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Peroxisome Proliferator-Activated Receptor Alpha Agonists Inhibit Cyclooxygenase 2 and Vascular Endothelial Growth Factor Transcriptional Activation in Human Colorectal Carcinoma Cells Via Inhibition of Activator Protein 1

Raquel Grau, Carmen Punzón, Manuel Fresno and Miguel A. Iñiguez
Centro de Biología Molecular "Severo Ochoa", Departamento de Biología Molecular. Universidad Autónoma de Madrid, 28049 Madrid, Spain.

Running title: Regulation of COX-2 and VEGF expression by PPARα

Address all correspondence and requests for reprints to: Miguel A. Iñiguez, Centro de Biología Molecular "Severo Ochoa". Departamento de Biología Molecular. Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain, Tel: +34-914978410; Fax: +34-914974799; E-mail: Mainiguez@cbm.uam.es

Abbreviations used are: LY, LY-171883; WY, WY-14,643; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; AP-1, activating protein-1; COX, Cyclooxygenase; GAPDH, glyceraldehide 3- phosphate dehydrogenase; IL, interleukin; Ion, A23187 calcium ionophore; JNK, c-Jun N-terminal kinase; LUC, luciferase; NF-κB, nuclear factor κB; C/EBP, CCAAT/enhancer binding protein; PMA, Phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome activated response element; VEGF, vascular endothelial growth factor.

SYNOPSIS

Recent evidence indicates that peroxisome proliferator activated receptor (PPAR) α ligands possess anti-inflammatory and anti-tumoral properties due to their inhibitory effects on the expression of genes involved in the inflammatory response. However, the precise molecular mechanisms underlying these effects are poorly understood. Here, we show that tumor promoter Phorbol 12-myristate 13-acetate (PMA) –mediated induction of genes that are significantly associated to inflammation, tumor growth and metastasis as Cyclooxygenase (COX)-2 and Vascular Endothelial Growth Factor (VEGF) is inhibited by PPARα ligands in the human colorectal carcinoma cell line SW620. PPARα activators LY-171883 and WY-14,643 were able to diminish transcriptional induction of COX-2 and VEGF by inhibiting AP-1 –mediated transcriptional activation induced by PMA or by c-Jun overexpression. The actions of these ligands on AP-1 activation and COX-2 and VEGF transcriptional induction were found to be dependent of PPARα expression. Our studies demonstrate the existence of a negative cross-talk between the PPARα and AP-1 dependent signaling pathways in these cells. PPARα interfered with at least two steps within the pathway leading to AP-1 activation. First, PPARα activation impaired AP-1 binding to a consensus DNA sequence. Second, PPARα ligands inhibited c-Jun transactivating activity. Taken together, these findings provide new insight into the anti-inflammatory and anti-tumoral properties of PPARα activation, through the inhibition of the induction of AP-1 –dependent genes involved in inflammation and tumor progression.

Key words: PPARα, COX-2, VEGF, SW620, LY-171883, WY-14,643

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor family of transcription factors, a diverse group of proteins that mediate ligand-dependent transcriptional activation and repression. They modulate gene transcription in response to specific ligands by binding as heterodimers with the retinoid X receptor (RXR) to specific peroxisome proliferator-response element (PPREs) on target genes (reviewed in [1]). So far, three distinct forms of PPARs have been described named PPARα, β (also called δ) and γ, each encoded by a different gene and showing a distinct tissue distribution [2]. In humans, PPARα is expressed in intestine, skeletal muscle, liver, kidney, adipose tissue and vascular endothelial cells [3, 4]. Several peroxisome proliferators have been shown to bind PPARα and to regulate transcriptional activity of target genes. These include the fibrate class of hypolipidemic drugs, non steroidal anti-inflammatory drugs (NSAIDs), fatty acids and eicosanoids (reviewed in [5]).

Interest in PPARs has increased dramatically since they were found to be involved in the regulation of processes as diverse as lipid and glucose metabolism, cell growth and inflammation. Therefore, in addition to their well known effects in diabetes, pharmacological agents that target PPARs may have therapeutic applications in cancer and inflammatory diseases [6, 7]. In this sense, several reports have shown the potentially beneficial chemopreventive effect of PPARα ligands in colon carcinogenesis [8-11]. Many of the anti-inflammatory and anti-neoplastic properties of PPAR ligands are due to their inhibitory effects on gene transcription [6, 12]. PPARα agonists are involved in the transcriptional repression of a variety of inflammatory genes [13-15]. These effects seem to be mediated by the inhibition of various transcription factors such as nuclear factor (NF)-κB, activator protein–1 (AP-1) [16] and Sp-1 [17].

On the other hand, a growing body of evidence has lighted the contribution COX-2 and VEGF genes in inflammation, tumor growth and angiogenesis (reviewed in [18, 19]). Cyclooxygenases, COX-1 and COX-2, catalyze the conversion of arachidonic acid (AA) to PGH_2, the key step in the biosynthesis of prostanoids. COX-1 is constitutively expressed in most tissues, whereas COX-2 expression is induced by cytokines, mitogens, and tumor promoters in a discrete number of cell types (reviewed in [20]). COX-2 is aberrantly overexpressed in many human cancers, most notably of colon origin [21], being considered to play an essential role in cancer progression, especially in colon carcinoma. Multiple studies have revealed a role of selective COX-2 inhibitors in decreasing the risk of developing colon cancer and in suppressing

tumor formation and growth in animal models [18, 22]. Besides, accumulating evidence supports a key role of VEGF in cancer, contributing to tumor neovascularization and dissemination. Increased expression of this factor has been found in most tumors and blockade of VEGF expression or activity ameliorate tumor growth in vivo (reviewed in [23, 24]).

Here, we have tested the effect of two structurally different PPARα activators as the hypolipidemic drug WY-14,643 and the leukotriene D4 antagonist LY-171883 in the regulation of COX-2 and VEGF gene expression in the colon carcinoma cell line SW620. Our results show that PPARα activators specifically suppressed transcriptional induction of COX-2 and VEGF by phorbol esters. These drugs inhibited up-regulation of COX-2 and VEGF by inhibiting AP-1 mediated transcriptional activation through a negative cross-talk between PPARα and AP-1 transcription factors

EXPERIMENTAL

Reagents

Opti-MEM, RPMI, glutamine and antibiotics were from Invitrogen. Fetal bovine serum (FBS) was purchased from Euroclone. Phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (Ion) were from Sigma (St. Louis, MO). The PPARα agonists LY-171883 and WY-14,643, the cyclopentenone prostaglandin 15d-$\Delta^{12,14}PGJ_2$ and the monoclonal COX-2 antibody were from Cayman Chemical. Horseradish peroxidase-coupled anti-mouse antibodies and the ECL detection system were from Pierce. Oligonucleotides were synthesized by Invitrogen. [^{32}P] ATP for radioactive labeling was from Amersham Biosciences. Reagents for DNA transfection and luciferase assays were from Promega. The most commonly used chemicals were from Sigma and Merck.

Cell Culture

The human colon carcinoma cell line SW620 was grown in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics. The human colon carcinoma cell line Caco-2 was grown in MEM culture medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non essential amino acids and antibiotics. The COS-7 cell line was cultured in standard conditions in DMEM culture medium. Cells were grown and maintained at 37ºC in a humidified atmosphere containing 5% CO_2 up to 70 % confluence and trypsinized with 0.25 % trypsin, 2 mM EDTA for experimental use. Cells were changed to medium with 0.5 % FBS prior to treatment with pharmacological reagents. No evidence of significant toxicity was observed at the doses used in any of our experiments as determined by the WST-1 cell viability assay.

