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MITOCHONDRIAL RESPIRATORY ACTIVITY AND SUPEROXIDE RADICAL GENERATION IN THE LIVER, BRAIN AND HEART AFTER CHRONIC ETHANOL INTAKE

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Abstract—Functional characteristics of mitochondria isolated from liver, brain and heart were studied in ethanol-fed rats using ethanol administration in drinking water as a model of moderate alcohol intoxication. Our results show a slight decrease in liver cytochrome aa_3 content, the mitochondrial alteration which is most consistently observed during chronic ethanol feeding. In liver and heart mitochondria, ethanol consumption led to an increase in state 3 respiration with NAD^+-linked substrates, whereas no changes were apparent in respiration rates with succinate as substrate. However a decrease was found in state 3 respiration with succinate in brain mitochondria isolated from ethanol-fed rats. Submitochondrial particles (SMP) were used to study the superoxide radical ($O_2^{\cdot-}$) production at the level of antimycin-inhibited regions of the respiratory chain. It appears that there is no clear correlation between ethanol effects on respiration and $O_2^{\cdot-}$ production. Whereas $O_2^{\cdot-}$ generation remained unchanged in heart mitochondria, an elevation of $O_2^{\cdot-}$ generation was observed in brain mitochondria, and in contrast, the rate of $O_2^{\cdot-}$ production was decreased in liver mitochondria of the ethanol-group in comparison to the control-group. Our findings support a tissue specificity for the toxic effects of ethanol towards the mitochondria and indicate that mitochondrial free radical mechanisms are involved in ethanol-induced toxicity in the brain.

Key words: ethanol; mitochondria; superoxide; liver; brain; heart

Growing evidence suggests that free radical mechanisms contribute to ethanol-induced liver injury [1]. An increased generation of partially reduced oxygen species such as superoxide ($O_2^{\cdot-}$) and H_2O_2 has been observed in liver microsomes from ethanol-treated rats [2–4], especially through the intervention of the ethanol-inducible cytochrome P450 (CYP2E1) [3, 4]. Free radical mechanisms also appear to be implicated in the toxicity of various extrahepatic tissues [5].

Mitochondria have been recognized as a major physiological source of reactive oxygen species which arise as a consequence of oxygen reduction [6]. The presence of several electron carriers and polyunsatured fatty acid rich membranes make this organelle highly susceptible to free radical attack leading to alterations of structural integrity and functions of mitochondria. Liver mitochondrial functions appear to be an early target for ethanol toxicity, whereas brain and cardiac mitochondria are much less susceptible to perturbations associated with chronic ethanol consumption [7, 8].

The aim of this study is to investigate comparative effects of chronic ethanol administration on mitochondrial respiratory activity and mitochondrial superoxide formation in liver, brain and heart of rats. This study is undertaken using ethanol administration in drinking water (10% v/v) [9] and therefore permits examination of the influence of moderate consumption of ethanol on rat mitochondrial functions.

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MATERIALS AND METHODS

Experimental animals. Male Sprague–Dawley rats weighing approximately 100 g at the start of the experiment were maintained on a standard laboratory diet (Iffa-Credo, U.A.R., France) containing 64% of energy as carbohydrate, 11% as lipid and 25% as protein. The vitamin E content of the diet was 150 mg/kg. Control rats had tap water as drinking fluid, whereas experimental animals received, as sole drinking fluid, an aqueous ethanol solution (10% v/v) for 4 weeks. The ethanol intake of these animals amounted to 7–9 g/kg body weight/day, representing about 18% of total energy intake. The body weight gains were similar in the control and experimental groups.

At the end of the experimental period, the rats were fasted for 16 hr. However, access to the ethanol drinking fluid was maintained to prevent any possible withdrawal stress. The animals were killed by decapitation, and the livers, brains and hearts were immediately removed and washed in ice-cold 0.15 M NaCl.

Mitochondrial preparations and respiratory studies. Rat liver mitochondria were prepared according to Beattie [10]. The rate of mitochondrial respiration was measured with a Gilson oxygraph at 25° using

Table 1. Effect of chronic ethanol administration on respiratory activity of rat liver mitochondria

