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Nitrated oleic acid up-regulates PPAR γ 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 (PPAR γ). Here we examined the therapeutic effects of 9- or 10-nitro-octadecenoic oleic acid (OA-NO₂) and native oleic acid (OA) in a mouse model of colitis. OA-NO₂ reduced the disease activity index and completely prevented dextran sulfate sodium-induced colon shortening and the increase in colonic p65 expression. Increased PPAR γ expression was observed in colon samples as well as in cells after OA-NO₂ administration, whereas no effect was seen with OA. This induction of PPAR γ expression was completely abolished by the PPAR γ antagonist GW9662. 5-Aminosalicylic acid, an anti-inflammatory drug routinely used in the management of inflammatory bowel disease, also increased PPAR γ expression but to a lesser extent. Altogether, these findings demonstrate that administration of OA-NO₂ attenuates colonic inflammation and improves clinical symptoms in experimental inflammatory bowel disease. This protection involves activation of colonic PPAR γ .

Keywords

Nitrated fatty acids; PPAR γ ; Experimental colitis; Nitric oxide; Free radicals

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 (PPAR γ) in IBD [2].

PPAR γ 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 PPAR γ 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- $\Delta^{12,14}$ -PGJ₂, and drugs including the thiazolidinedione derivatives troglitazone, rosiglitazone, and pioglitazone used for the treatment of type 2 diabetes [3]. PPAR $\gamma^{+/-}$ heterozygous mice exhibit an increased susceptibility to experimentally induced colitis [4], indicating a key role for PPAR γ in maintaining large intestine homeostasis. The fact that PPAR γ is highly expressed in colonic epithelium [5] makes it an attractive drug target for IBD therapy. Indeed, activation of PPAR γ 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- κ B (NF- κ B), 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 PPAR γ [16–18]. Although in vitro studies support 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-NO₂) in a murine model of IBD. We also explored whether any of the anti-inflammatory actions of OA-NO₂ involve activation of PPAR γ -dependent signaling.

Material and methods

Materials

Dextran sulfate sodium (DSS; 45 kDa) was from TdB Consultancy (Uppsala, Sweden). OA-NO₂ 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-NO₂). DSS, DSS + OA, and DSS + OA-NO₂ groups received 2% DSS (w/v) in the drinking water for 7 days. OA and OA-NO₂ (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- κ B 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- κ B 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-NO₂ 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 C₁₈ 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-NO₂ 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 ¹³C-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-NO₂, 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 C₁₈ reverse-phase column (150×2 mm, 3-μm particle size) with a mobile phase consisting of A (0.1% acetic acid in H₂O) 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-NO₂ was m/z 404.4/326.3 and for BME–[¹³C₁₈]OA-NO₂, 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% CO₂.

The regulation of PPAR γ by OA and/or OA-NO₂ was evaluated as follows: cells were treated with OA, OA-NO₂ (1 μ M, 24 h), or 5-ASA (3 μ M–30 mM, 16 h), in the absence or presence of the PPAR γ inhibitor GW9662 (1 μ M, 1 h pretreatment). 5-ASA (common treatment for IBD) was used as a control. The mRNA levels of PPAR γ 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 PPAR γ response elements (2 $\times$ Cyp-luc, kindly donated by V. Arulampalam), using the Fugene transfection reagent. The cells were further treated with OA, OA-NO₂ (1 μ M), or 5-ASA (300 μ M) in the presence or absence of GW9662 (1 μ M). Transcription levels of PPAR γ 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 PPAR γ , 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 hPPAR γ forward, 5'-CCTGATAGGCCCACTGTGT-3', and reverse, 5'-CAGGTGGGAGTGGACAAT-3'; mPPAR γ forward, 5'-TCACAAGAGCTGACCCAATGG-3', and reverse, 5'-GATCGCACTTTGGTATTCTTGA-3'; mSTAT-1 forward, 5'-CTTATTCCATGGACAAGGTTTG-3', and reverse, 5'-GGTGCTTCTTAATAGAGCTCTAGG-3'; mFABP2 forward, 5'-AAATGGGTGTTAATATAGTGAAAA-3', and reverse, 5'-CCTTCTTGTTGAATTGTCAGCTTC-3'; and β -actin forward, 5'-GCTCCTCCTGAGCGCAAGT-3', and reverse, 5'-GTGGACAGTGAGGCCAGGAT-3'. Thermal conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C, 30 s at 55°C, and 1 min at 72°C.

