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Oral Resolvin D1 attenuates early inflammation but not intimal hyperplasia in a rat carotid angioplasty model.

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Abstract

Inflammation ensuing from vascular injury promotes intimal hyperplasia (IH) and restenosis. Resolvin D1 (RvD1) is a lipid mediator that attenuates IH *in vivo* when delivered locally to the vessel wall in animal models. We tested the hypothesis that peri-procedural oral administration of RvD1 could blunt the local inflammatory response to angioplasty, and attenuate downstream IH. Carotid angioplasty was performed on rats fed with either RvD1 or vehicle through oral gavage, starting one day prior to injury until post-operative day (POD) 3 or 14 when arteries were harvested. To study pharmacokinetics and bioactivity of oral RvD1, we measured plasma RvD1 by ELISA, whole blood phagocytosis activity using flow cytometry, and cAMP levels in the thoracic aorta by ELISA. Carotid arteries were harvested on POD3 for staining (anti-CD45, anti-Myeloperoxidase (MPO), anti-Ki67 or dihydroethidium (DHE) for reactive oxygen species), mRNA expression of target genes (quantitative RT-PCR), or on POD14 for morphometry (elastin stain). RvD1 plasma concentration peaked 3h after gavage in rats, at which point we concurrently observed an increase in circulating monocyte phagocytosis activity and aortic cAMP levels in RvD1-treated rats vs. vehicle. Oral RvD1 attenuated local arterial inflammation after angioplasty by reducing CD45+, MPO+, Ki67+ cells, and DHE staining intensity. Oral RvD1 also reduced the expression of several pro-inflammatory genes within the injured vessels. However, oral RvD1 did not significantly reduce IH. Oral RvD1 attenuated acute inflammation within the arterial wall after angioplasty in rats, but did not significantly affect IH.

Keywords

Resolvin D1; Angioplasty; Inflammation; Intimal hyperplasia; Restenosis

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Introduction:

Peripheral arterial disease (PAD) is a major global cause of morbidity and mortality that is increasing in prevalence [1]. Although a broad range of revascularization options are available to treat PAD, long-term efficacy is diminished by excessive intimal hyperplasia (IH) leading to restenosis as a result of the surgical or endovascular injury to the treated vessel. Restenosis represents the most common complication following technically successful vascular interventions, frequently leading to repeat procedures and hospitalizations. Therefore, exploring new strategies to prevent restenosis has become crucial to fulfill one of the greatest unmet needs in vascular medicine [1–7].

Leukocyte recruitment, pro-inflammatory cytokine expression and reactive oxygen species (ROS) production are key early events in the inflammatory cascade that follows acute vascular injury [8,9]. Inflammation within the vascular wall promotes migration of vascular smooth muscle cells (VSMC) from the tunica intima to the tunica media and their proliferation which eventually leads to intimal hyperplasia (IH) and restenosis [10]. Currently, the most common approach to attenuate restenosis following peripheral angioplasty or stent placement is the use of potent anti-proliferative agents (e.g. paclitaxel) which carry significant cytotoxicity and delay re-endothelialization of the injured vessel [11,12]. Specialized pro-resolving lipid mediators (SPMs) are endogenously produced compounds that activate the resolution pathways, which in turn actively promote the return to homeostasis following acute inflammation within the vascular wall [13,14]. Resolvins constitute a family of SPMs derived from the omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA); both D-series (derived from DHA) and E-series (derived from EPA) resolvins mediate resolution of inflammation across a broad range of animal disease models, with resolvin E1 (RvE1) in clinical development for ophthalmic disease [15–17].

Resolvin D1 (RvD1) has been identified in a variety of human biological samples including arterial tissue [13,18,19]. RvD1 is produced in two stereoisomers depending on the enzymes involved in the first reaction of its synthetic pathway: 15-lipooxygenase (15-LOX) eventually determines the synthesis of 17S-RvD1, whereas cyclooxygenase-2, after its covalent modification by aspirin, determines the synthesis of 17R-RvD1 (or “aspirin-triggered”-RvD1, AT-RvD1). RvD1 exerts pro-resolving effects by decreasing neutrophil infiltration, and increasing the phagocytic activity of neutrophils and macrophages [17,20,21]. Both RvD1 isoforms modulate VSMC phenotype by decreasing migration and proliferation, factors which are crucial to the development of IH [22,23]. The anti-migratory action of 17R-RvD1 in VSMCs has been linked to the cAMP/PKA pathway, which has been extensively demonstrated to be protective against vascular inflammation [23–27]. We recently showed that 17S-RvD1, when delivered via a perivascular biodegradable film or via pluronic gel, attenuates IH formation in a rat carotid angioplasty model as well as in a rabbit carotid venous bypass model [28,29]. Recent studies have shown that RvD1 is rapidly absorbed after oral gavage administration in mice, attenuates acute inflammation in a mouse peritonitis model, and accelerates resolution by regulating the transcription in macrophages [30]. Therefore we sought to investigate if oral RvD1 could abrogate early inflammation and downstream IH in an established rat vascular injury model.

Materials and Methods:

Animals, cell culture and reagents:

Male Sprague- Dawley rats (400–500g; Charles River Laboratories, Wilmington, MA, USA) were used in compliance with an Institutional Animal Care and Use Committee (IACUC)-approved protocol (University of California, San Francisco #AN108115–01A). Rat arterial vascular smooth muscle cells (RASMCs) were isolated from uninjured control male Sprague-Dawley rats, as previously described [31]. 17S-RvD1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid), AT-RvD1 (7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) and the RvD1 enzyme immunoassay (ELISA) kits were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-CD45, anti-MPO and anti-Ki67 antibodies were purchased from Abcam (Cambridge, United Kingdom). Dihydroethidium (DHE) was purchased from Life Technologies (Grand Island, NY, USA).

Rat oral gavage and carotid artery angioplasty:

Rats were administered 17S-RvD1 (0.5μg/Kg bid), AT-RvD1 (0.5μg/Kg bid), or vehicle (ethanol) in 500μl of corn oil (Sigma-Aldrich St. Louis, MO, USA) through flexible oral gavage tubes (Instech Laboratories, Plymouth Meeting, PA, USA) inserted into the gastric cavity. Injury to the left common carotid artery was performed by balloon angioplasty as described previously [32]. A 2F balloon catheter (Edwards Lifesciences, Irvine, CA, USA) was inserted through an arteriotomy in the left external carotid artery, and was kept inflated at a pressure of 5ATM for 1 minute using a gauged inflation device (Merit Medical Endotek, West Merit Parkway South Jordan, UT, USA). After balloon deflation, the left external carotid artery was ligated and the wound closed. Rats where euthanized 3 hours after the last oral dose on post-operative day 3 (POD3) or 14 (POD14). Left common carotid arteries were harvested and immediately frozen after perfusion with heparinized saline (for the 3-day experiments) or fixed after perfusion with heparinized saline followed by 4% formaldehyde for paraffin embedding (for the 14-day experiments). Supplemental Figure 1 outlines the experimental timeline of the experiments. In the 14-day studies examining IH development, we tested two different oral dosing regimens. In a “short treatment” protocol rats received either oral 17S-RvD1 or vehicle bid starting from the day before surgery until POD3 (Suppl Fig 1B). In a “long treatment” protocol rats received either oral AT-RvD1 or vehicle bid starting from the day before surgery until POD14 (Suppl Fig 1C). We chose 17S-RvD1 for the short-term experiments because of the availability of a commercial ELISA to measure plasma levels; in contrast AT-RvD1 is believed to have a longer biological half-life so we selected this stereoisomer for the longer term strategy.