Plasmid Constructs

The COX-2 promoter construct COX-2-LUC contains the -1796 to +104 region of the human COX-2 gene in pXP2LUC plasmid. The 431-κB-COX-2-LUC mutant was generated by site-directed mutagenesis using the oligonucleotide 5´-GACAGGAGAGTGGtacCTACCCCCTCTGCTCCC-3´ (nucleotides -236 to -204 of the human COX-2 gene containing the NF-κB site as described previously [25]. The VEGF-LUC plasmid contains the region -1910 to +379 of the human VEGF promoter [26]. The -73Col-LUC plasmid

includes the AP-1 dependent region (73/+63bp) of the human collagenase promoter fused to the luciferase gene [27]. The NF-κB-Luc reporter plasmid contains a three tandem repeat of the NF-κB-binding motif of the H-2k gene upstream of the thymidine kinase minimal promoter [28]. The expression plasmid encoding PPARα was a generous gift of Dr. B Staels. [13]. PPARγ expression vector and those containing the transactivation ligand binding domains of PPARα or PPARγ fused to the GAL4 DBD (pCMX-Gal-L-mPPARα) were provided by Dr. R. M. Evans [29]. The reporter plasmid PPRE-LUC containing three copies of the peroxisome-proliferator response element (PPRE) of the acyl-CoA oxidase (ACO) was a generous gift from Dr. B. Belandia. The expression plasmid encoding human p65 was a generous gift of Dr. J. Alcamí. The expression plasmid pRSV-c-Jun has been previously described [30]. The GAL4-c-Jun plasmid expressing the first 166 amino acids of the human c-Jun fused to the DNA binding domain of the yeast GAL4 transcription factor (amino acids 1-147), was obtained from Dr. P. Angel. The GAL4-luciferase reporter plasmid contains five copies of GAL4 DNA binding sites fused to the luciferase gene.

mRNA Analysis

Total RNA was obtained from SW620 or Caco-2 cells by using the TriZol reagent (Invitrogen) and analyzed by quantitative real-time RT-PCR analysis. Reverse transcription of total RNA was performed using the components of the High Capacity cDNA Archive Kit (Applied Biosystems) and amplification of the COX-2 mRNA was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT instrument (Applied Biosystems). All samples were run in triplicate. COX-2 mRNA, VEGF mRNA, 18S rRNA specific primers and Taqman MGB probes were from Applied Biosystems. Relative quantitation of gene expression by real-time RT-PCR was calculated by the comparative threshold cycle ($\Delta\Delta C_T$) method following the manufacturer's software and instructions. Data were normalized to the endogenous control 18S rRNA to account for variability in the initial concentration of RNA and in the conversion efficiency of the reverse transcription reaction.

Immunoblot Analysis

SW620 or Caco-2 cells were disrupted in ice-cold lysis buffer (50 mM Tris-HCl (pH 8), 10 mM EDTA, 50 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Solubilized extracts (30 μg) were separated by SDS-PAGE on 10%

polyacrylamide gel, and transferred to nitrocellulose filters. The membranes were incubated overnight at 4°C with monoclonal mouse anti-COX-2 (1:1000) (Cayman Chemical) in blocking buffer. The filters were washed and incubated with rabbit anti-mouse IgG secondary Ab linked to horseradish peroxidase. The stained bands were visualized by the SuperSignal Substrate detection system (Pierce)

Transfection and Luciferase Assays

SW620 or COS-7 cells were transiently transfected by the LipofecAMINEPLUS reagent as recommended by the manufacturer (Invitrogen). Exponential growing cells were incubated for 4 h at 37^{o} C with a mixture of the correspondent reporter plasmid, LipofectAMINE and PLUS reagent in OptiMEM. In co-transfection experiments, different quantities of the correspondent expression plasmids were included as described in the figure legends. The total amount of transfected DNA was kept constant by using empty expression vectors. For transactivation assays, SW620 cells were cotransfected with GAL4-PPARα, GAL4-PPARγ o GAL4-c-Jun expression vectors together with a GAL4 luciferase reporter plasmid. Upon transfection, complete medium with 0.5 % FBS were added to the cells and incubated at 37^{o} C for additional 16 hours. Transfected cells were exposed to different stimuli as indicated. Then, cells were harvested and lysed. Luciferase activity was determined by using the luciferase assay system (Promega) with a luminometer Monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA). Protein measurements in extracts from transfected cells were performed with the BCA protein assay (Pierce). The data presented are expressed as the mean of the determinations in relative luciferase units (RLUs) ± SE per μg of total protein in the cell extract or as fold induction (observed experimental RLUs/ basal RLUs in absence of any stimulus).

Electrophoretic Mobility-Shift Assays

Nuclear extracts were prepared from SW620 cells as described previously [31]. Protein concentration was determined by the Bradford assay (Bio-Rad). 5 μg of nuclear protein was incubated with 1μg of poly(dI-dC) DNA carrier in DNA-binding buffer (2% (w/v) polyvinyl ethanol, 2.5% (v/v) glycerol, 10 mM Tris, pH 8, 0.5 mM EDTA, 0.5 mM dithiothreitol) with 6mM $MgCl_2$ for 10 min. The DNA binding reactions was performed by adding 50,000 cpm of ^{32}P-labeled double-stranded AP-1 consensus oligonucleotide (5´-CGCTTGATGAGTCAGCCGGAA-3´) (Promega) and incubated at room temperature for 15

min. A 30-fold molar excess of unlabeled oligonucleotide was added before the addition of the probe for competition when indicated. DNA–protein complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gels.

Cell Viability Assay

The viability of the cells was measured by the use of the tetrazolium salt WST-1 (Roche Molecular Biochemicals) as previously described [31]. SW620 cells were incubated for 3, 6 and 24 hours in the presence of increasing doses of PPARα ligands (10, 50 and 100 μM) and/or PMA + Ion and incubated with 10% WST-1 reagent. The formazan dye produced by metabolically active cells was quantified by spectrophotometrical measurement.

Statistical analysis

Data are expressed as mean ± SE and their statistical analysis was performed with Student´s t test. Ps< 0.05 were considered to be significant. All the experiments shown are either representative or the mean of triplicates of at least two independent ones performed in order to guarantee the reproducibility and the significance of the results**.**

RESULTS

PPARα ligands LY-171883 and WY-14,643 inhibit phorbol ester –induced COX-2 and VEGF expression in colon carcinoma cells

We first explored the influence of LY-171883 and WY-14,643 on gene expression in colon carcinoma cells by analyzing the expression of COX-2 and VEGF in SW620 and in Caco-2 colon carcinoma cell lines. COX-2 and VEGF mRNA levels was determined by quantitative real-time RT-PCR analysis. These cells express low levels of COX-2 or VEGF mRNAs that increased upon treatment with PMA (15 ng/ml) or PMA plus the A23187 calcium ionophore (Ion) (1 μM). Addition of LY-171883 or WY-14,643 resulted in a strong inhibition of the induction of COX-2 and VEGF mRNA levels by PMA or PMA plus Ion (Figure 1A). Hence, we next analyzed the effects of these PPARα ligands on COX-2 and VEGF protein expression in these cell lines. As shown in Figure 1B, LY-171883 or WY-14,643 treatment substantially reduced the induction of COX-2 protein expression elicited by PMA or PMA+Ion stimulation. Phorbol ester treatment induced an increase in VEGF protein production in the supernatants of SW620 or Caco-2 cells that was significantly inhibited by pretreatment with PPARα ligands (Figure 1C).

