Resolvin E1 (RvE1) Attenuates Atherosclerotic Plaque Formation in Diet and Inflammation-Induced Atherogenesis

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Objective—Epidemiological and recent clinical studies implicate periodontitis as an independent risk factor for cardiovascular disease. Previously, we demonstrated that rabbits with experimental periodontitis and cholesterol diet exhibit more aortic plaque compared with diet alone. We also showed that a proresolution mediator, Resolvin E1 (RvE1), reverses the experimental periodontitis. Here, we determined whether oral/topical application of RvE1 attenuates aortic atherosclerosis induced by both diet and periodontal inflammation.

Approach and Results—Thirty-nine rabbits on a 13-week regimen of 0.5% cholesterol diet were included. Periodontitis was induced by Porphyromonas gingivalis in 24 rabbits and 15 rabbits were placed in no-periodontitis groups. Interventions were no-treatment, vehicle, and RvE1 treatment (4 μg/site or 0.4 μg/site) topically applied 3× per week. At 13 weeks, both periodontitis and atherosclerotic plaques were quantified. Atherosclerotic plaques were assessed by Sudan IV staining, histology, and ex vivo MRI. Serum levels of C-reactive protein were evaluated as a measure of systemic inflammation. RvE1, used as an oral/topical agent, significantly diminished atherogenesis and prevented periodontitis (P<0.05). In the absence of periodontal inflammation, oral/topical application of RvE1 resulted in significantly less arterial plaque, a lower intima/media ratio, and decreased inflammatory cell infiltration compared with no-treatment (P<0.001). Local oral RvE1 application significantly reduced systemic levels of C-reactive protein (P<0.05).

Conclusions—The results suggest that oral/topical RvE1 attenuates enhanced atherogenesis induced by periodontitis and prevents vascular inflammation and atherosclerosis in the absence of periodontitis. The inhibition of vascular inflammation with endogenous mediators of resolution of inflammation provides a novel approach in the prevention of atherogenic events. (Arterioscler Thromb Vasc Biol. 2015;35:1123-1133. DOI: 10.1161/ATVBAHA.115.305324.)

Key Words: atherosclerosis ■ inflammation ■ models, animal

Atherosclerosis is a complex chronic vascular disease characterized by accumulation of plasma-derived lipids in large vessel walls. Cardiovascular diseases, including atherosclerosis, are a major health problem worldwide and account for 1 in every 4 deaths in the United States. Despite efforts aimed at reducing modifiable risk factors, such as cholesterol, smoking, hypertension, and diabetes mellitus, atherosclerosis continues to increase in the population. Additional pathologic factors must therefore contribute to the pathogenesis of the disease.

Advances in basic and experimental science have elucidated the role of inflammation and the underlying cellular and molecular mechanisms that contribute to atherogenesis. It is now recognized that inflammation is a pathological component at all stages of aortic plaque formation from initiation to rupture. Early plaques (type I) are characterized by accumulation of intimal macrophages and foam cells that progress to grossly visible fatty streaks (type II plaques). The factors that determine lesion progression to cause an atherothrombogenic event remain unclear; however, progression is strongly associated with persistent inflammation of the vascular wall, which suggests that chronic, unresolved inflammation may be an underlying mechanism for atherothrombogenesis.1

Resolution of inflammation is now understood to be a biochemically active process and a mechanism that self-limits a normal inflammatory challenge. Resolvin E1 (RvE1) is a potent mediator of active resolution derived from the ω-3 fatty acid, eicosapentaenoic acid. RvE1 is protective in rabbit periodontal disease,4 murine colitis,5 murine peritonitis,6 asthmatic airway inflammation,7 bacterial pneumonia, and acute lung injury in mice.8 Accumulating evidence suggests an important role for resolution phase agonists derived from dietary ω-3 polyunsaturated fatty acids, resolvins (ie, RvE1,
Periodontitis is an inflammatory disease of the tissues supporting the teeth (gingiva, alveolar bone, periodontal ligament, and cementum) in which microbial pathologic factors induce innate immune-mediated destruction of these tissues. Periodontitis has been implicated as a risk factor for systemic inflammation, in this case, periodontal disease, on initiation of atherosclerosis in the rabbit model. In addition, clinical studies demonstrate that people with periodontitis have elevated C-reactive protein (CRP), interleukin 6, haptoglobin, and fibrinogen. Postacute myocardial infarction patients with periodontitis have significantly higher CRP than acute myocardial infarction alone, suggesting that periodontal disease is an independent contributor to systemic inflammation.

Rabbit atherosclerosis models are advantageous for studying therapies to attenuate plaque progression or to reverse atherosclerosis because of their potential for translation to human disease. Rabbits provide a range of plaque stages that more closely resemble those in humans without genetic modification of the animal, and the plaque development can be modulated by diet. The rabbit is also a unique and valuable model for experimental periodontitis, which is induced by topical application of a human periodontal pathogen, Porphyromonas gingivalis, for 6 weeks. Similar to atherosclerotic plaque formation in the aorta, the periodontal disease closely resembles that in humans macroscopically and histologically. In a previous study with simultaneous induction of periodontitis and atherosclerosis in New Zealand White (NZW) rabbits >13 weeks, rabbits with experimentally induced periodontitis had more extensive accumulation of aortic lipids than did periodontitis-free animals.

In this study, we examine the therapeutic impact of Resolvin E1 on initiation of atherosclerosis in the rabbit model. In addition, we assess the impact of local, topical treatment of periodontitis on enhanced atherogenesis associated with periodontitis.

Materials and Methods
Materials and methods are available in the online-only Data Supplement.

