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Resveratrol protects against atherosclerosis, but does not add to the antiatherogenic 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 antiatherosclerotic drug due to its proposed hypolipidemic, anti-inflammatory and antioxidative 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 antiatherogenic effect of mild statin treatment, currently the most widely used antiatherogenic 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 (very)low-density lipoprotein cholesterol levels in all groups. Post hoc analyses showed that the antiatherogenic effect of atorvastatin could be explained by cholesterol lowering, while the antiatherosclerotic 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 antiatherogenic effect of atorvastatin.

Keywords: Atherosclerosis; Lipids; Oxidative stress; Inflammation; Resveratrol; Statin

Abbreviations: CETP, cholesteryl ester transfer protein *cox1/-2*, cyclooxygenase-1/-2; CXCL1, chemokine (C-X-C motif) ligand-1; *E3L*, *APOE*3-Leiden* transgenic; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; Ig, immunoglobulin; IL, interleukin; 8-*iso*-$PGF_{2\alpha}$, isoprostane 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$; *lox-1*, lectin-like oxidized low-density lipoprotein receptor-1; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; *mnsod*, manganese superoxide dismutase; oxLDL, oxidized LDL; *pon1*, paraoxonase-1; RT-PCR, real-time polymerase chain reaction; sE-selectin, soluble E-selectin; SMC, smooth muscle cell; TG, triglycerides; WTD, Western-type diet.

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1. Introduction

Dyslipidemia, characterized by high plasma levels of very low density lipoprotein (VLDL) and low-density lipoprotein (LDL) and low plasma levels of high-density lipoprotein (HDL), is a well-recognized risk factor for atherosclerosis, the main cause of 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 and results in up-regulation 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, in *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 lifespan 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 antiatherosclerotic activities, including hypolipidemic [6–8], antioxidative [9–12] and anti-inflammatory [9,11,13,14] properties.

Despite these promising and diverse antiatherosclerotic 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 antiatherosclerotic 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*$^{-/-}$) (reviewed in Ref. [21]). In addition, *ldlr*$^{-/-}$ mice are unable to up-regulate 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 antiatherogenic effect of atorvastatin.

2. Methods and materials

2.1. 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 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)-β,Δ-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 three to five 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.

2.2. 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 four 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, four sections with 50-μm intervals were used for atherosclerosis measurements. Sections were stained with hematoxylin–phloxine–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, USA) 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 (SMC) actin was used to quantify SMC area. Sirius Red was used to quantify collagen area.

2.3. Plasma lipid, lipoprotein and inflammatory marker analysis

Plasma was assayed for total cholesterol and triglycerides (TG) using commercially available enzymatic kits according to the manufacturer's protocols (236691 and 1488872; Roche Molecular Biochemicals, Indianapolis, IN, USA). 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.*, interleukin (IL)-1β, IL-6, IL-10, IL-12p70, tumor necrosis factor (TNF)α, chemokine (C-X-C motif) ligand-1 (CXCL1)] were assessed using a multiplex murine inflammatory cytokine profile immunoassay from Meso Scale Discovery (MSD) on an MSD 2400 plate reader according to the manufacturer's protocol (MSD, Gaithersburg, MD, USA). 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 (seven pools per group with two mice per pool).

2.4. 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