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OXIDATIVE STRESS AS A SIGNAL TO UP-REGULATE γ-CYSTATHIONASE IN THE FETAL-TO-NEONATAL TRANSITION IN RATS.

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Abstract – Hepatic γ-cystathionase, a rate-limiting enzyme for the synthesis of L-cysteine from L-methionine in the trans-sulphuration pathway, exhibits significantly higher activity in the newly born infant as compared to the fetus. The aim of this work was: 1) To determine whether the increase in γ-cystathionase activity occurring in the fetal-to-neonatal transition is due to up-regulation of its mRNA and protein, 2) To elucidate the mechanisms responsible for this increase in γ-cystathionase activity. Our results show that expression of γ-cystathionase at both the mRNA and protein levels was higher in newborn than in fetal liver. γ-Cystathionase activity in fetal hepatocytes *in vitro* increased when incubated with tert-butyl-hydroperoxide at low concentration (0.01 mM). Hence, moderate oxidative stress would act as a signal to up-regulate γ-cystathionase in the fetal to neonatal transition. Stress hormones, such as phenylephrine or glucagon also increased γ-cystathionase activity in fetal hepatocytes. We also report a competitive inhibition of purified γ-cystathionase by L-cysteine, which would help to maintain physiological low L-cysteine levels in hepatocytes. In conclusion, our results show that increased hepatic γ-cystathionase activity in the fetal-to-neonatal transition is due to up-regulation of its gene expression mediated by stress hormones together with the physiological oxidative stress that occurs at birth.

Key words: Glutathione, L-cysteine, tert-butylhydroperoxide, phenylephrine, glucagon, cAMP, oxidative stress, fetal-to-neonatal transition.

INTRODUCTION

Both, liver maturation and adaptive response to extra-uterine life are considered essential for the metabolic changes occurring in the fetal-to neonatal transition (1). The trans-sulphuration pathway is responsible for L-cysteine synthesis from L-methionine. One of its rate-limiting steps, synthesis of L-cysteine from cystathionine, is catalyzed by γ-cystathionase (international enzyme classification: EC 4.2.1.15). Newborn infants and rat pups exhibit higher hepatic γ-cystathionase activity than fetuses (2, 3). However, premature infants have lower hepatic γ-cystathionase activity than term infants (2, 4, 5). In fact, plasma L-cysteine as well as the metabolic flow through the trans-sulphuration pathway are significantly lower in premature infants than in full-term infants (5). In addition, γ-cystathionase also regulates the availability of L-cysteine for glutathione synthesis. Consequently, a deficiency in γ-cystathionase activity is associated with low glutathione levels in fetal life (2, 3), and in other situations, such as aging of the eye lenses (6, 7) surgical stress (8), cancer (9) and AIDS (10).

Mechanisms involved in the induction of γ-cystathionase activity at birth are not completely understood. Heinonen and Raïha reported that dexamethasone, glucagon or AMP increased γ-

Abbreviations: **AIDS**, Acquired Immunodeficiency Syndrome; **DEAE**, Diethylaminoethyl; **EDTA**, Ethylene diamine tetra acetic acid; **FPLC**, Fast protein liquid chromatography; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **PCR**, Polymerase chain reaction; **SDS**, Sodium dodecyl sulfate.

cystahionase activity in liver explants from human fetuses (11). The physiological oxidative stress that occurs in the fetal-to-neonatal transition may also be involved in this induction. Indeed, we found a marked increased glutathione oxidation in the liver of rat pups when compared with fetuses, as well as newborn infants (3, 12). The remarkable increase in arterial pO_2 –from 25 to 100 mmHg- that occurs at birth may be responsible for this physiological oxidative stress (13).

The aim of the present work was: 1) To determine whether the increase in γ-cystathionase activity that occurs in the fetal-to-neonatal transition is due to up-regulation of its mRNA and protein; 2) To elucidate the mechanisms responsible for this increase in γ-cystathionase activity; 3) To assess the effect of altering L-cysteine availability on γ-cystathionase activity.

MATERIALS AND METHODS

Animals

Wistar term pups (21 days of gestation) and newly born rats (< 12 hours of life) were used for studies on hepatic γ-cystathionase during the fetal to neonatal transition, and young adult rats (5 to 6 months old) were also used as controls for comparison. Number of rats and experiments is indicated in the figure legends.

