Vascular Endothelial Growth Factor Production in Human Prostate Cancer Cells Is Stimulated by Overexpression of Platelet 12-Lipoxygenase

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BACKGROUND. Elevated platelet 12-Lipoxygenase (P12-LOX) expression is associated with advanced stage and grade prostate cancer and overexpression in PC-3 cells promotes tumor growth and angiogenesis. The mechanisms underlying the role of P12-LOX in angiogenesis remain unclear.
METHODS. Enzyme linked immunosorbent assays were used to measure 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) and vascular endothelial growth factor (VEGF) in conditioned media of PC-3 cells stably overexpressing human P12-LOX. Immunoblotting was used to observe stimulation of signal transduction in prostate cancer cell lines following exposure to 12(S)-HETE.
RESULTS. P12-LOX overexpression promotes increased accumulation of 12(S)-HETE and VEGF in culture media leading to constitutive ERK1/2 phosphorylation. 12(S)-HETE stimulates ERK1/2 phosphorylation via a pertussis toxin sensitive G-protein coupled receptor (GPCR) and MEK; the inhibition of which reduces VEGF accumulation by 36% and 70%, respectively.
CONCLUSIONS. Our data provide insight into a possible mechanism by which prostate cancer cells with elevated expression of P12-LOX stimulate VEGF production, thus increasing their angiogenic potential. *Prostate 66: 779–787, 2006.* © 2006 Wiley-Liss, Inc.

KEY WORDS: **prostate; 12-lipoxygenase; VEGF**

INTRODUCTION

Prostate cancer is diagnosed in approximately 240,000 men in the United States per year making it the most common malignancy in men. While there is an inherent risk for men to develop prostate cancer, chances can be influenced by multiple factors including, but not limited to, age, race, and diet. Arachidonic acid, a polyunsaturated fatty acid found at high levels in red meats and high-fat dairy foods, is a major membrane component in mammalian cells. Mobilization of arachidonic acid from membrane phospholipid pools, as a result of stimulation, can lead to the production of eicosanoids via cyclooxygenases (COXs), lipoxygenases (LOXs), or cytochrome P450 epoxygenases. Eicosanoids are known to play significant roles in numerous physiological and pathological conditions. Recent studies have shown that certain metabolites of arachidonate, mainly the COX-2 product prostaglandin E2 (PGE2) and the 12-lipoxygenase product 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), play significant roles in the angiogenic and metastatic potentials of tumors [1].

LOX enzymes are found in a wide variety of plant and animal species and are designated 5-, 8-, 12-, and

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15-LOXs. In animals, there are three 12-LOX isoforms (platelet-, leukocyte-, and epidermal-type) that differ in tissue distribution, substrate specificity, regioselectivity, and product profile. Platelet-type 12-lipoxygenase was originally characterized in and thought to be exclusive to human platelets [2], but has more recently been linked to several cancers including prostate [3,4]. Platelet 12-Lipoxygenase (P12-LOX) protein expression has been confirmed in multiple prostate cancer cell lines including PC-3, DU145, and LNCaP [5]. Additionally, Gao et al. [6] reported a correlation between P12-LOX expression and clinical stage and grade in tissue from adenocarcinoma of the prostate. Moreover, PC-3 cells stably overexpressing P12-LOX promote enhanced tumor growth and angiogenesis in a mouse tumor model [7], but the mechanism of this influence remains elusive.

Angiogenesis, or the process of new blood vessel growth from extension of preexisting vasculature, is necessary for sustained tumor growth and metastasis [8]. For vascular endothelial cells of arterial, venous, or lymphatic origin, vascular endothelial growth factor (VEGF) is a potent mitogen [9], and withdrawal of VEGF leads to vascular regression in both physiological and pathological conditions [10]. Immunohistochemical analysis of human prostate cancer tissue indicates that cancer cells stain positive for VEGF and this staining correlates with microvascular density [11]. Solidifying the role of VEGF in prostate cancer, Borgstrom et al. [12] showed that prostate cancer induced angiogenesis is suppressed by blocking VEGF function with anti-VEGF monoclonal antibodies, thus limiting tumor growth to the prevascular phase.

Studies indicate that 12(S)-HETE has direct stimulatory effects on multiple processes associated with angiogenesis. For example, 12(S)-HETE has been shown to be a mitogenic factor for microvascular endothelial cells [13] and stimulates endothelial cell migration [14]. However, the potential of 12(S)-HETE as a significant stimulator of pathological angiogenesis may lie elsewhere. Indeed, 12(S)-HETE was shown to induce the expression of VEGF in human vascular smooth muscle cells [15]. The present studies were undertaken to determine whether overexpression of P12-LOX by prostate cancer cells leads to augmented production of VEGF. Our data demonstrates that elevated expression of P12-LOX leads to enhanced VEGF production in vitro. Additionally, we provide a potential mechanism for this phenomenon by reporting that 12(S)-HETE is capable of inducing signaling via the extracellular-signal related kinase1/2 (ERK1/2) mitogen-activated protein (MAP) kinase pathway in PC-3 and DU145 prostate cancer cell lines. These findings suggest that 12(S)-HETE production by prostate cancer cells stimulates VEGF production and may promote prostate cancer induced angiogenesis in vivo.

