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Citation

Arita, M., M. Yoshida, S. Hong, E. Tjonahen, J. N. Glickman, N. A. Petasis, R. S. Blumberg, and C. N. Serhan. 2005. "Resolvin E1, an Endogenous Lipid Mediator Derived from Omega-3 Eicosapentaenoic Acid, Protects against 2,4,6-Trinitrobenzene Sulfonic Acid-Induced Colitis." *Proceedings of the National Academy of Sciences* 102 (21): 7671–76. <https://doi.org/10.1073/pnas.0409271102>.

Published version

<https://doi.org/10.1073/pnas.0409271102>

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Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis

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Edited by Charles A. Dinarello, University of Colorado Health Sciences Center, Denver, CO, and approved April 7, 2005 (received for review December 13, 2004)

Resolvin E1 (RvE1; 5S,12R,18R-trihydroxyeicosapentaenoic acid) is an antiinflammatory lipid mediator derived from omega-3 fatty acid eicosapentaenoic acid (EPA). At the local site of inflammation, aspirin treatment enhances EPA conversion to 18R-oxygenated products, including RvE1, which carry potent antiinflammatory signals. Here, we obtained evidence for reduced leukocyte infiltration in a mouse peritonitis model, where the administration of EPA and aspirin initiated the generation of RvE1 in the exudates. Similar results were obtained with the administration of synthetic RvE1, which blocked leukocyte infiltration. RvE1 also protected against the development of 2,4,6-trinitrobenzene sulfonic acid-induced colitis. The beneficial effect was reflected by increased survival rates, sustained body weight, improvement of histologic scores, reduced serum anti-2,4,6-trinitrobenzene sulfonic acid IgG, decreased leukocyte infiltration, and proinflammatory gene expression, including IL-12 p40, TNF- α , and inducible nitric oxide synthase. Thus, the endogenous lipid mediator RvE1 counter-regulates leukocyte-mediated tissue injury and proinflammatory gene expression. These findings show an endogenous mechanism that may underlie the beneficial actions of omega-3 EPA and provide targeted approaches for the treatment of intestinal inflammation.

antiinflammation | aspirin-triggered lipid mediators | omega-3 PUFA | resolvins

In many chronic disorders, unresolved inflammation is a major mechanism of disease pathogenesis (1). Inflammation is a protective host response to foreign antigenic challenge or tissue injury that, if unopposed, could lead to loss of tissue structure as well as function. During the development of inflammation, the concerted actions of molecular signaling determine whether inflammatory cells undergo migration, activation, proliferation, differentiation, or clearance. Many inflammatory processes are self-limiting and self-resolving systems, suggesting the existence of endogenous antiinflammatory and/or proresolution mediators during the course of inflammation (for recent reviews, see refs. 6 and 15–17).

Omega-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid, which are enriched in fish oils, are held to be beneficial in a wide range of human inflammatory disorders, including cardiovascular diseases, rheumatoid arthritis, Alzheimer's disease, lung fibrosis, and inflammatory bowel disease (IBD) (2–5). These essential fatty acids are widely believed to act by means of several possible mechanisms, such as preventing conversion of arachidonate to proinflammatory eicosanoids or serving as an alternative substrate producing less potent products. Recently, we uncovered a series of oxygenated derivatives of omega-3 PUFA that possess potent antiinflammatory and immunoregulatory actions, suggesting an alternative and perhaps important role for these

essential fatty acids as precursors for potent bioactive protective mediators. The trivial name Resolvin (resolution phase interaction products) was introduced for these bioactive compounds (6, 7). Resolvin E1 (RvE1) is endogenously biosynthesized from EPA in the presence of aspirin during the spontaneous resolution phase of acute inflammation where specific cell–cell interactions occur. Recently, organic synthesis was achieved that permitted the complete stereochemical assignment of RvE1 as 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid (8). RvE1 possesses unique counterregulatory actions that inhibit polymorphonuclear leukocyte (PMN) transendothelial migration *in vitro* and also acts as a potent inhibitor of leukocyte infiltration, dendritic cell migration, and IL-12 production *in vivo* (7, 8).