PPARα signaling in SW620 colon carcinoma cells

LY-171883 and WY-14,643 are able to regulate gene expression by its ability to act as a PPARα activators [32]. Thus, we analyzed if these drugs behaved as PPARα agonist in SW620 cells. As shown in Figure 2A, SW620 colon carcinoma cells express low levels of PPARα mRNA that were induced by PMA treatment. Moreover, LY-171883 and WY-14,643 were able to transactivate a PPRE-dependent luciferase reporter (PPRE-LUC), pointing to transcriptional regulation through endogenous PPARα (Figure 2B). Transcription driven by this PPRE was strongly increased upon co-transfection of a PPARα expression vector in SW620 cells, which could not be further increased by PPARα ligands. On the other hand, in COS-7 cells, which lack detectable amounts of endogenous PPARα (not shown), PPAR agonists did not induce PPRE driven transcription unless PPARα expression vector was co-transfected (Figure 2C). Both LY-171883 and WY-14,643 were able to induce ligand-dependent transactivation of a GAL4-PPARα construct, further confirming the ability of these drugs to act as PPARα ligands in SW620 cells (Figure 2D). Conversely, LY-171883 did not exert any effect in the transactivation mediated by a

GAL4 PPARγ construct, which was efficiently induced by the PPARγ ligand 15-deoxy$\Delta^{12,14}$$PGJ_2$ (15d-PGJ_2) (Figure 2E). These results further confirmed that LY-171883 and WY-14,643 were able to positively regulate PPRE-driven transcription through binding to PPARα in the colon carcinoma cell line SW620.

PPARα ligands inhibit transcriptional induction of VEGF and COX-2 promoters

We next analyzed whether the effects of the PPARα ligands LY-171883 and WY-14,643 on PMA –mediated induction of COX-2 and VEGF were taking place at the transcriptional level. In agreement with data obtained with the mRNA and protein, PMA strongly induced the transcription driven by human COX-2 (pCOX-2 LUC) or VEGF (pVEGF-LUC) promoters in SW620 cells (Figure 3A and B). Similarly to the effect observed on COX-2 and VEGF expression, pretreatment with LY-171883 or WY-14,643 blunted PMA induction of COX-2 and VEGF promoters, reducing their activity to basal levels (Figure 3A and B).

As these results pointed to PPARα as a negative regulator of transcriptional induction of gene expression in these cells, we next explored the effect of increasing amounts of PPARα on PMA –mediated transcriptional induction of these genes. SW620 cells were transfected with increasing amounts of PPARα or PPARγ expression plasmids along with the COX-2 promoter construct. As shown in figure 3C, PPARα expression inhibited in a dose-dependent manner the induction of COX-2 promoter activity by PMA. More interestingly, PPARα expression enhanced the inhibitory effects elicited by PPARα ligands. On the other hand, transfection of high amounts of a PPARγ expression vector did not show any significant effect in the absence or in the presence of LY-171883 or WY-14,643.

Inhibition of AP-1 –mediated transcriptional induction by PPARα agonists

It is well known that most of the inhibitory effects on gene transcription of PPARα agonists are due to their ability to inhibit of various transcription factors such as nuclear factor (NF)-κB and activator protein–1 (AP-1) [16]. We determined the influence of AP-1 and NF-κB transcription factors on PMA -mediated transcriptional induction of COX-2 and VEGF in these cells as potential candidates to be affected by PPARα activation. As shown in Figure 4A, PMA treatment of SW620 cells was able to activate NF-κB transcription factor, increasing NF-κB -dependent transcription. However, mutation of the NF-κB response element in the COX-2

promoter (431-COX-2-κBmut) did not influence PMA driven COX-2 transcriptional activation (Fig 4A). Even more, whereas overexpression of p65 NF-κB clearly induced NF-κB-LUC reporter activity, it did not affect COX-2 or VEGF promoter. These results discarded an essential role of NF-KB in the regulation of COX-2 or VEGF expression upon PMA treatment in SW620 cells. Several reports have described the essential role of AP-1 activation in the regulation of COX-2 and VEGF gene expression in a variety of cell types including colon carcinoma cells [33, 34]. We have previously shown that over-expression of the AP-1 member c-Jun transactivated both COX-2 and VEGF promoters in a similar extent than the AP-1 -driven p-73Col-LUC reporter gene, thus confirming the involvement of this factor in the transcriptional induction of COX-2 and VEGF in SW620 cells [31]. Thus, we next analyzed the effect of PPARα agonists on c-Jun –mediated COX-2 promoter transcriptional induction. LY-171883 inhibited the c-Jun -mediated transactivation of COX-2 promoter both in the presence or absence of PMA which cooperated with c-Jun overexpression to further enhance transcriptional activation (Figure 4). Noteworthy, expression of increasing amounts of a c-Jun expression plasmid were able to substantially revert the effect elicited by LY-171-883, thus suggesting a negative cross-talk between c-Jun and PPARα signaling.

Transcriptional interference between AP-1 and PPARα in the regulation of gene expression in colon carcinoma cells

It is well known that PPARα can affect transcriptional activation through interference with other transcription factors [6, 12]. Interestingly, treatment of SW620 cells with LY-171883 or WY-14,643 inhibited the induction of the well-characterized AP-1 –dependent -73Col-LUC reporter by PMA (Figure 5A). Co-transfection of increasing amounts of PPARα further diminished the induction of the AP-1 reporter construct elicited by PMA. On the other hand, co-transfection with higher doses of a PPARγ expression vector did not elicited any substantial effect both in the presence or absence of PPARα agonists. These data pointed to a negative cross talk between AP-1 and PPARα signaling in the regulation of genes such as VEGF and COX-2 in colon carcinoma cells. In order to determine whether the transcriptional interference between PPARα and AP-1 activities occurs in a reciprocal manner, transfection assays were performed to test the effect of c-Jun on the PPARα -dependent activation of a PPRE -driven promoter. As expected, transfection with a PPARα expression vector consistently induced the PPRE reporter

activity. Co-transfection of increasing amounts of c-Jun expression vector led to a dose-dependent inhibition of reporter activity induced by PPARα, without affecting basal promoter activity in the absence of co-transfected receptor (Figure 5B). These results confirm the existence of a mutual antagonism between c-Jun and PPARα signaling.

PPARα ligands inhibit AP-1 activation at several levels

Once established that PPARα activation was inhibiting AP-1 –dependent transcriptional activation, we next addressed the fine mechanism by which PPARα agonists was inhibiting AP-1 mediated responses. First, we tested the influence of LY-171883 or WY-14,643 treatment on AP-1 binding to DNA by EMSA assays. As shown in Figure 6A, a retarded protein complex that bound specifically to the AP-1 consensus sequence was observed in unstimulated cells. PMA treatment induced a strong increase in the formation of the retarded complex, which was severely impaired in cells pretreated with LY-171883 or WY-14,643 prior to PMA stimulation. Transcriptional activation by c-Jun can be also modulated by the regulation of the activity of its intrinsic transactivation domain by serine phosphorylation [35]. As shown in Figure 6B, the PPARα agonists LY-171883 and WY-14,643 significantly reduced PMA-induced transactivating activity of the chimeric protein GAL4-c-Jun, that contains the c-Jun transactivation domain (1-166) fused to the DNA binding domain of the GAL4 yeast transcription factor.

