Resveratrol Protects against Atherosclerosis, but Does not Add to the Anti-Atherogenic Effect of Atorvastatin, in *APOE*3-Leiden.CETP* Mice

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Abstract

Resveratrol is a major constituent of traditional Asian medicinal herbs and red wine and is suggested to be a potential anti-atherosclerotic drug, due to its proposed hypolipidemic, anti-inflammatory and anti-oxidative properties. The aim of this study was to evaluate whether resveratrol protects against atherosclerosis development in *APOE*3-Leiden.CETP* (*E3L.CETP*) mice, and adds to the anti-atherogenic effect of mild statin treatment, currently the most widely used anti-atherogenic therapy. *E3L.CETP* mice were fed a cholesterol-rich diet without (control) or with resveratrol (0.01% w/w), atorvastatin (0.0027% w/w), or both for 14 weeks. During the study plasma lipid, inflammatory and oxidative stress parameters were determined. Resveratrol reduced atherosclerotic lesion area (-52%) in the aortic root, comparable to atorvastatin (-40%) and the combination of both drugs (-47%). The collagen/macrophage ratio in the atherosclerotic lesion, a marker of plaque stability, was increased by resveratrol (+108%), atorvastatin (+124%) and the combination (+154%). Resveratrol decreased plasma cholesterol levels (-19%) comparable to atorvastatin (-19%) and the combination (-22%), which was completely confined to (V)LDL-cholesterol levels in all groups. Posthoc analyses showed that the anti-atherogenic effect of atorvastatin could be explained by cholesterol lowering, while the anti-atherosclerotic effect of resveratrol could be attributed to factors additional to cholesterol lowering. Markers of inflammation and oxidative stress were not different, but resveratrol improved macrophage function. We conclude that resveratrol potently reduces atherosclerosis development and induces a more stable lesion phenotype in *E3L.CETP* mice. However, under the experimental conditions tested, resveratrol does not add to the anti-atherogenic effect of atorvastatin.

Introduction

Dyslipidemia, characterized by high plasma levels of VLDL and LDL, and low plasma levels of HDL, is a well recognized risk factor for atherosclerosis, the main cause for cardiovascular events. Current treatment of atherogenic dyslipidemia mainly aims at reducing plasma (V)LDL-cholesterol levels, for example by using statins, which effectively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. However, statin use prevents only 15-30% of all cardiovascular events.[1]

Atherosclerosis is considered to be a multifactorial inflammatory disease and optimal therapeutic treatment of atherosclerosis should thus encompass different approaches. In addition to dyslipidemia, oxidative stress, inflammation and macrophage foam cell formation are crucial processes in the development of atherosclerotic plaques.[2] (V)LDL particles are oxidized locally within the vascular wall, but also circulating oxidized (V)LDL can enter the vascular wall, and can trigger an inflammatory reaction by resident macrophages. This inflammatory reaction includes the production of cytokines, chemokines and reactive oxygen species (ROS), and results in upregulation of adhesion receptors on endothelial cells and recruitment of monocytes from the circulation.

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a phytoalexin produced in plants in response to stress, and is present in large amounts in some nuts, *Polygonum cuspidatum* (Japanese knotweed) and in the skin of red grapes. Consequently, resveratrol is a major constituent of traditional Asian medicinal herbs and red wine. Resveratrol has been shown to extend the life span of yeast[3] and mice,[4] to improve insulin sensitivity[4] and to prevent cancer development[5] in experimental models. Importantly, resveratrol has also been recognized to possess various anti-atherosclerotic activities, including hypolipidemic,[6-8], anti-oxidative[9-12] and anti-inflammatory[9,11,13,14] properties.

Despite these promising and diverse anti-atherosclerotic actions, studies addressing the effect of resveratrol on atherosclerosis are scarce. Recent preliminary reports suggest that resveratrol indeed reduces atherosclerosis development.[6,15,16] In the current study we investigated whether resveratrol protects against atherosclerosis development in *APOE*3-Leiden.CETP* (*E3L.CETP*) transgenic mice and whether resveratrol adds to mild atorvastatin treatment because of its proposed diverse anti-atherosclerotic properties. To address this, we used a dose of resveratrol (*i.e.* 11 mg/kg/day) similar to dosages in other experimental models (*i.e.* 2-20 mg/kg/day) in which resveratrol effectively reduced atherosclerosis.[6,15,16] To study a potential modulating effect of resveratrol on top of atorvastatin treatment, we used a mild atorvastatin dose (*i.e.* 2 mg/kg/day) aiming at a reduction in plasma cholesterol of 25%, which is approximately half of the maximal cholesterol-lowering effect of atorvastatin in this mouse model,[17,18] and similar to the reduction of plasma cholesterol in men. By using this strategy a (potentially) relevant additive effect of resveratrol could be better detected. *E3L.CETP* mice represent a unique murine atherosclerosis model for human-like lipoprotein metabolism that shows a similar response to lipid-lowering drugs including statins[19] and HDL-raising drugs[19,20] as humans. In contrast, classical atherosclerosis models have no human-like response to statins with respect to cholesterol lowering (*apoe*$^{-/-}$) or a variable response with respect to cholesterol

lowering and atherosclerosis development (*ldlr*$^{-/-}$).[21] In addition, *ldlr*$^{-/-}$ mice are unable to upregulate the LDLr after statin treatment, an important additional cholesterol-lowering characteristic of statin treatment. Therefore, the *E3L.CETP* model is thus a more suitable model than these classical models to study whether resveratrol is atheroprotective on top of atorvastatin treatment. Our results show that resveratrol markedly reduces atherosclerosis development, but does not add to the anti-atherogenic effect of atorvastatin.

