Chronic ethanol ingestion increases efficiency of oxidative phosphorylation in rat liver mitochondria

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Abstract The efficiency of oxidative phosphorylation was compared between rats chronically fed with ethanol and controls. (i) Results showed that the liver mitochondria state 4 respiratory rate was strongly inhibited, while the corresponding proton-motive force was not affected; (ii) the cytochrome oxidase content and activity were decreased and (iii) the oxidative-phosphorylation yield was increased in the ethanol exposed group. Furthermore, oxidative phosphorylation at coupling site II was not affected by ethanol. Cytochrome oxidase inhibition by sodium-azide mimicked the effects of ethanol intoxication in control mitochondria. This indicates that the decrease in cytochrome oxidase activity induced by ethanol intoxication directly increases the efficiency of oxidative phosphorylation.

Key words: Mitochondrion; Oxidative phosphorylation; Ethanol; Cytochrome oxidase; Proton-motive force; Sodium azide

1. Introduction

The effects of ethanol ingestion on mitochondrial metabolism are well known and include a decrease in the state 4 respiration rate ([1–4], for review see [5,6]) associated with a decrease in the cytochrome oxidase content [7–9]. The effect of ethanol on oxidative phosphorylation, however, remains unclear since the ATP/O ratio has been reported to be either unaffected [2,4,7] or decreased [10]. The question of a change in the efficiency of ethanol-induced oxidative phosphorylation is important for understanding the pathophysiology of alcoholic liver disease but also for the general understanding of the mechanisms of regulation of ATP synthesis and yield. Both proton pumps and mitochondrial inner membrane are responsible for the fine-tuning of oxidative phosphorylation. Two main mechanisms of energy waste have been described, namely, a proton leak across the mitochondrial inner membrane [11,12] or a decrease in proton-pump efficiency [13–18]. In contrast to previous reports [2,4,7,10], the present work shows that chronic ethanol feeding is responsible for an increase in ATP/O ratio, which seems to be due to a decrease in cytochrome oxidase activity. Indeed the addition of a low concentration of sodium-azide (a cytochrome oxidase inhibitor), precisely matching the effect of ethanol feeding on mitochondrial respiration was able to mimic the increased yield of oxidative phosphorylation observed in mitochondria from rats that were chronically fed ethanol.

2. Materials and methods

Male weaning Wistar rats (21 days old, 50–60 g) were fed with a semi-synthetic diet containing 72% of energy as carbohydrate, 6% as lipid (soya oil) and 22% as protein. In the ethanol group, ethanol was given in drinking water, at increasing concentrations (5–30%), over 6–10 weeks. Animals had free access to food and water.

After 6–10 weeks, liver mitochondria were prepared as previously described [19] in a medium consisting of 250 mM sucrose, 1 mM EGTA and 20 mM Tris–HCl (pH 7.2). Mitochondrial protein content was determined by the biuret method, with serum albumin as a standard.

Experiments were done in KCl medium at 37°C. It has been noted recently that KCl medium may be more pertinent for the study of oxidative phosphorylation since mitochondria in situ are exposed to salts [20].

For the determination of oxygen consumption, mitochondria were suspended in the following medium: 125 mM KCl, 1 mM EGTA, 5 mM Pi, 20 mM Tris–HCl (pH 7.2; 37°C), supplemented with either 5 mM succinate plus 0.5 mM malate plus 1.25 μM rotenone or 1 mM *N*,*N*,*N*′,*N*′-tetramethyl-*P*-phenylenediamine (TMPD) plus 5 mM ascorbate plus 0.3 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) plus 25 ng/ml antimycin. Mitochondrial respiration before and after addition of oligomycin 1.25 μg/mg proteins was measured polarographically using a Clark electrode. State 3 respiration was obtained after the addition of 1 mM ADP in the presence of succinate.

