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Opposing Effects of 15-Lipoxygenase-1 and -2 Metabolites on MAPK Signaling in Prostate

ALTERATION IN PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ*

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Linda C. Hsi, Leigh C. Wilson, and Thomas E. Eling‡

From the Eicosanoid Biochemistry Section, Laboratory of Molecular Carcinogenesis, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Human prostate tumors have elevated levels of 15-lipoxygenase-1 (15-LOX-1) and data suggest that 15-LOX-1 may play a role in the development of prostate cancer. In contrast, 15-LOX-2 expression is higher in normal rather than in tumor prostate tissue and appears to suppress cancer development. We recently reported that 13-(*S*)-HODE, the 15-LOX-1 metabolite, up-regulates the MAP kinase signaling pathway and subsequently down-regulates PPARγ in human colorectal carcinoma cells. To determine whether this mechanism is applicable to prostate cancer and what the effects of 15-LOX-2 are, we investigated the effect of 15-LOX-1, 15-LOX-2, and their metabolites on epidermal growth factor (EGF)- and insulin-like growth factor (IGF)-1 signaling in prostate carcinoma cells. In PC3 cells, 13-(*S*)-HODE, a 15-LOX-1 metabolite, up-regulated MAP kinase while in contrast 15-(*S*)-HETE, a 15-LOX-2 metabolite, down-regulated MAP kinase. As a result, 13-(*S*)-HODE increased PPARγ phosphorylation while a subsequent decrease in PPARγ phosphorylation was observed with 15-(*S*)-HETE. Thus, 15-LOX metabolites have opposing effects on the regulation of the MAP kinase signaling pathway and a downstream target of MAP kinase signaling like PPARγ. In addition to the EGF signaling pathway, the IGF signaling pathway appears to be linked to prostate cancer. 13-(*S*)-HODE and 15-(*S*)-HETE up-regulate or down-regulate, respectively, both the MAPK and Akt pathways after activation with IGF-1. Thus, the effect of these lipid metabolites is not solely restricted to EGF signaling and not solely restricted to MAPK signaling. These results provide a plausible mechanism to explain the apparent opposing effects 15-LOX-1 and 15-LOX-2 play in prostate cancer.

Lipoxygenases (LOXs)[1] are lipid peroxidizing enzymes that are categorized according to their position of oxygenation of arachidonic acid (1). For example, 15-LOXs oxygenate the substrate arachidonic acid at C-15. Two different human 15-LOXs have been identified that differ in tissue distribution and substrate preference. 15-LOX-1 is expressed in reticulocytes, eosinophils, macrophages, tracheobronchial epithelial cells, and skin (2). 15-LOX-2 has limited tissue distribution, with mRNA detected in prostate, lung, skin, and cornea (3). In terms of enzymatic characteristics, 15-LOX-1 preferentially metabolizes linoleic acid primarily to 13-(*S*)-HODE, but also metabolizes arachidonic acid to 15-(*S*)-HETE. 15-LOX-2, on the other hand, converts arachidonic acid to 15-(*S*)-HETE and metabolizes linoleic acid poorly (4).

Human prostate tumors have higher expression of 15-LOX-1 compared with normal adjacent tissue and this expression correlates with the Gleason score of the cancer (5). 13-(*S*)-HODE, the 15-LOX-1 metabolite, is detected in adenocarcinoma tissue (5). Furthermore, nude mice injected with 15-LOX-1-overexpressing prostate cells have increased frequency and size of tumors compared with nude mice injected with control cells (6). These data taken together suggest a possible pro-tumorigenic role for 15-LOX-1 in prostate tumor development. In contrast, 15-LOX-2 is expressed in normal prostate tissue, but poorly expressed in prostate tumors (3). The 15-LOX-2 expression is inversely correlated with the Gleason score of the tumor (3). Furthermore, 15-(*S*)-HETE is detected in benign prostate tissue samples, and 15-LOX-2 is a negative cell cycle regulator in normal prostate epithelial cells (3, 7). These results suggest there may be different, if not opposing, biological functions for 15-LOX-1 and 15-LOX-2 in the prostate.

Human prostate carcinomas express peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily (8). Ligand activation of this receptor causes many cancer cell lines to undergo a differentiative response and reverse their malignant phenotype (9). PPARγ agonists inhibit proliferation and potentially induce differentiation in many carcinoma cell lines, including breast (10, 11), colon (12), prostate (8, 13), lung (14, 15), esophageal (16), thyroid (17), and bladder (18). How the activation of PPARγ leads to growth inhibition is not known, but the data suggest that PPARγ could act as a tumor suppressor.

Metabolites of 15-LOX have recently been reported to serve as ligands for PPARγ. 13-(*S*)-HODE, 13-(*S*)-HpODE, and 15-(*S*)-HETE all show binding activity for PPARγ but at relatively high concentrations (19, 20). Also, recently, it was shown that exogenous 15-(*S*)-HETE could activate PPARγ using luciferase reporter assays and inhibit proliferation in prostate cells (8). Thus, based on activation of PPARγ, one could conclude that 15-LOX-1 may have antitumorigenic activity. However, this hypothesis is in conflict with the elevated 15-LOX-1 expression in tumors *versus* normal tissue and with observed increased

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‡ To whom correspondence should be addressed: NIEHS, P. O. Box 12233, Research Triangle Park, NC 27709. Tel.: 919-541-3911; Fax: 919-541-0146; E-mail: eling@niehs.nih.gov.

[1] The abbreviations used are: LOX, lipoxygenase; 15-LOX-1, 15-lipoxygenase-1; 15-LOX-2, 15-lipoxygenase-2; NaBT, sodium butyrate; 15-(*S*)-HETE, 15-(*S*)-hydroxyeicosatetraenoic acid; 13-(*S*)-HODE, 13-(*S*)-hydroxyoctadecadienoic acid; 13-(*S*)-HpODE, 13-(*S*)-hydroperoxyoctadecadienoic acid; NDGA, nordihydroguaiaretic acid; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular-regulated kinase; MEK, MAP kinase kinase; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; PPARγ, peroxisome proliferator-activated receptor γ; HPLC, high performance liquid chromatography; PI, phosphatidylinositol.

frequency and size of tumors in nude mice injected with 15-LOX-1-overexpressing cells compared with mice injected with control cells (6, 21).

Recently, we reported that 15-LOX-1 linoleic acid metabolites up-regulate the MAP kinase signaling pathway (22). MAP kinase can subsequently phosphorylate PPARγ, one potential downstream target of MAP kinase, and thus down-regulate PPARγ activity in human colorectal carcinoma cells (22). The up-regulation of the MAP kinase pathway provides a rationale to explain how 15-LOX-1 may play a protumorigenic role and may provide a clue to the difference between the biological function of 15-LOX-1 and 15-LOX-2. The aim of the present study is: 1) to compare the effects of 13-(*S*)-HODE, a 15-LOX-1 metabolite, to the effects of 15-(*S*)-HETE, a 15-LOX-2 metabolite, on MAP kinase signaling in prostate, 2) to determine whether changes in MAP kinase alter PPARγ in prostate, and 3) to determine whether any phenomenon observed is unique to EGF signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—The human prostate cell line PC3 was obtained from the American Type Culture Collection (ATCC). PC3 cells were cultivated in RPMI media (Invitrogen) supplemented with 10% fetal bovine serum (Summit), sodium pyruvate (Invitrogen), and gentamicin (1 mg/100 ml, Invitrogen). 15-LOX-1 or 15-LOX-2 constructs in pcDNA 3.1 vector (Invitrogen) were transfected into PC3 cells via LipofectAMINE (Invitrogen). Clones were selected in the presence of zeocin (Invitrogen). PC3 15-LOX-1 cells were cultivated in the same media as the normal PC3 cells plus the addition of zeocin.