Methods and materials

Animals

E3L mice were crossbred with mice expressing human cholesteryl ester transfer protein (CETP) under control of its natural flanking regions to generate heterozygous *E3L.CETP* mice.[19] Female *E3L.CETP* mice of 10-12 weeks of age were used for all studies. Mice were housed under standard conditions with a 12-h light/dark cycle and had free access to food and water. They were fed regular chow (Ssniff, Soest, Germany) or a Western-type diet (WTD) containing 15% (w/w) cacao butter (diet T, HopeFarms, Woerden, The Netherlands) supplemented with 0.15% (w/w) cholesterol (Sigma-Aldrich, Zwijndrecht, The Netherlands) with or without resveratrol (Sigma) and/or atorvastatin ([R-(R*,R*)]-2-(4-fluorophenyl)-ß,D-dihydroxy-5-(1-methylethyl)-3-phenyl-4[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid ($C_{33}H_{24}FN_2O_5$) (Lipitor®, Pfizer, Capelle a/d IJssel, The Netherlands). Body weight and food intake were monitored during the study. Food intake was monitored twice per week by determining the amount of diet consumed by the mice of each cage (each cage housed 3-5 mice), divided by the number of mice per cage. Unless indicated otherwise, blood was drawn after a 4-h fast in EDTA-containing cups by tail bleeding, and plasma was isolated by centrifugation and stored frozen at -80°C until further analyses. All animal experiments were approved by the Institutional Ethics Committee on Animal Care and Experimentation.

Atherosclerosis study and atherosclerosis quantification

During a run-in period of 5 weeks, all female *E3L.CETP* mice received the WTD (containing 0.15% w/w cholesterol). After matching into 4 groups based on age, body weight, and plasma cholesterol and triglyceride levels, the mice received the WTD either alone (control) or supplemented with resveratrol (0.01% w/w; 11 mg/kg/day), atorvastatin (0.0018% w/w; 2 mg/kg/day) or both, as described above. Since after 4 weeks of drug intervention the dose of atorvastatin did not result in an anticipated 25% reduction in plasma cholesterol levels, the dose of atorvastatin was adjusted to 0.0027% (w/w; 3 mg/kg/day) in the atorvastatin only group and in the combination group.

After 14 weeks of drug intervention, mice were killed by CO_2 inhalation. Blood was drawn via cardiac puncture for serum isolation and hearts were collected. Hearts were fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin, and perpendicular to the axis of the aorta cross-sectioned (5 µm) throughout the aortic root area starting from the appearance of open aortic valve leaflets. Per mouse, 4 sections with 50 µm intervals were used for atherosclerosis

measurements. Sections were stained with hematoxylin-phloxin-saffron for histological analysis. Lesions were categorized for severity according to the guidelines of the American Heart Association adapted for mice.[22] Various types of lesions were discerned; no lesions, mild lesions (types 1-3) and severe lesions (types 4-5). Lesion area was determined using Cell D imaging software (Olympus Soft Imaging Solutions, Münster, Germany). Immunohistochemistry for determination of lesion composition was performed as described previously.[22] Rabbit anti-mouse antibody AIA 31240 (1:3000; Accurate Chemical and Scientific, Westbury, NY) was used to quantify macrophage area and the number of monocytes adhering to the lesions.[23] Monoclonal mouse antibody M0851 (1:800; Dako, Heverlee, The Netherlands) against smooth muscle cell actin was used to quantify smooth muscle cell area. Sirius Red was used to quantify collagen area.

Plasma lipid, lipoprotein, and inflammatory marker analysis

Plasma was assayed for total cholesterol (TC) and triglycerides (TG) using commercially available enzymatic kits according to the manufacturer's protocols (236691 and 1488872, Roche Molecular Biochemicals, Indianapolis, IN). The cholesterol distribution over plasma lipoproteins was determined by fast performance liquid chromatography using pooled plasma (14-15 mice per pool).[24]

Plasma cytokine levels (*i.e.* IL-1ß, IL-6, IL-10, IL-12p70, TNFα, CXCL1) were assessed using a multiplex murine inflammatory cytokine profile immunoassay from Meso Scale Discovery (MSD) on a MSD 2400 plate reader according to the manufacturer's protocol (MSD, Gaithersburg, MD). Plasma levels of soluble E-selectin (sE-selectin; R&D systems, Abingdon, United Kingdom) and monocyte chemoattractant protein-1 (MCP-1; R&D systems) were determined according the manufacturers' instructions. Plasma levels of MCP-1 were determined in pooled plasma (7 pools per group with 2 mice per pool).

Hepatic and macrophage gene expression analysis

Total RNA from livers or from peritoneal macrophages was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. RNA quality of each sample was checked with the lab-on-a-chip technology using Experion Std Sens analysis kit (Biorad, Hercules, CA). One microgram of total RNA was converted to cDNA with iScript cDNA Synthesis kit (Biorad) and purified with Nucleospin Extract II kit (Macherey-Nagel). Real-Time PCR (RT-PCR) was conducted on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of hypoxanthine phosphoribosyltransferase (*hprt*) and cyclophilin (*cyclo*). Primer sequences are listed in Supplemental Table S1.

Analyses of oxidative stress markers

Serum levels of IgG1, IgG2a and IgM against oxidized LDL (oxLDL) were determined using the Mouse MonoAb ID kit (HRP) (Zymed Laboratories Inc, San Francisco, CA). Oxidized human LDL (5 µg/ml) dissolved in a $NaHCO_3$ buffer (pH 9.0) was coated overnight onto a flat-bottom 96-well high binding plate (Corning, NY). Subsequently, levels of IgG1, IgG2a and IgM in serum (dilution 1:3) were detected according to the manufacturer's instructions.

