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5-oxo-ETE activates migration of H295R adrenocortical cells via MAPK and PKC pathways

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Abstract

The OXE receptor is a GPCR activated by eicosanoids produced by the action of 5-lipoxygenase. We previously found that this membrane receptor participates in the regulation of cAMP-dependent and -independent steroidogenesis in human H295R adrenocortical carcinoma cells. In this study we analyzed the effects of the OXE receptor physiological activator 5-oxo-ETE on the growth and migration of H259R cells. While 5-oxo-ETE did not affect the growth of H295R cells, overexpression of OXE receptor caused an increase in cell proliferation, which was further increased by 5-oxo-ETE and blocked by 5-lipoxygenase inhibition. 5-oxo-ETE increased the migratory capacity of H295R cells in wound healing assays, but it did not induce the production of metalloproteases MMP-1, MMP-2, MMP-9 and MMP-10. The pro-migratory effect of 5-oxo-ETE was reduced by pharmacological inhibition of the MEK/ERK1/2, p38 and PKC pathways. 5-oxo-ETE caused significant activation of ERK and p38. ERK activation by the eicosanoid was reduced by the “pan” PKC inhibitor GF109203X but not by the classical PKC inhibitor Gö6976, suggesting the involvement of novel PKCs in this effect. Although H295R cells display detectable phosphorylation of Ser299 in PKCδ, a readout for the activation of this novel PKC, treatment with 5-oxo-ETE *per se* was unable to induce additional PKCδ activation. Our results revealed signaling effectors activated by 5-oxo-ETE in H295R cells and may have significant implications for our understanding of OXE receptor in adrenocortical cell pathophysiology.

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All authors listed have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Keywords

Oxoeicosanoid receptor OXE-R; 5-oxo-eicosatetraenoic acid; proliferation; migration; ERK1/2; PKC; H295R human adrenocortical cells

1. Introduction

It is well established that hormonal stimulation of steroid synthesis in adrenal zona fasciculata (ZF) and zona glomerulosa (ZG) cells as well as in testicular Leydig cells involves the release of arachidonic acid (AA) [1–3]. AA is metabolized through the lipoxygenase [4–6], epoxygenase [7], and cycloxygenase [8] pathways to generate a number of bioactive metabolites. Lipoxygenase products of AA metabolism can be readily detected in adrenal and Leydig cell cultures shortly after stimulation with adrenocorticotropin (ACTH), angiotensin II and luteinizing hormone (LH) [9–12]. 5-hydroxyeicosatetraenoic acid (5-HETE) and 5-hydroperoxyeicosatetraenoic acid (5-HPETE) produced by the action of 5-lipoxygenase (5-LOX) increase in response to LH in Leydig cells. These metabolites exert a positive effect on steroid biosynthesis by stimulating promoter activity of the mitochondrial regulatory StAR protein [13], which activates cholesterol accessibility to the complex P450 side chain cleavage [14, 15] for conversion to pregnenolone in the inner mitochondrial membrane.

The regulation of steroidogenesis in the adrenal ZF is predominantly controlled by the trophic hormone ACTH, whereas in the adrenal ZG it is controlled by angiotensin II, potassium and ACTH. ACTH activates the melanocortin 2 receptor (MC2R) [16], a G-protein-coupled receptor (GPCR) that stimulates cAMP production and activates protein kinase A (PKA) [17]. Angiotensin II binds to AT1 [18, 19], a GPCR that couples to the generation of the second messengers IP3, which promotes the elevation of cytosolic calcium, and diacylglycerol (DAG). This leads to the activation of protein kinase C (PKC) [17], a family of Ser/Thr kinases widely implicated in the control of cell growth, motility, and survival. PKCs can be classified into classical cPKCs (calcium-dependent PKCα, βI, βII, and γ), novel nPKCs (calcium independent PKCδ, ε, η, and θ), and atypical aPKCs (DAG/phorbol ester-insensitive, calcium-independent PKCζ and ι) [20]. Studies revealed that both cAMP/PKA- and DAG/calcium/PKC pathways promote the activation of the rate-limiting step of steroid production stimulation, *i.e.* the transport of cholesterol from the outer to the inner mitochondrial membrane [21].

The OXE receptor (OXE-R) is a GPCR encoded by the human gene *OXER1* (NCBI gene ID 165140) and activated by three eicosanoid products of 5-LOX metabolism, specifically 5-oxo-eicosatetraenoic acid (5-oxo-ETE), 5-HETE and 5-HPETE [22, 23]. 5-oxo-ETE has been described as the most potent agonist for the receptor. A very early report already supported the existence of a specific receptor for this compound [24]. 5-LOX products acting through the OXE-R were first identified as potent stimulators of leukocyte chemotaxis [24–27] and later implicated in asthma, allergy [28, 29] and the growth of certain cancers [30, 31]. OXE-R mediates the effects of these eicosanoids on cell migration and proliferation

[28–33]. The molecular mechanisms downstream of the activation of OXE-R are only partially understood.

We have previously shown that the steroid producing human cell line H295R expresses significant levels of OXE-R [34]. Studies also demonstrated that OXE-R is involved, at least in part, in the induction of StAR protein as well as in PKA- and PKC-dependent stimulation of steroidogenesis [34, 35]. In view of the limited information available on the role of OXE-R in adrenal cell physiology, here we investigated the effects of the activation of the OXE-R by 5-oxo-ETE on proliferation and migration in H295R cells. Our studies revealed that 5-oxo-ETE stimulates the migratory capacity of H295R cells, without having significant effects on proliferation. This eicosanoid also exerts a number of effects on key signaling pathways in H295R cells, suggesting that it may have significant implications for adrenocortical cell pathophysiology.

2. Materials and methods

2.1. Cell culture and reagents

H295R, an adrenal cell line with a steroid secretion pattern similar as that of freshly isolated adrenocortical cells [36–38], was purchased from ATCC and cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12, Gibco, Life Technologies) supplemented with 5% bovine Cosmic calf serum (HyClone, GE), 1% ITS+1 (Sigma), 200 UI/ml penicillin, and 200 μg/ml streptomycin sulfate (Gibco, Life Technologies) at 37 °C and 5% CO_2. For ectopic expression of the OXE-R in H295R cells, a 1.5-kb fragment of *OXER1* cDNA was cloned from this cell line and inserted into pBABE to generate pBABE-*OXER1*. This construct was used for the generation of the stable cell line H295R-OxeR1, as described elsewhere [35].

