Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator–activated receptor-γ

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5-aminosalicylic acid (5-ASA) is an antiinflammatory drug widely used in the treatment of inflammatory bowel diseases. It is known to inhibit the production of cytokines and inflammatory mediators, but the mechanism underlying the intestinal effects of 5-ASA remains unknown. Based on the common activities of peroxisome proliferator–activated receptor–γ (PPAR–γ) ligands and 5-ASA, we hypothesized that this nuclear receptor mediates 5-ASA therapeutic action. To test this possibility, colitis was induced in heterozygous PPAR–γ+/− mice and their wild-type littermates, which were then treated with 5-ASA. 5-ASA treatment had a beneficial effect on colitis only in wild-type and not in heterozygous mice. In epithelial cells, 5-ASA increased PPAR–γ expression, promoted its translocation from the cytoplasm to the nucleus, and induced a modification of its conformation permitting the recruitment of coactivators and the activation of a peroxisome-proliferator response element–driven gene. Validation of these results was obtained with organ cultures of human colonic biopsies. These data identify PPAR–γ as a target of 5-ASA underlying antiinflammatory effects in the colon.
PPAR-\(\gamma\) plays an important role in the maintenance of mucosal integrity in the intestine (12). Expressed at high levels in the colonic epithelium, PPAR-\(\gamma\) and its high-affinity synthetic ligands such as thiazolidinediones are involved in the regulation of colon inflammation (12–15). Based on the common activities of 5-ASA and PPAR-\(\gamma\) ligands in colonic epithelial cells, we hypothesized that PPAR-\(\gamma\) may be the molecular target of 5-ASA. In the present study, we report that chemically induced colitis in mice heterozygous at the PPAR-\(\gamma\) locus (PPAR-\(\gamma^{+/−}\)) was refractory to 5-ASA therapy. Using the HT-29 colon epithelial cell line, we found that 5-ASA induced PPAR-\(\gamma\) mRNA and protein expression. Furthermore, the ability of 5-ASA to bind and activate PPAR-\(\gamma\) in different experimental settings including receptor binding, transcriptional activation, and conformational changes indicated that 5-ASA exerts its effects in the colon through direct PPAR-\(\gamma\) activation.

RESULTS

Colon inflammation in PPAR-\(\gamma^{+/−}\) mice is refractory to 5-ASA therapy

Intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to male 129/Sv mice provoked a severe colitis, which represents a well-validated model that has many macroscopic and histologic similarities to human IBDs (Fig. 1 A) (16–18). In nontreated animals killed 5 d after colitis induction, weight loss and severe macroscopic and histologic lesions that would have resulted in death were observed in 50% of mice (Fig. 1). These lesions, characterized by large areas of ulceration, necrosis, and a transparietal neutrophilic infiltration, were scored by macroscopic and histologic evaluation as 5.2 ± 0.8 and 2.6 ± 1.2, respectively, according to the multiparametric Wallace and Ameho criteria (19, 20). The most clinically effective dosage of a controlled-release 5-ASA preparation given orally was investigated in these conventional 129/Sv mice by a detailed dose–response study of dosages ranging from 50 mg/kg/d to 1,000 mg/kg/d (Fig. 1, B–D). Evolution of weight curves was similar in control mice without colitis and mice receiving TNBS and treated with 5-ASA at 100, 500, and 1,000 mg/kg, but not with 5-ASA at 50 mg/kg (Fig. 1 B). These data were used to eliminate the 5-ASA dosage of 50 mg/kg. Because 5-ASA at 100 mg/kg was the concentration of 5-ASA closest to that used to treat IBDs in humans (21), and because the therapeutic effects of 5-ASA on body–weight changes (Fig. 1 B), mortality rate (Fig. 1 C), and macroscopic Wallace scores (Fig. 1 D) were statistically similar for the dosages of 100, 500, and 1,000 mg/kg, we selected 100 mg/kg as the optimal concentration of 5-ASA for the following study in PPAR-\(\gamma^{+/−}\) mice. At this concentration of 5-ASA, improvements in body–weight changes, mortality rate, and Wallace scores were similar those seen in the rosiglitazone-positive mice (Fig. 1, B–E). A significant decrease in colonic methyl peroxidase (MPO) and TNF-\(\alpha\) mRNA concentrations used as biological markers of inflammation paralleled this improve-