Urine from spontaneous urination upon handling of mice was collected in Eppendorf tubes and stored at -20°C until further analyses. The urinary excretion of 15(*S*)-8-*iso*-prostaglandin $F_2\alpha$ (8-*iso*-$PGF_2\alpha$) was determined in pooled urine samples (7 pools per group with 2 mice per pool) by GC-MS/MS after immunoaffinity column chromatography from Cayman Chemicals (Ann Arbor, MI) as described previously.[25] Values were corrected for urinary creatinine excretion, which was measured by GC-MS as described.[25]

Analyses of peritoneal macrophage function *in vitro*

Thioglycollate-elicited peritoneal macrophages from *E3L.CETP* mice were isolated and cholesterol efflux assay was performed as described previously.[26] Macrophages were loaded with [^{3}H]cholesterol-labeled acetylated LDL (AcLDL; 50 μg/ml) for 32 h and subsequently incubated overnight without or with the indicated concentrations of resveratrol. The next day these cholesterol-laden macrophages were incubated with lipid-free apoAI (10 μg protein/ml) or HDL (50 μg/ml) for 6 h and radioactivity was determined in the medium and cell lysates to determine the percentage of cholesterol efflux.

To study the effect of resveratrol on the uptake of AcLDL isolated peritoneal macrophages were incubated overnight without or with the indicated concentrations of resveratrol. The next day, the macrophages were incubated with [^{3}H]cholesteryl oleoyl ether-labeled AcLDL (10 μg protein/ml) for 6 h and radioactivity was measured in the cell lysates to determined the percentage of AcLDL uptake.

To determine the lipopolysaccharide (LPS)-induced TNFα response[27] peritoneal macrophages were incubated overnight with resveratrol (10 μM) and, subsequently, incubated for 4 h with the indicated concentrations of LPS (*Salmonella minnesota* Re595 LPS; Sigma). TNFα was determined in the medium by ELISA (OptEIA™ ELISA, BD Biosciences Pharmingen). For the macrophage assays, values were corrected for the cellular protein content.

Statistical analysis

Data are presented as mean ±SEM unless indicated otherwise. Differences were assessed by one-way ANOVA test followed by the Bonferroni posthoc test. Differences in plasma cholesterol levels were tested using the one-way ANOVA test followed by Dunnett's posthoc test. Two-way ANCOVA was performed to test for differences on atherosclerotic lesion area after controlling for the cholesterol-lowering capacity of the different treatments. The square root was taken of the atherosclerotic lesion area to linearize the relationship with plasma cholesterol exposure. A probability level (*P*) of 0.05 was considered significant. The Student's T-test was used to evaluate differences in the *in vitro* macrophage studies. SPSS 17.0 for Windows (SPSS, Chicago, IL) was used for statistical analysis.

Results

Resveratrol attenuates atherosclerosis development to a similar extent as atorvastatin

To study the effect of resveratrol on atherosclerosis development, *E3L.CETP* mice were fed a Western-type diet (WTD) without or with resveratrol, atorvastatin, or the combination of both

drugs. Neither of the treatments affected food intake and body weight during the study (not shown). Mice were sacrificed after 14 weeks of treatment and lesion size and lesion severity were determined in the valve area of the aortic root. Treatment with resveratrol reduced total atherosclerotic lesion area by -52% (P<0.01), whereas atorvastatin reduced this by -40% (P<0.05) as compared to control treated mice (Figure 1A). The combination of resveratrol and atorvastatin did not further reduce atherosclerotic lesion area (-47% as compared to the control group; P<0.01) as compared to single treatments. This reduction in lesion area was similar over all 4 sections of the sampled valve area of the aortic root (Figure 1B). Neither of the treatments significantly affected lesion severity as classified as mild (type 1-3) and severe (type 4-5) lesions (Figure 1C).

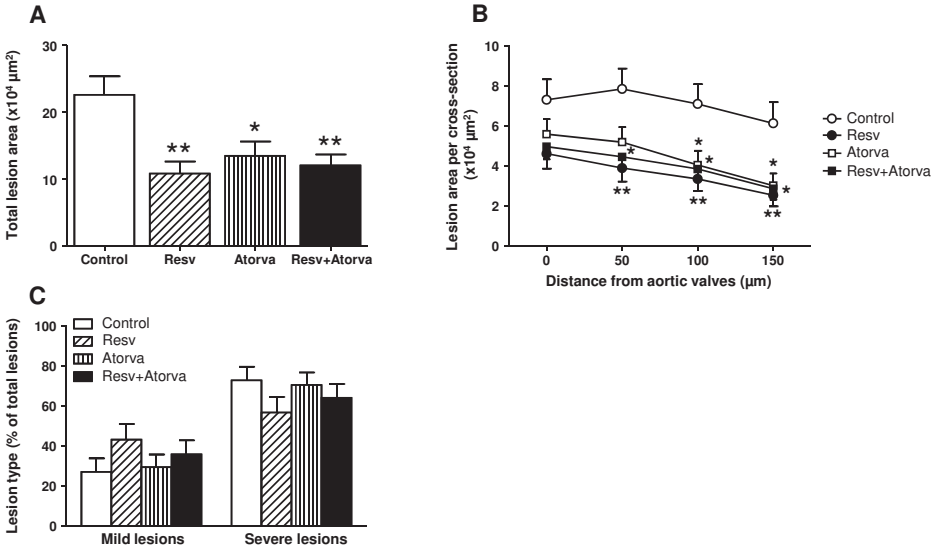

Fig. 1. Resveratrol, atorvastatin and the combination comparably reduce atherosclerosis development. Slides of the valve area of the aortic root were stained with hematoxylin-phloxin-saffron and total lesion area was calculated of 4 sections per mouse starting from the appearance of open aortic valve leaflets as described in *Methods and Materials* (A). Lesion area as a function of distance was determined by calculating the lesion area of each cross-section from A (B). The same 4 sections per mouse were categorized according to lesion severity (C). Values are means ±SEM (n=14-15). *P<0.05, **P<0.01 *vs* control group.

