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13-Oxo-ODE is an endogenous ligand for PPARγ in human colonic epithelial cells☆

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ABSTRACT

Background: The ligand activated nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ) induces transcriptional repression of pro-inflammatory factors. Activation of PPARγ is followed by amelioration of colitis in animal models of inflammatory bowel disease (IBD). A reduced expression of PPARγ was found in epithelial cells of patients with ulcerative colitis. The eicosanoids 13-HODE and 15-HETE are products of 12/15-lipoxygenase (LOX) and endogenous ligands for PPARγ. Dehydrogenation of 13-HODE by 13-HODE dehydrogenase results in formation of the 13-Oxo-ODE. Highest activity of 13-HODE dehydrogenase is found in colonic epithelial cells (CECs). We therefore investigated whether 13-Oxo-ODE is a new endogenous ligand of PPARγ in CECs.
Methods: LOX activity and 13-HODE dehydrogenase in CECs were investigated after stimulation with arachidonic or linoleic acid. LOX metabolites were identified by RP-18 reversed-phase HPLC. Binding of ^{14}C-labelled 13-Oxo-ODE was demonstrated using a His-tagged PPARγ.
Results: Stimulation of HT-29 and primary CECs homogenates with and without Ca-ionophor was followed by the formation of high amounts of the linoleic acid metabolite 13-Oxo-ODE (155 and 85 ng/ml). The decrease of IL-8 secretion from IEC was more pronounced after pre-incubation with 13-Oxo-ODE compared to the PPARγ agonist troglitazone and higher as with the known PPARγ ligands 13-HODE and 15-HETE. Binding assays with ^{14}C-labelled 13-Oxo-ODE clearly demonstrated a direct interaction.

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Abbreviations: 5-ASA, 5-aminosalicylic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; 13-Oxo-ODE, 13-oxooctadecadienoic acid; 15-HETE, hydroxyeicosatetraenoic acid; Ab, antibody; CECs, human primary colonic epithelial cells; COX, cyclooxygenase; cPLA$_2$, cytosolic phospholipase A2; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; DTT, dithiothreitol; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HPLC, high-performance liquid chromatography; IBD, inflammatory bowel disease; IEC, intestinal epithelial cells; IMACs, intestinal macrophages; IL, interleukin; LOX, lipoxygenase; LTB$_4$, leukotriene B$_4$; MHA, 1-hydroxy-8-methoxy-9,10-anthracenedione; MS, mass spectrometry; NF-κB, nuclear factor-kappaB; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PPARγ, peroxisome proliferator-activated receptor gamma; RT, reverse transcription; TAB, tetradecyltrimethylammonium bromide; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; TZDs, thiazolidinediones

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Conclusion: High amounts of 13-Oxo-ODE can be induced in CECs by stimulation of linoleic acid metabolism. 13-Oxo-ODE binds to PPARγ and has anti-inflammatory effects. 13-HODE dehydrogenase might be a therapeutic target in IBD.

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1. Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) is one of three members of the PPAR-family (besides PPARβ and PPARδ), which is a part of the nuclear hormone receptor superfamily [1–5]. Nuclear hormone receptors are transcription factors that are activated by the binding of small, lipophilic ligands [6–11].

PPARγ initially was identified for its role in adipocyte differentiation and the regulation of genes involved in lipid and glucose metabolism [12–15]. However, activation of PPARγ also can antagonise nuclear factor-kappaB (NF-κB) action in macrophages resulting in downregulation of pro-inflammatory cytokines [16–21]. Besides synthetic ligands known as thiazolidinediones (TZDs) [22] endogenous ligands of the eicosanoid- and prostaglandin-family have been identified [11,23]. The first endogenous ligand investigated was 15deoxy-Δ12,14-PGJ2 (15d-PGJ2) [24]. Glass and co-workers found the 12/15 lipoxygenase (LOX) products 13-HODE, 13-OXO and 15-HETE to be additional endogenous ligands for PPARγ in epithelial cell lines [25,26].

PPARγ has gained interest among gastroenterologists [27] as it could be consistently demonstrated that PPARγ ligands are capable of reducing the mucosal damage and prevent or downregulate the inflammatory response in several murine models of intestinal inflammation [28–34]. Recently, further evidence for an anti-inflammatory role of the TZD-PPARγ ligand rosiglitazone was found in IL-10 deficient mice in which rosiglitazone delayed onset of colitis [35] and in trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats in which it reduced mucosal ulceration and TNF secretion [36]. Overexpression of PPARγ by an adenoviral construct in mucosal epithelial cells in mice was associated with an amelioration of experimental inflammation [37].

However, not only TZDs could become important for a therapeutic use of PPARγ effects: activation of PPARγ by conjugated linoleic acids also protected mice from experimental colitis [38]. This effect was not seen in mice with a colon-specific deletion of the PPARγ-gene. PPARγ is highly expressed in intestinal epithelial cells [28]. A reduced expression of PPARγ in ulcerative colitis but not in Crohn's disease was reported [39]. A pilot study in patients with active ulcerative colitis refractory to standard medical therapy has shown some beneficial effects of TZDs [40]. However, systemic therapy with TZDs besides anti-inflammatory effects will have also metabolic side effects.

Linoleic acid is the major polyunsaturated fatty acid in human nutrition and diet [41]. The enzymatic oxidation of linoleic acid leads to the production of 13-hydroxyoctadecadienoic acid (13-HODE) [42–44] (see Fig. 1). As mentioned above, 13-HODE was identified as endogenous ligand for PPARγ. Subsequent dehydrogenation of 13-HODE by the NAD^+-dependent 13-HODE dehydrogenase results in the formation of the 2,4-dienone 13-oxooctadecadienoic acid (13-Oxo-ODE) [42,43] (Fig. 1). 13-HODE dehydrogenase has been discovered by Bull and co-workers [42,44–47]. The highest levels of 13-HODE dehydrogenase activity were observed in the colon and liver relative to other tissues [44,47,48]. Subsequent experiments demonstrated a strong positive correlation between the level of enzyme activity and the degree of differentiation both in vivo and in vitro [44,46–48]. Especially intestinal epithelial cells (IEC) show a high activity of 13-HODE dehydrogenase [48]. We therefore hypothesised that 13-Oxo-ODE could be a endogenous PPARγ ligand in primary IEC with anti-inflammatory properties. 13-Oxo-ODE was previously demonstrated to be an endogenous activator of PPARγ in macrophages [49].