![Figure 1. Colon inflammation in PPAR-\(\gamma\) heterozygous mice is refractory to 5-ASA therapy.](image)

...ment (Fig. 1 F). To determine whether the antiinflammatory effects of 5-ASA in the colon were mediated by PPAR-\(\gamma\), colitis was induced in 129/Sv PPAR-\(\gamma^{+/−}\) mice by intrarectal TNBS administration. Compared with their wild-type littermates, these heterozygous knockout mice had a mean 70 ± 8% decrease of PPAR-\(\gamma\) mRNA levels in the colon. As established previously (17), PPAR-\(\gamma^{+/−}\) mice exhibited more pronounced macroscopic/histologic lesions and higher concentrations of biological markers of inflammation than wild-type animals (Fig. 1, E and F). In contrast with control mice, 5-ASA treatment did not affect the severity of TNBS colitis in PPAR-\(\gamma^{+/−}\) mice, as assessed by macroscopic or histologic scores, colonic MPO, or TNF-\(\alpha\) concentrations (Fig. 1, E and F), indicating PPAR-\(\gamma\) may have a major role in mediating the antiinflammatory effect of 5-ASA.

5-ASA induces differentiation of 3T3-L1 cells

Most ligands of PPAR-\(\gamma\) are potent and effective at stimulat-

ing adipogenesis in preadipocyte cell lines expressing PPAR-\(\gamma\)
such as 3T3-L1 cells (22). To evaluate the role of 5-ASA as a PPAR-γ activator, we compared the effects of different concentrations of 5-ASA and of the thiazolidinedione rosiglitazone (rosi, $10^{-5}$ M) on confluent 3T3-L1 preadipocytes at day 8 after confluence. Optimal dosage of rosiglitazone ($10^{-5}$ M) enhanced intracytosolic accumulation of lipid as monitored by Oil Red O staining in 94 ± 3% of cells (Fig. 2). Treatment with 5-ASA also induced a marked differentiation of preadipocytes in a dose-dependent manner leading to lipid accumulation in 98 ± 1% of cells (Fig. 2). Lactate dehydrogenase activity remained low and was not modified in the presence of rosiglitazone or 5-ASA (unpublished data), ensuring that 5-ASA did not exert a direct cytotoxic effect on 3T3-L1 cells. These data establish that 5-ASA has an adipogenic capacity similar to that of rosiglitazone.

**Induction and activation of PPAR-γ expression in 5-ASA-treated epithelial cells**

Experiments in cultured preadipocyte suggest that activation of PPAR-γ by rosiglitazone modestly increases the expression of this receptor in a positive-feedback loop (23, 24). We then compared the capacity of 5-ASA and of rosiglitazone to induce PPAR-γ expression at the mRNA and protein levels in the HT-29 cell line. A threefold induction of PPAR-γ mRNA expression quantified by real-time PCR technique was found in colonic epithelial cells treated for 12 h with 5-ASA, 30 mM (Fig. 3 A). Western blot extended and confirmed these results, showing a threefold induction of PPAR-γ protein levels in cells treated by 5-ASA, 30 mM, and a twofold induction of PPAR-γ protein levels in cells treated by rosiglitazone, $10^{-5}$ M (Fig. 3 B).

We also investigated PPAR-γ transcriptional activity by transient transfections (13). Analysis of PPAR-γ activity in transfected HT-29 cells showed that 5-ASA at 30 mM increased the reporter-gene activity by threefold, thereby displaying an activity similar to that of rosiglitazone (Fig. 3 C).

**Binding of 5-ASA to PPAR-γ induces activation**

Activation of PPAR-γ results in a cascade of reactions including translocation or redistribution of PPAR-γ in the cell nucleus (25, 26), conformational changes within PPAR-γ (27, 28), recruitment of coactivators, and binding to specific DNA sequence elements termed peroxisome-proliferator response elements (PPRE) (29–31). The effects of 5-ASA-bound PPAR-γ were examined at these different steps.

