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OxeR1 regulates angiotensin II and cAMP-stimulated steroid production in human H295R adrenocortical cells

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ABSTRACT

Hormone-regulated steroidogenesis and StAR protein induction involve the action of lipoxygenated products. The products of 5-lipoxygenase act on inflammation and immunity by stimulation of a membrane receptor called OxeR1. The presence of OxeR1 in other systems has not been described up to date and little is known about its mechanism of action and other functions. In this context, the aim of this study was the identification and characterization of OxeR1 as a mediator of cAMP-dependent and independent pathways. Overexpression of OxeR1 in MA-10 Leydig cells increased cAMP-dependent progesterone production. Angiotensin II and cAMP stimulation of adrenocortical human H295R cells produced an increase in StAR protein induction and steroidogenesis in cells overexpressing OxeR1 as compared to mock-transfected cells. Additionally, activation of OxeR1 caused a time-dependent increase in ERK1/2 phosphorylation. In summary, membrane receptor OxeR1 is involved in StAR protein induction and activation of steroidogenesis triggered by cAMP or angiotensin II, acting, at least in part, through ERK1/2 activation.

1. Introduction

The regulation of steroidogenesis in the adrenal cortex is predominantly controlled by trophic hormone ACTH in adrenal zona fasciculate, and by angiotensin II, potassium and ACTH in adrenal zona glomerulosa. ACTH stimulates a G-protein-coupled receptor (GPCR) named melanocortin 2 receptor (MC2R) (Mountjoy et al., 1992). The activation of MC2R triggers the production of cAMP, which in turn activates protein kinase A (PKA). Angiotensin II stimulates membrane receptor type 1 (AT1) (Murphy et al., 1991; Sasaki et al., 1991), another GPCR, which increases the production of inositol trisphosphate (IP3), diacylglycerol (DAG), calcium and, finally, protein kinase C (PKC). Both cAMP/PKA- and IP3/DAG + Ca/PKC-dependent pathways finally allow 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, where it is converted to pregnenolone.

The stimulation of adrenal cortex cells and interstitial testicular Leydig cells by trophic hormones, acting through cAMP-dependent and independent pathways, leads to the release and metabolism of arachidonic acid (AA) (Maloberti et al., 2002; Moraga et al., 1997) through the lipoxygenase (LOX) pathway to hydroperoxyeicosatetraenoic acids, hydroxyeicosatetraenoic acids and oxo-eicosatetraenoic acids (HpETEs, HETEs and Oxo-ETEs, respectively) (Hirai et al., 1985; Reddy et al., 1992). 5-HpETE is the metabolite that activates steroid production and it is the product of 5-LOX, one of the three isoforms of the lipoxygenases (5- 12- and 15-LOX). In steroidogenic cells, trophic hormones rapidly increase StAR mRNA and protein levels. In turn, the induction of mitochondrial StAR protein increases the accessibility of cholesterol to the inner mitochondrial membrane. Despite the fact that 5-HpETE and 5-HETE have proven to be positive modulators of the StAR promoter (Wang et al., 2003b), and that the AA-responsive region is known to be located at the -67/-96 region of StAR promoter DNA, the mechanism by which 5-HpETE increases endogenous levels of StAR protein is still unknown.

In other systems such as eosinophils, nucleophils and platelets, a GPCR successively named TG1019, R527 and, more recently, OxeR1 (Brink et al., 2004; Hosoi et al., 2002, 2005; Jones et al., 2003) appears to be responsible for 5-HpETE action. This receptor has different levels of affinity to different lipidic compounds, and while 5-oxo-ETE is the lipoxygenated product with the highest activating capacity, the binding of a fatty acid such as docosahexanoic acid (DHA, 22:6) shows antagonistic actions (Hosoi et al., 2002). The expression of receptor OxeR1 has been detected in various types of tissue except for brain (Hosoi et al., 2002). Regarding its function, OxeR1 has been associated to the previously known effects of eicosatetraenoic acid derivatives on inflammatory and immune response (Grant et al., 2009), as well as on cell proliferation

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(O'Flaherty et al., 2005). However, the signal transduction pathway triggered by the activation of membrane receptor OxeR1 remains elusive.

The general purpose of our research is to describe the mechanism of action of lipoxygenated products in the activation of steroidogenesis. In particular, given that AA metabolism by LOX and StAR protein induction occur under cAMP-dependent and independent pathways (Mele et al., 1997; Omura et al., 1990; Shibata and Kojima, 1991), and that OxeR1 is present in steroidogenic cells (Cooke et al., 2013), the aim of this study was to analyze the involvement of OxeR1 in cAMP-dependent and independent activation of steroidogenesis and the signal transduction mechanism implicated in the process. The present study demonstrates that OxeR1 is a mediator of the hormonal response triggered by cAMP-dependent and independent pathways and that it acts, at least in part, via ERK1/2 activation.

