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Hydroxytyrosol Administration Enhances Atherosclerotic Lesion Development in Apo E Deficient Mice

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Hydroxytyrosol is a phenol found in olive oil. To verify the effect of hydroxytyrosol on the development of atherosclerosis, two groups of apo E deficient male mice on a standard chow diet were used: the control group receiving only water, and the second group an aqueous solution of hydroxytyrosol in order to provide a dose of 10 mg/kg/day to each mouse. This treatment was maintained for 10 weeks. At the moment of sacrifice, blood was drawn and heart removed. Plasma lipids, apolipoproteins and monocyte Mac-1 expression were assayed as well as aortic atherosclerotic areas in both groups. Data showed no significant changes in HDL cholesterol, paraoxonase, apolipoprotein B or triglyceride levels. However, hydroxytyrosol administration decreased apolipoprotein A-I and increased total cholesterol, atherosclerotic lesion areas and circulating monocytes expressing Mac-1. The latter was highly correlated with lesion areas ($r = 0.65$, $P < 0.01$). These results indicate that administration of hydroxytyrosol in low cholesterol diets increases atherosclerotic lesion associated with the degree of monocyte activation and remodelling of plasma lipoproteins. Our data supports the concept that phenolic-enriched products, out of the original matrix, could be not only non useful but also harmful. Our results suggest that the formulation of possible functional foods should approximate as much as possible the natural environment in which active molecules are found.

Key words: atherosclerosis, hydroxytyrosol, lipoproteins, olive oil, transgenic animal.

Atherosclerosis is a multifactorial disease that represents the primary cause of death in Western civilization (*1*). However, epidemiological studies have shown a lower incidence of coronary heart disease in Mediterranean countries (*2*). Mediterranean diet is largely vegetarian in nature and includes the consumption of large quantities of olive oil (*3*). The composition of the olive oil is primarily triacylglycerols and fatty acids, and secondarily unsaponifiable and soluble constituents where polyphenols are included (*4*).

There is compelling evidence that oxidative modification of low density lipoprotein plays a key role in the development of atherosclerosis (*5*). In human and animal dietary studies, virgin olive oil has been shown to reduce low density lipoprotein (LDL) susceptibility to oxidation (*6–8*). There is some available evidence from randomized, crossover, controlled studies, that phenolic compounds from olive oil could reduce risk factors for coronary heart disease (CHD) such as *in vivo* oxidized LDL, DNA oxidation, and thromboxane (TBX2) (*9*, *10*). Phenolic compounds are potent in vitro inhibitors of LDL oxidation and are capable of breaking the peroxidative chain reaction (*11*, *12*). Olive oil phenolic compounds also show other antiatherogenic activities, such as the inhibition of platelet aggregation (*13*) or the reduction of monocyte adhesion to the stimulated endothelium (*14*).

Hydroxytyrosol, as simple phenol or conjugate form, is one of the most important phenols present in olive oil (*15*). It is present in a particularly high concentration in extra virgin olive oil, representing around 10% of the total phenolic content of an olive oil (5 to 80 mg/kg) (*16*). Several studies have demonstrated that hydroxytyrosol is absorbed and excreted in urine as a response to the intake of virgin olive oil as well as to the intake of oily and aqueous preparations (*17–21*). Hydroxytyrosol has been recovered in all lipoprotein fractions, except in very low dense lipoproteins (VLDL), after extra virgin olive oil ingestion (*17*). In *in vivo* and *in vitro* studies, hydroxytyrosol and its metabolites have been shown to be strong antioxidants (*16*, *22–24*). However, phenolic compounds can have both anti- and pro-oxidant effects (*25*, *26*). Due to this, the *in vivo* anti-atherosclerotic potential of hydroxytyrosol merits to be investigated.

The aim of this study was to investigate the potential application of hydroxytyrosol isolated from its natural medium, the olive oil, as a pharmacological agent on the *in vivo* atherosclerosis process. The apoE-deficient mouse was used as a model of spontaneous atherosclerosis

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development. These animals develop severe atherosclerosis on a regular low-fat/low-cholesterol diet. The progression and histopathology of lesions in this animal model show similar features to those observed in humans and other species (*27*, *28*). Analyses of lipid, lipoproteins, and atherosclerotic lesions were performed to investigate the mechanisms involved.

MATERIAL AND METHODS

Animals—Homozygous apoE KO mice were bred in the *Unidad Mixta de Investigación*, Zaragoza. 21 males, aged two months, were fasted overnight, anesthetized with isofluorane, and blood samples obtained by retroorbital bleeding to estimate baseline plasma cholesterol and triglycerides. Two groups of 13 and 8 mice, of equal plasma cholesterol and triglycerides, were housed in sterile filter-top cages. Animals had ad libitum access to food and water. The protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza.

