Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 23 (2012) 829–837

Journal of Nutritional Biochemistry

Effects of long-term consumption of low doses of resveratrol on diet-induced mild hypercholesterolemia in pigs: a transcriptomic approach to disease prevention☆

María Azorín-Ortuño[a], María J. Yáñez-Gascón[a], Antonio González-Sarrías[a], Mar Larrosa[a], Fernando Vallejo[a], Francisco J. Pallarés[b], Ricardo Lucas[c], Juan C. Morales[c], Francisco A. Tomás-Barberán[a], María-Teresa García-Conesa[a,*], Juan C. Espín[a]

[a]Department Food Science and Technology, Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, P.O. Box 164, 30100 Campus de Espinardo, Murcia, Spain
[b]Department of Anatomy and Compared Pathology, School of Veterinary, University of Murcia, 30100, Campus de Espinardo, Murcia, Spain
[c]Department of Bioorganic Chemistry, Institute of Chemical Research, CSIC-University of Seville, 41092, Seville, Spain

Received 4 January 2011; received in revised form 28 March 2011; accepted 1 April 2011

Abstract

Metabolic and cardiovascular diseases (CVDs) have risen to alarming proportions, and there is a need for therapeutic and preventive measures. The polyphenol resveratrol (RES) protects against CVDs, but *in vivo* molecular mechanisms responsible for protection are not yet understood. Peripheral blood mononuclear cells (PBMNCs) are involved in the development of atherosclerosis and metabolic disorders. The identification of PBMNCs genes responding to dietary compounds might help to understand the mechanisms underlying the effects of polyphenols. We determined gene expression differences between PBMNCs from pigs fed a high-fat diet manifesting a mild increase of cholesterol and pigs fed a high-fat diet containing low doses of RES. Although the consumption of RES did not modify the levels of cholesterol, microarray analyses indicated that some of the differentially expressed genes, collagens (*COL1A, COL3A*), lipoprotein lipase (*LPL*) and fatty-acid binding proteins (*FABPs*) involved in CVDs and lipid metabolism were up-regulated by the high-fat diet and down-regulated by RES. Reverse transcriptase polymerase chain reaction confirmed that RES and RES-containing grape extract prevented the induction of *FABP4* in PBMNCs in female pigs fed a high-fat diet. Low micromolar concentrations of RES and its metabolite dihydroresveratrol exerted a minor but significant reducing effect on the induction of *FABP4* expression in human macrophages treated with oxidized low-density lipoprotein. Our results show that the consumption of low doses of RES modulates the expression of genes related to lipid metabolism and metabolic disorders that are affected by a high-fat diet and suggest that some of the circulating RES metabolites may contribute to these effects.

Keywords: FABP4; Macrophages; Oxidized LDL; Metabolites; Glucuronide; Dihydroresveratrol; Grape extract

1. Introduction

Obesity is attributed to a combination of high-energy diets, low-activity style of life and genetic make-up. Obesity-mediated inflammation plays a significant role in the pathogenesis of a cluster of chronic metabolic diseases such as endothelial dysfunction, atherosclerosis and cardiovascular diseases (CVDs), insulin resistance and type 2 diabetes, and nonalcoholic steatohepatitis. The incidence and impact of these major health threats have risen to alarming proportions in Western societies, and there is a great need for therapeutic and preventive measures [1]. Resveratrol (RES) is a naturally occurring polyphenol induced in some plants such as grapes and reported to be associated to the cardiovascular protective effects of red wine [2]. Other beneficial effects attributed to RES include anticancer, anti-inflammatory, antiaging and energy and lipid-metabolism modulatory effects [3]. Several molecular mechanisms have been proposed to explain the protective effects of RES including the induction of NAD^+-dependent deacetylase Sirt1, the down-regulation of NF-KB and AP-1 transcription, the interaction with drugs metabolizing enzymes, the activation of 5′-AMP-activated protein kinase (AMPK), the down-regulation of proinflammatory cytokines or the binding to estrogen receptors [2–4]. However, *in vivo* molecular mechanisms triggered by the consumption of RES have not yet been fully elucidated. It is hypothesized that the health benefits derived from RES consumption may be due to an interplay between RES and its *in vivo* metabolites with different molecular targets causing short-term (e.g., changes in enzymes activities) and long-term (e.g., changes in gene expression) effects [5].

The effects of polyphenols on gene expression have been largely studied in *in vitro* cell models using microarrays technology; however, not so many studies have investigated those effects *in vivo*. Peripheral

☆ Grants, sponsors and funding sources: This work has been supported by the Projects CICYT-BFU2007-60576 and Consolider Ingenio 2010, CSD2007-00063(Fun-C-Food). M.A.O. and M.L. are holders of a predoctoral JAE grant and JAE-DOC contract, respectively, from CSIC (Spain).
* Corresponding author. Tel.: +34 968 396276.
E-mail address: mtconesa@cebas.csic.es (M.-T. García-Conesa).

0955-2863/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2011.04.007

blood mononuclear cells (PBMNCs) are a subset of white blood cells, consisting of lymphocytes and monocytes readily accessible in animals and humans. Peripheral blood mononuclear cells express approximately 80% of the genes expressed in other tissues that can be altered in response to internal and external signals and can be used to investigate biological changes and novel biomarkers. Like this, PBMNCs gene expression has been used to characterize metabolic changes caused by the diet and its components including the consumption of polyphenol-rich products [6]. In addition, PBMNCs play a crucial role in the low-chronic inflammatory processes associated to atherosclerosis, and the gene expression profiles of these circulating cells can be regulated in response to lipid levels [7].

The identification of PBMNCs genes responding to RES consumption might offer insight on the molecular mechanisms underlying the beneficial action of this compound, particularly in the prevention against obesity-associated metabolic disorders and atherosclerosis. In this study, we fed pigs with a high-fat diet for 12 months to induce hypercholesterolemic conditions and investigated the putative preventive effects of prolonged supplementation with low doses of RES. We also examined the effects of grape extract (GE) containing an equivalent amount of RES and of GE alone to compare the specific contribution of RES against other polyphenols present in the GE. Our objectives were to (a) determine whether these low doses of RES were able to reduce the levels of serum lipids, (b) find genes that were affected in this animal model by a prolonged consumption of a high-fat diet and that were counteracted by the intake of RES and to (c) confirm and discuss the mechanistic role of selected genes whose expression is modulated by the consumption of RES. In addition, we investigated whether some of the circulating RES metabolites may be involved on those gene expression regulatory effects.

2. Materials and methods

2.1. Materials

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, >98.5% purity) **1** (Fig. 1A) was purchased from SeeBio Biotech Inc. (Shanghai, China). The GE and the RES-enriched grape extract (GE+RES) used in this study were kindly provided by Actafarma S.L. (Pozuelo de Alarcón, Madrid, Spain). Both extracts had a similar content of procyanidins (~110 mg/g), anthocyanins (~70 mg/g), flavonols (~3 mg/g) and hydroxycinnamic acids (~2 mg/g). Other stilbenes such as astringin, δ-viniferin and *trans*-piceid were detected only at trace levels. The GE+RES extract was from grapes