2. Materials and methods

2.1. Cell cultures

Human H295R adrenocortical cells (Rainey et al., 1994, 2004; Staels et al., 1993) were purchased from ATCC and cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12, Invitrogen) supplemented with 5% bovine Cosmic calf serum (HyClone, GE), 1% ITS+1 (Sigma), 200 UI/ml penicillin and 200 μg/ml streptomycin sulfate (Invitrogen) at 37 °C and 5% CO_2.

Mouse MA-10 Leydig cells were generously provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA) and cultured in Waymouth MB/752 (Sigma) medium supplemented with 20 mM HEPES, 1.12 g/l $NaHCO_3$, 15% horse serum (Invitrogen) and 50 mg/l gentamicin (Invitrogen) at 37 °C and 5% CO_2 as previously described (Ascoli, 1981).

2.2. Transient and stable transfection of OxeR1 cDNA in H295R and MA-10 cells

A 1.5-kb fragment of OxeR1 cDNA was cloned from human H295R adrenocortical cells into plasmids pRc/CMVi and pBABE, generating the pRc/CMVi-OxeR1 and pBABE-OxeR1 constructions. While plasmid pRc/CMVi-OxeR1 was used for transient transfections, plasmid pBABE-OxeR1 was used for the generation of the stable cell line.

For transient transfections, MA-10 cells were cultured in 6-well plates ($3.5–4 \times 10^5$ cells/well) overnight. pRc/CMVi-OxeR1 or the empty plasmid, pRc/CMVi-empty, were transfected (4 μg DNA) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

The same protocol was followed to generate stable cell lines transfecting H295R cells with pBABE-OxeR1 and subsequent selection using hygromycin B (50 μg/ml) for 30 days. H295R wild type or transfected with the empty plasmid, pBABE-empty, were used as controls.

2.3. Cell treatments

The MA-10 cell cultures were treated with or without 0.25 mM 8Br–cAMP (Sigma) and H295R cell cultures with or without 0.5 mM 8Br–cAMP or 10 nM angiotensin II (Sigma). All the aforementioned concentrations are in the submaximal range for steroidogenesis stimulation.

H295R cells were treated with OxeR1 agonist 5-oxo-ETE (Santa Cruz) with or without 50 μM PD98059 (Calbiochem). The concentration of 5-oxo-ETE and the length of treatments are indicated in the figures or in the text.

In all cases, stimulation was performed in a serum-free medium. At the end of experiments, progesterone production was determined in the culture media by radioimmunoassay (Cornejo Maciel et al., 2001).

2.4. RNA isolation and semiquantitative RT-PCR

Total cell RNA from the different treatment groups was isolated using TriReagent® following the manufacturer's instructions (MRC Inc.) (Cooke et al., 2013) and subjected to RT-PCR. Given that *OxeR1* is an intronless gene, a DNase treatment was performed prior to the reverse transcription to eliminate any genomic DNA contamination. RNA and RNase-free DNase1 (Invitrogen) were combined in a ratio 1–10 μg RNA/μl DNase, and the mix was processed following the manufacturer's instructions. The reverse transcription was performed using MMLV reverse transcriptase (Promega) following the manufacturer's instructions and a previously described protocol (Cooke et al., 2011). PCR reactions were performed under optimized conditions using a previously described protocol (Cooke et al., 2013) and the following specific sense/antisense primer pairs (synthesized by Integrated DNA Technologies) (Rozen and Skaletsky, 2000): *OxeR1:* 5′-CTCTTCATGCTGTCCACCAA-3′/5′-GAAGCCATG CCAAAGATGAT-3′ (amplicon size 449 bp); *StAR*: 5′-GGGG ACATTTAAGACGCAGA-3′/5′-CAGCCCTCTTGGTTGCTAAG-3′ (amplicon size 457 bp) and *L19* ribosomal protein, used as housekeeping gene (Chan et al., 1987): 5′-AGTATGCTCAGGCTTCAGAA-3′/5′-TTCCTTGGTCTTAGACCTGC-3′ (amplicon size 500 bp). The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification. Reaction products were resolved on 1.5% agarose gels (80 V) containing 0.5 μg/ml ethidium bromide to determine the molecular sizes of the *OxeR1*, *StAR* and *L19* amplicons. For comparisons of the amount of amplified *StAR* produced from different RNA samples, the amplified *L19* product of each sample was used as an internal standard. Gel images were acquired with a digital camera (Kodak Easyshare Z7121S). The levels of *StAR* and *L19* mRNA were quantitated using a computer-assisted image analyzer (Gel-Pro analyzer, IPS) and the PCR results for each sample were normalized by *L19* mRNA.

