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Original Article

Cardioprotective effect of chronic low dose ethanol drinking: Insights into the concept of ethanol preconditioning

Annabelle Guiraud, Michel de Lorgeril *, François Boucher, Corinne Berthonneche, Andry Rakotovao, Joël de Leiris

Laboratoire Nutrition, Vieillissement et Maladies Cardiovasculaires (NVMCV), Université Joseph Fourier, Grenoble, France

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Abstract

The reason why low-to-moderate alcohol drinking is associated with reduced cardiovascular mortality is not elucidated. *While data suggested that ethanol drinking may have a protective effect on global cardiac ischemia, the effect of chronic low dose ethanol drinking (CLEthD) on myocardial infarct size has not been evaluated in a model of regional ischemia*. Using an isolated rat heart model to exclude the effect of various *in vivo* confounders, we have studied the effect of CLEthD on infarct size (IS) and left ventricular function after 30 min of regional ischemia and 120 min of reperfusion. The effect of CLEthD was compared with ischemic preconditioning (IPC) and protein kinase C (PKC) isoforms were analysed in the myocardium before the 30-min ischemia. Ethanol-fed rats received 9% (v/v) ethanol in their drinking water for 7 weeks. Four groups of rats were studied: (1) control, (2) ethanol, (3) control + IPC, (4) ethanol + IPC. Compared with controls (59 ± 10), IS (as percent of risk zone) was smaller in the ethanol (39 ± 6) and IPC (31 ± 8) groups (both $p < 0.05$). Combination of ethanol and IPC in the same rats further decreased IS (–46% vs. ethanol, $p < 0.05$). PKC analyses did not show sustained ε isoform translocation in that model. These data indicate that chronic low dose ethanol drinking actually induces in the rat heart a chronic protective state that is independent from an effect on the traditional (lipid and coagulation) risk factors. Further studies are required to elucidate the mechanisms of that protection.

Keywords: Ethanol; Alcohol; Coronary heart disease; Myocardial infarction; Left ventricular function; Ischemic preconditioning; Protein kinase C

1. Introduction

A number of human studies have reported decreased cardiovascular disease among moderate alcohol drinkers as compared with abstainers.[1-8] Even very low consumption of alcohol (one drink per week in certain studies) appears to be protective, which is quite difficult to explain through the "alcohol-lipid-haemostasis" theory. Indeed, the protective effect of alcohol has been principally explained by an effect on blood lipids [9] and platelets [10,11], with increases in high density lipoproteins (HDL) and decreased platelet aggregation, resulting in a reduced rate of coronary artery obstruction. Other mechanisms are probably involved. For instance, recent clinical studies have shown that moderate drinking may improve early outcome after acute myocardial infarction [12–14] and prevent sudden cardiac death [15], suggesting a direct effect of ethanol on the ischemic myocardium. In fact, chronic ethanol consumption may mimic the classical ischemic preconditioning (IPC) and protect against ischemia and reperfusion injury [16]. Investigators, however, failed [17,18] to reproduce the ethanol cardioprotection reported by others in various experimental settings [19–27]. These conflicting data might be related to differences in animal species, experimental model (global versus regional ischemia, acute versus chronic ethanol exposure) and ethanol dosage. Also, in most studies reporting protection, only cardiac function recovery or cardiac enzyme release was studied [19–25] and a reduction in the size of the infarcted tissue was not measured raising doubt on the reality of a direct effect of ethanol drinking (especially at low dosage) on the ischemic myocardium [17,18].

The primary aim of this study was therefore to investigate the effect of chronic low dose ethanol drinking (CLEthD) on both infarct size and left ventricular function recovery in a rat model of regional ischemia and reperfusion. The rat species was selected because it is omnivorous and known to metabolise ethanol in a way similar to that of humans [28]. We used

* Corresponding author: Dr. M de Lorgeril, NVMCV, UFR de Médecine et Pharmacie, Domaine de la Merci, 38706 La Tronche (Grenoble), France.
E-mail address: michel.delorgeril@ujf-grenoble.fr (M. de Lorgeril).