Proton-motive force (Δp) measurements were performed as previously described [21] in the same KCl medium with 5 mM succinate plus 0.5 mM malate plus 1.25 μM rotenone supplemented by [^{3}H]TPMP$^+$ for $\Delta\Psi$ determinations, by [^{14}C]DMO for ΔpH determinations or by ^3H_2O and [^{14}C]mannitol for matrix volume determinations. In the case where coupling site II was functionally isolated, ferricyanide (1 mM) was used as the electron acceptor instead of oxygen in the presence of 0.4 mM KCN. In the latter case electron flux was assessed by ferricyanide reduction at 436 nm, considering that one electron is accepted by one ferricyanide.

The cytochrome content in isolated mitochondria was determined by spectrophotometry using a dual wavelength mode. The differences between reduced and oxidised spectra were analysed. The wavelength pairs and absorptivity coefficients used were: cytochrome $c+c_1$, 550–540 nm = 18 mM^{-1} cm^{-1}; cytochrome b: 563–575 nm = 18 mM^{-1} cm^{-1} and cytochrome $a+a_3$: 605–630 nm = 24 mM^{-1} cm^{-1} [22,23].

ATP/O ratios were determined in the presence of 5 mM Pi, 5 mM succinate plus 0.5 mM malate, 1.25 μM rotenone, 20 mM glucose, 1 mM $MgCl_2$ and 125 μM ATP. The rate of oxidative phosphorylation flux was modulated by different concentrations of hexokinase (0.2–2 U/ml). ATP production was monitored by glucose 6-phosphate formation, which was measured enzymatically by spectrophotometry according to Bergmeyer [24].

ATP, ADP, Pi and hexokinase were purchased from Roche (Meylan, France), succinate, rotenone, EGTA, CCCP, oligomycin and mal-

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onate from Sigma (L'Isle d'Abeau, France), Tris, HCl, malate and $MgCl_2$ from Merck (Nogent, France) and labelled compounds from Amersham (Les Ulis, France).

Results are expressed as mean ± S.E.M. Statistical analyses were made by non-paired Student's *t*-test.

3. Results

Oxidative phosphorylation parameters in isolated mitochondria from both control and chronic ethanol-fed rats are shown in Table 1. The state 4 respiratory rate was lower (−30%, $P < 0.001$) in liver mitochondria from chronic ethanol-fed rats and the significant decrease in oxygen uptake in this group persisted after oligomycin addition. Oxygen uptake in conditions of phosphorylation (state 3) as well as maximum cytochrome oxidase activity (uncoupled respiration rate in the presence of TMPD–ascorbate) was decreased in the ethanol group. This finding is consistent with the dramatic decrease in cytochrome $a+a_3$ in the chronic ethanol-fed group as compared to controls (0.045 ± 0.009 versus 0.088 ± 0.01 nmol/mg proteins, respectively, $n = 10$, $P < 0.01$). Cytochrome $c+c_1$ (0.233 ± 0.01 versus 0.225 ± 0.015 nmol/mg proteins, $n = 10$, ns) and cytochrome *b* (0.183 ± 0.015 versus 0.200 ± 0.012 nmol/mg proteins, $n = 10$, ns) were not affected.

While state 4 and state 3 respiratory rates were strongly inhibited, state 4 and state 3 proton-motive forces were not affected by chronic ethanol consumption suggesting a decrease in energy wastage processes. Indeed it has long been known that the relationship between respiration and proton-motive forces in non-phosphorylating mitochondria is not linear [12]. The curve exhibits both ohmic and non-ohmic parts, the latter being responsible for energy wastage due to either a non-ohmic increase in proton conductance of the inner membrane (non-ohmic leak) at high proton-motive force [11,12] or to a decrease in the efficiency of the respiratory chain (redox slipping) at high electron flux [13–16]. As shown in Fig. 1, we found a non-linear relationship both in mitochondria from chronic ethanol-fed and control rats. Moreover, the plots were completely overlapping but the non-ohmic part of the curve was decreased in the ethanol group. Hence, the highest proton-motive force which was identical in both groups was maintained with different respiratory rates: mitochondria from the ethanol group showed a lower respiratory rate as compared to controls. This finding indicates that chronic ethanol consumption decreases state 4 energy wastage.

