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Phenolics from Commercialized Grape Extracts Prevent Early Atherosclerotic Lesions in Hamsters by Mechanisms Other than Antioxidant Effect

CYRIL AUGER,[†,‡,§] PEGGY GÉRAIN,[†,‡] FLORENCE LAURENT-BICHON,[#] KARINE PORTET,[#] AURÉLIE BORNET,[§] BERTRAND CAPORICCIO,[‡] GÉRARD CROS,[#] PIERRE-LOUIS TEISSÉDRE,[§] AND JEAN-MAX ROUANET*[,‡]

Equipe d'Accueil 3762, Nutrition et Aliment, Département Agroressources and Procédés Biologiques, Université Montpellier II, Place E. Bataillon, 34095 Montpellier cedex 05, France; Laboratoire de Pharmacologie et Physiopathologie Expérimentales, Faculté de Pharmacie, Université Montpellier, Avenue Charles Flahault, 34093 Montpellier cedex 05, France; and UMR 1083 Sciences Pour l'Œnologie, Centre d'Œnologie, Faculté de Pharmacie, Avenue Charles Flahault, Université Montpellier I, 34093 Montpellier cedex 5, France

The aim of this study was to evaluate the antiatherosclerotic effect of commercially available phenolic-rich extracts from grape seeds (ExGrape seeds, EGS; grape seed extract, GSE) and marc (ExGrape total, EGT) in cholesterol-fed hamsters and to investigate possible operating mechanisms. These extracts fed at a moderate dose mimicking two glasses of red wine per meal reduced plasma cholesterol (−11% on average) but did not affect plasma antioxidant capacity of hamsters. The extracts prevented the development of aortic atherosclerosis by 68% (EGS), 63% (EGT), and 34% (GSE). Elsewhere, in an ex vivo experiment using rat aortic rings, EGS (7 μg/mL) induced 77% endothelium-dependent relaxation, whereas EGT and GSE (30 μg/mL) induced 84 and 72%, respectively. These results suggests that phenolic extracts from grape seeds and marc are beneficial in inhibiting atherosclerosis by indirect mechanism(s).

KEYWORDS: Phenolic extracts; atherosclerosis; hamster; rat aortic rings; vasorelaxation

INTRODUCTION

Epidemiologic evidence suggests that daily moderate wine consumption is associated with a lower incidence of cardiovascular diseases (*1*−*4*). The greater degree of cardioprotection is related to the ingestion of wine, particularly red wine with its high content of phenolic compounds (*5*). Usually, their antioxidant and free radical scavenging properties are thought to be responsible for their health benefits in humans (*6*) and in rats (*7*); these beneficial effects include decreased oxidation of low-density lipoproteins (LDL) (*8*−*13*). In addition, there is evidence that red wine phenolic compounds efficiently prevent early aortic atherosclerosis in hamsters (*14*, *15*). Development of atherosclerosis is characterized by dysfunction of endothelial cells, oxidation of LDL, and foam cell formation from macrophage, migration of vascular smooth muscle cells (VSMC) from arterial media into intima, excessive proliferation of VSMC in the neointima, and increased extracellular matrix deposition (*16*). Although phenolics can induce a direct antioxidant effect, they may also act by several other antiatherogenic mechanisms, such as inhibition of adhesion molecule expression through down-regulation of NF-κB activation in vascular endothelial cells (*17*), inhibition of platelet agregation (*18*), down-regulation of tissue factor expression (*19*), and particularly endothelium-dependent vasorelaxation through increased NO synthesis (*20*−*24*). Although the mechanisms responsible for the stimulatory effects of phenolics on endothelial NO production are not yet clearly clarified (*25*, *26*), it was recently demonstrated that a red wine phenolic extract up-regulates endothelial NO synthase expression and subsequent endothelial NO release, which is a pivotal vasoprotective molecule (*27*, *28*). In addition to this vasodilating feature, endothelial NO has antiatherosclerotic properties such as inhibition of platelet aggregation and adhesion to the vascular wall, smooth muscle cell proliferation, expression of genes involved in atherogenesis (*29*−*32*), and reduction of the influx of atherogenic monocytes and LDL through the endothelial cells into the artery wall (*33*).