γ-Cystathionase was purified from the liver of 8-12 month-old Wistar rats, and New Zealand rabbits were used to obtain antibodies against γ-cystathionase. All animals received humane care according to the criteria outlined in the "*Guide for the Care and Use of Laboratory Animals*" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985). This study was approved by the Research Committee of the School of Medicine (University of Valencia, Valencia, Spain).

Assay for γ-cystathionase activity

In rat liver γ-cystathionase activity was determined by the continuous spectrophotometric method of Flavin and Slaughter (14) with the modification described by Sastre *et al*. (7). Briefly, γ-cystathionase activity was assayed at 37^{o}C in 1 ml (final volume) of the following medium: 0.25 M Tris HCl, pH 8.1, 20 μM pyridoxal phosphate, 0.25 mM NADH, containing 30 I.U. of lactate dehydrogenase and using 30 mM cystathionine as substrate. The rate for the decrease in absorbance at 340 nm was measured before and after the addition of cystathionine. γ-Cystathionase activity was calculated as the rate for NADH consumption.

In hepatocytes isolated from rat fetuses, γ-cystathionase activity was measured as the rate of cysteine formation from cystathionine, as described by Sturman *et al*. (2). The continuous method was not used in isolated hepatocytes due to the high NADH oxidase activity that occurred in presence of *tert*-butyl-hydroperoxide. Hepatocytes were homogenized in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM EDTA, 1 mM di-thio-threitol and 20 μM pyridoxal phosphate. The homogenate was spun at 10,000 g for 30 min at 4^{o}C, and the supernatant was used to determine γ-cystathionase activity.

Purification of γ-cystathionase.

Following the method described by Bikel *et al*. (15), γ-cystathionase was purified approximately 1000 times from rat liver as described in (7).

Obtention of polyclonal antibodies against γ-cystathionase

Polyclonal antibodies against γ-cystathionase were obtained from rabbits following the procedure described by Harlow and Lane (16) as described in (7).

Western blotting of γ-cystathionase

γ-Cystathionase was detected by western blotting using the Protoblot Western Blot AP System (Biorad, Spain) and the specific rabbit antibodies obtained in our laboratory as previously indicated.

RT-PCR

Total RNA was isolated from liver from rat fetuses, newborns and young adults using the guanidium thiocyanate method described by Sambrook et al (17). Reverse transcription and polymerase chain reaction were performed in one step using the "Titan^{TM} One Tube RT-PCR" System (Boehringer Mannheim). The mRNA expression was studied by RT-PCR (Titan One Tube RT-PCR System) using specific oligonucleotides for γ-Cystathionase, 5'-ATCACACCACAGACCAAGCT-3' and 5'-AGGCTCTCAGCCAGAGCAAA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as internal control with the following specific oligonucleotides: 5'-GGT CCT CAG TGT AGC CCA AGA TG - 3' and 5'-CCT GGA GAA ACC TGC CAA GTA TG – 3'.

Statistical analysis

Results are expressed as mean ± S.D. One way analysis of variance (ANOVA) was performed first, and then the sets of data in which F was significant at the level of < 0.05 were examined by the Student t test.

RESULTS

Increase of hepatic γ-cystathionase activity in the fetal to neonatal transition

Figure 1 shows that γ-cystathionase activity in liver tissue was 40% higher ($P < 0.05$) 1 h after birth than in fetuses at term. It further increased at 6 h after birth if newborns were fed on maternal milk, reaching a value which was 67% higher than in fetuses (see Fig. 1). γ-cystathionase activity in liver tissue from adult rats was 80% higher than in fetuses at term and 30 % higher than in newborns not being breastfed. Hepatic γ-cystathionase activity was not significantly different between adult rats and newborns fed maternal milk.

