

Activation of Peroxisome Proliferator-activated Receptor α (PPAR α) Suppresses Hypoxia-inducible Factor-1 α (HIF-1 α) Signaling in Cancer Cells*

Received for publication, March 29, 2012, and in revised form, August 24, 2012. Published, JBC Papers in Press, August 29, 2012, DOI 10.1074/jbc.M112.367367

Jundong Zhou^{‡¶}, Shuyu Zhang^{§¶}, Jing Xue^{‡¶}, Jori Avery[¶], Jinchang Wu[‡], Stuart E. Lind[¶], and Wei-Qun Ding^{¶1}

From the [‡]Department of Radio-Oncology, Nanjing Medical University Affiliated Suzhou Hospital, and the [§]School of Radiation Medicine and Protection, Soochow University, Suzhou 205123, China, the [¶]Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, and the ¹Departments of Pathology and Medicine, University of Colorado Denver, Denver, Colorado 80217

Background: The mechanisms of PPAR α -mediated inhibition of tumor growth and angiogenesis remain unknown.

Results: Activation of PPAR α suppresses hypoxia-induced HIF-1 α signaling via promoting HIF-1 α degradation and diminishes hypoxia-induced VEGF secretion from cancer cells and tube formation by endothelial cells.

Conclusion: Activation of PPAR α suppresses the HIF-1 α signaling pathway in cancer cells.

Significance: The results support the development of PPAR α agonists as anticancer agents.

Activation of peroxisome proliferator-activated receptor α (PPAR α) has been demonstrated to inhibit tumor growth and angiogenesis, yet the mechanisms behind these actions remain to be characterized. In this study, we examined the effects of PPAR α activation on the hypoxia-inducible factor-1 α (HIF-1 α) signaling pathway in human breast (MCF-7) and ovarian (A2780) cancer cells under hypoxia. Incubation of cancer cells under 1% oxygen for 16 h significantly induced HIF-1 α expression and activity as assayed by Western blotting and reporter gene analysis. Treatment of the cells with PPAR α agonists, but not a PPAR γ agonist, prior to hypoxia diminished hypoxia-induced HIF-1 α expression and activity, and addition of a PPAR α antagonist attenuated the suppression of HIF-1 α signaling. Activation of PPAR α attenuated hypoxia-induced HA-tagged HIF-1 α protein expression without affecting the HA-tagged HIF-1 α mutant protein level, indicating that PPAR α activation promotes HIF-1 α degradation in these cells. This was further confirmed using proteasome inhibitors, which reversed PPAR α -mediated suppression of HIF-1 α expression under hypoxia. Using the co-immunoprecipitation technique, we found that activation of PPAR α enhances the binding of HIF-1 α to von Hippel-Lindau tumor suppressor (pVHL), a protein known to mediate HIF-1 α degradation through the ubiquitin-proteasome pathway. Following PPAR α -mediated suppression of HIF-1 α signaling, VEGF secretion from the cancer cells was significantly reduced, and tube formation by endothelial cells was dramatically impaired. Taken together, these findings demonstrate for the first time that activation of PPAR α suppresses hypoxia-induced HIF-1 α signaling

in cancer cells, providing novel insight into the anticancer properties of PPAR α agonists.

Hypoxia is an established characteristic of all solid tumors and is thought to be due to abnormal tumor microvasculature (1). To survive hypoxic conditions, tumor cells undergo a series of genetic and metabolic changes, such as enhanced glycolysis and survival factor overexpression (2, 3). Hypoxia-inducible factor-1 α (HIF-1 α),² a transcription factor, plays a key role in hypoxia-inducible gene expression. The expression of HIF-1 α is low under normoxia but highly inducible by hypoxia (3). Under hypoxic conditions, HIF-1 α translocates into the nucleus, dimerizes with HIF-1 β and other transcription factors, binds to the hypoxia response element (HRE), and transcriptionally activates hypoxia-inducible genes (4–6). HIF-1 α is associated with tumor growth, angiogenesis, metastasis, chemo/radioresistance, and poor prognosis (7). Compelling evidence indicates that blocking HIF-1 α activity or targeting HIF-1 α expression slows tumor growth and increases sensitivity of tumor cells to conventional therapy (1). Thus, targeting HIF-1 α is a strategy for cancer treatment.

Clofibrate and fenofibrate, two well known ligands for peroxisome proliferator-activated receptor α (PPAR α), have been widely used to control plasma levels of cholesterol and triglycerides, and they increase lipoprotein lipase activity (8). Recent studies demonstrated that these two drugs have anticancer properties in various model systems (9–11), but the mechanisms behind their actions remain to be elucidated. We have reported previously that activation of PPAR α mediates the anticancer action of docosahexaenoic acid, likely through down-regulation of hypoxia signaling (12, 13). We therefore hypothesized that the anticancer properties of PPAR α agonists

* This work was supported, in whole or in part, by National Institutes of Health Oklahoma IDeA Network of Biomedical Research Excellence (OK-INBRE) Program Grant 3P20RR016478-09S2. This work was also supported by American Cancer Society Grant CNE-117557, Susan G. Komen for the Cure Foundation Grant KG081083, and Oklahoma Center for the Advancement of Science and Technology Grant HR09-025.

¹ To whom correspondence should be addressed: University of Oklahoma Health Sciences Center, 975 NE 10th St., BRC 411A, Oklahoma City, OK 73104. Tel.: 405-271-1605; Fax: 405-271-3190; E-mail: wei-qun-ding@ouhsc.edu.

² The abbreviations used are: HIF-1 α , hypoxia-inducible factor-1 α ; HRE, hypoxia response element; PPAR α , peroxisome proliferator-activated receptor α ; RXR α , retinoid X receptor α ; HO-1, heme oxygenase 1; ANOVA, analysis of variance.

PPAR α Suppresses HIF-1 α Signaling

may similarly be due to suppression of HIF-1 α signaling in human cancer cells. Note that activation of the HIF-1 α gene has been reported to down-regulate the levels of retinoid X receptor α (RXR α), the obligate partner of PPAR α , which results in decreased DNA binding activity of PPAR α /RXR and reduced expression of the PPAR α -activated genes (14). To date, there have been no data examining the effects of PPAR α activation on HIF-1 α signaling in any experimental model systems. In this study, we report the effects of clofibrate and fenofibrate on HIF-1 α signaling in human breast and ovarian cancer cell model systems. We found that activation of PPAR α diminished hypoxia-induced HIF-1 α expression and activity, primarily through promoting HIF-1 α protein degradation. As HIF-1 α is known to enhance tumor growth, angiogenesis, and metastasis (15), our results support the development of PPAR α agonists as therapeutic anticancer agents.

EXPERIMENTAL PROCEDURES

Materials—The pGL3-HRE-luciferase reporter construct containing the HREs of the VEGF gene promoter was kindly provided by Dr. Konstantin Salnikow (Radiation Oncology Branch, NCI, Frederick, MD). The peroxisome proliferator response element-luciferase reporter construct was obtained from Dr. Bruce Spiegelman (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA), and the RXR α cDNA construct from Dr. Ronald C. Kahn (Joslin Diabetes Center, Harvard Medical School, Boston, MA). The pcDNA3-HA-HIF-1 α and pcDNA3-HA-HIF-1 α (P402A/P564A) (with two prolyl mutations) expression vectors were from Dr. William Kaelin (Dana-Farber Cancer Institute, Harvard Medical School). Anti-HIF-1 α antibody was from R&D Systems (Minneapolis, MN). Anti-HA tag and anti-pVHL antibodies were from Cell Signaling Technology, Inc. (Danvers, MA). Anti-GAPDH antibody was from ProMab Biotechnologies, Inc. (Albany, CA). Anti-heme oxygenase 1 (HO-1) antibody was from Stressgen (Ann Arbor, MI). The Dual-Luciferase reporter kit was purchased from Promega (Madison, WI). The VEGF ELISA kit was purchased from R&D Systems, Inc. Anti- β -actin antibody, clofibrate, fenofibrate, troglitazone, GW6471, GW9662, and other chemical agents were analytic grade and purchased from Sigma-Aldrich.