Results
Periodontitis Modifies the Course of Early Atherogenesis
A total of 26 male rabbits were used to assess the impact of local inflammation, in this case, periodontal disease, on atherosclerotic lesion development when induced by cholesterol-rich diet (Figure I in the online-only Data Supplement). Periodontal disease and atherosclerosis were simultaneously developed in 20 of the rabbits with a 0.5% cholesterol diet (CD) and application of topical P gingivalis to ligated teeth (+Pg). Four rabbits on CD received ligatures without P gingivalis and served as the atherosclerosis alone group. Negative controls included 2 rabbits fed a normal chow diet and kept free of P gingivalis (normal diet [ND]). P gingivalis application was stopped after 6 weeks of induction according to the established disease model. Treatments continued for another 7 weeks as did the CD. At the end of the 13-week period, the rabbits were euthanized and the mandibles as well as the entire aorta were dissected. The aorta was dissected from aortic arch to the iliac bifurcation, cut longitudinally, stained with Sudan IV, and imaged en face for quantification of aortic plaque deposition using ProImage software as previously described. Further, the aorta was embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathologic evaluation of intima, media, and adventitia. Intima/media ratio was calculated by the linear measurements of intima and media thickness. Sequential sections were stained with Masson’s trichrome for evaluation of collagen and the characteristics of atherogenesis, including inflammatory cell infiltration, medial fibrosis, and necrotic core, as previously described.

Macroscopic and microscopic evaluation of the mandibles revealed significant periodontal tissue destruction (both crestal and interproximal) similar to human periodontal disease in rabbits receiving tooth ligature and the human periodontal pathogen P gingivalis, (Figures 1A and 1B, +Pg compared with ND and CD). Evaluation of fatty streaking in the aortas of the animals that received both the CD and P gingivalis revealed that periodontal disease resulted in significantly more atheromatous plaque formation compared with CD alone (Figure 1C, aorta en face images). As previously shown, rabbits do not develop natural atherosclerotic changes; the rabbits on ND did not present with any fatty streaks (Figure 1C, ND). Histopathologic evaluation of aortic layers, tunica intima, tunica media, and tunica adventitia, revealed atherosclerotic changes with intimal thickening and lipid accumulation in cholesterol-fed animals, which was significantly greater in animals receiving P gingivalis (+Pg; n=5). Foam cell accumulation and invasion of smooth muscle cells within the tunica intima were obvious especially in animals with periodontal inflammation in addition to CD (Figure 1D; +Pg). Pathological thickening of the tunica intima and underlying atrophic media were also observed. A thin fibrous cap was visible overlying the smooth muscle cell layer facing the lumen containing macrophages and other inflammatory cells.

Quantitative measurements confirmed these observations. For periodontal disease, infrabony defect formation was significantly greater in P gingivalis induced cholesterol-fed animals (+Pg) compared with rabbits on CD alone (n=5; Figure 1E, upper left graph; P=0.0001). The histological bone loss measured is shown in the inset (Figure 1E, upper right graph). The animals that received P gingivalis...
in addition to CD had significantly greater bone loss compared with CD alone \( (P=0.007) \) and ND \( (P=0.0001) \). For aortic changes, the area covered by lipid was quantified as percentage of the entire aortic surface. Periodontal disease significantly increased the fatty deposits stained by Sudan IV (>75%; Figure 1E, lower left graph; \( P=0.01 \)). Calculation of intima/media ratio revealed that periodontal disease markedly increased intimal thickening (Figure 1E, lower right graph), however, the difference was not statistically significant compared with CD alone. As expected, CD alone showed significant changes in lipid deposition and intima/media ratio compared with healthy rabbits (ND). It seems that CD negatively affects periodontal tissues as seen with infrabony defect and histological bone loss assessments; however, the differences between ND were not statistically significant (Figure 1E).

**RvE1 Prevents Periodontal Inflammation and Attenuates Atheromatous Plaque**

Simultaneous treatments with vehicle or 0.4 \( \mu \)g RvE1 per site or 4 \( \mu \)g RvE1 per site were applied at the time of the disease induction \( (n=5 \text{ per group}) \). Periodontal disease induction was dramatic and clearly observed in all animals receiving *Porphyromonas gingivalis* and ligature without treatment. The initiation of periodontal disease by *P gingivalis* was unaffected by vehicle therapy and tissue destruction was at a level similar to the untreated rabbits (Figure 2A, upper panels). Oral-topical application of RvE1 demonstrated a dose/response inhibition of development of periodontal destruction. Descriptive histopathologic evaluations (hematoxylin and eosin staining) of periodontal tissues supported the clinical observations where RvE1- (4 \( \mu \)g/site) treated samples showed healthy histological architecture with intact bone and no inflammatory cell infiltration. Untreated (Figure 1B, +Pg) and vehicle-treated samples (Figure 2A, lower panels) showed classical histopathologic characteristics of periodontal disease with crestal bone loss, collagen degradation, and increased inflammatory cell infiltration. Quantitative morphological and histomorphometric assessments revealed the statistically significant impact of topical RvE1 treatment on prevention of periodontitis (Figure 2B). The CD alone group also exhibited mild interproximal bone loss compared with ND animals, but oral-topical RvE1 completely prevented periodontal tissue destruction in all groups with periodontal disease.

Evaluation of the impact of oral-topical RvE1 on atheromatous changes enhanced by periodontitis revealed that atherogenesis was prevented by RvE1 at both doses (Figure 2C). The animals on 0.5% CD with induced periodontal disease developed significantly more lipid accumulation that was
inhibited by RvE1 (Figure 2D; \( P < 0.05 \)). Vehicle treatment did not prevent these changes; animals in this group had large areas of lipid-rich plaque that covered the entire surface of aorta which was comparable with untreated (+Pg) group (Figure 2D). In fact, vehicle treatment tended to have worse outcomes. We do not think that 5% ethanol in saline would account for this effect. Although the results show more disease in vehicle-treated group, the difference between vehicle-treated group and the untreated group was not statistically significant. Histopathologic observations and histomorphometric measurements were similar to macroscopic assessments. CD alone, untreated, and vehicle-treated groups all showed signs of early atheroma formation (fatty streaks), including lipid-containing macrophages (foam cells). In both RvE1-treated groups, there was a marked decrease in plaque extent and thickness (Figure 2E, histology images). RvE1 (4 \( \mu g \)/site) resulted in significantly less total intimal surface calculated as the percent of total area indicating that RvE1 decreases atherogenesis characterized by thickening of intima and anthropic media layers (Figure 2F; \( P < 0.05 \)).