The present study was designed to compare the effects of three phenolic extracts from the agroindustry on plasma lipids and lipoprotein, on plasma antioxidant capacity (PAC), and on antiatherogenic properties in hamsters consuming a hypercho-

* Address correspondence to this author at Equipe d'Accueil 3762, Nutrition et Aliment, CC 023, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France [telephone (33) 04 67 14 35 21; fax (33) 04 67 63 36 49; e-mail rouanet@arpb.univ-montp2.fr].

† Both authors contributed equally.

‡ Equipe d'Accueil 3762, Université Montpellier II.

§ UMR 1083 Sciences Pour l'Œnologie, Université Montpellier I.

Laboratoire de Pharmacologie et Physiopathologie Expérimentales, Université Montpellier.

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lesterolemic diet. Hamsters were selected for this study because decreased plasma cholesterol has been reported in hamsters fed hypercholesterolemic diets following consumption of a red wine phenolic extract (*14*) or red wine or dealcoholized red wine (*15*). Furthermore, hamsters represent a useful model with respect to humans because they exhibit a similar manner of cholesterol metabolism (*34*, *35*) and have a lipoprotein profile similar to that found in humans when fed hypercholesterolemic diets (*36*). This model was also chosen because of its responsiveness to antiatherogenic interventions (*37*). To induce a peroxidative stress, the high-cholesterol and high-fat diet was rendered deficient in vitamins C and E and in selenium.

The aim of the present study was to trigger off the arterial wall response to such a stress (fatty streak formation and aortic atherosclerosis emergence) and then to look at the modulation of this effect by (i) a procyanidin-rich white grape seed extract (EGS), (ii) a marc extract from red and white grapes, rich in anthocyanidins (EGT), and (iii) a grape seed extract (GSE). Two possible mechanisms whereby these extracts could act were also investigated (i) by measurement of PAC in hamsters and (ii) by characterization of their vasorelaxant effects on rat aortic rings.

MATERIALS AND METHODS

Materials. The ExGrape seed (EGS) and ExGrape total (EGT) dry powder extracts from grape seeds and marc (Cabernet Sauvignon) were provided by Drs. N. Urban and E. Fesquet (La Gardonnenque S.C.A., Cruviers Lascours, France); another commercial grape seed extract (GSE) (confidential origin) was also tested.

Standards and HPLC Analysis. (+)-Catechin and (−)-epicatechin were obtained from Aldrich (St. Quentin Fallavier, France). (−)-Epigallocatechin, (−)-epigallocatechin 3-*O*-gallate, (−)-epicatechin 3-*O*-gallate, cyanidin 3-glucoside, malvidin 3-glucoside, cyanidin, and peonidin were obtained from Extra Synthèse (St. Quentin Fallavier, France). Procyanidin dimers B1, B2, B3, and B4 were obtained from grape seeds as previously reported (*38*). HPLC analysis with UV detection was performed using a Hewlett-Packard model 1090 with three low-pressure pumps and a diode array detector coupled to a Hewlett-Packard Chem Station for solvent delivery and detection. A Hewlett-Packard column packed with Nucleosil 100 C18 (250 × 4 mm, 5 μm particle size) was used for the stationary phase with a flow of 0.7 mL/min. The solvents used for separation (*39*) were as follows: solvent A, 50 mmol/L dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B, 20% A with 80% acetonitrile; solvent C, 200 mmol/L orthophosphoric acid adjusted with ammonia to pH 1.5. Elution was performed with a gradient previously described (*38*).

Animals. Forty male Golden Syrian hamsters (Janvier, Le Genest-St-Isle, France) weighing 60−80 g were randomly assigned to five groups groups (n = 8/group) with approximately equal mean group body weights. They were maintained in plastic cages in a temperature-controlled environment (23 ± 1 °C) subjected to a 12-h light/dark cycle (lights on at 7:00 a.m.) with free access to both food and water.

