Acute and Chronic Ethanol Treatment in Vivo Increases Malate-Aspartate Shuttle Capacity in Perfused Rat Liver*

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The effects of acute and chronic treatment with ethanol on transport of reducing equivalents into mitochondria via the malate-aspartate shuttle were studied in perfused rat liver. The shuttle capacity was estimated from the decrease in rates of glucose production from the reduced substrate sorbitol caused by an increase in the NADH/NAD+ ratio in the cytosol due to metabolism of ethanol. The greater the capacity of the malate-aspartate shuttle, the smaller the inhibition of glucose synthesis by ethanol. Glucose synthesis was decreased about 2-fold less in livers from fasted rats treated acutely 2.5 h earlier with ethanol than in untreated controls. Chronic treatment with ethanol for 3–5 weeks prevented completely the decrease in glucose synthesis from sorbitol due to ethanol oxidation.

Rates of ethanol uptake were elevated significantly from 69 ± 7 μmol/g/h in livers from control rats up to 92 ± 7 μmol/g/h in livers from SIAM rats. Similarly, rates of ethanol uptake were stimulated by chronic ethanol treatment from 71 ± 6 to 222 ± 15 μmol/g/h; this increase was largely sensitive to aminooxyacetate. Taken together, these data indicate that flux of reducing equivalents over the malate-aspartate shuttle is increased by both acute and chronic treatment with ethanol and that movement of reducing equivalents from the cytosol into the mitochondria via the malate-aspartate shuttle is an important rate determinant in hepatic ethanol oxidation.

It is well known that the mitochondrial inner membrane is impermeable to pyridine nucleotides (1); thus, a number of hydrogen shuttle mechanisms have been suggested to account for the bidirectional transport of reducing equivalents between the cytosol and mitochondria. One of the first proposals was the malate shuttle of Borst (2). NADH produced in the cytosol from the oxidation of ethanol converts cytosolic oxalacetate into malate via malate dehydrogenase, which enters mitochondria in exchange for a-ketoglutarate (see Fig. 3). Once inside the mitochondria, malate is converted into oxalacetate, resulting in the regeneration of NADH. Subsequently, oxalacetate is converted into aspartate and moves into the cytosol in exchange for glutamate. To complete the cycle, oxalacetate is regenerated in the cytosol from aspartate by transamination.

The malate-aspartate shuttle has been difficult to study because essential intermediates are distributed in both the cytosol and mitochondria (2). Recently, a new method was developed to estimate the capacity of the malate-aspartate shuttle mechanism based on inhibition of gluconeogenesis from a reduced substrate such as sorbitol due to ethanol oxidation in the perfused liver (3). By increasing NADH in the cytosol, ethanol increases competition for the movement of reducing equivalents produced by oxidation of sorbitol by sorbitol dehydrogenase into the mitochondria via the malate-aspartate shuttle, resulting in inhibition of gluconeogenesis from sorbitol (4). Thus, the greater the capacity of the malate-aspartate shuttle, the smaller the inhibition of glucose synthesis by ethanol. For example, in livers from normal rats, ethanol inhibited glucose production from sorbitol about 50% (3); however, if rats were exposed to cold for 3–6 weeks, ethanol failed to inhibit glucose formation. From such experiments it was concluded that the capacity of the malate-aspartate shuttle was increased by cold exposure (3).

Reoxidation of NADH is believed to be a major rate-determining step in hepatic ethanol oxidation (for review, see 5). Reducing equivalents generated from the metabolism of ethanol via alcohol dehydrogenase are moved into mitochondria predominantly by the malate aspartate shuttle (6), which has led to the hypothesis that shuttle capacity is also an important rate determinant for ethanol metabolism (7). It is well established that ethanol treatment stimulates ethanol oxidation (8, 9); however, whether associated changes in the capacity of the malate-aspartate shuttle occur has not been established clearly. Therefore, the purpose of the present study was to assess the effect of acute and chronic treatment of rats with ethanol on the capacity of the malate-aspartate shuttle in the perfused rat liver where complications caused by cell isolation are avoided. The results indicate that shuttle capacity is indeed increased dramatically by ethanol.