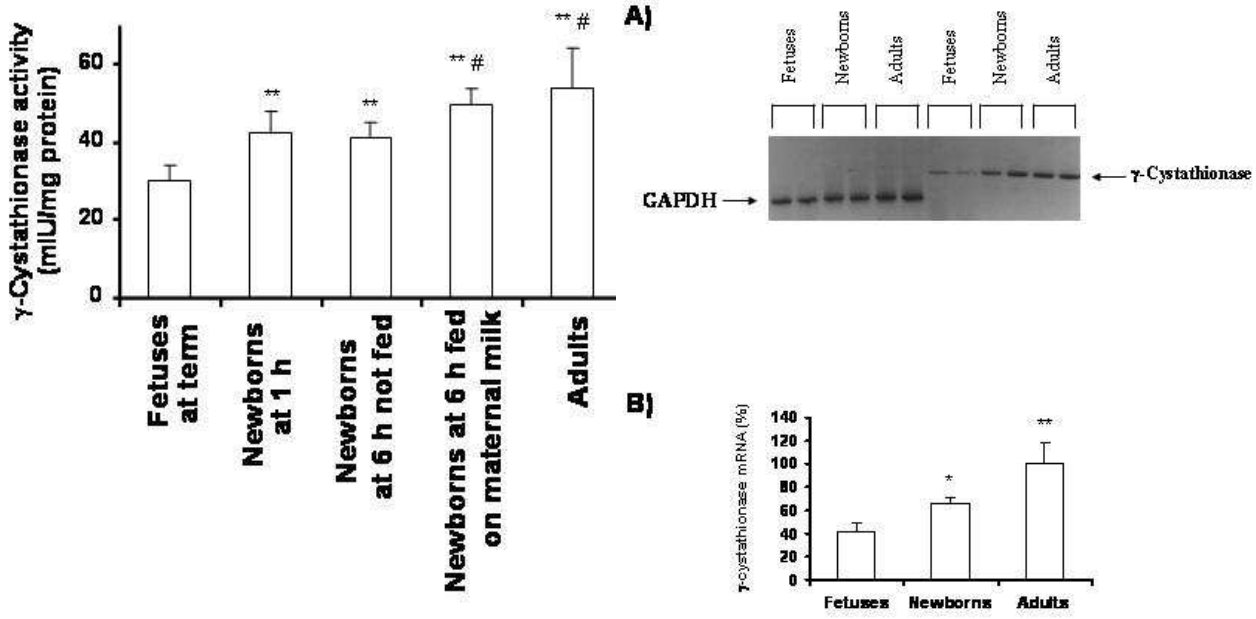

Figure 1. γ-cystathionase activity in the liver during the fetal-to-neonatal transition. γ-Cystathionase activity (mIU /mg prot.) was measured in liver tissue from rat fetuses, newborns and young adults. Results are mean ± S.D. for 3-11 experiments. Statistical difference is indicated as follows: ** P < 0.01 vs. "fetuses"; # P< 0.05 vs. "newborns at 6 h not fed".

Figure 2. Up-regulation of γ-cystathionase mRNA in the liver during the fetal-to-neonatal transition. The expression of γ-cystathionase mRNA was measured by RT-PCR in liver tissue from rat fetuses, newborns and young adults. The expression of glyceraldehyde-3 phosphate dehydrogenase mRNA was used as internal standard. Results are mean ± S.D. for 3 experiments. Statistical difference is indicated as follows: * P < 0.05; ** P < 0.01 vs. "fetuses".

Up-regulation of γ-cystathionase mRNA and protein in the fetal to neonatal transition

The expression of γ-cystathionase mRNA in the liver tissue was higher in adults than in newborns –not breastfed-, and it was also higher in the latter than in fetuses at term (Fig. 2). The mRNA for glyceraldehyde 3-phosphate dehydrogenase was used as control for the amount of RNA. The hepatic γ-cystathionase mRNA was 20% in fetuses and 52% in newborns when compared with 100% in adults.

Figure 3 shows that the expression of γ-cystathionase at the protein level was higher in newborn liver than in fetal liver. The enzyme expression increased further in adults when compared with newborns. Indeed, the densitometry revealed that γ-cystathionase expression in the liver tissue was 50% in fetuses and 75 % in newborns when compared with 100 % in adults. Furthermore, there is a linear correlation between the protein expression and the activity of the enzyme. This correlation demonstrates that the induction of the enzyme activity at birth is mainly due to up-regulation of the enzyme expression.