RvD1 measurement in plasma

Blood was collected through lateral tail vein puncture (200–300μl per draw) and plasma was obtained by means of centrifugation (1000g at 4°C). Plasma samples were mixed with methanol and water, acidified to pH 3.5, underwent solid-phase extraction (SPE; Sep-Pak Vac C18 cartridges; Waters, Milford, MA, USA) and were eluted in methyl formate. Methyl formate was evaporated under nitrogen flow and the extracted sample resuspended in ELISA buffer. 17S-RvD1 was quantified using an ELISA as described by the manufacturer. RvD1

extraction and measurement from rat blood were initially validated by comparing the measured versus the known values of 17S-RvD1 in raw plasma samples spiked with exogenous 17S-RvD1. To generate a concentration vs. time curve of 17S-RvD1 in rat plasma, rats underwent blood draws at different time-points after oral dosing, and samples were extracted and measured as described.

Whole blood phagocytosis assay

Blood was drawn from rats at baseline and 3 hours after a single dose of oral 17S-RvD1 (0.5μg/Kg). Each blood sample was divided into two aliquots (50μl each) and incubated with FITC-conjugated *E. Coli* particles either on ice or at 37°C for 1 hour. Red blood cells were lysed and the amount of phagocytosed *E. Coli* particles was quantified via flow cytometry (BD FACSVerse; BD Biosciences, San Jose, CA, USA) as median fluorescence intensity (MFI) analyzed by FlowJo software (FlowJo LLC, Ashland, OR, USA). Different populations of leukocytes were gated using forward scatter (FSC) and side scatter (SSC). The MFI from each aliquot at 37°C was divided by the MFI of the corresponding aliquot on ice; the MFI ratio of samples drawn post-17S-RvD1 administration was normalized to the MFI ratio of the baseline (pre-treatment) blood draw from the same rat.

cAMP levels in thoracic aorta

Thoracic aorta samples were harvested from rats 3 hours after a single oral dose of 17S-RvD1. Samples were snap-frozen in liquid nitrogen, pulverized and lysed in different volumes of HCl solution based on tissue weight. Lysates were spun at 12,000g for 10 minutes and the supernatants were collected. cAMP and total protein contents were measured via ELISA (Enzo Life Sciences, Farmingdale, NY, USA) and BCA assay (Thermo Fisher Scientific, Waltham, MA, USA), respectively, following the manufacturers' protocols. cAMP concentrations were calculated from absorbance values using a four-parameter logistic fit. Aortic cAMP levels were normalized to total protein content.

cGMP and cAMP levels in RASMCs

Confluent RASMCs were grown in 12-well plates and serum-starved overnight in DMEM containing 0.1% FBS. Cells were treated with 17S-RvD1 (10nM) and harvested at 5 and 15 minutes using a 0.1N HCl, 1% Triton X-100 lysis buffer. After spinning the lysates at 12,000g for 10 minutes and collecting the supernatants, cGMP, cAMP and total protein contents were measured via ELISA (Enzo Life Sciences, Farmingdale, NY, USA) and BCA assay (Thermo Fisher Scientific, Waltham, MA, USA), respectively, following the manufacturers' protocols. cGMP and cAMP concentrations in the lysates were calculated from the absorbance readouts using a four-parameter logistic fit and then normalized by total protein content.

Immunofluorescence and DHE staining

Freshly harvested arterial samples were snap-frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA), then subsequently sectioned and stained using dihydroethidium (DHE) or primary antibodies (see "Animals and reagents" above) against CD45, Myeloperoxidase (MPO) or Ki67. After incubation of a biotinylated-secondary

antibody (Life Technologies, Grand Island, NY, USA) and Streptavidin-FITC (Vector Laboratories, Burlingame, CA) incubation, the slides were mounted using a DAPI-containing mounting medium (Southern Biotech, Birmingham, AL, USA). FITC (CD45, MPO, Ki67) and DAPI (nuclei) signals were visualized by a fluorescence microscope and camera (model BX51 and DP70, Olympus, Shinjuku, Tokyo, Japan) in randomly selected fields (20X) from 3 different non-adjacent sections of each vessel. Images were analyzed using the software ImageJ (version 1.48, NIH; http://imagej.nih.gov/ij/download.html). CD45 and MPO-positive cells (FITC and DAPI double-positive cells) were counted and normalized by vessel area. Proliferative index was quantified as the percentage of Ki67-positive cells (FITC and DAPI double-positive cells) vs. total number of cells (DAPI positive cells). ROS production was quantified by measuring DHE fluorescence intensity which was then normalized to DAPI staining.

Quantitative gene expression (RT-PCR) of pro-inflammatory genes

Rats were treated as described and sacrificed on POD3 (Suppl Fig 1A). At the moment of harvest, carotid artery specimens (injured and contralateral uninjured control) were placed in RNAlater (Thermo Fisher Scientific, Waltham, MA) and frozen at −80°C until samples were ready for RNA extraction. Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Germantown, MD) with RNase-free DNase treatment according to the manufacturer's protocol. Total RNA from each specimen was used to generate complementary DNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for subsequent reverse transcription-PCR reactions. SYBR Green-amplified DNA was detected by incorporation of SYBR Green (Applied Biosystems). Dissociation curve analyses were performed to confirm the specificity of the SYBR Green signal. Data were normalized to two reference genes and subsequently to untreated bypass (bypass-only) grafts. PCR parameters included an initial 10-minute denaturation step at 95°C, followed by a cycling program of 95°C for 10 seconds and 60°C for 30 seconds for 40 cycles (CFX96 Real-Time System; Bio-Rad). Primers targeting rat genes coding for HO1, IL-1β, MCP1, TGF-1β, TNFα, IL-6 and IL-10 were used (Table 1).

Elastin stain and morphometric analysis

Common carotid arteries were harvested 14 days after balloon injury by perfusion-fixation as described above (Suppl Fig 1B and C). 6μm sections were taken from the paraffin-embedded common carotid arteries throughout the area of balloon injury. Deparaffinized sections were stained using an elastin staining kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Six non-adjacent sections within the area of balloon injury were analyzed per artery specimen using the software ImageJ. For each section, the luminal area, the area inside the internal elastic lamina and the area inside the external elastic lamina were measured and, in turn, utilized to extrapolate the neointimal area, the medial area and the mean neointimal thickness.

Statistical analysis

Data is shown as mean ± standard error of mean (SEM). Linear regression and Pearson r coefficient were used to study the correlation between the spiked and measured concentration of 17S-RvD1 in plasma samples. One-way analysis of variance (ANOVA) was

run on all the groups, followed by Sidak's or Dunnett's *post hoc* tests to account for multiple comparisons when appropriate. For all the data analyses, P values <0.05 were considered to be statistically significant.

Results:

17S-RvD1 plasma levels increase after oral administration in rats

To validate the solid-phase extraction of 17S-RvD1 from rat plasma we compared known concentrations of 17S-RvD1 in raw plasma samples with the ELISA results. We observed a strong correlation between spiked and measured concentrations of 17S-RvD1 ($r^2 = 0.9955$, $P < 0.0001$; Suppl Fig 2). Following a single oral administration of 17S-RvD1 to rats, we measured plasma levels of 17S-RvD1 over time and observed a peak of 446pg/ml (1.18nM) at 3 hours, which then returned to baseline levels between 6 and 24 hours with an estimated half-life of 1.24 hours (Fig 1). Half-life was extrapolated from the elimination rate constant which, in turn, was calculated from clearance and volume of distribution.

Oral 17S-RvD1 increases circulating monocyte phagocytosis activity

Leukocyte phagocytosis of fluorescently-labeled *E. Coli* particles was measured via flow cytometry from rat blood 3 hours after a single dose of oral 17S-RvD1. Leukocyte populations were differentiated by using forward scatter (FSC) and side scatter (SSC). Oral 17S-RvD1 treatment significantly increased monocyte phagocytosis activity in rat blood vs. vehicle (69% increase, P<0.05; Fig 2B and B') while we did not observe significant changes in phagocytic activity of circulating neutrophils (Fig 2A and A').

