Mechanisms Regulating Tumor Angiogenesis by 12-Lipoxygenase in Prostate Cancer Cells*

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12-Lipoxygenase utilizes arachidonic acid to synthesize 12(S)-hydroperoxyeicosatetraenoic acid, which is converted to the end product 12(S)-hydroxyeicosatetraenoic acid, an eicosanoid that promotes tumorigenesis and metastasis. Increased expression of 12-lipoxygenase has been documented in a number of carcinomas. When overexpressed in human prostate or breast cancer, 12-lipoxygenase promotes tumor angiogenesis and growth in vivo. The present study was undertaken to delineate the mechanisms by which 12-lipoxygenase enhances angiogenesis. Herein we report that nordihydroguaiaretic acid, a pan inhibitor of lipoxygenases and baicalin, a selective inhibitor of 12-lipoxygenase, reduced VEGF expression in human prostate cancer PC-3 cells. Overexpression of 12-lipoxygenase in PC-3 cells resulted in a 3-fold increase in VEGF protein level when compared with vector control cells. An increase in PI 3-kinase activity was found in 12-LOX-transfected PC-3 cells and inhibition of PI 3-kinase by LY294002 significantly reduced VEGF expression. Northern blot and real time PCR analyses revealed an elevated VEGF transcript level in PC-3 cells transfected with a 12-lipoxygenase expression construct. Using a VEGF promoter luciferase construct (∼1176/+54), we found a 10-fold increase in VEGF promoter activity in 12-lipoxygenase-transfected PC-3 cells. The region located between −88 and −66 of the VEGF promoter was identified as 12-lipoxygenase responsive using VEGF promoter-based luciferase assays. Further analysis with mutant constructs indicated Sp1 as a transcription factor required for 12-lipoxygenase stimulation of VEGF. Neutralization of VEGF by a function-blocking antibody significantly decreased the ability of 12-lipoxygenase-transfected PC-3 cells to stimulate endothelial cell migration, suggesting VEGF as an important effector for 12-lipoxygenase-mediated stimulation of tumor angiogenesis.

Arachidonic acid metabolism is catalyzed by two major groups of enzymes, cyclooxygenases (COX)3 and lipoxygenases (1). Metabolites of these pathways are involved in various steps of carcinogenesis by modulation of cell signaling cascades and mitogenic pathways (2, 3). Several lines of evidence indicate 12-lipoxygenase (12-LOX) as a key regulator of human cancer development. Overexpression of 12-LOX has been detected in a variety of tumors including breast, renal, pancreatic, and prostate cancer (4–6). In addition, anti-angiogenic factors also play a role in regulating the angiogenic phenotype by cancer cells is a pivotal point in the progression and metastasis of solid tumors. Angiogenesis, a complex process involving the formation of new blood vessels from the existing vasculature, is a seminal event in the field of tumor biology (12). Angiogenesis is mediated by a complex interplay of a variety of factors which include tumor and endothelial cells, pro-angiogenic factors i.e. vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), proteolytic enzymes, for example MMPs, cell surface molecules, for example integrins and many other factors (13). In addition, anti-angiogenic factors also play a role in regulating blood vessel formation and the balance between the pro- and anti-angiogenic factors determines the rate of formation of the neovasculature. Pro-angiogenic gene expression is activated by a multitude of factors, which include physiological stimuli such as hypoxia, oncogene activation, and tumor suppressor mutations (14). Several experimental observations have proved that VEGF is a potent tumor angiogenesis factor. Agents that target VEGF and its signaling pathways inhibit tumor growth and propagation in various experimental models (15). The promoter region of the VEGF gene has binding sites for transcription fac-
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Isolation of RNA, Northern Blotting, and Real Time PCR Analyses—Poly(A) mRNAs were isolated from wild-type PC-3, neo-α, neo-σ, nL2, and nL8 cells using an oligotex mRNA kit (Qiagen, Valencia, CA). For Northern blot analysis, aliquots of poly(A) mRNA (2.5 μg) were subjected to electrophoresis on a 1% agarose formaldehyde gel and transferred to Duralon-UV membranes (Stratagene). The membranes were subjected to UV cross-linking in a Stratagene Crosslinker (Stratagene). The VEGF cDNA was kindly provided by Dr. Shin Ohnishi (21). Membranes containing the transferred RNAs were prehybridized with QuikHyb solution (Stratagene) at 65°C for 1 h and then hybridized with denatured [32P]VEGF cDNA and salmon sperm DNA at 65°C for 2 h. Membranes were washed with 2× standard saline citrate (SSC)/0.1% SDS for 15 min at room temperature twice and then rinsed with 0.1× SSC/0.1% SDS for 5 min at 65°C. Radioactivity from the membranes was monitored with a Geiger counter until the background was at low levels, and the wet membranes were wrapped with plastic wrap and exposed to Kodak XAR-5 film for 24 h at –80°C. The membranes were reprobed with the 32P-labeled human glyceraldehyde-3-phosphate dehydrogenase (Clontech Laboratory Inc, Palo Alto, CA) as a loading control for mRNA levels in each sample.

