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Influence of the Phosphorylation State on Ethanol Oxidation and NADH Translocation in Isolated Rat Liver Cells

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The influence of the phosphorylation state on the rate of ethanol elimination, the redox state of the free NADH-NAD couple, and the transport of NADH through the mitochondrial membrane was studied in isolated liver cells from fed rats. The phosphorylation state was modified with the respiratory chain inhibitors, rotenone and antimycin A. NADH translocation through the mitochondrial membrane was inhibited with amino-oxyacetate, which is a malate-aspartate shuttle inhibitor.

As in earlier studies, ethanol was found to have no influence on the phosphorylation state. When the phosphorylation state was lowered with rotenone or antimycin A, the rate of ethanol elimination was correspondingly reduced, but when the NADH translocation between cytoplasm and mitochondria was interrupted with amino-oxyacetate, the correlation between the rate of ethanol elimination and the phosphorylation state was abolished. Amino-oxyacetate inhibited ethanol elimination by 45 %.

Rotenone and antimycin A administered together with ethanol increased the L/P ratio in the cytoplasm more than ethanol alone, suggesting that NADH is transferred out of mitochondria when the phosphorylation state is low. This transport of NADH out of mitochondria seems to proceed *via* the malate – aspartate shuttle, since amino-oxyacetate decreased the reducing effect of these respiratory chain inhibitors.

When rotenone and antimycin A are not present, NADH is transferred to mitochondria *via* the malate – aspartate shuttle and *via* a rotenone-insensitive shuttle.

When ethanol is oxidized in the liver, first to acetaldehyde in the cell cytoplasm and further to acetate in the mitochondria, 2 mol of NADH are formed from 1 mol of ethanol.[1] The NADH/NAD ratio of the cell changes to a more reduced level and in the cytoplasm this change in the redox state is linearly correlated with the rate of the alcohol dehydrogenase catalyzed reaction.[2] The NADH formed during ethanol oxidation is mainly reoxidized in the mitochondrial respiratory chain, since NADH-consuming reactions, like gluconeogenesis, are inhibited by ethanol.[3] The inner mitochondrial membrane is impermeable to NADH[4] and thus the translocation proceeds through specific shuttle mechanisms.

The main route for NADH seems to be the malate – aspartate shuttle, although other shuttles can contribute to the translocation as well.[5,6] In fasted rat liver this translocation of NADH seems to limit the rate of ethanol elimination[7,8] but in the liver of fed rat ethanol removal is limited by the rate of NADH reoxidation in the respiratory chain.[8,9]

Krebs and Veech were the first to demonstrate the interaction between the phosphorylation state, $ATP/(ADP \times HPO_4^{2-})$ and the redox state of the NADH/NAD couple in the cell cytoplasm[10] under different experimental conditions[11] and with different substrates.[12] In fasted rat the increase in the NADH/NAD ratio during ethanol elimination causes the phosphorylation state of the cell to increase as well.[12,13] In fed rat liver the situation is different; although ethanol increases the NADH/NAD ratio no changes in the phosphorylation state are observed.[2,13]

The present study was planned to further examine the connections between the phosphorylation state and the redox state of the NADH-NAD couple in fed rat liver during

ethanol oxidation. Fed rats were used since the rate of ethanol elimination is not limited by the rate of NADH translocation.[8] The free NADH/NAD ratio was changed to a more reduced level with ethanol and the phosphorylation state was modified with inhibitors of the respiratory chain. Amino-oxyacetate was used to inhibit NADH translocation through the malate-aspartate shuttle.[14]

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were used in all experiments. Rats, aged 4 – 5 months, had free access to food (Orion, Helsinki, Finland) and tap water. No fasting period preceded the experiments.

Reagents. Collagenase was purchased from Worthington Biochemical Corporation (Freehold, New Jersey, U.S.A.), rotenone, antimycin A and amino-oxyacetic acid from Sigma Chemical Company (St. Louis, Missouri, U.S.A.) and lactate dehydrogenase from Miles Seravac Ltd. (Maidenhead, Berkshire, England). The test kits for ATP, ADP/AMP were from Boehringer GmbH (Mannheim, W. Germany).