Experimental Conditions—PC3 cells were grown to 75–80% confluency and then serum deprived for 18 h. Cells were treated with 13-(*S*)-HODE (5 μM) (Cayman) or 15-(*S*)-HETE (10 μM) (Cayman) 45 min prior to the addition of EGF (10 ng/ml) (Collaborative Research Assoc.) unless otherwise noted. IGF-1 (100 ng/ml) (Diagnostic Systems Laboratories) was also used. The MEK inhibitor PD98059 (50 μM) (CalBiochem), when used, was added to cells 30 min prior to addition of 13-(*S*)-HODE or 15-(*S*)-HETE. In the case of the 15-LOX-1 PC3 cells, linoleic acid (30 μM) or arachidonic acid (30 μM) was added to the cells as described above in RPMI without phenol red prior to treatment with EGF. After stimulation with EGF or IGF-1, cells were harvested at the various time points indicated. Normal human tracheobronchial epithelial (NHTBE) cells, as previously described (23), were used as a positive control for the expression of 15-LOX. Differentiated 3T3-L1 cells, as previously described (24), were used as a positive control for the expression of PPARγ.

SDS-Polyacrylamide Gel Electrophoresis—For Western analysis, cells were lysed and normalized, and then NuPAGE sample buffer was added to the samples. 15-LOX-1 and -2, ERK-1, and -2, Akt, PPARγ, and actin proteins were separated on 4–12% Bis/Tris gradient NuPAGE gels (Invitrogen). Proteins were transferred onto nitrocellulose membrane (Invitrogen).

Immunoblot Analysis—Blots were blocked with 10% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and washed. The blots were then incubated in 1 or 5% milk in TBS-T with an anti-appropriate specific antibody. The following antibodies were used: CheY-human 15-LOX-1 (Ref. 25, kindly provided by Dr. E. Sigal), 15-LOX-2 (kindly provided by Dr. A. Brash), phosphospecific-MAP kinase (New England Biolab), MAP kinase (ERK-1 and ERK-2) (Santa Cruz Biotechnologies), PPARγ (Santa Cruz Biotechnologies), phosphospecific (Ser-437)-Akt (Cell Signaling Technology), Akt (Cell Signaling Technology), or actin (Santa Cruz Biotechnologies). After washing, blots were incubated with anti-rabbit IgG horseradish peroxidase-linked secondary antibody (Amersham Biosciences) for 15-LOX-1 and -2, MAP kinase, phospho-Akt, and Akt, anti-mouse IgG horseradish peroxidase-linked secondary antibody (Amersham Biosciences) for phospho-MAP kinase and PPARγ or anti-goat IgG horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology) for actin, respectively. After reacting by chemiluminescence (Amersham Biosciences ECL detection system), bands were detected by exposure to Hyperfilm-MP (Amersham Biosciences).

Analysis of Arachidonic Acid and Linoleic Acid Metabolites in Intact cells—PC3 or 15-LOX-1 clone cells cultured in 150-cm^2 dishes at each condition were washed with serum-free medium twice. 10 ml of phosphate-buffered saline supplemented with 10 μM $CaCl_2$ was then added to each plate, the appropriate treatments were added, and each plate was incubated for 15 min at 37 °C. Nordihydroguaiaretic acid (NDGA) was used at a concentration of 10 μM. Each plate was reacted with [^{3}H]arachidonic acid (3 μCi, 30 μM) (PerkinElmer Life Sciences) or [^{14}C]linoleic acid (3 μCi, 30 μM) (PerkinElmer Life Sciences) for 1 h. at 37 °C. The media were collected, and each plate was washed with 2 ml of MeOH and 2 ml of 1% acetic acid. The cells were scraped into this wash and added to the appropriate tube containing the media previously collected. The total collected media was acidified with acetic acid to pH 3 and applied to a C_{18}-PrepSep solid phase extraction column (Waters) pretreated with methanol. The samples were washed with acidified water, eluted with methanol, evaporated to dryness, and reconstituted with high pressure liquid chromatography (HPLC) solvent.

High Pressure Liquid Chromatography—Reverse-phase HPLC analysis was performed using an Ultrasphere ODS column (5 μm; 4.6 × 250 mm; Beckman). The solvent system consisted of a methanol/water gradient at a flow rate of 1.1 ml/min as previously described (26). Radioactivity was monitored using a Flow Scintillation Analyzer (Packard) with EcoLume (ICN Biochemicals) as the liquid scintillation mixture. Authentic standards of 13-(*S*)-HODE and 15-(*S*)-HETE (Cayman Chemical) were used.

Analysis of Densitometry Measurements—Autoradiograms from Western blots were scanned using a Umax™ Powerlook III™ scanner equipped with transparency adapter and scanning software. Bands were quantitated using Scion Image™ beta version 4.0.2. Western blot values were first corrected using their corresponding actin levels. Values shown are fold increases *versus* vehicle or zero hour as illustrated in the figure legends. Statistical analysis using Student's *t* test was also performed.

RESULTS

Endogenous 15-LOX-1, 15-LOX-2, and PPARγ Expression in PC3 Cells—PC3 cells, a human prostate cancer cell line, were used for the experimental studies to follow. Basal levels of 15-LOX-1, 15-LOX-2, and PPARγ expression were first confirmed by Western analysis (data not shown). PC3 cells lack endogenous 15-LOX-1 and 15-LOX-2 expression, while PPARγ1 is detectable. Differentiated 3T3-L1 cells, which express both PPARγ1 and PPARγ2 isoforms, were used as a positive control for PPARγ (27). No functional difference has been found between the two isotypes. PC3 cells express only the PPARγ1 isoform. From this point on, we will refer to PPARγ1 as PPARγ since all the subsequent experiments are done in PC3 cells.

Effect of 13-(S)-HODE or 15-(S)-HETE on MAPK Phosphorylation—Following serum deprivation, PC3 cells were pretreated with 5 μM 13-(*S*)-HODE or 10 μM 15-(*S*)-HETE for 45 min prior to treatment with EGF (10 ng/ml). The effect of 13-(*S*)-HODE or 15-(*S*)-HETE on MAP kinase after EGF stimulation was examined by Western analysis at the indicated time points (Fig. 1). Using a phosphospecific MAPK antibody, an increase in MAPK phosphorylation was observed in cells treated with 13-(*S*)-HODE compared with cells treated with EGF alone (Fig. 1*A*). A 2.2 ± 0.2-fold increase ($n = 3$) ($p < 0.001$) in MAPK phosphorylation could be detected after treatment. In contrast to this, a 2.8 ± 0.3-fold decrease ($n = 3$) ($p < 0.001$) in MAPK phosphorylation was observed in cells treated with 15-(*S*)-HETE compared with cells treated with EGF alone (Fig. 1*B*). Total MAP kinase expression levels were also examined by antibodies to ERK-1 and ERK-2 for this experiment and all subsequent experiments measuring phosphorylated MAPK. In all cases, total MAP kinase levels did not change, and thus only the levels of phosphorylated MAPK were altered by treatment with 13-(*S*)-HODE, 15-(*S*)-HETE, or the treatment indicated for each experiment (data not shown). The density of the phosphorylated proteins (ERK1/2) was measured, normalized to actin, and are reported in the parentheses below the blots.

