INFLAMMATORY BOWEL DISEASE

Peroxisome proliferator activated receptor γ in colonic epithelial cells protects against experimental inflammatory bowel disease

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Gut 2006;55:1104–1113. doi: 10.1136/gut.2005.081745

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Revised version received
4 December 2005
Accepted for publication
21 December 2005
Published online first
17 March 2006

Introduction: Peroxisome proliferator activated receptor γ (PPAR γ) is expressed in epithelial cells, macrophage, and T and B lymphocytes. Ligand induced activation of PPAR γ was reported to attenuate colitis activity but it is not clear whether this protection is mediated by epithelial or leucocyte PPAR γ .

Methods: Mice with targeted disruption of the PPAR γ gene in intestinal epithelial cells, generated using a villin-Cre transgene and floxed PPAR γ allele and designated PPAR $\gamma^{\Delta\text{EpC}}$, were compared with littermate mice having only the PPAR γ floxed allele with no Cre transgene that expressed PPAR γ in the gut, designated PPAR $\gamma^{\text{F/F}}$. Colitis was induced by administering dextran sodium sulphate (DSS) and the two mouse lines compared for typical symptoms of disease and expression of inflammatory cytokines.

Results: PPAR $\gamma^{\Delta\text{EpC}}$ mice displayed reduced expression of the PPAR γ target genes ADRP and FABP in the gut but were otherwise normal. Increased susceptibility to DSS induced colitis, as defined by body weight loss, colon length, diarrhoea, bleeding score, and altered histology, was found in PPAR $\gamma^{\Delta\text{EpC}}$ mice in comparison with PPAR $\gamma^{\text{F/F}}$ mice. Interleukin (IL)-6, IL-1 β , and tumour necrosis factor α mRNA levels in colons of PPAR $\gamma^{\Delta\text{EpC}}$ mice treated with DSS were higher than in similarly treated PPAR $\gamma^{\text{F/F}}$ mice. The PPAR γ ligand rosiglitazone decreased the severity of DSS induced colitis and suppressed cytokine production in both PPAR $\gamma^{\text{F/F}}$ and PPAR $\gamma^{\Delta\text{EpC}}$ mice.

Conclusions: These studies reveal that PPAR γ expressed in the colonic epithelium has an endogenous role in protection against DSS induced colitis and that rosiglitazone may act through a PPAR γ independent pathway to suppress inflammation.

Inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn's disease, is associated with chronic relapsing inflammation of the intestinal tract of unknown aetiology.^{1–3} Evidence suggests that IBD is triggered by a disturbance of the barrier to luminal antigens and pathogens in colonic epithelium and an aberrant immune response to enteric flora, leading to intestinal inflammation. The role of peroxisome proliferator activated receptor γ (PPAR γ) in the aetiology and treatment of IBD has been of great interest as ligands for this receptor have been in use for the treatment of type II diabetes and these ligands can attenuate inflammation under certain experimental conditions.

PPAR γ is a member of the nuclear receptor superfamily of transcription factors, most of which are ligand dependent transcriptional activators. PPAR γ is abundantly expressed in adipose tissue and colonic epithelium. Expression has also been observed in muscle, macrophage, and in T and B cells of humans and rodents.^{4–5} Ligands for PPAR γ include natural compounds with relatively low affinity such as polyunsaturated fatty acids, oxidised low density lipoprotein, certain eicosanoids, and 15deoxy- Δ 12,14-PGJ₂, and drugs including the thiazolidinedione derivatives troglitazone, rosiglitazone, and pioglitazone used for the treatment of type II diabetes.^{6–8} PPAR γ is the key transcription factor controlling adipogenesis associated with lipid storage, and ligand activation of PPAR γ is used to improve insulin sensitivity in type II diabetics.^{9–12} Genetic studies in mouse models have revealed a role for this receptor in the control of blood pressure¹³ and renal fluid retention^{14–15} in the kidney. PPAR γ was found to have tumour suppressor effects in the colon and other tissues^{16–21} although controversy still exists regarding its role in the colon in mouse

models of carcinogenesis.²² In humans, an allelic variant of PPAR γ that exhibits increased sensitivity to ligand activation and is associated with a reduced risk of type II diabetes²³ appears to protect against colon adenomas.^{24–25} The transcriptional coactivator Hic-5 was found to promote PPAR γ associated differentiation of colon epithelia thus suggesting a key role for PPAR γ in gut homeostasis.²⁶

Recently, thiazolidinediones were suggested to be of clinical benefit in the treatment of IBD. Indeed, 5-aminosalicylic acid, a drug used in the treatment of IBD patients, may exert its effects through PPAR γ .²⁷ Studies performed in vivo have shown that PPAR γ ligands suppress the inflammatory response by attenuating the production of chemokines and cytokines secreted from macrophage,²⁸ T and B lymphocytes,²⁹ and epithelial cells.³⁰ PPAR γ ligands also decrease the severity of colitis activity induced in mouse models.^{31–39} Furthermore, the observation that PPAR $\gamma^{+/-}$ heterozygous mice exhibit an increased susceptibility to experimentally induced colitis suggests that PPAR γ may have an important role in homeostasis within the gastrointestinal tract that affects the severity of colitis.³³ Other studies revealed that colonic epithelial cells from UC patients displayed dramatically

Abbreviations: ADRP, adipose differentiation related protein; DSS, dextran sodium sulphate; EpC, epithelial cell; FABP, fatty acid binding protein; F, floxed allele; IBD, inflammatory bowel disease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KLF4, kruppel-like factor 4; PPAR, peroxisome proliferator activated receptor; TNF- α , tumour necrosis factor α ; UC, ulcerative colitis; IL, interleukin; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPA, ribonuclease protection assays; DIG, digoxigenin; MIF, macrophage migration inhibitory factor

impaired expression of PPAR γ , in particular reduced expression and dependency on intestinal flora,³⁴ indicating that PPAR γ expressed in gut epithelium may have a protective effect against colon inflammation in humans. However, the colon contains several cell types that express PPAR γ and thus the role of epithelial cell PPAR γ in IBD and other diseases of the colonic remains unknown.