Tert-butyl hydroperoxide at low concentrations increases γ-cystathionase activity in vitro in fetal hepatocytes

Tert-butyl hydroperoxide was used as a pro-oxidant agent *in vitro* in isolated hepatocyes to induce glutathione oxidation as it occurs *in vivo* in the fetal to neonatal transition (Pallardó et al., 1991). The effect of tert-butyl hydroperoxide was studied *in vitro* at three different concentrations (0.01, 0.05 and 0.1 mM) in hepatocytes isolated from fetuses at term. γ-Cystathionase activity was increased by 15% in fetal hepatocytes after 1 h incubation with tert-butyl hydroperoxide at low concentration (0.01 mM) (see Fig. 4). However, γ-cystathionase activity decreased markedly (almost 50%) when fetal hepatocytes were exposed to a 10 times higher concentration of tert-butyl hydroperoxide (i.e. 0. 1 mM). No significant differences were found when using 0.05 mM tert-butyl hydroperoxide.

Phenylephrine, glucagon or cAMP increases γ-cystathionase activity in fetal hepatocytes

The effect of stress hormones on γ-cystathionase activity was tested *in vitro* in

hepatocytes from at term fetuses. 0.01 mM Phenylephrine and 0.001 mM glucagon induced a 34 % and 9% increase in γ-cystathionase activity respectively (see Fig. 5). A more intense stimuli triggered by 0.5 mM cAMP plus 0.5 mM teophylline –which inhibit phosphodiesterase- induced a 31 % increase in γ-cystathionase activity.