For Real Time PCR (RT-PCR) analysis, total RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturer’s protocol with an additional step of membrane DNase treatment (using an RNase-free DNase from Qiagen) to remove possible genomic DNA contamination. Total RNA was reverse-transcribed to cDNA using the SuperScript III first-strand synthesis system (Invitrogen) with oligo(dT)20 primer. For real time detection of target gene expression, TaqMan® Gene Expression assays (Applied Biosystems) were used. PCR was performed using Applied Biosystems 7000 Real-Time PCR System in a total reaction mixture of 25 μl containing 150 ng of cDNA, 1× ABI TaqMan PCR Master mix and 1.25 μl of probe/primer mixtures (TaqMan® Gene Expression assays). After denaturation at 95°C for 10 min, 40 cycles were performed at 95°C for 10 s, 60°C for 1 min (universal cycling conditions). Relative quantification was calculated using the comparative Ct method also known as the 2−ΔΔCt method (ΔΔCt = ΔCt sample − ΔCt reference) as described previously (22). Lower ΔCt values and lower ΔΔCt reflect a relatively higher amount of gene transcript.

Transient Transfection to Measure VEGF Promoter Activities—Cells were plated at 2 × 105 cells per 35-mm dish 2 days before transfection with the −1176/+54 VEGF promoter-luciferase construct, or other constructs as indicated, and a LacZ expression plasmid using GenePorter transfection reagent following the manufacturer’s protocol (Gene Therapy System). Cells were harvested for luciferase assay 36 h after transfection. Luciferase activity was measured using the luciferase assay kit (Promega, Madison, WI). In all co-transfection experiments, transfection efficiency was normalized by assaying β-galactosidase activity using the β-galactosidase assay kit (Invitrogen).

Immunoblotting—Immunoblotting was used to evaluate the level of 12-LOX protein expression, and phosphorylation of Akt. Semiconfluent (70–80%) cells were rinsed with ice-cold phosphate-buffered saline, scraped into lysis buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM leupeptin, 0.15 mM pepstatin A, 1 mM diithothreitol, and 1% Nonidet P-40. Protein concentration was measured using the BCA protein assay kit (Pierce) and 30-μg protein samples were mixed with 2× SDS sample buffer and subjected to electrophoresis in pre-made 4–20% gradient SDS-PAGE gels and transferred to a nitrocellulose membrane. After transfer, the membrane was blocked with TBS-T containing 5% low fat milk for 60 min, incubated with the primary antibody at the appropriate
RESULTS

Reduction of VEGF Expression by 12-LOX Inhibitors—Previous studies clearly have demonstrated that 12-LOX is expressed in prostate carcinoma cells (23) and the level of 12-LOX mRNA exhibits a positive correlation with tumor grade and stage (6). In addition, PC-3 cells overexpressing 12-LOX generate highly angiogenic tumors in mice (11). A similar observation, regarding the proangiogenic role for 12-LOX, also supports the view that 12-LOX plays a role in enhancing VEGF secretion from prostate cancer cells. To study whether 12-LOX regulates the production of VEGF, a putative angiogenic factor in prostate carcinoma, PC-3 cells were treated with graded levels of 12(S)-HETE, a pan-lipoxygenase inhibitor or baicalein (10 μM), a specific platelet-type 12-LOX inhibitor for 24 h and the culture medium tested for VEGF protein levels using enzyme immunoassay. As shown in Fig. 1A, both NDGA and baicalein significantly decreased VEGF secretion (p < 0.05 and **, p < 0.01) whereas a COX inhibitor, indomethacin (50 μM), increased VEGF levels but not significantly (p = 0.06). Shown here is a representative result from three independent experiments.

![FIGURE 1. Modulation of VEGF expression by LOX. A, reduction of VEGF protein levels by LOX inhibitors. The level of VEGF protein secreted from PC-3 cells treated with NDGA (50 μM), baicalein (10 μM), or indomethacin (50 μM) were determined using ELISA of the culture medium and normalized to the cell number. Values are means ± S.D. of three culture dishes. B, stimulation of VEGF secretion in PC-3 cells by 12(S)-HETE. PC-3 cells were treated with escalating concentrations of 12(S)-HETE as indicated for 48 h, and the protein levels of VEGF in the culture medium were measured using ELISA and normalized to the cell number. Values are means ± S.D. of three culture dishes. *p < 0.05. Shown here is a representative from three independent experiments.](https://www.jbc.org/content/281/27/18603/F1)

![Lipoxygenase Regulation of VEGF](https://www.jbc.org/content/281/27/18603/fig1a)