Diets and Feeding Procedures. Hamsters were fed a semipurified atherogenic diet that is described in detail in **Table 1**, in which the cholesterol content had been set at 0.5% and which was supplemented with 15% lard at the expense of starch and sucrose; this diet was free from selenium, vitamin C, and vitamin E. Animals were given food daily for 12 weeks, and uneaten food was weighed daily.

The hamsters of each group were additionally force-fed daily either tap water (control), a solution of EGS in water, a solution of EGT in water, or a solution of GSE in water. The volume of solutions force-fed was adjusted daily to the weight of the hamsters: it was established by extrapolating 500 mL/day average wine consumption, that is, about two glasses per meal (wine containing 2 g/L total phenolic compounds) for a 70 kg human to the equivalent for the daily weight of hamsters. This represents a volume of 7.14 mL/(kg of body wt·day). Because EGS, EGT, and GSE, respectively, contained ~864, 556, and 776 mg/g phenolic compounds (as gallic acid equivalent), hamsters from group EGS received 16.9 mg of EGS/(kg of body wt·day), hamsters from group EGT received 25.7 mg of EGT/(kg of body wt·day), and those from group GSE received 18.4 mg of GSE/(kg of body wt·day), dissolved in the above-mentioned volume of water.

Table 1. Composition of the Diet (Grams per Kilogram)

diet ingredient	exptl diet	diet ingredient	exptl diet
casein	200	mineral mix[a]	35
DL-methionine	3	vitamin mix[b]	10
cornstarch	393	lard	150
sucrose	154	cholesterol	5
cellulose	50		

[a] Mineral mixture contained (mg/kg of diet) the following: $CaHPO_4$, 17200; KCl, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_3, 120; $FeSO_4 \cdot 7H_2O$, 200; trace elements, 400 ($MnSO_4 \cdot H_2O$, 98; $CuSO_4 \cdot 5H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 80; $CoSO_4 \cdot 7H_2O$, 0.16; KI, 0.32; sufficient starch to bring to 40 g (per kg of diet). [b] Vitamin mixture contained (mg/kg of diet) the following: retinol, 12; cholecalciferol, 0.125; thiamin, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; menadione, 80; nicotinic acid, 200; choline, 2720; folic acid, 10; p-aminobenzoic acid, 100; biotin, 0.6; sufficient starch to bring to 20 g (per kg of diet).

Analytical Procedures. At the end of the 12 week experimental period, hamsters were deprived of food for 18 h and were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/mL at a dosage of 60 mg/kg of body wt). Blood was drawn by cardiac puncture with heparin-moistened syringes, and plasma was prepared by centrifugation at 2000*g* for 10 min at 4 °C and then stored at −80 °C until analysis. Plasma total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were determined by commercially available enzymatic methods (respectively, 401 and 352-4, Sigma Chemicals, Saint Quentin Fallavier, France). Plasma very low- and low-density lipoprotein cholesterol (nonHDL-C) was precipitated with phosphotungstate reagent (*40*), and HDL-C was measured in the supernatant. Plasma apolipoprotein A-1 (Apo A1) and apolipoprotein B (Apo B) concentrations were determined using Sigma turbidimetric immunoassay kits (356 and 357, respectively) as previously described (*41*, *42*).

Plasma Antioxidant Capacity. The PAC in hamsters was measured by the total antioxidant status method of Randox (Randox Laboratories Ltd., Crumlin, U.K.) using a commercial kit (NX2332). This assay is based on the ability of 2,2′-azinobis(3-ethylbenzothiazoline sulfonate) (ABTS) incubated with peroxidase (metmyoglobin) and hydrogen peroxide (H_2O_2) to produce the radical cation $ABTS^{\bullet+}$. This has a relatively stable blue-green color of which the absorbance is measured at 600 nm. Antioxidants in the added plasma cause suppression of this color production to a degree proportional to their concentration. This analytical procedure has been applied to physiological antioxidant compounds and radical-scavenging drugs, and an antioxidant ranking based on their reactivities relative to a 1.0 mmol/L Trolox standard has been established. The Trolox equivalent antioxidant capacity of plasma from an adult reference population has been measured and the method optimized and validated (*43*). We used this automated method to investigate the total PAC in hamsters.

