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A Dietary Resveratrol-Rich Grape Extract Prevents the Developing of Atherosclerotic Lesions in the Aorta of Pigs Fed an Atherogenic Diet

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Supporting Information

ABSTRACT: The presence of grape and wine polyphenol resveratrol (RES) in the diet is negligible. Therefore, the cardiovascular benefits of this molecule, in a dietary context, remain to be established. We aimed to investigate, through dietary intervention, the effects of a resveratrol-rich grape extract (GE-RES) on the prevention of early aortic lesions in pigs fed an atherogenic diet (AD). These effects were compared with those produced by a grape extract lacking RES (GE) or RES alone. Pigs fed the AD for 4 months showed early atherosclerotic lesions in the thoracic aorta: degeneration and fragmentation of elastic fibers, increase of intima thickness, subendothelial fibrosis, and accumulation of fatty cells and anion superoxide radicals. GE-RES was the most effective treatment and prevented the disruption of aortic elastic fibers, decreased their alteration (57%), and reduced the intima thickness (33%) and the accumulation of fatty cells (42%) and $O_2^{\bullet -}$ (38%) in aortic tissue. In addition, GE-RES moderately downregulated the expression of the suppressors of cytokine signaling 1 (*SOCS1*) and 3 (*SOCS3*), key regulators of vascular cell responses, in peripheral mononuclear blood cells. Our results suggest that the consumption of this GE-RES nutraceutical, in a dietary prevention context, could prevent early atherosclerotic events. The presence of RES in the grape extract strengthened these effects.

KEYWORDS: *atherosclerosis, resveratrol, oxidative stress, cardiovascular, nutraceutical, microarrays*

INTRODUCTION

According to the World Health Organization, the primary cause of morbi-mortality in the world is related to cardiovascular diseases (CVDs) (approximately one-third of all deaths are related to CVDs). The main underlying cause of CVDs is atherosclerosis, a complex multifactorial disease in which chronic inflammation and oxidative stress play crucial roles.[1] Endothelium is the major regulator of vascular homeostasis although peripheral blood mononuclear cells (PBMNCs) are also involved in the development of atherosclerosis and metabolic disorders. Endothelial dysfunction, an early marker for atherosclerosis, occurs when vascular homeostasis is disrupted.[2] A number of atherogenic and proinflammatory factors are involved in endothelial dysfunction, i.e. oxidative stress, low shear stress, smoking, hypertension, elevated and modified LDL, insulin resistance, elevated plasma homocysteine concentrations, genetic alterations, infectious microorganisms, or a combination of these and other factors.[1] Unbalanced diets can lead to obesity, a pathological condition that represents a state of low-grade inflammation, insulin resistance, and enlarged adipose tissue that needs urgent therapeutic and preventive actions.

The polyphenol *trans*-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene; RES), which naturally occurs in grapes and grape-derived foodstuffs such as red wine, has been reported to exert many different health-promoting effects including antioxidant, anti-inflammatory, antitumor, antiplatelet aggregation, cardioprotective, aging-delay, and antiobesity effects.[3] However, the vast majority of reported results are based on pharmacological approaches rather than nutritional ones, which use pathological models representative of human diseases with intraperitoneal or intravenous injections of RES or alternatively very high oral doses (hundreds of milligrams or grams) of this compound that cannot be reached through a common diet. Although the use of high RES doses has been reported to be safe in short trials with a low number of individuals,[4] the safety of long-term consumption of high RES doses has not yet been assessed. However, and essential from a dietary point of view, there is a need to ascertain whether long-term consumption of low doses of RES that may be achieved through the diet exert any of the beneficial effects attributed to this molecule.

The main dietary source of RES is red wine, with a highly variable content, ranging from "not detected" to 14 mg/L.[5] In Spain, the estimated dose of daily RES intake for wine drinkers is 0.19 ± 0.35 mg/day,[6] which suggests that the intake of RES

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from the diet, especially for non-red wine drinkers, is almost negligible.

RES is a phytoalexin, i.e. a stress-inducible metabolite synthesized in the plant to face unfavorable environmental conditions, which explains the high variability of RES in grapes and wines. However, this feature can be used to induce RES in grapes under controlled ultraviolet illumination,[7] which allows obtaining RES-rich grape extracts with low (mg/g) but safe and standardized RES contents.[8] We have previously reported the anti-inflammatory effects of a human equivalent dose (HED) of 10 mg of RES for a 70 kg person in an animal model of colitis.[9,10] More recently, we have described, in a mild hyperlipidemia pig model, that long-term consumption of resveratrol-rich grape extracts (HED RES of 8 mg for a 70 kg person) regulated some key genes in PBMNCs, such as *FAPB4* (fatty acid binding protein), associated with atherosclerosis and metabolic disorders.[8] To further investigate the dietary significance of resveratrol as a bioactive compound, the main aims of the present work were (i) to evaluate whether the intake of a grape extract supplement containing a low dose of RES exerts beneficial effects in a pig model with early atherosclerotic lesions in the aorta, and (ii) to ascertain the dietary relevance of this minor dose of RES compared to the rest of the polyphenols present in grape extracts.

Our results suggest that long-term consumption of a grape extract containing resveratrol, in a dietary prevention context, could prevent early atherosclerotic events by decreasing vascular oxidative stress and regulating some key regulators of vascular response.

MATERIALS AND METHODS

Materials. RES (3,5,4′-trihydroxy-*trans*-stilbene, >98.5% purity) was purchased from SeeBio Biotech Inc. (Shangai, China). Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), DL-1,4-dithiothreitol, and reduced glutathione (GSH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The conventional grape extract (GE) as well as the resveratrol-rich grape extract (GE-RES, Stilvid) were kindly provided by Actafarma S.L. (Pozuelo de Alarcon, Madrid, Spain). GE-RES (Stilvid) is the key ingredient included in the commercially available nutraceutical Revidox (Actafarma S.L.). The phenolic profile of both extracts, GE and GE-RES, was very similar (determined by HPLC−MS−MS) and contained ∼110 mg/g procyanidins, ∼70 mg/g anthocyanins, ∼3 mg/g flavonols and ∼2 mg/g hydroxycinnamic acids. However, GE-RES also contained ∼23 mg/g RES as well as other minor stilbenes (trace levels) because grapes were previously treated with UVC light.[7] Therefore, both extracts GE and GE-RES mainly differed in their RES content (Table S1 in the Supporting Information).

Organic solvents such as methanol (MeOH), acetone, acetonitrile, etc. were obtained from Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., USA) ultrapure water was used throughout this experiment. Tiletamine-zolazepam (Zoletil 50) was purchased from Virbac España S.A. (Esplugues de Llobregat, Spain) and sodium pentobarbital (Dolethal) from Vétoquinol (Alcobendas, Madrid, Spain). Coconut oil was purchased from Cailà & Parés (Barcelona, Spain) and cholic acid (98.5%) from Alfa Aesar GmbH & Co (Karlsruhe, Germany).

Animal Study Design. All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). The experimental design, included in the Spanish National Research Project BFU2007-60576, was approved by the Ethics Committee of the University of Murcia (Murcia, Spain) and by the Bioethics Committee-CSIC (Madrid, Spain). The ethics committees encouraged that the assays be conducted with the lowest possible number of animals.

Thirty minipigs (18 females and 12 males) were purchased from the Experimental Animal Service of the University of Cordoba (Spain). Housing and animal interventions were all carried out at the Veterinary Experimental Animal Farm of the University of Murcia (Murcia, Spain). Animals were exposed to a constant temperature of 25 °C and a natural day−night light cycle. This study is an extension of a previous experimentation in which the pigs were fed with a high-fat diet (diet supplemented with 20% beef tallow) and grape extracts for 12 months as described elsewhere.[8]

The animals (20 months old, weight 110 ± 21 kg) were penned separately depending on their gender and dietary intervention. A commercial cereal-based chow and beef tallow were purchased from a local supplier. Control animals (CT) were fed with commercial pig chow containing 5% of fat. The diet for animals fed the atherogenic diet (AD) was supplemented with 5.5% beef tallow (92.3% of fat content, including 280 mg of cholesterol/100 g), 10% coconut oil, and 1% cholic acid (to promote fat absorption). Coconut oil was composed of 6.9% caprylic acid (C_8), 6.7% capric acid (C_{10}), 49.9% lauric acid (C_{12}), 19.3% myristic acid (C_{14}), 8.5% palmitic acid (C_{16}), 2.2% stearic acid (C_{18}), 5.2% oleic acid ($C_{18:1}$) and 1.3% linoleic acid ($C_{18:2}$). Experimental diets were prepared weekly. The stability of phenolics in the diets was routinely monitored throughout the experiment. The chow of all the groups fed AD (except the control-AD group; AD) was supplemented either by grape extract (AD-GE), RES-rich grape extract (AD-GE-RES), or commercial RES alone (AD-RES). A group fed with normal chow was also supplemented with GE-RES (CT-GE-RES).

