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Expression and function of OXE receptor, an eicosanoid receptor, in steroidogenic cells

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ABSTRACT

Hormonal regulation of steroidogenesis involves arachidonic acid (AA) metabolism through the 5-lipoxygenase pathway. One of the products, 5-hydroperoxy-eicosatetraenoic acid (5-HpETE), acts as a modulator of the activity of the steroidogenic acute regulatory (StAR) protein promoter. Besides, an oxoeicosanoid receptor of the leukotriene receptor family named OXE-R is a membrane protein with high affinity and response to 5-HpETE, among other AA derivatives. The aim of our work was to elucidate whether this receptor may be involved in steroidogenesis. RT-PCR and western blot analysis demonstrated the presence of the mRNA and protein of the receptor in human H295R adrenocortical cells. The treatment of H295R or MA-10 cells (murine Leydig cell line) with 8Br-cAMP together with docosahexaenoic acid (DHA, an antagonist of the receptor) partially reduced StAR induction and steroidogenesis. On the contrary, 5-oxo-ETE – the prototypical agonist, with higher affinity and potency on the receptor – increased cAMP-dependent steroid production, StAR mRNA and protein levels. These results lead us to conclude that AA might modulate StAR induction and steroidogenesis, at least in part, through 5-HpETE production and activation of a membrane receptor, such as the OXE-R.

1. Introduction

In the acute phase of the stimulation of steroidogenesis, hormones such as ACTH and LH acting through a cAMP/PKA signaling pathway promote the transport of free cholesterol to the inner mitochondrial membrane. The steroidogenic acute regulatory (StAR) protein has a critical role in this cholesterol transfer (Clark et al., 1994; Lin et al., 1995). Several reports emphasize that arachidonic acid (AA) plays an essential role in the regulatory process (Maloberti et al., 2002; Wang et al., 2000). The stimulation of adrenal cells by ACTH (Solano et al., 1988) as well as Leydig cells by hCG (Moraga et al., 1997) stimulate AA release in a dose–response and time-dependent manner.

AA can be converted (Brash, 2001) to prostaglandins and thromboxanes (Needleman et al., 1986), to hydroxyeicosatetraenoic acids (Spector et al., 1988) and leukotrienes (Lewis and Austen, 1984), or to epoxyeicosatrienoic acids (Fitzpatrick and Murphy, 1988; Xu et al., 2011) via the cyclooxygenase, lipoxygenase, or cytochrome P450 pathways, respectively, depending on the cell type (Brash, 2001). The products of these reactions are generally released from the cell and exert their effects in an autocrine or paracrine fashion (Sala et al., 2010).

ACTH and LH/CG, also acting through a cAMP – dependent pathway rapidly increase StAR mRNA and protein levels. Several factors regulate StAR transcription (Manna et al., 2009; Stocco et al., 2001). Indeed, AA mediates its action on steroidogenesis through the regulation of StAR: inhibition of AA release decreased StAR gene expression and concomitant steroid production (Cornejo Maciel et al., 2005; Maloberti et al., 2005; Wang et al., 2000, 2002). Importantly, the inhibitory effects were reversed by addition of exogenous AA to the cell culture (Cornejo Maciel et al., 2005; Maloberti et al., 2005; Wang et al., 2000). The eicosatetraenoic acid derivatives of AA, 5-HpETE and 5-HETE, have stimulatory effects on StAR gene expression (Wang et al., 2003). The AA-responsive region is located at the −67/−96 region of the StAR promoter DNA.

In steroidogenic cells, the mobilization and metabolism of AA involves two enzymes, an acyl-CoA synthetase, named Acsl4, located in the mitochondrial associated membranes, and a mitochondrial acyl-CoA thioesterase named Acot2 (Finkielstein et al., 1998). Acsl4 levels are rapidly increased after hormone action, in cAMP dependent and independent fashion (Cornejo Maciel et al.,

Abbreviations: OXE-R, oxoeicosanoid receptor; *OXER1*, oxoeicosanoid receptor gene; AA, arachidonic acid; StAR, steroidogenic acute regulatory protein; 5-LOX, 5-lipoxygenase; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HpETE, 5-hydroperoxyeicosatetraenoic acid; 5-oxo-ETE, 5-oxoeicosatetraenoic acid; DHA, docosahexaenoic acid; GPCR, G-protein coupled receptor.

