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Interactions of ethanol drinking with *n*-3 fatty acids in rats: potential consequences for the cardiovascular system

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Moderate ethanol drinking (ED) and *n*-3 fatty acids have both been associated with low cardiac mortality. However, there are few data evaluating the interactions of ED with *n*-3. We recently reported that moderate ED results in increased *n*-3 in cardiac patients. The main aim of the present study was, through a well-controlled experimental model, to confirm that chronic ED actually results in increased *n*-3. Secondary aims were to examine the effects of chronic ED on cardiac mitochondria, cardiac function and experimental myocardial infarction. We studied the fatty acid profiles of plasma, cell membranes and cardiac mitochondria phospholipids in a rat model of chronic ED. In plasma and cell membranes, ED actually resulted in higher *n*-3 ($P=0{\cdot}005$). In mitochondria phospholipids of ED rats, *n*-3 were also increased ($P<0{\cdot}05$) but quite modestly. Cardiac mitochondrial function and left ventricular function were not significantly different in ED and control rats, while infarct size after 30 min ischaemia and reperfusion was smaller ($P<0{\cdot}0001$) in ED rats. This is the first animal study confirming interaction of alcohol drinking with *n*-3. We found no harmful effect of chronic ED on the heart in that model but a significant cardioprotection. Further studies are warranted to investigate the mechanisms by which moderate ED alters the metabolism of *n*-3 and whether *n*-3 are the mediators of the ED-induced cardioprotection.

Alcohol: *n*-3 Fatty acids: Phospholipids: Myocardial infarction: Mitochondria

Moderate ethanol drinking (ED) induces cardioprotection in animals[1,2] and is associated with reduced cardiac mortality in human subjects[3,4]. The mechanisms of this protection have not been elucidated to date. We recently reported that wine ED results in increased blood levels of *n*-3 fatty acids in coronary disease patients[5]. Since *n*-3 induce cardioprotection in animals[6,7] and reduce cardiac mortality in human subjects[8], we proposed that part of the ED-induced cardioprotection may be mediated through increased *n*-3. If confirmed, this effect of ED on *n*-3 may have major implications for the prevention of CVD. However, this new theory raises several questions and needs confirmation. The best way to confirm our human data[5] would be a long-term controlled trial in human subjects. However, such a trial is neither technically feasible nor ethically acceptable[9]. Another way is to examine whether the ED-induced increase in *n*-3 is reproducible in animal experiments where most potential confounders encountered in human studies can be controlled. This was the main aim of the present study.

Other questions are whether the increase in *n*-3 (observed in plasma of drinkers) is also observed in cells or cell structures (such as cardiac mitochondria) presumably involved in the previously described ED-induced cardiac resistance to ischaemia–reperfusion[1,2]. Alternatively, we wanted to examine whether chronic exposure to ethanol (e.g. during 18 weeks) may result in some form of cardiac toxicity or mitochondrial dysfunction, a possibility that has never been investigated in previous ethanol–heart studies in animals. Finally, we wanted to verify whether the cardioprotection observed after a short exposure to ethanol[1] might still be present after chronic exposure. A last question regarded the effect of ED on specific *n*-3. In human subjects, ED resulted in increased EPA, while the effect on DHA was borderline non-significant[5]. Since these *n*-3 fatty acids have different biological properties and physiological effects, it was important to examine whether ethanol may interact differently with each of them.

The main aim of the present study was therefore to confirm that chronic ED actually results in increased *n*-3 levels in well-controlled animal experiments. We first studied the plasma and cell membranes. We studied the membranes of erythrocytes, because of their relatively short half-life (compatible with dietary protocols in animals) and because they are known to reflect the fatty acid composition of cardiac

Abbreviations: ED, ethanol drinking; LV, left ventricular.

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cells[10]. We also studied the fatty acid composition of phospholipids in cardiac mitochondria with the objective to investigate whether the increase in *n*-3 may explain the protective effect of ED. We then examined the apparent contradiction that ED could be protective for the heart[1,2], possibly through an effect on mitochondria[11], and harmful for the mitochondria, as reported in specific contexts[12–14]. Indeed, the mitochondria seem to play critical roles in the ability of the heart to withstand ischaemia–reperfusion injuries[15,16]. Finally, we studied global cardiac function and mitochondrial function with a focus on functional coupling between mitochondrial creatine kinase and adenine nucleotide translocase[17]. Using an isolated heart model (which allows the study of the myocardium in a way that is independent of circulating blood cells and of non-cardiac factors such as neural and hormonal regulation), we examined the effect of chronic ED on the response of the heart to regional myocardial ischaemia and reperfusion.

Materials and methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals,* published by the US National Institute of Health, NIH Publication no. 85–23, revised 1996, National Academic Press, Washington, DC.