Diets—Normal mouse chow of low cholesterol content was Teklad Mouse/Rat Diet 2014 from Harlan Teklad (Harlan Ibérica, Barcelona, Spain). Two groups of study fed on a chow diet were established: a control group (13 mice) with water for drink and a hydroxytyrosol group (8 mice) receiving as beverage, an aqueous solution of hydroxytyrosol to provide a dose of 10 mg/kg/day per mouse. The purified product was obtained as previously described (*29*). Aqueous hydroxytyrosol solution was freshly replaced every day. Experimental agent was provided for 10 weeks and was well tolerated.

Biochemical Determinations—After the experimental period, animals were sacrificed by suffocation with CO_2 and blood was drawn from their hearts. Total plasma cholesterol and triglyceride concentrations were measured in a microtiter assay, using commercial kits from Sigma Chemical Co. (Madrid, Spain). High density lipoprotein (HDL) cholesterol was determined in a similar manner after phosphotungstic acid–$MnCl_2$ (Roche, Barcelona, Spain) precipitation of apo B containing particles (*30*). Measurement of the total isoprostane 8-iso-$PGF_2\alpha$ was carried out by immunoassay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Paraoxonase was assayed as arylesterase activity following the protocol previously described (*31*). Results were expressed as μmol phenylacetate hydrolyzed·min^{-1}·$liter^{-1}$ (IU·$liter^{-1}$).

Apolipoproteins A-I, A-II, A-IV and B were quantified by enzyme-linked immunosorbent assays using specific polyclonal antibodies (Biodesign, Saco, ME and Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described (*32*, *33*). All assays were done in triplicate, and all samples were processed in the same day. Intra-assay variability was lower than 4%.

To analyze lipoprotein profiles, 100 μl of pooled samples from each dietary group were subjected to fast protein liquid chromatography gel filtration using a Superose 6B column (Amersham Biosciences, Barcelona, Spain) as previously described (*34*).

Lipoprotein Oxidation Susceptibility—Lipoprotein oxidizability was measured as previously described (*35*). Briefly, 3 μl of lipoprotein fraction was diluted with 328 μl of phosphate-buffered saline (PBS). 3.3 μl of 1 mM $CuSO_4$ solution was added to start oxidation. The susceptibility of lipoproteins to oxidation was continuously monitored following changes in absorbance at 234 nm every 10 min for a period of 4 h and kinetics is shown. Alternatively, the presence of oxygen radicals was estimated by the 2′,7′-dichlorofluorescein diacetate (DCF) assay where LDL or VLDL (5 μg of cholesterol) were incubated, at 37°C, with 2 μg of DCF in a total volume of 150 μl and in presence of 25 μl of 0.1% sodium azide and 100 μl of PBS (*36*). Fluorescence, at 485 nm excitation and 535 nm emission, was measured after 3 h.

In Vitro *Incubation of Hydroxytyrosol with Murine Peritoneal Macrophages*—Peritoneal macrophages from apoE deficient mice were isolated and cultured as previously described (*37*). Cells were harvested by peritoneal lavage 5 days after intraperitoneal injection of 1 ml of 10% thioglycolate broth. Cells were then resuspended in culture RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and incubated at 37°C in a 5% CO_2 incubator for 1 h. The adherent cells were used in the experiments. Murine peritoneal macrophages (2×10^5 cells/well) were cultured in 24-well plates and incubated with different doses of hydroxytyrosol (2, 5 and 25 μM) at 37°C for 24 h. Then the media were aspirated and RNA extracted by addition of 200 μl of Trigent reagent MRC (Cincinatti, OH, USA) and following the manufacturer's instructions. Equal amounts of RNA from each plate were used in quantitative real-time RT-PCR analyses. First-strand cDNA synthesis and the PCR reactions were carried out using the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Madrid, Spain), according to the manufacturer's instructions. The following primers were used in real-time PCR: for *mac1*—sense, 5′-AGA CCC TGT CCG CTC ACG TA-3′; antisense, 5′-GAG ATC GTC TTG GCA GAT GCT-3′; and for β-*actin* as the invariant control—sense, 5′-CTG ACT GAC TAC CTC ATG AAG ATC CT-3′; antisense, 5′-CTT AAT GTC ACG CAC GAT TTC C-3′. Real time PCR reactions were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The relative amount of *mac1* mRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

Western-Blot—Fifteen or 6 μg of hepatic homogenate or plasma proteins were loaded onto a 10 or 7% SDS–polyacrylamide gels, respectively. Electrophoresis and protein transfer to PVDF membranes (Millipore, Madrid) were carried out, as previously described (*38*). To detect LDLr or SR-BI (scavenger receptor B1) protein expressions, rabbit polyclonal antibodies against mouse LDLr (Santa Cruz Biotechnology, Santa Cruz, CA) or SR-BI (Abcam, Cambridge, UK) receptors were used. Paraoxonase 1 (PON-1) protein bands were detected using a rabbit polyclonal antibody raised against a mouse oligopeptide (*33*), following current protocols of immunization (*39*). Detection was carried out using a secondary antibody anti-rabbit IgG conjugated to alkaline phosphatase and NTB/BCIP as enzyme substrate (Sigma Chemical Co., Madrid, Spain). Bands were scanned and analyzed using Quantity One software (BioRad, Madrid, Spain).