Liver cell preparation. Rats were anaesthetized with a pentobarbital (Nembutal,® 50 mg/kg, i.p.) and the liver cells were prepared as described by Seglen.[15] Cells were suspended either in Krebs-Henseleit bicarbonate buffer [16] which was 2 % in respect to bovine plasma albumin (fraction V), or in an organic biological buffer.[15] Phosphorus was omitted from the suspension medium. The viability of the cells was tested with toluidine blue and 85 – 95 % of the cells were found to be intact.

Incubation. Cells were incubated for 60 min at 37 °C with air as the gas phase. When indicated the buffers contained ethanol and inhibitors so that the final concentrations in the incubation mixture were as follows: ethanol 10 mM, amino-oxyacetate 0.5 mM, rotenone 20 μM and antimycin A 20 μM. Rotenone and antimycin A were dissolved in acetone and added to the incubation vessels as described by Grundin.[17] Incubations were stopped with perchloric acid. The protein precipitate was removed by centrifugation and the metabolites were determined from the neutralized supernatant within a few hours.

Analytical. The rate of ethanol elimination and the content of metabolites were determined by methods described previously.[9,18–21]

Units. The rate of ethanol elimination is expressed as μmol min^{-1} (g of liver cell wet $weight)^{-1}$. All the metabolites are μmol/g liver cell wet weight. The wet weight of the liver cells was estimated from the dry weight of the cell suspension, which was converted to wet weight as described by Krebs *et al.*[22]

Statistics. The paired *t*-test was used to calculate the differences between different groups. Cells from the same liver were compared. Linear regression analysis was performed on individual values.[23]

RESULTS

Ethanol elimination. Liver cells were isolated from fed rats and incubated both in Krebs-Henseleit bicarbonate buffer and in nonbicarbonate medium. The rate of ethanol elimination was significantly lower in the incubations not containing bicarbonate than in those containing it ($p < 0.05$) (Table 1). Amino-oxyacetate, which is an aspartate aminotransferase inhibitor,[14] and thus inhibits NADH translocation through the malate–aspartate shuttle, was used to test the activity of this shuttle in the translocation of NADH from the alcohol dehydrogenase catalyzed reaction. When bicarbonate was present, amino-oxyacetate inhibited the rate of ethanol elimination by 45 %, which is about the same as found *in vivo*,[6] but when the cells were suspended and incubated in nonbicarbonate medium no inhibition occurred, in agreement with the findings of Rognstad and Clark.[24]

Inhibition of NADH oxidation in the mitochondrial respiratory chain by rotenone and antimycin A markedly decreased the rate of ethanol elimination from the incubation medium (Table 1). Antimycin A appeared to be more efficient ($p < 0.001$) than rotenone. Combination of either rotenone or antimycin A with amino-oxyacetate gave results resembling the effect of amino-oxyacetate alone.

Cytoplasmic free NADH/free NAD ratio during ethanol oxidation. The cytoplasmic free NADH/free NAD ratio was measured as the lactate to pyruvate ratio which is in equilibrium with the free NADH-NAD couple in this cell compartment.[25] When rotenone or antimycin A was present the changes in the L/P ratio were significantly greater than when ethanol was present alone (Table 2). This is in accordance with the findings of Bremer and Davis,[26] who used isolated mitochondria to study NADH translocation between cytoplasm and mitochondria and reached the conclusion that when NADH reoxidation in the respiratory chain is inhibited the malate-aspartate shuttle transfers

Table 1. Rate of ethanol oxidation in isolated liver cells from fed rats. Liver cells (30–45 mg of dry weight) were incubated in bicarbonate medium for 60 min at 37 °C. The effect of bicarbonate on the ethanol oxidation rate was tested in bicarbonate-free medium. The concentrations at the beginning of the incubation were as follows: ethanol 10 mM, amino-oxyacetate (AOA) 0.5 mM, rotenone (R) 20 μM and antimycin A (A) 20 μM. The results are the mean ± S.D. of four experiments. Statistical differences were calculated with Student's *t*-test so that cells from the same liver could be compared.