13-(S)-HODE or 15-(S)-HETE Dose Response on MAPK Phosphorylation—In order to examine whether the response was dependent on the concentration of 13-(*S*)-HODE or 15-(*S*)-HETE, the effects of different 13-(*S*)-HODE or 15-(*S*)-HETE

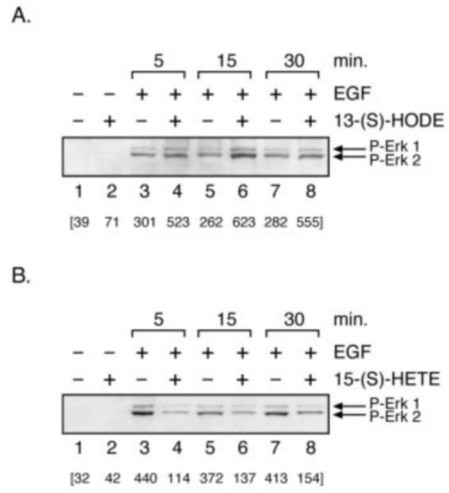

FIG. 1. **Effect of 13-(*S*)-HODE or 15-(*S*)-HETE on MAPK phosphorylation.** Western analysis of cell lysates demonstrating phosphorylated MAP kinase in PC3 cells after treatment with EGF (10 ng/ml) in the presence or absence of 13-(*S*)-HODE (5 μM) (*A*) or 15-(*S*)-HETE (10 μM) (*B*) for the indicated times. The data shown represent one of three separate experiments for each metabolite with similar results. 30 μg of total protein was loaded per lane for MAPK. Phosphorylation was measured by densitometry and normalized to actin. The values are reported in the parentheses below the gels.

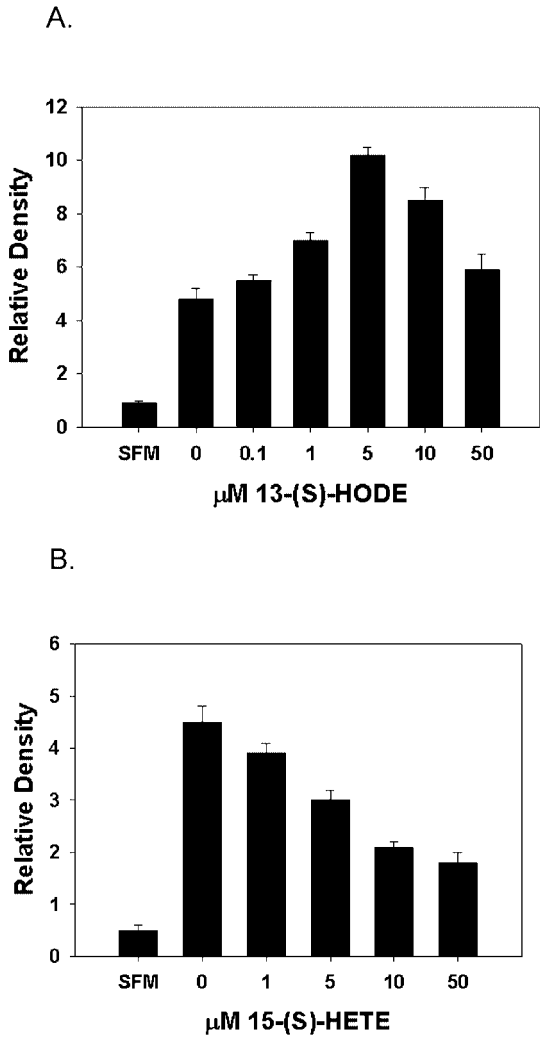

FIG. 2. **13-(*S*)-HODE or 15-(*S*)-HETE dose response on MAPK phosphorylation.** PC3 cells were treated with EGF (10 ng/ml) in the presence or absence of 13-(*S*)-HODE (*A*) or 15-(*S*)-HETE (*B*) at the indicated concentration for 15 min. Cell lysates were blotted for phosphorlyated MAPK and normalized to actin. Relative densities are reported. The data shown represent one of two separate experiments for each metabolite with similar results. 30 μg of total protein was loaded per lane for MAPK.

concentrations on MAP kinase phosphorylation were tested. Following serum deprivation, PC3 cells were pretreated with varying concentrations of 13-(*S*)-HODE or 15-(*S*)-HETE, ranging from 0.1–50 μM, for 45 min prior to treatment with EGF (10 ng/ml). The effect of the metabolites on MAP kinase phosphorylation after EGF stimulation was examined by Western analysis using a phosphospecific MAPK antibody. Fig. 2 reports the data for 13-(*S*)-HODE or 15-(*S*)-HETE EGF-dependent MAPK phosphorylation normalized to actin. An increase in MAPK phosphorylation was observed in cells treated with 13-(*S*)-HODE over the cells treated with EGF alone (Fig. 2*A*). An increase in MAPK phosphorylation was observed at concentrations as low as 0.1 μM, but the strongest increase in MAPK phosphorylation was detected at 5 μM 13-(*S*)-HODE. Conversely, a decrease in MAPK phosphorylation was observed in cells treated with 15-(*S*)-HETE compared with the cells treated with EGF alone (Fig. 2*B*). A decrease in MAPK phosphorylation was observed at 1 μM, but 10 μM was an optimal concentration. Total MAP kinase expression levels were also examined using antibodies to ERK-1 and ERK-2. No changes in total MAP kinase expression were noted. Thus, the response to lipid metabolites is to alter phosphorylation of MAP kinase. 5 μM 13-(*S*)-HODE or 10 μM 15-(*S*)-HETE are optimal concentrations to observe increases or decreases in MAPK phosphorylation in EGF-stimulated cells, respectively.

15-LOX-1 Overexpression and Activity—To determine if endogenous 15-LOX-1 metabolites could affect MAPK phosphorylation, a stable 15-LOX-1-overexpressing cell line was utilized. PC3 cells were transfected with either vector alone or the 15-LOX-1 cDNA. Individual clones were isolated and tested for 15-LOX-1 expression by Western analysis. The 15-LOX-1 PC3 cells were found to express 15-LOX-1 abundantly (data not shown). Parental PC3 cells expressed no 15-LOX-1. Actin was used as a control for the amount of protein loaded. We repeatedly tried to prepare PC3 cells overexpressing 15-LOX-2. Interestingly, in every attempt, the 15-LOX-2 transfected cells died while control vector-transfected cells grew normally. However, under the appropriate conditions, 15-LOX-1 will metabolize arachidonic acid to 15-(*S*)-HETE and thus, the 15-LOX-1 PC3 cells were used to test for the effects of endogenously formed 15-(*S*)-HETE.

To confirm metabolic activity of the cells, intact 15-LOX-1 PC3 cells were reacted with radiolabeled linoleic acid (30 μM), and 15-LOX-1 activity was examined by HPLC analysis of the metabolites. PC3 15-LOX-1 cells produced 13-(*S*)-HODE, the main metabolite, with a retention time of about 64 min (data not shown). This 15-LOX-1 activity was inhibited by NDGA, a lipoxygenase inhibitor, consistent with a 15-LOX-1 activity. Also, intact 15-LOX-1 PC3 cells reacted with radiolabeled arachidonic acid (30 μM) produced 15-(*S*)-HETE as the main metabolite (data not shown). In contrast, vector-transfected PC3 cells reacted with radiolabeled linoleic acid or arachidonic acid did not produce 15-LOX-1 metabolites. Hence, the 15-LOX-1 in the overexpressing cell line is active.