2.5. Western blotting

OxeR1, StAR, pERK1/2 and total ERK1/2 protein levels were analyzed in total cell lysates. The primary antibodies used were: OxeR1 rabbit polyclonal antibody (Cayman Chemical, Catalog No. 100025, Lot No. 197773, diluted 1/1000), StAR rabbit polyclonal antibody (Santa Cruz Biotechnologies, Catalog No. sc-25806, Lot No G1912, diluted 1/1500), rabbit polyclonal anti-pERK1/2 (Cell Signaling, Catalog No. 9101, Lot 28, diluted 1/4000), rabbit polyclonal anti-total ERK1/2 (Cell Signaling, Catalog No. 9102, Lot 23, diluted 1/4000), monoclonal anti-β-tubulin, clone AA2 (used as cell lysate loading control, Millipore, Catalog No. 05–661, Lot 2207268, diluted 1/4000). The secondary antibodies conjugated to peroxidase were goat anti-rabbit (BioRad, Catalog no. 170–6515, Control No. 350000928, diluted 1/4000) or goat anti-mouse immunoglobulin (BioRaD, Catalog No. 170–6516, Control No. 350002504, diluted 1/4000). The bound primary antibodies were detected using an enhanced chemiluminescence kit (Bio-Lumina, Kalium Technologies). Autoradiography gel images were acquired with a digital camera (Kodak Easyshare Z7121S). StAR and β-tubulin were quantitated using a computer-assisted image analyzer (Gel-Pro analyzer, IPS) and the results for each sample were normalized by β-tubulin.

2.6. Immunofluorescence analysis

H295R-empty and H295R-OxeR1 cells were grown to approximately 50% confluence on poly-D-lysine-coated cover glasses

(12 mm), fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.01% Triton X-100 for 10 min at 4 °C. After several washes with 1% PBS–0.05% Tween-20, cells were blocked with 1% albumin in PBS–0.05% Tween-20 for 60 min at room temperature. Cells were incubated with or without the specific primary antibody (OxeR1 rabbit polyclonal antibody, 1/500) overnight at 4 °C. After further washing, cells were incubated for 1 h at room temperature with the secondary goat anti-rabbit Cy2-conjugated antibody (1/400). Coverslips were mounted onto the slides using Fluorsave antifade reagent (Calbiochem) followed by examination using an Olympus FV300/BX6 laser-scanning confocal microscope.

2.7. Statistical analysis

All experiments were repeated independently at least three times. The figures show the results of representative gels. The data of steroid production are reported as means ± SEM of the three (or more) independent experiments. One-way ANOVA and Student–Newman–Kuels multiple comparison test were performed using GraphPad InStat software (GraphPad Software Inc.). Significant differences were defined as $P < 0.05$.

3. Results

3.1. OxeR1 expression and steroid production in MA-10 Leydig cells transfected with pRc/CMVi-OxeR1

First, we overexpressed OxeR1 in MA-10 rat Leydig cells by means of a transient transfection approach using a pRc/CMVi plasmid containing human OxeR1 cDNA (pRc/CMVi-OxeR1). Mock transfection was performed using the empty plasmid (pRc/CMVi-empty). Then, 24 h after transfection, OxeR1 protein levels were evaluated by Western blot in the cell lysates. As expected, OxeR1 migrated at a molecular weight of about 50 kDa in SDS–PAGE and, in basal non-stimulated conditions, transfection of the cDNA increased OxeR1 protein signal compared to endogenous expression (Fig. 1A). To analyze the effect of modifying OxeR1 protein levels on the final response of steroidogenic stimulation, we collected media from cells transfected with the constructs described earlier in basal and cAMP-stimulated conditions and determined steroid secretion. Overexpression of OxeR1 significantly increased cAMP-stimulated progesterone production compared to mock-transfected cells (Fig. 1B).

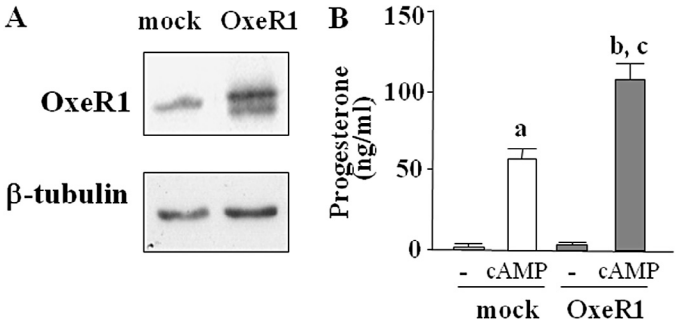

Fig. 1. OxeR1 expression and steroid production in MA-10 cells transfected with pRc/CMVi-OxeR1. (A) MA-10 cells were cultured and transiently transfected with an empty pRC/CMV (mock) or a pRC/CMV plasmid containing OxeR1 cDNA (OxeR1). Twenty-four hours after transfection, cells were collected and cell lysate proteins were analyzed by Western blot with specific antibodies for OxeR1 and β-tubulin (loading control). (B) Mock- or OxeR1-transfected MA-10 cells were cultured and incubated in serum-free medium in the presence or absence of 0.25 mM 8Br–cAMP for 1 h. Steroid production was evaluated by determining progesterone concentrations in the medium. Results are expressed in ng/ml as the mean ± SEM of four independent experiments. a, $P < 0.001$ vs. mock without cAMP; b, $P < 0.001$ vs. OxeR1 without cAMP; c, $P < 0.05$ vs. mock with cAMP.