IBD, including Crohn's disease and ulcerative colitis, is a chronic and relapsing inflammatory disorder characterized by abnormalities in mucosal responses to normally harmless bacterial antigens, abnormal cytokine production, and an inflammatory process associated with mucosal damage (9, 10). As such, IBD is characterized by colon inflammation associated with leukocytosis and proinflammatory gene expression. Results from human studies have suggested that fish oils rich in omega-3 PUFA are protective in reducing the rate of relapse in Crohn's disease (4), but the molecular mechanism underlying this beneficial effect remains to be elucidated.

Here, we report that RvE1, biosynthesized from EPA precursor, dramatically protects against the development of bowel inflammation in response to intrarectal antigenic hapten 2,4,6-trinitrobenzene sulfonic acid (TNBS) challenge, a well appreciated experimental colitis model. In short, RvE1 stopped leukocyte infiltration and down-regulated proinflammatory gene expression associated with this model *in vivo*, prolonging the life of these mice.

Methods

Mice. Six- to 8-week-old female BALB/c mice and male FvB mice were obtained from Charles River Laboratories. Mice were provided sterile food and water, kept in microisolator cages, and maintained in the animal facility of Harvard Medical School. All

This paper was submitted directly (Track II) to the PNAS office.

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Abbreviations: ATLA, aspirin-triggered lipoxin A₄ analog; COX, cyclooxygenase; EPA, eicosapentaenoic acid; IBD, inflammatory bowel disease; PMN, polymorphonuclear leukocyte; PUFA, polyunsaturated fatty acids; RvE1, resolvin E1; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNP, 2,4,6-trinitrophenyl.

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studies were performed under approval of the Harvard Medical School Standing Committee on Animals.

Induction of Peritonitis and Detection of RvE1. Inflammatory exudates were initiated with i.p. injection of 1 ml of zymosan A (1 mg/ml) into 6- to 8-week-old male FvB mice. Mice were pretreated with aspirin (0.5 mg) i.p., followed by EPA (0.3 mg) and zymosan. Peritoneal lavages were collected at 2 h, and cells were enumerated. Cell-free exudates were extracted by using C18 solid-phase extraction with deuterium-LTB₄ (Cayman Chemical, Ann Arbor, MI) as an internal standard for liquid chromatography–UV–tandem MS (MS/MS) analysis by using a liquid chromatography ion trap tandem mass spectrometer (LCQ, Finnigan-MAT, San Jose, CA) equipped with a 100 mm × 2 mm × 5 μm column (LUNA C18-2, Phenomenex, Torrance, CA) and UV diode array detector by using mobile phase (methanol:water:acetate at 65:35:0.01) from 0 to 8 min, ramped to methanol 8 to 30 min, with a 0.2 ml/min flow rate.

Induction of TNBS-Induced Colitis. To generate a more chronic T cell-mediated inflammation, BALB/c mice were sensitized with 150 μ l of the haptening agent TNBS (Sigma-Aldrich) of 2.5% in 50% ethanol by skin painting on day -7 . On day 0, 150 μ l of 1% TNBS in 50% ethanol was administered intrarectally by means of a 3.5-F catheter under anesthesia with tribromoethanol. To ensure distribution of the TNBS within the entire colon and cecum, mice were held in a vertical position for 1 min after

the instillation. On day 4, mice were killed, and immunopathologic characterization was performed as described in ref. 23.

In Vivo Treatment with RvE1. RvE1 was prepared by total organic synthesis and was qualified by both physical and biological properties (8). RvE1 was administered i.p. (1.0 μg per mouse; 50 $\mu\text{g}/\text{kg}$) on days -8 , -1 , and 0 before the induction of colitis (prevention mode). Aspirin-triggered lipoxin A_4 analog [ATLa; 15-epi-16-(*p*-fluoro)phenoxy-LXA $_4$] was given at the same dose as a direct comparison with RvE1.

Grading of Histologic Change. The degree of inflammation on microscopic cross sections of the colon was graded semiquantitatively. Severity of colitis was assessed based on five histologic criteria: mononuclear inflammation, neutrophilic infiltration/crypt abscesses, crypt hyperplasia, mucosal injury/ulceration, and mucosal hypervascularity. Each of the criteria was graded on a 0–3 scale (0, absent; 1, mild; 2, moderate; 3, severe). The five scores were summed to give a total score. Grading was performed in a blind fashion by the same pathologist (J.N.G.).

