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OXER1, a G protein-coupled oxoeicosatetraenoid receptor, mediates the survival-promoting effects of arachidonate 5-lipoxygenase in prostate cancer cells

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Abstract

Inhibition of 5-Lox induces apoptosis in prostate cancer cells by inactivating PKCε which is prevented by 5-oxoETE, and activators of PKCε prevent 5-Lox inhibition-induced apoptosis, suggesting that 5-Lox metabolites exert survival signaling via PKCε. However, mechanisms by which 5-Lox metabolites activate PKCε are not understood yet. We found that prostate cancer cells express high levels of OXER1, a G protein-coupled 5-oxoETE receptor, which delivers signal by generating diacyl-glycerol through phospholipase C-beta. Interestingly, we found that U73122, an inhibitor of PLC-beta, interrupts the apoptosis-preventing effect of 5-oxoETE, and exogenous diacyl-glycerol effectively prevents 5-Lox inhibition-induced apoptosis, suggesting that 5-oxoETE signals via OXER1 to promote prostate cancer cell survival.

Keywords

5-Lipoxygenase; Apoptosis; 5-OxoETE; OXER1; PLC-beta; PKC-epsilon

1. Introduction

Prostate cancer is more prevalent in “Western” countries and has emerged as the most common form of malignancy and second leading cause of cancer-related deaths in men in the United States [1]. Etiology of prostate cancer is still enigmatic. However, based on epidemiological studies and experiments with laboratory animals, a link between consumption of high-fat diets and occurrence of clinically evident prostate cancer has emerged [2–7], though the mechanism and role of dietary fatty acids or their metabolic products in the regulation of growth and survival characteristics of prostate cancer cells are yet to be fully understood. Arachidonic acid, an omega-6, polyunsaturated fatty acid, was found to strongly stimulate the growth of prostate cancer cells *in vitro* [8,9], demonstrating that diets high in animal fat (rich in arachidonic acid) may aggravate clinical prostate cancer. Interestingly, metabolic conversion through the 5-Lox pathway was observed to be required for the growth-promoting effects of arachidonic acid [8–10]. Later, it was observed that prostate cancer cells continuously generate 5-Lox metabolites, and inhibition of 5-Lox blocks production of these metabolites and triggers apoptosis both in androgen-sensitive as well as androgen-independent prostate cancer cells [11]. This apoptosis is prevented by

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exogenous 5(S)-HETE, and more effectively by its dehydrogenase-derivative 5-oxoETE, suggesting a pro-survival role of these 5-Lox metabolites in prostate cancer cells. However, downstream signaling mechanisms mediating the survival-promoting effects of 5-Lox metabolites in prostate cancer cells are yet to be fully characterized.

To understand the mechanisms underlying regulation of prostate cancer cell survival by 5-Lox, we systematically addressed the involvement of, (1) the phosphatidylinositol 3′-kinase-Akt/protein kinase B (PI3K-Akt), (2) the mitogen-activated protein kinase kinase-extracellular signal regulated kinase (MEK-ERK), and (3) the protein kinase C-epsilon (PKCε) pathway as potential mediator(s), because these pathways are known to promote survival and growth of a variety of cells including cancer cells [12– 14]. Interestingly, we observed that inhibition of 5-Lox induces apoptosis in prostate cancer cells without affecting the activities of Akt or ERK [15], however the activity of PKCε was rapidly decreased [16]. Among the PKC isoforms PKCε is known to be oncogenic which promotes tumor growth and recurrence by increasing cell proliferation as well as by decreasing apoptosis [17–21]. Thus, we examined whether PKCε is involved in the survival-promoting effects of 5-Lox in prostate cancer cells. Interestingly, we observed that the 5-Lox metabolite, 5-oxoETE, prevents both the 5-Lox inhibition-induced decrease of PKCε activity and induction of apoptosis, and 5-Lox inhibition-induced apoptosis is prevented by chemical- or specific peptide-activators of PKCε, suggesting that the 5-Lox activity regulates apoptosis in these cancer cells via signaling involving PKCε [16]. Under normal health condition, 5-Lox is only expressed in specific immune cells, but it is expressed in parenchyma body cells in disease conditions such as asthma, arthritis, psoriasis, and cancer [22–25], and increased expression and activity of 5-Lox has been found in prostate tumor tissues [26]. Thus, the 5-Lox pathway has emerged as a promising target for prostate cancer therapy. However, though 5-Lox plays an important role in promoting survival of prostate cancer cells, underlying mechanism by which the 5-Lox metabolites regulate the activity of PKCε to prevent apoptosis in prostate cancer cells is yet to be understood.

Existence of a G protein-coupled receptor (GPCR), for which 5-oxoETE serves as the major ligand, had been predicted by functional assays [27,28] and later has been identified and characterized [29–31]. The 5-oxoETE receptor has been renamed as OXER1 by the International Union of Pharmacology and has been included in a separate class in the GPCR family of proteins [32]. Documentation of the existence of OXER1 opened up a new avenue to better understand the role of 5-oxoETE and its signaling mechanisms. Using OXER1 gene-transfected cells (CHO cells, HEK cells, etc.) it was demonstrated that the GPCR, OXER1, signals through alpha(i) class of G proteins, generates calcium signal, inhibits cAMP production, and mediates 5-oxoETE-induced chemotaxis [29–32]. Identification of OXER1 mRNA in prostate cancer cells by RT-PCR followed by cloning and sequencing of the complete coding region has documented the expression of OXER1 gene in prostate cancer cells [33]. This finding paved the way to better understand the mechanism of action and role of 5-oxoETE and its receptor (OXER1) in the biology of prostate cancer. However, characterization of OXER1 proteins by biochemical and immunological techniques, and documentation of its downstream signaling mechanism in the regulation of prostate cancer cell survival had been lacking. Here, we report the expression of OXER1 proteins (∼48 kDa) in a panel of prostate cancer cells and in prostate tumor tissues. Interestingly we observed that, (1) in untreated prostate cancer cells OXER1, PLC-beta and PKCε are associated in a multi-protein complex, (2) treatment with MK591 interrupts the association of PLC-beta and PKCε with OXER1, (3) inhibition of PLC-beta prevents the apoptosis-preventing effects of 5-oxoETE, and (4) exogenous DAG prevents the 5-Lox inhibition-induced apoptosis in prostate cancer cells. These findings, together with the prevention of 5-Lox inhibition-induced down-regulation of PKCε by 5-oxoETE, and prevention of 5-Lox inhibition-induced apoptosis by specific activators of PKCε suggest that the 5-Lox

metabolite, 5-oxoETE, regulates prostate cancer cell survival via the GPCR, OXER1, and downstream signaling involving the oncogenic serine/threonine kinase, PKCε.

2. Materials and methods

2.1. Cell culture and reagents

LNCaP, PC3 and DU145 human prostate cancer cells were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI medium 1640 (Invitrogen, Carlsbad, CA). All the media were supplemented with 10% FBS and antibiotics. Antibodies against PKCε and PLC-beta, and lentiviral shRNA against 5-Lox and PKCε were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dominant-negative PKCε construct was a kind gift from Dr. Chaya Brodie, Department of Neurology, Henry Ford Health System). Beta-actin antibody, U73122, 1,2-dioleoyl-sn-glycerol, RO318220 and ibuprofen were purchased from Sigma (St. Louis, MO). 5-OxoETE was purchased from Cayman Chemical (Ann Arbor, MI). Antibody against OXER1 was developed by Anaspec (San Jose, CA) through contract. MK591 was obtained as a generous gift from Dr. Robert N. Young (Merck-Frosst Centre for Therapeutic Research, Quebec, Canada).