Inhibitor	Ethanol/ μmol g^{-1} min^{-1}	Inhibition/ %	Ethanol + AOA/ μmol g^{-1} min^{-1}	Inhibition/ %
–	1.32 ± 0.22	–	0.75 ± 0.13 [b]	44
R	0.66 ± 0.12 [b]	50	0.78 ± 0.13 [b]	41
A	0.42 ± 0.10 [c]	68	0.61 ± 0.06 [b]	54
R + A	0.36 ± 0.06 [c]	73	0.65 + 0.09 [b]	51
Bicarbonate-free medium	0.86 ± 0.31 [a]	35	0.82 ± 0.25 [a]	38

[a] = $p<0.05$. [b] = $p<0.01$. [c] = $p<0.001$ for statistical differences from the ethanol group.

NADH out from mitochondria. The present results show the situation to be similar in whole cell preparations and even when the cytoplasmic compartment is already reduced by ethanol (Table 2). The NADH transport was further studied in incubations where the malate–aspartate shuttle was blocked with amino-oxyacetate. The rise in the L/P ratio caused by rotenone and antimycin A was partially inhibited when amino-oxyacetate was added to the incubation mixture. In incubations containing antimycin A the inhibition was statistically significant ($p<0.01$), but not in incubations containing rotenone ($p>0.05$). When the cells were incubated in bicarbonate-free medium, which is known to reduce the capacity of the malate–aspartate shuttle,[7] the effect of amino-oxyacetate on the L/P ratio was even clearer (incubation with ethanol + rotenone: L/P = 132 ± 59 and incubation with ethanol + rotenone + amino-oxyacetate: L/P = 70 ± 65; statistical difference $p<0.05$, and incubation with ethanol + antimycin A: L/P = 165 ± 86 and incubation with ethanol + antimycin A + amino-oxyacetate: L/P = 53 ± 30; statistical difference $p<0.01$).

Table 2. Lactate/pyruvate ratios in isolated rat liver cells during oxidation of ethanol. Conditions and concentrations as in Table 1. Abbreviations: AOA = amino-oxyacetate, R = rotenone, A = antimycin A. The results are the mean ± S.D. of four experiments. Statistical differences from the ethanol group are calculated with the paired *t*-test.

Inhibitor	L/P AOA not present	L/P AOA present	Statistical differences between AOA and without AOA groups
No substrate			
–	9.4 ± 2.0	46.6 ± 12.9	$p<0.01$
Ethanol as substrate			
–	53 ± 16	62 ± 24	ns
R	270 ± 96 [a]	200 ± 78 [a]	ns
A	420 ± 170 [a]	190 ± 37 [b]	$p<0.01$
R + A	370 ± 110 [b]	230 ± 24 [b]	$p<0.05$

[a] = p<0.05. [b] = $p<0.01$ for statistical differences from the ethanol group.

Phosphorylation state. The phosphorylation state of the liver cells was determined as the ratio ATP/(ADP × HPO_4^{2-}).[10] 60 % of the total P_i was assumed to be in the form of HPO_4^{2-}.[11]

As previously found *in vivo*, ethanol did not affect the cell content of ATP or the phosphorylation state of the cells.[2,13] The inhibition of NADH transport *via* the malate–aspartate shuttle was likewise without effect (Table 3). Thus the oxidation of mitochondrial substrates or the transport of reducing equivalents *via* other shuttles, as suggested by Nordmann *et al.*,[6] can replace the malate–aspartate shuttle in fed rat liver cells.

When NADH oxidation in the respiratory chain was inhibited with rotenone, only a slight

Table 3. ATP, ADP, P_i and the phosphorylation state in isolated rat liver cells during oxidation of ethanol. Conditions and concentrations as in Table 1. Abbreviations: AOA = amino-oxyacetate, R = rotenone, A = antimycin A. The phosphorylation state is calculated as described in Ref. 25. The results are the mean ± S.D. of four experiments. Statistical differences from the ethanol group are calculated with the paired *t*-test.