MAPK Phosphorylation in 15-LOX-1 PC3 Cells—The exogenous addition of 13-(*S*)-HODE or 15-(*S*)-HETE followed by EGF stimulation of PC3 cells, respectively, either increased or decreased MAPK phosphorylation. Here, we investigate whether endogenous 13-(*S*)-HODE or 15-(*S*)-HETE has the same effect by utilizing 15-LOX-1-overexpressing cells. Following serum starvation, PC3 15-LOX-1 cells were pretreated with 30 μM

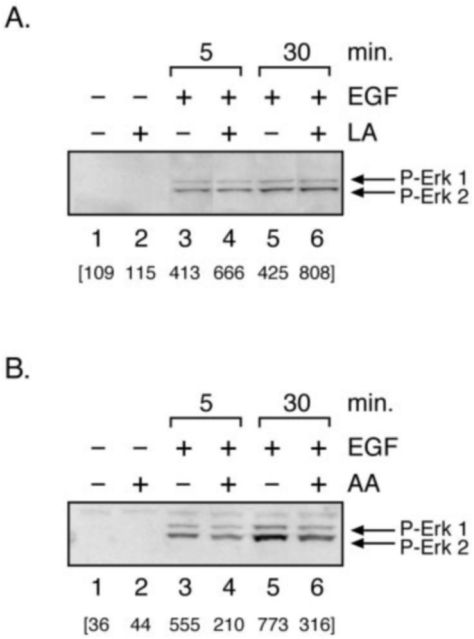

FIG. 3. **MAPK phosphorylation in 15-LOX-1 PC3 cells.** Western analysis of cell lysates demonstrating phosphorylated MAP kinase in 15-LOX-1 PC3 cells after treatment with EGF (10 ng/ml) in the presence or absence of linoleic acid (30 μM) (*A*) or arachidonic acid (30 μM) (*B*) for the indicated times. The data shown represent one of three separate experiments for each fatty acid with similar results. 30 μg of total protein was loaded per lane for MAPK. Phosphorylation was measured by densitometry and normalized to actin. The values are reported in the parentheses below the gels.

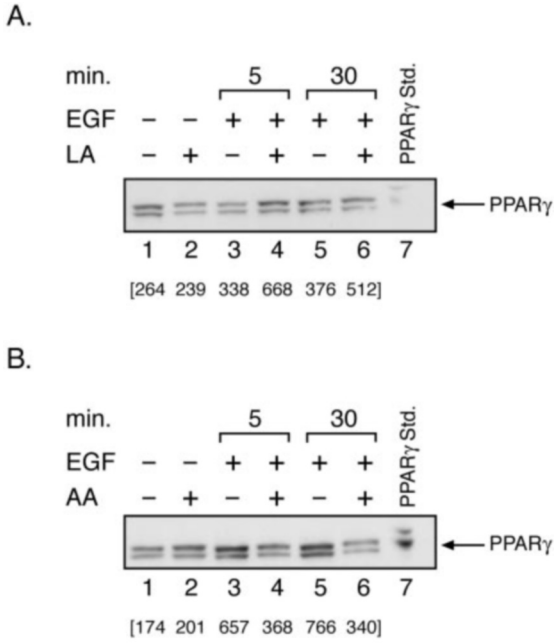

FIG. 5. **PPARγ phosphorylation in 15-LOX-1 PC3 cells.** Western analysis of cell lysates demonstrating phosphorylated PPARγ expression in 15-LOX-1 PC3 cells after treatment with EGF (10 ng/ml) in the presence or absence of linoleic acid (30 μM) (*A*) or arachidonic acid (30 μM) (*B*) for the indicated times. The data shown represent one of three separate experiments for each fatty acid with similar results. 30 μg of total protein was loaded per lane for PPARγ. 10 μg of PPARγ standard was used. Phosphorylation was measured by densitometry and normalized to actin. The values are reported in the parentheses below the gels.

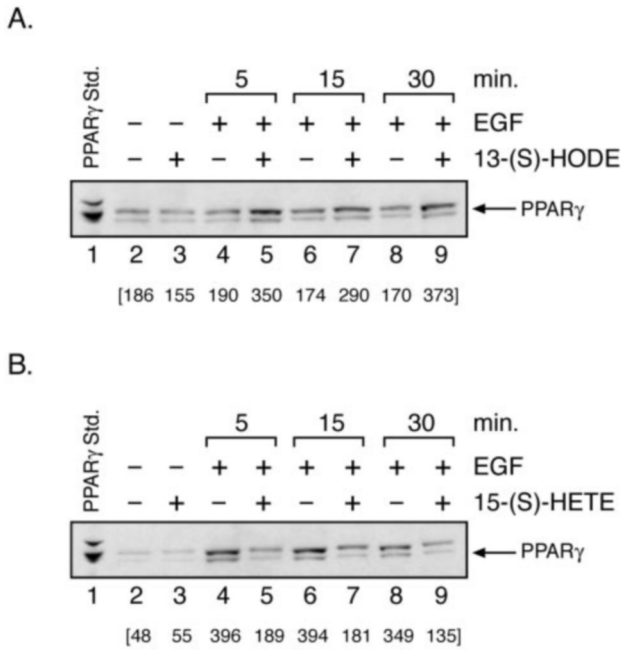

FIG. 4. **Effect of 13-(*S*)-HODE or 15-(*S*)-HETE on PPARγ phosphorylation.** Western analysis of cell lysates demonstrating phosphorylated PPARγ in PC3 cells after treatment with EGF (10 ng/ml) in the presence or absence of 13-(*S*)-HODE (5 μM) (*A*) or 15-(*S*)-HETE (10 μM) (*B*) for the indicated times. The data shown represent one of three separate experiments for each metabolite with similar results. 30 μg of total protein was loaded per lane for PPARγ. 10 μg of PPARγ standard was used. Phosphorylation was measured by densitometry and normalized to actin. The values are reported in the parentheses below the gels.

linoleic acid or 30 μM arachidonic acid for 45 min prior to treatment with EGF (10 ng/ml). MAP kinase phosphorylation after EGF stimulation was examined by Western analysis at the indicated time points (Fig. 3). Actin expression was measured and used to normalize the data. The normalized density measurements are reported in the brackets. A 1.9 ± 0.1-fold increase ($n = 3$) ($p < 0.002$) in MAPK phosphorylation was observed in cells treated with linoleic acid plus EGF over the only EGF-treated cells (Fig. 3*A*). In contrast, the addition of arachidonic acid plus EGF to 15-LOX-1 PC3 cells caused a 2.5 ± 0.1-fold decrease ($n = 3$) ($p < 0.002$) in MAPK phosphorylation compared with only EGF-treated cells (Fig. 3*B*). Thus, endogenously produced 15-LOX metabolites had a similar effect on MAPK as exogenously added metabolites.

Effect of 13-(S)-HODE or 15-(S)-HETE on PPARγ Phosphorylation—The 15-LOX metabolites altered MAPK phosphorylation. Since PPARγ is a potential downstream target for MAP kinase, we examined whether 13-(*S*)-HODE or 15-(*S*)-HETE alters PPARγ phosphorylation. Following serum deprivation, PC3 cells were pretreated with 5 μM 13-(*S*)-HODE or 10 μM 15-(*S*)-HETE for 45 min prior to treatment with EGF (10 ng/ml). PPARγ phosphorylation was examined by Western analysis at the indicated time points (Fig. 4). An increase in PPARγ phosphorylation was observed in 13-(*S*)-HODE plus EGF-treated cells over only EGF-treated cells (Fig. 4*A*). The upper band of the doublet observed is the phosphorylated form of PPARγ while the lower band of the doublet is the unphosphorylated form. The addition of 13-(*S*)-HODE increased PPARγ phosphorylation by 2.0 ± 0.2-fold ($n = 3$) ($p < 0.001$). In contrast, upon addition of 15-(*S*)-HETE to cells in the presence of EGF, a 2.3 ± 0.1-fold decrease ($n = 3$) ($p < 0.001$) in PPARγ phosphorylation was observed (Fig. 4*B*).

