

Differential Regulation of Vascular Endothelial Growth Factor Expression by Peroxisome Proliferator-activated Receptors in Bladder Cancer Cells*

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Sylvie Fauconnet^{‡§}, Isabelle Lascombe^{‡¶§}, Eric Chabannes^{||}, Gérard-Louis Adessi^{**},
Béatrice Desvergne[¶], Walter Wahli[¶], and Hugues Bittard^{||‡‡}

From the [‡]Institut d'Etudes et de Transfert de Gènes Bâtiment INSERM, 240 route de Dole, the ^{||}Service d'Urologie et d'Andrologie, Hôpital St Jacques, 2 place St Jacques, and the ^{**}Service d'Endocrinologie et Oncologie Moléculaires, Centre Hospitalier Universitaire Jean Minjot, 25000 Besançon, France, and the [¶]Institut de Biologie Animale, Université de Lausanne, CH-1015 Lausanne, Suisse

The growth of any solid tumor depends on angiogenesis. Vascular endothelial growth factor (VEGF) plays a prominent role in vesical tumor angiogenesis regulation. Previous studies have shown that the peroxisome proliferator-activated receptor γ (PPAR γ) was involved in the angiogenesis process. Here, we report for the first time that in two different human bladder cancer cell lines, RT4 (derived from grade I tumor) and T24 (derived from grade III tumor), VEGF (mRNA and protein) is differentially up-regulated by the three PPAR isotypes. Its expression is increased by PPAR α , β , and γ in RT4 cells and only by PPAR β in T24 cells via a transcriptional activation of the VEGF promoter through an indirect mechanism. This effect is potentiated by an RXR (retinoid-X-receptor), selective retinoid LG10068 providing support for a PPAR agonist-specific action on VEGF expression. While investigating the downstream signaling pathways involved in PPAR agonist-mediated up-regulation of VEGF, we found that only the MEK inhibitor PD98059 reduced PPAR ligand-induced expression of VEGF. These data contribute to a better understanding of the mechanisms by which PPARs regulate VEGF expression. They may lead to a new therapeutic approach to human bladder cancer in which excessive angiogenesis is a negative prognostic factor.

Bladder cancer comprises a wide range of tumors including transitional cell carcinoma (TCC)¹ (1, 2). This cancer represents the second cancer of the urinary tract in men. TCC is classified histopathologically into three types: superficial (pap-

illary tumors), confined to the bladder wall (pT1, pTa tumors), and invasive (stages T2–T4). Superficial bladder cancers represent a heterogeneous group of tumors, and about 60% of them will recur after transurethral resection (3). Some of them will progress to invasive and/or metastatic tumors and are therefore potentially lethal (4).

Angiogenesis, the process by which new vascular networks are formed from preexistent capillaries, is an essential component of the tumor growth and the metastatic pathway (5). Tumor angiogenesis is regulated by the production of angiogenic stimulators (6) including the vascular endothelial growth factor (VEGF), which has emerged as a key regulatory factor of the angiogenic process in either physiological or pathological conditions (7, 8, 9). VEGF is overexpressed in most human tumors such as kidney and bladder cancers (10). Elevated expression of VEGF in human tumor biopsies as well as the rise of VEGF levels in urine or serum have been reported to be independent prognostic and predictive factors of recurrence and disease progression in patients with superficial urothelial cancer (11–15).

Peroxisome proliferator-activated receptors (PPAR) belong to the steroid receptor superfamily and as such are ligand-activated transcription factors (16–19). They control gene expression by binding with their heterodimeric partner retinoid-X-receptor (RXR) (20) to peroxisome proliferator responsive elements (PPREs) (17, 20, 21). Three PPAR isotypes, PPAR α (NR1C1), PPAR β (NR1C2), and PPAR γ (NR1C3) (22) have been cloned and identified (17). PPAR α is predominantly found in the liver, heart, kidney, brown adipose tissue, and stomach mucosa; PPAR γ is primarily found in adipose tissue; PPAR β is ubiquitously expressed (23, 24). Fatty acid derivatives and eicosanoids were identified as natural ligands for PPARs. Furthermore, fibrates, including WY 14,643, are synthetic ligands for PPAR α that mediates the lipid-lowering activity of these drugs (25–28). The synthetic antidiabetic thiazolidinedione (TZD) agents are specific PPAR γ agonists (29–31). Recently, the L-165041 compound has been identified as being the first PPAR β -selective synthetic agonist (32). PPAR α plays an important role in fatty acid catabolism (33) and homeostasis in the liver as well as in the control of inflammatory response (25, 34). PPAR γ is involved in lipid metabolism, glucose metabolism, preadipocyte differentiation, inflammatory response, and macrophage differentiation (18, 35–38). The PPAR β function is poorly known. However, this receptor might be linked to colorectal cancer (39) and skin wound healing (40).

VEGF expression is regulated by many growth factors, environmental factors, and cytokines. A PPAR γ -mediated up-regu-

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§ These authors contributed equally to this work.

‡‡ To whom correspondence should be addressed: Service d'Urologie et d'Andrologie, Hôpital St. Jacques, 25000 Besançon, France. Tel.: 00-33-3-81-21-91-70; Fax: 00-33-3-81-21-91-73; E-mail: urologie@chu-besancon.fr.

¹ The abbreviations used are: TCC, transitional cell carcinoma; VEGF, vascular endothelial growth factor; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid-X-receptor; MAP, mitogen-activated protein; PPRE, peroxisome proliferator response element; ERK, extracellular signal-regulated kinase; PI, phosphatidylinositol; MEK, (MAP kinase)/ERK kinase; FABP, fatty acid-binding protein; Ox-LDL, oxidized low-density lipoprotein; RT, reverse transcriptase; ELISA, enzyme-linked immunosorbent assay; NLS, nuclear localization signal.

lation of VEGF (mRNA and protein secretion) has been established in human vascular smooth muscle cells (41). In addition, oxidized low-density lipoproteins (Ox-LDL) up-regulate VEGF expression in macrophages and endothelial cells, at least in part, through the activation of PPAR γ (42). Two of the major oxidized lipid components of Ox-LDL, 9-hydroxy-(S)-10,12-oxadecadienoic acid (9-HODE), and 13-hydroxy-(S)-10,12-oxadecadienoic acid (13-HODE) have been identified as endogenous activators and ligands of PPAR γ (39). All of these studies suggest that PPAR γ may be an important molecular target for the development of therapeutic inhibitors of angiogenesis in the treatment of cancer. No effect on VEGF expression has been observed in the presence of PPAR α and PPAR β agonists. So far, in human cancers, a PPAR-mediated regulation of VEGF expression has never been described.

Taking into account the importance of VEGF in the angiogenic process and its prognostic significance in the fate of TCC, the present investigation aimed to study VEGF gene regulation by the three PPAR isotypes (α , β , and γ) in RT4 cells (derived from grade I tumor) and T24 cells (derived from grade III tumor). Both groups of cells were derived from human bladder cancer and were used to clarify the intracellular signaling mechanisms involved. In this study, we uncovered a differential regulation of VEGF expression by PPARs according to the differentiated state of the cells. This regulated VEGF expression occurs through a transcriptional activation of the VEGF promoter *via* an indirect mechanism requiring an intermediary protein factor. In addition, the MAP kinase ERK 1/2 pathway modulates this regulation because an inhibition of PPAR-induced VEGF expression was observed only in the presence of PD98059 (MAP kinase/ERK 1/2 inhibitor).

EXPERIMENTAL PROCEDURES

Reagents—The hypolipidemic drug WY 14,643 came from Chemsyn Science Laboratories (Campro Scientific, Veenendaal, The Netherlands). L-165041, LG10068, and BRL 49653 compounds were a kind gift from Parke Davis. The MAP kinase/ERK 1/2 inhibitor PD98059 and the p38 MAP kinase-specific inhibitor SB203580 were purchased from Calbiochem (France Biochem, Meudon, France). Cycloheximide, actinomycin D, and wortmannin (specific PI 3-kinase inhibitor activity) were purchased from Sigma (La Verpillère, France). Ligands were dissolved in 100% Me₂SO or ethanol and added to cell cultures at a concentration of less than 0.1%.

