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ROLE OF THE MALATE–ASPARTATE SHUTTLE IN THE METABOLISM OF ETHANOL *IN VIVO*

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Abstract—In order to assess the role of the malate–aspartate shuttle during ethanol oxidation *in vivo*, the influence of aminooxyacetate administration was investigated on both the blood ethanol elimination rate and ethanol induced alterations of the liver redox state. Aminooxyacetate reduced by 50 per cent the blood ethanol elimination rate; it promoted at the same time the reduced state of the extramitochondrial space, whereas it induced a more oxidized state in the mitochondria. The comparison between the effect of either single or repeated administration of aminooxyacetate leads to the conclusion that the degree of inhibition of aspartate aminotransferase activity and the reduction of blood ethanol elimination rate are not directly connected. These findings suggest that an increased flux through other shuttle systems concerned with transport of reducing equivalents occurs when the malate–aspartate shuttle is inhibited.

Three different processes have been considered as rate limiting factors in the oxidation of ethanol by the liver: (a) dissociation of the alcohol dehydrogenase–NADH complex [1], (b) transfer of reducing equivalents from the cytosol into the mitochondria [2] and (c) rate of the mitochondrial NADH oxidation [3].

The implication of translocation of reducing equivalents and the special role of the malate–aspartate shuttle have been emphasized by Hassinen [4], who considers this shuttle as the main pathway for the transfer in the mitochondria of reducing equivalents originating in the cytosol during ethanol oxidation. Transamination of oxaloacetate to aspartate being involved in this shuttle [5], potent transaminase inhibitors, such as aminooxyacetate [6] or cycloserine [7], have been used to test the implication of the malate–aspartate shuttle in the transport of reducing equivalents. Williamson *et al.* [8], as well as Ylikahri *et al.* [9], showed by means of these inhibitors the involvement of the malate–aspartate shuttle during ethanol oxidation in perfused rat liver, whereas Rawat and Kuriyama [10] came to the same conclusion working on isolated liver mitochondria. However, no studies had been carried out to our knowledge to define the exact role played by the malate–aspartate shuttle in the overall metabolism of ethanol.

The purpose of the present study was, therefore, to investigate *in vivo* this role in the rat by testing the influence of aminooxyacetate on the blood ethanol elimination rate and on the ethanol induced alterations in the extra- and intra-mitochondrial liver redox states.

MATERIALS AND METHODS

Female Sprague–Dawley rats weighing approx. 200 g were maintained on a stock laboratory diet (IFFA) *ad lib.* No fasting period preceded the experiments.

Aminooxyacetate (Sigma Chemical Co.) was administered intraperitoneally (0·275 m-mole/kg body wt); when mentioned, this administration was repeated three times with an interval of 45 min between each injection. Ten min after the first administration of aminooxyacetate, rats were injected intraperitoneally with ethanol (10 m-moles/kg body wt). Control rats received equivalent volumes of saline.

For the determinations of liver metabolites, animals were lightly anesthesized with diethylether 30 min after the administration of ethanol and pieces of liver were removed immediately according to Wollenberger *et al.* [11]. Liver metabolites were determined enzymatically: α-glycerophosphate and lactate were assayed according to Hohorst [12, 13], pyruvate to Bücher *et al.* [14], acetoacetate and β-hydroxybutyrate to Williamson and Mellanby [15]. Prior to the determination of aspartate according to Pfleiderer [16], we ensured that the administration of aminooxyacetate did not interfere with the assay. Results are expressed as nmoles/g liver wet wt.

The rate of ethanol oxidation was studied in other animals by following the disappearance of ethanol from the blood. Heparinized blood samples from the ophthalmic plexus were collected under light anesthesia 5, 10, 15, 30, 45, 60, 90, 120 and 180 min after ethanol administration. Blood ethanol levels were determined by gas-chromatography according to Cooper [17].

The activity of aspartate aminotransferase was determined according to Karmen [18] in liver homogenates prepared from rats injected with aminooxyacetate.

All enzymatic determinations were carried out using reagents from Sigma Chemical Co., except β-hydroxybutyrate dehydrogenase which was purchased from Boehringer.