To determine if endogenously formed 13-(*S*)-HODE or 15-(*S*)-HETE also alters PPARγ phosphorylation, the 15-LOX-1-overexpressing PC3 cells were incubated with linoleic acid or arachidonic acid. PPARγ phosphorylation was increased by 1.7 ± 0.2-fold ($n = 3$) ($p < 0.009$) with linoleic acid (Fig. 5*A*). In contrast, incubation of the overexpressing cells with arachi-

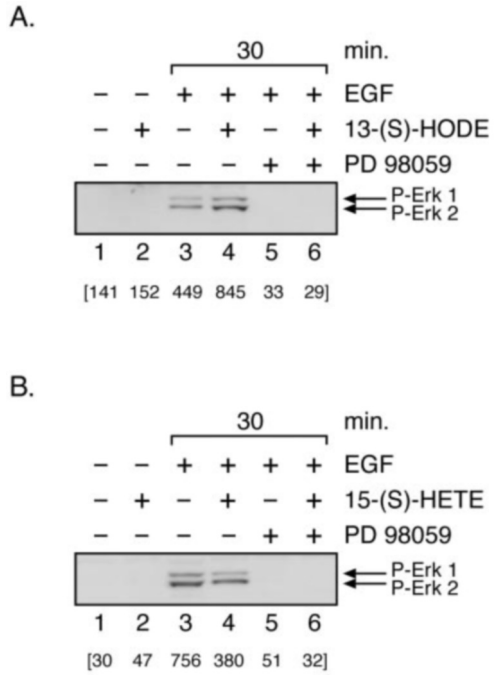

FIG. 6. **Effect of PD98059 on MAP kinase phosphorylation.** Western analysis of cell lysates demonstrating phosphorylated MAP kinase in PC3 cells after treatment with EGF (10 ng/ml) in the presence or absence of PD98059 (50 μM) with 13-(*S*)-HODE (5 μM) (*A*) or 15-(*S*)-HETE (10 μM) (*B*) for the indicated time. The data shown represent one of three separate experiments for each metabolite with similar results. 30 μg of total protein was loaded per lane for MAPK. Phosphorylation was measured by densitometry and normalized to actin. The values are reported in the parentheses below the gels.

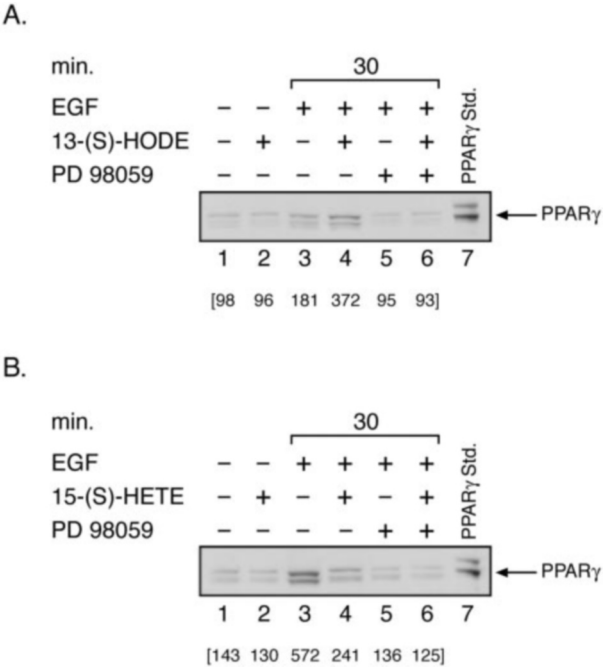

FIG. 7. **MAP kinase phosphorylation of PPARγ.** Western analysis of cell lysates demonstrating phosphorylated PPARγ in PC3 cells after treatment with EGF (10 ng/ml) in the presence or absence of PD98059 (50 μM) with 13-(*S*)-HODE (5 μM) (*A*) or 15-(*S*)-HETE (10 μM) (*B*) for the indicated time. The data shown represent one of three separate experiments for each metabolite with similar results. 30 μg of total protein was loaded per lane for PPARγ. Phosphorylation was measured by densitometry and normalized to actin. The values are reported in the parentheses below the gels.

donic acid caused a 2.1 ± 0.1-fold decrease ($n = 3$) ($p < 0.008$) in PPARγ phosphorylation (Fig. 5*B*). Thus, endogenously produced 13-(*S*)-HODE and 15-(*S*)-HETE had opposing effects on PPARγ phosphorylation. 13-(*S*)-HODE causes an increase in both MAPK and PPARγ phosphorylation and conversely, endogenously produced 15-(*S*)-HETE causes a decrease in both MAPK and PPARγ phosphorylation.

Phosphorylation of PPARγ Is MAPK-dependent—To confirm that the phosphorylation of PPARγ is dependent on MAP kinase activity, the effect of PD98059, a specific inhibitor of MEK, on MAPK and PPARγ phosphorylation was examined. Following serum deprivation, PC3 cells were pretreated with 5 μM 13-(*S*)-HODE or 10 μM 15-(*S*)-HETE for 45 min in the presence or absence of PD98059 (50 μM) prior to treatment with EGF (10 ng/ml). MAP kinase and PPARγ phosphorylation was examined by Western analysis at the indicated time points following EGF stimulation (Figs. 6 and 7). A 2.1 ± 0.3-fold increase ($n = 3$) ($p < 0.002$) in MAPK and a 2.0 ± 0.1-fold increase ($n = 3$) ($p < 0.001$) in PPARγ phosphorylation was observed in cells treated with 13-(*S*)-HODE plus EGF compared with only EGF-treated cells. A 2.1 ± 0.1-fold decrease ($n = 3$) ($p < 0.001$) in MAPK and a 2.3 ± 0.2-fold decrease ($n = 3$) ($p < 0.001$) in PPARγ phosphorylation was observed in cells treated with 15-(*S*)-HETE plus EGF compared with only EGF-treated cells. However, in the presence of the MEK inhibitor, PD98059, MAPK phosphorylation was abolished (Fig. 6). This is consistent with inhibition of MEK activity. Likewise, PPARγ phosphorylation was ablated (Fig. 7). Total MAP kinase levels did not change, only the levels of phosphorylated MAPK were altered by treatment with 13-(*S*)-HODE or 15-(*S*)-HETE (data not shown). Normalization of the phosphorylation density measurements was performed, and the values are reported in parentheses. These results are consistent with the hypothesis that phosphorylation of PPARγ observed upon treatment with 15-LOX metabolites in EGF-stimulated cells is dependent on MAP kinase activity in prostate cells.

Effect of 13-(S)-HODE or 15-(S)-HETE in IGF-1-treated Cells—In addition to the EGF signaling pathway, the IGF signaling pathway appears to be linked to prostate cancer (27–29). Thus, we examined the effect of 13-(*S*)-HODE or 15-(*S*)-HETE on MAPK and the subsequent phosphorylation of PPARγ with insulin-like growth factor-1 (IGF-1)-treated PC3 cells. Increased phosphorylation of MAPK (2.1 ± 0.1-fold, $n = 3$) ($p < 0.009$) and PPARγ (1.9 ± 0.1-fold, $n = 3$) ($p < 0.008$) with 13-(*S*)-HODE or decreased phosphorylation of MAPK (3.1 ± 0.2-fold, $n = 3$) ($p < 0.003$) and PPARγ (2.0 ± 0.1-fold, $n = 3$) ($p < 0.004$) with 15-(*S*)-HETE was also observed with IGF-1-activated MAPK signaling (Fig. 8). These findings suggest the effects of 15-LOX metabolites are not restricted to EGF signaling.