Intracellular localization of PPAR-γ in epithelial cells has not been characterized previously. In other cell types, distinct PPAR-γ localizations have been observed (25, 26, 32). Using fluorescence microscopy to visualize the cellular distribution of a GFP-tagged PPAR-γ, a predominantly cytoplasmic distribution of the fluorescent label was found in unstimulated HT-29 cells (Fig. 4 A). This cytoplasmic localization of PPAR-γ in epithelial cells has been noted previously ex vivo by immunohistochemistry in the colonic epithelial cells of patients (13). Exposure of epithelial cells to different concentrations of 5-ASA (1 mM, 5 mM, or 30...
pressed as fold activation (mean ± SEM) compared with untreated cells. (B) The level of PPAR-γ mRNA expression was quantified by real-time PCR in HT-29 ST cells incubated for 3, 6, 12, 18, 24, and 48 h with 5-ASA (30 mM) or rosiglitazone (10⁻⁵ M). The main induction of PPAR-γ mRNA expression was observed at 12 h in cells incubated with 5-ASA. Results were expressed as the mean ± SEM of six different experiments. (B) The level of PPAR-γ protein expression was evaluated by Western blot assay in untreated HT-29 cells (control) and after 24 h of 5-ASA (30 mM) or rosiglitazone (rosi, 10⁻⁵ M) treatment. OD values of PPAR-γ were given for each condition in proportion to the quantity of the internal control β-actin in the same sample. (C) Activation of PPAR-γ by 5-ASA. HT-29 cells transfected with the response element for PPAR-γ (2XCYP) and treated by 5-ASA (30 mM) or rosiglitazone (10⁻⁵ M) showed a similar, approximately threefold induction of PPAR-γ reporter gene activity indicating the ability of 5-ASA to induce PPAR-γ activation. Results are expressed as fold activation (mean ± SEM) compared with untreated cells.

mM) for 24 h resulted in a similar redistribution of GFP-tagged PPAR-γ to the nucleus (Fig. 4, A and B). The translocation of GFP-tagged PPAR-γ from the cytoplasm to the nucleus was also observed with rosiglitazone.

Next, to evaluate the conformational changes induced by the binding of 5-ASA to PPAR-γ, we performed a protease protection assay of [³⁵S]-PPAR-γ bound to either 5-ASA or rosiglitazone. Rosiglitazone binding did not lead to a radical change in the proteolytic pattern as compared with 5-ASA (Fig. 5). As previously described, rosiglitazone-positive con-
tightly with the PPAR-γ LBD interacting via hydrogen bonding with His-323, His-449, Tyr-473, and Ser-289, which are considered key determinants for molecular recognition and PPAR-γ activation (Fig. 8, C and D, and references 34, 39). The phenolic hydroxyl group of 5-ASA did not interact with the receptor, but its orientation favored an intramolecular hydrogen bond with the carboxyl group as shown in its crystal structure (40). Superimpositions of our 5-ASA model to other known PPAR-γ head group ligands such as the PPAR-γ–bound crystallographic conformations of tesaglitazar (AZ242) (39) and farglitazar (GI262570) (38) showed a similar PPAR-γ binding mode of 5-ASA and glitazars (Fig. 8 E).

5-ASA induces PPAR-γ expression and activation in human colonic mucosa

To validate the previous results obtained in vitro and in vivo in mice, we evaluated the effects of 5-ASA in organ cultures of right colonic biopsies taken in healthy mucosa of 12 untreated patients (6 non-IBD patients, 3 patients with Crohn’s
disease, and 3 patients with ulcerative colitis). After 24 h in a 5% CO₂ chamber, treatment of 5-ASA at 1, 30, and 50 mM increased expression of PPAR-γ mRNA and its target gene NGAL (Fig. 9 and reference 41). Release of lactic dehydrogenase from specimens in culture remained low and stable over the 24-h period. No change in lactic dehydrogenase release was induced by the presence of 5-ASA in the incubation medium.