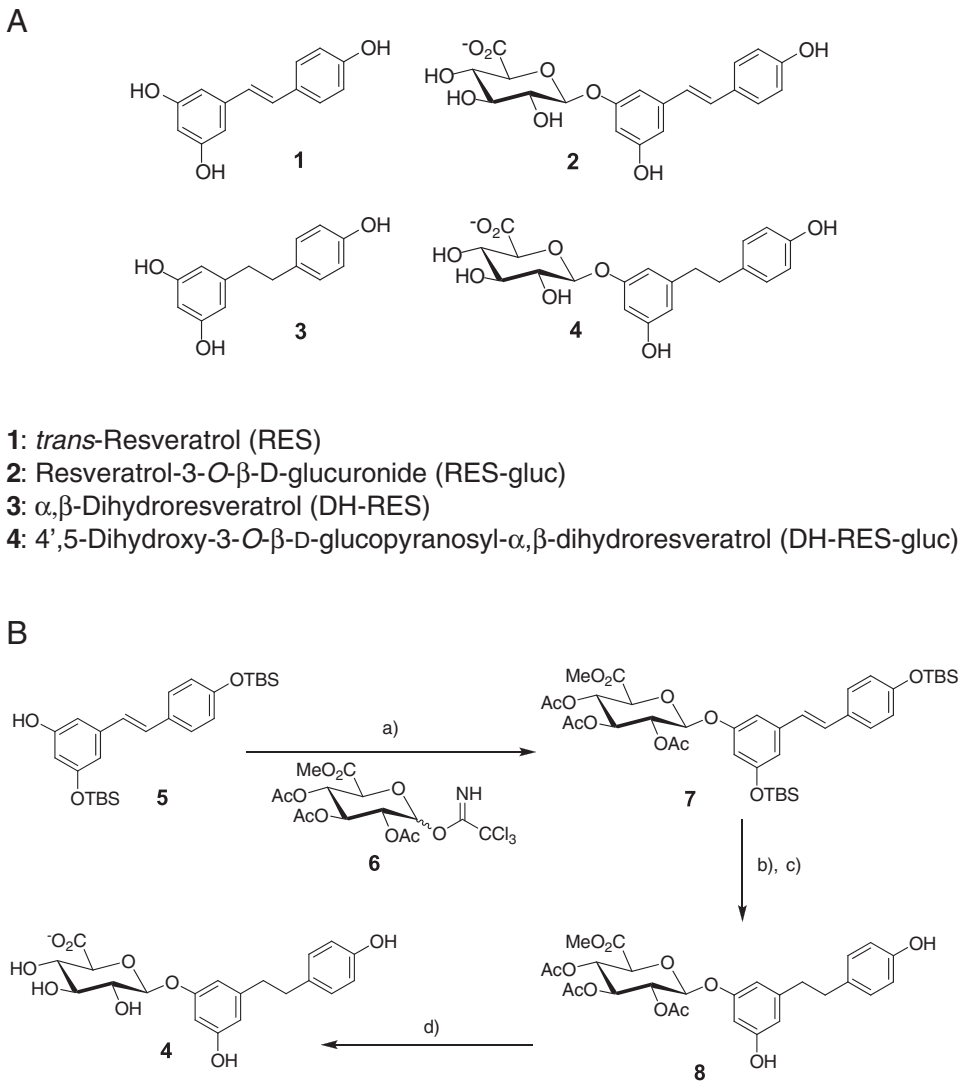

Fig. 1. (A) Chemical structure of *trans*-resveratrol (RES, **1**) and its metabolites, resveratrol-3-*O*-β-D-glucuronide (RES-gluc, **2**), α,β-dihydroresveratrol (DH-RES, **3**) and 4',5-dihydroxy-3-*O*-β-D-glucopyranosyl-α,β-dihydroresveratrol (DH-RES-gluc, **4**). (B) Synthesis of 4',5-dihydroxy-3-*O*-β-D-glucopyranosyl-α,β-dihydroresveratrol (DH-RES-gluc, **4**).

enriched in RES following a patented procedure (ES2177465, CSIC). This procedure is based on the specific induction in grapes of the content of phytoalexins (mostly RES) by controlled UV irradiation [8]. This procedure does not affect the content and nature of other phenolics present in the grapes, and thus, the two extracts used in this study, GE and GE+RES, differ essentially in the content of RES.

2.2. Synthesis of RES metabolites

Resveratrol-3-*O*-β-D-glucuronide **2** (RES-gluc) (Fig. 1A) was obtained from RES **1** following a published procedure [9]. α,β-Dihydroresveratrol **3** (DH-RES) (Fig. 1A) was prepared by catalytic hydrogenation of RES **1** as reported previously [10]. The synthesis of 4',5-dihydroxy-3-*O*-β-D-glucopyranosyl-α,β-dihydroresveratrol **4** (DH-RES-gluc) (Fig. 1A) was carried out from RES intermediate **5** following the procedure shown in Fig. 1B. Glycosylation of compound **5** with protected glucopyranosyl trichloroacetimidate **6** yielded intermediate **7**. Further hydrogenation of compound **7** and silyl deprotection with tetrabutylammonium fluoride gave 4',5-dihydroxy-3-*O*-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-α,β-dihydroresveratrol **8** as follows: compound **7** (500 mg, 0.64 mmol) was dissolved in 5 ml of tetrahydrofuran, and Pd/C was then added. Reaction mixture was stirred under a hydrogen atmosphere for 48 h. The reaction was then filtered off through Celite to afford the corresponding dihydroresveratrol derivative. The crude was dissolved in tetrahydrofuran (THF) (15 ml), and tetramethylammonium fluoride (TBAF) (2 ml) was then added. After 10 min, no starting material was detected. The reaction mixture was diluted with ethyl acetate (50 ml), and the organic phase was washed with saturated NH_4Cl and water. The resulting residue was purified by flash column chromatography (hexane: ethyl acetate from 1:1 to 1:3) to afford compound **8** (200 mg, 57%) as a yellow glassy solid; δ_H (300 MHz, $CDCl_3$) 6.87 (d, J=8.0 Hz, 2 H, Harom), 6.68 (d, J=8.0 Hz, 2 H, Harom), 6.30–6.28 (m, 2 H, Harom), 6.13 (s, 1 H, Harom), 5.30–5.15 (m, 3 H, H-3, H-4, H-1), 4.89 (m, 1 H, H-2), 4.02 (d, J=8.1 Hz, 1 H, H-5), 3.64 (s, 3 H, OCH_3), 2.66 (s, 4 H, 2×CH_2Ar), 1.99, 1.98, 1.97 (3s, 9 H, CH_3C=O). δ_C (75 MHz, $CDCl_3$) 170.7, 169.8, 169.7, 166.9 (C=O), 157.5, 156.8, 153.9, 144.7, 133.3, 129.7, 115.2, 109.5, 102.4, 99.1 (C-1), 71.0 (C-5), 69.2 (C-3), 65.9 (C-2), 62.5 (C-4), 53.0 (CH_3O), 37.9, 36.5 (CH_2Ar), 20.7, 20.6, 20.5 (CH_3C=O); ESI-MS (ES^+) calculated for $C_{27}H_{30}O_{12}$ (M+Na) 569.1, found: 568.8.

The dihydroresveratrol glucuronide **4** (DH-RES-gluc) was obtained after a final deprotection step under basic conditions: compound **8** (140 mg, 0.25 mmol) and Na_2CO_3 (68 mg, 0.64 mmol) were dissolved in a solution of methanol (4 ml) and H_2O (2 ml). The reaction mixture was stirred at room temperature for 16 h. After this period, water (1 ml) was added followed by addition of glacial acetic acid to reach pH 6.2. The solvents were then removed, and the residue was purified by reversed chromatography using an RP-C18 column and H_2O:MeOH mixtures as the mobile phase. Fractions containing the desired product were freeze-dried, affording the glucuronide **4** (90 mg, 90%). δ_H (300 MHz, D_2O) 6.96 (d, J=8.0 Hz, 2 H, Harom), 6.68 (d, J=8.1 Hz, 2 H, Harom), 6.44, 6.40, 6.31 (3s, 3 H, Harom), 4.80 (d, J=7.0 Hz, 1 H, H-1), 3.74 (m, 1 H, H-5), 3.52 (m, 3 H, H-3, H-4, H-2), 2.75 (s, 4 H, 2×CH_2Ar); δ_{13C} (75 MHz, D_2O) 176.6 (C=O), 160.2, 159.2, 156.4, 145.5, 134.0, 130.5, 116.1, 110.9, 109.5, 102.9, 102.5 (C-1), 77.7, 76.5, 74.6, 73.5 (C-2, C-3, C-4, C-5), 39.5, 37.9 (CH_2Ar). ESI-MS (ES^-) calculated for $C_{20}H_{22}O_9$ (M-H) 405.1, found: 405.0.

All chemicals were obtained from Aldrich Chemicals and used without further purification unless otherwise stated. All reactions were monitored by thin layer chromatography (TLC) on precoated Silica-Gel 60 plates F254 (Merck) and detected by heating with Mostain (500 ml of 10% H_2SO_4, 25g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 1g $Ce(SO_4)_2\cdot 4H_2O$). Reaction products were purified by flash chromatography with Merck Silica gel 60 (200–400 mesh). High-resolution FAB (+) mass spectra were obtained on a Micromass AutoSpec-Q spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on either a Bruker AVANCE 300 or ARX 400 or Bruker Advance DRX 500 MHz [300 or 400 MHz (1 H), 75 or 100 (^{13}C)] at room temperature for solutions in $CDCl_3$, D_2O or CD_3OD. Chemical shifts are referred to the solvent signal. Further NMR experiments (COSY, TOCSY, ROESY and HMQC) were done when necessary to assign the compound. Data were processed using manufacturer software, raw data were multiplied by shifted exponential window function prior to Fourier transform, and the baseline was corrected using polynomial fitting.