Effect of 13-(S)-HODE or 15-(S)-HETE on Akt in IGF-1-treated Cells—In addition to activating the MAPK signaling pathway, IGF-1 also can activate the PI3 kinase/Akt pathway. To determine whether this pathway is also affected by the 15-LOX metabolites, phosphorylated Akt was examined using an Akt-phosphospecific antibody. An increase in Akt phosphorylation was observed with 13-(*S*)-HODE plus IGF-1 (1.5 ± 0.1-fold, $n = 3$) ($p < 0.004$) while a decrease in Akt phosphorylation was observed with 15-(*S*)-HETE plus IGF-1 (1.4 ± 0.05-fold, $n = 3$) ($p < 0.008$) as compared with only IGF-1-stimulated cells (Fig. 9). Total Akt protein levels were not altered by the treatments (data not shown). Interestingly, the changes in phosphorylation of Akt by the 15-LOX metabolites was not as great as that observed on MAPK phosphorylation. A 2-fold or greater change in MAPK phosphorylation was observed upon addition of the metabolites in the presence of IGF-1. However, a less than 2-fold change in Akt phosphorylation was observed.

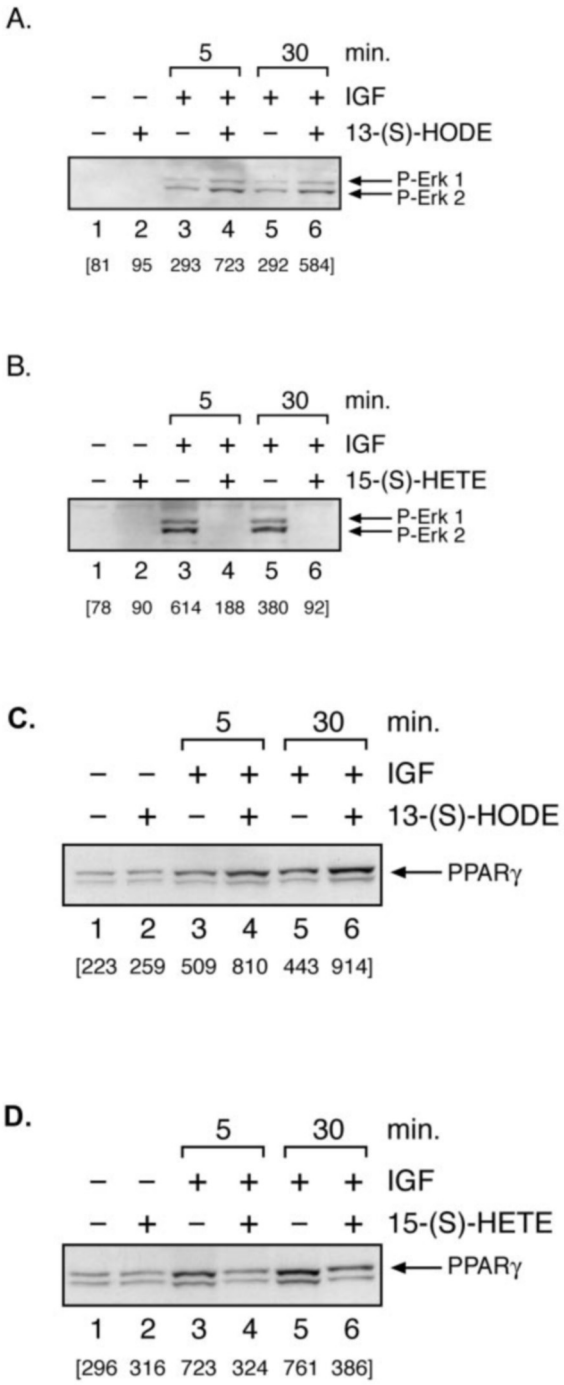

FIG. 8. **Effect of 13-(*S*)-HODE or 15-(*S*)-HETE on MAPK phosphorylation and PPARγ phosphorylation in IGF-treated cells.** Western analysis of cell lysates demonstrating phosphorylated MAP kinase or PPARγ in PC3 cells after treatment with IGF-1 (100 ng/ml) in the presence or absence of 13-(*S*)-HODE (5 μM) (*A* and *C*) or 15-(*S*)-HETE (10 μM) (*B* and *D*) for the indicated times. The data shown represent one of three separate experiments for each metabolite with similar results. 30 μg of total protein was loaded per lane for MAPK and PPARγ. Phosphorylation was measured by densitometry and normalized to actin. The values are reported in the parentheses below the gels.

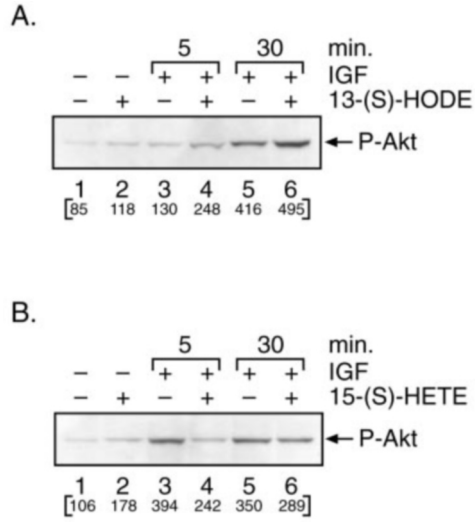

FIG. 9. **Effect of 13-(*S*)-HODE or 15-(*S*)-HETE on Akt phosphorylation in IGF-treated cells.** Western analysis of cell lysates demonstrating phosphorylated Akt in PC3 cells after treatment with IGF-1 (100 ng/ml) in the presence or absence of 13-(*S*)-HODE (5 μM) (*A*) or 15-(*S*)-HETE (10 μM) (*B*) for the indicated times. The data shown represent one of three separate experiments for each metabolite with similar results. 30 μg of total protein was loaded per lane for Akt. Phosphorylation was measured by densitometry and normalized to actin. The values are reported in the parentheses below the gels.

DISCUSSION

Previously, we have shown that the 15-LOX-1 metabolite, 13-HODE, increases EGF-dependent MAPK activity, which in turn can cause a decrease in PPARγ activity in colorectal cancer cells (22). In the current study, we have furthered our studies to include 15-LOX-2 and prostate cells since 15-LOX-1 and 15-LOX-2 differ in their expression in tumor and normal prostate tissue. The difference between 15-HETE, the major metabolite of 15-LOX-2, and 13-HODE, the major metabolite of 15-LOX-1, effects on MAPK activity was investigated. Both exogenous and endogenous 13-HODE increases MAPK activity and thus decreases PPARγ activity. In contrast, both exogenous and endogenous 15-HETE decreases MAPK activity, which in turn results in an increase in PPARγ activity. In addition, the effects of the 15-LOX metabolites on MAPK are not unique to EGF signaling, as the IGF signaling pathway and Akt phosphorylation are also affected in prostate cells. These findings are of particular interest given the fact that 15-LOX-1 expression is higher in tumors whereas 15-LOX-2 expression is higher in normal prostate tissue. Also, given the importance of the IGF/Akt pathway in prostate tissue, showing the metabolites alter this signaling pathway is particularly relevant to tumor development. Thus, the findings presented here advance our understanding of the two human 15-LOX enzymes, 15-LOX-1 and 15-LOX-2, which differ in their expression and their apparent biological activity in human prostate.

In prostate, higher expression of 15-LOX-1 in tumors is linked to Gleason score of the tumor whereas 15-LOX-2 is expressed in normal prostate tissue and inversely correlates with Gleason score (3, 5). Based upon several experimental findings, 15-LOX-1 appears to have a pro-tumorigenic activity while 15-LOX-2 appears to have anti-tumorigenic activity (6, 7). These results thus suggest opposing biological functions for 15-LOX-1 and 15-LOX-2 in the prostate. The results presented here provide a rationale for understanding the different biological activity between 15-LOX-1 and 15-LOX-2 in human prostate and is based, in part, on differences in substrate preference for the two enzymes. 15-LOX-1 preferentially metabolizes linoleic acid to 13-(*S*)-HODE while 15-LOX-2 metabolizes arachidonic acid to 15-(*S*)-HETE but poorly metabolizes linoleic acid (4). Thus, as normal tissue undergoes transformation to a tumor with the loss of 15-LOX-2 and gain of 15-LOX-1, the metabolites also shift from 15-(*S*)-HETE to 13-(*S*)-HODE.