Identification of the hydroperoxy derivatives of polyunsaturated fatty acids (HPOD) is usually accomplished by using gas chromatography with mass spectrometry (MS), which requires extensive derivatisation of the thermally unstable hydroperoxy group. Here we report a high-performance liquid chromatographic method in combination with ion trap MS that separates and characterises the HPOD isomers generated by HT-29 epithelial cells. The method does not require derivatisation of the hydroxyperoxide group. We further show that 13-Oxo-ODE is produced by intestinal epithelial cells (CECs), and it indeed binds to PPARγ, and downregulates cytokine production in CECs.

2. Materials and methods

2.1. Chemicals

McCoy's medium and fetal calf serum (FCS) were purchased from Gibco Life Technologies Co. Baker HPLC solvents (ultrahigh purity) were used for high-performance liquid chromatography (HPLC) analyses. Arachidonic and linoleic acid, prostaglandin B2 (PGB2) and all eicosanoid standards including 5-, 12-HETE, 13-HODE, 13-Oxo-ODE and leukotriene B_4 (LTB_4) were from *Cayman* Chemical Company (Ann Arbor, MI, USA). Solid-phase extraction columns, C18, were from J.T. Baker Inc. (Phillipsburg, NJ, USA).

2.2. LOX assay

Induction of LOX activity with calcium, arachidonic or linoleic acid and Ca-ionophore was investigated in intact HT-29 and whole cell lysates. The divalent cation ionophore A23187 induces the formation of oxidated arachidonic and linoleic metabolites from endogenous arachidonic and linoleic acid.

HT-29 cells were stimulated with Dulbecco's modified Eagle medium (DMEM; Biochrom FG 0415, Berlin) supplemented with $CaCl_2$ (final concentration 2.5 mM), NaCl (35 mM), arachidonic or linoleic acid (5 μM), Ca-ionophore A23187 (20 μM).

Enzyme activation was terminated after 1 h by addition of 3.0 ml inhibitor solution MeOH/CH_3CN (1:1, v/v) supplemented

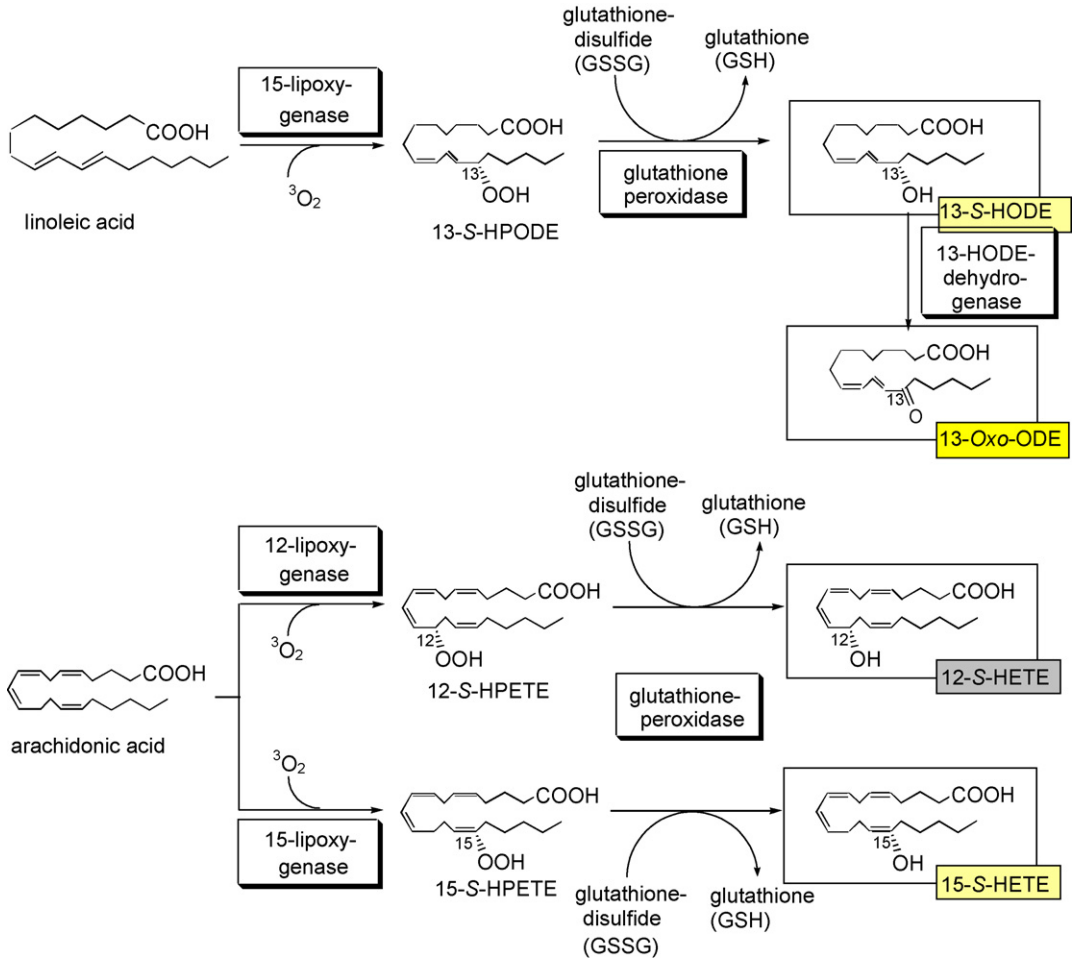

Fig. 1 – Oxidative metabolism of linoleic acid and arachidonic acid. The initial reactions in this pathways lead to the production of 13-S-HPODE and (12 or 13)-S-HPETE, respectively. Secondary metabolism of 13-S-HPODE by glutathione peroxidase and 13-HODE-dehydrogenase yields the 13-Oxo-ODE.

with the antioxidant nordihydroguaiaretic acid and 1-hydroxy-8-methoxy-9,10-anthracenedione plus PGB2 as chromatographic markers (final concentrations 0.3 and 30 μM, respectively). After stimulation medium containing secreted LOX and cyclooxygenase (COX) metabolites was collected and stored at −20 °C.

