folic acid casei medium (HiMedia Laboratories Pvt. Ltd, Mumbai, India) was inoculated with bacterial culture and incubated for 24 h at 37 °C. Folic acid casei medium was prepared according to the instruction of the manufacturer.

Human serum containing conjugase (EC 3.4.22.12, γ-glutamyl hydrolase) was obtained from 5.0 ml blood (Wilson & Horne 1982) taken from the medial cubital vein of volunteers. Blood was allowed to clot at room temperature. The clot was removed and the serum was centrifuged at 5000 g for 10 min. About 2.0 ml serum was dialyzed at 4 °C for 18 h versus 1.0 l of 0.1 M potassium phosphate buffer, pH 7.0, and containing 2 g acid-washed charcoal per 1.0 l, to remove endogenous folate. The dialysed serum was stored in 0.5 ml aliquots at −20 °C for later use. The test with L. casei showed no detectable folate.

Placental extracts were prepared ex tempore. The samples, 200 mg, were minced and placed in 2.0 ml 20 g/l solution of sodium ascorbate in a boiling water bath for 10 min, cooled in an ice bath, and homogenized with a Potter-Elvehjem homogenizer for 15 s. The samples were centrifuged at 4000 g for 20 min. The supernatant, 500 μl, was treated with 75 μl human serum conjugase and 5 μl 575 mM 2-mercaptoethanol solution. A drop of toluene was added to each tube and the tubes were incubated at 37 °C for 24 h, then for 5 min in a boiling water bath, and centrifuged to remove precipitated protein. The supernatants were used for the folic acid assay on the same day.

A solution of 10 mg folic acid (Kiev Vitamin Plant Ltd, Kiev, Ukraine) in 0.1 M NaOH was used as stock standard. A working standard solution was made by dilution of the stock standard solution to 1.0 ng/ml in folic acid casei medium. To obtain the standard curve, working standard solution was added in each experiment to ten tubes to get a range of concentrations 20–200 μg/ml. All tube volumes were adjusted up to 5 ml by folic acid casei medium without folic acid. Folic acid assay protocol included the following procedures: the medium was dispensed by 5.0 ml into culture tubes; to each tube, 100 μl placental extracts were added; the assay tubes and the tubes for standard curve were autoclaved at 1 atm for 15 min, cooled, inoculated with one drop of L. casei
in Lactobacilli Broth, and incubated at 37 °C for 48 h. The absorbance was measured at \( \lambda = 595 \) nm. The amount of folic acid in the samples was estimated on the basis of standard curve and calculated per milligram of tissue and tissue protein determined by the Bradford method (Bradford 1976).

Estimation of Met, Hcy, Cys, and GSH content by HPLC–coulochemical method

The content of Met and total (reduced and oxidized free and protein bound) Cys, Hcy, and GSH was estimated by an adapted method as described by Melnyk et al. (1999).

Placental samples (200 mg) were homogenized with 2.0 ml phosphate buffer. For determination of total aminothiols, the disulfide bonds were reduced by sodium borohydride. To 100 µl homogenate, 50 µl of 100 µM penicillamine (as internal standard) and 40 µl of 1.0 M sodium borohydride solution in 0.1 M NaOH were added, and after gentle mixing, the solution was incubated for 30 min at 50 °C with gentle shaking. To precipitate the proteins, 100 µl of 0.6 M perchloric acid–1.0 mM EDTA was added and the samples were incubated for 10 min on ice. After centrifugation at 14 000 g for 5 min, the supernatant was diluted 1:2 with the mobile phase and 50 µl were injected into the HPLC system (HP 1100, Hewlett-Packard, Waldbronn, Germany). Mobile phase consisted of 50 mM NaH₂PO₄, 1.0 mM ion-pairing reagent 1-octanesulfonic acid, and 5% acetonitrile (v/v), adjusted to pH 2.7 with 85% phosphoric acid (Houze et al. 2001). The Purospher STAR RP-18e column (250 × 4.6 mm, I.D., 5 µm; Merck) and the LiChrospher 100 RP-18 (4 × 4 mm I.D., 5 µm, Merck) served for analytical column and precolumn respectively. Isocratic elution was performed at ambient temperature at a flow rate of 0.8 ml/min and a pressure 1800–2100 psi.

Coulochemical detection

Following HPLC separation, Met, Hcy, Cys, and GSH were detected with a model 5200 A Coulochem II EC detector (ESA, Inc., Chelmsford, MA, USA) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). The guard cell was used to remove oxidizable impurities in the mobile phase that may interfere with baseline stability. For optimum detection of aminothiols, the electrode potentials of the guard cell was used to remove oxidizable impurities in the mobile phase, and the E1 and E2 electrodes of the analytical cell were set at +1400, +650, and +900 mV respectively. Peak area analysis and data storage for each compound were provided by the HP3D 3D ChemStation software (Hewlett-Packard, Waldbronn, Germany) based on calibration curves.

Calibration curves

Linear calibration curves consisting of six points for each compound were generated in the following ranges: 12.5–200.0 nmol/ml for Cys, 1.8–30.0 nmol/ml for GSH, and Hcy and 7.5–120.0 nmol/ml for Met. Calibration dilutions were prepared by serial 1:2 dilutions of the highest concentration with blank placental sample prepared by standard procedure. Point 0 referred to blank placental sample. In the mentioned concentration ranges, the parameters of calibration curves and their correlation coefficients \((r>0.99)\) revealed good linearity. HPLC–coulochemical detection met the precision, accuracy, and robustness of the method. For samples below the detection limit of compounds, a value of half of the detection limit was assigned.