3.2. OxeR1 expression in H295R adrenocortical cells transfected with pBABE-OxeR1

Next, we generated a human H295R adrenocortical cell line which stably expresses OxeR1 cDNA, named H295R-OxeR1 cells, by means of the transfection of a pBABE-OxeR1 plasmid followed by selection with hygromycin B. Mock transfection was performed using the empty plasmid (pBABE-empty). Then, OxeR1 expression was evaluated by RT-PCR, Western blot and immunocytochemistry. All analyses (RT-PCR in Fig. 2A, Western blot in Fig. 2B and immunocytochemistry in Fig. 2C) showed an increase in both OxeR1 mRNA and protein levels in H295R-OxeR1 cells as compared to H295R cells transfected with the empty plasmid, H295R-empty. Indeed, immunocytochemistry images analyzed by confocal microscopy showed a distribution similar to previously published data obtained from prostate cancer cells (Sarveswaran and Ghosh, 2013). The fluorescent signal appeared excluded from the nuclei and exhibited a punctuate appearance, with the strongest signal localized in the margin of the cell.

3.3. OxeR1 effects on H295R cell function: StAR expression and steroid production in H295R cells overexpressing OxeR1

As StAR abundance depends on the action of 5-LOX products, and our aim was testing the hypothesis that these AA metabolites act through the activation of the OxeR1 membrane receptor, we continued analyzing StAR protein levels in H295R-OxeR1 cells. The overexpression of OxeR1 significantly increased the expression of basal StAR mRNA, measured by RT-PCR, compared to mock-transfected cells (Fig. 3A). Consistent with the changes in mRNA levels, the basal expression of StAR protein, analyzed by Western blot, increased in the H295R-OxeR1 cell line compared to H295R-empty cells (Fig. 3B, lane 4 vs. 1).

In H295R cells, cholesterol transport across the mitochondrial membrane is regulated by three different stimuli: ACTH, mainly through the cAMP/protein kinase A system, and potassium and angiotensin II, which exert their actions through cAMP/PKA-independent pathways. Thus, we tested the effect of OxeR1 overexpression under both cAMP and angiotensin II stimulation. The stimulation of mock-transfected H295R cells with submaximal concentrations of 8Br–cAMP, permeable analog of the second messenger, or angiotensin II caused a widely described increase in StAR protein levels (Fig. 3B, lanes 2 and 3 vs. 1), and the same profile was obtained in H295R-OxeR1 cells (Fig. 3B, lanes 5 and 6 vs. 4). Moreover, the overexpression of OxeR1 further increased the expression of StAR protein in cAMP- and angiotensin II-treated H295R-OxeR1 cells as compared to mock-transfected cells (Fig. 3B, lanes 5 and 6 vs. 2 and 3).

Finally, we tested whether this receptor was involved in the activation of steroid production. For that purpose, we determined steroid production in the media of the cell cultures treated as described in the previous paragraph. Mock-transfected H295R cells increased progesterone production after treatment with both 8Br–cAMP and angiotensin II at submaximal concentrations (Fig. 4, white bars). The involvement of OxeR1 in cAMP- and angiotensin II-dependent pathways was evidenced by an increase in progesterone concentrations produced by cells overexpressing OxeR1 as compared to mock-transfected cells (Fig. 4, gray bars).

3.4. ERK1/2 phosphorylation in H295R cells overexpressing OxeR1

ERK1/2 phosphorylation has been described as a signal transduction pathway activated downstream OxeR1 activation in other