MATERIALS AND METHODS

Cell Culture and Reagents

The human prostate carcinoma cell lines PC-3 and DU145 were purchased from American Type Culture Collection (Manassas, VA). Both cell lines were routinely cultured in RPMI 1640 (Sigma, St. Louis, MO) with 5% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml). Human P12-LOX cDNA was kindly provided by Dr. Colin Funk (University of Pennsylvania, Philadelphia, PA) as pcDNA/6HisP12-LOX. Arachidonic acid and 12(S)-HETE were purchased from Cayman Chemical (Ann Arbor, MI). PD98059 and pertussis toxin were from Biomol (Plymouth Meeting, PA), U0126 was from LC Laboratories (Woburn, MA). PD158780 was a gift from Dr. James Elder (University of Michigan, Ann Arbor, MI). All other chemicals were obtained from Sigma unless otherwise stated.

Cell Transfection

The cDNA for 6HisP12-LOX was cloned into pTrex-dest30 using the Gateway system (Invitrogen, Carlsbad, CA). PC-3 cells at 40%–50% confluence in 60 mm dishes were transfected with 2 μg pTrex/6HisP12-LOX or empty control vector pcDNA3.1 (Invitrogen) using the Lipofectin reagent (Invitrogen) according to the manufacturer instructions. Stable transfectants were selected using 500 μg/ml G418 (Invitrogen) and individual colonies were picked using cloning cylinders. Clones were expanded and maintained in RPMI 1640, 5% FBS, and 250 μg/ml G418.

Cell Propagation

Cell growth of PC-3 mock and PC-3 pTrex/6HisP12-LOX transfected cells were monitored using the Aqueous MTS assay (Promega, Madison, WI). Briefly, 4×10^3 PC-3 control and P12-LOX overexpressing cells were plated per well of a 96-well plate in RPMI 1640 with 5% FBS and Pen/Strep. After 24 hr, a baseline measurement was taken for normalization. For growth analysis, the media was replaced with serum reduced RPMI (1% FBS). Cell growth was monitored at 24 hr intervals following treatment.

RNA Isolation and RT-PCR

PC-3 control and P12-LOX overexpressing cells (5×10^5 each group) were plated in 60 mm dishes in RPMI 1640 with 5% FBS and Pen/Strep then serum

starved for 24 hr. Total RNA was extracted using TRIZOL Reagent (Invitrogen). First strand synthesis was completed using oligo-dT and Superscript III reverse transcriptase (Invitrogen). This cDNA mixture was then subjected to PCR using the following primers: P12-LOX [16] sense 5′ gccaggtatgtggaggggatc 3′ and antisense 5′ ggcaccatgtctggctggcg 3′ yielding a 404-bp fragment. Reaction conditions were as follows: 30 cycles of 94°C for 30 sec, 70°C for 30 sec, and 72°C for 45 sec. β-actin was used as a control with primers from Invitrogen. PCR products were separated by electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

Treatments and Western Blot Analysis

PC-3 or DU145 cells were plated in 60 mm dishes at a density of 4×10^5 cells/dish. Cells were grown overnight in RPMI 1640 with 5% FBS and Pen/Strep. Cells were then serum starved for 24 hr in serum free RPMI with or without 0.5 μg/μl pertussis toxin. For recombinant human 6His tagged P12-LOX, expression was detected at this point. Otherwise, media was replaced again for 1 hr with serum free RPMI 1640 containing vehicle (0.1% DMSO) or with serum free RPMI 1640 containing PD98059 (50 μM for 1 hr), U0126 (10 μM for 30 min.), or PD158780 (10 μM for 20 min). Following this treatment, cells were washed twice in serum free RPMI, then treated with vehicle (0.1% EtOH) or 100, 300, or 500 nM 12(S)-HETE for various times. Cells were then placed in lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100, 2.0 mM EDTA, 1 mM sodium orthovanadate, 0.05 g sodium deoxycholate, 50 μg/ml pepstatin A, 50 μg/ml aprotinin, and 250 μM leupeptin), scraped and sonicated on ice. Protein concentration was determined using the BCA method (Pierce, Rockford, IL). Total protein (10 μg) was loaded and separated by electrophoresis in 4%–12% Tris-Glycine gels (Invitrogen) then transferred to nitrocellulose. For 6HisP12-LOX detection, blots were incubated for 1 hr with anti-HisG (Invitrogen) followed by 30 min with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma). For ERK studies, duplicate blots were probed in parallel with either anti-ERK or phospho-ERK primary antibodies followed by anti-rabbit HRP-conjugated antibodies (Cell Signaling Technology, Beverly, MA). Immunoreactivity was determined using enhanced chemiluminescense (Supersignal West Pico, Pierce). Blots were stripped and reprobed with anti-β-actin antibodies (Sigma) for band intensity normalization.