Liver RNA Preparation and Analysis—At the moment of sacrifice, livers were obtained and quickly frozen in liquid nitrogen. RNA was isolated using Trigent reagent MRC (Cincinatti, OH, USA) following the manufacturer's

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instructions. Total RNA was subjected to Northern blot analysis. The mouse clone for paraoxonase-1 (4158951 IMAGE Clone) was obtained from The MGC Geneservice, (Cambridge, UK). *Apoa1* and *Pon1* probes were previously described (*31*). A mouse β-*actin* was used as probe to normalize the amount of RNA loaded on the gel. Probes were labelled using [α-^{32}P]-dCTP and Rediprime. Filters were exposed to Biomax film (Kodak, Amersham) and films analyzed using a laser LKB 2202 densitometer (Amersham-Biosciences).

Evaluation of Atherosclerotic Lesions—The heart was perfused first with phosphate-buffered saline and later with phosphate-buffered formalin (4%, pH 7.4 Panreac, Barcelona, Spain) under physiological pressure. Hearts were dissected out. The aortic base of each heart was taken and transferred to liquid OCT (Bayer Diagnostic, Germany). Four serial criosections were made. Average lesion sizes were used for morphometric evaluations as previously described (*31*, *34*). Images were captured and digitized using a Nikon microscope equipped with a Cannon digital camera. Morphometric analyses were performed using the Scion Image software.

Monocyte Analysis of Surface Molecule Expression—After 10 weeks on diets, blood samples were obtained by retroorbital bleeding from overnight-fasted and isofluorane anesthetized mice. Approximately 1×10^6 white blood cells, resuspended in PBS supplemented with 0.1% (w/v) BSA and 10 mmol/liter sodium azide, were analysed for the expression of Mac-1 (Anti-CD11b from Beckton-Dikinson, Madrid, Spain) by fluorescence-activated cell sorter analysis. A total of 10,000 events accomplishing adequate FSvsSSC values were taken. Data were expressed as the percentage of marker-positive cells recovered in the region corresponding to monocytes.

Urine Hydroxytyrosol Analysis—Hydroxytyrosol and its biological metabolite 3-*O*-methyl-hydroxytyrosol (homovanillyl alcohol) were analysed by gas chromatography/mass spectrometry (GC/MS). Analyses were performed on a HP5980 gas chromatograph (Hewlett-Packard, Palo Alto, CA) coupled to an HP5973 mass spectrometer detector system and a HP7683 (Hewlett-Packard) series injector. Separation of hydroxytyrosol and tyrosol was carried out using a HP Ultra 2 (12.5 m × 0.2-mm i.d. and 0.33-μm film thickness) cross-linked 5% phenylmethyl silicone capillary column (Hewlett-Packard). Instrumental, hydrolytic, and extraction conditions of samples were previously described (*21*).

Statistical Analysis—Mann-Whitney U test was used for comparisons. Differences were considered significant when $P < 0.05$. Association between variables was assessed by Pearson's correlation coefficient using Instat 3.02 for Windows software (GraphPad, S. Diego, CA, USA).

RESULTS

Effect of Hydroxytyrosol on Plasma Parameters and Urine Hydroxytyrosol Derivatives—Table 1 shows concentrations of plasma lipids, PON enzymatic activity, isoprostanes and urine hydroxytyrosol and derivatives. After the 10 weeks of experimental period, higher plasma cholesterol levels were observed in the hydroxytyrosol group compared to the control group despite both groups presented similar cholesterolemia at the beginning of the study.

