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15-Lipoxygenase-1 Exerts Its Tumor Suppressive Role by Inhibiting Nuclear Factor-Kappa B Via Activation of PPAR Gamma

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ABSTRACT

15-Lipoxygenase-1 (15-LOX-1) is an enzyme of the inflammatory eicosanoid pathway whose expression is known to be lost in colorectal cancer (CRC). We have previously shown that reintroduction of the gene in CRC cell lines slows proliferation and induces apoptosis (Cimen et al. [2009] Cancer Sci 100: 2283–2291). We have hypothesized that 15-LOX-1 may be anti-tumorigenic by the inhibition of the anti-apoptotic inflammatory transcription factor nuclear factor kappa B. We show here that ectopic expression of 15-LOX-1 gene in HCT-116 and HT-29 CRC cell lines inhibited the degradation of inhibitor of kappa B (IκBα), decreased nuclear translocation of p65 and p50, decreased DNA binding in the nucleus and decreased transcriptional activity of Nuclear factor kappa B (NF-κB). As the 15-LOX-1 enzymatic product 13(S)-HODE is known to be a peroxisome proliferator-activated receptor gamma (PPARγ) agonist, and NF-κB can be inhibited by PPARγ, we examined whether activation of PPARγ was necessary for the abrogation of NF-κB activity. Our data show that the inhibition of both early and late stages of NF-κB activation could rescued by the PPARγ antagonist GW9662 indicating that the inhibition was most likely mediated via PPARγ. J. Cell. Biochem. 112: 2490–2501, 2011.

KEY WORDS: 15-LIPOXYGENASE-1; NF-κB; PPARγ; COLORECTAL CANCER

The eicosanoid pathway, described by the oxygenation of 20 carbon fatty acids by the inflammatory cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, has been implicated in cancer development [Cuendet and Pezzuto, 2000; Jones et al., 2003; Krishnamoorthy and Honn, 2008]. Unlike several pro-carcinogenic LOXs that have been described, 15-lipoxygenase-1 (15-LOX-1) has been shown to have a tumor suppressive nature in colorectal cancer (CRC) [Bhattacharya et al., 2009]. This enzyme preferentially catalyzes the conversion of linoleic acid to 13(S)-HODE [Nagy et al., 1998; Zuo et al., 2006] and a reduced level of 13(S)-HODE in vivo has been shown to be associated with CRC [Shureiqi et al., 1999, 2010]. Mechanisms for the tumor suppressive effects of 15-LOX-1 in CRC include loss of proliferation [Shureiqi et al., 1999; Cimen et al., 2009], induction of terminal differentiation and apoptosis [Shureiqi et al., 2005], and decreased motility, invasion, and migration of CRC cells [Cimen et al., 2009]. 15-LOX-1 expressing cells have been shown to undergo apoptosis via the downregulation of anti-apoptotic proteins [Cimen et al., 2009] and phosphorylation of p53 by DNA-PK [Zhu et al., 2008].

Nuclear factor kappa B (NF-κB) is an inflammatory transcription factor that is frequently dysregulated in many cancers including CRC, leading to increased cellular transformation, proliferation, and loss of apoptosis [Karin, 2006b; Wang et al., 2009]. In the absence of a stimulus, the NF-κB subunits bind to inhibitor of kappa B (IκB) and are sequestered away from the κB elements of target promoter regions [Karin, 2006a]. Following a pro-inflammatory stimulus, IκB is degraded in the proteosome following phosphorylation by inhibitor of kappa kinase (IKK), allowing the NF-κB subunits (most commonly p65 and p50) to favor nuclear localization. These proteins coordinate the transcription of several hundred target genes which have roles in processes as diverse as immune response, cellular differentiation, proliferation, and survival [Karin, 2006b; Hayden and Ghosh, 2008]. Therefore, it is not surprising that a large body of research has been devoted to identify

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antagonists of NF-κB activity [Bharti and Aggarwal, 2002; Van Waes, 2007].

The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor that is expressed in adipocytes and epithelial cells. Ligand-activated PPARγ heterodimerizes with retinoid X receptor (RXR) and this complex then recruits coactivators and corepressors before binding to peroxisome proliferator response elements (PPRE) in the promoters of target genes regulating lipid and carbohydrate metabolism and inflammation [Rumi et al., 2004]. A number of endogenous ligands/agonists for PPARγ such as fatty acid derivatives and synthetic ligands such as thiazolidinediones have been described [Michalik et al., 2004; Itoh et al., 2008]. PPARγ activation is anti-inflammatory in nature and has been shown to inhibit cellular proliferation, induce differentiation, and promote cell cycle arrest and apoptosis in colon cancer cell lines [Brockman et al., 1998; Sarraf et al., 1998; Yang and Frucht, 2001; Michalik et al., 2004]. Mechanistic studies on the anti-inflammatory actions of agonist activated PPARγ include inhibition of early events of NF-κB signaling, such as IκBα expression, phosphorylation and its subsequent degradation, as well as p65 nuclear translocation [Kim et al., 2007; Wan et al., 2008; Buroker et al., 2009]. PPARγ has also been shown to transrepress NF-κB and thereby inhibit the expression of inflammatory target genes such as iNOS [Pascual et al., 2005]. 13(S)-HODE and 15-HETE, the enzymatic products following oxygenation of linoleic acid and arachidonic acid, respectively, by 15-LOX-1 have been implicated as agonists for PPARγ in CRC cell lines [Bull et al., 2003; Nixon et al., 2003].

We have previously shown that forced expression of 15-LOX-1 resulted in decreased proliferation and increased apoptosis, as well as decreased cellular motility, anchorage independent growth, migration, and invasion through Matrigel in CRC cell lines [Cimen et al., 2009]. In this study, we have hypothesized that the pro-apoptotic effects of 15-LOX-1 may be mediated via an inhibition of the anti-apoptotic transcription factor NF-κB. We demonstrate here that forced expression of 15-LOX-1 in HCT-116 and HT-29 colon carcinoma cell lines resulted in the inhibition of NF-κB as shown by decreased nuclear translocation and binding of NF-κB subunits to the consensus sequences as well as decreased transcriptional activity. Many of these effects could be reversed when the cells were treated with the selective PPARγ antagonist GW9662. As PPARγ is reported to exert anti-inflammatory effects often by inhibiting the NF-κB pathway [Michalik et al., 2004; Wahli, 2008], we propose that 15-LOX-1 inhibits NF-κB at least in part via the 13(S)-HODE mediated activation of PPARγ.

MATERIALS AND METHODS

CELL CULTURE

HCT-116 human colon cancer cell line was obtained from the DSMZ (Braunschweig, Germany), HT-29 was purchased from ŞAP Enstitusu (Ankara, Turkey). The cells were grown in phenol red-free RPMI 1640 medium containing 10% fetal bovine serum (FBS) supplemented with 1% penicillin/streptomycin in a humidified atmosphere at 37°C and 5% CO_2. All cell culture media and supplements were purchased from Biochrom (Berlin, Germany).

PLASMIDS

The 15-LOX-1 cDNA cloned into a pcDNA3.1 vector with a zeocin mammalian marker was kindly provided by Dr Uddhav Kelavkar (Mercer University School of Medicine, Savannah Campus, GA) [Kelavkar et al., 1998]. The Pathdetect NF-κB *cis*-Reporting system (Stratagene Agilent, CA) was used according to manufacturer's instructions. The PPRE-3xTK-Luc, PPARγ1-LBD, and UAS_G-4xTK-Luc plasmids were kindly provided by Dr. Ronald Evans (Howard Hughes Medical Institute, CA) [Forman et al., 1995].

TRANSFECTIONS

The 15-LOX-1 vector was stably transfected into HCT-116 and transiently into HT-29 cells as described previously [Cimen et al., 2009]. Two monoclones of 15-LOX-1-transfected HCT-116 (1E7 and 1F4) were expanded and used for the experiments. HT-29 cells transiently transfected with the 15-LOX-1 construct using FuGENE HD (Roche, Mannheim, Germany) were collected after 48 h and processed for mRNA and protein extraction. The mRNA and protein were confirmed for the expression of 15-LOX-1 and used for the experiments. Control cells included the cells transfected with the empty pcDNA3.1 vector.