![Lipoxygenase Regulation of VEGF](https://www.jbc.org/content/281/27/18603/fig1b)
**Lipoxygenase Regulation of VEGF**

**FIGURE 2.** Increased VEGF protein levels in PC-3 cells as result of increased 12-LOX expression. A, Western blot analysis of 12-LOX transfectant (nL8) compared with the vector control (neo-α) and wild-type PC3 cells. A431 cell lysates were used as a positive control. β-Actin was used as the loading control. B, Increased VEGF production in PC-3 cells as result of 12-LOX overexpression. The levels of VEGF in the conditioned medium from 12-LOX-transfected PC-3 cells (nL8 and nL12), vector control cells (neo-α), and PC-3 parental cells were determined using ELISA and normalized to cell number. Values are means ± S.D. of three culture dishes. *, p < 0.01. Shown here is a representative result from three independent experiments. C, levels of IL-8 expression in PC-3 cells in 12-LOX-transfected PC-3 cells (nL2, nL8, and nL12) and their vector control cells (neo-α) as measured by ELISA of the culture medium and normalized to cell number. Note the lack of significant change in IL-8 production in PC-3 cells as result of 12-LOX expression. Values are means ± S.D. of three culture dishes. **, p < 0.01; ***, p < 0.001, when compared with the control.

**Increased VEGF Secretion in 12-LOX-overexpressing PC-3 Cells**—Previous studies demonstrated that tumors from 12-LOX-overexpressing PC-3 cells have greater angiogenic response compared with the control population (11). To investigate the mechanism of 12-LOX-mediated enhancement of angiogenesis, we used PC-3 cells stably transfected with the platelet-type 12-LOX expression vector. These 12-LOX-transfected PC-3 cells were found to express higher levels of 12-LOX compared with their vector control (Fig. 2A). The cells were characterized for the presence of higher levels of 12(S)-HETE production as described previously (11). ELISA for VEGF in the tumor-conditioned medium collected after 24 h of culture, demonstrated a higher secretion of VEGF by the nL8 and nL12 cells compared with empty vector-transfected cells (neo-α) and wild-type PC-3 cells (Fig. 2B). No significant changes in IL-8 levels were found in culture medium from 12-LOX-transfected PC-3 cells compared with the control cells (Fig. 2C). We also did not detect bFGF in culture medium from PC-3 or any other transfectants (data not shown). These findings suggest a selective modulation of VEGF secretion by 12-LOX in prostate cancer cells.

To verify that the observed increase in VEGF secretion is indeed an effect of 12-LOX enzymatic activity, we treated nL8 cells with NDGA (50 μM), baicalein (10 μM), or a COX inhibitor indomethacin (50 μM). Conditioned medium was collected from cells treated with each inhibitor, and the levels of VEGF were assayed by ELISA and normalized to cell number. We observed that NDGA or baicalein decreased VEGF secretion from nL8 cells compared with untreated nL8 cells (Fig. 2D). Also, we observed that indomethacin, a COX inhibitor, did not affect VEGF secretion from nL8 cells (Fig. 2D). The reduction of VEGF secretion in 12-LOX-overexpressing cells upon treatment with inhibitors suggests that the enzymatic activity of 12-LOX is required for VEGF secretion in these cells. These findings provide an explanation for our previous observation (11) of a higher angiogenic response in tumors formed from 12-LOX-overexpressing cells.

**Transcriptional Regulation of VEGF Expression by 12-LOX**—Regulation of VEGF gene expression occurs at transcriptional, post-transcriptional, and/or translational levels (16). To determine whether the increased secretion of VEGF in 12-LOX-overexpressing PC-3 cells results from an increase in the transcriptional rate of the VEGF gene, we performed Northern blot and Real Time PCR analyses of VEGF transcription in these cells. Northern blot analysis revealed a substantial increase in the steady state levels of VEGF mRNA in 12-LOX-transfected PC-3 cells (nL2 and nL8) when compared with the neo vector control cells (Fig. 3). Similarly, RT-PCR analysis also demonstrated that nL8 cells have higher levels of VEGF mRNA compared with the neo cells. The ΔCT values for VEGF mRNA were 8.3 and 9.14 in nL8 and neo cells, respectively, with a p value of 0.035. From these experiments, it is evident that 12-LOX-mediated increase in VEGF secretion involves an increase in the steady state level of VEGF mRNA in the 12-LOX-overexpressing cells.