EXPERIMENTAL PROCEDURES

Materials—Sorbitol, aminooxyacetate, alanine, lactate, and enzymes used for analyses of metabolites were from Sigma. All other chemicals were from standard sources and were of reagent grade.

Acute and Chronic Treatment with Ethanol—Female Sprague-Dawley rats (140–220 g) received laboratory chow and water ad libitum. Animals were given ethanol (5 g/kg i.g.) 2.5 h before surgery; controls were treated with saline. Some rats were fasted for 24 h before surgery as indicated in the figure legends.

For chronic treatment with ethanol, 24 rats (130–150 g, initial body weight) were divided into two groups. One group was given a chow diet and a solution of 25% sucrose ad libitum, and the second...
group received a solution of 25% sucrose containing 35% ethanol for 3–5 weeks (10).

Liver Perfusion—The nonrecirculating liver perfusion system used has been described in detail elsewhere (11). The perfusion fluid was Krebs-Henseleit bicarbonate buffer, pH 7.4, 37°C, saturated with \( \text{O}_2 : \text{CO}_2 \) (19:11). Oxygen uptake was calculated from influent minus effluent oxygen concentration differences measured with a Clark-type electrode (11), and fluorescence of pyridine nucleotides (excitation at 366 nm, emission at 450 nm) from the surface of the liver was monitored as described previously (12).

Analytical—Samples of perfusate were collected at 2–5-min intervals for analysis of glucose, lactate, pyruvate, and ethanol by standard enzymatic procedures (13). Rates were calculated from influent minus effluent concentration differences, flow rates, and liver wet weights. Statistical comparisons were performed with Student’s \( t \) test (14).

RESULTS

Effect of Acute Ethanol Treatment on Ethanol Uptake and Flux over the Malate-Aspartate Shuttle—Basal rates of oxygen uptake by livers from normal, fed rats were around 120 \( \mu \text{mol/g/h} \); rates were elevated to around 180 \( \mu \text{mol/g/h} \) in livers from rats treated 2.5 h before surgery with ethanol (SIAM) (Fig. 1, A and B). Infusion of the transaminase inhibitor aminooxyacetate (0.2 mM) decreased \( \text{O}_2 \) uptake 20–30 \( \mu \text{mol/g/h} \) in both groups (Fig. 1, A and B; Fig. 2). Concomitantly, pyridine nucleotide fluorescence detected from the surface of the liver increased transiently in livers from normal rats whereas the elevation was sustained in livers from SIAM rats (Fig. 1, A and B, top panels). When ethanol (2 mM) was infused, oxygen uptake increased about 15 \( \mu \text{mol/g/h} \) in livers from both groups (Figs. 1 and 2). Pyridine nucleotide fluorescence in livers from control rats was increased about 25% of the maximal response to anoxia produced by infusion of perfusate saturated with \( \text{N}_2 \); however, fluorescence was increased about 40% of the anoxic response in livers from SIAM rats (Fig. 1, A and B, top panels). When ethanol infusion was terminated, oxygen uptake declined, and pyridine nucleotide fluorescence diminished to basal levels in both groups of livers. After the infusion of aminooxyacetate was terminated, oxygen uptake increased to basal values whereas pyridine nucleotide fluorescence did not change.

Basal rates of lactate production of around 60 \( \mu \text{mol/g/h} \) in livers from control rats were diminished to about 25 \( \mu \text{mol/g/h} \) after acute exposure to ethanol 2.5 h before perfusion in livers from SIAM rats (Fig. 2) due to depletion of glycogen stores by ethanol (19). Upon infusion of aminooxyacetate, lactate production increased significantly to 95 \( \pm 12 \mu \text{mol/g/h} \) in control livers but was unaffected in livers from SIAM rats. This increase in livers from control rats was due most likely to elevated rates of conversion of pyruvate to lactate via lactate dehydrogenase caused by an increase in NADH redox state following infusion of aminooxyacetate. Glycolysis then increased because of the concomitant oxidation of NADH to NAD\(^+\) available for glyceraldehyde-3-phosphate dehydrogenase. When ethanol was infused subsequently, lactate production decreased dramatically in livers from control rats to about 20 \( \mu \text{mol/g/h} \) (Fig. 2). Rates were also diminished in livers from SIAM rats, but the absolute decrease was only around 10 \( \mu \text{mol/g/h} \) because glycogen stores were depleted by prior administration of ethanol (19, 20), and thus rates of glycolysis were minimal.

