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Preventive Effects of Nutritional Doses of Polyphenolic Molecules on Cardiac Fibrosis Associated with Metabolic Syndrome: Involvement of Osteopontin and Oxidative Stress

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We previously showed that grape extracts enriched in different polyphenolic families were similarly able to prevent reactive oxygen species (ROS) production, although having differential effects on various features of metabolic syndrome when administered at a dose of 21 mg/kg to the fructose (60%)-fed rat (a model of metabolic syndrome). In the present work, we analyzed on the same model the effect of pure polyphenolic molecules (catechin, resveratrol, delphinidin, and gallic acid) administered at a dose of 2.1 mg/kg. Delphinidin and gallic acid prevented insulin resistance, while gallic acid prevented the elevation of blood pressure. All molecules prevented cardiac ROS overproduction and NADPH overexpression. We also showed that fructose feeding was associated with cardiac fibrosis (accumulation of collagen I) and expression of osteopontin, a factor induced by ROS and a collagen I expression inducer. Collagen I and osteopontin expressions were prevented by the administration of all polyphenolic molecules. The potential use of polyphenols in the prevention of cardiac fibrosis should be further explored.

KEYWORDS: Polyphenols; fructose; rat; collagen I; osteopontin; ROS

INTRODUCTION

Metabolic syndrome classically associates three of the five following symptoms: obesity, hypertension, high triglycerides, low high-density lipoprotein (HDL) cholesterol, and moderate increase in glucose plasma levels (*1*). Patients featuring metabolic syndrome are at risk of developing cardiovascular diseases and diabetes. It is also known that a regular intake of polyphenols is susceptible to prevent cardiovascular complications (*2*).

A fructose (60%)-enriched diet administered to rats reproduces various features of the metabolic syndrome. Although those animals do not ingest high amounts of calories and do not become obese, they display hypertension associated with cardiac hypertrophy, along with biological signs of insulin resistance, such as high triglyceride, low HDL cholesterol plasma levels, and overproduction of reactive oxygen species (ROS) associated with overexpression of NADPH oxidase in various tissues, such as the heart and aorta (*3*). We have previously shown that various polyphenolic extracts from wine or grapes, administered at a dose of 21 mg/kg (the amount of total polyphenols equivalent to an intake of 500 mL of red wine rich in polyphenols by a 70 kg human) to rats fed a fructose (60%)-enriched diet, were similarly able to prevent ROS overproduction, although having differential effects on the various features of metabolic syndrome in those animals (*4*).

The aim of the present study was to determine whether pure polyphenolic molecules, chosen among different families of polyphenols (a tannin monomer, catechin; a stilbene, resveratrol; an anthocyanin, delphinidin; a phenolic acid, gallic acid) administered at a dose of 2.1 mg kg^{-1} day^{-1} (a dose equivalent of the amount of catechin theoretically ingested from the daily

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consumption of 500 mL of red wine by a 70 kg human), were able to reproduce those correcting effects on ROS overproduction and the various features of metabolic syndrome. In addition, we studied the effect of those molecules on the expression of cardiac collagen I and osteopontin, two proteins known to be involved in the development of cardiac fibrosis (*5*).

MATERIALS AND METHODS

Animal and Treatment Groups. Experiments were performed following European Community animal experiments ethical regulations. Sprague−Dawley rats (average weight of 200 g) were purchased from Harlan (Gannat, France) and divided into six groups of nine animals: one control group (C) fed with standard chow, one fructose-fed group (F) fed with fructose (60%)-enriched food (Harlan Teklad, Oxon, U.K.), and four groups of fructose-fed animals treated with the following polyphenolic molecules (2.1 mg/kg per day): delphinidin (FD), catechin (FC), gallic acid (FG), and resveratrol (FR). Diets and treatment of rats were initiated at the same time for 6 weeks. Polyphenols were administered once a day (10:00 a.m.) by gavage under the form of a water solution (10 mL/kg).