Hamster Aortic Tissue Processing. Following blood collection, the intact aorta was first perfused with phosphate-buffered saline containing 1 mmol/L $CaCl_2$ and 15 mmol/L glucose for 5 min and then with 0.1 mmol/L sodium cacodylate buffer (pH 7.4) containing 2.5 mmol/L $CaCl_2$, 830 mmol/L paraformaldehyde, and 150 mmol/L glutaraldehyde for the fixation of the vasculature as described by Simionescu et al. (*44*). The aorta was carefully dissected between sigmoid valves and the thoracic aorta and then thoroughly cleaned of loose adventitial tissue; the aortic arch was cut free, opened longitudinally along the outside of the arch, immersed in fresh fixative solution, and stored at 4 °C until staining. The aortic arches were then rinsed for 48 h in 0.1 mol/L sodium cacodylate buffer (pH 7.4) containing 30 mmol/L $CaCl_2$ and 250 mmol/L sucrose. They were then rinsed in distilled water, stained for 40 s in Harris hematoxylin, rinsed in distilled water again, and then quickly rinsed in 11.7 mol/L isopropyl alcohol; finally, they were stained in Oil Red O for 30 min according to the method of Nunnari et al.

Table 2. Phenolic Composition of the Dry Extracts (Milligrams per Gram of Powder)

compound[a]	EGS	EGT	GSE
EGC	86.77		
B3	60.57		22.49
B1	90.42	59.11	58.77
+(–)-catechin	172.66		47.62
B2	252.77		
B4			250.77
(–)-epicatechin	182.58	45.32	68.94
EGCG	30.97		
ECG			18.36
gallic acid		8.31	38.97
cyanidin 3-glucoside		7.92	
malvidin 3-glucoside		37.69	
cyanidin		3.87	
peonidin		8.53	

[a] EGC, (–)-epigallocatechin; B1, B2, B3, B4, procyanidin dimers; ECGC, (–)-epigallocatechin 3-*O*-gallate; ECG, (–)-epicatechin 3-*O*-gallate.

(*45*), rinsed in 11.7 mol/L isopropyl alcohol, and back to distilled water. Each aortic arch was then directly displayed on a glass slide, endothelium side up, covered with Aquamount mounting medium and cover slips, and observed *en face* by light microscopy. All segments were photographed using a video digitizer. The Oil Red O stained area was analyzed quantitatively using a computer-assisted morphometry system and expressed as a percentage of the total area surveyed.

Rat Aortic Preparation and Mounting for Vasorelaxation Studies. Male Wistar rats (Janvier, Le Genest-St-Isle, France) weighing 350−400 g were killed by cervical dislocation and exsanguination by carotid artery transection. The thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue in saline and cut into rings (4 mm long). The rings were then mounted on a standard organ bath comprising eight tanks filled with 4 mL of physiological buffered (pH 7.4) salt solution containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2 \cdot 2H_2O$, 25 mM $NaHCO_3$, and 11 mM glucose, maintained at 37 °C and bubbled with a 95% O_2/5% CO_2 mixture. Resting tension was adjusted to 1 g. Tension was measured with an isometric-force transducer connected to a Mac Lab V8e computerized system (AD Instruments, Castle Hill, Australia). After an equilibration period of 60 min, the vessels were contracted with noradrenaline (10^{-7} M) to test their contractile capacity. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (10 μM) to induce >65% relaxation of rings precontracted with noradrenaline (0.1 μM) as described by Furchgott and Zawadzki (*46*). Then the effect of each phenolic extract (EGS, EGT, and GSE) on aortic vasorelaxation was measured.