To assess the role of PPAR γ expressed in intestinal epithelium in the protection against experimentally induced IBD, conditional null mice with intestinal epithelial cell specific disruption of PPAR γ , designated PPAR $\gamma^{\Delta\text{IEpC}}$, were created, using a floxed PPAR γ allele⁴⁰ and Cre recombinase under the control of the villin gene promoter.⁴¹ PPAR $\gamma^{\Delta\text{IEpC}}$ mice displayed a significantly enhanced susceptibility to dextran sodium sulphate (DSS) induced colitis compared with their wild-type littermates, PPAR $\gamma^{\text{F/F}}$ mice. mRNA levels of interleukin (IL)-6, IL-1 β , and tumour necrosis factor α (TNF- α) in the colon in PPAR $\gamma^{\Delta\text{IEpC}}$ mice were also higher than in PPAR $\gamma^{\text{F/F}}$ mice. These studies reveal that PPAR γ in the colonic epithelium has a major protective effect against DSS induced colitis.

METHODS

Intestine specific PPAR $\gamma^{\Delta\text{IEpC}}$

PPAR $\gamma^{\text{F/F}}$ mice, produced as described previously,⁴⁰ were crossed with mice harbouring the Cre DNA recombinase under the control of the villin promoter (villin-Cre mice).⁴¹ Mice homozygous for the PPAR γ floxed allele and hemizygous for the villin-Cre transgene (designated PPAR $\gamma^{\Delta\text{IEpC}}$) and littermate control mice (designated PPAR $\gamma^{\text{F/F}}$) were generated. Subsequently, PPAR $\gamma^{\Delta\text{IEpC}}$ and PPAR $\gamma^{\text{F/F}}$ were interbred for at least six generations in order to produce littermates of the same mixed genetic background. Mice were reared on a 12 hour light/dark cycle and fed water and a pellet chow diet (NIH-07) ad libitum. All animal studies were carried out in accordance with Institute of Laboratory Animal Resources guidelines and approved by the National Cancer Institute Animal Care and Use Committee.

Quantitative real time polymerase chain reaction (PCR)

Total RNA was prepared from colon epithelium using Trizol reagent (Life Technology Inc., Rockville, Maryland, USA) following the manufacturer's instructions. cDNA was generated using 2.5 μg total RNA with the MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, California, USA). Primers were designed for real time PCR using Primer Express software (Applied Biosystems). Sequence and Genbank accession numbers for the forward and reverse primers used to quantify mRNAs were: FABP (NM_017399) forward 5'-CCA TGA ACT TCT CCG GCA AGT-3' and reverse 5'-TCC TTC CCT TTC TGG ATG AGG T-3'; ADRP (NM_007408) forward 5'-CAC AAA TTG CGG TTG CCA AT-3' and reverse 5'-ACT GGC AAC AAT CTC GGA CGT-3'; KLF4 (NM_010637) forward 5'-CCA GAC CAG ATG CAG TCA CAA-3' and reverse 5'-ACG ACC TTC TTC CCC TCT TTG-3'; keratin 20 (NM_023256) forward 5'-ATG AAG TCC TGG CCC AGA AGA-3' and reverse 5'-TTT CAT TTC AGC TCC TCC GTG TTC ACT-3'; PPAR β/δ (NM_011145) forward 5'-TTG AGC CCA AGT TCG AGT TTG CTG-3' and reverse 5'-ATT CTA GAG CCC GCA GAA TGG TGT-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (BC_095932) forward 5'-CAT GGC CTT CCG TGT TCC TA-3' and reverse 5'-GCG GCA CGT CAG ATC CA-3'. Real time PCR reactions were carried out using SYBR green PCR master mix (Finnzymes, Espoo, Finland) in the PTC-200 DNA Engine Cycler and detected using the CFD-3200 Opticon Detector (MJ Research, Waltham, Massachusetts, USA). The following conditions were used for PCR: 95°C for 15 seconds, 94°C for 10 seconds, 60°C for

30 seconds, and 72°C for 30 seconds, and repeated for 45 cycles. PCR included a no template control reaction to control for contamination and/or genomic amplification. All reactions had >90% efficiency. Relative expression levels of mRNA were normalised to GAPDH and analysed for statistical significance using the Student's *t* test (Prism 4.0).

Induction of colitis with DSS and rosiglitazone treatment

Female mice, 8–10 weeks old, PPAR $\gamma^{\Delta\text{IEpC}}$ mice or PPAR $\gamma^{\text{F/F}}$ mice, were administered 2.5% (wt/vol) DSS (molecular weight 35 000–44 000; ICN Biomedicals, Aurora, Ohio, USA) in the drinking water for seven days. For rosiglitazone (SmithKline Beecham Pharmaceuticals, West Sussex, UK) treatment, a powdered diet (AIN-93G; Dyets Inc., Bethlehem, Pennsylvania, USA) blended with the drug was administered for two days prior to DSS treatment to the end of DSS treatment to yield a dose of drug of approximately 20 mg/kg/day.

