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Original Contribution

Nitrated oleic acid up-regulates PPARγ and attenuates experimental inflammatory bowel disease

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ABSTRACT

Nitric oxide and its metabolites undergo nitration reactions with unsaturated fatty acids during oxidative inflammatory conditions, forming electrophilic nitro-fatty acid derivatives. These endogenous electrophilic mediators activate anti-inflammatory signaling reactions, serving as high-affinity ligands for peroxisome proliferator-activated receptor γ (PPARγ). Here we examined the therapeutic effects of 9- or 10-nitro-octadecenoic oleic acid (OA-NO_2) and native oleic acid (OA) in a mouse model of colitis. OA-NO_2 reduced the disease activity index and completely prevented dextran sulfate sodium-induced colon shortening and the increase in colonic p65 expression. Increased PPARγ expression was observed in colon samples as well as in cells after OA-NO_2 administration, whereas no effect was seen with OA. This induction of PPARγ expression was completely abolished by the PPARγ antagonist GW9662. 5-Aminosalicylic acid, an anti-inflammatory drug routinely used in the management of inflammatory bowel disease, also increased PPARγ expression but to a lesser extent. Altogether, these findings demonstrate that administration of OA-NO_2 attenuates colonic inflammation and improves clinical symptoms in experimental inflammatory bowel disease. This protection involves activation of colonic PPARγ.

Ulcerative colitis and Crohn disease are chronic relapsing inflammatory intestinal disorders of unknown etiology. Current treatments for inflammatory bowel disease (IBD)[2] include anti-inflammatory corticosteroids, aminosalicylates, and various immune modulators as well as the more recently developed anti-tumor necrosis factor-α [1]. Although therapies for IBD are improving they are still far from optimal for long-term disease management, and adverse side effects are common. In the search for novel therapeutic interventions attempts are being made to explore and selectively target the specific proinflammatory pathways that are central in IBD pathogenesis. Recent studies have indicated a role for peroxisome proliferator-activated receptor γ (PPARγ) in IBD [2].

PPARγ is a member of the nuclear receptor superfamily of transcription factors involved in the control of inflammation, cell proliferation, apoptosis, and metabolic functions. Ligands for PPARγ include natural compounds with relatively low affinity such as polyunsaturated fatty acids, oxidized low-density lipoprotein, certain eicosanoids, α, β-unsaturated keto derivatives of fatty acids, 15-deoxy-Δ12,14-PGJ_2, and drugs including the thiazolidinedione derivatives troglitazone, rosiglitazone, and pioglitazone used for the treatment of type 2 diabetes [3]. PPARγ$^{+/-}$ heterozygous mice exhibit an increased susceptibility to experimentally induced colitis [4], indicating a key role for PPARγ in maintaining large intestine homeostasis. The fact that PPARγ is highly expressed in colonic epithelium [5] makes it an attractive drug target for IBD therapy. Indeed, activation of PPARγ was recently shown to mediate the effects of aminosalicylates [6], anti-inflammatory agents routinely used in the treatment of IBD. In addition, thiazolidinedione depresses inflammation in murine models of IBD [7–9] and has also shown some benefit in recent clinical trials [9,10].

The free radical gas nitric oxide (NO) is generated by the inducible NO synthase during inflammation [11], and colonic NO formation is greatly enhanced in patients with active IBD [12]. It has been reported that nitration products of unsaturated fatty acids are formed via NO-dependent oxidative reactions [13]. These derivatives were initially viewed to be, like nitrotyrosine, a "footprint" of NO-dependent redox reactions [13,14]. More recently, it has been observed that electrophilic nitroalkene derivatives of unsaturated fatty acids mediate pluripotent cell signaling actions. In vitro cell studies have revealed potent anti-inflammatory actions of nitroalkenes via interaction with numerous pathways, including nuclear factor-κB (NF-κB), heme oxygenase-1, xanthine oxidase, and signal transducer and activator of transcription (STAT) signaling [15]. In addition, these electrophilic mediators were recently shown to be potent activators of PPARγ [16–18]. Although in vitro studies support

Abbreviations: DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; NO_2-FA, nitrated fatty acid; OA-NO_2, nitrated oleic acid; OA, native oleic acid; DAI, disease activity index; PPARγ, peroxisome proliferator-activated receptor γ; 5-ASA, 5-aminosalicylic acid.

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potential anti-inflammatory effects of nitrated fatty acids, little is known about their effects in vivo.

Here we studied the therapeutic effects of nitrated oleic acid (OA-NO_2) in a murine model of IBD. We also explored whether any of the anti-inflammatory actions of OA-NO_2 involve activation of PPARγ-dependent signaling.

Material and methods

Materials

Dextran sulfate sodium (DSS; 45 kDa) was from TdB Consultancy (Uppsala, Sweden). OA-NO_2 was synthesized as described previously [16]. 5-Aminosalicylic acid (5-ASA) and GW9662 were from Sigma (St. Louis, MO, USA). Fugene transfection reagent was from Roche (Mannheim, Germany). The Dual-Luciferase Reporter Assay System was from Promega (Madison, WI, USA). Trizol reagent and M-MLV reverse transcriptase were from Invitrogen (Carlsbad, CA, USA). SYBR green master mix was from Applied Biosystems (Foster City, CA, USA).

Animals

All experimental work on animals was approved by the Ethics Committee for Animal Experiments at the Karolinska Institutet. Female BALB/c mice, 7–8 weeks of age and weighing 19–22 g, were housed under temperature- and humidity-controlled conditions with a 12:12 h light:dark cycle and fed a standard pellet diet and tap water ad libitum. The mice were acclimatized for 2 weeks before the study was started.