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an isolated heart model to study the specific effect of ethanol on the myocardium itself, independent from the confounding effects of ethanol on other organs (liver) or blood components (lipids, leukocytes, platelets). Our second aim was to compare the effect of CLEthD and IPC and to examine whether their combination would result in synergy or antagonism. In the same line of reasoning, we have investigated the changes occurring in the sub-cellular localisation of the protein kinase C (PKC) isoforms because activation and translocation of PKC are thought to be involved both in IPC [29–31] and in response to ethanol [21–25] although there is no data so far *regarding CLEthD*.

2. Methods

All procedures were in accordance with recommendations published in the *Guide for the Care and Use of Laboratory Animals*, National Academic Press, Washington, DC, 1996. Adult male Wistar rats (IFFA Credo, France) were divided into ethanol drinking group ($n = 42$) and age-matched water drinking controls ($n = 24$). Ethanol-fed rats received 9% (v/v) ethanol in their drinking water for 7 weeks. Pilot studies in our laboratory have indeed suggested that 7 weeks of drinking and 9% ethanol are the minimal duration and dosage to see a protective effect without inducing metabolic abnormalities. Because we suspected (after the pilot studies) a smaller effect of chronic low dose ethanol drinking (compared with ethanol infusion) on infarct size, the groups drinking ethanol were larger ($n = 17$) than those drinking water ($n = 8$). All animals received standard solid food (UAR, France) ad libitum. The average consumption of ethanol (and other foods) was checked every 4 days and expressed as percent of total energy intake. Rats were weighed once a week and housed under conditions of constant temperature, humidity and standard light-dark cycle (12h/12h).

Heart preparation and perfusion were carried out according to methods described[32–34] with all animals being sacrificed between 8 and 10 am. Briefly, rats were anaesthetized with pentobarbital sodium (Sanofi; 40 mg/kg, i.p.), and heparinized (Sigma; 100 UI/rat, i.v.). Hearts were excised, washed in cold (+4°C) Krebs-Henseleit buffer and canulated via the aorta. After removing sinus node, heart was paced at 5 Hz (300 bpm) via a monopolar electrode placed on the left atrial wall and connected to a stimulator (6021 SRI, UK) and perfused at a constant pressure of 9.81 kPa (1m H_20) using the Langendorff mode with Krebs-Henseleit crystalloid buffer (containing in mM: NaCl 118; KCl 4.75; $NaHCO_3$ 25; $MgSO_4.7\ H_20$ 1.19; K H_2PO_4 1.18; $CaCL_2.2H_20$ 1.36 and glucose 11.1) and equilibrated with a mixture of O_2/CO_2 (95%/5%) at 37°C, pH 7.4. LV pressure was measured with a transducer (Statham P23ID, Gould, France) connected to a non-compliant water-filled ultra thin balloon introduced into the LV cavity with volume adjusted to preset a baseline end-diastolic pressure of 4 mmHg.

2.1. Experimental protocol

Two series of experiments were performed: one for hemodynamic and infarct size measurements ($n = 50$), and one to analyse PKC ($n = 16$). The hemodynamic-infarct study was performed in the following groups: controls ($n = 8$), ethanol ($n = 17$), IPC ($n = 8$) and ethanol+IPC ($n = 17$). The four groups were similar for all aspects of the protocol except for ethanol in the drinking water. A 5-0 silk snare was passed under the left-coronary artery close to its origin. After 15-min equilibration period and normoxic perfusion, the left-coronary artery was occluded by tightening the snare for 30 minutes and then reperfused for 120 minutes. In the IPC group, prior to the 30-minute regional ischemia, the hearts were submitted twice to 5-minute global ischemia and 10-minute reperfusion. All hearts were kept at 37°C in a thermostatically controlled glass chamber throughout the experimental protocol. For each heart, myocardial function was recorded after 15-min stabilization and then every 10 minutes. Regarding the PKC study, myocardial samples were obtained just before the 30-minute regional ischemia in each group, quickly frozen in liquid nitrogen and stored at –80°C until use.