An increase in VEGF mRNA levels may be attributed to either an increase in transcriptional rate or an increase in the stability of the VEGF mRNA (16, 18, 25) or both. To identify the influence of 12-LOX on the transcription of the VEGF gene, we employed promoter luciferase assays. The p1176-luc vector, a VEGF promoter luciferase vector used in these studies, consists of the VEGF promoter region (~1176/+54) fused upstream of a luciferase gene in a PGL2 vector (19). nL8,
nL12, and neo cells were transfected with the p1176-luc vector along with the LacZ plasmid to normalize transfection efficiency. Luciferase assay was performed on the lysed cells, and the values were normalized to β-gal enzyme activity measured using a β-gal assay kit. The assay revealed that there was an 10-fold increase in VEGF promoter activity in 12-LOX-overexpressing nL8 and nL12 cells compared with the empty vector control cells and the wild-type PC3 cells (Fig. 4A). These findings suggest that the observed increase in VEGF mRNA levels and secretion is a result of an increased transcriptional activity of the VEGF gene in 12-LOX-transfected PC-3 cells.

![Figure 3: Regulation of VEGF gene expression by 12-LOX.](Image)

Regulation of VEGF gene expression by 12-LOX. Northern blot analysis of VEGF mRNA levels in 12-LOX-transfected PC-3 cells (nL2 and nL8), their vector controls (neo-α and neo-α), and PC-3 parental cells. Poly(A)−RNA were isolated from 80% confluent cultures, subjected to electrophoresis on a 1% agarose formaldehyde gel, transferred to Duralon-UV membrane, and probed with 32P-labeled VEGF or G3PDH cDNA. Shown here is a typical result from two experiments. Note the increase in the levels of VEGF mRNA (3.8 and 4.3 kb isoforms), but not glyceraldehyde-3-phosphate dehydrogenase (G3PDH, loading control), in nL2 and nL8 as compared with their vector controls (neo-α and neo-α).

To further confirm the stimulation of VEGF promoter activity by 12-LOX, we examined whether or not a transient increase in 12-LOX expression could stimulate VEGF promoter activity. Wild-type PC3 cells were transiently transfected with a VEGF promoter construct and simultaneously, a 12-LOX expression construct or its vector control, pcDNA3.1. Co-transfection with the 12-LOX construct significantly increased 12-LOX expression (data not shown) and as shown in Fig. 4B, simultaneously, enhanced VEGF promoter activities, as compared with pcDNA3.1 vector control (p < 0.05). The results further confirmed the stimulation of VEGF promoter activity by 12-LOX.

The effects of 12(S)-HETE, the major eicosanoid product of 12-LOX, on VEGF promoter activity was then studied. Wild-type PC-3 cells were transfected with p1176 VEGF promoter construct and LacZ vectors and then treated with graded levels of 12(S)-HETE. As shown in Fig. 4C, after normalization for transfection efficiency, 12(S)-HETE increased VEGF promoter activity in PC-3 cells in a dose-dependent manner. The data suggest that 12(S)-HETE can modulate VEGF expression at transcriptional level in human prostate cancer cells. We also found that 12(S)-HETE treatment increased VEGF promoter activity in D145 cells (data not shown).

Involvement of an Sp1/AP2 Cluster in 12-LOX-stimulated VEGF Expression—VEGF transcription in human cells is regulated primarily by the transcription factors Sp1, AP2, HIF-1, and AP1. In addition, NF-κB regulates mouse VEGF promoter activity in mouse cell lines.
These factors bind to their respective recognition sites present in the promoter region of the VEGF gene (16). Systematic sequence analysis of the VEGF promoter region revealed that the region from −66 to −88 encompassed a GC box, which serves as the binding site for the transcription factors Sp1 and AP2. This region has two Sp1 and one AP-2 binding site (16).

To identify whether this region plays a role in 12-LOX-induced stimulation of VEGF promoter activity, deletional mutants of the p1176-luc vector with (−88/+54) and without (−66/+54) the region between −66 and −88 were used. Normalized luciferase activity results clearly demonstrated that the presence of the region between −66 and −88 is necessary for the 12-LOX-mediated VEGF promoter activity, and deletion of this region resulted in loss of activity (data not shown). To identify whether Sp1 or AP2 or both factors play a role in 12-LOX-mediated VEGF expression, VEGF promoter constructs (−88/+54) harboring site directed mutations targeting the binding sites for these two transcription factors were employed (19). The constructs had mutations in either both the Sp1 binding sites or the AP2 binding site or all the three binding sites (Fig. 4D) These constructs were co-transfected separately with the LacZ plasmid into nL8 and neo cells and compared with activity generated upon transfection of the −88/+54 promoter construct lacking any mutation. As shown in Fig. 4D, mutation of the AP2 binding site decreased, but did not abolish, the stimulation of promoter activity by 12-LOX (from 25-fold to 8-fold increase). In contrast, mutation of the two Sp1 binding sites dramatically decreased the stimulation of VEGF promoter activity by 12-LOX in which the increase of VEGF promoter activity in nL8 cells was no longer statistically significant. These studies clearly demonstrate that the transcription factor Sp1 and to a lesser extent AP2 are involved in 12-LOX-mediated up-regulation of VEGF promoter activity and gene expression.