Cell Culture—The breast cancer cell line MCF-7 and the human umbilical vein cell line EA.hy926 was purchased from American Type Culture Collection (Manassas, VA). The ovarian carcinoma cell line A2780 was a kind gift from Dr. Stephen Howell (University of California, San Diego, CA). MCF-7 and A2780 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a humidified environment containing 5% CO₂. EA.hy926 cells were cultivated in DMEM supplemented with 10% fetal bovine serum as specified by American Type Culture Collection. Cells were subcultivated twice per week in 75- or 150-mm flasks.

Transient Transfection and Luciferase Activity Assay—MCF-7 cells and A2780 cells were seeded into 100-mm cell culture dishes and reached 70–80% confluence 24 h after plating. The cells were then transfected with the pGL3-HRE-luciferase plasmid using FuGENE HD transfection reagent (Roche Applied Science) as described previously (16). The next day, cells were

split into 24-well plates at a density of 2×10^5 cells/well. 48 h after transfection, cells were treated for 4 h with clofibrate, fenofibrate, troglitazone, and other reagents at the indicated concentrations and durations and then placed into a hypoxia chamber (1% O₂ and 5% CO₂; ProOx model C21, Biospherix, Ltd., Lacona, NY) for 16 h. Cell lysate was prepared, and luciferase activity was assayed using the Dual-Luciferase reporter kit as described (16). The firefly luciferase activity was normalized for the amount of protein used for luciferase activity assay for each sample. The data are expressed as percentages of luciferase activity detected in untreated cells.

Overexpression of Wild-type and Mutant HIF-1 α in MCF-7 Cells—MCF-7 cells were seeded in 100-mm dishes and reached 70–80% confluence overnight. The cells were then transfected with the pcDNA3-HA-HIF-1 α and pcDNA3-HA-HIF-1 α (P402A/P564A) constructs using FuGENE HD transfection reagent as described previously (16). 72 h after transfection, overexpression of HA-tagged HIF-1 α and its mutant was verified using an antibody against HA. The cells transiently overexpressing HA-HIF-1 α and its mutant were subjected to the experiments testing the assumption that clofibrate may promote HIF-1 α degradation in these cells.

Western Blot Analysis—Western blotting was performed as described (17, 18). In brief, cells were lysed with the lysis buffer, sonicated on ice for three strokes of 10 s each, and centrifuged at $15,000 \times g$ for 15 min to remove insoluble material. 40 μ g of protein from each sample was separated on a 10% SDS-polyacrylamide gel; transferred to a PVDF membrane; and blotted with antibodies against HIF-1 α , HA, pVHL, HO-1, GAPDH, and β -actin.

Transient Knockdown of PPAR α and pVHL—siRNAs for PPAR α and pVHL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Each product is a pool of three target-specific 19–25-nucleotide siRNAs designed to knock down target gene expression. Scrambled siRNAs were applied as controls. siRNAs (50 or 100 pmol) were transfected into MCF-7 cells cultured in a 6-well plate using FuGENE HD transfection reagent according to the manufacturer's protocols. 48 h after the transfection, the cells were treated with 500 nM clofibrate for 4 h and placed into a hypoxia chamber or kept under normoxic conditions for 16 h. The knockdown was confirmed by Western blot analysis. Individual siRNAs in this siRNA pool were also purchased and used to demonstrate the knockdown of PPAR α and pVHL under multiple siRNA conditions in MCF-7 cells.

Co-immunoprecipitation—Co-immunoprecipitation was performed as described previously (16). In short, MCF-7 cells were treated with various agents under hypoxia. Before the cells were placed into the hypoxia chamber for 16 h, 10 mM MG132 was added to each dish (19). The cells were then washed with cold phosphate-buffered saline and harvested by adding 150 μ l of immunoprecipitation buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Cells were sonicated for 1 min with intervals on ice and centrifuged at $13,000 \times g$ for 30 min to remove insoluble material. Following preclearing for 1 h at 4 °C, total cell extract (200 μ g of protein) was incubated with anti-HIF-1 α antibody at 4 °C with gentle rotation overnight. The

antibody-protein complexes were precipitated by addition of 50 μ l of protein G-agarose and rotation for 2 h at 4 $^{\circ}$ C. The supernatants were then removed by centrifugation, and the pellets were washed with immunoprecipitation buffer and subjected to Western blotting with antibodies against pVHL and HIF-1 α .

RT-PCR—Total RNA was isolated from MCF-7 cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. RNA samples were reverse-transcribed with the SuperScript II kit (Invitrogen) as described previously (16). The cDNA was amplified by PCR using the following specific primers: HIF-1 α , 5'-CCT CAG TCT ACA CAG CCT G-3' (forward) and 5'-CAT ATC TGA AGA TTC AAC C-3' (reverse); VEGF, 5'-TCG GGC CTC CGA AAC CAT G-3' (forward) and 5'-CCT GGT GAG AGA TCT GGT TC-3' (reverse); and β -actin, 5'-GGA AAT CGT GCG TGA CAT TA-3' (forward) and 5'-GGA GCA ATG ATC TTG ATC TTC-3' (reverse). The samples were initially denatured at 94 $^{\circ}$ C for 2 min prior to thermal cycling. The thermal cycle for PCR was as follows: 94 $^{\circ}$ C for 15 s, 48 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min, for a total 30 cycles. The PCR products were separated on a 1% agarose gel containing ethidium bromide and visualized under ultraviolet light.

ELISA—Secretion of VEGF from MCF-7 cells was determined using an ELISA kit. Cells were seeded into 6-well plates at a density of 1×10^6 cells/well and treated with clofibrate or troglitazone for 4 h prior to placement into the hypoxia chamber for 16 h. The culture medium was then collected, and the level of VEGF in the medium was analyzed following the manufacturer's instructions. VEGF levels were normalized to cell numbers and are expressed as picograms/million liters of medium.

Tube Formation Analysis—The wells of a 96-well plate were coated with ice-cold BD MatrigelTM matrix gel solution (BD Biosciences) and allowed to polymerize for 1 h at 37 $^{\circ}$ C. Conditioned medium was prepared by treating MCF-7 cells with or without clofibrate under hypoxic conditions for 16 h. The medium was collected and centrifuged to remove damaged/detached cells prior to culturing endothelial cells. EA.hy926 cells, a human umbilical vein cell line with characteristics of differentiated endothelial cells, were plated into the polymerized cell matrix at a concentration of 1×10^4 cells in 150 μ l of conditioned medium/well. The formation of endothelial tubes were observed microscopically and photographed after 24 h of incubation. Three experiments were performed with triplicates each time. Tube formation was quantified using the "Pattern Recognition" method specified by the manufacturer.

Statistical Analysis—All statistical analysis was done with GraphPad Prism software. One-way analysis of variance (ANOVA) with Dunnett's post-test was used to determine differences among control and experimental groups, with $p < 0.05$ or 0.01 as the level of statistical significance.