Exogenous 13-(*S*)-HODE up-regulates the EGF- and IGF-1-dependent MAP kinase pathway in prostate cells. In contrast, exogenous 15-(*S*)-HETE down-regulates the EGF- and IGF-1-

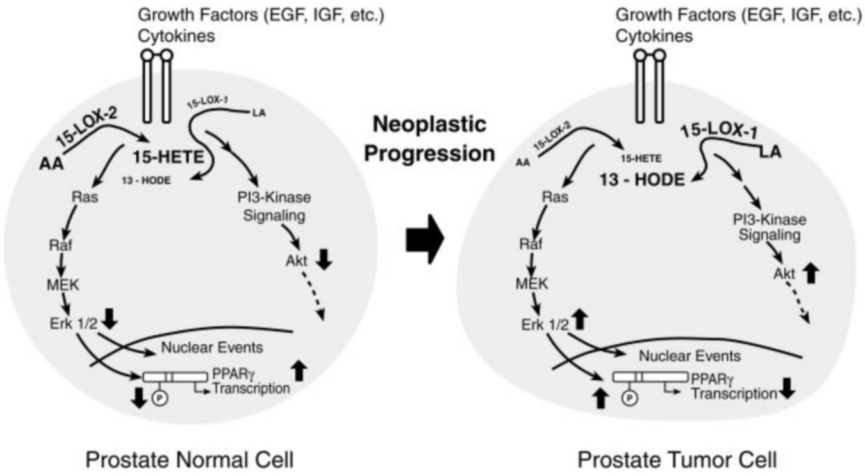

FIG. 10. **Proposed model for opposing effects of 15-LOX metabolites.** Growth factors (such as EGF or IGF) interact with their respective receptor that activates MAP kinase signaling. Endogenous 13-HODE up-regulates growth factor initiated MAP kinase activity and as a result, MAP kinase-dependent PPARγ phosphorylation is increased. This causes a down-regulation or loss of PPARγ transcriptional activity. In contrast, 15-HETE has the opposite effect, causing a down-regulation of MAPK activity. 13-HODE and 15-HETE are also thought to be endogenous ligands for PPARγ and cause activation of PPARγ receptor. However, a more likely scenario may be to regulate PPARγ through the MAPK signaling pathway. Thus a balance between these two opposing effects of 15-LOX metabolites may determine the role they play in the development of prostate cancer. Perhaps there is a shift in expression of 15-LOX-2 to 15-LOX-1 as a cell undergoes neoplastic progression from a normal cell to a tumor cell.

dependent MAP kinase pathway. To confirm that endogenous 15-LOX metabolites produce the same responses, we utilized stable 15-LOX-1 overexpressing PC3 cells. By treating these cells with linoleic acid or arachidonic acid, we found that endogenous 15-LOX metabolites had the same effect as addition of exogenous 13-(*S*)-HODE or 15-(*S*)-HETE. Thus, in PC3 cells, endogenous 15-LOX metabolites have opposing effects on the regulation of MAP kinase, a key pathway linked to cell proliferation and tumor development. Although it is possible that other 15-LOX products may be playing a role in the proposed action, we do know that the main metabolites generated are 13-(*S*)-HODE with linoleic acid as a substrate and 15-(*S*)-HETE with arachidonic acid as a substrate.

13-HODE and 15-HETE bind and activate PPARγ *in vitro* suggesting that the metabolites may function as endogenous ligands for PPARγ (19). However, these findings are observed with high concentrations of the metabolites (30–100 μM) and most of these studies employ only exogenous metabolites (19, 20). The physiological relevance of these lipid substances as regulators of PPARγ *in vivo* is not fully established. One potential downstream target of MAPK signaling is PPARγ. Phosphorylation of PPARγ results in a decrease in transcriptional activity (28–31). MAP kinase, a central regulator of cell growth, phosphorylates a key residue, Ser-82 on PPARγ1, which results in a decrease in PPARγ transcriptional activity (28, 29, 32). In PC3 cells, endogenous 15-LOX metabolites have opposing effects on the regulation of MAP kinase activity and have opposing effects on PPARγ activity. Based on data from this and previous studies (8, 22), it appears that down-regulation of PPARγ activity is specific for linoleic acid metabolites as 13-(*S*)-HODE, 13-(*R*)-HODE, and 13-(*S*)-HpODE all increase MAPK activity and hence PPARγ phosphorylation whereas 15-(*S*)-HETE, an arachidonic acid metabolite, has the opposite effect. These effects on MAP kinase are observed with endogenously generated metabolites and the addition of the 15-LOX metabolites alters MAP kinase at lower concentrations than required to observe PPARγ ligand binding *in vitro*. Thus, these findings indicate that endogenous 15-LOX metabolites alter PPARγ transcriptional activity via MAPK phosphorylation rather than acting as a ligand for this receptor. PPARγ agonists inhibit proliferation and potentially induce differentiation in many carcinoma cell lines (8, 10–18), suggesting that PPARγ could act as a tumor suppressor. The 15-LOX metabolites appear to modulate this activity via MAPK phosphorylation.

In addition to the EGF signaling, IGF-1 signaling appears to play an important role in prostate cancer. Epidemiologic studies suggest an association between increased serum levels of IGF-1 and an increased risk of prostate cancer (33). Furthermore, transgenic mice expressing human IGF-1 in basal epithelial cells of prostate led to activation of the IGF-1R and spontaneous tumorigenesis in prostate epithelium (34, 35). In response to IGF-1, the Raf-MEK-ERK and PI3K-Akt signaling pathways are often simultaneously activated and play important roles in IGF-1R-induced cellular proliferation and the inhibition of apoptosis. Traditionally, the Ras/Raf/MAP kinase pathway was thought to primarily mediate the cell proliferative response to IGF-1, whereas the PI3 kinase pathway, which activates Akt/PKB, was primarily implicated in mediating the anti-apoptotic effects of IGF-1 (36–38). However, recent studies have demonstrated a role for both pathways in mediating both responses. Coordination of the two pathways in a single cellular response may depend on cell type or the stage of differentiation (39–41).

In prostate PC3 cells, 13-(*S*)-HODE and 15-(*S*)-HETE up-regulate or down-regulate, respectively, both the MAPK and Akt pathways after activation with IGF-1. However, the magnitude of the response on MAPK is greater than on Akt. Thus, the effect of these lipid metabolites is not solely restricted to EGF signaling and not solely restricted to MAPK signaling. Exactly where in the signaling pathway the 15-LOX metabolites are regulating MAP kinase in the prostate carcinoma cells is not known and remains to be elucidated. Nonetheless, it appears that regulation of MAP kinase by 15-LOX metabolites may have important implications relevant to tumorigenesis, particularly in the prostate.