CBS assay

The expression of the CBS gene was estimated by standard RT-PCR and western blot analysis. Total RNA was isolated with the help of TRI Reagent – RNA Isolation Reagent (Molecular Research Center, USA) following the recommendations of the manufacturer, treated with DNAase I (MBI Fermentas) according to standard procedure and reverse transcribed using RNA RETROscript kit (Fermentas). The primers and conditions of amplification were taken from Persa et al. (2006). The size of the PCR product was 151 bp and its correspondence to the expected product was confirmed by sequence analysis.

Western blotting was performed using the Western Breeze Chemiluminescent Immunodetection System according to the protocol of the manufacturer (Invitrogen). Pieces of placental and liver tissues, 15 mg, were homogenized in lysis buffer (0.01 M Tris, pH 7.0, 1% SDS, 1 mM sodium orthovanadate, 1 × protease inhibitor cocktail (Roche)). The homogenate was centrifuged at 16 000 g, 4 °C, for 10 min. Total protein (20 µg) was resuspended in 4 × SDS reducing sample buffer, incubated at 70 °C, 10 min, and separated on a NuPAGE 4–12% Bis–Tris gel, transferred to a nitrocellulose membrane at 4 °C overnight, and visualized by Coomassie blue R-250. Membranes were blocked with 10% skimmed milk, 0.1% BSA in Tween Tris-buffered saline (TTBS; 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) for 6 h at room temperature, washed three times in TBS, and incubated with 1 µg/ml anti-CBS primary antibody (clone 3E1; Abnova Corporation, Taipei City, Taiwan) for 12 h at 4 °C. After incubation, the membranes were washed in TTBS and incubated with anti-mouse secondary antibodies conjugated with HRP for 30 min at room temperature. The membranes were developed using an UltraVision LP Detection System HRP Polymer & AEC Chromogen, 3-amino-9-ethylcarbazol (Labvision Corporation, Fremont, CA, USA) and Amersham Hyperfilm (GE Healthcare, Chalfont St Giles, UK). Thereafter, for loading normalization, the membrane was stripped and stained with Amido Black according to standard procedure. The general density of all the bands in each lane of stained membrane and of the specific bands at the developed films was measured at the scanner with the help of the program GelPro 3.1 (MediaCybernetics, Bethesda, MD, USA).

CBS enzyme activity was assayed following Persa et al. (2006). Placental samples (50 mg) were homogenized with 2.0 ml K-phosphate buffer (pH 8.0) and centrifuged for 5 min at 3000 g. The assay mixture had a total volume of 200 µl containing 250 mM Tris (pH 8.6), 0.25 mM pyridoxal 5′-phosphate, 0.5 mg/ml BSA, 0.38 mM S-adenosylmethionine, 30 mM [14C] serine (~ 50 000 d.p.m./mmol), 0.15 mM l-Hcy, and 50 µM cysteine and incubated at 37 °C for 30 min. The reaction mixture was preheated at 65 °C for 15 min and used as negative control. The reaction was stopped by adding 200 µl 10% trifluoroacetic acid (Sigma), followed by...
centrifugation at 14 000 g for 10 min. The supernatant (300 μl) was loaded onto a 1.5 x 12 cm column (2 ml AG-50Wx 8 resin (Bio-Rad)) and washed consecutively with 4 ml water (2 x), 4 ml 0.6 M HCl (6 x), and 4 ml water (4 x) under control of radioactivity. The end product of [U-13C] cystathionine was eluted with 2 ml 3 M NH4OH three times. Each fraction was neutralized with 0.35 ml of 11 M HCl, counted with a Beta Counter (Perkin Elmer Tri-Carb 2800 TR; Perkin Elmer, Waltham, MA, USA) and the radioactivity combined. The enzyme activity was expressed as mU/mg protein. One unit of CBS catalyzes the formation of 1 μmol cystathionine per minute at 37 °C. The column was regenerated by washing thoroughly with water, 3 M NH4OH, and then water (to pH 8.9), 6 M HCl and again water (to pH 4.5).

Cultivation of placental explants
Placental villous tissue from the third trimester of gestation (40 weeks) was collected and cultivated as described by Baczyk et al. (2006). Individual clumps of villi were dissected in sterile cold PBS under the microscope, transferred to culture media, and cultivated in a 6% ambient oxygen incubator at 37°C, 5% CO2 in serum-free media DMEM/Ham’s-F12 (PAA Laboratories GmbH, Pasching, Austria), 1% penicillin/ streptomycin, 1% amphotericin B, 2% glutamine (PAA Laboratories GmbH; control), and in the presence of additional 20, 40, or 80 μM Hcy. After 48 h, the explanted villi were removed from the medium and submitted to HPLC–coulochemical detection of Cys content and western blot analysis with primary antibodies to CBS according to standard procedures (see above).

The protein concentration was measured by standard procedure (Lowry et al. 1951).

Statistical analysis
All data were tested for normality and represented as medians (Me) with lower and upper quartiles (Lq–Uq) and as mean ± S.D. The significance of the differences between the groups and the correlation between the data was evaluated with the help of Mann–Whitney U, t-tests, comparison of confidence intervals for relative values, and with Spearman’s coefficients. The statistical analysis was performed in STATISTICA 7.0 (Stat-Soft, Inc., Tulsa, OK, USA).

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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