HT-29 cells were harvested by scraping. Following sonication enzyme activation was induced adding $CaCl_2$ (2.5 mM), NaCl (35 mM), arachidonic or linoleic acid (5 μM), Ca-ionophore A23187 (20 μM) for 1 h at 37 °C. Enzyme activation was terminated after 1 h by addition of 3.0 ml inhibitor solution.

2.3. *Solid-phase extraction*

Isolation of LOX and COX metabolites from non-soluble cell compounds and soluble polar inorganic and organic compounds was performed by solid-phase extraction with octadecylsilane (RP-18-columns).

Mixtures were kept 20 min on ice and centrifuged (5000 × *g*, 20 min, 0 °C). The supernatant was diluted with 10 ml of distilled water and passed through a pre-washed octadecylsilane reversed-phase cartridge (Bakerbond Octadecyl C-18 extraction columns, 500 mg, Baker), which had been washed with 5 ml CH_3OH and 5 ml water. The material was eluted with 250 μl CH_3OH.

2.4. *HPLC conditions*

Reversed-phase HPLC was performed on LiChrospher 100 RP-18 (5 μm particles: Merck, Darmstadt) 250 mm × 4 mm column (4 mm × 4 mm precolumn) combined with Merck LiChrospher 100 RP-18 (5 μm) 4 mm precolumn in manu-Cart "4".

Isocratic elution condition for hydroxyeicosatetraenoic acid and 13-HODE was 35% methanol, 30% acetonitrile, 35% H_2O and 0.1% acetic acid, pH 5.5, flow rate 1.0 ml/min monitored at 232 nm. Leukotrienes and 2,4-dienones were monitored at 270 nm with a HP1050 UV detector. Data were recorded on a Hewlett Packard data acquisition system and analysis was performed with the software "Peaks". An integration of the peak area was calculated.

2.5. *Quantification of oxidised fatty acid metabolites*

Quantification of arachidonic metabolites was performed using calibration curves. Calibration was performed by

comparing integrated areas of the peaks of external and internal standards detected in the HPLC-chromatograms.

Known amounts of authentic external standards of arachidonic metabolites were compared to the internal standards PGB2 and monomethoxychrysazin. Molar absorption coefficients given by Borgeat and co-workers [50] were used for calculations.

2.6. HPLC/ion trap MS

Analytical characterisation of 13-Oxo-ODE was performed using high-performance liquid chromatography coupled to ion trap mass spectrometry. The system used was an Agilent LC/MSD Ion Trap SL with an 11 binary pump and well plate autosampler. Interface HPLC → MS, 350 °C, ESI, Scan 100–1200 m/z, 5 μl/min.

2.7. RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Primers for human PPARγ (forward, 5′-CGTGAAGCTGTGTTGTCCTG-3′; reverse, 5′-GCTCCTAGGACT-GTCTTCTTC-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-TTAGCACCCCTGGCCAAGG-3′; reverse, 5′-CTTACTCCTTGGAGGCCATG-3′).

2.8. Transfection

Hela cells were transfected with pCR3.1-PPARγ-His vector (kind gift of Thomas M. McIntyre, University of Utah, USA) for 24 h with LipofectAMINE (#18324-111, Invitrogen life technologies, CA, USA) transfection reagent and then grown for 48 h. Controls were transfected with LipofectAMINE not containing plasmid.

2.9. Immunoblotting

Immunoblots were performed using antibodies to PPARγ, β-actin (sc-7196, H100 or sc-7273, E-80 Santa Cruz Biotechnology both) and β-actin (MAB1501, Chemicon) and peroxidase-conjugated secondary antibodies. Protein bands were visualised with a chemiluminescence detection kit (ECL Plus; Amersham Biosciences). The signals from three different immunoblots were quantified with the ImageQuant software (Version 5.2, Amersham Biosciences, Freiburg, Germany) and normalised to β-actin. Ratios were calculated defining the control signal as 1.0.

2.10. Determination of IL-8 protein

Supernatants of cell cultures were collected after 24 h and centrifuged. IL-8 concentration in the incubation medium was quantified by enzyme-linked immunosorbent assay (Biorad).

2.11. Binding assay

After transfection cells were washed twice with PBS, lysed in PBS containing 0.1% Triton X-100 and frozen at −70 °C until used. Transfection with PPARγ-His was assessed by immunoblotting with anti-PPARγ antibody. Thawed samples were incubated with 20 μM ^{14}C-13-Oxo-ODE or a mixture of 20 μM ^{14}C-13-Oxo-ODE and 400 μM unlabelled 13-Oxo-ODE in PBS for 2 h at 4 °C. Immunoprecipitation was performed with anti-His agarose. The 200 μl beads/sample were washed twice with TBS and added to the cell lysate/ligand mixture. Samples were rotated at 4 °C for 2 h, washed twice with PBS and retained ^{14}C was quantified with a scintillation detector.

2.12. Cells and stimulation

HT-29 cells were from American Type Culture Collection (Manassas, VA, USA). Hep-G2 cells were from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Human primary cells were prepared from colonic tissue obtained from patients undergoing surgical resection for colorectal carcinoma. The study was approved by the University of Regensburg Ethics Committee. Normal mucosa was taken at least 10 cm distant from the tumor. CECs were separated as described [51]. Fibroblasts were cultured and characterised by immunocytochemistry as described earlier [52] and used between passages 3 and 8. Intestinal macrophages (IMACs) were isolated as described in [53]. Before stimulation cells were starved for 24 h. TNF (10 ng/ml) served as control.

2.13. Statistical analysis

Data are expressed as mean (±S.D.). Statistical analyses were performed using the paired Student's *t*-test. Differences were considered significant at a p-value of <0.05.

3. Results

3.1. PPARγ expression in human primary cells and cell lines

PPARγ was amplified by RT-PCR from primary IECs, intestinal fibroblasts and IMACs (Fig. 2A, upper panel). A strong signal was also obtained with mRNA from the human cell lines HT-29 and HepG2. Integrity of the mRNA was verified by GAPDH RT-PCR (Fig. 2A, lower panel). The experiment was repeated three times with cells from different donors.