CELL TREATMENTS

HCT-116 cells were treated with 100 μM 13(S)-HODE (Cayman Chemical, MI) in ethanol and then processed for luciferase assay or immunofluorescence as described below. The final concentration of ethanol was kept below 0.5%. In order to inhibit PPARγ, the 15-LOX-1 expressing or control cells were incubated for 24 h with 1 μM of the PPARγ antagonist GW9662 (Sigma–Aldrich, Taufkirchen, Germany). The cells were treated with 1 μM PD146176 (Sigma–Aldrich) to inhibit 15-LOX-1, with 10 μM U0126 (Sigma–Aldrich) to inhibit ERK1/2 and with 32 μM SN-50 (Santa Cruz, CA) to inhibit NF-κB. GW9662, PD146176, and U0126 were dissolved in DMSO; SN-50 was dissolved in sterile deionized water. The final concentration of DMSO was kept below 0.1%.

WESTERN BLOT ANALYSIS

Nuclear and cytoplasmic extracts were isolated as described by Garneau et al. [2007] and stored at −80°C. The protein content was measured using the modified Bradford Assay using a Coomassie Plus protein assay reagent (Pierce, Rockford, IL). The proteins (50–80 μg) were separated in a 10% polyacrylamide gel and transferred onto a PVDF membrane (Roche). The membranes were blocked in 5% skim milk for 1 h and were incubated overnight with the appropriate primary antibodies: p65 (1:500 dilution), IκBα (1:500 dilution) p-IκBα (1:250 dilution), p-ERK1/2 (1:250 dilution), ERK1/2 (1:500 dilution), PPARγ (1:500 dilution), (all from Santa Cruz), and p-PPARγ (1:625 dilution; Abcam, Cambridge, MA) followed by incubation for 1 h with a horseradish peroxidase-conjugated goat anti-rabbit (1:2,000) or goat anti-mouse (1:2,000) secondary antibody. The bands were visualized by using the ECL Plus enhanced chemiluminescence kit (Pierce) according to the manufacturer's instructions.

LUCIFERASE REPORTER ASSAYS

15-LOX-1 expressing and control HCT-116 cells (2.5×10^5 cells/well) were plated in 12-well culture plates. The cells were transfected

with the Pathdetect NF-κB *cis*-Reporting system (Stratagene Agilent) plasmids using FuGENE HD (Roche). After 24 h, where indicated, the cells were treated with 100 μM 13(S)-HODE or 1 μM PD146176 or 1 μM of the PPARγ antagonist GW9662 (Sigma–Aldrich) for 24 h with or without pretreatment with TNFα (10 ng/ml) for 6 h. The cells were then collected and analyzed for luciferase activity. To confirm whether 13(S)-HODE was a ligand for PPARγ, cells were transfected with a PPARγ1LBD-GAL4DBD construct along with a UAS_G-4xTK-Luc construct containing the upstream activating sequence (UAS) of GAL4 upstream of a thymidine kinase (TK) driven luciferase reporter gene [Forman et al., 1995]. In the presence of a ligand, the PPARγ1LBD-GAL4DBD binds to the UAS_G-4xTK-Luc reporter gene thereby driving the transcription of the luciferase gene. To determine the PPARγ transcriptional activity, the PPRE-3xTK-LUC plasmid was transfected into 15-LOX-1 expressing and control HCT-116 cells (2.5×10^5 cells/well) for 24 h using FuGENE HD. Where indicated, the 15-LOX-1 expressing cells were treated with 1 μM GW9662 or parental HCT-116 cells were treated with 100 μM 13(S)-HODE for 24 h and collected and analyzed for luciferase activity.

Luciferase activity was assessed with Luciferase Reporter Gene Assay (Roche) according to the manufacturer's instructions using a Modulus luminometer (Turner Biosystems, CA). The enzyme activity was normalized for the efficiency of transfection in all assays on the basis of β-galactosidase activity levels and reported as normalized relative light units (RLU). All reporter assays were performed in four replicates in at least three independent experiments.

IMMUNOFLUORESCENCE STUDIES

15-LOX-1 expressing and empty vector (EV) transfected HCT-116 cells, or HCT-116 cells treated with 100 μM 13(S)-HODE or the vehicle (ethanol) control were cultured on glass coverslips for 24 h to 60–70% confluence. The cells were subsequently prepared for immunofluorescence to detect the NF-κB subunits p50 and p65. The cells were gently washed with sterile PBS, fixed with 4% paraformaldehyde for 10 min, washed, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Following this, the cells were washed, blocked with 1% BSA for 30 min at room temperature and incubated at 4°C overnight with p65 or p50 antibody (both at 1:100 dilution in 1% BSA). The cells were then washed with 0.1% Tween 20 in PBS, and incubated with Alexa Fluor488 (AF488)-labeled goat anti-rabbit secondary antibody (1:500 dilution), (Invitrogen, CA) for 1 h at 37°C. Nuclear staining was performed by incubating the cells in propidium iodide (PI; Applichem) for 30 min at room temperature. The slides were then photographed Zeiss LSM 510 (Jena, Germany) Confocal Laser Scanning Microscope. Image analysis was performed on the single-channel images to quantify the nuclear AF488/PI pixel intensity ratio of each dataset using ImageJ (www.rsb.info.nih.gov/ij/). The results are shown as the average of four independent experiments.

DNA-BINDING ELISA ASSAY

The NF-κB (human p50) Transcription Factor Assay Kit (Cayman Chemical) was used according to manufacturer's instructions. The kit consists of a double-stranded DNA sequence containing the NF-κB response element immobilized to the wells of a 96-well plate. Nuclear extracts of the HCT-116 1E7 clone and HT-29 transiently transfected with the 15-LOX-1 construct were applied to the wells and allowed to bind to the response element. NF-κB DNA binding was detected by the subsequent addition of a primary antibody against p50 and a horseradish peroxidase-conjugated secondary antibody. A colorimetric readout was obtained at 450 nm using a Bio-Rad plate reader.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The NF-κB DNA-binding activity was determined in the nuclear extracts of the 15-LOX-1 expressing and control cells using EMSA. EMSA experiments were performed by using the LightShift® Chemiluminescent EMSA Kit (Pierce) according to manufacturer's instructions. Nuclear extracts were isolated from HCT-116 cells stably transfected and HT-29 cells transiently transfected with the 15-LOX-1 vector. Oligonucleotides (4 pmol) containing the NF-κB consensus sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was end labeled with biotin by using the Biotin 3′ End DNA Labeling system (Pierce) with Terminal Deoxynucleotidyl Transferase (TdT) enzyme (Pierce) at 37°C for 30 min. The binding reaction (20 μl) had the following components added in the indicated order: 1× binding buffer, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl Poly (dI × dC), 0.05% NP-40, 20 fmol of labeled NF-κB consensus sequence oligonucleotide and 5 μg of nuclear extracts and incubated at room temperature for 20 min. Competition reactions were performed by using 200-fold molar excess of the unlabeled (cold) NF-κB consensus sequence oligonucleotides. For supershift assays, the reaction mix without labeled oligonucleotide was incubated with the p65 or p50 antibody (2 μl) first on ice for 10 min followed by another 20 min at room temperature after addition of the labeled oligonucleotide. Binding reactions were terminated by the addition of 5 μl of 5× loading dye supplied in the kit and loaded on an 8% nondenaturing polyacrylamide gel, which was prerun for 60 min and electrophoresed at 100 V for 1.5 h. The gel was electroblotted on a Biodyne-B precut Nylon membrane at 100 V for 45 min and crosslinked for 15 min using a UV transilluminator (Benda, Wiesloch, Germany). Bands were visualized by ECL plus substrate as described in Western blotting.

CELL PROLIFERATION ASSAY

Cell proliferation was measured using the Vybrant MTT assay kit (Invitrogen, Carlsbad, CA) according to manufacturer's guidelines. Briefly, 10,000 cells (15-LOX-1 expressing and EV-transfected control HCT-116 cells) were plated in a final volume of 100 μl in complete RPMI 1640 medium in 96-well tissue culture dishes. After 24 h, the 15-LOX-1 expressing and control cells were treated with 1 μM GW9662 for another 48 h. EV-transfected cells were treated with the NF-κB inhibitor 32 μM SN-50 for 48 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) labeling reagent was added after this, incubated for 4 h and solubilized with a 1% solution of SDS for 18 h. The absorbance was determined in a Bio-Rad microplate reader at 570 nm.