Myeloperoxidase Assay. Each tissue sample was assessed for PMN content and infiltration. Tissues were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, followed by three cycles of sonication and freeze-thawing. The particulate matter was removed by centrifugation ($16,000 \times g$ for 20 min), and 75 μ l of supernatant was added to 925 μ l of potassium phosphate buffer (pH 6.0) con-

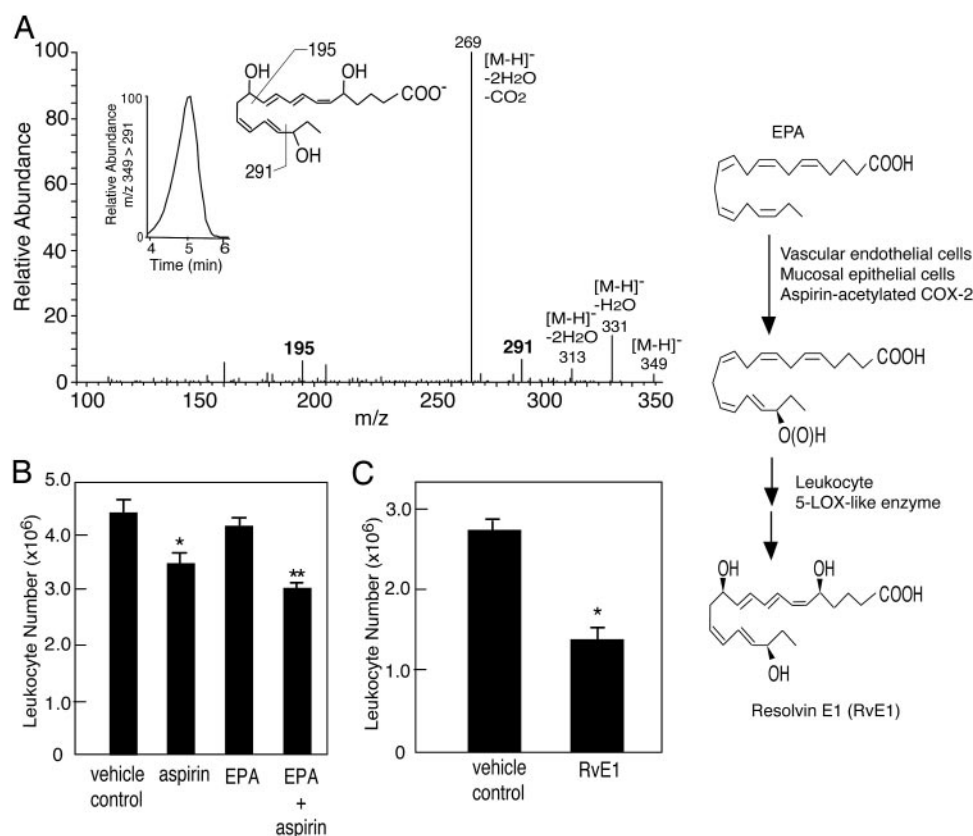


Fig. 1. RvE1 formation and antiinflammatory actions in peritonitis. (A) Inflammatory exudates from mice treated with EPA and aspirin generate RvE1 *in vivo*. (B) Inhibition of leukocyte infiltration in zymosan-induced peritonitis treated with EPA and aspirin. Mice were pretreated for 30 min with aspirin (0.5 mg) i.p., followed by EPA (0.3 mg) and zymosan. Peritoneal lavages were collected at 2 h, and cells were enumerated. ($n = 4$; *, $P < 0.05$, compared with vehicle control; **, $P < 0.05$, compared with aspirin alone). (C) Inhibition of leukocyte infiltration in zymosan-induced peritonitis. RvE1 (100 ng) was injected into tail veins, and zymosan A was injected into the peritoneum. Mice were killed, peritoneal lavages were collected (2 h), and cells were enumerated ($n = 3$; *, $P < 0.01$ compared with vehicle control).

taining 0.2 mg/ml *o*-dianisidine dihydrochloride (Sigma) and 0.0006% hydrogen peroxide. Changes in optical density were monitored at 460 nm at 25°C, at 30- and 90-s intervals. The calibration curve for conversion of myeloperoxidase activities to PMN number was performed as described in ref. 24.