Inhibitor	ATP/ μmol g^{-1}	ADP/ μmol g^{-1}	P_i/ μmol g^{-1}	$\frac{ATP}{ADP \times HPO_4^{2-}}$
No substrate				
–	1.52 ± 0.33	0.54 ± 0.17	8.15 ± 1.30	630 ± 240
Ethanol as substrate				
–	1.26 ± 0.30	0.42 ± 0.09	6.67 ± 1.81	800 ± 240
AOA	1.13 ± 0.24	0.49 ± 0.14	4.24 ± 0.94 [a]	960 ± 270
R	1.02 ± 0.23	0.46 ± 0.19	5.00 ± 2.29	640 ± 340
A	0.30 ± 0.17 [b]	0.45 ± 0.10	9.26 ± 2.48 [a]	130 ± 60 [b]
R+A	0.48 ± 0.07 [b]	0.61 ± 0.20 [a]	8.87 ± 2.41 [b]	160 ± 60 [b]
AOA+R	1.02 ± 0.18	0.51 ± 0.13	5.89 ± 1.80	610 ± 170 [a]
AOA+A	0.31 ± 0.09 [b]	0.61 ± 0.10 [a]	9.89 ± 2.69	90 ± 40 [b]
AOA+R+A	0.52 ± 0.09 [b]	0.68 ± 0.13 [b]	8.18 ± 2.12	160 ± 40 [b]

[a] $= p < 0.05$. [b] $= p < 0.01$ for statistical differences from the ethanol group.

and insignificant decrease in the cell content of ATP and in the phosphorylation state was found (Table 3), suggesting that the transport of NADH proceeds *via* rotenone-insensitive shuttles like the α-glycerophosphate shuttle. Antimycin A strongly decreased the ATP content and lowered the phosphorylation state.

Correlations between the phosphorylation state, redox state and the elimination rate of ethanol. A significant negative linear correlation was found between the cytoplasmic phosphorylation state and the L/P ratio during ethanol elimination in the presence of inhibitors of the respiratory chain (Table 4). Inhibition of the NADH translocation through the malate – aspartate shuttle did not affect this correlation. The phosphorylation state and the rate of ethanol elimination were positively correlated (Table 4), indicating the energy dependence of the NADH translocation between cytoplasm and mitochondria.[27,28] As found previously,[29,30] the L/P ratio and the rate of ethanol elimination were negatively correlated. Amino-oxyacetate abolished the latter two correlations.

Table 4. Correlation coefficients between phosphorylation state, lactate/pyruvate ratio, and the elimination rate of ethanol. The correlations between the phosphorylation state, the lactate/pyruvate ratio, and the rate of ethanol elimination were calculated with linear regression analysis from the results shown in the Tables 1, 2, and 3. Results of individual experiments from all incubations which were done in bicarbonate buffer were used in the calculation ($nf = 14$).

	Without amino-oxyacetate		With amino-oxyacetate	
	r	*p*	*r*	*p*
Phosphorylation state – L/P	−0.545	0.05	−0.687	0.01
Phosphorylation state – ethanol elimination rate	0.657	0.01	0.287	ns
L/P – ethanol elimination rate	−0.761	0.001	−0.080	ns

DISCUSSION

Interaction between the redox state and the phosphorylation state. Previous studies concerning the interaction between the redox state of the NADH – NAD couple and the phosphorylation state, which has been shown to be mediated *via* the glyceraldehyde-3-phosphate – 3-phosphoglycerate redox couple, have shown that different types of diets can induce parallel changes in these two ratios.[11] Also, when the NADH/NAD ratio is changed to a more reduced level, *e.g.* with ethanol, a simultaneous change is observed in the phosphorylation state in fasted rats[12,13] but not in fed ones.[2,13] It has therefore been concluded that the change in