DISCUSSION

Recent studies have suggested an anti-inflammatory and anti-tumoral role of PPAR activators in a variety of experimental models (reviewed in [6, 12, 36]. Many of these effects are mediated by their ability to inhibit gene transcription of genes involved in inflammation, cell growth and angiogenesis [1, 37, 38]. In the present study, we have shown that PPARα agonists severely diminished phorbol ester–mediated induction of VEGF and COX-2 expression in colon carcinoma cells. Accumulating evidence suggests a close relationship among inflammation, VEGF, COX-2, PPARs and cancer, particularly in the gastrointestinal tract. Chronic inflammation is a tumor promoter in almost all tissues and is implicated in the pathogenesis of several cancers, particularly those in the gastrointestinal tract. Indeed, patients with chronic inflammatory bowel diseases are at increased risk for developing colorectal cancer [39]. COX-2 and VEGF are crucial agents in inflammatory processes, cell proliferation and tumor growth, participating in promoting tumor –associated angiogenesis. These proteins are aberrantly expressed in colorectal carcinomas in comparison to normal intestinal epithelial cells and are associated with cell growth and tumor progression (reviewed in [18, 19]. Epidemiological studies have demonstrated that NSAIDs, agents inhibiting COX-2 –derived prostaglandin production, appear to be effective in cancer prevention [40]. Noticeably, some NSAIDs may act as PPARα and γ ligands suggesting that, in addition to inhibit prostaglandin production, they might also regulate gene expression as part of their anti-inflammatory and chemopreventive mechanisms [41]. PPARs are expressed in the intestine at various levels, playing an important role in the development of colon carcinomas [11, 42, 43]. Although PPARγ is the predominant isoform, PPARα is also expressed in the colon, being able to participate in the differentiation of malignant tumor cells [8, 44, 45]. Several reports point to PPARα ligands as potentially beneficial chemopreventive agents in colon carcinogenesis. Tanaka and cols have demonstrated that PPAR agonist including bezafibrate, a PPARα ligand are able to suppress chemically induced aberrant crypt foci formation in the rat colon [9, 11]. In addition, this PPARα agonist has been reported to suppress intestinal polyp formation in *Apc*-deficient mice [10]. Moreover, methylclofenapate, a drug displaying properties as a PPARα agonist is also able to reduce intestinal polyp size and number in $APC^{min/+}$ mice [8]. Kohno et al. [9] have recently reported that both, the COX-2 inhibitor Nimesulide and PPAR ligands inhibit colitis-related colon carcinogenesis. These authors reported that bezafibrate, a PPARα agonist, significantly reduced the incidence of chemically-

induced colon adenocarcinoma in mice. Interestingly, the suppressive effect of these drugs on the developing colonic adenocarcinoma was well correlated with lowered expression of COX-2 in the colonic malignancies. Accordingly, our results here, suggest that PPARα activators may display antineoplastic effects by their ability to inhibit genes involved in colonic inflammation such as COX-2 and VEGF among others. This may prevent the development of aberrant crypt foci, thus acting before the first steps of carcinogenesis occur. Anti-inflammatory action of PPARα has been ascribed to inhibition of genes involved in inflammation control in a variety of cell types [15, 46]. In addition, our results show the PPARα -mediated inhibition of COX-2 and VEGF transcriptional activation in a colon carcinoma cell line.

Several molecular mechanisms have been proposed to explain the inhibitory actions of PPARα on gene transcription. Negative regulation of gene expression by PPARs might occur either by competition for limiting amounts of essential coactivators or through direct physical interactions between PPARs and specific transcription factors [1, 7, 47]. Our results demonstrate that PPARα -mediated inhibition of COX-2 and VEGF transcriptional activation occurs, at least in part, by interfering with AP-1 –mediated activation. This interference is reciprocal, as expression of a transfected reporter gene linked to a PPRE was inhibited by cotransfection with a c-Jun expression plasmid. Accordingly, an excess of c-Jun was able to revert the repressive effect of PPAR on AP-1 mediated COX-2 transcription. Our findings suggest that PPARα interference on PMA-induced COX-2 and VEGF transcription in colon carcinoma cells occurs primarily through AP-1, discarding the involvement NF-κB in the regulation of these genes by PMA in the colon carcinoma cell line SW620. In this sense, Staels et al [15] have reported the inhibition of COX-2 transcriptional activation by IL-1 as a result of PPARα repression of NF-κB signaling in human aortic smooth-muscle cells. Negative crosstalk between PPARs and AP-1 has been extensively described. PPARs are able to inhibit the ability of c-Jun to activate transcription of Endothelin-1 [48, 49]. Conversely, c-Jun inhibits the ability of PPARs to activate PPRE –driven genes [16]. Direct interaction between PPARα and transcription factors has been identified as a mechanism for PPARα mediated repression of gene expression. Thus, previous reports have shown a direct interaction of PPARα with c-Jun [16]. Accordingly, our results are compatible with a direct interaction between c-Jun and PPAR. This interaction may explain the decrease in AP-1 binding to DNA found after PPARα agonist treatment in stimulated SW620 cells. In

addition to the interference on AP-1 binding to DNA, our results demonstrates that inhibition of AP-1 -dependent activity by PPARα activators may also occurs by diminishing the intrinsic c-Jun transactivating activity. AP-1 activity is dependent on the transcriptional and posttranscriptional activation of its components, members of Fos and Jun families [50]. c-Jun is considered the main component of AP-1 and its activity is regulated post-transcriptionally by c-Jun N-terminal kinase (JNK) phosphorylation at Ser 63 and 73 on its transactivating domain [50]. Interestingly, PPARα is able to interact with the JNK responsive part of c-Jun [16] and PPARα activators have been shown to be able to inhibit c-Jun phosphorylation by JNK [49]. PPARα -mediated interference on c-Jun -mediated transactivation may thus occur through interference with the phosphorylation of c-Jun in its transactivation domain what might also alter its association with critical co-activators necessary for transcriptional activation. Nevertheless, additional studies are necessary to define the precise molecular mechanisms involved in the negative regulation of AP-1 activation by PPARα in colon carcinoma cells.

Taken together, the present results and those findings of previous studies suggest that PPARα activation may be beneficial in the early stages of colon tumorigenesis through inhibition of AP-1 –mediated transcriptional activation of genes involved in inflammation such as COX-2 and VEGF. Inhibition of colonic inflammation by PPARα ligands might be responsible for their potential chemopreventive effects on inflammation-associated colon carcinogenesis. However, it must be taken into account that many of the reported effects of PPAR ligands in vitro await confirmation by additional basic and clinical research to ascertain whether they can be considered of pharmacological significance in vivo in humans.

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FIGURE LEGENDS

Figure 1. PPARα ligands LY-171883 and WY-14,643 regulate COX-2 and VEGF expression. LY-171883 (50 μM) or WY-14,643 (50 μM) were added to SW620 or Caco-2 cells 1 h with prior addition of PMA (15 ng/ml) or PMA plus Ion (1 μM) as indicated. A) Analysis of COX-2 and VEGF mRNA levels by quantitative real-time RT-PCR in cells treated with PMA or PMA+Ion for 16 h (SW620) or 6 h (Caco-2) in the presence or absence of PPARα ligands. B) COX-2 protein levels were analyzed by Western blot in extracts from cells treated with PMA or PMA+Ion for 16 h (SW620) or 6 h (Caco-2) in the presence or absence of LY-171883 or WY-14,643. C) Production of VEGF in the supernatants of SW620 or Caco-2 cells after the different treatments was determined by ELISA. Data expressed as mean ± SE (** $p< 0.01$, *** $p< 0.001$).