Flux over the malate-aspartate shuttle was evaluated by measuring the degree of inhibition of glucose production from the reduced substrate sorbitol by ethanol as described in detail elsewhere by Sugano et al. (3). Results of a typical experiment are shown in Fig. 3. The infusion of sorbitol (5 mM) increased rates of glucose production in livers from fasted rats significantly to 29 \( \pm 2 \mu \text{mol/g/h} \). The subsequent infusion of alanine (0.3 mM) increased rates of glucose formation to about 45 \( \mu \text{mol/g/h} \) by providing oxalacetate for the malate-aspartate shuttle and elevated oxygen uptake 10 \( \mu \text{mol/g/h} \) to values of around 110 \( \mu \text{mol/g/h} \). Alanine alone at concentrations up to 2.5 mM only increased rates of glucose production about 3–4
be rate limiting in livers from fasted rats even in the presence of 0.3 mM alanine, the concentration of alanine was elevated. That intermediates for the malate-aspartate shuttle could still be ascribed to its conversion directly to glucose. Ethanol (2 mM) decreased glucose production nearly completely and, after elevating oxygen uptake transiently, diminished rates about 10 μmol/g/h (Fig. 3, upper panel). When infusion of ethanol was terminated, rates of glucose production and oxygen uptake returned to basal values. Because it was possible that intermediates for the malate-aspartate shuttle could still be rate limiting in livers from fasted rats even in the presence of 0.3 mM alanine, the concentration of alanine was elevated. Upon infusion of 1.2 mM alanine, maximal rates of glucose production were still around 50 μmol/g/h (i.e. higher concentrations of alanine did not stimulate glucose synthesis), and oxygen uptake was elevated to values around 140 μmol/g/h (Fig. 3). Upon infusion of ethanol, rates of glucose production were inhibited about 45% of control values in livers from normal rats (Figs. 3 and 4). In contrast, rates of glucose formation were diminished only to about 75% of control in livers from ethanol-treated (SIAM) rats (Fig. 4, left, solid bar). Under these conditions, rates of ethanol uptake were significantly higher in livers from SIAM than control rats (92 ± 7 μmol/g/h, n = 5, p < 0.05).

Effect of Chronic Treatment with Ethanol on Malate-Aspartate Shuttle Capacity in Perfused Rat Liver—The effect of chronic treatment with ethanol on flux over the malate-aspartate shuttle was assessed by employing the experimental design depicted in Fig. 3. In the presence of 1.2 mM alanine, rates of glucose formation from sorbitol in livers from sucrose-treated rats were diminished by ethanol to about 40% of values measured in the absence of ethanol (Fig. 4, right, open bar). In contrast, infusion of ethanol inhibited glucose production by only around 4% in livers from ethanol-treated rats (Fig. 4, right, solid bar). Rates of ethanol uptake were elevated significantly from 71 ± 6 to 222 ± 15 μmol/g/h (n = 5, p < 0.01) by chronic ethanol treatment. Under these conditions, ethanol uptake was inhibited 90% by aminooxyacetate to 7 ± 2 μmol/g/h in livers from control rats and 75% (to 50 ± 4 μmol/g/h) in chronically ethanol-treated rats (n = 5, p < 0.05).

**DISCUSSION**

Acute Treatment with Ethanol Increases Malate-Aspartate Shuttle Capacity—The malate-aspartate shuttle utilizes reducing equivalents generated in the cytosol to convert oxaloacetate to malate via malate dehydrogenase (see Fig. 5). Malate is then transported into the mitochondria in exchange for α-ketoglutarate. The process is reversed in the mitochondria when malate dehydrogenase converts malate into oxaloacetate and NADH, and the latter is reoxidized to NAD⁺ with the concomitant consumption of oxygen. Carbon flow is balanced by transamination of mitochondrial oxaloacetate to aspartate, which is transported into the cytosol in exchange for glutamate (Fig. 5).