Statistical Analyses. Data are shown as the means ± SEM of eight measurements per group. Data were subjected to logarithmic transformation when necessary to achieve homogeneity of variances. Separately for each dietary treatment, statistical analysis of data was performed by one-way ANOVA followed by Fisher's protected least significant difference post-hoc procedure using a Stat View 512+ microcomputer program (Brain Power, Calabasas, CA). Differences were considered to be significant when $P < 0.05$.

RESULTS

Extract Composition. This composition, expressed as milligrams per gram of powder, is summarized in **Table 2**. ExGrape seed (EGS) and grape seed extract (GSE) are characterized by the absence of anthocyanins; EGS contained 54% flavanol monomers [as (−)-epigallocatechin, (−)-epicatechin 3-*O*-gallate, (−)-epigallocatechin 3-*O*-gallate, (+)-catechin, and (−)-epicatechin] and 46% procyanidin dimers (B1, B2, B3, and B4), whereas GSE contained 26.7% flavanol monomers [without (−)-epigallocatechin and (−)-epigallocatechin 3-*O*-gallate], 65.6% procyanidin dimers (without B3 and with high B4 concentration), and 7.7% gallic acid. ExGrape total (EGT) was rich in anthocyanins (34%) and also contained 26.5% (−)-epicatechin, 34.6% B1 as procyanidin dimer, and 4.8% gallic acid. Elsewhere, EGS, EGT, and GSE, respectively, contained 864, 556, and 776 mg/g phenolic compounds expressed as gallic acid equivalents.

Growth and Plasma Parameters. There were no significant differences in the final body weights and food intakes among the four groups (**Table 3**). Each extract significantly reduced plasma total cholesterol (TC) by 11% on average in comparison with controls (**Table 3**). Plasma Apo A1 and Apo B concentrations did not differ among the experimental groups (**Table 3**). PAC values are shown in **Table 3**; no significant effect of the extracts was observed.

Extent of Atherosclerosis in Hamsters. Average AFSA, measured as the percentage of Oil Red O staining relative to the total area surveyed (**Figure 1**), was significantly decreased in hamsters receiving EGS (68%), EGT (63%), and GSE (34%), in comparison with controls.

Relaxation in Rat Aortic Rings. All of the extracts used relaxed rat aorta with functional endothelium (**Figure 2**). The maximal percent relaxation values were 77.2 ± 3.0 (EGS), 84.5 ± 8.0 (EGT), and 72.3 ± 3.3 (GSE). These levels were reached at a lower extract concentration for EGS (7 μg/mL) than for EGT or GSE (30 μg/mL). The relaxations induced by these extracts were dependent on the presence of a functional endothelium. In endothelium-denuded vessels, slight relaxations occurred (between 10 and 13%) at concentrations 10−20-fold higher than those needed to obtain the same level in vessels with functional endothelium. Elsewhere, we have verified that the solvent used for extracts did not produce a relaxant effect and that in vessels with endothelium, blockade of endothelial NO synthesis by a NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) (10 μM), completely abolished endothelial-dependent relaxation but did not affect the endothelim-independent response to these extracts (results not shown here).

DISCUSSION

Procyanidins are known for their antioxidative activity in aqueous systems (*47*, *48*) and can inhibit LDL oxidation in vitro (*10*). Elsewhere, anthocyanins have attracted a lot of attention due to their health-promoting benefits in terms of reducing the risk of coronary heart disease and prevention of some chronic diseases (*5*, *49*). Anthocyanins may also prevent cholesterol-induced atherosclerosis (*50*) in rabbits and inhibit platelet agregation (*49*). The positive effect of these pigments could be related to their potent antioxidant activity demonstrated in various in vitro studies (*51*, *52*). In this work, the hamster model studied the effect of the extracts on early atherosclerosis with <10% foam cell coverage of the aorta; we showed that procyanidin-rich extract (ExGrape seeds) prevents early atherosclerosis in hypercholesterolemic Syrian hamsters and that the effect of an anthocyanin-rich extract (ExGrape total) was almost the same, both affecting plasma cholesterol but not apolipoprotein levels and plasma antioxidant capacity. These results demonstrate that these extracts are bioavailable. Grape seed extract, which contained fewer monomeric flavanols and procyanidin dimers (except B4) and no anthocyanins, exhibited a protective effect against atherosclerosis but to a lesser extent. According to the oxidative hypothesis of atherosclerosis (*16*, *53*−*55*), LDL entrapped in the subendothelial space of lesion-prone arterial sites is slowly oxidized through the action of resident vascular cells. The modification of LDL is associated with the recruitment of monocytes and macrophages that