**Signaling Pathways Linking 12-LOX Activity and VEGF Transcription**—A variety of signaling pathways have been identified to be involved in the regulation of VEGF expression in cells (26). Activation of the PI 3-kinase pathway has been linked to VEGF expression in cancer cells (16). It was found that 12-LOX-overexpressing PC-3 cells showed an increase in PI 3-kinase activity as indicated by an almost 2-fold increase in the formation of PI(3,4,5)P3 (PI3P) (Fig. 5A), which was inhibited by a PI 3-kinase inhibitor, LY294002 (20 μM). There also was a simultaneous increase in Akt phosphorylation in 12-LOX-transfected PC-3 cells (nL8) when compared with their vector control (neo-α) (Fig. 5B, left). The increased Akt phosphorylation was reduced by treatment of nL8 cells with a specific 12-LOX inhibitor, baicalein (Fig. 5B, right). There was a 35% decrease in the level of p-Akt upon baicalein treatment based on densitometric analysis (data not shown). The data suggest that there is an increase in PI 3-kinase activity in PC-3 cells as a result of 12-LOX overexpression. To identify whether 12-LOX-mediated increase in the activity of PI 3-kinase and Akt is responsible for the increased VEGF expression, we treated the cells with LY294002, a chemical inhibitor of PI 3-kinase. As shown in Fig. 5C, LY294002 (20 μM) treatment led to a dramatic decrease in VEGF secretion in both 12-LOX-transfected PC-3 cells (nL8 and nL12) and neo-controls (neo-α and neo-σ), as well as the PC-3 parental cell line. Transfection with a dominant negative form of Akt also was found to reduce VEGF expression in 12-LOX-transfected PC-3 cells (data not shown). These findings clearly demonstrate that 12-LOX enzyme activity drives the up-regulation of VEGF gene expression via the PI3K-Akt signaling pathway in 12-LOX-overexpressing prostate cancer cells.

**VEGF Secreted by 12-LOX-overexpressing PC-3 Cells as an Effector of Endothelial Cell Angiogenic Response**—Overexpression of 12-LOX in PC-3 cells results in highly angiogenic tumors (11). To study whether VEGF mediates the angiogenic activity of 12-LOX in prostate cancer cells, we evaluated the effect of neutralizing antibodies against VEGF or IL-8 on endothelial cell migration, as stimulated by the conditioned medium from 12-LOX-transfected PC-3 cells (nL8). As shown in Fig. 6, A and B, in the presence of control mouse IgG, the concentrated medium from 12-LOX-transfected PC-3 cells (nL8) stimulated endothelial cell migration (p < 0.01, Student’s t test) when compared with those from vector controls (neo-α). In the presence of neutralizing antibody against IL-8 as indicated by nL8, the concentrated medium from 12-LOX-transfected PC-3 cells still retained the ability to stimulate endothelial cells (p < 0.01, Student’s t test) (bottom panel in Fig. 6A; Fig. 6B). However, this increased ability was reduced to insignificant levels (p > 0.05) by pretreating the tumor cell-conditioned medium with a VEGF neutralizing antibody as indicated by nVEGF (middle panel in Fig. 6A; Fig. 6B). The data suggest that enhanced VEGF secretion from 12-LOX-overexpressing PC-3 cells can lead to an enhanced angiogenic response by stimulating endothelial cell migration.

**DISCUSSION**

The data presented in this study conclusively demonstrate that the enzyme 12-lipoxygenase and the metabolite 12(S)-HETE stimulate VEGF expression and secretion in prostate cancer cells. The results obtained from the experiments described in this work provide a strong basis for our previous observations of increased angiogenicity of PC3 cells overexpressing 12-LOX (11). By increasing VEGF secretion and in turn angiogenesis, 12-LOX may enhance the survival and promote...
Lipoxygenase Regulation of VEGF

Tumor angiogenesis is a complex phenomenon mediated by a variety of growth factors, cells, and cell surface molecules in the tumor stromal microenvironment. Presently, a large number of clinical trials are aimed at inhibiting angiogenesis in cancers using novel therapeutic approaches. VEGF has been documented as a major angiogenic factor involved in the survival and metastasis of solid tumors, and its expression has been correlated with poor patient survival in many neoplastic disorders. VEGF expression and secretion are stimulated by a vast multitude of factors, which originate from genetic and physiological alterations, commonly found in cancer cells (13, 15, 28, 29). As a key cytokine in tumor angiogenesis, the expression of VEGF is tightly regulated at the transcriptional, post-transcriptional, and/or translational levels (15, 16). It has been extensively reported that the expression of VEGF is regulated by the activation of oncoproteins such as Ras and Src, tumor suppressors such as P53, and physiological stressors such as hypoxia or hyperglycemia within the tumor microenvironment (16, 30).

