Metabolic inflammation in hepatic and vascular disorders

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Chapter 8

Resolvin E1 attenuates atherosclerosis in absence of cholesterol-lowering effects and on top of atorvastatin
Abstract

Aim: Besides LDL-cholesterol, local vascular inflammation plays a key role in atherogenesis. Efficient therapies to treat the inflammatory component of the disease have not been established. The discovery of specialized inflammation-resolving mediators, such as resolvins may provide new opportunities for treatment. This study examines whether the ω-3 fatty acid eicosapentaenoic acid-derived resolvin E1 (RvE1), can reduce atherosclerosis, when administered alone or in combination with a cholesterol-lowering statin. Methods and Results: ApoE*3Leiden mice were fed a hypercholesterolaemic diet for 9 weeks and subsequently treated with RvE1-low (1mg/kg/day), RvE1-high (5mg/kg/day), atorvastatin (1.5mg/kg/day) or the combination of atorvastatin and RvE1-low for the following 16 weeks. RvE1-low and RvE1-high reduced atherosclerotic lesion size to the same extent (-35%; p<0.05), attenuated the formation of severe lesions, also seen as a proportional increase in the presence of mild lesions, but did not alter plasma cholesterol levels. Cholesterol-lowering atorvastatin reduced atherosclerosis (-27%, p<0.05), and the combination of RvE1 and atorvastatin further attenuated lesion size (-51%, p<0.01) and increased the content of mild lesions. RvE1 had no effect on plasma levels of the systemic inflammatory marker SAA, but did down-regulate the local expression of pro-atherogenic genes in the aortae, (e.g. Cd74, Cd44, Ccl2, Ccr5 and Adam17) and significantly inactivated IFN-γ (p<0.001) and TNF-α (p<0.001) signalling pathways. Conclusions: RvE1 attenuates atherogenesis both alone and on top of a statin. The local effects of RvE1 are demonstrated by the modulated aortic expression of genes involved in inflammatory and immune responses, without altering plasma cholesterol or circulating SAA.
Introduction

Atherosclerotic plaque buildup is closely linked to an increased exposure to low-density lipoprotein (LDL) cholesterol, which has been the rationale for treatment with LDL-lowering statins over the last several decades. While the outcome of statin treatment undeniably is a reduction in cardiovascular disease (CVD) mortality, this still remains a major cause of death worldwide [1]. However, elevated LDL levels may be more important in the induction of disease, while plaque progression and later potential rupture is caused by vascular inflammation rather than high LDL levels per se. Intimal retention of LDL and its subsequent modification and oxidation provide a chronic trigger of vascular inflammation. This initially involves the innate immune system, with monocyte recruitment and macrophage and dendritic cell activation, which initiates later adaptive immune responses [2]. Due to the complexity of these local inflammatory events in CVD development, it is challenging to single out one pathway as a therapeutic target, when considering an immune-based approach. Statins, in addition to their LDL-lowering effects, have specific anti-inflammatory properties in the vasculature [3] but may not offer sufficient control [4], nor have other attempts at treating atherosclerosis with anti-inflammatory drugs been successful [5]. A major contributing factor to plaque build-up and instability is thought to be compromised clearance mechanisms at the plaque level, possibly suggesting a local imbalance between pro-inflammatory events and counter-acting resolution mechanisms of the immune system [6] suggesting that resolution may be dysfunctional.

The discovery of specialized pro-resolving mediators (SPMs) may represent a therapeutic alternative to classic anti-inflammatory drugs by harnessing the body’s own systems to regulate inflammation and promote homeostasis, including activation of endogenous clearance mechanisms [7]. Originally identified as oxygenation products of omega-3 fatty acids in resolving exudates of acute inflammation [8], these lipid-derived mediators with agonistic properties, such as resolvins, protectins, and maresins, are now established regulators of both acute and chronic inflammatory responses [9]. In a dual action these mediators dampen active pro-inflammatory pathways and concurrently activate pro-resolution functions such as phagocytic clearance and tissue repair mechanisms. Uniquely, they appear to do so without compromising host immune defense [9].