Furthermore, to characterize the atherosclerosis process, sequential sections of aorta were stained with Masson’s trichrome. Figure 3 demonstrates further atherosclerotic changes induced by CD and \( P. gingivalis \). Inflammatory infiltration and medial fibrosis were significantly increased by the induction of \( P. gingivalis \)--mediated periodontal inflammation. Necrotic core occupied 30% to 50% of the lesions where smooth muscle disorientation was observed. Fibrous cap surrounded the necrotic areas and foam cells were evident. No sign of plaque rupture was observed in any specimen. Vehicle treatment did not affect the atherosclerotic or inflammatory changes associated with the CD and \( P. gingivalis \). Treatment with RvE1 dramatically prevented inflammatory and atherosclerotic changes with minimal signs of necrotic areas, fibrotic changes in media, substantially lower infiltration of inflammatory cells, no sign of foam cells, and no evidence of fibrous cap or smooth muscle cell disorientation. RvE1 prevention of medial fibrosis was dose dependent; the higher dose showed statistically significant reductions in medial fibrosis compared with lower dose (4 \( \mu g \)/site versus 0.4 \( \mu g \)/site; \( P = 0.025 \)).

Oral-Topical RvE1 in the Absence of Periodontitis

To determine whether the impact on periodontitis enhanced atherogenesis of the proresolving agonist RvE1 is mediated through resolution of local inflammation alone, oral-topical local application of RvE1 was assessed in the CD model.
without concomitant periodontal disease. Simultaneous treatments with vehicle (n=3) or 0.4 μg RvE1 per site or 4 μg RvE1 per site were applied at the time of the CD initiation (n=5 per RvE1 group). The en face aortas of rabbits treated with oral-topical RvE1 showed smaller area coverage and less intense deposition of staining compared with aortas from untreated or vehicle-treated rabbits (Figure 4A and 4B, aorta images and graph). Histopathologic evaluations of thin slices confirmed these findings and showed lower intima/media ratios. There was also significant dose-dependent action of RvE1 on protection against atherogenesis (Figure 4C and 4D, histological images and graph; P<0.05). Results from this experimental arm demonstrated for the first time that locally applied oral-topical RvE1 has direct actions on the systemic inflammatory response and can prevent or control inflammation in the distant organ systems.

To determine the magnitude-of-impact of the RvE1 treatment on atherosclerotic changes as a result of cholesterol feeding with and without periodontitis, we compared percent lipid-covered area in all treatment groups to the CD alone (Figure 4E). A percentage of 0.5 cholesterol-rich diet resulted in lipid accumulation that covered on average 50±12% of the aorta surface (en face). When cholesterol-fed rabbits were simultaneously introduced to P gingivalis-mediated periodontal inflammation, lipid accumulation was ≈25±8% more (reached to >75% lipid-covered area). Both doses of RvE1 without added P gingivalis showed a dramatic preventive impact of ≤25% protection; the doses did not differ significantly. The group that received both P gingivalis and RvE1 showed ≈15% protection at the high dose and 10% at the low dose. These data suggest that periodontitis, as a local inflammatory insult, has significant systemic actions. In addition, as supported by the serum CRP levels, local oral-topical application of RvE1 has a significant impact on reducing vascular inflammation in the peripheral circulation.

High Field Ex Vivo MRI

Figure 5 shows representative high-field MR images that demonstrate differences in atherosclerotic plaques in a subset of 3 groups of rabbits (CD, +Pg, and +Pg with 4 μg RvE1 per site). CD (no periodontitis) images show a region without extensive plaque and no significant luminal narrowing. The magnetic transfer contrast image reveals a modest amount of thickening with fibrous protein and diffusion-weighted images and a low amount of lipid in the intima (Figure 5A). In the cholesterol-fed rabbit with periodontal disease (Figure 5B), there is an increase in lipid in a foamy macrophage region (an early type of lesion without an organized fibrous cap) with a protein mesh (gray). The red arrow shows a dark band characteristic of a fibrous cap and the lipid-enriched region. The hyper intense (bright regions) fat (triglyceride) deposits adherent to the adventitia that are not part of the plaque can be seen external to the vessel wall.
to the plaque in the diffusion-weighted images (Figure 5B). The images of an aortic segment of a rabbit treated with RvE1 shows a remarkably normal vessel wall with no sign of significant plaque in either MRI sequence (Figure 5C).

**Serum Lipid Level**

The lipid profiles including total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglyceride at each time point throughout the experiment were assessed to evaluate potential shifts in serum lipid levels induced by various treatments. Every group demonstrated high levels of circulating lipids as a result of the CD, which was statistically significant between baseline and 13 weeks (P<0.0001), except in the ND group. Although the serum lipid levels were similarly elevated in all groups (Table I in the online-only Data Supplement), this did not translate into plaque formation in the RvE1-treated groups, which supports the current literature suggesting that inflammation is an important contributor to plaque formation and eventually cardiovascular disease. Interestingly, the periodontal disease group showed smaller changes in all lipids compared with other groups (not statistically significant), but the atherosclerosis level was significantly greater compared with CD alone and RvE1-treated groups.