Statistics

Data are presented as means $\pm$ SEM. Statistical analysis was performed using Student's *t* test, the Mann–Whitney test, or one-way ANOVA. *P* < 0.05 was considered significant (GraphPad Software, Inc).

Results

Detection of OA-NO₂ in blood and colon tissue

OA-NO₂ measurements revealed that after 7 days of treatment, OA-NO₂ is present in both plasma and colon samples (Table 1) from the OA-NO₂ groups, while remaining undetectable in the untreated groups (control, DSS, and DSS + OA). The concentration of total OA-NO₂ in plasma was 14 nM after administration of 0.5 mg/kg/day and 270 nM with the higher dose of OA-NO₂ (5 mg/kg/day). Free OA-NO₂ in plasma was 8 nM after administration of 5 mg/kg/day OA-NO₂ and again undetectable in the other groups. Colon samples also revealed an increase in the concentration of total OA-NO₂ in the treated group compared with the untreated groups.

Attenuation of DSS-induced colitis by administration of nitrated oleic acid

Shortening of the large intestine is a typical feature of experimental colitis, and colon length is considered a robust marker of the severity of inflammation [23]. As shown in Fig. 1, DSS administration caused a 30% reduction in colon length compared with the control group. Coadministration of OA-NO₂ dose-dependently prevented the DSS-induced colon shortening (Fig. 1C). With the higher dose of OA-NO₂ (5 mg/kg/day) the colon length did not differ significantly from the controls not receiving DSS (Fig. 1B), demonstrating a protective effect of nitrated OA. The lower dose of OA (0.5 mg/kg/day, Fig. 1A) did not prevent colon shortening, whereas the higher dose had some protective effect.

To assess the symptoms associated with DSS-induced colitis, we recorded the DAI, which is achieved by scoring body weight loss, stool consistency, and rectal bleeding. The DSS group showed an increase in the DAI compared to the control group (Fig. 2), and this parameter was clearly reduced after OA-NO₂ coadministration. OA treatment did not cause any significant improvement in DAI.

Finally, colonic inflammation was also evaluated by measuring p65 (an NF- κ B subunit) levels in colon samples. As shown in Fig. 3, the expression of p65 was increased in the colonic mucosa of the DSS group and this increase was almost completely prevented in animals treated with 5 mg/kg/day OA-NO₂. Native oleic acid did not significantly attenuate the increase in DSS-induced p65 levels.

Activation of PPAR γ by nitrated oleic acid

A role for PPAR γ in the OA-NO₂ protection against DSS-induced colitis was hypothesized; thus treatment with OA-NO₂ was evaluated for an impact on this nuclear receptor.

OA-NO₂ increased the transcriptional expression of PPAR γ in colon homogenates from the DSS + OA-NO₂ group, whereas native oleic acid (DSS + OA) had no effect (Fig. 4). Moreover, the mRNA levels of two target genes of PPAR γ , STAT-1 and FABP2, were induced after OA-NO₂ (but not OA) administration, in the colon samples (Fig. 5). The mRNA expression of PPAR γ was also induced in SW480 cells after OA-NO₂ administration (Fig. 6), whereas OA had no effect. The addition of the PPAR γ inhibitor GW9662 completely reverted the increase in PPAR γ expression.

5-ASA is commonly used in the treatment of IBD and has recently been suggested to act via activation of PPAR γ [6]. Therefore we next analyzed the effect of 5-ASA on this nuclear receptor and compared it with the activation caused by OA-NO₂. SW480 cells were treated with increasing doses (3 μ M–30 mM) of 5-ASA, and only the dose of 300 μ M significantly increased the expression of PPAR γ (Table 2). This up-regulation of PPAR γ was abolished by GW9662 pretreatment. Interestingly, PPAR γ up-regulation was higher after the administration of 1 μ M OA-NO₂ than after 300 μ M 5-ASA (Fig. 7), demonstrating that OA-NO₂ is a considerably more potent activator of PPAR γ than 5-ASA.

To confirm the involvement of OA-NO₂ in the regulation of PPAR γ , SW480 cells were transiently transfected with the PPAR γ response element and treated with OA-NO₂ and OA in the presence or absence of GW9662 (Fig. 8). OA-NO₂ increased the transcriptional activity of PPAR γ 2.5-fold, whereas OA or vehicle had no effect. Pretreatment with GW9662 prevented the up-regulation of PPAR γ in the presence of OA-NO₂. Taken together, these results show that OA-NO₂ is a potent activator of PPAR γ in the colon. Such activation may explain the potent anti-inflammatory effects of OA-NO₂ observed in this study.