Western blot for PPARγ confirmed the results obtained by RT-PCR applying an anti-PPARγ rabbit polyclonal antibody (H100) with protein extracts from primary IECs, fibroblasts, monocytes and HT-29 (Fig. 2B, upper panel, *representative for three experiments*). PPARγ was further confirmed using an anti-PPARγ mouse monoclonal antibody (E80, Fig. 2B, middle panel, representative for three experiments). Signals were quantified and normalised to β-actin. The experiment was repeated three times with cells from different patients. The control signal from each immunoblot was defined as 1.0. Ratios were calculated from three blots. For H100 ratios were IECs 0.6: fibroblasts 0.5: monocytes 1.0: HT-29 0.6. For E80 ratios were IECs 0.8: fibroblasts 0.5: monocytes 0.3: HT-29 0.3.

3.2. Formation of linoleic and arachidonic acid metabolites in HT-29

To identify the capability of HT-29 to modify linoleic and arachidonic acid into LOX and COX metabolites a LOX assay was

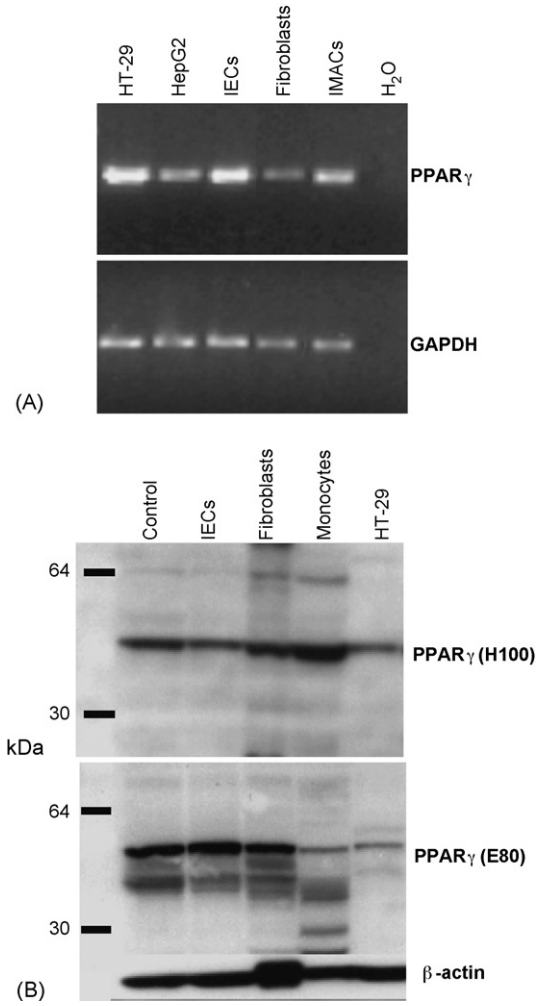

Fig. 2 – RT-PCR and Western blot analysis for PPARγ expression in human primary cells and cell lines. (A) PPARγ could be amplified with RT-PCR with mRNA from primary IECs, fibroblasts, IMACs and the cell lines HT-29 and HepG2 (upper lane). GAPDH RT-PCR (lower lane). (B) Western blot for PPARγ applying an anti-PPARγ rabbit polyclonal antibody (H100, upper lane) and mouse monoclonal antibody (E80, middle). PPARγ could be detected with IECs, fibroblasts, monocytes and HT-29. Recombinant protein was used as positive control. Western blotting for β-actin was performed for loading control. Both experiments were repeated three times with cells from different patients.

performed as described in Section 2. Formation of metabolites was determined by HPLC. Elution of 15-HETE, 12-HETE and 5-HETE was monitored at 232 nm (Fig. 3A) and LTB_4, 5,6,-DiHETE, 13-Oxo-ODE and 9-Oxo-ODE at 270 nm (Fig. 3B).

No LOX or COX metabolites were detected by HPLC in supernatants of HT-29 stimulated with both Ca^{2+} and arachidonic acid (Table 1) or Ca^{2+} alone (not shown). No metabolites were found in whole cell lysates.

HT-29 cells were stimulated with Ca^{2+}, arachidonic acid and Ca-ionophore. The supernatant was then subjected to HPLC analysis, 13-HODE, 15-, 12- and 5-HETE were detected (areas = 21.6, 148, 26.6 and 67.1, respectively, Table 1). When substituting arachidonic acid with linoleic acid in the LOX assay a considerable increase in formation of 15-, 12- and 5-HETE was monitored by HPLC (area = 689, 292 and 291, respectively) in supernatants of HT-29 cells. A further increase for 15-HETE was identified in whole cell lysate stimulated under the same conditions (area = 2045). 13-HODE was detected (area = 3521) but 12-HETE was decreased (area = 17.4) and 5-HODE was not detectable. The levels of free 13-Oxo-ODE lower those of its precursor 13-HODE as much of the end product might be bound to protein.

To investigate whether the Ca-ionophore was required, HT-29 cells were stimulated with Ca^{2+} and linoleic acid alone. 15-, 12- and 5-HETE was obtained from the supernatants of HT-29 cells (area = 170, 73 and 50, respectively, Table 1) suggesting that stimulation with Ca^{2+} is sufficient for the formation of those metabolites from linoleic acid. Increased amounts of 13-HODE, 15- and 12-HETE were measured in stimulated whole cell lysates (area = 3262, 7239 and 1652, respectively), because stimulation with Ca-ionophore may lead to the activation of enzymes that preferentially metabolize endogenous stores of arachidonic acid.

No LOX or COX metabolites were determined by HPLC in supernatants of HT-29 cells incubated with Ca^{2+} alone similar to the finding in whole cell lysates (not shown). After addition of both arachidonic acid and Ca^{2+}, LTB_4 was found in both supernatants and whole cell lysates (area = 8.4 and 6.2, respectively). Supernatants and cell lysates of HT-29 stimulated with Ca^{2+}, arachidonic acid and Ca-ionophore also contained only LTB_4 (area = 14.3 and 4.4, respectively).

