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Omega-3 Fatty Acid-Derived Mediators 17(R)-Hydroxy Docosahexaenoic Acid, Aspirin-Triggered Resolvin D1 and Resolvin D2 Prevent Experimental Colitis in Mice

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Resolvins of the D series are generated from docosahexaenoic acid, which are enriched in fish oils and are believed to exert beneficial roles on diverse inflammatory disorders, including inflammatory bowel disease (IBD). In this study, we investigated the anti-inflammatory effects of the aspirin-triggered resolvin D1 (AT-RvD1), its precursor (17(R)-hydroxy docosahexaenoic acid [17R-HDHA]) and resolvin D2 (RvD2) in dextran sulfate sodium (DSS)- or 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Our results showed that the systemic treatment with AT-RvD1, RvD2, or 17R-HDHA in a nanogram range greatly improved disease activity index, body weight loss, colonic damage, and polymorphonuclear infiltration in both colitis experimental models. Moreover, these treatments reduced colonic cytokine levels for TNF-α, IL-1β, MIP-2, and CXCL1/KC, as well as mRNA expression of NF-kB and the adhesion molecules VCAM-1, ICAM-1, and LFA-1. Furthermore, AT-RvD1, but not RvD2 or 17R-HDHA, depended on lipoxin A4 receptor (ALX) activation to inhibit IL-6, MCP-1, IFN-γ, and TNF-α levels in bone marrow-derived macrophages stimulated with LPS. Similarly, ALX blockade reversed the beneficial effects of AT-RvD1 in DSS-induced colitis. To our knowledge, our findings showed for the first time the anti-inflammatory effects of resolvins of the D series and precursor 17R-HDHA in preventing experimental colitis. We also demonstrated the relevant role exerted by ALX activation on proresolving action of AT-RvD1. Moreover, AT-RvD1 showed a higher potency than 17R-HDHA and RvD2 in preventing DSS-induced colitis. The results suggest that these lipid mediators possess a greater efficacy when compared with other currently used IBD therapies, such as monoclonal anti-TNF, and have the potential to be used for treating IBD. The Journal of Immunology, 2011, 187: 1957–1969.

During inflammatory disorders, tissue damage, or trauma leads to the release of endogenous chemical mediators that initiate inflammation, which is responsible for the pathogenesis and worsening of many chronic diseases (1, 2). However, most inflammatory processes are self-limiting and self-resolving systems, which are known as an active endogenous process aimed at protecting the host from exacerbated inflammation (2–4). It is now well established that in the process of resolution of inflammation, endogenous anti-inflammatory and/or proresolving mediators are released to control many important events, such as cell migration, activation, proliferation, and clearance of microorganisms and apoptotic cells (1, 2).

In this context, omega-3 (ω-3) polyunsaturated fatty acids (n-3 PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are enriched in some fish oils, are believed to exert beneficial effects on a wide range of human inflammatory disorders, such as inflammatory bowel disease (IBD), rheumatoid arthritis, asthma, cancer, and cardiovascular diseases (5–8). EPA and DHA originate the lipid mediators known as resolvins with two chemically unique structural forms, the E series and D series, respectively. These bioactive substances regulate critical cellular events in the resolution of inflammation (3, 9, 10). DHA is converted by human 15-lipoxygenase type I (15-LOX) to 17S-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-DHA (17S-HDHA, 17S-hydroxy-DHA or 17S-HDHA), which is the precursor of the D series resolvins such as resolvin D1 (RvD1) or 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-DHA and resolvin D2 (RvD2 or 7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-DHA). In the presence of aspirin, aspirin-acetylated cyclooxygenase-2 (COX-2) generates 17R-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-DHA (17R-hydroxy docosahexaenoic acid or 17R-HDHA), which following sequential oxygenation by 5-LOX, results in the production of 17-epi-RvD1 (17R-RvD1 or 7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E,19Z-DHA), also known as aspirin-triggered (AT)RvD1 (1, 11, 12). Furthermore, previous studies showed that the carbon 17S alcohol of RvD1 is enzymatically converted to 17-oxo-RvD1, which is essentially inactive, whereas the 17R alcohol configuration in its AT form (AT-RvD1) resists rapid inactivation (12).

Resolvins of the D series are reported to reduce inflammatory pain, block IL-1β transcripts induced by TNF-α in microglial

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Address correspondence and reprint requests to Dr. João B. Calixto, Departamento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88049-900, Florianópolis, Santa Catarina, Brazil. E-mail address: calixto@farmaco.ufsc.br or calixto3@terra.com.br

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Abbreviations used in this article: ω-3, omega-3; ALX, lipoxin A4 receptor; AT, aspirin-triggered; BMDM, bone marrow-derived macrophage; COX-2, cyclooxygenase-2; DAI, disease activity index; DHA, docosahexaenoic acid; DSS, dextran sulfate sodium; e.v., endovenously; EPA, eicosapentaenoic acid; GPR32, G protein-coupled receptor 32; IBD, inflammatory bowel disease; KC, keratinocyte-derived chemokine; 15-LOX, 15-lipoxygenase type I; MPO, myeloperoxidase; n-3 PUFA, ω-3 polyunsaturated fatty acid; NOS2, NO synthase 2; PMN, polymorphonuclear; 17S-HDHA, 17(R)-hydroxy docosahexaenoic acid; RvD1, resolvin D1; RvD2, resolvin D2; RvE1, resolvin E1; 17S-HDHA, 17S-hydroxy-docosahexaenoic acid; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

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cells, and also function as potent regulators limiting polymorphonuclear (PMN) infiltration into inflamed brain, skin, and peritoneum (11, 13–15). In addition, both 17R and 17S D series resolvins exhibit pronounced anti-inflammatory action in vivo (12, 13).