**RvE1 Treatment Reduces Serum CRP to Baseline Healthy Levels**

CRP is accepted as the gold standard clinical marker of vascular inflammation, atheroma plaque burden, and as a predictor of cardiovascular disease. Elevated serum CRP levels with periodontitis were reported by several clinical and preclinical studies. To evaluate the influence of local inflammation on the systemic inflammatory response and to determine whether pro-resolving actions of RvE1 alter systemic inflammation, serum CRP levels were measured at baseline, 6, and 13 weeks. CRP levels were significantly elevated in all cholesterol-fed rabbits (Figure 6A and 6B), but more dramatically in those who additionally had periodontal inflammation (Figure 6A; P<0.05). Of interest, topical treatment with RvE1, in both doses, significantly reduced the serum CRP levels; the reduction was more pronounced in the rabbits without periodontal inflammation with significant differences between 2 doses of RvE1 at 13 weeks (P=0.013). This finding supports the results of the measurements of lipid-covered area as well as histological evaluations, suggesting that local inflammation contributes to increased atheromatous plaque burden and systemic inflammation.

**Discussion**

In this article, we report the novel finding that preventive use of oral-topical RvE1, an endogenous mediator of resolution of...
inflammation, protected hypercholesterolemic rabbits against aortic atherosclerotic plaque formation. In addition, local oral-topical RvE1 treatment significantly prevented the extensive atheromatous plaque formation induced by the presence of a chronic local inflammation, periodontal disease. The goal was to investigate whether RvE1, a resolution phase lipid mediator known to prevent and treat periodontitis, provides protection from increased atherogenesis induced by periodontal disease in a prospective study. As previously shown by our group and others, rabbits develop significant lipid deposition and atherogenic changes in the aorta during a 13-week period when fed a 0.5% CD; local periodontal inflammation induced by

Figure 5. Ex vivo MRI. High field (11.7T) ex vivo images of rabbit aortic segments. A, Cholesterol-fed rabbit, early atherosclerotic changes are seen as thickening of intima and tunica media. B, Cholesterol-fed rabbit with periodontal disease; the red arrow shows a lipid-rich foamy macrophage region (an early type of lesion without an organized fibrous cap) within a protein mesh. C, Cholesterol-fed rabbit with periodontal disease with Resolvin E1 (RvE1) treatment. No detectable lipid is shown within the plaque; the bright regions external to the plaque are fat depots adherent to the adventitia in the segment examined. In the magnetic transfer images (top row), the dark regions depict a dense protein structure, whereas the diffusion-weighted images (DWI) images (obtained on the same slice corresponding to the magnetic transfer [MT] image) illustrate lipid (cholesteryl esters) as bright regions. Corresponding histological images from rabbits with or without treatment show similar findings. Hematoxylin and eosin stained sections show a thickened tunica media for the cholesterol-fed rabbit with periodontal disease (B) and a thin vessel wall for the RvE1-treated rabbit (C). CD indicates cholesterol diet, and +P gingivalis, CD diet + periodontal disease.

Figure 6. Local inflammation induces upregulation of C-reactive protein (CRP) in serum. Blood samples were collected at baseline, 6, and 13 weeks of disease/treatment. Serum CRP levels were measured using a rabbit-specific enzyme-linked immuno assay. CRP levels were elevated in all cholesterol-fed rabbits escalating significantly through 6 to 13 weeks (A and B; *P<0.05), but the upregulation was more dramatic in animals with periodontal inflammation (A; *P<0.001). Of interest, topical treatment with Resolvin E1 (RvE1; n=5/RvE1 group) significantly reduced the elevated CRP levels compared with untreated (n=5) and vehicle-treated (n=5) groups (A and B; #P<0.05). RvE1 dose dependently resulted in significant regression of CRP levels in cholesterol-fed rabbits without periodontal inflammation (B, **P<0.001 compared with RvE1 0.4 μg/site); CRP levels at 13 weeks were comparable with those on normal diet (ND), n=4; ANOVA with Bonferroni correction. CD indicates cholesterol diet.
*P. gingivalis* dramatically increases lipid deposition in this model.20 The data support a role for periodontitis, as a local, chronic inflammatory insult in the progression of cardiovascular disease and the role of inflammation in the initiation and progression of both periodontitis and cardiovascular disease. The prevention of both diseases by topical RvE1 suggests a potential therapeutic benefit.

Proinflammatory lipid mediators, such as leukotrienes, have been implicated in the promotion of atherosclerosis; it is noteworthy that people with periodontal disease have elevated levels of leukotriene in their gingival fluid.27 The leukotriene B4 receptor BLT-1 was identified on human vascular smooth muscle cells and colocalized with vascular smooth muscle cells in human atherosclerotic plaques. E series resolvins (RvE1 and RvE2) are antagonists for the leukotriene B4 receptor BLT-1, in addition to agonist signaling properties. Inhibition of leukotriene B4 receptor actions with BLT-1 receptor antagonists protects rabbits from intimal hyperplasia.28 Moreover, RvE1 is an agonist for the ChemR23 (ERV1) receptor, which was recently demonstrated to be expressed by human vascular smooth muscle cells. Binding of RvE1 to chemR23 modulated vascular smooth muscle cell migration via platelet-derived growth factor phosphorylation.29 These findings support the actions of RvE1 in the resolution vascular inflammation and protection from atherosclerotic lesions.

Perhaps the most striking observation is the marked inhibition of atherogenesis in the absence of periodontitis by local oral-topical application of relatively small doses of RvE1. These data suggest that RvE1 is rapidly absorbed through the mucosa into the circulation and that these low doses have a major impact of vascular inflammation. The findings are supported by significantly reduced levels of CRP after RvE1 treatment in both scenarios (with or without periodontal disease). The impact of RvE1 was more pronounced in rabbits without periodontal inflammation. The study results support the previous reports indicating periodontitis as an independent modifier of systemic inflammation.29,30 The therapeutic actions of RvE1 is comparable to those reported with statins in a recent report31 where CRP levels, but not low-density lipoprotein, were associated with atheroma plaque regression after statin therapy.