Food and fluid intakes were recorded daily. Blood pressure was measured by the tail cuff method on awake animals trained to restrainer immobilization using a Bioseb (Vitrolles, France) non-invasive blood pressure measurement apparatus.

Plasma Analyses. At the end of the treatment period, animals were sacrificed by decapitation, blood was collected on heparinized tubes and centrifuged, and plasma was collected and stored at −80 °C before processing. Plasma concentrations of glucose, total cholesterol (TC), HDL cholesterol (HDL-C), triglycerides (TG), and phospholipids (PLs) were determined using automatized enzymatic methods (Konelab DPC France, La Garenne Colombes, France). Plasma non-HDL cholesterol (non-HDL-C) was calculated from the difference between TC and HDL-C.

Plasma insulin was determined using a RIA rat insulin kit commercialized by Linco/Millipore (Molsheim, France). Insulin resistance was evaluated by the Homeostasis Model Assessment index (HOMA-IR) using the following formula: (insulin (mU/L) × glucose (mmol/L))/22.5.

Determination of Cardiac Superoxide Anion Production. Superoxide anion production was determined as previously described (*3*). Briefly, a piece of left ventricle (150 mg) was placed in Krebs buffer containing lucigenin (250 μM), and the intensity of luminescence was recorded on a luminometer (Perkin-Elmer Wallac, Victor, Turku, Finland). Results were expressed as count/mg of protein.

Immunoblotting. NADPH oxidase (p22phox and gp91phox subunits), osteopontin, and collagen I protein expressions were measured by western blotting as previously described (*3*). Briefly, proteins were extracted from the frozen left ventricles of rats. Samples were homogenized using an Ultra Turrax T25 basic (Irka-Werke) in an ice-cold extraction buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μM leupeptin, and 1 μM pepstatin. Proteins (50 μg) were separated with 12% SDS−polyacrylamide gel electrophoresis (PAGE) (p22phox and osteopontin), 10% SDS−PAGE (gp91phox), and 7.5% SDS−PAGE (collagen type I) and then transferred to a nitrocellulose membrane (90 min, 100 V). Membranes were blocked in 5% nonfat milk overnight at 4 °C. Then, membranes were incubated for 1 h with a primary antibody against p22phox, gp91phox, osteopontin, or collagen type I ($^1/_{400}$, Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer. After washes in TBS/Tween under gentle agitation, membranes were incubated for 1 h with horseradish-peroxidase-labeled secondary antibody ($^1/_{5000}$). After further washes, membranes were treated with enhanced chemiluminescence detection reagents (ECL, Amersham). β-Actin was used as loading references, and blot intensities were measured using the BIO-Profil 1D software (Fisher Bioblock).

Statistics. Statistical analyses were performed by analysis of variance (ANOVA) followed by a least significant difference test. The level of significance was set as $p \leq 0.05$.

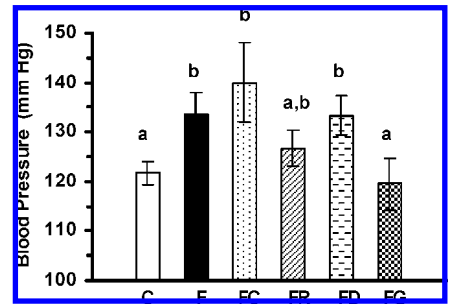

Figure 1. Effect of the fructose-enriched diet in the absence (F) or presence of the polyphenols (2.1 mg/kg/day) catechin (FC), resveratrol (FR), delphinidin (FD), and gallic acid (FG) on blood pressure levels after 6 weeks of a fructose-enriched diet, as compared to rats fed a normal diet (C). Columns with the same letters are not significantly different ($p \leq 0.05$).