Assessment of colitis

After DSS treatment was started, daily changes in body weight and clinical signs of colitis, such as rectal bleeding, diarrhoea, and piloerection, were examined. Disease activity index consisted of scoring for rectal bleeding (0–4) and diarrhoea (0–3) as previously reported.⁴² Hemocult SENSE (Beckman Coulter, Inc., Fullerton, California, USA) was used for examination of rectal bleeding. Macroscopic colonic damage was evaluated as previously described.⁴³

Southern blot analysis

Southern blot analysis to assess the floxed and recombined PPAR γ alleles was performed as previously described.⁴⁰ Genomic DNA isolated from epithelial cells from the colon, caecum, and small intestine, and total lung and kidney, were digested with BamHI, subjected to electrophoresis on a 0.5% agarose gel, and transferred to a GeneScreen Plus Hybridisation Transfer membrane (NEN Life Sciences, Boston, Massachusetts, USA). A 0.6 kb DNA fragment (SalI-EcoRI) derived from intron 2 of the PPAR γ gene (3'-probe) was used as a probe after labelling with ³²P by random priming in the presence of $\alpha^{32}\text{P}$ -dATP. Blots were exposed to a phosphorimager screen cassette followed by visualisation using a Molecular Dynamics Storm 860 Phosphorimager system (Sunnyvale, California, USA).

RNA analysis

RNA was extracted from total colon after DSS treatment using TRIzol reagent (Life Technology Inc.). Northern blot analysis was performed as previously described using standard protocols.⁴⁰ cDNA probes for PPAR γ (exon 2) and ribosomal protein 36B4 were amplified from a mouse cDNA library by PCR using gene specific primers and cloned into a pGEM-T Easy Vector (Promega Corp., Madison, Wisconsin, USA). The sequences were confirmed using a CEQ 2000XL DNA Analysis System (Beckman Coulter, Fullerton, California, USA). For ribonuclease protection assays (RPA), the Multiprobe RNase protection assay was performed according to the manufacturer's (Pharmingen, Philadelphia, Pennsylvania, USA) directions with modifications as previously described.⁴⁴ RNA was fractionated by electrophoresis through a formaldehyde-agarose gel and transferred to GeneScreen Plus membranes (DuPont, Wilmington, Delaware, USA) and the blots were hybridised at 42°C in Ultrahyb (Ambion, Austin, Texas, USA) with random primed labelled cDNA probes. Northern blots and RPA gels were visualised using a Molecular Dynamic Storm 860 Phosphorimager system and the signals quantified by

Figure 1 Recombination of deletion of the floxed peroxisome proliferator activated receptor γ ($\text{PPAR}\gamma$) allele (exon 2) in mice with intestinal epithelial cell specific disruption of $\text{PPAR}\gamma$ ($\text{PPAR}\gamma^{\Delta\text{EpC}}$ mice). (A) Southern blot analysis of BamHI digested genomic DNA isolated from epithelial cells of the colon (Co), caecum (Ce), small intestine (Si), lung (L), kidney (K), and brown adipose tissue (B) isolated from $\text{PPAR}\gamma^{\Delta\text{EpC}}$ or $\text{PPAR}\gamma^{F/F}$ mice. Floxed and deleted alleles migrated at approximately 10 and 8 kb, respectively. (B) Polymerase chain reaction (PCR) analysis of the recombination of $\text{PPAR}\gamma$ -loxP allele in genomic DNA isolated from epithelial cells of the colon (Co), caecum (Ce), small intestine (Si), lung (L), and kidney (K) in $\text{PPAR}\gamma^{\Delta\text{EpC}}$ or their wild-type littermates ($\text{PPAR}\gamma^{F/F}$ mice). The floxed and null alleles appear at 285 bp and 400 bp, respectively (10). (C) Northern blot analysis of $\text{PPAR}\gamma$ mRNA in $\text{PPAR}\gamma^{\Delta\text{EpC}}$ or $\text{PPAR}\gamma^{F/F}$ mice. Total RNA isolated from the colon (Co), caecum (Ce) and brown adipose tissue (B) were hybridised with riboprobes for $\text{PPAR}\gamma$ -exon 2 and 36B4. (D) Immunoblot analysis of $\text{PPAR}\gamma$ expression in nuclear extracts (10 μg) from colon epithelial cells isolated from two $\text{PPAR}\gamma^{\Delta\text{EpC}}$ mice and two $\text{PPAR}\gamma^{F/F}$ mice and CV-1 cells (10 μg) were subjected to western blot analysis and the blots developed by exposure to autoradiographic film. The blots were developed with antibody against $\text{PPAR}\gamma$ and GAPDH. NS, non-specific band that reacts with the $\text{PPAR}\gamma$ antibody. (E) Real time PCR analysis of adipose differentiation related protein (ADRP), fatty acid binding protein (FABP), keratin 20, kruppel-like factor 4 (KLF4), and $\text{PPAR}\beta/\delta$ mRNA in $\text{PPAR}\gamma^{F/F}$ (Cre $^{-}$) or $\text{PPAR}\gamma^{\Delta\text{EpC}}$ (Cre $^{+}$) mice. Total RNA isolated from colon epithelium was reverse transcribed and quantitative real time PCR analysis was used to quantify mRNA levels in both genotypes using 5 ng cDNA. * $p < 0.05$, Student's t test. Values are expressed as relative to the $\text{PPAR}\gamma^{F/F}$ control.