Homeostasis is a fundamental characteristic of living organisms and is often described as a process of balance. The physiological resolution of a well-orchestrated inflammatory response is essential to maintain homeostasis at the cellular and tissue level. This is accomplished in large part by specialized lipid pathways generating specific mediators that dampen the magnitude of the leukocyte and macrophage infiltrate during inflammation and promote resolution. There is a substantial body of evidence that proresolution molecules derived from ω-3 polyunsaturated fatty acids, the resolvins, and protectins, act to counter-regulate inflammation and promote resolution.31–33 RvE1 specifically interacts with its receptor, ERV1, on monocytes and macrophages to regulate leukocytes during inflammation, as well as local cytokines. A failure in the actions of these natural regulatory molecules in the pathogenesis of atherosclerosis has been hypothesized34,35 and a potential role in positively modifying the progression of disease has been suggested. For instance, Fredman et al36 demonstrate that in addition for modifying macrophage inflammatory phenotype, as reported by Bannenberg et al,37 resolvins play a major role in prevention of platelet aggregation. These findings when taken in the context of the experiments reported here are consistent with reduced macrophage accumulation in vessels and reduced lipid accumulation and inflammatory infiltration in vascular lesions.

Several classes of proresolving mediators exist, spanning from bioactive lipid mediators (like resolvins) to peptides (like annexin A1) and autacoids (like adenosine). In addition, therapies used for other inflammatory disease, particularly rheumatoid arthritis, have the potential to have beneficial effects in cardiovascular diseases. For instance, activation of the melanocortin receptor type 3 has been linked to protection from periodontitis in mice29 and is associated with decreased CRP levels in humans.24 In addition, a recent retrospective study has shown that statins may ameliorate periodontal inflammation in the same individuals who had cardiovascular disease and periodontal disease.39 This is likely related to the anti-inflammatory mechanism of statins that is linked to lipoxin and resolvin generation.40,41 Conversely, nonsteroidal anti-inflammatory drugs for arthritis do not have the same outcomes as RvE1, because nonsteroidal anti-inflammatory drugs actually interfere with resolution of inflammation pathways blocking synthesis of resolvins and lipoxins.42 Finally, interleukin-6 inhibition for treatment of rheumatoid arthritis has been suggested to provide cardiovascular protection, but this suggestion comes from in silico data analysis and has yet to be demonstrated in prospective studies in animals or man.42 Interleukin-6R antagonism in periodontal disease prevention is reported to have a positive effect in a single study, but the methods used in that study make the observations far from conclusive.43

For brief period of time, an infectious cause was considered as a potential cofactor for cardiovascular disease. The initial reports of an epidemiological association of periodontal disease and cardiovascular disease coincided with the *Chlamydia* observations in atherosclerotic lesions and spurred the idea that periodontal bacteria could be pathologic in cardiovascular disease.44–46 There are examples in the literature of studies demonstrating oral bacteria in atheromas,47 but the logic suggesting that antibiotic therapy would be expected to have an impact on disease progression may be flawed. Richardson et al48 reported that an infectious agent anywhere in the body might lead to coactivation of the innate immune system and this coactivation accelerated atherosclerosis in hypercholesterolemic rabbits that were having respiratory tract infections with *Pasteurella multocida*. Hence, infectious agents might be an indirect pathologic factor for cardiovascular disease providing the necessary inflammatory stimulus. However, human atheromas often lack any indication of the presence of infectious agents, and even if an infectious particle is present in the lesion, a pathogenic role is far from established for that particular organism.
It is more likely that the link between periodontitis and atherosclerotic disease is inflammation. A body of evidence in the cardiology literature suggests that a local inflammatory nidus can increase vascular inflammation systemically. Recent articles suggest that an elevated innate host response of any origin is a risk factor for cardiovascular disease as well as periodontitis, suggesting the inflammatory response as a common susceptibility determinant. A relatively recent study revealed that initiation of isolated inflammation in the murine dorsal air pouch model resulted in an upregulation of Cox-2 mRNA in the heart and lung supporting the hypothesis that the magnitude of the inflammatory response to insult or injury is a major determinant in the pathogenesis of inflammatory diseases, including periodontitis and cardiovascular disease.

In this study, MRI was valuable to further confirm our novel findings that periodontal disease, as a model of local inflammation, was capable of increasing atherosclerotic changes in the aorta and that topical use of RvE1 significantly reduces the extent of atherosclerotic changes induced by atherogenic diet or both atherogenic diet and periodontal disease in rabbits. A limitation of the rabbit model is the relatively short duration of the atherogenic diet. In rabbits, lesion morphology is altered by the percentage of cholesterol added to the diet and the duration of the diet. Short duration and a high percentage of cholesterol of >2% cause hypercholesterolemia, and atherogenic lesions rich in foam cells originating from macrophages. In contrast, a diet with a low cholesterol content and long duration causes atherosclerotic lesions, which are rich in smooth muscle cells and contain cholesterol deposits leading to lesions more similar to those of humans. In general, the typical diet induced atherosclerosis involves supplementation of 0.5% to 4% cholesterol per weight for 8 to 16 weeks and resulting lesions primarily consist of macrophage-derived foam cells. In our model of 0.5% cholesterol during a 13-week period, atherosclerotic lesions rich in foam cells and macrophages were developed, in addition to an increase in the intima media ratio. The histomorphometric analysis showed significant differences in the intima media ratio and atherosclerotic changes, including inflammatory infiltration, medial fibrosis, and necrotic core formation, between periodontitis and RvE1-treated groups indicating that the prevention of periodontal disease by RvE1 resulted in the prevention of atherogenesis. The interesting finding was that topical application of RvE1 in both doses protected the aortas from the atherogenic changes that are caused by the 0.5% CD in the absence of periodontal disease. Quantitatively, it seems that although periodontitis is a modifier of atherogenesis progression, prevention of inflammation is the overriding principle for the prevention of both diseases.

