The Role of Amino Acids and Their Transport Systems in the Regulation of Ureogenesis in the Primary Culture of Adult Rat Hepatocytes

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NAGAO, M., MORI, T., TSUCHIYAMA, A. and OYANAGI, K. *The Role of Amino Acids and Their Transport Systems in the Regulation of Ureogenesis in the Primary Culture of Adult Rat Hepatocytes*. Tohoku J. Exp. Med., 1989, **158** (4), 309-316 — The biosynthesis of urea, the metabolism of ammonia and the transport of amino acids were studied using the primary culture of hepatocytes from adult rats. The urea synthesis and ammonia detoxication were affected by the amino acids of urea cycle intermediates, such as ornithine, arginine and aspartate. When the hepatocytes were incubated in the medium containing 1 mM ammonium chloride, the transport activity of system-A, which was determined by the uptake of specific substrate methyl-2-amino isobutyric acid (MeAIB), did not change compared with the control level. However, the transport activity of ornithine was increased to a maximum after 4 hr of incubation with ammonia, and then decreased gradually to twice the control level. The activity of ornithine transcarbamylase (OTC) increased to twice the control. These results indicated that the amino acids of urea cycle intermediates, especially ornithine, can be the important regulators of ureogenesis. — ureogenesis; amino acid transport; primary culture of hepatocytes

The liver is the most important organ for amino acid metabolism and plays a significant role for the disposal of ammonia. Catabolism of amino acids results in the production of free ammonia. Ammonia is detoxified to urea through a series of enzyme reactions known as the urea cycle, which occurs primarily in the liver (Brown and Cohen 1959). Many reports on congenital hyperammonemia have been compiled and the clinical and biochemical features of the respective diseases have been ascertained (Walser 1983). However, the mechanism regulating ureogenesis and ammonia detoxication has not been well clarified. Many investigators have attempted to determine the factors involved in the regulation of ureogenesis. The close relationship between the hepatic levels of urea cycle

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enzymes and the extent of urea excretion has been investigated (Schimke 1962). In hepatic amino acid metabolism, recent evidence suggests that amino acid transport into liver cells is the rate limiting step for further metabolism (Shotwell et al. 1983). Saheki et al. (1977) reported that the level of urea synthesis varies with the concentration of urea cycle intermediates. In other words, the increase of urea formation is accompanied by an increase in acetylglutamate, ornithine and citrulline in the liver. So we have attempted to clarify the role of amino acids and their transport process in the ureogenesis. Recent studies have indicated that primary cultured hepatocytes isolated by the collagenase perfusion method retain many liver functions (Tanaka et al. 1978). Therefore it seems appropriate to use these cultured hepatocytes for the studies on ureogenesis. The present paper describes studies of the biosynthesis of urea, the metabolism of ammonia and the transport of amino acids, using the primary culture of hepatocytes from adult rats.

MATERIALS AND METHODS

Materials. The materials used for cell isolation and culture were as reported previously (Tanaka et al. 1978). Insulin and dexamethasone were obtained from Sigma (St. Louis, MO, USA). Aprotinin was from Bayer, Leverkusen. Collagenase for cell isolation was from Wako Pure Chemicals (Osaka). Radioisotope labeled amino acids, [^{14}C]-methyl-2-amino isobutyric acid (MeAIB), [^{14}C]-2-amino-bicyclo-[2, 2, 1]-heptane-2-carboxylic acid (BCH), [^{14}C]-ornithine and [^{14}C]-glutamine were purchased from New England Nuclear (Boston, MA, USA). [^3H]-Inulin was purchased from the Radiochemical Center, Amersham, Little Chalfont, U.K.

Animals. Adult male Wistar rats were obtained from Hokkaido Experimental Animals (Sapporo), weighed about 150 to 200 g and maintained on laboratory chow.