the NADH/NAD ratio does not to any great degree affect the phosphorylation state, and thus the phosphorylation state plays the more dominant role in this interaction. Accordingly, changes in the phosphorylation state should be reflected in the redox state. This was actually found to be the case during oxidation of ethanol in the present study, when the phosphorylation state was modified with rotenone or antimycin A. Similar results were obtained by Berger and Hommes,[31] who studied oxidation of pyruvate in fetal rat liver. They showed, like Veech *et al.*,[11–13] that the glyceraldehyde-3-phosphate – 3-phosphoglycerate redox couple is also reflected in the lowered redox state. Even though the glyceraldehyde-3-phosphate – 3-phosphoglycerate couple was not measured in the present study, it can be suggested that also during ethanol oxidation this couple transfers the effects of the phosphorylation state to the L/P ratio.

Redox state, phosphorylation state and the rate of ethanol elimination. Investigation of the correlation between the L/P ratio and the rate of ethanol elimination has produced two kinds of results. First, if the rate of ethanol elimination is inhibited with alcohol dehydrogenase inhibitors, such as pyrazole,[2] the ethanol-induced change in the L/P ratio is also decreased. Thus a positive correlation between the rate of ethanol elimination and the L/P ratio has been suggested to reflect the velocity of the alcohol dehydrogenase catalyzed reaction.[2] On the other hand, in fed and fasted rats,[29] differently treated rats,[30] and cells incubated with respiratory chain inhibitors (Table 4), the L/P ratio changes more when the elimination rate is low, *i.e.* the correlation is negative. This negative correlation seems to indicate that ethanol elimination is limited by the availability of NAD in the cytoplasm.

Inhibition of NADH reoxidation and thus lowering of the phosphorylation state also inhibits the rate of ethanol elimination. From these results it is not possible to conclude whether the decrement in the elimination rate is solely due to the lack of NAD or whether the inhibition of NADH translocation to mitochondria, which is known to be an energy-dependent reaction, is also involved.[27,28]

Effect of inhibitors on NADH translocation. The direction of NADH flow across the mitochondrial membrane depends strongly on the metabolic state of the liver. During conditions like starvation, when gluconeogenesis is strong, NADH is transferred from mitochondria to cytoplasm [3,32] and in conditions when large amounts of NADH are formed in cytoplasm, as during ethanol oxidation, the direction is from cytoplasm to mitochondria. Of the mechanisms that transfer NADH through the mitochondrial membrane, only the malate – aspartate shuttle can transfer NADH both into and out of mitochondria.[24] Accordingly it has been suggested to have a role in the formation and maintaining of the redox difference between mitochondria and cytoplasm.[26,33] In their studies with isolated mitochondria, Bremer and Davis [26] found that NADH was transferred out of mitochondria only when the respiratory chain was inhibited by rotenone. Similarly in the present study the cytoplasmic compartment was found to be more reduced when rotenone or antimycin A was present than when ethanol was present alone (Table 2). Thus when the phosphorylation state is lowered NADH is transferred out of mitochondria even though in the cytoplasmic compartment there is an oversupply of NADH because of ethanol oxidation. The involvement of the malate – aspartate shuttle in this reduction was directly demonstrated with amino-oxyacetate, which inhibits the malate – aspartate shuttle [14] and could modify the changes in the L/P ratio. Apparently the malate – aspartate shuttle has an important role in the regulation of the cytoplasmic redox state and it also forms a site of regulation for oxidation of reduced substrates in the liver cytoplasm.

Since rotenone appeared to be a less efficient inhibitor than antimycin A, it can be suggested that part of the NADH formed in the cytoplasm is translocated to the mitochondria for oxidation *via* a rotenone-insensitive pathway, like the α-glycerophosphate shuttle. Recently Nordmann *et al.*[6] reported that when the malate – aspartate shuttle is inhibited, other transport mechanisms take care of the transport. In favour of this suggestion it was found that the inhibition of the malate – aspartate shuttle slightly decreased the inhibitory action of rotenone (Table 1), even though the change was small and statistically insignificant.

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