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RS5444, a novel PPARγ agonist, regulates aspects of the differentiated phenotype in nontransformed intestinal epithelial cells

Lu Chen [a], Craig R. Bush [b], Brian M. Necela [a], Weidong Su [a], Masahiro Yanagisawa [a], Panos Z. Anastasiadis [a], Alan P. Fields [a], E. Aubrey Thompson [a,*]

[a] *Department of Cancer Biology, Mayo Clinic Comprehensive Cancer Center, 4500 San Pablo Road, Griffin Cancer Research Bldg., Rm 310, Jacksonville, FL 32224, United States*

[b] *University of Texas Medical Branch, Galveston, TX, United States*

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Abstract

Peroxisome proliferator-activated receptor-gamma (PPARγ) is expressed in the intestinal epithelium, yet little is known about the physiological role of PPARγ in the small bowel or the effects of PPARγ on small intestinal epithelial cells. The present studies investigate cellular and genomic effects of PPARγ in nontransformed rat intestinal epithelial cells (RIE). These cells were engineered to express mouse PPARγ1, and thereby to model the molecular phenotype that obtains upon induction of PPARγ at the crypt/villus junction in the small intestine. In these studies, we have used a novel third generation thiazolidinedione derivative, RS5444, which activates PPARγ with an EC50 about 1/50th that of rosiglitazone and has no effect on RIE cells that do not express PPARγ. We used Affymetrix oligonucleotide microarrays to identify potential PPARγ-regulated processes in RIE cells, including lipid metabolism, cell proliferation and differentiation, remodeling of the extracellular matrix, cell morphology, cell–cell adhesion, and motility. The genomic profile reflects cellular events that occur following PPARγ activation: RS5444 inhibited culture growth and caused irreversible G1 arrest, but did not induce apoptosis. In addition, RS5444 caused dramatic changes in cellular morphology which were associated with increased motility and diminished cellular adherence, but no increase in the ability of such cells to digest and invade Matrigel. Inhibition of proliferation, cell cycle arrest, increased motility, and altered adherence are aspects of the differentiated phenotype of villus epithelial cells, which withdraw from the cell cycle at the crypt/villus interface, migrate to the villus tips, and are subsequently shed by loss of contact with the epithelium and the underlying extracellular matrix. Our results are consistent with the hypothesis that PPARγ regulates critical aspects of differentiation in the small intestinal epithelium. Many nuclear receptors regulate differentiation. However, our results point to novel effects of PPARγ on cell–cell and cell–matrix interactions, which are not typical of other nuclear receptors.

Keywords: PPAR-gamma; Differentiation; Small intestine

1. Introduction

PPARγ, a member of the nuclear receptor superfamily, forms heterodimers with retinoid X receptors (principally RXRα), and regulates gene expression in a ligand-dependent manner (Miyata et al., 1994; Tontonoz et al., 1994). The PPARγ gene encodes three known transcripts which arise though alternative promoter utilization and alternative splicing and encode three PPARγ isoforms, called PPARγ1, γ2, and γ3 (Zhu et al., 1993, 1995; Greene et al., 1995; Mukherjee et al., 1997; Fajas et al., 1997, 1998). The two major isoforms, PPARγ1 and γ2, are identical in sequence except for an additional 30 amino-terminal amino acids in PPARγ2. The expression of PPARγ2 is largely restricted to adipocytes, where it functions as a master switch in differentiation (Mukherjee et al., 1997; Fajas et al., 1997). One of the best studied aspects of PPARγ action is regulation of genes that are involved in fatty acid transport and oxidation, and in energy metabolism. The role of PPARγ in regulating these metabolic processes has been studied in detail, largely because of the observation that thiazolidinedione agonists of PPARγ increase insulin sensitivity in individuals with type II diabetes (Lehmann et al., 1995; Saltiel and Olefsky, 1996; Willson et al., 1996).