Oral 17S-RvD1 treatment increases aortic cAMP levels

cAMP was measured from homogenized rat thoracic aorta samples (n=3) harvested 3 hours after a single dose of oral 17S-RvD1. Samples harvested from rats receiving 17S-RvD1 showed a significant increase in cAMP levels compared to those from rats receiving vehicle alone (cAMP/total protein ratio 0.23 of vehicle group vs. 0.46 of 17S-RvD1 group, 100% increase, P<0.05; Fig 3).

17S- RvD1 increases cGMP and cAMP in RASMCs *in vitro*

In primary cultured RASMCs, 17S-RvD1 (10nM) rapidly increased the levels of cGMP and cAMP 5 minutes after administration (cGMP/total protein ratio 0.2956×10^{-4} of vehicle group vs. 0.6259×10^{-4} of 17S-RvD1 group, 112% increase, P<0.05; cAMP/total protein ratio 3.913×10^{-4} of vehicle group vs. 6.512×10^{-4} of 17S-RvD1 group, 66.4% increase, P<0.05). While the increase in cGMP levels sustained at 15 minutes (cGMP/total protein ratio 0.2956×10^{-4} of vehicle group vs. 0.5851×10^{-4} of 17S-RvD1 group, 98% increase, P<0.05), cAMP levels quickly returned to baseline (Suppl Fig 3 A & B).

Oral 17S-RvD1 decreases early leukocyte infiltration following balloon angioplasty

Oral 17S-RvD1 treatment starting the day before balloon injury caused a significant reduction in leukocytes (i.e. CD45+ DAPI+ cells) identified within the vascular wall on

POD3 compared to oral vehicle (79% reduction, P<0.0001), as seen in representative images (Fig 4A) and quantitation of CD45+ DAPI+ cells normalized to vessel area (Fig 4B).

Oral 17S-RvD1 decreases early MPO expression following balloon angioplasty

Oral 17S-RvD1 treatment caused a significant reduction in MPO+ DAPI+ cells within the vascular wall on POD3 compared to oral vehicle (46% reduction, P<0.01) as seen in representative images (Fig 5A) and quantitation of MPO+ DAPI+ cells normalized to vessel area (Fig 5B).

Oral 17S-RvD1 decreases early ROS production following balloon angioplasty

Oral 17S-RvD1 treatment was associated with a significant reduction in ROS production (as visualized with DHE signal intensity) on POD3 within the vascular wall compared to oral vehicle (41% reduction, P<0.05) as seen in representative images (Fig 6A) and DHE signal intensity normalized to DAPI signal intensity (Fig 6B).

Oral 17S-RvD1 decreases early proliferative index in the artery wall following balloon injury

Oral 17S-RvD1 administered bid starting the day before balloon injury caused a significant reduction in proliferative index (defined as the percentage Ki67+ DAPI+ cells) on POD3 within the arterial wall compared to oral vehicle (70% reduction, P<0.001) as seen in representative images (Fig 7A) and quantitative analysis (Fig 7B).

Oral 17S-RvD1 modulates early inflammatory cytokine gene expression in the vascular wall following balloon injury

Oral 17S-RvD1 treatment reduced the vessel wall expression of several cytokines involved in the acute inflammatory process (HO1: 64% reduction, P<0.01; IL-1β: 80% reduction, P<0.001; MCP-1: 50% reduction, P<0.01; IL-10: 88% reduction, P<0.001) on POD3 compared to oral vehicle. On the other hand, gene expression of several other cytokines tested (TGF-1β , TNFα, IL-6) was not significantly affected by 17S-RvD1 (Fig 8).

Oral 17S-RvD1 and AT-RvD1 did not attenuate IH at 14 days following balloon injury

Oral 17S-RvD1 or AT-RvD1 were administered bid following a “short” or “long” treatment protocol (Suppl Fig 1B and C). Neither of the two treatment regimens, when compared to vehicle, significantly affected intimal area, medial area, neointimal area to medial area ratio (NI/M), mean neointimal thickness, or neointimal area to IEL area ratio (P>0.05) at 14 days after injury (Fig 9).

Discussion:

Acute vascular inflammation involves a complex series of events that ensues immediately after an injury (hours-days) such as balloon angioplasty. Physiologically, the resolution pathways mediated by the endogenous SPMs (including RvD1) abate acute inflammation. Chatterjee *et al* demonstrated that human arterial tissue is capable of synthesizing RvD1 as well as other D-series resolvins when exposed to DHA or its monohydroxy-metabolite 17-HDHA; moreover, they showed that isolated human endothelial cells and VSMCs produce

biologically active D-series resolvins when exposed to 17-HDHA [13]. If inflammation is not abated by the endogenous resolution pathways, it provides the substrate for aberrant remodeling, promoting IH formation and restenosis. Therefore, accelerating resolution following vascular injury may be a valuable strategy to promote repair and improve patency. In previous studies, we demonstrated that local perivascular delivery of the pro-resolving lipid mediator 17S-RvD1 inhibits IH formation in the rat carotid angioplasty model [28]. While local drug delivery is highly relevant to certain settings, oral treatment or pre-treatment strategies to augment SPM pathways is attractive for elective surgical and interventional settings. Although the effects of oral administration of SPM precursors (e.g. fish oil) have been described in different animal disease models [33,34], oral administration of a bioactive SPM (e.g. RvD1) is a novel strategy to directly enhance SPM bio-availability *in vivo*. To date, oral RvD1 treatment has been investigated in mice in a model of multiple sclerosis and a model of zymosan A-induced acute peritonitis [30,35]. To our knowledge, this is the first study testing oral administration of RvD1 in rats and in a setting of an acute vascular injury.

We found that plasma levels of 17S-RvD1 in rats peaked at a concentration of 446pg/ml (1.18nM) three hours following a single oral dose of 0.5μg/Kg. These levels fall within the concentration range of RvD1 (1–10 nM) observed to achieve anti-migratory and anti-proliferative effects in human and rat VSMCs *in vitro* [22,23,28]. This study also provides evidence of 17S-RvD1 bioactivity in rats concurrent with the plasma peak following a single oral dose; namely, increased phagocytic activity of circulating monocytes and increased aortic cAMP levels, both of which are biological effects of the SPM. However, measured plasma levels of 17S-RvD1 fell rapidly with a half-life of approximately 1.24 hours. Given the observed pharmacokinetics, if 17S-RvD1 were administered at the end of each half-life (impractical in the current study), a steady state concentration range of approximately 1.16nM - 2.32nM could be reached after 5–6 half-lives.

In this rat carotid angioplasty model, oral 17S-RvD1 blunted several events that characterize the acute inflammatory response within the vessel wall. Specifically, we observed diminished leukocyte infiltration (as suggested by a decreased number of CD45+ cells), reduced number of MPO+ cells, reduced ROS staining, and a lower proliferative index (i.e. percentage of Ki67+ cells) in arteries from treated animals on POD3. In addition, oral 17S-RvD1 treatment modulated the expression of several cytokines involved in the regulation of inflammation within the injured vessel. IL-1β and MCP-1 are well-known to promote inflammation within the vascular wall and facilitate the progression of restenosis following angioplasty [36–38]. In this study, both of these important cytokines were significantly downregulared by oral 17S-RvD1 treatment 3 days after angioplasty. IL-10 and HO-1, on the other hand, have been characterized as anti-inflammatory cytokines. 17S-RvD1 has been shown to increase IL-10 in bronchoalveolar lavage fluid in mice exposed to cigarette smoke [39]. RvD1 was also shown to protect mice from LPS-induced murine lung injury by enhancing HO-1 gene expression [40]. Surprisingly, gene expression of both HO-1 and IL-10 in the injured artery was significantly decreased following oral 17S-RvD1 treatment in this study. We speculate that this decrease in anti-inflammatory cytokine gene expression could represent a negative feedback induced by the reduction in upstream pro-inflammatory cytokines. One study conducted in primary human macrophages showed that 17S-RvD1

induced repolarization by decreasing the secretion of IL-1β and MCP-1; however IL-1β gene expression was not significantly affected. In the same study, no significant effects on macrophage gene expression and protein secretion were observed for IL-10 and HO-1 after treatment with 17S-RvD1 [41].