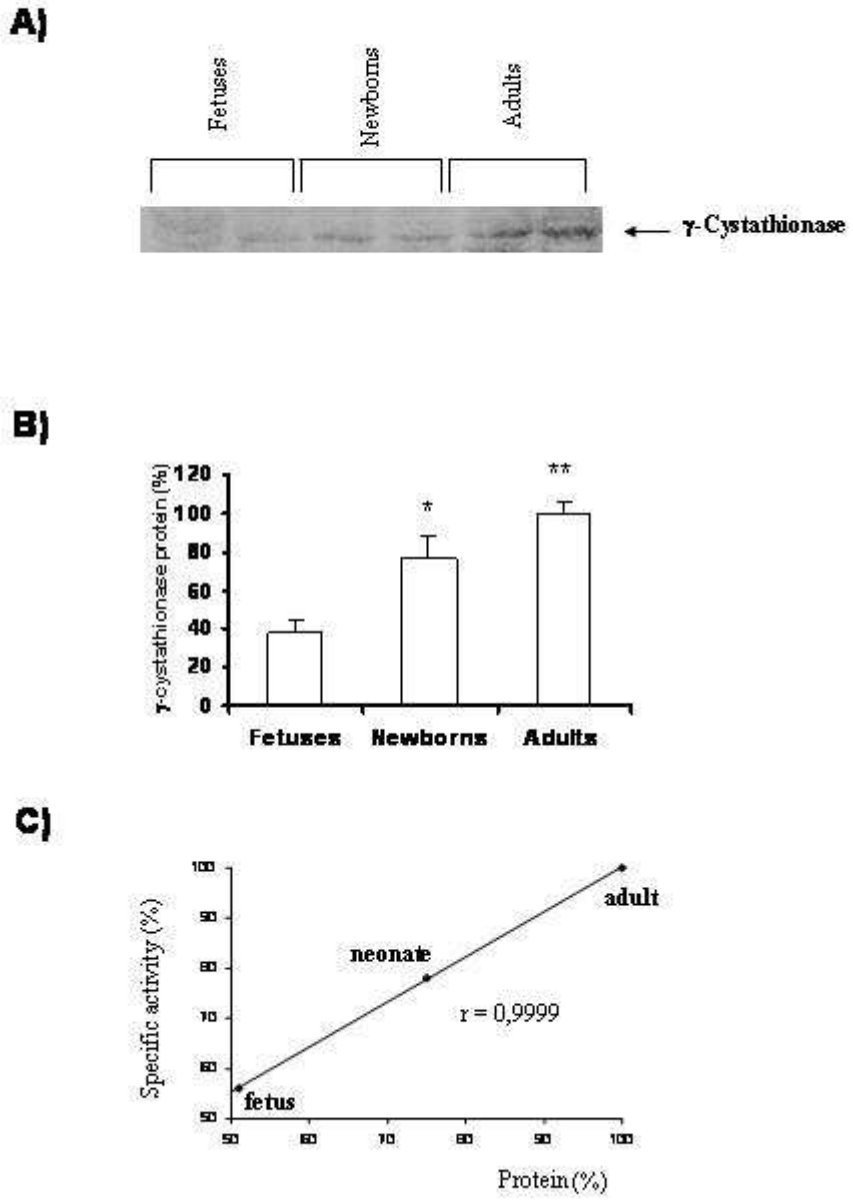

Figure 3. Up-regulation of γ-cystathionase protein in the liver during the fetal-to-neonatal transition. Panel **A** shows the expression of γ-cystathionase protein measured by western blotting in liver tissue from rat fetuses, newborns and young adults. Panel **B** shows the densitometric analysis of the western and panel **C** shows the correlation between γ-cystathionase activity and protein in liver tissue using the mean values for rat fetuses, newborns and young adults. Results are mean ± S.D. for 3 experiments. Statistical difference is indicated as follows: * $P < 0.05$;** $P < 0.01$ vs. "fetuses".

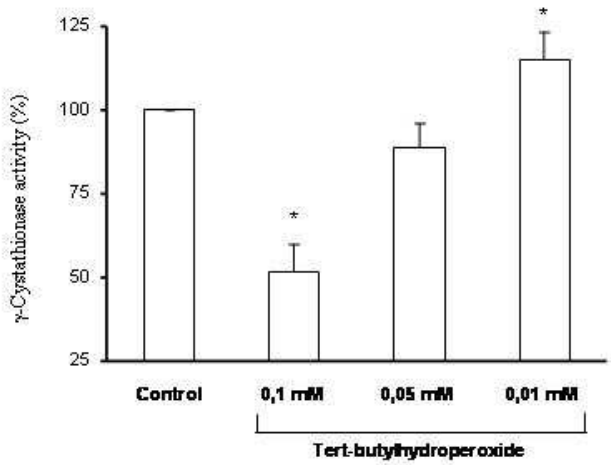

Figure 4. Effects of tert-butylhydroperoxide on γ-cystathionase activity *in vitro* in fetal hepatocytes. Fetal hepatocytes were cultured in presence of different concentrations of tert-butylhydroperoxide (0.1 mM, 0.05 mM and 0.01 mM). Results are mean ± S.D. for 3 experiments. Statistical difference is indicated as follows: * P < 0.05 vs. "controls".

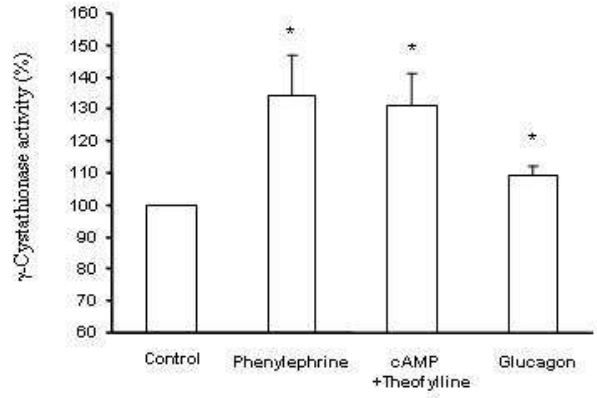

Figure 5. Effects of stress hormones on γ-cystathionase activity *in vitro* in fetal hepatocytes. Fetal hepatocytes were cultured in presence of phenylephrine, cAMP + teophylline or glucagon. Results are mean ± S.D. for 3 experiments. Statistical difference is indicated as follows: * P < 0.05 vs. "controls".

Inhibition of γ-cystathionase by L-cysteine

The inhibitory studies were performed with γ-cystathionase purified from rat liver, which exhibited the following kinetic parameters at different cystathionine concentrations: Vmax = 5.9 IU/mg protein; Km = 8.2 mM. In order to determine the type of inhibition by cysteine, a Lineweaver plot was performed using purified γ-cystathionase in presence or absence of cysteine with increasing concentrations of cystathionine. According to our results, the inhibition of γ-cystathionase by cysteine was competitive (See Fig. 6A).

γ-Cystathionase activity of the purified enzyme was diminished by 20 % in presence of 0.5 mM cysteine and by 50 % in presence of 1 mM cysteine when using 30 mM cystathionine as substrate (see Fig. 6B). Furthermore, γ-cystathionase activity was undetectable when using the physiological cystathionine-to-cysteine ratio (4:1) reported for rat liver (18). Addition of dithiotreitol, a potent reducing agent, restored the enzyme activity in presence of L-cysteine. Therefore, the inhibition of γ-cystathionase by cysteine seems to be mediated by its thiol group.