Conclusions

In an experimental model of periodontitis and atherogenesis, RvE1, used as a topical monotherapeutic agent, prevented atherosclerotic changes as well as periodontal bone loss induced by periodontal inflammation. Topically administered RvE1 to the gingiva was capable of protecting from atherogenic changes in 0.5% cholesterol-fed rabbits in the absence of oral lesions. Significant reduction in the circulating levels of CRP confirmed the anti-inflammatory changes induced by RvE1. These results show the potential benefits of RvE1 treatment in the attenuation or prevention of atherogenic changes in the aorta indicating a role for RvE1 in macrophage infiltration into intima. The mechanism of action of RvE1 in this scenario warrants further investigation. The study also supports ex vivo MRI as a reliable method of imaging that shows early atherogenic changes and a clear distinction between the foamy lipid area and the fibrous cap.

Taken together, the data suggest that topically applied RvE1, absorbed through dental tissues and oral mucosa, presents a unique therapeutic approach to control local and systemic inflammatory responses.

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Disclosures

Boston University is partly assigned patents on resolvins that are subject to potential consultant agreements for Dr Van Dyke. The other authors report no conflicts.

References


**Significance**

Epidemiological and recent clinical studies have implicated periodontitis as an independent risk factor for cardiovascular disease. Our data support a role for periodontitis in the progression of cardiovascular disease and the role of inflammation in the initiation and progression of both periodontitis and cardiovascular disease. We show that periodontal disease creates a chronic inflammatory state for local periodontal tissues that also complicates the pathogenesis of atherogenesis. The prevention of both diseases by topical Resolvin E1 suggests a potential therapeutic benefit for resolution agonists for the prevention and treatment of these two inflammatory diseases. Topically applied Resolvin E1, which is readily absorbed through dental tissues and oral mucosa, presents a unique therapeutic approach for the treatment of local and systemic inflammatory disease.
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**Materials and Methods**

**Animals**

New Zealand White (NZW) rabbits (41 males, 2.5-3.0 kg) were purchased from Pine Acre Research Farms (PARF, Inc., Norton, MA), kept in individual cages, received water *ad libitum*, and were fed standard rabbit chow at the Laboratory Animal Science Center at Boston University Medical Center for a week for acclimation. The study was approved by the Boston University Medical Center Institutional Animal Care and Use Committee prior to study initiation (Protocol #: AN-13948). In addition, the BUMC Institutional Biohazard Committee approved the use of *P. gingivalis* in this animal model to induce periodontal disease (Protocol #: 06-016).

**Bacteria Growth and Slurry Preparation**

*P. gingivalis* (a clinical human strain isolated from a periodontal lesion; A7436) was grown as previously described. Briefly, bacteria were cultured on agar plates containing trypsinase soy agar supplemented with 0.5% (w/v) yeast extract (Gibco Industries, Inc., Los Angeles), 5% defibrinated sheep red blood cells, 5 µg hemin, and 1 µg/ml vitamin K (Sigma-Aldrich Co., St. Louis, MO). Plates were incubated for 3 days at 37°C in jars anaerobically maintained through palladium catalyzed hydrogen/carbon dioxide envelopes (GasPak Plus, BD Microbiology Systems, Sparks, MD, USA). Colonies were randomly selected and anaerobically cultured overnight at 37°C in Wilkins-Chalgren Anaerobe Broth. Bacterial numbers were spectrophotometrically determined at 600 nm, adjusted to 10^9 CFU (0.8 optical density) in 1 ml broth. Slurry was prepared by adding sodium carboxymethylcellulose powder used as a thickening agent, into 1 ml broth with or without *P. gingivalis*. The slurry (2 ml/rabbit) was loaded into 3 ml syringes without needle and delivered to the ligatures (1 ml/site) as described below.

**Rabbit Model of *P. gingivalis* induced periodontitis**

Periodontitis was induced and established for a 6-week period using a previously established protocol 1,2. Briefly, a ligature (3-0 silk suture) was placed around the second premolar in both mandibular quadrants under general anesthesia (40 mg/kg ketamine; Ketaset®, Fort Dodge Animal Health, Fort Dodge, Iowa and 5 mg/kg xylazine; Anased®, Ben Venue Laboratories, Bedford, OH injections). The slurry containing *P. gingivalis* was topically applied to the ligatures every-other-day (Mon-Wed-Fri) over a 6-week period to induce periodontal inflammation. The control group received slurry without *P. gingivalis* correspondingly.

**Source and Preparation of RvE1**

RvE1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid) was a generous gift of Dr. Nicos Petasis, University of Southern California. RvE1 (1 mg) was provided in amber vials in ester form and concentrated in 1 ml of ethanol for stock solutions. Vials were sealed under nitrogen gas, tightly capped and stored at -80°C until the time of use. For each day of treatment, a vial was prepared by further dilution in saline to adjust for daily applied amounts (4 µg/site or 0.4 µg/site). To assure stability of the resolvins, the dilutions were prepared daily and kept on ice in dark glass bottles during the experiment.
Experimental Design