2.2. Cell viability assay

LNCaP prostate cancer cells (~5000 per well) were plated overnight in 96 well plates in complete growth medium (RPMI or DMEM plus 10% FBS) and treated with varying doses of MK591. Plates were incubated further for 72 h at 37 °C in the CO_2 incubator. Cell viability was measured by One Solution MTS/PES Cell Titer assay from Promega (Madison, WI) as described before [8,11].

2.3. Immunohistochemistry and immunocytochemistry

Formalin-fixed paraffin-embedded human prostate tumor tissue sections (Gleason 5–7) were deparaffinized and soaked in graded concentrations of methanol. Then the sections were treated with antigen-retrieval buffer in the microwave (Power setting: High) for 1 min. After washing, sections were blocked in 10% horse serum for 1 h at RT and then treated either with control rabbit-IgG or with primary rabbit-polyclonal OXER1 antibody (1:50) overnight at 4°C in the presence or absence of 50-fold extra free antigenic peptide (Anaspec, San Jose, CA). After washing slides were stained for 1 min using DAB as substrate and then counter-stained with hematoxylin (Harris) for 30 s. Blocking, antibody treatment and color development were done using Vectastain Elite Impression kit (Cat.# MP-7401; Vector Laboratories, Burlingame, CA). For immunocytochemistry, plate-adhered cells were fixed in 4% paraformaldehyde in PBS and stained with anti-OXER1 antibody as described above. Photographs were taken with a Nikon digital camera attached to a Leica fluorescence microscope at ×400.

2.4. Western blot

Cells (~3×10^5) were plated and allowed to grow for 48 h. The old medium was then replaced with 2 ml fresh RPMI medium and the cells were treated with inhibitors. After treatment, cells were harvested, washed, and lysed in lysis buffer (50 mM HEPES, pH 7.4,150 mM NaCl, 1 mM EDTA, 1 mM orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1% NP-40, and a cocktail of protease inhibitors). Proteins were separated by 12% SDS–PAGE and transferred to nitrocellulose or PVDF membranes. Membranes were blocked with 5% nonfat-milk solution and then blotted with appropriate primary antibody followed by peroxidase-labeled secondary antibody. Bands were visualized by enhanced chemiluminescence detection kit from Pierce Biotech (Rockford, IL) and analyzed with a densitometer using Kodak imaging software. Unless otherwise mentioned, blots of proteins of interest were analyzed in three separate experiments.

2.5. Reverse-transcriptase polymerase chain reaction (RT-PCR)

To examine expression of OXER1 mRNA, total RNAs were isolated from prostate cancer cells using Qiagen RN-Easy Mini kit (Qiagen, Valencia, CA). First strand cDNA synthesis and PCR were performed using a kit from Invitrogen (Carlsbad, CA). GAPDH was used as control to normalize amount of template used in the reaction mixture. For PCR, the following primer pairs were used: OXER1 (Forward: 5′-CAG TGG CTG CGA GAA TGC TGA TG-3′; Reverse: 5′-TGG GAA TGC CAT CCT GGA CAC-3′) and GAPDH: (Forward: 5′-TGA AGG TCG GAG TCA ACG GAT TTG G-3′; Reverse: 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′) for 30 cycles and 28 cycles respectively. PCR products were resolved in 2% agarose gel and bands were visualized by ethidium bromide under UV illumination. Photographs were taken and analyzed with Eagle Eye II Darkroom Cabinet still video imaging system using EagleSightv3.1 software (Stratagene, La Jolla, CA). Band intensities of the receptor were normalized to that of GAPDH.

2.6. Measurement of DNA degradation

Apoptosis was quantitatively measured by detecting degradation of nuclear DNA to nucleosomal fragments by sandwich-ELISA. LNCaP cells ($\sim 3 \times 10^5$) were plated in 60 mm dishes and allowed to grow for 48 h. Cells were then treated either with the experimental agents or the solvent vehicle for varying periods of time up to 24 h. At the end of incubation periods, cells were lysed and the degradation of chromatin-DNA to nucleosomal fragments was measured by Cell Death Detection ELISA^{plus} kit from Roche (Indianapolis, IN) as described before [11,15,16].

2.7. Measurement of PKCε activity by IP-kinase assay

Prostate cancer cells ($\sim 1 \times 10^6$) were plated in 100 mm diameter plates and allowed to grow for 48 h. The old culture medium was then replaced with fresh 5 ml RPMI medium and the cells were treated with inhibitors or solvent vehicle (0.2% DMSO) for varying periods of time up to 6 h. Then the cells were lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride) containing 0.4% Triton-X100, 10% glycerol, and a cocktail of protease inhibitors. Enzymatic activity of PKCε was measured by an ELISA method using biotin-labeled peptide substrate (Cell Signaling Technology, Danvers, MA) as published before [16]. Briefly, cell lysates were cleared by centrifugation at 12,000g for 10 min at 4 °C and the supernatants (~500 μg proteins) were used to immunoprecipitate (IP) PKCε using 4 μg polyclonal anti-PKCε antibody. The tubes were rotated overnight at 4 °C and the immunecomplexes were precipitated using anti-rabbit IgG-coated magnetic beads (Invitrogen) for 2 h at 4 °C. Then the beads were washed five times with lysis buffer containing 0.1% Triton-X100 and finally suspended in 25 μl of 1× kinase assay buffer. Enzymatic reactions in 50 μl were carried out for 15 min at RT using 10 μl of IP-slurry with beads, and stopped with 50 μl of 50 mM EDTA. Aliquots of reaction mixtures (25 μl) were placed into streptavidin-coated 8-well strips and incubated for 60 min at RT. Wells were washed and phosphorylation of biotinylated-peptide substrate (cAMP response element-binding protein or CREB at Ser-133) was detected by specific anti-phosphoserine primary antibody followed by secondary HRP-labeled anti-rabbit antibody. After washing, color was developed using ABTS (2,2′-azino-di (3-ethylbenzthiazoline-6-sulfonate) as substrate for 15 min at RT. Absorbance was measured at 405 nm in a digital plate reader (Bio-Tek Instruments).

2.8. Fluorescence and LASER-confocal microscopy

Prostate cancer cells ($\sim 3 \times 10^5$) were plated overnight in RPMI medium 1640 supplemented with 10% FBS onto 60 mm diameter tissue culture plates (Falcon). Then the cells were

transfected with a dominant-negative GFP-labeled PKCε construct and allowed to grow for 72 h. On the day of experiment, live cells were observed under a LEICA fluorescence microscope. Photographs were taken with a Nikon digital camera attached to a Leica fluorescence microscope at ×400. Image acquisition and data processing were done with a Dell computer attached to the microscope using SPOT-Advanced software. For LASER confocal microscopy, a glycerol mounted antibody-stained slide was observed under a Zeiss Micro Systems LSM-410 invert microscope. Photographs were taken at the bottom plane and at various planes of one micrometer increment from the bottom.

2.9. Statistical analysis

Significance of the difference between values in treated and untreated groups were calculated by two-tailed student's *t*-test using GraphPad Instat Software. A p value of <0.05 was considered to be statistically significant.