By substitution of arachidonic acid with linoleic acid in the LOX assay, formation of LTB_4, 5,6-DiHETE, 13-Oxo-ODE and 9-Oxo-ODE was demonstrated by HPLC in supernatants and whole cell lysates (area = 8, 35.6, 129, 58 and 11.5, 81.8, 140, 44.2, respectively). Additionally, HT-29 cells were stimulated with Ca^{2+} and linoleic acid without Ca-ionophore. The linoleic acid metabolites 5,6-DiHETE, 13-Oxo-ODE and 9-Oxo-ODE were found in both the supernatants and whole cell lysates (area = 5.5, 12.2, 7.8 and 219, 329 and 70, respectively). LTB_4 was not detected under these conditions.

3.3. *Quantification of linoleic and arachidonic acid metabolites in HT-29 cells*

To calculate the concentration of metabolites in whole cell lysates a calibration curve for 13-Oxo-ODE (Fig. 3C), 15-HETE, 5-HETE and 12-HETE (Fig. 3D) was generated measuring the peak areas of 0.3125, 0.625, 1.25, 2.5, 5 ng/ml metabolite by HPLC analysis. The concentration of 13-Oxo-ODE identified in whole cell lysates from HT-29 stimulated with linoleic acid, Ca^{2+} and Ca-ionophore was calculated as 155 ng/ml. The concentration of 13-Oxo-ODE found after stimulation with linoleic acid and Ca^{2+} only was 85 ng/ml. The concentrations of the metabolites 15-HETE and 5-HETE identified in whole cell lysates from HT-29 cells stimulated with arachidonic acid, Ca^{2+} and Ca-ionophore were calculated as 142 and 64 ng/ml, respectively. These data indicate that 13-Oxo-ODE is a very prominent LOX product in intestinal epithelial cells. Concentration of 12-HETE was beyond the calibration curve and was assessed as 33 ng/ml.

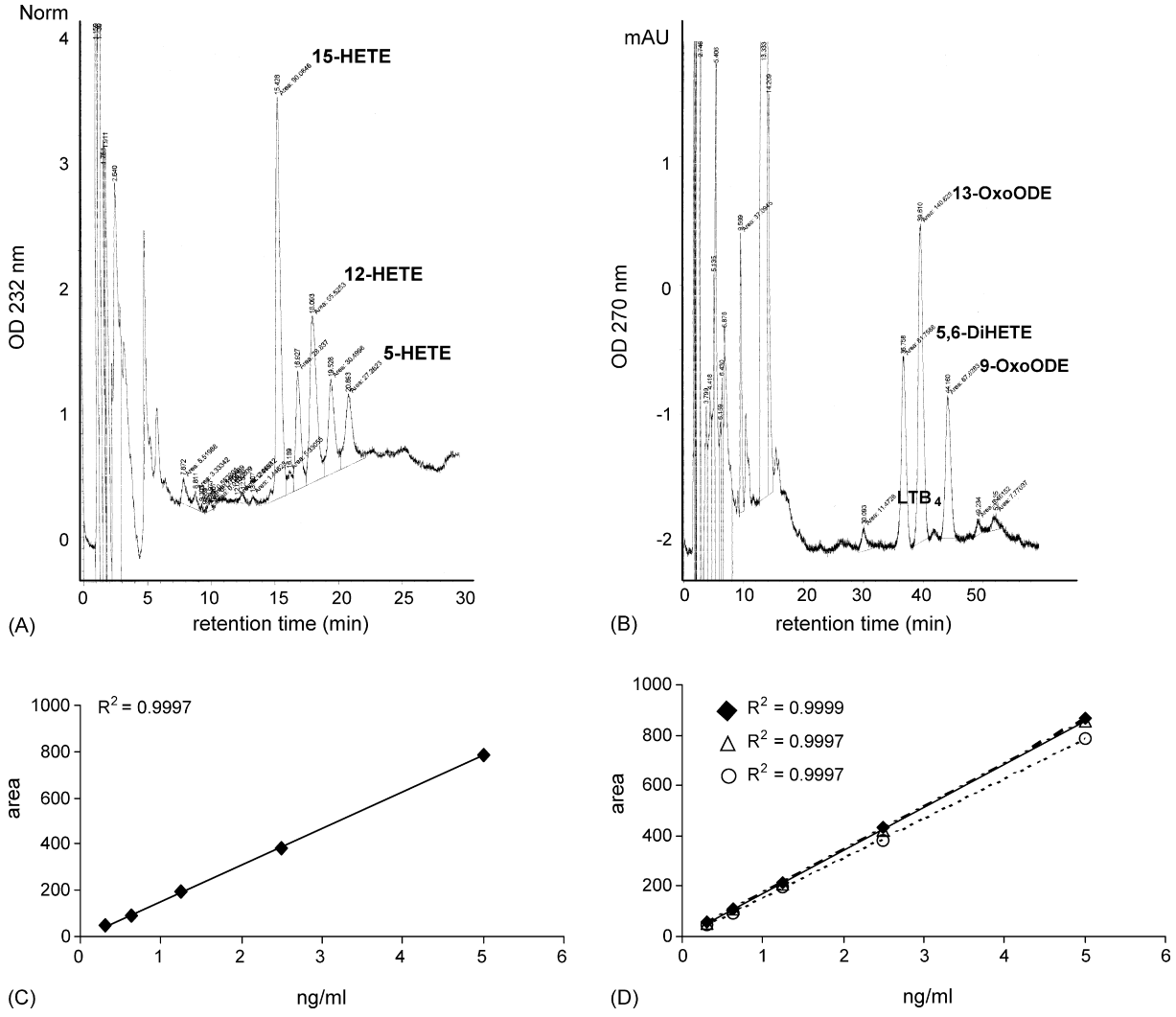

Fig. 3 – HPLC analysis of LOX and COX metabolites from linoleic and arachidonic acid. (A) Elution of 15-HETE, 12-HETE and 5-HETE was monitored at 232 nm (retention time 15.30 ± 0.41, 16.96 ± 0.95 and 20.59 ± 0.33 min, respectively). (B) Elution of LTB_4, 5,6,-DiHETE, 13-Oxo-ODE and 9-Oxo-ODE at 270 nm (retention time 29.72 ± 0.22, 35.7 ± 1.64, 38.45 ± 1.77 and 43.05 ± 2.15 min, respectively). (C) Calibration curve for 13-Oxo-ODE. (D) Calibration curve for (◆) 15-HETE, (△) 5-HETE and (○) 12-HETE.