Figure 2. LY-171883 and WY-14,643 mediate PPARα-dependent transcriptional activation in SW620 cells. A) RT-PCR analysis of PPARα mRNA expression in SW620 cells. An aliquot of the amplified DNA was separated on an agarose gel and stained with ethidium bromide for qualitative comparison. (B) Cells were cultured in the absence or presence of PMA (15 ng/ml) for 16 h. (B) SW620 cells or COS-7 cells (in C) were transfected with the PPAR responsive reporter plasmid PPRE-LUC along with a PPARα expression vector. After transfection, cells were treated for 16 h with LY-171883 (50 μM) or WY-14,643 (50 μM) and luciferase activity was determined. (D) SW620 cells were transiently transfected with a GAL4-LUC reporter plus expression vectors for the chimeric construct GAL4-PPARα or GAL4-PPARγ (in E). Cells were incubated with PPARα agonists LY-171883 (50 μM) and WY-14,643 (50 μM) or with the PPAR-γ agonist 15d-PGJ_2 (1 μM) for 16 h. Data expressed as mean ± SE (** $p< 0.01$, *** $p< 0.001$)

Figure 3. Effects of PPARα agonists on COX-2 and VEGF promoter activity. SW620 cells transfected with pCOX-2-LUC (A) or pVEGF-LUC (B) reporters, were treated for 1 hr with LY-171883 (50 μM) or WY-14,643 (50 μM), and then stimulated with PMA (15 ng/ml) for 16 hr. Results are the means ± SE expressed as RLUs per μg of total protein in the cell extract. C) SW620 cells were transiently transfected with the COX-2-LUC reporter along with an empty vector, or different quantities of PPARα or PPARγ expression plasmids as indicated. After transfection, cells were treated for 1 h with LY-171883 (50 μM) or WY-14,643 (50 μM), prior to

PMA treatment. Cells were lysed and luciferase activity was determined. Results are shown as percentage of activation by PMA considering 100 % the induction of promoter activity in the absence of treatment with PPAR vectors and ligands. Data expressed as mean ± SE (* $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$).

Figure 4. PPARα ligands inhibit AP-1 –mediated transcriptional induction of COX-2. (A) SW620 cells were transfected with the NF-κB-LUC, 431-COX-2-LUC or 431-COX-2(κBmut)-LUC reporter genes in the presence or absence of PMA (15ng/ml) for 16 h.(B) SW620 cells were co-transfected with the COX-2-LUC, VEGF-LUC and NF-κB-LUC reporter plasmids along with 10 to 50 ng of an expression vector for p65 NF-κB. (C) Cells were co-transfected with pCOX-2-LUC reporter plasmid along with 10 to 50 ng of the RSV-c-Jun expression plasmid. Cells were grown in the presence or absence of LY-171883 (50 μM) and stimulated with PMA (15 ng/ml) for 16 h. Results are the means ± SE.

Figure 5. Negative cross-talk between PPARα and AP-1. A) SW620 cells were transiently transfected with the AP-1 reporter construct -73-Col-LUC together with different quantities of PPARα or PPARγ expression plasmids as indicated. After transfection, cells were treated for 1 h with LY-171883 (50 μM) or WY-14,643 (50 μM), prior to PMA treatment. Cells were lysed and luciferase activity was determined. Results are shown as percentage of activation by PMA considering 100 % the induction of promoter activity in the absence of treatment with PPAR ligands. B) SW620 cells were transiently transfected with the PPRE reporter construct PPRE-LUC along with increasing quantities of the c-Jun expression plasmid in the presence or absence of a PPARα expression plasmid. After transfection, cells were lysed and luciferase activity was determined. Results are shown as fold induction over the observed RLUs in absence of co-transfection of PPARα and c-Jun. Data are expressed as mean ± SE (* $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$).

Figure 6. PPARα ligands inhibit AP-1 -mediated signaling. (A) Nuclear extracts were obtained from cells incubated with LY-171883 (50 μM) or WY-14,643 (50 μM) for 2 h and then stimulated with PMA (15 ng/ml) for 4 h. Binding to a consensus AP-1 labeled probe was evaluated by EMSA. A 30-fold molar excess of unlabeled AP-1 consensus oligonucleotide was added to determine the specific binding (comp). The lower panel shows the densitometry of the

radioactive bands in arbitrary units (a.u.). B) Cells were transiently co-transfected with the reporter plasmid Gal4-LUC along with a GAL4-c-Jun expression vector. Cells were treated with PMA for 16 h in the absence or presence of LY-171883 (50 μM) or WY-14,643 (50 μM) and luciferase activity was determined. Results are represented as the percentage of activation by PMA considering 100 % the induction of reporter activity in the absence of treatment with PPAR ligands. Data expressed as mean ± SE (** $p < 0.01$, *** $p < 0.001$).