ImageQuant software package (Molecular Dynamics, Sunnyvale, California, USA).

Western blot analysis

Epithelial cells were isolated from colon and cell extracts prepared by sonication. Monkey kidney CV-1 cells transfected with a mouse $\text{PPAR}\gamma 1$ expression vector⁴⁵ were used as a positive control. Nuclear extracts were prepared as described previously⁴⁵ and protein concentrations were determined using the BCA Kit (Pierce Biotechnology Inc, Rockford, Illinois, USA). Nuclear extract proteins (10 μg) from each sample were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis through 10% polyacrylamide gels and transferring onto nitrocellulose membranes (Schleicher and Schuell, Keene, New Hampshire, USA). Staining was carried out using mouse anti- $\text{PPAR}\gamma$ monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) and peroxidase conjugated goat antimouse IgG horseradish peroxidase secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Staining for mouse GAPDH was carried out using a goat antimouse GAPDH (Chemicon, Temecula, California, USA). Blots were developed with an enhanced chemiluminescence

detection kit (Pierce) and visualised using autoradiographic film.

Histological analysis

For histological analysis, tissue samples were fixed in 10% neutral buffered formalin overnight at 4°C and paraffin embedded. Thereafter, 4 μm sections were cut, deparaffinised with xylene, and stained with haematoxylin-eosin. Alcian blue staining was carried out using the MicroProbe System Alcian blue Stain Kit (Fisher Scientific Company, Virginia, USA). Nuclei were counterstained by Nuclear Fast Red (Kernechtrot).

In situ hybridisation

In situ hybridisation was carried out using digoxigenin (DIG) labelled cRNA probes and DIG RNA Labelling Mix (Roche Applied Science, Indianapolis, Indiana, USA) according to the manufacturer's instruction with a slight modification. In brief, 5 μM frozen sections were incubated in 2 $\mu\text{g}/\text{ml}$ proteinase K for 30 minutes at 37°C and then 4% paraformaldehyde for 15 minutes to stop the reaction, followed by incubation in 0.2 M HCl for 10 minutes. Sections were washed twice in PBS containing 0.1% active diethyl

Figure 2 (A, B) In situ hybridisation analysis of peroxisome proliferator activated receptor γ (PPAR γ) mRNA expression in mice with intestinal epithelial cell specific disruption of PPAR γ (PPAR $\gamma^{\Delta EPC}$ mice) (A) and their wild-type littermates (PPAR $\gamma^{F/F}$ mice) (B). The inset in (B) is in situ hybridisation for the PPAR γ sense probe. (C–G) Histological analysis of PPAR $\gamma^{\Delta EPC}$ or PPAR $\gamma^{F/F}$ mouse colons. Haematoxylin-eosin staining was performed on colon sections prepared from paraffin embedded samples from PPAR $\gamma^{\Delta EPC}$ (B, F) or PPAR $\gamma^{F/F}$ (C) mice. Alcian blue staining was performed on colon sections prepared from PPAR $\gamma^{\Delta EPC}$ (D) or PPAR $\gamma^{F/F}$ (E) mice. (A–G) 400 $\times$ magnification. Arrows refer to points discussed in the results section.

pyrocarbonate (Sigma-Aldrich Co., St Louis, Missouri, USA) for 15 minutes and equilibrated in 5 $\times$ SSC for 15 minutes. Sections were then subjected to prehybridisation with hybridisation buffer (5 $\times$ SSC, 50% formamide, 40 μ g/ml salmon sperm DNA) for two hours at 50 $^{\circ}$ C, followed by hybridisation with a PPAR γ (exon 2) probe overnight at 50 $^{\circ}$ C. A PPAR γ specific probe was generated from mouse PPAR γ exon 2 cDNA inserted into pGEM (Promega Corp). The antisense and sense probes were synthesised by T7 and SP6 polymerases, respectively, after linearisation of the vector with HindIII and EcoRI, respectively. Sections were serially

washed in 2 $\times$ SSC, 1 $\times$ SSC, 0.5 $\times$ SSC, and 0.1 $\times$ SSC for one hour each at room temperature. They were then equilibrated in buffer I (100 mM Tris, 150 mM NaCl, pH 7.5) for five minutes and treated with 1:1000 dilution of anti-DIG antibody (Roche Applied Science) in buffer I containing 0.5% blocking reagent for two hours at room temperature. Sections were washed twice in buffer I for five minutes and incubated with biotinylated anti-sheep IgG (Vector Laboratories, Burlingame, California, USA) for 30 minutes at room temperature. Sections were then incubated with alkaline phosphatase conjugated avidin and biotin complex

for 30 minutes at room temperature and subjected to incubation with buffer II (100 mM Tris, 150 mM NaCl, 50 mM MgCl₂, pH 9.5) for five minutes. Buffer II containing 450 µg/ml of 4-nitroblue tetrazolium chloride and 175 µg/ml of 5-bromo-4-chloro-3-indolyl-phosphate was applied to each section and the reaction was stopped in TE buffer.

Statistical analysis

All values in the figures and text are expressed as mean (SEM). Statistical comparisons were examined with ANOVA followed by Fisher's protected least significant difference test. Results were considered significantly different at $p < 0.05$.