Crohn’s disease and ulcerative colitis are idiopathic inflammatory bowel disorders that lead to long term and occasionally irreversible impairment of gastrointestinal structure and function (16, 17). IBD are characterized by strong leukocyte activation and infiltration into the intestinal tissues, the release of proinflammatory cytokines (18) and enzymes, and the formation of reactive oxygen species. All these events may induce an extensive and unbalanced activation of the mucosal immune system driven by the commensal flora (19).

Recent evidence has suggested a role of PUFAs on ameliorating disease activity in both human and experimental IBD (20–24). These beneficial effects on disease severity have been in most cases, associated with a reduction in the production of arachidonic acid-derived eicosanoids, such as PGE₂, and downregulation of proinflammatory mediators, such as TNF-α, IL-1β, and leukotriene B₄ (20, 23, 24). Furthermore, the ω-3 fatty acid-derived mediator resolvin E1 (RvE1) has shown important positive effects on dextran sulfate sodium (DSS)- and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, reducing mortality and colon damage in addition to reducing PMN infiltration and production of TNF-α and IL-1β in colonic tissue (7, 25).

To further explore the beneficial effects of n-3 PUFAs and their derivatives on IBD, we investigated the anti-inflammatory properties and some of the molecular mechanisms underlying the effects of 17R-HDHA, AT-RvD1, and RvD2 in experimental models of colitis in mice. To our knowledge, our findings showed, for the first time, that 17R-HDHA, AT-RvD1, and RvD2 caused pronounced systemic anti-inflammatory effects in both DSS- and TNBS-induced colitis and suggested that these effects are primarily associated with the reduction in PMN infiltration as well as inhibition of release and/or expression of cytokines, chemokines, adhesion molecules, and transcription factors in the colonic tissue. Moreover, our results also suggested that AT-RvD1, but not RvD2 or 17R-HDHA, exerted its effects via a lipoxin A4 receptor (ALX)-dependent mechanism in vitro and in vivo.

Materials and Methods

Animals

Male BALB/c mice (8–10 wk of age) were obtained from the Laboratório de Farmacologia Experimental animal house, Universidade Federal de Santa Catarina (Florianópolis, Santa Catarina, Brazil) and housed in collective cages at 22 ± 1°C under a 12-h light/dark cycle (lights on at 07:00 h) with free access to laboratory chow and tap water. Experiments were performed during the light phase of the cycle. The experimental procedures were previously approved by the Universidade Federal de Santa Catarina’s Committee on the Ethical Use of Animals, where the study was carried out, and were conducted in accordance with Brazilian regulations on animal welfare.

Induction and assessment of DSS-induced colitis

Male BALB/c mice (n = 5–7/group) were provided with a solution of filtered water containing 3% DSS (w/v, 36,000–50,000) ad libitum over a 5 d period. Every other day, the DSS solution was replenished. Following this 5-d period, DSS was replaced with normal drinking water for 2 d, and at the end of the seventh day, the animals were euthanized. Control mice received only drinking water. All animals were examined once a day, and the disease activity index (DAI) was assessed as described previously (26, 27). DAI was the combined score of weight loss, stool consistency, and bleeding. Scores were defined as follows: stool consistency was graded 0 for no diarrhea, 2 for loose stool that did not stick to the anus, and 4 for liquid stool that did stick to the anus. The presence of fecal blood was graded 0 for none, 2 for moderate, and 4 for gross bleeding. For weight loss, a value of 0 was assigned if body weight remained within 1% of baseline or higher, 1 for a 1–5% loss, 2 for a 5–10% loss, 3 for a 10–15% loss, and 4 for weight loss >15%. At the end of the 7-d period, the colons were removed and examined for the consistency of the stool found within as well as the gross macroscopic appearance and length, which was measured from 1 cm above the anus to the top of the cecum. This macroscopic scoring was performed as previously described (Supplemental Table I) (28). The three scores for each animal were summed to provide the total macroscopic score. In another set of experiments, each excised portion of the distal colon was fixed immediately in 4% formaldehyde solution. Tissues were embedded in paraffin, sectioned (5 μm), mounted on glass slides, and deparaffinized. For a general histological analysis, slices were stained using H&E standard techniques. Samples were analyzed by light microscopy and scored as described previously (Supplemental Table II) (28). In this case, the experiments were performed in a double-blind manner.