The following reagents were used: 5-oxo-ETE (Santa Cruz), 8Br-cAMP (Sigma), angiotensin II (Sigma), phorbol 12-myristate 13-acetate (PMA, LC Laboratories), zileuton (5-LOX inhibitor, Cayman Chemical), H89 (PKA inhibitor, Calbiochem), PD98059 (MEK1/2 inhibitor, Calbiochem), SB203580 (p38 inhibitor, Cell Signaling Technology), wortmannin (PI3K inhibitor, Sigma), Gö6976 (inhibitor of classical PKCs, Tocris Bioscience), GF109203X ("pan" PKC inhibitor, Tocris Bioscience). Concentrations are indicated in the corresponding figure legends. 5-oxo-ETE is provided by Santa Cruz at a concentration of 0.3 mM in ethanol (vehicle).

2.2. Trypan blue exclusion

For assessment of cell viability, H295R cell cultures were trypsinized, resuspended in growth medium, and stained with 0.2% trypan blue [39]. Cell suspensions were loaded into a hemocytometer chamber provided with the Countess™ II FL Automated Cell Counter (Thermo Fisher), and the viable cell count process was performed following the manufacturer's instructions.

2.3. Crystal violet staining

Subconfluent H295R cell cultures were fixed with 10% formaldehyde for 15 min at room temperature and stained with 0.05% crystal violet in 10% ethanol for 30 min at room temperature [40]. After thorough washing with water to remove excess stain, plates were air-dried at room temperature. The adsorbed dye was solubilized using 0.1% glacial acetic acid in 50% ethanol for 10 min at ambient temperature [41]. The crystal violet solution was then diluted in a 1:5 ratio with the previously mentioned solubilizing solution in a new plate. Absorbance at 590 nm, which correlates with the number of viable cells, was measured in a plate-reader spectrophotometer (Synergy, BioTek Instruments, Inc.)

2.4. BrdU incorporation assay

DNA synthesis was assessed using a colorimetric immunoassay based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation. Briefly, H295R cell cultures were subjected to BrdU incorporation (4 h) and fixed for BrdU immunodetection (90 min) using a commercial kit (Cell proliferation ELISA, BrdU, Roche Diagnostics), according to the manufacturer's instructions. Non-seeded wells containing medium alone, seeded wells with cells stimulated with vehicle, and cells not treated with BrdU were included as negative controls. Measurements were done using a spectrophotometer microplate reader at 450 nm (Synergy, BioTek Instruments, Inc.).

2.5. Cell cycle analysis

Propidium iodine (PI)-stained cellular DNA content was analyzed by flow cytometry. Briefly, H295R cell cultures were trypsinized, washed, resuspended, and fixed in ice-cold 70% ethanol for 30 min, and then stained with PI (40 μg/ml) in the presence of RNAse A (100 μg/ml) for 30 min at 37°C. Cell cycle distribution was analyzed with a fluorescence activated cell sorting FACScalibur (Becton Dickinson). Ten thousand events were counted for each analysis. Data from cell debris and aggregated cells were excluded. The CytoSpec® 9.0 software (Purdue University Cytometry Laboratories, http://www.cyto.purdue.edu/Purdue_software) and a free limited version of ModFit LT™ were used to analyze cell cycle distribution into G0/G1, S and G2/M phases.

2.6. Wound healing assay

Confluent cultures of H295R cells were serum starved for 24 h, and a wound was generated using a sterile p10 tip. Cells were subjected to different treatments, and wounds analyzed 24 h later. Micrographs were taken using an Eclipse E200 Nikon microscopy (40X magnification). The width of wounds was measured using the Micrometrics® SE Premium imaging software (Accu-Scope, NY, USA). Migration was determined by the difference (in μm) between the initial wound widths (0 h) and the final wound widths (24 h) and normalized to the migration of the control cells. At least three independent experiments were performed.

2.7. Western blotting

Cells were lysed in a buffer containing 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue, and extracts were subjected to SDS-

polyacrylamide gel electrophoresis (PAGE), as previously described [42]. For Western blotting, the primary antibodies used were: anti-phospho-ERK1/2 (Thr202/Tyr204), anti-total ERK1/2, anti-PKCδ, anti-PKCα, anti-PKCε , anti-PKCη, anti-PKCζ, anti-PKCι, anti-phospho-p38, anti-total p38, anti-phospho-Akt, anti-total Akt (Cell Signaling Technology), anti-phospho-Ser299-PKCδ (Abcam), anti-actin and anti-vinculin (Bio-Rad Laboratories). As secondary antibodies we used goat anti-mouse or goat anti-rabbit antibodies conjugated to peroxidase (Bio-Rad Laboratories). Bands were visualized by enhanced chemiluminescence. Images were captured using an Odyssey Fc system (LI-COR Biosciences). Image processing and densitometry analysis were carried out using the Image Studio Lite software (LI-COR Biosciences).

2.8. Quantitative RT-PCR (qPCR)

Total RNA was extracted from subconfluent plates of H295R cells using the RNeasy kit (Qiagen). One μg of RNA per sample was reverse transcribed using random hexamers as primers and the Taqman reverse transcription reagent kit (Applied Biosystems). PCR amplifications were performed using an ABI PRISM 7300 Detection System in a total volume of 20 μl containing Taqman Universal PCR Master Mix (Applied Biosystems), 1 μl of cDNA, the fluorescent probe (200 nM), and commercial target primers for the target genes (300 nM). PCR product formation was continuously monitored using the Sequence Detection System software version 1.7 (Applied Biosystems). The FAM signal was normalized to endogenous UBC (housekeeping gene). MMP-1, MMP-2, MMP-9, MMP-10, and UBC primers, as well as probes 5′ end-labeled with 6-carboxyfluorescein (6-FAM) were purchased from Applied Biosystems.