2.3. Animal study design

The experimental design was included in the Spanish National Research Project BFU2007-60576 and approved by the Ethics Committee of the University of Murcia (Murcia, Spain) and by the Bioethics Committee-CSIC (Madrid, Spain). All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). A total of 30 mini pigs (18 ♀ and 12 ♂) 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 and fed a standard chow before any experimental procedure.

Eight-month-old animals were penned separately by sex and by dietary group: (a) control group (CT; n=10, 6 ♀ and 4 ♂), (b) high-fat group (H-F; n=5, 3 ♀ and 2 ♂), (c) high-fat+GE (H-F+GE; n=5, 3 ♀ and 2 ♂) [1.6 g GE/70-kg body weight (BW) per day], (d) high-fat+RES-enriched GE (H-F+GE+RES; n=5, 3 ♀ and 2 ♂) [1.6 g GE +RES/70-kg BW per day, equivalent to 8 mg of RES/70-kg animal BW per day; human equivalent dose (HED)~7 mg RES assuming a 70-kg person] and (e) high-fat+RES group (H-F+RES; n=5, 3 ♀ and 2 ♂) (8 mg RES/70-kg animal BW per day). A commercial cereal-based chow and beef tallow were purchased from a local supplier. Control animals were fed with normal chow containing 5% fat. For animals fed the H-F diet, beef tallow was added to the chow to increase the fat percentage up to 20%. The beef tallow used in this study (92.3% of fat content) contained a mixture of saturated and unsaturated fatty acids (ratio saturated/unsaturated=0.62) and 280 mg of cholesterol/100 g. Experimental diets were prepared weekly. On average, the animals consumed a daily amount of 1 kg of diet per animal. Animals were fed with the experimental diets for 12 months.

2.4. Serum biochemistry analysis

Pigs were bled at baseline (before starting treatment) and then at 6 months and 9 months after the initiation of the study for biochemical determinations. Blood was collected after 12-h fasting by carotid puncture using a Vacutainer system and immediately centrifuged at 14,000*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, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), triglycerides (TG) and the serum hepatic leakage enzymes alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyltransferase (GGT) were measured on an Olympus AU600 autoanalyzer (Olympus Diagnostica, Hamburg, Germany).

2.5. Peripheral blood mononuclear cells isolation

Heparinized blood (BD Vacutainer, Franklin Lakes, NJ, USA) was processed within 2 h after extraction and used to isolate PBMNCs. The PBMNCs isolation was carried out under sterile conditions to avoid the activation of monocytes. Blood was diluted 1:1 with RPMI 1640 cell culture medium and processed by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions. The total number of cells isolated ($14.3\pm5.0\times10^6$, n=30) and their viability (95%–100%) were estimated by trypan blue. Isolated mononuclear cells were also analyzed by flow cytometry (FACSort, BD, San José, CA, USA) using the parameters size and granularity to estimate the proportion of lymphocytes, monocytes and possible contamination with granulocytes. In agreement with reported values [11], the mean percentage of the different cell populations was as follows: lymphocytes, 79.4±5.5; monocytes, 14.0±4.8 and granulocytes, 5.3±3.0. The PBMNCs were obtained at baseline and at 6, 9 and 12 months of the dietary intervention. After washing the cells (×2) with ice-cold phosphate buffer solution (PBS), the PBMNCs were lysed using RNeasy Plus lysis buffer (Qiagen, Madrid, Spain) and stored at −80°C for RNA extraction.

2.6. Cultured cells studies

Human monocytic leukemia THP-1 cells were purchased from the American Type Culture (ATCC, Manassas, VA, USA). THP-1 cells were cultured in RPMI 1640 medium (Gibco, Invitrogen, Barcelona, Spain) supplemented with 10% fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and glutamine (2 mM) at 37°C in 5% CO_2. THP-1 monocytes were treated with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Madrid, Spain) for 72 h to facilitate differentiation into macrophages. After treatment, the adherent macrophages were washed (×3) with PBS and incubated with cell culture medium (0.1% FCS) for 16 h at 37°C prior to incubation for 1 h with each of the tested RES metabolites followed by 24-h co-treatment with human oxidized LDL (oxLDL, 20 μg/ml; Biomedical Technologies Inc., Stoughton, MA, USA). The oxidation degree of LDL was estimated by the formation of thiobarbituric-acid-reacting substances (TBARS) using malondialdehyde (TBARS=32.4±4.7 nmol/mg). All the tested compounds were added to the culture medium in a minimal volume (0.1%) of DMSO. Control cells were treated with the corresponding amount of vehicle. For each metabolite, two independent experiments were performed (n=3 to 6 wells per experiment per concentration). Cells were washed, lysed with RNeasy Plus lysis buffer (Qiagen, Madrid, Spain) and frozen at −80°C prior to RNA extraction.

2.7. RNA extraction protocols

Total RNA was isolated from PBMNCs or macrophages using the RNeasy Plus Mini kit (Qiagen, Madrid, Spain) following the manufacturer's recommendations. RNA concentration and purity were checked using the Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies). Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 were used for microarrays and (or) reverse transcriptase polymerase chain reaction (RT-PCR) experiments. The integrity of the ribosomal RNA was further checked using agarose gel electrophoresis (1%). Pure RNA samples were divided in aliquots and frozen at −80°C until further analysis.

2.8. Porcine microarray 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 H-F

diet or to the H-F+RES diet was performed using Affymetrix microarray chips. Biotinylated cRNA was prepared according to the One-Cycle target IVT labeling Affymetrix protocol from 4 μg total RNA (Expression Analysis Technical Manual, 2004, Affymetrix). Following fragmentation, 15 μg cRNA was hybridized for 16 h at 45C° on GeneChip Porcine Genome Arrays (Affymetrix, Santa Clara, CA, USA). GeneChips were washed and stained in the Affymetrix Fluidics Station 450 and scanned using the GeneChip Scanner 3000. This array contains 23,937 probe sets that interrogate approximately 23,256 transcripts from 20,201 *Sus scrofa* genes. Since the Affymetrix porcine genome microarray is not fully annotated, we have also used information from a published annotation database that describes approximately 82% of the probe sets [11]. In addition, some probe sets were verified by BLAST comparison of the Affymetrix target sequence against the GeneBank NR database. The number of microarrays performed was as follows: (a) CT group, time 0, n=10 arrays (5 females, 5 males; basal expression levels); (b) CT group, time 9 months, n=4 arrays (2 females, 2 males); (c) H-F group, time 9 months, n=4 arrays (2 females, 2 males); (d) H-F+RES group, time 9 months, n=4 arrays (2 females, 2 males).

2.9. Bioinformatic tools for statistical and functional analysis

The CEL files obtained from GCOS software (Affymetrix) were used to analyze the data with Robust Multichip Average (RMA) implemented with the Affymetrix Expression console (v.1.1). The RMA-normalized data were tested for differential gene expression between groups using an empirical Bayes method that fits a hierarchical mixture model [12] implemented with the software Gene Expression Pattern Analysis Suite (GEPAS) (http://gepas.bioinfo.cipf.es/). Using this model, probabilities for each expression pattern (DE, differentially expressed; EE, equivalently expressed) are calculated for every gene. These probabilities are referred to as posterior probabilities, and a threshold can be set to control the false discovery rate (FDR). In the present study, DE genes were defined as those with a posterior probability exceeding 0.99 that controls the FDR at 0.01. Minimum Information About a Microarray Experiment compliant, the complete data set, both RMA normalized and original CEL files, from control and treated PBMNCs after exposure to the H-F diet or to the H-F+RES diet has been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE23503. 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 to responsive genes.

2.10. 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 probe Ssc.1089.1.S1_at annotated as *FABP4* (fatty acid binding protein 4, adipocyte). TaqMan FAM-labeled, primers–probe sets were purchased from Assays-on-demand (ABI, Madrid, Spain): Ss03373313_m1 for pig *FABP4* and Hs00609791_m1 for human *FABP4*. Expression changes were determined in RNA samples extracted from (a) PBMNCs isolated from pigs' blood at 0-, 6-, 9- and 12-month time points of the experimental procedure and from (b) human macrophages after exposure to oxLDL and the RES metabolites. 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. The fold change in the expression levels of *FABP4* in the PBMNCs isolated from pigs was normalized to the levels of the endogenous control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Ss03373286_u1) utilizing the $2^{-\Delta\Delta Ct}$ method for relative quantification of gene expression. The Ct values for replicates of each group were averaged, and the ΔCt value was determined by subtracting the average *GAPDH* Ct value from the average target Ct value. Each ΔΔCt was calculated as the difference in ΔCt between the sample of interest (each treatment group) and the ΔCt calibrator value (CT group). The expression levels of *FABP4* in human macrophages were also normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1) utilizing a standard curve method for quantification.