RESULTS

Generation of intestinal epithelial cell specific PPAR γ null mice

To study the functional role of PPAR γ in intestinal epithelial cells in IBD, PPAR γ -floxed mice (PPAR $\gamma^{F/F}$) mice were crossed with villin-Cre transgenic mice to generate mice lacking expression of the receptor in the intestinal epithelia. PPAR $\gamma^{\Delta EpC}$ mice were born at the expected Mendelian frequencies and exhibited no overt abnormalities compared with PPAR $\gamma^{F/F}$ littermates. To estimate the extent of intestinal epithelium specific deletion of the PPAR γ floxed exon 2, recombination was examined by PCR and Southern blot analyses, as described previously.⁴⁰ By PCR analysis, the null allele from the recombined PPAR γ floxed allele, amplified as a 400 bp product, was detected in epithelial cells of the colon, caecum, and small intestine isolated from PPAR $\gamma^{\Delta EpC}$ mice and was not detected in DNA isolated from the lung, kidney, and brown adipose tissue of these mice, other known sites of significant PPAR γ expression (fig 1A).

The intact floxed allele, which was amplified as a 285 bp product, was not detected in epithelial cells of the colon, caecum, or small intestine isolated from PPAR $\gamma^{\Delta EpC}$ mice. By

Southern blot analysis, an 8 kb fragment that represents the PPAR γ gene lacking exon 2 was only detected in DNA isolated from epithelial cells of the colon, caecum, and small intestine isolated from PPAR $\gamma^{\Delta EpC}$ mice; this fragment was not found in DNA isolated from the lung, kidney, and brown adipose tissue (fig 1B). The intact PPAR γ exon 2 floxed allele, represented by a 10 kb hybridising band, was not detected in DNA isolated from epithelial cells of the colon, caecum, and small intestine of PPAR $\gamma^{\Delta EpC}$ mice but was found in DNA from the lung, kidney, and brown adipose tissue. These data suggest that epithelial cells of the colon, caecum, and small intestine of PPAR $\gamma^{\Delta EpC}$ mice had almost complete recombination of the PPAR γ floxed allele, while no recombination was found in the lung, kidney, or brown adipose.

This degree of specificity is in agreement with an earlier report showing, using a villin-beta gal as well as a villin-Cre transgenic mouse crossed with the R26R indicator strain, that expression of the villin gene promoter was restricted to intestinal epithelial cells.⁴¹ As shown by northern blot analysis using a probe for PPAR γ exon 2, the colon and caecum of PPAR $\gamma^{\Delta EpC}$ mice had essentially no expression of PPAR γ mRNA whereas BAT of PPAR $\gamma^{\Delta EpC}$ mice showed a similar degree of expression of PPAR γ mRNA to brown adipose tissue of PPAR $\gamma^{F/F}$ mice (fig 1C). The reason that no hybridising bands were detected in the colon and caecum of PPAR $\gamma^{\Delta EpC}$ mice is likely due to the fact that cells other than epithelial cells in the colon do not express significant amounts of PPAR γ mRNA.

Western blot analysis revealed that PPAR γ expression was extinguished in colon epithelial cells from PPAR $\gamma^{\Delta EpC}$ mice; the receptor protein was readily detected in PPAR $\gamma^{F/F}$ mice and mobility corresponded with PPAR $\gamma 1$ (fig 1D). GAPDH protein was used as a loading control. The identity of the non-specific band (fig 1D, NS) that reacts with the anti-monoclonal antibody is not known.

Figure 3 Lack of endogenous peroxisome proliferator activated receptor γ (PPAR γ) expression in colonic epithelial cells results in increased susceptibility to dextran sodium sulphate (DSS) induced colitis. Clinical features of mice with intestinal epithelial cell specific disruption of PPAR γ (PPAR $\gamma^{\Delta EpC}$ mice) with DSS induced colitis. (A) Daily changes in body weight following DSS induction of colitis. PPAR $\gamma^{\Delta EpC}$ mice ($n = 20$) displayed a higher rate of body weight loss than their wild-type littermates (PPAR $\gamma^{F/F}$ mice) ($n = 16$). PPAR $\gamma^{\Delta EpC}$ mice ($n = 20$) showed more severe clinical features, as defined by body weight loss at day 7 after DSS treatment (B), colon length, which was shortened by severe colitis (C), diarrhoea score (D), bleeding score (E), and macroscopic damage (F) in comparison with PPAR $\gamma^{F/F}$ mice ($n = 16$). Data are mean (SEM). * $p < 0.05$.

Figure 4 Macroscopic and histological findings in mice with intestinal epithelial cell specific disruption of peroxisome proliferator activated receptor γ (PPAR $\gamma^{\Delta IEpC}$ mice) and their wild-type littermates (PPAR $\gamma^{F/F}$ mice) after dextran sodium sulphate (DSS) induced colitis. (A) Macroscopic findings. Picture of the colon isolated from PPAR $\gamma^{\Delta IEpC}$ mice and PPAR $\gamma^{F/F}$ mice after DSS treatment for seven days. (B–E) Histology of colons seven days after DSS treatment without rosiglitazone treatment from PPAR $\gamma^{\Delta IEpC}$ mice (B, C) and PPAR $\gamma^{F/F}$ mice (D, E). Haematoxylin-eosin staining was performed on colon sections prepared from paraffin embedded samples. (B) and (D) are 40 $\times$ magnification; (C) and (E) are 100 $\times$ magnification.