Table 1. **Effects of hydroxytyrosol on ApoE knockout mice plasma and urine parameters.**

	Chow (n = 13)	Hydroxytyrosol (n = 8)
Initial plasma cholesterol (mmol·liter^{-1})	16 ± 5	16 ± 4
Plasma cholesterol (mmol·liter^{-1})	15.7 ± 2.7	18.4 ± 1.1*
HDL cholesterol (mmol·liter^{-1})	1.1 ± 0.13	1.2 ± 0.12
Plasma triglycerides (mmol·liter^{-1})	1 ± 0.4	1.3 ± 0.1
Apo A-I (mg·liter^{-1})	320 ± 60	240 ± 60*
Apo A-II (AU·liter^{-1})	1.1 ± 0.2	1.1 ± 0.2
Apo A-IV (AU·liter^{-1})	1.0 ± 0.2	1.0 ± 0.2
Apo B (AU·liter^{-1})	0.7 ± 0.03	0.7 ± 0.04
8-Iso-prostaglandin $F_2\alpha$ (pg·ml^{-1})	76 ± 4	173 ± 20*
Arylesterase activity × 10^3 (UI·ml^{-1})	100 ± 13	95 ± 12
Urine hydroxytyrosol (ng·ml^{-1})	867 ± 761	5,293 ± 2,332*
Urine homovanillyl alcohol (ng·ml^{-1})	187 ± 9	411 ± 91*

Results are expressed as mean ± SD. Mice were fed chow or hydroxytyrosol diet for 10 weeks and fasted overnight before blood collection. Statistical analyses were done according to Mann-Whitney U test. *$P < 0.05$ *vs.* control.

Hydroxytyrosol administration did not induce any statistically significant changes in the values of plasma apo B, arylesterase activity of paraoxonase and triglycerides. Despite no significant change in HDL cholesterol levels, serum apolipoprotein A-I levels were significantly decreased by hydroxytyrosol administration while apolipoproteins A-II and A-IV were not altered.

The distribution of cholesterol among the different plasma lipoproteins after the experimental period in control and hydroxytyrosol groups is shown in Fig. 1. Dietary hydroxytyrosol increased VLDL and LDL cholesterol (Fig. 1, a and b) in agreement with the cholesterol increase shown in Table 1, and indicating particles carrying more cholesterol due to the maintenance of apo B and triglyceride levels. In order to investigate for the elevation of LDL cholesterol, LDL receptor protein was estimated in liver by Western blotting. Hydroxytyrosol administration did not induce any significant change in the hepatic protein (1993 ± 205 arbitrary units of absorbance for control *vs.* 1903 ± 147 for hydroxytyrosol-treated animals).

In order to verify hydroxytyrosol absorption, urine hydroxytyrosol and homovanillyl alcohol concentrations were carried out. As shown in Table 1, the excretion of hydroxytyrosol and its major metabolite, homovanillyl alcohol, was higher in animals receiving hydroxytyrosol.

Evaluation of Susceptibility to Oxidation and Oxidative Status—Figure 2 shows the susceptibility to oxidation of VLDL and LDL by two different methods. When both lipoproteins were incubated with $CuSO_4$, a different pattern was observed. Thus, while VLDL (Fig. 2A) from hydroxytyrosol-treated animals were more resistant to oxidation than those of control animals, the opposite was observed for LDL from hydroxytyrosol-treated animals (Fig. 2B). In agreement with the latter finding, LDL from mice receiving hydroxytyrosol showed an increased fluorescence when incubated with dichlorofluorescein what indicated an elevated level of oxygen radicals in these particles (Fig. 2C). The general oxidative status was evaluated by estimating plasma 8-iso-prostaglandin

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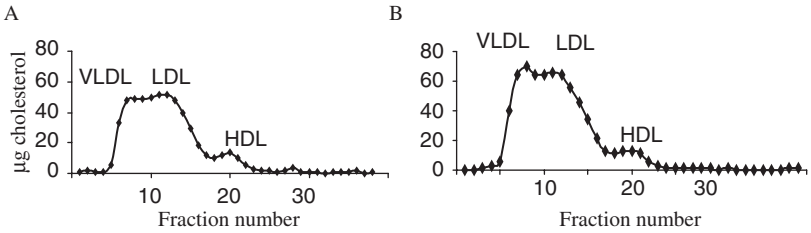

Fig. 1. **FPLC plasma lipoprotein profile from apoE knockout mice following the different experimental conditions.** Representative pooled plasma samples were fractionated by Superose 6B FPLC column chromatography as described in "MATERIALS AND METHODS" and fractions analyzed for cholesterol. Results are shown as μg of cholesterol per isolated fraction. (a) and (b) mice on chow and hydroxytyrosol diet, respectively.