Sugano et al. (3) demonstrated that the capacity of the malate-aspartate substrate shuttle system to move reducing equivalents generated in the cytosol into mitochondria could...
The presence of the transaminase inhibitor aminooxyacetate were reduces oxalacetate (OAA) to malate (MAL), and NADH is oxidized of glutamate (GLUZ') to a-ketoglutarate, and aspartate is transported increases in NADH fluorescence caused by ethanol in the deamination of alanine to pyruvate and carboxylation of pyruvate acetate (AOA). Mitochondrial oxalacetate can also be formed by the acetate and a-ketoglutarate to glutamate with consumption of molecular oxygen. Mitochondrial oxalacetate is NADH, which is then reoxidized via the electron transport chain respectively, and reduction of NAD+ to NADH. Malate dehydrogenase then moves hydrogen from NADH to the cytosol, and in exchange for glutamate. The transamination of aspartate to oxalacetate via malate dehydrogenase, and NADH is reduced to NADH, which is then reoxidized via the electron transport chain with consumption of molecular oxygen. Mitochondrial oxalacetate is transaminated to aspartate (ASP) with the concomitant deamination of glutamate (GLUT) to a-ketoglutarate, and aspartate is transported from mitochondria into the cytosol by a Ca2+-sensitive translocator in exchange for glutamate. The transamination of aspartate to oxalacetate and a-ketoglutarate to glutamate is inhibited by aminooxyacetate (AOA). Mitochondrial oxalacetate can also be formed by the deamination of alanine to pyruvate and carboxylation of pyruvate into oxalacetate.

be assessed qualitatively by measuring the effects of ethanol on rates of glucose synthesis from a reduced substrate (i.e., sorbitol) in the presence of catalytic amounts of alanine which generate oxalacetate in the mitochondria (see Fig. 5). Infusion of ethanol increases the generation of reducing equivalents in the cytosol via alcohol dehydrogenase, which competes for movement of NADH produced by the oxidation of sorbitol with sorbitol dehydrogenase through the malate-aspartate shuttle into the mitochondria and thus inhibits glucose synthesis. However, if malate-aspartate shuttle capacity is increased, such as in livers from cold-adapted rats (3), the capacity to move hydrogen from NADH from the cytosol into mitochondria is increased, and thus inhibition of glucose synthesis from sorbitol by ethanol is diminished.

Pretreatment for 3-4 h with ethanol causes a swift increase in alcohol metabolism (SIAM) in rats (15; see “Results”), mice (16), ADH-positive deer mice (17) and humans (18). The mechanism of SIAM involves more rapid reoxidation of NADH to NAD+ in mitochondria due to relatively increased rates of ADP production as a result of decreased glycolysis (19). Since reoxidation of NADH is postulated to be rate limiting for ethanol oxidation via ADH, it is likely that malate-aspartate shuttle capacity is increased by acute ethanol treatment. Indeed, four observations support this idea. First, rates of oxygen uptake were elevated about 50% by acute ethanol treatment (Figs. 1 and 2 and Ref. 19). Second, increases in NADH fluorescence caused by ethanol in the presence of the transaminase inhibitor aminooxyacetate were about twice as great in livers from SIAM rats as in livers from controls (Figs. 1 and 2). Third, rates of export of reducing equivalents in the form of lactate were not increased by infusion of aminooxyacetate in livers from SIAM animals (Fig. 3, right panel). In contrast, lactate production was elevated significantly by inhibition of transaminases in livers from control rats, indicating increased export of reducing equivalents as lactate under these conditions. Fourth, glucose production from sorbitol was inhibited much less in livers from ethanol-treated (SIAM) rats than in controls (Fig. 4), indicating that competition for movement of reducing equivalents generated by oxidation of ethanol and sorbitol dehydrogenase into mitochondria was diminished. Taken together, these data indicate that malate aspartate shuttle capacity is increased by acute treatment with ethanol.