DISCUSSION

Although 5-ASA therapy has been widely used in patients with IBD, the general mechanism underlying its antiinflammatory effects in the colon remain incompletely characterized. In the present study, we demonstrate that PPAR-γ is the key receptor for 5-ASA that mediates its main effects in the colon. This determination is based on multiple functional, pharmacological, and chemical lines of evidence and was validated in vivo in a murine model of IBD and with human clinical samples.

After oral or rectal administration into the colon, some 5-ASA is absorbed by colonic epithelial cells, but most remains within the lumen and is passed in the stool (3–5). In IBD patients receiving standard 5-ASA maintenance treatment, the median mucosal concentrations of 5-ASA are 16 ng/mg, ranging from 3 to 50 ng/mg of wet colonic tissues (2). The therapeutic effect of 5-ASA depends more on the direct contact of the molecule with the epithelium of the colon than on the tissue concentration of 5-ASA in the colon, indicating that a high perimucosal concentration of 5-ASA is a prereq-
uit site for its action. It has been previously reported in patients conventionally treated with 5-ASA that stool concentrations of 5-ASA are in the median order of 30 mM, ranging from 10 to 100 mM; these concentrations correspond to luminal concentrations of 5-ASA 100 times greater than the concentrations in the colonic mucosa (2, 7, 34, 42, 43). In vitro, these millimolar concentrations of 5-ASA (1–50 mM) are able to inhibit signal transduction (44), cell proliferation (45), and expression of the inducible nitric oxide synthase (46).

Taken together, these data indicate that the 5-ASA concentrations between 1 and 50 mM used in the present study are clinically and biologically relevant and may be even below the 5-ASA concentrations found intraluminally in IBD patients receiving 5-ASA maintenance treatment.

We have previously shown that PPAR-γ is expressed at the highest levels in adipose tissue and the colonic epithelium (12, 13, 47, 48). Best characterized as a regulator of cellular metabolism and adipocyte differentiation, PPAR-γ is also capable of inhibiting the inflammatory response in several experimental models of colitis induced either by the administration of dextran sodium sulfate (14, 15) or 2, 4, 6-TNBS (17) and by ischemia-reperfusion (49). Based on the common antiinflammatory functions of 5-ASA and PPAR-γ in epithelial cells and the high expression of PPAR-γ by the colon epithelial cells that 5-ASA targets, we hypothesized that 5-ASA may be a new functional ligand of PPAR-γ. Using clinically relevant 5-ASA concentrations (36, 37), we showed by transfection studies using GFP-tagged PPAR-γ, a PPAR-γ-sensitive reporter gene, protease protection assays, and GST pull-down assays that 5-ASA is able to bind PPAR-γ, to induce its translocation from the cytosol of epithelial cells to the nucleus, to promote a PPAR-γ conformational change, and to induce recruitment of a coactivator.

Evidence supporting 5-ASA as a direct functional agonist of PPAR-γ was obtained with a ligand-binding assay and by using 3T3-L1 cells. PPAR-γ is a master regulator of adipocyte differentiation, and this effect has been well demonstrated with the 3T3-L1 cells, which required PPAR-γ activation for their differentiation (22). We showed that, like the PPAR-γ ligand rosiglitazone, 5-ASA is an inducer of adipogenesis resulting in the conversion of 3T3-L1 preadipocytes into adipocytes. The relevance of these in vitro data was further demonstrated in an experimental model of colitis in mice treated with a controlled-release 5-ASA preparation developed to deliver the drug in the colon and to minimize its systemic absorption in the proximal intestine (3–5). Indeed, PPAR-γ+/− mice with colitis induced by intrarectal administration of TNBS were completely refractory to 5-ASA preventive treatment, whereas the same dosage of 5-ASA given to wild-type mice with the same genetic background significantly attenuated the inflammatory response in the colon. Besides this therapeutic effect of 5-ASA given preventively, we and others have also shown that, after induction of colitis by dextran sodium sulfate or TNBS, administration of PPAR-γ ligands is always effective in reducing lesion intensity and mortality compared with untreated mice, despite destruction of the epithelial cell layer (14, 17). The mechanisms involved in the therapeutic effects of PPAR-γ ligands in established intestinal lesions remain partially unknown. It is possible that expression of PPAR-γ in the colon by immune cells such as macrophages and lymphocytes residing in the lamina propria may play a role (50, 51). Because the spontaneous fluorescent properties of 5-ASA enable its localization in macrophages and lymphocytes of the lamina propria after oral or rectal administration (52), we can hypothesize that during 5-ASA administration, PPAR-γ expressed by macrophages and T cells may be activated in vivo and may induce antiinflammatory effects in the gut, as previously described (15, 53).

