Liver intracellular L-cysteine concentration is maintained after inhibition of the trans-sulfuration pathway by propargylglycine in rats

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To study the fate of L-cysteine and amino acid homeostasis in liver after the inhibition of the trans-sulfuration pathway, rats were treated with propargylglycine (PPG). At 4 h after the administration of PPG, liver cystathionase (*EC* 4.4.1.1) activity was undetectable, L-cystathionine levels were significantly higher, L-cysteine was unchanged and GSH concentration was significantly lower than values found in livers from control rats injected intraperitoneally with 0·15 M-NaCl. The hepatic levels of amino acids that are intermediates of the urea cycle, L-ornithine, L-citrulline and L-arginine and blood urea were significantly greater. Urea excretion was also higher in PPG-treated rats when compared with control rats. These data suggest a stimulation of ureagenesis in PPG-treated rats. The inhibition of γ-cystathionase was reflected in the blood levels of amino acids, because the L-methionine : L-cyst(e)ine ratio was significantly higher in PPG-treated rats than in control rats; blood concentration of cystathionine was also greater. Histological examination of liver and kidney showed no changes in PPG-treated rats when compared with controls. The administration of N-acetylcysteine (NAC) to PPG-treated rats reversed the changes in blood urea and in liver GSH. These data suggest that when liver L-cysteine production was impaired by the blockage of the trans-sulfuration pathway, the concentration of this amino acid was maintained mainly by an increase in protein degradation and by a depletion in GSH concentration that may spare L-cysteine.

Liver: Cysteine: Propargylglycine: Cystathionase

The maintenance of intracellular L-cysteine concentration in liver is achieved by several mechanisms: (1) the trans-sulfuration pathway, (2) the uptake from plasma, (3) proteolysis, (4) the rate of L-cysteine incorporation into GSH and (5) the conversion to taurine. The availability of L-cysteine is the rate-limiting step for the synthesis of GSH by mammalian cells (Tateishi *et al.* 1974; Beutler, 1989); therefore, the capacity of tissues, mainly the liver, to synthesize L-cysteine from L-methionine is important to cell homeostasis (Rose & Wixom, 1955). It has been estimated that 50–80% of the dietary L-methionine requirements can be dispensed with if the diet is supplemented with L-cysteine, and for this reason the latter has been called a conditionally indispensable amino acid (Laidlaw & Kopple, 1987) in those situations in which cystathionase activity is low.

The daily turnover of L-methionine which includes the incorporation into and release from protein and the conversion of methionine to homocysteine and its remethylation, is approximately double the dietary intake (Nunn, 1987), therefore the recycling of L-methionine from homocysteine is important (Finkelstein, 1990). However, homocysteine is

at a branch point because it can also undergo condensation with serine to form cystathionine through the trans-sulfuration pathway due to the action of cystathionine-β-synthase (*EC* 4.2.1.22). This enzyme uses vitamin B_6 as a cofactor and it is activated by S-adenosylmethionine. The trans-sulfuration sequence is completed by the breakdown of L-cystathionine to L-cysteine and α-ketobutyrate by γ-cystathionase (L-cystathionine cysteine-lyase; *EC* 4.4.1.1) which also uses vitamin B_6 as a cofactor.

It has been shown that DL-2-amino-4-pentynoic acid (propargylglycine; PPG) rapidly inactivates rat cystathionase (Washtien & Abeles, 1977) *in vivo* because the decrease in the cystathionase activity is detected within 1 h and this enzyme activity is maintained at a low level for 1 d. The specificity and efficacy of PPG in inactivating the enzyme appears to give information related to the metabolic importance of this enzyme in the trans-sulfuration pathway. It has been shown that inhibition of the enzyme by PPG produces an increase of L-cystathionine in liver, kidney and brain, and when the enzyme activity is restored the concentration of L-cystathionine drops to the normal value (Reed, 1995).