12(S)-HETE and VEGF Quantitation

PC-3 control and P12-LOX overexpressing cells were seeded in 12-well plates at a density of 1×10^5 cells/well. Cells were grown to approximately 60% confluence in RPMI 1640 with 5% FBS and Pen/Strep. Media was then replaced with equal volumes per well of serum reduced media (1% FBS) for 24 hr. The amount of 12(S)-HETE present in conditioned culture media was determined using the Correlate-EIA for 12(S)-HETE Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer instructions. Similarly, the concentration of VEGF in conditioned media was determined by a Human VEGF Accucyte EIA (Oncogene, Boston, MA).

Statistics

Data are expressed as mean $\pm$ SD. Data were statistically analyzed using the Student's unpaired *t*-test.

RESULTS

P12-LOX Overexpression by PC-3 Cells Confers No In Vitro Growth Advantage

P12-LOX expression has been shown to correlate with stage and grade of prostate cancer [6]. Nie et al. [7] found that P12-LOX overexpression in prostate cells resulted in a growth advantage in vivo but conferred no proliferative benefits in vitro. To expand our knowledge of the role of P12-LOX in prostate cancer, we began by overexpressing P12-LOX in PC-3 cells by stable transfection. Figure 1A shows, as determined by RT-PCR, the increase in P12-LOX mRNA expression by PC-3 cells following stable transfection. Protein expression of 6HisP12-LOX by several PC-3 transfected clones (P12-LOX1, P12-LOX4, and P12-LOX5) is shown in Figure 1B. PC-3 clone P12-LOX4 was chosen for further studies. The MTS proliferation assay indicated that overexpression of P12-LOX confers no in vitro growth advantage to PC-3 cells (Fig. 1C), thus confirming the earlier report [7]. Furthermore, treatment of PC-3 cells with escalating doses of the P12-LOX product 12(S)-HETE (100, 300, and 500 nM) had no effect on the growth of PC-3 cells (data not shown). These results indicate that 6HisP12-LOX is expressed stably in transfected PC-3 clones and that P12-LOX overexpression confers no in vitro growth advantage to these cells.

P12-LOX Overexpression in Prostate Cancer Cells Stimulates Production of 12(S)-HETE and VEGF

As P12-LOX metabolizes arachidonic acid into almost exclusively 12(S)-HETE [3], one can speculate that the in vivo growth effects observed by Nie et al. [7] are a result of increased production of this bioactive lipid. To investigate the effects of P12-LOX overexpression on the production of 12(S)-HETE, we