2.2. Risk zone and infarct size

After 120 minutes of reperfusion and retightening of the coronary snare, a solution of Evans Blue was injected through the aorta to delineate the non-stained risk zone. The hearts were then briefly frozen in liquid nitrogen and stored at –20°C. They were then cut into 6 to 7 transverse slices of 1 mm thickness. Slices were incubated in 1% triphenyltetrazolium chloride in sodium phosphate buffer at 37°C during 20 minutes to stain viable cells in the risk zone. Volume of infarct and risk zones was calculated using image software (NIH AutoExtractor 1.51). Risk zone was expressed as percent of total ventricular volume and infarct size as percent of risk zone.

2.3. PKC isoform assay

PKC activation is associated with translocation from the soluble to the particulate fraction [29–31]. So, to test whether cardioprotection induced by CLEthD is mediated by PKC activation in our model, we determined the sub-cellular distribution of α, δ and ε PKC in the soluble (S) and particulate (P) fractions of hearts from the 4 groups of rats. Subcellular fractionation study was made at 4°C. Frozen hearts (about 200 mg) were minced and homogenized in TEE buffer (50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 0,3% β-mercaptoethanol, 10 mM benzamidine and 50 μg/ml PMSF, pH 7,5) with a homogeniser at its maximal speed three times. Homogenates were centrifuged at 100,000 g for 60 min and the supernatant was frozen at –80°C. The pellet was resuspended in TEET buffer (TEE buffer with 0.2% Triton X-100), incubated for 60 min and centrifuged at

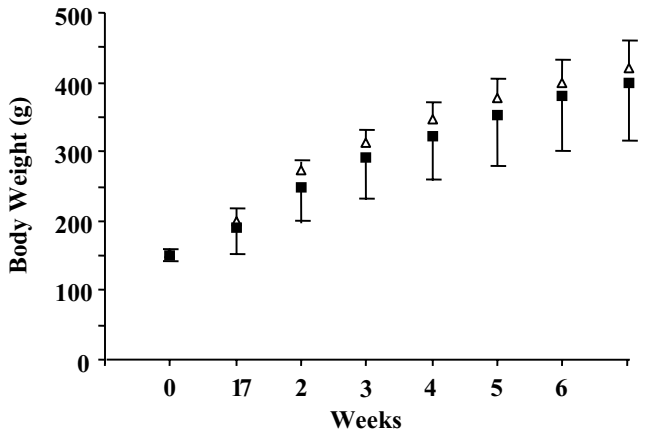

Fig. 1. Effect of ethanol drinking on body weight in ethanol-fed and control groups. **Open triangles**, control; **closed squares**, ethanol-fed rats.

100,000 g for 60 min. The supernatant was stored in ice and the pellet was resuspended in TEET buffer, incubated for 60 min and centrifuged at 100,000 g for 20 min. The TEE and TEET supernatants were designated as soluble (S) and particulate (P) fractions. The total protein concentration in each fraction was determined with a kit (Pierce, Rockford, UK). To quantitate PKC isoforms, the S and P fractions were boiled with 50% glycerol, 5% SDS, 250mM Tris base, 50mM Tris.HCl, 5% β-mercaptoethanol and 5% bromophenol blue, pH 6,8. Each fraction (15 μg) was loaded on 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane. The membrane was incubated with primary antibodies (Transduction Laboratories, France) for one night at 4°C. After washing in TBS-T buffer (20 mM Tris base, 137mM NaCl and 0,05% Tween 20, pH 7,4) with 1% non fat milk, the membranes were incubated with peroxidase-linked secondary antibodies (Interchim, France) for one hour. After being washed in TBS-T (4 times for 7 minutes each), PKC isoforms were detected with a chimiluminesent detection kit (Super Signal West Pico, Pierce). The amounts of PKC isoforms on the immunoblots were quantitated using image software (NIH AutoExtractor 1.51).

2.4. Statistics

Data are expressed as mean ± SEM. End points included cardiac function parameters and infarct size in the 4 groups. Measurements were analysed by ANOVA with between-group differences tested by post-hoc application of Tukey's test. For all tests, $p < 0.05$ was considered significant.

3. Results

As shown in Figure 1, the increase in body weight was not different in the ethanol-fed and control groups: 399 ± 83 g and 421 ± 40 g respectively after 7 weks. Also, there was no difference between groups in blood lipids (total cholesterol: 0.64 ± 0.09 g/L in controls and 0.51 ± 0.09 in the ethanol group; triglycerides: 1.47 ± 0.35 g/L vs. 1.63 ± 0.97 and HDL cholesterol: 0.32 ± 0.05 g/L vs. 0.29 ± 0.07 in the ethanol and control groups respectively) and blood ethanol levels averaged 1.7 ± 0.1 mg/dL in ethanol-fed rats at the time of sacrifice.