Resveratrol and atorvastatin induce stable lesions

Subsequently, we assessed whether the different treatments affected monocyte recruitment and lesion composition. With respect to lesion composition we characterized macrophage content, a destabilizing component in the lesions, and collagen and smooth muscle cell content, both stabilizing components in the lesions.[2] As shown in Figure 2, neither of the treatments affected the number of adhering monocytes to the endothelium. However, resveratrol tended to decrease macrophage content, whereas atorvastatin significantly decreased it (Figure 2B). Resveratrol enhanced smooth muscle cell and collagen content to a similar extent as

atorvastatin (Figure 2C+D). Both resveratrol and atorvastatin thus induced more stable lesions as indicated by an enhanced collagen/macrophage ratio (2.2-fold and 2.4-fold, respectively) (Figure 2E). The combination treatment did not have a significant additional effect on lesion composition as compared to the single treatments.

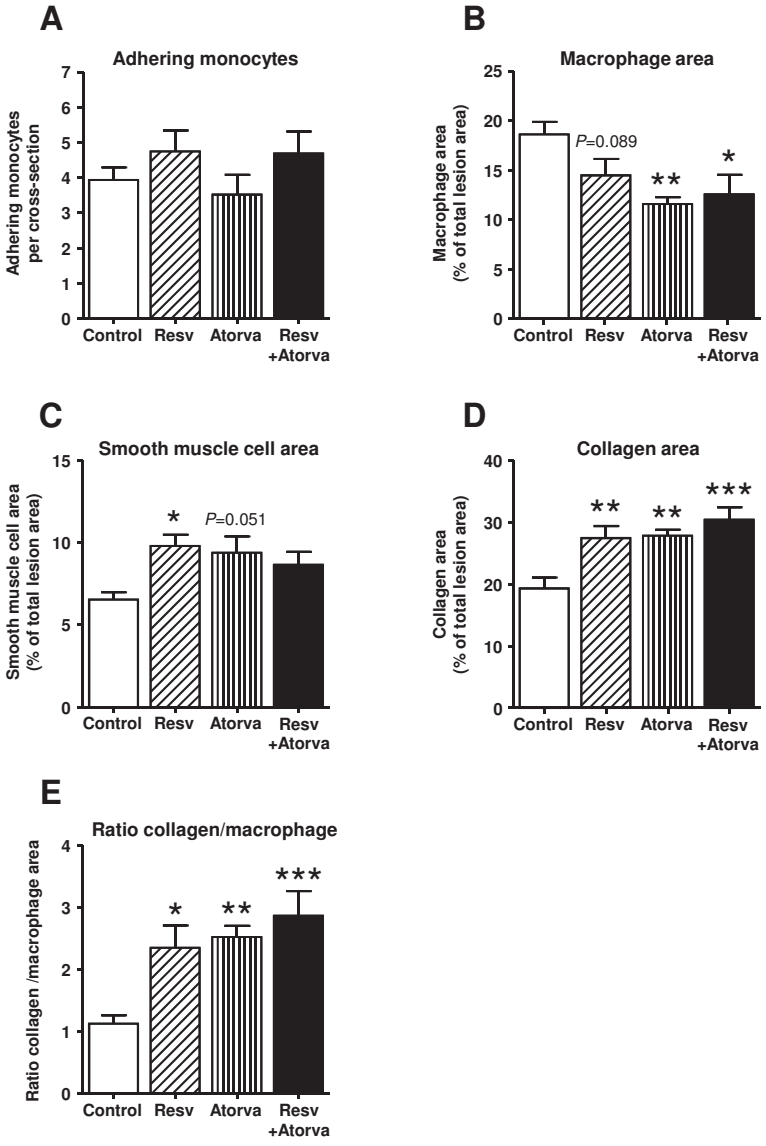

Fig. 2. Resveratrol, atorvastatin and the combination induce more stable lesions. The adhesion of monocytes to the lesions in the aortic root was determined (A) as were the macrophage content (B), collagen content (C), smooth muscle cell (SMC) content (D) and collagen/macrophage ratio (E) of the lesions. Values are means ±SEM (n=14-15). **P*<0.05, ***P*<0.01, ****P*<0.001 *vs* control group.

Resveratrol and atorvastatin reduce (V)LDL levels to a similar extent

To study whether the anti-atherogenic effect of the different treatments could be the result of hypolipidemic, anti-inflammatory and/or anti-oxidant properties of resveratrol and atorvastatin, we first determined the effect of the different treatments on plasma lipid levels. Plasma cholesterol levels were similarly reduced by resveratrol (-19%; *P*=0.077) and atorvastatin (-22%; *P*<0.05) as compared to the control group (Figure 3A). The combination treatment did not further reduce plasma cholesterol levels (-21% as compared to control-treated mice; *P*<0.05). Lipoprotein fractionation showed that for all treatments the reduction in plasma cholesterol levels was confined to the (V)LDL fraction, while HDL-cholesterol levels were not affected (Figure 3B).