Prior work in our lab has demonstrated that the anti-migratory effects of RvD1 on growth factor stimulated human VSMC is receptor-dependent and involves the cAMP/Protein Kinase A pathway [23]. In the present study, oral RvD1 induced increased levels of cAMP in the thoracic aorta. Furthermore, in primary cultured RASMCs, we observed that 17S-RvD1 rapidly increased the levels of both cAMP and cGMP (Suppl Fig 3 A and B). cGMP is a well-known mediator of VSMC relaxation [42]. These findings warrant further investigation on the cyclic nucleotide signaling pathways responsive to RvD1 in VSMC, and how they may modulate physiologic responses in vascular tissue.

Despite the observed effects of oral RvD1 on early inflammation within the injured artery wall, we did not observe a significant effect on IH development at 14 days with either treatment regimen. In the limited dose study, we employed oral 17S-RvD1 administered bid through POD3 to test the hypothesis that early augmentation of resolution pathways could influence downstream vessel healing. In the second protocol, we extended oral dosing for 14 days using the “aspirin-triggered” epimer of RvD1 (i.e. AT-RvD1) given its increased resistance to metabolic inactivation compared to 17S-RvD1 [15,43]. The lack of efficacy observed with both approaches suggests that the effects observed on acute inflammation were not sufficient to alter IH formation. Our previous experience with perivascular delivery of 17S-RvD1 demonstrated a significant reduction in IH formation in the same rat carotid angioplasty model (using a local dose of 200ng ≈ 0.531nmoles loaded per perivascular device), and also in a rabbit vein graft model (local dose of 1μg ≈ 2.656nmoles loaded per perivascular device) [28,29]. A perivascular drug-eluting approach has the inherent advantage of sustained, higher tissue concentrations selectively at the injury site. An oral administration strategy for RvD1 would likely require a significant increase in both dose frequency (as suggested by the short half-life of 1.24 hours) and dose amount, to achieve similar tissue levels as a direct local treatment. Moreover, the average peak plasma concentration of 17S-RvD1 (446pg/ml) we achieved was low in relation to the dose given orally (0.5μg/Kg which equals to 200ng for a 400g rat). A previous study estimated the plasma volume of male Sprague-Dawley rats to be ~4ml for each 100g of total body weight [44]. This means that only ~3.6% of the oral dose administered was measured in the plasma at 3 hours. The reasons underlying this poor bioavailability are likely to be related to gastrointestinal absorption of RvD1, its degradation, or both. In recent studies, targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used with radio-labeled RvD1 to study its pharmacokinetics in tissue and plasma while differentiating the administered and the endogenous RvD1 [45,46]. The use of radio-labeled RvD1 might be helpful to better characterize the pharmacokinetics of RvD1 in future *in vivo* studies.

We speculate that sustained local vascular levels of RvD1 are particularly critical for its effects on resident cells, especially VSMC, in comparison to more rapid effects on circulating leukocytes. In this regard the direct anti-migratory, anti-inflammatory effects of SPM on VSMC previously reported are of fundamental importance to IH development

following angioplasty, and may account for the disparate results observed between oral and perivascular RvD1 administration in this rat angioplasty model. We are unable to directly assess VSMC migration *in vivo* to support this hypothesis.

In conclusion, bioactive RvD1 plasma levels may be achieved by oral administration in the rat, as demonstrated by effects on circulating leukocyte phagocytosis and aortic cAMP production, as well as attenuation of acute inflammation at the site of vascular injury. However, these acute changes did not correlate with downstream vascular healing and IH development. Other observed effects such as enhanced phagocytosis activity may be of relevance to the host response to injury, and provide foundation for further study. Pharmacokinetics of oral RvD1 may be challenging for translation, and alternative formulations with longer half-live would be advantageous. Further studies using alternative compounds, analogues, precursors, or dosing strategies are needed to test the concept of therapeutic SPM administration to blunt the inflammatory response after vascular injury and influence downstream vessel remodeling and restenosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- RvD1 increased rapidly in rat plasma after oral gavage.
- A single dose of oral RvD1 increased monocyte phagocytosis and aortic cAMP levels.
- Oral RvD1 decreased acute inflammation within the arterial wall after angioplasty.
- Oral RvD1 did not affect intimal hyperplasia development 14 days after angioplasty.

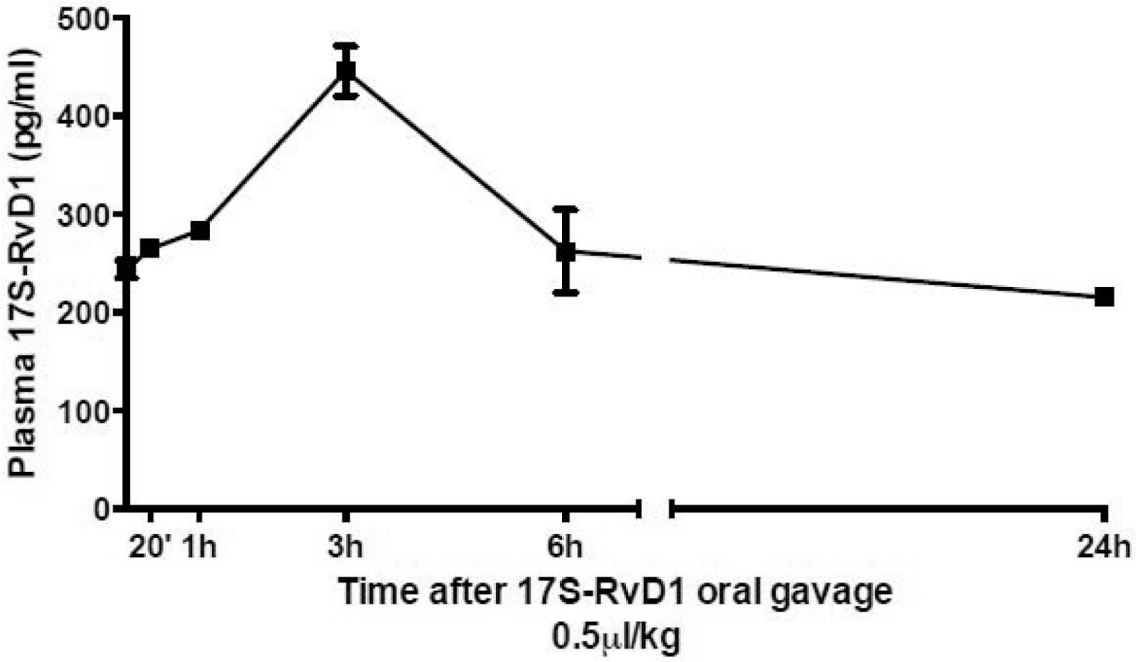

Figure 1. Pharmacokinetics of oral 17S-RvD1 in rats.

After a single oral dose of 17S-RvD1 in 500μl of corn oil, rats were subjected to multiple blood draws at different time points. Plasma samples underwent SPE and 17S-RvD1 quantified by EIA. 17S-RvD1 plasma concentration increased gradually, peaked at 3 hours reaching a max concentration of 446pg/ml (1.18nM), and went back to baseline within 24 hours (n=3).