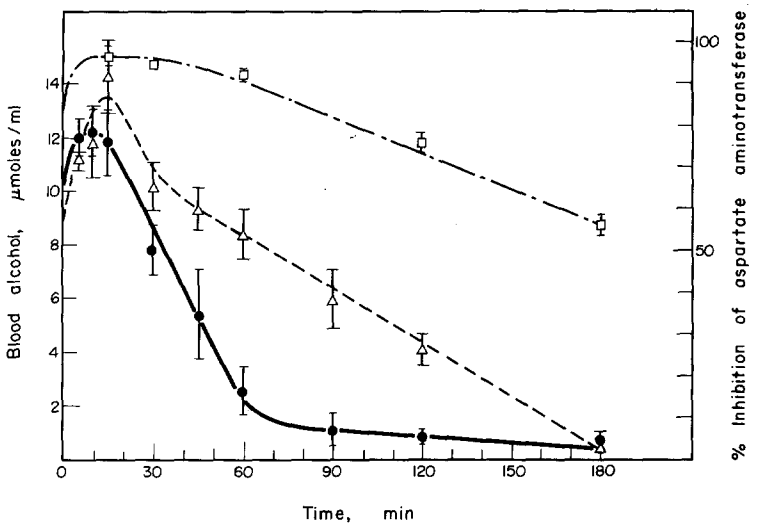

Fig. 1. Effect of a single administration of aminooxyacetate on blood ethanol and hepatic aspartate aminotransferase. Ethanol (10 m-moles/kg) was administered i.p. 10 min after i.p. injection of aminooxyacetate (0·275 m-mole/kg) or saline). (—●—●—) Blood ethanol in rats receiving no aminooxyacetate. (– – △– – △– –) Blood ethanol in aminooxyacetate treated rats. (– · □– · –□ · –) Liver aspartate aminotransferase activity in aminooxyacetate treated rats. Each point is the mean of six animals. Vertical bars represent 2 S.E.M.

Results are given as the mean ± S.E.M. Significance was determined by Student's *t*-test.

RESULTS

Effect of aminooxyacetate on the overall metabolism of ethanol. In order to test the inhibitory effect of aminooxyacetate on the malate–aspartate shuttle as well as the influence of such an inhibition on the overall metabolism of ethanol *in vivo*, we have studied the action of aminooxyacetate on both aspartate aminotransferase activity and the blood ethanol elimination rate.

When aminooxyacetate was administered in a single dose prior to ethanol (Fig. 1), the rate of fall of blood alcohol was reduced by about 50 per cent (0·56 m-mole/100 ml per hr in ethanol + aminooxyacetate treated rats instead of 1·17 m-moles/100 ml per hr in those treated with ethanol alone). The amount of alcohol eliminated from the body (calculated using Widmark's formula [19]; $b = \beta . r . p$) was respectively 3·46 and 8·00 m-moles/kg body wt per hr in animals treated with and without aminooxyacetate.

Under this condition of aminooxyacetate administration, the percentage of inhibition of liver aspartate aminotransferase activity was about 90 per cent during the first hour and decreased to 60 per cent during the 2 following hours.

Repeated administration of aminooxyacetate failed to promote the degree of inhibition induced by a single dose of aminooxyacetate on ethanol metabolism, although the activity of liver aspartate aminotransferase was almost completely inhibited during the whole experimental period (Fig. 2).

Effect of aminooxyacetate on alterations of the hepatic redox state induced by ethanol. Lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios were measured in order to assess the modifications of the extra- and intra-mitochondrial redox states induced by aminooxyacetate and/or ethanol. The α-glycerophosphate level was used as an index of activity of the α-glycerophosphate shuttle [20]. The hepatic aspartate level was also determined because of its implication in the malate–aspartate shuttle [21].

As shown in Table 1, the administration of aminooxyacetate induced in the liver a five-fold increase in the lactate/pyruvate ratio, which was mainly due to a three-fold increase in the lactate level. On the contrary, this administration produced a decrease of the β-hydroxybutyrate/acetoacetate ratio, which is related to a predominant decrease of the β-hydroxybutyrate level.

Administration of ethanol alone induced also an increase in the lactate/pyruvate ratio (3-fold), but failed to modify significantly the β-hydroxybutyrate/acetoacetate ratio.

When ethanol was administered to aminooxyacetate-treated rats, the alterations induced by aminooxyacetate alone on the extramitochondrial redox state were enhanced: a four-fold increase in lactate occurred, whereas pyruvate decreased to less than one-third of the control values, yielding a more than ten-fold increase in the lactate/pyruvate ratio. On the contrary, administration of both aminooxyacetate and ethanol induced a significant decrease in the β-hydrox-