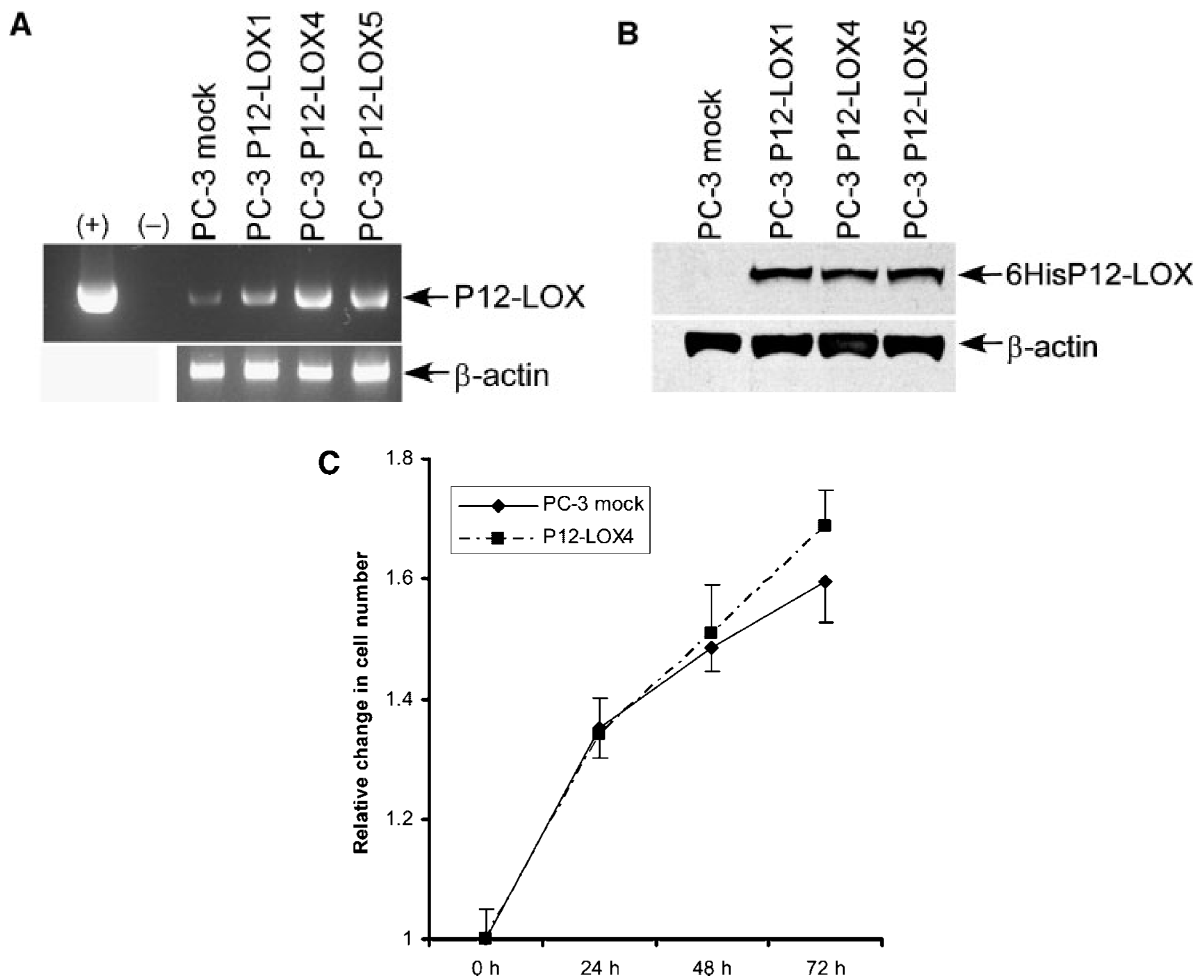

Fig. 1. Platelet-type 12-lipoxygenase expression of several stably transfected human PC-3 prostate cancer cell clones. **A**: 5×10^5 cells were plated in 60 mm dishes and grown to ~90% confluence. Total RNA was extracted and reverse transcribed. The *P12-LOX* gene was amplified using PCR and gene specific primers. PCR products were separated using 2.0% agarose gel electorphoresis and visualized by ethidium bromide staining. Lanes: (+), positive control (pTrex/6HisP12-LOX plasmid); (–), negative control; PC-3 mock (transfected with empty vector); P12-LOX1, -4, -5, individual clones. **B**: Expression of 6HisP12-LOX protein was analyzed by Western blotting. Ten microgram of whole cell lysates were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose. 6HisP12-LOX protein was detected using an anti-6HisGly antibody; β-actin was detected using anti-β-actin antibody. Lane assignments are similar to those used in (A). **C**: In vitro growth effects of P12-LOX overexpression by PC-3 cells. Cell growth rates for PC-3 mock (n = 3) and P12-LOX4 cells (n = 3) were analyzed by MTS assay. A baseline measurement was taken 24 hr after plating (0 time point). Subsequent time points (24, 48, and 72 hr) were measured as a percent change in cell number relative to time point 0.

analyzed the conditioned media from P12-LOX4 cells by ELISA. As indicated in Table I, P12-LOX overexpression leads to increased production of 12(S)-HETE in serum free conditions. P12-LOX4 cells secrete >threefold 12(S)-HETE compared to PC-3 controls after 30 and 60 min in serum free media. Exposure to 10 ng/ml arachidonic acid increased the amount of 12(S)-HETE secreted by both cell lines with a more

TABLE I. 12(S)-HETE and VEGF Present in Conditioned Media (pg/10^5 Cells)

	12(S)-HETE				VEGF
	30 min	30 min + aa	60 min	60 min + aa	24 hr
PC-3	200.56 ± 0.14	6,285.80 ± 830.54	289.04 ± 58.32	9,632.40 ± 330.05	127.71 ± 17.22
P12-LOX4	626.11 ± 91.40	16,352.02 ± 5,419.03	917.72 ± 299.56	21,524.45 ± 4,037.04	1,642.60 ± 396.01

pronounced effect noticeable for P12-LOX4 cells. Although 12(S)-HETE was shown to promote angiogenic responses such as endothelial cell retraction and mitogenesis [1], it is controversial as to whether 12(S)-HETE can stimulate significant angiogenesis alone. However, 12(S)-HETE can induce the expression of VEGF in human vascular smooth muscle cells [17]. We found that conditioned media of PC-3 cells overexpressing P12-LOX contained approximately 13-fold more VEGF ($P < 0.01$) than that of untransfected controls (Table I) indicating that increased expression of P12-LOX can be linked to augmented VEGF secretion by prostate cancer cells.