STATISTICAL ANALYSES

Data analysis and graphing was performed using the GraphPad Prism 5 software package (Prism, CA). Data are expressed as

mean ± SEM. Statistical analyses between experimental results were based on one-way ANOVA using Tukey's multiple comparison test or by Mann–Whitney *U*-test. Significant difference was statistically considered at the level of $P < 0.05$.

RESULTS

15-LOX-1 EXPRESSION REDUCES NUCLEAR TRANSLOCATION OF NF-κB

15-Lipoxygenase-1 is not expressed in both HCT-116 and HT-29 CRC cell lines [Cimen et al., 2009]. However, when transfected with a pcDNA3.1-15-LOX-1 vector, the protein was expressed in both cell lines and was enzymatically active producing significantly more 13(S)-HODE than the cells transfected with the EV [Cimen et al., 2009].

Nuclear extracts isolated from the 15-LOX-1 expressing cells were probed for translocation of the NF-κB subunit p65 using Western blot (Fig. 1A). The results indicate that forced expression of 15-LOX-1 in both HCT-116 and HT-29 cell lines could reduce the nuclear translocation of the NF-κB subunit p65 when compared to the EV-transfected cells. Additionally, preincubation of the 15-LOX-1 expressing cells with 1 μM PD146176, a specific inhibitor of 15-LOX-1 without any nonspecific antioxidant activity [Sendobry et al., 1997], could reverse the inhibition in nuclear translocation of p65. NF-κB is normally held in the cytoplasm by IκB proteins and following a pro-inflammatory stimulus, the IκB proteins are phosphorylated and degraded in the proteosome. We therefore assayed the expression of IκBα and phosphorylated IκBα in the cytoplasmic extracts of the 15-LOX-1 expressing HCT-116 and HT-29 as well as control cells by Western blot (Fig. 1B). Our data indicate a higher level of IκBα in the cytoplasm of 15-LOX-1 expressing HCT-116 and HT-29 cells when compared to EV-transfected cells. Additionally, the phosphorylated form of IκBα was found to be much lower in the 15-LOX-1 expressing cells when compared to the control cells, indicating that degradation of IκBα and release of active NF-κB was inhibited when 15-LOX-1 was expressed. When the 15-LOX-1 expressing cells were treated with 1 μM PD146176 and probed for the expression of IκBα and its phosphorylated form, we observed a reduction of IκBα level along with an increase in its phosphorylated form. This further confirms that the retention of the NF-κB subunits by IκBα in the cytoplasm most likely resulted from the expression of 15-LOX-1.

15-LOX-1 EXPRESSION REDUCES THE DNA-BINDING ACTIVITY OF NF-κB

We then wanted to examine whether the loss of nuclear translocation of the NF-κB subunits was also associated with a decrease in the DNA-binding activity of the transcription factor. For

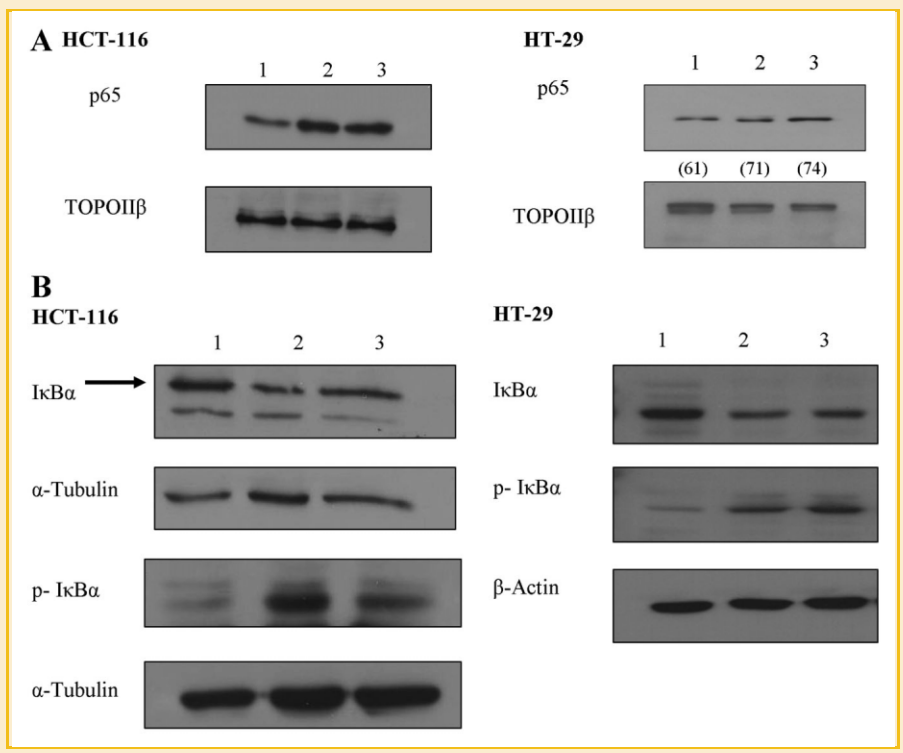

Fig. 1. 15-LOX-1 expression reduces NF-κB p65 nuclear translocation. A: Reduced translocation of p65 was observed in nuclear extracts isolated from HCT-116 (left panel) and HT-29 (right panel) cells with forced expression of 15-LOX-1 (lane 1) compared to control empty vector (EV) transfected cells (lane 2). This effect was reversed when the cells were treated with 1 μM 15-LOX-1 inhibitor PD146176 (lane 3). B: Cytoplasmic extracts from 15-LOX-1 expressing cells were probed for IκBα and p-IκBα using Western blot. Stable (HCT-116, left panel) or transient (HT-29, right panel) transfection with the 15-LOX-1 plasmid resulted in increased levels of IκBα (lane 1) and decreased levels of phosphorylated IκBα (lane 1) in the cytoplasm when compared to control EV cells (lane 2). This effect was also reversed upon treatment with 1 μM PD146176 (lane 3).

this purpose, we determined the DNA-binding activity of the p50 subunit using the NF-κB (human p50) Transcription Factor Assay Kit. Nuclear extracts from 15-LOX-1 expressing HCT-116 cells were incubated in a 96-well plate containing the NF-κB consensus sequence. Once the binding reaction was complete, colorimetric detection of the active (i.e., DNA bound) p50 was carried out with a primary antibody against p50 followed by a horseradish peroxidase-conjugated secondary antibody. Our data (Fig. 2A) indicate that the amount of active DNA bound p50 protein was significantly ($^{*}P < 0.05$) lower in the 15-LOX-1 expressing cells when compared to the control cells. Additionally, when the cells were incubated with PD146176, the DNA bound active form of p50 increased to the same level as the control EV-transfected cells, further confirming the specificity of 15-LOX-1 in inhibiting the DNA-binding activity of NF-κB. In order to further confirm that this effect was not limited solely to HCT-116 cells, nuclear extracts were collected from HT-29 cells transiently transfected with the 15-LOX-1 vector and applied to the NF-κB (human p50) Transcription Factor Assay Kit as described above. Our data (Fig. 2A) indicate that 15-LOX-1 expression in HT-29 cells could also significantly ($^{***}P < 0.001$) reduce the p50 DNA-binding activity when compared to the control EV or mock transfected cells.

Additionally, we carried out a nonradioactive EMSA to determine binding of the NF-κB subunits to the κB consensus oligonucleotides. For this purpose, nuclear extracts were isolated from cells transfected either stably (HCT-116) or transiently (HT-29) with the 15-LOX-1 vector. The proteins were then incubated with the biotin-labeled κB consensus oligonucleotides and processed for EMSA as described in the Materials and Methods section. The data (Fig. 2B) indicate that expression of 15-LOX-1 resulted in reduced mobility shift and thereby reduced κB consensus DNA binding in the nucleus when compared to EV-transfected or 15-LOX-1 expressing cells treated with PD146176. Additionally, for both cell lines, the specificity of the reaction was confirmed by incubating the reaction mixture with 200-fold excess of the cold probe which resulted in a loss of mobility shift (Fig. 2B, lane 5, left and right panels) as well as by incubating with 2 μl of p65 and p50 antibodies which resulted in a supershift (Fig. 2B, lanes 7 and 8 respectively, left and right panels). In order to ensure that the transfection efficiencies were equal, the cytoplasmic extracts were immunoblotted with a 15-LOX-1 antibody (Fig. 2C). The 15-LOX-1 expression was found to be slightly higher for the HCT-116 cell line, which may have been responsible for the differences in signal observed in the mobility shift for the two cell lines.