LV function at baseline during normoxic perfusion and before ischemia was not different in the ethanol and control groups (Table 1). After 120 min of reperfusion (Table 2), there was no significant difference between groups in coronary flow, LVDevP and diastolic pressure.

The risk zone was not different in the four groups (Figure 2A). As shown in Figure 2B, infarct size (as percent of risk zone) was significantly ($p < 0.05$) smaller in the IPC (31 ± 8) and ethanol (39 ± 6) groups compared with the controls (59 ± 10). The combination of ethanol and IPC further

Table 1
Left ventricular function at baseline in the four groups of rats

Experimental groups					
	Control (n = 8)	**Ethanol (n = 17)**	**IPC (n = 8)**	**Ethanol+IPC (n = 17)**	**ANOVA P**
LVDevP (mmHg)	130.2 ± 6.0	128.0 ± 2.0	117.8 ± 2.0	127.8 ± 3.2	0.34
Diastolic pressure (mmHg)	4.44 ± 0.26	4.29 ± 0.14	4.00 ± 0.00	4.18 ± 0.10	0.49
+dp/dt (mmHg/s)	6375 ± 683	5915 ± 235	5444 ± 383	5766 ± 237	0.93
–dp/dt (mmHg/s)	4531 ± 310	3929 ± 168	3838 ± 92	3909 ± 164	0.67
Coronary Flow (mL/s)	16.85 ± 0.36	15.24 ± 0.55	14.75 ± 0.67	15.82 ± 0.44	0.64

LVDevP: left ventricular developed pressure.

Table 2
Hemodynamics after 120 min of reperfusion in the four groups of rats

Experimental groups					
	Control (n = 8))	**Ethanol (n = 17))**	**IPC (n = 8)**	**Ethanol+IPC (n = 17)**	**ANOVA P**
LVDevP (mmHg)	66.3 ± 4.3	67.3 ± 2.4	56.6 ± 5.8	63.4 ± 2.1	0.16
Diastolic pressure (mmHg)	33.63 ± 2.88	29.47 ± 2.08	33.00 ± 7.93	23.77 ± 3.71	0.31
+dp/dt (mmHg/s)	3219 ± 305	3463 ± 248	2813 ± 246	3300 ± 167	0.37
–dp/dt (mmHg)	2519 ± 164	2175 ± 178	2006 ± 211	2206 ± 142	0.43
Coronary flow (mL/min)	10.00 ± 0.57	9.62 ± 0.57	8.94 ± 0.82	9.26 ± 0.45	0.73

LVDevP: left ventricular developed pressure.

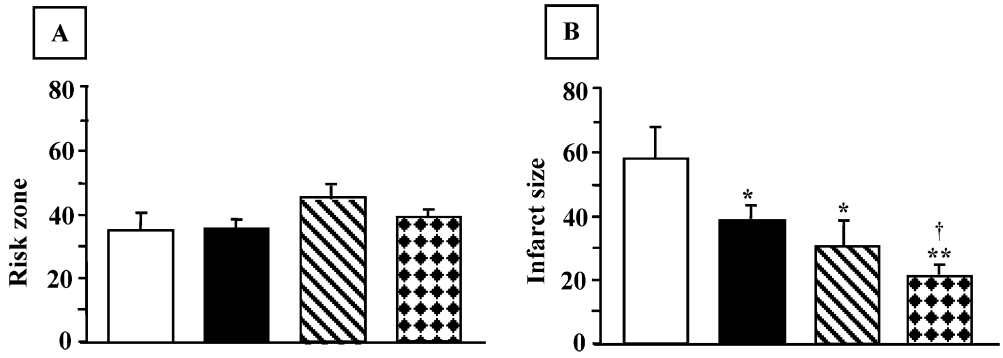

Fig. 2. Comparison of risk zone in the 4 experimental groups, from left to right Control, Ethanol, IPC, and Ethanol + IPC, respectively (A). Effect of IPC and ethanol drinking on infarct size (B).
*:$p < 0.05$; **: $p < 0.001$ vs. controls and †: $p < 0.05$ vs. ethanol.

decreased infarct size (21 ± 3, $p < 0.05$ vs. ethanol). In fact, the effect of ethanol on infarct size was the same in the absence (–34% vs. controls) or presence (a further decrease of 32%) of IPC and the effect of IPC on infarct size was the same when rats were drinkers (a further decrease of 46%) or not (–47% vs. controls).