2.9. Statistical analysis

Independent experiments were performed at least three times. Statistical significance was done with Student's *t*-test or ANOVA using GraphPad Prism 5.0. In all cases, a value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Effect of 5-oxo-ETE on H295R cell proliferation

In the first set of experiments, we evaluated if activation of the OXE-R had any impact on the growth of H295R cells. Trypan blue exclusion experiments showed that treatment with 5-oxo-ETE (500 nM) for 96 h did not significantly affect cell viability relative to vehicle-treated control cells (Fig. 1A). When we assessed the effect of 5-oxo-ETE on proliferation of asynchronously growing H295R cells using BrdU incorporation, cell cycle distribution analysis and crystal violet assay, we found that 5-oxo-ETE caused only minor effects, which were not statistically significant (Fig. 1B, 1C, and 1D, Suppl. Fig. 1A). Similar results were observed when 5-oxo-ETE was added every 24 h for 96 h (Suppl. Fig 1B). Thus, the eicosanoid did not trigger any obvious changes in H295R cell growth.

Next, we took advantage of a cell line that we generated in which we overexpressed the OXE-R (H295R-OxeR1). We found that this cell line has an elevated basal proliferative rate compared to parental cells, and treatment with 5-oxo-ETE further enhanced their

proliferative rate (Fig. 1E). Treatment of H295R-OxeR1 cells with the 5-LOX inhibitor zileuton reduced proliferation (Fig. 1E). Thus, although OXE-R activation may not have a proliferative effect *per se*, it may be possible that this receptor participates in the modulation of the effect of other proliferative agents or in specific circumstances when the receptor or its agonists are supraphysiologically produced.

Given that PKA- and PKC-dependent pathways are known to play relevant roles in adrenocortical cell physiology, we examined the effects of 8Br-cAMP, which directly activates PKA, and angiotensin II, which couples to the DAG/Ca/PKC pathway [17]. We found that treatment of H295R cells with 8Br-cAMP markedly reduced cell number (Fig. 1A). Along the same line, we observed significant inhibition by 8Br-cAMP in BrdU incorporation (Fig 1B) and crystal violet assays (Fig. 1D). The inhibitory growth effect of 8Br-cAMP was confirmed by the accumulation of cells in G2/M as determined by FACS analysis (Fig. 1C). Despite a minor effect of angiotensin II on H295R cell viability, we did not observe any significant effect on cell proliferation or cell cycle distribution.

3.2. Effect of 5-oxo-ETE on cell migration

Next, we analyzed the effect of 5-oxo-ETE on the ability of H295R cells to migrate. For these studies, we used a wound healing assay [43], which has been used before in this cellular model [44]. Interestingly, we found that 5-oxo-ETE caused a significant increase in cell migration relative to control (vehicle-treated). A representative experiment is shown in Fig. 2A. A quantitative analysis of the 5-oxo-ETE effect revealed that motility was increased by ~70% in response to this eicosanoid (Fig. 2B, see also Suppl. Fig 2). On the other hand, angiotensin II had no effect on the migration of H295R cells (Fig. 2B). 8Br-cAMP caused a slight inhibition in cell migration, but this effect was not statistically significant (Fig. 2B).

To investigate the mechanisms behind the pro-migratory action of 5-oxo-ETE in H295R cells, we examined the effect of different signal transduction pathway inhibitors (Fig. 2C). PD98059, an inhibitor of MEK (the kinase responsible for ERK activation), caused a major inhibition (~70%) of migration stimulated by 5-oxo-ETE, thus implying the involvement of the MEK/ERK pathway in this effect. We also observed a significant inhibition (~45%) by SB203580, an inhibitor of the MAPK p38. On the other hand, inhibition of PI3K using wortmannin did not affect cell motility induced by 5-oxo-ETE. The PKA inhibitor H89 slightly enhanced migration; however, this effect was not statistically significant. Since PKC isozymes have been widely implicated in cell motility in a number of cancer cells [45], we examined the effects of two PKC inhibitors, GF109203X (a ‘pan” PKC inhibitor) and Gö6976 (which preferentially inhibits classical PKCs). These experiments revealed that GF109203X reduced cell migration induced by 5-oxo-ETE by ~40%, whereas Gö6976 had no effect. None of the inhibitors have any detectable effect on the migration of H295R cells in the absence of 5-oxo-ETE.

3.3. Effect of 5-oxo-ETE on the activation of signaling pathways

To determine if 5-oxo-ETE activates the ERK1/2 and p38 pathways, we determined phospho-ERK1/2 and phospho-p38 levels by Western blot. These experiments showed that 5-oxo-ETE caused a time-dependent increase in ERK1/2 and p38 phosphorylation without

any effect on Akt phosphorylation (Fig. 3A). Given that PKC has been shown to be upstream for ERK activation in many cell types, including adrenal cells [46], we hypothesized that ERK1/2 activation by 5-oxo-ETE could be sensitive to PKC inhibition. As shown in Fig. 3B, ERK1/2 phosphorylation was totally inhibited by GF109203X, whereas Gö6976 had no effect on 5-oxo-ETE. This result indicates that activation of ERK1/2 by 5-oxo-ETE is not mediated by the classical PKCs and suggests that other novel nPKCs might be responsible for this effect.

In addition to the classical PKCα, the novel PKCδ and PKCε isozymes are the most commonly expressed PKCs in most cell types [47]. We analyzed the expression of PKCs in H295R cells by Western blot, using as controls two unrelated cell lines (PC3 prostate cancer cells and A549 lung cancer cells). In addition to classical PKCα and atypical PKCζ and ι (which are ubiquitously expressed), we found significant expression of novel PKCs δ, ε, and η (Fig. 4A).

