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Effects of chronic alcohol consumption on the steady-state kinetics properties of cytochrome oxidase in rat liver

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The effect of chronic alcohol consumption on steady-state kinetic characteristics of cytochrome oxidase in rat liver was studied using submitochondrial particles prepared from ethanol-fed and control rats. Preparations from both control and alcoholic rats had equivalent apparent K_m values for cytochrome *c* of 13 μM in the presence of phenazine methosulfate or 19 μM with *N,N,N′,N′*-tetramethylphenylene diamine as oxidation-reduction mediators at physiological ionic strength. Both preparations showed comparable stimulation (approx. 3-fold) of oxidase activity following detergent solubilization of the membrane and similar temperature dependence for oxidase activity. Under all conditions, preparations from alcohol-fed rats displayed 30 to 50% lower rats of cytochrome oxidase activity per unit membrane protein than those from control rats. The diminution in specific activity per mg protein was accompanied by a similar decline in heme aa_3 content, as has been noted in previous studies. When expressed on a turnover number basis, the molecular activity of cytochrome oxidase (natoms O/min per nmol heme *a*) was equivalent in both alcoholic and control preparations. The results indicate that the intrinsic kinetic characteristics of cytochrome oxidase are not changed by alcohol consumption. The data suggest that the characteristic decline in heme aa_3 content and cytochrome oxidase specific activity seen in ethanol-fed rats does not arise from alterations in the accessibility of the oxidase towards cytochrome *c*, or from changes in bulk phase lipid composition or physical properties. The results support the conclusion that ethanol consumption decreases the membrane content of functionally active oxidase molecules, but does not change the catalytic properties of these oxidase molecules.

Introduction

Chronic alcohol consumption alters mitochondrial morphology [1,2] and leads to distinctive alterations in the enzymatic activity and content of certain proteins of the mitochondrial inner membrane, particularly cytochromes aa_3 [2–8], cytochromes *b* [8,9], NADH dehydrogenase [10] and ATPase [6,11]. Most widely reported among these is the effect on cytochrome oxidase. Several studies have noted decreased heme aa_3 content, measured spectrophotometrically, in liver mitochondria, submitochondrial particles and hepatocytes isolated form ethanol-fed animals as compared to pair-fed controls [4–8]. Studies of respiration at Site III utilizing ascorbate and redox mediators as electron donors to endogenous cytochrome *c* have likewise noted consistent decreases in the rate of oxygen utilization per unit membrane protein in preparations from ethanol-fed rats [6,12]. Decreased electron flow at Site III also contributes to the characteristic diminution in respiratory rates with NAD-linked substrates [13] seen in membrane preparations from ethanol-fed rats [12].

Previous studies of the effects of ethanol consumption on cytochrome oxidase have relied on comparisons of oxidase activity measured with alcoholic and control preparations under a standardized set of assay conditions. Such an approach is valid only if kinetic parameters (e.g., K_m) are the same for both types of preparation. Only one early study tested this point, briefly reporting identical K_m values for cytochrome *c* with mitochondria from both alcohol-fed and control rats under a single assay condition [5]. Recently, we have presented immunochemical evidence suggesting the existence of an inactive form of cytochrome oxidase in submitochondrial particles from alcoholic rats [14]. One

Abbreviations: DOC, deoxycholate; PMS, phenazine methosulfate; TMPD, *N,N,N′,N′*-tetramethylphenylene diamine.

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possible way in which the enzyme might become inactive is if it were inaccessible to cytochrome *c*, its substrate. This might potentially come about because of alcohol-linked changes in the properties of membrane lipids [15,16] leading, in turn, to incorrect assembly or insertion of the oxidase protein complex into the membrane. Such a situation might be expected to be reflected by alterations in the interaction of the oxidase with cytochrome *c* or in a differential response toward detergent solubilization of the membrane.