Cell Lines and Culture Conditions—The RT4 and T24 cell lines were purchased from the American Type Culture Collection (Biovalley, Conches, France). The cells were maintained at 37 °C in a 5% CO₂ atmosphere in phenol red-free Mc COY's 5a medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 1% antibiotic antimycotic mixture (10 mg/ml streptomycin, 10,000 units/ml penicillin, 25 μ g/ml amphotericin B), 2 mM glutamine, and 15 mM Hepes (Sigma). The cells were tested for the absence of mycoplasma before the experiments were started. For the VEGF expression studies, cells were grown to 100% confluence to avoid any variation in VEGF expression in Mc COY's 5a medium supplemented with 5% deplemented fetal calf serum, 2 mM glutamine, and 15 mM Hepes. Before stimulation, cells were washed three times for 24 h with serum-free Mc COY's 5a medium in order to prevent any remaining serum effect. For stimulation with the PPAR ligands (WY 14,643 or L-165041 or BRL 49653) and RXR ligand LG10068, cells were incubated for 24 h in serum-free Mc COY's 5a medium. In the inhibitory experiments of protein synthesis and cellular signaling pathways, confluent cells were incubated for 24 h with 10 μ g/ml cycloheximide, 1 or 20 μ M PD98059, 100 nM wortmannin, or 10 μ M SB203580 alone or in the presence of PPAR agonists. The VEGF mRNA expression analysis was then measured by Northern blotting as described below.

Plasmid Constructions—The pSG5 hPPAR α , pBS hPPAR β , and pBS hPPAR γ plasmids were a kind gift from L. Michalik (IBA, Lausanne, Switzerland). They were used as positive controls in RT-PCR assays, generating fragments of 125-bp, 100-bp, and 130-bp lengths, respectively, corresponding to the coding region from the A/B domain of each nuclear receptor. The reporter plasmid Cyp2XPAL-LUC (26) was also a kind gift from L. Michalik. The VEGF promoter-luciferase reporter construct was a kind gift from A. Weisz (Istituto di Patologia generale

e Oncologia, Facoltà di Medicina e chirurgica, seconda Università di Napoli, Naples, Italy). This pGL2 basic vector contains the human VEGF promoter from -2279 to +56, linked to the firefly luciferase reporter gene (43). The eukaryotic expression vector pSG Δ 2 containing the NLS LacZ gene from pMMuLV NLS LacZ (NLS LacZ construct) (44) was used as an internal control of transfection efficiency and was called hereafter the β -gal plasmid.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assays—Total RNA from RT4 and T24 cells was isolated using TRIzol[®] reagent purchased from Invitrogen. Contaminating genomic DNA was removed with RNase-free DNase I (Invitrogen) according to the manufacturer's instructions. Total RNA from human tissues was used as a positive control and was provided by CLONTECH (Saint Quentin Yvelines, France). The synthesis of cDNA was performed in a total volume of 20 μ l using 6 μ g of total RNA extracted from human liver (positive control for PPAR α and PPAR γ) and human kidney (positive control for PPAR β) or 1 μ g of total RNA extracted from RT4 or T24 cells. The reaction was performed in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen) and 0.5 μ g of oligo(dT)₁₂₋₁₈ (Invitrogen). Subsequent amplifications of the partial cDNA encoding hPPAR α , hPPAR β , and hPPAR γ were performed using 6 μ l of reverse-transcribed mixture, which was one-third diluted as a template with specific oligonucleotide primers, as follows: hPPAR α sense, 5'-ACTCTGCCCTCTCGCCACTC-3' and antisense, 5'-GCCAAAGCTTCCAGAACTATCCTC-3'; hPPAR β sense, 5'-GAGCAGCCACAGGAGGAAGCC-3' and antisense, 5'-GCTGTGGTCCCTCCCA-T-3'; hPPAR γ sense, 5'-AGAGATGCCATTCTGGCCAC-3' and antisense, 5'-GTGGAGTAGAAATGCTGGAGA-3'. PCR reactions were performed in a total volume of 20 μ l in the presence of 100 pmol of each oligonucleotide primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 μ M dNTP, 1.5 mM MgCl₂, and 5 units of Taq DNA recombinant polymerase (Invitrogen). The expected sizes of PCR products for hPPAR α , β , and γ were 125, 100, and 130 base pairs, respectively. Negative controls for reverse transcription and PCR amplifications were included. For the plasmid controls, 0.5 μ g of plasmid was used. The PCR mixtures were subjected to 30 cycles of amplifications by denaturation (30 s at 94 °C), hybridization (30 s at 60 °C), and elongation (20 s at 72 °C). The PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide.

RNA Isolation and Northern Blotting Analysis—Total RNA from confluent cells was isolated using a commercially available kit TRI reagent (Molecular Research Center, Euromedex, Souffelwiersheim, France) according to the manufacturer's recommendations. The RNA (30 μ g) was size-fractionated by electrophoresis on a 1.2% agarose gel and transferred to a nylon membrane (Zeta-Probe GT Genomic (Bio-Rad)) using a vacuum blotting system. The filters were prehybridized for 5 min at 42 °C in a solution containing 50% formamide, 0.25 M NaCl, 7% SDS, and 0.12 M Na₂HPO₄ (pH 7.2). The hybridizations were performed for 48 h in the same solution at 42 °C with the VEGF cDNA probe (45) labeled with [α -³²P]dCTP (PerkinElmer Life Sciences) using the random hexamer labeling method (Prime-a-gene labeling system, Promega, Lyon, France). After a rapid wash in 2 \times SSC solution at room temperature, two washes were performed for 15 min at room temperature in 2 \times SSC, 0.1% SDS and 0.5 \times SSC, 0.1% SDS, respectively. The final wash was performed for 15 min at 55 °C in 0.1 \times SSC, 0.1% SDS. To check the loading of equivalent amounts of total RNA and to normalize the experiments, the filters were hybridized with a 1200-bp mouse β -actin probe labeled with [α -³²P]dCTP by the random hexamer labeling method. The VEGF and β -actin mRNA were quantitated using PhosphorImager analysis (Molecular Imager[®] System, GS-505, Bio-Rad).

VEGF Protein Levels in RT4 and T24 Cell-conditioned Media—After a serum-free period of 24 h, confluent cells were stimulated for 24 h in the presence of 50 μ M WY 14,643 or 25 μ M L-165041 or 10 μ M BRL 49653 or vehicle. VEGF protein levels in cell-conditioned medium were determined by ELISA, using a human VEGF immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Data are expressed in ng/mg of total cellular proteins and are the mean values of three independent experiments in quadruplicate. The total cellular protein concentration was determined using a protein assay according to the Bradford method (Bio-Rad).

Determination of VEGF mRNA Stability—To evaluate VEGF mRNA stability in RT4 and T24 cells, we measured the half-life of VEGF mRNA in the cells after 24 h of incubation in the presence of PPAR ligands. The transcription inhibitor actinomycin D (5 μ g/ml) (Sigma) was added to the culture to block further gene transcription. Cells were harvested at 30 min, and 1, 2, 3, 4, and 6 h after the addition of actinomycin D. The amount of VEGF and β -actin at each time point was



FIG. 1. Expression of PPAR isotypes in RT4 and T24 cells. RT-PCR analysis using specific primers for hPPAR α , hPPAR β , and hPPAR γ as described in detail under "Experimental Procedures." Specific cDNAs were synthesized from human liver and kidney RNA (positive controls), plasmids (positive controls), and from RT4 and T24 RNAs using oligo(dT)₁₂₋₁₈ in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase. PCR products were resolved on a 1.5% agarose gel.

quantified after Northern blotting using phosphorimager analysis; the amount of VEGF mRNA was corrected for loading differences using the amount of β -actin mRNA.

VEGF Promoter Activity in Response to PPAR Ligand Stimulation—For functional studies, T24 cells were seeded in 6-well plates at a concentration of 1.5×10^5 cells per well in Mc COY's 5a medium supplemented with 5% delipidated serum. All transient transfections were performed using Lipofectin[®] Reagent (Invitrogen) according to the manufacturer's recommended protocol. A total amount of 4 μ g of DNA (2 μ g of β -gal plasmid and 2 μ g of reporter plasmid) was transfected with Lipofectin reagent (2 μ g/ μ g plasmid DNA). After 24 h, cells were incubated for 12 h in Mc COY's 5a medium supplemented with 5% delipidated serum and then stimulated with test drugs in the absence of serum for 24 h more. Cells were harvested using reporter lysis buffer purchased from Promega. Luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer's recommendations. The β -galactosidase activity was spectrophotometrically measured using orthonitrophenyl β -D-galactopyranoside as substrate. Luciferase activity values were normalized to a β -galactosidase activity content, and -fold activation was calculated.