2.11. Statistics

Where indicated, experimental results are shown as the mean±S.D. Statistical analyses were performed by the Student's t test, and significance was defined as P<.05 or P<.01.

3. Results

3.1. Serum biochemical parameters

All animals survived all procedures and completed the study. At the end of the 12-month experimental period, no significant differences in the mean weight of each animal group were found (data not shown). The sequential changes in the levels of glucose, total cholesterol, LDL-c, HDL-c, TG, ALP, GGT, AST and ALT are listed in Supplementary Tables 1 and 2 for male and female pigs, respectively. A summary of the plasma lipids changes is represented in Fig. 2. In general, the levels of serum LDL-c, HDL-c and TG were significantly lower (P<.05) in male pigs than in female pigs. Consumption of the H-F diet increased the levels of LDL-c and HDL-c by approximately 50% and 30% in males and females, respectively (only significant in some female groups, P<.05), whereas TG levels were not altered. Addition of RES, GE+RES or GE to the H-F diet did not have a significant effect on the serum levels of these parameters. Glucose levels were also lower in male pigs than in female pigs, and none of the treatments had an effect on the levels of this sugar. Regarding the liver enzymes, the serum levels of ALP and GGT were higher in male pigs than in female pigs (Fig. 3), whereas the levels of ALT were similar or slightly lower in male animals than in female animals. Differences in the ALP, GGT and ALT levels between the dietary groups were not significant, indicating a lack of effect after exposure of the animals to the high-fat diet or to any of the treatments with RES, GE+RES or GE alone. The serum levels of AST exhibited a very high variability between individuals, in particular, in the female groups. No significant effects of any of the tested diets on AST levels were detected.

3.2. Microarray data

In order to validate our microarray analysis method, we first determined sex differential gene expression between female and

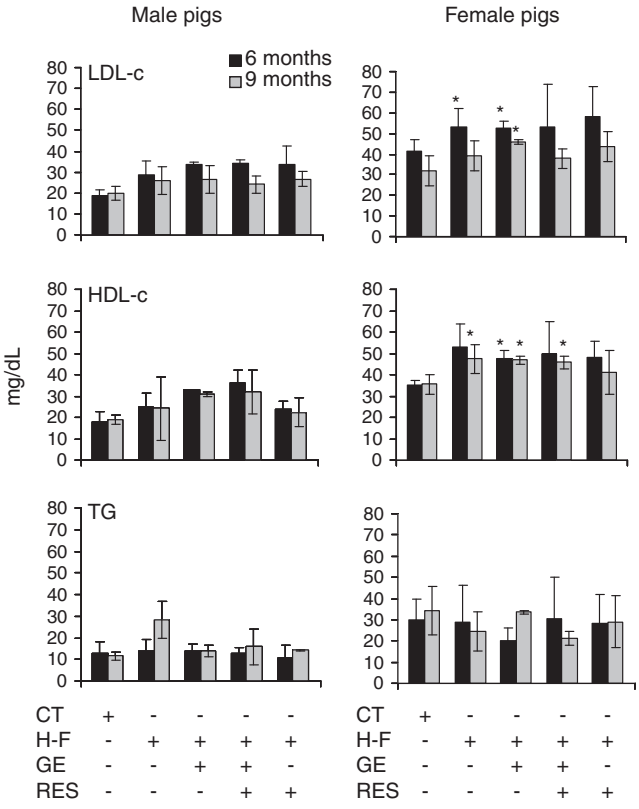

Fig. 2. Effect of the experimental diets on plasma lipids in male and female pigs. Treatments are indicated as follows: (1) CT, animals on a control diet; (2) H-F, animals on a high-fat diet; (3) H-F+GE, animals on a high-fat diet supplemented with GE (1.6 g GE/70-kg BW per day); (4) H-F+GE+RES, animals on a high-fat diet supplemented with GE containing RES (1.6 g GE+RES/70-kg BW per day, equivalent to 8 mg of RES/70-kg animal BW per day); (5) H-F+RES, animals on a high-fat diet supplemented with RES (8 mg RES/70-kg animal BW per day). Data are mean values±S.D. from fasting plasma samples after 6 and 9 months of treatment (n=2 to 5 per group) (*P<.05 with respect to control group, CT).

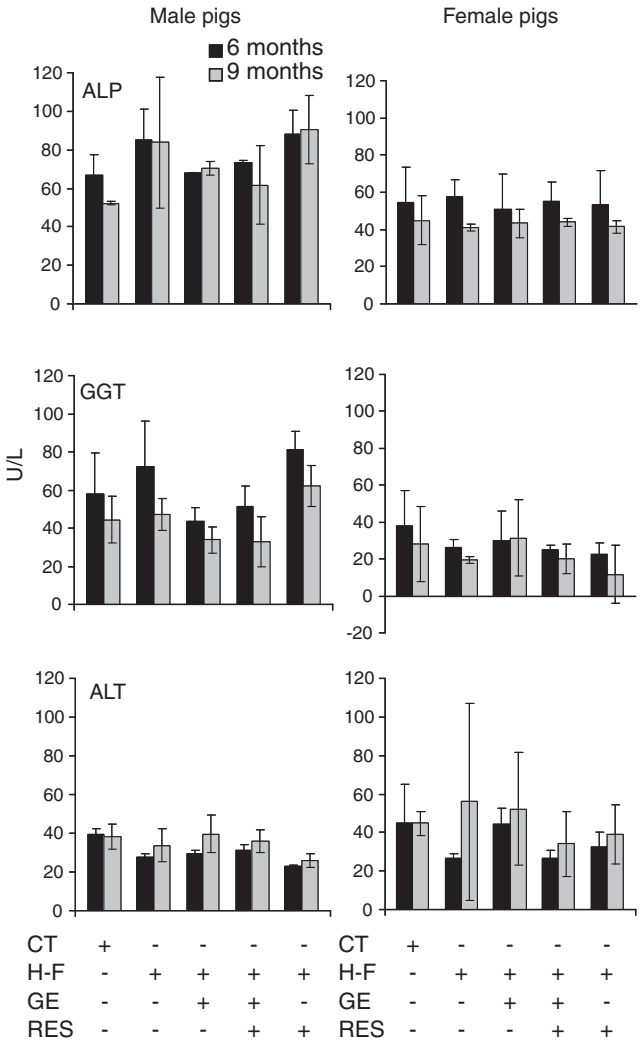

Fig. 3. Effect of the experimental diets on the serum levels of liver enzymes in male and female pigs. Treatments are indicated as follows: (1) CT, animals on a control diet; (2) H-F, animals on a high-fat diet; (3) H-F+GE, animals on a high-fat diet supplemented with GE (1.6 g GE/70-kg BW per day); (4) H-F+GE+RES, animals on a high-fat diet supplemented with GE containing RES (1.6 g GE+RES/70-kg BW per day, equivalent to 8 mg of RES/70-kg animal BW per day); (5) H-F+RES, animals on a high-fat diet supplemented with RES (8 mg RES/70-kg animal BW per day). Data are mean values±S.D. from fasting plasma samples after 6 and 9 months of treatment (n=2 to 5 per group).

male pigs within each experimental group. The complete lists of probe sets that exhibited significant sex-biased expression together with tentative annotations are provided in Supplementary Tables 3 to 6. By comparing results across all the groups using the 'compare data' tool in the IPA software, we were able to identify the overlapping sex-biased genes (Table 1). Probe sets representing X-linked genes like *XIST* were more expressed in the PBMNCs from female pigs, whereas Y-linked genes *DDX3Y*, *EIF1AY*, *EIF2S3Y*, *JARID1D* and *UTY* were more expressed in the PBMNCs from male pigs. The X-linked gene *TMSB4X* was found to be down-regulated in females. We also identified other sexually differentiated genes such as genes encoding members of the cytochrome P450s family, *CYP1B1*; genes involved in inflammatory processes, *CXCL14*, *AMCF-II*; genes encoding several matricellular glycoproteins, *THBS1*, *SEPP1* and *MAMDC2*; as well as a component of the cytoskeleton, *ACTG2*. These results are in good agreement with previous reports [13,14] and evidence that the hierarchical Bayes analysis method used in our study had sufficient statistical power to detect differentially expressed genes.