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2005; Mele et al., 2012). The result of the concerted action of Acsl4 and Acot2 is the release of AA in a compartmentalized fashion, directing the metabolism to the lipoxygenase (LOX) pathway (Maloberti et al., 2002). Products of the LOX pathway have been demonstrated to modulate steroidogenesis in the adrenal cortex (Antonipillai et al., 1988; Hirai et al., 1985; Jones et al., 1987; Kojima et al., 1985; Nishikawa et al., 1994; Omura et al., 1990; Shibata and Kojima, 1991; Solano et al., 1987), ovaries (Milvae et al., 1986, 1984; Reich et al., 1983; Van Voorhis et al., 1993) and testes (Cooke, 1999; Dix et al., 1984; Majercik and Puett, 1991; Mele et al., 1997; Reddy et al., 1993). Several reports indicate that the initial products of the lipoxygenase pathway, the eicosatetraenoic acids HpETEs and HETEs, may be involved. Their formation and effects on steroidogenesis have been reported in rat testis (Reddy et al., 1992, 1993) and MA-10 Leydig cells (Majercik and Puett, 1991) and in adrenal cells (Antonipillai et al., 1988; Hirai et al., 1985; Shibata and Kojima, 1991). In adrenal cells, there is an ACTH-stimulated 5-HpETE production and inhibition of the 5-LOX pathway inhibited ACTH-stimulated steroid biosynthesis. Similarly, Leydig cells generate 5-HpETE and 5-HETE in a cAMP-dependent fashion. However, the mechanisms of action of the products of this pathway have not been elucidated.

Although the most familiar products of the metabolism of AA by the actions of 5-LOX are the leukotrienes, another metabolite of AA associated with the 5-LOX pathway is the 5-oxo-ETE (Grant et al., 2009). The major route of production of 5-oxo-ETE is from the precursor 5-HETE by the actions of a specific dehydrogenase. The eicosanoid 5-oxo-ETE has recently been identified as the ligand for the oxoeicosanoid receptor. This is a G-protein coupled receptor (GPCR) of the leukotriene receptor subfamily, activated by the ligands 5-oxo-ETE, 5-HpETE and 5-HETE (Hosoi et al., 2002; Jones et al., 2003) and named oxoeicosanoid receptor (OXE-R) (Brink et al., 2004). The gene encoding OXE-R, named *OXER*1, is expressed in various human tissues except the brain, with preferential expression in peripheral leukocyte, lung, spleen, liver, and kidney, although somewhat less intense signals were observed in mRNA from adrenal, placenta, ovary and testis (Hosoi et al., 2002). Although *OXER*1 has been described in many species (*Bos taurus*, *Pan troglodytes, Pan paniscus, Pongo abelii, Macaca mulatta, Callithrix jacchus, Otolemur garnettii, Canis lupus familiaris* and *Sarcophilus harrisii),* there are no reports describing the gene or protein in mice or rats.

In this study we tested whether the OXE receptor is present in steroidogenic cells and whether this receptor is involved in the action of lipoxygenated products of AA metabolism on the acute regulation of steroidogenesis. Here, we report the expression of 5-oxo-ETE receptor in adrenocortical human cells. We also show that the activation of the receptor by the agonist 5-oxo-ETE, as well as its inhibition by the antagonist docosahexaenoic acid (DHA) are reflected in the levels of StAR mRNA and protein and in the rate of steroid production, suggesting that metabolism of AA by 5-LOX promotes StAR protein induction and steroidogenesis signaling through a membrane receptor, the OXE receptor.

2. Materials and methods

2.1. Cell cultures and treatments

H295R adrenocortical cells (Gazdar et al., 1990; Rainey et al., 2004) were purchased to the ATCC and cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12) (Invitrogen) supplemented with 5% bovine Cosmic calf serum (HyClone), 1% ITS + 1 (Sigma), 200 UI/ml penicillin and 200 μg/ml streptomicin sulphate (Invitrogen) at 37 °C and 5% CO_2.

MA-10 mouse Leydig tumor 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).

The cell cultures were treated with or without 8Br-cAMP at the submaximal concentration of 0.5 mM. The stimulations were performed in the presence and absence of the OXE-R agonist 5-oxo-ETE (Santa Cruz) or in the presence and absence of the OXE-R antagonist, docosahexaenoic acid (DHA) (Tocris), in a serum-free medium. The concentrations of 5-oxo-ETE and DHA, as well as the length of the treatments, are indicated in the figures or in the text. At the end of all the experiments, progesterone or aldosterone

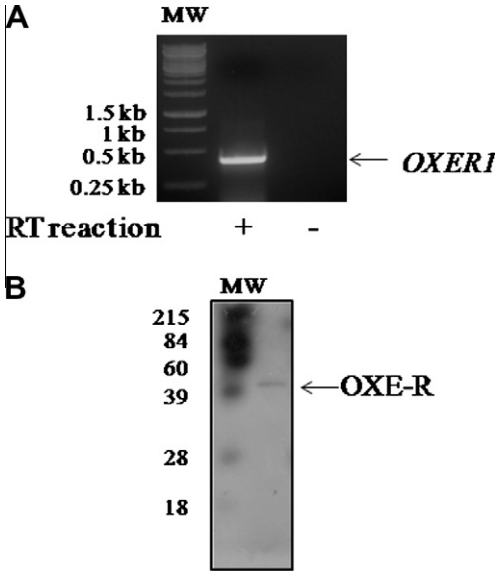

Fig. 1. Expression of the OXE receptor in human steroidogenic H295R adrenocortical cells. (A) RT reaction was performed on DNase-digested RNA extracted from adrenocortical H295R cell cultures. PCR using specific primers for human *OXER*1 and *L*19 cDNA as loading control was performed. PCR products were resolved in ethidium bromide-stained agarose gels. Molecular weight standards are indicated on the margin. The figure shows a representative gel of three independent experiments. (B) Western blot analysis was performed on total cell lysates of human H295R adrenocortical cells. Western blot was developed with a commercially available specific antibody against OXE-R. Molecular weight standards are indicated on the margin. The figure shows a representative immunoblot of three independent experiments.