Our studies suggest that 12-LOX is able to regulate VEGF gene expression at the transcriptional level. First, Northern blot analysis revealed an increase in the steady state levels of VEGF mRNA in 12-LOX-transfected PC-3 cells, indicating that there is an enhanced transcription or stabilization of VEGF mRNA. This finding was supported by the results from the RT-PCR analysis. Second, transfection with a VEGF promoter luciferase construct (-1176/+54) elicited a more than 10-fold increase in VEGF promoter activity in 12-LOX-transfected PC-3 cells as compared with their vector controls, suggesting that there was an increased VEGF promoter activity in PC-3 cells as a result of increased 12-LOX expression. Finally, transient cotransfection with a 12-LOX expression construct enhanced VEGF promoter activity in PC-3 cells. Although 12-LOX is able to stimulate VEGF expression at the transcriptional level, further studies are required to determine whether 12-LOX is involved in enhancing the stability or translation of VEGF mRNA.

Both NDGA and baicalein reduced VEGF expression, indicating that the enzymatic activity of 12-LOX plays a significant role in the regulation of VEGF gene expression. Exogenously added 12(S)-HETE was found to increase VEGF expression at both protein and promoter levels. It should be noted, however, that the extent of stimulation of VEGF expression by 12(S)-HETE is much less than that observed in PC-3 cells stably expressing 12-LOX. This may be caused by the inability of exogenously added 12(S)-HETE to mimic the cellular activities of endogenously produced 12(S)-HETE because of temporal and spatial limitations. PC-3 cells stably transfected with 12-LOX maintain a constant production of 12(S)-HETE. On the other hand, exogenously added 12(S)-HETE may be rapidly uptaken by cells and trapped in the phospholipid pool making it unavailable for biological responses. Thus, 12(S)-HETE is greatly diminished for the treatment time of 48 h, as compared with the stable transfectants, which maintain a sustained production of the eicosanoid. Yet another reason for the lessened activity of exogenous 12(S)-HETE could be the fact that the majority of the biological activity of 12-LOX may be mediated by the immediate metabolites 12(S)-HPETE, and 12(S)-HETE the metabolite derived from degradation of 12(S)-HPETE contributes only a smaller percentage. Because 12(S)-HPETE is a short lived compound it is not practical to use this metabolite for exogenous cell treatment experiments. In addition to 12-LOX and 12(S)-HETE, other eicosanoids or eicosanoid producing enzymes have been found involved in the regulation of VEGF expression. For example, VEGF induction by cobalt chloride-simulated hypoxia is maintained by a concomitant, persistent induction of COX-2 expression and sustained elevation of prostaglandin E2 synthesis in a human metastatic prostate cancer cell line (31).

metastasis of tumor cells from the primary site. Previous studies have shown that 12-LOX is a positive modulator of tumor cell survival and stimulates the activity of various anti-apoptotic factors in tumor cells (8–10, 27). The present observations have revealed that 12-LOX can potentially stimulate tumor cell survival and angiogenesis by modulating VEGF expression.

FIGURE 6. Increased angiogenicity of 12-LOX-transfected PC-3 cells is partially mediated by VEGF. A, stimulation of endothelial cell migration by nL8 is blocked by a VEGF neutralizing antibody but not by an IL-8-neutralizing antibody. Conditioned medium from 12-LOX-transfected PC-3 cells (nL8) and their vector controls (neo-th) were harvested and concentrated 6 X using Centricon-10. HUVEC migration assay, in response to the conditioned medium, was conducted as described under “Experimental Procedures.” Shown here are migrated endothelial cells (as indicated by purple or dark purple staining) in response to the conditioned medium. Note that the stimulation of endothelial cell migration by nL8 when compared with its vector control, neo-th (top panel). This increased stimulation of endothelial cell migration was blocked by the pretreatment with a VEGF neutralizing antibody (as indicated by nVEGF) (middle panel), but not by an IL-8-neutralizing antibody (bottom panel). B, enumeration of endothelial cell migration. The migrated endothelial cells in response to different stimuli were counted in a double blind approach and the migration of endothelial cells was expressed as the percentage of the medium control in which no conditioned medium was added. Column, the average percentage of migration when compared with the medium control; bar, S.D. from six membranes. a, p < 0.01; b, p > 0.05 according to Student’s t test.
Inhibition of COX-2 activity reduced hypoxia-induced VEGF expression (31). In addition to COX-2, 15-LOX-1 and 5-LOX of the lipoxygenase family are also implicated in the regulation of VEGF expression. Overexpression of 15-LOX-1 in PC-3 cells stimulates tumor growth via upregulation of VEGF (32). Inhibition of 5-LOX by MK886 reduces VEGF expression and increases survival in malignant mesothelial cells (33). However, the mechanistic basis underlying eicosanoid regulation of VEGF expression has not been examined in detail. Here we present data for the first time that 12-LOX and 12(S)-HETE can regulate VEGF gene expression at transcriptional level. It will be interesting to see whether or not other eicosanoids regulate VEGF expression at the transcriptional level similar to 12-LOX or 12(S)-HETE.