Figure 1

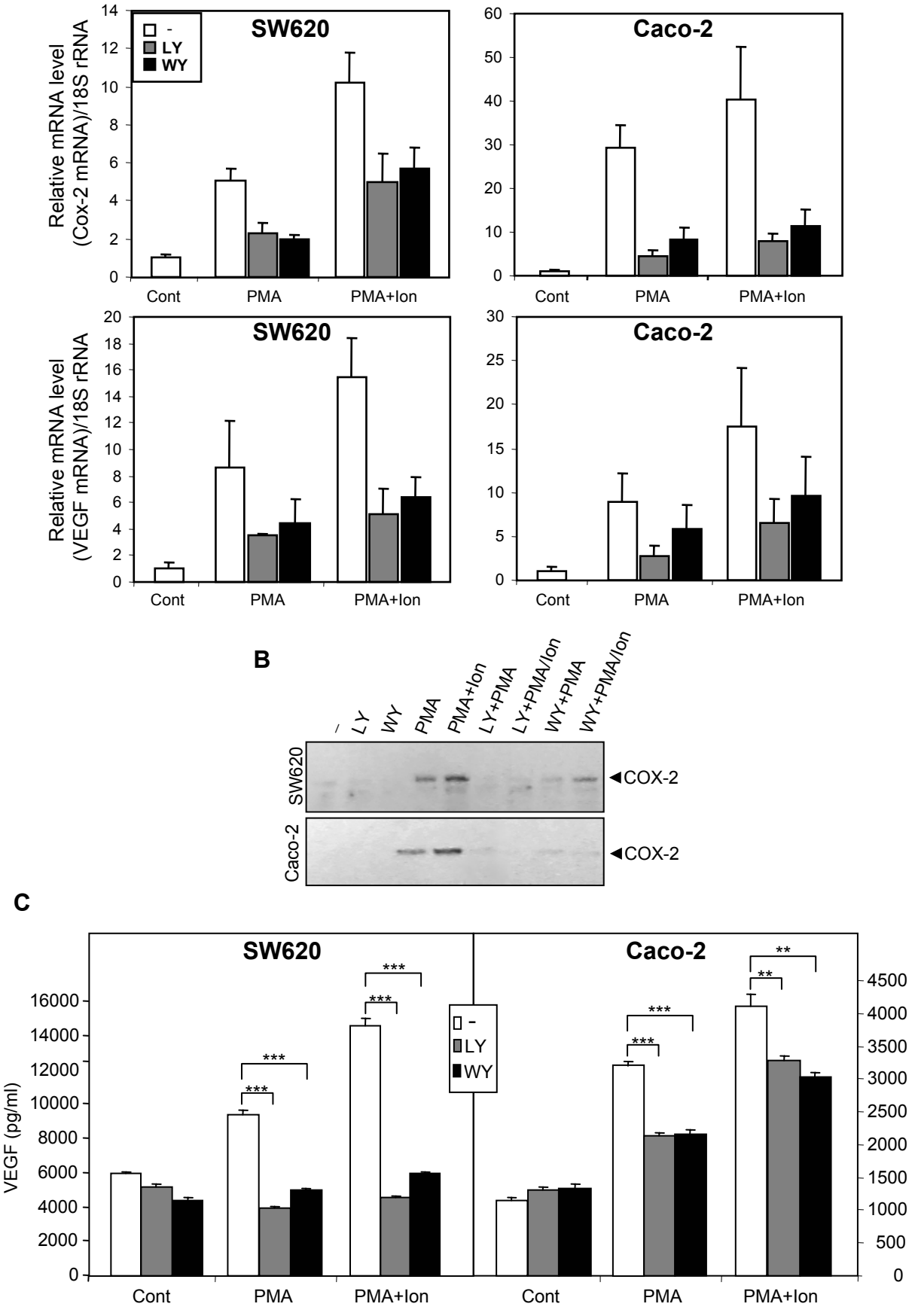

A
SW620
Caco-2
- LY WY
Relative mRNA level (Cox-2 mRNA)/18S rRNA
Relative mRNA level (VEGF mRNA)/18S rRNA
Cont
PMA
PMA+Ion
B
- LY WY PMA PMA+Ion LY+PMA LY+PMA/Ion WY+PMA WY+PMA/Ion
SW620
Caco-2
COX-2
C
SW620
Caco-2
VEGF (pg/ml)
- LY WY
Cont
PMA
PMA+Ion

Figure 2

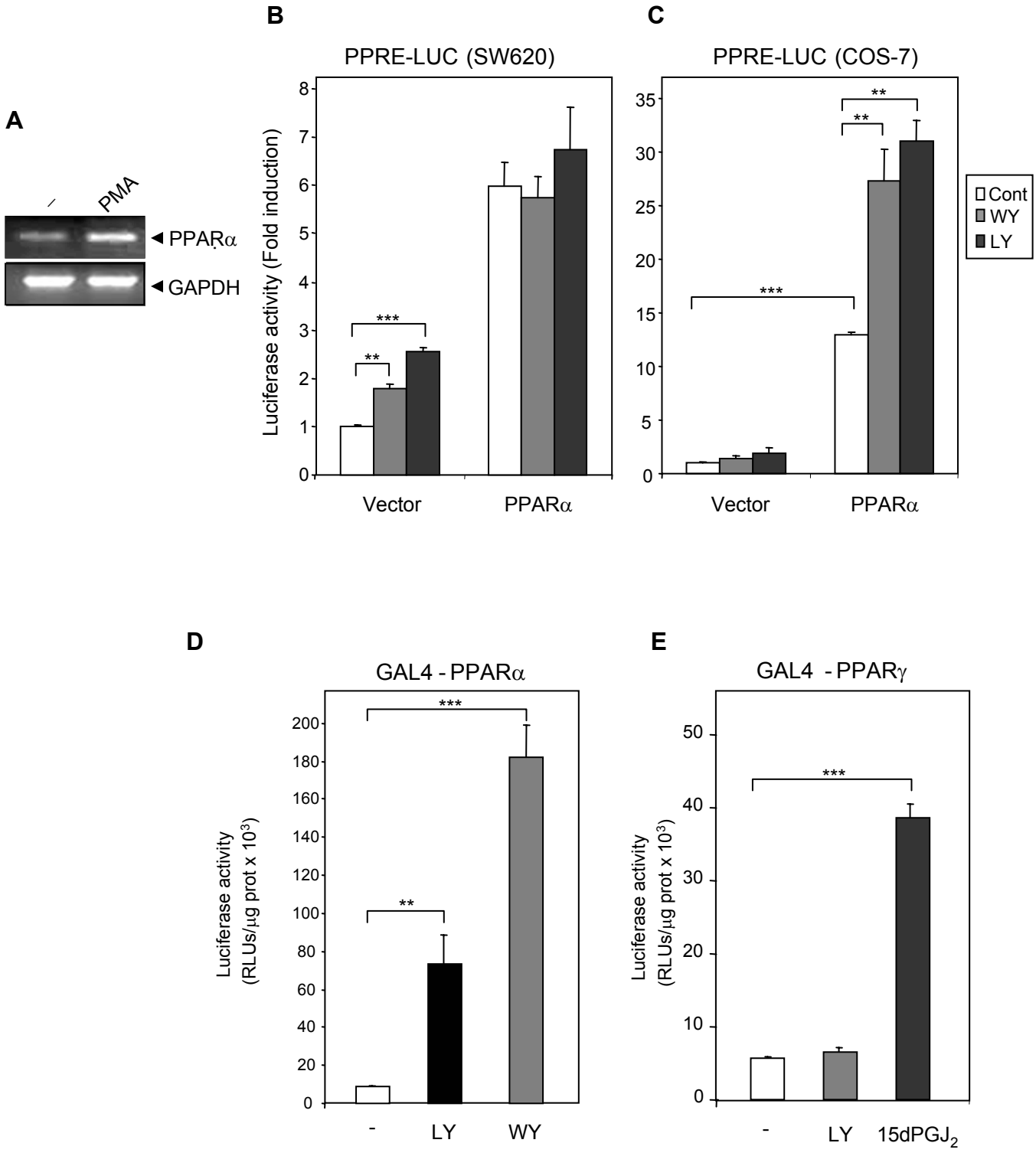

A
PMA
PPARα
GAPDH
B
PPRE-LUC (SW620)
Luciferase activity (Fold induction)
Vector
PPARα
C
PPRE-LUC (COS-7)
Cont
WY
LY
Vector
PPARα
D
GAL4 - PPARα
Luciferase activity (RLUs/μg prot x 10³)
-
LY
WY
E
GAL4 - PPARγ
Luciferase activity (RLUs/μg prot x 10³)
-
LY
15dPGJ2