Induction and assessment of TNBS-induced colitis

Colitis was induced by intracolonial administration of TNBS, as described previously (29). Briefly, 1-d fasted mice were slightly anesthetized with an i.p. injection of 10 mg/kg xylazine and 80 mg/kg ketamine. TNBS (1 mg in 100 μl 35% ethanol) was administered intrarectally using a polyethylene PE-50 catheter slowly inserted into the colon 4 cm proximal to the anus. The animals were kept in a head-down vertical position for 2 min. Control mice received 100 μl sterile 0.9% NaCl solution. Four hours later, the animals were given free access to food and water. Throughout the experiment, mice were monitored for body weight loss and overall mortality. Three days following TNBS administration, mice were sacrificed, and the colonic tissues were excised longitudinally. They were then rinsed with saline and scored for macroscopic damage as described previously (30, 31). Macroscopic damage was evaluated using the following scoring system: 0, no damage; 1, hyperemia without ulcers; 2, hyperemia with bowel wall thickening but no ulcers; 3, one site of ulceration without bowel wall thickening; 4, two or more sites of ulceration or inflammation; 5, 0.5 cm of inflammation and major damage; 6, at least 1 cm of major damage (for every additional 0.5 cm of damage, the score was increased by one to a maximum of 10); plus 1 for presence of diarrhea; plus 2 for presence of mild or severe adhesions, respectively (31). In another set of experiments, each excised portion of distal colon was immediately fixed in 4% formaldehyde solution. All tissues were embedded in paraffin and sectioned to 5-μm thickness, mounted on glass slides, and deparaffinized. For general histology and morphometric analysis, slices were stained using H&E standard techniques. Samples were analyzed by light microscopy.

Treatments

In DSS-induced colitis, animals were endovenously (e.v.) treated with 0.1, 0.3, or 1 μg/animal/17R-HDHA (17R-hydroxy-4Z,7Z,10Z,13Z,15Z,19Z-DHA), AT-RvD1 (7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15Z,19Z-DHA), or RvD2 (7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-DHA) from Cayman Chemical (Ann Arbor, MI) once a day from days 0 to 7. In another set of experiments, animals received an e.v. injection of N-BOC-PHE-GLY-PHE-LEU-GLY (BOC-1) (2 μg/kg/animal, e.v.) or vehicle, starting 1 h before TNBS instillation. All animals were sacrificed by cervical dislocation at 72 h after TNBS administration (i.e., 4 h after receiving the last treatment).

Myeloperoxidase assay

Neutrophil infiltration into colonic tissue was assessed indirectly by measuring myeloperoxidase (MPO) activity. MPO was performed as described previously (29, 30). On day 7 (DSS protocol) or day 3 (TNBS protocol), animals were killed, and colon tissue segments were homogenized in 5% EDTA/NaCl buffer (pH 4.7) and centrifuged at 10,000 × g for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyl trimethyl ammonium bromide buffer (pH 5.4), and the samples were frozen in liquid nitrogen and thawed three times. Upon thawing, the samples were similarly centrifuged, and 25 μl of the supernatant was used for the MPO enzymatic reaction was was added by the addition of 1.6 mM tetramethylbenzidine, 80 mM NaPO₄, and 0.3 mM hydrogen peroxide (H₂O₂). The absorbance was measured spectrophotometrically at 690 nm, and the results are expressed in OD per milligram of tissue.
**Determination of cytokine levels**

Briefly, colon segments were homogenized in phosphate buffer containing 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzothiazonium chloride, 10 mM EDTA, and 20 IU aprotinin A. The homogenate was centrifuged at 3000 × g for 10 min, and the supernatants were stored at −70°C for further analysis. TNF-α, IL-1β, MIP-2, keratinocyte-derived chemokine (CXC1/KC), IL-17, and IL-10 levels were evaluated using ELISA kits from R&D Systems (Minneapolis, MN), according to the manufacturer’s recommendations. The amount of protein in each sample was measured using the Bradford method (34).

**RNA extraction and real-time PCR**

Total RNA from colon or cells was extracted using the SV Total RNA Isolation System (Z3100; Promega, Madison, WI), according to the manufacturer’s instructions. The RNA concentration was determined using a NanoDrop 1100 (NanoDrop Technologies, Wilmington, DE). An amount of 50 ng (colony tissue) or 10 ng (cell culture) of total RNA was used for cDNA synthesis. A reverse transcription assay was performed as described in the Moloney murine leukemia virus reverse transcriptase protocol, according to the manufacturer’s instructions. cDNA was amplified in duplicate using the TaqMan Universal PCR Master Mix Kit with specific TaqMan Gene Expression target genes, the 3′-quencher MGB and FAM-labeled probes for mouse NF-κB (Mm00476361_m1), VCAM-1 (Mm01320970_m1), ICAM-1 (Mm005616024_g1), LFA-1 (Mm01278854_m1), COX-2 (Mm01307334_g1), NO synthase 2 (NOS2), inducible (Mm01309898_m1), and GAPDH (NM_000884.2) that was used as an endogenous control for normalization. The PCRs were performed in a 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA). The amplification reactions were incubated at 50°C for 2 min, 95°C for 10 min, 50 cycles of 95°C for 15 s, and 60°C for 1 min. Expression of the target genes was calibrated against conditions found in control animals (i.e., animals that received vehicle).