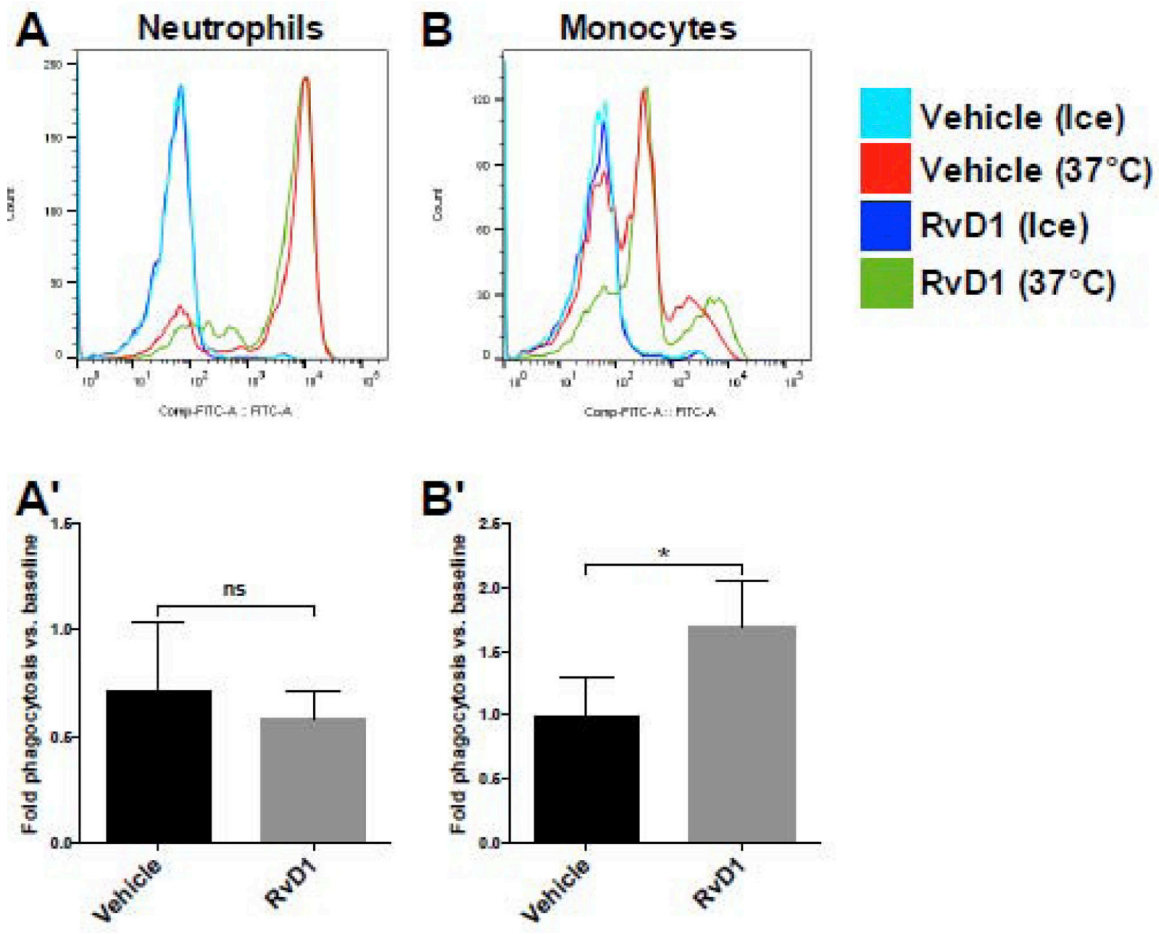

Figure 2. Oral 17S-RvD1 increases monocyte phagocytosis in rat blood.

Different populations of WBC were gated using FSC and SSC. Monocytes (B & B') showed a significant increase in phagocytic activity 3 hours after a single oral dose of 17S-RvD1 compared to vehicle. However, neutrophils (A & A') did not significantly change their phagocytic activity after 17S-RvD1 treatment (n=4). *P<0.05

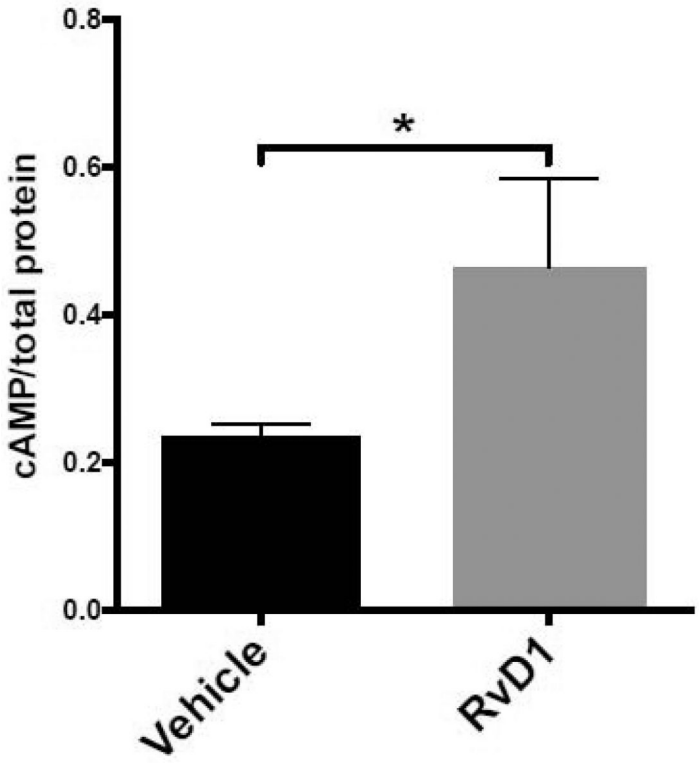

Figure 3. Oral 17S-RvD1 increases cAMP levels in rat thoracic aorta.

Homogenized thoracic aortas harvested from rats that received a single oral dose of 17S-RvD1 showed significantly higher cAMP levels compared to vehicle-treated rats 3 hours after administration (n=3). *P<0.05