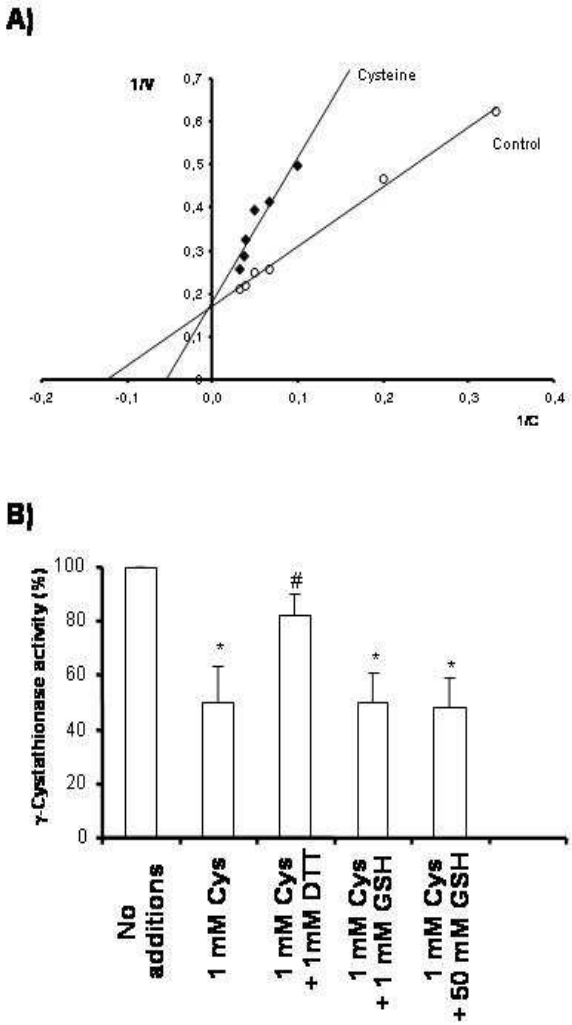

Figure 6. **A)** Competitive inhibition of purified γ-cystathionase by L-cysteine. γ-Cystathionase activity was measured using the continuous method (see Methods) with the enzyme purified from rat liver in presence or absence of 0.5 mM L-cysteine. Abbreviations used: V = γ-cystathionase activity (IU./mg protein); C = cystathionine concentration (mM). **B)** Inhibition of γ-cystathionase activity by L-cysteine and reversal by DTT. Results are mean ± S.D. for 3-4 experiments. Statistical difference is indicated as follows: * P < 0.05 vs. control (no additions).

DISCUSSION

The trans-sulphuration pathway, indispensable for conversion of methionine into L-cysteine and subsequently glutathione synthesis is restricted to those tissues containing both cystathionine β-synthase and γ-cystathionase activities, being the liver where these activities are found to be at highest (19, 20, 21). Moreover, hepatic γ-cystathionase activity is higher in newborn than in fetuses both in rat pups and humans (2, 3). However, Levonen and colleagues reported that γ-cystathionase mRNA was abundantly expressed prior to birth in humans, and no significant changes were found in at term fetuses when compared with newborn or adults (22). Consequently, a post-transcriptional regulation seems to be responsible for the induction of γ-cystathionase activity upon birth in humans. Our results show that an up-regulation of γ-cystathionase is responsible for the increase in the enzyme activity in the fetal-to-neonatal transition in rats. Thus, both the mRNA and the protein for γ-cystathionase increase at birth in rat liver. Furthermore, there is a linear relationship between the mRNA and the protein expression of this enzyme, indicating that the up-regulation is triggered at the transcriptional level and consequently, no accumulation of the mRNA occurs in the fetus at term, as it is the case for other enzymes such as superoxide dismutase, catalase or glutathione peroxidase (23).

Hormones, such as cortisol and glucagon, are considered responsible for the induction of different enzymes in the fetal-to-neonatal transition (1, 24) and γ-cystathionase is one of them. Thus, Heinonen and Raïha reported that dexamethasone, glucagon or dibutyryl cyclic AMP plus teophylline increased γ-cystathionase activity in liver explants from human fetuses (11). In agreement with these results, we report here that phenylephrine, glucagon or di-butyryl cyclic cAMP plus teophylline raise γ-cystathionase activity in fetal hepatocytes.