**Murine bone marrow-derived macrophage culture**

BALB/c mice were sacrificed by cervical dislocation. Total bone marrow was obtained from mice by flushing the femurs and tibiae with DMEM. The decanted citrate buffer of bone marrow-derived macrophages (BMDM) has been described previously (35). Briefly, bone marrow mononuclear phagocyte precursor cells were propagated in suspension by culturing in macrophage medium (DMEM containing glucose, supplemented with 2 mM Glutamine, 10% FCS, 10 mM HEPES, 100 U/ml penicillin, and 100 U/ml streptomycin) and 10% L929 cell-conditioned medium. Cells were harvested on day 7, and 2 × 10^5 cells/ml were cultured in a 96-well culture plate for 24 h. Afterward, adherent cells were stimulated for 24 h with LPS (1 μg/ml) in the presence or absence of 17R-HDHA (100 and 300 nM), AT-RvD1 (100 and 300 nM), RvD2 (100 and 300 nM), BOC-1 (10 μM), and BOC-1 (10 μM) plus 17R-HDHA (300 nM), AT-RvD1 (300 nM), or RvD2 (300 nM) per well in a total volume of 200 μl. After stimulation, the plate was centrifuged (200 × g, 10 min), and the cell-free supernatant was collected and stored at −70°C for cytokine determination. A cytokine bead array Mouse Inflammation Kit was used to measure IL-6, MCP-1, IFN-γ, TNF-α, and IL-10 using the BD FACSCanto II (BD Biosciences, San Diego, CA). Real-time PCR was performed for COX-2 and NOS2 using the adherent cells as described above.

**Immunohistochemistry analysis**

Immunohistochemical reaction was performed using the colon according to previously described methods (36). Slices (5 μm) were stained using the following primary Ab and respective dilution: monoclonal mouse anti–phospho-p65 NF-κB (1:50). High-temperature Ag retrieval was performed by the immersion of the slides in a water bath at 95–98°C in a 10 mM trisodium citrate buffer of pH 6.0 for 45 min. Non-specific binding was blocked by incubating sections for 1 h with goat normal serum diluted in PBS. After overnight incubation at 4°C with primary Ab, the slices were washed with PBS and incubated with the secondary Ab EnVision Plus (ready-to-use) for 1 h at room temperature. After the appropriate biotinylated secondary Ab, immune complexes were visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DakoCytomation, Glostrup, Denmark) in 0.03% H2O2 in PBS (for the exact amount of time: 1 min). The reaction was stopped by thorough washing in water and counterstained with Harris’ hematoxylin. Besides staining untreated animals as negative controls, sections were incubated with isotype-matched primary Ab of irrelevant specificity, or the primary Ab was omitted. Despite Ag retrieval, these controls resulted in little or no staining, principally because peroxide pretreatment (inactivation of endogenous peroxidase) appears to destroy the epitopes to which antimouse (secondary) Ab otherwise binds. Images of colon sections stained with Abs to anti–phospho-p65 NF-κB were obtained using a Sight DS-5M-L1 digital camera connected to an Eclipse 50i light microscope (both from Nikon, Melville, NY). Settings for image acquisition were identical for control and experimental tissues. Four ocular fields per section (five to eight mice per group) were captured, and a threshold OD that best discriminated staining from the background was obtained using the NIH Image J 1.36b imaging software (National Institutes of Health, Bethesda, MD). The total pixel intensity was determined, and data were expressed as OD, using a counting grid at x40 magnification.
PUFA-derived mediators AT-RvD1, RvD2, and 17R-HDHA decrease leukocyte influx and improve microscopic colon damage

DSS-induced colon damage is associated with an influx of inflammatory cells, such as neutrophils, into the intestinal mucosa (17). Therefore, we assessed whether the protective effect of the lipid mediators AT-RvD1, RvD2, or 17R-HDHA in DSS-mediated colitis was associated with alterations in the inflammatory cell infiltration of the intestinal mucosa. Seven days after the initiation of DSS treatment, mucosal neutrophil infiltration into the colon were indirectly assessed by measuring MPO activity. DSS-treated mice displayed a relevant increase in colonic MPO levels compared with control animals (Fig. 2A–C). Systemic treatment with AT-RvD1 (A), RvD2 (B), or 17R-HDHA (C) reduced body weight loss, improved the DAI, ameliorated colon macroscopic damage, and enhanced colon length when compared with mice from the DSS alone group. Representative photographs of colons from control mice (control), DSS-treated mice (DSS 3%), and mice treated with AT-RvD1–, RvD2–, and 17R-HDHA–treated mice (1 μg/animal, e.v.) (Fig. 2D). Data are reported as means ± SEM of five to seven mice per group. #p < 0.05 versus the control group, *p < 0.05 versus the DSS-treated group.

To further confirm the results from the MPO analysis, colons were processed for histological observation. In colons from the control group, no histopathological changes were observed. In contrast, the representative sections of H&E staining revealed a pronounced cell infiltration into the lamina propria and colonic mucosa from DSS-treated mice. Moreover, the colonic tissue from DSS-treated mice appeared thick and sometimes ulcerated, showing a distortion of crypts, which resulted in microscopic damage (Fig. 2D, 2E). Interestingly, the histological evaluation of colons from AT-RvD1–, RvD2–, or 17R-HDHA–treated mice (1 μg/animal, e.v.) revealed a clear reduction in the inflammatory response, resulting in a prominent decrease in microscopic tissue damage compared with colons from DSS-treated mice (Fig. 2D, 2E).

AT-RvD1, RvD2, and 17R-HDHA treatment reduce colonic protein levels and mRNA expression of inflammatory mediators

Accumulating data in the literature suggest that cytokines and chemokines, such as TNF-α, IL-1β, MIP-2, and keratinocyte-derived chemokine (CXCL-1/KC), are critically involved in in-
Inflammatory cell recruitment (29, 37). Therefore, on the seventh day after the induction of colitis, we assessed the colonic protein levels of these inflammatory mediators. DSS administration resulted in a pronounced increase in colonic TNF-α, IL-1β, MIP-2, and CXCL1/KC protein levels (Fig. 3A–D). Systemic treatment with AT-RvD1, RvD2, or 17R-HDHA (1 mg/animal, e.v.) resulted in a significant decrease in the protein levels of these mediators (Fig. 3A–D).