The trans-sulfuration pathway is important for the synthesis of L-cysteine and GSH from L-methionine in liver. There are situations where cystathionase activity is almost absent such as in the first hours of neonatal life and in the premature neonate (Sturman *et al.* 1970; Pallardó *et al.* 1991) and during surgical stress (Viña *et al.* 1992; Crespo *et al* 1997). This deficiency in cystathionase activity produces an increased blood L-methionine : L-cyst(e)ine ratio when compared with controls. Therefore, the aim of the present study was to evaluate the metabolism of L-cyst(e)ine when the trans-sulfuration pathway is inhibited and to study how cells cope with a deficiency in this enzyme. To that end, we used, as an experimental model, rats injected with PPG, a specific inhibitor of cystathionase.

In the present work we found that in PPG-treated rats, liver cystathionase was undetectable and L-cystathionine increased significantly but L-cysteine concentration was kept constant. Our results suggest that this was achieved by increased protein degradation, followed by higher release of L-cysteine and a lower GSH concentration. This mechanism of adaptation produces an increase in the urinary excretion and blood urea levels. The administration of N-acetyl-L-cysteine (NAC) reversed the changes in plasma urea and liver GSH levels. These data show that the liver keeps the concentration of L-cysteine within normal values despite the impairment of the trans-sulfuration pathway.

MATERIALS AND METHODS

Rats

Mature male Wistar rats of 200–250 g body weight were bought from Charles River (Barcelona, Spain). The rats had free access to a commercial laboratory diet (Letica, S.A., Barcelona, Spain) and tap water. This diet contains (g/kg diet): carbohydrates 590, lipids 30 and protein 160. All rats were maintained on a 12 h light–12 h dark cycle and under controlled conditions of temperature (22°).

Chemicals

PPG was purchased from Sigma Chemical Co., St Louis, MO, USA. N-acetylcysteine was obtained at the pharmacy of our Medical School and was originally obtained from Zambón (Sta Perpetua De Mogoda, Barcelona, Spain).

Rat treatment and sampling procedures

For the measurements of blood amino acids and liver amino acid and GSH concentrations, food was withdrawn after treatment with saline (0·15 M-NaCl) (control rats) or PPG (50 mg/kg injected intraperitoneally). The experiments were performed 4 h later. Rats were anaesthetized with pentothal (50 mg/kg body weight, intraperitoneally) and maintained at 37° with a homeothermic blanket. Liver samples and blood from the aorta (collected in heparinized syringes) were taken. A group of PPG-treated rats was treated simultaneously with an intragastric dose of NAC (500 mg/kg).

For the determination of urinary urea excretion, animals were placed in individual metabolic cages for 48 h. At 8, 16, 24 and 48 h after treatment (a daily injection at 08.00 hours of PPG or saline) urine samples were collected into 25 ml test tubes containing 1 ml 6 M-HCl. In these experiments animals were fasting because the substrate availability exerts immediate control over the velocity of urea synthesis *in vivo* (Beliveau-Carey *et al.* 1993).

Urea was also measured in plasma from retro-orbital plexus blood after 4, 8, 12, 20 and 24 h treatment in a different set of rats; this experiment was carried out on control rats, rats injected with PPG and PPG-treated rats injected intraperitoneally with NAC (80 mg/kg three times in 24 h). In these experiments food was withdrawn after treatment with saline, PPG or PPG + NAC.

Liver and kidney biopsies were taken from rats treated with PPG for 4 h and from control rats, fixed in phosphate-buffered formaldehyde and blocked in paraffin for sectioning. The liver and kidney sections were stained with haematoxylin and eosin.