An animal model of chronic ethanol drinking

Male Wistar rats (IFFA Credo, France) were divided into an ED group (*n* 92) and an age-matched, water-drinking control group (*n* 68). For each experiment, rats were randomly distributed into the control water-drinking or ED groups. In the first experiments evaluating the effects of ED on *n*-3, sixty-one rats (twenty-one controls and forty ED) were available to study the effects of 7-week ED and thirty-eight rats (eighteen controls and twenty ED) were available to study the 18-week ED. To study the effects of ED on infarct size and left ventricular (LV) function, nineteen rats (ten controls and nine ED) were available and to study the effects of ED on mitochondria twenty-four rats (twelve controls and twelve ED) were available. After including the rats lost for any reason before the end of each experiment, a total of 160 rats were used for that study. ED rats received 6 % or 12 % (v/v) ethanol in their drinking water for 7 or 18 weeks. Such concentrations of ethanol were not expected to change the drinking habits of rats[1]. That way of ethanol supplementation was preferred to gavage because rats are known to spontaneously regulate the amounts of ethanol they drink without being sick or inducing toxicity. To quantify the amounts of ethanol actually drunk by the rats, daily water or alcohol drinking was monitored in each group. All animals received standard solid food (UAR, France) *ad libitum*. The same food pellet formula was used in all groups of rats. The intakes of solid food and beverages were also monitored in each group in order to calculate total energy intake, energy intake from solid food and energy intake from ethanol per d per rat in each group of rats. It was also possible to calculate the average intake of specific fatty acids, including plant and marine *n*-3, in each group. The rats were weighed once per week and housed under conditions of constant temperature, humidity and standard light–dark cycle.

Experiments to study the response of the heart to ischaemia and reperfusion

Heart preparation and perfusion were carried out according to described methods[1]. Briefly, the rats were anaesthetized with sodium pentobarbital and heparinized. Their hearts were excised, canulated via the aorta, paced at 5 Hz (300 beats per min) and infused at a constant pressure of 9·81 kPa using the Langendorff mode with Krebs–Henseleit crystalloid buffer[1]. The hearts were equilibrated with a mixture of O_2/CO_2 (95 %/5 %) at 37°C, pH 7·4. LV pressure was measured with an intraventricular non-compliant balloon (initial diastolic pressure 4 mmHg). A 5-0 silk snare was passed under the left coronary artery at 1 to 2 mm from its origin and the artery was occluded for 30 min. After 120-min reperfusion, the ligation was retightened and a solution of Evans blue was injected to delineate the risk zone. Infarct size was measured using triphenyl tetrazolium chloride staining as described[1]. The risk zone was expressed as a percentage of total ventricular volume and infarct size as percentage of risk zone.

Mitochondria preparation and respiration tests

Cardiac mitochondria were isolated as described[18]. Briefly, LV tissue was minced, washed, suspended in an isolation medium (10 ml)[19], subjected to mild trypsin digestion for 30 min at 4°C and then diluted in the isolation medium containing bovine serum albumin and trypsin inhibitor. The homogenate was centrifuged for 10 min at 600 ***g*** and the supernatant was decanted and centrifuged at 8000 ***g*** for 15 min to obtain a mitochondrial pellet. The upper layer was discarded and the tightly packed dark pellet was resuspended and washed three times. For lipid analyses, the mitochondria were purified on a discontinuous gradient containing 6 % Percoll and 17 % and 35 % metrizamide[19]. After 30 min centrifugation at 50 500 ***g***, a narrow band was recovered to sediment mitochondria for 10 min at 8000 ***g***. The respiration rates of isolated cardiac mitochondria were measured polarographically in a specific medium as described[20]. The reaction was initiated by the addition of mitochondria. After adding respiratory substrates (5 mM-glutamate, 2 mM-malate), increasing amounts of ATP were added. The stimulatory effect of ATP was calculated from the respiration rates measured in the presence of a given concentration of ATP minus the value in the absence of ATP (state 2). The half saturation constant for ATP ($K_{1/2}$ (ATP)) and the V_{max} value in the presence and absence of 20 mM-creatine were calculated from double-reciprocal plots of the dependence of respiration on the concentration of ATP[20]. Functional coupling between adenine nucleotide translocase and mitochondrial creatine kinase was expressed by the ratio: $(V_{max}/K_{1/2})$cr/$(V_{max}/K_{1/2})$ATP, in which $(V_{max}/K_{1/2})$cr is the catalytic efficiency of the stimulation of respiration by ATP in the presence of 20 mM-creatine and $(V_{max}/K_{1/2})$ATP is the catalytic efficiency of the stimulation of respiration by ATP in the absence of creatine[17,20].