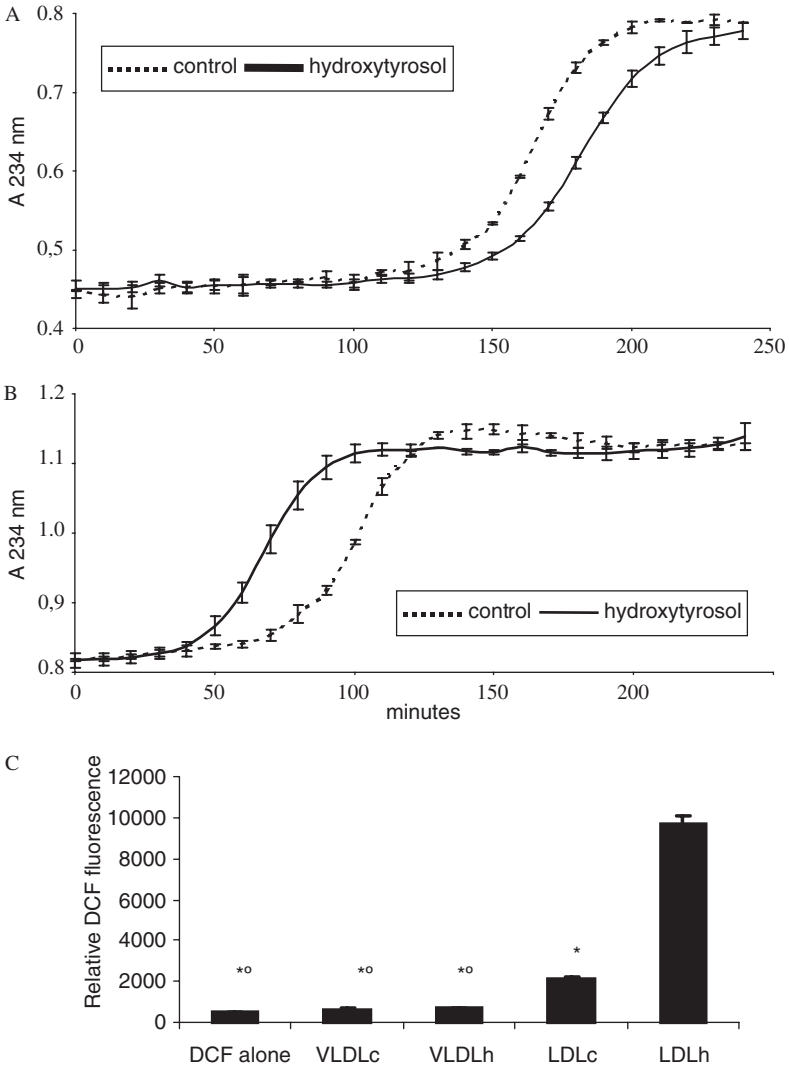

Fig. 2. **Oxidability and oxidative status of lipoproteins following the different experimental conditions.** (A) VLDL from control and hydroxytyrosol groups were incubated with 10 μM $CuSO_4$ at 37°C to induce oxidation. Absorbance at 234 nm was continuously monitored and kinetics is shown. (B) LDL oxidation in similar experiment. (C) The presence of oxygen radicals in lipoproteins was estimated by fluorescence emission of LDL or VLDL (5 μg of cholesterol) from control (c) and hydroxytyrosol (h) incubated with 2 μg of 2′,7′-dichlorofluorescein diacetate in a total volume of 150 μl and in presence of 25 μl of 0.1% sodium azide and 100 μl of PBS. Data are mean ± SEM. * and ° $p < 0.001$ *vs.* LDLc and LDLh respectively according to one-way ANOVA.

$F_2\alpha$ levels, which also appeared increased in hydroxytyrosol-treated mice (Table 1).

In order to assess the effect of hydroxytyrosol on the antioxidative mechanisms, we measured paraoxonase, an antioxidant enzyme presents in HDL. The arylesterase activity of paraoxonase is shown in Table 1. According to our results, hydroxytyrosol administration did not change the activity of the enzyme. Similar results were observed, when the mass of the enzyme was estimated by an immunoassay with a specific anti–mouse PON-1 antibody (1,498 ± 286 arbitrary units of absorbance for control *vs.* 1,355 ± 147 for hydroxytyrosol-treated animals).

Quantification of Lesion Area—Figure 3 shows the atherosclerotic lesion area in mice killed at the end of the study. A foam cell infiltration into the intima was observed in all animals killed at 18 weeks of age (data not shown). The administration of aqueous solution of hydroxytyrosol significantly increased the area of the atherosclerotic lesions.

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Mac-1 Analysis—In order to verify whether variations in the degree of circulating monocytes activation could be implicated in the lesion area development, circulating monocytes expressing Mac-1 were analysed. Mac-1 (CD11b) is an integrin molecule, referred to as a ligand for ICAM-1 and 2, and involved in recruitment of monocytes in atherosclerosis development. The percentage of monocytes expressing Mac1 in both groups is displayed in Fig. 4A and was significantly higher in hydroxytyrosol fed animals. A significant association ($r = 0.658$, $P < 0.01$) between the percentage of monocytes expressing Mac-1 and the aortic atherosclerotic lesion areas was observed (Fig. 4B).

