Accumulation of Cystathionine, Cystathionine Ketimine, and Perhydro-1,4-Thiazepine-3,5-Dicarboxylic Acid in Whole Brain and Various Regions of the Brain of D,L-Propargylglycine–Treated Rats

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Experimental cystathioninuria was induced in rats by administration of the cystathionine γ-lyase inhibitor, D,L-propargylglycine. The cystathionine metabolites, cystathionine ketimine (CK) and perhydro-1,4-thiazepine-3,5-dicarboxylic acid (PHTZDC), were identified in whole brain and various regions of the brain in D,L-propargylglycine–treated rats. The concentration of CK and PHTZDC in whole brain and various regions of the brain increased gradually after administration of D,L-propargylglycine, and reached the highest value at about 20 hours. CK and PHTZDC accumulated in whole brain and various regions of the brain in proportion to the amount of accumulated cystathionine after D,L-propargylglycine administration. The concentration of these compounds in the cerebellum was higher versus the other regions of the rat brain.

CYSTATHIONINURIA is an autosomal recessive hereditary disorder involving the persistent excretion of a large amount of cystathionine in the urine due to cystathionine γ-lyase deficiency.[1] It has been reported that experimental cystathioninuria induced in rats by administration of D,L-propargylglycine resulted in the accumulation of large amounts of cystathionine in several tissues[2,3] and various regions of the brain.[4] Cystathionine is well known as an important intermediate of the transsulfuration pathway in mammalian tissues. Tallan et al[5] showed that the human brain at autopsy contains a high concentration of cystathionine. In other mammalian species, the concentration of cystathionine in the pool of free amino acids was higher in the brain versus any other tissue.[6] High levels of cystathionine in the human brain were also demonstrated by Okumura et al.[7] It was reported that cystathionine accumulated in the brain of rats that were fed diets deficient in pyridoxine, with the concentration varying among different regions of the brain.[8-9] The relative activity of transsulfuration enzymes and concentration of cystathionine in the whole brain and various regions of the brain have also been studied.[10] Sturman et al[11] suggested that the relative activity of cystathionine β-synthase and cystathionine alone could not account for the accumulation of cystathionine in the brain.

Cystathionine metabolites have been identified in the urine from a patient with cystathioninuria[12] and several tissues from D,L-propargylglycine–treated rats.[2] The activity of cystathionine β-synthase in rats was evenly distributed following treatment with D,L-propargylglycine. On the other hand, cystathionine concentrations were unevenly distributed in the brain of both normal and D,L-propargylglycine–treated rats.[4]

One of the cystathionine metabolites, cystathionine ketimine (CK), has been identified as a product of the enzymatic monodeamination of cystathionine by a transaminase, recently identified as glutamine transaminase.[13] CK has been found in bovine cerebellum, human urine,[14-15] and urine from rats with cystathioninuria.[16] These findings clearly indicate the presence in mammalian tissues of enzymatic mechanisms for the cyclization of cystathionine followed by enzymatic reduction of the produced CK. Nardini et al[17] reported the purification of a NAD(P)H-dependent reductase from bovine brain that actively reduces a class of ketimines. Ketimine reductase activity was located primarily in the cerebellum and cerebral cortex. The discovery of the ketimine-reducing enzyme acting on sulfur ketimine compounds clarifies the biosynthetic pathway of 1,4-thiomorpholine-3,5-dicarboxylic acid (the reduction product of lanthionine ketimine) and perhydro-1,4-thiazepine-3,5-dicarboxylic acid ([PHTZDC] the reduction product of CK) in mammalian brain.

We have suggested that in rats with experimental cystathioninuria induced by injection of D,L-propargylglycine, the main route of cystathionine metabolism is from cystathionine monooxo acids to PHTZDC through CK[16] (Fig 1). This metabolic pathway is supported by our present results. In this study, we determined the time course for accumulation of cystathionine, CK, and PHTZDC in whole brain and various regions of the brain.