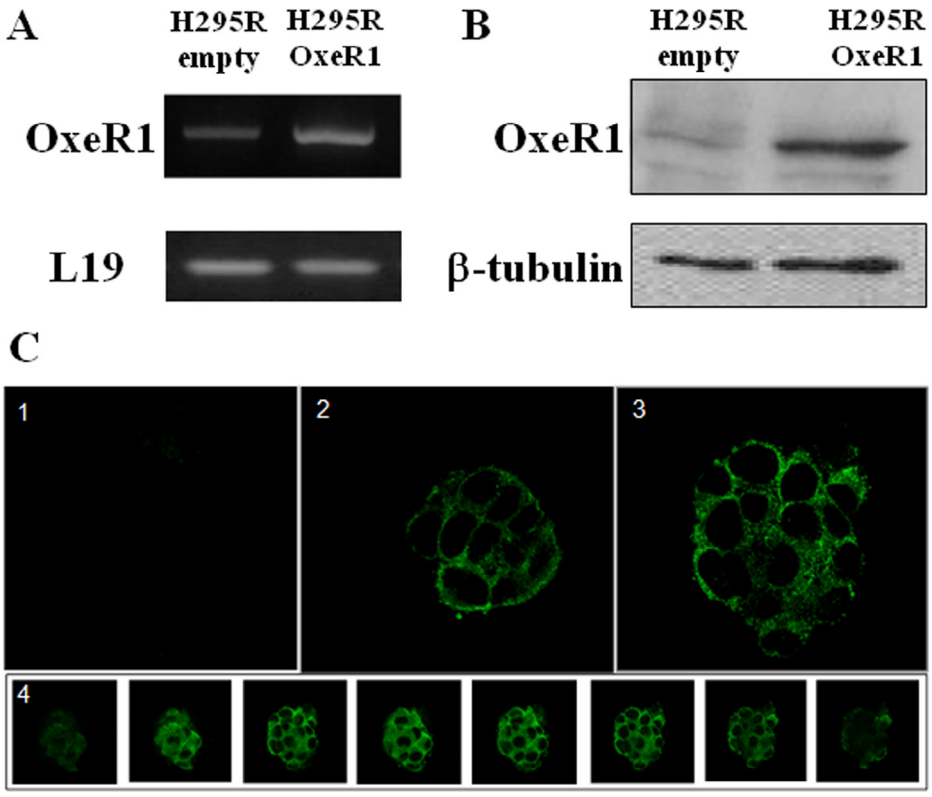

Fig. 2. OxeR1 expression in H295R transfected with pBABE-OxeR1. H295R cells were transfected with an empty pBABE or a pBABE plasmid containing OxeR1 cDNA. After selection with hygromycin B, the resulting cells, H295R-empty and H295R-OxeR1, were cultured and cell lysates were obtained for analyses. (A) Total RNA was isolated and used in semiquantitative RT-PCR with specific primers for *OxeR1* cDNA and *L19* cDNA. PCR products were resolved in ethidium bromide-stained agarose gels. (B) Cell lysate proteins were studied by Western blot with antibodies that specifically recognize OxeR1 and β-tubulin (loading control). (C) Cells were cultured in poly-D-lysine-coated coverslips and immunocytochemical assays were conducted with the antibodies that specifically recognize OxeR1. 1: background signal in the absence of antibody; 2: H295R-empty cells; 3 and 4: H295R-OxeR1 cells; 4: different planes of 3 at 1-μm increment from the bottom.

cell types. Thus, to initiate the characterization of the mechanism triggered by the activation of OxeR1 in steroidogenic cells, we determined the effect of agonist 5-oxo-ETE on the phosphorylation state of ERK1/2 in H295R cells. A time-dependent increase was observed in ERK1/2 phosphorylation, with a peak at 5 min of 5-oxo-ETE action on H295R-empty cells (Fig. 5). Cells overexpressing OxeR1 showed a similar profile with higher activity of ERK1/2 at all times tested (Fig. 5).

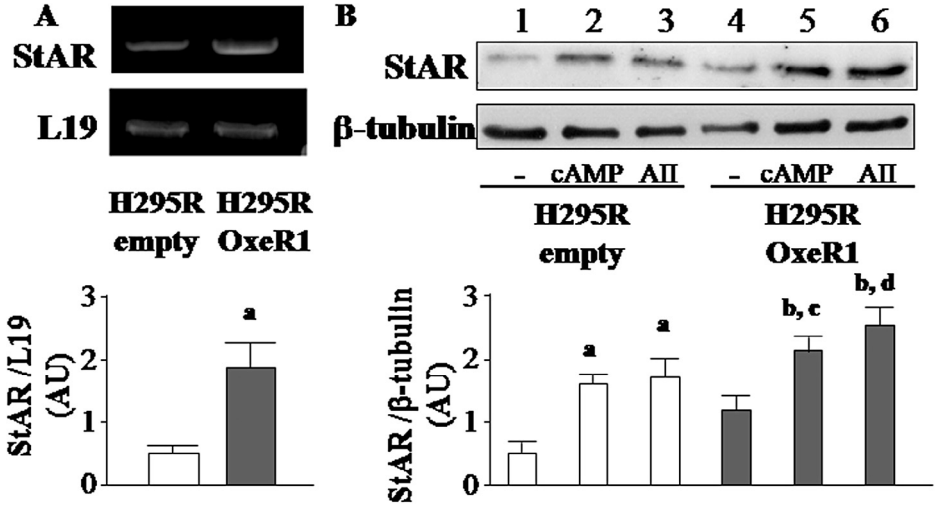

Fig. 3. StAR expression in H295R cells overexpressing OxeR1. (A) Total RNA was isolated from cultured H295R-empty and H295R-OxeR1 cells and used in semiquantitative RT-PCR with specific primers for *StAR* and *L19* cDNAs. PCR products were resolved in ethidium bromide-stained agarose gels. a, $P < 0.01$ vs. H295R-empty. (B) Cultured H295R-empty and H295R-OxeR1 cells were incubated in serum-free medium in the presence or absence of 5.10^{-4} M 8Br–cAMP (cAMP) or 10^{-8} M angiotensin II (AII) for 6 h. Cells were collected and cell lysate proteins were analyzed by Western blot with antibodies that specifically recognize StAR and β-tubulin. a, $P < 0.01$ vs. H295R-empty alone; b, $P < 0.01$ vs. H295R-OxeR1 alone; c, $P < 0.05$ vs. H295R-empty with 8Br–cAMP; d, $P < 0.01$ vs. H295R-empty with AII.