To prove a direct interaction of hydroxytyrosol with macrophages in the induction of Mac-1 expression and whether this increase was mediated by changes in gene expression, macrophages of apoE deficient mice were prepared and incubated in presence of variable concentrations of hydroxytyrosol. As shown in Fig. 4C, this compound induced the expression of *mac-1* in a dose response between 2 and 25 μM.

Hepatic Expression of Paraoxonase 1 and Apolipoprotein A-I—Data in Fig. 5 is expressed as arbitrary units and referred to the level of β-*actin* determined by Northern blot analyses. Hydroxytyrosol administration induced an increase in the hepatic expression of *apoa1* (Fig. 5A). A similar pattern was found for the expression of *pon1* (Fig. 5B), although the increase did not reach significance.

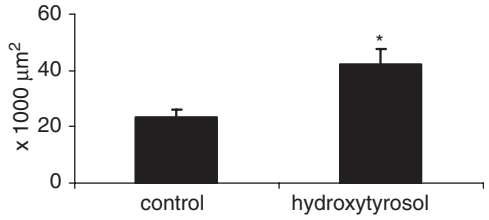

Fig. 3. **Effects of hydroxytyrosol on atherosclerotic lesions in apoE knockout mice.** Cross-sectional analysis of aortic lesions from animals were stained, computerized and analyzed. Data are mean ± SEM for each group. Statistical analyses were done according to Mann-Whitney U test. *$P < 0.05$ *vs.* control.

DISCUSSION

This study was aimed at investigating whether a phenolic compound, with well-known antioxidant properties as

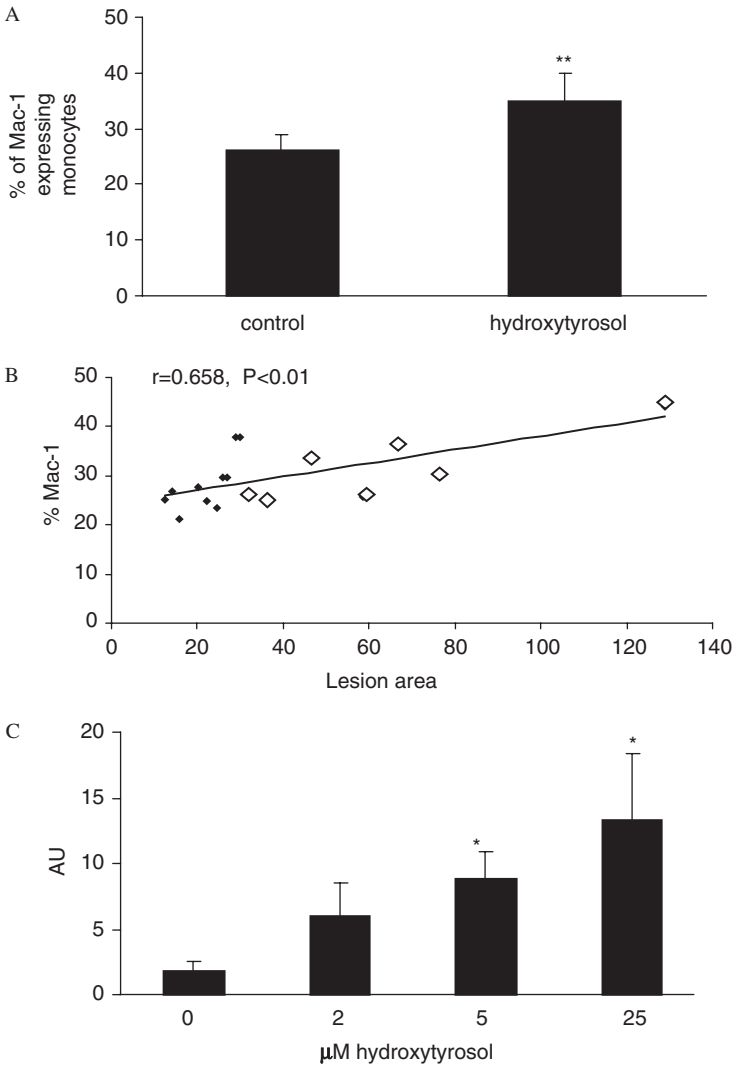

Fig. 4. **Effects of hydroxytyrosol on Mac-1 expression.** (A) Flow cytometry analysis of monocyte Mac-1 (CD11b) positive cells was carried out. Data are mean ± SEM for each group. Statistical analyses were done according to Mann-Whitney U test. **$P < 0.05$ *vs.* control. (B) Relationship between the lesion area and percentage of Mac-1 expressing monocytes. Correlation coefficient was calculated according to Pearson's test. (C) *In vitro* incubation of murine macrophages with hydroxytyrosol at different doses for 24 h. Data of *mac1* mRNA expression are reflected as arbitrary units normalized to the β-actin gene expression. Data are mean ± SEM for each concentration. Statistical analyses were done according to Mann-Whitney U test. *$P < 0.05$ *vs.* control without hydroxytyrosol.

