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Research Article

# Activation of PPARγ is not involved in butyrate-induced epithelial cell differentiation

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**Abstract**

Histone deacetylase-inhibitors affect growth and differentiation of intestinal epithelial cells by inducing expression of several transcription factors, e.g. Peroxisome proliferator-activated receptor γ (PPARγ) or vitamin D receptor (VDR). While activation of VDR by butyrate mainly seems to be responsible for cellular differentiation, the activation of PPARγ in intestinal cells remains to be elucidated. The aim of this study was to determine the role of PPARγ in butyrate-induced cell growth inhibition and differentiation induction in Caco-2 cells. Treatment with PPARγ ligands ciglitazone and BADGE (bisphenol A diglycidyl) enhanced butyrate-induced cell growth inhibition in a dose- and time-dependent manner, whereas cell differentiation was unaffected after treatment with PPARγ ligands rosiglitazone and MCC-555. Experiments were further performed in dominant-negative PPARγ mutant cells leading to an increase in cell growth whereas butyrate-induced cell differentiation was again unaffected. The present study clearly demonstrated that PPARγ is involved in butyrate-induced inhibition of cell growth, but seems not to play an essential role in butyrate-induced cell differentiation.
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*Keywords:* Butyrate; PPARγ; Colon cancer; Proliferation; Differentiation

## Introduction

Although data reported in humans are still controversial [1], evidence has been provided that alimentary fibers exert a protective effect against colon carcinogenesis in rodents [2–4]. The protective effect is linked to the capacity of fibers to be metabolized into short chain fatty acids as butyrate [5]. Whereas in normal epithelial cells butyrate is the preferred oxidative fuel and stimulates growth [6,7], it has been shown to inhibit proliferation and induce rapid cell differentiation in colon carcinoma cell lines [7–9].

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily, also including the receptors for thyroid hormone, retinoids, steroid hormones and vitamin D [10,11]. They occur in three different isotypes termed α, β (also called δ, NUC-1 or FAAR) and γ. Moreover, in man, three PPARγ-mRNA isoforms have been described, PPARγ1, γ2 and γ3 [12]. Upon activation, PPARs heterodimerize with another nuclear receptor, the 9-*cis*-retinoid X receptor (RXR), and alter the transcription of numerous target genes after binding to a specific DNA binding site termed PPRE (peroxisome proliferator responsive element) [13,14]. It has been demonstrated that 15-deoxy-12,14-prostaglandin J2 (15d-PGJ2), a metabolite of prostaglandin D2, is a potential endogenous ligand for PPARγ [15], and that thiazolidinediones (synthetic anti-diabetic agents) such as troglitazone, rosiglitazone, ciglitazone and MCC-555 are specific exogenous ligands for PPARγ [16]. Other PPARγ-ligands has been described,

*Abbreviations:* AP, alkaline phosphatase; $APC^{MIN}$, adenomatous polyposis; BADGE, bisphenol A diglycidyl; HDAC, histone deacetylase; PPARγ, peroxidase proliferator-activated receptor γ; PPRE, peroxisome proliferator responsive element; RXR, 9-*cis*-retinoid X receptor; VDR, vitamin D receptor; 15d-PGJ2, 15deoxy-12,14-prostaglandin $J_2$.

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including eicosanoids [17] and non-steroidal anti-inflammatory drugs [18].

PPARγ is expressed at high levels in colonic epithelial cells and colon cancer cells [19,20]. Sarraf and colleagues [21] showed that activators of PPARγ suppress the growth response of colon cancer cells. Brockmann and colleagues reported that activation of PPARγ leads to inhibition of anchorage independent growth of colon cancer cells [22]. On the other hand, Seaz and colleagues [23] and Lefebvre and colleagues [24] showed in $APC^{MIN}$ (adenomatous polyposis coli) mice, an animal model of familial polyposis, that treatment with PPARγ ligands increases the frequency and size of colon polyps. Although the results of these in vitro and in vivo studies are contradictory, it is very likely that the PPARγ pathway can modulate the growth response of colon epithelial cells and colon cancer cells.