In addition, the modulation of colonic proinflammatory cytokine IL-17 and anti-inflammatory cytokine IL-10 was also investigated. DSS treatment did not significantly alter protein levels of IL-17 compared with control mice (Fig. 3E). However, DSS resulted in a significant reduction of IL-10 protein levels in the mouse colon (Fig. 3F). Treatment with AT-RvD1, RvD2, or 17R-HDHA (1 μg/animal, e.v.) resulted in a significant decrease in the protein levels of these mediators (Fig. 3A–D).

In addition, the modulation of colonic proinflammatory cytokine IL-17 and anti-inflammatory cytokine IL-10 was also investigated. DSS treatment did not significantly alter protein levels of IL-17 compared with control mice (Fig. 3E). However, DSS resulted in a significant reduction of IL-10 protein levels in the mouse colon (Fig. 3F). Treatment with AT-RvD1, RvD2, or 17R-HDHA (1 μg/animal, e.v.) resulted in a significant decrease in the protein levels of these mediators (Fig. 3A–D).

Furthermore, we evaluated the mRNA expression of two important inflammatory mediators, COX-2 and NOS2, directly in BMDM culture stimulated with LPS, because macrophages are one of the most important inflammatory cells in the pathogenesis of human and experimental IBD (17). LPS incubation for 24 h induced strong mRNA expression of both COX-2 and NOS2 in macrophages (Fig. 3G, 3H). The presence of 100 nM (data not shown) or 300 nM AT-RvD1, RvD2, or 17R-HDHA significantly reduced COX-2 mRNA expression but interestingly did not alter NOS2 mRNA expression when compared with the group treated with LPS alone (Fig. 3G, 3H).

AT-RvD1, RvD2, and 17R-HDHA inhibit the NF-κB pathway and decrease expression of adhesion molecule in mouse colon with DSS-induced colitis

Activation of NF-κB is implicated in the pathogenesis of experimental colitis (36) and involves the transcription of several inflammatory genes, such as adhesion molecules in numerous inflammatory disorders (38). Thus, we evaluated whether systemic treatment with AT-RvD1, RvD2, or 17R-HDHA could regulate NF-κB mRNA expression and p65 NF-κB subunit protein activation by immunohistochemistry in DSS-induced colitis. Furthermore, we also assessed mRNA expression of the adhesion molecules VCAM-1, ICAM-1, and LFA-1 in mouse colon. Our results showed a significant reduction of NF-κB mRNA expression and protein activation in animals treated with AT-RvD1, RvD2, or 17R-HDHA compared with DSS-treated animals (Fig. 4A–C). Interestingly, this reduction of NF-κB activation in AT-RvD1–, RvD2–, or 17R-HDHA–treated animals was accompanied by a significant reduction in VCAM-1, ICAM-1, and LFA-1 mRNA expression compared with the group treated with DSS alone (Fig. 4D–F). These data suggest that the impairment of NF-κB activation by the DHA-derived mediators could suppress the
expression of adhesion molecules and indirectly reduce PMN infiltration of intestinal mucosa during experimental colitis.

Treatment with AT-RvD1, RvD2, or 17R-HDHA protect mice against TNBS-induced colitis

Recently, our group has shown that 72 h after TNBS administration, mice developed severe diarrhea, striking hyperemia, necrosis, and inflammation in the gut accompanied by an extensive wasting disease, rectal prolapsed, and sustained weight loss (29). To evaluate the anti-inflammatory effects of AT-RvD1, RvD2, or 17R-HDHA (1 μg/animal) once per day reduced colonic levels of TNF-α (A), IL-1β (B), MIP-2 (C), and CXCL1/KC (D) but did not alter IL-17 (E) and IL-10 (F) protein levels as analyzed by ELISA. In addition, macrophages from bone marrow (G, H) of naive mice were stimulated with LPS (1 μg/well) in the presence or absence of AT-RvD1, RvD2, or 17R-HDHA (300 nM/well) for 24 h, and the adherent cells were analyzed for COX-2 and NOS2 mRNA expression by real-time PCR. AT-RvD1, RvD2, or 17R-HDHA incubation significantly reduced (G) mRNA COX-2 but did not inhibit (H) NOS2 mRNA when compared with the LPS-treated group. Real-time PCR assay was performed in duplicate, and GAPDH mRNA was used to normalize the relative amount of mRNA. Data are reported as means ± SEM of five to seven mice. *p < 0.05 versus the control group, **p < 0.05 versus the DSS-treated group.

AT-RvD1 reduces proinflammatory cytokine and chemokine production in vitro through ALX activation

Recent findings have shown that RvD1 exerts part of its pro-resolving actions via interactions with ALX, also known as formyl peptide receptor 2, present in the inflammatory cells such as macrophages (39). For this reason, we verified whether the ALX-selective antagonist, BOC-1, is capable of blocking the effects of the lipid mediators AT-RvD1, RvD2, or 17R-HDHA on cytokine and chemokine release from BMDM culture stimulated with LPS. Our results showed that in vitro LPS stimulation (1 μg/ml, for 24 h) produced a prominent increase protein expression of the cytokines IL-6, MCP-1, IFN-γ, and TNF-α (Fig. 6). The in vitro
treatment with AT-RvD1 at 100 nM (data not shown) and 300 nM significantly reduced IL-6, MCP-1, IFN-γ, and TNF-α (Fig. 6A–D) released from the macrophages, when compared with the group treated with LPS alone. The treatment with BOC-1 (10 μM) did not change cytokine levels when compared with the macrophages treated with LPS alone, but it significantly reversed the inhibitory effect of AT-RvD1 on cytokine release (Fig. 6A–D).