Six different groups were followed for 4 months, i.e., control group (CT; n = 5, 3 females ♀ and 2 males ♂), atherogenic diet-group (AD, n = 5, 3 ♀ and 2 ♂), AD + grape extract group (AD-GE; n = 5, 3 ♀ and 2 ♂; 1 g of GE/70 kg body weight (bw) per day), AD + RES-rich grape extract group (AD-GE-RES; n = 5, 3 ♀ and 2 ♂; 1 g of GE + RES/70 kg bw per day, equivalent to 18 mg of RES/70 kg animal bw per day), AD + RES group (AD-RES; n = 5, 3 ♀ and 2 ♂; 18 mg of RES/70 kg animal bw per day), and finally, CT + GE-RES group (CT-GE-RES; n = 5, 3 ♀ and 2 ♂). The human equivalent dose (HED) was 900 mg of grape extract and 16 mg of RES assuming a 70 kg person.[11] After the 4 month intervention period, fasted pigs were sedated with tiletamine-zolazepam (Virbac) and sacrificed using a lethal dose of sodium pentobarbital (Vétoquinol).

Just before euthanasia blood samples were collected from the jugular vein in BD Vacutainer tubes without anticoagulant for serum and BD Vacutainer lithium heparin tubes for whole blood (BD, Franklin Lakes, NJ).

Aortas were obtained from the thoracic cavity (from the base of the heart to the bifurcation of the iliac arteries in the pelvis). Aortas were rinsed in phosphate buffered saline and macroscopically evaluated. Adjacent portions, next to the aortic arc, were obtained. Part of each aortic sample was fixed in 10% neutral formalin for histopathology and immunohistochemistry. Another portion of aorta was frozen in 2-methylbutane, cooled in liquid nitrogen for lipids staining, another part was incubated with hydroethidine prior to being frozen in liquid nitrogen, and another part was frozen at −80 °C to determine grape polyphenols or derived metabolites.

Serum Biochemical Analysis. Blood samples were immediately centrifuged at 14000*g* for 15 min at 4 °C in a Sigma 1-13 microcentrifuge (Braun Biotech. International, Germany). The serum was immediately frozen at −80 °C for further analysis. Levels of total glucose, cholesterol, HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c), triglycerides, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), amylase, phosphorus, calcium, creatinine, bilirubin, urea, albumin, haptoglobin, total proteins, creatin phosphokinase (CPK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyltransferase (GGT) were measured using an Olympus AU600 autoanalyzer (Olympus Diagnostica, Hamburg, Germany).

Histological Analyses. The formalin-fixed samples of aortic tissue were embedded in paraffin wax and sectioned at 4 μm with an RM 2155 Leica microtome (Leica Microsystems GmbH, Wetzlar, Germany). Adjacent sections were stained with hematoxylin and

eosin, Masson trichrome for distinguishing muscular and connective tissues and orcein for elastic fibers. The frozen samples in 2-methylbutane were sectioned at 8 μm with a CM 1900 Leica cryostat (Leica Microsystems GmbH), and sections were stained with Sudan IV for lipids.

Aortic lesions were evaluated by two expert anatomopathologists that were blind to the different treatments. Both experts used the same scores (marks from 0 to 3) and gave independent marks for each lesion. The mean of the evaluations from the two anatomopathologists was used as the final value for each lesion.

The thickening of the aortic subendothelial intima was scored from 0 to 3, i.e. 0, absence of intima thickness; 1, mild intima thickness; 2, moderate intima thickness; 3, intense intima thickness. The maximum thickness of the intima was recorded using the photomicroscope Leica DM6000B (Leica Microsystems GmbH) for image capturing and the Leica Qwin Pro imaging software for parameter measurement. The presence of lipid drops in the intima of the aorta stained with Sudan IV was scored from 0 to 3, i.e. 0, absence of lipid drops; 1, low number of lipid drops; 2, moderate number of lipid drops and 3, high number of lipid drops. The alterations in the structure of the elastic fibers were scored from 0 to 3, i.e. 0, elastic fibers not altered; 1, mild flattening of the wavy structure and irregular arrangement; 2, moderate flattening of the wavy structure and irregular arrangement; 3, severe flattening of the wavy structure, irregular arrangement, and hyaline degeneration and disruption of fibers. The extension of the lesion in the aortic subendothelial intima of each sample was scored from 0 to 3, i.e. 0, absence of lesion; 1, lesion comprising less than 15% of the extension of the sample; 2, lesion comprising between 15% and 50% of the extension of the sample; 3, lesion comprising more than 50% of the extension of the sample.

In Situ Detection of Superoxide Anion Radical. Hydroethidine (HE), an oxidative fluorescent dye, was used to detect superoxide anion ($O_2^{\bullet-}$) production in situ as previously reported.[12] HE is permeable to cells and, in the presence of $O_2^{\bullet-}$, is oxidized to fluorescent ethidium bromide (EtBr), which is trapped by intercalation with DNA. EtBr is excited at 488 nm, with a maximum emission at 610 nm. Aortic ring segments were incubated with HE (Sigma Aldrich) (10 μM, at 37 °C for 60 min), washed three times, embedded in OCT medium (Electron Microscopy Sciences, Hatfield, PA), and frozen at −80 °C. Frozen ring segments were cut into sections 10 μm thick in a Leica cryostat and examined on a DM6000B fluorescence Leica microscope, using the same imaging setting in each case. Fluorescent images were collected using a 590 nm long-pass filter. Images were captured (DFC280, Leica) at 20× magnification and analyzed using the Leica Qwin Pro imaging software. Ten to sixteen fields per aortic segment were analyzed (n = 4−5 for each group), and the number of fluorescent ethidium bromide (EB)-stained nuclei was counted per section and normalized for surface area for each section. Segments coincubated with Tiron (10 mM) were used as negative control.

Peripheral Blood Mononuclear Cells Isolation and RNA Extraction. PBMNCs were isolated from heparinized blood (BD Vacutainer, Franklin Lakes, NJ, USA) within two hours after extraction. Isolation was carried out under sterile conditions using density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, Madrid, Spain) as previously described.[8] PBMNC cells were obtained from animals from all the experimental groups at the end of the dietary intervention, lysed using RNeasy Plus lysis buffer (Qiagen, Madrid, Spain), and stored at −80 °C for RNA extraction. Total RNA was isolated from PBMNCs using the RNeasy Plus Mini kit (Qiagen), and RNA concentration was checked using the Nanodrop ND-1000 UV−vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 were used for microarrays and (or) RT-PCR experiments. The integrity of the rRNA was further checked using agarose gel electrophoresis (1%). Pure RNA samples were divided into aliquots and frozen at −80 °C until further analysis.

Porcine Microarray and Functional Analyses. A search for potential candidate genes expressed in the PBMNCs for which transcription levels may have been modulated after exposure of the animals to the different diets was performed using GeneChip Porcine Genome Arrays (Affymetrix, Santa Clara, CA) as previously described.[8] Since the Affymetrix porcine genome microarray is not fully annotated, we have used information from a published annotation database that describes approximately 82% of the probe sets.[13] Specific probe sets were additionally verified by BLAST comparison of the Affymetrix target sequence against the GeneBank NR database. The number of microarrays performed was as follows: (i) CT group, n = 4 arrays (2 females, 2 males); (ii) AD group, n = 4 arrays (2 females, 2 males); (iii) AD-GE group, n = 4 arrays (2 females, 2 males); (iv) AD-GE-RES group, n = 4 arrays (2 females, 2 males); (v) AD-RES group, n = 4 arrays (2 females, 2 males); (vi) CT-GE-RES group, n = 4 arrays (2 females, 2 males).