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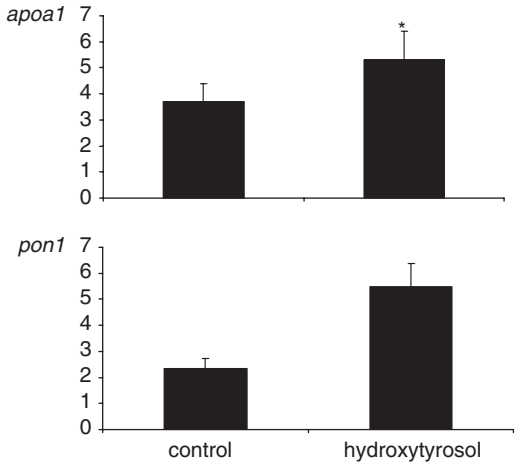

Fig. 5. **Differential effects of hydroxytyrosol on hepatic apoa1 and pon1 mRNA expressions.** Results are expressed as mean ± SEM of arbitrary absorbance units normalized to the β-actin gene expression. 5 μg of total liver RNA from animals in each group were subjected to Northern analysis as described in "MATERIALS AND METHODS." Statistical analyses were done according to Mann-Whitney U test. *$P < 0.05$ *vs.* control.

hydroxytyrosol, isolated from its natural medium—olive oil—could prevent atherosclerosis development and in consequence to be a potential pharmacological agent. From our results, an aqueous solution of hydroxytyrosol increased the atherosclerotic lesion area in apoE knockout mice. This increase was accompanied by an increase in VLDL- and LDL-cholesterol, LDL oxidability, and in the activation of circulating monocytes. In addition, plasma apoA-I was decreased what supposes a failure of antioxidant protective mechanisms and other functions attributed to this apolipoprotein.

The urinary increase in hydroxytyrosol and its biological metabolite, the homovanillyl alcohol, observed in the hydroxytyrosol-treated group (Table 1) indicates that our experimental design of aqueous hydroxytyrosol administration was effective promoting its absorption in mice. Besides, the use of gas chromatography/mass spectrometry confirms that the molecule was intact and rejects a potential destruction by gut flora (*40*). These results are in agreement with previous studies that have demonstrated hydroxytyrosol absorption and urine excretion in animal and humans after olive oil ingestion (*17*, *21*).

Little is known about the effects of hydroxytyrosol on lipid plasma parameters. The present results indicate that in hydroxytyrosol-treated mice plasma total cholesterol increased, a change also reflected in the plasma lipoprotein profile (Fig. 1). Interestingly, the similar plasma apoB concentrations in both groups of animals (Table 1)—in agreement with the lack of change in hepatic levels of LDL receptor—and the absence of changes in the distribution of apoB between VLDL and LDL (data not shown) indicate that apoB containing particles of hydroxytyrosol-treated animals carry more cholesterol. Bigger LDL particles, with difficulties to enter toward the subendothelial space, remain in plasma longer, increasing their plasma half-life. This fact could explain both the higher LDL susceptibility to oxidation (Fig 2B) and the observed increase of oxygen radicals in LDL (Fig 2C) in the hydroxytyrosol-treated animals. Currently, bigger LDL are considered less atherogenic in presence of normal apoA-I levels (*41*). However, our data provides a new paradigm to be considered, since a 24% decrease in apoA-I containing particles could also contribute to the higher oxidability of LDL in hydroxytyrosol-treated mice due to a decrease in the antioxidant properties of apoA-I (*42*) regardless of HDL paraoxonase activity. In addition, other functions attributed to the apoA-I such as a role in reverse transport (*43*) could also be affected and contributing to the observed increase in atherosclerosis. In fact, decreased apoA-I levels augmented the atherosclerosis development of mice having increased LDL levels (*44*).

The increase in hepatic *apoa1* mRNA expression (Fig. 5A) was not reflected in the intracellular protein levels (data not shown) pointing out to posttranscriptional mechanisms, such as the efficiency of translation (*45*), might be regulated by hydroxytyrosol. Once in plasma, the decrease in apolipoprotein A-I (Table 1) was observed in HDL fraction (fraction numbers 18–24 of Fig. 1) and in fraction numbers 28–31 (Fig. 1) corresponding to free apolipoprotein A-I (data not shown). This fact may indicate an impaired hepatic secretion, a secretion of a bigger particle explaining the disappearance of the second small peak or a selective uptake of HDL holoparticles and a blocked removal of cholesterol from HDL apoA-I containing particles. Given that the decrease in plasma apoA-I in hydroxytyrosol-treated animals (Table 1) occurs in the absence of changes in hepatic concentrations of apoA-I and SR-B1 receptor—a cholesterol acceptor, (data not shown)—the first two possibilities are more plausible to explain the present findings. Posttranscriptional or posttranslational mechanisms of paraoxonase expression may also be influenced by hydroxytyrosol administration. The increased hepatic *pon1* expression, in agreement with data from other dietary phenols (*46*), was not paralleled by plasma paraoxonase activity or enzyme protein levels. In fact, a transcriptional coordinate regulation of *apoa1* and *pon1* genes in hepatocytes has been proposed (*47*) and our results may indicate that in vivo both genes also behave similarly regarding hydroxytyrosol administration.