RESULTS

Activation of PPAR α by Clofibrate and Fenofibrate in MCF-7 Cells—To understand whether the PPAR α ligands clofibrate and fenofibrate activate PPAR α signaling in MCF-7 cells, cells were transfected with 3 μ g of the peroxisome proliferator

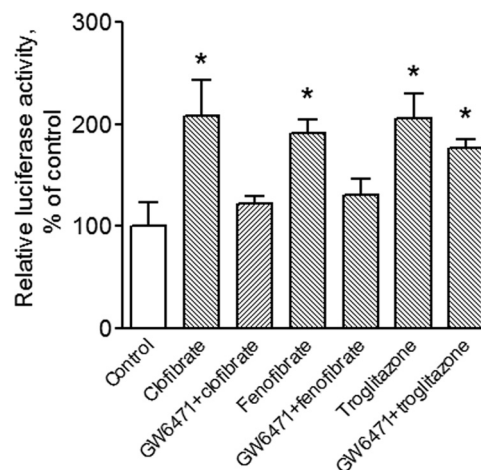


FIGURE 1. Clofibrate and fenofibrate activate PPAR α signaling in MCF-7 cells. Cells were transfected with the peroxisome proliferator response element-luciferase reporter and RXR α cDNA constructs and treated with 500 μ M clofibrate, 100 μ M fenofibrate, and 20 μ M troglitazone in the presence or absence of 5 μ M GW6471 for 4 h. Cell lysates were prepared, and luciferase activity was assayed. Data (mean \pm S.E., $n = 3$) are expressed as percentages of the luciferase activity detected in untreated control cells. *, $p < 0.05$ compared with control cells using one-way ANOVA, followed by Dunnett's analysis.

response element-luciferase plasmid construct and 1 μ g of RXR α cDNA (13) and treated with 500 μ M clofibrate, 100 μ M fenofibrate (two established PPAR α agonists), or 20 μ M troglitazone (a PPAR γ agonist) for 4 h. The concentrations of the compounds were chosen according to previous studies (20, 21). As expected, all three compounds activated peroxisome proliferator response element-driven reporter gene activity in MCF-7 cells (Fig. 1). However, pretreatment of the cells with a well established PPAR α antagonist, GW6471 (5 μ M), reversed PPAR activation by clofibrate and fenofibrate but had no significant effect on troglitazone-induced reporter gene activity, indicating that clofibrate and fenofibrate activate PPAR α in this model system.

Activation of PPAR α Suppresses Hypoxia-induced HIF-1 α Expression and Activity—To investigate the effects of activation of PPAR α on hypoxia-induced HIF-1 α expression and activity, MCF-7 cells were treated with clofibrate at various concentrations for 4 h prior to placing the cells into a hypoxia chamber with a gas mixture of 94% N₂, 5% CO₂, and 1% O₂ for 16 h. As shown in Fig. 2 (A and B), hypoxia induced a dramatic increase in HIF-1 α expression as assayed by Western blotting and in HRE-driven reporter activity as analyzed using the reporter gene technique. Addition of clofibrate suppressed hypoxia-induced HIF-1 α expression and activity in a concentration-dependent manner (Fig. 2, A and B). To confirm that this suppression of HIF-1 α signaling is not a cell type-related event, A2780 cells were also subjected to the same experimental conditions. Hypoxia-induced HIF-1 α expression and activity were also suppressed by clofibrate in A2780 cells (Fig. 2, C and D), similar to the results obtained in MCF-7 cells.

To further establish that activation of PPAR α suppresses HIF-1 α expression and activity, fenofibrate and troglitazone, two well established PPAR agonists, were applied to MCF-7 cells. HRE-luciferase reporter assay and Western blot analysis showed that hypoxia-induced HIF-1 α expression and activity

PPAR α Suppresses HIF-1 α Signaling

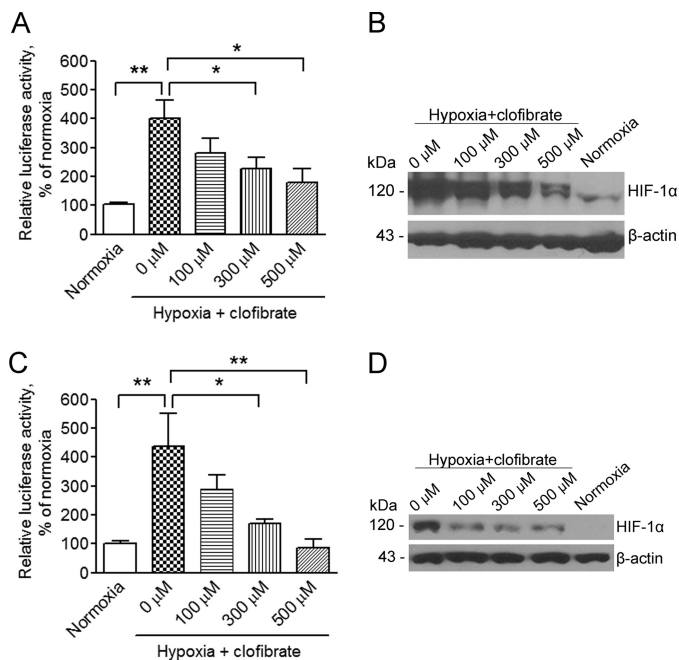


FIGURE 2. Clofibrate suppresses hypoxia-induced HIF-1 α expression and activity in a concentration-dependent manner. *A*, MCF-7 cells were transfected with the pGL3-HRE-luciferase reporter construct. Cells were treated with different concentrations of clofibrate for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and luciferase activity was assayed. Data (mean \pm S.E., $n = 3$) are expressed as percentages of the luciferase activity detected in untreated cells under normoxia. *, $p < 0.05$; **, $p < 0.01$ compared with untreated cells using one-way ANOVA, followed by Dunnett's analysis. *B*, MCF-7 cells were treated with different concentrations of clofibrate for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and Western blotting was performed using antibodies against HIF-1 α and β -actin. Shown are representative images of three individual experiments. *C*, A2780 cells were transfected with the pGL3-HRE-luciferase reporter construct. Cells were treated with different concentrations of clofibrate for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and luciferase activity was assayed. Data (mean \pm S.E., $n = 3$) are expressed as percentages of the luciferase activity detected in untreated cells under normoxia. *, $p < 0.05$; **, $p < 0.01$ compared with untreated cells using one-way ANOVA, followed by Dunnett's analysis. *D*, A2780 cells were treated with different concentrations of clofibrate for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and Western blotting was performed using antibodies against HIF-1 α and β -actin. Shown are representative images of three individual experiments.

were significantly down-regulated by fenofibrate, a PPAR α agonist, but not by troglitazone, a PPAR γ agonist (Fig. 3, *A* and *B*). In addition, the inhibitory effects of clofibrate were reversed by GW6471 (5 μ M), a potent PPAR α antagonist. GW9662, a PPAR γ antagonist, did not alter the inhibitory effects of clofibrate on hypoxia-induced HIF-1 α expression or activity (Fig. 3, *C* and *D*). Furthermore, transient knockdown of PPAR α using siRNA partially reversed clofibrate-induced suppression of HIF-1 α expression in MCF-7 cells. The knockdown of PPAR α was confirmed using multiple siRNA conditions (Fig. 3, *E* and *F*). Taken together, these findings indicate that it is the activation of PPAR α that accounts for the suppression of hypoxia-induced HIF-1 α expression and activity by clofibrate and fenofibrate.