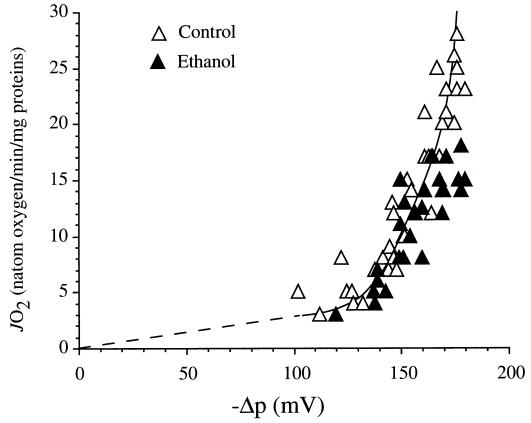

Fig. 1. Relationship between oxygen consumption rate (J_{O_2}) and proton-motive force (Δp) in non-phosphorylating liver mitochondria isolated from control and chronic ethanol-fed rats. Rat liver mitochondria (4 mg/ml) were suspended in the following medium: 125 mM KCl, 1 mM EGTA, 5 mM Pi, 20 mM Tris–HCl (pH 7.2; 37°C), supplemented with 5 mM succinate plus 0.5 mM malate plus 1.25 μM rotenone plus oligomycin 1.25 μg/mg proteins. The respiration rate was modulated by addition of malonate (0–10 mM). Δp measurements were performed in parallel experiments in the same medium supplemented by [^{3}H]TPMP$^+$ for $\Delta\Psi$ determinations, by [^{14}C]DMO for ΔpH determinations, or by ^3H_2O and [^{14}C]mannitol for matrix volume determinations. Each determination was performed in duplicate.

Since a similar state 3 proton-motive force was sustained in controls and ethanol group despite a large decrease in respiratory rate energy in the latter group, we have investigated the efficiency of oxidative phosphorylation. This was achieved by studying the relationship between ATP synthesis and oxygen consumption. For this purpose, the rate of oxidative phosphorylation was modulated by a change in ADP turnover induced by hexokinase addition in the presence of glucose, and ATP synthesis was assessed from glucose 6-phosphate synthesis. As compared to classical determination of state 3 ATP/O ratio, this method allows us to study ATP synthesis without a saturating concentration of free ADP (i.e. very low phosphate potential, $\Delta G'p$). As shown in Fig. 2A, the relationships between *J* glucose 6-phosphate (assessing J_{ATP}) and

Table 1
Oxygen consumption (J_{O_2}), matrix volume and proton-motive force (Δp) in isolated mitochondria of control and chronic ethanol-fed rats

	J_{O_2} (natom oxygen/min/mg proteins)				Matrix volume (μl/mg proteins) ($n = 20$)	Proton-motive force (mV)					
	succinate			TMPD		state 4			state 3		
	state 4 ($n = 10$)	oligomycin ($n = 10$)	state 3 ($n = 6$)	CCCP ($n = 6$)		ΔpH ($n = 8$)	$\Delta\Psi$ ($n = 8$)	Δp ($n = 8$)	ΔpH ($n = 8$)	$\Delta\Psi$ ($n = 8$)	Δp ($n = 8$)
Control	31.2 ± 1.1	25.9 ± 1.1	151 ± 6	637 ± 17	0.94 ± 0.05	−42 ± 3	−136 ± 5	−178 ± 5	−38 ± 4	−109 ± 4	−147 ± 6
Ethanol	21.8 ± 1.1	15.5 ± 1.1	102 ± 10	486 ± 12	1.01 ± 0.06	−37 ± 2	−135 ± 2	−172 ± 5	−39 ± 3	−115 ± 4	−154 ± 5
	***	***	***	***	NS	NS	NS	NS	NS	NS	NS