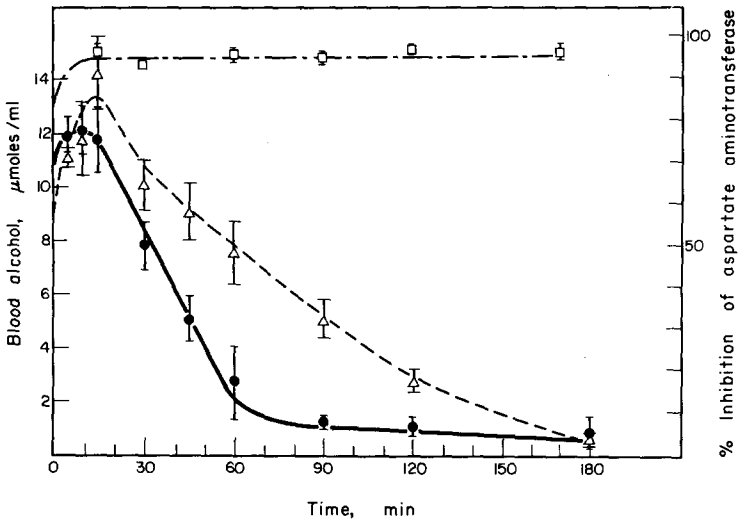

Fig. 2. Effect of repeated administration of aminooxyacetate on blood ethanol and hepatic aspartate aminotransferase. Ethanol (10 m-moles/kg) was administered i.p. 10 min after the first i.p. injection of aminooxyacetate (0·275 m-mole/kg). The same administration of aminooxyacetate was repeated three times with an interval of 45 min between each injection. Animals treated with ethanol alone received saline instead of aminooxyacetate. Standard symbols as in Fig. 1. Each point is the mean of eight animals. Vertical bars represent 2 S.E.M.

ybutyrate/acetoacetate ratio compared with the results obtained in rats treated with ethanol alone: this drop is, however, not significant when compared with the values observed in animals receiving aminooxyacetate without ethanol. Total ketone bodies were significantly reduced in this condition, as well as in rats treated with aminooxyacetate alone.

The effects of aminooxyacetate and/or ethanol on the hepatic levels of aspartate and α-glycerophosphate are shown in Table 2. The results indicate that α-glycerophosphate was enhanced to about the same extent by aminooxyacetate or ethanol administration. On the contrary, the administration of either aminooxyacetate or ethanol affected in opposite ways the aspartate level, which was strongly depressed (70 per cent) by aminooxyacetate and slightly enhanced (35 per cent) by ethanol; this last effect of ethanol on the aspartate level is in discrepancy with our previous finding in

Table 1. Effect of aminooxyacetate (AOA), ethanol or aminooxyacetate and ethanol on the hepatic lactate, pyruvate, β-hydroxybutyrate and acetoacetate levels

Hepatic levels (nmoles/g wet wt)	Treatment			
	Saline	AOA	Ethanol	AOA + Ethanol
Lactate	1047 ± 233 (9)	3109 ± 484 (9) (P < 0·001)	773 ± 225 (9) (0·05 < P < 0·1)	4167 ± 1020 (9) (P < 0·001)
Pyruvate	158 ± 26 (9)	90 ± 11 (9) (P < 0·001)	40 ± 7 (9) (P < 0·001)	52 ± 4 (9) (P < 0·001)
β-Hydroxybutyrate	129 ± 37 (8)	31 ± 13 (8) (P < 0·001)	149 ± 24 (8) (0·3 < P < 0·4)	50 ± 11 (8) (0·001 < P < 0·01)
Acetoacetate	51 ± 18 (8)	29 ± 9 (8) (0·02 < P < 0·05)	44 ± 10 (8) (0·4 < P < 0·5)	38 ± 18 (8) (0·3 < P < 0·4)
Lactate / Pyruvate	7 ± 1 (9)	35 ± 5 (9) (P < 0·001)	20 ± 7 (9) (0·001 < P < 0·01)	82 ± 21 (9) (P < 0·001)
β-Hydroxybutyrate / Acetoacetate	2·9 ± 1·0 (8)	1·2 ± 0·5 (8) (0·01 < P < 0·02)	3·7 ± 1·0 (8) (0·3 < P < 0·4)	1·7 ± 0·6 (8) (0·05 < P < 0·1)

Animals were sacrificed 40 min after the administration of aminooxyacetate and/or 30 min after the administration of ethanol.

Aminooxyacetate (0·275 m-mole/kg body wt) and ethanol (10 m-mols/kg body wt) were administered intraperitoneally.

Results are given as means ± S.E.M., with the number of determinations in parentheses. The statistical significance refers to the control saline group.

Table 2. Effect of aminooxyacetate (AOA), ethanol or aminooxyacetate and ethanol on the hepatic aspartate and α-glycerophosphate levels

Hepatic levels (nmoles/g wet wt)	Treatment			
	Saline	AOA	Ethanol	AOA + Ethanol
Aspartate	1092 ± 139 (10)	370 ± 58 (10) ($P < 0.001$)	1465 ± 70 (10) ($P < 0.001$)	272 ± 35 (7) ($P < 0.001$)
α-Glycerophosphate	166 ± 24 (7)	475 ± 153 (7) ($0.001 < P < 0.01$)	435 ± 104 (7) ($P < 0.001$)	2463 ± 615 (7) ($P < 0.001$)

Experimental conditions as in Table 1. Results are given as means ± S.E.M. with the number of determinations in parentheses.

fasted rats [22]. When both aminooxyacetate and ethanol were administered, α-glycerophosphate was considerably increased, reaching more than 14 times the control values, whereas aspartate was markedly decreased as in the case of aminooxyacetate administered alone.

