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STUDIES ON UREA SYNTHESIS IN THE LIVER OF RATS TREATED CHRONICALLY WITH ETHANOL USING PERFUSED LIVERS, ISOLATED HEPATOCYTES, AND MITOCHONDRIA

KAYO ADACHI,* TATSUO MATSUHASHI,* YUJI NISHIZAWA,* JIRO USUKURA,* JERZY POPINIGIS† and TAKASHI WAKABAYASHI*‡

*Department of Cell Biology and Molecular Pathology, Nagoya University School of Medicine, Nagoya 466, Japan; and †Department of Bioenergetics, "Jedrzej Sniadecki" Academy of Physical Education, 80-336, Gdansk-Oliwa, Poland

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Abstract—Changes in urea synthesis in the liver of rats treated with 32% ethanol in the drinking water for up to 6 months were studied using perfused livers, isolated hepatocytes, and mitochondria. Results obtained from ethanol-treated rats are summarized as follows: (1) the mitochondria of the hepatocytes of rats treated with ethanol for 2 months or longer became enlarged to various degrees, (2) the levels of ammonia in the serum remained within a normal range, while those in liver tissue were elevated compared with the control, (3) urea synthesis from ammonia in perfused livers was decreased markedly, while that from citrulline remained in the normal range, (4) the activities of carbamyl phosphate synthetase (CPS; EC 2.7.2.5) and ornithine transcarbamylase (OTC; EC 2.1.3.3) in mitochondria were unchanged compared with those of the control, and (5) the levels of ATP in liver tissue and the ability of mitochondria to synthesize ATP were decreased markedly compared with the control. Both the level of ATP in the hepatocytes and the synthesis of urea from ammonia by perfused livers of rats treated with ethanol were resistant to externally added ethanol, while those of control animals were severely affected. These results suggest that the intracellular level of ATP is intimately related to urea synthesis in both control and ethanol-treated animals, and lowered levels of ATP may be a key factor in the suppression of urea synthesis in ethanol-treated animals.

Key words: chronic ethanol intoxication; hepatic megamitochondria; urea synthesis; perfused livers; hepatocyte; intracellular ATP

Structural and functional changes of the liver in chronic ethanol intoxication have been studied extensively by many investigators [1–8]. One of the most distinct ultrastructural changes of hepatocytes in chronic ethanol intoxication is the formation of megamitochondria. Besides being affected by ethanol intoxication, mitochondria become gigantic in various tissues under both physiological and pathological conditions concomitant with changes in the intracellular milieu [9–14]. It is essential to study the mechanism and pathophysiological meaning of the formation of megamitochondria in order to determine the relationship between the changes in structure and those in the function of mitochondria in various pathological conditions.

In our laboratory, we have been studying the mechanism of the formation of hepatic megamitochondria, using hydrazine and alkyl alcohols, including ethanol, as experimental models [15–23]. We have stressed the role of membrane fusion in the mechanism of the formation of megamitochondria based on physiochemical, biochemical, and morphological analyses of mitochondria of hydrazine-treated rat liver [16–19]. We have also found that alkyl alcohols, such as propanol and butanol, induce morphological changes in mitochondria of the liver similar to those induced by ethanol, including the formation of megamitochondria [20–23]. Furthermore, we have found that the phospholipid composition and the ratio of unsaturated fatty acids to saturated fatty acids in mitochondrial membranes are altered markedly by chronic ethanol intoxication, resulting in changes in the thermotropic behavior of mitochondrial membranes revealed by differential scanning calorimetry and fluorescence polarization measurements [21].

One of the serious questions concerning the phenomenon of megamitochondrial formation is how functions of the cell are altered or affected by the presence of megamitochondria. There is a body of data available in the literature dealing with the function of megamitochondria [15, 16, 19, 21–23]. However, most studies on giant mitochondria have been carried out on pellets of normal-sized mitochondria or those enlarged and thus do not reflect the sizes of megamitochondria *in situ*, suggesting that megamitochondria are broken into smaller ones when they are isolated [32]. Thus, information about the function of megamitochondria is limited and will remain such until a methodology is developed that permits isolation of structurally and metabolically intact pure megamitochondria.

In the present study, we have focused on urea synthesis in the liver during chronic ethanol intoxication, since an important part of the process occurs within mitochondria and the initial step of the process requires ATP. We have combined data obtained from isolated mitochondria, hepatocytes, and perfused livers in an effort to clarify the functional state of megamitochondria, and to determine how hepatocyte functions are altered by the presence of megamitochondria.

‡ Corresponding author: Takashi Wakabayashi, M.D., Department of Cell Biology and Molecular Pathology, Nagoya University School of Medicine, 65, Tsurumai-cho, Showa-ku, Nagoya 466, Japan. Tel. 052-741-2111, Ext. 2011; FAX: 052-741-2631.

MATERIALS AND METHODS

Chemicals

Nucleotides were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). HEPES was obtained from the Dojin-Kagaku Laboratory (Kumamoto, Japan). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd.

Perfusion of the liver

Perfusion of the liver was carried out essentially by the method of Iijima [33]. The basic perfusate was a KH§ solution containing 120 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.6 mM $CaCl_2$, and 25 mM $NaHCO_3$, pH 7.4. It was bubbled with 95% O_2 + 5% CO_2 at 37° for at least 2 hr prior to the experiment.