Opposing biological actions by eicosanoids is frequently observed. For example, the conversion of arachidonic acid to thromboxane A_2 promotes platelet aggregation and vasoconstriction, whereas the formation of prostacyclin inhibits platelet aggregation and promotes vasodilation (42). The two lipoxygenases, 15-LOX-1 and 15-LOX-2, are other examples where the balance between two enzymes that metabolize cis-unsatur-

ated fatty acids appear to antithetically modulate the activity of a key pathway regulating biological events. In this particular case, a balance between two opposing effects could determine the role 15-LOX plays in the development of prostate cancer. Perhaps there is a shift in expression of 15-LOX-2 to 15-LOX-1, and presumably a shift from 15-(*S*)-HETE to 13-(*S*)-HODE, as a cell undergoes neoplastic progression from a normal cell to a tumor cell (Fig. 10). These metabolites exert a dramatically opposite effect on EGF and IGF-1 signaling pathways leading to an increase or decrease in MAP kinase activity. MAP kinase may be a key player in determining whether a pro-tumorigenic activity or an anti-tumorigenic response is observed. Loss of 15-LOX-2 expression and gain of 15-LOX-1 may contribute to prostate cancer development and progression. Thus, a shift from 15-LOX-2 expression to 15-LOX-1 expression may serve as a useful marker of prostate cancer development. Further investigation of 15-LOXs in prostate will need to be done to clarify their function in cancer development.

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REFERENCES

1. Yamamoto, S. (1992) *Biochim. Biophys. Acta* **1128,** 117–131
2. Funk, C. D. (1996) *Biochim. Biophys. Acta* **1304,** 65–84
3. Shappell, S. B., Boeglin, W. E., Olson, S. J., Kasper, S., and Brash, A. R. (1999) *Am. J. Path.* **155,** 235–245
4. Brash, A. R., Boeglin, W. E., and Chang, M. S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6148–6152
5. Kelavkar, U. P., Cohen, C., Kamitani, H., Eling, T. E., and Badr, K. F. (2000) *Carcinogenesis* **21,** 1777–1787
6. Kelavkar, U. P., Nixon, J. B., Cohen, C., Dillehay, D., Eling, T. E., and Badr, K. (2001) *Carcinogenesis* **22,** 1765–1773
7. Tang, S., Bhatia, B., Maldonado, C. J., Yang, P., Newman, R. A., Liu, J., Chandra, D., Traag, J., Klein, R. D., Fischer, S. M., Chopra, D., Shen, J., Zhau, H., Chung, L. W. K., and Tang, D. G. (2002) *J. Biol. Chem.* **277,** 16189–16201
8. Shappell, S. B., Gupta, R. A., Manning, S., Whitehead, R., Boeglin, W. E., Schneider, C., Case, T., Price, J., Jack, G. S., Wheeler, T. M., Matusik, R. J., Brash, A. R., and DuBois, R. N. (2001) *Cancer Res.* **61,** 497–503
9. Sarraf, P., Mueller, E., Jones, D., King, F. J., DeAngelo, D. J., Partridge, J. B., Holden, S. A., Chen, L. B., Singer, S., Fletcher, C., and Spiegelman, B. M. (1998) *Nat. Med.* **4,** 1046–1052
10. Elstner, E., Muller, C., Koshizuka, K., Williamson, E. A., Park, D., Asou, H., Shintaku, P., Said, J. W., Heber, D., and Koeffler, H. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8806–8811
11. Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M., Fletcher, C., Singer, S., and Spiegelman, B. M. (1998) *Mol. Cell* **1,** 465–470
12. Brockman, J. A., Gupta, R. A., and DuBois, R. N. (1998) *Gastroenterology* **115,** 1049–1055
13. Butler, R., Mitchell, S. H., Tindall, D. J., and Young, C. Y. (2000) *Cell Growth & Differ.* **11,** 49–61
14. Chang, T. H., and Szabo, E. (2000) *Cancer Res.* **60,** 1129–1138
15. Tsubouchi, Y., Sano, H., Kawahito, Y., Mukai, S., and Yamada, R. (2000) *Biochem. Biophys. Res. Commun.* **270,** 400–405
16. Takashima, T., Fujiwara, Y., Higuchi, K., Arakawa, T., Yano, Y., Hasuma, T., and Otani, S. (2001) *Int. J. Oncol.* **19,** 465–471
17. Ohta, K., Endo, T., Haraguchi, K., Hershman, J. M., and Onaya, T. (2001) *J. Clin. Endocrinol. Metab.* **86,** 2170–2177
18. Guan, Y., Zhang, Y., Breyer, R. M., Davis, L., and Breyer, M. D. (1999) *Neoplasia* **1,** 330–339
19. Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H., and Evans, R. M. (1998) *Cell* **93,** 229–240
20. Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Willson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D., and Glass, C. K. (1999) *Nature* **400,** 378–382
21. Ikawa, H., Kamitani, H., Calvo, B. F., Foley, J. F., and Eling T. E. (1999) *Cancer Res.* **59,** 360–366
22. Hsi, L. C., Wilson, L., Nixon, J., and Eling, T. E. (2001) *J. Biol. Chem.* **276,** 34545–34552
23. Hill, E., Eling, T., and Nettesheim, P. (1998) *Am. J. Respir. Cell Mol. Biol.* **18,** 662–669
24. Schwarz, E. J., Reginato, M. J., Shao, D., Krakow, S. L., and Lazar, M. A. (1997) *Mol. Cell Biol.* **17,** 1552–1561
25. Sigal, E., Grunberger, D., Highland, E. Gross, C., Dixon, R. A. F., and Craik, C. S. (1990) *J. Biol. Chem.* **265,** 5113–5120
26. Henke, D. C., Kouzan, S., and Eling, T. E. (1984) *Anal. Biochem.* **162,** 156–159
27. Totonez, P., Graves, R., Budavari, A., Erdjument-Bromage, M., Hu, E., Tempst, P., and Spiegelman, B. (1994) *Nucleic Acids Res.* **22,** 5623–5634
28. Camp, H. S., and Tafuri, S. R. (1997) *J. Biol. Chem.* **272,** 10811–10816
29. Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996) *Science* **274,** 2100–2103
30. Reginato, M. J., Krakow, S. L., Bailey, S. T., and Lazar, M. A. (1998) *J. Biol. Chem.* **273,** 1855–1858
31. Zhang, B., Berger, J., Zhou, G., Elbrecht, A., Biswas, S., White-Carrington, S., Szalkowski, D., and Moller, D. E. (1996) *J. Biol. Chem.* **271,** 31771–31774
32. Adams, M., Reginato, M. J., Shao, D., Lazar, M. A., and Chatterjee, V. K. (1997) *J. Biol. Chem.* **272,** 5128–5132
33. Wolk, A., Mantzoros, C. S., Andersson, S-O., Bergstrom, R., Signorello, L. B., Lagiou, P., Adami, H. O., and Trichopoulos, D. (1998) *J. Natl. Can. Inst.* **90,** 911–915
34. DiGiovanni, J., Kiguchi, K., Frijhoff, A., Wilker, E., Bol, D. K., Beltrán, L., Moats, S., Ramirez, A., Jorcano, J., and Conti, C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3455–3460
35. DiGiovanni, J., Bol, D. K., Wilker, E., Beltran, L., Carbajal, S., Moats, S., Ramirez, A., Jorcano, J., and Kiguchi, K. (2000) *Cancer Res.* **60,** 1561–1570
36. Kulik, G., Klippel, A., and Weber, M. J. (1997) *Mol. Cell. Biol.* **17,** 1595–1606
37. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91,** 231–241
38. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) *Science* **275,** 661–665
39. Alblas, J., Slager-Davidov, R., Steenbergh, P. H., Sussenbach, J. S., and van der Burg, B. (1998) *Oncogene* **16,** 131–139
40. Dufourny, B., Alblas, J., van Teeffelen, H. A., van Schaik, F. M., van der Burg, B., Steenbergh, P. H., and Sussenbach, J. S. (1997) *J. Biol. Chem.* **272,** 31163–31171
41. Jiang, B. H., Aoki, M., Zheng, J. Z., Li, J., and Vogt, P. K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2077–2081
42. Ullrich, V., Zou, M. H., and Bachschmid, M. (2001) *Biochim. Biophys. Acta.*, **1532,** 1–14