3. Results

3.1. Inhibition of 5-Lox kills prostate cancer cells

An important role of 5-Lox activity in the survival of prostate cancer cells has been perceived from the work in various laboratories using a range of chemical inhibitors of 5-Lox [8–11,15,16,34– 36]. We observed that treatment either with MK591, a chemical inhibitor of 5-Lox, or lentiviral shRNA (to block 5-Lox expression) effectively kills prostate cancer cells, suggesting that 5-Lox plays a role in the survival of prostate cancer cells (Fig. 1a–g). Recently, we reported that 5-Lox inhibition-induced apoptosis in prostate cancer cells occur via rapid down-regulation of PKCε [16], but without affecting Akt [15], and that 5-oxoETE, a metabolite of 5-Lox, prevents both the 5-Lox inhibition-induced loss of PKCε and induction of apoptosis [16]. Altogether, these findings suggest that 5-Lox activity may regulate the survival of prostate cancer cells via signaling through an Akt-independent, PKCε-dependent mechanism.

3.2. The 5-Lox metabolite, 5-oxoETE, prevents 5-Lox inhibition-induced loss of PKCε activity, but does not stimulate the activity of PKCε by direct interaction

Our recent observations that inhibition of 5-Lox induces apoptosis in prostate cancer cells via down-regulation of PKCε, and prevention of 5-Lox inhibition-induced apoptosis by specific peptide or chemical activators of PKCε, suggested that 5-Lox metabolites regulate apoptosis in prostate cancer cells via signaling involving PKCε [16]. However, how 5-oxoETE may regulate the activity of PKCε in prostate cancer cells is an intriguing but unknown question. Here, we show that though the 5-Lox metabolite, 5-oxoETE, prevents 5-Lox inhibition-induced loss of PKCε activity in whole cells, it does not stimulate the activity of isolated PKCε by direct interaction (Fig. 2a and b), suggesting that 5-oxoETE may regulate the activity PKCε via signaling through an indirect mechanism.

3.3. Both prostate tumor tissues and prostate cancer cells express OXER1 which is localized on cell surface

The GPCR, OXER1, has been identified and characterized from human genome analysis by reverse pharmacology, and found that the 5-Lox metabolite, 5-oxoETE, serves as the major ligand for this receptor [29–31]. This finding raised the possibility that in prostate cancer cells 5-oxoETE may exert survival-signaling via OXER1. However, detection and characterization of OXER1 proteins from prostate cancer cells had been lacking. Here, for the first time, we show that both prostate tumor tissues and cultured prostate cancer cells express high-levels of OXER1 proteins (Fig. 3a and b). By RT-PCR we also found that prostate cancer cells express OXER1 mRNA (Fig. 3d). These findings provide material

support to the concept that 5-oxoETE may exert its pro-survival effects in prostate cancer cells via signaling through its cognate receptor, OXER1. By immunocytochemistry using an N-terminal extracellular domain-specific antibody we observed a strong fluorescence signal in intact (non-permeabilized) prostate cancer cells, which suggests that OXER1 is localized on cell surface (Fig. 3f and g). A LASER-confocal-microscopic image-analysis also showed that the GPCR, OXER1, is localized on the plasma membrane of prostate cancer cells (Fig. 3h). These findings are in congruence with published reports and conform to the idea that 5-oxoETE may deliver survival signals in prostate cancer cells via OXER1 by functioning as a ligand-receptor complex.

3.4. The 5-Lox metabolite, 5-oxoETE, prevents apoptosis in prostate cancer cells via a G protein-coupled receptor-mediated signaling

If 5-oxoETE exerts its survival signaling via OXER1 then it is expected that inhibition of OXER1 will also kill prostate cancer cells. Since, no chemical inhibitor of OXER1 is available as of today we used siRNA against OXER1 and showed that treatment with OXER1 siRNA kills prostate cancer cells similar to that of inhibition of 5-Lox, documenting an important role of OXER1 in the survival of prostate cancer cells [33]. It is known that OXER1-mediated signaling involves activation of PLC-beta and production of diacyl-glycerol (DAG) from membrane phospholipids [29–32]. Thus, here we examined whether the apoptosis-preventing effect of 5-oxoETE in prostate cancer cells is dependent on the function of PLC-beta. We found that while the MK591-induced apoptosis in prostate cancer cells is prevented by 5-oxoETE, co-treatment with U73122, an inhibitor of phospholipase C-beta, interrupts the pro-survival effects of 5-oxoETE, and that exogenous DAG (1,2-dioleoyl-sn-glycerol) dramatically reduces the apoptosis-inducing effects of 5-Lox inhibition (Fig. 4a and b). Moreover, we observed that treatment of prostate cancer cells with U73122 alone also induces apoptosis in prostate cancer cells, which is prevented by exogenous DAG (Fig. 4c and d). These findings suggest that 5-oxo-ETE may regulate prostate cancer cell survival via a GPCR-mediated signaling.

3.5. Prostate cancer cells maintain catalytically active PKCε which is associated with OXER1 and PLC-beta (PLC-β) in a complex

A critical role of PLC-beta in the survival of prostate cancer cells indicates that these cells may continuously generate and use DAG for downstream signaling to promote their survival. This finding also suggests that prostate cancer cells may also harbor a continuously active species of PKCε which plays an important role in the survival of these cancer cells. We observed that untreated prostate cancer cells in serum-free medium (without any exogenous stimuli) maintain high levels of catalytically active PKCε, which supports the concept that prostate cancer cells may use OXER1- and PLC-beta-mediated signals to maintain a continuously active species of PKCε for their survival (Fig. 5a–e).

Since the GPCR, OXER1, is known to exert signals via PLC-beta by generating DAG which in turn activates PKCε, we hypothesized that these molecules may associate to form a complex for better functional interaction. We tested this hypothesis by immunoprecipitation of OXER1 followed by Western blot analysis of proteins which revealed that in untreated prostate cancer cells both PLC-beta and PKCε are associated with OXER1 in a multi-protein complex which is consistent with the idea that these three molecules are associated together presumably for efficient signaling (Fig. 5f). Interestingly, we observed that treatment with MK591 disrupts the association of PLC-beta and PKCε with OXER1, suggesting that the association of these proteins depend on the activity of 5-Lox. Survivin, a pro-survival protein regulated by 5-Lox signaling, was found neither to be associated with the OXER1 complex, nor it pulled down PLC-beta or PKCε when immunoprecipitated.

3.6. Inhibition of PKCε kills prostate cancer cells, and activation of PKCε prevents 5-Lox inhibition-induced apoptosis

Down-regulation of PKCε in prostate cancer cells by inhibition of 5-Lox, its reversal by 5-oxoETE, and the 5-Lox-dependent association of catalytically active PKCε with OXER1 in a complex, indicated that 5-Lox activity may regulate prostate cancer cell survival via activation of PKCε. However, if 5-Lox metabolites regulate survival of prostate cancer cells via signaling through PKCε, then it is expected that direct inhibition of PKCε will also interfere with prostate cancer cell survival. Interestingly, we observed that over-expression of a dominant-negative PKCε construct, or treatment with PKCε-specific shRNA (short hairpin RNA), kills prostate cancer cells, suggesting that the activity of PKCε plays an important role in the survival of prostate cancer cells (Fig. 6a–c). Moreover, we observed that treatment of prostate cancer cells with a chemical inhibitor of PKCε, RO318220, effectively kills prostate cancer cells (Fig. 6d). Finally, co-treatment of cells with KAE1-1, a TAT-labeled specific octa-peptide activator of PKCε, was observed to effectively prevent 5-Lox inhibition-induced apoptosis, whereas KIE1-1 (a specific octa-peptide inhibitor of PKCε) failed to prevent apoptosis, suggesting that PKCε plays an important role in the survival of prostate cancer cells presumably as a mediator of signals down-stream of 5-Lox activity (Fig. 6e). Altogether, our current findings, along with other published data [8,11,15,16,33], suggest that prostate cancer cells metabolize arachidonic acid via 5-Lox to generate 5-oxoETE, and that 5-oxoETE signals through its cognate GPCR, OXER1, to promote survival of prostate cancer cells via a PKCε-dependent mechanism (Fig. 7).