Detection of 2,4,6-Trinitrophenyl (TNP)-Specific Antibodies with ELISA. Sera were collected on day 4, and anti-TNP antibodies were determined by ELISA as described (with a minor modification) in ref. 25. Briefly, 100 mg of ovalbumin was dissolved in 4 ml of 0.05 M carbonate-bicarbonate buffer at pH 9.6, followed by the addition of 1 ml of TNBS 5% solution and incubation for 2 h at room temperature. TNP-ovalbumin was dialyzed in PBS and then stored at -80°C until use. For the detection of TNP-specific antibodies, TNP-ovalbumin was dissolved at the concentration of 0.1 mg/ml in 0.05 M carbonate-bicarbonate buffer at pH 9.6, and 100 μl was placed in each well of 96-well microtiter plates for coating. After blocking with 1% BSA in PBS, plates were incubated with appropriately diluted serum. For detection, horseradish peroxidase-conjugated secondary Abs against mouse IgG (Southern Biotechnology Associates), followed by the incubation of TMB microwell peroxidase substrate (SureBlue, Kirkegaard & Perry Laboratories) as a substrate for horseradish peroxidase, were used. One unit, which was judged by using the serum of mice that were immunized by 1% TNBS emulsion with Freund's complete adjuvant (Sigma) as an internal control, was used.

Quantitative Real-Time PCR. Total RNA from colon was prepared by using TRIzol reagent (Life Technologies, Rockville, MD). Real-time PCR was performed by using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) according to manufacturer's protocol. Primers for IFN- γ , IL-12p40, TNF- α , IL-4, TGF- β , IL-10, inducible nitric oxide synthase, and GAPDH were designed as described in ref. 26. Primers for cyclooxygenase (COX)-2 were designed as follows: 5'-AGAAGGAAATGGCTGCAGAA-3' and 5'-GCTCGGCTTCCAGTATTGAG-3'. Quantities of specific mRNA in the sample were measured according to the corresponding gene-specific standard curve. The relative expression of each gene was normalized against the housekeeping gene GAPDH.

RT-PCR. Amplification of mouse ChemR23 and GAPDH was carried out with HotStartTaq DNA polymerase (Qiagen, Valencia, CA) by using specific primers 5'-CTGATCCCCGTCTTCATCAT-3' and 5'-TGGTGAAGCTCCTGTGACTG-3', which amplify 376-bp product for ChemR23; and 5'-GACCACAGTCCATGCCATCACT-3' and 5'-TCCACCACCT GTTGTCTGTAG-3', which amplify 430-bp product for GAPDH.

Statistical Analysis. All results in the figures and text are expressed as mean $\pm$ SE of n mice per group. Statistical significance was determined by Student's t test. $P < 0.05$ was considered significant.

Results

RvE1 Formation in Peritoneal Inflammatory Exudates. During the course of an acute inflammatory challenge associated with administration of zymosan i.p., we documented the formation of RvE1 in the inflammatory exudates by using a liquid chromatography-UV-MS/MS mediator-lipidomic analysis after injection of EPA and aspirin (Fig. 1A). Selected ion chromatograms and ions present within the MS/MS were consistent with the production of RvE1 with a parent ion at m/z 349 = [M-H] $^{-}$ and characteristic product ions at m/z 291 and 195 that are denoted in Fig. 1A *Inset*. These findings are consistent with previous work (7) indicating that murine inflammatory exudates exposed