Activation of PPAR α Promotes HIF-1 α Protein Degradation—Activation of PPAR α may suppress HIF-1 α expression through transcriptional or post-transcriptional regulation. We therefore performed RT-PCR analysis to test whether HIF-1 α mRNA expression is regulated by PPAR α activation. At con-

centrations that suppressed hypoxia-induced HIF-1 α protein expression, neither clofibrate nor fenofibrate significantly altered HIF-1 α mRNA levels (Fig. 4*A*), suggesting that the suppression takes place at the protein level. MG132, a proteasome inhibitor that prevents degradation of ubiquitinated HIF-1 α protein (19), and bortezomib, a structurally unrelated proteasome inhibitor known to enhance HIF-1 α protein levels in cancer cells (22), were then used to treat MCF-7 cells prior to hypoxia. Both MG132 and bortezomib reversed the clofibrate-induced suppression of HIF-1 α expression (Fig. 4*B*), suggesting that clofibrate suppresses hypoxia-induced HIF-1 α expression by promoting HIF-1 α degradation.

It is well established that degradation of HIF-1 α protein is regulated by oxygen-dependent prolyl hydroxylation (23). Hydroxylation of prolines 402 and 564 in HIF-1 α by prolyl hydroxylases is required for the recognition of HIF-1 α protein by pVHL. The recognition of HIF-1 α by pVHL allows ubiquitination and subsequent HIF-1 α degradation (24–26). To investigate whether degradation of HIF-1 α induced by PPAR α activation is involved with hydroxylation of Pro-402 and Pro-564 of HIF-1 α are essential for this effect.

Because the interaction of HIF-1 α and pVHL is critical for degradation of HIF-1 α , we performed co-immunoprecipitation to test whether clofibrate affects the binding of HIF-1 α to pVHL under hypoxia, using MG132 to block HIF-1 α degradation (Fig. 4*D*). pVHL expression was detected after immunoprecipitation with anti-HIF-1 α antibody, confirming an interaction of HIF-1 α with pVHL in this model system. Semiquantification of the Western blots by densitometric analysis showed that this interaction was enhanced by clofibrate treatment (Fig. 4*D* and Table 1), an observation consistent with the conclusion that activation of PPAR α promotes HIF-1 α degradation, thereby suppressing HIF-1 α signaling in cancer cells. To further confirm the involvement of pVHL in PPAR α -mediated suppression of HIF-1 α signaling, we knocked down pVHL expression in MCF-7 cells using multiple siRNA conditions (Fig. 5*A*). The suppression of HIF-1 α signaling by clofibrate was attenuated by knocking down pVHL (Fig. 5*B*), supporting a critical role of pVHL in PPAR α -mediated down-regulation of HIF-1 α signaling.

Activation of PPAR α Inhibits Expression of the Hypoxia-inducible Genes and Tube Formation by EA.hy926 Cells—To understand the consequences of the suppression of HIF-1 α signaling through activation of PPAR α , we analyzed the expression of VEGF (15), HO-1 (27), and GAPDH (28), three well recognized HIF-1 α target genes in MCF-7 cells. Under hypoxic conditions, the level of VEGF in the medium after culturing MCF-7 cells was significantly higher than that under normoxia. Treatment with clofibrate, but not troglitazone, reversed hypoxia-induced VEGF secretion from MCF-7 cells (Fig. 6*A*).

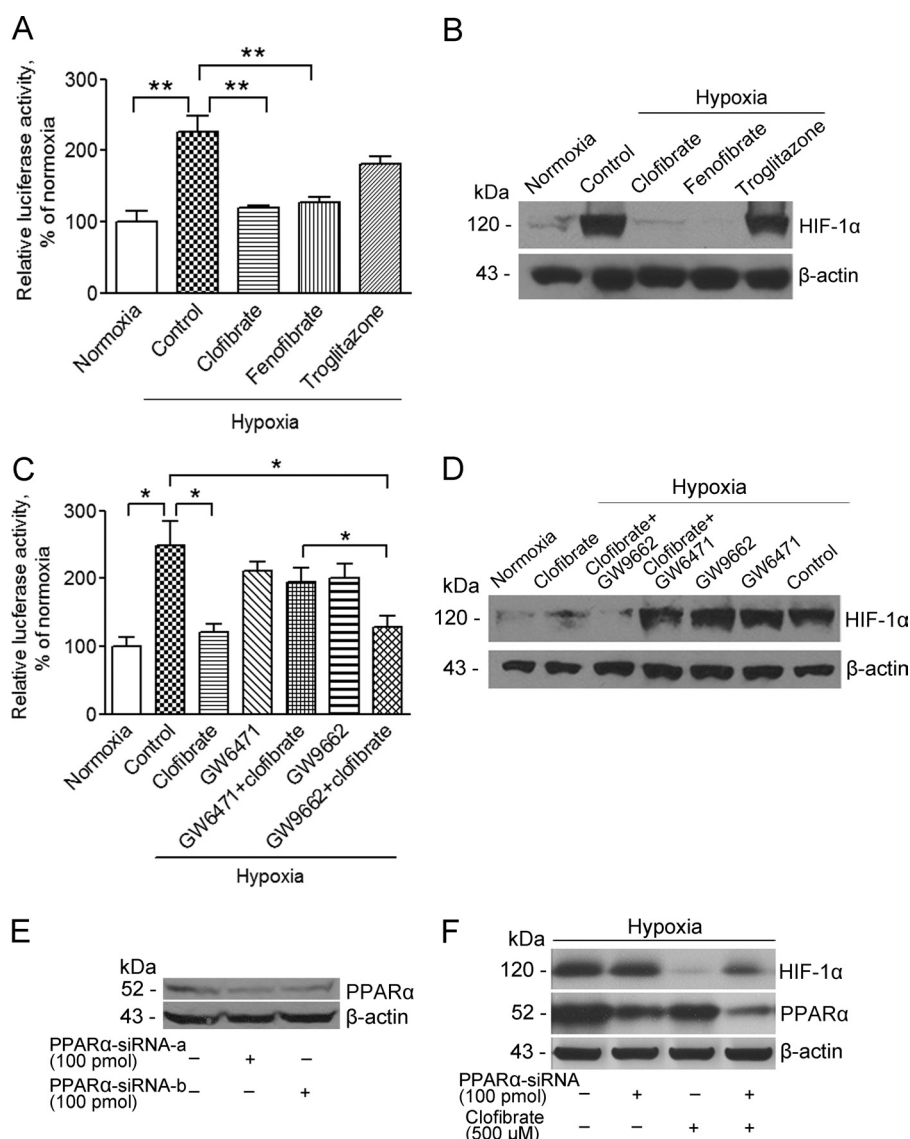


FIGURE 3. Clofibrate suppresses hypoxia-induced HIF-1 α expression and activity through PPAR α activation. *A*, MCF-7 cells were transfected with the pGL3-HRE-luciferase reporter construct. Cells were treated with 500 μ M clofibrate, 100 μ M fenofibrate, and 20 μ M troglitazone for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and luciferase activity was assayed. Data (mean \pm S.E., $n = 3$) are expressed as percentages of the luciferase activity detected in untreated cells under normoxia. **, $p < 0.01$ compared with untreated cells using one-way ANOVA, followed by Dunnett's analysis. *B*, MCF-7 cells were treated with 500 μ M clofibrate, 100 μ M fenofibrate, and 20 μ M troglitazone for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and Western blotting was performed using antibodies against HIF-1 α and β -actin. Shown are representative images of three individual experiments. *C*, MCF-7 cells were transfected with the pGL3-HRE-luciferase reporter construct. Cells were pretreated with 5 μ M GW6471 or 10 μ M GW9662 for 15 min prior to addition of 500 μ M clofibrate for another 4 h. The cells were then placed into a hypoxia chamber for 16 h. Cell lysate was prepared, and luciferase activity was assayed. Data (mean \pm S.E., $n = 3-5$) are expressed as percentages of the luciferase activity detected in untreated cells under normoxia. *, $p < 0.05$ compared with untreated cells using one-way ANOVA, followed by Dunnett's analysis. *D*, MCF-7 cells were pretreated with 5 μ M GW6471 or 10 μ M GW9662 for 15 min prior to addition of 500 μ M clofibrate for another 4 h. The cells were then placed into a hypoxia chamber for 16 h. Cell lysates were prepared, and Western blotting was performed using antibodies against HIF-1 α and β -actin. Shown are representative images of three individual experiments. *E*, MCF-7 cells were transfected with individual PPAR α siRNAs (100 pmol/ml); siRNA-a or siRNA-b) for 48 h. Cell lysates were prepared, and Western blotting was performed using antibodies against PPAR α and β -actin. *F*, MCF-7 cells were transfected with a pool of PPAR α siRNAs (100 pmol/ml; siRNA-a, siRNA-b, and siRNA-c) prior to treatment with 500 μ M clofibrate for 4 h. The cells were then placed into a hypoxia chamber for 16 h. Cell lysates were prepared, and Western blotting was performed using antibodies against HIF-1 α , PPAR α , and β -actin. Shown are representative images of three individual experiments.