Using the same empirical method, we next analyzed and compared microarrays results between the CT, the H-F and the H-F +RES experimental groups in female and male pigs separately. A very small number of probe sets (<1%) were found to exhibit significant expression changes (Supplementary Tables 7 to 10), and the data sets show a different profile of changing genes between females and males after exposure to the H-F diet or to the H-F diet plus RES. To identify common genes across sexes and diets, the four data sets were analyzed with the 'compare data' tool in the IPA software. We identified a number of genes that were commonly regulated by high-fat consumption and counteracted by RES supplementation. A selection of these genes is listed in Table 2. Four of these genes, *COL3A1*, *FBN1*, *FSTL1* and *LPL*, appeared in all four data sets and were found to be up-regulated by the H-F diet and down-regulated by the H-F diet supplemented with RES in both female and male pigs. Other four candidate genes from this list were found to be significantly regulated in at least three of the experimental groups, mostly up-regulated by the fat intake and down-regulated by RES supplementation: *COL1A1*, *COL1A2*, *DCN* and *SPARC*. Other genes of interest were also affected by the intake of fat and counteracted by RES in either female or male pigs including some members of the *FABP* family. A search in the literature indicated that most of these candidate genes have been associated to CVDs diseases and (or) lipid metabolism. The IPA software was also used to provide insights about the functional relevance of differentially expressed genes between experimental groups. We used a Fisher's Exact Test to identify common enriched

Table 1
Sexually dimorphic significant expressed genes in PMNBCs isolated from pigs (Bayes test, posterior probability>.99, FDR<0.01)

Probe sets	Gene identification (most significant alignment) *	Gene symbol
Up-regulated in females		
Ssc.13426.1.A1_at	X (inactive)-specific transcript (non-protein coding)	*XIST*
Ssc.4897.1.A1_at		
Ssc.6667.1.A1_at		
Ssc.2434.1.A1_at		
Ssc.21512.1.A1_at		
Ssc.31029.1.A1_at		
Ssc.10316.1.s1_at	Actin, gamma 2, smooth muscle	*ACTG2*
Up-regulated in males		
Ssc.7473.1.A1_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked (DDX3Y) on chromosome Y	*DDX3Y (DBY)*
Ssc.26799.1.S1_at	Eukaryotic translation initiation factor 1A, Y-linked	*EIF1AY*
Ssc.16426.1.S1_at	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa, Y-linked	*EIF2S3Y*
Ssc.21814.1.S1_at	Lysine (K)-specific demethylase 5D Y-linked	*JARID1D (SCMY, KDM5D)*
Ssc.27304.1.S1_at	Thymosin beta 4, X-linked	*TMSB4X*
Ssc.4122.1.S1_at		
Ssc.27304.3.S1_s_at		
Ssc.15821.1	Ubiquitously transcribed tetratricopeptide repeat gene, Y-linked	*UTY*
Ssc.27236.1		
Ssc.10406.1.A1_at	Thrombospondin 1	*THBS1*
Ssc.924.2.A1_at		
Ssc.921.3.A1_at		
Ssc.26702.1.A1_at		
Ssc.29636.1	MAM domain containing 2	*MAMDC2*
Ssc.26228.1.S1_at	Zinc finger protein, X-linked	*ZFX*
Ssc.4894.1.S1_at	Chemokine (C-X-C motif) ligand 14	*CXCL14*
Ssc.20578.1.S1_at		
Ssc.719. 1.S1_at	Alveolar macrophage-derived chemotactic factor-II	*AMCF-II*
Ssc.719. 1.S1_a_at		
Ssc.12616.1.A1_at	Cytochrome P450, family 1, subfamily B, polypeptide 1	*CYP1B1*
Ssc.8767.1.A1_at		
Ssc.8479.1.A1_at	Selenoprotein P	*SEPP1*

* Based on BLAST comparison of Affymetrix target sequences against GenBank NR database.

Table 2
Genes associated to CVDs and lipid metabolism that were up- or down-regulated in isolated PBMNCs from female and male pigs after exposure to H-F or to H-F+RES (Bayes test, posterior probability>.99, FDR<0.01)

Probe sets ID	Gene Symbol	Gene name	Biological function(s) associated to CVDs and (or) lipid metabolism	H-F		H-F +RES	
				♀	♂	♀	♂
Ssc.1091.1.S1_at	*COL1A1*	Collagen alpha 1(I) chain precursor.	Primary constituents of ECM involved in vascular remodeling. Overexpressed in pig arteries exposed to high pressure/flow [15].	↑	↑	-	↓
Ssc.21011.1.S1_at	*COL1A2*	Collagen alpha 2(I) chain precursor.		↑	↑	-	↓
Ssc.11302.1.S1_at Ssc.11302.1.S2_at	*COL3A1*	Collagen alpha 1(III) chain precursor		↑	↑	↓	↓
Ssc.10245.2.A1_a_at	*DCN*	Decorin precursor (bone proteoglycan II)	Small matrix proteoglycan that binds to type I collagen fibrils and plays a role in matrix assembly. Involved in atherosclerotic lesions and binding of LDL to collagen [16].	↑	↑	-	↓
Ssc.16045.1.S1_at	*FBN1*	Fibrillin 1 precursor.	Glycoprotein constituent of microfibrils that interacts with *DCN* and affects arterial stiffness [17].	↑	↑	↓*	↓
Ssc.23242.1.A1_at	*FSTL1*	Follistatin-related protein 1 precursor	Overexpressed in heart failure, localized to endothelium and smooth muscle cells, associated to ECM-related proteins [18].	↑	↑	↓	↓
Ssc.1458.3.A1_a_at	*SPARC*	Secreted protein, acidic, cysteine-rich (osteonectin)	Collagen-binding protein mediates cell–matrix interactions in wound healing. Involved in vascular remodeling and up-regulated in diabetes vascular hypertrophy [19].	↑	↑	-	↓
Ssc.16335.1.S2_at Ssc.1147.2.S1_at	*LPL*	Lipoprotein lipase precursor	Key enzyme in the catabolism of TGs from VLDL and chylomicrons. It also acts as a cholesteryl ester transferase [20].	↑	↑	↓	↓
Ssc.676.1.S1_at	*FABP6*	Ileal lipid-binding protein	Role in transport of bile acids and metabolism of certain hormones. Expressed in the enterocytes of ileum, ovary and adrenal gland [21].	-	↑	-	↓
Ssc.1089.1.S1_at	*FABP4*	Fatty acid-binding protein, adipocyte	Highly expressed in adipocytes and macrophages. Inhibition of FABP4 prevents metabolic diseases [22].	↑	-	↓	-

* Bayes test, posterior probability>.98.

pathways in the four data sets. We found only one pathway, 'atherosclerosis signaling', whose *P* value was <.05 after Benjamin–Hochberg (B-H) multiple testing correction. This observation was mainly attributed to some of those genes included in Table 2 (*COL3A1*, *COL1A1*, *COL1A2*, *LPL*). We then used the IPA system to examine the potential biological functions that may have been significantly affected (B-H multiple testing correction, *P* value <.05) in the four experimental groups. As a result, we found that many of the significant changing genes listed in the four data sets fell into the following categories: (a) cardiovascular disease, cardiovascular system development and function; (b) hematological disease, hematological system development and function; and (c) lipid metabolism and metabolic disease.

3.3. RT-PCR in PBMNCs

The RT-PCR analysis confirmed that the consumption of the H-F diet increased the levels of expression of *FABP4* in PBMNCs isolated from female pigs at 6 months after initiating the diet (Fig. 4). This induction was even more pronounced after 9 and 12 months of the experiment. Resveratrol or RES-containing GE significantly prevented the induction of *FABP4* expression already after 6 months of dietary supplementation. In addition, we found that long-term supplementation with GE without RES also reduced the levels of *FABP4* mRNA. No changes in the expression of *FABP4* were detected in male animals (data not shown).

3.4. Cell culture studies

In THP-1 monocytes, very low levels of *FABP4* transcripts were detected. However, in PMA-differentiated macrophages, the expression levels of *FABP4* were higher and were further increased after treatment with 10 μg/ml and 20 μg/ml of oxLDL for 24 h. The highest induction (~4.5-fold) was observed after exposure to 20 μg/ml of oxLDL for 24 h. Under these conditions, we investigated whether RES and some of its derived metabolites might have a reducing effect on the expression of *FABP4*. We examined the effects of RES aglycone (0.01–2.0 μM), RES-gluc (0.1–15 μM), DH-RES (0.1–2.0 μM) and DH-RES-gluc (0.1–15 μM) (Fig. 5). The expression levels of *FABP4* in oxLDL-treated macrophages were in general not affected by most of the metabolites and concentrations assayed. However, a small but significant reduction (*P*<.05) was observed when cells were treated with RES 0.1 μM (~15% reduction) or with DH-RES 0.1 μM (~20% reduction).