Statistical Analysis—Each experiment, subjected to a statistical analysis, was performed independently at least three times with similar results. The significance of the data was determined using Student's *t* test (two-tailed). $p < 0.05$ was deemed significant. The data presented consist of mean \pm S.D.

RESULTS

Expression of the Three PPAR Isotypes in RT4 and T24 Cells—RT-PCR was performed to demonstrate the expression of all three hPPAR (α , β , and γ) mRNAs in RT4 and T24 cells cultured *in vitro*. Based on the primers used in this study to amplify the cDNA of hPPAR α , hPPAR β , and hPPAR γ , fragments were expected to be 125, 100, and 130 base pairs in length, respectively. RNA samples from human liver and kidney were used as positive controls as well as plasmids containing the fragments of 125, 100, and 130 bp of hPPAR α , hPPAR β , and hPPAR γ , respectively. As shown in Fig. 1, all three PPAR mRNAs were expressed in both cell lines. Negative controls, performed in the absence of mRNA or directly on mRNA, yielded no detectable band (data not shown). Although the expression of hPPAR γ mRNA and protein in T24 cells has been reported previously (46), this study demonstrates for the first time the expression of hPPAR α and hPPAR β mRNAs in RT4 and T24 cells and that of hPPAR γ mRNA in RT4 cells.

Enhancement of VEGF mRNA Expression by Synthetic PPAR Agonists—To examine the regulation of VEGF expression in RT4 (derived from grade I tumor) and T24 (derived from grade III tumor) bladder cancer cells, we first investigated the ability of these tumor cells to express the VEGF gene constitutively. Total RNA was extracted from these cells and was subjected to Northern blot analysis. On the Northern blots (Fig. 2, upper panels), three bands at \sim 5.2, 4.5, and 1.7 kb were observed with the VEGF-A cDNA probe. Thus, RT4 and T24 cell lines express VEGF-A. The basal VEGF mRNA levels were lower in T24 cells than in RT4 cells. In RT4 cells after the ligand-dependent activation of the three PPAR isotypes (WY 14,643 for

PPAR α , L-165041 for PPAR β , and BRL 49653 for PPAR γ) for 24 h, we observed a significant induced VEGF mRNA expression in each case for each of the VEGF transcripts (Fig. 2). The 5.2-kb transcript level was increased 5.5- and 5.3-fold with WY 14,643 (50 μ M) and L-165041 (25 μ M), respectively. In the case of the 4.5-kb transcript, PPAR α and β agonists increased its expression 5.6- and 6.2-fold, respectively. For the 1.7-kb transcript, PPAR α and β ligands stimulated this transcript expression to the same extent with 4.8- and 4.6-fold increases, respectively. The thiazolidinedione BRL 49653, a PPAR γ ligand, induced the three VEGF transcripts to a lower extent than the other two ligands, WY 14,643 and L-165041, with 2.7-, 2.7-, and 2.5-fold inductions for the 5.2-, 4.5-, and 1.7-kb transcripts, respectively. These results contrast with the expression of VEGF observed in T24 cells. Indeed, in these cells no effect of PPAR α and γ ligands on VEGF expression was observed; only PPAR β regulated the VEGF gene with 2-, 3.6-, and 2.9-fold inductions for the 5.2-, 4.5-, and 1.7-kb transcripts, respectively.

In conclusion, in cells derived from grade I bladder cancer, VEGF expression is regulated by PPAR α , β , and γ . In contrast, in cells derived from grade III bladder cancer, PPAR α and PPAR γ -mediated up-regulation of VEGF expression cannot be found despite the presence of receptors in these cells. VEGF expression is induced only by PPAR β . Thus, for the first time we demonstrate a differential up-regulation of the expression of VEGF mRNA by PPAR agonists in bladder cancer cells according to the differentiation state of the cells.

PPAR Ligands Increase VEGF Protein Levels in Bladder Cancer Cell-conditioned Medium—To determine whether the up-regulation of VEGF mRNA levels by PPAR ligands correlates with higher VEGF protein levels in RT4- and T24 cell-conditioned media, we treated cells with vehicle alone or with 50 μ M WY 14,643, or 25 μ M L-165041, or 10 μ M BRL 49653 for 24 h. Then, we performed an enzyme-linked immunosorbent assay analysis of RT4 and T24 cell-conditioned media (Fig. 3). The amount of VEGF proteins was greater in RT4 cell-conditioned medium than that measured in T24 cell-conditioned medium. We found that the conditioned media of RT4 and T24 control cells (in the presence of vehicle alone) contained 3.7 ± 0.6 and 1.1 ± 0.2 ng/mg total cellular proteins, respectively. The PPAR activators WY 14,643, L-165041, and BRL 49653 significantly increased VEGF protein levels by 2.6-, 3-, and 1.7-fold, respectively, after 24 h stimulation of RT4 cells. In T24 cell-conditioned medium, only the PPAR β activator L-165041 increased VEGF protein levels by 4.4-fold. Thus, the PPAR agonist-dependent increase in VEGF gene expression correlates with increased levels of VEGF protein in the culture medium.

Increased PPAR Agonist-dependent Stimulation of VEGF mRNA Expression in RT4 Cells by the RXR Ligand LG10068—To examine the efficacy of a retinoid in potentiating the PPAR ligand effect on VEGF mRNA expression, and thus to confirm the specificity of the effect of PPAR agonists WY 14,643, L-165041, and BRL 49653, we treated cells with 1 μ M LG10068, a RXR-selective ligand, alone or in the presence of PPAR ligands. PPARs are known to activate cis-acting elements in the promoters of target genes as heterodimers with RXR (20). As shown in Fig. 4, no VEGF transcript was induced by LG10068 alone after 24 h of stimulation. The RXR agonist potentiated the effects of WY 14,643, L-165041, and BRL 49653 for the three VEGF transcripts. This result indicates the involvement of the RXR/PPAR heterodimer complex in the regulation of VEGF expression in RT4 cells.

PPAR Ligands Have No Effect on VEGF mRNA Half-Life—To determine whether PPAR synthetic ligands can in-