The CEL files obtained from GCOS software (Affymetrix) were used to analyze the data with Robust Multichip Average (RMA) implemented with the Affymetrix GeneChip Expression console (RMA Sketch for 1.0ST arrays). RMA-normalized data were tested for differential gene expression between groups using an empirical Bayes method (Limma)[14] implemented with Babelomics (http://babelomics.bioinfo.cipf.es/) which performs well for small n microarrays.[15] Using this model, differentially expressed genes were defined as those with a Limma P-value <0.01 and with ratios (treated/control) >1.2 (upregulation) and <0.8 (downregulation). About Microarray Experiment (MIAME) compliance, the complete data set, both RMA normalized and original CEL files, from PBMNC control and after exposure to the different experimental diets, has been deposited in NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE30874. Ingenuity Pathways Analysis (IPA) (http://www.ingenuity.com/) was used to identify molecules commonly regulated in the different groups as well as significantly altered biological pathways and top regulated functions associated with responsive genes.

Quantitative RT-PCR. We used one-step quantitative RT-PCR (Taqman system, Applied Biosystems, ABI, Madrid, Spain) to validate differential expression between groups for the following probes: Ssc.2827.1.S1_at annotated as *SOCS3* (suppressor of cytokine signaling 3); Ssc.26216.1.S1_at and Ssc.26216.2.A1_at annotated as *SOCS1* (suppressor of cytokine signaling 1); Ssc.7953.1.A1_at and Ssc.9789.1.A1_at annotated as *VCL* (vinculin or metavinculin); Ssc.22761.1.S1_at, Ssc.7645.1.A1_at and Ssc.7645.2.S1_at annotated as *CFL2* (Cofilin or Cofilin 2); and Ssc.16031.1.A1_at annotated as *EGR1* (early growth response protein 1). TaqMan FAM-labeled, primer−probe sets were purchased from Assays-on-demand (ABI, Madrid, Spain): Ss03387992_u1 for *SOCS3*, Ss03391019_m1 for *VCL*, Ss03391732_g1 for *CFL2*, and Ss03373483_s1 for *EGR1*. The primer−probe set for *SOCS1* was designed based on its mRNA sequence (GenBank: GQ421919): forward primer sequence (CGCGCATCCCCCTCAA); reverse primer sequence (GCGGCCGATCATATCTGGAA), and reporter sequence (CCTCCGCGATTACTTG). The one-step real-time RT-PCRs were run on the ABI 7500 system following manufacturer's conditions, using a total reaction volume of 25 μL in a MicroAmp Optical 96-well plate covered by optical adhesive covers and using Taqman Universal Master Mix (ABI, Madrid, Spain). All assays were undertaken at the same time under identical conditions and in triplicate. Gene expression levels were normalized to the levels of the endogenous control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Ss03373286_u1) utilizing a standard curve method for quantification.

Immunohistochemistry. The avidin−biotin−peroxidase complex technique was used for the detection of SOCS3 in aortic tissue according to Gupta et al.[16] The primary antibody was a rabbit anti-SOCS3 (Abcam, Cambridge, U.K.) diluted 1:100 in TBS, and the secondary antibody, a peroxidase conjugate anti-rabbit IgG (Sigma-Aldrich) diluted 1:300 in TBS. Positive labeling was detected using 3,3′-diaminobenzidine tetrahydrochloride (Dako). Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. Negative controls consisted of replacement of the primary antibody by blocking solution, normal serum, and isotype-matched reagents of irrelevant specificity. Images were captured (Leica DM6000B, Leica Microsystems GmbH) and analyzed using the ImageJ free software

(http://rsbweb.nih.gov/ij/) and the plugin "color deconvolution".[17] The integrated optical density was calculated. The results are expressed as the mean ± SE (5 samples from each animal). Five independent fields were analyzed in each sample.

Interleukin-6 (IL-6) and Endothelin-1 Analysis. IL-6 was measured in pig serum and aortic tissue using the pig ELISA kit ab100755 from Abcam (Cambridge, U.K.) according to the manufacturer's instructions. Endothelin-1 was measured in pig aortic tissue using the EIA kit EK-023-01 from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA) following manufacturer's specifications. To measure IL-6 or endothelin-1 in aortic tissue, 100 mg of aorta was homogenized in RIPA buffer using an Ultra-Turrax IKA T10 (IKA-Werke GmbH & Co. KG, Staufen, Germany), centrifuged at 14000*g* for 15 min at 4 °C in a Sigma 1-13 microcentrifuge (Braun Biotech. Int.). Protein concentration was measured using the DC Protein Assay kit (Biorad, Barcelona, Spain) and following the manufacturer's instructions.

Phenolics, Homocysteine, and Malondialdehyde Determination in Pig Samples. Aortic tissue (500 mg) and plasma (300 μL) were processed according to Azorín-Ortuño et al.[18] to determine the presence of phenolics (including RES) and derived metabolites. Detection was achieved using LC-DAD−ESI ion trap (1100 series, Agilent Technologies, Waldbronn, Germany) and UHPLC−triple quadrupole (QqQ) MS detection (1290 Infinity, Agilent) according to Tomás-Barberán et al.[19] and Azorín-Ortuño et al.[18]

Total HCys and GSH were determined in plasma. Plasma samples were processed as described by Hellmuth et al.[20] and analyzed in the UHPLC−MS-QqQ system. EDTA-treated plasma (20 μL) was spiked with the internal standard (9 μM) d_8-homocysteine (ClinMass, Munich, Germany) to check daily ionization in the UHPLC−MS-QqQ system. Chromatographic separations for HCys and GSH were those described in Hellmuth et al.[20] MS data were collected in multiple reaction monitoring (MRM) mode by monitoring specific transitions of parent and product ions for each metabolite in the positive ionization mode (Hcys 136 → 90 and 136 → 56; GSH 308 → 233, 308 → 179 and 308 → 162; d_8-homocysteine 277 → 140, 277 → 122, 277 → 94; d_4-homocysteine (after reduction) 140 → 94, 140 → 77, 140 → 59. The quantification of both total HCys and GSH was carried out using authentic standards and calibration curves in the range from 0.01 to 20 μM in spiked plasma.

Malondialdehyde was analyzed in aortic tissue (100 mg), plasma (50 μL), and serum (50 μL) as described by Mateos et al.[21] using an Elite LaChrom-Hitachi HPLC system.

Statistics. Unless otherwise stated, experimental results are shown as the mean ± SE. Statistical analysis of ordinal variables was carried out with the Mann−Whitney *U* nonparametric test. The rest of the analyses were performed by the Student *t*-test. Significance level was defined as $P < 0.05$.

RESULTS

Serum Biochemical Variables. No significant differences were found in the mean weight of the animals (data not shown). In general, a high variability was observed in the serum biochemical parameters analyzed with no significant differences detected between groups at the end of the 4 month experimental period (Table S2 in the Supporting Information). It should be noted, however, that the atherogenic diet (AD) caused an increase (although not significant) in the levels of the hepatic enzymes, ALP and GGT, especially in males, ALT in females, and AST in both males and females. These changes were attenuated in the animals supplemented with RES-rich grape extracts (AD-GE-RES) and with RES (AD-RES) in the case of ALP and GGT (Figure S1 in the Supporting Information) but not in ALT and AST.