As one approach for examining the molecular basis for alterations of cytochrome oxidase associated with alcohol consumption, we have compared the steady-state kinetics properties of the oxidase in membrane preparations obtained from control and ethanol-fed rats under a variety of experimental conditions. Cytochrome oxidase exhibits complex behavior in classical steady-state kinetic analyses [17–23]. However, recent studies have clarified the effects of pH, ionic strength, detergents, buffer anions and method of assay (polarographic versus spectrophotometric) on kinetic parameters of the oxidase [21–23]. In our study, we have examined the effects of detergent-solubilization, various redox mediators, and assay temperature to evaluate the role of accessibility of cytochrome *c* towards the oxidase in the characteristic alcohol effects. The results support the conclusion that alcohol consumption results in a mixture of fully active and fully inactive enzyme forms within the membrane, but does not alter the intrinsic catalytic properties of active oxidase molecules.

Experimental procedures

Animals

Male, Sprague-Dawley, littermate rats (Charles River Breeding Laboratories, Raleigh, NC), initial weight 100 to 120 g, were pair-fed a totally liquid diet [24] (Bio-Serv, Frenchtown, NJ) containing ethanol as 36% of total calories for 40 to 45 days as previously described [6]. Control rats received an isocaloric amount of control liquid diet having carbohydrate in place of ethanol [24].

Preparations

Rat liver submitochondrial particles were prepared by sonication of mitoplasts as previously described [6]. Protein concentration was measured by the cyanide biuret procedure [25]. Heme aa_3 contents were determined from ΔA_{605} of reduced-minus-oxidized difference spectra [8]. Extinction coefficient values of 12.0 $mM^{-1} \cdot cm^{-1}$ for heme *a* (24.0 $mM^{-1} \cdot cm^{-1}$ for heme aa_3) were used [8,26].

Kinetic studies

Cytochrome oxidase activity was determined polarographically with an oxygen electrode at 25°C. The medium was 120 mM KCl, 20 mM Na^+-Hepes and 5 mM $MgCl_2$ (pH 7.5). For K_m determinations, 20 mM ascorbate, either 2 μM PMS or 600 μM TMPD, varying concentrations of cytochrome *c* (4.85 to 194 μM), and 0.2 mg submitochondrial particle protein were present in a final volume of 1.65 ml. Horse-heart cytochrome *c* (Type VI, Sigma, St. Louis, MO) was used for all experiments. For detergent activation of the oxidase, submitochondrial particles were diluted to 10 mg protein/ml in 0.25 M sucrose and treated with 0.3% (w/v) deoxycholate (DOC) prior to assay [27]. Rates of oxygen uptake were corrected for autooxidation measured in the absence of submitochondrial particles. Kinetic parameters were determined graphically from Lineweaver-Burk double-reciprocal plots [28]. Student's *t*-test was used to evaluate differences between groups [29]. Values of $P < 0.05$ were considered significantly different.

Results

Comparison of PMS versus TMPD as redox mediator for cytochrome oxidase in submitochondrial particles

Polarographic assays of cytochrome oxidase utilize a redox mediator dye to facilitate transfer of reducing equivalents between the exogenous reductant ascorbate and cytochrome *c* [30]. Rat liver submitochondrial particles, prepared by the method we have described [6], have a membrane sidedness inverted with respect to intact mitochondria [6]. Thus, endogenous cytochrome *c* is located on the inner side of the submitochondrial particle membrane. For this reason, differential permeability of redox mediators can influence the measured cytochrome oxidase activity and kinetic parameters. To verify that the redox mediator concentration was not limiting for oxidase activity, we first titrated the mediators PMS and TMPD in the absence and presence of added cytochrome *c* (Fig. 1). With added cytochrome *c*, concentrations of 2 μM PMS and 600 μM TMPD produced near maximal rates of oxygen uptake and yet had minimal background autooxidation rates. Thus, these concentrations were chosen for use in subsequent cytochrome *c* titrations. By comparison, these particular mediator concentrations elicited less than maximal rates of respiration through Site III with endogenous cytochrome *c* (Fig. 1). PMS was more effective than TMPD in facilitating oxidation of endogenous cytochrome *c*, both in terms of requiring use at a lower concentration and in producing noticeably higher rates of oxygen uptake. This preference for PMS over TMPD with respect to measurement of Site III respiration in submitochondrial particles probably reflects a difference in the membrane permeability characteristics of these two mediators. By contrast, for oxidation of exogenous cytochrome *c*, TMPD promoted slightly higher rates of oxygen uptake than PMS. Submitochondrial