Animal treatments and assessment of colitis

Mice were divided in four groups (eight animals per group: control, DSS, DSS + OA (native oleic acid), and DSS + OA-NO_2). DSS, DSS + OA, and DSS + OA-NO_2 groups received 2% DSS (w/v) in the drinking water for 7 days. OA and OA-NO_2 (0.5 or 5 mg /kg/day) were given subcutaneously using Alzet osmotic minipumps (Model 1007D; Durect Corp., Cupertino, CA, USA) from the first day of the experiment. Control animals received tap water only.

Mice were examined daily by a blinded investigator to determine the disease activity index (DAI). The DAI was achieved by scoring body weight loss, stool consistency, and bleeding as described [19]. At the end of the study period mice were anesthetized with isoflurane, and blood was collected by heart puncture, followed by cervical dislocation. The colon was removed and its length was recorded. Tissues and plasma samples were frozen and kept at −80°C until analysis. Levels of the NF-κB subunit p65 were assessed by immunohistochemical staining of colon biopsies, as described but with slight modifications [20]. In this case, the primary antibody was the NF-κB p65 monoclonal antibody (sc-8008; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the secondary antibody was the biotinylated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA). The intensity of the staining was scored (0–2) by a blinded investigator.

Measurements of nitro-fatty acids

Acetonitrile extraction was used to isolate lipids from murine intestine and plasma stored at −80°C until processing. Quantitative analysis of free OA-NO_2 levels was conducted by high-performance liquid chromatography–electrospray ionization mass spectrometry using a triple-quadrupole mass spectrometer (API 5000; Applied Biosystems/MDS Sciex, Framingham, MA, USA) [21,22]. Samples were resolved by high-performance liquid chromatography (HPLC) using a 150×2-mm C_{18} Luna reversed-phase column (particle size 3 mm; Phenomenex, Belmont, CA, USA) at a flow rate of 0.25 ml/min and a linear gradient of acetonitrile (+0.1% acetic acid) in water (45–80% in 45 min). Mass spectrometric detection of OA-NO_2 was performed using multiple reaction monitoring (MRM) mode by monitoring the mass transition m/z 326/46 for the native compound and m/z 344/46 for the ^{13}C-labeled internal standard. Quantitative analysis of protein-adducted molecules was performed in the MRM scan mode. Separately, β-mercaptoethanol (BME) was utilized to capture net free and protein-adducted OA-NO_2, and then the data were calculated for the pools of free and adducted species. Lipid extracts were treated with 500 mM BME for 30 min at 37°C and analyzed by HPLC-ESI-MS/MS. HPLC separations were performed on a C_{18} reverse-phase column (150×2 mm, 3-μm particle size) with a mobile phase consisting of A (0.1% acetic acid in H_2O) and B (0.1% acetic acid in acetonitrile). Products were resolved from the column and compared with standards using the following gradient program: 45% B for 1 min, 45–80% B over 44 min, 80–100% B over 1 min, 100% B for 7 min, 100–45% B over 6 s, and 45% B for 10 min to reequilibrate the column by monitoring for molecules that undergo an $M^-/[M–BME]^-$ transition. The transition used for BME–OA-NO_2 was m/z 404.4/326.3 and for BME–[$^{13}C_{18}$]OA-NO_2, m/z 422.4/344.3. The declustering potentials were −50 V for BME adducts and collision energies were set at −17 for BME adducts. Zero-grade air was used as source gas, and nitrogen was used in the collision chamber. Data were acquired and analyzed using Analyst 1.4.2 software (Applied Biosystems). Quantification was achieved by comparing peak area ratios between analytes and their corresponding internal standards and then calculating analyte concentration using an internal standard curve. Concentrations were normalized to plasma volume or wet tissue weight.

Cell culture assays

Colonocyte cell lines (SW480) were kindly donated by V. Arulampalam. Cultures were maintained in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS in a humidified incubator at 37°C and 0.5% CO_2.

The regulation of PPARγ by OA and/or OA-NO_2 was evaluated as follows: cells were treated with OA, OA-NO_2 (1 μM, 24 h), or 5-ASA (3 μM–30 mM, 16 h), in the absence or presence of the PPARγ inhibitor GW9662 (1 μM, 1 h pretreatment). 5-ASA (common treatment for IBD) was used as a control. The mRNA levels of PPARγ were further analyzed by quantitative RT-PCR. In continuation, the cells were transiently transfected with a plasmid containing the luciferase gene under the control of two tandem PPARγ response elements (2× Cyp-luc, kindly donated by V. Arulampalam), using the Fugene transfection reagent. The cells were further treated with OA, OA-NO_2 (1 μM), or 5-ASA (300 μM) in the presence or absence of GW9662 (1 μM). Transcription levels of PPARγ were measured by luciferase assay using the Dual-Luciferase Reporter Assay System. The transfection efficiency was normalized to the *Renilla* luciferase activity expressed by the pRL-TK plasmid (Promega).

RNA isolation and quantitative RT-PCR

Total RNA was extracted from cells and frozen tissues using a power homogenizer (KEBO-Lab, Stockholm, Sweden) and Trizol reagent. One microgram of total RNA was reverse transcribed with M-MLV reverse transcriptase. The relative expression levels of PPARγ, STAT-1, and fatty acid binding protein 2 (FABP2) were determined by real-time PCR in a 7900 sequence detection system (Applied Biosystems). Primers were designed with Primer Express software (Applied Biosystems) and amplification was carried out with SYBR green master mix. Sequences of the primers used were hPPARγ forward, 5′-CCTGATAGGCCCCACTGTGT-3′, and reverse, 5′-CAGGTGG-GAGTGGAACAAT-3′; mPPARγ forward, 5′-TCACAAGAGCTGACC-CAATGG-3′, and reverse, 5′-GATCGCACTTTGGTATTCTTGGA-3′;