The ApoE*3Leiden transgenic mouse has a humanised lipoprotein profile with elevated plasma cholesterol levels confined mainly to the VLDL/LDL-sized lipoprotein fractions [10]. The
model is well-established to quantify the build-up of lesions and their potential amelioration by
drug intervention, and is well-characterized for statins [11]. Using this model, we herein
investigated the efficacy of resolvin E1 (RvE1), an endogenous SPM derived from
eicosapentaenoic acid, in attenuating atherosclerotic lesion development. A putative anti-
atherogenic effect of RvE1 was studied at two doses, and in combination with atorvastatin. A
reference group was treated with atorvastatin alone.

Materials and methods

Ethics statement
Experiments were approved by an independent Ethical Committee on Animal Care and
Experimentation (Zeist, the Netherlands; approval number DEC2680) and were performed in
compliance with the European Commission Directive on the use of animals for scientific
purposes.

Animals and treatments
Eighty female ApoE*3Leiden transgenic mice were fed an atherogenic Western-type diet (HC)
for a nine-week run-in period. This diet contains 40.5% sucrose, 20% acid casein, 15% cocoa
butter, 10% corn starch, 5.45% cellulose, 5.1% mineral mixture, 1% choline chloride, 1% corn
oil, 0.2% methionine and 0.4% cholesterol (all w/w) (AB-diets, Woerden, the Netherlands).
After the run-in period, in which plasma cholesterol increased from 2.8±0.4 mM to
17.5±2.5 mM as expected for ApoE*3Leiden transgenic mice, mice were divided into 5
treatment groups that were matched for total plasma cholesterol (n=16/group) continuing on
HC with 1) vehicle control, 2) 1.5 mg/kg/day atorvastatin, 3) 1 mg/kg/day RvE1 (RvE1-low), 4)
5 mg/kg/day RvE1 (RvE1-high), 5) or a combination of atorvastatin 1.5 mg/kg/day and RvE1 at
1 mg/kg/day (combination group), for the following 16 weeks. Atorvastatin (Lipitor, Pfizer,
Capelle a/d IJssel, the Netherlands) was supplemented to the diet (0.0015% w/w), and RvE1 and
vehicle were administered daily by oral gavage between 9 and 10 am. Resolvin E1 (RvE1; RX-
10001) was provided by Resolvyx Pharmaceuticals Inc. (Cambridge, MA, USA). The dose RvE1
used in this study was defined based on previous experiments in humans and mice. Briefly,
pharmacokinetics analysis of a single dose, 10 mg, 14C-labeled RvE1 (approximately
0.15mg/kg) in a Phase 1 clinical study showed a half-life of about 7 hours (Resolvyx
unpublished; trial number NCT00941018). Furthermore, administration of repeated doses of
100 mg and higher in the same study were well tolerated and without safety related issues. In
mice, 14C-labeled RvE1 at 1mg/kg resulted in plasma concentrations greater than 3 nM, which
is the EC50 defined in in vitro experiments. These levels (>3 nM) were maintained for a period of 8 hours post administration (Resolvyx unpublished).

All animals had free access to water and food. Body weight and food intake were monitored throughout the study. Animals were sacrificed by CO₂ asphyxiation after 16 weeks of treatment to collect hearts including the aortic roots which were fixed in formalin and embedded in paraffin for atherosclerosis analysis.

Prior to the main study described above, a pilot experiment was performed using the same experimental conditions but with 9 weeks of RvE1 intervention. This experiment served to investigate whether a dose of 1 mg/kg is efficacious in a setting of experimental diet-induced atherosclerosis and to establish a microarray gene expression dataset of aortae.