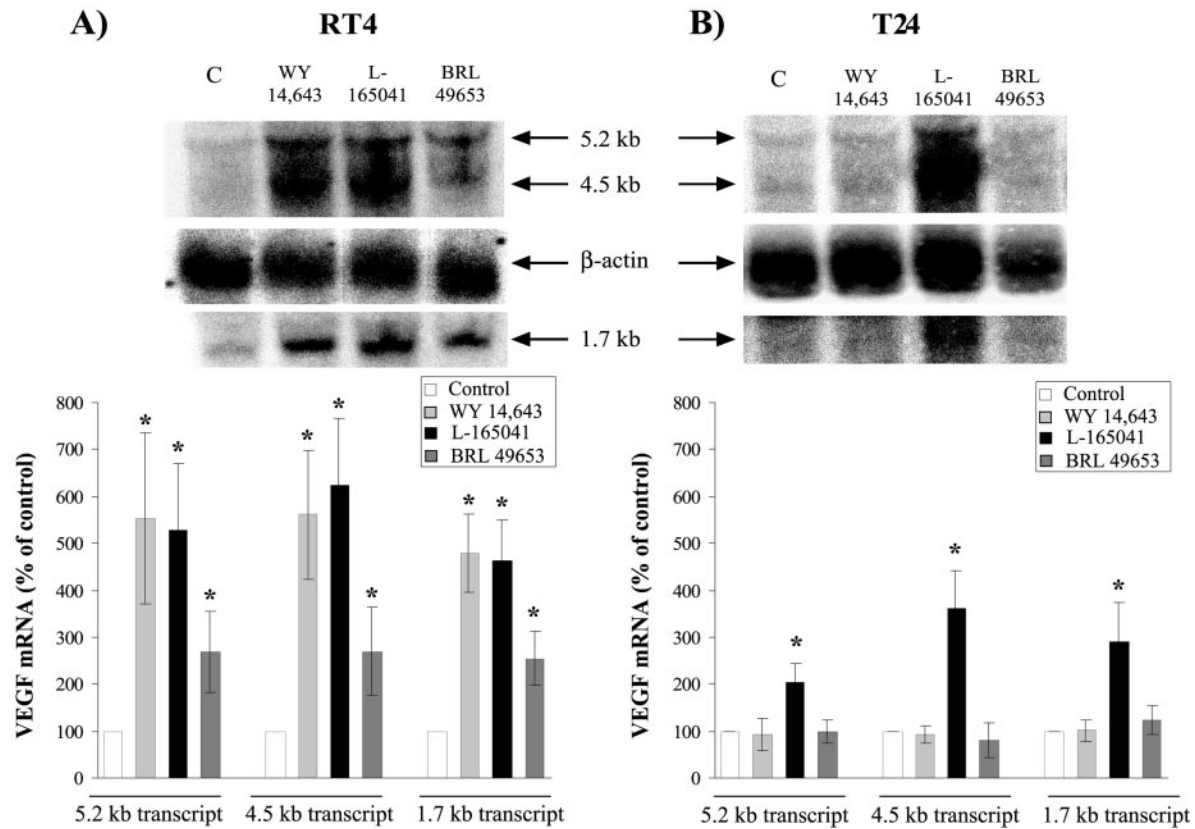


FIG. 2. Induction of VEGF mRNA by synthetic PPAR ligands in RT4 and T24 cells. A confluent monolayer of cells was cultured in serum-free Mc COY's 5a medium with vehicle alone (less than 0.1% ethanol or Me₂SO), 50 μ M WY 14,643, 25 μ M L-165041, or 10 μ M BRL 49653 for 24 h. Total RNA was extracted, and 30 μ g of RNA were subjected to Northern blot analysis performed with [α -³²P]dCTP-labeled probes for VEGF and β -actin as described in detail under "Experimental Procedures." *A, upper panel*, Northern blot of VEGF mRNA in RT4 cells revealing three bands of 5.2, 4.5, and 1.7 kb, respectively, and enhanced expression of VEGF mRNA by PPAR agonists. *Lower panel*, densitometric quantitation of the 5.2-, 4.5-, and 1.7-kb bands of VEGF mRNA in RT4 cells. *B, upper panel*, Northern blot of VEGF mRNA in T24 cells. *Lower panel*, densitometric quantitation of the enhanced expression of the 5.2-, 4.5-, and 1.7-kb bands of VEGF mRNA by PPAR agonists. ³²P-VEGF and β -actin signals were quantified by phosphorimager analysis. Data represent the fold inductions between treated and untreated cells calculated after normalization of VEGF mRNA signals to β -actin signals from each lane. The mRNA level in untreated cells was set at 100. Values are the mean \pm S.D. of three experiments in quadruplicate for each treatment. *, $p < 0.05$.

crease the stability of VEGF mRNA, cells were left untreated or treated with 50 μ M WY 14,643 or 25 μ M L-165041 or 10 μ M BRL 49653 for 24 h prior to the addition of the transcriptional inhibitor actinomycin D (5 μ g/ml). Then, the VEGF mRNA half-life was estimated with quantitative Northern blot analysis. As shown in Fig. 5, in the control cells there was a rapid decay of VEGF mRNA for the 4.5- and 1.7-kb transcripts with a half-life of 0.6 h. After 2 h of treatment with actinomycin D, there was no more VEGF mRNA. On the contrary, we observed a longer half-life (2 h) for the 5.2-kb transcript and the disappearance of total VEGF mRNA after 4 h of treatment with actinomycin D. In the presence of WY 14,643, L-165041, and BRL 49653, there was no increase of the half-life of VEGF mRNA compared with the control cells. Thus, the synthetic PPAR ligands used in this study did not modify the stability of VEGF mRNA. From these results we conclude that PPAR ligands regulate the VEGF gene at the transcriptional level.

Effects of Synthetic PPAR Ligands on VEGF Promoter Activity—The ability of PPARs to affect VEGF gene expression at the promoter level was investigated using the VEGF-1 plasmid construction containing the VEGF promoter from -2279 to +56 that was introduced upstream from the luciferase gene. After transfection of this construct, the cells were treated with vehicle alone (less than 0.1% ethanol or Me₂SO) or with the indicated concentrations of synthetic PPAR ligands (Fig. 6). As shown in Fig. 6B, these treatments did not enhance luciferase activity compared with the control. However, the treatment of

the three PPAR isotypes with these activators stimulated expression from the PPRE-driven luciferase construct. The PPRE reporter construct (Cyp2XP_{al}-Luc) exhibited a 1.8-, 2-, and 2.5-fold induction with the PPAR α , PPAR β , and PPAR γ activators, respectively (Fig. 6A). This result confirms that the three PPAR isotypes are present and functional in the T24 cell line. Thus, the absence of any effect of PPAR activators on the VEGF-1 promoter might indicate an absence of PPREs in the promoter region located from -2279 to +56. Therefore, we can conclude that if the VEGF gene is a direct target of PPARs, the PPRE is most likely located outside of the analyzed promoter region. Alternatively, the PPAR ligand-dependent stimulation of the VEGF gene might be indirect, related to the stimulation of a factor, which in turn mediates VEGF transcription.

Induction of VEGF mRNA by PPAR Agonists Requires de Novo Protein Synthesis—To determine whether the synthesis of new proteins is involved in PPAR ligand-induced VEGF mRNA transcription, cells were untreated or treated for 24 h with 50 μ M WY 14,643 or 25 μ M L-165041 or 10 μ M BRL 49653 in the absence or presence of 10 μ g/ml cycloheximide, a protein synthesis inhibitor. As shown in Fig. 7, in human bladder cancer cell lines RT4 (Fig. 7A) and T24 (Fig. 7B), the treatment with cycloheximide completely inhibited PPAR ligand-induced VEGF mRNA expression. These data demonstrate that the stimulation of VEGF mRNA expression by synthetic PPAR ligands is induced by the increased synthesis of new proteins such as regulatory proteins.

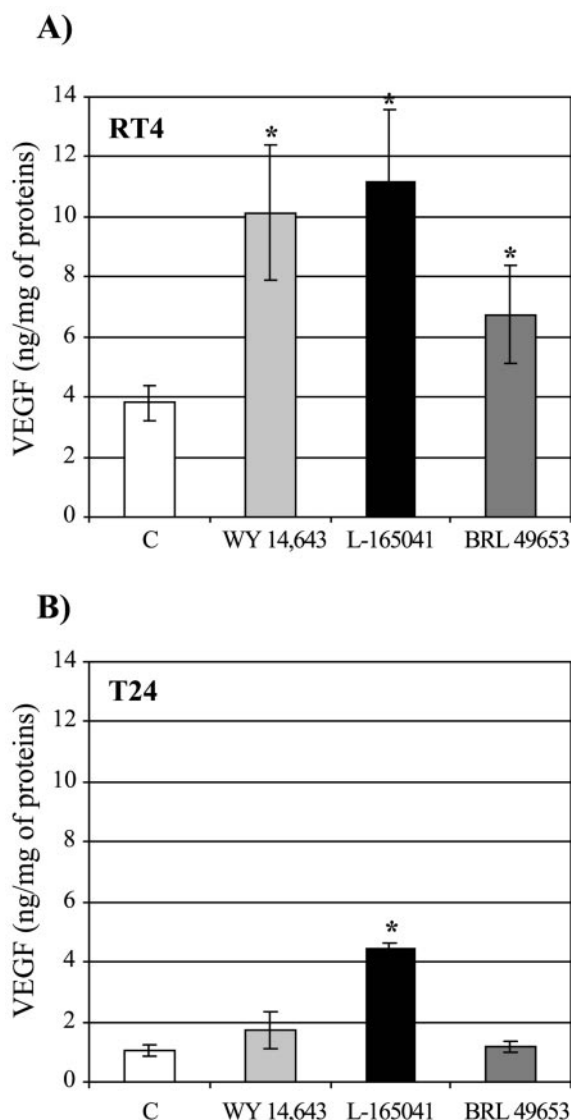


FIG. 3. Some synthetic PPAR ligands increase VEGF protein level in bladder cancer cell-conditioned medium. Confluent RT4 (A) and T24 (B) cells were untreated or treated with 50 μM WY 14,643, or 25 μM L-165041, or 10 μM BRL 49653 for 24 h. Subsequently, conditioned media were collected. The concentration of VEGF was measured by ELISA. Data are expressed in ng/mg total cellular proteins per well. Values represent the mean \pm S.D. for $n = 12$ measurements from three independent experiments. *, $p < 0.05$.

