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Control of state 3 respiration in liver mitochondria from rats subjected to chronic ethanol consumption

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Male Sprague-Dawley rats were pair-fed a liquid diet containing 36% of calories as ethanol for at least 31 days. Mitochondria were isolated from the livers and assayed for state 3, state 4 and uncoupled respiration at all three coupling sites. Assay conditions were established that maximized state 3 respiration with each substrate while maintaining a high respiratory control ratio. In mitochondria from ethanol-fed animals, state 3 respiratory rates were decreased at all three coupling sites. The decreased state 3 rate observed at site III was still significantly higher than the state 3 rates observed at site II in mitochondria from either ethanol-fed or control animals. Moreover, the maximal (FCCP-uncoupled) rates with succinate and α-ketoglutarate were the same in mitochondria from ethanol-fed and control animals, whereas with glutamate-malate as substrate it was lowered 23% by chronic ethanol consumption. To investigate the role of cytochrome oxidase in modulating the respiratory rate with site I and site II substrates, the effects of cyanide on state 3 and FCCP-uncoupled respiration were determined. When the mitochondria were uncoupled there was no decrease in the rate of succinate oxidation until the rates of ascorbate and succinate oxidation became equivalent. Conversely, parallel inhibition of ascorbate, succinate and glutamate-malate state 3 respiratory rates were observed at all concentrations (1–50 μM) of cyanide utilized. These observations suggest strongly that in coupled mitochondria ethanol-elicited decreases in cytochrome oxidase activity depress the state 3 respiratory rates with site I and II substrates.

Introduction

Chronic ethanol consumption has been shown to elicit pronounced alterations in the energy metabolism of rat liver tissue. Among those changes are a dramatically lowered ATP content [1,2], decreased adenine nucleotide translocase activity [2,3] due to lowered concentrations of exchangeable ATP in mitochondria [3,4], and a depressed rate of ATP synthase activity [3–7]. Also observed are decreased rates of respiration using NADH-linked [5,8], FAD-linked [3,9–11] and cytochrome-*c*-linked substrates [5,8]. Decreases in the content of respiratory chain components [12,13] and in cytochrome *c* oxidase activity [3,14,15] have also been documented. Recently, Thayer and Rubin [16] have reported that the decrease in cytochrome oxidase content observed earlier in mitochondria from ethanol-fed animals [12,13] was due to a deficit in heme a rather than

Abbreviations: RCR, respiratory control ratio; FCCP, carbonylcyanide *p*-trifluorophenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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to a loss in the polypeptides comprising the multisubunit complex.

It is possible that the marked decreases in cytochrome oxidase activity might be primarily responsible for the lowered rate of respiration observed at the other energy-conserving sites after chronic ethanol feeding. Alternatively, the decreased activity of the ATP synthase complex could result in the lowered respiratory rates observed when respiration is coupled to ATP synthesis (state 3 respiration). In this study, both coupled and uncoupled respiration at all three coupling sites have been examined, and evidence is presented that the ethanol-induced change at the cytochrome oxidase portion of the respiratory chain (site III) depresses coupled respiration with FAD- and NADH-linked substrates. Moreover, data are presented which suggest that the ethanol-elicited lesions in the NADH-ubiquinone segment of the chain [17] limit electron transport through coupling site I.

Experimental procedures

Materials

Male Sprague-Dawley rats and many of the reagents were obtained from the sources listed previously [3,4,18]. The cytochrome *c*, Hepes, l-malic acid, l-glutamic acid, α-ketoglutaric acid, EGTA and TMPD were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. L-Ascorbic acid was obtained from Fisher Scientific Co., Raleigh, NC, U.S.A.

Methods

Male rats weighing 150–250 g were pair-fed for either 1 month, 8 months or 1 year on a nutritionally adequate liquid diet [19] in which ethanol provided 36% of the total energy. Pair-fed control rats received the same diet, but with maltose-dextrin isocalorically substituted for ethanol. Tightly coupled mitochondria were prepared as described previously [4], except that the final centrifugation sediment was resuspended in 0.25 M sucrose/1.0 mM EGTA. Respiratory rates were measured as indicated previously [4] using a Clark oxygen electrode. Aliquots of mitochondria maintained at 0 °C were taken for assay, and all respiratory rate measurements were completed within 3 h after the organelles were prepared. Both state 3 and state 4 respiration were relatively constant for all substrates utilized over the period during which measurements were taken.