The hypoxia-induced mRNA expression of the VEGF gene was also diminished by clofibrate and fenofibrate (data not shown), indicating that activation of PPAR α suppresses expression of the HIF-1 α target genes. This was further confirmed by the observation that protein expression of both HO-1 and GAPDH induced by hypoxia was reversed by treatment with clofibrate and fenofibrate in this model system (Fig. 6*B*).

To further elucidate the consequences of PPAR α -mediated suppression of HIF-1 α signaling, we examined the formation of

tubes by the endothelial cell line EA.hy926, utilizing a well recognized *in vitro* angiogenesis model system (5, 29–32). Tube formation by EA.hy926 cells was significantly increased when they were cultured in medium conditioned by the prior growth of MCF-7 cells grown under hypoxic conditions. If the conditioned medium was obtained from hypoxic MCF-7 cells treated with clofibrate or fenofibrate, tube formation was not enhanced, consistent with a decreased content of VEGF in the conditioned medium. The presence of troglitazone during the period when

PPAR α Suppresses HIF-1 α Signaling

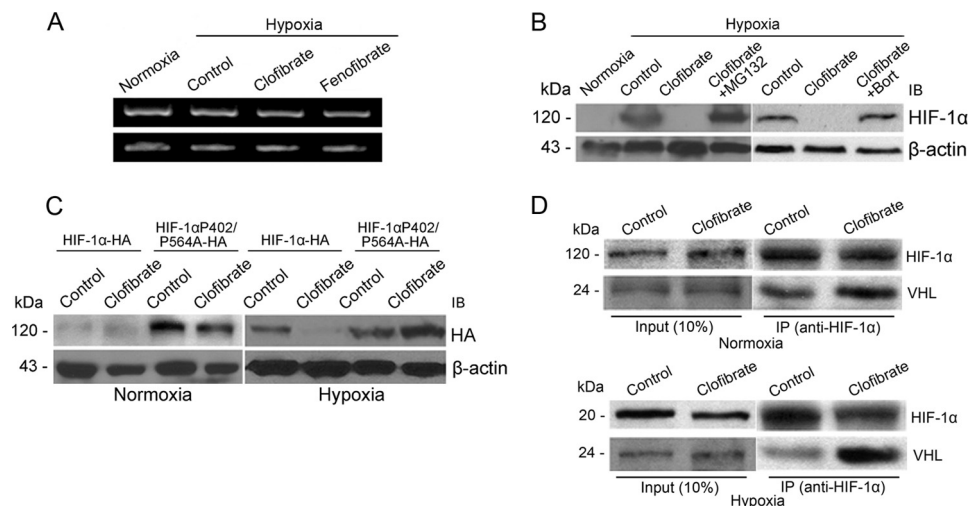


FIGURE 4. Clofibrate promotes HIF-1 α proteasomal degradation. *A*, MCF-7 cells were treated with 500 μ M clofibrate and 100 μ M fenofibrate for 4 h prior to placement in a hypoxia chamber for 16 h. RT-PCR was performed to determine the mRNA levels of HIF-1 α and β -actin. Shown are representative images of three individual experiments. *B*, MCF-7 cells were treated with 500 μ M clofibrate in the presence or absence of 10 μ M MG132 or 1 μ M bortezomib (*Bort*) for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and Western blotting was performed using antibodies against HIF-1 α and β -actin. Shown are representative images of three individual experiments. *IB*, immunoblot. *C*, MCF-7 cells were transfected with either HA-HIF-1 α expression vector or HA-HIF-1 α (P402A/P564A) mutant vector. After 48 h of transfection, cells were treated with 500 μ M clofibrate for 4 h prior to placement in a hypoxia chamber or kept under normoxic conditions for 16 h. Cell lysates were prepared, and Western blotting was performed using antibodies against HA and β -actin. Shown are representative images of three individual experiments. *D*, MCF-7 cells were treated with 500 μ M clofibrate for 4 h prior to addition of 10 μ M MG132. The cells were then either placed into a hypoxia chamber or kept under normoxic conditions for 16 h. Cell extracts were prepared, and equal amounts of cell extracts from each sample were immunoprecipitated (*IP*) with HIF-1 α antibody. The immunoprecipitates were separated on a 10% SDS-polyacrylamide gel and blotted with antibodies against HIF-1 α and pVHL. Shown are representative images of three individual experiments.

TABLE 1

Clofibrate enhances the interaction of HIF-1 α and pVHL (densitometric analysis of the Western blot data presented in Fig. 4D)

Intensities were normalized to input levels of the same proteins and are expressed as levels relative to controls under normoxia ($n = 3$).

	Normoxia		Hypoxia	
	Control	Clofibrate	Control	Clofibrate
pVHL	1.0	1.3 \pm 0.3	0.9 \pm 0.3	1.6 \pm 0.4 ^a
HIF-1 α	1.0	0.8 \pm 0.2	1.2 \pm 0.3	0.9 \pm 0.2

^a $p < 0.05$ compared with the control using one-way ANOVA.

hypoxic MCF-7 cells conditioned the medium did not alter tube formation by the EA.hy926 cells (Fig. 7). This observation is consistent with the suppression of hypoxia-induced VEGF secretion from MCF-7 cells, suggesting that activation of PPAR α inhibits tumor angiogenesis.

DISCUSSION

PPAR α is a nuclear receptor that belongs to the PPAR family, the members of which function as transcription factors regulating expression of many genes (33). Activation of PPAR α has been shown to play a key role in lipid metabolism, fatty acid oxidation, glucose homeostasis, and the inflammatory process (34). In recent years, PPAR α -specific agonists have been reported to inhibit the growth of various cancer cells in cultured cell lines and in xenograft nude mouse models (35–37). However, little is known about the molecular and cellular mechanisms of PPAR α -mediated growth inhibition of cancer cells. In this study, we demonstrated for the first time that activation of PPAR α suppresses hypoxia-induced HIF-1 α expression and activity and that this suppression is mediated through promoting HIF-1 α protein degradation in human cancer cells. These novel observations improve our understanding of the interaction of PPAR α and HIF-1 α signaling in human cancer cells.