Hepatic cystathionine β-synthase, another key enzyme in trans-sulphuration, is also regulated by glucagon, glucocorticoids, or cAMP, which increase its gene expression (25, 26, 27). Therefore, the trans-sulphuration pathway is hormonally regulated mainly by stress hormones, such as glucagon, glucocorticoids and phenylephrine.

The flux through the trans-sulphuration pathway seems to be also regulated by oxidative stress, at least in the fetal-to-neonatal transition. Our results show that γ-cystathionase activity in fetal hepatocytes is increased by a moderate oxidative stress induced by a low tert-butylhydroperoxide concentration, which simulates the physiological oxidative stress that occurs in the fetal to neonatal transition. Hence, moderate oxidative stress would serve as a signal to up-regulate γ-cystathionase in the fetal to neonatal transition. On the other hand, the flux through cystathionine β-synthase increases under exposure to H_2O_2 or tert-butylhydroperoxide via heme oxidation (25,28). Hence, a low or moderate oxidative stress increases the generation of cysteine from methionine. Since the intracellular cysteine levels are generally rate-limiting for glutathione synthesis, the up-regulation of the trans-sulphuration pathway by oxidative stress would be an adaptive mechanism to enhance GSH levels and consequently the antioxidant cellular capacity.

It is well-known that newborn animals of many species are more resistant to hyperoxia than adult animals (29, 30). This can be explained because newborn animals can adapt to hyperoxia by up-regulating antioxidant enzymes (30, 31). Accordingly, adult animals had no increases in their lung antioxidant enzyme levels during exposure to hyperoxia, whereas neonatal animals exhibited rapid increases in their antioxidant enzyme levels during the hyperoxic challenge (30). This adaptive mechanism seems to be present only during a short period after birth (32). On the contrary, an excessive oxidative stress may cause enzyme inactivation instead of adaptive up-regulation. Indeed, our results show that an intense oxidative stress - induced by high concentrations of tert-butylhydroperoxide- decreases γ-cystathionase activity. Deleterious effects of an intense oxidative stress should be taken into account especially in hypoxic newborns and prematures. Newborn babies accumulate great amounts of hypoxanthine, the substrate of xanthine oxidase, in tissue and body fluids during hypoxia. Upon reoxygenation a burst of oxygen free radicals is produced overwhelming the antioxidant defense systems causing oxidative stress and even tissue damage (13, 31, 33). This situation may be aggravated in preterm babies, where the capacity of these defense systems is significantly reduced (34). In fact, Saugstad has proposed that oxygen radicals are responsible for the development of some neonatal diseases, such as bronchopulmonary dysplasia, retinopathy of

prematurity, necrotizing enterocolitis and ductus arteriosus (35). The deficiency of γ-cystathionase reported in premature infants (2, 4, 5) may be due to absence of γ-cystathionase up-regulation when the liver maturation is not completed, or to an excessive pro-oxidant situation.

Our results also highlight the importance of the nutritional status for the up-regulation of γ-cystathionase and hence the trans-sulphuration pathway. Hepatic γ-cystathionase activity increased rapidly at birth and it rose further on in newborns fed on maternal milk. Consequently, the complete induction of the trans-sulphuration pathway in the fetal-to-neonatal transition depends on milk feeding, which should be initiated as soon as possible in newborns.

On the other hand, the competitive inhibition of purified γ-cystathionase by L-cysteine that we report here has physiological relevance since it would serve to maintain low cysteine concentrations in hepatocytes. Indeed, the enzyme activity is inhibited at the cystathionine-cysteine ratio occurring in the liver. This inhibition would prevent an excessive accumulation of intracellular cysteine, which may cause cysteine auto-oxidation leading to pro-oxidant and deleterious effects (36).

In conclusion, our results show that the increase in hepatic γ-cystathionase activity in the fetal-to-neonatal transition is due to up-regulation of its gene expression, which is mediated by the physiological oxidative stress that occurs at birth together with stress hormones, such as phenylephrine and glucagon. Moreover, a complete induction of the enzyme activity is obtained in neonates once milk feeding is established. Competitive inhibition of the enzyme with its product L-cysteine helps to maintain low L-cysteine levels in hepatocytes.

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