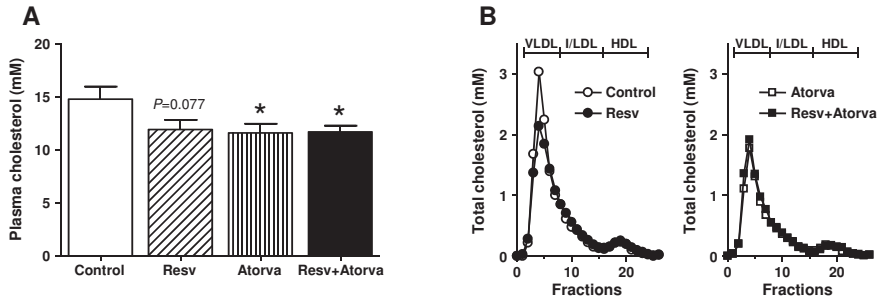

Fig. 3. Resveratrol, atorvastatin and the combination similarly lower plasma (V)LDL-cholesterol levels. Plasma cholesterol was determined after 8 weeks of treatment (A). The cholesterol distribution over lipoproteins was determined in pooled plasma (B). Values are means ±SEM (n=14-15). **P*<0.05 *vs* control group.

To investigate whether this reduction in (V)LDL could be caused by reduced hepatic production of VLDL and/or increased clearance of (V)LDL from plasma, we subsequently determined the expression of hepatic genes involved in cholesterol and TG metabolism (Supplemental Table S2). Resveratrol treatment did not affect hepatic mRNA expression of genes involved in cholesterol metabolism (*i.e. ldlr*, *sr-b1*, *abca1* and *abcg1*) and VLDL metabolism (*i.e. apob, mttp*, *fasn* and *lpl*). Treatment with atorvastatin and/or the combination altered the expression of genes involved in cholesterol metabolism (*i.e.* increased: *ldlr*; decreased: *abca1, abcg1* and *sr-b1*), but not those involved in VLDL metabolism, with the exception of a decreased expression of *lpl*.

Unlike for atorvastatin, the anti-atherogenic effect of resveratrol may not be solely explained by its plasma cholesterol-lowering capacity

Since our collective data suggest that the anti-atherogenic effect of resveratrol and atorvastatin may mainly depend on their ability to reduce plasma cholesterol, we performed 2-way ANCOVA analyses in which we controlled for the cholesterol-lowering effect of the treatments by using the cumulative plasma cholesterol exposure during the study (*i.e.* area under the

curve). The effect of resveratrol on atherosclerotic lesion area reduction remained significant (F = 5.34; P<0.05), whereas for atorvastatin treatment significance was lost (F = 0.88; P=0.36). This indicates that the reduction in atherosclerosis development upon atorvastatin treatment can be explained by its cholesterol lowering effect, while additional mechanism(s) could be involved in the anti-atherogenic effect of resveratrol.

Resveratrol and atorvastatin do not affect inflammatory parameters

To elucidate by which additional mechanism(s) resveratrol may be atheroprotective, we assessed the effect of the different treatments on inflammatory parameters in liver and plasma. Neither of the treatments affected hepatic mRNA levels of *il-1ß* and *il-6* (Supplemental Table S2) or a panel of plasma cytokine levels (*i.e.* IL-1ß, IL-6, IL-10, IL-12p70, TNFα and CXCL1, the murine homologue of IL-8), with the exception that the combination treatment reduced CXCL1 levels by -33% (P<0.05; not shown). Similarly, resveratrol and atorvastatin did not affect plasma levels of the acute-phase proteins soluble E-selectin (sE-selectin) and MCP-1, but the combination reduced plasma MCP-1 by -30% (P<0.05; not shown). These findings indicate that both resveratrol and atorvastatin do not substantially affect the hepatic and systemic inflammatory status of *E3L.CETP* mice.

Resveratrol and atorvastatin do not affect oxidative stress parameters

We next investigated the effect of resveratrol, atorvastatin and their combination on markers of oxidative stress. Resveratrol did not affect hepatic mRNA expression of enzymes involved in oxidative stress (*i.e. mnsod, pon1, cox1, cox2, lox1*) (Supplemental Table S2). Treatment with atorvastatin and/or the combination decreased the expression of *cox2*, *lox1* and *mnsod*, but did not affect expression of *pon1* and *cox1*. Furthermore, circulating levels of IgG1, IgG2a and IgM directed against oxLDL were not different between the four groups (not shown). Likewise, neither of the treatments affected urinary excretion of the isoprostane 15(*S*)-8-*iso*-prostaglandin $F_2\alpha$ (8-*iso*-$PGF_2\alpha$) (not shown), a biomarker of lipid peroxidation.[28]

Resveratrol beneficially influences macrophage function

Since macrophages are the most prominent inflammatory cells in the atherosclerotic lesion and involved in both the initiation, progression and regression of the atherosclerotic lesion,[2] we assessed the effect of resveratrol on macrophage function, *i.e.* cholesterol efflux and release of proinflammatory mediators. Resveratrol enhanced the cholesterol efflux from primary macrophages from *E3L.CETP* mice to apoAI in a dose-dependent manner up to +243% (P<0.05), but not to HDL (Figure 4). Moreover, resveratrol reduced the uptake of AcLDL up to -24% (P<0.05) as compared to vehicle-treated primary macrophages (Figure 5), without affecting the expression of genes involved in cholesterol efflux and/or uptake (*i.e. abca1, abcg1, sr-b1, sr-a1, cd36, lpl*) (not shown). In addition, resveratrol reduced the lipopolysaccharide (LPS)-induced TNFα secretion up to -63% (P<0.05) as compared to untreated primary macrophages (Figure 6). These data show that resveratrol beneficially influences macrophage function, indicating that resveratrol may have local anti-atherosclerotic effects in the vessel wall, which may explain its cholesterol-independent effect on atherosclerosis.