Effect of the Inhibition of the PI 3-Kinase and p38 Kinase Pathways on PPAR-dependent VEGF mRNA Expression—To elucidate the signal transduction pathway(s) responsible for VEGF induction by synthetic PPAR ligands, we have investigated the contribution of PI 3-kinase to VEGF regulation and examined the role of several MAP kinase family members using pharmacological inhibitors. As shown in Fig. 8, the treatment with the drug wortmannin (100 nM), which is a specific inhibitor of PI 3-kinase activity, did not inhibit VEGF mRNA induction by PPAR ligands in RT4 cells. Similar results were obtained in T24 cells stimulated by L-165041 in the presence of wortmannin (data not shown). To establish whether p38 kinase activation was required for PPAR ligand effects on VEGF mRNA expression, we treated RT4 cells with the p38-specific inhibitor SB203580. As seen in Fig. 8, the treatment with SB203580 (10 μM) did not suppress VEGF induction by WY 14,643, L-165041, and BRL 49653. Such an absence of inhibition was observed in T24 cells stimulated by L-165041 in the presence of SB203580 (data not shown). Taken together, these

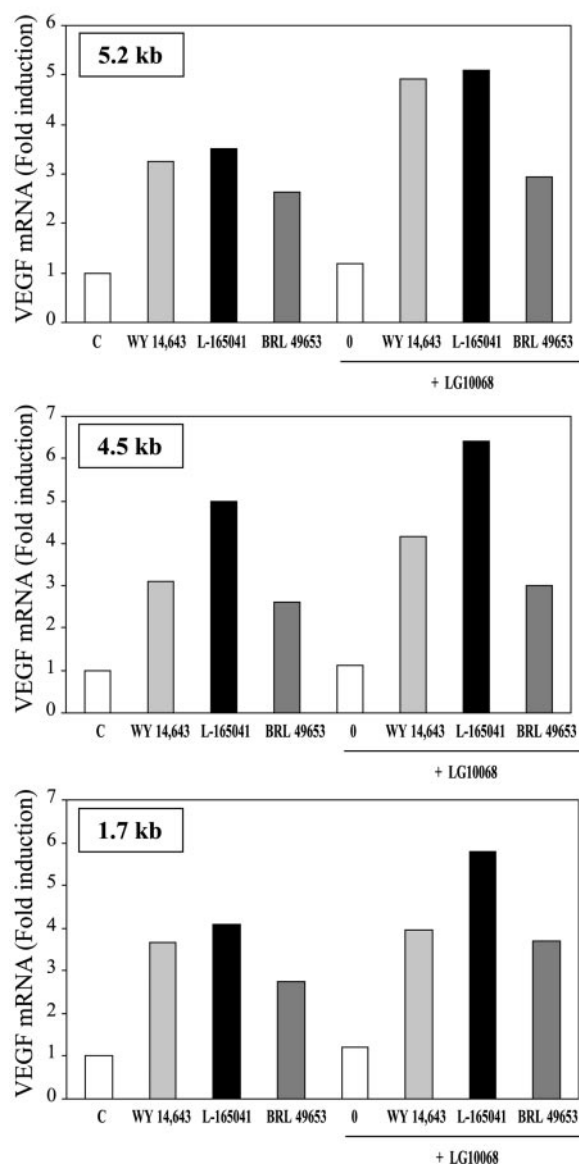


FIG. 4. The RXR-selective ligand LG10068 raises the PPAR agonist effect on VEGF mRNA expression in RT4 cells. At confluence, RT4 cells were cultured in serum-free Mc COY's 5a medium with vehicle alone (less than 0.1% ethanol or Me_2SO), 50 μM WY 14,643 (WY), 25 μM L-165041 (L), or 10 μM BRL 49653. Each was used alone or combined with 1 μM RXR-selective ligand LG10068 for 24 h. Total RNA was isolated and analyzed by Northern blot and performed with [α - ^{32}P]dCTP-labeled probes for VEGF and β -actin as described in detail under "Experimental Procedures." Densitometric analysis was performed after quantitation of the 5.2-, 4.5-, and 1.7-kb bands of VEGF mRNA ($n = 4$). ^{32}P -VEGF and β -actin signals were quantified by phosphorimager analysis. Mean control value in untreated cells was set at 1. Data represent the fold inductions between treated and untreated cells, calculated after normalization of VEGF mRNA signals to β -actin signals from each lane. Values are the means of four measurements for each treatment from one experiment.

results clearly indicate that the PI 3-kinase and p38 kinase pathways do not transduce the PPAR ligand signal on VEGF mRNA induction in the human bladder cancer cell lines studied.

The MAP (ERK 1/2) Kinase Pathway Is Involved in PPAR Ligand-induced VEGF mRNA Expression—Next, we tested whether the MAP kinases ERK 1/2 are involved in the stimulation of VEGF expression by PPAR agonists. Therefore, we treated the cells with PPAR agonists alone or in the presence of different concentrations of the MAP kinase/ERK 1/2 (MEK 1) inhibitor PD98059. As shown in Fig. 9, at 20 μM PD98059