Regulation of PPAR-γ expression in epithelial cells remains completely unknown. There is evidence that activation of PPAR-γ by thiazolidinedione in preadipocytes can increase modestly the expression of this receptor in a positive-feedback loop (23, 24). In HT-29 colonic epithelial cells treated with 5-ASA, we observed a threefold induction of PPAR-γ expression at the mRNA and protein levels. These results obtained by quantitative real-time PCR paralleled those found in organ cultures of human colonic biopsies and by Western blot analysis at the protein level in epithelial cell line. These data suggest that 5-ASA is able to bind and activate PPAR-γ and also may enhance its expression. This point is particularly important in patients with ulcerative colitis characterized by a chronic inflammation of the colon in which PPAR-γ expression is decreased in colonic epithelial cells (13).

Taken together, these data make a compelling case that 5-ASA is a novel PPAR-γ agonist and show that PPAR-γ is the major functional receptor mediating the common 5-ASA activities in IBD. Several X-ray studies of PPAR-γ crystal structures revealed the binding mode of synthetic

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**Figure 9.** 5-ASA induces PPAR-γ expression and activation in organ cultures of human colonic biopsies. Levels of PPAR-γ mRNA (red) and its target gene NGAL (blue) quantified by real-time PCR in human colonic biopsies after 24 h of culture with medium alone and three concentrations of 5-ASA (1, 30, and 50 mM). Results are expressed as the fold induction (mean ± SEM) compared with control (medium). The number of patients and statistical significance are indicated. *, P < 0.05 compared with control (medium); †, P < 0.05 compared with 5-ASA (1 mM); ‡, P < 0.05 compared with 5-ASA (30 mM).
PPAR-γ agonists such as glitazones and glitazar s in the LBD of the receptor (38, 39). In our study, we identified 5-ASA as a new synthetic PPAR-γ ligand interacting with the LBD. Because 5-ASA was originally developed without any knowledge of its molecular target, there is hope that the research described here will lead to the rational optimization or development of better PPAR-γ ligands. Improvements in efficacy may reside in a new compound with higher affinity or an association of agents with additive or synergic effects on the PPAR-γ/retinoid x receptor (RXR) heterodimer. Studies are also in progress to evaluate if quantification of PPAR-γ expression in the intestinal mucosa of patients may be a marker for monitoring therapeutic effectiveness of 5-ASA. By analogy with glitazones or glitazar s, other studies should also be done to assess whether 5-ASA could have therapeutic effects in patients with metabolic disorders.

**MATERIALS AND METHODS**

5-ASA, GW1929 (N-aryl tyrosine activator), and 2, 4, 6-TNBS were purchased at Sigma-Aldrich. Rosiglitazone was acquired at Sphio. [3H]rosiglitazone was synthesized at Isobio.