To test whether chronic cardioprotection induced by CLEthD is associated with PKC activation, we determined the sub-cellular distribution of α, δ and ε PKC in the soluble (S) and particulate (P) fractions of hearts from the 4 groups of rats. Table 3 shows each PKC isoform in each fraction. Compared with controls, the drinking groups (as well as the IPC group) did not show significant difference for the ε isoform although there was a small (borderline significant) difference in the ethanol+IPC group regarding the α isoform.

4. Discussion

The present data show that chronic *and* low dose ethanol drinking actually results in a significant reduction of infarct size in the ex vivo heart rat model, a protective effect similar to that observed with IPC.

4.1. Ethanol and Infarct size

Published data about ethanol and the ischemic myocardium are confusing. Most studies reporting a protective effect of ethanol were in fact conducted in models of global ischemia and infarct size was not measured. In studies evaluating the effect of ethanol on infarct size in models of regional ischemia, results were negative [17,18] or, when they were positive, investigators used acute (not chronic) ethanol exposure of the myocardium [34] or high (not low) dose of ethanol [26,27]. Regarding ethanol dosage (a major point in terms of clinical implication), the dosage tested in the present study corresponds to less than 15% of total daily energy intake, when expressing ethanol drinking in terms of human nutrition. Thus, it is the effect of low-to-moderate chronic drinking that is studied here whereas investigators who reported a significant effect on infarct size had used very high dosage, higher than 30% of total energy intake [26,27]. Such high dosages correspond to heavy drinking in humans, a condition often associated with major clinical and biological side effects and a poor overall prognosis [35]. This is reminiscent of studies testing the effect of acute ethanol exposure on infarct size [18,34] where ethanol was intravenously infused prior to ischemia and failed to affect infarct size. In contrast, ethanol exposure of perfused hearts followed by washout or sufficient time to metabolise the alcohol prior to ischemia induced a significant reduction of infarct size [34]. Thus, if present in sufficient concentration throughout ischemia, ethanol was not protective and even abolished the protection induced by IPC [34]. This also probably explains the failure to show any protective effect of ethanol using in vivo model of myocardial ischemia where average blood

Table 3
Distribution of PKC isoforms in the four groups of rats

	PKCε		PKCδ		PKCα	
	Soluble	Particulate	Soluble	Particulate	Soluble	Particulate
Control	49.7 ± 8.7	50.3 ± 8.7	81.9 ± 5.0	18.1 ± 5.0	93.2 ± 1.6	6.8 ± 1.6
Ethanol	46.3 ± 13.2	53.7 ± 13.2	64.3 ± 15.0	35.7 ± 15.0	64.1 ± 12.8	35.9 ± 12.8
IPC	43.9 ± 7.7	56.1 ± 7.7	69.4 ± 14.1	30.6 ± 14.1	75.3 ± 7.3	24.7 ± 7.3
IPC+Ethanol	13.2 ± 10.9	86.8 ± 10.9	56.8 ± 9.5	43.2 ± 9.5	53.7 ± 4.3*	46.3 ± 4.3*
ANOVA p for trend	ns	ns	ns	ns	<0,05	<0,05

Each PKC fraction is expressed as percentage of total (particulate + soluble) of this isoform.
* : $p < 0.05$ versus control.

alcohol level during ischemia was very high [17]. Taken together, these data (including ours) are in mutual agreement and underline the importance of the way of drinking alcohol (regular versus binging, moderate versus heavy) to induce cardiac protection. The point should be constantly in mind when "celebrating" the health benefits of alcohol drinking.

Another point is that, in this study, there was no significant difference between groups in blood lipids. This is an indirect confirmation that ethanol intake was actually low and did not result in obvious metabolic alteration in these animals. This is in line with most human data since significant changes in blood lipids (or liver enzymes) usually are only observed beyond a certain level of ethanol consumption.

4.2. Mechanism of ethanol preconditioning

In our isolated heart model, we used a crystalloid buffer, therefore eliminating the problem of the presence of ethanol during ischemia [34]. Additionally, the effect of ethanol in the present study was independent from an effect on lipoproteins, haemostasis and other circulating blood factors (that are often presented as the mediators of the protective effect induced by moderate drinking) since they were not present in the perfusion buffer. Thus, CLEthD actually induced a direct protective effect on the ischemic myocardium that resembles IPC. However, IPC is a response to an acute stress whereas the protective effect of CLEthD is the consequence of a regular exposure. This raises the question of whether IPC and ethanol preconditioning involve the same biological mechanism(s) or, in other words, the same signalling pathway.

Although the present study was not designed to fully investigate the mechanism of ethanol preconditioning, it is noteworthy that several investigators have proposed that both ethanol and IPC activate PKC and that PKC activation is responsible for the protection through its effect on ATP-dependent potassium channels [19–31]. As a matter of fact, the degree of protection (of reduction of infarct size) obtained with low dose ethanol drinking in our model is in the same range as the protection observed with IPC. In addition, both IPC and CLEthD were associated with similar subcellular fractionation profiles of PKC isoforms. For instance, in our model, both types of preconditioning were not associated with obvious change of the ε isoform although previous investigators claimed that this specific isoform might play a primary role in both IPC and ethanol preconditioning [21,23–25,29–31].

One possibility, as cardiac PKC activity has been shown [36,37] to translocate very rapidly (as early as 1 minute after the onset of ischemia), but transiently (with a rapid relocation to cytosol after every cycle of reperfusion), is that a sustained translocation of the ε isoform is not necessary to induce cardioprotection in that model [36,37]. This suggests that the two short episodes of ischemia that were used in our IPC protocol (as well as the low ethanol dosage that was tested in this study) were not sufficient to induce sustained translocation of the ε isoform. In that view, the ε isoform may serve as a trigger (sustained activation-translocation is not required to induce cardioprotection) rather than a mediator (sustained activation is required) of the IPC- or ethanol-induced cardioprotection. Also, we have no explanation for the change in the α isoform observed in one group and not in the other groups with reduction of infarct size. Further studies are required to re-examine this (and other) possible mechanism(s).

4.3. Ethanol and left ventricular function

In this study, there was no difference in LVDevP and diastolic pressure between the IPC and ethanol groups in one side and the control group in the other side. This may be quite surprising. However, in many other studies (for instance in [34]), limitation of infarct size (following either ethanol or ischemic preconditioning) was not associated with improvement in cardiac function. The point has been commented in some review articles such as, for instance, by Kloner and Jennings [38]. Actually, brief episodes of ischemia (preconditioning ischemia) can have both a negative effect on the heart in terms of function (the stunning phenomenon) and a protective effect against cell necrosis (the so-called ischemic preconditioning). Thus, in our study, despite a smaller infarct size (compared with the control group), the preconditioned hearts (following brief ischemia or chronic ethanol drinking) might have developed a certain degree of myocardial stunning that could have masked the protective effect resulting from tissue salvage. As a result, no difference in cardiac function was detected between the groups in our study.

4.4. Clinical implication and conclusion

During the past 25 years, many drugs have been claimed to protect the ischemic myocardium, but few of these results have been reproducible and none has been translated into clinical therapies. Furthermore, any potentially effective drug needs to be given prior to the acute coronary attack (prior to ischemia) in order to protect the myocardium. This form of drug treatment is usually not possible in most patients with acute myocardial infarction. These difficulties have prompted many investigators to try developing non-pharmacologic approaches aimed at maintaining the heart in a chronically protected state, such that ischemic damage would be attenuated whenever ischemia occurs. To this end, some groups are using gene therapy to emulate the genetic changes that underlie the late phase of IPC. The present study, in association with a large body of evidence provided by clinical and epidemiological studies [1–15], suggests that chronic low dose ethanol drinking may represent a simple way to induce a chronic cardioprotective state.

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