4. Discussion

Our findings and evidence from various laboratories showed that the 5-Lox pathway plays an essential role in the survival of prostate cancer cells (Fig. 1) [8–11,15,16,34–36]. However, signaling mechanisms underlying regulation of prostate cancer cell survival by 5-Lox are yet to be understood. We addressed this question by examining the effects of 5-Lox inhibition on major signaling pathways that are known to play important roles in cell-viability. Recently, we reported that inhibition of 5-Lox induces apoptosis in prostate cancer cells without inhibition of Akt or ERK1/2, two well known pro-survival mechanisms which play important roles in the viability of a variety of cells, including cancer cells [12–15]. Interestingly, the activity of PKCε was found to be severely down-regulated when 5-Lox was inhibited, and activators of PKCε prevented 5-Lox inhibition-induced apoptosis [16]. These findings indicated that the 5-Lox metabolites may promote survival of prostate cancer cells through a PKCε-dependent mechanism, and suggested that prostate cancer cells are equipped with additional survival mechanisms which help them to bypass chemotherapies that are directed against Akt and ERK, two well-characterized anti-cancer molecular targets. Existence of an Akt-independent survival mechanism in LNCaP prostate cancer cells was also reported previously using a panel of survival/growth factors in the presence or absence of inhibitors of PI3K-Akt [37]. Though the role of PKCε is well-known to promote survival and growth of a variety of cells including prostate cancer cells [14,20,21,38,39], mechanism of the regulation of PKCε by metabolites of 5-Lox in prostate cancer cell survival are yet to be characterized.

We observed that 5-oxoETE effectively prevents 5-Lox inhibition-induced loss of PKCε activity, suggesting that the activity of PKCε is positively regulated by 5-oxoETE (Fig. 2a). However, we also found that 5-oxoETE does not stimulate the activity of isolated PKCε in cell-free system by direct interaction, suggesting that 5-oxoETE may regulate PKCε via an indirect mechanism (Fig. 2b). A GPCR (now called OXER1) has been characterized, which transduces signal of the 5-Lox metabolite 5-oxoETE (and to a lesser extent 5(S)-HETE), by working as a ligand-receptor complex [27–29]. GPCRs are seven transmembrane receptors which can signal via activation of PLC-beta and generation of DAG [14,40–42]. Previously,

we reported mRNA expression and a role of OXER1 in prostate cancer cell survival [33]. In this report, for the first time, we show that both prostate tumors and cultured prostate cancer cells express high levels of OXER1 proteins (Fig. 3a–c). Interestingly, it was observed that the androgen-independent, distant-metastatic prostate cancer cells express higher levels of OXER1 than the androgen-sensitive prostate cancer cells (DU145 > PC3 > LNCaP). OXER1 has been characterized to be localized on the plasma membrane of immune cells, and our detection of OXER1 immunoreactivity by an extracellular domain-specific antibody in non-permeabilized prostate cancer cells demonstrates that OXER1 is localized on prostate cancer cell surface (Fig. 3f–h), suggesting that OXER1 may be activated by 5-oxoETE both in an autocrine as well as in a paracrine fashion.

An important role of OXER1 in the survival of prostate cancer cells has been demonstrated by treating prostate cancer cells with specific siRNAs [33]. The GPCR, OXER1, has been functionally characterized to signal via generation of DAG through the action of PLC-beta [29–32]. This concept signified that the pro-survival effects of 5-oxoETE/OXER1 may also depend on the activity of PLC-beta. Thus, we examined whether inhibition of PLC-beta can interrupt the survival-signaling delivered by 5-oxoETE. Interestingly, we observed that pre-treatment of prostate cancer cells with a PLC-beta inhibitor (U73122) dramatically inhibits the pro-survival effect of 5-oxoETE (Fig. 4a). Moreover, effective prevention of 5-Lox inhibition-induced apoptosis by exogenous DAG, the product of PLC-beta, confirmed the critical role of a GPCR-mediated signaling in the regulation of prostate cancer cell survival by 5-Lox activity (Fig. 4b). We also observed that U73122 triggers apoptosis in prostate cancer cells when used individually and this apoptosis is also prevented by DAG (Fig. 4c and d), signifying a critical role of a GPCR-mediated signaling in the survival of prostate cancer cells.

Our observation of the existence of catalytically active species of PKCε in untreated prostate cancer cells suggests that harboring a continuously active form of PKCε is a characteristic feature of prostate cancer cells (Fig. 5a–e). This feature was previously demonstrated in prostate cancer cells and tumor tissues by immunohistochemistry showing membrane localization of PKCε, and also by its co-localization, phosphorylation and activation of the transcription factor Stat3 [16,43]. However, upstream signals that can feed PKCε for its continuously active state in prostate cancer cells are yet to be identified and characterized. Our observations of a continuous generation of 5-Lox metabolites by prostate cancer cells from arachidonic acid [8,9,11,16], may ideally provide the signals needed for the continuous activation of PKCε. In this report, we present evidence that the apoptosis-triggering effects of 5-Lox inhibition in prostate cancer cells occur via down-regulation of PKCε and this down-regulation is prevented by 5-oxoETE (Fig. 2a). We also observed that immunoprecipitation of OXER1 co-precipitates PLC-beta and PKCε, and the association of PLC-beta and PKCε with OXER1 is disrupted when the cells are treated with 5-Lox inhibitor (Fig. 5f). PLC-beta is well known to activate PKCε via generation of DAG [14,40,41]. And, we found that DAG can effectively prevent the 5-Lox inhibition-induced apoptosis in prostate cancer cells (Fig. 4b). These findings are consistent with the idea that the apoptosis-preventing effects of 5-Lox in prostate cancer cells is dependent on the function of PLC-beta which in untreated prostate cancer cells remain associated with OXER1. Thus, it appears that prostate cancer cells use 5-oxoETE to regulate the activity of PKCε via signaling through OXER1 and PLC-beta.

Co-precipitation of PLC-beta and PKCε with OXER1 signifies that in prostate cancer cells OXER1, PLC-beta and PKCε exist as a multi-protein complex presumably to improve signaling efficiency for promoting survival of these cancer cells. However, this finding also raised the question whether direct inhibition of PKCε may also interrupt the cascade of survival signaling in these cells. Inhibition of PKCε by dominant-negative constructs,

lentiviral shRNA, or chemical inhibitor was observed to effectively kill prostate cancer cells, suggesting that indeed PKCε plays an important role in the survival of prostate cancer cells (Fig. 6a–d). Finally, prevention of 5-Lox inhibition-induced apoptosis by specific peptide-activator of PKCε (KAE1–1) confirms the concept that the survival-promoting effect of 5-Lox in prostate cancer cells is mediated, at least partially, via signaling through PKCε (Fig. 6e). Altogether, these findings suggest that 5-Lox metabolites may promote survival of prostate cancer cells by activating PKCε via signaling through OXER1 and consequent production of DAG by phospholipase C-beta (PLC-β). Thus, a fundamental mechanism of cell survival has been uncovered which is continuously active in prostate cancer cells and is fueled by metabolism of arachidonic acid through 5-Lox (Fig. 7). PKCε is a transforming oncogene and is well characterized to promote cell survival by increasing resistance against apoptosis [18–21,38–42,44–48]. Thus, regulation of PKCε activity in prostate cancer cells by inhibitors and metabolites of 5-Lox opens up a new avenue to explore the activation mechanism and role of PKCε in prostate and probably other types of cancer cells.