To further demonstrate that deletion of PPAR γ in the colon resulted in functional changes in PPAR γ target gene expression, quantitative real time PCR was performed using RNA isolated from colon epithelium. Indeed, deletion of PPAR γ mediated by villin-Cre resulted in a significant decrease in the constitutive expression of mRNA encoding fatty acid binding protein (FABP), a PPAR γ target gene (fig 1E). Constitutive expression of mRNA encoding adipocyte related protein (ADRP) had a trend towards lower expression without statistical significance ($p = 0.06$) in PPAR $\gamma^{\Delta IEpC}$ compared with PPAR $\gamma^{F/F}$ mice (fig 1D). Expression of mRNA encoding two epithelial cell differentiation markers, keratin 20 and kruppel-like factor 4 (KLF4), was unchanged by deletion of PPAR γ in the colon epithelia (fig 1D); expression of PPAR β/δ mRNA was also not different between genotypes.

In situ hybridisation analysis revealed that PPAR γ mRNA was not expressed in colon epithelial cells of PPAR $\gamma^{\Delta IEpC}$ mice (fig 2A) compared with clear expression found in the colon of PPAR $\gamma^{F/F}$ mice (fig 2B). Histological observations with haematoxylin-eosin staining revealed no gross differences in whole colon epithelia, including goblet cells, between PPAR $\gamma^{\Delta IEpC}$ mice and PPAR $\gamma^{F/F}$ mice in the absence of DSS treatment. Occasionally, colon epithelia from PPAR $\gamma^{\Delta IEpC}$ mice exhibited abnormal narrowing with

apparent accumulation of mucin in cells towards the lumen (fig 2E v 2D). In fact, Alcian blue staining uncovered regions of the colon epithelia in PPAR $\gamma^{\Delta IEpC}$ mice that secreted more mucin (fig 2E, arrows) compared with PPAR $\gamma^{F/F}$ mice (fig 2F). Furthermore, a few small inflammatory lesions were detected in the colon of PPAR $\gamma^{\Delta IEpC}$ mice without DSS treatment (fig 2G, arrow), whereas there were no inflammatory lesions in PPAR $\gamma^{F/F}$ mice. These inflammatory lesions were distinct from lesions having more mucin staining in the colon of PPAR $\gamma^{\Delta IEpC}$ mice.

Susceptibility of PPAR $\gamma^{\Delta IEpC}$ to DSS induced IBD

PPAR $\gamma^{\Delta IEpC}$ mice showed increased susceptibility to DSS induced colitis in comparison with PPAR $\gamma^{F/F}$ mice. Administration of 2.5% DSS in drinking water for seven days induced colitis, as revealed by clinical symptoms (loss of body weight, diarrhoea, and bloody faeces) in PPAR $\gamma^{\Delta IEpC}$ and PPAR $\gamma^{F/F}$ mice (fig 3). PPAR $\gamma^{\Delta IEpC}$ mice showed significant body weight loss in comparison with mice PPAR $\gamma^{F/F}$ mice at 4, 5, 6, and 7 days from the beginning of DSS treatment (19.1 (1.1)% and 11.2 (1.0)% of initial body weight at day 7 for PPAR $\gamma^{\Delta IEpC}$ and PPAR $\gamma^{F/F}$ mice, respectively) (fig 3A, B). Colon length of PPAR $\gamma^{\Delta IEpC}$ mice on treatment with DSS for seven days was considerably shortened compared with PPAR $\gamma^{F/F}$ mice, which indicates

Figure 5 Rosiglitazone (Rosi) treatment improved dextran sodium sulphate (DSS) induced colitis in mice with intestinal epithelial cell specific disruption of peroxisome proliferator activated receptor γ (PPAR γ^{AIEpC} mice) and their wild-type littermates (PPAR $\gamma^{F/F}$ mice). Rosiglitazone administration (20 mg/kg/day), beginning two days before DSS treatment, significantly attenuated disease activity, body weight loss at day 7 after DSS treatment (A), colon length (B), bleeding score (C), and macroscopic damage (D) in PPAR γ^{AIEpC} mice and PPAR $\gamma^{F/F}$ mice. For the control (without rosiglitazone) group: PPAR γ^{AIEpC} mice, n = 8; PPAR $\gamma^{F/F}$ mice, n = 8; rosiglitazone treatment group: PPAR γ^{AIEpC} mice, n = 8; PPAR $\gamma^{F/F}$ mice, n = 10. Data are mean (SEM). *p < 0.05. In (B), p values are ^{ab}p < 0.05; ^{cd}p < 0.05; ^{ac}p = 0.07; ^{bd}p = 0.06.

that PPAR γ^{AIEpC} mice exhibited more severe colitis than PPAR $\gamma^{F/F}$ mice (fig 3C, 4A). Compared with PPAR $\gamma^{F/F}$ mice, PPAR γ^{AIEpC} mice also showed worsening of clinical symptoms, represented by the relatively poor diarrhoea score and bleeding score (fig 3D, E) and increased severity of the macroscopic observations and histological signs of colon injury (fig 3F, 4B–E). Histological analysis of PPAR γ^{AIEpC} mice showed absence of epithelium and intensive submucosal infiltration of inflammatory cells in comparison with PPAR $\gamma^{F/F}$ mice (fig 4B–E). These findings indicate that, in the absence of an exogenous ligand, PPAR γ in colonic epithelium has a protective effect against DSS induced colitis.

Attenuation of DSS induced IBD by PPAR γ ligands

Administration of the PPAR γ ligand rosiglitazone (20 mg/kg/day), beginning two days before commencing DSS treatment, resulted in a decrease in the severity of symptoms in both PPAR $\gamma^{F/F}$ and PPAR γ^{AIEpC} mice. In PPAR $\gamma^{F/F}$ mice, rosiglitazone reduced body weight loss (10.8 (1.3)% and 7.8 (0.4)% of initial body weight at day 7, without and with rosiglitazone, respectively) (fig 5A). In PPAR γ^{AIEpC} mice, rosiglitazone treatment also reversed the body weight loss (20.5 (1.4)% and 15.2 (1.1)% of initial body weight at day 7, without and with rosiglitazone, respectively) and decreased the severity of other symptoms associated with DSS induced colitis. Colon length, diarrhoea, and bleeding scores as well as histological signs of colon injury were improved by ligand treatment (fig 5B–D).