4. Discussion

Resveratrol is considered one of the most promising natural molecules with beneficial effects against cardiovascular and metabolic disorders as evidenced by many *in vitro* and *in vivo* studies [2,3]. However, the mechanisms underlying the prevention of the

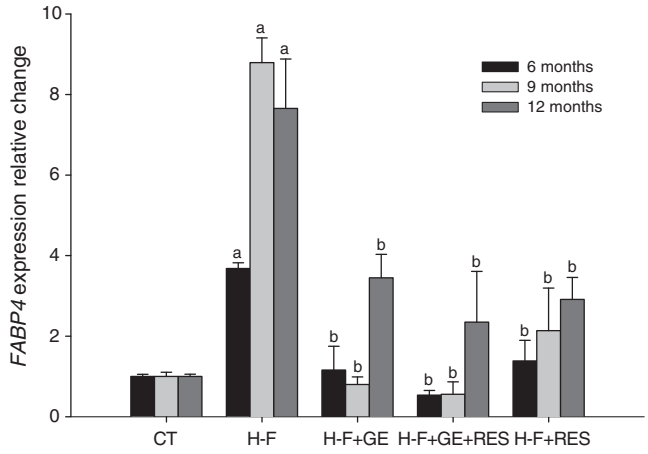

Fig. 4. Effect of the experimental diets (H-F, H-F+GE, H-F+GE+RES, H-F+RES) on the *FABP4* expression in PBMNCs from female pigs. Gene expression was determined by one-step quantitative RT-PCR and was normalized to *GAPDH* and expressed relative to gene expression in the control group (CT). Each column represents the mean values ±S.D. from *n*=3 replicates. a: *P*<.05 with respect to CT; b: *P*<.05 with respect to H-F.

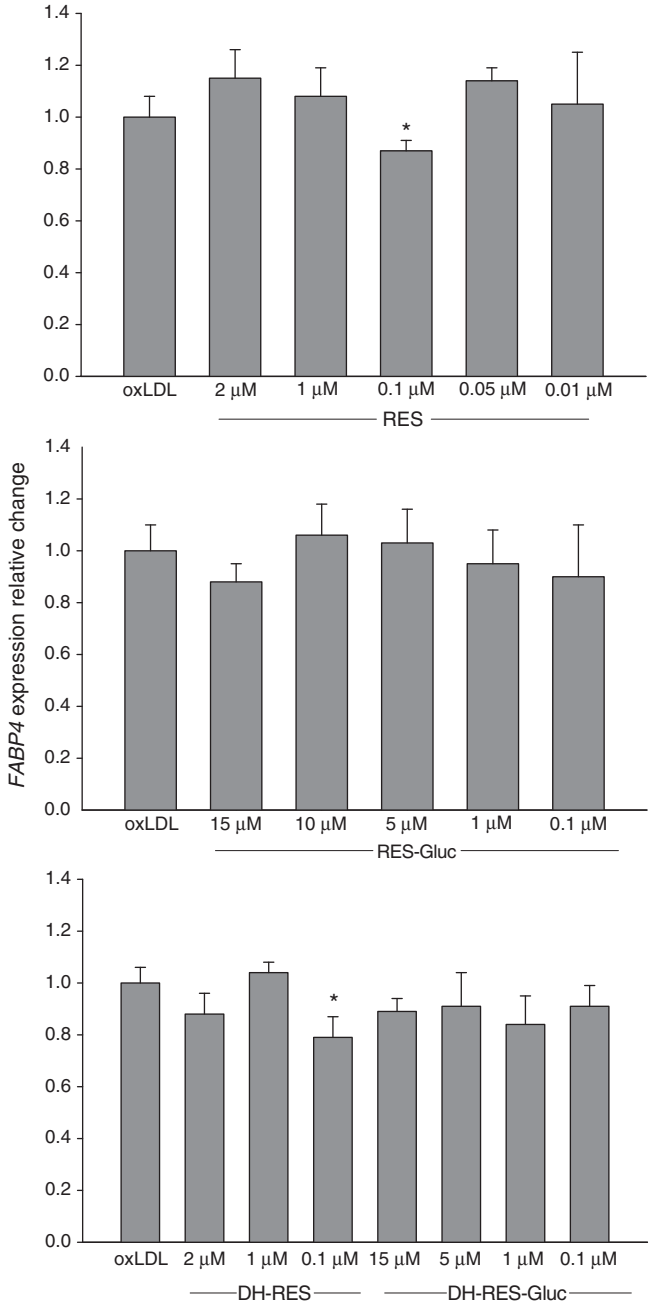

Fig. 5. Effect of various concentrations of RES and the different RES metabolites, RES-gluc, DH-RES and DH-RES-gluc, on the gene expression of *FABP4* in human macrophages. Cells were incubated for 1 h with each of the tested metabolites followed by 24-h co-treatment with human oxLDL (20 μg/ml). Gene expression was determined by one-step quantitative RT-PCR and was normalized to *GAPDH* and expressed relative to gene expression in the oxLDL group (macrophages treated only with oxLDL). Each column represents the mean values±S.D. from two separate experiments (each experiment $n=3$ to 6 replicates). *$P<.05$ with respect to the oxLDL-treated group.

development of these chronic disorders by the consumption of dietary doses of RES are poorly understood. This is due, in part, to the lack of suitable humanoid models. Swine are considered a good model of lipid metabolism and cardiovascular studies. They have lipoprotein profiles and metabolism similar to humans, and when fed cholesterol- and lipid-containing diets, they develop a human-like atherosclerosis [23]. To determine whether dietary doses of RES had any beneficial preventive effect against early stages of the development of lipid disorders, we compared a group of high-fat-induced mild hypercholesterolemic pigs against a group of animals supplemented with low doses of RES for several months. We also examined and compared the effects of RES-containing GE and of GE alone.

We first determined differences between the experimental groups in the serum lipid profile, glucose levels and liver enzymes leakage. In agreement with previously reported data in pigs [23,24] and in rodents [25,26], our results show an increase in the levels of LDL-c and HDL-c but not of TG after consumption of the H-F diet. Unlike previous results, we observed a mild increase in the cholesterol levels likely due to differences in the amount of cholesterol provided with the diet (0.56 g cholesterol/kg in our study vs. 15–40 g/kg in other studies) [23,24] or the lack of cholate (to increase fat absorption) in our experimental diet. Resveratrol has been repeatedly shown to have no effects on the levels of TG in rodent models of diet-induced dyslipidemia, but the results on cholesterol-reducing properties appear to be dose and time dependent [25,26]. In a recent study in pigs [24], the effects of RES against high levels of cholesterol induced by a hypercholesterolemic diet were investigated. After 7 weeks of supplementation with RES, the levels of total serum cholesterol were greatly reduced. In our study, supplementation of the H-F diet with RES for up to 9 months did not have any significant effect on the levels of LDL-c or HDL-c. These results may be partially explained by the different doses of RES used. Robich et al. [24] fed the pigs with a very high dose of RES, 100 mg/kg BW (HED ~6400 mg/70-kg person). Although adverse effects of RES have only been documented at very high doses (3000 mg/kg BW) [5], a recommended dosage of 5–10 mg/kg BW per day not only is toxicologically safe but is also more representative of dietary doses of RES. In our study, we administered a daily amount of 8 mg of RES for a 70-kg animal, which is ~800-fold lower than the more pharmacological dose used by Robich et al. [24].

Resveratrol has also been reported to counteract diet-induced hyperglycemic effects [2]. We measured the levels of plasma fasting glucose to determine an effect of the H-F intake and of RES supplementation on glucose homeostasis. Although some investigators have reported a significant increase in fasting plasma glucose in pigs after the consumption of a high-fat diet for 3 months [27], we and others [23] have found that the intake of a high-fat diet for periods of up to 12 months was not sufficient to alter the glucose homeostasis in pigs. Some studies have also shown that RES was able to reduce fasted plasma glucose levels [28]. However, in our study, RES supplementation did not modify the levels of glucose in any of the experimental groups. The explanation for these results may be found again in the doses of RES used but also in the physiological state of the experimental animals. In diet-induced obesity mice, RES administration significantly reduced the levels of fasted glucose after 3 days, but the dose administered was 1000 mg/kg BW (HED ~6300 mg/70-kg person) [28]. In a more recent study [29], the effects of a much lower oral dose of RES 5 mg/kg BW (HED~45 mg/60-kg person) were compared between control and diabetic-induced rats. Resveratrol reduced the levels of fasting blood glucose in the diabetic rats, while it did not have any effect in the control healthy animals.