Unlike PPARγ2, PPARγ1 is expressed in many epithelial and immune cells. The gut is a major site of PPARγ expression, and the abundance of PPARγ1 in the colon is similar to that observed in adipocytes (Fajas et al., 1997). PPARγ1 mRNA is

* Corresponding author. Tel.: +1 904 953 6226; fax: +1 904 9530277.
E-mail address: Thompson.aubrey@mayo.edu (E.A. Thompson).

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expressed in the small intestine of rodents and humans (Lefebvre et al., 1999; Escher et al., 2001; Huin et al., 2000; Fajas et al., 1997). PPARγ2 mRNA is expressed at very much lower abundance in these tissues (Fajas et al., 1997). Rosiglitazone, a thiazolindiedione PPARγ agonist, inhibits ischemia reperfusion injury in the small intestine (Nakajima et al., 2001), indicating that PPARγ is functional in that tissue. Furthermore, two studies have shown that PPARγ agonists suppress the number and size of polyps that form in the small intestine of mice with APC mutations (Niho et al., 2003; Yang et al., 2005), although two other reports indicated that PPARγ agonists had no effect on polyp formation in the small intestine of APC mice (Lefebvre et al., 1998; Saez et al., 1998). Although there is some controversy, the preponderance of data indicate that PPARγ is expressed and is functional in epithelial cells of the small intestine, where this receptor may play an important role in transformation and/or inflammation. However, the physiological functions of PPARγ in the small bowel are unclear.

One possible clue concerning the role of PPARγ in the intestine comes from studies of human colon cancer cell lines. PPARγ1 is highly expressed in some human colon cancer cell lines (DuBois et al., 1998), and PPARγ agonists have been shown to induce aspects of differentiation of some colon cancer cells (Sarraf et al., 1998; Kitamura et al., 1999; Chang and Szabo, 2000; Gupta et al., 2003; Kato et al., 2004; Yoshizumi et al., 2004; Brockman et al., 1998). PPARγ has also been implicated in embryonic development of the small intestine (Drori et al., 2005). These data suggest that PPARγ may regulate differentiation of intestinal epithelial cells. We have therefore carried out a series of studies to determine the cellular and genomic consequences of PPARγ activation in nontransformed rat intestinal epithelial cells (RIE). RIE cells exhibit contact inhibition (Ko et al., 1998), do not form colonies in soft agar (Zhang et al., 2004), and are not tumorigenic in nude mice (Sheng et al., 1999). RIE cells are believed to be derived from the proliferative transit amplifying cells within the crypts of Lieberkühn, and these cells have been widely studied as a model of transforming growth factor-beta (TGFβ) regulation of enterocyte proliferation (Ko et al., 1998; Smith, 1994; Sheng et al., 1997; Winesett et al., 1996).

Immunohistochemical analysis of PPARγ in the adult mouse indicates that this receptor is expressed at very low levels in the transit amplifying cells within the crypts of Lieberkühn (Drori et al., 2005). RIE cells, which are derived from this compartment, also express very low levels of PPARγ. However PPARγ is induced at the crypt/villus junction, where intestinal epithelial cells cease to proliferate and begin to differentiate. Therefore, we engineered RIE cells to express mouse PPARγ1 so as to model the transition that occurs at the crypt/villus junction and to determine how induction of this receptor affects the properties of cells in the villus epithelium of the small intestine.

Since induction of PPARγ coincides with differentiation of intestinal epithelial cells during embryogenesis (Drori et al., 2005), our initial goal was to test the hypothesis that PPARγ regulates differentiation of intestinal epithelial cells in culture. Our functional definition of differentiation includes global changes in gene expression, inhibition of proliferation, and cytodifferentiation. We report here that PPARγ regulates a large cohort of genes that regulate metabolism, signal transduction, proliferation, adhesion, migration, and morphology. The cellular response to activation of PPARγ recapitulates the genomic profile, in that PPARγ agonists cause irreversible G1 arrest of RIE cells, with profound changes in cellular morphology, motility, and adhesion. These responses reflect aspects of the differentiated phenotype of normal intestinal epithelial cells and indicate that PPARγ regulates processes that control renewal of the intestinal epithelium. These studies represent, to our knowledge, the first systematic attempt to define the physiological role of PPARγ in nontransformed epithelial cells from the small intestine, and our observations reveal important potential PPARγ functions in the small bowel. Furthermore, our observations reveal a novel aspect of PPARγ action: regulation of migration, motility, and adhesion of intestinal epithelial cells. These processes are generally not associated with activation of nuclear receptors.