Table 3. Effect of Force-Feeding Phenolic Extracts on Growth, Food Intake, Plasma Total Cholesterol and Apolipoprotein Concentrations and Plasma Antioxidant Capacity in Hamsters Fed an Atherogenic Diet for 12 Weeks[a]

dietary group	control	EGS	EGT	GSE
final body wt (g)	112.6 ± 2.4	115.1 ± 1.4	117.8 ± 3.5	113.3 ± 2.0
food intake (g/day)	4.51 ± 0.05	4.32 ± 0.08	4.60 ± 0.10	4.39 ± 0.06
TC[b] (mmol/L)	9.38 ± 0.40a	8.14 ± 0.35b	8.55 ± 0.42b	8.39 ± 0.47b
Apo A1 (g/L)	1.610 ± 0.052	1.658 ± 0.032	1.644 ± 0.013	1.633 ± 0.050
Apo B (g/L)	0.282 ± 0.039	0.300 ± 0.029	0.274 ± 0.031	0.330 ± 0.038
Apo A1/Apo B	6.07 ± 0.85	5.25 ± 0.37	6.08 ± 0.71	5.26 ± 0.90
PAC[b] (mmol/L)	0.936 ± 0.064	0.964 ± 0.040	0.979 ± 0.018	0.992 ± 0.062

[a] Values are mean ± SEM, $n = 8$. Data were analyzed by one-way ANOVA followed by the least significant difference test. For each dietary treatment, means in a column with different letters differ, $P < 0.05$. [b] TC, total cholesterol; PAC, plasma antioxidant capacity.

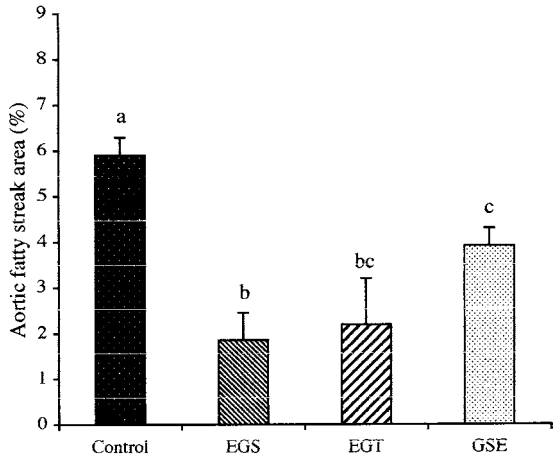

Figure 1. Effects of force-feeding water (control), a procyanidin-rich white grape seed extract (EGS), a marc extract from red and white grapes, rich in anthocyanins (EGT), or a grape seed extract (GSE) on aortic fatty streak area (AFSA) in hamsters fed an atherogenic diet for 12 weeks. AFSA is expressed as a percentage of the total aortic area surveyed. Each bar represents mean ± SEM from eight hamsters. Bars with different letters differ, $P < 0.05$.