Most available phospho-PKC antibodies are either non-selective and/or do not correlate with PKC activation status, except for an anti-phospho-Ser299-PKCδ that has been recently characterized [42, 48] (see also Suppl. Fig. 3). Fig. 4B shows that H259R cells display detectable basal levels PKCδ phosphorylation in position Ser299, suggesting an elevated endogenous basal activation. Phospho-Ser299-PKCδ levels could be further enhanced by a maximal concentration of the phorbol ester PMA, a typical PKC activator, as reported previously in other models [42]. However, phospho-Ser-PKCδ levels were not modified by 5-oxo-ETE treatment at times in which ERK1/2 activation was readily detected. This finding suggests that although PKCδ (and probably other PKCs) may be basally activated in H295R cells, stimulation of OXE-R by its ligand may not lead to DAG generation and subsequent PKC activation. It is likely that ERK1/2 activation downstream of OXE-R in these cells may depend on the constitutive basal activation of a novel PKC.

3.4. Effect of 5-oxo-ETE on the induction of metalloproteases (MMPs)

Previous studies in eosinophils and basophils demonstrated a role for 5-oxo-ETE in the production of metalloproteases (MMPs), such as MMP-9 [49, 50]. Since MMPs are known to play important roles in cell migration and tumor invasion, we evaluated if 5-oxo-ETE could induce the production of MMPs in H295R cells. As an approach, we used qPCR to measure mRNA levels of four key MMPs (MMP-1, MMP-2, MMP-9, and MMP-10). We used PMA as a control, as this phorbol ester induces MMP production in multiple cancer cell models [51]. As shown in Fig. 5, PMA induced the production of MMP-1, MMP-9, and MMP-10. MMP-2 mRNA levels were not significantly modified in response to the phorbol ester. When we examined the effect of 5-oxo-ETE, we found that this eicosanoid was unable to induce MMP-1, MMP-2, MMP-9 or MMP-10 in H295R cells.

4. Discussion

The results of this study provide an insight into the role of OXE-R in the biology of adrenocortical cells and the effectors of this pathway. We found that 5-oxo-ETE, an eicosanoid produced by the 5-LOX pathway, induces the migration of H295R adrenocortical cells.

ACTH and angiotensin II are well known stimuli for steroid production in the adrenal gland, although their role in promoting growth has been controversial [17]. *In vivo*, ACTH promotes hypersecretion of steroids and hypertrophy of the gland, however, it inhibits proliferation in adrenal cell cultures [52, 53]. Particularly, the action and signaling pathways of ACTH on adrenocortical cell growth *in vitro* are still a subject of debate [54]. Whereas antimitogenic and pro-apoptotic actions of the cAMP/PKA dependent pathway has been described in many types of cancer cells [55], there has been also evidence for the contribution of this pathway in tumor growth [56]. In agreement with other study [57], our results support the notion that the cAMP pathway exerts an anti-proliferative effect in human adrenocortical tumor H295R cells. With regard to angiotensin II, studies have shown both proliferative [58–62] and anti-proliferative effects [63]. In this study, we did not find any significant effect of angiotensin II on the proliferation of H295R cells.

There is increasing evidence for the involvement of 5-oxo-ETE and its receptor in cell proliferation. 5-oxo-ETE promotes cell growth and survival in breast and prostate cancer cells [32], while silencing the OXE-R decreases cell viability [30]. Unlike these studies, we could not find any significant effect in the proliferation of H295R cells in response to 5-oxo-ETE. It is interesting that enhanced cell proliferation could be observed in H295R cells that overexpress OXE-R, an effect that is further increased by 5-oxo-ETE and reduced by the inhibitor of the 5-LOX pathway zileuton. These results suggest two important conclusions. First, in wild-type H295R cells, the magnitude of stimulation of the endogenous OXE-R is not sufficient to promote mitogenesis. Of course, we cannot rule out that in a physiological setting synergistic effects of OXE-R with other receptors may have an impact on cell growth, an area of research that is currently under investigation. The second conclusion is that endogenous production of 5-oxo-ETE and/or other 5-LOX metabolites through autocrine and/or paracrine mechanisms may activate OXE-R. Conceivably, OXE-R may be involved in adrenal tumor formation and/or growth.

Pro-migratory effects of 5-oxo-ETE, 5-HETE and 5-HPETE have been previously described in some models [26]. Indeed, the demonstration of the biological relationship between these eicosanoids and OXE-R came from studies of 5-oxo-ETE-induced chemotaxis in Chinese hamster ovary (CHO) cells [64] and subsequently in leukocytes [29]. More recently, it was reported that 5-oxo-ETE rearranges the actin cytoskeleton and induces migration in prostate cancer cells [32]. In breast cancer cells, overexpression of ACSL4, an enzyme involved in the regulation of intracellular levels of AA, results in an aggressive phenotype, and their high migratory rate is sensitive to 5-LOX inhibition, suggesting a potential role for eicosanoids and OXE-R [65]. Along the same line, it was found that breast cancer cells express high levels of OXE-R and that 5-oxo-ETE exerts a proliferative action [31]. Unlike a previous finding in eosinophils [49], we found that in H295R cells 5-oxo-ETE does not induce the production of MMPs, proteases that play important roles in the degradation of the extracellular matrix.

MAPK signaling pathways play a vital role in regulating adrenocortical cell function, including steroid production [66, 67]. Here we show that upon OXE-R stimulation there is activation of the ERK1/2 and p38 pathways. We found that these pathways are important mediations of 5-oxo-ETE–induced cell migration. Inhibition of pathways leading to ERK1/2

activation should have significant therapeutic impact. In fact, a very interesting recent report by Pereira *et al.* [68] highlighted the potential of modulating the ERK pathway for the targeting of adrenal cancer. We found that ERK activation by 5-oxo-ETE is dependent on PKC activity, since it is impaired by the "pan" PKC inhibitor GF109203X. The lack of effect of Gö6976, an inhibitor of the classical PKCs is indicative of the involvement of novel PKCs. This is consistent with the requirement of PKCε for 5-oxo-ETE responses in prostate cancer cells [69]. Here, we showed that H295R cells express the novel PKCs δ, ε, and η. It is interesting that despite the lack of PKC activation by 5-oxo-ETE, PKC activity is still required for ERK activation as well as for migration. It is likely that novel PKCs are basally activated in H295R cells, which is consistent with the high phospho-Ser299-PKCδ signal detected in these cells under basal conditions (see Fig. 5B), which is indicative of nPKC activation [42, 48]. The characterization of discrete PKC isozymes involved in the effect of 5-oxo-ETE would require RNAi silencing or CRISPR approaches currently underway in our laboratory.