It is interesting to note that under normal health conditions, 5-Lox is only expressed in specific immune cells such as neutrophils, eosinophils, basophils, macrophages (not in T cells), but not in non-immune parenchyma body cells [49–51]. However, over-expression of 5-Lox has been implicated in inflammatory diseases, such as asthma, arthritis, psoriasis, and a variety of cancers, such as cancer of the prostate, lung, pancreas, brain [22–26,49–52]. Thus, agents that specifically block the activity of 5-Lox may turn out to be attractive tools to treat these diseases. Over-expression of 5-Lox was reported in prostate cancer [26], and recently we observed that while prostate tumor tissues express high levels of 5-Lox, its expression in normal, non-tumor prostate glands is undetectable (Sarveswaran et al.; Unpublished observations). Thus, expression of 5-Lox in cancerous prostate epithelial cells together with a critical role of 5-Lox in the survival of prostate cancer cells suggest for a pivotal and possibly an indispensable role of 5-Lox in the development and progression of prostate cancer. Since arachidonic acid (an omega-6 polyunsaturated fatty acid) is abundant in “Western-diets” (where prostate cancer is also more common) [2–7], our novel findings suggest that prostate cancer cells gain a clear survival advantage from high-fat, “Western-style” diets via metabolic conversion of arachidonic acid through the 5-Lox pathway, and downstream signaling via OXER1, leading to the activation of PKCε, an oncogenic serine/threonine kinase well known to promote cell survival and prevent apoptosis.

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Abbreviations

5-Lox	5-lipoxygenase
5-oxoETE	5-oxoeicosatetraenoic acid
GPCR	G protein-coupled receptor
OXER1	5-oxoETE receptor
PLC-β	phospholipase C-beta
PKCε	protein kinase C-epsilon

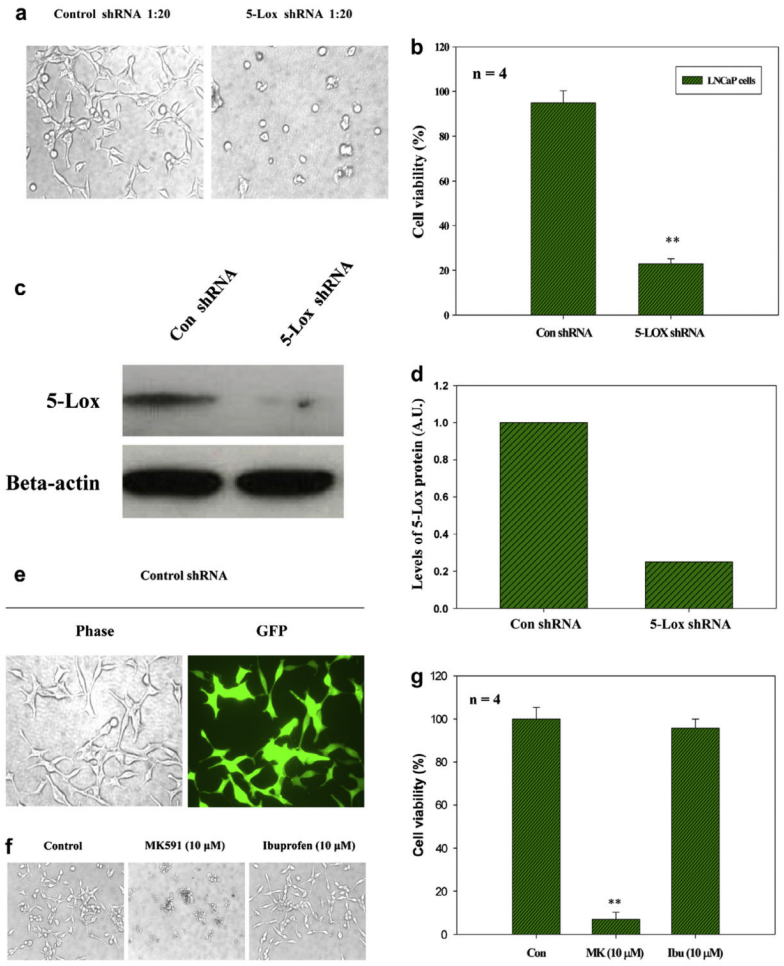


Fig. 1.

Effect of 5-Lox inhibition on prostate cancer cell survival. In (a–e), LNCaP cells (∼40,000 per well) were plated in 12 well tissue culture plates in complete growth medium and treated with control or 5-Lox shRNA lentiviral particles (cell to virus ratio = 1:20). After 5 days, photographs were taken at ×400 (a), and cell viability was measured by MTS/PES Cell Titer assay (*$p < 0.05$, $n = 4$) (b). Expression of 5-Lox was analyzed by Western blot (c). In (d), relative levels of 5-Lox protein is shown by densitometric analysis of bands (AU = arbitrary unit). Transfection efficiency of lentivirus is shown in (e) using GFP-labeled control shRNA plasmids. *Note:* More than 90% of cells are transfected using lentiviral particles. In (f and g), LNCaP prostate cancer cells (∼30,000 per well) were plated in 24 well tissue culture plates in RPMI medium and treated with MK591 or ibuprofen. Plates were incubated for 24 h at 37 °C in the CO_2 incubator and photographs were taken at ×400 (f). Cell viability was measured by MTS/PES Cell Titer assay from Promega (g). Results are presented as mean value of each data point ± standard error (*$p < 0.05$, $n = 4$).