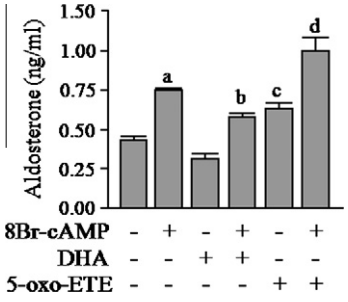

Fig. 2. Effect of DHA and 5-oxo-ETE on steroidogenesis in human H295R adrenocortical cells. H295R cells were cultured and incubated in a serum-free medium in the presence or absence of 50 μM DHA or 500 nM 5-oxo-ETE. Stimulation was performed with 0.5 mM 8Br-cAMP for 3 h. Steroid production was evaluated by determining aldosterone concentrations in the medium. The results are expressed in ng/ml as the means ± SEM of four independent experiments. a, $P < 0.001$ vs. non-treated cells; b, $P < 0.05$ vs. 8Br-cAMP-treated cells; c, $P < 0.05$ vs. non-treated cells; d, $P < 0.001$ vs. 8Br-cAMP-treated cells.

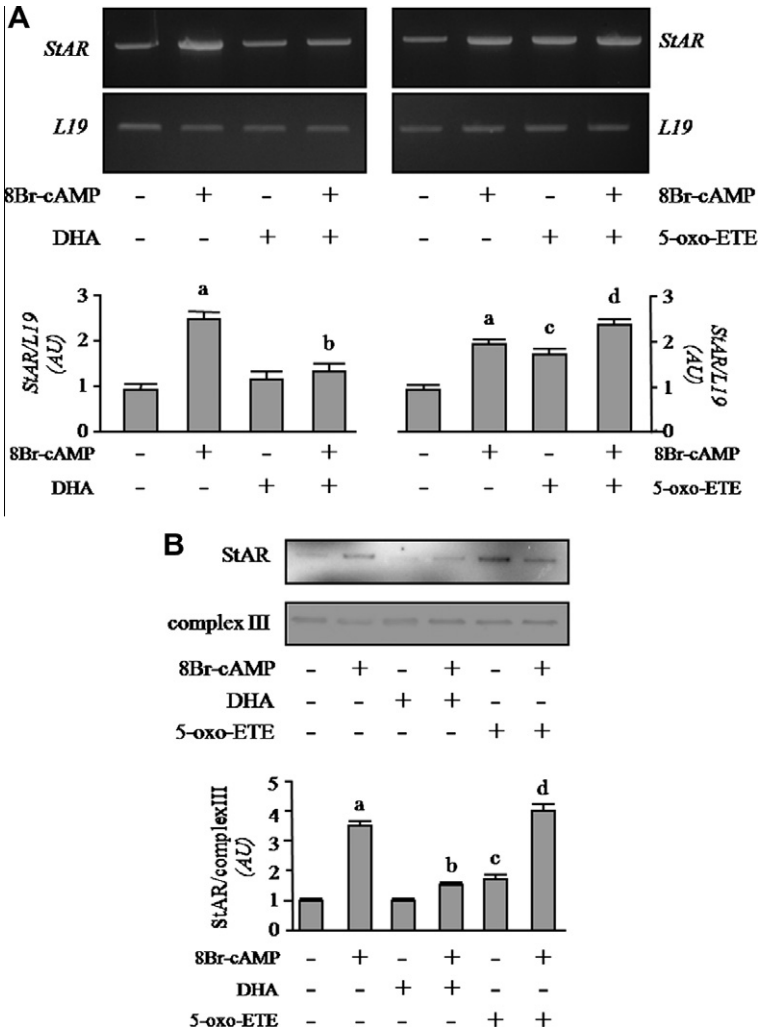

Fig. 3. Effect of DHA and 5-oxo-ETE on StAR protein expression in human H295R adrenocortical cells. H295R cells were cultured and incubated in a serum-free medium in the presence or absence of 50 μM DHA or 500 nM 5-oxo-ETE. Stimulation was performed with 0.5 mM 8Br-cAMP for 3 h. (A) Total RNA was isolated and used in semiquantitative RT-PCR with specific primers for human *StAR* cDNA and *L*19 cDNA as loading control. PCR products were resolved in ethidium bromide-stained agarose gels. The figure shows a representative gel of three independent experiments. The integrated optical density of each band was quantitated. *StAR* values were normalized against the corresponding *L*19 band and expressed as arbitrary units (AUs). The results are expressed as the means ± SEM. a, $P < 0.001$ vs. non-treated cells; b, $P < 0.01$ vs. 8Br-cAMP treated cells; c, $P < 0.01$ vs. non-treated cells; d, $P < 0.05$ vs. 8Br-cAMP-treated cells. (B) Cells were collected and the cell lysate proteins were used to obtain the mitochondrial fraction in order to analyze StAR expression by western blot with specific antibodies. The figure shows a representative immunoblot of three independent experiments. The integrated optical density of each band was quantitated. StAR values were normalized against the corresponding mitochondrial complex III band and expressed as arbitrary units (AU). The results are expressed as the means ± SEM. a, $P < 0.001$ vs. non-treated cells; b, $P < 0.001$ vs. 8Br-cAMP-treated cells; c, $P < 0.05$ vs. non-treated cells; d, $P < 0.05$ vs. 8Br-cAMP-treated cells.