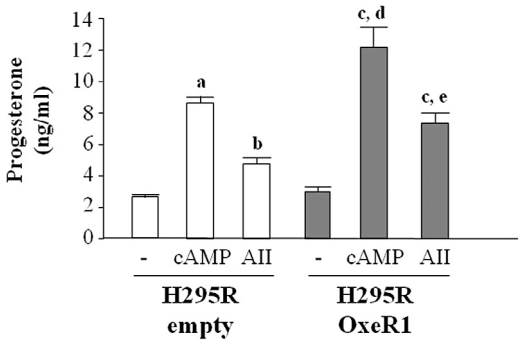

Fig. 4. Steroid production in H295R cells overexpressing OxeR1. H295R-empty and H295R-OxeR1 cells were cultured and incubated in serum-free medium in the presence or absence of 5.10^{-4} M 8Br–cAMP (cAMP) or 10^{-8} M angiotensin II (AII) for 6 h. Steroid production was evaluated by determining progesterone concentrations in the culture medium. Results are expressed in ng/ml as the mean ± SEM of four independent experiments. a, $P < 0.001$ vs. H295R-empty alone; b, $P < 0.05$ vs. H295R-empty alone; c, $P < 0.001$ vs. H295R-OxeR1 alone; d, $P < 0.01$ vs. H295R-empty with 8Br–cAMP; e, $P < 0.01$ vs. H295R-empty with AII.

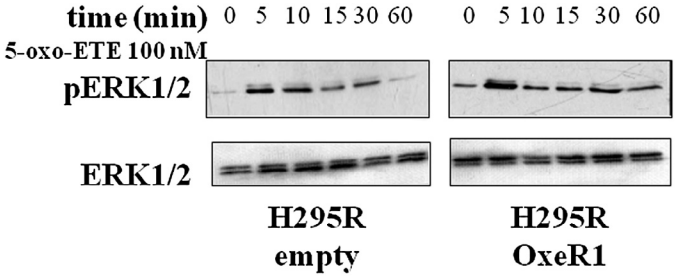

Fig. 5. ERK1/2 phosphorylation under OxeR1 activation in H295R cells. H295R-empty and H295R-OxeR1 cells were cultured and incubated in serum-free medium for 24 h, previous to treatment with 100 nM 5-oxo-ETE for the indicated times. Cells were collected and cell lysate proteins were used to analyze ERK1/2 phosphorylation by Western blot with specific antibodies that recognize total and phosphorylated forms of ERK1/2.

3.5. Effect of ERK1/2 inhibition on 5-oxo-ETE-stimulated StAR protein induction

In order to analyze the relationship between 5-oxo-ETE/OxeR1-dependent ERK1/2 activation and StAR induction, we stimulated H295R cells with 100 nM 5-oxo-ETE in the presence of PD98059, an inhibitor of ERK1/2 activation. Stimulation with 5-oxo-ETE caused a onefold (1.0 ± 0.2) increase in pERK phosphorylation compared to non-stimulated cells (Fig. 6A). This phosphorylation was decreased in a 36 ± 4% by PD98059, reaching values statistically not different from control cells. Thus, PD98059 indeed decreased the degree of MAPK phosphorylation produced by 5 min of stimulation with agonist 5-oxo-ETE.

StAR protein levels in cells stimulated with 5-oxo-ETE were 0.56 ± 0.04 times higher than in control cells (Fig. 6B). This induction decreased by 25 ± 6% when the stimulation was performed in the presence of PD98059. Values obtained in cells stimulated with 5-oxo-ETE and PD98059 were not different from the control. Thus, as already shown (Cooke et al., 2013), treatment with 5-oxo-ETE during 6 h increased StAR protein expression as compared to non-stimulated cells, while treatment with the inhibitor of ERK1/2 activation decreased such induction (Fig. 6B).

4. Discussion

Based on previous findings showing (i) the essential role of 5-LOX derivatives of AA in the acute increase in steroidogenesis (Cooke, 1999; Mele et al., 1997; Solano et al., 1987; Wang et al., 2000, 2003b), (ii) the existence of OxeR1, typical seven transmembrane GPCR for 5-oxo-ETE, 5-HpETE and 5-HETE (Hosoi et al., 2002; Jones et al., 2003), (iii) the presence of OxeR1 in human adrenocortical cells (Cooke et al., 2013), and (iv) the pharmacological evidence of its involvement in the activation of steroidogenesis triggered by cAMP (Cooke et al., 2013), this study further tested the hypothesis that 5-LOX derivatives of AA may signal through their own receptor in regulating steroidogenesis activated by cAMP-dependent and independent pathways. To such end, we used a plasmid-mediated gene transfer to increase the expression of OxeR1 in rat Leydig cells of the MA-10 cell line and in human adrenocortical cells of the H295R cell line. In summary, the results obtained in this study indicate that: (i) membrane receptor OxeR1 participates in the regulation of StAR protein induction and steroidogenesis after the activation of