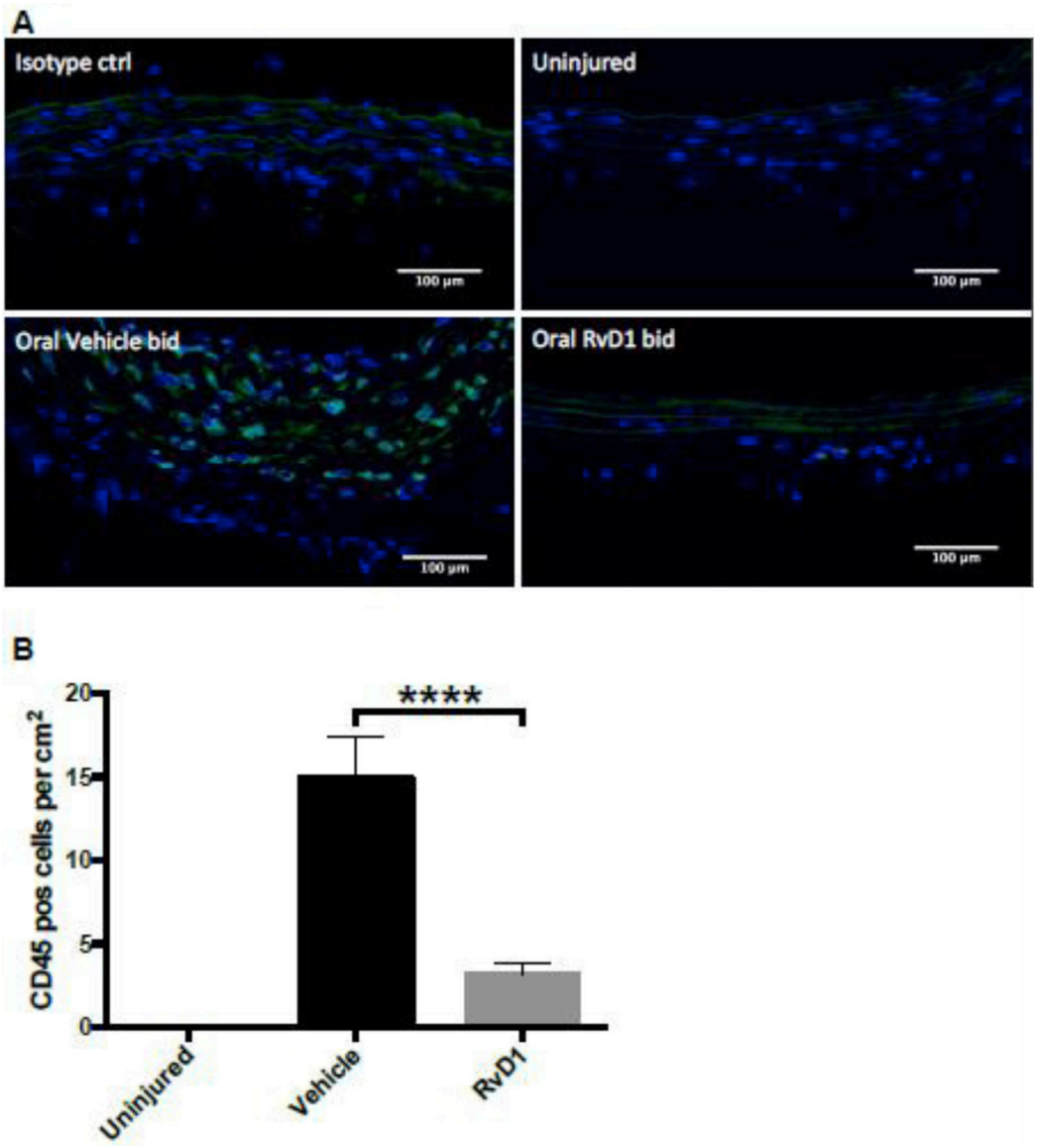

Figure 4. Oral 17S-RvD1 decreases CD45+ cells within the injured vascular wall.

CD45+ DAPI+ cells were counted and normalized by vessel area. Arteries harvested from rats treated with oral 17S-RvD1 showed a significant reduction of CD45+ cells within the vascular wall compared to those from the vehicle-treated rats (n=5; A & B). ****P<0.0001

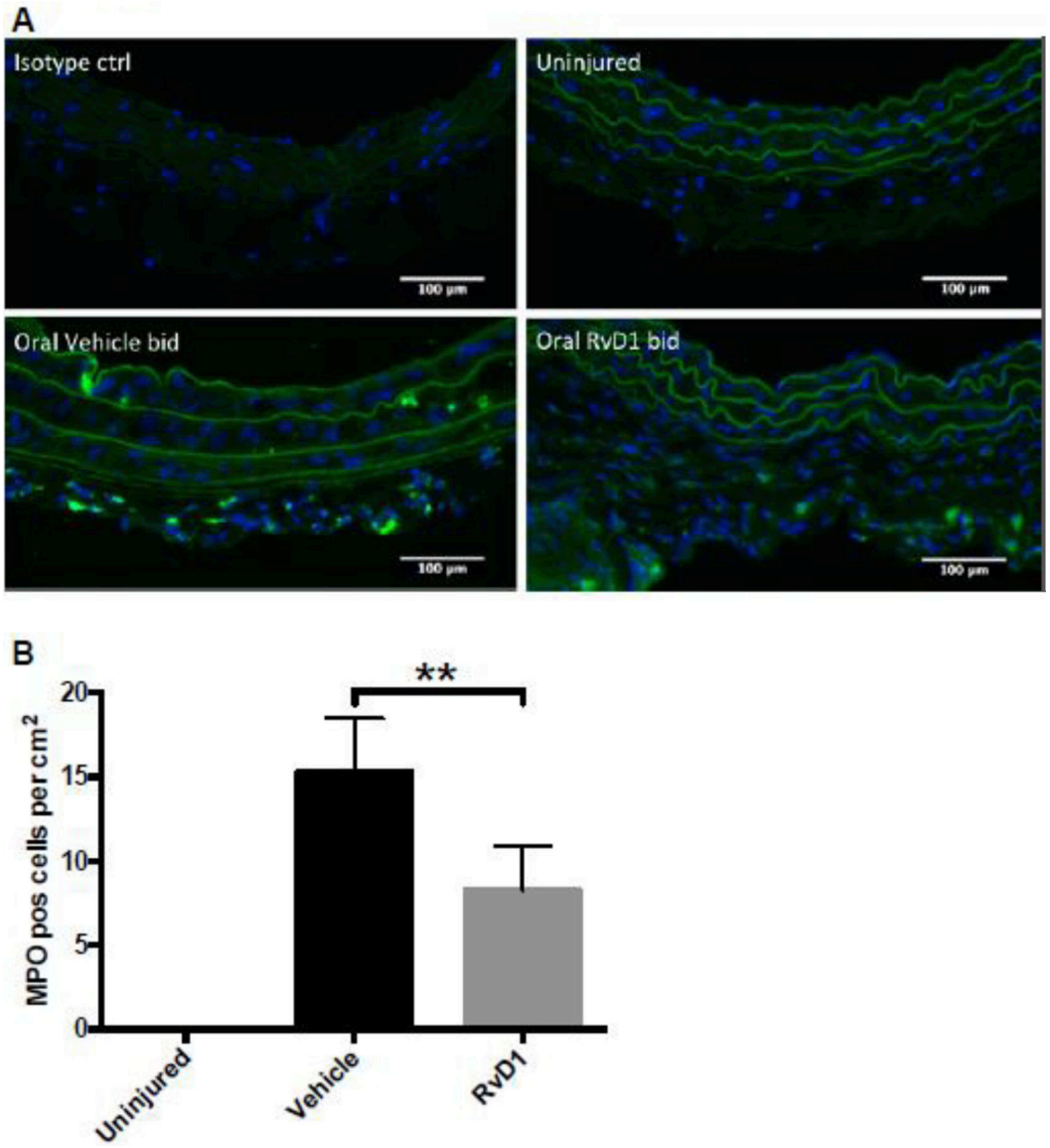

Figure 5. Oral 17S-RvD1 decreases MPO+ cells within the injured vascular wall.

MPO+ DAPI+ cells were counted and normalized by vessel area. Arteries harvested from rats treated with oral 17S-RvD1 showed a significant reduction of MPO+ cells within the vascular wall compared to those from the vehicle-treated rats (n=5; A & B). **P<0.01