Animals were injected i.p. with pentobarbital (50 mg/kg of body weight), the abdominal cavities were opened, and livers were perfused with the KH solution via the portal vein. Then livers were removed from the abdominal cavities and were perfused with various solutions at a flow rate of 3.5 to 4.0 mL/min/g liver weight (a flow-through method).

Estimation of urea synthesis

The rate of urea synthesis by the perfused liver was measured by the method of Derr and Zieve [34]. Citrulline is synthesized within the matrix of mitochondria using various substrates: First, CP is synthesized from NH^+, CO_2 and ATP, which reaction is catalyzed by CPS. Second, citrulline is synthesized from ornithine and CP, which is catalyzed by OTC. Citrulline thus synthesized leaves the mitochondria, and urea is formed in the cytoplasm from citrulline and aspartate. Thus, the following three different substrates for urea synthesis were tested: (1) 15 mM ammonium chloride plus 4 mM ornithine; (2) 15 mM CP plus 4 mM ornithine; and (3) 15 mM citrulline. When ammonia or CP was used as a substrate for urea synthesis, the amount of urea formed was measured in the presence and absence of urease (EC 3.5.1.5) by a thiosemicarbazide-diacetylmonoxine method [35] using the Urea N-Test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). When citrulline was used, the amount of urea formed was measured by a urease-indophenol method [36] using the Urea NB-Test kit (Wako Pure Chemical Industries, Ltd.).

Isolation of hepatocytes

Isolation of rat hepatocytes was carried out by the method of Harris [37] using a collagenase perfusion procedure. The liver was perfused first with a KH (Ca^{2+}-free) solution and then was perfused further with a KH (+2.6 mM Ca^{2+}) solution containing collagenase (EC 3.4.24.3) (Millipore Corp., Freehold, NJ, U.S.A.). Next, the liver was removed and part of it was used for the isolation of hepatocytes. Hepatocytes were finally suspended in a KH (+2 mM Ca^{2+}) solution saturated with 95% O_2 + 5% CO_2. The viability of the hepatocytes was examined under a light microscope using trypan blue. Hepatocytes were intact in more than 85% of the population 2 hr after isolation.

Isolation of mitochondria and microsomes

After measuring the wet weight of the liver, it was rinsed in a medium containing 2 mM HEPES, pH 7.4, 70 mM sucrose, 220 mM mannitol, 0.1 mM EDTA, and 0.05% bovine serum albumin (fatty acid free). The mitochondrial fraction was obtained by a differential centrifugation method described previously [15]. The microsomal fraction was obtained by centrifuging the post-mitochondrial fraction at 105,000 *g* for 1 hr. Protein was determined by the procedure of Lowry *et al.* [38].

Enzyme activities

The activity of the MEOS was determined by the method of Teschke *et al.* [39]. The aldehydes bound to the semicarbazide were determined at 224 nm after an overnight diffusion period at room temperature using a Hitachi U-3200 spectrophotometer. Activities of CPS (EC 2.7.2.5) and OTC (EC 2.1.3.3) were measured by the method of Jones [40] using the mitochondrial fraction. Citrulline was determined spectrophotometrically at 466 nm by the diacetylmonoxime method [41].

Estimation of ammonia and urea in the serum and liver homogenate

The amounts of ammonia and urea in the 105,000 *g* supernatant of liver homogenate and in the serum were determined using the Ammonia-Test kit (Wako Pure Chemical Industries, Ltd.) for the former and the Urea NB-Test kit (Wako Pure Chemical Industries, Ltd.) for the latter.

Oxygen uptake by mitochondria

The coupling efficiency of mitochondria was measured using a Clark-type oxygen electrode (Beckman Co., Fullerton CA, U.S.A.) as described previously [15]. The respiration medium contained 220 mM mannitol, 70 mM sucrose, 5 mM Tris–HCl, pH 7.4, 0.1 mM EDTA (the MSTE medium) and 3 mM $MgCl_2$. The efficiency of oxidative phosphorylation by mitochondria was calculated from the oxygen trace.

ATP synthesis by mitochondria and the contents of ATP in isolated hepatocytes and liver homogenate

ATP synthesis by mitochondria was measured by the luciferin-luciferase method [42], as described previously [43]. The incubation medium (5 mL) was the MSTE medium containing 3 mM $MgCl_2$, 5 mM sodium glutamate, 5 mM sodium malate, 5 mM potassium phosphate and 0.2 mg/mL of mitochondria. The reaction was started by the addition of 500 nmol ADP to the reaction mixture at 30°. After 0, 15, 30, 45, and 60 sec, a 20-μL portion of the sample was withdrawn, and put into a test tube containing 980 μL of the cold MSTE medium. The standard concentration curve for ATP was obtained from a standard ATP solution (1×10^{-7}–2.5×10^{-6} M) using a TD-4000 lumiphotometer (Labo Science, Tokyo, Japan).