Meanwhile, incubation with RvD2 or 17R-HDHA at 100 nM (data not shown) and 300 nM also reduced IL-6, MCP-1, IFN-γ, and TNF-α protein release from LPS-stimulated macrophages, but their inhibitory effects on cytokine levels were not affected in the presence of the ALX antagonist. These results suggest that the anti-inflammatory effect displayed by AT-RvD1, but not RvD2 or 17R-HDHA, in experimental colitis can be partially mediated by the interaction of AT-RvD1 with the ALX receptor. It is of note that no significant differences between 100 and 300 nM concentrations of n-3 PUFA-derived mediators were observed on cytokine levels.

Pharmacological blockade of ALX reverses AT-RvD1 beneficial effects in DSS-induced colitis

RvD1 has been shown earlier to be a selective agonist of the ALX receptor (39), and the activation of the ALX receptor has been demonstrated to decrease colon inflammation in different experimental models (25, 40). In accordance with our in vitro results and previous reports, we investigated whether an ALX-selective antagonist could prevent the anti-inflammatory effect of AT-RvD1 in DSS-induced colitis. For this purpose, mice were treated once a day with the selective ALX receptor antagonist BOC-1 (2 mg/kg, e.v.) alone or in combination with AT-RvD1 (0.3 μg/animal, e.v.) for 7 d. The treatment with BOC-1 alone did not alter the inflammatory parameters observed in the DSS-treated group (Fig. 7). However, BOC-1 notably reversed the protective effect of AT-RvD1 against body weight loss, DAI, macroscopic colon damage, and colon length reduction (Fig. 7), indicating a functional ALX-dependent mechanism in the amelioration of inflammatory signals of DSS-induced colitis (Fig. 8).

Discussion

Human necessity for the nutritional supply of essential polyunsaturated fatty acids, such as ω-3, have been highlighted in recent years because these fatty acids are precursors of lipid mediators that are critical for a variety of cellular signaling pathways and extremely important for the resolution of many inflammatory disorders (10, 25). In this context, the current study demonstrated for the first time, to our knowledge, that systemic

FIGURE 4. AT-RvD1, RvD2, and 17R-HDHA inhibit the NF-κB pathway and decrease expression of adhesion molecules in mouse colon with DSS-induced colitis. Following a 7-d AT-RvD1, RvD2, or 17R-HDHA treatment, colon samples were processed for immunohistochemistry analysis or mRNA expression. Systemic treatment with AT-RvD1, RvD2, or 17R-HDHA (1 μg/animal) significantly reduced phospho (p)-p65 (an NF-κB subunit) immunostaining, compared with the DSS-alone group (A, B). At the same dose, the lipid mediators suppressed NF-κB (C), VCAM-1 (D), ICAM-1 (E), and LFA-1 mRNA (F) expression in mouse colon with DSS-induced colitis. The mean intensity for p-p65 NF-κB staining was determined from image analysis and is represented as arbitrary units. Original magnification ×400. Real-time PCR assay was performed in duplicate, and GAPDH mRNA was used to normalize the relative amount of mRNA. Data are reported as means ± SEM of five to seven mice. #p < 0.05 versus the control group, *p < 0.05 versus the DSS-treated group.
treatment with ω-3 fatty acid-derived mediators AT-RvD1, its precursor 17R-HDHA and RvD2 protected mice from the severity of both DSS and TNBS-induced colitis and were associated with a reduction of proinflammatory mediators and consequent amelioration of disease signals.

IBDs represent dysregulated immune response to commensal microbiota in a genetically susceptible host (7) and are known as debilitating diseases of the gastrointestinal tract that affect millions of people worldwide. Experimentally, the administration of DSS polymers in drinking water leads to acute colitis characterized by bloody diarrhea, body weight loss, ulcerations, and infiltrations with granulocytes (41). In this paper, we showed that the treatment with AT-RvD1, RvD2, or 17R-HDHA consistently decreased the DAI, body weight loss, colonic tissue damage, and cellular infiltration of epithelial mucosa in DSS-induced colitis. In addition, our results also demonstrated a prominent improvement in survival, body weight loss, and macro- and microscopic damage in TNBS-induced colitis. In fact, PUFAs are potent anti-inflammatory mediators in controlling disease activity in DSS-induced colitis. For example, oral administration of DHA has been shown to result in a significant inhibition of body weight loss, colon shortening, and histological damage (20). Furthermore, systemic treatment with RvE1, derived from EPA, has shown marked reduction in inflammatory signals in DSS- and TNBS-induced colitis (25), which were associated with a decrease in PMN infiltration in mouse colon (25).