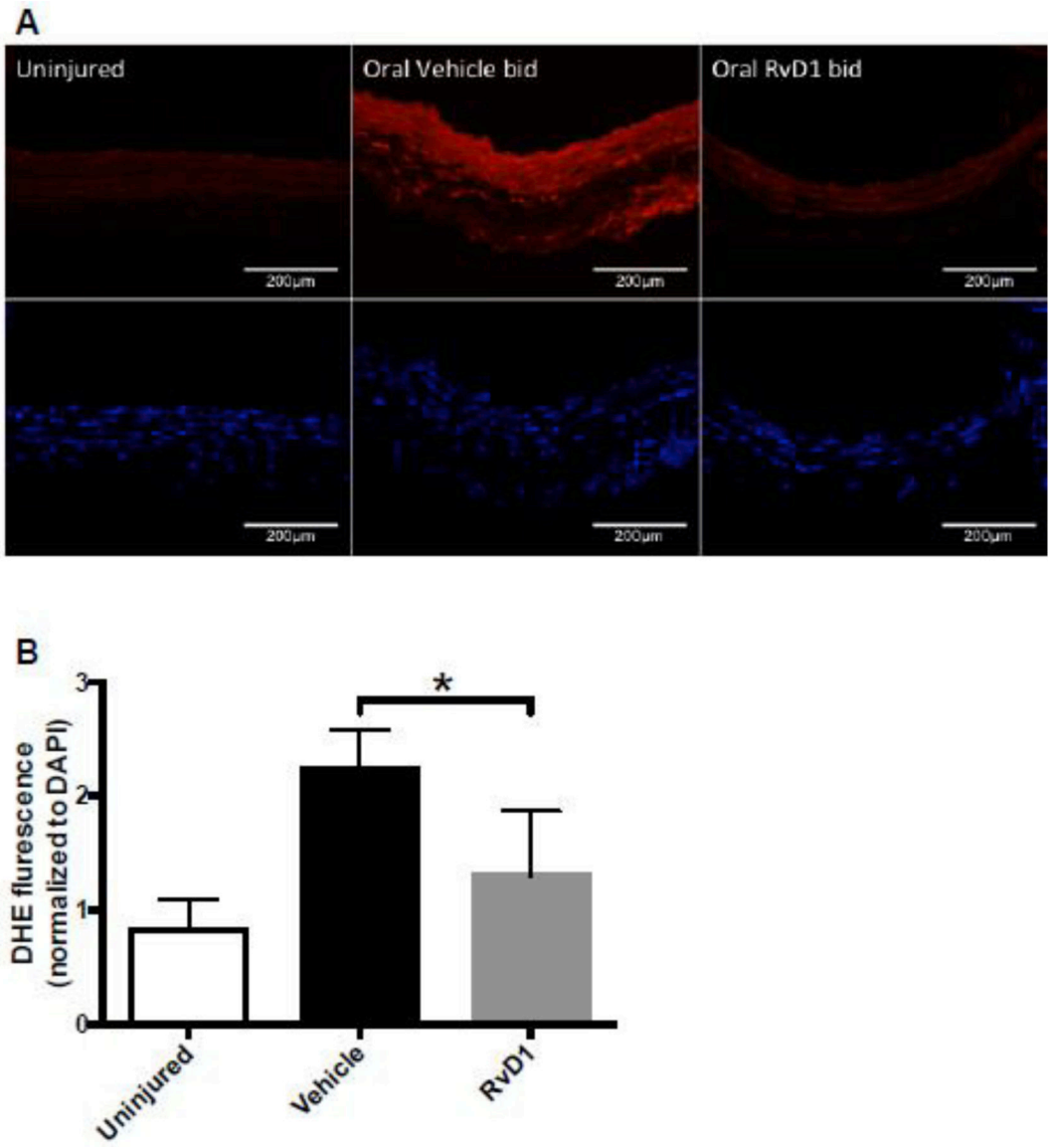

Figure 6. Oral 17S-RvD1 decreases ROS production within the injured vascular wall.

DHE fluorescence intensity was quantified and normalized by DAPI fluorescence intensity. Arteries harvested from rats treated with oral 17S-RvD1 showed a significant reduction in DHE signal within the vascular wall compared to those from the vehicle-treated rats (n=5; A & B). *P<0.05

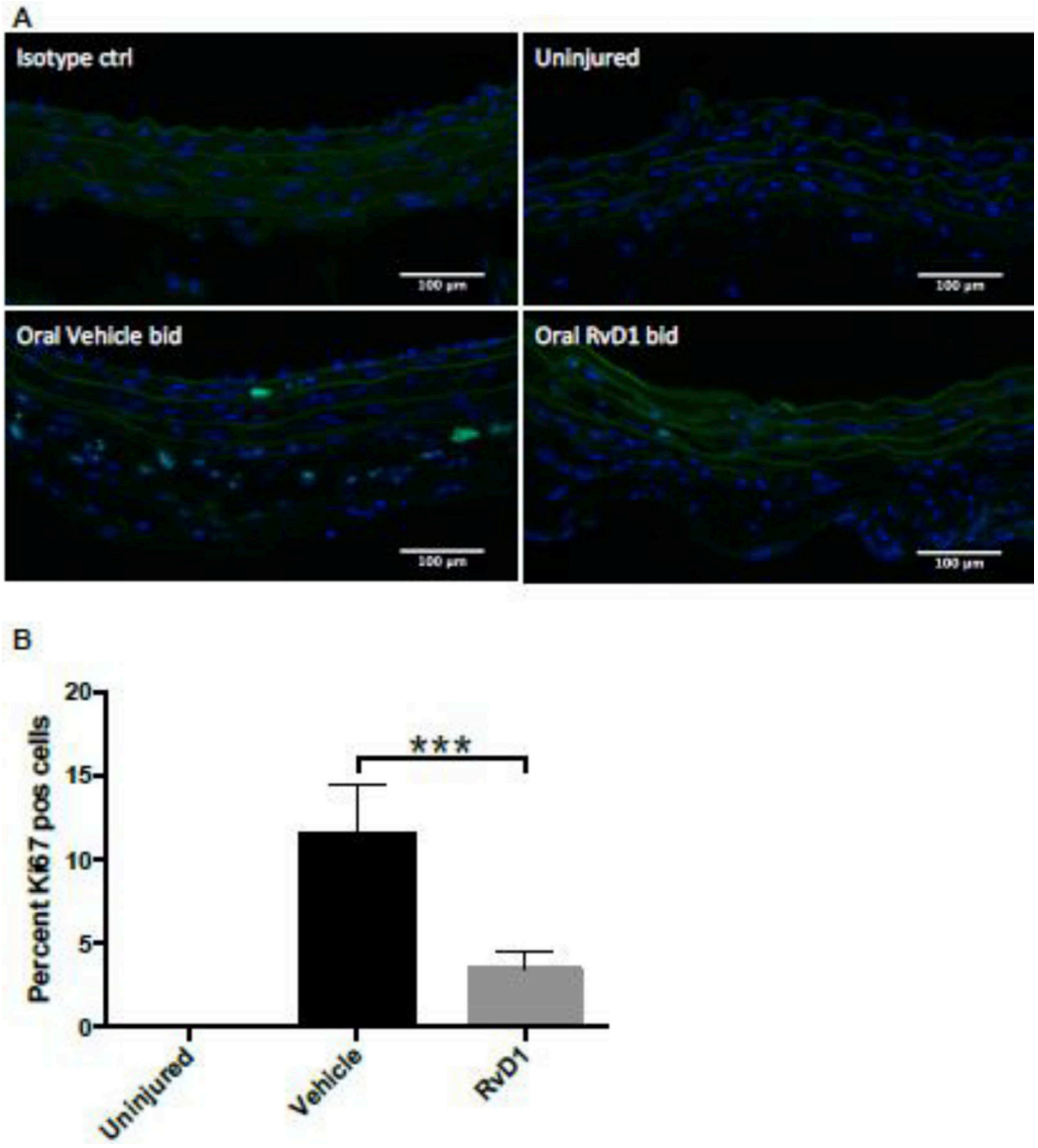

Figure 7. Oral 17S-RvD1 decreases Ki67+ cells within the injured vascular wall.