The assay conditions for maximal state 3 respiratory rates and respiratory control ratios were determined experimentally. The KCl concentration for maximal state 3 respiration and respiratory control was determined to be 130 mM utilizing glutamate-malate, α-ketoglutarate and succinate as oxidizable substrates. The concentrations of substrates which gave maximal state 3 respiration and respiratory control ratios were determined in preliminary experiments. These measurements were important, since the maximal state 3 respiratory rates with various oxidizable substrates were being compared in this study. With glutamate-malate the highest state 3 rates and respiratory control ratios were obtained when the substrates were equimolar; concentrations of 5 mM were therefore selected for glutamate and malate. The α-ketoglutarate concentration in the assay mixture giving high state 3 respiration and respiratory control was also 5 mM. As in previous studies [4], a concentration of 11 mM succinate was used in the present study upon being confirmed as optimal in state 3 and respiratory control ratio measurements. The optimal concentration of ascorbate was determined to be 10 mM. State 3 and state 4 respiration increased continuously as a function of TMPD concentration, with respiratory control at site III remaining relatively constant. A TMPD concentration of 500 μM was selected to mediate electron flow from ascorbate to cytochrome *c*. Under the above assay conditions ascorbate oxidation was inhibited 96% by 1 mM NaCN.

Utilizing the concentrations of oxidizable substrates and TMPD designated above, assays were performed at 30 °C in a mixture containing 130 mM KCl, 2 mM potassium phosphate, 3 mM Hepes, 2 mM $MgCl_2$, 1 mM EGTA (pH 7.2). Additions of 2.4, 0.6 and 0.3 mg mitochondrial protein, and 0.27, 0.19 and 0.14 μmol ADP were used in assays for NADH-linked substrates, succinate, and ascorbate-TMPD, respectively, in all assays except those with cyanide. When cyanide was included, 0.6 mg of mitochondrial protein was utilized, and ADP concentrations are designated

in the legend. Uncoupled respiratory rates were measured in an assay mixture containing 0.54 μM FCCP. Cytochrome *c* oxidase activity was measured spectrophotometrically at 30 °C by a modification of the procedure described by Sottocasa et al. [20]. A solution of cytochrome *c* (Sigma, Type III) was reduced with excess ascorbate and adjusted to pH 7.0; the ascorbate was then removed by dialysis against H_2O. The assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.1% Tween 80, 35 μM reduced cytochrome *c* and 12–25 μg mitochondrial protein in a final volume of 1 ml. Mitochondrial preparations (approx. 30 mg protein/ml) were diluted 1 : 20 and sonicated 15 s in a sonicator bath before being assayed by the above procedure.

The values reported in the tables and figures are the averages ± S.E. for the numbers indicated. Statistically significant differences were determined utilizing the paired *t*-test, with one exception. The *t*-test for two means was used in Table II to compare respiratory rates of preparations from control and ethanol-fed animals. In some experiments the data from animal pairs fed for two different time periods were pooled. In these cases the differences observed between control and ethanol-fed animals were also significant when each feeding group was analyzed separately utilizing the paired *t*-test.

Results

Preliminary experiments were designed to determine the assay conditions which would allow state 3 respiration to proceed maximally. Measurement of maximal state 3 respiratory rates was essential to determine the quantitative relationships between ethanol-related losses in state 3 respiration with site I, site II, and site III substrates. The appropriate respiratory control media can vary markedly in composition, depending on the type of mitochondria being assayed and the properties to be measured [21]. For the present study, the previously used respiratory control assay medium [4] was modified to maximize state 3 respiratory rates in preparations of rat liver mitochondria without compromising the respiratory control ratio. The results of these measurements were summarized in the Experimental procedures section. The conditions established were utilized for the experiments which follow.