The oxidized LDL may contribute to the increase in the general oxidative status in hydroxytyrosol-treated mice as estimated by the plasma 8-iso-prostaglandin $F_2\alpha$ levels (Table 1)—considered a specific and reliable marker of oxidant stress *in vivo* (*48*)—, although it may not be through direct generation from LDL given its low arachidonic content. Fontana *et al.* (*49*) found that F_2-isoprostanes activate Mac-1 (CD11b) expression through a very restricted signalling pathway. Our *in vivo* results are in agreement with this study; hydroxytyrosol administration significantly increased isoprostane levels as well as Mac-1 on circulating monocyte expression (Fig. 4A). However, *in vitro* incubation of macrophages with hydroxytyrosol at concentrations observed *in vivo* (*18*) also indicates a direct interaction of this compound with macrophages in line with recent experiments (*50*, *51*) and a consequent increase in *mac1* mRNA levels (Fig. 4C). Overall, these mechanisms could contribute to the increased *in vivo* expression of Mac1 on circulating monocytes of treated mice. This activation

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could be a potential explanation for the high correlation (r = 0.65, P < 0.01) observed between atherosclerotic lesion area and monocytes expressing Mac-1. Being this mechanism more relevant in the atherosclerosis development than the cholesterol changes considering the poor association between plasma cholesterol levels and lesion area (r = 0.1, ns).

In contrast to the anti-atherosclerotic effects hypothesized for phenolic compounds, the present study suggests that oral hydroxytyrosol administration to apoE-KO mice promotes atherosclerosis. These results agree with previous studies using other phenolic compounds (resveratrol, caffeic acid and synaptic acid) in rabbits (*52*) and in golden Syrian hamsters (*40*). One explanation for this could be that antioxidant molecules can also act as oxidants. The pro-oxidant effect of catecol-phenolics has been linked to the formation of reactive electrophilic quinone-type metabolites, although a two sequential one-electron oxidation steps of the aromatic π-system has also been proposed (*53*). Furthermore, phenolic antioxidants, such as hydroxytyrosol, *in vivo* do not act in an isolated way but as an intricate antioxidant network together with ascorbate or glutathione. High quantities or long term treatments with a phenolic compound can deplete the reduced glutathione, ascorbic acid, or urate pool, turning an antioxidant effect into pro-oxidant, as has been reported for quercetin (*54*). Thus, supplementing high-risk individuals with an antioxidant (*i.e.* vitamin E) alone promoted rather than reduced lipid peroxidation (*55*) while the combination of vitamin E and vitamin C was effective in reducing atherosclerosis in human trials (*56*). Vitamin C can regenerate the oxidized form of vitamin E, the α-tocopheroxyl radical, to its reduced form, the α-tocopherol (*57*). However, a month intervention with hydroxytyrosol in rabbits evidenced a beneficial anti-atherosclerosis effect (*58*). Three important factors differentiate the latter work from ours: first, the length of treatment, second, the effect was observed when hydroxytyrosol was administered in hypercholesterolemic diets, which may indicate an interaction with dietary cholesterol, which would be absent in the present low-cholesterol diets and third the differences in HDL between both animal models. Thus, rabbits do not produce hepatic apoA-I and lack plasma apoA-II (*59*), whereas our animal model lacks apoE. Discrepancies between models fuel the notion of specific dietary responses modulated by a specific genetic makeup (*28*). Considering that atherosclerosis is a slow progressing complex disease in which the oxidative insult could be exerted by various oxidants, acting over different pathways and/or mechanisms, further experiments are required to clarify the influences of regimen of administration, experimental design, species-specific effects or doses, among other factors, on the experimental outcome.

In conclusion, our data supports the concept that hydroxytyrosol-enriched products, out of the original food matrix, could have detrimental effects on atherosclerosis development, and suggest that the formulation of functional foods may require maintaining the natural environment in which these molecules are found. In addition, potential interactions with other nutrients may exist and specific genetic backgrounds may modify the benefit of hydroxytyrosol administration.

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