High levels of dietary lipids and hypercholesterolemia also increase serum levels of the liver enzymes ALT, AST and ALP, although the results may be variable [30]. Several studies have shown a therapeutic effect of RES against liver diseases, where the administration of this compound causes a decrease of the liver enzymes, mostly of ALT and AST [31]. In our study, the mild H-F diet-induced hypercholesterolemia or the RES supplementation did not modify the serum levels of hepatic enzymes. A plausible explanation may be yet again the different experimental approach since those previous studies are more of a pharmacological nature where the animals were all severely treated to induce liver damage, and, in most cases, RES was administered via intraperitoneal or intravenous routes at much higher doses than those attainable through the diet. Our results show that prolonged administration of low doses of RES to healthy animals

subjected to a mild diet-induced hypercholesterolemic condition has no apparent effects on the cholesterol, glucose or liver enzymes levels.

In an attempt to find molecular changes induced by the H-F diet that may be indicative of lipid metabolism alterations as well as changes caused by the RES supplementation that may reveal putative early regulatory effects of this molecule, we searched for gene expression changes in PBMNCs that might reflect an initial response to the H-F diet and to RES. Since there is evidence that differential gene expression may be at the molecular base of sex differences in physiological and pathological responses and, in particular, in metabolic and cardiovascular diseases [32], female and male microarrays were analyzed separately. Consistent with previous findings in human, mouse and swine independent studies [13,14], chromosome X-linked and Y-linked genes and several other genes were some of the most abundant ones among the sexually dimorphic genes identified in our study. Overall, these results represent reliable controls of our experimental setup and also indicate that highly sex-biased genes detected in different tissues in the pig [14] are also represented in the PBMNCs isolated from pig blood.

Analysis of differential expression between the experimental groups shows that exposure to the H-F diet or to the H-F diet+RES affected a small and different set of genes in female and male pigs. We found, however, some commonly regulated genes all related to inflammatory processes, atherosclerosis and lipid metabolism disorders (Table 2). In particular, a group of genes that are part of the extracellular matrix (ECM) and (or) are associated with ECM proteins and matrix remodeling (*COL1A1*, *COL1A2*, *COL3A1*, *DCN*, *FBN1*, *FSTL1* and *SPARC*) was all up-regulated by the H-F diet and down-regulated by RES consumption. Some of these proteins, COL1A, DCN, FBN1 and SPARC, have been shown to interact and bind to each other during fibrosis and ECM remodeling and to be affected in response to vascular damage [15–19]. In addition, some of these genes have also been reported to be expressed and regulated in adipose tissue and to be associated with obesity. In a recent review of microarrays analyses searching for obesity-specific gene profiles [33], a number of genes expressed in adipose tissue and functionally related to inflammation, cell adhesion and lipid metabolism were identified as potential therapeutic targets. Among those, *LPL* and *FABP5* were both diet-induced in rodents. In our study, *LPL* and some members of the *FABP* family, in particular, *FABP6* and *FABP4*, were also found to be up-regulated by the H-F diet and down-regulated by RES in males and females, respectively (Table 2).

Fatty-acid binding proteins are a family of proteins that bind hydrophobic ligands such as fatty acids, steroids and bile acids to enable their transport in biological fluids and for intracellular trafficking. [21]. Fatty-acid binding protein 4 has emerged as a novel risk biomarker of metabolic disorders and atherosclerosis since it is involved in inflammatory and metabolic responses in adipocytes and macrophages, and specific inhibition of FABP4 has been demonstrated to be an effective therapy against atherosclerosis [22]. Our results show that the intake of the H-F diet for several months induced the mRNA levels of *FABP4* in PBMNCs from female pigs and that RES counteracted this effect. A putative mechanism underlying this effect may be the activation by RES of NAD-dependent histone deacetylase SIRT1 that has been reported to be involved in the regulation of lipid metabolism by repressing peroxisome proliferator-activated receptor (PPAR)γ and down-regulating the expression of FABP4 and FABP3 in adipocytes [34]. We double searched our microarrays results looking for changes (even if these did not surpass the cutoff criteria of the analysis) in the levels of expression of the PPARs (PPARγ, PPARα and PPARδ) and of all the sirtuins (SIRT1, SIRT2, SIRT3, SIRT5 and SIRT6) represented on the chip, but no effects were induced on any of these genes by the H-F diet or the H-F+RES (fold change ~1.0). The influence of RES on sirtuins is currently only partially understood. Some studies in rats and mice have reported the induction of sirtuins in the colon by low oral doses of RES [35,36]. However, and more in consonance with our results, other studies on the regulation of sirtuins expression in mice following prolonged consumption of low levels of RES showed either no effect or even a down-regulation of the levels of SIRT1, SIRT2, SIRT3 and SIRT5 in various tissues [37,38]. Our RT-PCR results also showed that the expression of *FABP4* was down-regulated after exposure to the RES-containing GE extract or to the GE alone and indicated that different polyphenols present in grapes may induce common regulatory mechanisms. Indeed, other polyphenols found in GEs such as catechin and epigallocatechin-gallate have been shown to down-regulate the expression of *LPL* and *FABP4* in the aorta of mice deficient in apolipoprotein E [39] and in the white adipose tissue of mice fed a high-fat diet [40], respectively.

In order to understand the *in vivo* effects of dietary polyphenols, we need to investigate and determine the bioactivity of their metabolites. Macrophages play a key role in inflammation and atherosclerosis, with exposure to oxLDL being a critical event that triggers the regulation of the expression of multiple genes and proteins, amongst which FABP4 is highly up-regulated [41]. We have used cultured macrophages as a model to determine whether RES and some of its circulating metabolites, to which PBMNCs may be truly exposed following consumption of RES, were able to reduce the expression of *FABP4* in oxLDL-challenged macrophages and might have contributed to the down-regulation of *FABP4* in the mononuclear cells. Recently, the bioactivity of several sulfate-conjugated RES metabolites was investigated using some *in vitro* tests, but effects were tested at rather high nonphysiological concentrations [42,43]. In our study, we have examined the effects of RES and of some of its main metabolites at a range of concentrations that are representative of *in vivo* plasma values reported in human (up to 4.2 μM RES and 17 μM RES-3-*O*-glucuronide) [44], pig (up to 5.5 μM RES-3-*O*-glucuronide) [45] and rodent (up to 1 μM DH-RES and 33 μM DH-RES-gluc) pharmacokinetic studies [46]. Our results show a very modest but significant reduction (10%–20%) on *FABP4* expression only at some of the concentrations tested, i.e., 0.1 μM for the aglycones. In agreement with previous studies comparing the effects on gene expression of other phenolic compounds and their conjugates [47], it does appear that the aglycones are usually more efficient than the conjugates, that the changes induced at the concentrations found in plasma are quite moderate (<30%) and that effects are often observed at an optimal concentration within the physiological range.

There are some limitations to our study, in particular, the relatively small number of pigs in each group due to the difficulty in keeping and handling these animals. Therefore, our results should be interpreted in this context. Although we can not discard that other parameters not considered here may have been affected by the experimental diets, the present study corroborates the great difficulty in finding phenotypic evidences associated to a protective effect in long-term nutritional studies. In these studies, healthy individuals or individuals with a mild condition are dosed with low levels of the test compound (in our case, RES), in opposition to experimental pathological models in which usually severely damaged individuals are administered with much higher and more effective pharmacological doses of the compound. We have also shown that, in response to an H-F diet and to RES supplementation, PBMNCs express and reveal changes in genes that are associated to vascular damage and lipid metabolism and suggest that the intake of the H-F diet might be associated to the development of some early lesions in the pig arteries and that supplementation with RES may reduce or avoid this initial damage. However, and although PBMNCs are considered a potential model to assess changes that may occur in other parts of the body, we can not discard that the levels of other genes such as those of sirtuins may have been affected in other tissues and that these changes have not been reflected in the PBMNCs. Finally, our *in vitro* data indicate

that the *in vivo* circulating concentrations of RES aglycone and of DH-RES metabolite may have a small contribution to the observed *in vivo* down-regulation of *FABP4*.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.04.007.

Acknowledgments

Authors are grateful to Dr. Alberto Quiles and Dr. Cándido Gutiérrez for their assistance in some parts of the study.