Chronic ethanol feeding resulted in significant decreases in the state 3 respiratory rates at all three coupling sites in rat liver mitochondria (Table I). State 4 rates were unchanged, except with ascorbate-TMPD, where it was significantly lowered. The state 3 respiratory rate through cytochrome oxidase in ethanol fed rats, while reduced, was nevertheless much higher than the state 3 rate with site I and site II substrates. For example, state 3 respiration with ascorbate-TMPD in ethanol-fed animals was lowered 28%, but was still significantly higher ($P < 0.01$) than state 3 respiratory rates in control animals with succinate as substrate. Due to the decrease in state 3 respiratory rates with all the substrates utilized, the resulting respiratory control ratios were also lowered (Table I). The ethanol-related decrease in respiratory control through site III was less pronounced due to both state 3 and state 4 respiration being lowered with ascorbate-TMPD as substrate.

The results in Table I are qualitatively similar to those observed by Thayer and Rubin [5] with liver submitochondrial particles prepared from liquid diet control and ethanol-fed animals. The ratio of ascorbate to succinate state 3 respiratory rates measured with control mitochondria was 1.8 in the present study (Table I); in contrast, the same ratio calculated from their earlier investigation was 0.9. The lower rate of ascorbate oxidation relative to that of succinate reported earlier [5] may be due to the type and concentration of electron mediator utilized in assaying cytochrome oxidase activity. In the present investigation, 500 μM TMPD was utilized, whereas in the earlier study 3.1 μM phenazine methosulfate was employed.

Respiratory measurements in the presence and absence of uncoupler (Table II) demonstrated that, while there was a significant decrease in the activity of cytochrome oxidase in uncoupled ethanol mitochondria, it was still sufficient to catalyze maximal electron transfer from site I and site II substrates at uncoupler stimulated rates. As shown in Table II, the uncoupled respiratory rate through site III in mitochondria from ethanol-fed animals was significantly higher than the uncoupler-stimulated respiration with the site I substrates

TABLE I

THE EFFECT OF CHRONIC ETHANOL CONSUMPTION ON RESPIRATORY RATES AND THE RESPIRATORY CONTROL RATIO

Respiratory rates are expressed as μgatom O/min per mg mitochondrial protein ± S.E., n.s., not significant.

Respiratory activity	Substrate			
	glutamate-malate [a]	α-ketoglutarate [b]	succinate [c]	ascorbate-TMPD [c]
State 3 rate				
Control	0.117 ± 0.004	0.105 ± 0.003	0.194 ± 0.013	0.349 ± 0.017
Ethanol-fed	0.092 ± 0.004	0.092 ± 0.004	0.122 ± 0.009	0.253 ± 0.012
% decrease	21	12	37	28
P	< 0.0005	< 0.005	< 0.0005	< 0.005
State 4 rate				
Control	0.018 ± 0.001	0.017 ± 0.001	0.036 ± 0.004	0.234 ± 0.013
Ethanol-fed	0.020 ± 0.001	0.019 ± 0.001	0.037 ± 0.003	0.182 ± 0.006
% decrease	−11	−12	−3	22
P	n.s.	n.s.	n.s.	< 0.005
Respiratory control ratio				
Control	6.59 ± 0.21	6.45 ± 0.17	5.60 ± 0.30	1.50 ± 0.03
Ethanol-fed	4.83 ± 0.27	5.10 ± 0.26	3.53 ± 0.37	1.39 ± 0.05
% decrease	27	21	37	7
P	< 0.0005	< 0.001	< 0.005	< 0.05

[a] Combined data from animals fed for 1 month and for 1 year ($n = 23$ pairs).
[b] Data from animals fed for 1 month ($n = 18$ pairs).
[c] Combined data from animals fed for 8 months and for 1 year ($n = 8$ pairs).

and succinate. Moreover, in mitochondria from ethanol-fed animals the respiratory rate in the presence of uncoupler was elevated significantly as compared with the state 3 respiratory rate; this was the case with both succinate and the site I substrates. Notably, with α-ketoglutarate the state 3 and uncoupler-stimulated rates in control mitochondria were the same and were equal to the uncoupled respiratory rate exhibited by mitochondria from ethanol-fed animals. In contrast with α-ketoglutarate and succinate, the uncoupler-stimulated respiration with glutamate-malate was lower in preparations fromethanol-fed animals than in control mitochondria.