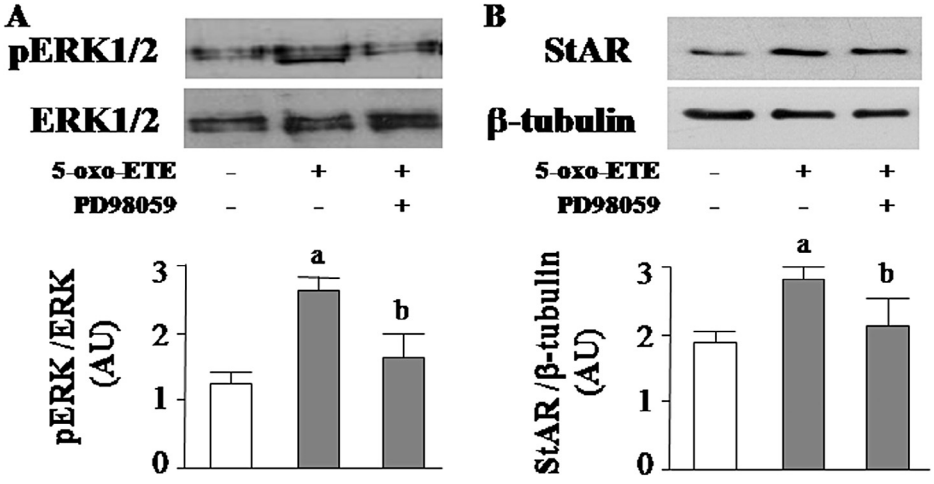

Fig. 6. StAR induction triggered by OxeR1 activation under inhibition of ERK1/2. H295R cells were cultured and incubated in serum-free medium for 24 h, previous to treatment with 100 nM 5-oxo-ETE for 5 min (panel A) or 6 h (panel B). Cells were collected and cell lysate proteins were used for analyses. (A) ERK1/2 phosphorylation was studied by Western blot with specific antibodies that recognize total and phosphorylated forms of ERK1/2. a, $P < 0.01$ vs. control cells; b, $P < 0.01$ vs. 5-oxo-ETE alone. (B) StAR protein and β-tubulin levels were studied by Western blot with specific antibodies. a, $P < 0.05$ vs. control cells; b, $P < 0.05$ vs. 5-oxo-ETE alone.

steroidogenic cells, (ii) the participation of OxeR1 is shared by stimuli acting through different signal transduction pathways in the regulatory mechanism of steroidogenesis, and (iii) there is activation of the ERK1/2 pathway downstream OxeR1 activation.

The transfection of OxeR1 cDNA in mouse MA-10 Leydig cells caused an increase in OxeR1 protein signal as compared to endogenous expression and a double band in the transfected lane in Fig. 1. Although the analysis of this finding is outside the scope of this project, it can be speculated that differences from endogenous expression might be caused by the translation of the transfected cDNA, as the transfected cells are MA-10 of mouse origin, while the transfected plasmid contains OxeR1 cDNA of human origin.

8Br-, dibutyryl- and monobutyryl-cAMP are cell-permeant cAMP derivatives, widely used as stimulators of cAMP/PKA dependent pathways. According to a previous report (Samandari et al., 2007), H295R cells do not express high levels of the ACTH receptor and, accordingly, steroidogenesis may not be stimulated by ACTH. By contrast, stimulation with cAMP permeant analogs directly increases steroid production, indicating that cAMP signaling is intact. In this same cell line, the AT1 receptor exhibits high expression and angiotensin II has been proven to stimulate the steroidogenic response (Samandari et al., 2007).

The transfection of OxeR1 cDNA caused changes in StAR protein levels and steroidogenesis. The results obtained in this study are in agreement with our previous analysis using a pharmacological approach. In the basal state, the increase in OxeR1 expression caused an increase in StAR expression without changes in steroidogenesis, which had also happened previously in the pharmacological analysis when cells were stimulated with 5-oxo-ETE. A possible explanation may be that the activation by 5-oxo-ETE and the increase in OxeR1 expression both trigger, by themselves, StAR protein induction but not the activation of kinases necessary for the post-translational modifications of this mitochondrial protein. It is known that StAR protein has to be present and phosphorylated by PKA (Arakane et al., 1997) and ERK1/2 (Poderoso et al., 2008) to increase cholesterol transport through the mitochondrial membranes. Thus, the stimulation of cells overexpressing OxeR1 is needed to trigger post-translational modifications of StAR, causing its activation, and increased steroidogenesis compared to mock-transfected cells. In H295R cells, this effect is observed under cAMP and angiotensin II stimulation: while cAMP activates the PKA cascade, angiotensin II triggers the PKC signal transduction pathway. In both cases, common intermediates and enzymes lead to AA release, metabolism (Mele et al., 2012) and production of 5-LOX metabolites (Nadler et al., 1987). Thus, OxeR1 might be activated by 5-LOX metabolites produced through cAMP- dependent and independent pathways.