The cellular infiltrate have pathogenic roles in animal models of IBD, and its control is extremely important for the attenuation of colitis (29). Recently, studies have demonstrated that RvD1 and RvD2 are potent regulators of leukocyte activation and migration (33, 39), suggesting a possible action on cell infiltration in experimental colitis. In our results, colon from DSS-treated mice showed high MPO activity, indicating strong PMN infiltration. Interestingly, we showed that systemic treatment with AT-RvD1, RvD2, or 17R-HDHA significantly blocked MPO activity in DSS- and TNBS-induced colitis, suggesting an impairment of PMN infiltration in colonic tissue. Likewise, fish oil rich in both EPA and DHA has been demonstrated to significantly reduce MPO activity in mouse colon (22, 23). In addition, RvE1 has been shown to suppress PMN infiltration of colonic tissue with TNBS-induced colitis (25), as well as diminish MPO activity in mouse paws treated with carrageenan (15).

Our results showed that the prevention of cell infiltration and colon damage observed in mice systemically treated with AT-RvD1, RvD2, or 17R-HDHA were likely to be associated with reduced levels of the proinflammatory cytokines TNF-α and IL-1β and the chemokines MIP-2 and CXCL1/KC, soluble mediators involved in cellular migration and adhesion molecule upregulation (37). The cytokines TNF-α and IL-1β play a pivotal role in pathogenesis of human and experimental colitis (42, 43), and Abs against TNF-α are largely used in the clinical treatment of IBD (44). Previous reports have demonstrated that RvE1 treatment

FIGURE 5. Treatment with AT-RvD1, RvD2, and 17R-HDHA protect mice against TNBS-induced colitis. After 1 d of fasting, mice were anesthetized and given 100 μl TNBS (1 mg/animal in 35% ethanol) into the colon. Systemic treatment with AT-RvD1, RvD2, or 17R-HDHA (1 μg/animal) per day from days 0 to 3 significantly improved survival (A), body weight loss (B), colonic macroscopic damage (C), reduced MPO activity (D), and microscopic damage (E), when compared with the TNBS-alone group. F, Representative paraffin sections of colons from control mice (control), TNBS-treated mice (TNBS), and mice treated with AT-RvD1, RvD2, or 17R-HDHA (1 μg/animal, e.v.) stained with H&E. Original magnification ×100. Data are reported as means ± SEM of six to eight mice per group. #p < 0.05 versus vehicle-treated control group, *p < 0.05 versus TNBS-treated group.
abolishes colonic mRNA expression of TNF-α and IL-1β in DSS- and TNBS-induced colitis (7, 25), which are associated with a dramatic inhibition of PMN infiltration of the intestinal mucosa. Furthermore, the reduction of MIP-2 and CXCL1/KC levels observed in our results could be associated with diminished MPO activity, because these chemokines are implicated in PMN migration to the inflamed colon (29).

Some evidence has emerged indicating that cytokines and chemokines can upregulate adhesion molecule expression (45, 46), which are critical for leukocyte adhesion and transmigration from blood to inflamed tissue (47). In this paper, we demonstrated an apparent association between cytokine/chemokine levels and adhesion molecule expression, because systemic treatment with AT-RvD1, RvD2, or 17R-HDHA reduced the cytokines and chemokines described earlier and mRNA expression for the adhesion molecules VCAM-1, ICAM-1, and LFA-1 in mouse colon with DSS-induced colitis. The expression of VCAM-1 and ICAM-1 is a major determinant of leukocyte recruitment to the inflamed intestine (48). However, VCAM-1 immunoneutralization has shown higher therapeutic effects than that of ICAM-1 in experimental colitis (49). Furthermore, our findings are in line with previous studies that have shown that RvE1 and RvD1 reduce PMN transmigration across choroidal retinal endothelial cells stimulated with IL-1β and that this effect is dependent on VCAM-1 expression (50). In addition, in vitro incubation with EPA or DHA has been shown to decrease VCAM-1, ICAM-1, and LFA-1 expression in human epithelial cells stimulated with LPS (51).

The expression of adhesion molecules can be regulated by proinflammatory cytokines in an NF-κB–dependent manner (38), which is activated in experimental colitis (36). Therefore, we investigated whether the effect of PUFA-derived mediators on the reduction of inflammatory mediators was associated with the inhibition of NF-κB. Our data clearly demonstrated that the systemic administration of AT-RvD1, RvD2, or 17R-HDHA markedly inhibited NF-κB protein phosphorylation and mRNA expression in mouse colon with DSS-induced colitis. Our data confirm and extend earlier studies that have demonstrated that NF-κB is downregulated in the presence of DHA or RvE1 (7, 52) and that systemic treatment with 17R-HDHA or AT-RvD1 decrease NF-κB activation that is associated with inhibition of TNF-α and IL-1β in a rat hind paw with experimental arthritis (53).