Figure 3

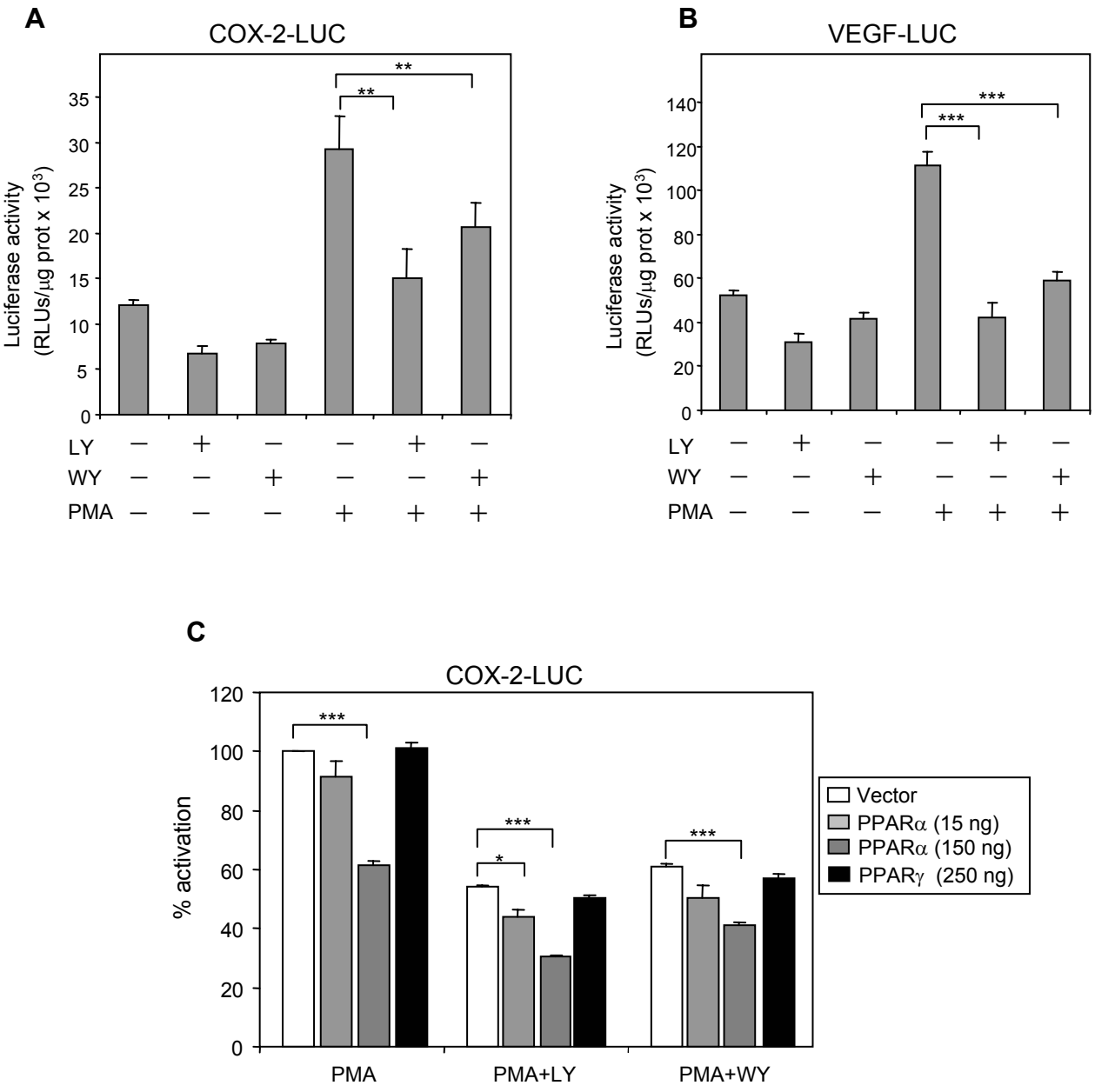

A
COX-2-LUC
Luciferase activity (RLUs/μg prot x 10³)
LY
WY
PMA
B
VEGF-LUC
Luciferase activity (RLUs/μg prot x 10³)
LY
WY
PMA
C
COX-2-LUC
% activation
PMA
PMA+LY
PMA+WY
Vector
PPARα (15 ng)
PPARα (150 ng)
PPARγ (250 ng)

Figure 4

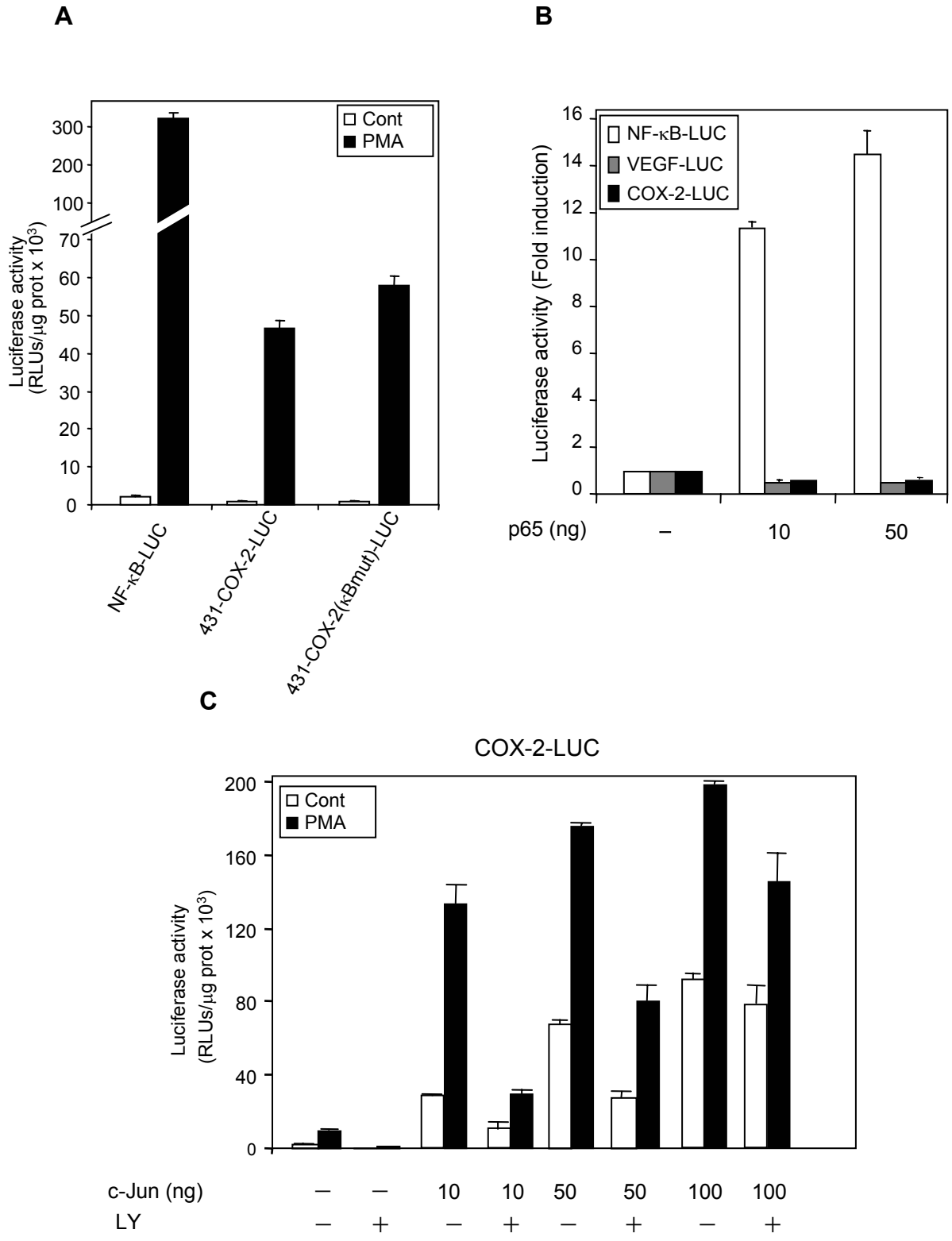

A
B
C
Cont
PMA
Luciferase activity (RLUs/μg prot x 10³)
NF-κB-LUC
431-COX-2-LUC
431-COX-2(κBmut)-LUC
NF-κB-LUC
VEGF-LUC
COX-2-LUC
Luciferase activity (Fold induction)
p65 (ng)
–
10
50
COX-2-LUC
c-Jun (ng)
LY