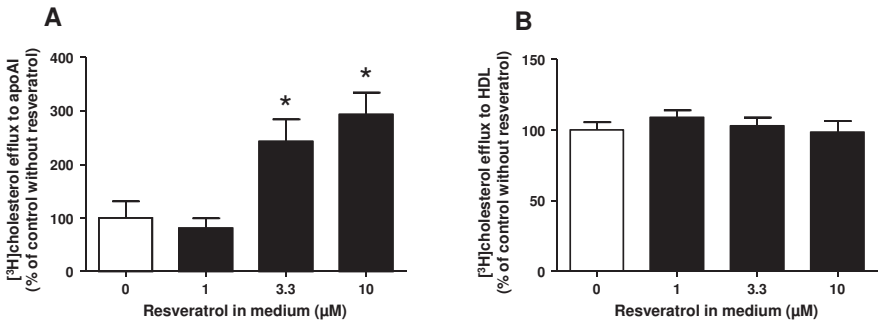

Fig. 4. Resveratrol enhances the cholesterol efflux from macrophages. [^{3}H]cholesterol-laden peritoneal macrophages from *E3L.CETP* mice were equilibrated overnight without or with the indicated concentrations resveratrol. The cholesterol efflux was determined after incubation without or with lipid-free apoAI (10 μg/ml) (A) and HDL (50 μg protein/ml) (B) as percentage of ^{3}H-activity released into the medium per μg cell protein. The data of 2 separate experiments were combined and expressed as percentage relative to the control without addition of resveratrol (white bar) ±SEM (n=8). *P<0.05 *vs* control.

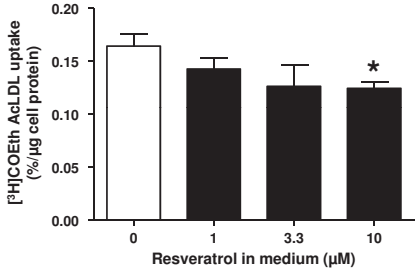

Fig. 5. Resveratrol reduces the uptake of AcLDL by macrophages. Peritoneal macrophages from *E3L.CETP* mice were equilibrated overnight without or with the indicated concentrations of resveratrol. The uptake of [^{3}H]cholesteryl oleoyl ether-labeled AcLDL ([^{3}H]COEth AcLDL; 10 μg protein/ml) was determined after 6 h. Values are expressed as the mean percentage of ^{3}H-activity per μg cell protein ±SEM (n=4). *P<0.05 *vs* control.

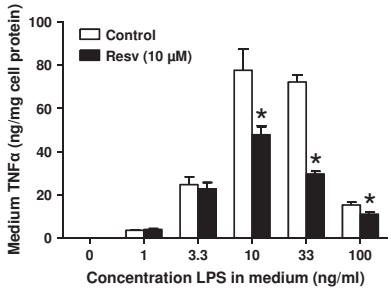

Fig. 6. Resveratrol reduces the LPS-induced inflammatory response of macrophages. Peritoneal macrophages from *E3L.CETP* mice were equilibrated overnight without or with resveratrol (10 μM). Subsequently, cells were incubated for 4 h with the indicated concentrations LPS, and TNFα secretion into the medium was determined. Values are expressed as means per mg cell protein ±SEM (n=4). *P<0.05 *vs* without resveratrol.

Discussion

In the present study we assessed the anti-atherosclerotic potential of resveratrol, either alone or in combination with atorvastatin. We showed that resveratrol reduces atherosclerosis development and improves plaque stability to a similar extent as atorvastatin in *E3L.CETP* mice. Whereas the anti-atherogenic effect of atorvastatin could be explained by its cholesterol-lowering effect, additional mechanisms may be involved in the anti-atherogenic effect of resveratrol, which may involve beneficial modulation of macrophage function. However, the combination of resveratrol and atorvastatin did not additively reduce atherosclerosis.

Our finding that resveratrol efficiently reduces atherosclerotic lesion development in *E3L.CETP* mice is in accordance with preliminary reports in literature. The first report suggested only qualitatively, that resveratrol reduced atherosclerosis development in the aortas of apoE-deficient mice.[6] The second report used a very limited number of mice (*i.e.* n=4-7), but nevertheless showed a significant reduction in atherosclerosis development in apoE/LDLr-double deficient mice.[15] The most recent report observed that resveratrol reduced the intima/media area ratio in New Zealand rabbits fed a 1% cholesterol diet.[16] The fact that resveratrol reduces atherosclerosis supports the hypothesis that resveratrol contributes to the "French Paradox", *i.e.* the notion that moderate and prolonged consumption of red wine is associated with decreased cardiovascular morbidity and mortality in the French population despite high dietary saturated fat consumption.[29] Resveratrol is a major constituent of the skin of red grapes and, therefore, abundantly present in red wine with concentrations ranging from 1-12 mg/L (*i.e.* 0.2-2 mg/glass).[30]

To assess the potential anti-atherosclerotic actions of resveratrol responsible for the protection against atherosclerosis, plasma lipid levels as well as inflammatory and oxidative stress parameters were studied. We found no relevant effect of resveratrol treatment on hepatic and systemic inflammation as indicated by hepatic mRNA expression of cytokines and circulating cytokine and acute-phase protein levels, respectively. Similarly, there was no effect of resveratrol on oxidative stress, as evidenced by unaltered circulating antibodies against oxLDL as well as unaltered urinary excretion of the isoprostane 8-*iso*-$PGF_2\alpha$. The latter is a biomarker of lipid peroxidation[28] and as such has been associated with coronary heart disease.[31] The fact that in our studies resveratrol treatment did not affect systemic inflammatory and oxidative stress parameters may be the result of the mild treatment strategy as compared to most other studies in which a reducing effect of resveratrol on these parameters was observed. We treated the mice chronically with 0.01% (w/w) resveratrol in the diet, comparable to 11 mg/kg/day, whereas most other acute or chronic experimental studies used at least 2.5- to 10-fold higher dosages.[6,9,10,13] We chose for this mild treatment strategy since comparable dosages (2-20 mg/kg/day) effectively reduced atherosclerosis development in the above mentioned preliminary reports.[6,15,16] Furthermore, this dose is similar to the recommended dosage of resveratrol supplementation in humans (ranging from 1-20 mg/kg/day; *e.g.* www.resveratrol.info; www.resveratrolreference.com). The anti-inflammatory and anti-oxidative effect of resveratrol *in vivo* may in particular be apparent after challenging the mice with inflammatory stimuli, since various reports describing the anti-inflammatory effect of resveratrol *in vivo* used such a

experimental set-up, *e.g.* by lipopolysaccharide (LPS) injection[13] or by inducing experimental colitis.[11]