FIGURE 6. AT-RvD1 reduces proinflammatory cytokine and chemokine production in macrophages stimulated with LPS through ALX activation. Bone marrow-derived macrophages from naive mice were stimulated with LPS (1 μg/ml) in the presence or absence of AT-RvD1, RvD2, or 17R-HDHA (300 nM/well) or in combination with BOC-1, an ALX selective antagonist (10 μM/well) for 24 h, and the culture supernatants were analyzed for cytokine levels using a cytokine bead array kit. AT-RvD1 reduced production of IL-6, MCP-1, IFN-γ, and TNF-α (A–D), which was significantly reversed in the presence of BOC-1. The incubation with RvD2 (E–H) or 17R-HDHA (I–L) also reduced production of IL-6, MCP-1, IFN-γ, and TNF-α, when compared with the group treated with LPS alone, but this effect was not reversed in the presence of BOC-1 (E). Data are reported as means ± SEM (n = 3/group). #p < 0.05 versus control group, *p < 0.05 versus LPS-treated group, Δp < 0.05 versus the AT-RvD1–treated group.
Considering the above findings and our data, it is tempting to suggest that the production of cytokines/chemokines and down-regulation of the NF-κB pathway could be just a consequence of decreased cell migration to the colon. For this reason, we also tested the direct effects of AT-RvD1, RvD2, or 17R-HDHA on macrophages stimulated with LPS. Macrophages develop an important pathological role in IBD, which is associated with the production of high levels of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β (54), as well as the expression and activity of COX-2 and NOS2 (55). Our results showed that AT-RvD1, RvD2, or 17R-HDHA in vitro incubation significantly reduced COX-2 but not NOS2 mRNA expression in BMDMs stimulated with LPS. The reduction of COX-2 and NOS2 expression by PUFAs has been described in experimental colitis (22, 25), and it has been reported that these mediators contribute to the pathogenesis of IBD (56). However, our results for NOS2 expression are in agreement with a previous report that showed that DHA did not inhibit NOS2 expression in vascular smooth muscle cells stimulated with LPS (57). Furthermore, data from the literature suggest NOS2 and NO are critical mediators of cell apoptosis in phagocytosis and intracellular killing of bacteria (58, 59).

Next, we performed another set of experiments to clarify the pathway underlying AT-RvD1–, RvD2–, or 17R-HDHA–mediated reduction of inflammatory mediators. We stimulated BMDM cultures with LPS and incubated with AT-RvD1, RvD2, or 17R-HDHA in the presence or absence of BOC-1 (ALX selective antagonist), because previous literature suggest that RvD1 seems to act and bind to the ALX receptor (39). Our results demonstrated that AT-RvD1, but not RvD2 or 17R-HDHA, inhibited cytokine
levels of IL-6, MCP-1, IFN-γ, and TNF-α in LPS-stimulated macrophages in an ALX-dependent manner. On the basis of the above data, we also investigated whether systemic BOC-1 administration could reverse the beneficial effects of AT-RvD1 in DSS-induced colitis. Consistent with our in vitro results, treatment with AT-RvD1 plus BOC-1 prevented the anti-inflammatory effects of AT-RvD1 on DAI, body weight loss, colon damage, and shortening. A recent study showed that pretreatment with BOC-1 significantly reversed the positive effects of RvD1 on LPS-induced acute lung injury in mice (60). In this way, our results confirm the intrinsic interaction of ALX with AT-RvD1 to exert their anti-inflammatory and proresolution activities.

As mentioned earlier, AT-RvD1 is a metabolite of 17R-HDHA created in the presence of aspirin by a series of reactions that include enzymatic epoxidation and hydrolysis (12). Both RvD1 and AT-RvD1 have shown to exhibit an equipotent dose-dependent decrease in PMN infiltration in murine peritonitis, and consequently, no significant differences in their potencies have been observed, suggesting that they share a common site of action on PMNs (12). In contrast, a previous study has suggested that 17R-HDHA could generate different AT resolvins, such as AT-RvD1–4 (1). Furthermore, a previous report demonstrated that the colon tissue of fat-1 transgenic mice, which have an increased n-3 PUFA status, generates higher levels of bioactive n-3 PUFA-derived lipid mediators, such as RvE1 and RvD3, and showed protection against DSS-induced colitis (61). These data support the hypothesis that the proresolving actions of 17R-HDHA during intestinal inflammation could be, at least partly, due to conversion of 17R-HDHA in resolvins of the D series. Surprisingly, we did not observe any reversion of 17R-HDHA effects on decreasing cytokine production by ALX blockade in LPS-stimulated macrophages.

Another target of AT-RvD1 is the G protein-coupled receptor 32 (GPR32), an orphan receptor (39). Earlier data have demonstrated that ALX or GPR32 increases the ability of RvD1 to enhance phagocytosis, suggesting that the RvD1 response is both ALX and GPR32 dependent (39). However, human GPR32 has not revealed apparent candidates with significant sequence homology, and the murine counterparts of human GPR32 remain unknown, which makes it difficult to clarify the real role of GPR32 in RvD1 action (39). In addition, recent findings have demonstrated that RvD2 protects mice in a cecal ligation and puncture model by reducing excessive leukocyte infiltration and cytokine production as well as enhancing microbe clearance, thus preventing sepsis-induced lethality (33). Nevertheless, the possible receptor or target interactions for RvD2 remain unknown.

In conclusion, we have demonstrated for the first time, to our knowledge, that the n-3 PUFA-derived mediator AT-RvD1, RvD2, and 17R-HDHA are effective in preventing colitis in two different models of intestinal inflammation. Our results showed that their beneficial actions were mainly associated with their ability to inhibit PMN infiltration, downregulate NF-κB, and reduce proinflammatory cytokines, chemokines, and some adhesion molecules. Furthermore, we have established the first experimental evidence, to our knowledge, that the epimer of RvD1, AT-RvD1, exerts its positive effects in an ALX-dependent way (Fig. 8).
8). Taken together, our findings strongly suggest that the precursor of resolvin s of the D series, 17R-HDHA, and its products, AT-RvD1 and RvD2, have therapeutic potential to be used in the treatment of human IBD.

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Disclosures
The authors have no financial conflicts of interest.

References