Ki-67+ DAPI+ cells were counted and normalized by the total number of cells (i.e. proliferative index). Arteries harvested from rats treated with oral 17S-RvD1 showed a significant reduction of Ki-67+ cells within the vascular wall compared to those from the vehicle-treated rats (n=5; A & B). ***P<0.001

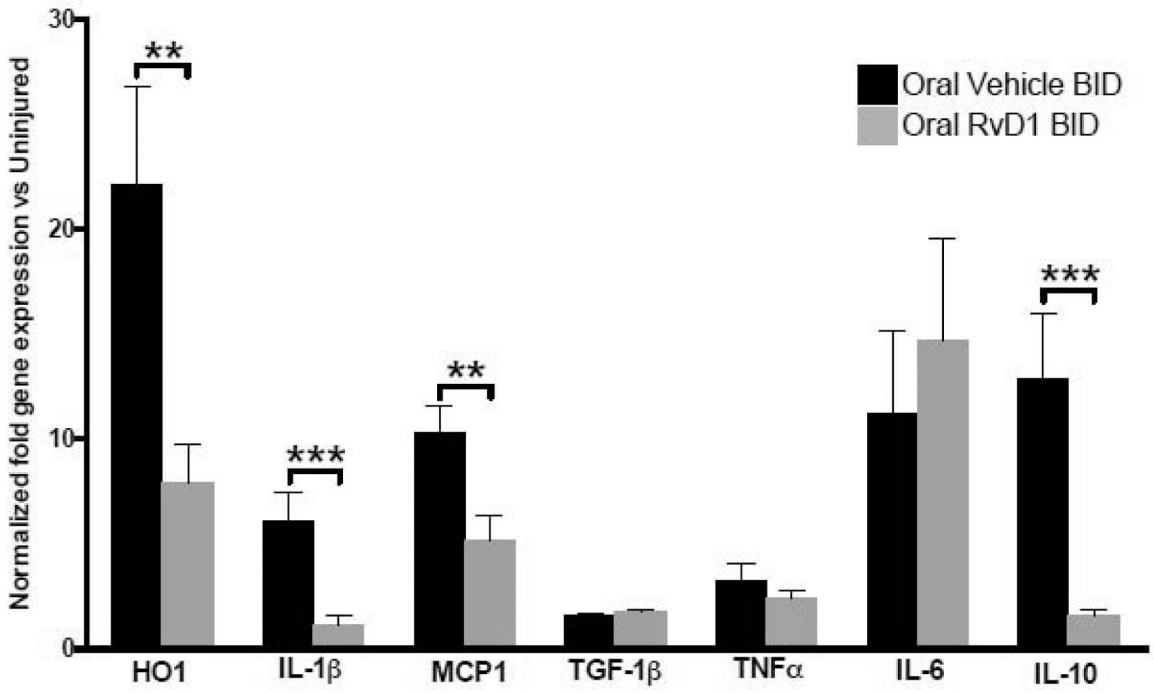

Figure 8. Oral 17S-RvD1 modulates cytokine gene expression within the injured vascular wall.

Fold gene expression from each injured artery was calculated by using the ΔΔCT method and was further normalized by the contralateral uninjured control. Rats treated with oral 17S-RvD1 showed a significant reduction in HO1, IL-1β, MCP1 and IL-10 gene expression within the injured vessel wall compared to the vehicle-treated rats. On the other hand, TGF-1β, TNFα and IL-6 gene expression did not show a significant change (n=12–16). **P<0.01, ***P<0.001

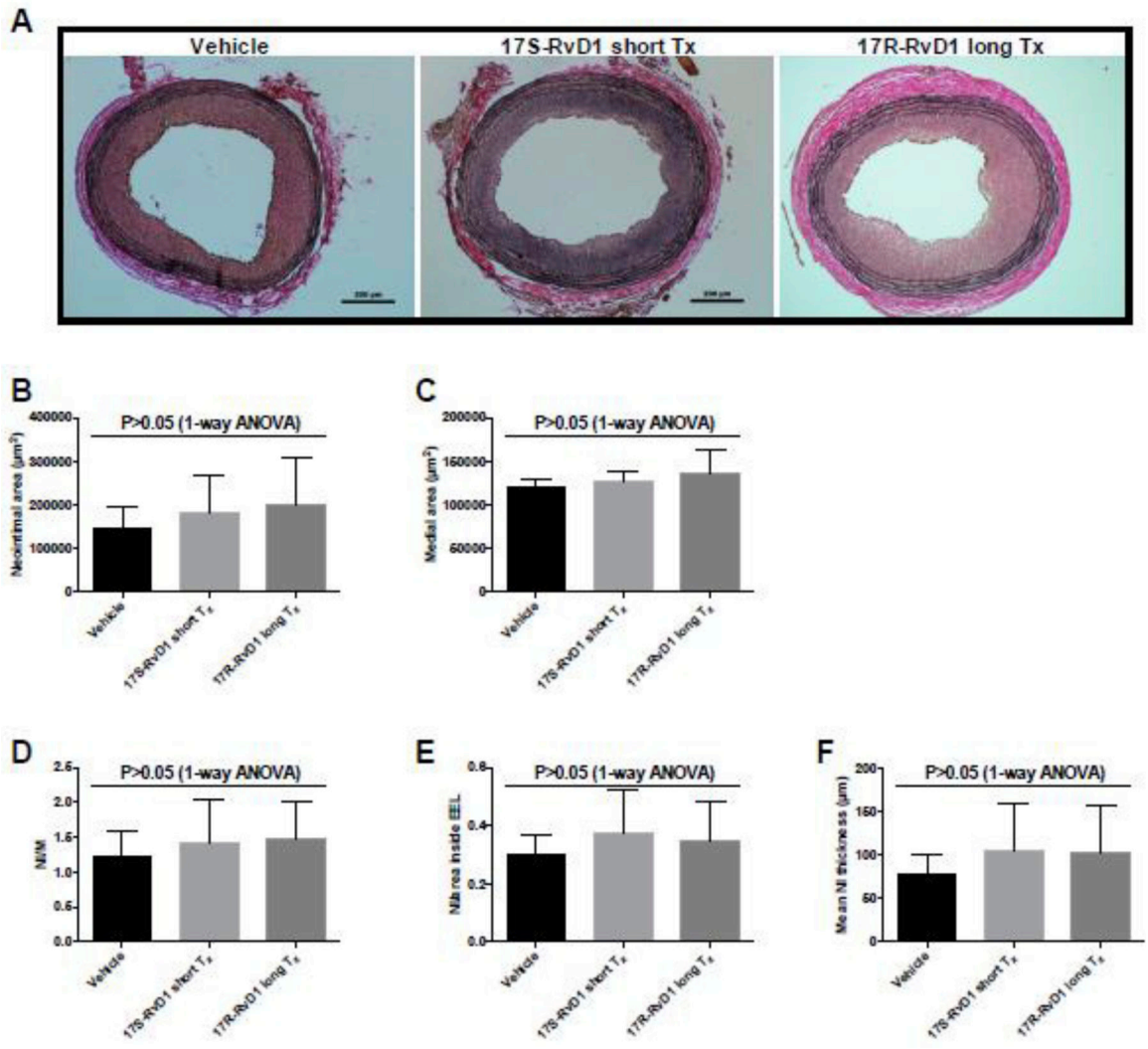

Figure 9. Oral 17S-RvD1 did not significantly affect and IH formation 14 days after balloon angioplasty.

Elastin stain was used to carry out morphometric analysis in rat carotid artery samples on POD14 (A). Whether the rats were treated with RvD1 (short or long treatment) or vehicle, injured carotid arteries did not show any significant difference in neointimal area (B), medial area (C), neointimal area normalized by medial area (D), neointimal area normalized by area inside the external elastic lamina (E) or mean neointimal thickness (F; n=5). P>0.05

Table 1:

Primers used in quantitative RT-PCR

Primer	Forward sequence	Reverse sequence
HO1	ACAGAGTTTCTTCGCCAGAGG	GGTCGCCAACAGGAAACTGA
TGF-β1	GAAGTCACCCGCGTGCTAAT	GCACTGCTTCCCGAATGTCT
MCP1	GCCTGTTGTTCACAGTTGCT	GTTCTCCAGCCGACTCATTG
TNFα	CCAAATGGGCTCCCTCTCAT	GGCTTGTCACTCGAGTTTTGA
IL-1β	AGGCTGACAGACCCCAAAAG	GTCGAGATGCTGCTGTGAGA
IL-6	AGCGATGATGCACTGTCAGA	TCCAGAAGACCAGAGCAGAT
IL-10	CCCTCTGGATACAGCTGCGA	ATGGCCTTGTAGACACCTTTGT
HPRT	TGACCTGCTGGATTACATTAAAGC	GTCATTACAGTAGCTCTTCAGTCTGATAAA