TABLE II

COMPARISON OF STATE 3 AND UNCOUPLER-STIMULATED RESPIRATION

The uncoupler utilized with glutamate-malate, α-ketoglutarate, and succinate-driven respiration was FCCP (0.54 μM). When reduced cytochrome *c* was utilized as substrate, mitochondria were uncoupled by addition of 0.1% Tween 80.

Animal type	Respiratory rate (μgatom O/min per mg protein)						
	glutamate-malate		α-ketoglutarate		succinate		reduced cytochrome *c*
	state 3	uncoupled	state 3	uncoupled	state 3	uncoupled	uncoupled
Control	0.116 ± 0.007	0.213 ± 0.018	0.102 ± 0.004	0.109 ± 0.004	0.231 ± 0.010	0.344 ± 0.014	0.854 ± 0.141
	$P < 0.001$ [a], $n = 12$		n.s., $n = 11$		$P < 0.001$, $n = 11$		$n = 10$
Ethanol-fed	0.088 ± 0.006 [b]	0.165 ± 0.011 [b]	0.086 ± 0.005 [b]	0.110 ± 0.008	0.151 ± 0.010 [b]	0.370 ± 0.018	0.507 ± 0.061 [b,c]
	$P < 0.001$, $n = 9$		$P < 0.005$, $n = 9$		$P < 0.001$, $n = 9$		$n = 9$

[a] The *P* values shown indicate the significance of the difference between state 3 and uncoupler-stimulated respiratory rates.
[b] Significant difference between preparations from control and ethanol-fed animals using the *t*-test for two means.
[c] *P* for difference from ethanol-fed, succinate, uncoupled = 0.06, paired *t*-test.

The data in Fig. 1 confirm that in rat liver mitochondria the catalytic capacity of cytochrome oxidase is in excess of that needed to maintain maximal respiration with succinate. In this experiment the inhibition of ascorbate and succinate oxidation was followed in uncoupled mitochondria as a function of cyanide concentration. Succinate was chosen for assay since it elicits higher respiratory activity than do the NADH-linked substrates. Oxygen utilization remained constant with succinate as a substrate until the rate of ascorbate oxidation decreased to that with succinate. At that point the rate of oxidation of both substrates was equivalent at each cyanide concentration, with succinate oxidation decreasing as the molarity of the inhibitor was increased. There were no significant differences in the rates of ascorbate and succinate oxidation at the higher cyanide concentrations.

In contrast with the inhibition pattern observed with uncoupled mitochondria, ascorbate and succinate oxidation decreased in parallel by increasing concentrations of cyanide when state 3 respiration was measured in coupled mitochondria (Fig. 2A). For example, the concentration of cyanide which inhibited ascorbate oxidation 28% (the percentage inhibition of ascorbate state 3 respiration in mitochondria from ethanol-fed animals (Table I)) also depressed the rate of succinate state 3 respiration by 27.5%. State 3 respiration with glutamate-malate also decreased similarly to that with ascorbate when inhibition of oxidation of these substrates with cyanide was compared (Fig. 2B). With both the site I and site III substrates, the inhibition was proportional to cyanide concentration. In this latter experiment, the range of cyanide utilized was extended in order to show substantial inhibition of state 3 respiration with glutamate-malate. Notably, the inhibition of