Table 1. Effect of the Fructose-Enriched Diet in the Absence (F) or Presence of the Polyphenols (2.1 mg kg^{-1} Day^{-1}) Catechin (FC), Resveratrol (FR), Delphinidin (FD), and Gallic Acid (FG) on Carbohydrate Metabolic Parameters after 6 Weeks of a Fructose-Enriched Diet, as Compared to Rats Fed a Normal Diet (C)[a]

groups	glucose (mM)	insulin (mM)	HOMA-IR
C	6.21 ± 0.34 a	3.37 ± 0.45 a	21.99 ± 2.62 a
F	6.90 ± 0.29 a	4.11 ± 0.58 a	32.60 ± 4.82 b
FC	6.18 ± 0.37 a	4.32 ± 0.78 a	30.06 ± 5.32 a,b
FR	6.17 ± 0.45 a	3.31 ± 0.72 a	23.14 ± 4.96 a,b
FD	6.34 ± 0.47 a	2.90 ± 0.40 a	20.76 ± 3.20 a
FG	5.91 ± 0.40 a	2.67 ± 0.23 a	18.13 ± 3.10 a

[a] Values with the same letter are not significantly different ($p \leq 0.05$).

RESULTS

No significant difference was observed between groups regarding body weight, fluid, or food intake (not shown). As expected, blood pressure (**Figure 1**) was moderately elevated in F as compared to the C group. In treated groups, blood pressure was higher in FC or FD than in C and not significantly different from F groups; it was lower in FG than in the F group and not significantly different from the C group. In the FR group, it was intermediate between and not significantly different from C or F groups.

As shown in **Table 1**, plasma levels of glucose or insulin were not significantly different within groups. However, the HOMA-IR index for insulin resistance was significantly higher in F than in the C group, as previously reported. Among polyphenol-treated groups, FD and FG HOMA-IR indexes were significantly lower than F and not significantly different from C groups.

Table 2 indicates plasma lipid levels in the various treatment groups. As expected, the F group was characterized by high TG, low HDL-C, high non-HDL-C, high TC, and high PL, along with a high TG/HDL-C ratio. This lipid profile is classically recorded in the various models of metabolic syndrome and notably the fructose-fed rat. The various polyphenolic molecule treatments had only a mild effect on those changes: TG and PL were unchanged, and HDL-C was only modestly increased by delphinidin, while HDL-C/TG ratios from FD and FG groups were intermediate between and not significantly different from C or F groups.

Parts **A**−**C** of **Figure 2** illustrate the effect of polyphenolic molecules on the superoxide anion production (**Figure 2A**) and NADPH gp91 phox (**Figure 2B**) and p22 phox (**Figure 2C**) subunits expression from hearts collected from the different treatment groups. As shown previously, superoxide anion

Table 2. Effect of the Fructose-Enriched Diet in the Absence (F) or Presence of the Polyphenols (2.1 mg kg^{-1} Day^{-1}) Catechin (FC), Resveratrol (FR), Delphinidin (FD), and Gallic Acid (FG) on Plasma Lipid Parameters after 6 Weeks of a Fructose-Enriched Diet, as Compared to Rats Fed a Normal Diet (C)[a]

groups	TG (mM)	phospholipids (mM)	TC (mM)	HDL-C (mM)	non-HDL-C (mM)	TG/HDL-C ratio
C	2.25 ± 0.19 a	2.13 ± 0.14 a	1.91 ± 0.12 a	1.27 ± 0.10 a	0.74 ± 0.09 a	1.91 ± 0.17 a
F	5.93 ± 0.59 b	2.93 ± 0.14 b,c	2.35 ± 0.13 b	0.80 ± 0.11 b	1.67 ± 0.11 b	9.38 ± 1.91 b
FC	5.26 ± 0.58 b	3.08 ± 0.15 b,c	2.51 ± 0.12 b	0.89 ± 0.17 b	1.61 ± 0.16 b	9.01 ± 2.49 b
FR	5.65 ± 0.88 b	3.19 ± 0.16 c	2.72 ± 0.10 c	0.91 ± 0.13 b	1.89 ± 0.20 b	8.78 ± 2.56 b
FD	4.78 ± 0.54 b	2.95 ± 0.11 b,c	2.62 ± 0.07 b	1.03 ± 0.11 a,b	1.59 ± 0.12 b	5.71 ± 1.35 a,b
FG	4.39 ± 0.37 b	2.79 ± 0.12 b	2.41 ± 0.12 b	0.89 ± 0.11 b	1.52 ± 0.12 b	5.98 ± 1.41 a,b

[a] Abbreviations: TG, triglycerides; TC, total cholesterol; HDL-C, HDL cholesterol; non-HDL-C, non-HDL cholesterol. Values with the same letter are not significantly different ($p \leq 0.05$).