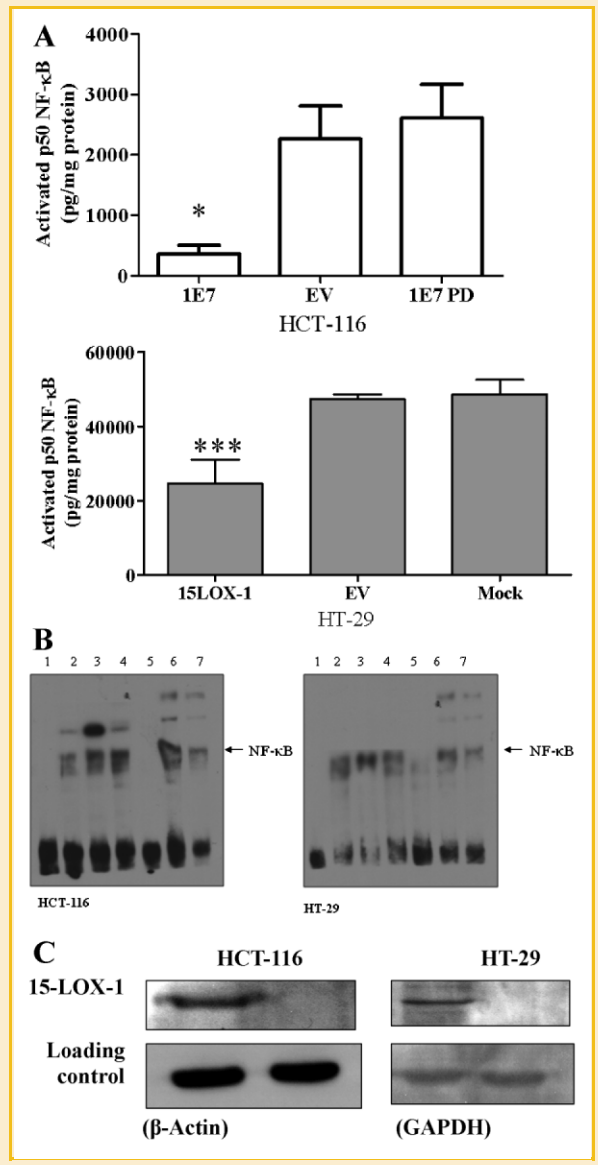

Fig. 2. 15-LOX-1 expression reduces NF-κB DNA binding. A: Nuclear extracts were applied on the NF-κB (human p50) Transcription Factor Assay Kit. HCT-116 and HT-29 expressing 15-LOX-1 stably or transiently respectively showed significantly reduced p50 DNA binding when compared to control EV cells, or when the 15-LOX-1 expressing cells were treated with PD146176. B: Reduced DNA binding in nuclear extracts was also observed by EMSA in HCT-116 (left panel) and HT-29 (right panel) expressing 15-LOX-1. Lane 1: free probe, lane 2: 15-LOX-1 expressing cells, lane 3: 15-LOX-1 expressing cells treated with PD146176, lane 4: empty vector transfected cells, lane 5: reaction mixture incubated with 200-fold excess cold probe, lane 6: supershift with p65 antibody, lane 7: supershift with p50 antibody. C: An immunoblot of the cytoplasmic extracts for 15-LOX-1 indicating the expression of 15-LOX-1 in both the cell lines.

15-LOX-1 EXPRESSION REDUCES THE TRANSCRIPTIONAL ACTIVITY OF NF-κB

We next determined the transcriptional activity of NF-κB in the 15-LOX-1 expressing cells. For this purpose, we transfected the 15-LOX-1 expressing and control cells with the PathDetect *cis*-Reporting system (Stratagene Agilent), collected the cells and assayed them for luciferase activity. This assay works on the principle of the transcription of a luciferase reporter gene in the presence of a TATA box promoter and an NF-κB *cis*-enhancer element. Our data (Fig. 3) indicate that two monoclones of 15-LOX-1 expressing HCT-116 cells (1E7 and 1F4) could significantly reduce the transcriptional activity of NF-κB when compared to control EV-transfected cells ($^{**}P < 0.01$ for 1E7 compared to control cells (EV) and $^{***}P < 0.001$ for 1F4 compared to EV). Additionally, this inhibitory effect of 15-LOX-1 on NF-κB transcriptional activity was observed even when the cells were treated with TNFα for 6 h in order to activate NF-κB ($^{**}P < 0.01$ for 1E7 compared to EV and

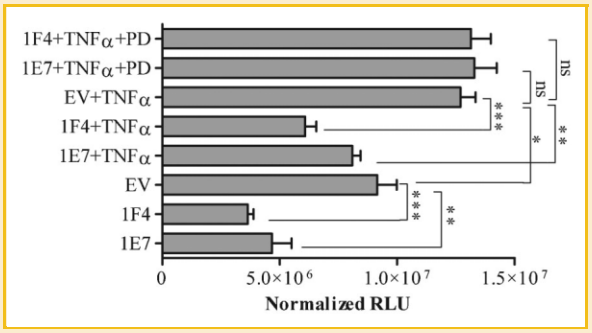

Fig. 3. Luciferase assays indicate reduced NF-κB transcriptional activity in 15-LOX-1 expressing cells. Two monoclones of HCT-116 cells (1E7 and 1F4) stably expressing 15-LOX-1 was transfected with the NF-κB Pathdetect *cis*-Reporting plasmid and the cells were collected 24 h later. Significantly reduced NF-κB transcriptional activity was observed in 15-LOX-1 expressing cells compared to the control cells (EV). This inhibition persisted even when the NF-κB pathway in the cells was activated with treatment with TNFα for 6 h. Treatment of the cells with 1 μM PD146176 could reverse this inhibition and the transcriptional activity could reach the levels similar to the control (EV) cells. β-Galactosidase was used for normalization. Statistical comparisons were carried out using one-way ANOVA with Tukey's multiple comparison test.

$^{***}P < 0.001$ for 1F4 compared to EV). Moreover, when the cells were treated with 1 μM PD146176, the transcriptional activity of NF-κB in 1E7 and 1F4 cells returned to the level observed with the EV-transfected control cells ($P > 0.05$ for both comparisons), thereby further attesting the role of 15-LOX-1 in inhibiting the transcriptional activity of NF-κB.

13(S)-HODE IS A PPARγ LIGAND AND INCREASES ITS TRANSCRIPTIONAL ACTIVITY

We then wanted to determine the mechanism of NF-κB inhibition by 15-LOX-1. PPARγ is known to have antineoplastic effects in CRC, one of which is by the inhibition of NF-κB [Straus and Glass, 2007]. In order to ascertain whether PPARγ was activated in 15-LOX-1 expressing cells, we wanted to first confirm whether 13(S)-HODE was a ligand for PPARγ. To that purpose we transfected 15-LOX-1 expressing cells with a PPARγ1LBD-GAL4DBD construct along with a UAS_G-4xTK-Luc construct. Our data (Fig. 4A, left panel) indicate that both 1E7 and 1F4 had significantly ($^{***}P < 0.001$) higher luciferase activity compared to the EV-transfected cells, indicating that the endogenous 13(S)-HODE produced in these cell lines (owing to the forced expression of 15-LOX-1) could act as a ligand for