MATERIALS AND METHODS

Chemicals

Authentic cystathionine and D,L-propargylglycine were purchased from Sigma (St Louis, MO). CK was prepared according to the method of Ricci et al.[18] PHTZDC was kindly provided by Dr S. Duprè, Dipartimento di Scienze Biochimiche, Università di Roma ''La Sapienza'' (Rome, Italy). Phenyl isothiocyanate (PITC) and other reagents were obtained from Wako Pure Chemicals (Osaka, Japan).

Animals and Treatment

Male Wistar rats (mean body weight, ~200 g) were injected intraperitoneally with 20 mg D,L-propargylglycine/200 g body weight. Control rats received the same volume of physiological saline solution. The rats were decapitated at various times after the injection. The brain was removed quickly and the cerebellum, mesencephalon, thalamus plus hypothalamus, caudatum, hippocampus, and cerebral cortex were dissected out and immediately stored at −80°C until analysis.

The Animal Care Committee of Kochi Medical School approved the experimental protocol.

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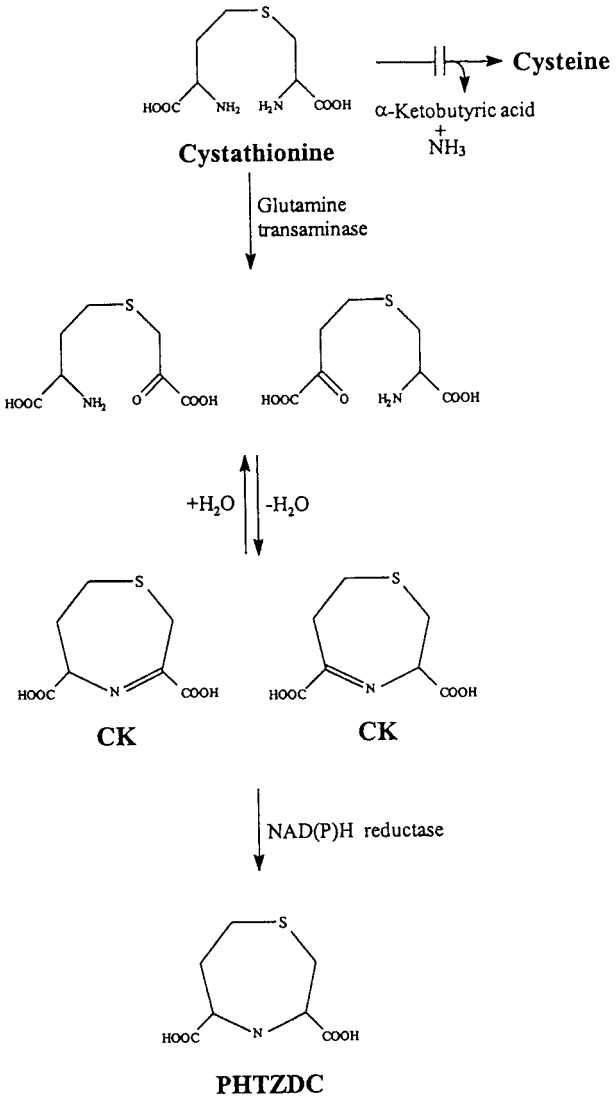

Fig 1. Metabolic pathway of CK from cystathionine.

Analysis of Amino Acids

Each tissue sample (~1 g) was homogenized in 4 vol 2% sulfosalicylic acid for 5 minutes and then centrifuged at 3,000 × *g* for 15 minutes. The supernatant was applied to a Diaion SK-1 column (H-form, 1 × 10 cm), washed with 20 mL water, and CK was eluted with 180 mL water. After elution of CK, cystathionine was eluted with 30 mL 2-mol/L ammonium hydroxide. Both the water and 2-mol/L ammonium hydroxide fractions were dried under reduced pressure at 45°C. The residue was dissolved in water (0.5 mL/g brain) for the determination of cystathionine using an amino acid analyzer. The water fraction residue was resuspended in 0.4 mL water, and then 1.2 mL coupling buffer (acetonitrile:pyridine:triethylamine:water 10:5:2:3) and 80 µL PITC were added. After standing for 60 minutes at room temperature, the solution was dried under reduced pressure at 45°C. The residue was dissolved in 0.4 mL 0.01-mol/L potassium phosphate buffer (pH 8.0), and 100 mL of the sample was analyzed by high-performance liquid chromatography (HPLC).[15] The determination of PHTZDC in brain using liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS) was described previously.[19]