Estimation of ATP in isolated hepatocytes was carried out within 2 hr after preparation. Hepatocytes were suspended in KH (Ca^{2+}-free) medium (5 mg/mL) at 37°, and ethanol at final concentrations of 5 and 50 mM was added to the medium. After 5 min, 100 μL of the sample was reacted with 100 μL of ATP-releasing agent (Labo

§ Abbreviations: KH, Krebs–Henseleit–bicarbonate; OTC, ornithine transcarbamylase; CPS, carbamyl phosphate synthetase; CP, carbamyl phosphate; MEOS, microsomal ethanol-oxidizing system; and N-AGA, *N*-acetylglutamate.

Science), and the content of ATP was measured. To measure the content of ATP in the liver tissue, a portion of the liver (2 g) was frozen in liquid nitrogen. It was thawed to 4° just prior to the experiment. The tissue was cut into small pieces in 1 mL of a medium containing 1 M Tris–HCl, pH 7.8, 10 mM $MgCl_2$ and 0.1 mM EDTA, and then 60% perchloric acid (one-tenth portion, v/v) was added to the medium. The tissue was then homogenized with Polytron and centrifuged for 5 min at 1200 *g*. To the supernatant (3 mL), 1 mL of 3 N KOH was added, and the mixture was centrifuged for 5 min at 1200 *g*. The supernatant was diluted 100 times with 20 mM HEPES (pH 7.8), and the content of ATP in the supernatant was estimated.

Electron microscopy

Liver tissue and isolated mitochondria were prepared for electron microscopy as described previously [15]. Thin sections were cut on a Reichert ultracut N, stained with lead citrate, and examined in a Hitachi H-800 electron microscope operated at 100 kV.

RESULTS

Ultrastructural changes in the hepatocytes of rats chronically treated with ethanol

The present study reconfirmed a previous report from our laboratory [20] concerning ultrastructural changes in the mitochondria of hepatocytes of rats treated with ethanol for various lengths of time. Thus, hepatocytes of animals given ethanol for 2 months or longer were characterized by the presence of mitochondria enlarged to various degrees with poorly developed cristae (Fig. 1).

Functional changes in the hepatocytes of rats chronically treated with ethanol

Changes in MEOS activities. MEOS activity was elevated markedly in rats treated with ethanol for 1 week, and the level of the activity remained high for at least 3 months thereafter (Fig. 2).

Changes in urea synthesis. The major purpose of the present study was to examine the effects of chronic ethanol intoxication on urea synthesis in hepatocytes. When we consider the level of ammonia in the serum, both the amount of ammonia released by the degradation of proteins and the ability of the hepatocytes to synthesize urea from ammonia must be taken into account. Thus, we measured the levels of ammonia and urea in the serum and in liver tissues obtained from ethanol-treated rats (Fig. 3). The level of ammonia in the serum of ethanol-treated animals was almost the same as that of the control animals, while that of ammonia in the liver tissues was elevated in the former animals. In contrast, levels of urea in both the serum and liver tissues of ethanol-treated animals were definitely lower than those of the control animals. Thus, we examined the ability of hepatocytes to synthesize urea using perfused liver and various substrates for urea synthesis. Since urea synthesis takes place both inside and outside of mitochondria in hepatocytes, we tested ammonia, CP and citrulline as