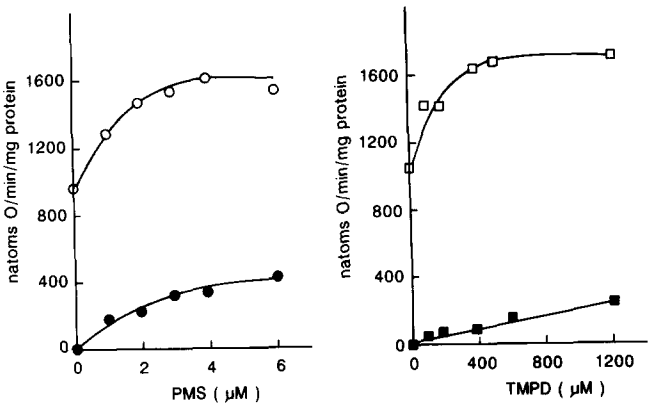

Fig. 1. Effect of redox mediator concentration on oxidation of endogenous and exogenous cytochrome *c* in rat liver submitochondrial particles. The net rate of oxygen uptake by control rat liver submitochondrial particles with ascorbate (20 mM) as substrate was determined as described in Experimental procedures in the absence (solid symbols) or presence (open symbols) of added cytochrome *c* (48.5 μM) and various concentrations of PMS (circles, left) or TMPD (squares, right), as indicated.

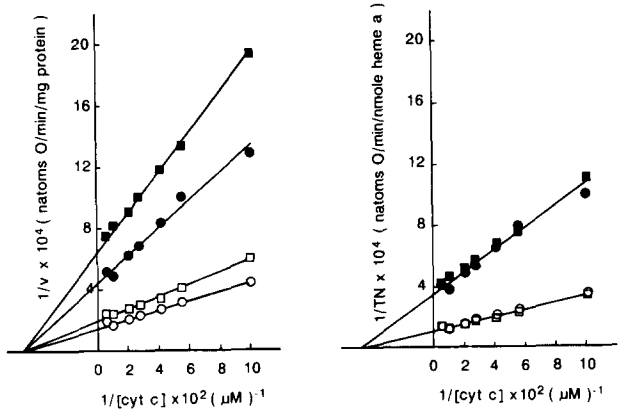

Fig. 2. Representative double-reciprocal plots illustrating the effect of alcohol consumption on the steady state kinetics behavior of cytochrome oxidase in rat-liver submitochondrial particles. The rate of oxidation of ascorbate (20 mM) in the presence of TMPD (0.6 mM) and various concentrations of cytochrome *c* was measured with submitochondrial particles from an alcoholic rat (squares) and pair-fed control rat (circles). Submitochondrial particles were either pretreated with 0.3% deoxycholate (open symbols) or not pretreated (solid symbols). In the left panel, data have been plotted on the basis of total membrane protein in the assay, whereas in the right panel the same data have been calculated with respect to heme *a* concentration, determined spectrophotometrically, of the respective alcoholic and control submitochondrial particles. Symbols: ■, alcoholic, −DOC; □, alcoholic, +DOC; ●, control, −DOC; ○, control, +DOC.

particles from alcohol-fed rats displayed similar patterns in such mediator titrations (not shown).