production was determined in the culture media by radioimmunoanalysis (Cornejo Maciel et al., 2001; Mele et al., 2012).

2.2. RNA isolation and semiquantitative RT-PCR

Total cell RNA from the different treatment groups was isolated by the guanidium thiocyanate –phenol–chloroform method using TriReagent® following the manufacturer's instructions (MRC Inc.), quantified spectrophotometrically (OD260/280), treated with DNase, and subjected to RT-PCR. Given that *OXER*1 is an intronless gene, previous to the reverse transcription, a DNAse treatment was performed to eliminate any genomic DNA contamination. RNA and DNase1 RNase-free DNase (Invitrogen) were combined in a ratio 1–10 μg RNA/μl DNase, and the mix was processed following the instructions of the manufacturer. cDNA was synthesized incubating 2 μg of extracted RNA with 0.5 μg of random primer hexamers (Promega) in a total volume of 15 μl at 70 °C for 5 min. Then, 10 μl of reverse transcription buffer containing 1.25 mM of each of all four dNTPs (Promega), 25 U RNase inhibitor RNAsin® (Promega) and 200 U MMLV reverse transcriptase (Promega) were added for further incubation at 37 °C for 60 min. cDNA generated was used to perform the amplification in a 25 μl reaction buffer containing 0.1 μg cDNA (1–2 μl), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 2.5 U of Taq polymerase (GoTaq Polymerase, Promega), and 50 pmol of each specific primer pairs. PCR reactions were performed under optimized conditions using the following primer pairs (synthesized by Integrated DNA Technologies). For amplification of *OXER*1, the sense primer 5′-ctcttcatgctgtccaccaa-3′ and the antisense primer 5′-gaagccatgccaaagatgat-3′ were used (amplicon size 449 bp) (Rozen and Skaletsky, 2000). For amplification of human *StAR*, the sense primer 5′-GGGGACATTTAAGACGCAGA-3′ and the antisense primer 5′-CAGCCCTCTTGGTTGCTAAG-3′ were used (amplicon size 457 bp) (Rozen and Skaletsky, 2000). For amplification of murine *StAR*, the sense primer 5′-GGGACGAAGTGCTAAGTAAGATGG-3′ and the antisense primer 5′-GGTCAATGTGGTGGACAGTCC-3′ were used (amplicon size 566 bp) (Cooke et al., 2011). *L*19 ribosomal

protein, used as housekeeping gene (Chan et al., 1987) was amplified in human samples using the sense primer 5′-AGTATGCTCAGGCTTCAGAA-3′ and the antisense primer 5′-ttccttggtcttagacctgc-3′ (amplicon size 500 bp) and in mouse samples using the sense primer 5′-GAAATCGCCAATGCCAACTC-3′ and the antisense primer 5′-TCTTAGACCTGCGAGCCTCA-3′ (amplicon size 405 bp). RT-PCR was conducted with a MultiGene™ Thermal Cycler (Labnet International, Inc.). PCR cycles were: for *OXER*1, 30 s at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C, and then 2.5 min at 72 °C; for human *StAR*, 5 min at 94 °C followed by 26 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C; for murine StAR, 5 min at 94 °C followed by 26 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C. Human L19 mRNA was concurrently assayed with the same RT and the following PCR cycle: 5 min at 94 °C, followed by 26 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. Murine L19 mRNA was concurrently assayed with the same RT and mouse StAR PCR cycle. In all cases, the PCR finished with a cycle at 72 °C for 10 min and holding at 4 °C. 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 of ethidium bromide to determine the molecular sizes of the *OXER*1, *StAR* and *L*19 amplicons. The gel images were acquired with a digital camera (Kodak Easyshare Z7121S). The levels of murine and human *StAR* and *L*19 mRNA were quantitated using a computer-assisted image analyzer (Gel-Pro analyzer, IPS) and the PCR results for each sample were normalized by *L*19 mRNA. Products were subjected to sequence analysis (Macrogen).