Liver amino acid uptake

Hepatic amino acid uptake was studied in control rats and in rats after short-term (4 h) and long-term (52 h) inhibition of γ-cystathionase by PPG . In the short-term experiments food was withdrawn after saline or PPG was injected and the blood was collected 4 h later. In the long-term experiments the PPG-treated group received a daily PPG injection in the morning (08.00 hours) for 3 d. Control rats were injected for 3 d with saline. The experiments were performed 4 h after the last injection when food was withdrawn. Food intake was not significantly different in the PPG treated rats over 52 h when compared with controls. For the liver amino acid uptake, blood was collected from the hepatic and portal veins and then from the aorta in controls and in rats injected with PPG. Amino acids taken up by the liver were estimated by assuming in both the control and PPG-treated rats that portal vein and hepatic artery represent 70 % and 30 % of the afferent hepatic blood flow respectively (Rémésy *et al.* 1978).

Metabolite and γ-cystathionase activity determination

Blood and liver amino acid composition was determined on an LKB 3201 amino acid analyser as described previously (Barber *et al.* 1985). GSH was measured by the GSH-transferase (*EC* 2.5.1.18) method (Brigelius *et al.* 1983). Urea was measured by the method of Nuzum & Snodgrass (1976). L-Cysteine was determined by a spectrophotometric method (Gaitonde, 1967). γ-Cystathionase was measured by the method of Heinonen (1973).

Statistics

For Tables 1, 2, and 3 Student's *t* test was used. For Table 4 and Fig. 1, the analyses were conducted by the least-significant difference test, which consists of two steps. First, an ANOVA was performed. The null hypothesis was accepted for all numbers of those sets in which F was non-significant at the level of $P < 0{\cdot}05$. Second, sets of data in which F was significant were examined by the modified t-statistic ($t = (X_1 - X_2)/s(1/n_1 + 1/n_2)^{1/2}$ where s^2, the mean square within groups, is taken from the ANOVA) at $P < 0{\cdot}05$ (Wallenstein *et al.* 1980). Values in the text are means with their standard errors.

RESULTS

Effect of propargylglycine administration for 4 h on liver and whole-blood free amino acid concentration

In PPG-treated rats whole-blood amino acid levels (μmol/l) were similar to those in controls except for L-cystathionine, L-glutamate and L-isoleucine which were significantly higher (in the control group: 3 (SE 2), 183 (SE 8), 66 (SE 5) respectively; in the PPG treated group: 24 (SE 5), 482 (SE 28), 94 (SE 6), respectively, $P < 0{\cdot}05$). The L-methionine : L-cyst(e)ine ratio was significantly lower ($P < 0{\cdot}05$) in the control group (mean 1·4 (SE 0·1) *n* 9) than in the PPG-group (mean 2·4 (SE 0·3), *n* 5).

When PPG was administered for 4 h the liver cystathionase activity was undetectable and the concentration of L-cystathionine was significantly greater than control values. The concentrations of those amino acids related to the urea cycle, such as L-ornithine, L-citrulline and L-arginine were also higher in the PPG-treated group than in controls. The concentration of L-alanine was lower and that of L-glutamate was higher when compared with controls (Table 1).

Time-course of plasma urea concentration in control rats, propargylglycine-treated rats and in propargylglycine-treated rats injected with N-acetyl-L-cysteine

Plasma urea concentration was measured during 24 h in control rats, PPG-treated rats and in PPG-treated rats injected with NAC. After the PPG injection, plasma urea concentration increased to values which remained significantly higher than in controls. When NAC was administered to PPG-treated rats the plasma urea levels were similar to those of controls (Fig. 1). These differences were maintained after a period of 48 h (results not shown). Creatinine clearance was determined and no difference was observed between control rats, PPG-treated rats and PPG-treated rats injected with NAC (results not shown).

Urinary excretion of urea during 48 h in control and propargylglycine-treated rats

The amount of urea excreted was measured during 48 h in both control and PPG-treated rats. For the first 24 h, the urea excretion was measured every 8 h. The excretion of urea was significantly higher after 16 h of the PPG treatment when compared with controls (Table 2). These values are in agreement with the urea plasma values found in PPG-treated rats when compared with controls (Fig. 1).