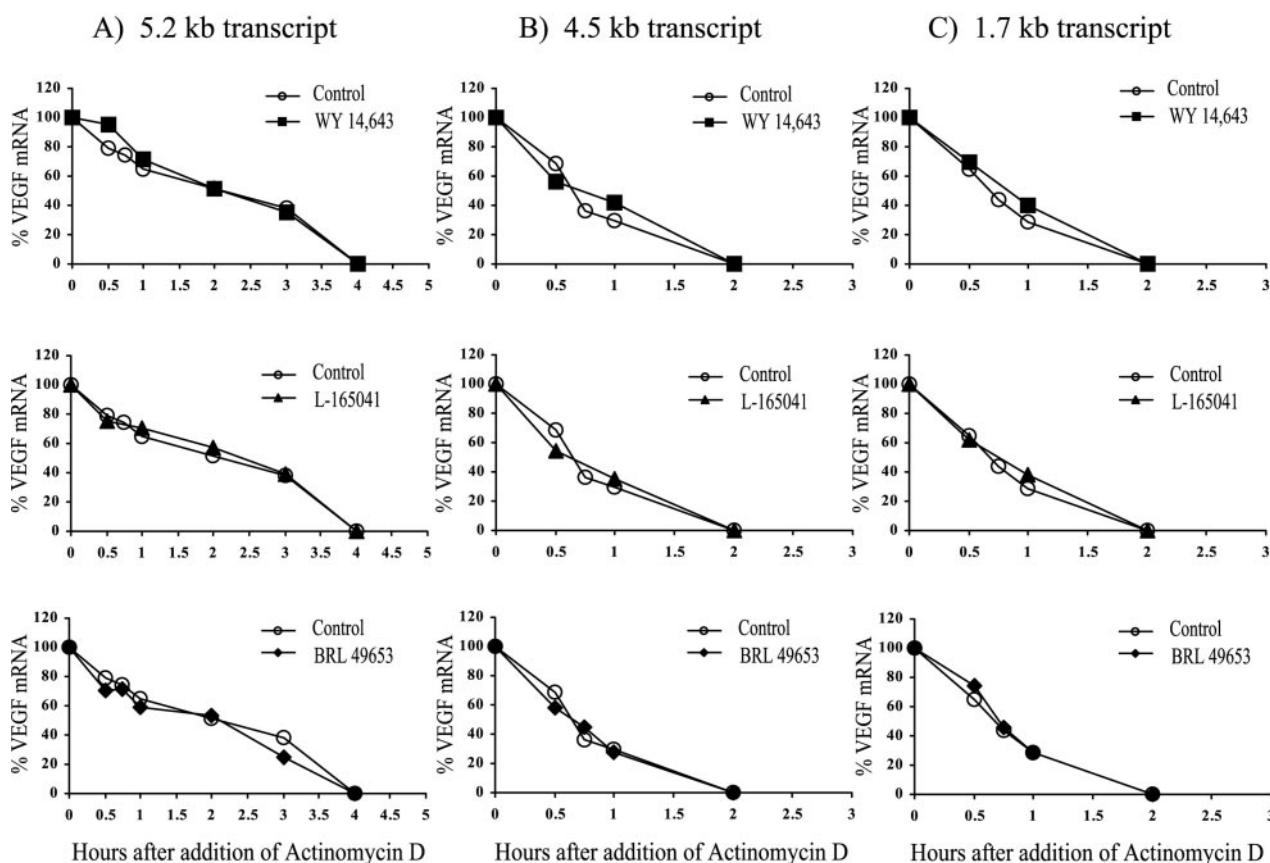


FIG. 5. **VEGF mRNA half-life study.** Confluent RT4 cells were incubated in serum-free Mc COY's 5a medium for 24 h and then stimulated for 24 h more with vehicle alone or 50 μM WY 14,643, or 25 μM L-165041, or 10 μM BRL 49653 before actinomycin D (5 $\mu\text{g}/\text{ml}$) was added. Total RNA was extracted at the indicated times after the transcription inhibitor addition and assayed for Northern blotting analysis. Membranes were hybridized with [α - ^{32}P]dCTP-labeled probes for VEGF and β -actin. ^{32}P -VEGF and β -actin signals were quantified by phosphorimager analysis. VEGF mRNA signals were normalized to the β -actin signals from each lane. Indicated percentages represent levels of the three VEGF mRNA transcripts of 5.2 kb (A), 4.5 kb (B), and 1.7 kb (C) in PPAR ligand-treated cells or untreated cells relative to the appropriate control (vehicle or PPAR ligands at time 0) in the absence of actinomycin D. Data correspond to one experiment in quadruplicate for each treatment. Similar results were obtained from another independent experiment.

decreased the stimulative effect of the PPAR α ligand on the expression of the three VEGF transcripts as well as the effect of the PPAR β and γ agonists. In T24 cells, as seen in Fig. 10 (A and B), PD98059 diminished the L-165041 effect on VEGF mRNA expression. As indicated in the *inset* (Fig. 10B), PD98059 decreased the L-165041-induced VEGF mRNA expression by 25, 20, and 30% for the 5.2-, 4.5-, and 1.7-kb transcripts, respectively, at a concentration of 1 μM . At 20 μM , PD98059 decreased VEGF mRNA expression to a greater extent. We observed a reduction of 45, 30, and 35% for the 5.2-, 4.5-, and 1.7-kb transcripts, respectively. Thus, the ERK 1/2 pathway is involved in PPAR agonist-induced VEGF expression in the two human bladder cancer cell lines studied.

DISCUSSION

In human bladder cancer cells, the signal transduction pathways involved in the VEGF regulation remain largely unknown. Recently, in human bladder tumors and cell lines it has been demonstrated that components of the hypoxia response pathway, including HIF-1 α (hypoxia inducible factor) and HIF-2 α , are important cofactors in the regulation of VEGF (47). Furthermore, in previous studies we have shown an up-regulation of VEGF expression by PMA (phorbol 12-myristate 13-acetate) in the human bladder cancer cell line RT4 (48). Here, we report on the regulation by PPARs of VEGF expression in two human bladder cancer cell lines, RT4 and T24. The RT4 cell line was established from a differentiated papillary tumor in which P53 and H-Ras were not mutated (49). In contrast, the

T24 cell line, which was established from an undifferentiated carcinoma (50), expressed mutated P53 and H-Ras. In this work we have shown that RT4 cells expressed VEGF at levels four times higher than the T24 cells did. These observations agree with those of O'Brien *et al.* (11). Indeed, they demonstrated that in superficial tumors VEGF was 4-fold higher than in invasive tumors and 10-fold higher than in normal bladder. The high level of VEGF in well differentiated cells is not restricted to bladder cancer because it was also observed in endometrial cancers (51).

PPARs are expressed in several human tissues, among them the urinary tract. Indeed, mRNAs for the three PPAR isotypes have been found in the normal ureter and bladder (52). Recently, PPAR γ has been reported to be highly expressed in human TCCs as well as in the T24 cell line (46). In the present study we have confirmed the expression of the γ isotype in the T24 cell line, and we have demonstrated for the first time the expression of PPAR α and β in T24 and RT4 cells and that of PPAR γ in RT4 cells.

In this report we have shown a PPAR γ -enhanced VEGF expression in well differentiated RT4 cells, and we have established for the very first time a PPAR α - and PPAR β -mediated up-regulation of VEGF expression. In contrast, in undifferentiated T24 cells VEGF expression is induced only by PPAR β . In RT4 cells, the up-regulation of VEGF (mRNA and protein) expression by PPAR α and β was greater than that by PPAR γ . Furthermore, the activation by PPAR β was higher in these

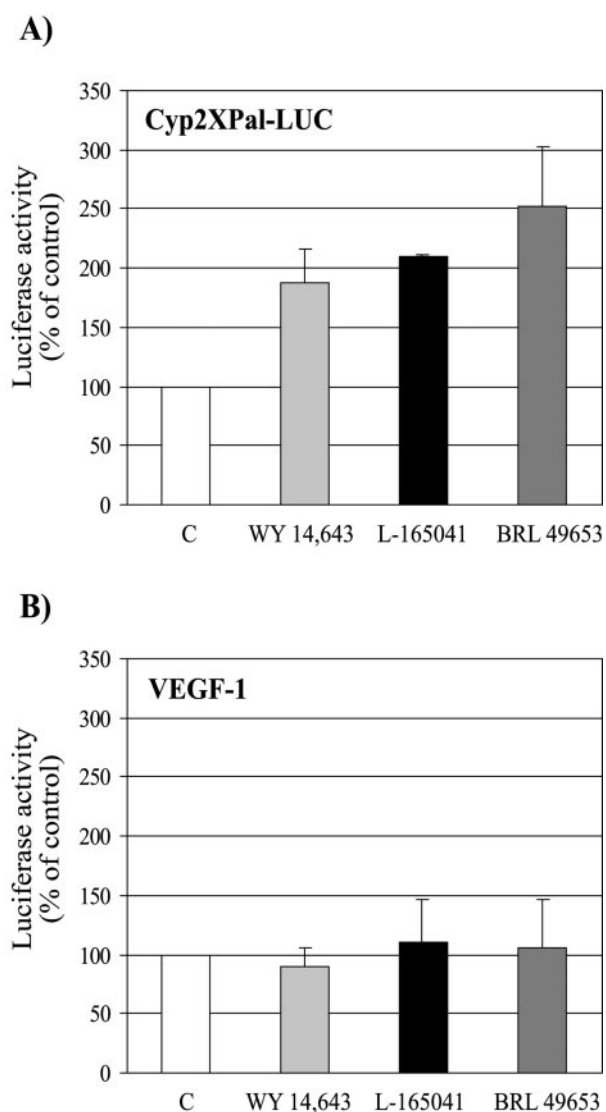


FIG. 6. The VEGF-1 plasmid construction is not induced by synthetic PPAR ligands. T24 cells were cotransfected with the β -gal plasmid (as an internal control for transfection efficiency) and the CYP2XPal-Luc reporter construct (A) containing two copies of the CYP4A6 PPRE cloned in palindromic orientation upstream of the minimal herpes simplex virus thymidine kinase promoter or the VEGF-1 plasmid (B) containing the human VEGF promoter from -2279 to $+56$ upstream of the luciferase gene. Cells were treated with vehicle alone (less than 0.1% ethanol or Me_2SO) (C), $50 \mu\text{M}$ WY 14,643, $25 \mu\text{M}$ L-165041, or $10 \mu\text{M}$ BRL 49653 for 24 h. Cell extracts were subsequently assayed for luciferase activity. Normalized luciferase activity was represented as fold increase over control conditions. Data represent the mean \pm S.D. of assays performed in quadruplicate from two independent experiments.