However, after administration of rosiglitazone, PPAR γ^{AIEpC} mice still showed more severe disease symptoms than PPAR $\gamma^{F/F}$ mice treated with rosiglitazone. These data indicate that ligand activated PPAR γ attenuates colitis regardless of the presence or absence of PPAR γ in the colonic epithelium.

Analysis of cytokine expression in DSS induced IBD and effect of PPAR γ ligands

As another indication of the extent of IBD, cytokines were analysed. IL-1 β , IL-6, and TNF- α , secreted from colonic epithelium as well as macrophage and T cells, are increased during the course of IBD.^{1 46 47} A ribonuclease protection assay for tissue associated cytokines was performed on RNA isolated from whole colon after seven days of DSS treatment. Marked induction of IL-6 (fig 6A, B, F, G), IL-1 β (fig 6C, H) and TNF- α (p = 0.065) (fig 6D, I) in the colon of PPAR γ^{AIEpC} mice was observed while no differences in expression of IL-15 (fig 6A, E) or macrophage migration inhibitory factor (MIF) (fig 6D, J) mRNAs were noted between PPAR γ^{AIEpC} and PPAR $\gamma^{F/F}$ mice. Thus lack of PPAR γ expression in the colonic epithelium resulted in enhanced induction of IL-6, IL-1 β , and TNF- α mRNA expression by DSS treatment. Administration of rosiglitazone reduced IL-6, IL-1 β , and TNF- α mRNA levels in both PPAR γ^{AIEpC} and PPAR $\gamma^{F/F}$ mice. However, PPAR γ^{AIEpC} mice treated with rosiglitazone had higher IL-6, IL-1 β , and TNF- α mRNA levels than did similarly treated PPAR $\gamma^{F/F}$ mice after DSS treatment.

Figure 6 Loss of epithelial peroxisome proliferator activated receptor γ (PPAR γ) function results in increased interleukin (IL)-6, IL-1 β , and tumour necrosis factor α (TNF- α) levels in the whole colon after dextran sodium sulphate (DSS) administration. (A–D) RNA was extracted from the whole colon of mice with intestinal epithelial cell specific disruption of PPAR γ (PPAR $\gamma^{\Delta/\Delta}$ mice) and Cre $^{-/-}$ mice, and then analysed by RNase protection using antisense riboprobes for IL-6 and IL-15 (A), IL-6 (B), IL-1 β (C), TNF- α and macrophage migration inhibitory factor (MIF) (D), and L32 (A–D). (E–J) Quantitation of mRNAs encoding IL-15 (E), IL-6 (F, G), IL-1 β (H), TNF- α (I), and MIF (J) normalised to mRNA for L32. (A, D) PPAR $\gamma^{\Delta/\Delta}$ mice, n=7 and PPAR $\gamma^{F/F}$ mice, n=7. (B, C) PPAR $\gamma^{\Delta/\Delta}$ mice, n=7 and PPAR $\gamma^{F/F}$ mice, n=6. Data are mean (SEM). * $p < 0.05$. Rosi, rosiglitazone.

DISCUSSION

Activation of PPAR γ has been reported to ameliorate IBD disease activity in rodent DSS, trinitrobenzene sulphonic acid, and ischaemic colitis models.^{32–35, 36} The role of PPAR γ in attenuating inflammation has been investigated^{34, 48} and a clinical trial of rosiglitazone in UC patients has been carried out.⁴⁹ During the progress of inflammation, PPAR γ is expressed in epithelial cells as well as macrophages and T and B cells infiltrating colon tissue. It is generally thought that alterations of epithelial cells and activation of macrophages cause the extensive tissue injury associated with experimental colitis, followed by a dysregulated immune response of CD4 $^{+}$ T cells. Although PPAR γ is expressed in colonic epithelium, the role of this receptor in colonic epithelial cells has remained unclear. Evidence for a role of PPAR γ in colitis has emerged from the use of PPAR γ ligands in colon cancer cell lines.³⁰ PPAR γ ligands reduce nuclear factor κ B signalling, leading to a decrease in the production of cytokines in colon cancer cell lines that are derived from

the colon epithelium.³⁰ In addition, in the clinical setting, expression of PPAR γ in colonic epithelium is impaired in UC patients while expression of PPAR γ in inflammatory cells remains normal.⁵⁰

However, the precise contribution of PPAR γ in epithelia versus immune cells had not been elucidated prior to this study.^{32–35} PPAR γ in macrophage may also have a role in the inflammation associated with IBD. Indeed, PPAR γ ligands have been shown to reduce expression of cytokines in activated macrophage,²⁸ an effect that appears to be independent of PPAR γ expression at high ligand concentrations.⁵¹ Of interest, PPAR $\gamma^{+/-}$ heterozygous mice exhibit an increased susceptibility to experimental colitis, as revealed by loss of body weight data, indicating that endogenous expression of PPAR γ , in the absence of endogenous ligands, may have an effective role in the control of colitis.³³ However, in another study using mice with disruption of the PPAR γ gene in epithelial tissues using a mouse mammary tumour virus promoter, there were no apparent differences in the

extent of colitis symptoms between the floxed and knockout mice.³⁶ It should be noted that these mice do not have disruption of the PPAR γ gene specifically in gut epithelial cells and thus could have functional PPAR γ expression in T and B cells.^{52–53}

Thus the issue of site of action of PPAR γ in ameliorating the effect of ligands in IBD remains a point of contention.⁵⁴ In addition, the role of this receptor in protecting against induced colitis is still unclear; is it due to an anti-inflammatory effect of PPAR γ in macrophage or due to its expression in the colon epithelia?