2.3. Western blotting

OXE-R protein levels were analyzed in a total cell lysate and StAR protein levels in the mitochondrial fraction. The primary antibodies used were: OXE-R rabbit polyclonal antibody (Cayman), rabbit antisera anti-StAR (generously provided by Dr. Doug Stocco), monoclonal anti-β-tubulin (used as cell lysate loading control, Millipore) and monoclonal anti-Ox-Phos complex III core 2 subunit (used as mitochondrial loading control, Invitrogen). The secondary antibodies were goat anti-rabbit or anti-mouse immunoglobulin conjugated to peroxidase. The bound primary antibodies were detected using an enhanced chemiluminescence kit (GE Healthcare). The autoradiography gel images were acquired with a digital camera (Kodak Easyshare Z7121S). The levels of StAR and complex III were quantitated using an image analyzer (Gel-Pro analyzer, IPS) and the results for each sample were normalized by complex III.

2.4. Statistical analysis

All experiments were run in duplicate and repeated independently at least three times. The data 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., San Diego, CA). Significant differences from compared value were defined as $P < 0.05$.

3. Results

3.1. Expression of the OXE receptor in the human steroidogenic H295R adrenocortical cells

In order to understand the mechanism of action of eicosanoids in steroidogenic cells, we wanted to know whether these cells express OXE-R. The expression of the OXE receptor was tested in the human adrenocortical H295R cells by means of mRNA detection by RT-PCR. Given that *OXER*1 is an intronless gene, genomic DNA contamination had to be specially considered. Thus, previous to the reverse transcription, a DNAse treatment was performed on RNA samples to eliminate any genomic DNA contamination. Fig. 1A shows the result of the electrophoretic analysis of the PCR products of a representative RT-PCR. The effectiveness of the DNAse digestion is confirmed by the fact that after the treatment with the enzyme and in the absence of reverse transcription, no fragment is amplified (Fig. 1A, lane RT−). The primer set specific for *OXER*1 amplified a fragment of the predicted size (Fig. 1A, lane RT+).

The expression of the OXE receptor at the protein level was tested by western blot (Fig. 1B). A commercially available specific antibody detected a positive band of approximately 46 kDa, coinciding with the predicted size of the OXE-R (accession number NP_683765.1), thereby confirming that human steroidogenic H295R cells express OXE-R.

3.2. Involvement of the OXE receptor in the activation of steroidogenesis and StAR protein expression in human H295R adrenocortical cells

Once the presence of the receptor was demonstrated in H295R cells, we tested whether the receptor is involved in the activation of steroid production. To that end, decreased or increased activity of the receptor was achieved by means of exogenous addition of an antagonist or agonist to the cell cultures and steroid concentration was determined in the culture media. H295R adrenocortical cells produce aldosterone under the stimulation of ACTH, angiotensin II or K^+, which act through different signaling cascades. We analyzed the involvement of OXE-R in the c-AMP-dependent pathway. The results are shown in Fig. 2. While treatment of H295R cells with DHA, an antagonist of the receptor, partially inhibited 8Br-cAMP-stimulated aldosterone production, 5-oxo-ETE – the most potent agonist of the receptor – increased steroid production. 5-oxo-ETE slightly but significantly increased basal aldosterone production, indicating that 5-oxo-ETE may have an action by itself. The concentration of progesterone, an intermediary steroid of the biosynthetic pathway, in the culture media rendered a similar profile (data not shown). These findings support our hypothesis that the steroidogenic-promoting effects of eicosanoids may be mediated through its receptor, OXE-R.

To characterize the mechanism by which the OXE-R is involved in steroid secretion, we determined the effect of DHA on the expression of StAR gene, involved in the acute steroidogenic re-

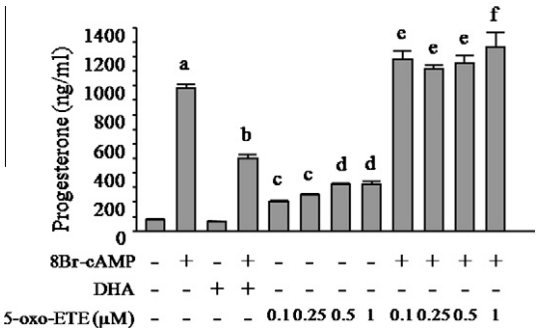

Fig. 4. Effect of DHA and 5-oxo-ETE on steroidogenesis in mouse MA-10 Leydig cells. MA-10 cells were cultured and incubated in serum-free medium in the presence or absence of 50 μM DHA or the indicated concentrations of 5-oxo-ETE. Stimulation was performed with 0.5 mM 8Br-cAMP for 1 h. Steroid production was evaluated by determining progesterone concentrations in the medium. The results are expressed in ng/ml as the means ± SEM of four independent experiments. a, $P < 0.001$ vs. non-treated cells; b, $P < 0.001$ vs. 8Br-cAMP-treated cells; c, $P < 0.05$ vs. non-treated cells; d, $P < 0.01$ vs. non-treated cells; e, $P < 0.05$ vs. 8Br-cAMP-treated cells; f, $P < 0.01$ vs. non-treated cells.