For oxygen consumption rate determination, rat liver mitochondria (2 mg/ml) were suspended in the following medium: 125 mM KCl, 1 mM EGTA, 5 mM Pi, 20 mM Tris–HCl (pH 7.2; 37°C), supplemented with either 5 mM succinate plus 0.5 mM malate plus 1.25 μM rotenone or 1 mM TMPD plus 5 mM ascorbate plus 0.3 μM CCCP plus 25 ng/ml antimycin. Respiration after addition of oligomycin 1.25 μg/mg proteins was measured. State 3 respiration was obtained after the addition of 1 mM ADP. Δp measurements were performed in the same medium with 5 mM succinate plus 0.5 mM malate plus 1.25 μM rotenone supplemented by [^{3}H]TPMP$^+$ for $\Delta\Psi$ determinations, by [^{14}C]DMO for ΔpH determinations, or by ^3H_2O and [^{14}C]mannitol for matrix volume determinations. Each determination was performed in duplicate from at least three preparations. Results are mean ± S.E.M. ***$P < 0.001$ vs control, unpaired Student's *t*-test.

oxygen consumption rate were linear and parallel in both groups, but the curve of the chronic ethanol group was shifted to the left. In other words, whatever the respiratory rate, ATP synthesis was higher in the ethanol group. This increased ATP synthesis was due to an increased proton-motive force as shown in Fig. 2B. Indeed the relationship between respiratory rate and proton-motive force of the ethanol group was shifted to the right: for a given respiratory rate, the proton-motive force was always higher.

Hence, these results show that chronic ethanol consumption induces a decrease in cytochrome oxidase content and activity when state 4 energy wastage was decreased and oxidative phosphorylation efficiency was increased. Taken together these findings suggest that a cytochrome oxidase decrease is responsible for the increase in the yield of oxidative phosphorylation. Nevertheless an effect located on the coupling site II cannot be completely ruled out, therefore we have investigated the role of ethanol exposure on the functionally isolated coupling site II of the respiratory chain by using ferricyanide as an electron acceptor. In the ethanol group as compared to controls, we found no significant effect on either proton-motive force (151 ± 14 versus 161 ± 8 mV, $n = 3$, ns) or P/2e$^-$ (0.25 ± 0.13 versus 0.21 ± 0.07, $n = 3$, ns) indicating that this effect is indeed located on the cytochrome oxidase. This was further confirmed by mimicking the ethanol-exposure condition by sodium azide (a known quasi-irreversible cytochrome oxidase inhibitor). The concentration used (75 μM) permitted close matching of the decrease in respiratory rate observed in mitochondria prepared from ethanol-fed rats. We found that