3.4. *MS analysis of linoleic acid metabolite-formation in HT-29 cells*

To confirm the HPLC-results of the enzymatic modification of linoleic acid in HT-29 cells MS was performed. Therefore, a LOX assay was performed with whole cell lysates of HT-29 cells stimulated with Ca^{2+}, Ca-ionophore and linoleic acid (calculated mass $M = 294.4$) as substrate for 1 h at 37 °C as described in Section 2. Enzyme activation was terminated and LOX/COX metabolites were isolated by solid-phase extraction with octadecylsilane. For analysis one sample was directly infused into an Agilent SL-Trap with an ESI interface in positive ion mode. The presence of 13-Oxo-ODE was confirmed at an $m/z = 294.3$ (Fig. 4A, resulting from a positively charged M and a corresponding chlorine-adduct at $m/z = 329.5$). The trapped $m/z = 294.3$ showed in the MS^2 two major peaks at $m/z = 249.3$ and 275.2 resulting from a loss of (CO_2H) and (H_3O) (Fig. 4B). The MS^3 from $m/z = 249.3$ led to a couple of mass tracks (e.g. 193.2, 177.1 or 179, Fig. 4C). MS^3 from $m/z = 275.2$ generated a main peak at $m/z = 189.1$ (Fig. 4D).

3.5. *Influence of different PPARγ ligands on IL-8 secretion of HT-29 cells*

The decrease in the secretion of the pro-inflammatory cytokine IL-8 from HT-29 cells was used as a surrogate to quantify the anti-inflammatory potential of PPARγ ligands. HT-29 cells were starved for 24 h in serum-free medium and cultured for 24 h in the presence of 10 ng/ml TNF and 5-HETE, 15-HETE, 13-Oxo-ODE and 13-HODE (3.12, 6.25, 12.5, 25, 50 or 100 μM, respectively) or troglitazone and 15D-PGJ2 (1.56, 3.12, 6.25, 12.5, 25 or 50 μM, respectively). Secretion of IL-8 into the culture medium was measured by ELISA (Fig. 5). All ligands

Table 1 – HPLC analysis of 15-, 12- and 5-HETE, LTB_4, 5,6,-DiHETE, 13-Oxo-ODE and 9-Oxo-ODE

	Ca^{2+} arachidonic acid		Ca^{2+} Ca-ionophore arachidonic acid		Ca^{2+} Ca-ionophore linoleic acid		Ca^{2+} linoleic acid	
	Supernatant	Whole cell lysate	Supernatant	Whole cell lysate	Supernatant	Whole cell lysate	Supernatant	Whole cell lysate
13-HODE	0	0	21.6	0	0	3521	0	3262
15-HETE	0	0	148	0	689	2045	170	7239
12-HETE	0	0	26.6	0	292	17.4	73	1652
5-HETE	0	0	67.1	0	291	0	50	0
LTB_4	8.4	6.2	14.3	4.4	8	11.5	0	0
5,6,-DiHETE	0	0	0	0	35.6	81.8	5.5	219
13-Oxo-ODE	0	0	0	0	129	140	12.2	329
9-Oxo-ODE	0	0	0	0	58	44.2	7.8	70

LOX or COX metabolites were determined in supernatant or whole cell lysate of HT-29 stimulated with Ca-ionophore, arachidonic and linoleic acid. Numerical value = integrated peak area.