The primary endpoint of this study was the measurement of aortic plaque deposition in rabbits with or without periodontal disease, with or without cholesterol diet, and with or without RvE1 treatment (Supplemental Figure 1, Experimental Design). In the periodontitis and atherogenesis groups, rabbits (n=20) were fed a 0.5% cholesterol diet for 13 weeks and received slurry with *P. gingivalis* for the first 6 weeks enabling *in vivo* assessment of both periodontal disease initiation and atherogenesis in the same animal. An additional group was fed the 0.5% cholesterol diet, but did not receive *P. gingivalis* (received slurry without *P. gingivalis*) and was designated as cholesterol diet alone, CD. (n=4). The 20 rabbits on cholesterol diet and topical *P. gingivalis* were further divided into four groups of 5 animals each: 1) No treatment; 2) RvE1-treated (4 μg in 5% ethanol in saline/site); 3) RvE1-treated (0.4 μg in 5% ethanol in saline/site) and 4) Vehicle-treated (5% ethanol in saline). The first group received no treatment (periodontitis group, +*Pg*). The second two groups received RvE1 in two separate doses as a local, topical mono-therapy applied directly to the ligature to prevent local periodontal inflammation. Application of 5% ethanol in saline served as the fourth group (vehicle group). The topical applications of RvE1 and vehicle were on the same schedule as the *P. gingivalis* application (every-other-day) for the first six weeks; *P. gingivalis* application stopped at 6 weeks, but ligatures were kept and treatments continued until weeks 13, which was the end of the study.

In the atherogenesis only groups (without periodontitis), 15 rabbits received 0.5% cholesterol diet, while 2 rabbits served as diet controls and received a normal rabbit chow diet (total of 17 rabbits). Ligatures were placed around second premolars in all animals including rabbits on normal diet, but no *P. gingivalis* was added. Three animals were in the no treatment (cholesterol diet alone, CD) group and the three treatment groups (vehicle, RvE1-4μg/site and RvE1-0.4μg/site) consisted of 4 animals each.

Rabbits were bled via the central ear artery for blood (5ml) collection at baseline, 6 weeks and 13 weeks. Serum was separated by 2800 rpm centrifugation and stored at -80°C. Circulating lipids including total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglyceride were measured using a commercial laboratory (Quest Diagnostics Veterinary Labs, Wyomissing, PA). C-reactive protein (CRP) levels were measured in serum samples using a rabbit-specific ELISA kit following the manufacturer’s instructions (Alpco®; Salem, NH 03079).

At the end of the 13-week period, all animals were euthanized using an overdose of pentobarbital (Euthanasia-5 Solution; Veterinary Laboratories, Inc., Lenexa, Kansas, 120 mg/kg) injected via marginal ear vein to sedated animals. Mandibles were dissected for macroscopic, radiological and histological evaluation of periodontal disease as previously described. The entire aorta was dissected, cut longitudinally, stained with Sudan IV and displayed *en face* for quantification of aortic plaque deposition as previously described. In addition, sections used for analysis of intima/media ratio were taken from the same region of the thoracic branch of the aorta of each animal; and 5 μm cross sections (n=3) were taken from the aortic segment (2 cm in length) and standardized between the groups for analysis. In all groups aorta segments were fixed in formalin, embedded in paraffin, sectioned and stained with Hematoxylin & Eosin (H&E) and Masson’s trichrome for histological evaluation.
En face preparation and quantification of aortic surface area covered by atherosclerotic lesions

The aorta was dissected out under a dissecting microscope using dissection scissors and forceps. All adventitial tissue was removed by careful dissection. The aorta was removed from the heart until 5 mm after the iliac bifurcation. The dissected aortas were cut longitudinally and pinned en face onto a standard black wax dissection pan using short anodized pins (Fine Science Tools, Inc. CA, USA). The pan containing the pinned aortas was stained using the following procedure: The aortas were immersed in 70% ethanol for 5 minutes, drained and the Sudan IV solution was placed into the pan for 15 minutes, and then drained. The aortas were destained using 80% ethanol for 3 minutes. Ethanol was drained and the stained vessels were rinsed under running water to remove all ethanol. The drained pan was then filled with filtered PBS and drained just before imaging using the dissecting microscope (Stemi 2000-C, Zeiss) with an inverted camera (AxioCam HRc, Zeiss). The extent of atherosclerosis was determined using ProImage software.

Histological preparation and assessments of aorta layers in H&E stained sections

The whole aorta was sectioned across the lumen and embedded in paraffin. Thin sections (5 μm) were cut and stained with H&E or Masson’s Trichrome for histopathological evaluations. Images were taken at 2.5x, 10x and 20x magnifications using an inverted light microscope (Axio Observer A1, Zeiss). Image analysis (ImageJ) was used to outline the endothelium, internal elastic lamina (IEL) and external elastic lamina (EEL). Inflammatory cell infiltrate (mainly macrophages and foam cells) in the layers of aorta were evaluated. Area measurements were calculated using an image software program (ProImage 4). Intima area (between the endothelium and IEL) and media area (between the IEL and EEL) were measured for each aorta and averaged for each group. The total cross-sectional intimal area was measured between the endothelial cell monolayer and the IEL, and the total cross-sectional medial area was measured between the EEL and the IEL. The intimal and medial surface areas as well as the intima/media ratio were taken as measures of the severity of atherosclerosis as described previously. The entire area of the media and the entire area of the intima were measured, and then averaged for each animal. The mean values were used to calculate the ratio between the intima and the media and compared between groups. Masson trichrome stained sections were evaluated for medial fibrosis, pathologic intima thickening, signs of necrotic core, fibrous cap and inflammatory cell infiltration. Medial fibrosis was semiquantitatively measured using a scale of 0-4, where 0 referred to no sign of fibrotic changes, 1 referred to mild evidence of fibrosis (less than 25% of the area showed sign of fibrotic changes), 2 referred to moderate evidence of fibrosis (between 25-50% of the area showed sign of fibrotic changes), 3 referred to advanced evidence of fibrosis (between 50-75% of the area showed sign of fibrotic changes), and 4 corresponded to severe evidence of fibrosis (more than 75% of the area showed sign of fibrotic changes). Likewise, inflammatory infiltrate was quantified using the same scale of 0-4 corresponding to “none”, “mild”, “moderate”, “advanced”, and “severe” inflammatory cell infiltration. Necrotic core area was measured as the percentage of total vessel area demonstrating necrosis. Smooth muscle fiber orientation and disorientation, collagen content, foam cells, and fibrous cap were studied as further measures of atherosclerotic changes in aorta.