Effects on Aortic Lesions. Aortic tissue-diet associated changes showed a similar tendency both in female and in male animals and were analyzed together. The Masson trichrome staining revealed a significant increase ($P < 0.05$) in the thickness of the aortic intima (due to proliferation of connective tissue) in the pigs fed the atherogenic diet (AD) vs the groups fed with the normal chow (CT and CT-GE-RES) (Figure 1). Among all the pigs fed with the AD, only the group supplemented with GE-RES (AD-GE-RES) exhibited a significant reduction of the aortic intima thickness. In particular, the minimum thickness measured in one of the animals from the AD-GE-RES group was only 34.55 μm, which indicated a reduction in the aortic intima thickness of up to 90% in

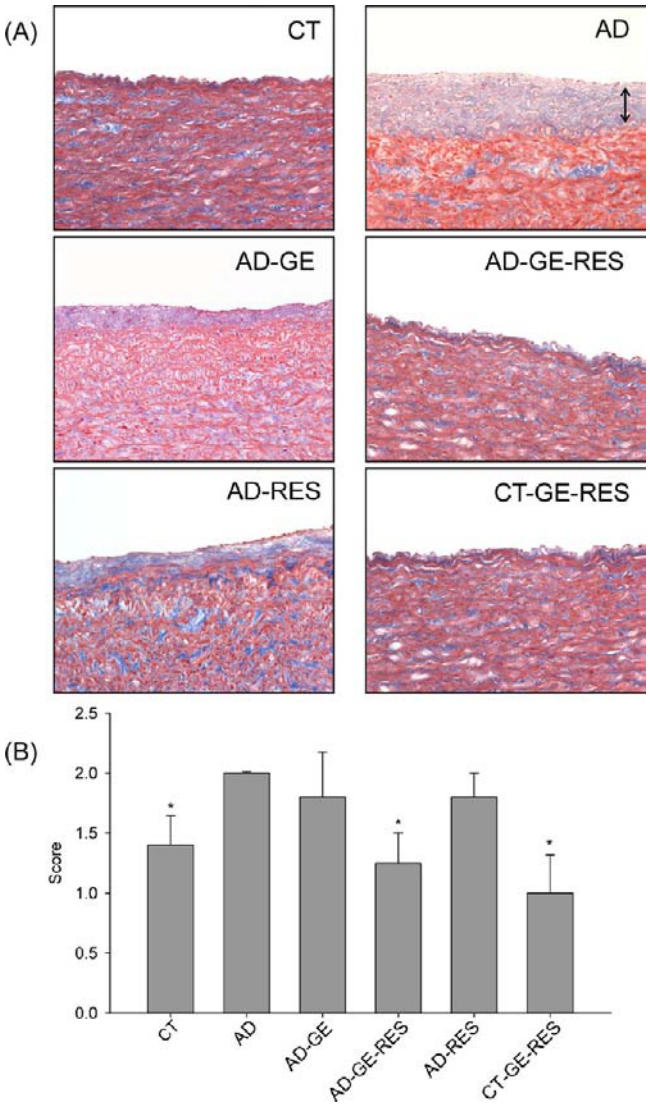

Figure 1. GE-RES prevented collagen deposition and subendothelial thickness of aortic tissue. (A) Representative photomicrographs of pig aortic tissue sections stained with Masson's trichrome staining. Connective tissue (mainly collagen deposition, in blue) in subendothelial area was prominently present in AD (marked with the arrow) over the control. (B) Quantification of subendothelial thickness in the different groups. Results are shown as the mean ± SE of two independent evaluations from 4 different tissue preparations for each pig in each group ($n = 5$). CT, control group fed with normal chow; CT-GE-RES, control group fed with normal chow and supplemented with RES-rich grape extract; AD, control group fed with atherogenic diet; AD-GE, group fed with AD plus grape extract; AD-GE-RES, group fed with AD plus RES-rich grape extract; AD-RES, group fed with AD plus RES (RES) alone. Asterisks designate significant difference ($P < 0.05$) over AD group.

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comparison with the maximum thickness values measured in the AD group (356.98 μm).

The presence of lipid drops in the intima of the aorta was observed in all experimental groups including the CT and CT-GE-RES groups (Figure 2). However, lipid drops were significantly lower ($P < 0.05$) in the AD-GE, AD-GE-RES, and CT-GE-RES supplemented groups in comparison to pigs from the AD group.

With respect to the orcein staining, flattening of the wavy structure and irregular arrangement of the internal elastic lamina as well as of the more internal elastic fibers of the tunica media were observed in all experimental groups. Hyaline degeneration and disruption of fibers were detected only in the groups AD, AD-GE, and AD-RES (Figure 3). Significant differences in the score of elastic fiber alterations ($P < 0.05$)

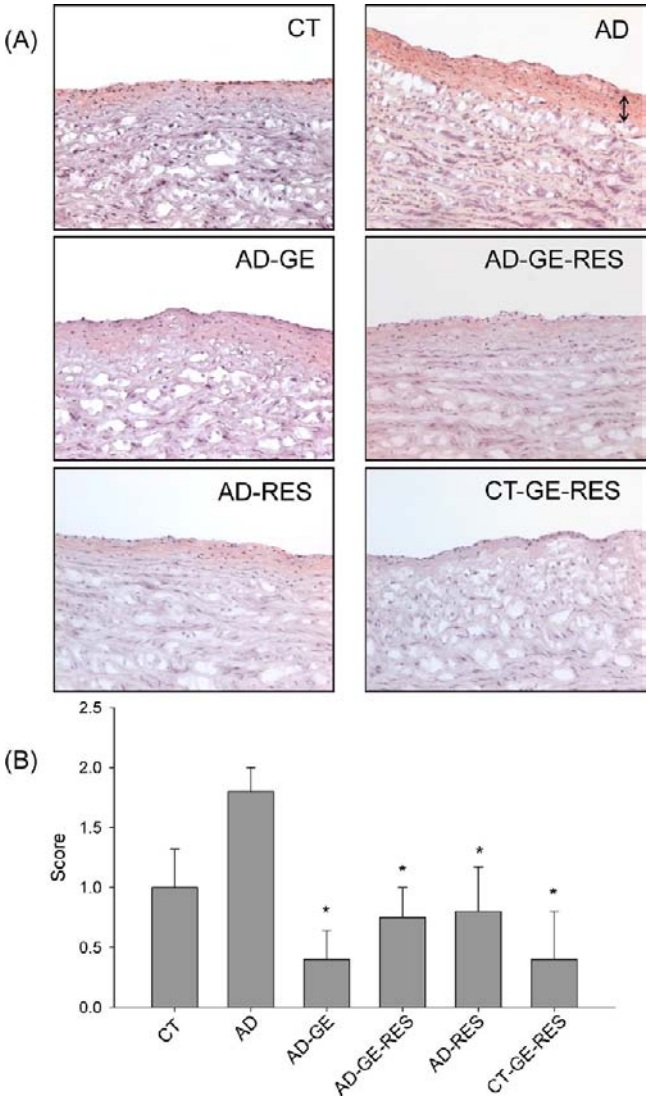

Figure 2. Effects on lipid drop deposition in the aortic intima. (A) Pig aortic tissue sections showing lipid drops (reddish color) in the intima. Samples were stained with Sudan IV staining. Accumulation of lipid drops was evident in all the groups, especially in the AD group (designated by the arrow). (B) Quantification of lipid drop accumulation. Results are shown as the mean ± SE of two independent evaluations from 4 different tissue preparations for each pig in each group ($n = 5$). Groups are those defined in Figure 1. All the treatments decreased the accumulation of lipid drops, especially in the AD-GE and CT-GE-RES groups. Asterisks designate significant difference ($P < 0.05$) over the AD group.