Effect of propargylglycine administration on liver amino acid uptake and/or release

Inhibition of γ-cystathionase for 52 h increased liver L-cystathionine and this was followed by a significantly high release of L-cystathionine from liver when compared with control

Table 1. *Hepatic amino acid concentrations (nmol/g liver) in control and propargylglycine (PPG)-treated rats*

(Mean values with their standard errors for five to seven rats)

	Control		PPG	
Amino acid	Mean	SEM	Mean	SEM
L-Taurine	2331	814	1859	349
L-Threonine	446	7	654*	61
L-Serine	957	166	1181	130
L-Asparagine	143	27	178	23
LGlutamate	1772	135	2316*	146
L-Glutamine	5369	466	4325	546
L-Proline	194	15	213	17
Glycine	2249	191	2392	183
L-Alanine	3239	275	2186*	286
L-Citrulline	75	21	484*	90
L-Valine	215	19	293*	19
L-Cystine	13	2	23	4
L-Homocysteine	62	18	42	6
L-Methionine	62	10	63	7
L-Cystathionine	34	6	3252*	278
L-Isoleucine	142	21	176	15
L-Leucine	221	23	262	10
L-Tyrosine	131	16	105	9
L-Phenylalanine	87	14	114	11
L-Ornithine	238	18	1004*	115
L-Lysine	476	55	610	60
L-Histidine	697	44	773	49
L-Arginine	32	3	54*	4

* Mean values were significantly different from the corresponding control values, $P < 0.05$.

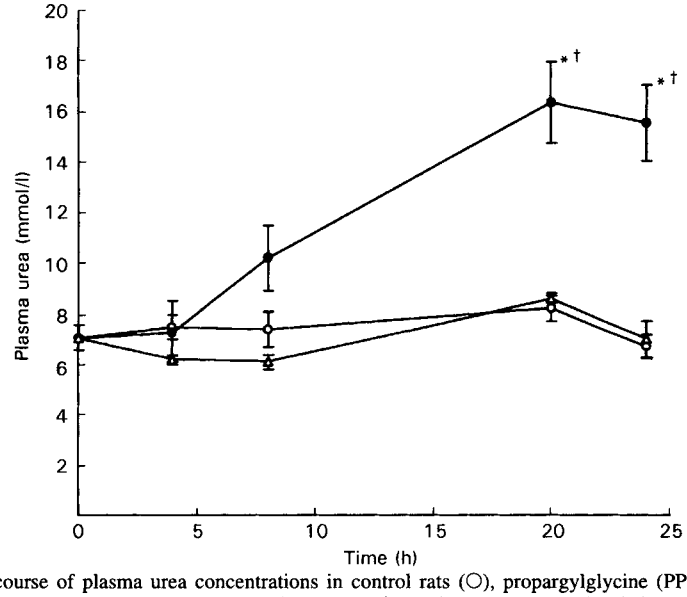

Fig. 1. Time-course of plasma urea concentrations in control rats (○), propargylglycine (PPG)-treated rats (●) and PPG-treated rats injected with N-acetyl-L-cysteine (NAC)(△). Values are means with their standard errors represented by vertical bars. The experiments were: at 4, 8 and 20 h, n 3; at 0 and 24 h, n 5. Mean values were significantly different from the corresponding control values, $P < 0.05$. † Values in PPG-treated rats were significantly different from those in PPG + NAC rats, $P < 0.05$.

Table 2. *Urinary urea excretion (mmol) over 48 h in control rats and propargylglycine(PPG)-treated rats*

(Mean values with their standard errors for five rats)

	Control		PPG	
Time period (h)	Mean	SEM	Mean	SEM
0–8	2·3	0·4	2·6	0·1
8–16	2·2	0·2	2·9	0·3
16–24	2·0	0·3	3·0*	0·2
24–48	5·1	0·4	7·4*	0·7

* Mean values were significantly different from the corresponding control values, $P < 0·05$.

rats (Table 3). In control rats L-cystine was taken up by liver; however, in PPG-treated rats L-cystine was significantly released from liver. The uptake of the branched-chain amino acids was reduced by PPG treatment. However, it was only significantly different for L-leucine. Similar results were found after 4 h inhibition of γ-cystathionase by PPG (results not shown).