DISCUSSION

The transfer of reducing equivalents from cytoplasm to mitochondria is essential in the hepatic metabolism of reduced substrates such as ethanol. It is generally considered that various shuttle mechanisms must mediate this translocation [23], since liver mitochondria are impermeable to NADH. The role of the α-glycerophosphate shuttle in hepatic metabolism has been discussed and still remains uncertain [4, 23], whereas the malate–aspartate shuttle [9] and the fatty acid elongation cycle [24, 25] are assumed to play an important role in the oxidation of cytoplasmic reducing equivalents by hepatocytes [26]. Beside these mechanisms, the extramitochondrial reduction of pyruvate into lactate contributes also to the regeneration of cytoplasmic NAD^+.

The present data, showing that ethanol induces in the liver a large increase in the lactate/pyruvate ratio as well as in the α-glycerophosphate level, confirm the well-known lowering effect of ethanol on the cytoplasmic NAD^+/NADH ratio [22, 27]. However, no significant alteration of the β-hydroxybutyrate/acetoacetate ratio occurs, indicating that the mitochondrial redox state is unchanged by ethanol under our experimental conditions. These effects suggest that shuttle systems are rate limiting factors in the transfer of reducing equivalents resulting from ethanol oxidation in the cytosol.

Among these shuttles, the malate–aspartate has been shown by several authors to be involved during the oxidation of ethanol in isolated liver mitochondria and in perfused rat liver [4, 8–10, 27]. In order to assess the contribution of the malate–aspartate shuttle *in vivo* in the transport of extramitochondrial NADH to the respiratory chain during ethanol oxidation, we have studied the effect of aminooxyacetate which inhibits the aminotransferase steps involved in this shuttle. Our results show that a single administration of aminooxyacetate not only reduces the aspartate level but also affects the cytoplasmic and the mitochondrial redox states in opposite ways. An increased lactate/pyruvate ratio indicates a more reduced state in the extra-mitochondrial compartment, whereas a decreased β-hydroxybutyrate/acetoacetate ratio shows an increased oxidation state in the mitochondria. These opposite intra- and extra-mitochondrial disturbances, proving that aminooxyacetate alters *in vivo* the transport of reducing equivalents through impairment of the malate–aspartate shuttle, support the previous report of Williamson *et al.* [8] which states that the malate–aspartate shuttle plays an important part in the intracellular transport of reducing equivalents.

This control is relevant to the problem of ethanol metabolism in the liver. The present data, showing that inhibition of the malate–aspartate shuttle by aminooxyacetate promotes the ethanol induced disturbances of the cytosolic redox state, provide evidence for the *in vivo* involvement of the malate–aspartate shuttle as a rate limiting factor in the transport of reducing equivalents resulting from ethanol oxidation in the liver.

Such a role is further confirmed by the results of Fig. 1 showing a relationship between inhibition of aspartate aminotransferase on one hand and inhibition of ethanol metabolism on the other. It is, however, noteworthy that the decrease of the blood ethanol elimination rate is not enhanced when an almost complete and lasting inhibition of this transaminase is induced by repeated administration of aminooxyacetate (Fig. 2).

The most probable explanation for this finding is that the malate–aspartate shuttle deficiency is followed by an increased contribution from other transfer systems. This could be the case for the fatty acid elongation cycle, which has been recently recognized as energically favorable for inward transport of reducing equivalents [24, 25], as well as for the α-glycerophosphate shuttle, which is considered as one of the major system for the oxidation of liver cytoplasmic reducing equivalents when the α-glycerophosphate level is increased [20]. The present observation, showing that α-glycerophosphate is increased in the liver of rats treated by aminooxyacetate + ethanol, favours the concept that the α-glycerophosphate shuttle activity is enhanced following inhibition of the malate–aspartate one. A similar substitution of increased flux through the α-glycerophosphate shuttle upon inhibition of the malate–aspartate shuttle, associated with increased tissue α-

glycerophosphate levels, has been observed in rat hearts perfused with glucose [28].

In conclusion, the present report indicates that the inhibition of the malate–aspartate shuttle reduces to a considerable extent the oxidation of ethanol *in vivo*; it suggests, furthermore, that increased contribution from other shuttle mechanisms occurs when the malate–aspartate shuttle is inhibited.

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