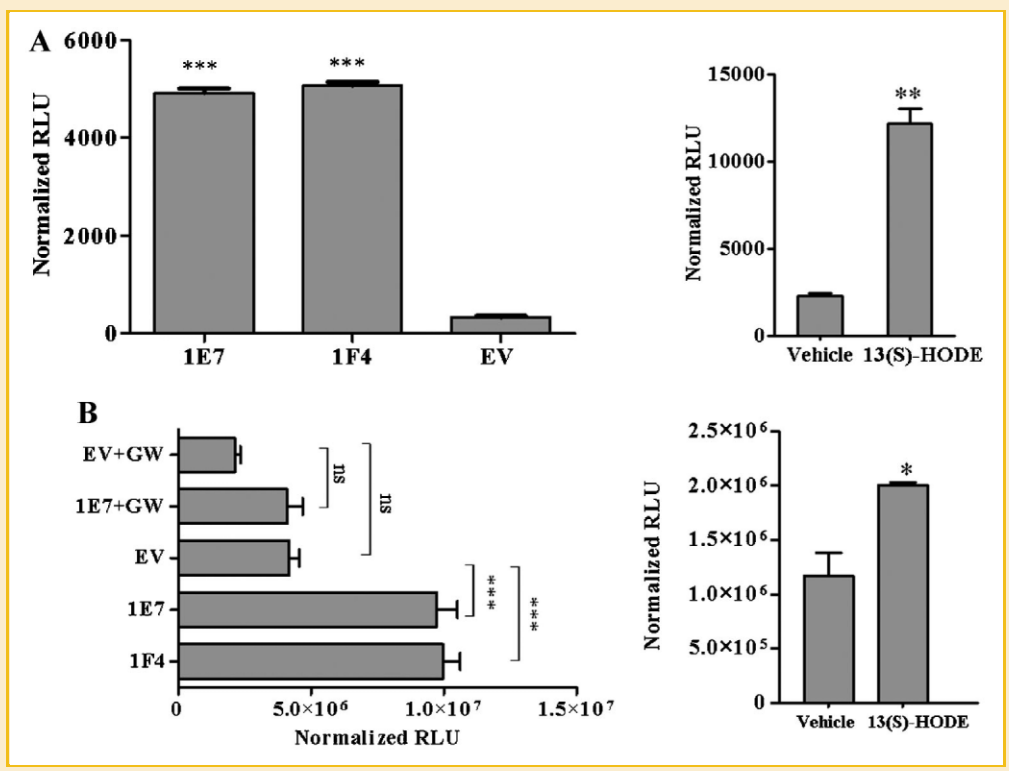

Fig. 4. PPARγ is activated in the 15-LOX-1 expressing cells. A: The 15-LOX-1 expressing cells (1E7 and 1F4) were transfected with the PPARγ1LBD-GAL4DBD and UAS_G-4xTK-Luc constructs. In the presence of a ligand, the PPARγ1LBD-GAL4DBD binds to the UAS_G-4xTK-Luc and drives the expression of luciferase. 15-LOX-1 expressing cells, with higher endogenous levels of the enzymatic product 13(S)-HODE, showed significantly higher luciferase activity compared to the control cells indicating the 13(S)-HODE acts as a ligand for PPARγ (left panel). Parental HCT-116 cells transfected with the PPARγ1LBD-GAL4DBD and UAS_G-4xTK-Luc constructs and treated with 100 μM 13(S)-HODE showed an increase in the luciferase activity, further confirming that 13(S)-HODE acts as a ligand for PPARγ (right panel). B: The 15-LOX-1 expressing cells showed higher PPARγ transcriptional activity compared to the control cells. The HCT-116 cells were transfected with a PPRE-3xTK-Luc construct. 1E7 and 1F4 cells showed significantly increased transcriptional activity of PPARγ, likely resulting from the increased levels of the PPARγ ligand 13(S)-HODE in these cells compared to the control (EV) cells. Treatment of the cells with 1 μM of the PPARγ specific antagonist GW9662 (GW) could reverse the increase in luciferase activity (left panel). Parental HCT-116 cells treated with 100 μM 13(S)-HODE and transfected with the PPRE-3xTK-Luc showed a significant increase in the PPARγ transcriptional activity when compared to vehicle-treated cells (right panel). β-Galactosidase was used for normalization. Statistical comparisons were carried out using one-way ANOVA with Tukey's multiple comparison test. The 13(S)-HODE treatment data were analyzed for statistical significance using Mann–Whitney *U*-test.

PPARγ. Additionally, when 13(S)-HODE (100 μM) was exogenously added to parental HCT-116 cells for 24 h after transfection with the reporter constructs, it also resulted in a significant increase ($^{**}P < 0.01$) in the luciferase signals when compared to cells treated with ethanol (vehicle) alone, further confirming that 13(S)-HODE could act as a ligand for PPARγ (Fig. 4A, right panel).

Next, the PPARγ transcriptional activity was determined by transfecting the 15-LOX-1 expressing and control cells with the PPRE-3xTK-Luc construct which has three copies of the PPARγ response element (PPRE) upstream of a TK driven luciferase reporter gene. Our data (Fig. 4B, left panel) indicate that both 1E7 and 1F4 15-LOX-1 expressing cells could increase the transcriptional activity of PPARγ when compared to the EV-transfected cells. This increase in activity is most likely due to the production of 13(S)-HODE in the 15-LOX-1 expressing cell lines and this endogenous 13(S)-HODE could serve as a ligand for the activation of PPARγ. When the 1E7 cells were incubated with the PPARγ antagonist GW9662, the PPARγ transcriptional activity was reduced and reached levels similar to the EV-transfected cells treated with GW9662 ($P > 0.05$). Additionally, the decrease in PPARγ activity in the GW9662-treated EV-transfected cells was found to be statistically not significant when compared to the untreated EV cells ($P > 0.05$). Parental HCT-116 treated with 100 μM of exogenously added 13(S)-HODE for 24 h after transfection with the PPRE-3xTK-Luc construct also showed a significant increase ($^{*}P < 0.05$) in the transcriptional activity of PPARγ (Fig. 4B, right panel) when compared to vehicle-treated cells.

PPARγ INHIBITION ACTIVATES NF-κB NUCLEAR TRANSLOCATION IN 15-LOX-1 EXPRESSING CELLS

Previous studies have indicated that PPARγ activation is associated with an inhibition of NF-κB activity in the colon [Chen et al., 2003; Wahli, 2008]. Our data indicated that cells with forced expression of 15-LOX-1 showed an inhibition of NF-κB nuclear translocation, DNA binding and transcriptional activity, along with a transcriptional activation of PPARγ. We therefore wanted to examine if the two events were related. We first wanted to determine whether PPARγ inhibition by the antagonist GW9662 could rescue the loss of phosphorylation of IκBα when 15-LOX-1 is expressed. Western blot (Fig. 5A) showed that treatment of the 1E7 cells with GW9662 resulted in a recovery of phosphorylation IκBα (lane 2) when compared to untreated 1E7 cells (lane 1). No such change was observed when the EV-transfected cells were treated with GW9662 (lane 3 vs. lane 4). Next, in order to determine whether the inhibition in nuclear translocation of p65 observed by Western blot (Fig. 1) in the 15-LOX-1 expressing cells was due to PPARγ activation, we incubated the HCT-116 cells with the PPARγ specific antagonist GW9662 (1 μM) for 24 h and then collected the cells for immunofluorescence studies. The images were analyzed by Image J to obtain nuclear AF488/PI pixel intensity ratio in order to quantify the NF-κB nuclear translocation. Our data confirm that 15-LOX-1 expression in both 1E7 (Fig. 5B,C) and 1F4 (Supplementary Fig. S1A,B) HCT-116 cells could significantly decrease nuclear translocation of both p50 and p65 when compared to EV-transfected cells ($^{*}P < 0.05$ for all comparisons). This inhibition is most likely due to endogenous production of 13(S)-HODE in the 15-LOX-1 expressing cells, since immunofluorescence studies of parental HCT-116 cells treated with 100 μM exogenously added 13(S)-HODE resulted in a significant decrease ($^{*}P < 0.05$) in nuclear translocation of p50 and p65 when compared to cells treated with ethanol only (Supplementary data Fig. S2C,D). Moreover, the inhibition in nuclear translocation of NF-κB in 15-LOX-1 expressing cells could be rescued by the incubation of the cells with 1 μM of the 15-LOX-1-specific inhibitor PD146176. Additionally, when the cells were treated with 1 μM of GW9662, a similar reversal of the inhibition in translocation of both p50 and p65 could be observed, indicating that attenuation of PPARγ activity in the 15-LOX-1 expressing cells could rescue the inhibition of NF-κB.