Lipid and fatty acid analyses

Plasma lipids and erythrocyte phospholipids were extracted in hexane–isopropanol as described[21]. Fatty acids were first

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methylated; after extraction with hexane, methyl esters were separated and quantified by GC (Hewlett Packard 5890 Series Gas Chromatography). Methyl ester peaks were identified by comparing their retention times to those of a standard mixture. Each fatty acid, including palmitate, and each *n*-3 fatty acid was expressed as a percentage of total fatty acids.

Mitochondrial lipids were extracted in hexane–isopropanol and dissolved in chloroform–methanol containing cholesteryl acetate as an internal standard. Total phospholipids, neutral lipids and the internal standard were separated by TLC followed by flame-ionization detection in an Iatroscan TLC-FID (Iatron Laboratory, Tokyo, Japan).

Fatty acid composition of mitochondrial phospholipids

After lipid extraction, mitochondrial phospholipids were separated from the total lipid extract by TLC on silica gel plates with a chloroform–methanol–water solvent. Phosphatidylcholine, phosphatidylethanolamine and cardiolipin spots were scraped directly into test tubes, dissolved in chloroform–methanol, evaporated under N_2 and mixed with 0·1 mg/ml heptadecanoic acid. The same method used for blood fatty acids analysis was then applied.

Statistics

Data are expressed as means with their standard errors. The measurements were analysed by ANOVA with inter-group differences tested by a *post hoc* application of Tukey's test and by Student's *t* test when comparing two groups. Repeated-measures ANOVA was used to compare the evolution of body weight in the different groups of rats. $P<0·05$ was considered significant.

Results

Effects of 7-week ethanol drinking on plasma fatty acids

We first studied the effects of 6 % and 12 % ED on plasma lipids and fatty acid composition over 7 weeks with the aim to confirm the effect of ED on *n*-3 observed in our human study[5]. Along the 7 weeks, total energy intake was not significantly different in the three groups; 419·7 (SEM 38·0), 360·3 (SEM 35·1) and 349·4 (SEM 40·9) kJ/d per rat respectively in the control, 6 % and 12 % ED groups. The mean consumption of pure ethanol was of 0·9 (SEM 0·1) and 1·7 (SEM 0·1) g/d per rat in the 6 % and 12 % ED groups, corresponding to 7·5 (SEM 0·6) and 14·6 (SEM 0·4) % total energy intake. Total lipid intake was (not significantly) higher in control rats (723·2 (SEM 65·2) mg/d per rat) compared with drinkers (574·5 (SEM 58·4) and 515·4 (SEM 66·3) in the 6 % and 12 % ED rats) while plant *n*-3 intake (α-linolenic acid, 27·5 (SEM 2·5) mg/d per rat in controls *v.* 21·8 (SEM 2·2) and 19·6 (SEM 2·5) in the 6 % and 12 % ED groups) and marine *n*-3 intake (EPA 8·0 (SEM 0·7), 6·3 (SEM 0·6) and 5·7 (SEM 0·7); DHA 13·0 (SEM 1·2), 10·3 (SEM 1·1) and 9·3 (SEM 1·2) mg/d per rat) were slightly (but not significantly) higher in controls compared with the two groups of drinkers. As expected, ED had no significant effect on body weight (Fig. 1 (A)) but had significant effects on blood lipids with lower cholesterol and TAG levels with higher ethanol

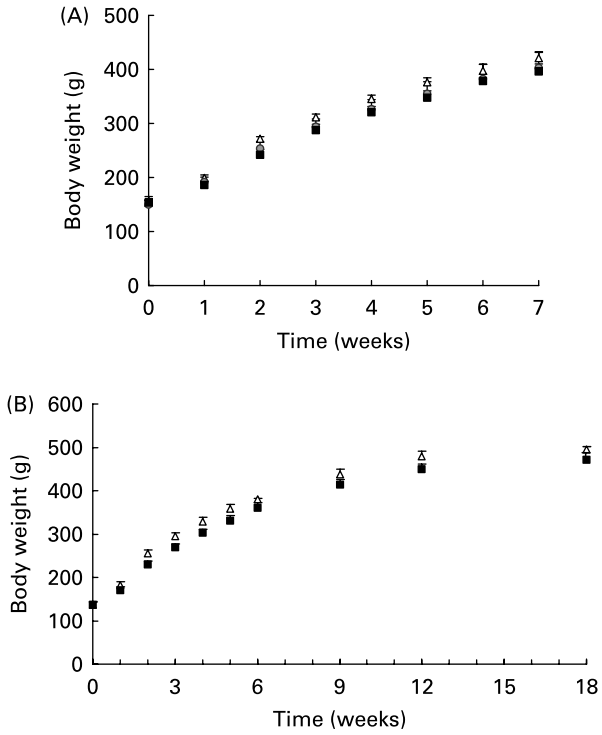

Fig. 1. Effect of 7-week ethanol drinking (ED) (6 and 12 %) (A) △, controls (*n* 21); ●, ethanol 6 % (*n* 20); ■, ethanol 12 % (*n* 20) and of 18-week ED (12 %) (B) △, controls (*n* 18); ■, ethanol (*n* 20).