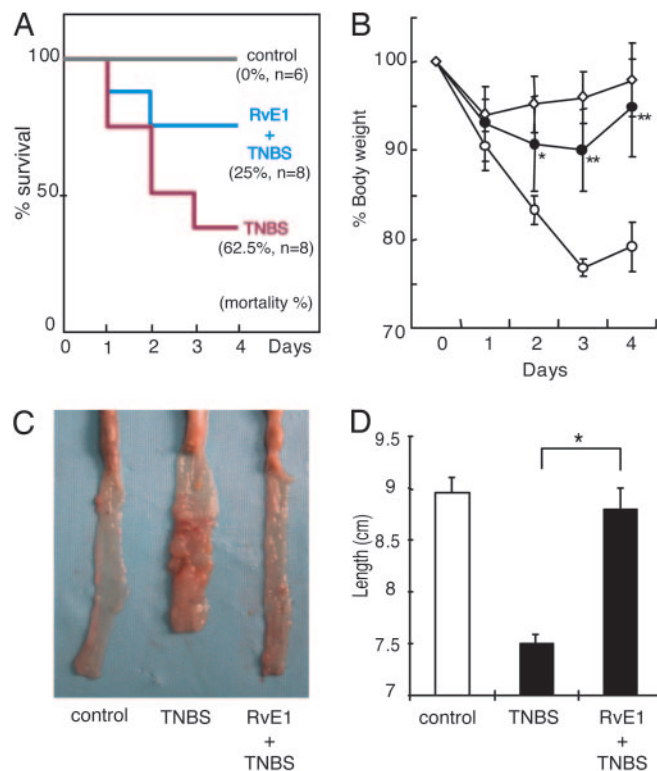


Fig. 2. RvE1 dramatically attenuates TNBS-induced colitis. (A) The survival rate of RvE1-treated mice was significantly higher than that of nontreated mice. (B) Wasting disease measured by body weight loss in mice with TNBS colitis is improved by RvE1 treatment. Open diamonds, vehicle control; open circles, TNBS only; filled circles, TNBS treated with RvE1 (1 μg per mouse or 0.05 mg/kg). *, $P < 0.01$; **, $P < 0.001$ compared with vehicle control ($n = 6$). (C) Macroscopic appearance of the colon of mice receiving vehicle, TNBS only, or RvE1 (0.05 mg/kg). (D) Length of the inflamed colons of mice treated with either TNBS alone or RvE1. *, $P < 0.01$, compared with TNBS alone.

in vivo to EPA and aspirin produced the trihydroxy-containing product RvE1, presumably by means of the activities of leukocyte 5-lipoxygenase and aspirin-acetylated COX-2. Peritoneal inflammatory cells, predominantly PMNs, were 30% lower in EPA- and aspirin-treated mice when endogenous RvE1 was present in the peritoneal exudates (Fig. 1B). Moreover, exogenous administration of as little as 100 ng per mouse of RvE1 reduced leukocyte infiltration by 50% (Fig. 1C). In contrast, reduction product 18R-HEPE from the metabolic precursor 18R-H(p)EPE was much less potent. RvE1 proved to be more potent than indomethacin, a well characterized nonsteroidal antiinflammatory drug (11), which reduced leukocyte numbers in the exudate by $\approx 20\%$ in this model.

RvE1 Protects Mice from TNBS Colitis. Next, we assessed the effects of RvE1 in the TNBS colitis model. After sensitization to TNBS by skin painting, male BALB/c mice (6–8 weeks old) were subjected to intrarectal administration of TNBS (1.5 mg per mouse in 50% ethanol). Severe illness that was characterized by bloody diarrhea and severe wasting disease was observed. The treatment of mice with RvE1 (1.0 μg per mouse; 0.05 mg/kg) reduced overall mortality, which was 25% and 62.5%, respectively (RvE1 treatment, compared with the TNBS groups alone) (Fig. 2A). Animals that received TNBS in association with control vehicle experienced severe weight loss (Fig. 2B, open circles). In contrast, mice that received RvE1 experienced less weight loss (Fig. 2B, filled circles). These differences are directly reflected in the levels of macroscopic