PPARγ INHIBITION LEADS TO NF-κB DNA BINDING IN 15-LOX-1 EXPRESSING CELLS

In order to determine whether PPARγ is involved in the inhibition of NF-κB DNA binding in the 15-LOX-1 expressing cells, we conducted EMSA to determine nuclear DNA binding. Our data (Fig. 6) indicate that pretreatment of the cells with 1 μM of the PPARγ antagonist GW9662 (lane 3) could revert the inhibition in DNA binding observed with the expression of 15-LOX-1 (Figs. 2B and 6, lane 1).

PPARγ INHIBITION ENHANCES TRANSCRIPTIONAL ACTIVITY OF NF-κB IN 15-LOX-1 EXPRESSING CELLS

The transcriptional activity of NF-κB was next determined in 15-LOX-1 expressing and control cells in the presence or not of 1 μM of the PPARγ antagonist GW9662 using the Pathdetect NF-κB *cis*-Reporting system. Our data (Fig. 7, left panel) indicate that treatment of the 15-LOX-1 expressing 1F4 cells with GW9662 resulted in a significant ($^{*}P < 0.05$) increase in the NF-κB transcriptional activity when compared to untreated 1F4 cells. However, no such increase in transcriptional activity was observed when the EV-transfected cells were treated with GW9662 ($P > 0.05$). Additionally, the NF-κB transcriptional activity in GW9662-treated 1F4 cells reached the same level as the EV cells ($P > 0.05$). Treatment of parental HCT-116 with 100 μM 13(S)-HODE for 24 h after transfection with the reporter construct also resulted in a significant loss of NF-κB transcriptional activity ($^{**}P < 0.01$) when compared to cells treated with the vehicle ethanol (Fig. 7, right panel). Moreover, when the HCT-116 cells were treated with 1 μM GW9662 for 24 h following the treatment with 13(S)-HODE, a recovery of the NF-κB transcriptional activity was observed.

PPARγ AND ERK1/2 ARE PHOSPHORYLATED IN 15-LOX-1 EXPRESSING CELLS

Previous reports have indicated that treatment of HCT-116 CRC cells and PC3 prostate cancer cells with 13(S)-HODE could increase PPARγ phosphorylation and that this phosphorylation was mediated by extracellular signal regulated kinase (ERK) 1/2 [Hsi et al., 2001, 2002]. Additionally, phosphorylated PPARγ has been shown to inhibit NF-κB [Chen et al., 2003]. We therefore examined the phosphorylation status of PPARγ and ERK1/2 in HCT-116 cells stably expressing 15-LOX-1. As the HCT-116 cell line has a mutation in *Ras*, the MAPK pathway is constitutively active in these cells. Western blot using an antibody against phosphorylated ERK1/2 indicated increased phosphorylation in cells that express 15-LOX-

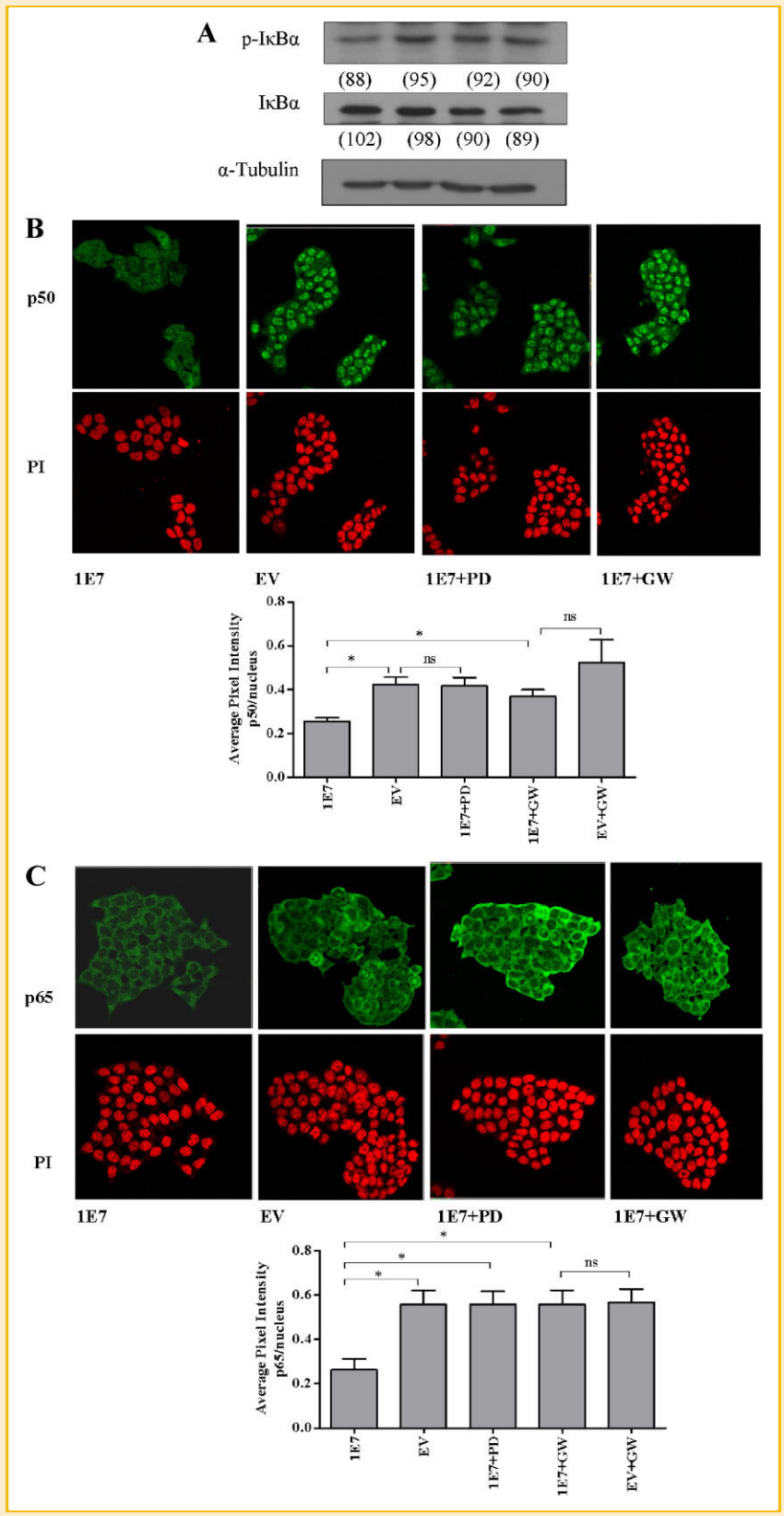

Fig. 5. Inhibition of NF-κB in 15-LOX-1 expressing cells could be reversed by treatment with the PPARγ-specific antagonist GW9662 (GW). A: 15-LOX-1 expressing (1E7, lane 1), control cells (EV, lane 2), 1E7 cells (lane 3) and EV cells (lane 4) treated with 1 μM GW were probed for phosphorylated and total IκBα using Western blot. For immunofluorescence assays, the cells were incubated with either a p50 (B) or p65 (C) antibody and counterstained with an Alexa Fluor 488 secondary antibody. Propidium iodide (PI) was used to visualize the cell nucleus. The fluorescence intensity of p65 (B) or p50 (C) staining was quantified by AF488/PI pixel intensity ratio. A significantly reduced nuclear translocation of NF-κB was observed in 15-LOX-1 expressing 1E7 cells when compared to the control cells (EV). This inhibition could be reversed by the treatment of 1E7 cells with 1 μM of either the 15-LOX-1 inhibitor PD146176 (PD) or GW9662 (GW). Statistical comparisons were carried out using Mann–Whitney *U*-test.

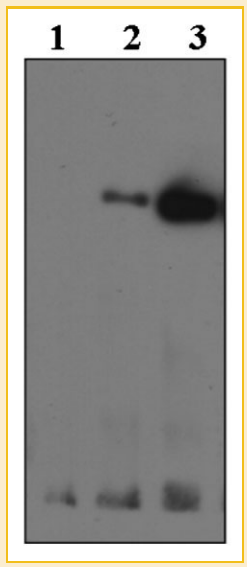

Fig. 6. EMSA indicating increased NF-κB DNA binding when nuclear extracts from the 15-LOX-1 expressing cells were treated with 1 μM of the PPARγ-specific antagonist GW9662. Lane 1: Free probe, lane 2: 15-LOX-1 expressing 1E7 cells, lane 3: 1E7 cells treated with 1 μM GW9662.