Figure 5

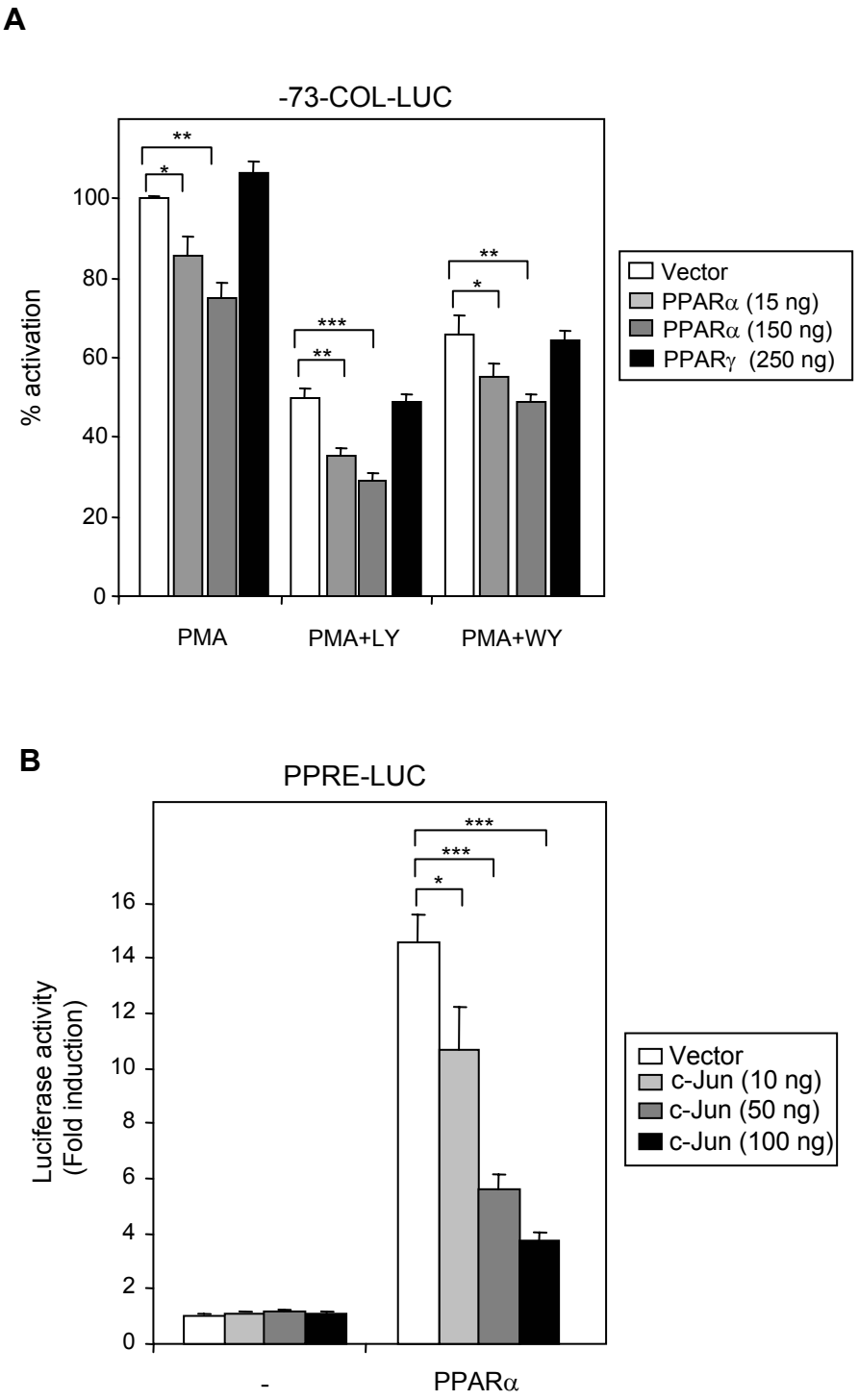
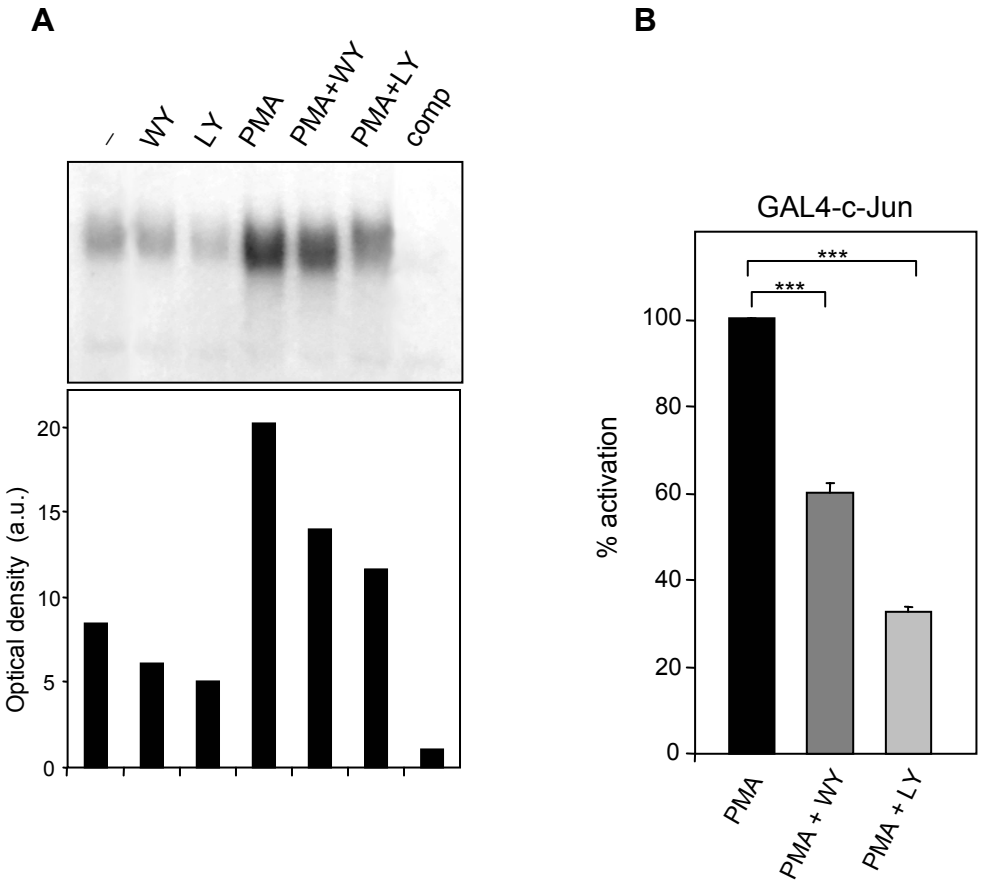

A
-73-COL-LUC
% activation
0
20
40
60
80
100
PMA
PMA+LY
PMA+WY
Vector
PPARα (15 ng)
PPARα (150 ng)
PPARγ (250 ng)
B
PPRE-LUC
Luciferase activity
(Fold induction)
0
2
4
6
8
10
12
14
16
-
PPARα
Vector
c-Jun (10 ng)
c-Jun (50 ng)
c-Jun (100 ng)

Figure 6


A
-
WY
LY
PMA
PMA+WY
PMA+LY
comp
Optical density (a.u.)
0
5
10
15
20
B
GAL4-c-Jun
***
***
% activation
0
20
40
60
80
100
PMA
PMA + WY
PMA + LY