The relationship between 5-oxo-ETE/OXE-R and cell migration has been primarily described in highly migratory cells such as leukocytes and cancer cells. Given that H295R cells are derived from a human adrenocortical tumor, the results from our study may be relevant for adrenal cancer progression. From a development perspective, ZG cells in the outer region of the adult adrenal cortex serve as precursors that migrate towards the inner ZF during tissue homeostasis and regeneration [70, 71]. The search for stimulators of migration in the adrenal cortex has however so far produced meager results. We speculate that 5-LOX products acting through OXE-R could have a role in zonation induction.

In summary, our results show that 5-oxo-ETE promotes migration of H295R cells without significantly impacting on cell growth. Dissecting the signaling events downstream of the OXE-R in adrenal cells would greatly help in our understanding of the molecular effectors of this GPCR. This will help addressing the potential roles of OXE-R and its physiological activators in adrenal development and pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-LOX	5-lipoxygenase
5-oxo-ETE	5-oxo-eicosatetraenoic acid
8Br-cAMP	8Bromo-cyclic AMP
AA	arachidonic acid

ACTH	adrenocorticotropin
ANGII	angiotensin II
ERK	extracellular signal-regulated protein kinases
GPCR	G protein coupled receptor
MMP	metalloprotease
OXE-R	oxoeicosanoid receptor
PKA	protein kinase A
PKC	protein kinase C
ZG	adrenal zona fasciculate
ZF	adrenal zona glomerulosa

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Highlights

- 5-oxo-ETE induces migration without affecting proliferation in adrenal H295R cells.
- H295R cell migration depends on PKC, ERK1/2 and p38 pathways.
- H295R cell migration is not mediated by classical PKCs.
- 5-oxo-ETE does not induce the expression of metalloproteases in H295R cells.

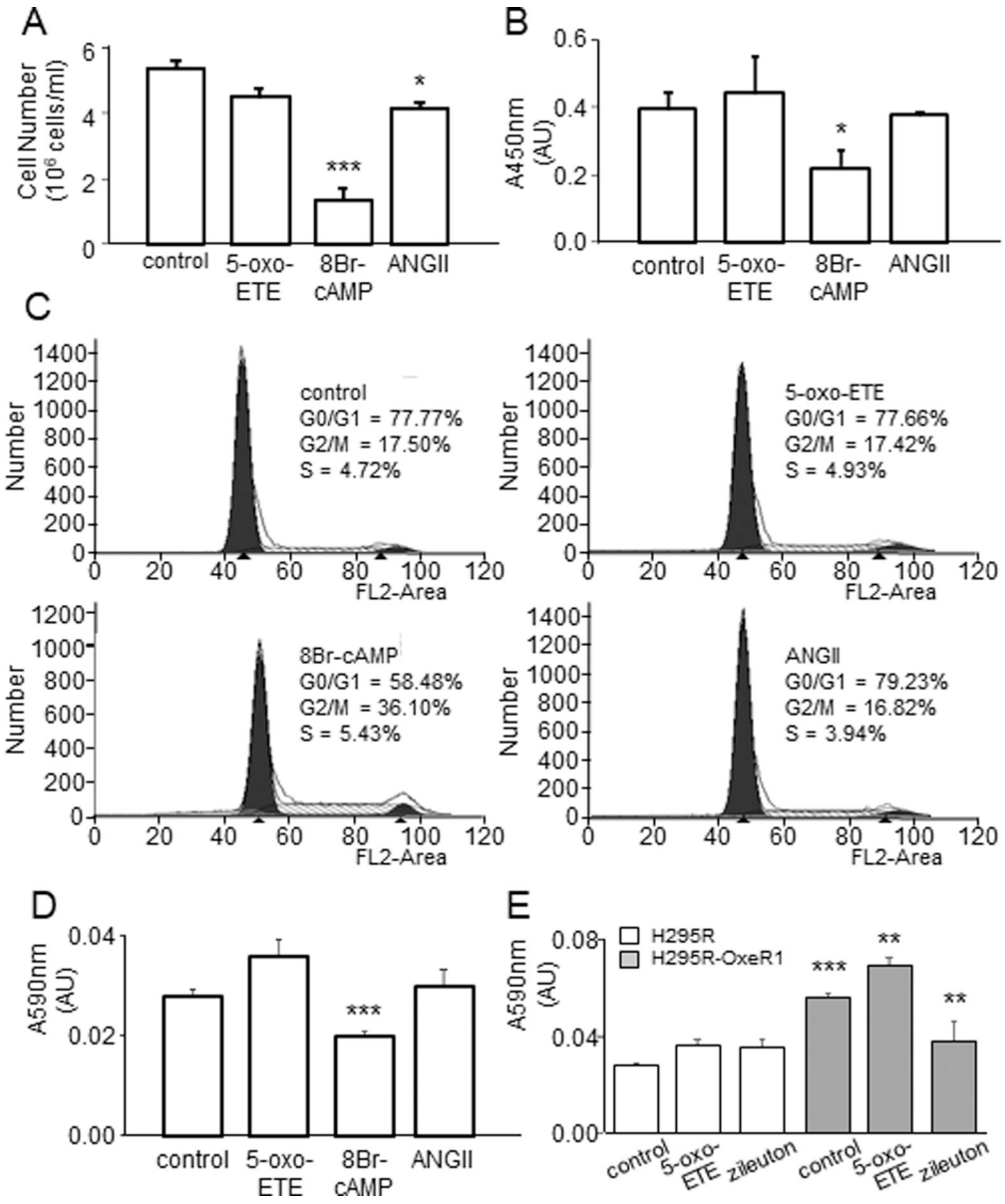

Fig. 1: Effects of 5-oxo-ETE, 8Br-cAMP and angiotensin II on proliferation of H295R cells.