($P<0·05$ when comparing the control group with either the 6 % or the 12 % ED group). Regarding fatty acids (Table 1), there was a significant increase in total *n*-3 ($P=0·0007$) with a progressive increase in EPA (+65 %, $P=0·0001$) at increasing ethanol doses. For total SFA, total *n*-3 and EPA, the differences were significant ($P<0·05$) when comparing the control group with either the 6 % or the 12 % ED group. The increase in DHA was less consistent but still highly significant (+19 %, $P=0·0007$), although there was no statistically significant difference between the controls and the 6 % ED group (Table 1). The increase in *n*-3 was at the expense of SFA ($P=0·007$). In view of these results, with a significant effect on plasma DHA seen only with the 12 % dose, we decided to select the 12 % dose of ethanol for an 18-week test (corresponding to the half-life of erythrocytes) to study the effect of ED on cell membrane *n*-3. We used the same model to study mitochondrial function, global LV function and the heart response to regional ischaemia and reperfusion.

Effects of 18-week ethanol drinking

We examined the effects of 12 % ED on erythrocyte fatty acids. The use of cardiac tissue was reserved for the isolation and functional study of mitochondria. In that model of long-term chronic drinking, results were quite different (compared with the 7-week experiment) with a significantly ($P<0·0005$) lower total energy intake among drinkers compared with controls (293·1 (SEM 4·6) kJ/d per rat *v.* 348·6 (SEM 5·0)). The mean consumption of ethanol was 1·7 (SEM 0·1) g/d per rat of pure ethanol corresponding to 17·1 (SEM 0·5) % total energy intake. Also, the intake of plant *n*-3 was significantly

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Table 1. Blood lipids and plasma fatty acids (expressed as % total fatty acids) after 7 weeks of ethanol drinking (6 % and 12 %, v/v) *v.* water-drinking controls*

(Mean values with their standard errors)

	Controls (*n* 21)		Ethanol 6 % (*n* 20)		Ethanol 12 % (*n* 20)		
	Mean	SEM	Mean	SEM	Mean	SEM	*P* ANOVA
Blood lipids (g/l)							
Total cholesterol	0·64	0·02	0·53	0·02	0·47	0·02	0·0001
HDL-cholesterol	0·32	0·01	0·32	0·01	0·26	0·02	0·04
TAG	1·40	0·06	1·24	007	1·03	0·08	0·005
Plasma fatty acids (%)							
Total SFA	29·4	0·4	26·1	0·8	27·8	0·4	0·007
Total MUFA	25·0	0·7	26·5	1·4	24·0	1·4	0·20
Total PUFA	46·1	0·9	46·9	1·6	48·4	1·5	0·20
Total *n*-6	41·3	0·8	41·6	1·4	42·4	1·4	0·40
Total *n*-3	4·7	0·2	5·3	0·3	6·0	0·2	0·0007
18 : 3*n*-3 (ALA)	1·00	0·06	1·30	0·12	1·20	0·09	0·30
20 : 5*n*-3 (EPA)	0·49	0·02	0·63	0·05	0·81	0·06	0·0001
22 : 5*n*-3 (DPA)	0·56	0·04	0·66	0·05	0·77	0·03	0·01
22 : 6*n*-3 (DHA)	2·69	0·11	2·69	0·13	3·20	0·08	0·0007

ALA, α-linolenic acid.
* For details of animals and procedures, see Materials and methods.

($P<0·0001$) lower in drinkers (15·2 (SEM 0·3) mg/d per rat) compared with controls (22·8 (SEM 0·4)) and the intake of marine *n*-3 (EPA 4·4 (SEM 0·1) *v.* 6·6 (SEM 0·1) mg/d per rat and DHA 7·2 (SEM 0·1) *v.* 10·8 (SEM 0·2)) was significantly ($P<0·0001$) lower in drinkers. However, there was no significant difference between groups for body weight (Fig. 1 (B)), while the weight of the rats tended to reach a plateau after the eighth week of follow-up. This slower growth of the rats after the eighth week probably explains why the average energy intake was lower in the 18-week experiments than in the 7-week experiments. This also explains why the same 12 % ethanol dose resulted in relatively higher ethanol intake (when expressed as % energy intake) in the 18-week than in the 7-week experiments. We found a significant ($P=0·007$) increase in total *n*-3 in erythrocyte (Table 2). The increase was less impressive than in plasma with a borderline non-significant increase for EPA and a 10 % increase for DHA ($P=0·02$).