Biochemical, histological and microarray gene expression analyses
A detailed description of biochemical, histological and aortic microarray analyses is provided in Supplemental methods. In brief, plasma parameters (total cholesterol, serum amyloid A, E-selectin and alanine aminotransferase) were quantified using commercially available assays as described previously [12, 13]. Atherosclerosis was scored blindly in 4 serial cross-sections (at 50 μm intervals) of the aortic root. Morphometric analysis of lesion number and area was performed using cell^D software (version 2.7; Olympus Soft Imaging Solutions, Hamburg, Germany), and lesion severity was scored according to the established classification of the American Heart Association [14, 15]. Aortic genome-wide gene expression analyses were performed using Illumina microarray analysis followed by established normalisation and quality control protocols and pathway analysis as described [16, 17].

Statistical analysis
Significance of differences was tested using one-way ANOVA with LSD post-hoc test. Statistical tests were performed using SPSS software (version 20, IBM, Armonk USA). A p value ≤0.05 was considered statistically significant. All data are presented as mean ± SEM.

Results

RvE1 treatment reduces atherosclerotic lesion area
ApoE*3Leiden transgenic mice were fed the HC diet for 9 weeks to induce atherosclerotic lesions, after which treatment with RvE1 was started and continued for another 16 weeks until the end of the study. Analysis of atherosclerotic lesions in the valve area of the aortic root (Fig. 1A) revealed a clear treatment effect of RvE1 relative to vehicle in which the total lesion
area was 163200±17410 \( \mu \text{m}^2 \). RvE1-low (105300±12200 \( \mu \text{m}^2 \)) and RvE1-high (103700±17640 \( \mu \text{m}^2 \)) significantly attenuated lesion area by 35% compared with vehicle control (both \( p<0.05 \) vs vehicle; Fig. 1B). The reference compound atorvastatin (119400±16410 \( \mu \text{m}^2 \)) reduced atherosclerosis to a comparable extent as RvE1 (27% reduction; \( p<0.05 \) vs vehicle). Notably, the combination treatment had the strongest effect on atherosclerosis (82300±1522 \( \mu \text{m}^2 \)) equalling a 51% reduction (\( p<0.001 \) vs vehicle). The lesion area of this group tended to be 31% smaller than with atorvastatin (\( p=0.11 \) vs atorvastatin).

**Figure 1. Treatment with RvE1 reduces atherosclerotic lesion area.** Representative images (A) of haematoxylin-phloxine-saffron stained cross-sections of the aortic root after 16 weeks of treatment with vehicle, RvE1-low 1mg/kg/day, RvE1-high 5mg/kg/day, atorvastatin 1.5mg/kg/day, combination of atorvastatin 1.5mg/kg/day and RvE1 1mg/kg/day. (B) Quantification of total atherosclerotic lesion area in serial cross-sections of the aortic root after 16 weeks of treatment. Results are shown as mean±SEM (\( n=15-16 \)/group). \( * p<0.05, ** p<0.01 \) vs. the vehicle group.

In the ApoE*3Leiden transgenic mice model, the reduction in lesion area upon statin treatment is closely linked to the cholesterol lowering effects of these drugs [10, 11]. In the current study, atorvastatin reduced plasma cholesterol levels from 17.5±0.3 mM before the start of treatment
to 12.0±0.4 mM at endpoint (18% reduction vs vehicle; p<0.001) (Fig. 2A). RvE1 had no effect on plasma cholesterol levels at either dose. Combined treatment with RvE1 and atorvastatin reduced cholesterol levels to the same degree as atorvastatin alone (18% reduction at endpoint; p<0.01 vs vehicle) (Fig. 2A). Atorvastatin and the combination treatment reduced the total cholesterol exposure, calculated over the entire treatment period, by 21% and 17% (both p<0.001 vs vehicle) respectively (Fig. 2B), while cholesterol exposure was not affected by RvE1.