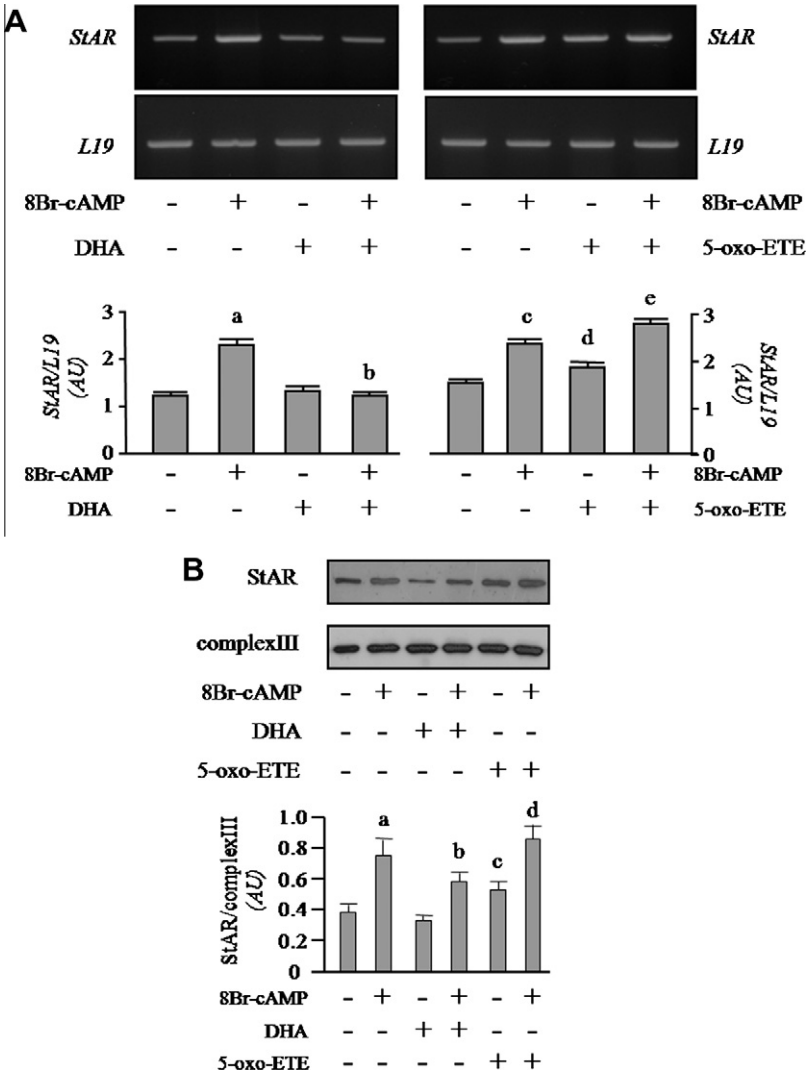

Fig. 5. Effect of DHA and 5-oxo-ETE on StAR protein expression in mouse MA-10 Leydig cells. MA-10 cells were cultured and incubated in a serum-free medium in the presence or absence of 50 μM DHA or 500 nM 5-oxo-ETE. Stimulation was performed with 0.5 mM 8Br-cAMP for 3 h. (A) Total RNA was isolated and used in semiquantitative RT-PCR with specific primers for *StAR* cDNA and *L*19 cDNA as loading control. PCR products were resolved in ethidium bromide-stained agarose gels. The figure shows a representative gel of three independent experiments. The integrated optical density of each band was quantitated. *StAR* values were normalized against the corresponding *L*19 band and expressed as arbitrary units (AU). The results are expressed as the means ± SEM. a, $P < 0.001$ vs. non-treated cells; b, $P < 0.001$ vs. 8Br-cAMP treated cells; c, $P < 0.01$ vs. non-treated cells; d, $P < 0.05$ vs. non-treated cells; e, $P < 0.05$ vs. cAMP-treated cells. (B) Cells were collected and the cell lysate proteins were used to obtain the mitochondrial fraction in order to analyze StAR expression by western blot with specific antibodies. The figure shows a representative immunoblot of three independent experiments. The integrated optical density of each band was quantitated. StAR values were normalized against the corresponding mitochondrial complex III band and expressed as arbitrary units (AU). The results are expressed as the means ± SEM. a, $P < 0.001$ vs. non-treated-cells; b, $P < 0.01$ vs. 8Br-cAMP-treated cells; c, $P < 0.05$ vs. non-treated cells; d, $P < 0.05$ vs. 8Br-cAMP-treated cells.

sponse. The relative abundance of StAR mRNA levels was determined by semiquantitative RT-PCR and StAR protein levels by western blot. StAR mRNA (Fig. 3A) and protein (Fig. 3B) levels where decreased in DHA-treated H295R cells. Next, we sought to determine whether 5-oxo-ETE has a causative role in increasing StAR expression. H295R cells were treated for 3 h with 0.5 mM 8Br-cAMP with or without 5-oxo-ETE and total RNA was isolated for semiquantitative analysis by RT-PCR. As shown in Fig. 3A, 5-oxo-ETE increased StAR mRNA levels by itself, an effect that is also observed in 8Br-cAMP-stimulated H295R cells. These effects were also observed when the protein levels were analyzed by western blot (Fig. 3B).