Respiration state	Control (N = 12)	Ethanol-fed (N = 12)	% Change	P
Succinate (6 mM)				
State 4	10.72 ± 0.62	10.31 ± 0.90	− 4	NS
State 3	46.26 ± 2.70	47.08 ± 2.17	+ 2	NS
RCR	4.31 ± 0.32	4.58 ± 0.20	+ 6	NS
ADP/O	1.84 ± 0.04	1.77 ± 0.07	− 4	NS
β-Hydroxybutyrate (14 mM)				
State 4	4.91 ± 0.43	5.10 ± 0.39	+ 4	NS
State 3	16.74 ± 0.43	19.10 ± 1.24	+14	$P < 0.025$
RCR	3.42 ± 1.11	3.77 ± 0.48	+ 9	NS
ADP/O	2.85 ± 0.11	2.84 ± 0.09	− 1	NS
Glutamate/malate (2.25 mM/0.25 mM)				
State 4	5.16 ± 1.31	4.78 ± 0.58	− 8	NS
State 3	30.29 ± 1.46	34.03 ± 1.57	+12	$P < 0.005$
RCR	6.34 ± 1.06	7.39 ± 1.08	+16	NS
ADP/O	2.92 ± 0.12	2.90 ± 0.08	− 1	NS

Results, except RCR and ADP/O, are expressed as nmol oxygen consumed per min per mg protein. Values are means ± SEM. NS, not significantly different.

a Clark oxygen electrode. The reaction medium (pH 7.4) consisted of 58 mM KCl, 25 mM NaCl, 6 mM $MgCl_2$, 13 mM K_2HPO_4, 3 mM KH_2PO_4 and mitochondria equivalent to 2.5–3.0 mg protein (final volume 1.6 mL) according to Chance and Williams [11]. Final concentrations of added respiratory substrates were: sodium succinate 6 mM, sodium β-hydroxybutyrate 14 mM, or sodium glutamate 2.25 mM + sodium L-malate 0.25 mM. Respiration was initiated by the addition of the substrates (state 4), after which 150 μM ADP was added to induce an immediate increase in the rate of oxygen utilization (state 3 as defined by Chance and Williams [11]). This state was maintained until the added ADP was phosphorylated. At this time, the rate of oxygen consumption decreased (state 4′). State 3, 4 and 4′ activities were determined by calculating the oxygen consumed per minute and per mg of mitochondrial protein. The respiratory control ratio (RCR) was determined by dividing state 3 activity by that of state 4′. The ADP/O ratio was calculated by dividing nmol of ADP phosphorylated by ng-atoms of O consumed during each interval at state 3.

Rat brain mitochondria were prepared according to Braughler *et al.* [12] without Ficoll treatment. The reaction medium (pH 7.4) consisted of 160 mM KCl, 0.25 mM EGTA, 10 mM KH_2PO_4, and mitochondria equivalent to 1–2 mg protein (final volume 1.6 mL) as described in Braughler *et al.* [12]. Final concentrations of added respiratory substrates were: sodium succinate 18 mM, sodium pyruvate 1 mM + sodium L-malate 2.5 mM, or sodium glutamate 10 mM + sodium L-malate 10 mM. State 3 was initiated by the addition of 190 μM ADP containing 1 mM $MgCl_2$. State 3 and 4 activities, RCR and ADP/O were determined as described above.

Rat heart mitochondria were prepared according to Mela and Seitz [13]. The reaction medium (pH 7.4) consisted of 250 mM sucrose, 10 mM KCl, 10 mM KH_2PO_4, 15 mM Tris and mitochondria equivalent to 1–2 mg (final volume 1.6 mL) as described in Williams and Li [14]. Final concentrations of added respiratory substrates were: sodium succinate 12 mM, sodium β-hydroxybutyrate 12 mM, or sodium glutamate 6 mM + sodium L-malate 6 mM. State 3 was initiated by the addition of 150 μM ADP. State 3 and 4 activities, RCR and ADP/O were determined as described above.

Cytochrome contents. Freshly thawed mitochondrial suspensions were diluted with an equal volume of 4% Triton X-100 and 0.4 M phosphate buffer (pH 7) to a final concentration of approximatively 5 mg/mL. After centrifugation at 5000 *g* for 15 min, the supernatant fraction was used for determination of cytochrome contents as described in Vanneste [15].

Superoxide production. Superoxide production was measured in SOD* free submitochondrial particles (SMP) prepared as described by Sottocasa *et al.* [16] from isolated mitochondria. To quantify mitochondrial superoxide generation, the inhibitory effect of SOD on the superoxide-induced oxidation of epinephrine to adrenochrome was studied in the presence of succinate and antimycin A [17].

Expression of results. Protein concentration was determined by the method of Lowry *et al.* [18]. Results were expressed as means ±SEM. Values obtained in ethanol-fed and control groups were compared by Student's *t*-test.

RESULTS

Effects of chronic ethanol treatment on liver mitochondria

Whereas determination of hepatic mitochondrial respiration with succinate as substrate indicates no

* Abbreviations: CoQ, coenzyme Q; RCR, respiratory control ratio; SMP, submitochondrial particles; SOD, superoxide dismutase.