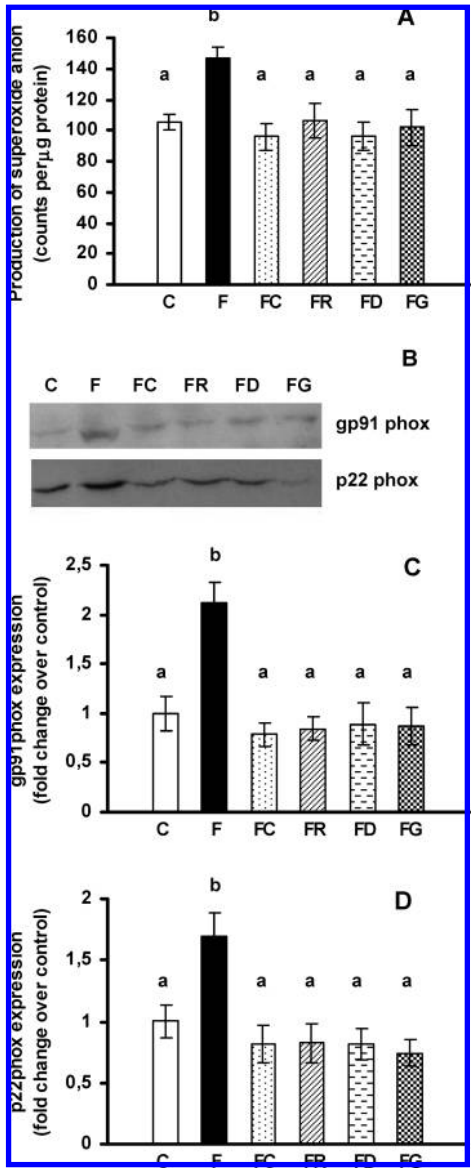

Figure 2. (A−D) Effect of the fructose-enriched diet in the absence (F) or presence of the polyphenols (2.1 mg kg^{-1} day^{-1}) catechin (FC), resveratrol (FR), delphinidin (FD), and gallic acid (FG) on superoxide anion production (A) and NADPH oxidase subunits gp91 phox and p22 phox (B−D) protein expression, as compared to rats fed a normal diet (C). B illustrates a representative gel of six different rats from each group, while C and D correspond to the quantification of the ECL signal for gp91phox and gp22phox, respectively, expressed as mean ± standard error of the mean (SEM) ($n = 6$ per group). Columns with the same letters are not significantly different ($p \leq 0.05$).

production was enhanced by the fructose-enriched diet. Treatment with all polyphenolic molecules was able to prevent the

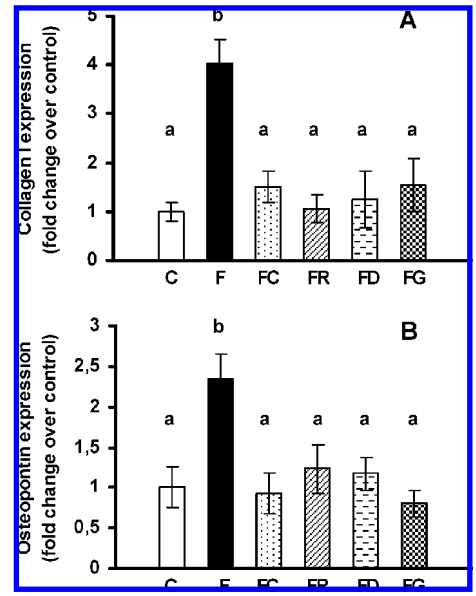

Figure 3. (A and B) Effect of the fructose-enriched diet in the absence (F) or presence of the polyphenols catechin (FC), resveratrol (FR), delphinidin (FD), or gallic acid (FG) on the protein expressions of collagen I (A) and osteopontin (B) as compared to rats fed a normal diet (C). Data correspond to the quantification of the ECL signal expressed as mean ± SEM ($n = 6$ per group). Columns with the same letters are not significantly different ($p \leq 0.05$).