3.3. Effects of DHA and 5-oxo-ETE on MA-10 Leydig cell function

The study was extended to a second steroidogenic cell line. We used murine MA-10 Leydig cells that also respond through a cAMP-dependent pathway. The effects of DHA and 5-oxo-ETE on MA-10 Leydig cell function are shown in Figs. 4 and 5. Treatment of MA-10 cells with 0.5 mM 8Br-cAMP alone resulted in the already known increases in progesterone production (Fig. 4), StAR mRNA (Fig. 5A) and protein (Fig. 5B) levels. Treatment of the cell cultures with DHA significantly and partially decreased progesterone production (Fig. 4). This effect on steroidogenesis may be mediated by an action on StAR expression, since DHA inhibited cAMP-dependent StAR mRNA (Fig. 5A) and protein induction (Fig. 5B). Moreover, the inhibitory action of DHA could also be observed on cAMP-stimulated StAR promoter activity (data not shown).

To examine the role of the OXE-R in MA-10 Leydig cells, we performed a dose–response study on the effects of 5-oxo-ETE on steroid production by incubating cells with increasing concentrations of 5-oxo-ETE in medium containing 0.5 mM 8Br-cAMP. Fig. 4 shows that progesterone concentration in the incubation media increased at all concentrations tested after 1 h of cAMP action. The

effect could also be detected at the 3-h time point (data not shown). The agonist of the OXE-R also increased StAR mRNA (Fig. 5A) and protein levels (Fig. 5B), suggesting an effect on StAR gene expression.

4. Discussion

Based on previous findings about the essential role of 5-LOX derivatives of AA in the acute increase of steroidogenesis (Cooke, 1999; Mele et al., 1997; Solano et al., 1987; Wang et al., 2000, 2003), and published reports about the existence of typical seven transmembrane G-protein-coupled receptors for 5-oxo, 5-hydroperoxy and 5-hydroxyeicosatetraenoic acids (5-oxo-ETE, 5-HpETE and 5-HETE respectively) (Hosoi et al., 2002; Jones et al., 2003), we hypothesized that 5-LOX derivatives of AA may signal through their own receptor in regulating steroidogenesis.

We found that the oxoeicosanoid receptor, OXE-R, is present in the steroidogenic H295R adrenocortical cells of human origin. Using primer sets based on the published sequences of *OXER*1 and commercially available antibodies we were able to detect the *OXER*1 mRNA and protein in human H295R adrenocortical cells. Our data, for the first time, document the expression of OXE-R in steroidogenic cells. In order to evaluate the function of OXE-R, we developed a pharmacological approach, inhibiting or activating the receptor by means of docosahexaenoic acid (DHA) and 5-oxo-ETE respectively, and we extended the functional study to a second steroidogenic cell line, the MA-10 mouse Leydig cells. DHA, the inhibitor of the OXE-R, reduced StAR protein levels and steroidogenesis not only in the human steroidogenic adrenocortical cells but also in mouse MA-10 Leydig cells. The partial inhibition observed with DHA suggests that the action of the lipoxygenated products could be mediated at least in part by the activation of the OXE-R. In line with these results, 5-oxo-ETE, the prototypical and most potent agonist of the receptor, increased the expression of StAR, at the level of the protein and mRNA. Through the action on StAR protein levels, 5-oxo-ETE finally increased progesterone production in both cell lines. It is worth noting that, although 5-oxo-ETE by itself increased StAR mRNA and protein levels, it did not promote full steroidogenesis, possibly due to the lack of StAR protein phosphorylation. StAR protein has to be present and phosphorylated by PKA (Arakane et al., 1997) and ERK1/2 (Poderoso et al., 2008) to positively regulate cholesterol transport through the mitochondrial membranes. In order to reach full steroidogenesis, the stimulation of the cAMP/PKA cascade is necessary for the formation of an active mitochondrial protein complex including TSPO, PAP7, PKA, MEK and ERK proteins. This multi-protein interaction leads to the phosphorylation of StAR, a process essential for its full activity (Liu et al., 2003, 2006).

Collectively, these findings demonstrate for the first time that lipoxygenated metabolites of AA participate in the acute regulation of steroidogenesis through a mechanism that involves, at least in part, the autocrine or paracrine activation of an eicosanoid receptor, the OXE-R. This finding is in agreement with the identification of 5-HpETE and 5-HETE as two cAMP-induced AA metabolites, and the demonstration of their stimulatory effects on StAR gene expression and steroid hormone production. Moreover, AA enhanced the binding of nuclear protein(s) to the −67/−96 region of the StAR promoter DNA suggesting that the AA-responsive element is located within this DNA sequence (Wang et al., 2003).