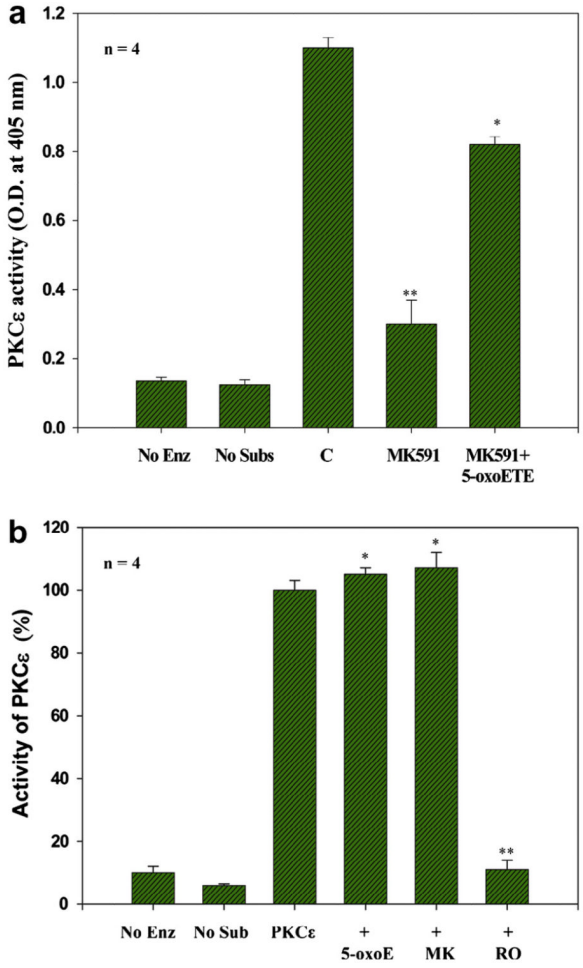
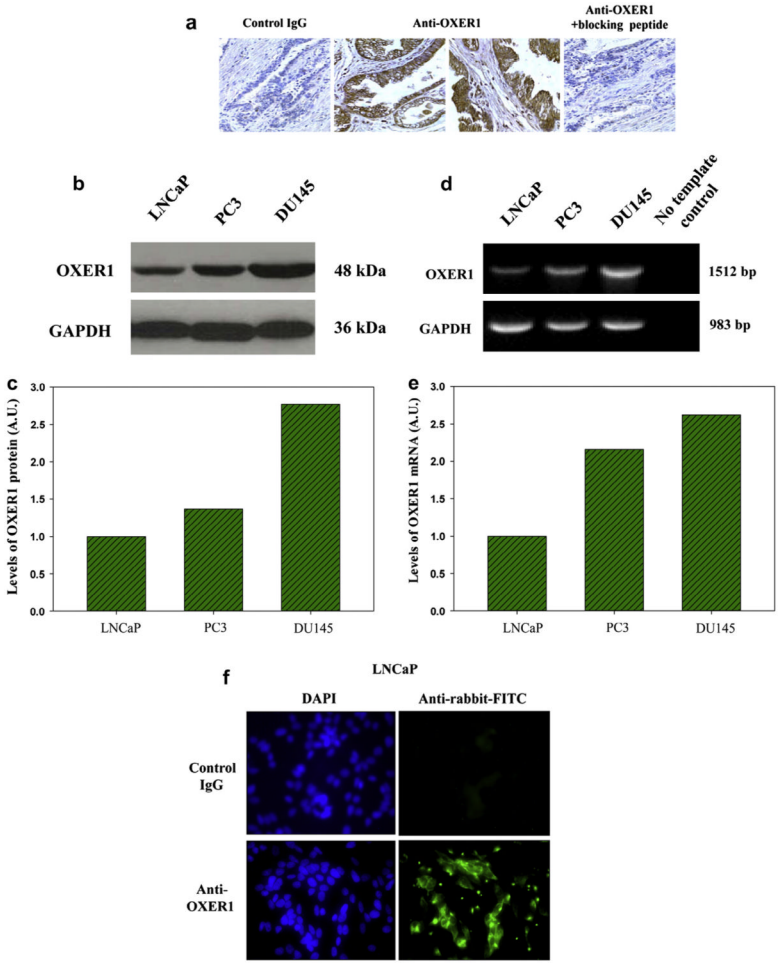


Fig. 2.

Effects of 5-oxoETE on MK591-induced loss of PKCε activity in prostate cancer cells and on the activity of isolated PKCε in cell-free system. In (a), LNCaP prostate cancer cells (1×10^6 per plate) were plated and allowed to grow for 48 h. Then the cells were treated with MK591 (10 μM) with or without 5-oxoETE (10 μM) for 8 h. Control cells were treated with the vehicle only (0.2% DMSO). At the end of treatment period, cells were lysed and the enzymatic activity of PKCε was determined by IP-kinase assay. In (b), PKCε was first isolated from untreated LNCaP cells and then treated with 5-oxoETE or MK591 (10 μM) for 10 min in the assay buffer. RO-318220 (10 μM), an inhibitor of PKC, was used as positive control. Enzymatic reaction was started by adding substrate to the assay mixture. After 15 min of reaction, enzyme activity was calculated as described in Section 2. Results are presented as mean value of each data point ± standard error ($n = 4$).



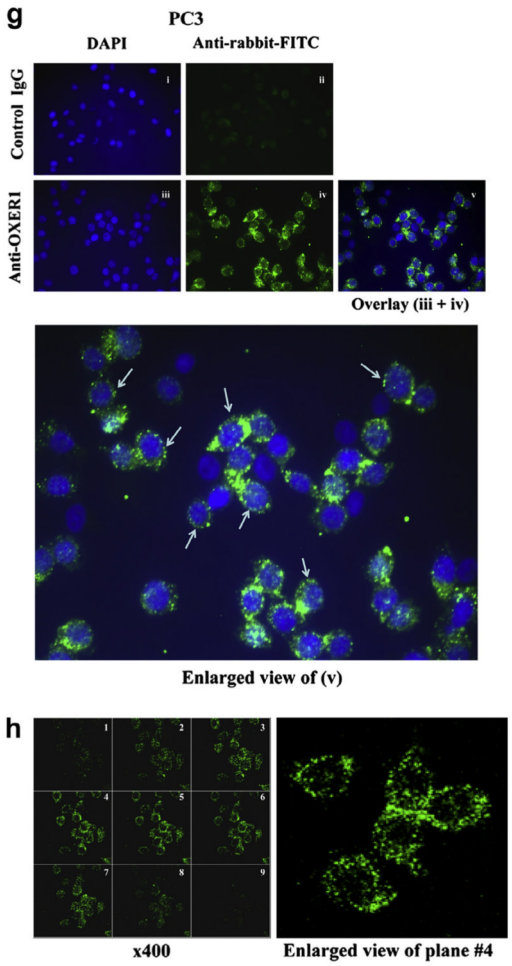


Fig. 3.

Expression of OXER1 in prostate tumors and prostate cancer cells. In (a), prostate tumor tissue slides were processed and treated with rabbit anti-OXER1 antibody (1:50) and detected by IHC as described in Section 2. Photographs were taken with a Nikon digital camera at ×400. Representative pictures of Gleason 5–7 tumors are shown here (total number of tumors analyzed = 5; all positive). *Note:* Intermediate grade tumors with multilayered epithelium and intra-lacunar intrusion are visible. A non-specific rabbit IgG was used as negative control. The specificity of OXER1 antibody was confirmed by mixing with 50-fold excess of the peptide against which the antibody was generated. In (b), LNCaP, PC3 and DU145 prostate cancer cells (1×10^6) were plated in 100 mm diameter plates and allowed to grow for 48 h. Then the cells were lysed in lysis buffer and cell lysate proteins (∼100 μg per lane) were separated in 12% SDS–PAGE to detect expression of OXER1 by Western blot. (c) Shows relative expression of OXER1 protein (normalized to GAPDH) by densitometry. In (d), expression of OXER1 mRNA in prostate cancer cells was detected by RT-PCR using gene-specific primer sets as described in Section 2. Relative expression of OXER1 mRNA (normalized to GAPDH) is shown in (e) by densitometric analysis. Surface localization of OXER1 in prostate cancer cells. LNCaP (f), and PC3 (g), cells were fixed in 4% paraformaldehyde in PBS, washed in PBS, and treated with anti-OXER1 antibody (1:50) for 1 h at RT with gentle shaking. Non-specific rabbit IgG (1:50) was used as negative control. After washing cells were treated with secondary FITC-labeled anti-rabbit IgG and observed under a fluorescence microscope. Photographs were taken with a Nikon digital camera attached to a Leica fluorescence microscope at ×400. Arrows in the enlarged plate

indicate localization of OXER1 at the boundary of cells. In (h), PC3 cells were stained as in (g) and analyzed by a Zeiss LASER-confocal microscope to show different planes at 1 micron increment from the bottom. On the right, an enlarged view of plate #4 (at a plane of 4 μm up from the bottom) is presented showing surface localization of OXER1.