We then studied the effects of 12 % ED on cardiac mitochondrial fatty acids. We found significant increases (Table 3) in total *n*-3 in cardiac phosphatidylcholine and cardiolipin ($P<0·05$ and $P<0·0005$). The main effects ($P<0·01$) were seen for DHA (+48 %) and α-linolenic acid (+60 %) in cardiolipin.

We finally studied the effect of 12 % ED on the respiration rate of isolated cardiac mitochondria. Before the functional coupling experiments, cytochrome c tests were carried out, showing that, in all cases, the prepared mitochondria had an intact outer membrane[18,20]. Fig. 2 shows double-reciprocal plot linearization of the dependence of respiration rate on the concentration of ATP and the effect of creatine on respiration. No difference was observed between groups in either case. The creatine-induced decrease in the apparent $K_{1/2}$ (Table 4) indicates the preservation of functional coupling between adenine nucleotide translocase and mitochondrial creatine kinase and the absence of significant effect of 12 % ED. A quantitative estimate of functional coupling is the ratio $(V_{max}/K_{1/2})$cr:$(V_{max}/K_{1/2})$ATP. This ratio was not different in the ED and control groups (3·4 (SEM 0·1) and 3·2 (SEM 0·2) respectively).

Effects of 12 % ethanol drinking on left ventricular function and regional ischaemia–reperfusion injury

We studied global LV function after 18 weeks at baseline (Table 5), during ischaemia (data not shown) and after a 30 min regional ischaemia followed by reperfusion (Table 6). We found no difference between groups at any time, indicating that 12 % chronic (18-week) ED was apparently not toxic for the myocardium. In addition (Fig. 3), infarct size was reduced by ED (30·2 (SEM 2·0) % of risk zone against 48·4 (SEM 2·8) % in controls; $P<0·0001$) confirming that the myocardium of ED rats is more resistant to ischaemia and

Table 2. Blood lipids and erythrocyte fatty acids (as % total fatty acids) after 18 weeks of ethanol drinking (12 %, v/v) *v.* water-drinking controls*

(Mean values with their standard errors)

	Controls (*n* 18)		Ethanol 12 % (*n* 20)		
	Mean	SEM	Mean	SEM	*P*
Blood lipids (g/l)					
Total cholesterol	0·56	0·03	0·51	0·02	0·16
HDL-cholesterol	0·37	0·01	0·38	0·02	0·82
TAG	1·69	0·12	1·03	0·09	0·007
Erythrocyte fatty acids (%)					
Total SFA	38·7	0·5	36·6	0·3	0·0007
Total MUFA	11·6	0·5	11·7	0·8	0·89
Total PUFA	49·7	0·7	51·7	0·4	0·01
Total *n*-6	42·2	0·6	43·6	0·4	0·04
Total *n*-3	7·4	0·1	8·1	0·1	0·007
18 : 3*n*-3	0·20	0·02	0·17	0·01	0·28
20 : 5*n*-3	0·76	0·04	0·86	0·04	0·08
22 : 5*n*-3	2·01	0·06	2·18	0·05	0·05
22 : 6*n*-3	4·52	0·08	4·89	0·11	0·02

* For details of animals and procedures, see Materials and methods.

Table 3. Individual fatty acids (as % of total fatty acids) in cardiac mitochondria phospholipids after 18-week ethanol drinking *v.* water-drinking controls†

(Mean values with their standard errors)

	Phosphatidylcholine				Phosphatidylethanolamine				Cardiolipin			
	Controls (*n* 6)		Ethanol 12 % (*n* 6)		Controls (*n* 6)		Ethanol 12 % (*n* 6)		Controls (*n* 6)		Ethanol 12 % (*n* 6)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
SFA	46·70	0·70	46·90	0·40	41·00	1·30	42·20	0·50	12·80	0·60	13·20	0·50
MUFA	12·60	0·50	13·00	0·40	9·10	0·60	8·90	0·20	8·80	0·20	8·20	0·30
Total *n*-6	37·60	0·90	36·10	0·50	30·90	0·30	28·80	0·60	77·20	0·70	77·00	0·60
Total *n*-3	2·80	0·20	3·50*	0·20	18·70	0·50	19·70	0·90	0·80	0·02	1·10**	0·05
18 : 3*n*-3	0·08	0·01	0·14*	0·02	0·13	0·01	0·17	0·02	0·15	0·01	0·24**	0·01
20 : 5*n*-3	0·29	0·04	0·32	0·04	0·30	0·02	0·32	0·02	0·19	0·02	0·17	0·01
22 : 5*n*-3	0·63	0·06	0·71	0·06	1·98	0·04	1·90	0·05	0·21	0·01	0·28	0·03
22 : 6*n*-3	1·82	0·09	2·37*	0·11	16·28	0·43	17·30	0·90	0·29	0·02	0·43**	0·01

Mean values were significantly different from controls: *$P<0·05$; **$P<0·0005$.
† For details of animals and procedures, see Materials and methods.

reperfusion than the myocardium of non-drinker rats even after long-term (18 weeks) chronic exposure.