We have made several interesting findings in the context of PPAR α and HIF-1 α signaling in cancer cells. The first relates to the clear demonstration that activation of PPAR α suppresses HIF-1 α signaling in both breast and ovarian cancer cells. The interaction of PPAR α and HIF-1 α signaling has been previously investigated in other model systems. An early study demonstrated that HIF-1 α mediates the inhibition of PPAR α expression under hypoxia in epithelial cells *in vitro* and *in vivo* (38). The down-regulation of PPAR α by hypoxia was recently observed in a study using human placentas (39). HIF-1 α was also shown to inhibit PPAR α signaling by reducing its DNA binding activity in cardiomyocytes (14). However, how PPAR α might regulate HIF-1 α signaling has not been described in any model systems, and the relationship between these two important nuclear receptor signaling pathways in cancer cells has been less characterized. The facts that PPAR α agonists are reported to inhibit tumor growth in various cancer model systems (10, 35–37) and that HIF-1 α is an established regulator of tumor growth, angiogenesis, and metastasis (15) led us to examine how activation of PPAR α might affect HIF-1 α expression and activity in cancer cells. We have shown that the PPAR α agonists clofibrate and fenofibrate diminish hypoxia-induced HIF-1 α expression and activity in our model systems. This is the first demonstration that activation of PPAR α suppresses HIF-1 α expression and activity, thus providing novel insight into the interaction of these two important signaling pathways in cancer.

Our second new finding relates to the observation that clofibrate promotes the degradation of HIF-1 α protein in cancer cells. It is well known that HIF-1 α protein degradation is regulated by oxygen (27). Under normoxia, HIF-1 α is hydroxylated by prolyl hydroxylases at Pro-402 and Pro-564, present in the

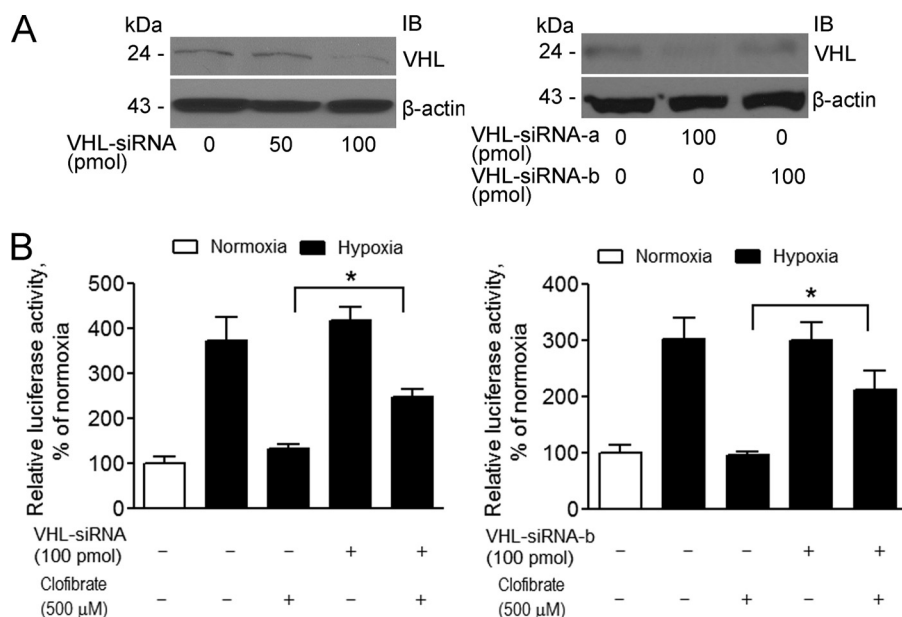


FIGURE 5. Knockdown of pVHL attenuates clofibrate-induced suppression of HIF-1 α signaling. *A, left panel*, MCF-7 cells were transfected with a pool of pVHL siRNAs (50–100 pmol/ml; siRNA-a, siRNA-b, and siRNA-c) for 48 h. *Right panel*, MCF-7 cells were transfected with individual pVHL siRNAs (100 pmol/ml; siRNA-a or siRNA-b) for 48 h. Cell lysates were prepared, and Western blotting was performed using antibodies against pVHL and β -actin. Shown are representative images of three individual experiments. *IB*, immunoblot. *B, left panel*, MCF-7 cells were cotransfected with a pool of pVHL siRNAs (100 pmol/ml) and the pGL3-HRE-luciferase reporter construct. *Right panel*, MCF-7 cells were cotransfected with pVHL siRNA-b (100 pmol/ml) and the pGL3-HRE-luciferase reporter construct. 48 h after transfection, cells were treated with 500 μ M clofibrate for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and luciferase activity was assayed. Data (mean \pm S.E., $n = 3$) are expressed as percentages of the luciferase activity detected in untreated cells under normoxia. *, $p < 0.05$ analyzed by one-way ANOVA analysis.

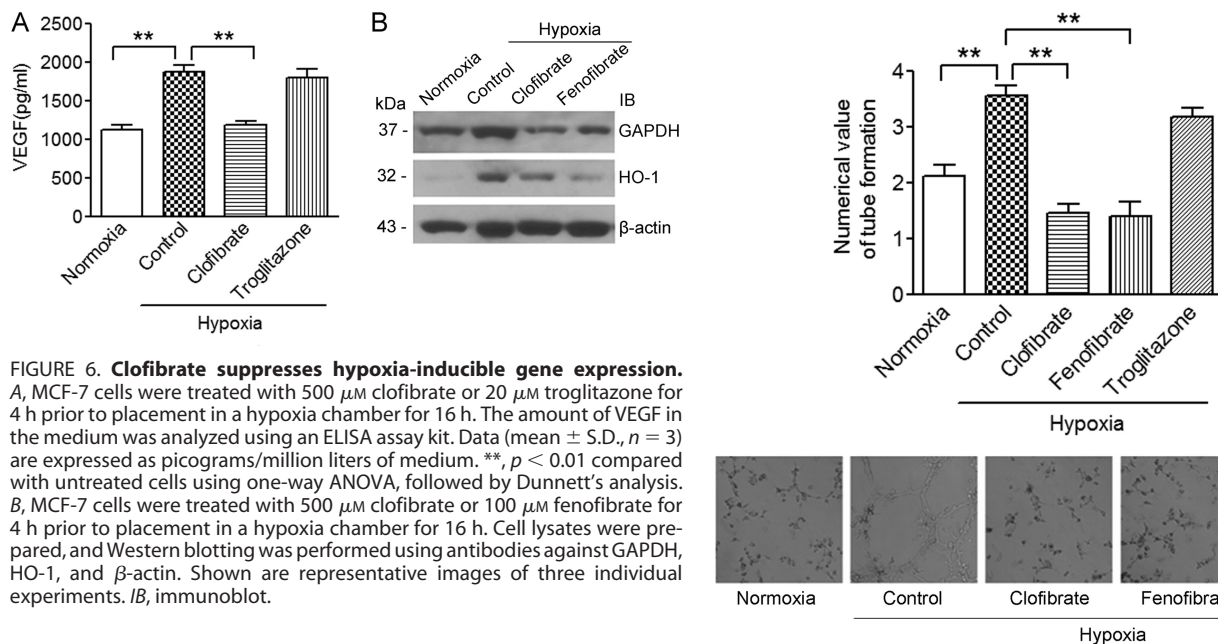


FIGURE 6. Clofibrate suppresses hypoxia-inducible gene expression. *A*, MCF-7 cells were treated with 500 μ M clofibrate or 20 μ M troglitazone for 4 h prior to placement in a hypoxia chamber for 16 h. The amount of VEGF in the medium was analyzed using an ELISA assay kit. Data (mean \pm S.D., $n = 3$) are expressed as picograms/million liters of medium. **, $p < 0.01$ compared with untreated cells using one-way ANOVA, followed by Dunnett's analysis. *B*, MCF-7 cells were treated with 500 μ M clofibrate or 100 μ M fenofibrate for 4 h prior to placement in a hypoxia chamber for 16 h. Cell lysates were prepared, and Western blotting was performed using antibodies against GAPDH, HO-1, and β -actin. Shown are representative images of three individual experiments. *IB*, immunoblot.