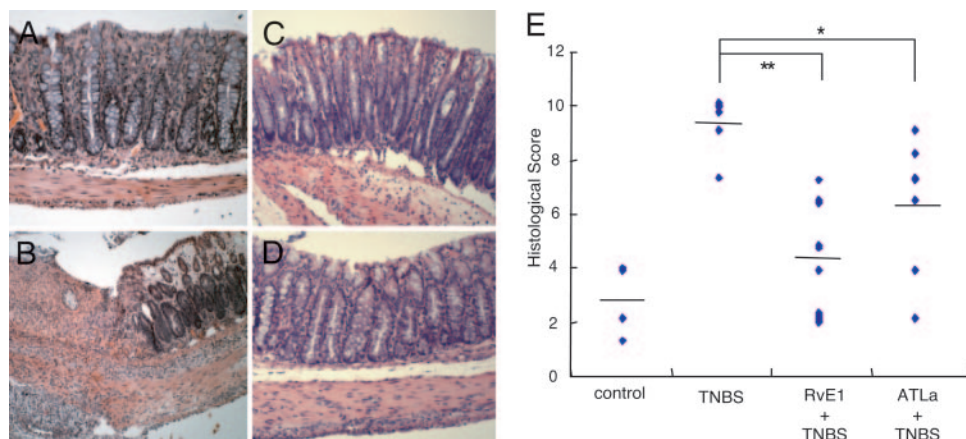


Fig. 3. Colon histopathology from RvE1- or ATLa-treated mice. (A–D) Colon section of mouse treated with vehicle (A), day 4 after TNB-induced colitis (B), RvE1-treated mouse (C), and ATLa-treated mouse (D). (E) Histological scores of the colon of mice receiving vehicle, TNBS only, TNBS plus RvE1, or TNBS plus ATLa. * and **, $P < 0.05$ and $P < 0.01$, respectively.

injury observed in that mice treated with RvE1 did not exhibit significant shortening and thickening of the colon (Fig. 2 C and D). Consistent with these macroscopic changes, mice treated with control vehicle exhibited marked transmural infiltration with inflammatory cells such as PMNs, monocytes, and lymphocytes and injury with ulceration (Fig. 3B). In contrast, mice treated with RvE1 exhibited less severe histologic features of colitis (Fig. 3C). For the purpose of direct comparison, ATLa, another aspirin-triggered lipid mediator that proved to be protective against dextran sodium sulfate-induced colitis (12), also provided dramatic protection in the TNBS-colitis model (Fig. 3D). When quantified by a histologic scoring system for evidence of inflammation and injury, these histologic differences were highly significant (Fig. 3E).

In addition to the histological scores, mice treated with RvE1 exhibited significantly lower levels of myeloperoxidase activity, compared with mice treated with the control vehicle, suggesting reduced leukocyte infiltration in colon tissues (Fig. 4A). The level of serum anti-TNBS IgG also was decreased by RvE1 treatment, suggesting attenuation of antigen presentation and B cell production of IgG (Fig. 4B), a measure of the level of adaptive immunity. To determine whether this RvE1-mediated protection from colitis was associated with alterations in proinflammatory gene expression, mRNA levels in colon were determined by quantitative real-time PCR (Fig. 4C). This study revealed a significant reduction in TNF- α , IL-12 p40, inducible nitric oxide synthase, and COX-2 from mice that received RvE1. Interestingly, direct effects were not observed for the T cell cytokines such as IFN- γ , IL-4, and IL-10. Also, TGF- β did not show significant differences. Recently, an RvE1 receptor was identified in human and mouse as a G protein-coupled receptor, ChemR23 (8). Murine ChemR23 mRNA was expressed in mouse colon and was slightly increased in levels in colons obtained from TNBS-treated mice (Fig. 5).

Discussion

Here, we demonstrate that RvE1, an “endogenous” lipid mediator that exhibits potent antiinflammatory activity, is generated in the course of inflammation *in vivo* from EPA when aspirin is administered. RvE1 reduced leukocyte infiltration, turned off proinflammatory gene expression, and prevented the development of severe experimental colitis in mice. Together, these observations suggest a therapeutic potential of resolving intestinal inflammation *in vivo*.