cells than the effect observed in T24 cells. Several studies have described the role of PPAR γ activation in the angiogenesis process. Indeed, one of them reported that PPAR γ ligands suppressed human umbilical vein endothelial cells (HUVEC) differentiation into tube-like structures in three-dimensional collagen gels *in vitro* and that they inhibited VEGF-induced angiogenesis in rat cornea *in vivo*. However, they did not modify the VEGF expression in HUVEC (53). Some recent findings established that PPAR γ , unlike the isotypes α and β , increased VEGF expression in human vascular smooth muscle cells (41). Besides, Ox-LDL up-regulates VEGF expression in macrophages and endothelial cells, at least in part, through the activation of PPAR γ (42). In our study, we have also simulated RT4 cells in the presence of other PPAR γ agonists such as

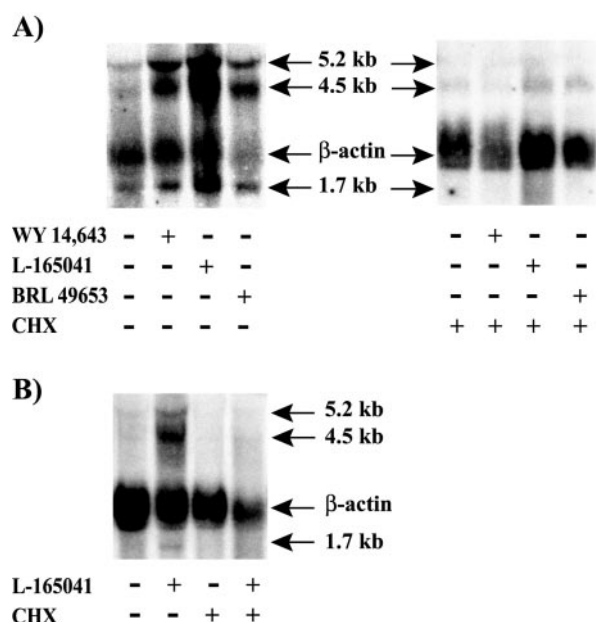


FIG. 7. Induction of VEGF mRNA by synthetic PPAR ligands depends on the synthesis of new proteins in RT4 and T24 cells. The confluent monolayer of RT4 (A) and T24 (B) cells was incubated in serum-free Mc COY's 5a medium for 24 h and then untreated or treated for 24 h with $50 \mu\text{M}$ WY 14,643, $25 \mu\text{M}$ L-165041, $10 \mu\text{M}$ BRL 49653 for RT4 cells and $25 \mu\text{M}$ L-165041 for T24 cells in the absence or presence of $10 \mu\text{g/ml}$ cycloheximide (CHX). Total RNA was isolated and analyzed by Northern blot for the expression of VEGF and β -actin as described in detail under "Experimental Procedures." This experiment has been performed in quadruplicate for each treatment and repeated two times with similar results.

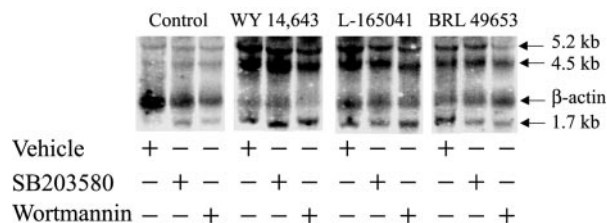


FIG. 8. The p38 MAP kinase and the PI 3-kinase pathways are not involved in the induction of VEGF mRNA by synthetic PPAR ligands in RT4 cells. At confluence, RT4 cells were incubated for 24 h in serum-free Mc COY's 5a medium. Then, they were untreated or treated with $50 \mu\text{M}$ WY 14,643, or $25 \mu\text{M}$ L-165041, or $10 \mu\text{M}$ BRL 49653 in the absence or presence of p38 inhibitor SB203580 ($10 \mu\text{M}$) or PI 3-kinase inhibitor wortmannin (100 nM) for 24 h. Total RNA was extracted, and $30 \mu\text{g}$ of RNA were subjected to Northern blotting analysis for the expression of VEGF. Membranes were hybridized to [α - ^{32}P]dCTP-labeled probes for VEGF and β -actin as described in detail under "Experimental Procedures." This experiment has been performed in quadruplicate for each treatment and repeated two times with similar results.

troglitazone and 15d-PGJ2 (data not shown). VEGF rise (mRNA and protein) was induced by troglitazone but to a lower extent than it was in the presence of BRL 49653. We also observed an increase in cell death after the treatment with 15d-PGJ2, as previously described by Guan *et al.* (46).

The absence of an effect of PPAR α and γ on VEGF expression in T24 cells is due neither to an insufficient number of endogenous receptors nor to nonfunctional ones. Actually, in transient transfection experiments, the PPAR ligands WY 14,643 and BRL 49653 stimulated the activity of a reporter gene containing two PPAR-binding sites (PPRE) in its promoter. This result provides additional evidence for the presence of PPAR α and γ in these cells. Moreover, they are sufficiently abundant to stimulate the PPRE-driven reporter construct.

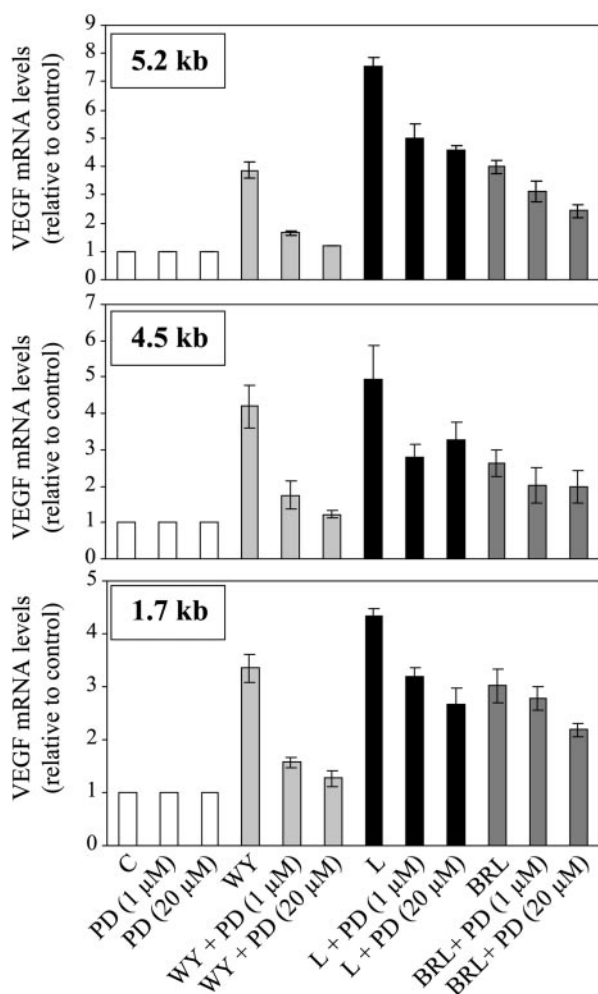


FIG. 9. The MAP kinase pathway inhibitor PD98059 decreases PPAR agonist-induced VEGF mRNA expression in RT4 cells. At confluence, RT4 cells were cultured in serum-free Mc COY's 5a medium with vehicle alone (less than 0.1% ethanol or Me₂SO), 1 μM or 20 μM PD98059, 50 μM WY 14,643 (WY), 25 μM L-165041 (L), 10 μM BRL 49653. (BRL) alone or in the presence of 1 μM or 20 μM PD98059 for 24 h. Total RNA was extracted, and 30 μg of RNA were subjected to Northern blotting analysis performed with [³²P]dCTP-labeled probes for VEGF and β-actin as described in detail under "Experimental Procedures." Densitometric quantitation of the 5.2-, 4.5-, and 1.7-kb bands of VEGF mRNA was performed. ³²P-VEGF and β-actin signals were quantified by phosphorimager analysis. Data represent the VEGF mRNA level values relative to the appropriate control value set at 1. These values were calculated after normalization of VEGF mRNA signals to β-actin signals from each lane. Values are the mean ± S.D. of four measurements for each treatment from one experiment.