oxygen-dependent degradation domain of the protein. These modifications allow HIF-1 α to be recognized by pVHL, which is the recognition component of an E3 ubiquitin ligase, leading to HIF-1 α ubiquitination and subsequent proteasomal degradation (40). Several lines of evidence from our study support the conclusion that activation of PPAR α enhances HIF-1 α degradation in cancer cells. First, we have shown that HIF-1 α mRNA expression was unchanged after addition of clofibrate under hypoxic conditions, indicating that transcription of the HIF-1 α gene is not affected by activation of PPAR α . Second, when MG132 and bortezomib, two well established proteasome

FIGURE 7. Tube formation of EA.hy926 cells cultured in conditioned media. EA.hy926 cells resuspended in conditioned media were plated onto 96-well plates coated with Matrigel. The conditioned media were prepared from the media used to culture MCF-7 cells under various conditions (normoxia, hypoxia, hypoxia plus clofibrate, hypoxia plus fenofibrate, and hypoxia plus troglitazone). Tube formation of EA.hy926 cells was recorded 24 h after plating. Shown are representative images of three individual experiments (*lower panels*) and quantitative analysis following the manufacturer's formula (*upper panel*). **, $p < 0.01$ analyzed by one-way ANOVA, followed by Dunnett's analysis.

inhibitors (22, 41, 42), were applied to the cells under hypoxia, clofibrate-induced suppression of HIF-1 α expression was reversed, indicating that HIF-1 α protein degradation is tar-

PPAR α Suppresses HIF-1 α Signaling

geted by clofibrate. Third, we demonstrated that when HIF-1 α was overexpressed in MCF-7 cells, clofibrate treatment suppressed wild-type HIF-1 α expression but had no effect on mutant HIF-1 α (with mutation of Pro-402 and Pro-564) expression, strongly indicating that activation of PPAR α promotes HIF-1 α protein degradation. Finally, our co-immunoprecipitation experiments showed that clofibrate enhanced the interaction of HIF-1 α with pVHL under hypoxic conditions, suggesting that more HIF-1 α proteins are processed into the ubiquitin-proteasome degradation pathway upon activation of PPAR α in our model system. This was further confirmed by the observation that knockdown of pVHL significantly attenuated clofibrate-induced suppression of HIF-1 α signaling. Given the importance of both PPAR α and HIF-1 α signaling in cancer as well as in other diseases, the detailed mechanisms of how PPAR α activation leads to an enhanced interaction of pVHL with HIF-1 α protein, resulting in rapid degradation of HIF-1 α under hypoxia, merit further investigation.

Another interesting finding of this study relates to the consequences of PPAR α -mediated suppression of hypoxia-induced HIF-1 α expression and activity. This includes primarily the demonstration that clofibrate treatment down-regulates hypoxia-induced expression of VEGF in and secretion of VEGF from MCF-7 cells and suppresses hypoxia-induced tube formation (angiogenesis) by endothelial cells. The VEGF gene is a well established target of HIF-1 α signaling (15). Suppression of VEGF expression by PPAR α agonists has been described in other cancer model systems (43–46). Our findings complement previous reports and provide a mechanistic explanation of how PPAR α agonists suppress VEGF expression and angiogenesis, thus supporting the development of PPAR α agonists as effective anti-angiogenic agents for cancer therapy.

In conclusion, we have demonstrated that activation of PPAR α suppresses hypoxia-induced HIF-1 α signaling via promotion of HIF-1 α degradation in human cancer cells. These findings provide new insight into our understanding of the two important signaling pathways in cancer cells and support the use of PPAR α agonists as therapeutic anticancer agents.

Acknowledgment—We thank Dr. Minghui Zou (Department of Medicine, University of Oklahoma Health Sciences Center) for use of the hypoxia chamber.

REFERENCES

1. Poon, E., Harris, A. L., and Ashcroft, M. (2009) Targeting the hypoxia-inducible factor (HIF) pathway in cancer. *Expert Rev. Mol. Med.* **11**, e26
2. Kunz, M., and Ibrahim, S. M. (2003) Molecular responses to hypoxia in tumor cells. *Mol. Cancer* **2**, 23
3. Harris, A. L. (2002) Hypoxia—a key regulatory factor in tumor growth. *Nat. Rev. Cancer* **2**, 38–47
4. Kaelin, W. G., Jr., and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol. Cell* **30**, 393–402
5. Manalo, D. J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B. D., Ye, S. Q., Garcia, J. G., and Semenza, G. L. (2005) Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* **105**, 659–669
6. Elvidge, G. P., Glenny, L., Appelhoff, R. J., Ratcliffe, P. J., Ragoussis, J., and Gleadow, J. M. (2006) Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1 α , HIF-2 α , and other pathways. *J. Biol. Chem.* **281**, 15215–15226
7. Brown, J. M., and Wilson, W. R. (2004) Exploiting tumor hypoxia in cancer treatment. *Nat. Rev. Cancer* **4**, 437–447
8. Urbanska, K., Pannizzo, P., Grabacka, M., Croul, S., Del Valle, L., Khalili, K., and Reiss, K. (2008) Activation of PPAR α inhibits IGF-I-mediated growth and survival responses in medulloblastoma cell lines. *Int. J. Cancer* **123**, 1015–1024
9. Zak, Z., Gelebart, P., and Lai, R. (2010) Fenofibrate induces effective apoptosis in mantle cell lymphoma by inhibiting the TNF α /NF- κ B signaling axis. *Leukemia* **24**, 1476–1486
10. Yokoyama, Y., Xin, B., Shigeto, T., Umemoto, M., Kasai-Sakamoto, A., Futagami, M., Tsuchida, S., Al-Mulla, F., and Mizunuma, H. (2007) Clofibrinic acid, a peroxisome proliferator-activated receptor α ligand, inhibits growth of human ovarian cancer. *Mol. Cancer Ther.* **6**, 1379–1386
11. Grabacka, M., Placha, W., Plonka, P. M., Pajak, S., Urbanska, K., Laidler, P., and Slominski, A. (2004) Inhibition of melanoma metastases by fenofibrate. *Arch. Dermatol. Res.* **296**, 54–58
12. Tuller, E. R., Beavers, C. T., Lou, J. R., Ihnat, M. A., Benbrook, D. M., and Ding, W. Q. (2009) Docosahexaenoic acid inhibits superoxide dismutase 1 gene transcription in human cancer cells: the involvement of peroxisome proliferator-activated receptor α and hypoxia-inducible factor-2 α signaling. *Mol. Pharmacol.* **76**, 588–595
13. Tuller, E. R., Brock, A. L., Yu, H., Lou, J. R., Benbrook, D. M., and Ding, W. Q. (2009) PPAR α signaling mediates the synergistic cytotoxicity of clioquinol and docosahexaenoic acid in human cancer cells. *Biochem. Pharmacol.* **77**, 1480–1486
14. Belanger, A. J., Luo, Z., Vincent, K. A., Akita, G. Y., Cheng, S. H., Gregory, R. J., and Jiang, C. (2007) Hypoxia-inducible factor-1 mediates hypoxia-induced cardiomyocyte lipid accumulation by reducing the DNA binding activity of peroxisome proliferator-activated receptor α /retinoid X receptor. *Biochem. Biophys. Res. Commun.* **364**, 567–572
15. Rankin, E. B., and Giaccia, A. J. (2008) The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ.* **15**, 678–685
16. Zhang, X., Yu, H., Lou, J. R., Zheng, J., Zhu, H., Popescu, N. I., Lupu, F., Lind, S. E., and Ding, W. Q. (2011) MicroRNA-19 (miR-19) regulates tissue factor expression in breast cancer cells. *J. Biol. Chem.* **286**, 1429–1435
17. Ding, W. Q., Liu, B., Vaught, J. L., Palmiter, R. D., and Lind, S. E. (2006) Clioquinol and docosahexaenoic acid act synergistically to kill tumor cells. *Mol. Cancer Ther.* **5**, 1864–1872
18. Ding, W. Q., Liu, B., Vaught, J. L., Yamauchi, H., and Lind, S. E. (2005) Anticancer activity of the antibiotic clioquinol. *Cancer Res.* **65**, 3389–3395
19. Nardinocchi, L., Pantisano, V., Puca, R., Porru, M., Aiello, A., Grasselli, A., Leonetti, C., Safran, M., Rechavi, G., Givol, D., Farsetti, A., and D'Orazi, G. (2010) Zinc down-regulates HIF-1 α and inhibits its activity in tumor cells *in vitro* and *in vivo*. *PLoS ONE* **5**, e15048
20. Göttlicher, M., Widmark, E., Li, Q., and Gustafsson, J. A. (1992) Fatty acids activate a chimera of the clofibrinic acid-activated receptor and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4653–4657
21. Adams, M., Montague, C. T., Prins, J. B., Holder, J. C., Smith, S. A., Sanders, L., Digby, J. E., Sewter, C. P., Lazar, M. A., Chatterjee, V. K., and O'Rahilly, S. (1997) Activators of peroxisome proliferator-activated receptor γ have depot-specific effects on human preadipocyte differentiation. *J. Clin. Invest.* **100**, 3149–3153
22. Birle, D. C., and Hedley, D. W. (2007) Suppression of the hypoxia-inducible factor-1 response in cervical carcinoma xenografts by proteasome inhibitors. *Cancer Res.* **67**, 1735–1743
23. Brahimi-Horn, C., and Pouyssegur, J. (2006) The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. *Bull. Cancer* **93**, E73–E80
24. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001) HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* **292**, 464–468
25. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hy-