The concept that endogenous counterregulatory pathways of antiinflammation occur *in vivo* through generation of resolvins is of

interest given the potency of RvE1 observed herein. Hence, understanding the regulation of these natural endogenous antiinflammatory products is important to optimize the potential utility of this pathway *in vivo*. During acute inflammation, inflammatory cells

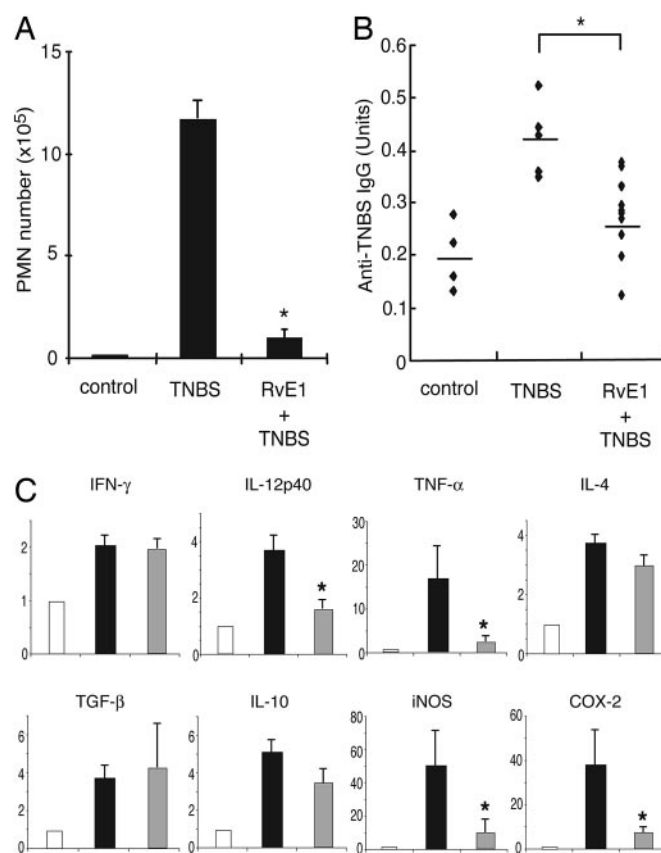


Fig. 4. RvE1 reduces leukocyte infiltration and proinflammatory mediators in colitis. (A) Myeloperoxidase activity of colons of mice treated with TNBS alone or RvE1. *, $P < 0.0001$ vs. mice treated with TNBS alone. (B) Anti-TNBS IgG level in serum on day 4 from mice treated with TNBS alone or RvE1 as noted. *, $P < 0.01$. (C) Quantitative real-time PCR analysis of mRNA expression of inflammatory mediators in colons obtained on day 4 from control mice (white column), mice treated with TNBS alone (black column), or mice receiving TNBS plus RvE1 (gray column). *, $P < 0.01$.

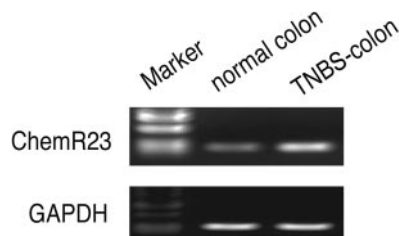


Fig. 5. ChemR23 mRNA is expressed in mouse colon. Total RNA was isolated from control and TNBS-treated colons, and RT-PCR was performed as described in *Methods*.

expressing COX-2 treated with aspirin transform EPA by means of insertion of molecular oxygen in the *R* configuration to yield 18*R*-H(p)EPE (7). Acetylation of COX-2 with aspirin treatment promotes the generation of 18*R*-HEPE, which also may account for some of the bioactivity profile of aspirin. Without aspirin, 18*R*-HEPE also could be generated by bacterial cytochrome P450 monooxygenase (7, 13). Once formed, 18*R*-HEPE is then further converted by means of cell–cell interactions and the sequential action of the leukocyte lipoxygenase reaction that leads to the formation of 5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid (RvE1) (8).