Discussion

The main objective of the present study in rats was to examine whether chronic moderate ED may influence *n*-3 blood and cell levels, an effect already reported in a human study and requiring confirmation[5]. A period of 7-week ED actually resulted in higher plasma *n*-3 levels (+65 % for EPA, +19 % for DHA) in rats. Thus, the fish-like effect of moderate ED appeared to be easily reproducible in a well-controlled experimental model. As recently discussed, this may have major clinical implications because such increases in blood *n*-3 levels are probably associated with cardioprotection and reduced cardiac mortality in patients[5,8,22–24].

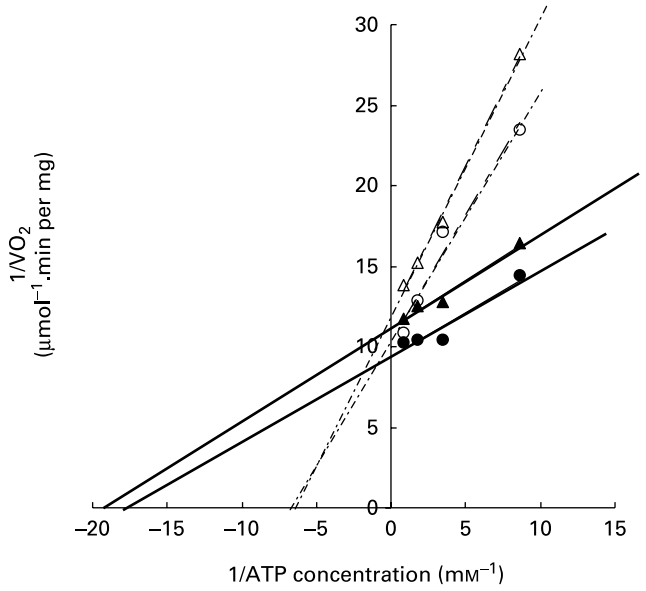

Fig. 2. Dependence of respiration on ATP concentration. Double-reciprocal plots in the presence or absence of creatine. ○, controls; ●, controls + creatine; △, ethanol drinking (ED) group; ▲, ED group + creatine.

Ethanol, lipids and essential fatty acids

In the present study, ED resulted in a highly significant increase in DHA levels, an effect that was borderline non-significant in our previous human study[5]. Thus, the effect of ED on *n*-3 seems not to be confined to EPA, at least in rats. This is important for future research because DHA has specific endogenous metabolism and biological and physiological effects compared with EPA. In contrast, ethanol had no significant effect on α-linolenic acid (the main plant *n*-3) and on linoleic acid (the main plant *n*-6) while the latter was decreased in our human study in response to wine drinking[5]. Since the main difference between our study in patients and the present study in rats is that the rats were drinking pure ethanol (while our French patients were essentially drinking wine), we cannot exclude the possibility that non-ethanolic components of wine also interact with the metabolism of essential fatty acids, a hypothesis that requires further investigation. These data nonetheless suggest that the overall effect of ethanol on essential PUFA may be different in rats and human subjects. This is not surprising as cholesterol and TAG, two other major blood lipids, tended to decrease in rat plasma while they are known to increase in human subjects in response to ED[25]. Thus, extrapolating the effect of ED on fatty acids in rats to human subjects requires some caution. In that context, the very similar effects of ED on *n*-3 in human subjects and rats and in the different experiments reported in the present study with different doses of ethanol, different durations of exposure and different biological measurements (cardiac mitochondria, erythrocyte membranes and plasma) strongly support the theory of a fish-like effect of ED[5].