Table 2. Cytochromes in liver mitochondria from alcohol and control rats

	Cytochrome *b*	Cytochromes ($c + c_1$)	Cytochrome aa_3
Control	0.112 ± 0.06	0.321 ± 0.013	0.166 ± 0.010
Ethanol-fed	0.106 ± 0.06	0.326 ± 0.016	0.144 ± 0.010
P	NS	NS	$P < 0.005$

Results are expressed as nmol per mg of protein. Values are means ± SEM with 24 animals in each group. NS, not significantly different

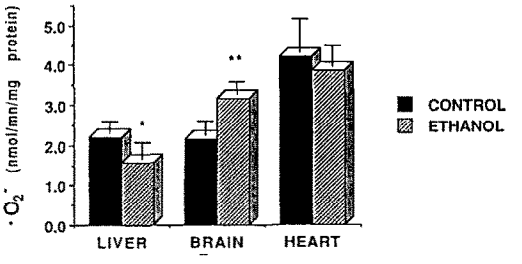

Fig. 1. Effect of chronic ethanol administration on superoxide generation from submitochondrial particles. The results are expressed as means ± SEM with nine animals in each group. *P <0.05; **P <0.01, versus control value.

differences in state 3 or state 4, a significant increase in O_2 uptake in state 3 appears in ethanol-treated rats, as compared to the control ones, when β-hydroxybutyrate or glutamate/malate is used as substrate (+14% or +12%, respectively) (Table 1). However, the efficiency of coupling between oxidation and phosphorylation activities, as shown through measurement of the RCR, is not affected nor is the capacity for oxidative phosphorylation (ADP/O ratio) (Table 1).

The cytochrome contents of the mitochondrial preparations are shown in Table 2. Liver mitochondria lose almost 14% of their cytochrome aa_3 after chronic ethanol feeding. In contrast the content of cytochromes *b* and ($c + c_1$) is not affected.

The O_2^- production in SMP at the level of the antimycin-inhibited region of the respiratory chain is significantly decreased, by 30%, in the ethanol-treated group in comparison to the control group (Fig. 1).

Effects of chronic ethanol treatment on brain mitochondria

When succinate is employed as respiratory substrate, only state 3 is decreased significantly (−16%) in brain mitochondria from ethanol-fed rats (Table 3). In contrast, rates of oxidation in state 3 and state 4 with pyruvate/malate or glutamate/malate as substrate, are similar in mitochondria from ethanol-fed and control rats (Table 3). Accordingly, neither RCR nor ADP/O are modified.

The O_2^- production in SMP is significantly enhanced (+48%) in the ethanol group (Fig. 1).

Effects of chronic ethanol treatment on heart mitochondria

As shown in Table 4, state 3 respiration with NAD^+-linked substrates is enhanced significantly (+34% with β-hydroxybutyrate or +23% with glutamate/malate) in heart mitochondria isolated from ethanol-fed rats, although no changes are seen in RCR or ADP/O ratio. In contrast, ethanol consumption did not modify mitochondrial respiration, degree of coupling (RCR) or oxidative phosphorylation (ADP/O) with succinate as substrate.

The O_2^- production by SMP of the ethanol group was unchanged (Fig. 1).

DISCUSSION

One of the earliest manifestations of the effects of ethanol consumption on the liver is a change in the structure of the liver mitochondria. Also, chronic ethanol consumption (36% calories as ethanol) results in a generalized depression in hepatic mitochondrial energy metabolism (see Ref. 19 for summaries). Moderate chronic ethanol consumption in the drinking water also produces liver mitochondrial dysfunctions [20, 21]. The ethanol-elicited alteration observed most consistently in liver mitochondria after a one month feeding period, is a decrease in cytochrome aa_3 content. Our results also show such an effect, although somewhat less in magnitude, when compared with previous reports [8, 20–25]. However, this decrease in cytochrome aa_3 content did not result in any changes in oxygen consumption. The same effect has been observed previously by Adachi *et al.* [25] at the beginning of chronic ethanol consumption in drinking water (32% v/v), whereas ultrastructural changes of hepatic mitochondria associated with a decrease in cytochrome oxidase activity, as well as in oxygen consumption, appeared later. The lack of decreased respiratory rate observed here in the presence of NAD^+-linked substrates or succinate can be related to the composition of the diet. It was observed by Wahid *et al.* [26] that liver mitochondrial function was altered by chronic ethanol ingestion only when the diet contained a high fat and adequate protein content (41% fat and 19% protein) and not when the diet had a low fat and high protein content (10% fat and 28% protein). An increased respiratory rate with β-hydroxybutyrate as substrate was observed after long-term alcohol feeding in drinking water in male Sprague–Dawley rats but not in male Wistar