![Figure 2. Treatment with RvE1 does not reduce plasma cholesterol. (A) Plasma cholesterol levels over time in the different treatment groups from t=0 to t=25 weeks. (B) Cholesterol exposure during the 16-week treatment period. Results are shown as mean±SEM (n=15-16/group). * p<0.05, ** p<0.01, *** p<0.001 vs. the vehicle group.](image)

To further characterise the anti-atherogenic effect of RvE1, we measured circulating markers of inflammation that are involved in atherogenesis. Serum amyloid A (SAA) is a liver-derived inflammation marker that can exert pro-atherogenic effects in the vasculature [18], and E-selectin is expressed on activated endothelial cells and involved in immune cell adhesion and recruitment [19]. SAA levels rose in the vehicle group from 4.7±0.3 μg/ml at the beginning of the study (data not shown) to 6.2±0.7 μg/ml at the end of the study. SAA levels were not affected by RvE1-low (6.4±0.5 μg/ml), RvE1-high (6.9±0.4 μg/ml), atorvastatin (5.4±0.3 μg/ml), or the combination treatment (5.9±0.6 μg/ml) (Table 1). E-selectin also increased over time in the vehicle control group, from 47.3±1.8 ng/ml at the start of the study (not shown) to 68.1±2.8 ng/ml at the end of the study. E-selectin levels were not affected by RvE1-low (65.7±3.0 ng/ml) or RvE1-high (67.5±1.5 ng/ml) (Table 1). Atorvastatin significantly reduced E-selectin (53.2±1.7 ng/ml; p<0.001 vs vehicle), an effect that was not further enhanced by combination with RvE1 (51.1±2.4 ng/ml; p<0.001 vs vehicle) (Table 1).
Table 1. Plasma parameters at the end of the study (t=25 weeks)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>RvE1 low</th>
<th>RvE1 high</th>
<th>Atorva</th>
<th>Combi</th>
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<tr>
<td>SAA (μg/ml)</td>
<td>6.2 ± 0.7</td>
<td>6.4 ± 0.5</td>
<td>6.9 ± 0.4</td>
<td>5.4 ± 0.3</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td>E-selectin (ng/ml)</td>
<td>68.1 ± 2.8</td>
<td>65.7 ± 3.0</td>
<td>67.5 ± 1.5</td>
<td>53.2 ± 1.7***</td>
<td>51.1 ± 2.4***</td>
</tr>
<tr>
<td>ALAT (U/l)</td>
<td>139.4 ± 24.8</td>
<td>116.3 ± 12.3</td>
<td>108.6 ± 24.4</td>
<td>76.5 ± 12.6*</td>
<td>67.5 ± 12.5</td>
</tr>
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Results are shown as mean±SEM (n=15-16/group). * p<0.05, *** p<0.001 vs. the vehicle group.

Alanine aminotransferase (ALAT), a liver integrity marker, increased during atherogenic diet feeding from 53.3±2.1 U/L at baseline, to 139.4±24.8 U/L at the end of the study in the vehicle control group. Treatment with RvE1 did not affect ALAT levels (RvE1-low 116.3±12.3; RvE1-high 108.6±24.4). Plasma ALAT levels were significantly lower in atorvastatin treated animals (76.5±12.6 U/L; p<0.05 vs vehicle) and the combination treatment also resulted in attenuated ALAT levels (67.5±12.5 U/L, p<0.05 vs vehicle, n.s. vs atorva) which were comparable to the atorvastatin group.

**RvE1 treatment improves lesion severity**

To further elucidate how RvE1 interferes with the progression of atherosclerosis, aortic segments were analysed for presence or absence of lesions and the number of lesion-free segments was determined (Fig. 3A). When lesions were present, each individual lesion was graded to determine its severity according to the classification system developed by the American Heart Association [14, 15] (Fig. 3B).

In the vehicle group, 8±3% of all aortic segments were free of lesions (Fig. 3A). A higher content of lesion-free segments was observed with RvE1 (RvE1-low 13.3±3% and RvE1-high 15±5%) and atorvastatin (11±4%) but these effects were not significant. The combination treatment had the highest content of lesion-free segments (18±4%, p=0.07 vs vehicle).