12(S)-HETE Induces ERK1/2 Phosphorylation in Prostate Cancer Cells

Regulation of VEGF protein expression has previously been associated with ERK1/2 MAPK activation [18–20] and 12(S)-HETE has been shown to induce phosphorylation of ERK1/2 in several cell lines [21,22]. We found that under serum free conditions P12-LOX overexpressing PC-3 cells have high constitutive levels of ERK1/2 phosphorylation (Fig. 2A). As we have shown that P12-LOX overexpression leads to accumulation of 12(S)-HETE in culture medium, we investigated the potential of 12(S)-HETE as a stimulator of ERK1/2 phosphorylation in PC-3 prostate cancer cells by treating parental PC-3 cells with escalating doses of 12(S)-HETE. As shown in Figure 2B, 12(S)-HETE induces a transient, concentration dependent increase in ERK1/2 phosphorylation in parental PC-3 cells with a peak at 10 min post-exposure. Hence, it is reasonable to speculate that abundant 12(S)-HETE production by P12-LOX overexpressing cells, thus facilitating chronic exposure to high concentrations of 12(S)-HETE, can result in constitutive activation of the ERK1/2 MAPK pathway and increase secretion of VEGF.

It appears as though these effects are not specific to PC-3 prostate cancer cells, as the transient increase in ERK1/2 phosphorylation induced by 12(S)-HETE in PC-3 cells was mirrored in DU145 cells (not shown); however, due to high basal levels of ERK1/2 phosphorylation in DU145 cells, the effect was less pronounced. High basal levels of ERK1/2 phosphorylation were previously noted in DU145 cells, an effect related