Effect of alcohol consumption on kinetic parameters of cytochrome oxidase

Fig. 2 shows representative steady state kinetics data in the form of a double-reciprocal plot of rates of oxygen uptake at various concentrations of cytochrome *c* in a high (physiological) ionic strength medium with TMPD as a redox mediator. Under these conditions the data fit a single K_m value of about 19 μM for cytochrome *c*, in agreement with other studies [21]. Treatment of the submitochondrial particle preparations with deoxycholate prior to assay activated cytochrome oxidase activity [27], but did not change the apparent K_m value for cytochrome *c* (Fig. 2). The degree of activation of both alcoholic and control preparations was about 3-fold. V_{max} values of the alcoholic samples were about 60% those of controls, both with and without deoxycholate treatment, when expressed on the basis of total membrane protein in the assay (natom O/min per mg protein). We also measured heme aa_3 content spectrophotometrically on the same submitochondrial particle preparations and recalculated the kinetics data on a turnover number basis (natom O/min per nmol heme *a*). For the particular preparations used here, heme *a* concentrations were 0.83 ± 0.04 (control) and 0.59 ± 0.02 (alcoholic) nmol/mg membrane protein, respectively (mean ± S.D., $n = 4$, significantly different at $P < 0.01$). When the kinetics data are expressed on a per heme *a* basis, it is clear that both alcoholic and control preparations exhibit similar molecular activity (Table I). These results indicate that the catalytic efficiency of the active oxidase molecules is unchanged by alcohol consumption, although the content of biochemically active

TABLE I

Effect of chronic alcohol consumption on kinetic parameters of cytochrome oxidase

The maximum rates for oxidation of exogenous cytochrome *c* were determined in the presence of either PMS (2 μM) or TMPD (600 μM) using rat liver submitochondrial particles which had been pretreated with deoxycholate (+DOC) or not pretreated (−DOC). Values indicate mean ± S.D. for three sets of kinetic experiments similar to those in Fig. 2 that were conducted with independent pairs of submitochondrial particle preparations from control and alcohol-fed rats.

Redox mediator	DOC pretreatment	V_{max} (natom O/min per mg protein)		TN_{max} (natom O/min per nmol heme *a*)	
		control	alcoholic	control	alcoholic
PMS	−	1950 ± 179	1270 ± 95	2433 ± 192	2198 ± 196
	+	4769 ± 556	3327 ± 211	6224 ± 608	6016 ± 387
TMPD	−	2395 ± 132	1434 ± 65	2867 ± 247	2473 ± 198
	+	7325 ± 543	4509 ± 523	8199 ± 176	7723 ± 596

enzyme is decreased in membranes from alcoholic animals. This finding is fully consistent with previous data [14].

Similar experiments were conducted using phenazine methosulfate (PMS) as a redox mediator. Again, preparations from alcoholic rats showed lower V_{max} compared to those from controls when expressed on a per mg membrane protein basis. On a per heme *a* basis, both alcoholic and control preparations exhibited similar turnover numbers (Table I). For both types of preparation, comparable apparent K_m values for cytochrome *c* were also found.

With PMS as mediator, the apparent K_m value for cytochrome *c* was 13.0 ± 2.2 μM ($n = 6$), a value significantly ($P < 0.02$) lower than the 19.1 ± 4.5 μM ($n = 6$) found when TMPD was used as mediator. This difference in K_m probably reflects more efficient catalysis of electron transfer between exogenous and endogenous cytochrome *c* by PMS as compared to TMPD (cf. Fig. 1), rather than more efficient transfer of reducing equivalents between ascorbate and exogenous cytochrome *c*.

A similar pattern of results was also observed when experiments were conducted without any redox mediators present. Alcoholic submitochondrial particles exhibited lower V_{max} values compared to control submitochondrial particles on a per mg protein basis, but similar turnover numbers per heme *a*. In the absence of redox mediators, higher apparent K_m values for cytochrome *c*, in the range of 40 μM, were seen both without and with deoxycholate activation of the oxidase.

Use of Triton X-100 as an activating agent in place of deoxycholate gave comparable results. Namely, cytochrome oxidase activity was stimulated to extents similar to those produced by deoxycholate in both alcoholic and control submitochondrial particles. Likewise, turnover numbers per heme *a* were similar in both preparations.