Hepatic glutathione and L-cysteine concentrations

The GSH concentration was significantly lower in the PPG-treated rats when compared with controls. When NAC was administered to PPG-treated rats, the GSH value was similar to that of controls. Free L-cysteine concentration in liver was similar in the three situations studied (Table 4).

Table 3. *Hepatic amino acid uptake (%) in control and propargylglycine (PPG)-treated rats*

(Mean values with their standard errors for four rats)

	Control		PPG	
Amino acid	Mean	SEM	Mean	SEM
L-Taurine	8	3	8	2
L-Threonine	11	6	11	2
L-Serine	18	7	18	7
L-Glutamate	− 3	6	0	2
L-Glutamine	3	4	10	3
L-Proline	13	5	13	7
Glycine	11	6	16	4
L-Alanine	23	6	33	5
L-Citrulline	− 2	1	− 7	5
L-Valine	15	4	− 3	6
L-Cystine	27	3	− 2*	5
L-Methionine	29	27	20	9
L-Cystathionine	0	0	− 6*	2
L-Isoleucine	21	13	− 8	9
L-Leucine	28	6	5*	4
L-Tyrosine	20	7	19	11
L-Phenylalanine	18	6	24	2
L-Ornithine	− 8	18	− 16	1
L-Lysine	13	8	11	1
L-Histidine	17	15	13	5
L-Arginine	17	8	5	3

* Mean values were significantly different from the corresponding control values, $P < 0·05$.

Table 4. *Hepatic concentrations of GSH and L-cysteine in control rats, propargylglycine (PPG)-treated rats and rats treated with PPG + N-acetyl-L-cysteine (NAC)*

(Means values with their standard errors, number of experiments in parentheses)

	Control		PPG		PPG + NAC	
	Mean	SEM	Mean	SEM	Mean	SEM
GSH (μmol/g liver)	6·0	0·4(20)	3·8*†	0·3(18)	6·3	1·2(6)
L-Cysteine (nmol/g liver)	119	9(16)	122	9(14)	164	34(5)

* Mean value was significantly different from the corresponding control value, $P < 0·05$.
† Mean value was significantly different from that for the PPG + NAC group, $P < 0·05$.

Liver and kidney histology

The livers and kidneys of four rats treated with PPG for 4 h and four control rats were examined by an experienced pathologist who was unaware of the treatment group involved. No abnormalities were detected in liver and kidney of any rat studied (results not shown).

DISCUSSION

The enzyme γ-cystathionase is responsible for the cleavage of L-cystathionine to L-cysteine, 2-oxobutyrate and NH_4^+; therefore, it catalyses an essential step in the trans-sulfuration pathway which allows the synthesis of L-cysteine from L-methionine. Several pathways of L-cysteine catabolism function efficiently to prevent toxic accumulation of L-cysteine, because a high rate of catabolism forms a thiazolidine derivate with vitamin B_6 which may deplete cells of this vitamin (Griffith, 1987). An excess of L-cysteine is also cytotoxic due to the generation of H_2O_2, $OH^{\cdot}$ and $O_2^{\cdot -}$ during rapid auto-oxidation of L-cysteine to the corresponding disulfide (Viña *et al.* 1983). GSH is a tripeptide widely distributed in mammalian cells and essential for cell physiology. One of its functions is its role as a physiological reservoir of L-cysteine.