Movement of NADH from Cytosol into Mitochondria via the Malate-Aspartate Shuttle Is an Important Rate Determinant of Hepatic Ethanol Oxidation—It is well documented that chronic treatment with ethanol increases rates of hepatic ethanol oxidation (8, 9). In the present study, rates of ethanol uptake were elevated by chronic treatment with ethanol and were inhibited almost completely by the transaminase inhibitor, aminooxyacetate (see “Results”), consistent with other observations that ethanol elimination in vivo was decreased significantly by the inhibitor (21). These data are consistent with the hypothesis that reoxidation of NADH to NAD+ in mitochondria via the malate-aspartate shuttle is a key rate determinant in ethanol oxidation in perfused rat liver. It also indicates that flux of reducing equivalents over the malate-aspartate shuttle is increased after chronic treatment with ethanol. In support of this latter idea, glucose production from sorbitol was unaffected by infusion of ethanol in perfused livers from ethanol-treated rats (Fig. 4, right, solid bar). In contrast, rates were diminished more than 50% in livers from control rats (Fig. 4, right, open bar), indicating a greater capacity to move reducing equivalents between the cytosol and the mitochondria in livers from chronically ethanol-treated rats than in untreated controls. Moreover, rates of ethanol oxidation were more rapid in livers from chronically treated rats where flux over the malate-aspartate shuttle was greater than in livers from rats treated acutely with ethanol (Fig. 4 and “Results”), lending further support to the hypothesis that movement of reducing equivalents from cytosol into mitochondria via the malate-aspartate shuttle is a key rate determinant of hepatic ethanol metabolism.

How Does Ethanol Treatment Increase Malate-Aspartate Shuttle Capacity?—The data presented in this study clearly demonstrate that both acute and chronic treatments with ethanol elevate hepatic malate-aspartate shuttle capacity; however, the mechanism by which this occurs is not yet clear. It is well known that ethanol elevates blood levels of adrenergic hormones such as epinephrine and norepinephrine (20). In addition, norepinephrine increases malate-aspartate shuttle capacity in perfused livers (22), most likely by increasing levels of mitochondrial Ca2+ which stimulates aspartate efflux from mitochondria via the glutamate-aspartate translocator (23). The resultant elevation in cytosolic aspartate would in turn increase the capacity of the malate-aspartate shuttle by elevating levels of cytosolic oxalacetate. Also, rates of ethanol oxidation and malate-aspartate shuttle capacity are elevated in livers from cold-adapted rats due to increased circulating levels of adrenergic hormones (3). Further, increases in oxygen uptake and ethanol metabolism in perfused rat livers caused by acute treatment with ethanol are blocked by adrenalectomy (19). Taken together, these data indicate that malate-aspartate shuttle capacity is elevated by ethanol due

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**Fig. 5.** Mechanism of movement of reducing equivalents from the cytosol into mitochondria by the malate-aspartate shuttle. Sorbitol or ethanol is oxidized by cytosolic dehydrogenases with the concomitant formation of fructose or acetaldehyde, respectively, and reduction of NAD+ to NADH. Malate dehydrogenase then reduces oxalacetate (OAA) to malate (MAL), and NADH is oxidized to NAD+. Malate is transported into the mitochondria in exchange for a-ketoglutarate (aKG). Mitochondrial malate is then oxidized to oxalacetate via malate dehydrogenase, and NADH is reduced to NADH, which is then reoxidized via the electron transport chain with consumption of molecular oxygen. Mitochondrial oxalacetate is transaminated to aspartate (ASP) with the concomitant deamination of glutamate (GLUT) to a-ketoglutarate, and aspartate is transported from mitochondria into the cytosol by a Ca2+-sensitive translocator in exchange for glutamate. The transamination of aspartate to oxalacetate and a-ketoglutarate to glutamate is inhibited by aminooxyacetate (AOA). Mitochondrial oxalacetate can also be formed by the deamination of alanine to pyruvate and carboxylation of pyruvate into oxalacetate.
Ethanol Increases Malate-Aspartate Shuttle


Ethanol increases malate-aspartate shuttle to hormone-mediated increases in Ca²⁺ levels which stimulate aspartate efflux from mitochondria. Thus, the malate-aspartate shuttle is an important rate determinant in hepatic ethanol metabolism. It follows from this reasoning that nutritional and hormonal factors that affect hepatic intracellular calcium will influence ethanol metabolism via actions on the malate-aspartate shuttle.
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