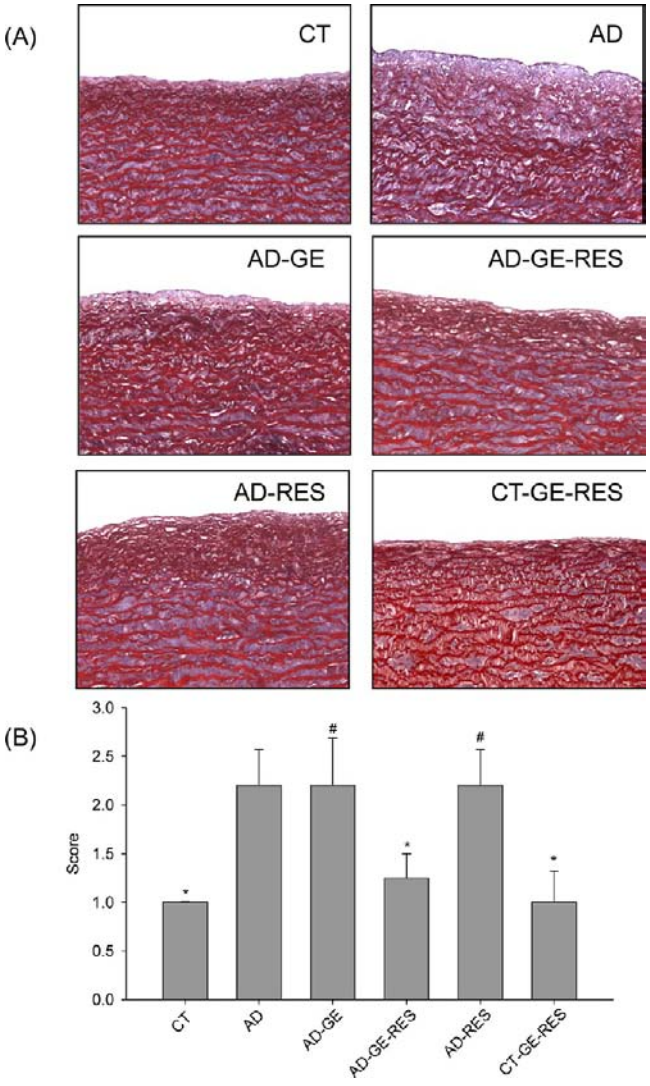

Figure 3. GE-RES decreases the alteration of elastic fibers in aortic tissue. (A) Aortic tissue sections stained with orcein. Elastic fibers (brownish color) were remarkably damaged in AD, AD-GE, and AD-RES groups. (B) Quantification of alterations in the structure of the elastic fibers in the different groups. Results are shown as the mean ± SE of two independent evaluations from 4 different tissue preparations for each pig in each group ($n = 5$). *Significant difference ($P < 0.05$) over the AD group; #significant difference ($P < 0.05$) over the CT group. The asterisks also designate the absence of elastic fibers disruption (CT, AD-GE-RES, and CT-GE-RES groups). Groups are those defined in Figure 1.

were found in the CT and CT-GE-RES groups in comparison with the AD group. Pigs from the AD-GE-RES group were partially protected from the alteration of the elastic fibers (Figure 3). Interestingly, pigs from the AD-GE-RES group were the only animals fed AD that did not show hyaline degeneration and disruption of elastic fibers (Figure 3).

The extension of the lesion in the aortic subendothelial intima was significantly higher ($P < 0.05$) in the animals of the AD group than in the pigs of the CT groups (CT and CT-GE-RES, no fat added to the diet) clearly evidencing the damage caused in the endothelium of the animals following the intake

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of the atherogenic diet. All of the treatments reduced this observed aortic damage more significantly ($P < 0.05$) in the animals supplemented with GE or GE-RES (Figure S2 in the Supporting Information). It should be noted that the lowest lesion extension was observed in the AD-GE-RES group.

Grape Extracts Prevent Superoxide Anion Accumulation in Aortic Tissue. Consumption of the atherogenic diet (AD) for 4 months was associated with elevated $O_2^{\bullet-}$ levels in the aortic tissue of both female and male pigs as revealed by red-stained nuclei (Figure 4). The presence of $O_2^{\bullet-}$ (expressed as the number of stained nuclei per mm^2) was significantly higher (3.5-fold) in the aortic samples from the AD group than in those from the CT groups (CT and CT-GE-RES, no fat added to the diet) ($P < 0.05$). This increase was significantly and equally reduced in both the AD-GE and AD-GE-RES groups ($P < 0.05$) whereas the reduction of $O_2^{\bullet-}$ was not statistically significant in the AD-RES group. Interestingly, the $O_2^{\bullet-}$ levels in the aorta from pigs fed with normal chow supplemented with GE-RES (CT-GE-RES) were 2-fold lower ($P < 0.05$) than in the CT group (Figure 4). No sex-dependent differences were observed.

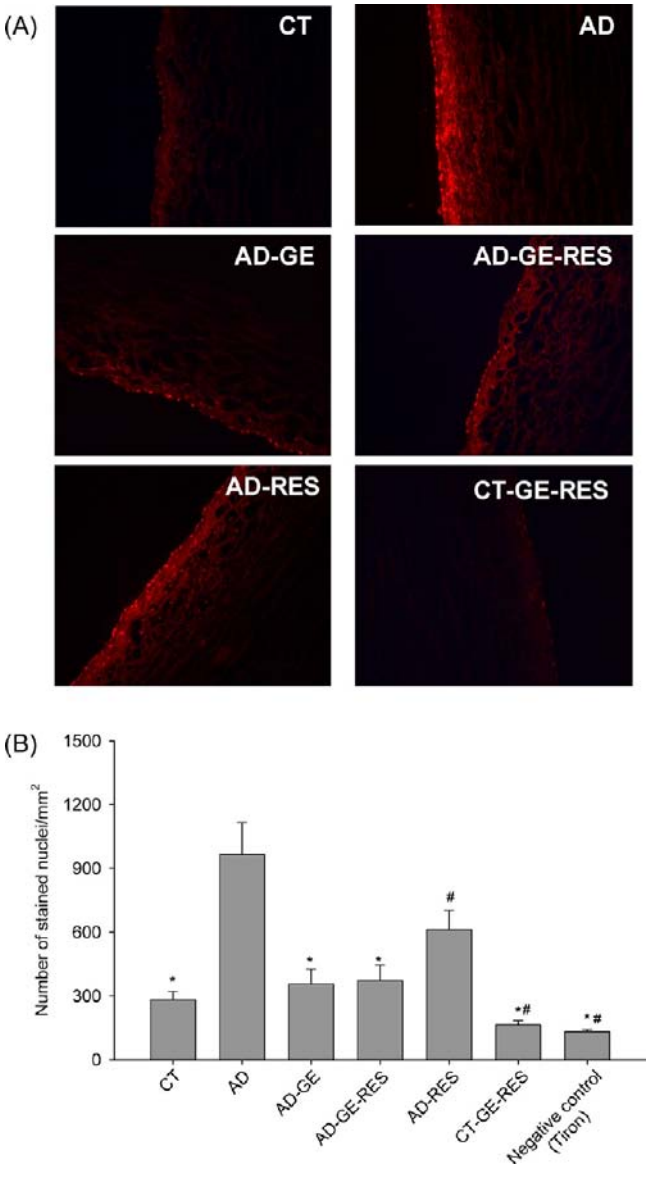

Figure 4. GE and GE-RES decreased vascular oxidative stress. (A) Superoxide anion ($O_2^{\bullet-}$) accumulation in pig aortic tissues. Superoxide anion levels were detected in cell nuclei by measuring the red fluoresce as described in Materials and Methods. (B) Quantification of red nuclei in the different groups. Results are shown as the mean ± SE from 4 different tissue preparations for each pig in each group ($n = 5$). Groups are those defined in Figure 1. *Significant difference ($P < 0.05$) over the AD group; #significant difference ($P < 0.05$) over the CT group.

Microarray Data. We next analyzed and compared microarray results between the different experimental groups for female and male pigs separately. A small number of probe sets (1−7%) were found to surpass the cutoff criteria and display significant expression changes (Tables S3 and S4 in the Supporting Information). The data sets showed different profiles of changing genes between females and males after exposure to the AD diet or to the AD diet supplemented with GE, GE-RES, or RES. To identify common genes across the experimental diets, the data sets were analyzed with the "compare data" tool in the IPA software. We identified a number of genes that were commonly regulated by the AD and counteracted by GE, GE-RES, or RES supplementation in females and males (Tables S5 and S6 in the Supporting Information). IPA software was also used to provide insights about the functional relevance of the differentially expressed genes between the experimental groups. Among other functions, we found that some of the significant changing genes in both female and male pigs fell into the following categories: (1) cardiovascular system development and function (endothelial cell chemotaxis, adhesion, morphogenesis, and development; blood vessel formation; vascular smooth muscle cell growth and adhesion; recruitment and organization of collagen fibrils and osteoclasts); (2) lipid metabolism and metabolic disease; (3) macrophage differentiation, recruitment, and adhesion; (4) coronary artery disease. A search in the literature corroborated that a selection of these candidate genes were associated with atherosclerosis and (or) aortic tissue damage (Table 1) and were further investigated by RT-PCR.