A more refined analysis of lesion severity showed that all experimental groups exhibited lesions ranging from mild (I) to most severe (V) (Fig. 3B). However, there was a striking difference in distribution of severity scores in favour of milder lesions in the RvE1-treated groups. Most notably the content of mild type I lesions (early fatty streaks) was almost doubled in the low-dose RvE1 group, with 22±5% type I lesions, compared with 12±2% in the vehicle group (p=0.17, Fig. 3B). The effect of RvE1 seemed to be dose-dependent as this percentage was further increased to 32±7% in the high-dose group (p<0.01 vs vehicle). Although reference atorvastatin alone did not affect the content of type I lesions (14±4%, n.s. vs vehicle), the combination treatment resulted in significantly more of these mild lesions (32±7%, combination vs vehicle p<0.01, combination vs atorvastatin p<0.05, Fig. 3B) indicating that the observed attenuation of lesion severity can be ascribed to RvE1.
In the vehicle control group 31±6% of the lesions were severe type V lesions. RvE1-treated groups contained less type V lesions (RvE1-low 18±3%, n.s. vs vehicle and RvE1-high 16±4%, p<0.05 vs vehicle) which represents a reduction of 42% and 48%, respectively (Fig. 3B). The content of type V lesions was also reduced by atorvastatin (15±4%, p<0.01 vs vehicle), and the combination treatment had the lowest content of type V lesions (10±3%, p<0.01 vs vehicle) equaling a reduction of 67%.

**RvE1 did not reduce macrophage content in the aortic lesions**

Immunohistochemical staining showed that MAC-3-positive cells (macrophages) were present in aortic lesions of the vehicle control group (MAC-3 positive area 19437±2666 μm²). RvE1 treatment did not affect this area (RvE1-low dose 15754±2024 μm²; RvE1-high dose 18674±3067 μm²). The reference, atorvastatin, reduced the MAC-3-positive area by 42% (11243±1975 μm², p<0.05) (Fig. 4) which is in line with reported effects of statins on macrophage content in the ApoE*3Leiden transgenic mice model[20, 21]. A similar reduction was observed in the combination group (12738±1965 μm²).

Targeted analysis of gene expression levels of cell surface receptors commonly associated with pro-inflammatory macrophages (Cxc10, Cxcl11, Ccr7) as well as receptors associated with macrophages contributing to resolution (Cd163, Mrc1) did not reveal an effect of RvE1 treatment (RT-PCR analysis; not shown). Atorvastatin did also not cause any change in the expression of these receptors.
Figure 4. RvE1 treatment does not affect macrophage content in the aortic lesions. Quantification of immunohistochemically stained MAC-3 positive macrophage area of atherosclerotic lesions after 16 weeks of treatment. Results are shown as mean±SEM (n=15-16/group). * p<0.05, ** p<0.01 vs. the vehicle group.

**RvE1 down-regulates inflammatory genes and pathways in the vasculature**

To evaluate potential early gene expression changes in aorta, aortae collected from ApoE*3Leiden mice treated with RvE1 (1 mg/kg) or vehicle for a period of 9 weeks were used for a comprehensive gene expression analysis by microarray. RvE1 significantly affected the expression of 73 genes involved in the biological process “Inflammation” and 62 genes in “Immune cell trafficking” (not shown). Among the genes down-regulated with RvE1 several are known to be involved in atherogenesis, e.g. Ctss, Cd74, Cd44, Hpse, Ifr5, Ccl2, Csp1, Il20rb, Ccr5 and Adam17. Also, genes involved in antigen presentation and dendritic cell maturation, including Lilrb3, Lair1, Casp1, Ctss, Irf5, Lat2, Tyrobp, Hla-dqb1, Hla-Dma, Cd74, Hla-Drb5 were down-regulated by RvE1. Subsequent pathway analysis revealed a significant inactivation of the pathways downstream of IFN-γ (Z-score -4.05; p<0.001) and TNF-α (Z-score -3.10; p<0.001) in aortae of RvE1-treated mice. These results indicate pro-resolution of inflammation by RvE1 in atherogenesis.