Resveratrol treatment reduced plasma (V)LDL-cholesterol levels similarly as atorvastatin treatment. This hypolipidemic effect of resveratrol is in accordance with previous reports,[6-8] and can result from several mechanisms. First, resveratrol could reduce hepatic VLDL secretion, since resveratrol reduced apoB secretion in human HepG2 cells.[32] However, we did not observe differences in expression of several hepatic genes involved in VLDL biogenesis (*i.e. fasn*, *mttp*, *apob*), suggesting that resveratrol did not lower plasma lipids by reducing hepatic VLDL production in the present study. Nevertheless, one has to keep in mind that this does not exclude a potential effect of resveratrol on posttranslational modification of the proteins involved in VLDL biogenesis. Second, resveratrol treatment could enhance VLDL catabolism and/or uptake. However, the lack of an effect of resveratrol treatment on key hepatic genes involved in VLDL clearance (*i.e. ldlr*, *lpl*), the lack of a reduction in plasma TG levels (not shown) and the lack of studies in literature, so far do not provide further support for this mechanism. Third, resveratrol may inhibit HMG-CoA reductase, possibly via enhancing cytochrome P450 27-hydroxylase (CYP27A1) activity which syntheses the HMG-CoA reductase-inhibitor 27-hydroxycholesterol.[33] Resveratrol was shown to attenuate the expression of HMG-CoA reductase in hamsters[34] and reduce HMG-CoA reductase activity in mice.[6] Although this could contribute to the hypocholesterolemic effect in the present study, it is unlikely to be the prevailing mechanism. Resveratrol treatment did not alter the hepatic cholesterol content (*i.e.* free cholesterol and cholesteryl esters; Supplemental Figure S1), while treatment with the HMG-CoA reductase inhibitor atorvastatin did result in the expected reduction of hepatic cholesterol. Finally, we speculate that resveratrol treatment may reduce intestinal cholesterol absorption, but this has not been studied so far. Therefore, further studies are necessary to reveal the prevailing mechanism by which resveratrol lowers plasma cholesterol levels. Resveratrol was also suggested to increase plasma HDL-cholesterol levels in apoE-deficient mice.[6] However, it had no HDL-raising effect in our *E3L.CETP* model, which is otherwise highly responsive to HDL-cholesterol increasing drugs.[19,20]

The above considerations show that resveratrol mainly reduces atherosclerosis development as a result of lowering plasma (V)LDL-cholesterol levels. However, controlling for this cholesterol-lowering effect of resveratrol in the statistical analyses showed that additional anti-atherogenic mechanism(s) could be involved. If so, it is likely that resveratrol exerts these additional mechanism(s) locally in the vessel wall by beneficially altering macrophage function. We found that resveratrol improved at least three of the most important characteristics of macrophages related to atherosclerosis, *i.e.* potency to efflux cholesterol, formation of foam cells and the release of proinflammatory mediators. These anti-atherogenic actions of resveratrol in primary macrophages of *E3L.CETP* mice together indicate a potential local effect of resveratrol at the level of macrophages in the vessel wall in addition to its hypolipidemic effect. Our finding that resveratrol enhances the cholesterol efflux from primary macrophages is in line with previous findings by others.[33,35,36] They reported that resveratrol enhanced cholesterol efflux from macrophages via upregulation of the gene and protein expression of ABCA1 and

ABCG1. Upregulation of ABCG1 was not consistent in different cell types, which could explain the lack of an effect of resveratrol on cholesterol efflux towards HDL in our studies. Moreover, Voloshyna *et al*.[33] found that these effects were PPARγ- and adenosine 2A receptor-dependent. However, we were unable to observe an increase in ABCA1 expression. Thus, it is likely that resveratrol upregulated ABCA1 protein posttranscriptionally. Alternatively, resveratrol could have increased CYP27A1 activity,[33] which stimulates the efflux of cholesterol to apoAI by a yet undefined ABCA1-independent pathway.[37]

Resveratrol effectively protected against atherosclerosis development. Despite the suggested differences in underlying mechanisms, treatment with resveratrol did not add to atorvastatin treatment with respect to reducing atherosclerosis in our model. Since resveratrol may act on atherosclerosis by mechanism(s) in addition to cholesterol-lowering, these mechanistic pathways may become more prominent at higher dosage.

In summary, we have demonstrated that resveratrol potently reduces atherosclerosis development and results in more stable lesions in *E3L.CETP* mice, comparable to atorvastatin treatment. This anti-atherogenic effect of resveratrol is mainly explained by its plasma (V)LDL-cholesterol-lowering capacity, but may also include local anti-atherogenic effects in the vessel wall such as on macrophage function. Nonetheless, in the current experimental set-up resveratrol did not add to the effects of atorvastatin with respect to protection against atherosclerosis development and underlying atherosclerotic risk factors.

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Supplemental data

Supplemental Table S1. Primers used for quantitative RT-PCR analysis.