RT-PCR in PBMNCs. RT-PCR analyses confirmed that the expression of *SOCS3* in PBMNCs isolated from female pigs fed the AD increased by 2-fold ($P < 0.05$) over the control group (CT) and that this induction was significantly reversed by treatments with GE-RES and RES ($P < 0.05$, Figure 5). RT-PCR also indicated that, in male pigs, the expression of *SOCS1* was induced in the AD group over the CT group, and partially prevented in the AD-GE-RES and AD-RES groups. In addition, the expression of *CFL2* was also confirmed to be induced by the AD diet in PBMNCs isolated from males and this induction was partially repressed in animals supplemented with GE-RES or RES alone (these changes were not statistically confirmed since there were only two male animals per group). No significant differences were detected by RT-PCR in the expression of *VCL* or *EGR1* between any of the experimental groups, and none of the selected targets exhibited expression differences between the CT and the CT-GE-RES group (results not shown).

Interleukin-6 (IL-6) and Endothelin-1 Levels. No detectable IL-6 levels were found in the aortic tissue of pigs whereas a high variability was found in pig serum (results not shown). In the case of endothelin-1, no differences in aortic tissue levels were observed among the groups except for males from the CT-GE-RES group, in which the levels were 2-fold lower than for the rest of the groups (results not shown).

Table 1. Selected Genes (Microarray Results) Associated with Atherosclerosis and (or) Aortic Tissue Damage in Pig PBMNCs[a]

probe set ID	gene symbol	gene name	biological function(s) associated with atherosclerosis and (or) aortic tissue damage	AD vs CT	AD-GE vs AD	AD-GE-RES vs AD	AD-RES vs AD
			Females				
Ssc.7953.1.A1_at	*VCL*	vinculin	It binds actin and is involved in adhesion. Its expression is reduced in atherosclerotic plaques.[38]	↓[b]	↑[b]	↑[b]	↑[b]
Ssc.9789.1.A1_at				↓[b]	NC	↑[c]	NC
Ssc.16031.1.A1_at	*EGR1*	early growth response factor 1	Transcription factor activated in vascular stress. Upregulated in atherosclerotic lesions.[39]	↑[b]	↓[d]	↓[b]	↓[c]
Ssc.2827.1.S1_at	*SOCS3*	suppressor of cytokine signaling 3	Involved in negative regulation of cytokines. Expressed in macrophages and ECs. Highly expressed in inflammation and in atherosclerotic lesions.[42]	↑[b]	↓[c]	↓[c]	↓[b]
			Males				
Ssc.22761.1.S1_at	*CFL2*	cofilin 2	Regulator of actin dynamics. Its expression is decreased in damaged aortic tissue. It is repressed by anti-inflammatory cytokines.[48]	↑[c]	↓[c]	NC	↓[c]
Ssc.7645.1.A1_at				↑[c]	↓[c]	↓[b]	↓[b]
Ssc.7645.2.S1_at				↑[b]	↓[b]	↓[c]	↓[c]
Ssc.26216.1.S1_at	*SOCS1*	suppressor of cytokine signaling 1	Member of the SOCS family involved in negative regulation of cytokines. Important role in inflammation and CVDs.[40]	↑[c]	↓[c]	↓[c]	↓[b]
Ssc.26216.2.A1_at				↑[b]	↓[c]	↓[b]	↓[b]

[a]NC, no change; PBMNCs, peripheral blood mononuclear cells; AD, atherogenic diet; GE, grape extract; GE-RES, resveratrol-rich grape extract; RES, resveratrol. [b]Limma *P*-value < 0.01. [c]Limma *P*-value < 0.1. [d]Not significant.

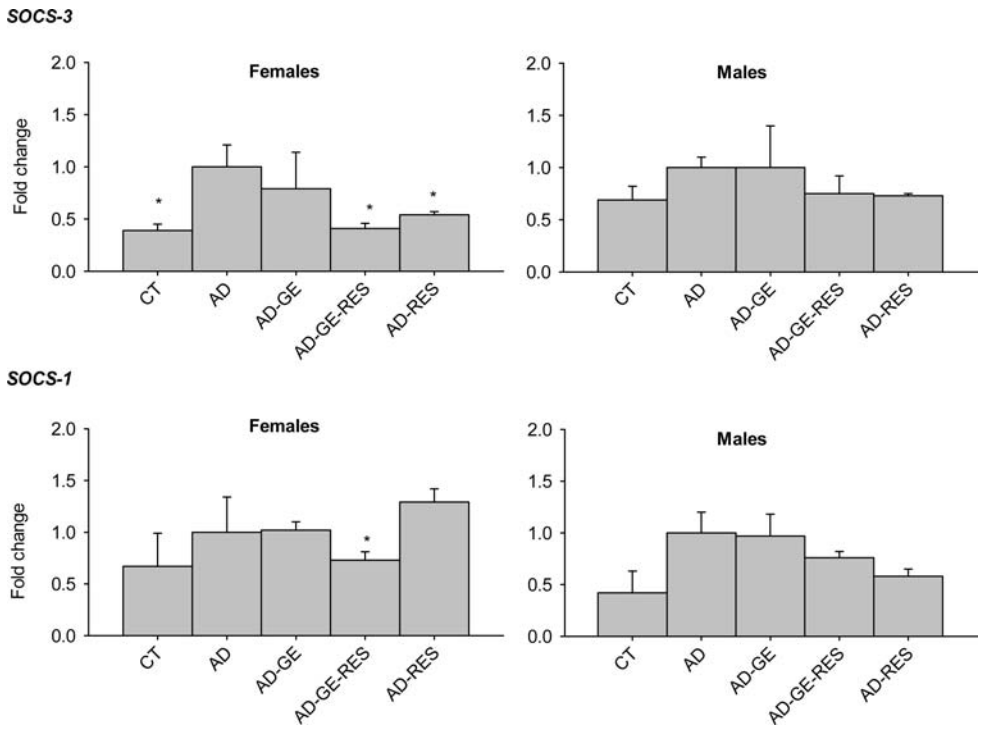

Figure 5. GE-RES decreased *SOCS3* and *SOCS1* expression in pig PBMNCs (peripheral blood mononuclear cells). Groups are those defined in Figure 1. Gene expression was determined after 4 months of dietary intervention by one-step quantitative RT-PCR and was normalized to *GAPDH* and expressed relative to gene expression in the AD group. In females ($n = 3$), the asterisk designates significant difference ($P < 0.05$) over the AD group. Each bar represents the mean values ± SD from $n = 3$ replicates.

SOCS3 Levels in Aortic Tissue. A high variability was detected in the levels of SOCS3 in the aortic tissue from the AD group although these values were, on average, higher than in the rest of the experimental groups. Unlike the results obtained for *SOCS3* expression in PBMNCs, no differences were found in the levels of SOCS3 between the experimental groups (Figure S3 in the Supporting Information). In addition, no sex-dependent differences were observed.

Grape Phenolics in Pig Plasma and Aorta. None of the phenolic compounds found in grapes (anthocyanins, flavan-3-ols, etc.) or any of their derived metabolites were detected in any of the pig plasma and aortic samples analyzed. RES and derived metabolites were not detected in plasma either. However, RES and some of its derived metabolites were detected in the aorta of all the animals fed with RES or RES-rich grape extract (AD-GE-RES, AD-RES, and CT-GE-RES) at very low concentrations (nM range). No differences were observed in the aortic metabolic profile of RES between the different groups or between sexes. Co-elution with authentic standards and monitoring of the specific transitions of RES and derived metabolites using the multiple reaction monitoring mode allowed for the identification of RES 3-*O*-glucuronide

(403 m/z^-, MS/MS 227), RES 3-*O*-sulfate (307 m/z^-, MS/MS 227), and RES (227 m/z^-, MS/MS 185, 143) (Figure S4 in the Supporting Information). The microbiota-derived RES metabolite dihydroresveratrol as well as its conjugates were not detected in any of the samples.

Total Homocysteine (Hcys), Malondialdehyde (MDA), and Reduced Glutathione (GSH) Levels. Total Hcys levels were higher in males than in females and slightly higher in the AD group than in the CT group, although no significant differences were observed among groups (Figure S5A in the Supporting Information). No MDA was detected in any of the serum or plasma samples. MDA was detected in pig aortas although the consumption of the atherogenic diet did not significantly increase MDA content. Likewise, no significant changes were observed among groups and no differences were found between sexes (Figure S5B in the Supporting Information). In the case of GSH, the consumption of the atherogenic diet slightly decreased the GSH levels, especially in male pigs, although no significant differences were observed among groups (Figure S5C in the Supporting Information).