**Induction of TNBS colitis and study design.** Animal experiments were performed in accredited establishments at the Institut de Génétique et de Biologie Moléculaire et Cellulaire in Strasbourg (B39-108 and B 67–218-5) and at the Institut Pasteur from Lille (86/609/CEE) according to governmental guidelines. Animals were housed five per cage and had free access to standard mouse chow and tap water. For colitis induction, mice were anesthetized for 90–120 min and received an intrarectal administration of TNBS (40 µl, 150 mg/kg) dissolved in a 1:1 mixture of 0.9% NaCl with 100% ethanol (13, 18). Control mice received a 1:1 mixture of 0.9% NaCl with 100% ethanol or a saline solution using the same technique. Animals were killed 5 d after TNBS administration. The antiinflammatory effects of 5-ASA were evaluated by administration of different dosages of a control preparation (50 mg/kg/d–1,000 mg/kg/d) and compared with the PPAR-γ ligand rosiglitazone used at the optimal dosage of 20 mg/kg in 129/Sv wild-type and PPAR-γ/+ mice (Fig. 1 and reference 17). 5-ASA was administered orally once daily, starting 2 d before colitis induction. Body-weight changes and macroscopic and histological indications of colitis were evaluated blindly by two investigators. The colon of each mouse was examined under a dissecting microscope (at a magnification of 5) to evaluate the macroscopic lesions according to the Wallace criteria. The Wallace score rates macroscopic lesions on a scale from 0 to 10 based on features reflecting inflammation, such as hyperenu, thickening of the bowel, and extent of ulceration (19). A colon specimen located precisely 2 cm above the anal canal was used for histological evaluation according to the Ameba criteria (20). This grading on a scale from 0 to 6 takes into account the degree of inflammation infiltrate, the presence of erosion, ulceration, or necrosis, and the depth and surface extension of lesions. The other part of the colon were frozen and used to quantify PPAR-γ mRNA levels, MPO, and TNF-α mRNA levels (16, 17).

**Cell lines.** 3T3-L1 preadipocytes (ATCC CC-CL173) were grown to confluence in DME (Invitrogen) with 10% FCS and antibiotics. The medium was supplemented with 1 µM dexamethasone, 0.2 mM isobutyl methyl xanthine, 10 µg/ml insulin, and 10% FCS for 2 d (27). The effects of 5-ASA (10⁻⁴–M–10⁻² M) on the differentiation of 3T3-L1 preadipocytes into adipocytes were compared with the effects of rosiglitazone (10⁻⁵ M) on confluent cells over 8 d. Medium, 5-ASA, and rosiglitazone were changed every 2 d. Cell viability was assessed by testing lactate dehydrogenase activity in the culture medium taken during the course of the differentiation process or on mature 3T3-L1 cells, treated or not with rosiglitazone and 5-ASA. Adipogenesis was scored by staining lipids with Oil Red O. Counts of at least 500 cells/sample were systematically performed blindly in four different experiments. Results were expressed as the mean ± SEM number of stained cells per experiment.

The colon carcinoma cell line HT-29 STD (American Type Culture Collection; HTB-38) was routinely grown in DME supplemented with 10% heat-FCS and antibiotics. Cells were grown in monolayers, incubated in 5% CO₂ at 37°C and 95% relative humidity.

**Real-time mRNA quantification.** Total RNA was isolated from cells and colonic tissues using Rnaseasy kit (Macherey Nagel) according to the manufacturer’s instructions. RNA quantification was performed using spectrophotometry. After treatment at 37°C for 30 min with 20–50 units of RNase-free DNase I (Roche Diagnostics Corp.), oligo-dT primers (Roche Diagnostics Corp.) were used to synthesize single-stranded cDNA. mRNAs were quantified using SYBR green Master Mix (Applera Corp.) with specific mouse oligonucleotides: TNF-α, forward, 5’-TGGAGATGACCAAGTGCAACCC-3’, reverse, 5’-CATCCTTCTCAATTCGACTGACAA-3’; and human oligonucleotides: PPAR-γ, forward, 5’-CCTGTATAGGCCCATCTGTG-3’, reverse, 5’-CAGTGGAGGTGGAAC-CAAT-3’ and NGAL, forward, 5’-TCACGGAGGGCCCAAGA-3’, reverse, 5’-CCTCTACGGAGAAACCAAGG-3’ in a GeneAmp Abiprism 7000 (Applera Corp.). In each assay, calibrated and no-template controls were included. Each sample was run in triplicate. SYBR green dye intensity was analyzed using the Abiprism 7000 SDS software (Applera Corp.). All results were normalized to the unaltered housekeeping gene β-actin: mouse β-actin, forward, 5’-GGGTCAAGAAATCCCTATG-3’, reverse, 5’-GGTCCTAACATGATCGTG-3’ and human β-actin, forward, 5’-TCACCAACATCGTGGCCCATCAGG-3’, reverse, 5’-CAGCCGAAA-CGGCTATTGCAAG-3’ (16).