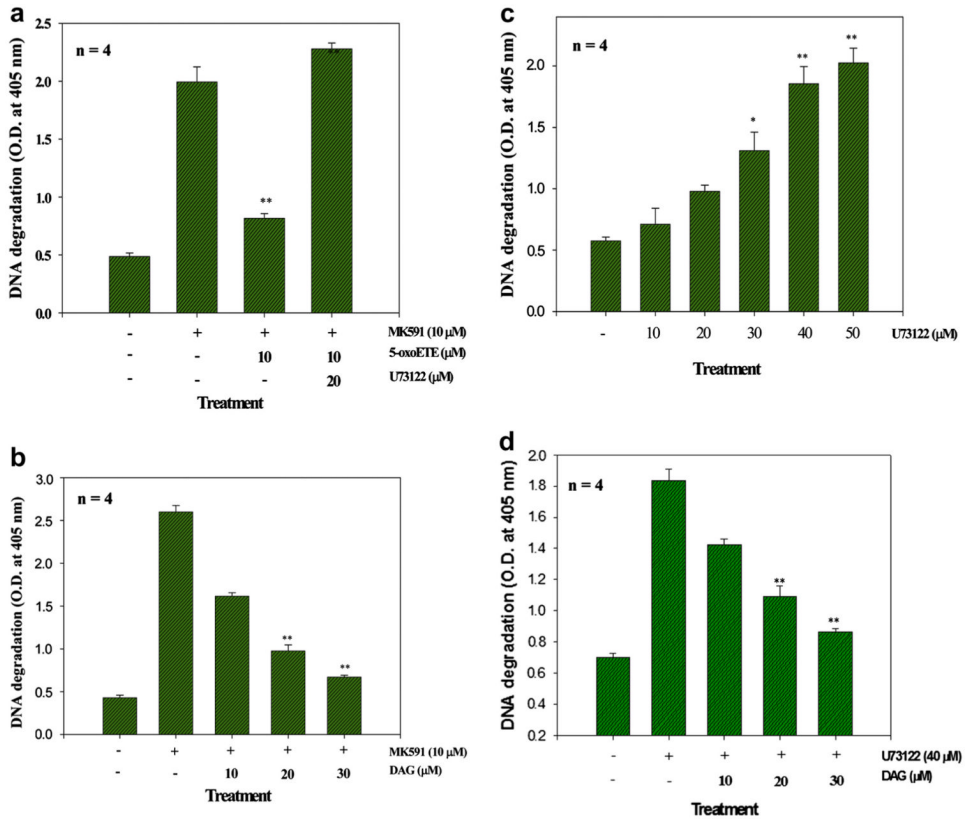


Fig. 4.

Effect of U73122 on the prevention of MK591-induced apoptosis by 5-oxoETE. In (a), cells were plated as in Fig. 1, pre-treated for 30 min with U73122 (20 μM), and then treated with 10 μM MK591 with or without 10 μM 5-oxoETE for 16 h. Control cells were treated with the vehicle only (0.2% DMSO). At the end of incubation period, apoptosis was measured by cell death ELISA. Results represent mean values of each data point ± standard error (n = 4). In (b), cells were pre-treated with varying doses of 1,2-dioleoyl-sn-glycerol for 30 min and then treated with 10 μM MK591 for 16 h. Apoptosis was measured by ELISA. In (c), cells were treated with varying doses of U73122 for 16 h and then the apoptosis was measured by ELISA. In (d), cells were pre-treated with varying doses of 1,2-dioleoyl-sn-glycerol for 30 min and then treated with U73122 (40 μM) for 16 h. After incubation, apoptosis was measured by ELISA. Results are presented as mean value of each data point ± standard error (n = 4).

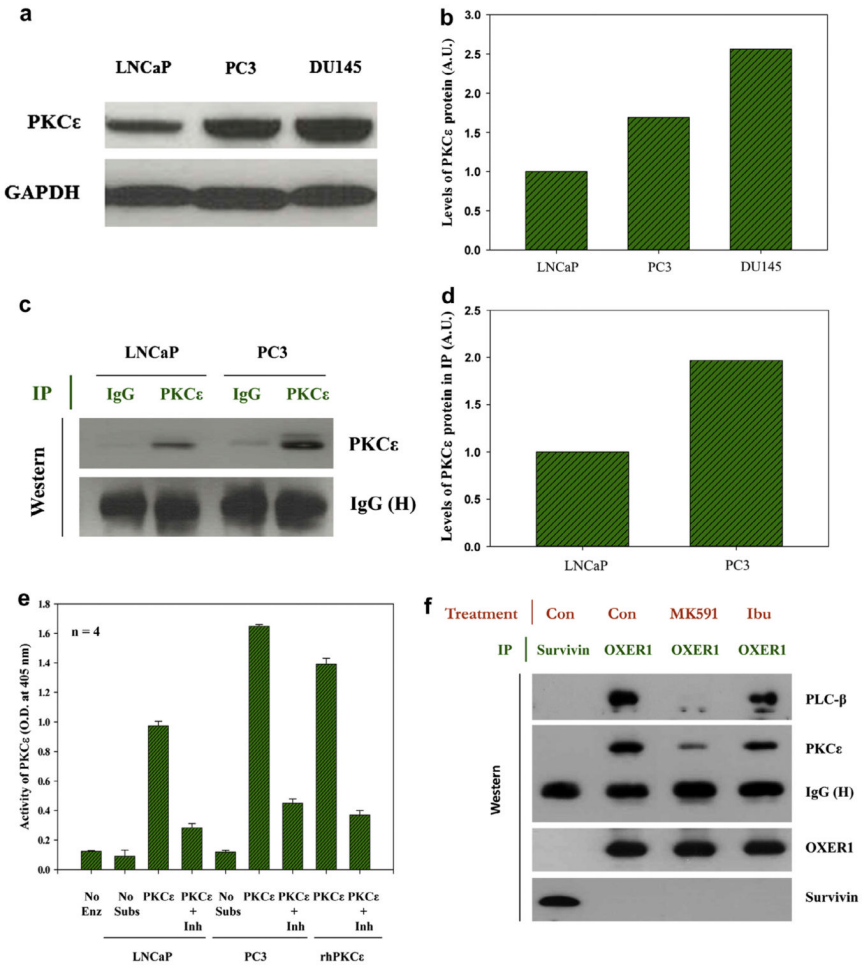


Fig. 5.

Expression and enzymatic activity of PKCε in cultured prostate cancer cells. LNCaP, PC3 and DU145 prostate cancer cells (1×10^6) were plated in 100 mm diameter plates and allowed to grow for 48 h. Then the cells were washed and lysed in lysis buffer. In (a), cell lysate proteins (100 μg per lane) were separated in 12% SDS–PAGE and expression of PKCε was detected by Western blot. In (c), PKCε was immunoprecipitated from whole cell lysates (500 μg protein per sample) using 2 μg/ml anti-PKCε antibody (or control rabbit IgG antibody) and then detected by Western blot. Relative levels of PKCε protein in IP was measured by densitometry and normalized to IgG heavy chains (H) which is detected in all rabbit antibodies (d). In (e), kinase activity of PKCε was measured by IP-kinase assay as described in Section 2. No Enz (=no enzyme added) and No Subs (=no peptide substrate added) were used as negative controls, and a recombinant human PKCε (20 ng per assay) was used in parallel as positive control. Activity of PKCε was confirmed using 40 μM of the specific peptide inhibitor KIE1–1. Results are shown as mean value ± standard error ($n = 4$). In(f), cells were treated either with vehicle only (Con), or 10 μM MK591, or 10 μM Ibuprofen for 16 h and whole cell lysates were made. Then, OXER1 proteins were immunoprecipitated from cell lysates (equivalent to 800 μg of proteins in each sample) using 2 μg/ml rabbit anti-OXER1 antibody and analyzed by Western blot. A rabbit anti-survivin antibody (2 μg/ml) was used in parallel to make control IP. *Note:* IgG heavy chains (H) are detected in both anti-OXER1 and anti-survivin rabbit antibodies.