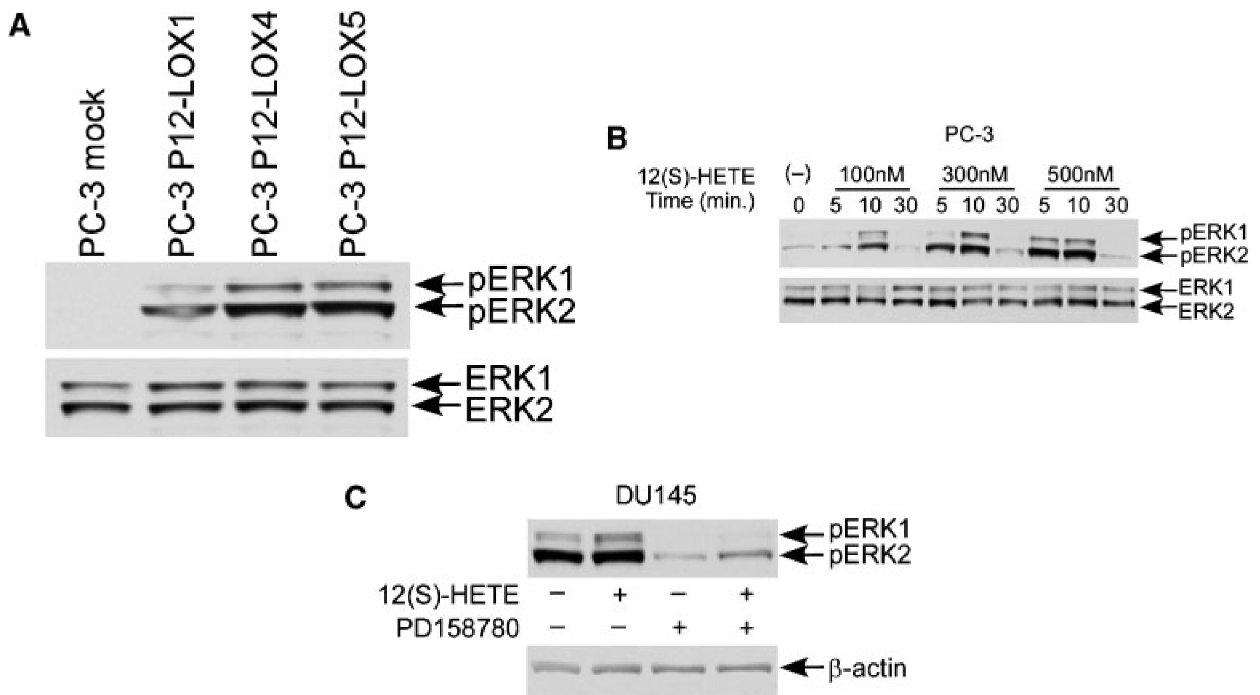

Fig. 2. 5×10^5 cells were plated in 60 mm dishes in RPMI containing 5% FBS. Cells were grown to 60%–70% confluence and serum deprived for 24 hr in RPMI. **A**: PC-3 mock and P12-LOX1, -4, -5 clones were lysed in lysis buffer and sonicated on ice. Ten microgram total protein per well was separated by polyacrylamide gel electrophoresis (in duplicate) and transferred to nitrocellulose. Phosphorylated and total ERK1/2 were detected with antibodies specific for each respective form of ERK1/2. **B**: PC-3 cells were exposed to 100, 300, or 500 nM 12(S)-HETE for 5, 10, or 30 min. Following treatment, cells were lysed, separated, and analyzed as in (A). **C**: Effects of EGFR inhibitor treatment on the 12(S)-HETE induced phosphorylation state of ERK1/2 in DU145 cells. Cells were plated at a density of 5×10^5 cells per 60 mm dish in 5% FBS and grown till 60%–70% confluent. Cells were serum deprived overnight in RPMI then pretreated with PD158780 (10 μM) for 20 min followed by treatment with either vehicle (0.1% EtOH) or 12(S)-HETE (100 nM) for 10 min. Protein was harvested and detected by Western blotting as described in Materials and Methods. Treatments in (A) and (C) were performed in triplicate with a representative blot shown for each.

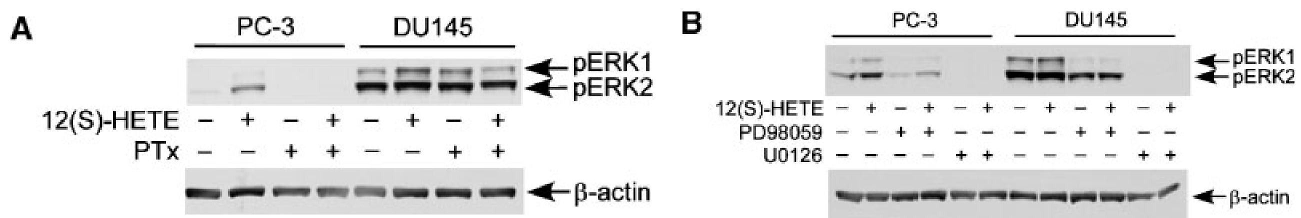

Fig. 3. Western blot analysis of the phosphorylation state of ERK1/2 following treatment with inhibitors of signal transduction pathways. **A**: Effects of pertussis toxin treatment on 12(S)-HETE induced phosphorylation of ERK1/2 in PC-3 and DU145 cells. Cells were plated at a density of 5×10^5 cells per 60 mm dish in 5% FBS and grown till 60%–70% confluent. Media was replaced with serum free media (with or without 0.5 μg/μl PTx) and cells were incubated for 18 hr. Cells were then either treated with vehicle (0.1% EtOH) or 100 nM 12(S)-HETE for 10 min. **B**: Effects of MEK inhibition on 12(S)-HETE induced phosphorylation of ERK1/2 in PC-3 and DU145 cells. Cells were plated and grown to 60%–70% confluence as in (A). Following 24 hr serum of deprivation, cells were pretreated with vehicle (0.1% DMSO for 30 min), PD98059 (50 μM for 1 hr) or U0126 (10 μM for 30 min) followed by treatment with vehicle (0.1% EtOH) or 100 nM 12(S)-HETE for 10 min. Protein was harvested and detected by Western blotting as described in Materials and Methods. Treatments were performed in triplicate with a representative blot shown for each.

to increased production of epidermal growth factor receptor (EGFR) and secretion of the EGFR ligands epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) [23]. Indeed, pretreatment with the EGFR inhibitor PD158780 almost completely blocked basal ERK1/2 phosphorylation but had no effect on 12(S)-HETE induced ERK1/2 phosphorylation (Fig. 2C) indicating that 12(S)-HETE does not induce ERK1/2 phosphorylation through any EGFR-dependent pathway in these cells.

12(S)-HETE Induces ERK1/2 Phosphorylation Through a Pertussis Toxin Sensitive G-Protein Coupled Receptor and MEK

12(S)-HETE was shown to initiate MAPK signaling through a pertussis toxin sensitive GPCR ($G_{i/o}$) in human epidermoid carcinoma A431 cells [24]. To delineate the mechanism of 12(S)-HETE induced phosphorylation of ERK1/2 in PC-3 and DU145 cells, we treated cells with PTx and inhibitors of MEK, the upstream kinase of ERK1/2. Treatment of PC-3 and DU145 cells with 0.5 μg/uL pertussis toxin for 18 hr blocked ERK1/2 phosphorylation induction by 12(S)-HETE (Fig. 3A), indicating that exogenous 12(S)-HETE induction of ERK1/2 phosphorylation is dependent upon 12(S)-HETE stimulation of pertussis toxin sensitive GPCRs. To determine if 12(S)-HETE stimulated ERK1/2 phosphorylation is dependent upon MEK, we pretreated PC-3 and DU145 cells with two structurally different inhibitors of MEK; PD98059 and U0126. As seen in Figure 3B, both PD98059 and U0126 can inhibit 12(S)-HETE induced phosphorylation of ERK1/2 in PC-3 and DU145 cells. U0126 appeared to be a much more potent MEK inhibitor as it eliminated both basal and inducible ERK1/2 phosphorylation. This may be due to U0126's ability to inhibit both MEK1 and MEK2, while PD98059 inhibition is specific for MEK1 only.