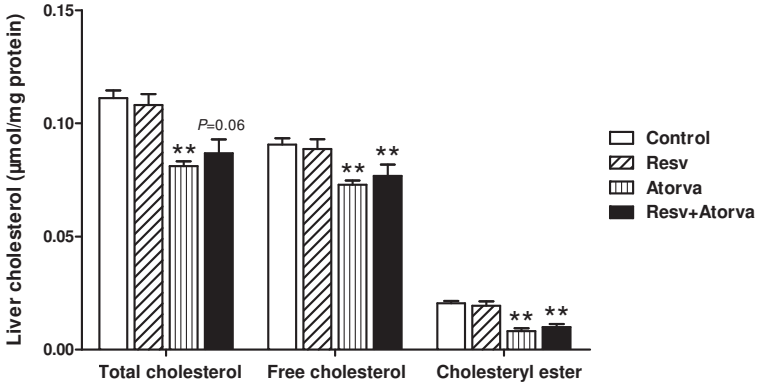
Gene	Forward primer	Reverse primer
Abca1	CCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
Abcg1	GGTCATCATCACTTTGGTCCTTG	TCTCTCGAAGTGAATGAAATTTATCG
Apob	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
Cd36	GCAAAGAACAGCAGCAAATC	CAGTGAAGGCTCAAAGATGG
Cox1	TGGGGTGCCCTCACCAGTCAA	TGGGGCCTGAGTAGCCCGTG
Cox2	GGCCATGGAGTGGACTTAAA	ACTGCAGGTTCTCAGGGATG
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Fasn	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTCACTGCAGCC
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Il-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il-6	CCGGAGAGGAGACTTCACAG	TTCTGCAAGTGCATCATCGT
Ldlr	GCATCAGCTTGGACAAGGTGT	GGGAACAGCCACCATTGTTG
Lox1	CGGCAGACCTGCCAATCTTTGG	GGCCAGGCTTCTTCCGATGCA
Lpl	TTTTCTGGGACTGAGGATGG	GTCAGGCCAGCTGAAGTAGG
Mnsod	TCGCTGTGTCCTTGCGGACG	GCCTCCTGCCCGTGCTGC
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Pon1	TCCTCCCGGCTCAGAGGTGC	GTGGTGCCTTGCAGGACGGT
Sr-a1	CAGTCCAGGAACATGGGAAT	ACGTGCGCTTGTTCTTCTTT
Sr-b1	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA

Abca1/abcg1, ATP-binding cassette transporter a1/g1; *Apob*, apolipoprotein B; *Cox1 / 2*, cyclooxygenase-1 / -2; *Cyclo*, cyclophilin; *Fasn*, fatty acid synthase; *Hprt*, hypoxanthine phosphoribosyl-transferase; *Il-1β / -6*, interleukin-1β / -6; *Ldlr*, low-density lipoprotein receptor; *Lox-1*, lectin-like oxidized low-density lipoprotein receptor-1; *Lpl*, lipoprotein lipase; *Mnsod*, manganese superoxide dismutase; *Mttp*, microsomal triglyceride transfer protein; *Pon1*, paraoxonase-1; *Sr-a1 / -b1*, scavenger receptor-A1 / -B1.

Supplemental Table S2. Effect of resveratrol and atorvastatin on hepatic gene expression.

Gene	Control	Resv	Atorva	Resv + Atorva
Lipid metabolism				
Abca1	1.00 ± 0.21	0.93 ± 0.19	0.69 ± 0.15*	0.76 ± 0.19
Abcg1	1.00 ± 0.22	0.81 ± 0.24	0.54 ± 0.19**	0.62 ± 0.05**
Apob	1.00 ± 0.28	0.94 ± 0.35	0.92 ± 0.29	1.00 ± 0.23
Fasn	1.00 ± 0.24	1.73 ± 1.14	1.59 ± 0.49	1.49 ± 0.91
Ldlr	1.00 ± 0.24	0.87 ± 0.37	1.36 ± 0.49	1.74 ± 0.55*
Lpl	1.00 ± 0.35	0.83 ± 0.16	0.54 ± 0.09**	0.55 ± 0.24**
Mttp	1.00 ± 0.18	0.94 ± 0.29	1.10 ± 0.26	1.05 ± 0.16
Sr-b1	1.00 ± 0.25	0.92 ± 0.29	0.62 ± 0.18*	0.73 ± 0.14
Inflammation				
IL-1β	1.00 ± 0.37	0.61 ± 0.24	0.56 ± 0.24	0.76 ± 0.51
IL-6	1.00 ± 0.34	0.81 ± 0.35	1.04 ± 0.55	1.36 ± 0.47
Oxidative stress				
Cox1	1.00 ± 0.32	1.01 ± 0.35	0.78 ± 0.21	0.80 ± 0.40
Cox2	1.00 ± 0.95	0.57 ± 0.45	0.37 ± 0.24	0.38 ± 0.16*
Lox1	1.00 ± 0.72	0.56 ± 0.30	0.42 ± 0.17**	0.36 ± 0.10**
Mnsod	1.00 ± 0.22	0.92 ± 0.26	0.77 ± 0.22	0.67 ± 0.24*
Pon1	1.00 ± 0.15	0.94 ± 0.10	1.21 ± 0.32	1.08 ± 0.32

See the legend of Supplemental Table S1 for explanation of abbreviations. Statistical differences were assessed with one-way ANOVA followed by Bonferroni posthoc test. *P<0.05, **P<0.01 *vs* control group.



Supplemental Fig. S1. Resveratrol does not reduce the hepatic cholesterol content. The hepatic total cholesterol, free cholesterol and cholesteryl ester content were determined according to Wong *et al*. [Wong MC 2012 Atherosclerosis] in livers of *E3L.CETP* mice that were treated with either no drug (control), resveratrol (Resv), atorvastatin (Atorva) or the combination (Resv+Atorva) for 14 weeks. Values are means ± SEM (n=14-15). Statistical differences were assessed with one-way ANOVA followed by Bonferroni posthoc test. **P<0.01 *vs* control group.