We identified the PI 3-kinase pathway as an important signaling pathway for 12-LOX to increase VEGF expression in human prostate cancer cells as illustrated in Fig. 5. Increased 12-LOX expression or activity leads to the increased formation of 12(S)-HETE, which subsequently activates PI 3-kinase (34). Increased PI 3-kinase activity was observed in 12-LOX-transfected PC-3 cells. In congruence with the observed increase in PI 3-kinase, we found an increase in the activation of Akt, a downstream effector of PI 3-kinase, in 12-LOX-transfected PC-3 cells as measured by the level of phosphorylation of Ser473 in Akt. Further, the increased level of active Akt in 12-LOX-transfected PC-3 cells was attenuated by baicalein, a specific inhibitor of 12-LOX. Activated PI 3-kinase stimulates VEGF gene expression through the activation of various downstream effectors. It has been reported that PI 3-kinase is required for hypoxia-induced VEGF expression in H-ras-transformed NIH3T3 cells (35). In prostate cancer, in addition to its role in cell survival in LNCaP cells (36), PI 3-kinase/Akt signaling pathway has been found to regulate VEGF expression through the expression or activation of hypoxia inducible factor 1α (37). In this study, we found that inhibition of PI 3-kinase with a pharmacological inhibitor LY294002 or by a dominant negative inhibitor of Akt reduced VEGF gene expression.

One of the downstream effectors of PI 3-kinase, Akt, was found to be involved in stimulation of VEGF expression by 12-LOX. The results suggest that the PI 3-kinase/Akt signaling pathway is involved in the stimulation of VEGF expression by 12-LOX. In addition to Akt, other downstream effectors of PI 3-kinase such as PKCζ are known to be activated by PI3 and PDK (38, 39). PKCζ has been shown to regulate VEGF gene expression by interacting with the transcription factor Sp1 in renal carcinoma cells (40). Experiments are ongoing to determine whether PKCζ is activated by PI 3-kinase in prostate cancer cells and whether PKCζ activity is increased in 12-LOX-transfected PC-3 cells and if so, what role PKCζ plays in increased VEGF expression.

Finally it is worthwhile to note that the cellular signaling from PI 3-kinase can be attenuated by PTEN/MMAC1 tumor suppressor protein, a lipid phosphatase that functions as a negative regulator of the PI 3-kinase/Akt pathway (41). In primary prostate cancer, loss of PTEN expression correlates with high Gleason score and advanced stage (42, 43). Inactivation of PTEN tumor suppressor gene has been found associated with increased angiogenesis in localized prostate carcinoma (44). It remains to be determined, however, whether PTEN attenuates 12-LOX stimulation of prostate cancer angiogenesis and progression and whether inactivation of PTEN or loss of PTEN expression augment the stimulation of tumor angiogenesis by 12-LOX. The above discussion is summarized in Fig. 7.

In summary, the present study describes a novel link between 12-LOX and VEGF expression in prostate cancer cells. We demonstrated VEGF as an important effector in 12-LOX-enhanced angiogenicity of prostate tumor cells. We further showed an important role for PI 3-kinase-dependent signaling pathway in 12-LOX-stimulated VEGF gene expression. The findings reported herein provide a novel insight into how eicosanoids modulate tumor angiogenesis and progression.