**Discussion**

Herein we report that intervention with RvE1, an endogenous oxidation product of the ω-3 PUFA eicosapentaenoic acid (EPA) with inflammation resolution properties [22], attenuated atherosclerotic lesion development when administered orally once a daily over a period of 16 weeks in mice with established progressive atherosclerosis. RvE1 reduced total lesion area by 35%, and in animals treated with RvE1 the proportion of atherosclerotic lesions were significantly skewed towards mild (type I) lesions over more severe (type IV and V) lesions. RvE1 had no effect on plasma cholesterol levels, which is in marked contrast to atorvastatin, the
Resolvin E1 attenuates atherosclerosis

active comparator used. Atorvastatin attenuated atherosclerosis by 27% and reduced plasma cholesterol concentrations by 18%, but had no effect on lesion distribution towards milder lesions. Combination treatment with RvE1 and atorvastatin caused a more pronounced reduction of atherosclerotic lesion area (by 51%) with retained reduction in lesion severity, as observed with RvE1 alone. Gene expression analysis of aortae showed that RvE1 reduced activation of specific inflammatory pathways (IFN-γ and TNF-α) and diminished the expression of specific pro-atherogenic inflammatory factors.

In the current study we used atorvastatin as reference compound. The hypolipidaemic effects of statins, among which atorvastatin, in the ApoE*3Leiden transgenic mouse model have been described in many studies ([10, 11] and references therein). The anti-atherosclerotic activities of statins in ApoE*3Leiden transgenic mice are strongly related to their (V)LDL cholesterol-lowering effects[10], and high statin doses are needed to substantially quench inflammation [20, 23, 24]. The dose of atorvastatin employed in the present study was low (1.5 mg/kg/day) and plasma cholesterol was lowered by 18%. This hypolipidaemic effect is in a clinically relevant range, i.e. also observed in atorvastatin treated patients ([11] and references therein). Under the experimental conditions employed, atorvastatin reduced atherosclerosis by 27%. Notably, the potency of RvE1 monotherapy to attenuate atherosclerotic lesion development was even more pronounced. Importantly, RvE1 significantly reduced lesion load and improved lesion severity in absence of an effect on plasma cholesterol which remained unaltered and high (~16 mM). RvE1 increased the content of mild type I lesions, an effect that was not observed in the case of atorvastatin, and hence discriminates RvE1 from atorvastatin.

The clinical benefit of statins, in particular when employed at high doses, may be attributed to some extent to an anti-inflammatory action [4]. However, anti-inflammatory agents in current clinical use, including NSAIDs, do not seem to improve cardiovascular outcomes, or at least their therapeutic benefit is controversial [5], yet inflammation indisputably contributes to progression of disease [5, 25]. Some of these anti-inflammatory agents may shut down pathways that are dependent on cyclooxygenase-2 (Cox-2) or 12/15-lipoxygenase (12/15-LOX), enzymes that are necessary for pro-resolution activation. In fact, RvE1 is formed in the presence of aspirin-mediated acetylation of Cox-2, which will favor EPA over arachidonic acid as Cox-2 enzyme substrate [8], while current selective Cox-2 inhibitors will prevent access of any substrate to Cox-2 ([26, 27] and references therein). A major difference between RvE1 (and other SPM), and anti-inflammatory agents currently available is that SPM act as agonists, not inhibitors, with the potential to actively shift immune response balance from pro-inflammation
to pro-resolution [9]. The observed anti-atherosclerotic effect of RvE1 may thus be attributable to the combined action of inhibiting pro-inflammatory and activating pro-resolution pathways including upregulation of macrophage clearance mechanisms during active inflammation, which is a hallmark feature of the SPM class of mediators [7, 28]. Our findings of activating pro-resolving mechanisms for a treatment of atherosclerosis are also supported by a recent study using the peptide Ac2-26 acting on the N-formyl peptide receptor 2 (FPR2/ALX), which is also the receptor for the SPM lipoxin-A4 [29].