Isolation and monolayer culture of adult rat hepatocytes. Adult rat hepatocytes were isolated from male Wistar rats by the in situ perfusion method reported previously (Seglen 1976). The isolated cells were plated at near confluent density (4×10^5 cells/cm²) and grown as monolayers in Williams medium E (WE) with 5% calf serum, 10^{-9} M insulin in 2-cm diameter wells of Linbro multi-well plastic dishes coated with collagen type I. Five hours after seeding, the medium was changed to WE containing 5 U/ml aprotinin (Nakamura et al. 1984), 10^{-9} M dexamethasone and 10^{-9} M insulin. This medium change was carried out once every day.

Measurement of amino acid uptake. The uptake of amino acid in hepatocytes attached to the culture dish was measured by the method of Kletzien et al. (1976). The plate was rinsed with 10 to 15 ml of warm (37°C) Hanks'-Hepes salt solution (Hanks' balanced salt solution minus glucose at pH 7.4 with 20 mM Hepes buffer). This was followed by incubation in 1.0 ml of Hanks' complete-Hepes solution (8 mM glucose added to Hanks'-Hepes salt solution) at 37°C containing radiolabeled amino acid (0.2 mM, 0.1 $\mu\text{Ci/ml}$) for the various length of time (30 sec, 1, 2, 5 and 10 min). Incubation was terminated by aspirating off the medium and rinsing the cells several times with phosphate buffered saline (PBS). Cells were digested in 0.5 N NaOH and aliquots of the digest were taken for the protein determination and liquid scintillation counting. [^3H]-Inulin was used for the determination of the extracellular trapping. The data were corrected for the extracellular trapping and expressed as pmoles of amino acids/mg protein/30 sec.

Assay procedure. Urea synthesis and ammonia detoxication by the hepatocytes were determined as follows. After 24 hr of culture in serum free WE, the hepatocytes were washed with PBS, then incubated for 12 hr in Hanks'-Hepes salt solution (1.0 ml) containing 1 mM ammonium chloride and further addition of various amino acids. Samples of

incubation media were taken and the amount of urea formed was measured by the method of Ceriotti and Spandrio (1963). The results were expressed as μ moles of urea formed per 10^6 cells per 12 hr. The concentration of ammonia in the incubation medium was assayed by the indophenol method (Chaney and Marbach 1973). The enzyme activity of ornithine transcarbamylase (E.C. 2. 1. 3. 3.) was measured by the method of Schimke. (1962). Cellular protein was measured by the method of Lowry et al. (1951).

RESULTS

Effects of amino acids on urea synthesis from ammonium chloride

It is accepted that urea synthesis in vivo requires a supply of amino acids as the source of the urea cycle intermediates and energy metabolism (Saheki et al. 1978). We have examined the effects of various amino acids on urea synthesis using primary cultured hepatocytes. The results are shown in Table 1. The same batch of hepatocytes was used under each conditions to allow for direct comparisons. Each amino acid was added at the concentration of 2 mM in Hanks'-Hepes solution containing 1 mM ammonium chloride. The rates of urea synthesis were high when the amino acids of urea cycle intermediates (ornithine, aspartate and arginine) were added to the medium. Lysine also stimulated urea synthesis. Though glutamine inhibited the elimination of ammonia from the medium, it increased urea synthesis most of all the amino acids tested. The concentrations of ammonia in the incubation media containing the urea cycle intermediates decreased more than it did in the medium with any other amino acids. The consumption of each amino acids was measured at the same time.