(A-D) H295R cells were treated with 500 nM 5-oxo-ETE, 1 mM 8Br-cAMP or 100 nM angiotensin II (ANGII) for 96 h. (A) Trypan blue exclusion assay. ***, p<0.001 *vs.* control; *, p<0.05 *vs.* control; (B) BrdU incorporation. *, p<0,05 *vs.* control; (C) Propidium iodide (PI) staining of DNA detected by flow cytometry; (D) Crystal violet staining. ***, p<0.001 *vs.* control. (E) H295R and H295R-OxeR1 cells were treated with 500 nM 5-oxo-ETE or 0.5 mM zileuton and analyzed by crystal violet staining. *** p<0.001 *vs.* parental H295R cells; ** p<0.01 *vs.* parental H295R-OxeR1 cells. (A), (B), (D) and (E) Results are expressed as mean ± SEM (n=3). (C) similar results were observed in two additional independent experiments.

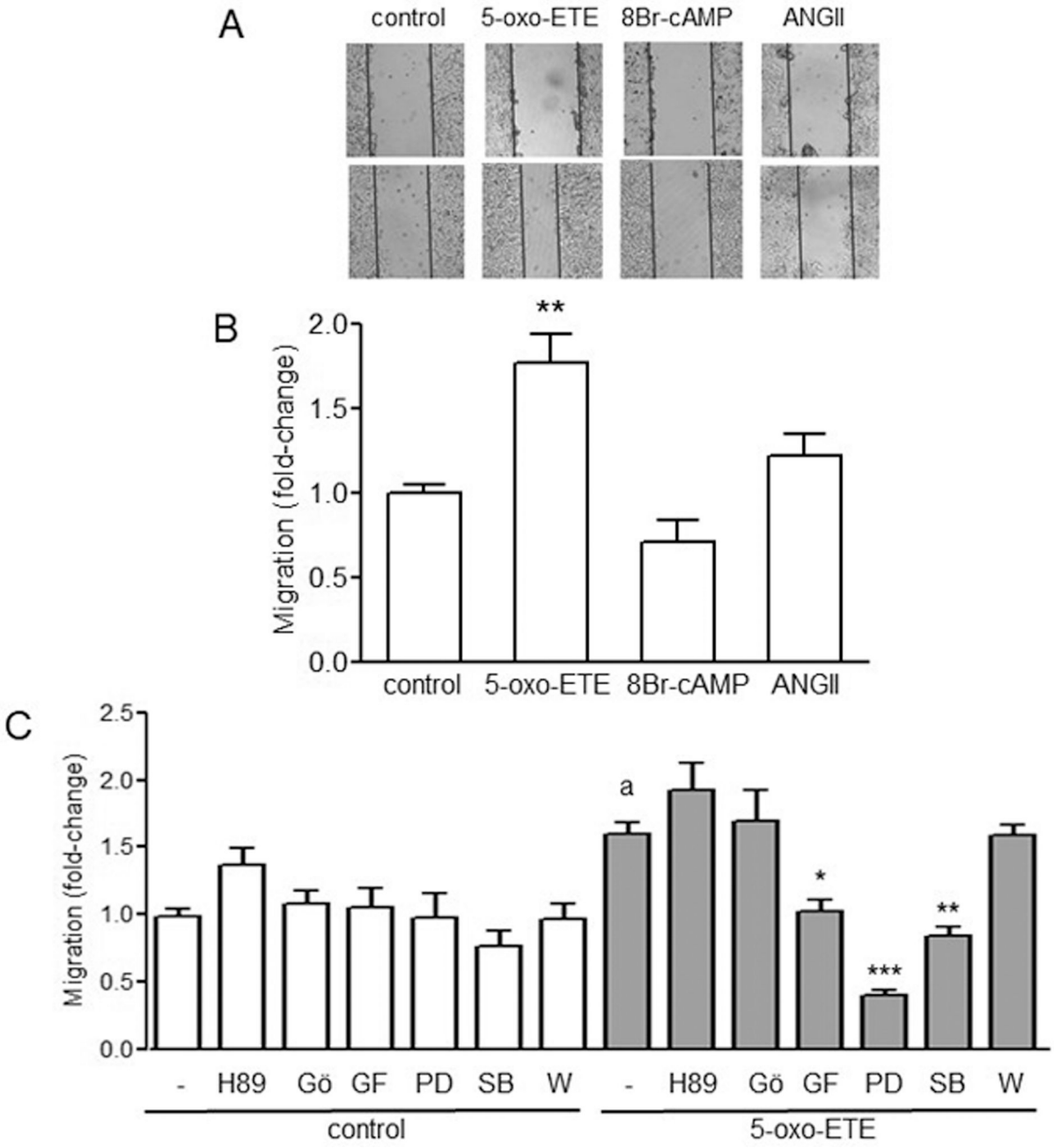

Fig. 2: Effects of 5-oxo-ETE, 8Br-cAMP, angiotensin II and signaling inhibitors on migration of H295R cells.

(A) and (B) Cells were treated with 500 nM 5-oxo-ETE, 1 mM 8Br-cAMP or 100 nM angiotensin II (ANGII) for 24 h, and migration assessed using a wound healing assay. (A) Representative micrographs. (B) Quantification of cell migration: Data (mean ± SEM, n=3) are expressed as fold increase relative to control (vehicle-treated) cells. **, $p<0.01$ *vs.* control. (C) Effect of pathway inhibitors on migration induced by 500 nM 5-oxo-ETE. The following inhibitors were used: 20 μM H89, 3 μM Gö6976 (Gö), 3 μM GF109203X (GF), 50 μM PD98059 (PD), 10 μM SB203580 (SB), and 50 nM wortmannin (W). Quantification of cell migration: Data (mean ± SEM, n=3) are expressed as fold increase relative to control

cells. *a*, p<0.01 *vs*. control; *, p<0.05 *vs*. 5-oxo-ETE; **, p<0.01 *vs*. 5-oxo-ETE; ***, p<0.001 *vs*. 5-oxo-ETE.