On the basis of our published finding, that NaB (sodium butyrate) upregulates PPARγ in colon cancer cells [25], this study is addressed to elucidate the role of PPARγ in butyrate-induced differentiation and/or growth response.

## Materials and methods

### *Cell culture*

The human colorectal cancer cell line Caco-2 was obtained from the European Collection of cell cultures (ECACC). The stock was maintained in 175 $cm^2$ flasks in a humidified incubator at 37°C in an atmosphere of 95% air and 5% $CO_2$. Caco-2 cells of passage 40–50 were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids and 1% penicillin/streptomycin. For experiments, cells were seeded on plastic cell culture wells in DMEM until nearly confluent. Medium was then removed and replaced by a medium containing either the solvent or butyrate [1–2 mmol/L], calcitriol/1,25$(OH)_2D_3$ [$10^{-6}$ mol/L], rosiglitazone [50 μmol/L] or MCC-555 [30 μmol/L]. Butyrate (Merck-Schuchardt, Hohenbrunn, Germany) was solubilized in PBS (phosphate buffered saline) and added to the medium. Calcitriol (Biomol, Hamburg, Germany) was solubilized in ethanol, rosiglitazone and MCC-555 (all from Cayman Chemicals, U.S.) in DMSO.

Cytotoxicity was excluded by commercial kit measuring lactate dehydrogenase activity in the supernatant of damaged cells (Merck, Darmstadt, Germany).

### *Cell counts*

Cells were suspended and cultured on 96-well dishes at a density of $10^4$/well (0.28 $cm^2$). 24 h after plating, cells were incubated for 24 h–72 h with substances. At given time points following treatment, cell numbers were assessed by crystal violet staining. Medium was removed from the plates and cells were fixed with 5% formaldehyde for 5 min. After washing with PBS, cells were stained with 0.5% crystal violet for 10 min, washed again with PBS and unstained with 33% acetic acid. Absorption, which correlates with the cell number, was measured at 620 nm.

### *Assay for cell proliferation*

The effect of the PPARγ ligands on DNA synthesis of cells was assessed using a cell proliferation ELISA kit (Roche Diagnostics, Tokyo, Japan). This assay is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis, and is a non-radioactive alternative to the [$^3$H]-thymidine incorporation assay. Incorporated BrdU was measured colorimetrically.

### *Cell death detection assay*

To determine and quantify the induction of apoptosis by sodium butyrate and PPARγ agonist MCC-555 in Caco-2-wild-type cells, cytoplasmic histone-associated DNA fragments were measured using a commercially available Cell Death Detection ELISA kit (Roche Molecular Biochemicals). 24 h after treatment with the mentioned substances, 1 μg of cell lysate was used for the ELISA procedure, following the manufacturer's instructions. DNA fragmentation was quantified photometrically at 405 nm.

### *Transfection assay*

The following plasmids were used for transfection: pcDNA3 (Invitrogen), as an empty vector for control transfection and the plasmid pcDNA3-$PPAR_{\gamma L468A/E471A}$, a dominant-negative PPARγ double-mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) [26]. These constructs were transfected into subconfluent Caco-2 cells with lipofectamine 2000 (Invitrogen) in serum-free conditions. After 6 h, the cells were fed with fresh medium containing 10% FCS. 24 h later, the cells were fed with medium containing G418 (400 μg/mL) and culture medium supplemented with G418 was replaced twice a week. G418-resistant colonies were collected and used for further analysis.

### *RT (reverse transcription) competitive multiplex PCR (polymerase chain reaction)*

Total cellular RNA was isolated by RNAzol B™ (Wak-Chemie, Bad Homburg, Germany), phenol/chloroform extracted, isopropanol precipitated and reconstituted in diethylpyrocarbonate-treated water, as recently published by our group [27]. RNA was reverse transcribed into cDNA by Superscript II-reverse transcriptase (Life technologies). Aliquots of a master mix containing KCl, Tris–HCl, $MgCl_2$, dNTPs, primers for the target genes and 0.5 μL of cDNA per

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