- droxylation. *Science* **292**, 468–472
26. Hoffman, M. A., Ohh, M., Yang, H., Klco, J. M., Ivan, M., and Kaelin, W. G., Jr. (2001) von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to down-regulate HIF. *Hum. Mol. Genet.* **10**, 1019–1027
 27. Semenza, G. L. (2003) Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer* **3**, 721–732
 28. Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y., and Semenza, G. L. (1998) Cellular and developmental control of O₂ homeostasis by hypoxia-inducible factor-1 α . *Genes Dev.* **12**, 149–162
 29. Bauer, J., Margolis, M., Schreiner, C., Edgell, C. J., Azizkhan, J., Lazarowski, E., and Juliano, R. L. (1992) *In vitro* model of angiogenesis using a human endothelium-derived permanent cell line: contributions of induced gene expression, G-proteins, and integrins. *J. Cell. Physiol.* **153**, 437–449
 30. Benndorf, R., Böger, R. H., Ergün, S., Steenpass, A., and Wieland, T. (2003) Angiotensin II type 2 receptor inhibits vascular endothelial growth factor-induced migration and *in vitro* tube formation of human endothelial cells. *Circ. Res.* **93**, 438–447
 31. Shim, J. S., Matsui, Y., Bhat, S., Nacev, B. A., Xu, J., Bhang, H. E., Dhara, S., Han, K. C., Chong, C. R., Pomper, M. G., So, A., and Liu, J. O. (2010) Effect of nitroxoline on angiogenesis and growth of human bladder cancer. *J. Natl. Cancer Inst.* **102**, 1855–1873
 32. Cui, Y., Han, Z., Hu, Y., Song, G., Hao, C., Xia, H., and Ma, X. (2012) MicroRNA-181b and microRNA-9 mediate arsenic-induced angiogenesis via NRP1. *J. Cell. Physiol.* **227**, 772–783
 33. Schoonjans, K., Staels, B., and Auwerx, J. (1996) The peroxisome proliferator-activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. *Biochim. Biophys. Acta* **1302**, 93–109
 34. Desvergne, B., and Wahli, W. (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr. Rev.* **20**, 649–688
 35. Shigeto, T., Yokoyama, Y., Xin, B., and Mizunuma, H. (2007) Peroxisome proliferator-activated receptor α and γ ligands inhibit the growth of human ovarian cancer. *Oncol. Rep.* **18**, 833–840
 36. Drukala, J., Urbanska, K., Wilk, A., Grabacka, M., Wybieralska, E., Del Valle, L., Madeja, Z., and Reiss, K. (2010) ROS accumulation and IGF-IR inhibition contribute to fenofibrate/PPAR α -mediated inhibition of glioma cell motility *in vitro*. *Mol. Cancer* **9**, 159
 37. Grabacka, M., Plonka, P. M., Urbanska, K., and Reiss, K. (2006) Peroxisome proliferator-activated receptor α activation decreases metastatic potential of melanoma cells *in vitro* via down-regulation of Akt. *Clin. Cancer Res.* **12**, 3028–3036
 38. Narravula, S., and Colgan, S. P. (2001) Hypoxia-inducible factor-1-mediated inhibition of peroxisome proliferator-activated receptor α expression during hypoxia. *J. Immunol.* **166**, 7543–7548
 39. Chang, T. T., Shyu, M. K., Huang, M. C., Hsu, C. C., Yeh, S. Y., Chen, M. R., and Lin, C. J. (2011) Hypoxia-mediated down-regulation of OCTN2 and PPAR α expression in human placentas and in BeWo cells. *Mol. Pharmacol.* **8**, 117–125
 40. Pouysségur, J., Dayan, F., and Mazure, N. M. (2006) Hypoxia signaling in cancer and approaches to enforce tumor regression. *Nature* **441**, 437–443
 41. Meriin, A. B., Gabai, V. L., Yaglom, J., Shifrin, V. I., and Sherman, M. Y. (1998) Proteasome inhibitors activate stress kinases and induce Hsp72. Diverse effects on apoptosis. *J. Biol. Chem.* **273**, 6373–6379
 42. Pan, J. A., Ullman, E., Dou, Z., and Zong, W. X. (2011) Inhibition of protein degradation induces apoptosis through a microtubule-associated protein 1 light chain 3-mediated activation of caspase-8 at intracellular membranes. *Mol. Cell. Biol.* **31**, 3158–3170
 43. Meissner, M., Stein, M., Urbich, C., Reisinger, K., Suske, G., Staels, B., Kaufmann, R., and Gille, J. (2004) PPAR α activators inhibit vascular endothelial growth factor receptor 2 expression by repressing Sp1-dependent DNA binding and transactivation. *Circ. Res.* **94**, 324–332
 44. Panigrahy, D., Kaipainen, A., Huang, S., Butterfield, C. E., Barnés, C. M., Fannon, M., Laforme, A. M., Chaponis, D. M., Folkman, J., and Kieran, M. W. (2008) PPAR α agonist fenofibrate suppresses tumor growth through direct and indirect angiogenesis inhibition. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 985–990
 45. Grau, R., Punzón, C., Fresno, M., and Iñiguez, M. A. (2006) Peroxisome proliferator-activated receptor α agonists inhibit cyclooxygenase 2 and vascular endothelial growth factor transcriptional activation in human colorectal carcinoma cells via inhibition of activator protein 1. *Biochem. J.* **395**, 81–88
 46. Grau, R., Díaz-Muñoz, M. D., Cacheiro-Llaguno, C., Fresno, M., and Iñiguez, M. A. (2008) Role of peroxisome proliferator-activated receptor α in the control of cyclooxygenase 2 and vascular endothelial growth factor: involvement in tumor growth. *PPAR Res.* **2008**, 352437