Instrumentation

A Hitachi (Tokyo, Japan) L-6200 high-performance liquid chromatograph equipped with a Hitachi L-4200 UV-VIS detector and a 5-µm Inertsil ODS-2 column (150 × 4.6 mm ID) was used. The mobile phase was as follows: A, 0.05 mol/L ammonium acetate; B, 0.05 mol/L ammonium acetate:acetonitrile (65:35, vol/vol); and C, acetonitrile: water (70:30, vol/vol). After washing with solvent C for 20 minutes, the column was preconditioned with solvent A for 30 minutes before sample loading, and then a linear gradient from A to 100% B for 30 minutes was produced with a flow rate of 1.0 mL/min. Detection was performed at 380 nm.

The amino acid analyzer was a Hitachi model 835 liquid chromatograph. The operating conditions for LC-APCI-MS were as described previously.[19]

RESULTS

To study the physiological role of cystathionine metabolites in the brain, we investigated cystathionine, CK, and PHTZDC in the whole brain and various regions of the brain of D,L-propargylglycine–treated rats. Figure 2B represents a typical HPLC elution profile of CK in the whole brain of D,L-propargylglycine–treated rats. The detection was performed at 380 nm, which is the characteristic absorption maximum of the PITC derivative of CK. When the authentic CK was added to

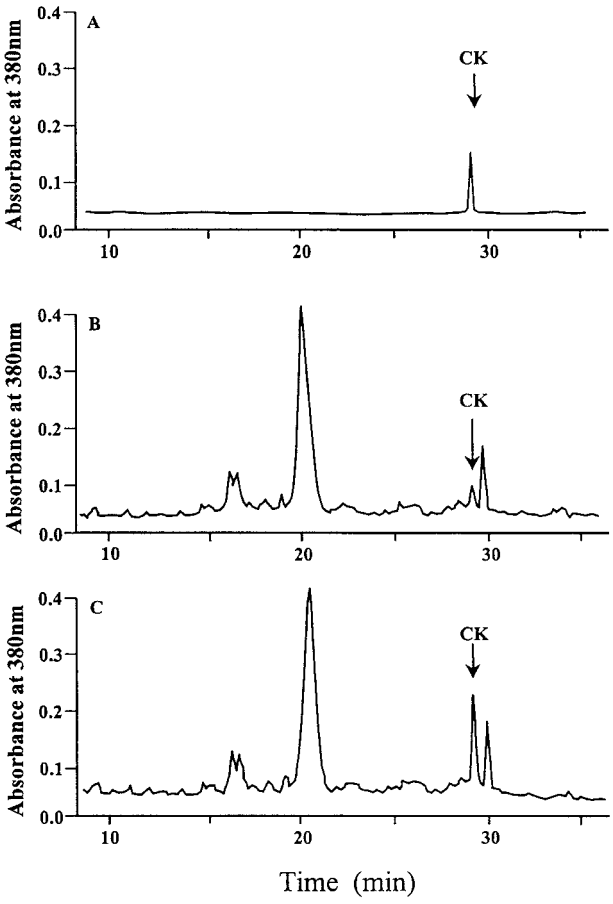

Fig 2. HPLC elution profile of CK in rat whole brain at 20 hours after D,L-propargylglycine administration. (A) Chromatogram of authentic CK, (B) sample corresponding to 0.5 g original brain from D,L-propargylglycine–treated rat, (C) A plus B.

the whole-brain sample prior to the derivatization procedure, the peak with a retention time of 29 minutes co-eluted with authentic CK (Fig 2C), the retention time of which corresponded to that of the authentic CK (Fig 2A). This result indicates the presence of CK in the whole brain from D,L-propargylglycine–treated rats.