1 (1E7, lane 1) compared to EV-transfected cells (Fig. 8, lane 2). This phosphorylation was decreased when the 1E7 cells were treated with the 15-LOX-1-specific inhibitor PD146176 (lane 3). Additionally, treatment of 1E7 cells with the ERK1/2 inhibitor U0126 decreased the phosphorylation of ERK1/2. When the proteins were probed with an antibody against p-PPARγ, the phosphorylation of PPARγ was seen to be higher in the 15-LOX-1 expressing 1E7 cells (lane 1) when compared to the EV-transfected cells (lane 2). Treatment with both PD146176 and U0126 could reduce the phosphorylation of PPARγ, indicating that the phosphorylation was a specific effect of 15-LOX-1 expression and that it was via the kinase activity of ERK1/2. The levels of total PPARγ were stable in 15-LOX-1 expressing and control cells. Interestingly, the levels of total ERK1/2 were seen to be lower in the 15-LOX-1 expressing cells when compared to the EV-transfected cells. Thus, although overall ERK1/2 levels are seen to decrease in 15-LOX-1 expressing cells, this ERK1/2 appears to be more active, with PPARγ as one of its targets.

INHIBITION OF PPARγ INCREASES CELLULAR PROLIFERATION

In order to determine whether the inhibition of NF-κB via activation of PPARγ was reflected functionally in the cells, we treated HCT-116 cells stably transfected with the 15-LOX-1 construct with the PPARγ antagonist GW9662 (1 μM). Our data (Fig. 9) show that inhibition of PPARγ in the 15-LOX-1 expressing cells could increase the proliferation of the cells significantly ($^{***}P < 0.001$) to a level similar to the control EV-transfected cells. Treatment of the EV cells with GW9662 (EV + GW) did not change their proliferation, most likely because PPARγ is not activated in these cells ($P > 0.05$). On the other hand, treatment of the EV cells with 32 μM of SN-50 (EV + SN-50), specific peptide inhibitor of NF-κB, resulted in a dramatic reduction of proliferation. As NF-κB activation provides mitogenic signals, inhibition of NF-κB is likely to cause a growth arrest, as is also seen when the cells express 15-LOX-1. The growth inhibition with SN-50, however, appears to be more pronounced than that with 15-LOX-1 expression.

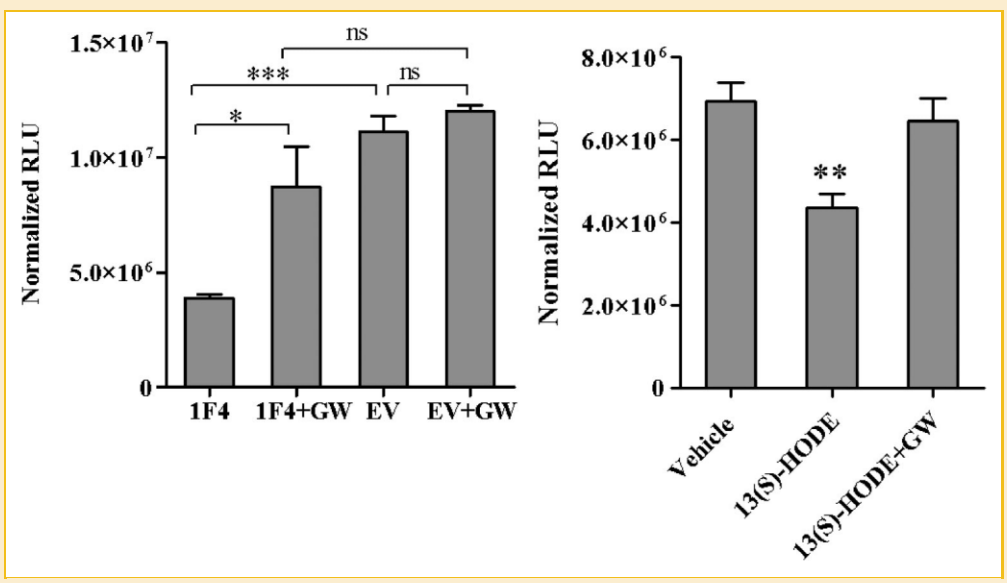

Fig. 7. Luciferase assays indicating increased transcriptional activity of NF-κB when the 15-LOX-1 expressing HCT-116 cells (1F4, left panel) or wild-type HCT-116 cells incubated with 100 μM 13(S)-HODE (right panel) were treated with the PPARγ-specific antagonist GW9662 (GW). The cells were transfected with the NF-κB Pathdetect *cis*-Reporting plasmid for 24 h, treated with 100 μM 13(S)-HODE for 24 h (right panel only) and 1 μM GW9662 for 24 h after which the cells were collected and processed for luciferase activity. β-Galactosidase was used for normalization. Statistical comparisons were carried out using one-way ANOVA with Tukey's multiple comparison test. The 13(S)-HODE treatment data were analyzed for statistical significance using Mann–Whitney *U*-test.

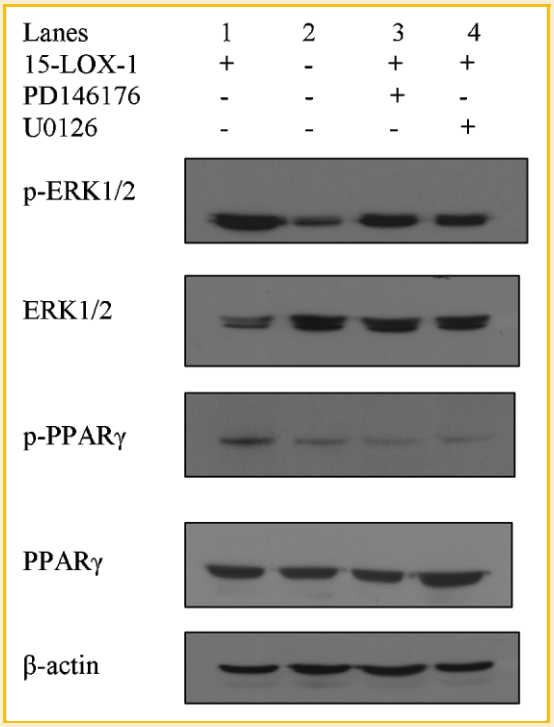

Fig. 8. 15-LOX-1 expression results in phosphorylation of ERK1/2 and PPARγ. Whole cell extracts were probed with the ERK1/2, p-ERK1/2, PPARγ, and p-PPARγ antibodies. Increased phosphorylation of ERK1/2 and PPARγ was observed in 15-LOX-1 expressing cells (lane 1) when compared to the control empty vector transfected cells (lane 2). Treatment of 15-LOX-1 expressing cells with PD146176 (1 μM, lane 3) or with the ERK1/2 inhibitor U0126 (10 μM, lane 4) could reverse the phosphorylation of both proteins. Total ERK1/2 was seen to decrease in the 15-LOX-1 expressing cells (lane 1) when compared to the control cells (lane 2). No change in total PPARγ expression was observed with 15-LOX-1 expression.

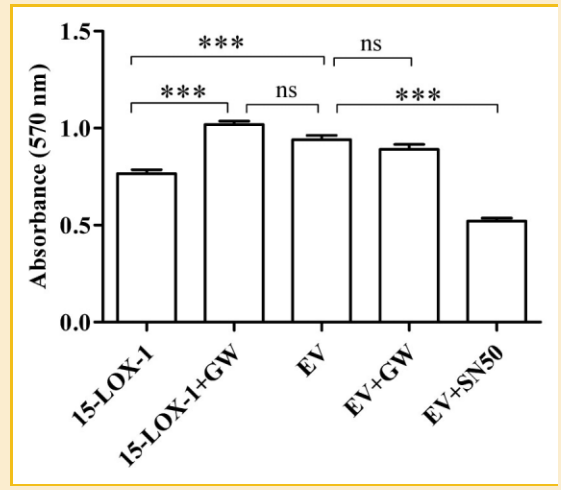

Fig. 9. The proliferation of 15-LOX-1 expressing cells increases when PPARγ is inhibited. MTT assay indicates that 15-LOX-1 expressing 1E7 cells, but not the empty vector-transfected control cells, treated with 1 μM GW9662 results in a significant increase in the proliferation. Treatment of control cells with 32 μM SN-50, a specific peptide inhibitor for NF-κB resulted in a decrease in proliferation. This decrease, however, was more pronounced than the inhibition in proliferation observed with the expression of 15-LOX-1 alone. The data for eight replicates is shown here and statistical comparisons were carried out using one-way ANOVA with Tukey's multiple comparison test.