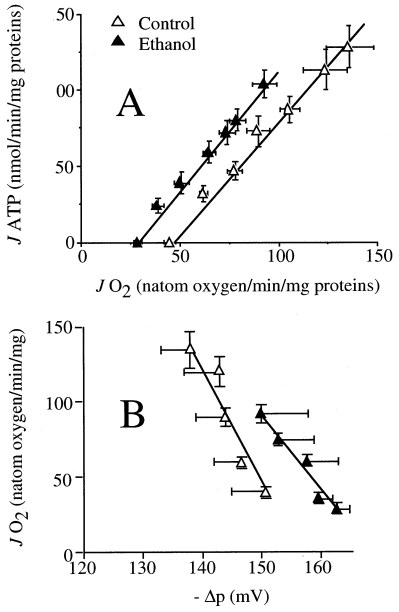

Fig. 2. Relationship between (A) ATP synthesis (J_{ATP}) and oxygen consumption rate (J_{O_2}) and (B) oxygen consumption rate (J_{O_2}) and proton-motive force (Δp) in liver mitochondria isolated from control and chronic ethanol-fed rats. Rat liver mitochondria (2 mg/ml) were suspended in the following medium: 125 mM KCl, 1 mM EGTA, 5 mM Pi, 20 mM Tris–HCl (pH 7.2; 37°C), supplemented with 5 mM succinate plus 0.5 mM malate, 1.25 μM rotenone, 20 mM glucose, 1 mM $MgCl_2$ and 125 μM ATP. ATP synthesis flux was modulated by different concentrations of hexokinase (0.2–2 U/ml). ATP production was monitored by glucose 6-phosphate formation, which was measured enzymatically. Δp was measured as described in Fig. 1. Results are expressed as mean ± S.E.M., $n = 7$ for (A); $n = 3$ preparation in duplicate for (B).

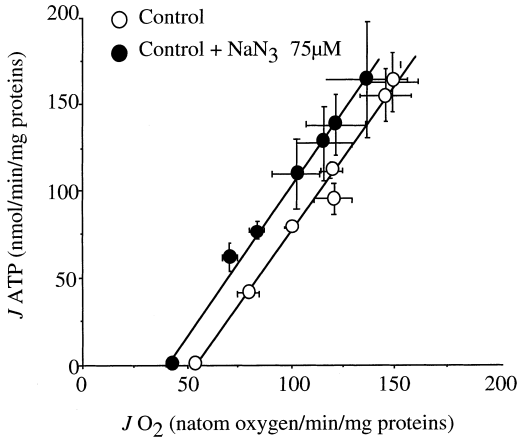

Fig. 3. Relationship between ATP synthesis (J_{ATP}) and oxygen consumption rate (J_{O_2}) in liver mitochondria incubated with or without sodium azide (NaN_3). Rat liver mitochondria (2 mg/ml) were suspended as described in Fig. 2 with or without NaN_3 75 μM. Results are expressed as mean ± S.E.M., $n = 3$.

such pharmacological inhibition of cytochrome oxidase induced a similar shift in the relationship between J_{ATP} and the oxygen consumption rate (Fig. 3).

4. Discussion

The main finding reported in this work is an increased proton-motive force and ATP/O ratio by chronic ethanol feeding. This result contradicts previous reports, which either found no difference [2,4,7] or a slight decrease [10] in ATP/O ratio. This discrepancy is most probably due to the method used for assessing ATP/O ratio, i.e. saturating ADP concentrations (state 3) versus variable and non-saturating ADP concentrations regenerated by glucose 6-phosphate synthesis in presence of hexokinase. We have previously shown in a model of polyunsaturated fatty acid deficiency that ATP/O ratio was unchanged under ADP saturating concentrations (state 3) whereas it was decreased when measured in a more physiological manner with hexokinase-regenerating system and non-saturating ADP concentrations [16,25]. In intact liver cells isolated from PUFA deficient animals we have previously reported a similar respiratory rate associated with a significant decrease in cellular ATP/ADP ratio [26], which is in agreement with the decreased ATP/O ratio measured with the hexokinase method in mitochondria isolated from PUFA-deficient rats [25]. This led us to consider this hexokinase method to be more appropriate. Indeed as compared to the hexokinase system, state 3 ADP concentration results in very low mitochondrial phosphate and membrane potentials and in a very high ATP synthesis rate, a particular condition where energy wastes are kept artificially low [13,25].

A change in proton leak across the mitochondrial membrane due to ethanol exposure can be excluded since (i) there was a unique relationship between respiratory rate and proton-motive force in state 4 (Fig. 1) and (ii) no difference was seen when coupling site II was functionally isolated. It has been recently demonstrated that cytochrome oxidase H^+/e^- stoichiometry is variable and represents a main location for energy waste at the level of the respiratory chain [14,27]. We therefore conclude that ethanol feeding is responsible for an increased oxidative phosphorylation efficiency by decreasing

cytochrome oxidase content and activity. This was confirmed by the effect of sodium-azide, which was similar to ethanol responsible for an increased yield of oxidative phosphorylation.