The time course for cystathionine, CK, and PHTZDC levels in the whole brain of rats treated with D,L-propargylglycine was measured (Fig 3). CK and PHTZDC accumulated in the rat brain in proportion to the concentration of cystathionine after D,L-propargylglycine administration. When D,L-propargylglycine was injected in the rats, the concentration of cystathionine, CK, and PHTZDC increased rapidly. CK reached a maximal level (15.88 ng/g) at 20 hours after treatment with D,L-propargylglycine. In the brain of control rats and treated rats at 4 hours after D,L-propargylglycine, CK was not detected. On the other hand, both cystathionine and PHTZDC were found in the brain of control rats at a concentration of 13 µg/g and 371 ng/g, respectively. Cystathionine and PHTZDC values reached the highest levels at 24 hours after D,L-propargylglycine treatment, which were about nine and 1.6 times the control values. As compared with the brain, the accumulation of these compounds in the kidney and liver was faster: at 20 hours after D,L-propargylglycine administration, the concentration of CK in the kidney and liver reached 326.2 and 183.58 ng/g, which are 20 and 10 times higher versus the level in rat brain, respectively.

We examined the distribution of cystathionine and the cystathionine metabolites, CK and PHTZDC, in the six regions of the brain from D,L-propargylglycine–treated rats: cerebellum, mesencephalon, thalamus plus hypothalamus, caudatum, hippocampus, and cerebral cortex. HPLC elution profiles of PITC derivatives of the cerebellum from control and D,L-propargylglycine–treated rats are shown in Fig 4. In the cerebellum of both control rats and D,L-propargylglycine–treated rats, peaks with the same retention time as that of the authentic CK were detected (Fig 4A and B). When authentic CK was added to the cerebellum from D,L-propargylglycine–treated rats, the peak retention time, which corresponds to that of authentic CK, increased (Fig 4C). The time course of cystathionine, CK, and PHTZDC in various regions of the brain from control rats and

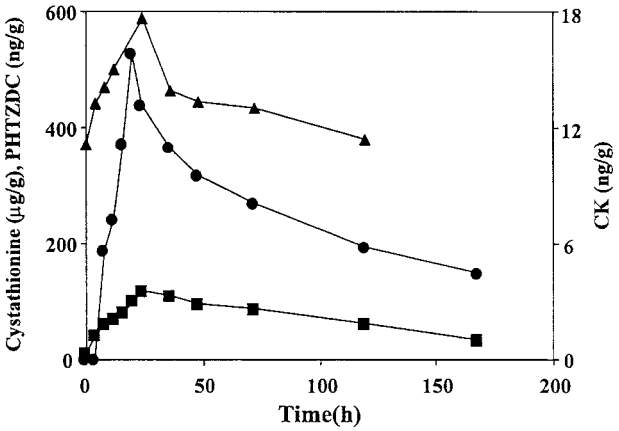

Fig 3. Accumulation of (■) cystathionine, (●) CK, and (▲) PHTZDC in the whole brain of rats treated with D,L-propargylglycine. All curves are the mean of at least 3 independent experiments.