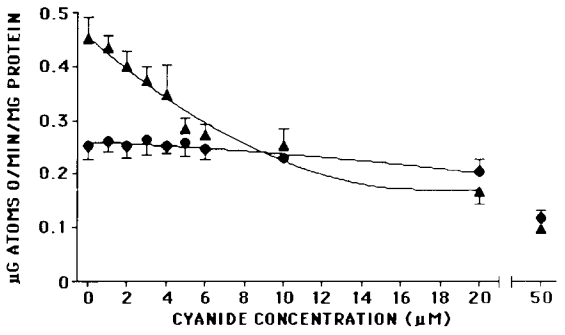

Fig. 1. Cyanide inhibition of ascorbate and succinate oxidation in uncoupled mitochondria. Coupled mitochondria were incubated at 0 °C for 30 min with the concentrations of NaCN indicated in the figure. Aliquots of mitochondria were then assayed for rates of ascorbate (▲) and succinate (●) oxidation in the presence of cyanide at the concentrations utilized in the incubation mixture. Thus, the mitochondria were maintained at constant cyanide concentrations during both the incubation and assay periods. All assays were carried out as described in Experimental procedures, with the mitochondria being uncoupled by adjusting the assay mixture to 0.54 µM FCCP. Each point is an average from either three or four separate mitochondria preparations obtained from pellet-fed animals, with the bars indicating S.E. Points without bars were values with S.E. encompassed within the height of the symbol.

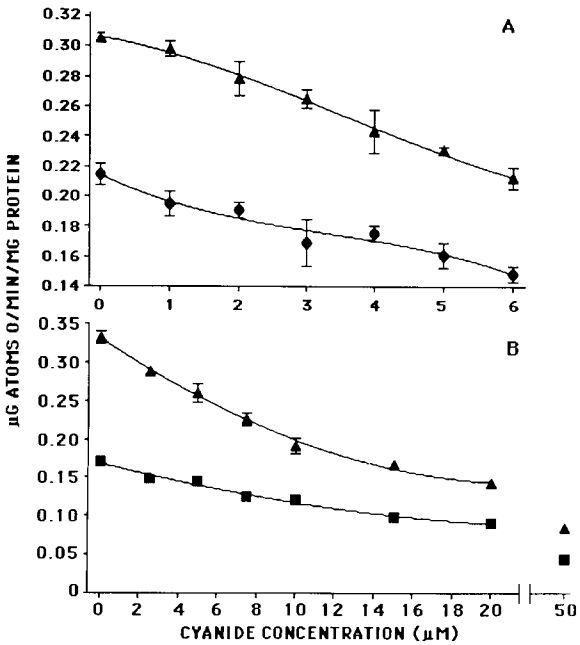

Fig. 2. Cyanide inhibition of state 3 respiration. (A) Coupled mitochondria were assayed in the presence of cyanide at the concentrations indicated for state 3 respiratory rates with ascorbate (▲) and succinate (●) as oxidizable substrates. State 3 respiration was initiated by addition of 0.68 µmol ADP when succinate was substrate, and with 1.35 µmol ADP when ascorbate oxidation was followed. (B) Coupled mitochondria were assayed as in (A) for state 3 respiration with ascorbate (▲) and glutamate-malate (■) as oxidizable substrates. Incubations with cyanide were as in Fig. 1. State 3 respiration with glutamate-malate was initiated by addition of 2.0 µmol ADP. In both (A) and (B), each point is the average from three separate mitochondria preparations obtained from pellet-fed animals, with the bars indicating S.E. Points without bars were values with S.E. encompassed within the height of the symbol.

ascorbate oxidation by cyanide in the two separate experiments was similar; the activity remaining at 6 μM cyanide was 69% and 73% in Fig. 2A and B, respectively.

Discussion

A major objective of this study was to determine whether the dramatic decreases in cytochrome oxidase activity [3,14,15] contributed to the declines in state 3 respiratory activity observed with site I and site II substrates [3,5,8–11]. The data in this investigation indicate that cytochrome oxidase in mitochondria from ethanol-fed animals has sufficient catalytic capacity to maintain normal respiratory rates with succinate and the site I substrates under conditions where energy conservation is dissociated from electron transport. This was illustrated in Table II, where the uncoupler-stimulated rates were the same in mitochondria from control and ethanol-fed animals with either α-ketoglutarate or succinate as substrate. Further evidence that cytochrome oxidase activity was not rate-limiting in uncoupled mitochondria from ethanol-fed animals comes from the inhibition profiles of succinate and ascorbate oxidation (Fig. 1). However, when the cyanide inhibition patterns for state 3 respiration with ascorbate, succinate, and glutamate-malate were compared, it was apparent that in coupled mitochondria small decreases in cytochrome oxidase activity were paralleled by losses in succinate or glutamate-malate-driven respiration. These inhibition profiles for state 3 respiration are consistent with the earlier observation of Groen et al. [22] that cytochrome oxidase influences the state 3 respiratory rate, exhibiting a flux control coefficient [23] of 0.17 in mitochondria isolated from chow-fed rats. The results in Fig. 2 also confirm that in coupled mitochondria the rate of substrate oxidation is controlled by a multimolecular process involving two or more components of the oxidative phosphorylation system [22].