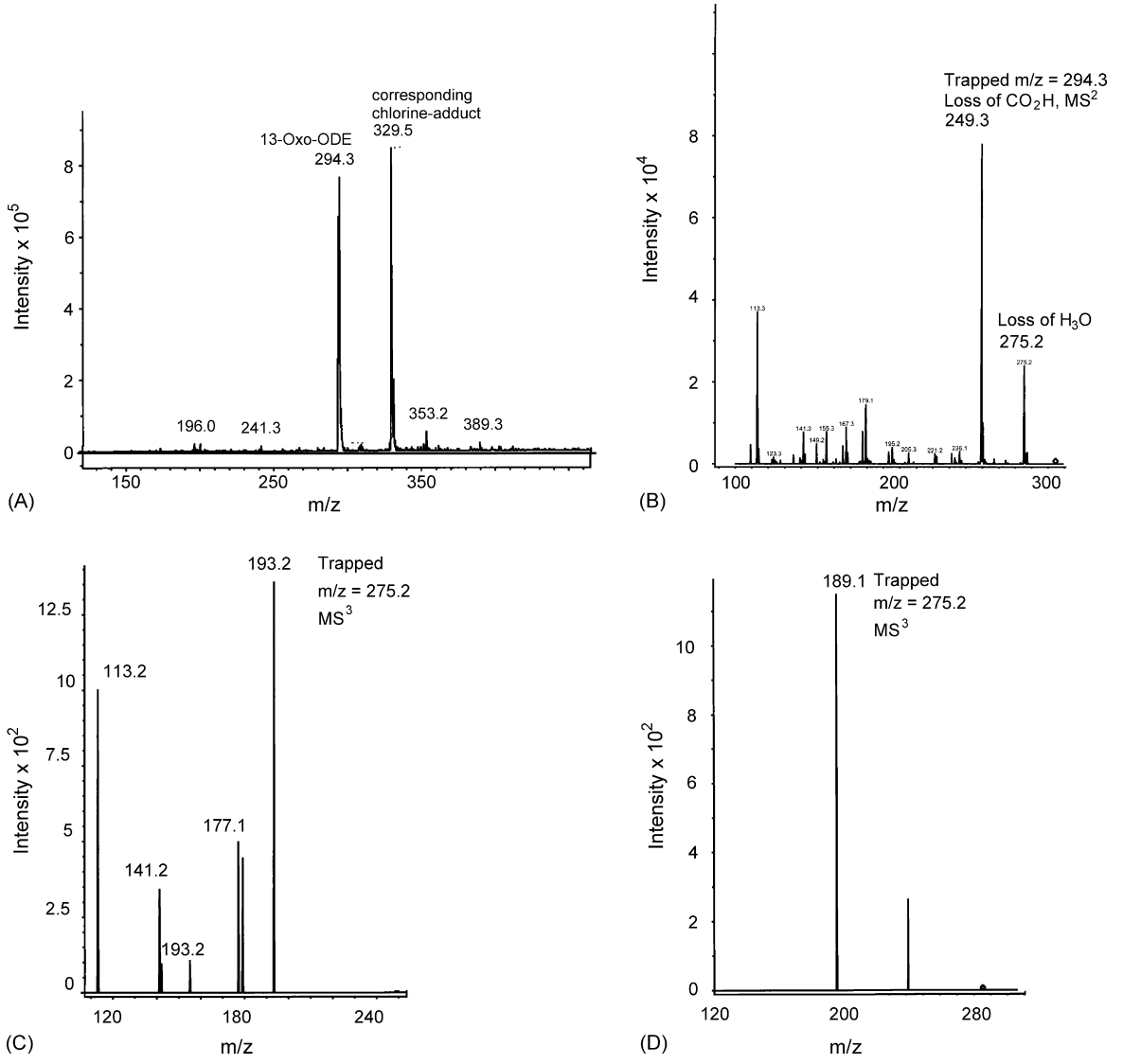

Fig. 4 – Identification of 13-Oxo-ODE as metabolite of linoleic acid by ion trap MS. Whole cell lysate of HT-29 were stimulated with Ca^{2+}, Ca-ionophore and linoleic acid. (A) Presence of 13-Oxo-ODE at *M* = 294.3. (B) 13-Oxo-ODE fragments at least into two peaks at *M* = 249.3 and 275.2 as determined by ion trapping. A MS^3 of the two peaks revealed degradation products at (C) *M* = 179.3 and 193.3 and (D) *M* = 189.1; *x*-axis = *m*/*z*, *y*-axis = relative intensity.

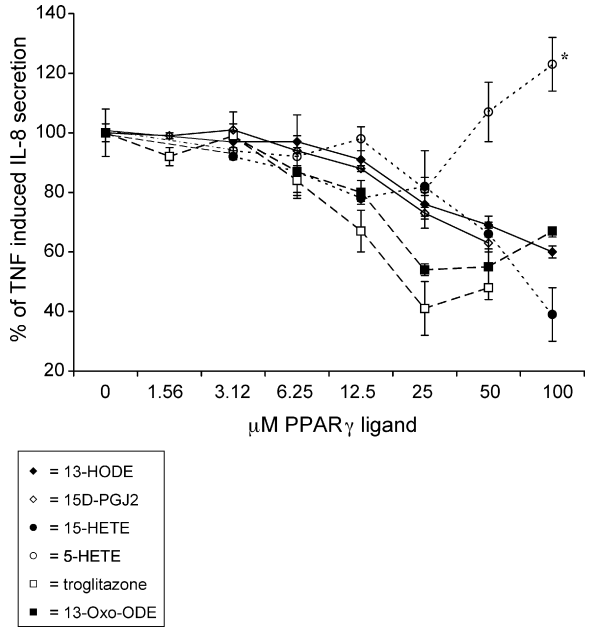

Fig. 5 – IL-8 secretion of HT-29 cells after incubation with TNF and PPARγ ligands. Cells were cultured for 24 h in the presence of 10 ng/ml TNF and 5-HETE (open circle), 15-HETE (filled circle), troglitazone (open square), 13-Oxo-ODE (filled square), 15D-PGJ2 (open diamond), 13-HODE (filled diamond). Secretion of IL-8 into the culture medium was measured by ELISA. The curve progression of the 5-HETE-treated group is significantly different from the other five groups ($p < 0.05$). TNF stimulation alone = 100%. Stimulation with ligand alone served as control and was subtracted from the result of costimulation. Bars represent mean ± S.E.M.

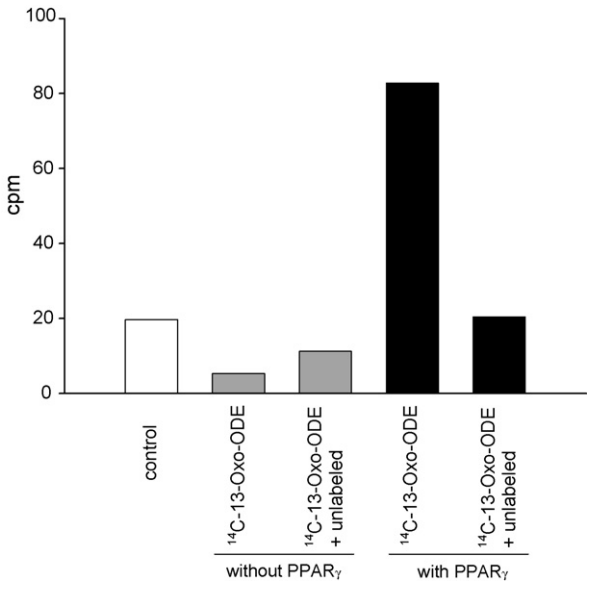

Fig. 6 – Evidence for specific binding of 13-Oxo-ODE to PPARγ in HeLa cells. HeLa cells were transfected with pCR3.1-PPARγ-His as described in Section 2. Control cells were treated with LipofectAMINE not containing the plasmid. Cell lysates were incubated with 20 μM ^{14}C-labelled 13-Oxo-ODE alone or 20 μM ^{14}C-13-Oxo-ODE and an excess (400 μM) of unlabelled 13-Oxo-ODE. No increase in cpm could be detected in non-transfected, LipofectAMINE treated HeLa cells (light grey bars). If cells expressed PPARγ an up to fivefold increase in cpm was observed which was abolished by the addition of unlabelled 13-Oxo-ODE indicating specific binding of 13-Oxo-ODE (dark grey bars). The data shown are representative for two more independent experiments.

dose-dependently inhibited IL-8 secretion. However, after stimulation with 50 μM PPARγ ligand, IL-8 secretion of 5-HETE-treated cells was significantly different from all other conditions ($p < 0.05$). The 5-HETE-induced IL-8 secretion was further increased after stimulation with 100 μM PPARγ ligand ($p < 0.05$) compared to 13-HODE, 13-Oxo-ODE and 15-HETE. The curve progression of the 5-HETE-treated group is significantly different from the other five stimulations with PPARγ ligand ($p < 0.05$).