The less impressive increase in marine *n*-3 (EPA and DHA) in cell membranes and mitochondrial phospholipids than in plasma was not unexpected, considering the quite short duration of exposure (18 weeks) in the current study, the well-known tight regulation of fatty acid metabolism in cell membrane phospholipids[26–28] and the fact that in the 18-week experiments the dietary intakes of both plant and marine *n*-3 were significantly lower among drinkers because of the reduced solid food intake. All these things, in particular the lower intakes of EPA and DHA, might have weakened the

Table 4. Apparent $K_{1/2}$ (μM) and V_{max} (nmol O_2/min per mg) of ATP for respiration in the presence or in absence of 20 mM-creatine in control and ethanol drinking (18 weeks, 12 %) rats*

(Mean values with their standard errors)

	ATP					ATP + creatine				
	Controls (*n* 6)		Ethanol (*n* 6)			Controls (*n* 6)		Ethanol (*n* 6)		
	Mean	SEM	Mean	SEM	*P*	Mean	SEM	Mean	SEM	*P*
Apparent $K_{1/2}$	154	11	158	12	0·66	52	8	49	4	0·74
V_{max}	97	12	83	13	0·24	106	12	88	11	0·09

* For details of animals and procedures, see Materials and methods.

effect of ED on *n*-3 levels and hence indirectly confirms that ED actually results in a quite strong effect on marine *n*-3.

Ethanol and the heart

Our knowledge about the effect of ED on cardiac function remains elusive[29]. A second aim of this study was therefore to examine the direct effect of chronic moderate ED on the heart of rats. For that purpose, we used an isolated heart model independent from the confounding effects of ethanol on the brain–heart neurological connections and on other organs (liver) or blood components (HDL, platelets, leucocytes, hormones) potentially involved in the resistance (or sensitivity) of the heart to ischaemia and reperfusion.

We found no effect of chronic ED on global cardiac function before, during ischaemia and after 30 min of reperfusion. Thus, at least in that model, chronic ED was apparently not toxic for the myocardium. This is not unexpected because in that 12 % dose model, the real consumption of ethanol over the 18-week period was calculated to represent about 17 % of total energy intake. According to our human data[29,30], it is unlikely that such doses of ethanol could result in myocardial toxicity.

On the other hand, infarct size was actually reduced after 18-week 12 % ED. This was a confirmation of our previous data with a shorter (7-week) exposure to ethanol and a lower (9 %) ethanol dosage[1].

The important changes in *n*-3 levels in the plasma of ED rats cannot explain the state of myocardial resistance to ischaemia and reperfusion, the so-called 'ethanol preconditioning'[1], since we used an isolated heart model with no plasma in the system. Whether the alterations in the fatty acid composition of cardiac cells and mitochondrial phospholipids may have mediated the responses to ischaemia and reperfusion is the next question. At first glance, it seems difficult to explain this resistance to ischaemia–reperfusion by the small increases in cell membrane *n*-3 recorded in ED rats. This does not mean that the increase in *n*-3 does not play any role in the reduced cardiac mortality more generally observed in human drinkers (compared with abstainers), especially the reduced risk of sudden cardiac death[3,4]. Higher *n*-3 plasma levels, with higher amounts of free *n*-3 available to preserve some critical cell structures during ischaemia[8], may explain part of the clinical protection. It is nonetheless difficult (from these data) to claim that the reduction in infarct size is mediated through the small increases in cell membrane *n*-3 observed in the current study and further studies are needed to solve the question.

Moderate ethanol drinking and cardiac mitochondria

A third aim of this study was to investigate the effect of ED on mitochondrial function. In fact, our question was: can chronic ED be protective for the heart in case of ischaemia–reperfusion[1,2] and at the same time harmful for the mitochondria as previously described[12–14]? There is no evidence to support this apparent contradiction, since we have found no harmful effect of chronic ED on the mitochondrial respiration rate. On the other hand, these changes in mitochondria *n*-3 did not result in significant changes in respiration rate. Are these data in line with the current literature about the effects of marine *n*-3 on mitochondrial function? In fact, there is little

Table 5. Left ventricular function and coronary flow at baseline in the 12 %-ethanol drinking rats and controls after 18 weeks*

(Mean values with their standard errors)

	Controls (*n* 10)		Ethanol (*n* 9)		
	Mean	SEM	Mean	SEM	*P*
Coronary flow (ml/min)	15·40	0·74	14·28	0·76	0·31
Diastolic pressure (mmHg)	4·85	0·19	5·07	0·39	0·19
LVDevP (mmHg)	139·0	5·8	127·7	6·8	0·52
+dP/dt (mmHg/s)	4391	201	3848	143	0·06
−dP/dt (mmHg/s)	2583	142	2243	87	0·06

LVDevP, left ventricular developed pressure.
* For details of animals and procedures, see Materials and methods.

Table 6. Left ventricular function recovery and coronary flow after 30 min of reperfusion in the 12 %-ethanol drinking rats and controls after 18 weeks*

(Mean values with their standard errors)

	Controls (*n* 10)		Ethanol (*n* 9)		
	Mean	SEM	Mean	SEM	*P*
Coronary flow (% baseline)	78·5	2·4	84·0	3·5	0·96
Diastolic pressure (mmHg)	16·50	3·91	16·84	3·63	0·95
LVDevP (% baseline)	72·2	3·8	74·3	4·8	0·74
+dP/dt (% baseline)	67·4	4·7	77·5	4·8	0·15
−dP/dt (% baseline)	72·8	3·8	73·4	5·0	0·93

LVDevP, left ventricular developed pressure.
* For details of animals and procedures, see Materials and methods.