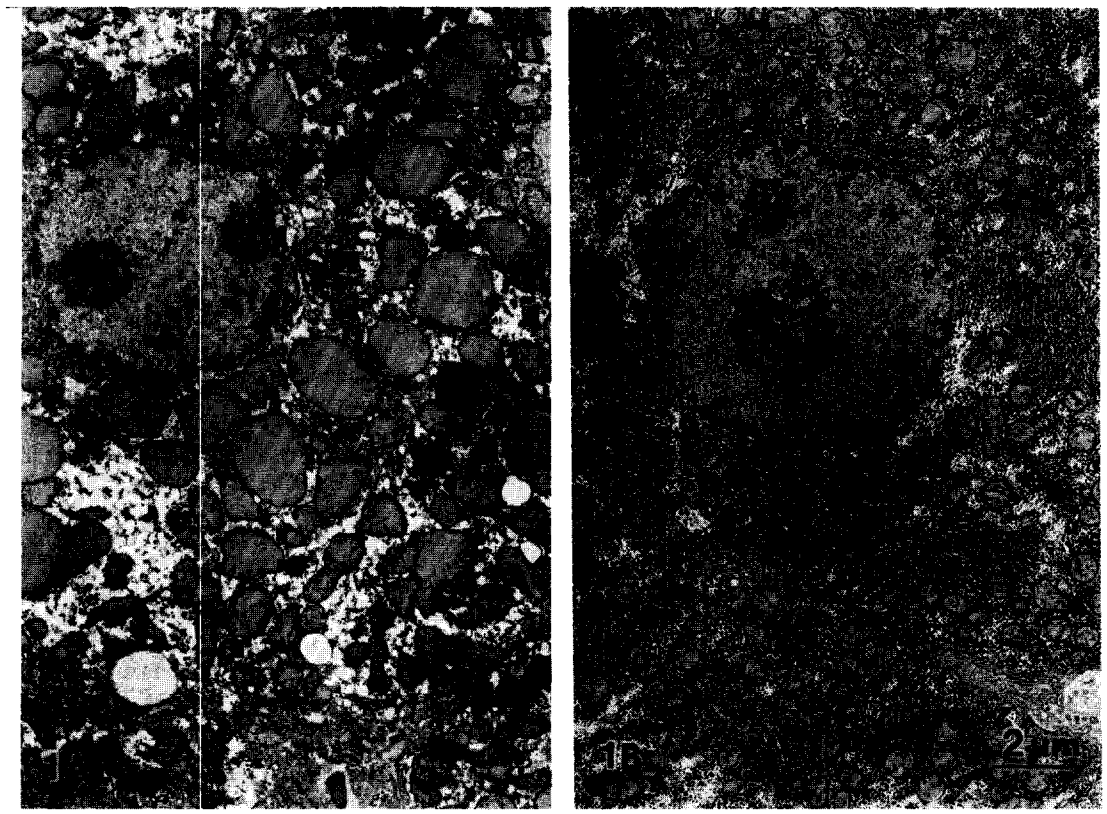

Fig. 1. Effects of ethanol on the ultrastructure of hepatic mitochondria. (A) A typical electron micrograph obtained from a rat given 32% ethanol in the drinking water for 6 months, demonstrating enlarged mitochondria with poorly developed cristae. Magnification: 7800×. (B) Paired-control. Magnification: 7800×.

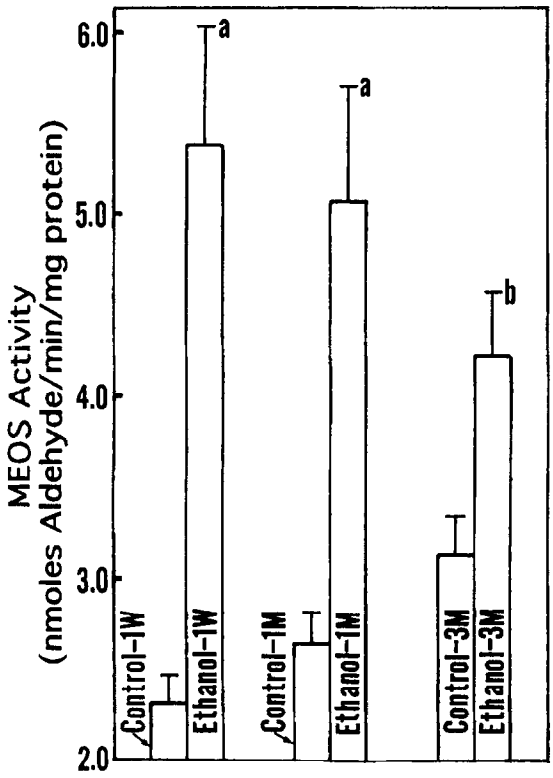

Fig. 2. Activity of the microsomal ethanol oxidizing system (MEOS) in ethanol-treated rat livers. Microsomes were obtained from the liver of control rats and those given 32% ethanol in the drinking water for 1 week (1W), 1 month (1M) or 3 months (3M). Microsomes (5 mg) were preincubated with ethanol for 10 min at 37° in the outer chamber of a 50-mL Erlenmeyer flask with a center well containing 0.6 mL of 15 mM semicarbazide in 0.1 M phosphate buffer, pH 7.4, in the inner chamber. The incubation medium (2.7 mL) in the outer chamber of the flask contained 0.1 M phosphate buffer, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 8 mM sodium isocitrate, 0.34 U/mL of isocitrate dehydrogenase and 50 mM ethanol. The reaction was started by the addition of 0.3 mL of 4 mM $NADP^+$ to the incubation mixture. The reaction was carried out for 5 min at 37°. The aldehydes bound to semicarbazide were determined spectrophotometrically at 224 nm. Values are the means ± SEM of five different experiments. Key: ([a]) statistically different from the control ($0.001 < P < 0.01$); and ([b]) statistically different from the control ($0.02 < P < 0.05$).

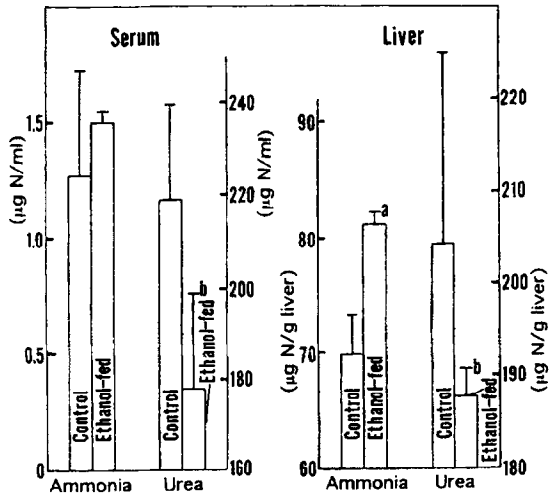

Fig. 3. Levels of ammonia and urea in the serum and liver of ethanol-treated rats. The serum and liver were obtained from rats given 32% ethanol in the drinking water for 6 months. A part of the liver (2 g) was homogenized in 40 mL of distilled water. The supernatant obtained by centrifuging the homogenate for 30 min at 105,000 *g* was used for analyses. The amounts of ammonia and urea in the serum and in the 105,000 *g* supernatant were determined using an Ammonia-Test kit and a Urea NB-Test kit, respectively. Values are the means ± SEM of five different experiments. Key: ([a]) statistically different from the control ($0.01 < P < 0.02$); and ([b]) statistically different from the control ($0.02 < P < 0.05$).

substrates to detect which steps of urea synthesis were disturbed by the ethanol intoxication (Fig. 4). The rate of urea synthesis in the perfused livers of ethanol-treated rats was decreased markedly compared with that of the control animals when ammonia was used as the substrate.