VEGF Expression Is Reduced by Inhibition of PTx Sensitive G-Protein Coupled Receptors and MEK

In this study, we have shown that P12-LOX overexpression leads to increased VEGF production. To determine if the observed increase in secreted VEGF was due to 12(S)-HETE activation of the ERK1/2 MAPK pathway, we treated P12-LOX4 cells with PTx to block signaling induced by 12(S)-HETE stimulation of PTx sensitive GPCRs and with the MEK1/2 inhibitor U0126 and quantified, by ELISA, secreted VEGF protein 24 hr post-initiation of treatment. As shown in Figure 4, treatment of P12LOX-4 cells with PTx

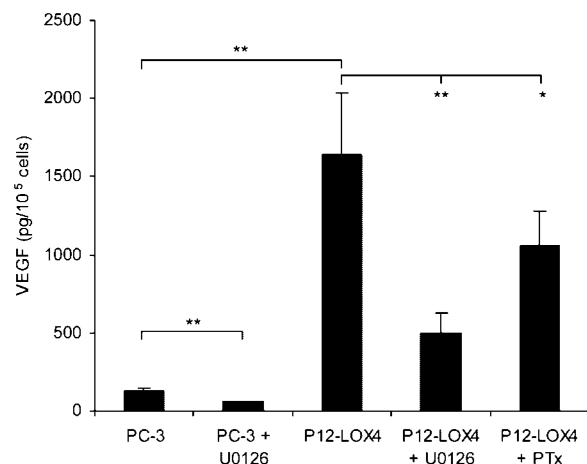

Fig. 4. VEGF protein from conditioned media of P12-LOX4 cells. PC-3 control and P12-LOX4 cells were seeded in RPMI with 5% FBS at a density of 1×10^5 cells per well in a 12-well dish and grown to ~60% confluence. Cells were incubated for 24 hr in serum reduced media (1% FBS) with or without PTx (0.5 μg/μl). Media was then replaced with fresh serum reduced media containing vehicle (0.1% DMSO), U0126 (10 μM), or PTx (0.5 μg/μl) for 24 hr. Conditioned media was collected and analyzed for VEGF content by ELISA. Data are shown as mean ± SD (untreated, n = 6 each group; U0126 and PTx, n = 3 each group). **Statistically significant ($P < 0.01$). *Statistically significant ($P < 0.05$).

reduced the level of VEGF in conditioned media by 36%. Interestingly, U0126 reduced VEGF accumulation by 70% for P12-LOX4 cells and by 56% in control PC-3 cells indicating MEK as a central player in the production of VEGF. This data indicate that chronic exposure to 12(S)-HETE is only partially responsible for the observed increase in VEGF expression as a result of P12-LOX overexpression in PC-3 cells.

DISCUSSION

Eicosanoids derived from aracidonic acid metabolism are known to play significant roles in a multitude of physiological and pathological conditions. A recent review indicates that eicosanoid products of COX-2 and 12-lipoxygenase modulate tumor growth by enhancing the angiogenic potential of multiple carcinomas [1]. The role of COX-2 in prostate cancer progression as a regulator of angiogenesis has been extensively investigated [25,26]; however, the role of 12-LOX is less clearly defined.

Platelet-type 12-lipoxygenase metabolizes arachidonic acid into the eicosanoid 12(S)-HETE. P12-LOX is expressed by platelets, several endothelial cell types [14,27], as well as in several types of animal and human tumors including prostate [3]. In 1995, Gao et al. [6] found that P12-LOX mRNA expression was significantly higher in prostate adenocarcinoma tissue compared to matched normal prostate epithelium, and P12-LOX expression could be correlated with carcinoma stage and grade. Stable overexpression of P12-LOX by PC-3 cells was shown to promote enhanced tumor growth and angiogenesis in the subcutaneous mouse model [7]; however, the underlying mechanism behind this phenomenon was not reported. Interestingly, in vitro studies show that P12-LOX overexpression by PC-3 cells confers no growth advantage (herein and [7]). It appears that in vivo, overexpression of P12-LOX results in some sort of alteration within the tumor microenvironment that promotes tumor growth, possibly by augmenting the tumor's angiogenic potential.