Our previous pharmacological analysis included the decrease in OxeR1 function. We used docosahexaenoic acid (DHA) as an antagonist of the receptor, a compound which was characterized in the original description of OxeR1 (Hosoi et al., 2002). DHA decreased 8Br–cAMP-induced StAR protein levels by about 45% and steroidogenesis by about 25% in H295R cells (Cooke et al., 2013). This antagonist was also tested in mouse MA-10 Leydig cells, again rendering a reduction in cAMP-stimulated StAR protein induction and steroidogenesis (Cooke et al., 2013). These results, together with the present report, support the hypothesis that OxeR1 is activated after stimulation of the cells.

The involvement of a membrane receptor has been also described in the action of other metabolites of AA on steroidogenesis. The cyclooxygenase-2 appears to be responsible for a tonic inhibition of StAR gene expression and steroid production (Wang et al., 2003a), while thromboxane A2, an AA metabolite generated by cyclooxygenase-2 and thromboxane A synthase, has an inhibitory effect on steroidogenesis (Wang et al., 2008). The existence of a receptor for thromboxane A2 suggests an autocrine loop involving cyclooxygenase-2, thromboxane A synthase and thromboxane A2 and its receptor in cyclooxygenase-2-dependent inhibition of the StAR gene (Pandey et al., 2009).

The 5-LOX derivatives of AA play an important role in the inflammatory and immune response as they activate polymorphonuclear neutrophils (O'Flaherty et al., 2005) and neutrophil and eosinophil chemotaxis (Powell et al., 1993, 1995). These metabolites also affect growth and survival of a variety of cancer cells (Bishayee and Khuda-Bukhsh, 2013; Ghosh and Myers, 1997, 1998). Early descriptions of the effects of 5-LOX products reported that responses were sensitive to pertussis toxin, suggesting that the effects were mediated by a Gαi-coupled GPCR (O'Flaherty et al., 1998). Indeed, these assumptions were confirmed once the OxeR1 was cloned (Hosoi et al., 2002, 2005; Jones et al., 2003), although Gαi activation on its own is not sufficient to explain chemotaxis, as cell migration is initiated only after the liberation of the Gβγ heterodimer (Neptune et al., 1999).

A biased OxeR1 antagonist, named Gue1654, has been recently identified and proven to inhibit Gβγ but not Gα signaling triggered by OxeR1 activation (Blättermann et al., 2012). Moreover, a further characterization of this compound has thoroughly discussed which of these GPCR-coupled signaling pathways – Gαi, Gβγ or β-arrestin – are truly important for chemoattractant functions in leukocytes (Konya et al., 2014).

Additionally, the signal transduction pathway triggered by OxeR1 downstream the activation of G proteins is scarcely described. It has been postulated that survival-promoting effects of 5-LOX metabolites in prostate cancer cells are exerted by the activation of OxeR1 signaling through PKCε, a pro-survival serine/threonine kinase, via an indirect mechanism (Sarveswaran et al., 2011). Pertussis toxin has been proven to only partially inhibit respiratory burst in granulocytes induced by 5-oxo-ETE, which suggests that the action of OxeR1 relies not only on the activation of Gαi but also on the action of other components of the GPCR system. The OxeR1 signaling pathway, as a member of the seven transmembrane GPCR family, may comprise another component, the β-arrestins, which are only now being extensively analyzed (Shenoy and Lefkowitz, 2011). In this aspect, β-arrestin-dependent, G protein-independent activation of ERK1/2 has been described after β2-adrenergic receptor activation (Shenoy et al., 2006). OxeR1 signal may then include such a G protein-independent pathway, as Gue1654 blocks β-arrestin2 recruitment in cells overexpressing OxeR1 and pertussis toxin-insensitive ERK1/2 phosphorylation in human eosinophils and neutrophils (Konya et al., 2014). In line with this observation, our study shows that, in steroidogenic cells, ERK1/2 phosphorylation occurs shortly after OxeR1 activation by 5-oxo-ETE. Moreover, the inhibition of this MAPK activation decreases the action of 5-oxo-ETE on StAR protein induction, relating the OxeR1 signal transduction pathway with this key regulatory protein in the acute stimulation of steroidogenesis.

Although no rodent ortholog of OxeR1 has been detected to date (Cooke et al., 2013), the fact that pharmacological inhibition and activation, as well as the overexpression of OxeR1, have functional consequences in murine MA-10 Leydig cells has led us to hypothesize about receptor involvement from a functional point of view. Additionally, we report the generation of a human steroidogenic cell line that stably overexpresses OxeR1, a useful tool to further study the signal transduction pathways involved in the cascade triggered by the activation of this membrane receptor.

In conclusion, the activation of steroidogenesis by cAMP-dependent and independent pathways involves signaling through the autocrine/paracrine loop of 5-LOX/5-eicosatetraenoic acid derivatives/OxeR1, in which 5-HpETE and/or 5-HETE and/or 5-oxo-ETE are released from steroidogenic cells and then bind to their receptors in the same or adjacent cells. The activated 5-oxo-ETE receptor induces downstream signaling which includes, at least, ERK1/2 activation.

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