To look for the reasons for the decreased utilization of ammonia in mitochondria of ethanol-treated rat hepatocytes, we measured the activity of CPS together with that of OTC in mitochondria (Fig. 5). The activity of neither enzyme was affected by the ethanol treatment. Next, we focused on the intracellular levels of ATP since the synthesis of CP from ammonia requires ATP. The content of ATP in the liver tissues of ethanol-treated animals was found to be extremely low compared with that of the control animals (Fig. 6). These results suggested that the major reason for the lowered rate of urea synthesis from ammonia in ethanol-treated rat hepatocytes was the low levels of ATP. To determine the reasons for the lowered levels of ATP in the hepatocytes, we first examined the respiratory activity of mitochondria obtained from the livers of experimental animals (Table 1) and their ability to synthesize ATP (Fig. 7). As expected from the ultrastructural appearances of hepatic mitochondria of animals, the respiratory rates of mitochondria of ethanol-treated animals were decreased markedly when either succinate or glutamate was the oxidizable substrate. When succinate was the substrate, the rates of state 3 respiration and state 4 respiration were decreased to the same extent, resulting in the respiratory control index being unchanged. On the other hand, when glutamate was the substrate, the rate of state 3 respiration decreased significantly, whereas that of state 4 respiration had a tendency to be increased, resulting in a lowering of the respiratory control index. Thus, the coupling efficiencies of mitochondria of ethanol-intoxicated rat livers were decreased when glutamate was the oxidizable substrate.

Hepatic mitochondria isolated from control animals converted externally added ADP into ATP almost completely after 1 min of addition, whereas those from ethanol-treated rats converted only half of the externally added ADP into ATP after 1 min of addition (Fig. 7).

Second, *in vitro* effects of ethanol on ATP synthesis by mitochondria were examined. As shown in Fig. 7, preincubation of hepatic mitochondria isolated from either ethanol-treated or control animals with 50 mM ethanol caused decreases in their ability to synthesize ATP. Since mitochondria are not equipped with an ethanol-oxidizing system, these results reflect a direct effect of ethanol on mitochondrial ATP synthesis. Thus, we have

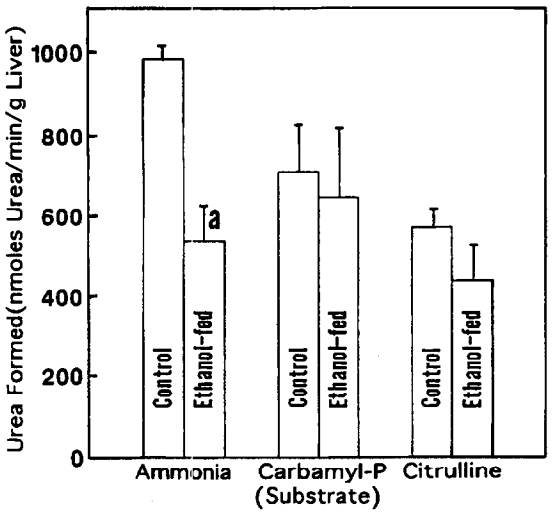

Fig. 4. Urea synthesis from ammonia, carbamyl phosphate (carbamyl-P), or citrulline in the perfused liver of ethanol-treated rats. Livers were perfused with KH medium in the presence of 15 mM ammonium chloride + 4 mM ornithine, 15 mM carbamyl-P + 4 mM ornithine, or 15 mM citrulline. Details of the experimental conditions are described in Materials and Methods. Livers were obtained from the rats given 32% ethanol in the drinking water for 6 months and from the control animals. Values are the means ± SEM of five different experiments. Key: ([a]) statistically different from the control at $P < 0.01$.

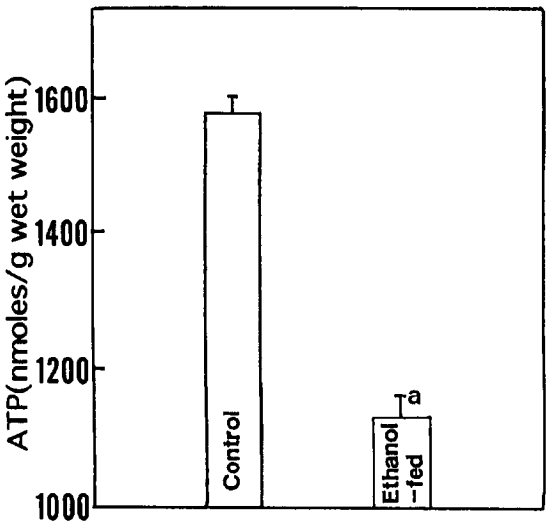

Fig. 6. Hepatic ATP levels in ethanol-treated rats. Details of experimental conditions are described in Materials and Methods. Livers were obtained from rats given 32% ethanol in the drinking water for 6 months and from the control rats. Values are the means ± SEM of five different experiments. Key: ([a]) statistically different from the control at $P < 0.001$.