Discussion

Diverse nitration products of unsaturated fatty acids (nitroalkenes) are formed endogenously via NO-dependent oxidative inflammatory reactions [22,24]. Studies in cell cultures [15] have demonstrated pluripotent anti-inflammatory signaling actions of nitrated fatty acids (NO₂-FAs), prompting the evaluation of the capability of these compounds to suppress inflammation *in vivo*.

Here we show that systemic administration of OA-NO₂ attenuates DSS-induced colitis in mice. This protective effect is associated with *in vivo* activation of colonic PPAR γ and concomitant down-regulation of NF- κ B, a key mediator of the proinflammatory response. Although activation of PPAR γ by OA-NO₂ may represent a central mechanism for the observed protection, interactions with other anti-inflammatory pathways might also contribute [15]. These findings are concordant with recent studies suggesting that activation of PPAR γ is associated with anti-inflammatory effects [2,5,25]. Indeed, activation of PPAR γ , e.g., by thiazolidinedione derivatives, inhibits the inflammatory response in experimental colitis [5,6,8,26]. Moreover, recent data have suggested that 5-ASA also acts through the activation of PPAR γ . 5-ASA is also able to serve as a PPAR γ ligand and promote its translocation from the cytoplasm to the nucleus, perhaps in a positive feedback loop [6]. Our data confirm that 5-ASA activates PPAR γ expression, and this effect is almost abolished in the presence of the PPAR γ antagonist GW9662. However, our results clearly demonstrate that nitrated oleic acid is a considerably more potent activator of PPAR γ than 5-ASA.

Although NO₂-FAs may release some NO under certain circumstances, current data indicate that NO and cGMP do not significantly transduce NO₂-FA signaling actions. Rather, the strongly electron-withdrawing nitro (NO₂) group renders nitroalkene fatty acid derivatives electrophilic. This supports a kinetically rapid and reversible covalent adduction of nucleophilic amino acids, primarily cysteine [27]. As a consequence, NO₂-FAs can posttranslationally modify critical redox-reactive thiols of various signaling mediators and transcription factors to alter their function [15]. The activation of PPAR γ is a consequence of such electrophilic protein adduction by NO₂-FAs [17,28].

Herein, native oleic acid administration as a control for OA-NO₂ failed to significantly affect DAI or colonic expression of NF- κ B and PPAR γ . However, at a higher dose (5 mg/kg/day), OA had some protective effect, as revealed by the prevention of colon shortening. At present, the mechanism behind this effect is unclear. Recently, Chen and colleagues [29] demonstrated protective effects of dietary OA in the same DSS-induced model of colitis. They found that DSS caused an inhibition of hepatic stearoyl-CoA desaturase 1, thereby decreasing oleic acid biogenesis. Apparently, this inhibition exacerbated proinflammatory responses to DSS challenge, an effect limited by dietary oleic acid supplementation. It is possible that a greater protective effect of OA would have been revealed if we had increased the dose. Indeed, the total dose of OA in the study by Chen and colleagues was close to 100 mg per day, i.e., ~1000-fold higher than the dose used in the present study. It is also plausible that the effects of OA and the previously observed protective effects of monounsaturated lipids [30] at some stage involve endogenous nitration of the fatty acids. Conditions for such reactions seem particularly favorable when using the oral administration route. Thus, in the acidic stomach salivary-derived nitrite (NO₂⁻) continuously meets gastric acid, thereby forming potent nitrating species including the nitrogen dioxide radical (NO₂ $\cdot$) [31–33]. Thus, the weak protective effect of OA could possibly be explained by some degree of endogenous nitration.

Consumption of monounsaturated fat, such as in the olive-oil-rich Mediterranean diet, is related to benefits for diseases associated with chronic inflammation, including cardiovascular disease, rheumatoid arthritis, and gastrointestinal inflammation [34]. Oleic acid is also the major constituent of olive oil. Future studies will reveal whether dietary interventions can be used to increase the circulating levels of NO₂-FAs and if this would offer any long-term protection against chronic inflammatory disorders, including IBD.