Ex vivo Magnetic Resonance Imaging (MRI)

Histology is the conventional method to classify the different stages of atherosclerosis. Although histological analysis is limited to samples taken out of the body and introduces
artifacts of preparation and staining, it is valuable for a semi-quantitative assessment of lipid accumulation in the aorta and is essential to perform for comparisons with other histology studies. In order to complement the histology results, we applied ex vivo MRI to vessel segments that were not embedded in paraffin. Ex vivo MRI does not require longitudinal slicing or thin sectioning, exposure to solvents or addition of stains.

Rabbit aortic segments were prepared as described previously and transferred to a phosphate buffer solution for imaging. Segments from three rabbits on three different protocols (cholesterol diet with and without periodontal disease, and 1.0 mg/ml RvE1 treatment of cholesterol diet with periodontitis) were examined with ex-vivo MRI. Investigators (N-G, J-H) were blinded as to their identity until the MRI analysis was completed. Ex vivo MRI was performed in a vertical-bore Bruker Avance spectrometer (11.7-Tesla) fitted with gradient coils (bore size= 89 mm; maximum gradient strength = 906.6 mT/m). Aortic segments were imaged in phosphate buffered solution using a 10 mm birdcage transmitter/receiver coil. During data acquisition, the samples were maintained at 37º C, using a thermocouple-heating element. 2-dimentional (2D) T2-weighted spin-echo images and 2D T1-weighted spoiled-gradient-echo images with and without magnetization transfer, and 2D diffusion-weighted images (DWI) were acquired as previously described in detail. The magnetic transfer contrast sequence, as optimized in the previous work detects organized fibrous protein as the most intense region (darkest) while less dense and less organized protein is seen as gray. Lipid is not detected with this sequence. Diffusion-weighted imaging (DWI) applied in this study was optimized to selectively detect liquid (cholesterol esters), which are bright against a dark background of all other plaque components.

Statistical Analysis

Mean values for direct bone measurements performed on methylene blue-stained mandibles were used to determine the changes in alveolar bone level evaluated as crestal bone loss and infrabony defects. Similarly, mean values for measurements of lipid-covered area of aortas, intima/media ratio (%), inflammatory cell infiltration, medial fibrosis, and necrotic core (% area) were used to test the impact of periodontal inflammation on atherosclerosis in cholesterol-fed rabbits and to determine whether RvE1 treatment can reduce the atherosclerotic changes compared to untreated and vehicle-treated groups. The differences in all comparisons were tested using ANOVA with Bonferroni correction (α=0.05). Further, mean values for CRP levels were measured before and after treatment. Statistical analysis was performed using nonparametric tests. Comparisons between the five study groups in each condition (with or without P. gingivalis induction) were performed using the Kruskal-Wallis test. In case of significant differences, post hoc two-group comparisons were made with the χ² test. Statistical significance was set at p≤0.05.


Supplementary Material

Supplementary Figure 1. Experimental design. Atherosclerotic plaques were induced in 39 NZW rabbits using 0.5% cholesterol-enriched diet over 13 weeks. Simultaneously, periodontal disease was induced in 20 rabbits (4 groups; 5 rabbits each) using a silk ligature tied around second premolars and a human periodontopathogen, *P. gingivalis*, which was topically applied in a carboxymethylcellulose slurry preparation during the first 6 weeks of atherosclerosis induction. Treatment groups included vehicle-treated, RvE1-treated -0.4µg, RvE1-treated- 4µg/site, and untreated (+Pg). An additional group with 4 rabbits on cholesterol diet served as control without periodontal disease (CD). The remaining 15 rabbits did not receive *P. gingivalis* slurry but were treated with 1) Vehicle (n=3); 2) RvE1-0.4µg/site (n=5); and 3) RvE1-4µg/site (n=5). Two additional rabbits without atherosclerosis or periodontitis induction were included to serve as healthy control (ND). Treatments continued for 13 weeks where all animals were euthanatized for tissue harvesting and analyses. Blood was collected to test for total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglyceride at baseline and repeated at 2, 6 and 13 weeks.
Supplemental Table I. Serum Lipid Levels

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Cholesterol (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
<th>LDL-C (mg/dL)</th>
<th>Triglyceride (mg/dL)</th>
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<tr>
<td></td>
<td>Time Point</td>
<td>Baseline</td>
<td>13 weeks</td>
<td>Baseline</td>
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<tr>
<td>Normal Diet</td>
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<td>36±1.9</td>
<td>33±0.0</td>
<td>26±1.9</td>
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<tr>
<td>Cholesterol Diet</td>
<td></td>
<td>33±4.6</td>
<td>1934±196*</td>
<td>27.5±7.3</td>
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<tr>
<td>+ P. gingivalis</td>
<td></td>
<td>33.2±4.6</td>
<td>1215±55*</td>
<td>18.2±2.7</td>
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<tr>
<td>Vehicle-treated</td>
<td></td>
<td>32.4±3.6</td>
<td>1818±170*</td>
<td>20±2.5</td>
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<tr>
<td>RvE1-treated 0.4µg/site</td>
<td></td>
<td>33.2±4.6</td>
<td>1215±55*</td>
<td>18.2±2.7</td>
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<td>RvE1-treated 4µg/site</td>
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<td>33.8±3.8</td>
<td>1669±310*</td>
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<td>Vehicle-treated without Pg</td>
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<td>34.5±9.9</td>
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<td>1826±327*</td>
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<tr>
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<td>28.8±2.1</td>
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</table>

*Statistically significant compared to baseline; p<0.0001; ANOVA with Bonferroni post hoc test; Pg: P. gingivalis; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; **under the detection limit of the test.