also examined the *in vitro* effects of ethanol on the intracellular levels of ATP using isolated hepatocytes that were equipped with an ethanol-oxidizing system (Fig. 8). The content of ATP in isolated hepatocytes obtained from control animals was decreased as the concentration of externally added ethanol was increased, while that in isolated hepatocytes obtained from ethanol-treated animals decreased to a lesser extent (by 19%) when 5 mM ethanol was added externally, and remained at about the same level as the concentration of ethanol was increased.

Third, we studied the *in vitro* effects of ethanol on urea synthesis from ammonia using the perfused livers in an effort to clarify the relationship between urea synthesis and intracellular levels of ATP in ethanol-treated rats (Fig. 9). In the control animals, urea synthesis from ammonia in the perfused livers was decreases as the concentration of externally added ethanol was increased, while in ethanol-treated animals externally added ethanol had no effect on urea synthesis.

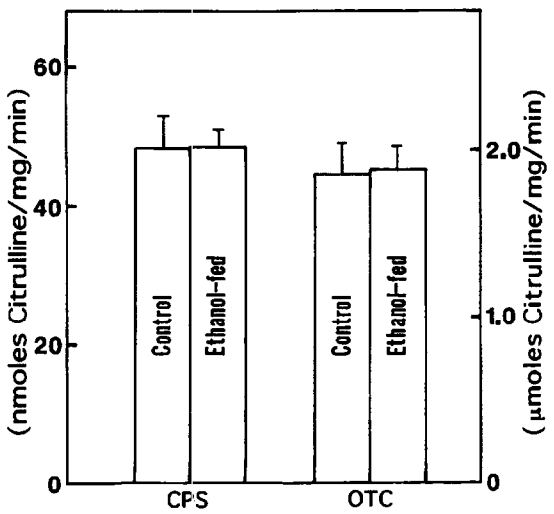

Fig. 5. Activities of carbamyl phosphate synthetase (CPS) and ornithine transcarbamylase (OTC) in ethanol-treated rat liver mitochondria. Mitochondria were isolated from the livers of rats given 32% ethanol in the drinking water for 6 months and from the livers of control rats. Value are the means ± SEM of five different experiments.

DISCUSSION

A body of data is available in the literature concerning changes in carbohydrate, lipid, protein and drug metabolism caused by chronic ethanol intoxication. On the other hand, little information has been published dealing with urea synthesis during acute and chronic ethanol intoxication. Among these studies are: *in vitro* effects of ethanol on urea synthesis by isolated normal rat hepatocytes [44, 45], and urea or citrulline synthesis by isolated hepatocytes, liver slices, or mitochondria of rats treated chronically with ethanol [46–48]. Thus, the purpose of the present study was to investigate systematically the effects of chronic ethanol intoxication on urea synthesis in perfused liver, isolated hepatocytes and liver mitochondria with the aim being to determine the role of megamitochondria in pathophysiological aspects of ethanol intoxication, since the major purpose in urea synthesis takes place within mitochondria.

Previously, Titheradge and Haynes [49] and Wanders *et al.* [50] using mitochondria and hepatocytes of normal rats reported that the intramitochondrial levels of ATP are related intimately to the levels of citrulline or urea. We paid attention to their experimental data and focused on the effects of chronic ethanol intoxication on urea and ATP syntheses in hepatic mitochondria.

Table 1. Coupling efficiencies of ethanol-treated rat liver mitochondria

Treatment	Substrate: Succinate State 3 (natom O_2/mg/min)	Succinate State 4 (natom O_2/mg/min)	Succinate Respiratory control index	Glutamate (+malate) State 3 (natom O_2/mg/min)	Glutamate (+malate) State 4 (natom O_2/mg/min)	Glutamate (+malate) Respiratory control index
Paired control	130.6 ± 4.6	30.4 ± 0.7	4.31 ± 0.23	87.9 ± 6.0	15.2 ± 0.5	5.80 ± 0.47
Ethanol 1 week	141.2 ± 1.1	34.4 ± 1.9	4.01 ± 0.12	101.6 ± 6.5	19.4 ± 1.7*	5.26 ± 0.18
Paired control	129.9 ± 2.5	31.3 ± 1.6	4.26 ± 0.23	87.2 ± 2.8	15.9 ± 0.9	5.56 ± 0.41
Ethanol 1 month	78.8 ± 3.8†	17.1 ± 0.9†	4.62 ± 0.13	66.9 ± 3.8‡	17.0 ± 0.6	3.92 ± 0.17‡
Paired control	121.0 ± 4.6	24.2 ± 1.4	5.06 ± 0.37	78.8 ± 3.5	10.8 ± 1.1	7.63 ± 0.96
Ethanol 3 months	46.1 ± 3.0†	11.1 ± 0.8†	4.19 ± 0.11	48.9 ± 2.2†	11.2 ± 0.9	4.42 ± 0.24‡

Mitochondria were isolated from the liver of rats given 32% ethanol in the drinking water for 3 months. The coupling efficiency of mitochondria was measured using a Clark-type oxygen electrode as described previously [15]. The respiration medium (a 4.5-mL system) contained 220 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, pH 7.4, 0.1 mM EDTA and 3 mM $MgCl_2$. Oxidizable substrate was 5 mM succinate or 5 mM glutamate (+2.5 mM malate). The rate of respiration was measured at 30°. Values are the means ± SEM of five different experiments.