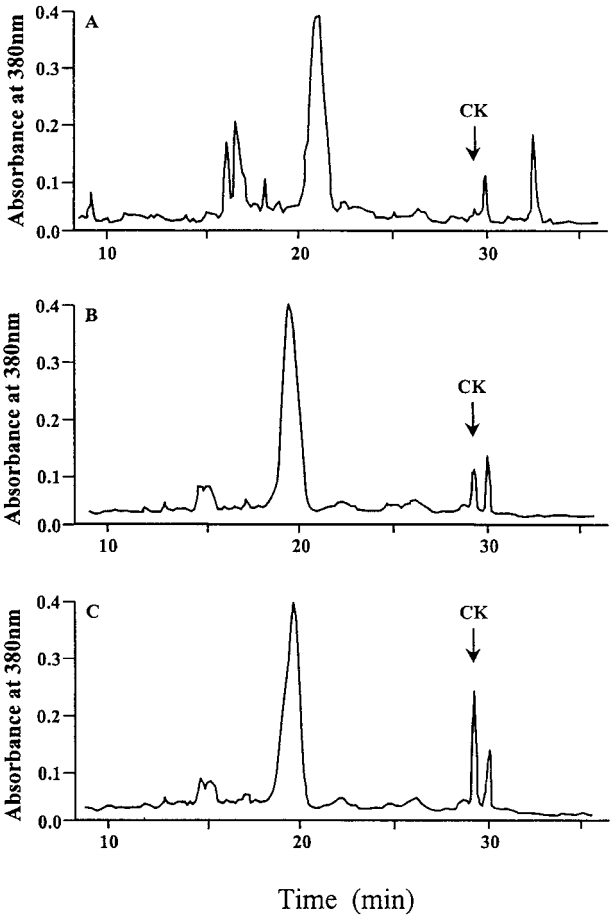

Fig 4. HPLC elution profile of CK in cerebellum from control rats and at 20 hours in D,L-propargylglycine–treated rats. (A) Sample corresponding to 0.5 g cerebellum from control rats, (B) sample corresponding to 0.5 g cerebellum from D,L-propargylglycine–treated rats, (C) B plus 50 ng authentic CK.

rats treated with D,L-propargylglycine is shown in Fig 5. The administration of D,L-propargylglycine induces the accumulation of these compounds in all six regions of the rat brain. A maximal effect was found at 20 hours following treatment. The concentration of cystathionine and PHTZDC was relatively constant in five areas of the brain, with the cerebellum being a higher outlier. The concentration of cystathionine was approximately 19 times greater in the cerebellum from control rats versus the cerebral cortex; this difference decreased to about four-fold at 20 hours after D,L-propargylglycine treatment. On the other hand, the difference in PHTZDC concentrations between control and D,L-propargylglycine–treated rats remained nearly the same (1.94- and 1.76-fold, respectively). CK concentrations were low in the cerebral cortex and constant in other areas. In the cerebellum, CK increased faster versus the other regions of rat brain treated with D,L-propargylglycine, and reached 22.54 ng/g at 20 hours after D,L-propargylglycine treatment.

DISCUSSION

Cystathionine, the parent compound necessary for the production of CK, is an important intermediate in the transsulfuration