We conclude that administration of nitrated oleic acid attenuates inflammation in experimental inflammatory bowel disease. This protection involves activation of colonic PPAR γ .

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Abbreviations

DSS	dextran sulfate sodium
IBD	inflammatory bowel disease
NO₂-FA	nitrated fatty acid
OA-NO₂	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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Fig. 1. Colon length measurements after administration of DSS, in the absence or presence of OA or OA-NO₂. (A) 0.5 mg/kg/day and (B) 5 mg/kg/day. (C) DSS-induced reduction in colon length compared to the control group (0 mg/kg is the DSS group). Mann-Whitney test, *** $P < 0.0001$; ** $P < 0.004$; * $P < 0.02$.

Fig. 2.

General assessment of colitis based on the DAI. DAI was reduced after treatment with OA-NO₂ (5 mg/kg/day), compared with the DSS group. Mann-Whitney test; * $P < 0.02$.

Fig. 3. The DSS-induced increase in the expression of p65 is attenuated by OA-NO₂ (5 mg/kg/day). (A) Immunohistochemistry staining and (B) quantification of the staining (a.u., arbitrary units). Mann–Whitney test; ** $P < 0.004$.

Fig. 4.

Nitrated oleic acid up-regulates PPAR expression in vivo. PPAR gene expression was measured by quantitative RT-PCR in the colon samples from the four experimental groups (OA-NO₂: 5 mg/kg/day). Student *t* test; **P* < 0.01.

Fig. 5. Analysis of PPAR γ target genes. Gene expression of STAT-1 and FABP2 was measured in the colon samples from the four experimental groups (OA-NO₂: 5 mg/kg/day) by quantitative RT-PCR. Student *t* test; **P* < 0.01.

Fig. 6. Nitrated oleic acid up-regulates PPAR γ expression in vitro. SW480 cells were treated with OA or OA-NO₂ (1 μ M), in the absence or presence of GW9662 (1 μ M). PPAR gene expression was measured by quantitative RT-PCR. Student *t* test; ****P* < 0.001.

Fig. 7. OA-NO₂ vs 5-ASA in the regulation of PPAR γ expression. SW480 cells were treated with OA (1 μ M), OA-NO₂ (1 μ M), or 5-ASA (300 μ M). PPAR gene expression was measured by quantitative RT-PCR. Student *t* test; ****P* < 0.001.

Fig. 8.

PPAR γ is transcriptionally activated by OA-NO₂ but not by OA in vitro. SW480 cells were transiently transfected with the PPAR γ response element and treated with OA, OA-NO₂ (1 μ M), or 5-ASA (300 μ M) in the presence or absence of GW9662 (1 μ M). Expression levels of PPAR γ were measured by luciferase assay. Student *t* test; **P* < 0.01.

Table 1

Nitrated oleic acid concentration in plasma and colon samples

OA-NO ₂	Free OA-NO ₂ in plasma (nM)	Total OA-NO ₂ in plasma (nM)	Total OA-NO ₂ in colon (ng/g tissue)
Untreated	0	0	0
0.5 mg/kg/day	0.64±0.10	13.85±4.49	0.59 ^{**} ±0.13
5 mg/kg/day	8.06 [*] ±1.31	269.8±29.35	nm

Nitrated oleic acid concentration in plasma and colon samples from the OA-NO₂-treated groups (0.5 and 5 mg/kg/day) vs the untreated groups. nm, not measured.

^{*} $P < 0.001$, one-way ANOVA, post hoc Tukey.

^{**} $P < 0.01$, Student *t* test.

Table 2**5-ASA up-regulates PPAR γ expression**

Experiment	PPAR γ expression (fold)
Expt 1	
5-ASA (0 μ M)	1
5-ASA (3 μ M)	1.21
5-ASA (30 μ M)	0.93
5-ASA (300 μ M)	2.7*
5-ASA (3 mM)	0.91
5-ASA (30 mM)	1.44
Expt 2	
5-ASA (300 μ M)	2.7
GW (1 μ M) + 5-ASA (300 μ M)	0.4*

Expt 1: SW480 cells were treated with increasing doses of 5-ASA. PPAR γ expression was significantly higher with the 300 μ M dose. Expt 2: SW480 cells were treated with 5-ASA in the absence or presence of GW9662. PPAR γ gene expression was measured by quantitative RT-PCR.

* $P < 0.01$, Student t test.