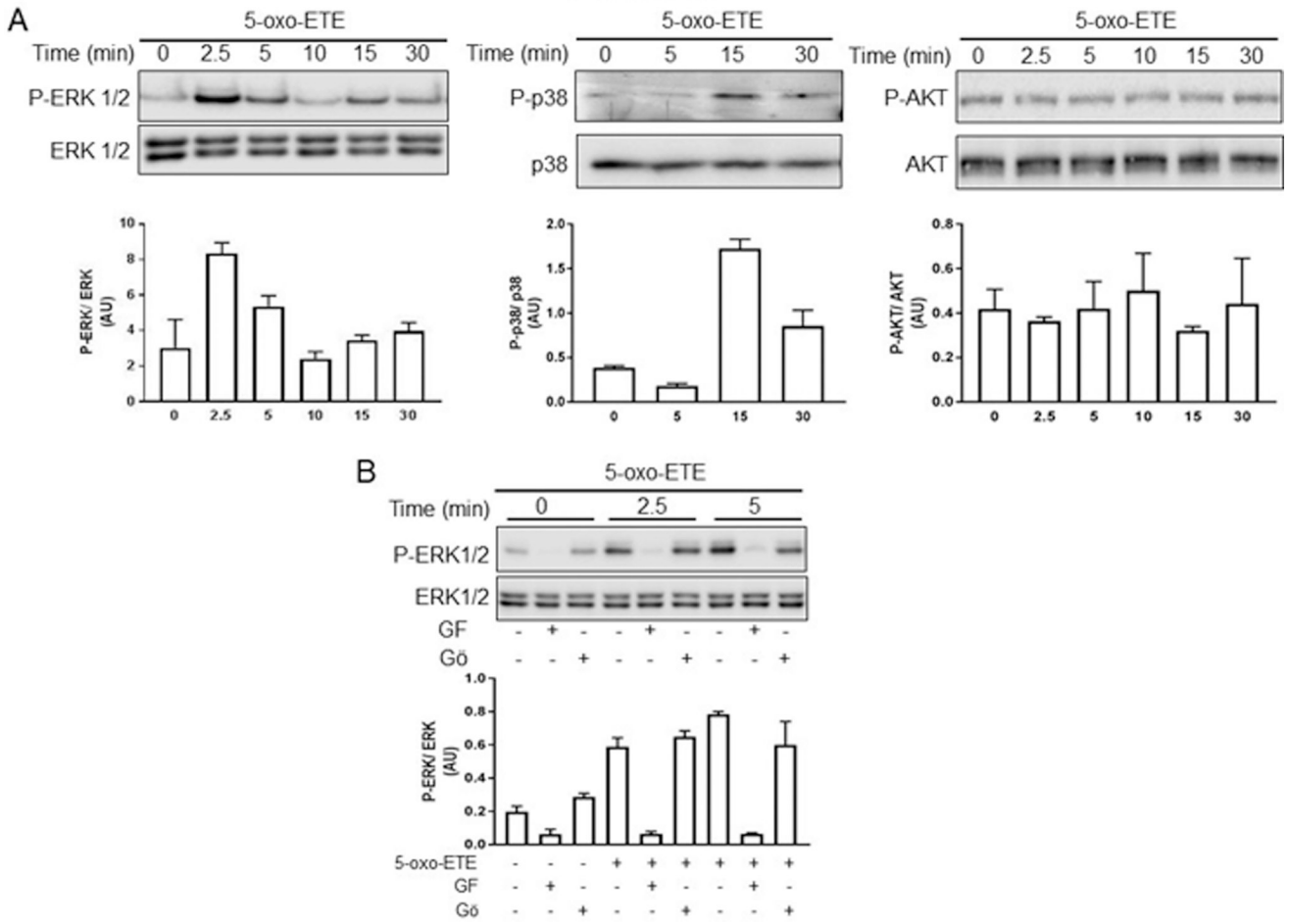

Fig. 3: Activation of signaling pathways in H295R cells by 5-oxo-ETE.

Cells were treated with 500 nM 5-oxo-ETE in the absence (A) or in the presence (B) of 3 μM Gö6976 (Gö) or 3 μM GF109203X (GF). Phospho-ERK1/2 (A and B), phospho-p38 and phospho-AKT (A) were analyzed by Western blot. The figure shows representative Western blots and the quantification of three independent experiments. Results are expressed as mean ± SEM.