However, the presence of the *OXER*1 mRNA and protein could not be detected in murine MA-10 cells. The different origins of the two cell lines may explain the negative result in the RT-PCR and western blot analysis. The mouse ortholog of the human *OXER*1 is a niacin receptor, Niacr1, with low similarity in the nucleotide and aminoacid sequences. A BLAST analysis of the human

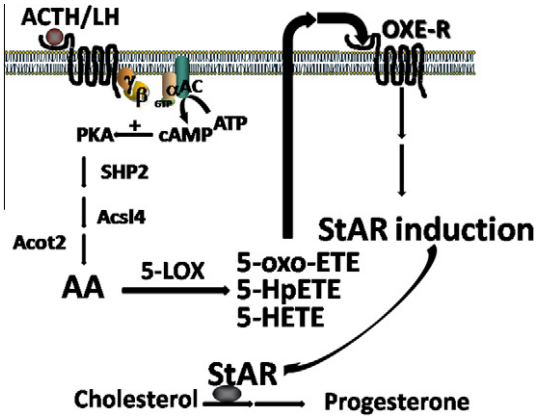

Fig. 6. Proposed model of the action of 5-LOX derivatives of AA on steroidogenesis. The figure depicts the cAMP/PKA dependent cascade initiated by the activation of the ACTH or LH/CG membrane receptors. It involves the activity of the tyrosine phosphatase SHP2 and the concerted action of Acsl4 and Acot2 to generate mitochondrial AA. The metabolism by 5-LOX would render 5-HETE, 5HpETE, and/or 5-oxo-ETE, which would be released from the cell and exert their effect in an autocrine or paracrine fashion on the OXE receptor to regulate StAR protein expression and steroidogenesis.

*OXER*1 nucleotide sequence compared with the mouse genomic and transcript sequences demonstrated similarity only with a very short fragment (query coverage 24%) of Gpr81. In our study, the primers designed to recognize the human *OXER*1 were used to amplify murine samples. We had negative results, and this is in agreement with the fact that the human sequence of the receptor demonstrates poor homology when compared to the sequence in the mouse genome. However, the fact that both, agonist and antagonist, clearly affect steroidogenesis and StAR protein and mRNA levels in murine cells at the same concentration as in human cells lead us to suggest the involvement of the receptor from a functional point of view. Further experiments, out of the scope of this study, should be performed to establish the molecular characterization of the murine receptor of oxoeicosanoids. Both, mouse Niacr1 and Gpr81 belong to the hydroxycarboxylic family of the G-protein-coupled receptors (GPCRs). Human HCA1, HCA2 and HCA3 – previously known as GPR81, GPR109A and GPR109B respectively – belong to the hydroxycarboxylic acid receptor family (formerly nicotinic acid receptor family) (Offermanns et al., 2011). OXE-R is one of the GPCRs most closely related to the HCA receptor family, sharing an arginine residue in a transmembrane domain, which would anchor the carboxylic group present in oxoeicosanoic acids as well as in HCA receptor ligands (Tunaru et al., 2005). Since OXE-R ligands may structurally resemble HCA receptor ligands, OXE-R, a receptor of the leukotriene family, for a polyunsaturated long chain carboxylic acid with an oxo, hydroxy or hydroperoxy substitution in the 5-position resembles HCA receptors also with regard to its agonistic ligand. OXE-R might well be regarded as another member of the hydroxycarboxylic acid receptor family (Ahmed et al., 2009).

5-oxo-ETE is a product of AA metabolism in a variety of inflammatory cells and can also be formed from 5-HETE by structural cells, possibly by transcellular biosynthesis. 5-oxo-ETE is a potent chemoattractant for eosinophils (Powell et al., 1995) and has similar effects on neutrophils, basophils and monocytes (Sturm et al., 2005). It elicits infiltration of eosinophils and, to a lesser extent, neutrophils into the skin after intradermal injection in humans. It also promotes the survival of tumor cells (Ghosh and Myers, 1998). Among other intracellular processes, phospholipase C activity, activation of PI3 K and phosphorylation of Akt and ERK1/2 appear to be involved in the chemoattractant effects of 5-oxo-ETE in

CHO cells transfected with the OXE-R (Hosoi et al., 2005). The biological actions of 5-oxo-ETE are mediated by the highly selective OXE receptor, which is expressed on a variety of inflammatory cells (Hosoi et al., 2002) as well as tumor cells (Sundaram and Ghosh, 2006). Although the pathophysiological functions of 5-oxo-ETE and its receptor are still not well understood, they may play important roles in asthma (Jones, 2005) and allergic diseases, cancer (O'Flaherty et al., 2005; Sundaram and Ghosh, 2006), and cardiovascular disease. Here we report for the first time not only the presence of OXE-R in a new cell type but also its involvement in an additional physiological function: OXE-R has a role in the regulation of steroid production stimulated through a cAMP-dependent pathway in adrenocortical and Leydig cells. Altogether, the findings included in this report suggest that eicosanoids exert their steroidogenic promoting effects through an OXE receptor-mediated pathway. Studing the downstream signaling mechanisms of OXE-R in the regulation of steroidogenesis remains a major challenge for future research. The proposed model is depicted in Fig. 6.

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