DISCUSSION

15-Lipoxygenase-1 has been convincingly shown to have tumor suppressive properties in the colon in the recent years [reviewed by Bhattacharya et al., 2009]. We and others have shown that expression of this protein reduces proliferation, increases apoptosis, and reduces cellular motility in colon cancer cell lines [Shureiqi et al., 1999; Zhu et al., 2008; Cimen et al., 2009]. Although several NF-κB target genes such as IL-1β, TNFα, etc. are implicated in pro-inflammatory effects, the transcription factor also regulates cell proliferation, apoptosis, and cell migration. We therefore hypothesized that the antineoplastic effects of 15-LOX-1 in the colon may manifest itself via the inhibition of NF-κB.

NF-κB transcription is dependent on several factors such as phosphorylation status of IκB, nuclear translocation of the NF-κB subunits and DNA binding [Mayo and Baldwin, 2000]. In cancer cells, NF-κB can be constitutively active, resulting in the activation of inflammatory and anti-apoptotic pathways. Several pro-inflammatory and pro-carcinogenic lipoxygenases such as 5-LO and 12-LO have been shown to activate NF-κB [Bonizzi et al., 1999; Kandouz et al., 2003]. However, the effect of the 15-LOX-1 pathway on NF-κB activity is not known. A recent report by Shureiqi et al. [2010] indicated that 15-LOX-1 expression in LoVo CRC cells resulted in a decrease of the expression of the NF-κB target gene IL-1β. In T cells, 13-HODE, but not 15-HETE, could effectively reduce DNA binding and transcriptional activity of NF-κB as shown by EMSA and luciferase assays, respectively [Yang et al., 2002]. Additionally, Kandouz et al. [2003] reported that 13(S)-HODE treatment of PC-3 cells could effectively inhibit NF-κB DNA binding.

In this study, we have introduced 15-LOX-1 in HCT-116 and HT-29 colon cancer cell lines and examined its effect on NF-κB activity. We have shown here for the first time that the expression of 15-LOX-1 inhibited the nuclear translocation of NF-κB by inhibiting the phosphorylation and degradation of IκBα. A similar loss in nuclear translocation of NF-κB was observed when the cells were treated with exogenous 13(S)-HODE. Additionally, this loss of nuclear translocation was accompanied by an inhibition of the DNA binding ability of p50 and p65. Moreover, luciferase assays indicated that 15-LOX-1 expression could inhibit the transcriptional activity of NF-κB even when the cells were activated with TNFα. All these effects were rescued when the cells were preincubated with the 15-LOX-1 inhibitor PD146176, indicating the specificity of the inhibition.

We next wanted to determine a possible mechanism by which 15-LOX-1 caused the inhibition of NF-κB. One of the characteristics of oxidized lipids such as 13(S)-HODE is their ability to act as ligands for nuclear receptors such as PPARγ [Itoh et al., 2008]. PPARγ has characteristics of a colon cancer tumor suppressor provided the *APC* gene is of wild-type [Sarraf et al., 1999]. Using luciferase reporter gene assays, we have shown that 13(S)-HODE

produced endogenously, or added exogenously, could act as a ligand and increase the transcriptional activity of PPARγ in HCT-116 cells with a wild-type *APC* gene. 15-LOX-1 expression in HCT-116 cells, however, did not change the mRNA (data not shown) or total protein expression of PPARγ (Fig. 8).

Having confirmed that the 15-LOX-1 expressing cells had an activated PPARγ, we proceeded to examine whether the inhibition of NF-κB observed in these cells was via PPARγ. Inhibition of PPARγ with the antagonist GW9662 resulted in a rescue of the phosphorylation of IκBα, as well as recovery of the loss of nuclear translocation, DNA binding and transcriptional activity of NF-κB in 15-LOX-1 expressing cells. Several cell-specific mechanisms have been proposed for the inhibition of NF-κB by PPARγ, including suppression of IκB kinase activity in pancreatic acinar AR42J cells [Wan et al., 2008], inhibition of nuclear translocation and DNA binding of p65 in rat pancreatic beta cells [Kim et al., 2007], as well as transrepression of NF-κB transcriptional activity by SUMOylated PPARγ [Pascual et al., 2005]. A recent study has indicated that in rat glomerular mesangial cells, unliganded PPARγ was found to physically associate with p65 and was necessary for the transcriptional activity of NF-κB. In the presence of 15-PGJ_2, a natural agonist of PPARγ, this physical association was disrupted, leading to a loss of NF-κB activity [Wen et al., 2010].

Previous reports indicated that 15-LOX-1 expression resulted in phosphorylation of ERK1/2 and PPARγ, thereby assigning a pro-tumorigenic role to 15-LOX-1 [Hsi et al., 2001; Yoshinaga et al., 2004]. It was also claimed that the phosphorylated PPARγ was transcriptionally less active based on previous reports in the literature; however, no PPARγ transcriptional activity assays were reported in that study [Hsi et al., 2001]. We have confirmed in this study that 15-LOX-1 expression could indeed lead to the phosphorylation of ERK1/2 which could in turn phosphorylate PPARγ, although the overall levels of total ERK1/2 were low (Fig. 8). However, we have observed that two monoclones of HCT-116 cells stably expressing 15-LOX-1 show a higher transcriptional activity of PPARγ (Fig. 4B) when compared to EV-transfected cells. Additionally, it has been shown that PPARγ, phosphorylated via ERK1/2, can physically associate with p65 and inhibit its transcriptional activity [Chen et al., 2003]. We therefore propose that while 15-LOX-1 expression can enhance the transcriptional activation of PPARγ; it can also activate the MAPK pathway leading to the phosphorylation of PPARγ. This phosphorylated form of PPARγ may associate with p65 and inhibit NF-κB activity. We believe that this work has, at least in part, addressed the controversy regarding the alleged pro-tumorigenic nature of 15-LOX-1 with regard to MAPK activation and PPARγ phosphorylation.

Finally, we also established that the inhibition of NF-κB via the activation of PPARγ was functionally reflected in the proliferation of the cells. Inhibition of PPARγ with GW9662 in the 15-LOX-1 expressing cells, but not control cells, resulted in a significant increase in the proliferation to levels similar to the control cells. Additionally, treatment of the control cells with SN-50, a specific peptide inhibitor of NF-κB, resulted in a significant decrease in proliferation (EV + SN-50). This decrease, however, was more pronounced than the decrease in proliferation observed when the cells were forced to express 15-LOX-1. This may be because SN-50 is a direct inhibitor of NF-κB which acts by antagonizing the translocation of p65 to the nucleus. 15-LOX-1, on the other hand, is an indirect inhibitor of NF-κB, acting via PPARγ.

Taken together, we have shown in this study that: (i) 15-LOX-1 expression increases the cytoplasmic levels of IκBα and decreases its phosphorylation and degradation; (ii) cells expressing 15-LOX-1 show decreased nuclear translocation of p65 and p50, which can be reversed by incubation with the 15-LOX-1-specific inhibitor PD146176 and the PPARγ antagonist GW9662; (iii) 15-LOX-1 expression results in decreased binding of NF-κB subunits p50 and p65 to their consensus DNA-binding sequences and decreased NF-κB transcriptional activity, both of which could be reversed by GW9662; (iv) cells expressing 15-LOX-1 show increased phosphorylation of PPARγ via ERK1/2. Based on previous reports, this phosphorylated PPARγ may associate with p65 and inhibit NF-κB. (v) Functionally, the inhibition of PPARγ in the 15-LOX-1 expressing cells, but not the control cells, resulted in a significant increase in cellular proliferation. These properties of 15-LOX-1 further emphasize the importance of this protein as a possible therapeutic option in colorectal carcinogenesis.

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