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REFERENCES
1. Funk, C. D. (2001) Science 294, 1871–1875
2. Honn, K. V., Tang, D. G., Gao, X., Butovich, I. A., Liu, B., Timar, J., and Haggmann, W. (1994) Cancer Metastasis Rev. 13, 365–396
3. Tang, D. G., Renaud, C., Stojakovic, S., Diglio, C. A., Porter, A., and Honn, K. V. (1995) Biochem. Biophys. Res. Commun. 211, 462–468
4. Yoshimura, R., Inoue, K., Kawahito, Y., Miyashita, M., Tsuchida, K., Matsuyama, M., Sano, H., and Nakatani, T. (2004) Int. J. Mol. Med. 13, 41–46
5. Natarajan, R., and Nadler, J. (1998) Front. Biosci. 3, E81–E88
6. Gao, X., Grignon, D. J., Chibbi, T., Zacharek, A., Chen, Y. Q., Sakr, W., Porter, A. T.,
7. Nithipatikom, K., Isbell, M. A., See, W. A., and Campbell, W. B. (2005) Cancer Lett. 233, 219–225
8. Tang, D. G., Chen, Y. Q., and Honn, K. V. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5241–5246
9. Pidgeon, G. P., Kandouz, M., Meram, A., and Honn, K. V. (2002) Cancer Res. 62, 2721–2727
10. Pidgeon, G. P., Tang, K., Cai, Y. L., Piasentin, E., and Honn, K. V. (2003) Cancer Res. 63, 4258–4267
11. Nie, D., Hillman, G. G., Geddes, T., Tang, K., Pierson, C., Grignon, D. J., and Honn, K. V. (1998) Cancer Res. 58, 4047–4051
12. Hanahan, D., and Folkman, J. (1996) Cell 86, 353–364
13. Carmeliet, P. (2000) Nat. Med. 6, 564–573
14. Bergers, G., and Benjamin, L. E. (2003) Nat. Rev. Cancer 3, 401–410
15. Pages, G., and Pouyssegur, J. (2005) Cardiovasc. Res. 65, 564–573
16. Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., and Semenza, G. L. (1996) Mol. Cell. Biol. 16, 4604–4613
17. Tiav, J., Bar, F., Iyer, N. V., Zhang, D., Zhang, D. L., and Semenza, G. L. (2000) Mol. Cell. Biol. 20, 18165–18172
18. Miura, H., Miyazaki, T., Kuroda, M., Oka, T., Machinami, R., Kodama, T., Shibuya, M., Makuchii, M., Yaziaki, Y., and Ohnishi, S. (1997) J. Hepatol. 27, 854–861
19. Lei, E., Chen, M. G., Breier, G., and Risau, W. (1995) J. Biol. Chem. 270, 19761–19766
20. Liu, X. H., Kirschenbaum, A., Yao, S., Stearns, M. E., Holland, J. F., Claffey, K., and Levine, A. C. (1999) Clin. Exp. Metastasis 17, 687–694
21. Giri, D., and Ittmann, M. (1999) J. Biol. Chem. 274, 26277–26280
22. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyer, C. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15587–15591
23. McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Lodis, M., and Sellers, W. R. (1999) Cancer Res. 59, 4291–4296
24. Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., Said, J. W., Isaacs, W. B., and Sawyer, C. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5246–5250
25. Gupta, V. K., Jaskowiak, N. T., Beckett, M. A., Auceri, H. J., Grunstein, J., Johnson, R. S., Calvin, D. A., Nodzenski, E., Pejovic, M., Kufe, D. W., Posner, M. C., and Weichselbaum, R. R. (2002) Cancer J. 8, 47–54
26. Lara, P. N., Jr., Twardowski, P., and Quinn, D. I. (2004) Clin. Prostate Cancer 3, 165–173
27. Harris, A. L. (2002) Nat. Rev. Cancer 2, 38–47
28. Gupta, V. K., Jaskowiak, N. T., Beckett, M. A., Auceri, H. J., Grunstein, J., Johnson, R. S., Calvin, D. A., Nodzenski, E., Pejovic, M., Kufe, D. W., Posner, M. C., and Weichselbaum, R. R. (2002) Cancer J. 8, 47–54
29. Lara, P. N., Jr., Twardowski, P., and Quinn, D. I. (2004) Clin. Prostate Cancer 3, 165–173
30. Harris, A. L. (2002) Nat. Rev. Cancer 2, 38–47
31. Liu, X. H., Kirschenbaum, A., Yao, S., Stearns, M. E., Holland, J. F., Claffey, K., and Levine, A. C. (1999) Clin. Exp. Metastasis 17, 687–694
32. Kelavkar, U. P., Nixon, J. B., Cohen, C., Dillehay, D., Eling, T. E., and Baud, K. F. (2001) Cardiovasc. Res. 65, 564–573
33. Romano, M., Catalanp, A., Nutini, M., D’Urbano, E., Crescenzi, C., Clarisa, J., Libner, R., Davi, G., and Procopio, A. (2001) FASEB J. 15, 2326–2336
34. Szekeres, C. K., Trika, K., Nie, D., and Honn, K. V. (2000) Biochem. Biophys. Res. Commun. 275, 690–695
35. Mazure, N. M., Chen, E. Y., Laderoute, K. R., and Giaccia, A. J. (1997) J. Biol. Chem. 272, 3322–3331
36. Lin, J., Adam, R. M., Santistevan, E., and Freeman, M. R. (1999) Cancer Res. 59, 2891–2897
37. Zhong, H., De Marzo, A. M., Auufghe, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L., and Simons, J. W. (1999) Cancer Res. 59, 5830–5835
38. Chou, M. H., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S., and Toker, A. (1998) Curr. Biol. 8, 1069–1077
39. Le Good, J. A., Ziegler, W. H., Parekh, D., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) Science 281, 2042–2045
40. Pal, S., Claffey, K. P., Cohen, H. T., and Mukhopadhyay, D. (1998) J. Biol. Chem. 273, 26277–26280
41. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyer, C. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15587–15591
42. McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Lodis, M., and Sellers, W. R. (1999) Cancer Res. 59, 4291–4296
43. Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., Said, J. W., Isaacs, W. B., and Sawyer, C. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5246–5250
44. Giri, D., and Ittmann, M. (1999) Hum. Pathol. 30, 419–424