3.6. *Binding of 13-Oxo-ODE to PPARγ*

HeLa cells were transfected with pCR3.1-PPARγ-His to overexpress PPARγ as described in Section 2. Protein in cell lysates was controlled by immunoblotting (data not shown). Cell lysates with or without PPARγ overexpression were either incubated with 20 μM ^{14}C-labelled 13-Oxo-ODE alone or 20 μM ^{14}C-13-Oxo-ODE with an excess (400 μM) of unlabelled 13-Oxo-ODE to estimate unspecific binding. After immunoprecipitation (anti-His-tag) no increase in cpm compared to control could be detected in non-transfected, LipofectAMINE treated HeLa cells (Fig. 6). If cells were transfected with pCR3.1-PPARγ-His and expressed PPARγ an up to fivefold increase in cpm was observed which was abolished by the addition of unlabelled 13-Oxo-ODE indicating specific binding of this LOX metabolite to PPARγ (Fig. 6).

The addition of different concentrations of ^{14}C-13-Oxo-ODE showed saturation of the specific binding at a concentration of about 20 μM (Fig. 7). In non-transfected cells some non-specific binding is evident (Fig. 7).

4. Discussion

The present manuscript provides evidence that PPARγ is expressed in IEC and the HT-29 cell line. A detailed analysis of the endogenous PPARγ ligands produced by IEC is provided. Among those LOX products produced in HT-29 cells 13-Oxo-ODE is the most prominent. Similar to other endogenous PPARγ ligands 13-Oxo-ODE had anti-inflammatory effects and reduced IL-8 secretion from HT-29 cells significantly. Further, we provide evidence that 13-Oxo-ODE directly binds to PPARγ. The increase in HETE observed in cells stimulated with linoleic acid must be derived from the liberation of endogenous stores of arachidonic acid considering the expenditure of time for elongation of linoleate to arachidonate. Taken together our experiments identify a new endogenous PPARγ ligand being specific for intestinal epithelial cells as IEC express high

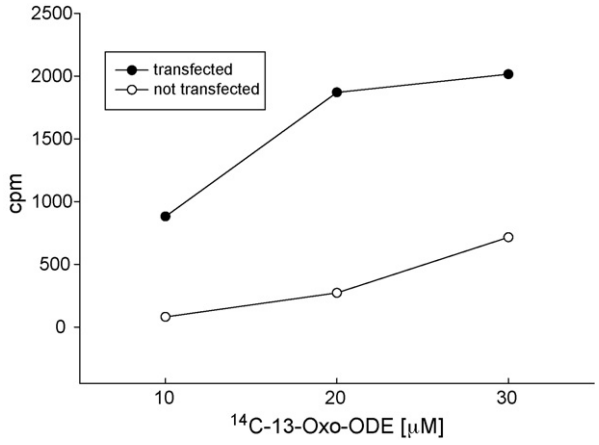

Fig. 7 – Evidence for saturable binding of 13-Oxo-ODE to PPARγ. HeLa cells were transfected with pCR3.1-PPARγ-His as described in Section 2. Control cells were treated with LipofectAMINE not containing the plasmid. Cell lysates were incubated with 20 μM ^{14}C-labelled 13-Oxo-ODE. The addition of 10, 20 and 30 μM ^{14}C-13-Oxo-ODE showed a saturation of the binding at 20 μM. The data shown are representative for one more independent experiment.

activity of 13-HODE dehydrogenase [48]. This might be an explanation for the important role of PPARγ in IEC that has recently been demonstrated. *We provide an explanation why the PPARγ expression in intestinal epithelial cells is crucial for mucosal homeostasis despite the fact that PPARγ expression itself is not increased in this specific cell population. The specific presence of an active endogenous ligand on one hand explains those findings mentioned above. On the other hand it provides a therapeutic target for the treatment of inflammatory bowel disease with high tissue specifity.*

Ligand mediated activation of PPARγ is followed by a reduction of the mucosal damage and prevention or down-regulation of inflammation in several murine models of colitis [28–34]. Rosiglitazone delayed the onset of colitis in IL-10 deficient mice [35] and reduced mucosal damage in TNBS-induced colitis in rats [36]. Overexpression of PPARγ in epithelial cells ameliorated mucosal inflammation [37]. An increase of the mucosal concentration of endogenous PPARγ ligands could reduce mucosal inflammation as data on the supplementation with conjugated linoleic acids suggest [38].

Rousseaux et al. presented evidence that the therapeutic effect of 5-aminosalicylic acid (5-ASA) may be mediated by PPARγ [54]. 5-ASA treatment had beneficial effects on colitis in wild-type mice but not in heterozygous PPARγ knock out animals [54]. In epithelial cells, 5-ASA increased PPARγ expression and promoted its translocation to the nucleus where it induced a modification allowing the recruitment of co-factors for the regulation of transcription.

Contradictory results have been published on the cell type in which PPARγ expression is important or altered during mucosal inflammation. Katayama et al. demonstrated a reduction of PPARγ expression during colitis in lamina propria lymphocytes and macrophages, whereas colitis did not alter expression in CECs [37]. In contrast, Dubuquoy et al. presented evidence that PPARγ is expressed primarily in mucosal epithelial cells with only low expression in lamina propria macrophages and almost no expression in lymphocytes [39]. Recently Adachi et al. presented evidence that PPARγ expression in mucosal epithelial cells is crucial for its anti-inflammatory effects with respect to colitis [55]. The data summarised above raised the question why expression of PPARγ was important or crucial in epithelial cells but only to a lesser extent in macrophages. It is generally assumed that macrophage activation is more relevant for the initiation of intestinal inflammation than epithelial cell activation. Our investigation allows the speculation that PPARγ expression in epithelial cells may be of selective importance because they produce and contain an additional endogenous ligand in high concentrations. Production of 13-Oxo-ODE might be stimulated by pharmacological intervention. Another option could be a local application by enema. Future research has to evaluate the therapeutic potential of 13-Oxo-ODE.

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