Temperature dependence of cytochrome oxidase activity

To examine the role of changes in bulk membrane lipids in affecting oxidase function, we studied the temperature dependence of oxidase activity with submitochondrial particles (Fig. 3). At all temperatures within the range of 10 to 40 °C, alcoholic and control preparations exhibited approximately parallel lines in plots of log respiration rate per mg protein versus reciprocal (absolute) temperature. For both types of preparation, such plots exhibited changes in slope ('breaks') near 29 °C. Rates for the alcoholic preparations were about 60% those of the controls. When adjusted for heme *a* content, equivalent turnover number values were observed at each temperature for both alcoholic and control preparations. Similar patterns for the alcohol effect were found in the presence of different redox mediators as well as with or without deoxycholate pretreatment of the submitochondrial particles to activate the oxidase.

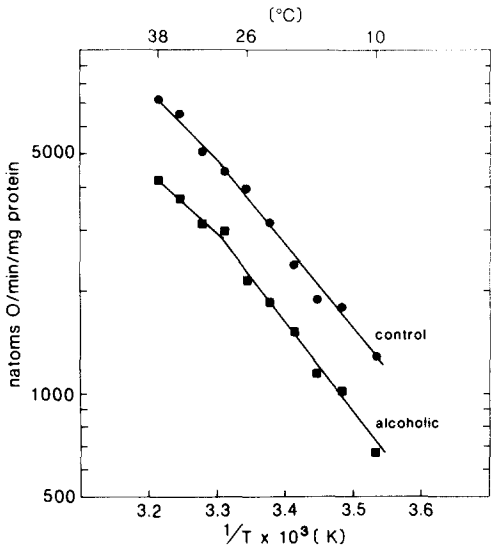

Fig. 3. Temperature dependence of cytochrome oxidase activity in rat liver submitochondrial particles. The net rate of oxidation of ascorbate (20 mM) in the presence of PMS (2 μM) and cytochrome *c* (97 μM) was determined at various temperatures in the medium described under Experimental procedures. The oxygen concentration of the medium at each temperature was calibrated by recording the stoichiometric oxidation of a limiting amount of spectrophotometrically-standardized NADH by submitochondrial particles in a separate experiment. Results from a representative experiment are shown. Symbols: ■, alcoholic; ●, control.

Effect of alcohol consumption on site III respiratory activity

For comparison, we also measured the rate of Site III respiration with endogenous cytochrome *c* in the same submitochondrial particle preparations as used for the kinetics studies (Table II). The data indicated a diminution of about 50% on a total membrane protein basis in preparations from alcoholic rats, using either PMS or TMPD as the redox mediator. Again, on a per heme *a*

TABLE II

Effect of chronic ethanol consumption on Site III respiratory rates of rat liver submitochondrial particles

The rate of oxidation of ascorbate (20 mM) in the presence of either PMS (2 μM) or TMPD (600 μM) by liver submitochondrial particles from control or alcoholic rats was determined at 25 °C in the medium described in Experimental procedures. Values indicate mean ± S.D. ($n = 4$). The data were obtained using the same submitochondrial particle preparations as used for the kinetics studies reported in Table I.

Redox mediator	Respiratory rate (natoms O/min per mg protein)	
	control	alcoholic
PMS	323 ± 68	182 ± 54
TMPD	165 ± 30	95 ± 19

basis, the rates were not significantly different between alcoholic and control preparations.