* Statistically different from the control ($0.02 < P < 0.05$).

† Statistically different from the control ($P < 0.001$).

‡ Statistically different from the control ($0.001 < P < 0.01$).

Site of disturbances in urea synthesis in ethanol-treated rat hepatocytes

The levels of ammonia in both the serum and liver depend upon the amount of ammonia released by the degradation of proteins and the capacity of hepatocytes to synthesize urea. As was shown, the level of ammonia in the serum of ethanol-treated animals was almost the same as that of control animals, while the level of ammonia in the liver tissues of ethanol-treated rats was definitely higher than that of the control animals. On the other hand, the levels of urea in both the serum and the liver of experimental animals showed a tendency to be lower than those of untreated control animals. These data agree with those of Vemuri and Indira [51], who gave

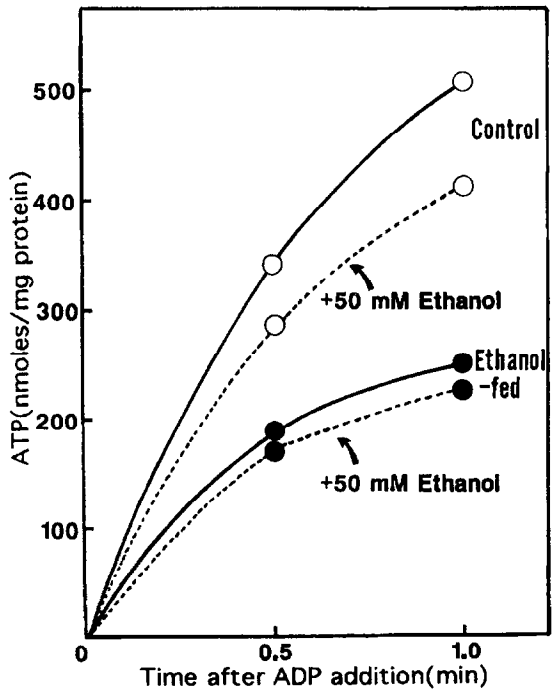

Fig. 7. ATP synthesis by hepatic mitochondria in ethanol-treated rats. Liver mitochondria obtained from rats given 32% ethanol in the drinking water for 6 months and those from the control rats were incubated in an MSTE (+3 mM $MgCl_2$) medium for 5 min at 30° in the presence (----) and absence (—) of ethanol at a concentration of 50 mM. Details of the experimental conditions are described in Materials and Methods. Data were obtained from one experiment representative of three different experiments.

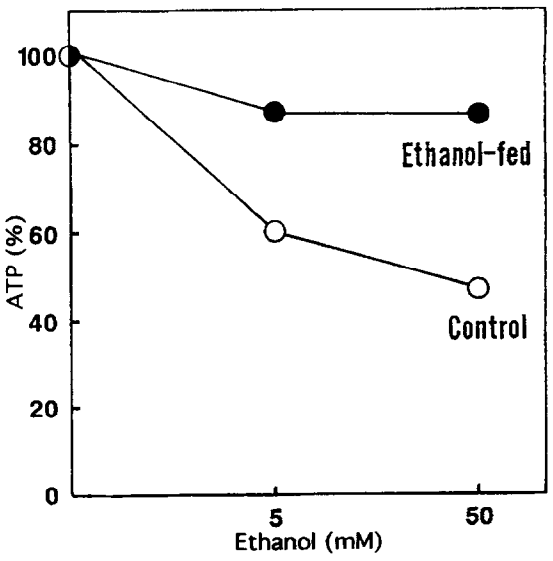

Fig. 8. *In vitro* effects of ethanol on the ATP content of hepatocytes obtained from rats given 32% ethanol in the drinking water for 6 months and those of the hepatocytes from control animals. Hepatocytes were incubated in a KH (Ca^{2+}-free) medium for 5 min at 37° in the absence and in the presence of ethanol (5 mM, 50 mM). The ratio of the ATP levels in the hepatocytes after 5 min of incubation in the presence of ethanol to those of the hepatocytes after 5 min of incubation in the absence of ethanol was expressed as ATP (%). Data were obtained from one experiment representative of three different experiments.