References

[1] Dixon JB. The effect of obesity on health outcomes. Mol Cell Endocrin 2010;316:104–8.
[2] Das M, Das DK. Resveratrol and cardiovascular health. Mol Aspects Med 2010;31:503–12.
[3] Szkudelska K, Szkudelski T. Resveratrol, obesity and diabetes. Eur J Pharmacol 2010;635:1–8.
[4] Queen BL, Tollefsbol TO. Polyphenols and aging. Curr Aging Sci 2010;3:34–42.
[5] Cottart CH, Nivet-Antoine V, Laguillier-Morizot C, Beaudeux JL. Resveratrol bioavailability and toxicity in humans. Mol Nutr Food Res 2010;54:7–16.
[6] Khymenets O, Fitó M, Covas MI, et al. Mononuclear cell transcriptome response after sustained virgin olive oil consumption in humans: a nutrigenomics study. OMICS 2009;13:7–19.
[7] Ma J, Dempsey AA, Stamatiou D, Marshall KW, Liew CC. Identifying leukocyte gene expression patterns associated with plasma lipid levels in human subjects. Atherosclerosis 2007;191:63–72.
[8] Cantos E, Espín JC, Tomas-Barberan FA. Postharvest induction modeling method using UV irradiation pulses for obtaining resveratrol-enriched table grapes: a new "functional" fruit? J Agric Food Chem 2001;49:5052–8.
[9] Lucas R, Alcantara D, Morales JC. A concise synthesis of glucuronide metabolites of urolithin-B, resveratrol and hydroxytyrosol. Carbohydrate Res 2009;344:1340–6.
[10] Stivala LA, Savio M, Carafoli F, et al. Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. J Biol Chem 2001;276:22586–94.
[11] Tsai S, Cassady JP, Freking BA, Nonneman DJ, Rohrer GA, Piedrahita JA. Annotation of the Affymetrix porcine genome microarray. Animal Genetics 2006;37:422–31.
[12] Kendziorski CM, Newton MA, Lan H, Gould MN. On parametric empirical Bayes methods for comparing multiple groups using replicated gene expression profiles. Statist Med 2003;22:3899–914.
[13] Isensee J, Witt H, Pregla R, Hetzer R, Regitz-Zagrosek V, Ruiz-Noppinger P. Sexually dimorphic gene expression in the heart of mice and men. J Mol Med 2008;86:61–74.
[14] Ferraz ALJ, Ojeda A, López-Béjar M, et al. Transcriptome architecture across tissues in the pig. BMC Genomics 2008;9:173–93.
[15] Medhora M, Bousamra IIM, Zhu D, Somberg L, Jacobs ER. Upregulation of collagens detected by gene array in a model of flow-induced pulmonary vascular remodeling. Am J Physiol Heart Circ Physiol 2002;282:H414–22.
[16] Tannock LR, King VL. Proteoglycan mediated lipoprotein retention: a mechanism of diabetic atherosclerosis. Rev Endocr Metab Disord 2008;9:289–300.
[17] Ramirez F, Sakai LY. Biogenesis and function of fibrillin assemblies. Cell Tissue Res 2010;339:71–82.
[18] Lara-Pezzi E, Felkin LE, Birks EJ, et al. Expression of follistatin-related genes is altered in heart failure. Endocrinol 2008;149:5822–7.
[19] McCurdy S, Baicu CF, Heymans S, Bradshaw AD. Cardiac extracellular matrix remodelling: fibrillar collagens and secreted protein acidic and rich in cysteine (SPARC). J Mol Cell Cardiol 2010;48:544–9.
[20] Stein Y, Stein O. Lipoprotein lipase and atherosclerosis. Atherosclerosis 2003;170:1–9.
[21] Ono T, Odani S. Initial studies of the cytoplasmic FABP superfamily. Proc Jpn Acad Ser B 2010;86:220–8.
[22] Furuhashi M, Tuncman G, Görgün CZ, et al. Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. Nature 2007;447:959–65.
[23] Gerrity RG, Natarajan R, Nadler JL, Kimsey T. Diabetes-induced accelerated atherosclerosis in swine. Diabetes 2001;50:1654–65.
[24] Robich MP, Chu LM, Chaudray M, et al. Anti-angiogenic effect of high-dose resveratrol in swine model of metabolic syndrome. Surgery 2010;148:453–62.
[25] Ahn J, Cho I, Kim S, Kwon D, Ha T. Dietary resveratrol alters lipids metabolism-related gene expression of mice on an atherogenic diet. J Hepathol 2008;49:1019–28.
[26] Rocha KKR, Souza GA, Seiva FRF, Cataneo AC, Novelli ELB. Resveratrol toxicity: effects on risks factors for atherosclerosis and hepatic oxidative stress in standard and high-fat diets. Food Chem Toxicol 2009;47:1362–7.
[27] Larsen MO, Rolin B, Wilken M, Carr RD, Svendsen O. High-fat high-energy feeding impairs fasting glucose and increases fasting insulin levels in the Göttingen minipig. Ann NY Acad Sci 2002;967:414–23.
[28] Smith JJ, Kenney RD, Gagne DJ, et al. Small molecule activators of SIRT replicate signalling pathways triggered by calorie restriction in vivo. BMC Systems Biol 2009;3:31.
[29] Palsamy P, Subramanian S. Ameliorative potential of resveratrol on proinflammatory cytokines, hyperglycemia mediated oxidative stress, and pancreatic β-cell dysfunction in streptozotocin-nicotinamide-induced diabetic rats. J Cell Physiol 2010;224:423–32.
[30] Prasad K. Effects of vitamin E on serum enzymes and electrolytes in hypercholesterolemia. Mol Cell Biochem 2010;335:67–74.
[31] Bishayee A, Darvesh AS, Politis T, McGory R. Resveratrol and liver disease: form bench to bedside and community. Liver Int 2010;30:1103–14.
[32] Kautzky-Willer A, Handisurya A. Metabolic diseases and associated complications: sex and gender matter. Eur J Clin Invest 2009;39:631–48.
[33] Kim Y, Park T. DNA microarrays to define and search for genes associated with obesity. Biotechnol J 2010;5:99–112.
[34] Shan TZ, Ren Y, Wu T, Liu CX, Wang YZ. Regulatory role of Sirt1 on the gene expression of fatty acid-binding protein 3 in cultured porcine adipocytes. J Cell Biochem 2009;107:984–91.
[35] Larrosa M, Yañez-Gascón MJ, Selma MV, et al. Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. J Agric Food Chem 2009;57:2211–20.
[36] Singh UP, Singh NP, Singh B, et al. Resveratrol (trans-3.5.4'-trihydroxystiblbene) induces silent mating type information regulation-1 and down-regulates nuclear transcription factor-kappaB activation to abrogate dextran sulfate sodium-induced colitis. J Pharmacol Exp Ther 2010;332:829–39.
[37] Barger JL, Kayo T, Vann JM, et al. A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. PLoS ONE 2008;3:e2264.
[38] Barger JL, Kayo T, Pugh TD, Prolla TA, Weindruch R. Short-term consumption of a resveratrol-containing nutraceutical mixture mimics gene expression of long-term caloric restriction in mouse heart. Exp Gerontol 2008;43:859–66.
[39] Auclair S, Milenkovic D, Besson C, et al. Catechin reduces atherosclerotic lesion development in apo E-deficient mice: a transcriptomic study. Atherosclerosis 2009;204:e21–7.
[40] Lee MS, Kim CT, Kim Y. Green tea (−)-epigallocatechin-3-gallate reduces body weight with regulation of multiple genes expression in adipose tissue of diet-induced obese mice. Ann Nutr Metab 2009;54:151–7.
[41] Fach EM, Garulacan LA, Gao J, et al. In vitro biomarker discovery for atherosclerosis by proteomics. Mol Cell Proteom 2004;3:1200–10.
[42] Hoshino J, Park EJ, Kondratyuk TP, et al. Selective synthesis and biological evaluation of sulfate-conjugated resveratrol metabolites. J Med Chem 2010;53:5033–43.
[43] Calamini B, Ratia K, Malkowski MG, et al. Pleiotropic mechanisms facilitated by resveratrol and its metabolites. Biochem J 2010;429:273–82.
[44] Brown VA, Patel KR, Viskaduraki M, et al. Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics and effect on the insulin-like growth factor axis. Cancer Res 2010;70:9003–11.
[45] Azorín-Ortuño M, Yañéz-Gascón MJ, Pallarés FJ, et al. Pharmacokinetic study of trans-resveratrol in adult pigs. J Agric Food Chem 2010;58:11165–71.
[46] Juan ME, Alfaras I, Planas JM. Determination of dihydroresveratrol in rat plasma by HPLC. J Agric Food Chem 2010;58:7472–5.
[47] Tribolo S, Lodi F, Connor C, et al. Comparative effects of quercetin and its predominant human metabolites on adhesion molecules expression in activated human vascular endothelial cells. Atherosclerosis 2008;197:50–6.