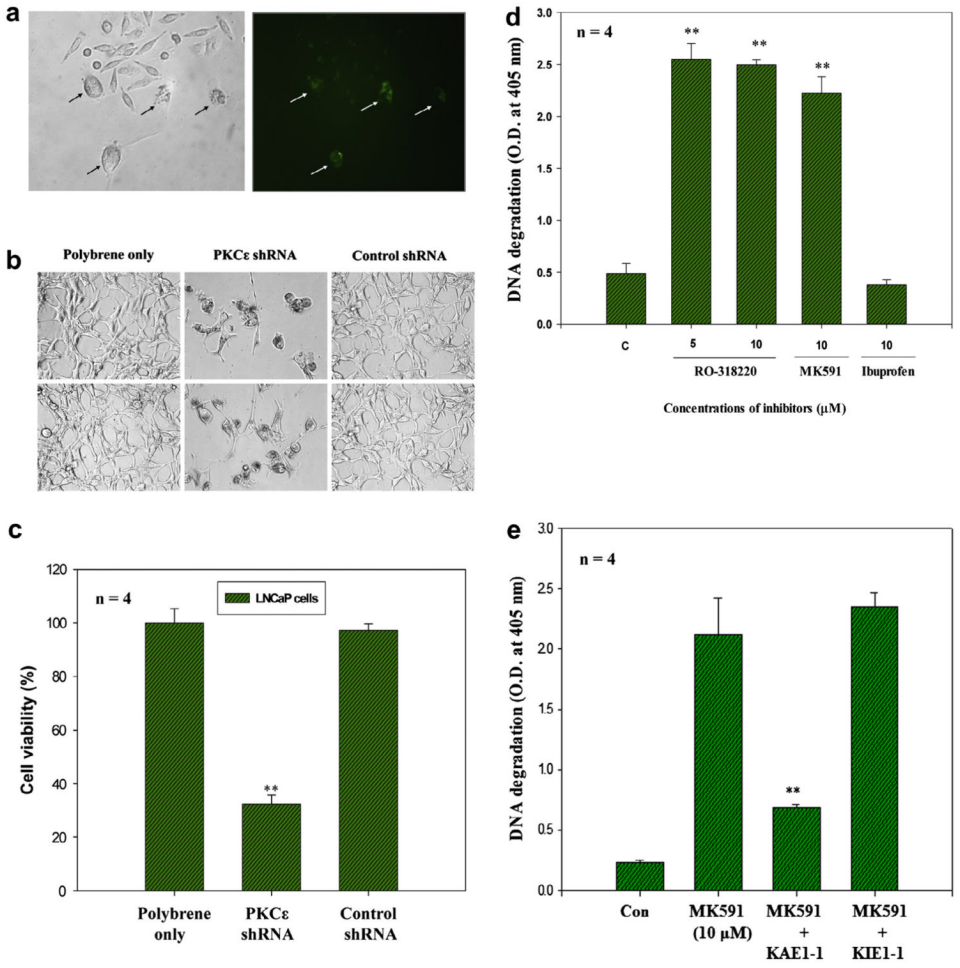


Fig. 6.

Effects of inhibitors and activators of PKCε on prostate cancer cell survival. In (a), PC3 cells were plated overnight and transfected with a dominant-negative PKCε-GFP construct. At 72 h post-transfection the cells were photographed under a fluorescence microscope at ×400. *Note:* Only cells over-expressing d/n-PKCε (arrows) are affected. In (b) and (c), LNCaP cells were treated with a lentiviral construct against PKCε shRNA or control shRNA and incubated at 37 °C for 4 days. At the end of incubation period, cells were photographed under a microscope at ×400 (b). Cell viability was measured by MTS/PES Cell Titer assay (c). In (d), cells were treated with a PKC chemical inhibitor (RO-318220) as indicated and apoptosis was measured by histone-ELISA. MK591 and Ibuprofen were used as positive and negative controls respectively. Results represent mean values of each data point ± standard error ($n = 4$). In (e), LNCaP cells (3×10^5) were plated in 60 mm diameter plates and allowed to grow for 48 h. Then the old medium was replaced by 2 ml fresh RPMI medium and the cells were treated with MK591 (10 μM) with or without a peptide activator (KAE1-1) or inhibitor (KIE1-1) of PKCε (10 μM) at 37 °C for 8 h. Control cells were treated with solvent only (0.2% DMSO). At the end of incubation period, apoptosis was measured by cell death ELISA. Data presented as mean ± standard error ($n = 4$).

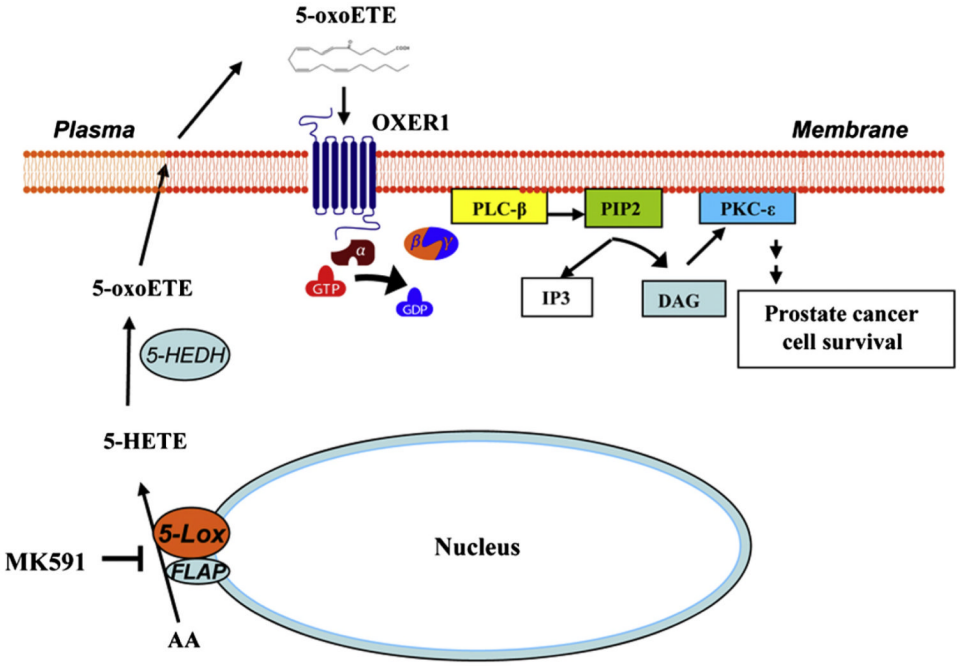


Fig. 7.

Proposed model for the regulation of prostate cancer cell survival by 5-Lox. Arachidonic acid is metabolized by 5-Lox to generate 5(S)-HETE which is converted to 5-oxoETE by a dehydrogenase. The ligand (5-oxoETE) binds with the GPCR (OXER1) on the cell surface and activates PLC-beta to generate DAG. DAG in turn activates PKCε and regulates prostate cancer cell survival by preventing apoptosis.