superoxide anion overproduction associated with the fructose-enriched diet. In accordance with those data, NADPH gp91 phox (**Figure 2B**) and p22 phox (**Figure 2C**) subunits were overexpressed in the F group and overexpression of both subunits was prevented in all polyphenol-treated animals.

Parts **A** and **B** of **Figure 3** illustrate the effect of the fructose-enriched diet, in the absence or presence of polyphenolic molecules, on the expression of two proteins involved in cardiac fibrosis, collagen I (**Figure 3A**) and osteopontin (**Figure 3B**). Both proteins were overexpressed in the hearts from fructose-fed rats, and overexpression was prevented by the various polyphenolic treatments.

DISCUSSION

The aim of the present work was to evaluate pure polyphenolic molecules administered at a dose (2.1 mg/kg) compatible with their daily intake, for their potential to prevent the various features of metabolic syndrome in the fructose-fed rat. We previously used this model to study the effects of polyphenolic extracts (21 mg/kg), such as a total red wine polyphenolic extract (*4*) or two extracts enriched in different families of grape polyphenols (anthocyanins or procyanidins) (*6*). Our previous data indicated that extracts enriched in different families had differential effects on the various features of metabolic syn-

drome. Indeed, a procyanidin-enriched extract (also enriched in gallo-tanins, see below) prevented insulin resistance, although anthocyanin-enriched extract prevented high blood pressure and cardiac hypertrophy, while a total wine extract had a less pronounced effect on all parameters. Interestingly, all extracts had the same preventing effect on superoxide anion overproduction and NADPH oxidase overexpression, two features highly reproducible in the fructose-fed rat model (*3, 6*). Those data suggested that polyphenolic molecules may display some specific properties in addition to their well-known antioxidant activity. It is why the potential of pure polyphenolic molecules belonging to different polyphenolic families deserved to be explored in the same model.

Our present study confirmed the occurrence in the fructose-fed rat of various features of the metabolic syndrome linked to insulin resistance, including hypertension and dyslipidemia, along with an increased production of ROS. Some of those features were differentially modulated by the administration of pure polyphenolic molecules.

With regard to insulin resistance, delphinidin and gallic acid prevented fructose-induced increase of the HOMA-IR index. Our previous data also indicated that an extract enriched in gallo-tanins (and procyanidins) was also able to correct the HOMA-IR index in the same fructose-fed model (*6*). Moreover, gallic-acid-enriched molecules were described as insulin sensitizers (*7*). Because gallic acid is a degradation product of delphinidin (*8*), it is possible that both molecules share insulin resistance, correcting properties through similar degradation products or metabolites when administered *in vivo*.

Blood pressure increase was prevented by gallic acid. No specific work has described this particular property of pure gallic acid administered *in vivo*. Interestingly, another *in vivo* study has shown that plant extracts (green tea) enriched in gallic acid or gallic-acid-containing molecules were able to prevent hypertension in the SHR rat when administered chronically (*10*), although *in vitro* studies showed that gallic acid induces vasoconstriction (*9*). It is however possible that the vascular effect of gallic acid may depend upon the feeding state, as shown in human subjects with tea (*11*). Another possibility is that some insulin-sensitizing properties of gallic acid may participate in its antihypertensive activity, as previously shown using the insulin-sensitizing drugs metformin (*12*) or thiazolidine diones (*13*).