A defective immune-cell mediated clearance in atherosclerotic lesions has been suggested as a major component in progressive disease, and is not restored by statin treatment [6]. In this respect, and in marked contrast to atorvastatin, RvE1 did not reduce the macrophage content in lesions. This is consistent with observations in vivo with another SPM, neuroprotectin D1, where resolution of inflammation-driven retinal neovascularization in a model of wet AMD occurred in the absence of change in numbers of microglia but with clear conversion to a pro-resolution ramified shape [30]. We investigated a potential change in the gene expression of macrophage cell surface receptors that would allow differentiation between different subsets of macrophages, but we could not detect any difference between control or RvE1 treated animals. Importantly, many of the processes thought to be resolved by SPMs are natural endogenous processes with a relative short occurrence (a few weeks) during the entire process of atherogenesis (>20 weeks). For instance, the process of neutrophil infiltration into the vasculature takes place at around 10 weeks of atherogenic diet feeding and lasts only for a few weeks, i.e. it cannot be studied at later time points [12, 31]. Thus, it is likely that any histological analysis of immune cells in aortic lesions at endpoint does not allow identification of the inflammatory event during atherogenesis that was resolved by RvE1.

Furthermore, it is also increasingly recognized that macrophage cell surface molecule expression may not fully reflect the functional stage of a macrophage [32], and for technical reasons we were limited to a few markers. Thus, it is unlikely that immunohistochemical analysis of putative macrophage cell surface markers in aortic lesions at a 25-week endpoint would allow us to identify in full or even partially the macrophage effector profile contributing to lesion reduction in the presence of RvE1.

Microarray analysis of gene expression changes in aortae collected earlier in time (18 weeks) revealed that RvE1-treated animals displayed reduced expression of genes related to inflammation and immune cell trafficking compared with the untreated controls. For instance, RvE1 down-regulated the expression of chemokines or chemokine receptors (e.g. Ccl2, Ccr5)
suggesting a dampening effect on immune cell trafficking and innate immunity. Furthermore, RvE1 reduced the expression of MHC class II molecules suggesting a role for RvE1 in regulation of T-cell responses in atherosclerosis by reducing antigen presentation. Consistent with this, RvE1 was shown to regulate expression of co-stimulatory molecules, CD-80 and CD-86[33], and also dampen TH1 and TH17 T-cell responses, while upregulating IL-10 to promote corneal clearance in herpes simplex virus-induced ocular keratitis [34]. Novel findings in the present study were that several genes, whose products are known to be involved in atherogenesis, were beneficially affected by RvE1, including \textit{Cd74}, \textit{Casp1}, and \textit{Cd44}. An integrated analysis of gene expression patterns across pathways showed that RvE1 significantly reduced activation of the inflammatory signaling routes triggered by TNF-α and IFN-γ, both of which are known to control crucial pro-atherogenic pathways during lesion development [35, 36]. The observed effects of RvE1 in downregulating pro-inflammatory mediators is concordant with previous studies on RvE1 that described attenuated secretion of pro-inflammatory cytokines, including IFN-γ, IL-6, IL-1β and TNF-α, and the attenuation of neutrophil and macrophage infiltration at sites of inflammation [37, 38]. Consistent with this and our findings, topical gingival treatment of periodontitis with RvE1 also reduced atherosclerosis in a rabbit model of disease, which was accompanied by a dampening effect on inflammation [39].

Taken together, our results demonstrate for the first time the efficacy of an SPM, RvE1, in an experimental model of diet-induced atherosclerosis, the ApoE*3Leiden transgenic mouse, and further support the concept that defective resolution of inflammation may contribute to progression of atherosclerotic disease. While RvE1 showed comparable efficacy to that of an established statin in attenuating atherosclerosis, the more pronounced effect of their combined actions is indicative of a therapeutic potential of SPM in atherosclerosis, and the added benefit of activating pro-resolution pathways on top of existing first line treatment.