Discussion

Data presented in this paper indicate that the steady-state kinetics characteristics of cytochrome oxidase are similar in submitochondrial particles from both alcoholic and control rats. Apparent K_m values for cytochrome *c* are about 19 μM with TMPD, or 13 μM with PMS, in both cases when measured in a medium having ionic strength approx. 0.15 M. This single K_m value is considered to be that of the 'high-affinity' site, and indeed the only site, for interaction of cytochrome *c* with the oxidase at physiologically relevant ionic strength conditions [21]. The similar degree of activation of the oxidase by detergents in both alcoholic and control preparations, as well as the similar temperature dependence of activity, further suggests that the environment of the active enzyme molecules in both alcoholic and control membranes is equivalent. Both types of preparation exhibit similar accessibility of the oxidase towards cytochrome *c* as judged by these kinetic properties. Thus, the orientation of the oxidase protein within the liver mitochondrial membrane of ethanol-fed rats, at least of the active portion of the oxidase molecules, must be the same as that of controls.

These results are consistent with the suggestion that chronic alcohol consumption decreases the membrane content of active oxidase molecules, but does not change the kinetic properties of the residual functional oxidase molecules. In the same experimental system (i.e., submitochondrial particles), a previous study based on immunoinhibition titrations with antiserum against rat liver cytochrome oxidase has demonstrated equal cytochrome oxidase immunochemical reactivity per unit membrane protein in both alcoholic and control rats [14]. In addition, earlier studies have established that the relative purity of the submitochondrial particles and their recovery during preparation from mitochondria are comparable between alcoholic and control rats [8]. Together, such biochemical and immunochemical data provide evidence for the existence of an additional population of inactive oxidase molecules within the membranes of alcoholic animals [14]. Results of the present study indicate that the inferred inactive oxidase population is totally inert kinetically, however, and does not influence the interaction of the active oxidase molecules with cytochrome *c*. The findings reported in this paper support an 'all or none' mode for the effect of ethanol consumption on the oxidase. Mitochondrial membranes of alcoholic animals apparently contain a mixture of fully active and fully inactive forms of cytochrome oxidase.

It remains possible that the inactive form of the oxidase in alcoholic animals may arise from an alteration of the tightly bound lipids, particularly cardiolipin, required for catalytic activity [31,32]. In addition to effects on proteins, chronic alcohol consumption also affects the composition and properties of lipid components of the mitochondrial inner membrane [7,15,16,33]. Although phospholipid headgroup composition appears similar, subtle shifts toward greater saturation of acyl chains, especially for cardiolipin, have been found [15,33]. Cytochrome oxidase is influenced by its membrane lipid environment and demonstrates a requirement for tightly bound cardiolipin for catalysis of oxygen reduction [31,32]. Thus, alcohol-related lipid changes could, in principle, play a role in inactivating cytochrome oxidase in alcoholic animals. Indeed, it has been demonstrated that lipids extracted from liver mitochondria of alcoholic baboons were less efficient at reactivating a delipidated preparation of bovine heart cytochrome oxidase than those extracted from baboons fed a control diet [34]. Data presented in this paper suggest, however, that alcohol-induced changes in bulk phase lipid composition or properties are not responsible for the lower oxidase activity per unit membrane protein seen in alcoholic animals. In addition, electrophoretic analysis of immunoprecipitates [14], as well as recent immunoblot studies (unpublished data), indicate that the subunit composition of the oxidase is not altered following chronic ethanol consumption. Defects in heme aa_3 attachment remain a possible explanation for the existence of the inactive form of oxidase in alcoholic animals, however. Alternatively, inactivation of the enzyme by a toxic product derived from metabolism of ethanol, such as by formation of an acetaldehyde adduct [35], may occur.

It is interesting to note that several mitochondrial myopathies associated with defects in respiratory chain proteins, particularly cytochrome oxidase, have been identified in humans within the past few years [36–38]. While some of these clinical cases have been ascribed to an absence of specific oxidase subunits, others have noted decreased cytochrome oxidase functional activity without any loss of subunits [37,38]. The latter situation is similar to that found in ethanol-fed animals. Thus, chronic alcohol consumption may provide an example of a toxin-induced 'disease' which alters mitochondrial structure and function in a manner similar to that of such genetic diseases as the mitochondrial myopathies. Determining how such abnormalities come about at the molecular level will be important for understanding the pathobiology of 'mitochondrial diseases' in the future.

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