2. Materials and methods

2.1. Reagents, cell lines, and immunological assays

RS5444 was provided by Sankyo Ltd., Tokyo, Japan. Troglitazone and rosiglitazone were from obtained from our colleague Dr. Al Copland (Mayo Clinic). Carbaprostacyclin (cPGI), 15-deoxy-$\Delta^{12,14}$-prostglandin J2 (15-d-Δ-PGJ2) and linoleic acid (LA) were purchased from Cayman. Dimethyl sulphoxide (DMSO) and fenofibrate were purchased from Sigma. Transforming growth factor-beta1 (TGF-β1) was from purchased from R&D Systems and dissolved in 4 mM HCl, 0.1% BSA. Mouse PPARγ1 expression vector pSG5-mPPARγ1 (Lehmann et al., 1995) and the PPARγ reporter $PPRE_3$-TK-LUC (Kliewer et al., 1992) were provided by Dr. Steven A. Kliewer; pSG5-PPARα and pSG5-PPARδ were obtained from Dr. Al Copland. RIE/Smad3 (RIE/S3) and RIE/PKCβII (RIE/βII) cells were generated by infecting RIE-1 cells with retrovirus pBabe-puro3-Flag-Smad3 or pBabe-puro3-PKCβII as previously described (Conery et al., 2004; Yu et al., 2003). Stable RIE/S3 and RIE/βII cells were selected and maintained in 5 μg/ml of puromycin. RIE/γ1, RIE/S3γ1, and RIE/βIIγ1 cells were generated by transfecting RIE-1, RIE/S3, and RIE/βII cells, respectively, with pSG5-mPPARγ1 and pREP4 (Invitrogen) using calcium phosphate coprecipitation. RIE/γ1, RIE/S3γ1, and RIE/βIIγ1 cells that were hygromycin B resistant were screened for stable PPARγ expression. For immunoblotting, cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed by sonication in protein lysis buffer consisting of 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 100 mM NaF, and protease inhibitor cocktail (Sigma–Aldrich). Protein concentration was determined by the method of Bradford (Bio-Rad). An aliquot of total protein (5 μg) was resolved by electrophoresis in 10% Tris–glycine gels (Invitrogen) and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% nonfat milk in 10 mM Tris–HCl pH 7.4, 150 mM NaCl, and 0.1% Tween 20 (TBST) overnight at 4 °C. Thereafter, the membrane was incubated with antibodies to PPARγ (Santa Cruz), or actin (Santa Cruz) diluted in 1% milk/TBST at room temperature for 2 h, after which the membrane was washed in TBST three times for 10 min each. The membrane was then incubated with horseradish peroxidase-conjugated anti-mouse (Santa Cruz), or anti-goat (Santa Cruz) secondary antibodies in 1% milk/TBST at room temperature for 1 h. The membrane was washed three times in TBST for 10 min each. Antigen–antibody complexes were detected using the ECL Plus chemiluminescent system (Amersham Bioscience).

For immunofluorescence analysis, cells cultured on glass cover slips were fixed in 3% paraformaldehyde for 30 min then permeabilized in 0.2% Triton X-100 for 5 min. Cells were blocked with 3% nonfat milk/PBS for 10 min at room temperature, and then incubated with anti PPARγ (Santa Cruz) and anti β-catenin (whole antiserum developed in rabbits, Sigma) diluted in 3% nonfat milk/PBS for 30 min at room temperature. PBS-washed cover slips were incubated with biotin–XX goat anti-mouse IgG (Molecular Probes) for 15 min, and