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Declaration of interests

PG and LW were employees of Resolvyx Pharmaceuticals Inc.

References

8. Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-


Supplemental data

Supplement 1. Detailed materials and methods

Plasma analyses: Total plasma cholesterol was measured by a commercially available enzymatic assay (cholesterol CHOD-PAP, Roche Diagnostics, Almere, the Netherlands). Serum amyloid A (SAA) and E-selectin were measured by ELISA (Life Technologies, Bleiswijk, the Netherlands for SAA; R&D Systems, Abingdon, UK for E-selectin) and alanine aminotransferase (ALAT) activity was determined using a spectrophotometric activity assay (Reflotron Plus system, Roche Diagnostics).

Atherosclerotic lesion analysis: Serial cross sections (5 μm) from the valve area of the aortic root were stained with haematoxylin-phloxine-saffron and atherosclerosis was analysed blindly in 4 serial cross sections (at 50 μm intervals) from each specimen. Cell-D software (Olympus Soft Imagine Solutions GmbH, Hamburg, Germany) was used for morphometric computer-assisted quantification of lesion area and lesion number; lesion severity was scored according to the established classification of the American Heart Association [1, 2]. Five lesion types are distinguished using this scoring system: Type I (early fatty streak): up to ten foam cells present in the intima; Type II (regular fatty streak): more than ten foam cells in the intima; Type III (mild plaque): foam cells in the intima with appearance of a fibrotic cap; Type IV (moderate plaque): infiltration of foam cells in the media, elastic fibres intact; Type V (severe plaque): structure of media severely disrupted with fragmented elastic fibres, cholesterol crystals, mineralization and possible presence of necrosis.

RNA extraction and microarray analysis: RNA extraction was performed as described previously in detail [3]. Briefly, total RNA was extracted from individual aortae (n=6/group) using glass beads and RNAzol (Campro Scientific, Veenendaal, the Netherlands). RNA integrity was confirmed using the RNA6000 Nano Lab-on-a-Chip kit and a Bioanalyzer 2100 (Agilent Technologies, Amstelveen, the Netherlands). The one-cycle Target Labeling and control reagent kit and the protocols optimized by Affymetrix were used to prepare biotinylated cRNA (from 5μg of total RNA) for microarray hybridization. The quality of intermediate products (that is, biotin-labeled cRNA and fragmented cRNA) was again controlled using the RNA 600 Nano Lab-on-a-Chip kit and a Bioanalyzer 2100. Microarray analysis was carried out using an Affymetrix technology platform and Affymetrix GeneChips® mouse genome 430_2.0 arrays. The hybridization and probe array washing and staining procedures were executed as described in the Affymetrix protocols, and probe arrays were
scanned with Hewlett-Packard Gene Array Scanner (Leiden Genome Technology Center, Leiden, the Netherlands).

**Microarray data analysis:** The probe-level background-subtracted expression values were used as input for the lumi package [4] of the R/Bioconductor [http://www.bioconductor.org; http://www.r-project.org] to perform quality control and quantile normalization. Unexpressed probes (p>0.01 in all experiments) were removed from further analysis. Differentially expressed probes were identified using the limma package of R/Bioconductor [5]. The calculated p-values <0.01 were used as the threshold for significance. Differentially expressed probes (DEPs) were used as an input for pathway analysis through Ingenuity Pathway Analysis suite (www.ingenuity.com, accessed 2014). In silico prediction of upstream regulator analysis was performed using the Ingenuity Pathway Analysis (IPA) software. This analysis determines the activation state of upstream regulators (molecules) such as transcription factors based on the observed differential gene expression. This results in an overlap p-value and an activation z-score for each molecule in the IPA knowledge base. The overlap p-value indicates the significance of the overlap between the known target genes of a transcription factor and the differentially expressed genes measured in an experiment. The activation z-score indicates activation (positive z-score) or inhibition (negative z-score) of a particular transcription factor. An activation z-score >2 or <-2 indicates significant activation or inhibition of a pathway or process.

References Supplement 1: