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Red Wine Polyphenols Alone or in Association with Ethanol Prevent Hypertension, Cardiac Hypertrophy, and Production of Reactive Oxygen Species in the Insulin-Resistant Fructose-Fed Rat

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The effects of a red wine polyphenolic extract (RWPE), ethanol, or both combined were evaluated in insulin resistant rats. Rats were fed for 6 weeks with fructose (60%)-enriched food and force-fed with (a) water only (F group), (b) aqueous solution of RWPE (100 mg/kg, FP group), (c) 10% (v/v) mixture of ethanol and water (FE group), or (d) solution containing the same amount of the RWPE and ethanol (FPE group). Animals fed a standard chow (C group) were used for comparison purpose. After 6 weeks, blood pressure was higher in F (130.0 ×b1 1.7 mm Hg) than in C animals (109.6 ×b1 0.9 mm Hg) and similar to the C group in all other fructose-fed treatment groups. Relative heart weight was higher in F (3.10 ×b1 0.05) than in C (2.78 ×b1 0.07) and significantly lower in FP (2.92 ×b1 0.04) and FPE (2.87 ×b1 0.08 mg/g) than in F animals. Left ventricle and aorta productions of reactive oxygen species ($O_2^{\bullet -}$) were higher in F than in C groups and lowered by the RWPE but not by the ethanol treatment. Ethanol but not the RWPE treatment reduced the degree of insulin resistance in the fructose-fed rats. In summary, our study showed that polyphenols are able to prevent cardiac hypertrophy and production of reactive oxygen species in the insulin resistant fructose-fed rat.

KEYWORDS: Polyphenols; ethanol; hypertension; cardiac hypertrophy; oxidative stress; insulin resistance

INTRODUCTION

Oxidative stress and oxidative damage to tissues have been involved in the pathogenesis of diabetic complications, through the effects of free radicals on lipids and proteins (*1*−*3*). But few data are available concerning the relationship between insulin resistance and oxidative stress.

Feeding rats with a fructose (60%)-enriched diet has been reported to generate many metabolic alterations representing most features of the syndrome X (*4*) (or metabolic syndrome), which associates glucose intolerance, visceral obesity, hypertension, insulin resistance, and dyslipidemia, and predisposes to type II diabetes development and atherosclerotic cardiovascular diseases (*5*, *6*). Indeed, a fructose diet increases blood pressure and insulinemia without hyperglycemia (*7*), along with the development of insulin resistance and hypertriglyceridemia. It is widely used as a nonobese model for studying the role of insulin resistance in hypertension. In this model, both insulin sensitizer and antihypertensive drugs prevent hypertension (*8*, *9*). Moreover, this model exhibits a significant degree of oxidative stress. Indeed, Faure et al. (*10*) reported a greater lipid peroxidation and a defect in the free radical defense system. Similarly, Anurag and Anuradha (*11*) observed a higher lipid peroxide content in fructose fed rats. Furthermore, we recently found that high fructose feeding is associated with a precocious increase in ROS production by aorta and heart, associated with enhanced markers of oxidative stress. We also found that high fructose feeding was associated with the progressive development of cardiac hypertrophy along with increased cardiac superoxide anion production, suggesting that reactive oxygen species (ROS) could be a causal event in the development of cardiovascular complications of insulin resistance (S.D., Eleni Paizani, Theophile Dimo, J.-P.C., G.C., and J.A., personal communication).

Polyphenols are natural compounds found in most vegetables and fruits, being responsible for their taste and pigmentation,

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and are highly concentrated in red wine. They represent a wide family of molecules, mainly known for their antioxidant properties. Polyphenols are reducing agents, and similarly to vitamins C or E, protect body tissues against oxidative stress and associated pathologies such as cancer, coronary heart disease, and inflammation (*12*). Administering some antioxidant molecules such as polyphenols could therefore decrease insulin resistance-related oxidative stress and hypertension, as previously shown with other antioxidants such as vitamin E, which was shown to improve the free radical defense system potential and insulin sensitivity in fructose-fed rats (*10*). In addition, in a previous study, we found that red wine polyphenols or ethanol were both able to lower glycemia in streptozotocin-induced diabetic rats either when administered separately or when administered together (*13*).

In man, moderate and regular consumption of alcoholic beverages has been shown to favorably influence cardiovascular morbidity and mortality. For example, reduced mortality from coronary artery disease is well-established (*14*−*16*). Some studies have related this protective effect of alcoholic beverages to their ability to reduce LDL cholesterol and fibrinogen and to increase HDL cholesterol levels (*17*, *18*). However, the respective roles of ethanol or other major wine constituents (i.e., polyphenols) in the protective effects of wine are not totally established. Ethanol itself was shown to lower the degree of insulin resistance, in both humans (*19*−*20*) and animals (*21*).

Using the fructose-fed rat, the present study was designed to evaluate the potential of a red wine polyphenolic extract, administered in the absence or presence of ethanol, to prevent the cardiovascular complications and tissular oxidative stress associated with insulin resistance.

MATERIALS AND METHODS

Preparation and Characterization of Red Wine Polyphenolic Extract. Preparation and characterization of the polyphenolic extract (RWPE) from a red French wine (Corbières, A. O. C.) was prepared as described previously (*22*, *23*). One liter of red wine produced 2.9 g of extract, which contained 471 mg/g total phenolic compounds expressed as gallic acid. Phenolic levels in the extract were obtained according to HPLC analysis procedure. In particular, the extract contained 8.6 mg/g catechin, 8.7 mg/g epicatechin, dimers (B1: 6.9 mg/g; B2: 8.0 mg/g; B3: 20.7 mg/g; and B4: 0.7 mg/g), anthocyanins (malvidin-3-glucoside: 11.7 mg/g; peonidin-3-glucoside: 0.66 mg/g; and cyanidin-3-glucoside: 0.06 mg/g), and phenolic acids (gallic acid: 5.0 mg/g; caffeic acid 2.5 mg/g; and caftaric acid: 12.5 mg/g).

Animals and Treatments. Male Sprague−Dawley rats (Iffa-Credo, Larbresle, France) weighing 185−220 g were maintained on a 12 h light/dark cycle, in a temperature and humidity controlled environment. After an adaptation period of one week, they were subdivided into five groups of homogeneous weights of nine animals each (three rats per cage): a control group (C) fed a standard chow, a fructose-fed group (F) fed commercial fructose-enriched diet (see next), a RWPE-treated fructose-fed group (FP), an ethanol-treated fructose-fed group (FE), and a RWPE + ethanol-treated fructose-fed group (FPE). Animals were allowed free access to water and food. Standard chow contained 60% vegetable starch, 11% fat, and 29% protein (UAR Company, France), whereas fructose-fed rats received a diet containing 66% fructose, 22% proteins, and 12% fat (Harlan Teklad Company). Mineral and vitamin content of the two diets were similar. Rats received RWPE and/or ethanol once daily by gavage for 6 weeks. Doses were calculated on the basis of a daily human (70 kg weight) consumption of 0.5 L of red wine (containing 3000 mg/L total polyphenols). FP group received a 10 mL/kg solution of RWPE (10.50 g/L, containing 5 g/L polyphenols) for a dose of 50 mg/kg/day of polyphenols. E group received 10 mL/kg of a 10% ethanol solution in water (i.e., 1 mL of ethanol/kg/day), the FEP group receiving a 10 mL/kg solution of 10.50 g/L RWPE diluted in a 10% ethanol solution.

Metabolic Parameters and Cardiovascular Changes. Animal weights were recorded twice a week. Systolic blood pressure was measured once a week by the tail-cuff method using a Letica Scientific Instrument (Barcelona, Spain) electrosphygomanometer (Lc 5002 Storage Pressure Meter) composed of a thermoregulated room containing six restrainers and a microprocessor, after the rats were warmed at 35 °C for 10 min in a Le 5650/6 Heater and Scanner. Blood pressure was measured under conscious conditions. In this method, the reappearance of pulsation on a digital display of the blood pressure cuff is detected by a pressure transducer, amplified, and recorded digitally as the systolic blood pressure (SBP). The average of three pressure readings was recorded for each measurement.

At the end of the treatment period, blood was collected on heparin coated tubes, and the thoracic aorta was immediately removed, cleaned of adherent fat, washed in an ice-cold bicarbonate buffer, and kept at 4 °C until measurement of superoxide anion production. The heart was removed and weighed for the calculation of heart to body weight ratio (Heart Weight Index, mg/g), and the left ventricular tissue was used for the detection of superoxide anion production.

Biochemical Analysis and Markers of Oxidative Stress. Plasma glucose was determined using a Konelab automatic plasma analyzer. Plasma insulin was evaluated by radioimmunoassay using the Kit Rat Insulin RIA, (ref. RI-13K, Linco Research Inc.). In each animal, the insulin resistance and β cell function were estimated according to the method described by Matthews et al. (*24*). The insulin resistance score HOMA:ir was computed with the following formula: plasma glucose $\times$ serum insulin/22.5, with plasma glucose in mmol/L and serum insulin in mU/L.

ASAT (aspartate aminotransferase) and ALAT (alanine aminotransferase) activity and urea were measured on final blood sampling with an HITACHI 704 apparatus, using Kits 851124, 851132, and 1489364, respectively, from Roche/Hitachi, Roche Diagnostics, and Gmbh-D-68298 Mannheim.

Determination of Superoxide Anion Production. Superoxide anion production was evaluated in tissues as previously described (*25*) by chemiluminescence using lucigenin bis-*N*-methyl acridinium. Briefly, the thoracic aorta or left ventricle (150 mg) was placed in Krebs buffer containing 250 μM lucigenin, the intensity of luminescence was measured on a luminometer (Wallac LKB 1251, Finland), and chemoluminescence was measured for 10 s for the aorta and 60 s for the left ventricle. This concentration of lucigenin was found not to affect $O_2^{\bullet -}$ production when compared to lower concentrations (5−30 μM) (*26*). Results were expressed as mV/g tissue.

Expression of Results and Statistics. Data are shown as the mean $\pm$ SEM. Statistical comparisons were performed with the Statgraphics software (Uniware, Paris, France) using a multiple range test after ANOVA analysis. When ANOVA indicated a significant difference between groups ($p \leq 0.05$), a Newmann−Keuls test was used to compare each pair of means.

RESULTS

General Features. No mortality was observed in any treatment group. No significant difference appeared in body weight, ASAT, ALAT, or urea between the treatment groups (not illustrated).

Table 1 shows mean plasma glucose, insulin, and index of insulin-resistance (HOMA:ir). No significant difference was observed regarding plasma glucose and insulin, although higher levels were noted for both parameters in fructose-fed as compared to control rats. When both glucose and insulin were taken into account to measure the degree of insulin resistance, the HOMA:ir index was significantly increased in group F as compared to group C. Treatment with polyphenols (FP group) lowered HOMA:ir to a level intermediary between and not significantly different from C or F rats, while ethanol alone (FE group) or its association with polyphenols (FEP group) normalized HOMA:ir to the control (C group) level.

Cardiovascular Changes. Figure 1 illustrates the change in mean systolic blood pressure during the study. The high-

Table 1. Metabolic Parameters in Rats from Control (C), Fructose (F), Fructose + RWPE (FP), Fructose + Ethanol (FE), and Fructose + Ethanol + RWPE (FPE)[a]

treatment group	glucose (mM)	insulin (ng/mL)	HOMA:ir
C	7.83 ± 0.3	2.42 ± 0.28	12.56 ± 4.59a
F	8.93 ± 0.4	4.00 ± 0.61	38.69 ± 5.6b
FP	8.00 ± 0.4	3.40 ± 0.53	29.22 ± 4.24ab
FE	8.37 ± 0.3	2.67 ± 0.46	22.91 ± 3.90a
FPE	7.54 ± 0.2	2.98 ± 0.74	24.22 ± 6.70a

[a] Values are means ×b1SEM, $n = 9$. For each treatment group, means in a column with different letters differ ($p \leq 0.05$).

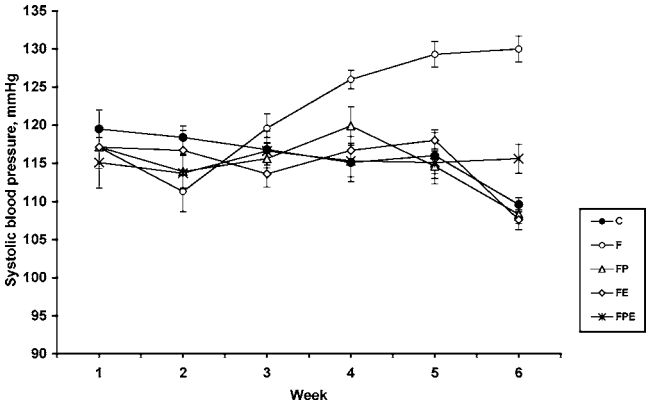

Figure 1. Effect of the various treatments on blood pressure as a function of time. Blood pressure was recorded using the tail-cuff method in control (C group) or fructose-fed animals force-fed with water only (F group), red wine polyphenolic extract (FP group), ethanol (FE group), or red wine polyphenolic extract plus ethanol (FPE group). Values are means ×b1SEM, $n = 9$.

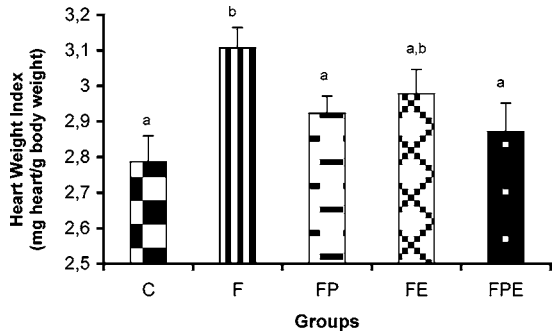

Figure 2. Heart weight index of rats from control (C), fructose (F), fructose + red wine polyphenolic extract (FP), fructose + ethanol (FE), and fructose + red wine polyphenolic extract + ethanol (FPE) treated groups after 6 weeks of fructose diet. Values are means ×b1SEM, $n = 9$. Bars without a common letter differ ($p \leq 0.05$).

fructose diet (F group) was associated with a progressive increase in blood pressure, which became significantly higher than that of C group at week four of the study. Treatment with polyphenolic RWPE (FP group), ethanol (FE group), or both RWPE + ethanol (FPE group) prevented the development of fructose-induced hypertension.

As depicted in the **Figure 2**, rats fed fructose (F group) developed a significant cardiac hypertrophy as demonstrated by the increase in heart to body weight ratio as compared with C rats.

Treatment with polyphenolic extract or the association of polyphenols and ethanol but not with ethanol alone significantly inhibited the development of cardiac hypertrophy.

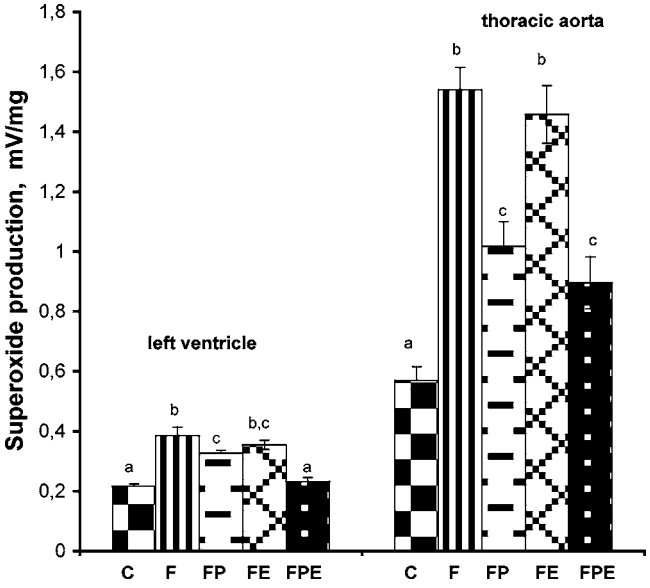

Figure 3. Production of superoxide anion per milligram of tissue (left ventricle or thoracic aorta) of rats from control (C), fructose (F), fructose + red wine polyphenolic extract (FP), fructose + ethanol (FE), and fructose + red wine polyphenolic extract + ethanol (FPE) treated groups after 6 weeks of fructose diet. Values are means ×b1SEM, $n = 9$. Bars without a common letter differ ($p \leq 0.05$).

Superoxide Anion Production. As depicted in **Figure 3**, high fructose feeding induced the overproduction of superoxide anion both in aorta or heart tissues. The polyphenolic extract, the association of polyphenols and ethanol, but not ethanol alone, significantly reduced the enhanced production of superoxide anion in the heart. The polyphenolic extract or the association of ethanol and polyphenols, but not ethanol alone, reduced the enhanced production of superoxide anion in the aorta.

DISCUSSION

The aim of the present study was to evaluate the respective roles of a red wine polyphenolic extract, ethanol, or both, in the prevention of cardiovascular complications and tissular oxidative stress in a model of insulin resistance, the fructose-fed rat.

In agreement with previous studies (*8*, *9*, *27*, *28*), fructose-fed animals displayed insulin resistance and hypertension. Fructose-fed rats progressively developed high blood pressure and cardiac hypertrophy in association with an increased production of superoxide anion in heart and aorta. Those data support the hypothesis of the existence of a vicious circle between hyperinsulinemia, free radicals, and insulin resistance (*29*).

According to previous studies suggesting that flavonoids of various plants produce beneficial effects in cardiovascular diseases and hypertension, RWPE treatment prevented the development of hypertension. Various studies have also shown that endothelium-dependent vasorelaxation is impaired in the fructose-fed rat and is associated with an overproduction of $O_2^{\bullet -}$ through an activation of endothelial NADH/NADPH oxidase (*30*), which can be inhibited by polyphenols (*31*). Our results showing treatment with polyphenols reduces the overproduction of $O_2^{\bullet -}$ by the aorta suggest that the prevention of high blood pressure by polyphenols could be linked to the prevention of endothelial dysfunction. The vasorelaxing properties of polyphenols (*32*) could also be a possible mechanism explaining their antihypertensive activity. Indeed, the vasorelaxing proper-

ties of the same phenolic extract were previously shown (*33*) and related to a possible NO liberation by the vascular endothelium. Whether or not this vascular activity is linked to an antioxidant activity is still under debate (*34*).