There do, indeed, appear to be other factors in addition to lowered cytochrome oxidase activity that affect succinate-driven state 3 respiration in mitochondria from ethanol-fed animals, since the decrease in ascorbate oxidation in these organelles was only 28% (Table I), whereas that for succinate oxidation was 37% (Table I) and 38% (Table II). These differences between the ethanol-elicited depressions of ascorbate and succinate oxidation are statistically significant (Table I data, $P < 0.025$, paired t-test). Another component of the oxidative phosphorylation system which is altered dramatically as a result of ethanol consumption is the ATP synthase; there are ethanol-elicited decreases in the ATPase [3,6] and ATP–P_i exchange activities [7], and in oligomycin sensitivity [24], of the ATP synthase in intact liver mitochondria. It is possible, therefore, that the rate of state 3 respiration with succinate as substrate could also be influenced by the ethanol-elicited decrease in the activity of this enzyme complex.

Previous studies have demonstrated that determinations of the influence of the ATP synthase on the state 3 respiratory rate are functions of the conditions employed to measure the relationship between the synthase and state 3 respiration [25–28]. Under certain experimental conditions [26,28] it was observed that any depression in the activity of the ATP synthase in coupled mitochondria resulted in a decrease in state 3 respiration. These latter observaitons provide for the possibility that the ethanol-elicited depression in the activity of the synthase complex could be partially responsible for the decreased respiratory activity in mitochondria from ethanol-fed animals.

In contrast with succinate and α-ketoglutarate, the rate observed in mitochondria from ethanol-fed animals with glutamate-malate as substrate remained depressed in the presence of uncoupler (Table I). This observation is consistent with earlier reports of ethanol-elicited decreases in NADH dehydrogenase activity [17,29] and alterations in the iron-sulfur centers associated with the flavoprotein [17]. These lesions in NADH dehydrogenase are likely to limit the rate of electron flow through site I and may also affect energy conservation in this region of the respiratory chain [5,7,8].

The differential response of glutamate-malate and α-ketoglutarate respiration to the presence of the uncoupler (Table II) may be related to the difference in the rate of electron transport elicited by these two substrate systems. It is likely that the rate of α-ketoglutarate respiration, which in uncoupled mitochondria is significantly lower than

that with glutamate-malate, is limited by events that occur prior to electron transfer through the NADH dehydrogenase complex. These would include metabolite transport through the inner mitochondrial membrane and oxidation of α-ketoglutarate by the α-ketoglutarate dehydrogenase complex.

The observations in this communication suggest that ethanol-elicited lesions in cytochrome oxidase are rate-limiting for site I and site II substrates. They also provide for the possibility that alterations in the ATP synthase complex may have a minor effect on the respiratory rate with succinate. Furthermore, the results emphasize the potential of the ethanol-induced lesions in both NADH dehydrogenase [17] and metabolic transport mechanisms [22] to limit electron transport through site I. These observations are consistent with those made in submitochondrial particles by Thayer and Rubin [13] which indicated that ethanol-elicited alterations in cytochrome oxidase and NADH dehydrogenase influence the rate of respiration in mitochondria from ethanol-fed rats.

The results in this study indicate, therefore, that ethanol-elicited alterations in state 3 respiration are controlled by more than one component of the oxidative phosphorylation system. In contrast with normal mitochondria [22], however, the components limiting the respiratory rate may exert their effects as a function of the degree to which their catalytic properties have been depressed by chronic alcohol consumption. Notably, cytochrome oxidase, NADH dehydrogenase, and the ATP synthase all contain subunits which are mitochondrial gene products [30,31]. As earlier suggested [13], it is possible that state 3 respiration, and thus the rate of ATP synthesis, is limited by ethanol-elicited effects on the mitochondrial protein-synthesizing system.

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