Angiogenesis, the process of new blood vessel formation from preexisting vasculature, is a complex process mediated by endothelial cells of the vasculature in response to external stimuli. Significant data alludes to a link between the production of 12(S)-HETE and the processes of tumor induced angiogenesis. For example, 12(S)-HETE possesses mitogenic properties for microvascular endothelial cells, promotes endothelial cell migration [3], and facilitates wound healing in scratch injured cell monolayers [13]. In addition, overexpression of P12-LOX in CD4 endothelial cells promotes cell migration and tube differentiation [14]. We found that overexpression of P12-LOX by PC-3 cells led to increased 12(S)-HETE production and accumulation within the culture media in as little as 30 min under serum free conditions. Addition of the P12-LOX substrate, arachidonic acid, further enhanced 12(S)-HETE production and accumulation. It is easy to speculate that cells overexpressing P12-LOX possess an enhanced angiogenic phenotype as a result of elevated 12(S)-HETE production. Moreover, 12(S)-HETE was shown recently to stimulate the production of the angiogenic factor VEGF in human vascular smooth muscle cells [15]. Earlier studies demonstrated the significance of VEGF in tumor angiogenesis by showing that inhibition of VEGF signaling results in impaired tumor angiogenesis and consequently tumor growth [28,29]. We found that P12-LOX overexpression in PC-3 cells resulted in a 13-fold increase in VEGF accumulation in culture media under serum free conditions. These results led us to inquire about a possible mechanism by which P12-LOX, presumably acting through 12(S)-HETE, could modulate the expression of VEGF.

Hypoxia is considered to be a major stimulator of VEGF expression [30]. In addition to hypoxia, various growth factor stimulated receptor tyrosine kinases can initiate signal transduction cascades leading to the transcription of VEGF [31–33]. One such signal transduction cascade is the MAPK pathway, particularly the ERK1/2 cascade [19,20]. We found that exposure of PC-3 and DU145 cells to 12(S)-HETE stimulates phosphorylation of ERK1/2 in a dose and time-dependant manner and that blockade of MEK, the kinase that phosphorylates ERK1/2, by two structurally unrelated pharmacological inhibitors, PD98059 and U0129, inhibited ERK1/2 phosphorylation in PC-3 and DU145 cells. LNCaP cells have been indicated to be affected similarly upon exposure to 12(S)-HETE [34]. Similar effects were noted previously in pancreatic β cells [35] and human epidermoid carcinoma A431 cells [24], indicating that this effect may be mirrored in a broad spectrum of cell types.

Signaling induced by 12(S)-HETE was previously shown to be mediated by a subset of transmembrane GPCRs that are sensitive to PTx [21,36]. Indeed, we found that 12(S)-HETE signals through a PTx sensitive GPCR ($G_{i/o}$) as treatment with PTx blocks ERK1/2 phosphorylation by 12(S)-HETE. Interestingly, blockade of 12(S)-HETE signaling through this GPCR resulted in only a 36% decrease in VEGF present in the conditioned media of cells overexpressing P12-LOX, whereas inhibition of MEK resulted in a 70% decrease in VEGF present in the conditioned media. These results indicate that 12(S)-HETE signaling through a PTx sensitive GPCR is only partially responsible for this increased VEGF presence and that other mechanisms of ERK1/2 stimulation are at work, such as 12(S)-HETE interaction with some intracellular,

as yet uncharacterized, receptor of 12(S)-HETE. In fact 12(S)-HETE can bind to a 50-kDa protein [37] that exists as part of a high molecular weight cytosolic binding complex [38]; however, the functional implications of binding to this receptor remain to be determined. In addition, it is possible that 12(S)-HETE may promote VEGF transcription by binding to the nuclear receptor peroxisome proliferated activating receptor gamma (PPARγ) [39], thus bypassing signaling through MAPKs completely. The role of PPARγ as an initiator of VEGF transcription is well known [40,41]. Also, we cannot rule out the possibility that P12-LOX overexpression promotes the expression of some other protein, such as COX-2, which in turn enhances VEGF expression [42] or that an initial burst of VEGF expression can initiate further VEGF expression via some uncharacterized VEGF autocrine pathway. Thus, we cannot conclude with absolute certainty that the observed increase in VEGF secretion is a result of 12-LOX enzymatic activity. Mechanisms of stimulating VEGF transcription such as these, as well as whether 12(S)-HETE stimulates VEGF transcription or promotes VEGF transcript stability in prostate cancer cells are currently being addressed in our laboratory.

CONCLUSIONS

Our results indicate that increased expression of P12-LOX in prostate cancer can result in enhanced production and secretion of the bioactive lipid 12(S)-HETE, which can stimulate ERK MAPK signaling through a pertussis toxin sensitive G-protein coupled receptor (GPCR). Activation of ERK1/2 by 12(S)-HETE in prostate cancer cells can mediate the expression of at least one angiogenic factor, shown herein to be VEGF. Increased expression of P12-LOX by prostate cancer cells results in augmented production of 12(S)-HETE and VEGF, two factors known to promote blood vessel formation, which can lead to enhanced tumor growth and metastasis by inducing extension of local vasculature. We believe that use of P12-LOX inhibitors as part of a combined anti-angiogenesis therapy regimen may prove beneficial in the treatment of prostate cancer.

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