DISCUSSION

Arteriosclerosis is defined as chronic arterial change consisting in hardening, loss of elasticity, and luminal narrowing. Atherosclerosis, the vascular disease of greatest importance in human beings and the main underlying cause of CVDs, is a specific type of arteriosclerosis that involves both chronic inflammatory and oxidative stress status.

The use of pigs as a model to understand human atherosclerosis shows some advantages such as the physiological and anatomical similarities to humans and the development of spontaneous atherosclerosis, particularly in the aorta. In contrast, the need for long dietary interventions to induce lipid disorders, the difficulties associated with the care of the animals, and the high maintenance cost and the scarce availability of commercially antibodies against pig markers make this model difficult to use.[22,23] The use of pathological models that represent a specific cardiovascular damage and are exposed to high (pharmacological) doses of dietary compounds are often useful to show the responses induced by the tested compounds but are not representative of a nutritional approach. The conclusions drawn from these pharmacological studies cannot be extrapolated to human healthy individuals that regularly consume low doses of the compounds with the diet. In this context, the aim of the present study was to apply an animal model representative of the development of atherosclerosis by the diet to investigate the putative preventive effects of daily low doses of resveratrol. For this purpose, we fed minipigs with an atherogenic diet (AD) to try to induce early atherosclerotic events such as initial aortic damage and to evaluate whether low doses of RES, against other grape polyphenols, may reduce or prevent this damage. Unlike the pharmacological approaches, this dietary intervention poses the difficulty of detecting and measuring small to moderate responses.

RES has been previously reported to exert antiatherogenic properties through a number of mechanisms including the decrease of oxidative stress, inhibition of LDL oxidation, regulation of serum lipid profile, decrease of inflammatory cytokines, etc.[24] Most of these properties have been mainly described in rodents exposed to high concentrations of the isolated compound. For example, intraperitoneal injection of RES (approximately 500 mg HED) prevented the development of abdominal aneurysm in mice in association with reduced inflammation, oxidative stress, neoangiogenesis, and extracellular matrix disruption.[25] From a dietary point of view, however, RES is a minor polyphenol present in grapes and wines in combination with other groups of polyphenols and with a high variability in its content. This implies that the current assumptions that the benefits of red wine are mainly due to its RES content and that the consumption of grapes and (or) wine ensures the intake of enough RES to exert some beneficial effects are not fully true. In fact, the antiatherogenic properties of other grape phenolics, in the absence of RES, have been previously reported in rodents.[26] Our objectives were to investigate the antiatherogenic properties of a dietary resveratrol-rich grape extract, and to evaluate the specific contribution of a low RES content, within the polyphenolic profile of grape extracts, to the prevention of early atherosclerotic lesions in the aorta of pigs fed an atherogenic diet (AD). Our dietary approach together with the use of pigs, physiologically much closer to humans than rodents, strengthens the conclusions obtained.

The present study is an extension of that recently reported by Azorín-Ortuño et al.[8] In that previous study both female and male pigs were fed a high-fat diet (H-F) (supplemented with beef tallow, 20% fat) that induced a mild hyperlipidemia after 12 months of intervention. RES (8 mg of RES/70 kg animal bw per day) was tested for its capacity to ameliorate the effects of the H-F diet. In general, the pigs were resistant to some of the metabolic changes associated with a high-fat diet since no apparent effects on the cholesterol, glucose, or liver enzyme levels were observed. In addition, supplementation with RES did not affect any of those parameters. In view of these results, in the present study, we decided to increase the atherogenic potential of the diet by including coconut oil and cholic acid.[27] After 4 months of experimentation, the pigs fed with AD did not increase significantly their weight and only minor changes were observed in serum biochemical parameters, which suggested the difficulty of inducing metabolic changes in this pig model despite the atherogenic diet (Table S1 in the Supporting Information).

The accumulation of foam cells in the intima and the increase of the intima-media thickness in abdominal aorta and carotid artery have been described as an early stage of atherosclerotic vascular disease in a porcine model using a high-fat diet.[28] Another study carried out in domestic pigs using a hyperlipidemic diet failed to induce advanced coronary atherosclerotic lesions, and only early stages of disease characterized by the presence of foam cells in the intima without severe luminal narrowing were induced.[29] The changes observed in the aorta of the pigs included in our experiment can be classified as early stages of atherosclerotic vascular disease. The presence of some proliferation of connective tissue and lipids in the intima of the aorta in the pigs of the CT group gives some evidence for possible arteriosclerotic changes caused in these animals by aging (the pigs were 20 months old). The increase in the severity of the lesions found in the aorta of the animals from the AD group can be entirely attributed to the atherogenic diet.

The internal elastic lamina constitutes a barrier between the intima and the media with a protective role since it may affect the transport of LDL particles across the intima-media and their accumulation and it may also prevent the migration of medial smooth muscle cells to the intima.[30] Therefore, defects in this structure could contribute to the development of atherosclerotic lesions and neointima formation in arteries.[31] In our study

we have found a direct relationship between the degree of loss of the normal structure of elastic fibers and the neointima formation since the groups in which degeneration and rupture of elastic fibers were observed (AD, AD-GE, and AD-RES) also presented the highest degree of thickening of the intima, and, in the groups in which flattening of the wavy structure and irregularity, but not rupture, in the outline occurred (CT, AD-GE-RES and CT-GE-RES), the thickening was lower (Figures 1−3). In a previous report in which abdominal aortic aneurysm was induced in mice by periaortic application of $CaCl_2$,[25] the animals treated with an intraperitoneal injection of RES (HED approximately 500 mg/day RES for a 70 kg person) exhibited a normal morphology of the elastic lamellae while untreated animals presented destruction of the normal wavy morphology. Under the conditions of our study, exposure of the animals to GE-RES induced some changes in the normal elastic fiber morphology but of minor importance versus the other groups (Figure 3).

Reactive oxygen species (ROS) formation is a process inherent of metabolism. However, excessive ROS production in the vascular wall, namely, generation of superoxide anion ($O_2^{\bullet-}$), is an early triggering mechanism for atherosclerosis by promoting oxidative stress and activation of proinflammatory signaling cascades, and also contributes to the decrease of nitric oxide (NO) bioavailability.[32] A previous study described that dietary wine phenolics (including resveratrol) decreased the aortic fatty streak accumulation in hypercholesterolemic hamsters independently of the plasma antioxidant capacity, suggesting that other indirect mechanism may take place.[33] In the present study, we clearly observed that oxidative stress could be involved in the aortic lesions, and it seems that the prevention of vascular oxidative stress by the intake of GE-RES was clearly shown and may be part of the antiatherogenic mechanisms activated by this product. Our results suggest that the observed antioxidant activity was mainly due to other polyphenols present in the grape extract and not to the content of RES since both the grape extract alone (GE) and the grape extract enriched in RES (GE-RES) equally prevented the production of $O_2^{\bullet-}$ in the endothelium whereas RES alone was less effective (Figure 4). Interestingly, the dietary administration of GE-RES also prevented the $O_2^{\bullet-}$ production in pigs fed with a normal chow (CT-GE-RES; no fat added, Figure 4), which suggests that the regular intake of this GE-RES may even have a preventive effect of vascular oxidative stress in the context of a normal healthy diet. However, whereas both GE and GE-RES were equally effective in the reduction of endothelial $O_2^{\bullet-}$ accumulation, GE-RES was more efficient than the GE alone in the prevention of the fat-induced aortic lesions. Since the combination of therapies reversing both oxidative stress and vascular inflammation have been suggested to exert a synergistic benefit against vascular damage,[34] GE-RES may constitute a more efficient product against atherosclerosis development than grape extracts lacking RES.