Table 3. Effect of chronic ethanol administration on respiratory activity of rat brain mitochondria

Respiration state	Control (N = 12)	Ethanol-fed (N = 12)	% Change	P
Succinate (18 mM)				
State 4	22.78 ± 2.39	21.27 ± 1.69	− 7	NS
State 3	62.14 ± 5.12	52.66 ± 2.96	−16	P < 0.005
RCR	2.75 ± 0.21	2.50 ± 0.22	+10	NS
ADP/O	1.92 ± 0.14	1.92 ± 0.10	—	
Pyruvate/malate (1 mM/2.5 mM)				
State 4	16.15 ± 2.32	14.65 ± 1.67	−10	NS
State 3	60.82 ± 3.50	59.63 ± 4.13	− 2	NS
RCR	3.99 ± 0.67	4.25 ± 0.68	+ 6	NS
ADP/O	2.79 ± 0.07	2.75 ± 0.08	− 2	NS
Glutamate/malate (10 mM/10 mM)				
State 4	14.80 ± 1.85	15.24 ± 1.93	+ 3	NS
State 3	44.61 ± 3.34	44.49 ± 1.58	− 1	NS
RCR	3.03 ± 0.21	2.97 ± 0.40	− 2	NS
ADP/O	2.83 ± 0.11	2.78 ± 0.10	− 2	NS

Results, except RCR and ADP/O, are expressed as nmol oxygen consumed per min per mg protein. Values are means ± SEM. NS, not significantly different.

Table 4. Effect of chronic ethanol administration on respiratory activity of rat heart mitochondria

Respiration state	Control (N = 12)	Ethanol-fed (N = 12)	% Change	P
Succinate (12 mM)				
State 4	42.75 ± 2.54	43.33 ± 3.00	+ 1	NS
State 3	112.58 ± 9.32	116.35 ± 5.10	+ 3	NS
RCR	2.60 ± 0.16	2.70 ± 0.18	+ 4	NS
ADP/O	1.60 ± 0.09	1.62 ± 0.05	+ 1	NS
β-Hydroxybutyrate (12 mM)				
State 4	11.26 ± 1.05	12.39 ± 1.14	+10	NS
State 3	30.16 ± 1.85	40.50 ± 6.74	+34	P < 0.01
RCR	2.90 ± 0.40	3.30 ± 0.30	+13	NS
ADP/O	2.88 ± 0.26	2.64 ± 0.07	− 8	NS
Glutamate/malate (6 mM/6 mM)				
State 4	16.66 ± 1.42	18.79 ± 2.18	+12	NS
State 3	56.53 ± 3.50	69.74 ± 9.29	+23	P < 0.01
RCR	3.41 ± 0.25	3.70 ± 0.20	+ 8	NS
ADP/O	2.51 ± 0.26	2.45 ± 0.20	− 2	NS

Results, except RCR and ADP/O, are expressed as nmol oxygen consumed per min per mg protein. Values are means ± SEM. NS, not significantly different.

rats [27]. It appears that mitochondrial functions are not only dependent on the composition of the diet, but also on the strain of rat and this can explain discrepancies observed in other studies using moderate ethanol consumption [20, 21]. One can suggest that the increase in respiratory rate at state 3 with NAD^+-linked substrates observed here represents an adaptive response to ethanol exposure linked to a raising of reduced pyridine nucleotides induced by ethanol metabolism in the liver. These reducing equivalents produced in the cytosol by alcohol dehydrogenase (ADH) are moved into mitochondria via shuttles. The increased flux of reducing equivalents observed after chronic ethanol treatment [28] could induce NAD^+-linked dehydrogenases and consequently a greater consumption of oxygen for oxidizing NADH via the mitochondrial electron transfer chain. Indeed, Jenkins and Peters [29] observed that liver mitochondria of alcoholics without cirrhosis show increased levels in the enzymes associated with the mitochondrial matrix, such as glutamate dehydrogenase and malate dehydrogenase, whereas enzymes associated with the mitochondrial membranes show normal levels, in particular succinate dehydrogenase.