γ-Cystathionase can be inhibited by PPG and *in vivo* administration of this compound decreases the activity of the enzyme by 90% (Reed, 1995). PPG also inhibits alanine aminotransferase (*EC* 2.6.1.2) (Burnett *et al.* 1980; Cornell *et al.* 1984), which probably explains why the concentration of L-alanine was lower and that of L-glutamate was higher in PPG-treated rats when compared with controls. Using rat hepatocytes, treatment with PPG at 2 mmol/l produced a 76% inhibition of γ-cystathionase and a significant inhibition of the metabolism of [^{35}S]methionine to [^{35}S]glutathione by 93%, to sulfate by 88% and to L-cysteine by 89% while L-cystathionine accumulation in these incubation systems was significantly higher than in controls (Rao *et al.* 1990).

In the present experiments, the γ-cystathionase activity was undetectable after 4 h of PPG administration and whole-blood and liver L-cystathionine levels were significantly higher than in control rats. Therefore, the extent of inhibition of cystathionase allows gathering of information about the physiological importance of this reaction *in vivo*. The availability of L-cysteine is the rate-limiting step for the synthesis of GSH by mammalian cells, therefore the capacity of tissues, mainly the liver, to synthesize L-cysteine from L-methionine is important to cell metabolism. When cystathionase was inhibited, L-cystathionine levels rose significantly but liver L-cysteine concentration remained constant. Maintaining hepatic L-cysteine concentration can be achieved by three means: an increased liver uptake of blood L-cysteine, an acceleration of the proteolysis and a decreased L-

cysteine utilization. In our case, the release of L-cystine by liver and the urinary excretion of urea were significantly higher after PPG treatment; the liver GSH concentration was significantly lower in the PPG-treated rats than in the controls which suggests that the synthesis from L-cysteine could be impaired. The synthesis and degradation of GSH are mediated by the γ-glutamyl cycle. The first enzyme of GSH degradation is γ-glutamyltranspeptidase (*EC* 2.3.2.1; GGT) which is the only enzyme capable of the cleavage of the γ-glutamyl bond of GSH. Its activity in liver is very low which explains why GSH degradation in liver is negligible.

The higher urinary urea excretion is in accordance with the high plasma urea values found in the PPG-treated rats when compared with controls and the rise in the hepatic levels of the intermediary amino acids of the urea cycle. All these results suggest that the increased liver protein degradation is one of the important pathways for keeping L-cysteine concentration within a normal range when the trans-sulfuration pathway is impaired. This is in agreement with the work of Lu *et al.* (1991) which shows that cellular L-cysteine level is unchanged despite the significant fall in GSH after glucagon or phenylephrine treatment.

The fact that GSH was significantly lower after injecting PPG , even though L-cysteine was not, shows that when γ-cystathionase activity was inhibited, the increased L-cysteine availability from proteolysis was channelled to the rest of the tissues by a higher release from the liver. N-acetyl-L-cysteine administration brought the plasma urea and liver GSH levels to normal values and this shows that the metabolic derangement found in PPG-treated rats is not a side effect of the L-cystathionine inhibitor used, but rather an adaptation to ensure a steady supply of L-cyst(e)ine to the different tissues.

The low cystathionase activity in situations such as surgical stress (Viña *et al.* 1992) or in premature neonates (Sturman *et al.* 1970; Pallardó *et al.* 1991; Viña *et al.* 1995) is significantly reflected in the blood levels of amino acid, because the blood L-methionine : L-cyst(e)ine ratio was significantly lower in the controls than in these situations. In the experimental inhibition of cystathionase by PPG similar results were found because the ratio was lower in controls. Thus, the ratio can be taken as another indication of the activity of the trans-sulfuration pathway.

All the results discussed here show the importance of N-acetyl-L-cysteine administration in those situations in which cystathionase is inhibited because increased protein breakdown occurs to meet the requirements for L-cysteine. Therefore, L-cysteine may be considered to be an indispensable amino acid in those situations where cystathionase is inactive or not fully expressed.

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