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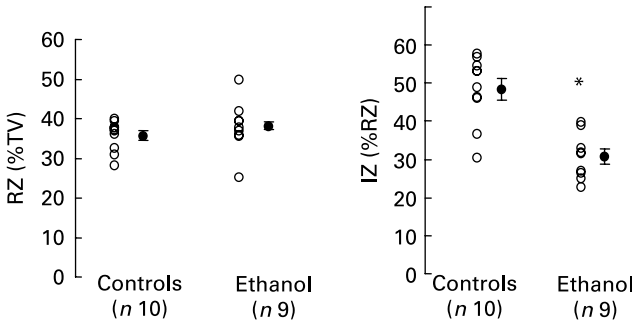

Fig. 3. Risk zone (RZ) and infarct size (IZ) after 18 weeks of 12 % ethanol drinking (ED) *v.* controls. RZ was 35·7 (SEM 1·2) % total ventricular volume (TV) in controls and 38 (SEM 2·2) % in ED rats (NS). Individual data and group means with their standard errors. *Mean values were significantly different; $P<0·0001$.

agreement among the various studies. Certain studies report no effect[31] while others describe a decreased[32] or an improved[33] function. However, the increases in *n*-3 levels were much larger in these previous studies than in the present one. Thus, our own data suggest that the small changes in mitochondrial *n*-3 levels following chronic ED probably had no significant effect on the mitochondrial respiration rate, at least in normoxic conditions. Also, the myocardial resistance to ischaemia–reperfusion resulting from chronic ED was apparently not mediated by quantitative changes in mitochondrial function as measured in normoxic conditions. Whether the increases in cardiac *n*-3 in the present study were sufficient to induce protection during ischaemia–reperfusion is questionable. While the increases in marine *n*-3 levels in mitochondrial phospholipids were highly significant, the relative contribution of each marine *n*-3 to total fatty acids remained small (less than 0·5 % total fatty acids in cardiolipin, for instance) and their potential role during ischaemia might be speculative. On the other hand, *n*-3 are molecules with potent biophysical properties that can interdigitate, even at low molecular percent concentrations, into specific micro domains of biological membranes (e.g. ion channels) and exert potentially profound effects on heart cell physiology[7,8] in the case of ischaemia even at very low concentrations[34]. Thus, the assumption of a protective effect resulting from small changes in *n*-3 is not unrealistic. Further studies are required to examine that issue, knowing that we need to exactly mimic the complex *in vivo* environment that mitochondria experience during ischaemia and reperfusion. A major challenge is to evaluate the function of mitochondria in their cellular environment, an approach that has been limited so far by major technical difficulties.

Limitations

Some limitations of the present study deserve mention. First, as discussed earlier, we did not investigate the effect of chronic ED on mitochondrial function during ischaemia and reperfusion. Thus, we cannot say that the respiration rate of cardiac mitochondria of ED rats was not different from that of control rats during ischaemia. In fact, our aim was to study mitochondria in the absence of ischaemia, because ethanol has been reported to damage mitochondria even in the absence of ischaemia[11–13]. Using a specific protocol of chronic ED, we just cannot confirm the harmful effect of ethanol on cardiac mitochondria. However, our studies on isolated cardiac mitochondria are very useful to study structural (fatty acid composition) and (potentially detrimental) functional changes induced by *in vivo* chronic ED with various oxygen concentrations.

Second, the current study was not designed to fully explore the potential mechanisms by which chronic ED may induce protection against ischaemia and reperfusion[15,16]. Alternative pathways, such as the permeability transition pore, the opening of which is sensitive to fatty acids[35], should be investigated in future studies.

Third, a major question is in regard to the mechanism(s) by which chronic ED results in increased *n*-3 levels. Further studies are warranted to understand at which level of their respective metabolisms (intestinal absorption, regulation of lipolysis and lipogenesis, whole-body fuel utilization, regulation of *n*-3 elongation and desaturation) ethanol and *n*-3 interact.

In conclusion, we have shown for the first time in rats that chronic ED results in increased *n*-3 levels in plasma, cell membranes and cardiac mitochondria phospholipids. This confirms the fish-like effect of ED reported in a previous human study[5]. These changes were associated with protection against ischaemia–reperfusion, but not with a significant effect on mitochondrial function measured in normoxic conditions. Further studies are required to investigate whether *n*-3 are the mediators of the ED-induced cardioprotection.

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