**Evaluation of PPARγ, β-actin, and MPO by Western blot analysis.** Total proteins were obtained by cell or colon homogenization in an extraction buffer consisting of PBS with 2% triton, 100 mM PMSF, and a classical protease-inhibitor cocktail. Total proteins were then separated by PAGE and electroblotted. Polyanvylidenefluoride membranes were incubated overnight with rabbit polyclonal primary antibody directed against PPAR-γ (dilution 1/500, Tebu Bio) or MPO (dilution 1/500; DakoCytomation) (13, 17). β-actin was detected using a rabbit monoclonal primary antibody diluted at 1/10,000 (Sigma-Aldrich). Immunodetection with a swine secondary peroxidase-conjugated antibody (1/1,000; DakoCytomation) and chemiluminescence were performed according to manufacturer’s protocol (ECL, Amersham Biosciences). MPO results were expressed as units of OD per quantity of total protein. OD values of PPAR-γ were given for each condition in proportion to the quantity of the internal control β-actin in the sample.

**GFP-tagged PPAR-γ.** 1 µg of GFP-tagged PPAR-γ vector was transfected in HT-29 STD cells using the Effectene transfection reagent (QiAGEN). After 24 h incubation, cells were treated with 5-ASA (1 mM, 5 mM, or 30 mM) or with rosiglitazone (10⁻⁵ M) for 1 d. After two PBS washes, cells were fixed by 4% paraformaldehyde. Nuclei were stained with Hoechst 33342 solution, 0.125 mg/ml (Sigma-Aldrich). Slides were mounted and visualized under a fluorescence microscope (Leica).

**Protease digestion assay.** [³⁵S]Methionine-labeled full-length human PPAR-γ was generated using transcription/translation system (Promega) according to the manufacturer’s instructions. 1 µl of labeled PPAR-γ2, diluted in binding buffer (10 mM Heps, pH 7.4; 100 mM KCl; 2 mM diithiothreitol; 10% glycerol), was incubated with 5-ASA (5 mM) or rosiglitazone (10⁻⁵ M) for 20 min at 4°C and submitted to chymotrypsin digestion as previously described (54). This high concentration of rosiglitazone was used to ensure complete receptor saturation. Different concentrations of chymotrypsin (0, 1, 3, 5, 6, 9, and 12 U; Sigma-Aldrich) were added to the mix for 30 min at 25°C. The reaction was terminated by the addition of 15
μl of 3X denaturing gel loading buffer and by boiling for 1 min. Proteolysis products were separated by electrophoresis through a 12% SDS-PAGE gel. After electrophoresis, gels were dried, and radioactivity was detected with a Storm 860 phosphorimager (Molecular Dynamics).

GST pull-down experiments. The protocol used has been published elsewhere (55). In brief, 5–10 pmol (5 μl) of [35]S-labeled human PPAR-γ was incubated with the indicated concentration of ligand in a 200-μl final volume. The binding buffer consisted of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100. After 1 h incubation at 20°C, 40 μl of GST-DRIP slurry was added to the mix and agitated slowly on a rotating wheel for 90 min at 20°C. Unbound material was removed by three successive washes of sepharose beads by 10 volumes of 1X phosphate-buffered saline 0.1% Triton X-100. Resin-bound receptors were then resolved by SDS-PAGE on a 10% gel and detected by autoradiograph with a Dupont Storm 860 phosphorimager (Molecular Dynamics). The amount of PPAR-γ bound to coactivator in the presence of the indicated ligands was expressed as fold-induction relative to that measured in the absence of ligand (defined as 1). Each sample was processed in duplicate.