Treatment of fructose-fed rats by RWPE also prevented cardiac hypertrophy and lowered the production of reactive oxygen species (ROS) from the heart. In a recent time-course study (S.D., Eleni Paizani, Theophile Dimo, J.P.C., G.C., and J.A., personal communication), we observed that cardiac hypertrophy in the fructose-fed rat was preceded by an increased production of ROS. Previous studies have also shown that pharmacological intervention prevented both $O_2^{\bullet -}$ production and cardiac hypertrophy in the angiotensin II-induced hypertension (*25*). Our results obtained in the fructose-fed rat reinforce the hypothesis of the pathogenic role of oxidative stress in cardiac hypertrophy.

Our results also indicate that ethanol treatment with a moderate dose (1 mL/kg/day) prevented blood pressure increase and insulin resistance but not cardiac/hypertrophy. No effect on overproduction of tissue reactive oxygen species was observed with ethanol alone, although a decrease was noted in the presence of polyphenols. A correction of hypertension with ethanol was previously shown in spontaneously hypertensive rats (SHR). The secretion of atrial natriuretic peptide (ANP) and the modulation of the aldosterone and plasma arginin vasopressin systems (*35*), the increase in NO-dependent vasorelaxant responses to adenosine receptor activation in SHR animals (*36*), or the decrease in tissue aldehyde conjugates and cytosolic free calcium (*37*) could account in part for the ethanol antihypertensive effect. However, similarly to the fructose-fed rat, the SHR rat is responsive to insulin sensitizers (*38*, *39*). Therefore, the possible insulin sensitizing effect of ethanol, previously found by Furuya et al. (*21*) using a dose of ethanol similar to that used in the present study, should also be evoked as a possible mechanism of the antihypertensive activity of ethanol in the fructose-fed rat.

Treatment of the fructose-fed rat by the combination of polyphenols and ethanol prevented hypertension, cardiac hypertrophy, left ventricle or aorta production of oxidative species, and insulin resistance, as expected. Our data showed that the effect of ethanol on cardiac hypertrophy or ROS was observed in the presence of polyphenols only, suggesting that ethanol may amplify the in vivo effects of polyphenols, possibly by modulating the bioavailability of polyphenols, as previously suggested (*40*). Recently, mechanisms responsible for the superoxide anion production with ethanol and phenolics were reported on healthy rat model (*41*). Effects on antioxidant enzymes were reported and could be attributed to ethanol, but an increase in the plasma antioxidant capacity and reduced glutathione (GSH)/glutathione disulfide (GSSH) ratio was attributed to the nonalcoholic components of red wine. Despite the finding that renal (Na + K)-ATPase activity was upregulated by ethanol, it was not altered by either red wine or alcohol-free red wine. Red wine elevated the activity of CAT (catalase) and GSH-Px (glutathione peroxidase), and it was suggested that there is an enhancement of the antioxidant defense potential in kidney and plasma after chronic red wine consumption. Moreover, in another work, with a healthy rat model (*42*), diets rich in either dealcoholated red wine, quercetin, or catechin induced endothelium-dependent vasorelaxation in rat aorta in a resting state through the enhancement of nitric oxide ((*)NO) production, without modifying $O_2^{\bullet -}$ generation; thus, the bioavailability of (*)NO was increased. The increase in the (*)NO-cyclic GMP (guanosine-3′,5′-monophosphate) pathway could explains the beneficial effect of flavonoids at vascular level.

In summary, our study confirmed in a model of insulin resistance associated with hypertension, the fructose-fed rat, that a red wine polyphenolic extract (100 mg/kg), ethanol (1 mL/kg), or the combination of both prevented the development of high blood pressure. In addition, we show here for the first time that polyphenols or the association of polyphenols and ethanol prevented both cardiac hypertrophy and ROS overproduction by heart or aorta, while ethanol corrected insulin resistance but was not able to significantly reduce cardiac hypertrophy. These data illustrate the differential mechanisms of polyphenols and ethanol in regulating blood pressure and further suggest that cardiac hypertrophy is linked to free radical production. Further studies will determine the mechanism of the effect of polyphenols on the production of superoxide anions and their possible effects on the regulation of NADPH oxidase expression.

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