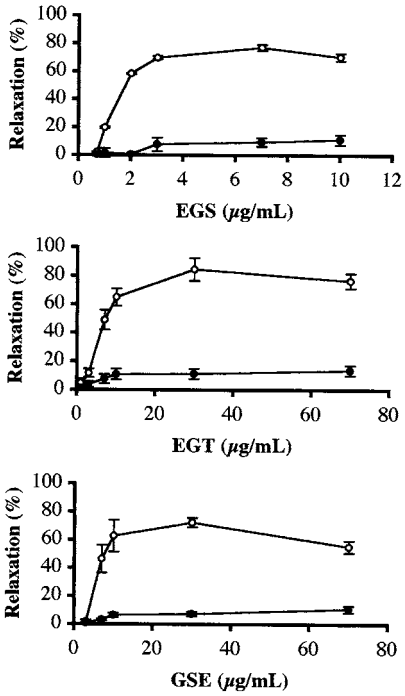

Figure 2. Concentration–response curves for the relaxation of isolated aortic rings of rat by a procyanidin-rich white grape seed extract (EGS), a marc extract from red and white grapes, rich in anthocyanins (EGT), and a grape seed extract (GSE) in the presence (○) or in the absence (●) of endothelium. Each point represents mean ± SEM from eight rats.

internalize modified LDL, leading to foam cell formation and the development of fatty streaks. Oxidation of LDL in the arterial wall is thought to be a very important step in atherogenesis. Here, no improvement of PAC by phenolic extracts was observed, whereas AFSA development was prevented. All of these results suggest that these extracts acted by other mechanism(s) operating outside a hypolipemic or especially an antioxidant effect. As shown by the data in **Figure 2**, at least one of them could be vasorelaxation.

Indeed, our findings provided direct evidence that a mixture of phenolic compounds present in the extracts triggered off an endothelium-dependent relaxation, suggesting an increase in NO content in the rat aorta. The involvement of NO in endothelium-dependent vasorelaxation appeared for low concentrations of phenolics. Moreover, 10−20 times higher concentrations for extracts are needed to cause endothelium-independent vasorelaxation by a different and undetermined mechanism. Similar results have been previously reported (*21*) using red wine phenolic extract and leucoanthocyanidol; these authors reported that some phenolics, with specific structural requirement, induce an endothelium-dependent relaxation via an enhancement of endothelial NO synthesis and that the antioxidant properties of polyphenols are unlikely to be involved in this effect. This could be related here to the unmodified values of PAC measured in the experiment performed on hamsters.

Some years ago, a procyanidin-rich extract from grape seeds (73% procyanidins, 5.6% monomeric flavanols) was shown to attenuate the development of aortic atherosclerosis in cholesterol-fed rabbits (*56*), and it was suggested that this effect was related to prevention of LDL oxidation in the arterial wall. EGS used in our study contained 46% procyanidins and 54% monomeric flavanols, and EGT contained 34% anthocyanins, 35% procyanidins, and 27% monomeric flavanols. In a previous study (*14*), we showed that a red wine phenolic extract, shown by others to induce an endothelium-dependent relaxation via an enhencement of endothelial NO synthesis (*21*) and containing 42% procyanidins, 20% monomeric flavanols, 14% anthocyanins and 24% phenolic acids, prevented early atherosclerosis in hamsters by a hypolipemic and an antioxidant effect, but also by an indirect mechanism such as glutathione peroxidase economy.

More recently, Bagchi et al. (*57*) reported that in hypercholesterolemic hamsters a grape seed procyanidin extract (GSPE) reduced the percentage of aorta covered with foam cells by 50 and 63% following supplementation of these animals with 50 and 100 mg/kg GSPE, respectively. Moreover, plasma total cholesterol (TC) was reduced by 25 and 23% and triglyceride (TG) level was reduced by 10 and 34% after the hamsters were fed 50 and 100 mg/kg GSPE, respectively; GSPE contained 75−

80% oligomeric procyanidins and 3−5% monomeric procyanidins. In our study, EGS and EGT reduced AFS by 68 and 63%, respectively, reducing TC by 11%, without modifying the plasma TG level; it must be pointed out that hamsters received EGS at 17 mg/kg and EGT at 27 mg/kg. All of these reports emphasized the complexity of numerous possible operating mechanisms of action depending on the presence and combination of different families of phenolic compounds, in both nature and quantity.

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