Transient transfection with PPAR-γ and stimulation of cells. HT-29 STD cells were transiently transfected using the Effectene transfection reagent (QIAGEN) according to instructions from the manufacturer. To test PPAR-γ activation, we performed transfection with 500 ng of a minimal promoter construct containing two copies of PPRE obtained from the cytomegoc p450 4A (2XCPY) (13). The renilla luciferase plasmid (0.1 μg/well) was also transfected as an internal control for monitoring transfection efficiency and for normalizing the firefly luciferase activity. Transfected cells were incubated for 48 h at 37°C. Stimulations were performed for 6 h with 5-ASA 30 mM or with the PPAR-γ synthetic ligand rosiglitazone 10−6 M used as positive control. Total cell extracts were prepared using the Passive Lysis Buffer (Promega). Luciferase activity was assayed in 20 μl of the extract using Promega’s Dual Luciferase assay system according to the manufacturer’s protocol. Transfections were assayed in triplicate in at least three separate experiments. The luciferase activity was expressed as fold of the activity obtained in cells treated with 5-ASA dividing by luciferase activity from nonstimulated cells.

Competition binding assay for PPAR-γ. Ligand-binding assays were processed as described previously (56). In brief, purified PPAR-γ-LBD (In-vitrogen) was incubated at 4°C for 2–3 h in 100 μl of buffer A (50 mM Tris, pH 8.0, 100 mM KCl, 100 μg/ml OVA, 0.3% [3-cholamidopropyl dimethylammonio]-1-propanesulfonate), and 10 mM diethiothreitol with 40 nM [3H]rosiglitazone in the presence of increasing concentrations of unlabeled 5-ASA (0–100 mM) or the known PPAR-γ agonist GW1929 (0–800 nM, Sigma-Aldrich) used as positive control. 40 nM is the concentration of [3H]rosiglitazone usually required for competition experiments, yielding a reversible 80% saturation of the receptor (27). Bound radioactive ligand was separated from free radioactive ligand by incubating samples for 10 min at 4°C with 50 μl of a charcoal–dextran suspension (3% charcoal, 0.3% dextran in buffer A) and was quantified by liquid scintillation counting. Each assay was performed in triplicate. Kd values were calculated according to the equation of Cheng and Prusoff corresponding to \( K_d = \frac{IC_{50}}{1 + \left(\frac{[L]}{K_L}\right)} \) where \( L \) is the concentration of [3H]rosiglitazone (40 nM), and the Kd for rosiglitzone is 45 nM (57).

Molecular modeling. Molecular modeling studies were performed using SYBYL software version 6.9 (Tripos Associates Inc.) running on Silicon Graphics workstations. A three-dimensional model of the zwitterion form of 5-ASA was built from a standard fragments library, and its geometry was subsequently optimized using the Tripos force field (56) including the electrostatic term calculated from Gasteiger and Hückel atomic charges. Powell’s method, available in Maximin 2 (SYBYL 6.9; Tripos Associates) procedure, was used for energy minimization until the gradient value was smaller than 0.001 kcal/mol Å. The structure of the human PPAR-γ ligand-binding domain was obtained from its complexed X-ray crystal structure with the rosiglitazone available in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (1FM6) (58). Flexible docking of 5-ASA into the receptor active site was performed using GOLD software (Cambridge Crystallographic Data Centre; reference 59). The most stable docking models were selected according to the best-scored conformation predicted by the GoldScore (59) and X-Score scoring functions (60). The complexes were energy-minimized using the Powell method available in Maximin2 procedure with the Tripos force field and a dielectric constant of 4.0 until the gradient value reached 0.01 kcal/mol Å. The anneal function was used to define a 10-Å hot region and a 15-Å region of interest around the ligand.

Patients and organ culture. Six endoscopically normal right colonic biopsies were taken with an endoscopic forceps on surgical specimens from 12 untreated patients operated on for Crohn’s disease (n = 3), ulcerative colitis (n = 3), or colon cancer (n = 6). The study was approved by the local ethics committee. Biopsies were immediately placed in HBSS–calcium or magnesium free supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. After washing, biopsies were incubated in a humidified atmosphere for 1 d at 37°C in six-well tissue-culture plates containing 2 ml of RPMI 1640 supplemented with 1, 30, or 50 mM 5-ASA (61). Supernatants were removed, filtered, and stored at −80°C for determination of lactate dehydrogenase release, and biopsies were processed to quantify PPAR-γ and NGAL mRNA.

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