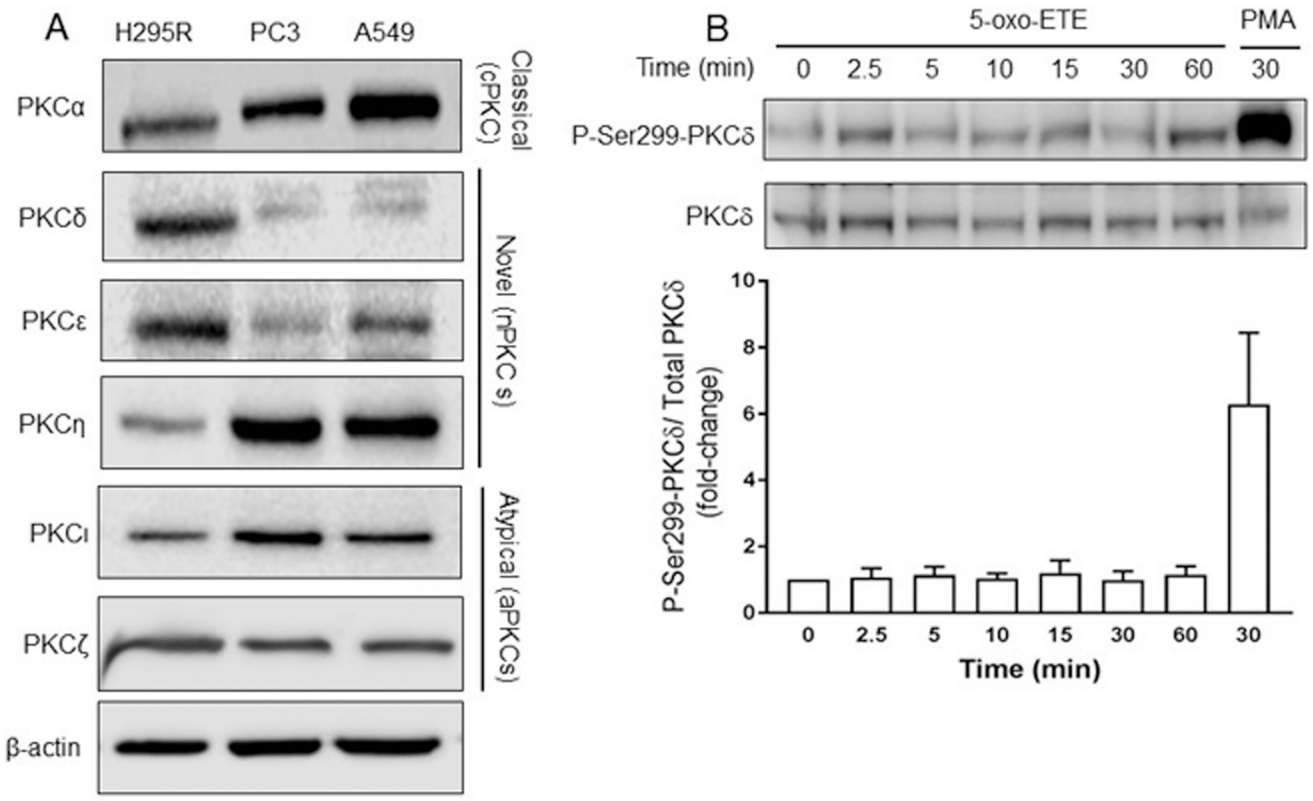

Fig. 4: PKCs expression and phosphorylation state of PKCδ in H295R cells.

(A) Expression of PKC isozymes was analyzed by Western blot in total lysates of the indicated cell lines. (B) Cells were treated with 500 nM 5-oxo-ETE for the indicated times, and phospho-Ser299-PKCδ levels were analyzed by Western blot. The figure shows a representative Western blot and the quantification of three independent experiments. Results are expressed as mean ± SEM.

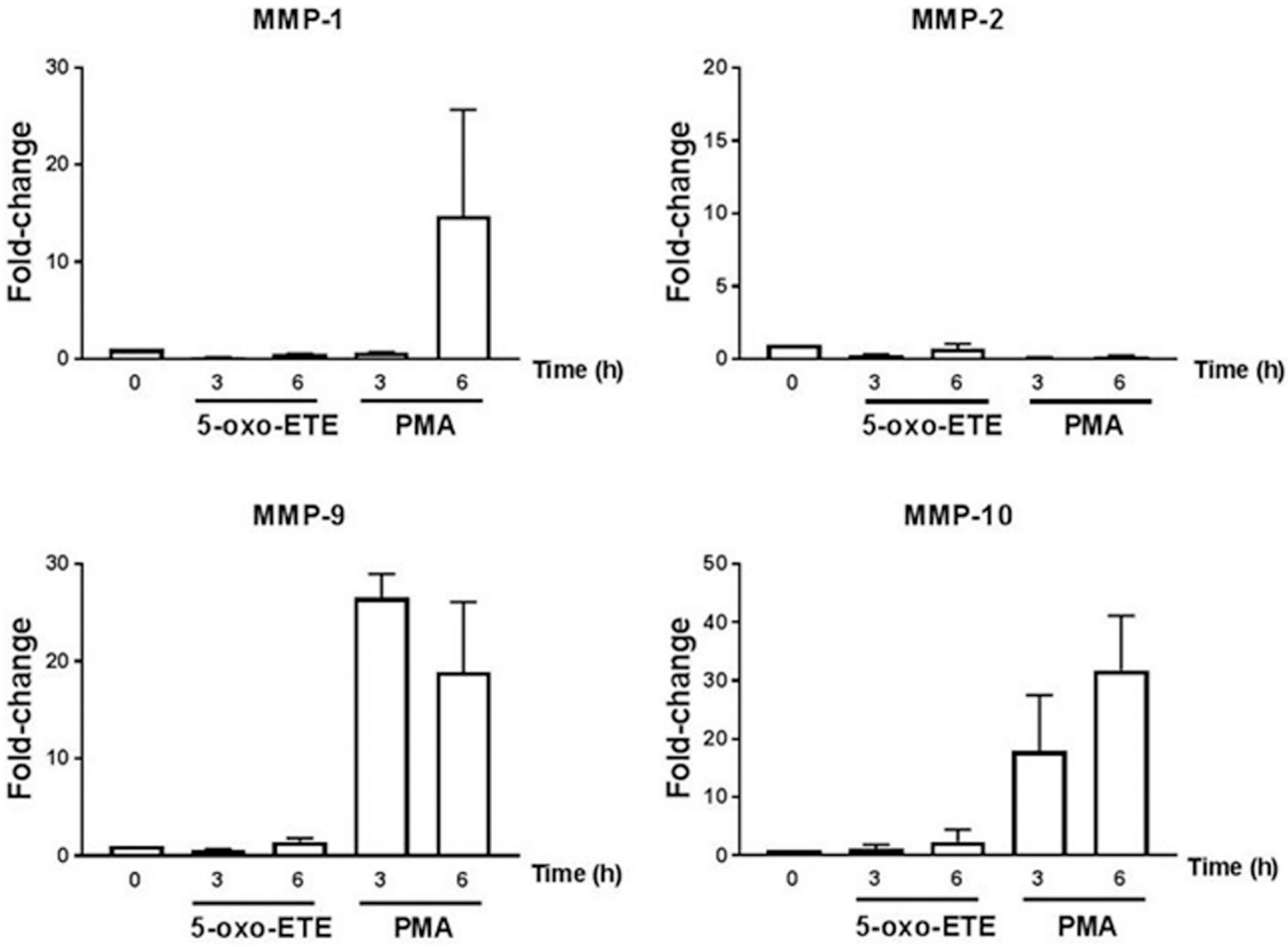

Fig. 5: Effect of 5-oxo-ETE on the expression of metalloproteases in H295R cells.

Cells were treated with 500 nM 5-oxo-ETE or 100 nM PMA for 1 h, and samples were obtained 3 and 6 h later for determining the expression of metalloproteases (MMPs) by qPCR, as described in Materials and Methods. Results are presented as fold-change relative to untreated cells. Data are expressed as mean ± SD of triplicate samples. Two additional independent experiments gave similar results.