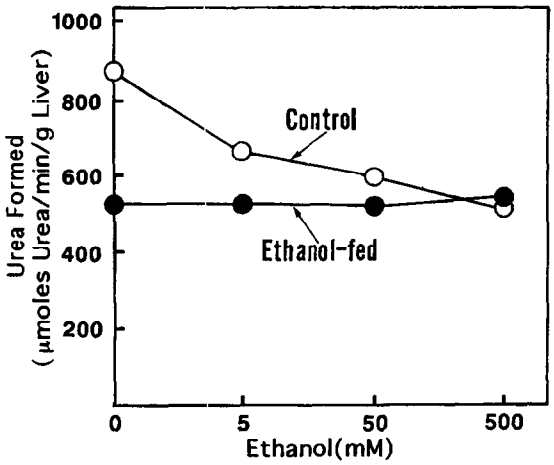

Fig. 9. *In vitro* effects of ethanol on urea synthesis from ammonia in perfused livers obtained from rats given 32% ethanol in the drinking water for 6 months and from control animals. Data were obtained from one experiment representative of three different experiments.

ethanol to rats orally at a level of 5 g/kg of body weight, daily for 5 days.

Among three different substrates for urea synthesis, we found that urea synthesis from ammonia was disturbed (Fig. 4). Factors regulating citrulline synthesis within hepatic mitochondria are the level of N-AGA as a co-factor of CPS, the ratio of ATP/ADP, and the activities of CPS and OTC. It has been reported that an increase in the intramitochondrial levels of N-AGA enhances the activity of CPS and also raises intramitochondrial ATP/ADP ratios, resulting in enhanced cirtulline synthesis [51, 52]. Increases in the level of N-AGA were reported to be induced by glucagon [53] or ammonia [54]. In contrast, decreases in the level of N-AGA were reported to be caused by propionate [55]. Hensgens *et al.* [53] have reported that the incubation of isolated hepatocytes with ammonia, ornithine, lactic acid, and oleic acid induces an 8-fold elevation of N-AGA levels within 10 min. N-AGA may play an important role in the regulation of urea synthesis concomitant with changes in the levels of ammonia. In the case of ethanol intoxication, the oxidation of ethanol causes the elevation of intracellular NADH/NAD^+ ratios, resulting in the reductive amination of α-ketoglutarate to glutamate, utilizing NADH and NH_4^+. The formation of acetyl CoA is accelerated as a result of ethanol degradation. Thus, ethanol intoxication possibly causes an increase in intracellular glutamate and acetyl CoA, resulting in enhanced production of N-AGA from glutamate and acetyl CoA. Although the level of N-AGA was not estimated in the present study, it may be reasonable to assume that the synthesis of N-AGA was enhanced in chronic ethanol intoxication.

As shown in the present study, the activity of CPS localized in the matrix space of hepatic mitochondria remained in the normal range, although chronic ethanol intoxication causes changes in various mitochondrial enzyme activities [3–5, 7, 8, 22, 48]. Therefore, we speculated that lowered levels of intracellular ATP/ADP ratios would be the major reason for decreased citrulline (urea) synthesis. The present study has shown that the ATP levels in the liver tissue of ethanol-intoxicated animals are actually extremely low. Furthermore, coupling efficiencies and the ability to synthesize ATP were diminished in mitochondria of the livers of ethanol-intoxicated animals. Concerning the utilization of ATP by hepatocytes, Israel *et al.* [46] have shown that chronic ethanol intoxication activates (Na^+ + K^+)-ATPase, resulting in the enhanced consumption of intracellular ATP. The present study showed that the levels of ATP in the hepatocytes of control animals incubated with 50 mM ethanol decreased to less than 50% of control levels, whereas those of hepatocytes obtained from ethanol-treated animals were not affected as much (Fig. 8). The difference in *in vitro* effects of ethanol on the ATP levels of hepatocytes between control and ethanol-treated animals may be explained as follows: in ethanol-treated animals, the ability of hepatocytes to oxidize ethanol was enhanced because of increased MEOS activities, and the concentration of ethanol within the hepatocytes was not sufficient to affect the ATP synthesis of mitochondria further. Also, the membranes of mitochondria obtained from ethanol-treated rats become more resistant to externally added ethanol [56].

Correlation between ATP synthesis and urea formation in ethanol-treated rat hepatocytes

Since the low levels of intracellular ATP were found to be the most probable cause of the lowered synthesis of urea from ammonia in ethanol-treated rat livers, we tried to obtain experimental data to correlate these phenomena. Externally added ethanol was shown to suppress both ATP synthesis and urea formation to exactly the same degree in control animals, whereas it had little effect on either ATP synthesis or the formation of urea in ethanol-treated animals. Titheradge and Haynes [49] and Wanders *et al.* [50] obtained similar data on the effects of externally added ethanol on ATP synthesis and on the formation of urea in normal rat liver. These findings indicate that lowered levels of ATP in control rat hepatocytes caused by externally added ethanol exist in ethanol-intoxicated rat hepatocytes *in vivo*, resulting in a decrease in the formation of urea in the latter animals, although the decrease was not significant since serum levels of ammonia stayed within normal ranges in these animals. However, these results by no means lead to the conclusion that chronic ethanol intoxication is free from hyperammonemia. We suspect that hyperammonemia may be induced easily in ethanol-intoxicated animals under conditions in which the production of ammonia is increased by enhanced proteolysis (e.g. in high protein diet, starvation, or overload of physical exercise) or by an hypoxic environment that lowers ATP synthesis because of the limited ability to synthesize urea from ammonia.

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