To directly examine the role of PPAR γ expressed in gut epithelial in the inflammatory colitis model, intestinal epithelium specific PPAR γ null mice were generated using the Cre/loxP strategy and the PPAR γ floxed allele described in an earlier report.⁴⁰ PPAR $\gamma^{\Delta\text{IEpC}}$ mice exhibited almost complete deletion of PPAR γ expression in colonic epithelium. The functional significance of this deletion was demonstrated by showing that expression of mRNA encoding FABP, a known PPAR target gene, was significantly lower in PPAR $\gamma^{\Delta\text{IEpC}}$ mice compared with PPAR $\gamma^{\text{F/F}}$ mice. While others have shown that heterozygous PPAR $\gamma^{+/-}$ mice exhibit reduced constitutive expression of mRNAs encoding keratin 20 and KLF4,²⁶ expression of keratin 20 and KLF4 were unchanged in PPAR $\gamma^{\Delta\text{IEpC}}$ mice compared with PPAR $\gamma^{\text{F/F}}$ mice. The reason for this difference is uncertain but may be due to the influence of PPAR γ in other cell types.

There were some lesions with more mucin in total colon tissue of PPAR $\gamma^{\Delta\text{IEpC}}$ mice. The role of PPAR γ in mucin accumulation is not known and requires further investigation. In addition, it is not clear from these studies whether mucin influences the response to DSS induced colitis. There is a possibility that hypersecretion of mucin induced the increased activity of proteinase for mucin, which interferes with defence in the colon mucosa. PPAR $\gamma^{\Delta\text{IEpC}}$ mice without DSS treatment also had a few small inflammatory lesions in the colon, suggesting that PPAR $\gamma^{\Delta\text{IEpC}}$ mice had a weakened or abnormal immune system in colon mucosa possibly against certain types of commensal bacteria. Further analysis is needed.

After receiving DSS treatment for seven days, PPAR $\gamma^{\Delta\text{IEpC}}$ mice exhibited more severe colitis, as revealed by loss of body weight, shortened colon length, diarrhoea, and bleeding scores, and macroscopic and histological analysis in comparison with PPAR $\gamma^{\text{F/F}}$ mice with or without rosiglitazone administration. This finding suggests that endogenous PPAR γ in colonic epithelial cells might be involved in maintaining the homeostasis of the colon and preventing initiation of colon injury and subsequent development of inflammation. Thus the possibility exists that UC patients with impaired expression of PPAR γ have more severe inflammation in the colon. It is especially noteworthy that attenuation of disease activity was noted in both PPAR $\gamma^{\text{F/F}}$ and PPAR $\gamma^{\Delta\text{IEpC}}$ mice on rosiglitazone treatment. This suggests that the response to rosiglitazone may be independent of PPAR γ , as suggested by earlier studies.³¹ This may not be surprising in view of a recent study revealing that troglitazone is able to effectively inhibit the activity of myeloperoxidase through a PPAR γ independent mechanism.⁵⁵ This could account for the PPAR γ independent effects of rosiglitazone on PPAR $\gamma^{\Delta\text{IEpC}}$ mice observed in the current study. While recombination is virtually complete and PPAR γ mRNA protein not detectable in epithelial cells, the possibility cannot be excluded that rosiglitazone activated PPAR γ expressed in non-colon epithelial cells, such as macrophage, and that attenuation of colitis may be due to PPAR γ in infiltrating macrophages that inhibit the production of cytokines²⁸ resulting in a decrease in the severity of colitis.

The finding that PPAR $\gamma^{\Delta\text{IEpC}}$ mice exhibited higher levels of IL-6, IL-1 β , and TNF- α than PPAR $\gamma^{\text{F/F}}$ mice either without or with rosiglitazone treatment suggests the possibility that PPAR γ in epithelial cells normally suppresses the pathway producing these cytokines. This hypothesis is supported by previous data showing that PPAR γ ligands inhibit the activation of nuclear factor κB ,⁴⁸ and that TLR4-activated PPAR γ in epithelium attenuates nuclear factor κB signalling in an I κB dependent fashion.⁵⁰ However, the composition of inflammatory cells between PPAR $\gamma^{\Delta\text{IEpC}}$ and PPAR $\gamma^{\text{F/F}}$ mice remains to be determined. Epithelial PPAR γ may also modulate mucosal barrier function and alter the control of apoptosis of epithelial cells resulting in the accumulation and activation of immune cells and upregulation of proinflammatory cytokines.

In summary, disruption of PPAR γ in colonic epithelial cells resulted in increased susceptibility to DSS induced colitis associated with an elevation in IL-6, IL-1 β , and TNF- α levels. These data suggest that endogenous PPAR γ in colonic epithelium has a protective effect against IBD. Further studies are warranted to determine the mechanism of this protective effect.

ACKNOWLEDGEMENTS

We thank Walter Wahli for the mouse PPAR γ 1 cDNA. The National Cancer Institute Intramural Research Program of the National Institutes of Health supported this study.

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Conflict of interest: None declared.

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