As previously reported (*3*), fructose-fed rats developed an atherogenic lipid profile. None of the polyphenolic molecules had any significant effect on lipids, except delphinidine, which had some correcting effect on HDL-C. In previous studies (*4*), we found that an extract enriched in anthocyanins (also containing 3 mg of delphinidin; P.-L. Teissèdre, personal communication) had a correcting effect on low HDL cholesterol levels in the fructose-fed rat. Kwon et al. (*14*) also described a correcting effect of black soybean anthocyanins, used as a supplement included in food (0.037% or approximately 3−4 mg/kg), on the lipid profile in rats fed a high fat diet. It is therefore possible that anthocyanin polyphenols possess some particular effect on lipid metabolism.

Our data also confirmed the inhibitory effects of all four polyphenolic molecules on ROS production and NADPH oxidase overexpression, as previously obtained with the various extracts tested, suggesting that the antioxidant activity, a property found with all molecules tested thus far, was responsible for both the inhibition of ROS production and NADPH protein overexpression. It is known that activation of NADPH oxidase induces an auto-amplification loop, leading to the increase expression of the enzyme (*15*). It is therefore probable that the antioxidant activity of polyphenolic molecules will block the auto-amplification loop, as shown not only with polyphenols in the fructose-fed rat but also with other antioxidants, such as *N*-acetyl cystein (NAC) in the streptozotocin-induced diabetic rat (*16*). It is of note, however, that polyphenolic molecules appear as very potent regulators of NADPH expression, because their dose (2.1 mg/kg) was 10 times lower than that used in studies using extracts (21 mg/kg) and almost 1000 times lower than that of NAC (1.4−1.5 g/kg).

We also describe for the first time that collagen I and osteopontin (OPN) are overexpressed in the heart of fructose-fed animals and that the various polyphenolic molecules normalize the expression of those proteins. The accumulation of collagen I, a fibrillar protein of the extracellular matrix, characterizes cardiac fibrosis. Progressive cardiac fibrosis contributes to an increase in cardiac muscle stiffness occurring during inflammatory or healing processes (such as myocardial infarction) or chronic wall stretch (such as hypertension) (*17*−*22*). Collagen I accumulation is stimulated by various pro-inflammatory factors, such as OPN. OPN itself is not expressed in adult tissues, except during inflammatory or healing processes, because its expression is stimulated by ROS (*23*). After its secretion under a phosphorylated form, OPN is severed by thrombin and interacts with integrin receptors from target cells, leading to an increase in collagen expression. In the heart, OPN expression is stimulated during mechanical stress and hypoxia and its accumulation induces excessive stiffness and impairment of relaxation. The role of angiotensin II and aldosterone in collagen deposition has recently be highlighted, with a succession of events involving (a) the stimulation of NADPH expression by mineralocorticoids, (b) the stimulation of OPN expression by superoxide anion produced by NADPH, and (c) the stimulation of collagen expression by OPN (*24*−*27*).

We found that polyphenolic molecules were able to prevent the cardiac accumulation of collagen and osteopontin. It is therefore tempting to speculate that their mechanism is related to an antioxidant effect. Thus, it could be hypothetized that the oxidative stress associated with the fructose diet induces the expression of OPN and subsequently that of collagen I and that polyphenols prevent both OPN and collagen I overexpression through their antioxidant activity.

In summary, the present study allowed us to demonstrate that various pure polyphenolic molecules, administered at a dose as low as 2.1 mg/kg, possess differential effects on the various features of metabolic syndrome associated with high fructose feeding in rats. In particular, delphinidin and gallic acid were found to act on insulin resistance, while gallic acid was the most potent for preventing high blood pressure. In addition, all molecules prevented cardiac ROS overproduction and NADPH overexpression. We also showed that fructose feeding was associated with the accumulation of collagen I (cardiac fibrosis) and osteopontin (a factor induced by ROS and a collagen I expression inducer), and that cardiac fibrosis and overproduction of ROS are preventable by the administration of antioxidant polyphenols administered at nutritional doses. The long-term functional consequence of cardiac accumulation of collagen I and its possible prevention by polyphenolic molecules should be further studied.

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