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References

[1] Cunningham, C.C., Kouri, D.L., Beeker, K.R. and Spach, P.I. (1989) Alcohol Clin. Exp. Res. 13, 58–65.
[2] Cederbaum, A.I., Lieber, C.S. and Rubin, E. (1974) Arch. Biochem. Biophys. 165, 560–569.
[3] Murphy, M.P. and Tipton, K.F. (1992) Biochem. Pharmacol. 43, 2663–2667.
[4] Spach, P.I., Bottenus, R.E. and Cunningham, C.C. (1982) Biochem. J. 202, 445–452.
[5] Cunningham, C.C., Coleman, W.B. and Spach, P.I. (1990) Alcohol 25, 127–136.
[6] Hoek, J.B. (1994) Curr. Top. Bioenerg. 17, 197–241.
[7] Ribiere, C., Hininger, I., Saffar-Boccara, C., Sabourault, D. and Nordmann, R. (1994) Biochem. Pharmacol. 47, 1827–1833.
[8] Thayer, W.S. and Rubin, E. (1981) J. Biol. Chem. 256, 6090–6097.
[9] Thayer, W.S. (1987) Ann. NY Acad. Sci. 492, 193–206.
[10] Thayer, W.S. and Rubin, E. (1979) J. Biol. Chem. 254, 7717–7723.
[11] Brand, M.D., Chien, L., Ainscow, E.K., Rolfe, D.F.S. and Porter, R.K. (1994) Biochim. Biophys. Acta 1187, 132–139.
[12] Nicholls, D.G. (1974) Eur. J. Biochem. 50, 305–315.
[13] Fontaine, E., Devin, A., Rigoulet, M. and Leverve, X.M. (1997) Biochem. Biophys. Res. Commun. 232, 532–535.
[14] Papa, S., Capitanio, N., Capitanio, G., De Nitto, E. and Minuto, M. (1991) FEBS Lett. 288, 183–186.
[15] Pietrobon, D., Zoratti, M. and Azzone, G.F. (1983) Biochim. Biophys. Acta 723, 317–321.
[16] Rigoulet, M., Leverve, X., Fontaine, E., Ouhabi, R. and Guérin, B. (1998) Mol. Cell. Biochem. 184, 35–52.
[17] Rigoulet, M., Ouhabi, R., Leverve, X., Putod-Paramelle, F. and Guérin, B. (1989) Biochim. Biophys. Acta 975, 325–329.
[18] Rigoulet, M., Fraisse, L., Ouhabi, R., Guérin, B., Fontaine, E. and Leverve, X. (1990) Biochim. Biophys. Acta 1018, 91–97.
[19] Klingenberg, M. and Slenczka, W. (1959) Biochem. Z. 331, 486–495.
[20] Devin, A., Guérin, B. and Rigoulet, M. (1997) Biochim. Biophys. Acta 1319, 293–300.
[21] Devin, A., Guérin, B. and Rigoulet, M. (1996) Biochim. Biophys. Acta 1273, 13–20.
[22] Ohnishi, T., Kawagushi, K. and Hagihara, B. (1966) J. Biol. Chem 241, 797–806.
[23] Vanneste, W.H. (1966) Biochim. Biophys. Acta 113, 175–182.
[24] Bergmeyer, H.U. (1974), pp. 1238–1243, 2nd ed. Verlag Chemie Weinheim/Academic Press, New York.
[25] Fontaine, E., Moussa, M., Devin, A., Garcia, J., Ghisolfi, J., Rigoulet, M. and Leverve, X. (1996) Biochim. Biophys. Acta 1276, 181–187.
[26] Piquet, M.A., Fontaine, E., Sibille, B., Filippi, C., Keriel, K. and Leverve, X.M. (1996) Biochem. J. 317, 667–674.
[27] Capitanio, N., Capitanio, G., De Nitto, E., Villani, G. and Papa, S. (1991) FEBS Lett. 288, 179–182.