TABLE 1. *Effects of various amino acids on urea synthesis and the changes in ammonia concentration during the culture of hepatocytes*

Conditions of culture	Urea synthesis (μ mole/ 10^6 cells/12 hr)	Decrease in ammonia concentration (mM)	Amino acid consumption (%)
NH ₄ Cl + alanine	0.42 ± 0.06	0.49 ± 0.02	6.0 ± 1.2
NH ₄ Cl + arginine	0.96 ± 0.08	0.68 ± 0.01	29.5 ± 3.2
NH ₄ Cl + aspartate	1.15 ± 0.07	0.75 ± 0.01	33.2 ± 7.6
NH ₄ Cl + ornithine	1.67 ± 0.19	0.79 ± 0.03	39.1 ± 4.4
NH ₄ Cl + glutamine	2.26 ± 0.22	0.16 ± 0.04	52.4 ± 3.8
NH ₄ Cl + lysine	0.98 ± 0.16	0.62 ± 0.01	19.5 ± 7.2
NH ₄ Cl + leucine	0.56 ± 0.10	0.52 ± 0.04	4.4 ± 2.2
NH ₄ Cl	0.36 ± 0.11	0.42 ± 0.01	

Various amino acids were added at a concentration of 2 mM in Hanks'-Hepes salt solution containing 1 mM ammonium chloride. The urea synthesis and the decreased in ammonia concentration were measured after 12 hr of incubation. The concentration of amino acid in the medium was measured using the automatic amino acid analyzer (L-8500; Hitachi, Tokyo). Values are the means $\pm$ s.d. of three experiments.

The concentration of glutamine decreased most. The consumption of ornithine, aspartate and arginine amounted to 30–40% of the prior concentration, while those of alanine and leucine were less than 10% of the amounts present in the incubation media.

Changes of amino acid transport activity

We examined the effects of ammonia on the cellular transport activity of amino acids (Table 2). After 12 hr of culture in Hanks'-Hepes salt solution containing 1 mM ammonium chloride, transport activity was assayed as mentioned above. To monitor the transport activity of system-A, which is reactive with neutral amino acids, such as alanine and glycine (Kletzien et al. 1976), we used methyl-2-amino isobutyric acid (MeAIB) a nonmetabolizable alanine analog. The transport activity of system-L, which is most reactive with branched-chain and aromatic amino acids (Nadziejko and Reichberg 1984), was assayed using the nonmetabolizable analog 2-amino-bicyclo-[2, 2, 1]-hepatane-2-carboxylic acid (BCH). We further analyzed the transport activity of ornithine, arginine, lysine and glutamine. The uptake of MeAIB by hepatocytes cultured in ammonia-containing medium was approximately the same level as the control. The uptake of BCH increased slightly compared with the control level. The transport activity of ornithine increased to twice that of the control cells. In addition, those of arginine and lysine were apparently stimulated. However, the uptake of glutamine decreased to about 60 percent of the control levels.

Ornithine transport and ornithine transcarbamylase activity

We have indicated that urea synthesis from ammonium chloride in hepatocytes is stimulated by adding amino acids of urea cycle intermediates to the medium. At the same time, the membrane transport of these amino acids is activated. In particular, ornithine had the most stimulating effect on urea synthesis in these amino acids. Therefore, we measured the rate of ornithine

TABLE 2. *Effects of ammonia on amino acid uptake*

Substrate	Amino acid uptake (pmoles/mg protein/30 sec)	
	NH ₄ Cl	Control
MeAIB	116.4±3.3	113.1±8.7
BCH	81.5±9.5	62.7±1.8
Ornithine	194.3±5.5	102.4±4.8
Arginine	174.5±4.2	125.4±3.3
Lysine	342.8±3.0	212.9±7.5
Glutamine	225.9±5.8	426.1±13.3

Uptake of amino acid was measured as described under "Materials and Methods". Values are means±s.d. of three experiments.

Fig. 1. Effects of ammonia on the time course of ornithine transport in cultured hepatocytes. The time course of ornithine uptake was measured in the hepatocytes cultured in the incubation medium containing 1 mM ammonium chloride (●, ammonia). The uptake of ornithine in the incubation medium without ammonia are also shown (○, control). The values are means $\pm$ s.d. of three experiments.