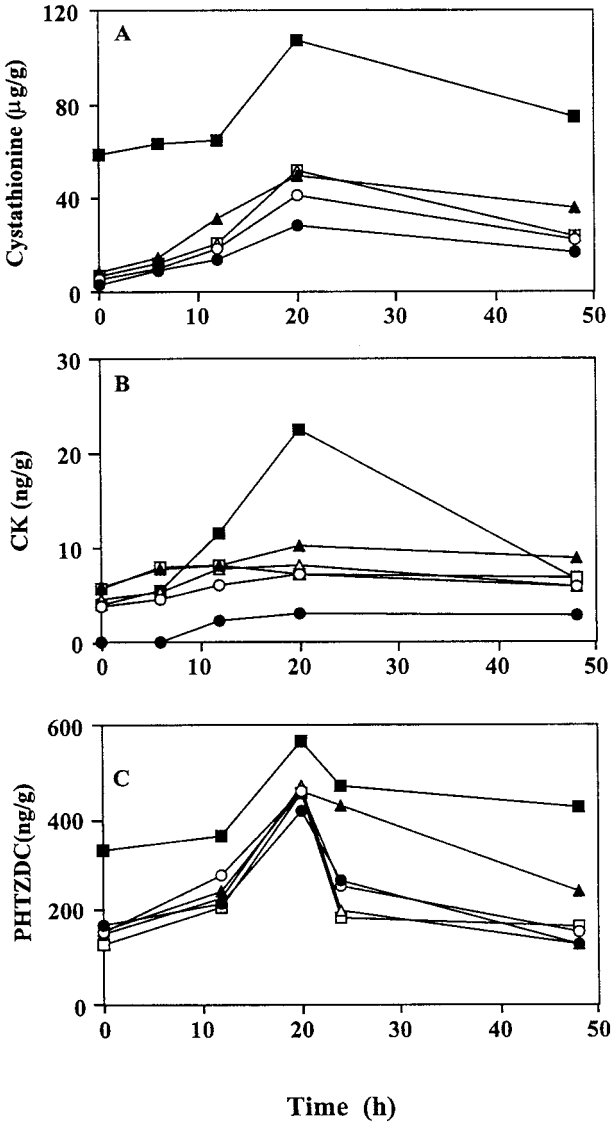

Fig 5. Accumulation of (A) cystathionine, (B) CK, and (C) PHTZDC in the various regions of rat brain treated with D,L-propargylglycine. (■) Cerebellum, (▲) mesencephalon, (△) thalamus plus hypothalamus, (○) caudatum, (□) hippocampus, (●) cerebral cortex. All curves are the mean of at least 3 independent experiments.

pathway from methionine to cysteine in mammalian tissues. CK has been identified as a product of the enzymatic monodeamination of cystathionine by a glutamine transaminase in liver, kidney, and brain.[13,14] We have reported that cystathionine accumulates in various regions of the brain of D,L-propargylglycine–treated rats,[4] and in patients with cystathioninuria, several cystathionine metabolites were excreted in the urine.[12,19] Normally, cystathionine is synthesized from serine and homocysteine by cystathionine β-synthase and is degraded to cysteine, α-ketobutyric acid, and ammonia by cystathionine γ-lyase in mammalian tissues. Injection of D,L-propargylglycine in rats led to a decrease of cystathionine γ-lyase activity in the liver and kidney. We have also reported that cystathionine β-synthase was not affected by the administration of D,L-propargylglycine.[20] The detection of CK in bovine cerebellum and the presence in the brain of an enzyme[13] that converts cystathionine to the cyclic ketimine suggest a metabolic pathway for this amino acid based on an initial transamination step (Fig 1). This biosynthetic pathway is supported by the presence of PHTZDC, the reduced product of CK, in bovine brain and in normal human urine[21] and by the excretion of a very large amount of PHTZDC in the urine of patients with cystathioninuria[12,19] and experimentally cystathioninuric rats[4,16] induced by injection of D,L-propargylglycine. Cystathionine in normal rat brain was distributed unevenly[8,9] and the concentration was greatest in the cerebellum, where it was approximately 10 times higher versus the cerebral cortex.[4] The present results also indicate that the concentration of cystathionine, CK, and PHTZDC in the six regions of the rat brain was different, and was higher in the cerebellum versus the other five regions at 20 hours after administration of D,L-propargylglycine (Fig 5). This finding further supports the results reported previously.[8,9]

In the present study, we found an accumulation of cystathionine, CK, and PHTZDC in the brain of rats treated with D,L-propargylglycine. The PHTZDC level in the whole brain and six regions of the brain increased correspondingly with cystathionine and CK concentrations in rats after treatment with D,L-propargylglycine. By an alternative metabolic pathway, cystathionine is monodeaminated by glutamine transaminase.[13] Monodeaminated cystathionine mono-oxo acids form CK through a cyclization process,[18] and CK is reduced to PHTZDC by a NAD(P)H-dependent reductase.[17] These results suggest that the conversion of CK to PHTZDC together with the prior monodeamination of cystathionine is a significant route of cystathionine metabolism in the brain of D,L-propargylglycine–treated rats, as reported previously.[16]

The biochemical properties of sulfur-containing cyclic ketimines and their reduced products, such as CK and PHTZDC, are now beginning to be understood. The metabolism of these compounds in the brain of D,L-propargylglycine–treated rats was similar to that found in the urine of patients with hereditary cystathioninuria and in the urine of D,L-propargylglycine–treated rats. These findings may make it possible to use D,L-propargylglycine–treated rats as an animal model of cystathioninuria for clarification of the mechanisms of cystathionine and the physiological functions of cystathionine and its metabolites in mammals.

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