In Crohn's disease, neutrophil recruitment to the intestinal wall and an excessive activation of macrophages and T helper 1 cells leads to the enhanced production of proinflammatory cytokines such as TNF- α . This cytokine milieu favors an amplification of the inflammatory cascade of additional inflammatory mediators, destructive enzymes, and free radicals that cause tissue damage (9, 10). The relapsing and remitting course of IBD, together with the spontaneous resolution, implies the existence of an endogenous resolution signal. In addition to resolvins, it is now appreciated that several new families of endogenous antiinflammatory and/or proresolution mediators are generated during a host response, including TGF- β , IL-10, peroxisome proliferator-activated receptor (PPAR) γ agonists, and lipoxins (14–17). Studies in humans suggested that omega-3 PUFA such as EPA and docosahexaenoic acid are protective in reducing the rates of relapse in Crohn's disease (4, 5). These results raise the intriguing possibility that omega-3 PUFA is a precursor of endogenous resolution signals. Indeed, the present results demonstrate the endogenous conversion of EPA and the formation of the resolvin mediator RvE1 in inflamed loci. Moreover, we provide evidence that RvE1, when administered exogenously, protects against the development of TNBS-colitis by reducing local inflammation. We also confirmed the protective actions of RvE1 in another epithelial-related inflammatory disease model, namely rabbit periodontitis (H. Hasturk, I. A. Kantarci, M.A., T. Ohira, N. Ebrahimi, N.A.P., B. Levy, C.N.S., and T. E. Van Dyke, unpublished data).

Hapten-induced colitis such as TNBS colitis is a useful model to study the early or initiating events in the development of mucosal inflammation (18). In this model, intestinal inflammation develops as a result of the covalent binding of the haptizing agent to

autologous host proteins, with subsequent stimulation of a delayed-type hypersensitivity to TNBS-modified self-antigens in the context of an exaggerated innate immune response generating IL-12 (19). Although the relationship of this hapten-induced colitis model displays Crohn's disease-like features, most notably transmural leukocytic inflammation and predominant T helper 1 cell activity of mucosal leukocytes. RvE1 treatment dramatically reduced leukocyte infiltration, proinflammatory gene expression (i.e., IL-12 p40, TNF- α), and the level of anti-TNBS IgG, suggesting the immunoregulatory action of RvE1 in both innate and acquired limbs of the mucosal immune response. It is of interest to note that 15-epi-lipoxin A₄, aspirin-triggered lipoxin A₄ (ATL), also is protective against colonic damage induced by dextran sodium sulfate (12). Along these lines, during the course of the present manuscript submission, the second-generation 3-oxa-lipoxin A₄/ATL analogue was shown to be protective against TNBS colitis (20). RvE1 and ATL have different receptors, namely ChemR23 and ALX, which share structural similarity but different tissue distributions (8). Both receptors are expressed in colon (Fig. 5 and cf. ref. 20). Hence, these aspirin-triggered lipid mediators, namely RvE1 and ATL, act at different sites to protect colon; whether these lipid mediators are additive or synergistic in their actions in colon protection is a question that remains for further study.

Results from both human and animal studies indicate that TNF- α plays a critical role in pathogenesis of IBD. For example, the overexpression of TNF- α in mice results in the development of a Crohn's disease-like phenotype. Also, TNF- α deficient mice as well as anti-TNF- α treatment reduces intestinal inflammation in several animal models (21). More importantly, blockade of TNF- α is now a widely used therapeutic strategy in the management of Crohn's disease and rheumatoid arthritis (22). TNF- α directly induces NF- κ B-mediated up-regulation of adhesion molecules and inflammatory mediators and participates in apoptosis. The dramatic decrease in TNF- α and inducible nitric oxide synthase expression by as little as 0.05 mg/kg of RvE1 and complete protection against the development of TNBS colitis argue strongly that formation as well as administration of RvE1 and related compounds can offer an additional avenue for the protection of mucosal inflammation and injury.

Taken together, our findings offer evidence of an endogenous antiinflammatory lipid mediator RvE1 whose activity may form the basis for some of the beneficial actions of omega-3 EPA in human diseases. The potent bioactivity of RvE1 may offer an alternative approach for human IBD and other inflammatory disorders and also suggests the existence of a potentially useful target (e.g., RvE1 receptors) (8) for new therapeutic interventions in a wide range of human diseases.

We thank M. H. Small for helpful manuscript preparation and K. Gotlinger, A. Rubin, and K. Kanno for skillful technical assistance. This study was supported in part by the Uehara Memorial Foundation (M.A.), Research Fellowships from the Crohn's and Colitis Foundation of America (to M.Y.), and National Institutes of Health Grants DK44319 (to R.S.B.), DK51362 (to R.S.B.), GM38765 (to C.N.S.), and 1-P50-DE016191 (to C.N.S.).

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