Then how can the differential regulation of VEGF expression between the RT4 and T24 cell lines be explained? On the one hand we could assume that the differential regulation of VEGF by PPAR depends on the differentiated state of the cells and/or the fact that the products of the antioncogene p53 and the protooncogene H-Ras are mutated or not. On the other hand, recent data have demonstrated that fatty acids and hypolipidemic drugs regulated PPAR α - and γ -mediated gene expression via liver fatty acid-binding protein, (L-FABP) (54). Wolfrum *et al.* (54) have provided evidence that L-FABP interacts with PPAR α and PPAR γ , but not with PPAR β , through protein/protein contacts. L-FABP might be a possible candidate for allowing signaling molecules to reach the nuclear receptors. Furthermore, a loss of A-FABP (adipocyte-type fatty acid-binding protein) is associated with the progression of human bladder TCC (55). In fact, the percentage of tumors expressing A-FABP is very high in low grade lesions but decreased dras-

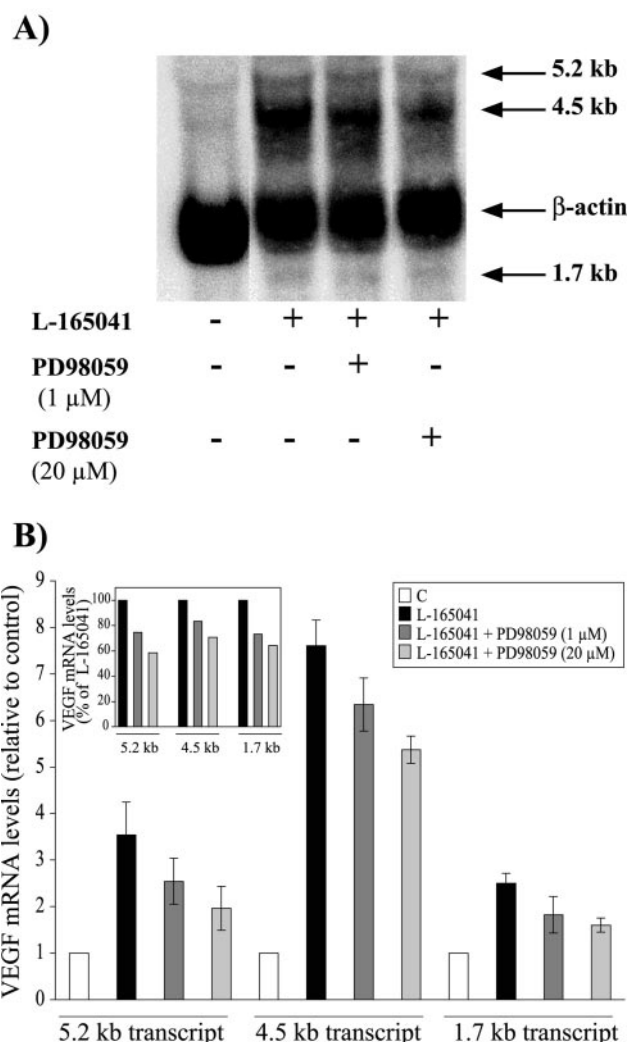


FIG. 10. The MEK 1 inhibitor PD98059 decreases L-165041-induced VEGF mRNA expression in T24 cells. The confluent monolayer of T24 cells was cultured in serum-free Mc COY's 5a medium with vehicle alone (less than 0.1% ethanol or Me₂SO), 25 μM L-165041, 1 μM or 20 μM PD98059, or L-165041 plus PD98059 (1 μM or 20 μM) for 24 h. Total RNA was isolated and used for Northern blot analysis performed with [³²P]dCTP-labeled probes for VEGF and β-actin as described in detail under "Experimental Procedures." A, Northern blot of VEGF mRNA with the three bands of 5.2-, 4.5-, and 1.7-kb, respectively. B, densitometric quantitation of the 5.2-, 4.5-, and 1.7-kb bands of VEGF mRNA. ³²P-VEGF and β-actin signals were quantified by phosphorimager analysis. Data represent the VEGF mRNA level values relative to the appropriate control value set at 1. These values were calculated after normalization of VEGF mRNA signals to β-actin signals from each lane. *Inset*, the VEGF mRNA level value of L-165041-treated cells was set at 100% for each transcript, and the VEGF mRNA level values in the presence of the kinase inhibitor PD98059 were calculated as percentages of the value obtained in the L-165041-treated cells. Values are the mean ± S.D. of four measurements for each treatment from one experiment.

tically in grade III and IV neoplasms. A-FABP seems to be a biomarker on which diagnosis and prognosis in TCC progression could be grounded.

In our model we hypothesize that the loss of expression of a protein related to the FABP family in grade III tumor-derived T24 cells leads to the absence of PPAR α and PPAR γ transactivation, which could also explain the absence of VEGF regulation by the PPAR α and γ isotypes in these cells. This FABP family protein would be present in low grade tumor-derived RT4 cells, allowing PPAR α and γ activation and leading to enhanced VEGF expression by both PPAR subtypes.

Obviously VEGF expression is tightly regulated by both

transcriptional and post-transcriptional mechanisms (56–58). As indicated by our half-life VEGF mRNA study, there was no stabilization of VEGF mRNA that suggested a regulation by PPAR at the transcriptional level. The transfection of the VEGF-1 plasmid containing the VEGF promoter located from –2279 to +56 revealed the absence of a cis-regulatory DNA sequence required for PPAR transcriptional activity in this region since luciferase activity was not induced by PPAR. The experiments in the presence of the protein synthesis inhibitor, cycloheximide, suggest that the up-regulation of VEGF mRNA expression by PPAR agonists requires the synthesis of new proteins. This indicates an indirect mechanism of VEGF gene regulation by PPARs. Thus, a regulatory protein could be induced by PPAR and then interact with the promoter of the VEGF gene. Further experiments in the presence of other promoter constructs are necessary to better understand the molecular mechanism involved in PPAR ligand-mediated VEGF mRNA expression. A time course of VEGF mRNA expression was performed in T24 cells (data not shown). The VEGF mRNA level was determined after cells were treated with L-165041 for 30 min, 4 h, and 24 h. The PPAR β agonist significantly enhanced VEGF mRNA expression after 24 h of stimulation, providing support for an indirect effect of PPAR on VEGF gene regulation.

Nevertheless, the PPAR ligand-induced VEGF expression seems to be PPAR-specific, because activation of the heterodimeric partner of these nuclear receptors potentiated the effect of PPAR agonists. The RXR-specific ligand LG10068 had no intrinsic effect on VEGF expression, but when combined with PPAR agonists, it had a greater effect on VEGF expression than with PPAR ligands alone.

PPAR α and PPAR γ are phosphoproteins. Their regulatory activity is dependent on their phosphorylated state in addition to ligand binding. The phosphorylation of these nuclear receptors is mediated by MAP kinase pathways (59, 60). The inhibition of transcriptional PPAR activity by MAP kinase inhibitors has already been reported in several studies (61, 62). The MAP kinase pathway is interesting to explore because it has been reported that angiostatin, an endogenous inhibitor of angiogenesis (63), diminished activation of the MAP kinases ERK1 and ERK2 in human dermal microvascular cells (64). We have subsequently analyzed the role of the signal-transducing molecules PI 3-kinase and MAP kinase. Our study revealed that the regulation of VEGF expression by PPAR was inhibited only by the ERK 1/2 inhibitor PD98059, suggesting that the MAP kinase pathway was involved in PPAR agonist-mediated VEGF mRNA induction.

In short, we have demonstrated for the first time a differential up-regulation of VEGF mRNA expression by PPAR agonists in human bladder cancer cells according to the differentiation state of the cells. This PPAR ligand-mediated effect is specific to PPAR and involves an indirect mechanism requiring an intermediary regulatory protein through the MAP (ERK 1/2) kinase pathway, probably by a modulation of the phosphorylation state of the receptors. Synthetic ligands for both PPAR α (fibrates) and PPAR γ (thiazolidinediones) are useful in the treatment of metabolic disorders such as hyperlipidemia, atherosclerosis, diabetes, and obesity. Our results demonstrate that these molecules are potential activators of angiogenesis. This effect has never been shown before in tumor cells. Because a lot of patients take anti-diabetic drugs and hypolipidemic agents, further exploration of the role of PPARs in human bladder cancer biology is crucial.

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