In the myocardium, the increase in state 3 respiratory rate with NAD^+-linked substrates could not be related to ethanol metabolism since ADH was very low. However, it was clearly demonstrated that the redox state of mitochondrial NADH serves as a regulatory site for oxidative phosphorylation [30, 31]. Denton and McCormack [32, 33] proposed

that hormonal activation of the heart stimulated respiration by increasing the NADH redox state secondary to Ca^{2+} activation of mitochondrial dehydrogenases. As ethanol elevates blood levels of adrenergic hormones such as epinephrine and norepinephrine [34], hormonal stimulation of NAD^+-linked dehydrogenases could explain the increase in state 3 respiratory rate.

In the brain, such a mechanism cannot be invoked in respiratory regulation, as an enhanced β-adrenergic sensitivity to catecholamines during chronic ethanol feeding has been reported specifically in the myocardium [35]. Furthermore, in contrast to the heart, chronic ethanol feeding does not increase calcium uptake of brain mitochondria and synaptosomes [36, 37]. While changes are not evident in pyruvate/malate-induced or glutamate/malate-induced respiration in the brain, a reduction of the succinate-induced respiration appears. This effect is probably linked to an enhanced $O_2^{\cdot-}$ production in brain SMP observed here after chronic ethanol treatment. Superoxide generated *in vitro* inhibits mitochondrial respiration [12, 38, 39]. Furthermore, it appears that the membrane-bound enzymes which are closer to the radical generating system are more sensitive to free radical attack [38]. The differential response of energy-linked respiration by brain mitochondria during chronic ethanol intake may be related to the facts that (i) the NAD^+-linked dehydrogenases (such as pyruvate dehydrogenase, glutamate dehydrogenase or malate dehydrogenase) are soluble enzymes found in the matrix of mitochondria and distant from the membranous radical generating system and (ii) succinate dehydrogenase is located in the inner membrane of mitochondria like the respiratory complexes and is near the membranous radical generating system of mitochondria.

Our results concerning $O_2^{\cdot-}$ generation in the tissues were unexpected as Boveris and Chance [40] showed that the rate of superoxide formation is directly proportional to the rate of mitochondrial oxygen consumption. The difference observed between tissues in mitochondrial univalent reduction of O_2 after chronic ethanol feeding could be the results of modifications in the content or the activity of respiratory chain components involved specifically in free radical production, or in the content of some mitochondrial anti-oxidants like coenzyme Q (CoQ) and vitamin E, which may modulate this process [41–43]. Nowadays there is experimental data about the anti-oxidant functions of CoQ in biological membranes in addition to its well-established role as a redox compound of the inner mitochondrial respiratory chain [41–45]. Among the anti-oxidant properties of the reduced form of CoQ, could be implied the quenching of the $O_2^{\cdot-}$ radical, or carbon-centered lipid radicals or the sparing of vitamin E by reducing the α-tocopheroxyl radical. It has been shown that CoQ or vitamin E can modulate free radical production *in vitro* or in SMP through scavenging activities [44, 46–48]. We have observed in our conditions of ethanol feeding, that vitamin E contents were either increased in the liver [to be published], decreased in the brain [49] or not altered in the myocardium [50]. Specific tissue changes in the content of mitochondrial anti-oxidants induced by chronic ethanol feeding can therefore modulate free radical production.

The decrease in $O_2^{\cdot-}$ production by rat liver mitochondria following chronic ethanol intoxication contrasts with our previous report concerning an enhancement of liver mitochondrial $O_2^{\cdot-}$ generation following acute ethanol load [51]. However, prolonged chronic ethanol feeding (36% of calories as ethanol) to rats elicits a decrease of H_2O_2 production by intact liver mitochondria [52]. It thus appears that a decrease in free radical production by liver mitochondria is an early effect of ethanol intoxication which could also be apparent during moderate intoxication. It thus appears that liver mitochondria cannot be implicated in the development of oxidative stress in the liver. The increase in reactive oxygen intermediates by microsomes and to a lesser extent by nuclei, can be considered in hepatic toxicity after chronic ethanol treatment [2–4, 53].

In contrast, our results indicate that the increase in $O_2^{\cdot-}$ generation might herald the appearance of an oxidative stress in the brain. In the aging process, when comparing brain, liver and heart, the brain is the first organ to exhibit a significant increase in mitochondrial superoxide production [54]. In the present study, using a moderate alcohol drinking model, the increase in the brain mitochondrial $O_2^{\cdot-}$ generation indicates that this tissue may represent an early target for free radical attack.

The lack of change in mitochondrial $O_2^{\cdot-}$ generation in rat myocardium observed after chronic ethanol treatment does not exclude the risk of oxidative stress in the heart. In fact, we have previously reported myocardial disturbances in the antioxidant defence systems in ethanol-treated rats leading to an oxidation of protein thiols, a marker of free radical mechanism [50].

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