Fig. 2. Effects of ammonia on the time course of ornithine transcarbamylase activity in cultured hepatocytes. OTC activity in the hepatocytes cultured in ammonia-containing medium was measured during incubation (●, ammonia). Control values were obtained in the hepatocytes cultured in the incubation medium without ammonia (○, control). The values are means $\pm$ s.d. of three experiments.

transport and ornithine transcarbamylase (OTC) activity during the time of incubation with ammonia. The uptake of ornithine increased rapidly more than three times the control level after 4 hr of incubation, then it decreased gradually to twice the control (Fig. 1). The time course of OTC activity is shown in Fig. 2. When ammonium chloride was added to the medium at a final concentration of 1 mM, OTC activity was induced gradually, reaching twice the control after 12 hr of incubation. However, OTC activity in control hepatocytes was approximately constant over this period.

DISCUSSION

The experiments described in this paper were designed to clarify some aspects of ureogenesis. In addition to urea cycle enzymes, the transport processes of amino acids are important in urea synthesis. Transport across the plasma membrane or mitochondrial membrane is not usually considered, but it is the first step by which amino acid is committed to further metabolism. It is not well known how the amino acid transport systems are concerned in the regulation of urea synthesis in the liver. It is reported that urea synthesis is regulated by the amounts of amino acids transported to the liver (Saheki et al. 1977). When the serum level of ammonia increases and a great deal of ammonia is transported to the liver, it is expected that role of amino acids in the urea synthesis and ammonia disposal might be greater. The present data indicated that amino acids of urea cycle intermediates (ornithine, aspartate and arginine) stimulated urea synthesis and ammonia removal much more than the other amino acids did. Arginine is known as one of the regulators of ureogenesis (Brown and Cohen 1959). It stimulates the activity of the N-acetylglutamate synthetase (NAGS) which synthesize the allosteric activator of carbamyl phosphate synthetase (CPS), N-acetylglutamate (Shigesada and Tatibana 1971). It is also accepted that the synthesis of carbamyl phosphate is the rate limiting step for ureogenesis, and arginine can be the key substrate (Brown and Cohen 1959). However, Cohen et al. (1982) reported that ornithine also could be the regulator of ureogenesis. It is also observed that the ornithine content in the liver increased on injection of ammonium chloride (Saheki et al. 1978). The present data using the primary cultured hepatocytes indicated that the urea synthesis was stimulated by ornithine. Therefore we had an interest in the ornithine transport and OTC activity in the hepatocytes loaded with high concentration of ammonia. We have approved that in the range of physiological concentration of ammonia in the medium (0–50 μ M), synthesis of urea in hepatocytes was saturated at the endogeneous concentration of ornithine (0.11 ± 0.02 mM, unpublished data). When the hepatocytes were incubated in a high concentration of ammonia (1 mM), the rate of ornithine transport was accelerated to twice the control. This suggests that the intracellular concentration of ornithine was increased to amplify the urea cycle. It may be that ornithine transport at the mitochondrial membrane is stimulated at the

same time. OTC activity was also induced to about twice the control. The mechanism of OTC induction in cultured hepatocytes is suggested to be substrate induction (Pierson et al. 1977), the increase of ornithine and carbamyl phosphate. The inhibition of glutamine transport is another aspect in the regulation of amino acid transport.

Free ammonia released from tissue were synthesized to glutamine and metabolized to urea in the liver. However, when the concentration of ammonia in the extracellular space was high, glutamine transport into hepatocytes was suppressed. It is often observed that the serum concentration of glutamine is consistently high in the patients with congenital hyperammonemia. The suppression of glutamine uptake by hepatocytes may account for some part of this.

The present results indicated that amino acids of urea cycle intermediates, especially ornithine, play an important role in the regulation of urea synthesis. With high concentration of ammonia in the extracellular space, the synthesis of urea in the hepatocytes is determined by the supply of ornithine to the cells. This is in keeping with the phenomenon that the ornithine transport at the plasma membrane and OTC activity are activated. It is also suggested that the transport processes of amino acids must be taken into consideration as the short time regulator of ureogenesis. We are now attempting to clarify the molecular mechanism of the regulation of amino acid transport using our culture system.

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