As previously suggested, other mechanisms may be taking place. In an attempt to determine some of the molecular mechanisms which may underlay the antiatherosclerotic effects of GE and GE-RES, we first investigated the levels of endothelin-1 and IL-6 in aortic tissue since both are known to be involved in the production of $O_2^{\bullet-}$.[35] In the present study, no clear relationship was observed between vascular $O_2^{\bullet-}$ accumulation, endothelin-1, and IL-6. We then decided to use multiple gene expression analysis using microarrays to try to detect other possible molecular CVD markers and related functions and pathways that may have been regulated by the different treatments and that may support the effects found in the fat-induced aortic lesions. Our first approach was to perform microarray analyses in RNA from aortic tissue and to search for molecular differences associated with the observed tissue changes. However, we were not able to obtain RNA from the isolated segments of pig aortic tissue by the methods found in the bibliography.[36] It is known that PBMNCs play a crucial role in the inflammatory processes associated with atherosclerosis and that the gene expression profiles of these cells can be regulated in response to dietary components.[37] Indeed, in our previous study where the pigs were fed a H-F diet alone or supplemented with RES, we already indicated that PBMNCs exhibited altered levels in the expression of genes related to inflammation, atherosclerosis, and lipid metabolic disorders which were induced by the diet and regulated by RES intake.[8] At the end of this second intervention we decided to analyze again the expression of PBMNC genes in the search for further significant changes associated with the AD consumption and with a regulatory effect of RES, GE, or GE-RES.

Among the genes found to be affected by the intake of the AD and counteracted by GE, GE-RES, and/or RES in PBMNCs, we selected several targets which have all been found to be expressed and altered in aortic lesions, mostly in endothelial cells (ECs) or in vascular smooth muscle cells (VSMCs).[38] All these genes are also expressed in circulating lymphocytes, monocytes, and macrophages,[39] and changes in their expression in PBMNCs may be useful surrogate markers of early atherosclerotic lesions. From the selected genes, RT-PCR analysis confirmed the induction of *SOCS3* and *SOCS1* in PBMNCs from female and male pigs, respectively, fed an AD. This induction was partially reversed by some of the treatments, most significantly by GE-RES. *SOCS3* and *SOCS1* are key regulators of vascular cell responses and are highly expressed in ECs, VSMCs, and macrophages in the inflammatory regions of atherosclerotic plaques.[40] Of particular interest, the expression of *SOCS3* was found to be decreased in SMCs from injured arteries in female minipigs, indicating an association between this protein and vascular damage in these animals.[41] The regulation of *SOCS3* by dietary components might be of interest in the treatment of atherosclerosis. In a recent human study, the expression of *SOCS3* was found to be induced after a high-fat, high-carbohydrate meal, and this increase was absent after a meal rich in fiber and fruit.[42] To further confirm a modulatory effect of the experimental diets on *SOCS3* in the pigs, we investigated the expression levels of this molecule in ECs and in aortic tissue. We extracted small quantities of RNA from scraped aortic ECs from the pigs' artery and measured the expression of *SOCS3*, however, we did not find differences between any of the experimental groups investigated (data not shown). We next determined SOCS3 levels in aortic tissue using anti-rabbit SOCS3 antibodies that had been reported to show cross-reactivity with pig.[16] SOCS3 protein levels were higher in the AD group than in the CT group (Figure S3 in the Supporting Information), which supported the results obtained in PBMNCs. However, unlike in the PBMNCs, no differences were observed among the rest of the groups which showed similar values to CT group (Figure S3 in the Supporting Information). We detected differences in the regulation of *SOCS3* and *SOCS1* between sexes in PBMNCs but not in the aortic tissue at protein level. A link between sex steroids, estrogens and testosterone, growth hormone, and SOCS has been reported.[43] However, there is

not a clearly established sexual dimorphism for *SOCS* expression which may explain the differences found between female and male pigs at gene level. This deserves further research.

Elevation of total homocysteine is an independent risk factor for atherosclerotic disease,[44] and hyperhomocysteinemia has been reported to increase lipid peroxidation products, such as MDA, in pigs.[45] In addition, GSH is an important regulator of Akt signaling pathway[46] in relation to insulin sensitivity. However, in our pig model, no significant changes were detected in Hcys, MDA, and GSH (Figure S4 in the Supporting Information), which suggested that these markers did not play an important role in the developing of early atherosclerotic lesions in the pig aorta induced by the atherogenic diet.

The metabolites and tissue distribution of RES in the pig have been recently reported by Azorín-Ortuño et al.[18] In the present study, we were not able to detect RES or any of its derived metabolites in the plasma of any of the animals investigated, which may be due to the fasting period prior to blood extraction and to the low RES dose used (~30-fold lower than in the previously reported study[18]). However, we detected some RES-derived metabolites in the aortic tissue from the AD-RES, AD-GE-RES, and CT-GE-RES treated animals (Figure S5 in the Supporting Information). It is possible that these metabolites may be involved in the aortic changes observed in these groups. However, taking into account the different effects on the aorta in the GE-RES and RES groups, the presence of RES-derived metabolites was not enough to justify the effects observed. Therefore, other mechanisms may be also contributing to the observed effects, i.e. effects of other nondetected grape metabolites, signaling cascades with systemic effects not specifically occurring in aortic tissue, etc. The low bioavailability of RES vs its pleiotropic effects, a phenomenon designated as the "RES paradox",[18] is still a contradictory issue under research and debate. It has been proposed that the effects of RES may be partially mediated by the triggering of signaling cascades or through sensory stimulation from the gastrointestinal tract to target organs such as the brain.[47]

Our results support that a daily intake of a grape nutraceutical containing low doses of RES exerts benefits against high-fat-induced atherosclerosis development by preventing early vascular events. These effects seemed to be mediated, at least in part, by a reduction in vascular oxidative stress and the regulation of the expression of the suppressors of cytokine signaling 1 and 3 (SOCS1 and SOCS3). These effects are not exclusive of RES since other grape polyphenols exert some similar beneficial outcomes. In fact, a combination of RES and grape extract, as present in GE-RES, does appear to potentiate these results.

We must assume a number of limitations to our study: (i) a relatively small number of pigs was allocated to each group, which is due to the ethics committee's advice, as well as to the cost and difficulty in keeping and handling these animals; (ii) although the aortic lesions were similar in both males and females, some of the changes at gene level were, however, sex-dependent, which made it more difficult to obtain statistically significant results; (iii) the commercial availability of pig antibodies is scarce in comparison to those raised against human, mouse, or rat, which severely limits the number of markers that can be studied. Importantly, we have shown these benefits using a nutritional approach rather than a pharmacological one. In this regard, we must highlight that dietary interventions can only exert moderate effects. In addition, this approach must involve the experimentation in mild pathological models (early stages). However, and consequently, this can limit severely the unraveling of precise molecular mechanisms. To the best of our knowledge, this is the first report in which dietary supplementation with a grape extract containing resveratrol is shown to contribute to the prevention of early atherosclerotic lesions in the pig, a big mammal physiologically close to humans.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental details including tables (Table S1, phenolic composition of the grape extracts GE and GE-RES; Table S2, serum biochemical parameters after 4 months upon feeding with different diets; Tables S3 and S4, genes with altered levels of expression in PBMNCs from female pigs and male pigs; Tables S5 and S6, genes with altered levels of expression in PBMNCs from female pigs and male pigs fed an atherogenic diet (AD) and counteracted by supplementation of the diet with GE, GE-RES or RES) and figures (Figure S1, ALP and GGT levels in male and female pigs after 4 months; Figure S2, extension of aortic lesion in pig groups after 4 months; Figure S3, SOCS-3 levels detected in aortic tissue; Figure S4, RES-derived metabolites detected in the aorta of pigs; Figure S5, total Hcys and GSH (plasma) and MDA (aortic tissue) levels. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): MAO, MJYG, FJP, JR, AGS, ML, FV and MTGC have declared that no competing interests exist. FTB and JCE are co-inventors of the patent ES 2177465 that describes the process to increase the resveratrol content in grapes using UVC light.

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■ ABBREVIATIONS USED

AD, atherogenic diet; CVD, cardiovascular disease; ECs, endothelial cells; GE, grape extract; GE-RES, resveratrol-rich grape extract; GSH, reduced glutathione; HCys, homocysteine; HED, human equivalent dose; MDA, malondialdehyde; PBMNCs, peripheral blood mononuclear cells; RES, *trans*-

resveratrol; SOCs, suppressors of cytokine signaling; VSMCs, vascular smooth muscle cells

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