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Cloning and expression of the estrogen receptor-α (Esr1) from the Harderian gland of the sea turtle (*Lepidochelys olivacea*)

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ABSTRACT

The effects of estradiol on the Harderian gland (HG) are believed to be partially regulated by the transcriptional regulation of the estrogen-related genes via estrogen receptor (ER). In reptiles, however, it has not been well established whether the HG contains or expresses steroid nuclear receptors. As a first step toward investigating the molecular mechanisms of estrogen signalling in the HG, we isolated the cDNA for ERα in the sea turtle *Lepidochelys olivacea*. ERα was cloned using RT-PCR coupled with 5′ and 3′ RACE procedures. The cDNA contains a complete open reading frame encoding 588 amino acid residues. Comparative analysis of this amino acid sequence showed moderate to strong conservation of the ERα (Esr1) gene within divergent vertebrate groups. In transfection studies, the cloned ER displayed high affinity K_d = 0.25 nM and high specificity for 17β-estradiol. Binding assays using sucrose density gradients demonstrated a specific 7–7.5 S binding component in the HG cytosolic fractions. RT-qPCR analysis showed significant ERα mRNA expression in the liver, HG, lung and brain. Altogether, these results provide evidence for the expression of intracellular ERs in the HG of the sea turtle and suggest that ERα may be an important modulator of the estrogen-mediated response in the HG of reptiles.

1. Introduction

Over the last several years, various experimental reports have documented the effects of estrogen on the Harderian gland (HG). With the exception of a few species including the horse, cat, human, and bat, these tubulo-alveolar glands occur in all groups of vertebrates, from anuran amphibians to mammals (Sakai, 1981).

In reptiles, the HG is always present. Interestingly the chelonians have a well-developed HG that has a single duct (Cowan, 1969). In snakes and lizards, the HG is very large, and its secretions are directed into the vomero-nasal organ of Jackobson; hence, its function is thought to involve corneal lubrication and vomerolfaction (Kennedy, 1970; Rehorek, 1997). Nonetheless, the activity of this gland has been linked to other physiological processes, such as pheromone production, orbital lubrication, immunoglobulin secretion, steroid biosynthesis, and modulation of reproductive function (Olcese and Wesche, 1989; Payne, 1994; Vilchis et al., 2002). Moreover, the presence of "salt-secreting cells" in the glandular epithelium of some turtle species (*Malaclemys* and *Pseudemys* for example) has suggested that this organ plays a prominent role in osmoregulation (Cowan, 1969; Chieffi-Baccari et al., 1996).

Previous reports have indicated that sex steroids are involved in the differentiation, growth, and function of the HG. The role of estrogen in the histological changes of the gland was originally noticed after estrogenic treatment of gonadectomised hamsters (Woolley and Worley, 1954). Subsequent observations indicated that the ovarian hormones are required to maintain its structure and secretory activity (Hoffman, 1971). There are also reports that the cyclical changes that occur within the HG correspond with the oestrous cycle, pregnancy and lactation (Payne et al., 1979; Vilchis et al., 2006). In this same species, the glandular expression of a hormone-induced gene (FGH22) appears to be up-regulated by estrogen (Varriale et al., 1996).

Autoradiographic studies performed with armadillos indicated a specific nuclear uptake of [H^3]17β-estradiol (17β-E_2) by the periductal mucosal cells of the mucus-secreting lobules in the HG (Weaker et al., 1983). In birds, estrogen replacement induces mitosis and alters the secretions and cellular composition of the male and female glands (Gupta and Maiti, 1983), while estrogen may be associated with certain dimorphic HG features in amphibians and reptiles (Varriale and Chieffi, 1997; Huang et al., 2006; Serino et al., 2007; Erickson, 2007).

At the cellular level, most of the biological effects of 17β-E_2 are mediated through two distinct intracellular receptors, ERα and ERβ, which are encoded by unique genes (Hall et al., 2001). Tissue

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localisation studies have revealed that ERα is the predominant form in the uterus, cervix, vagina, and other target organs, whereas ERβ is expressed primarily in the gonadal tissue, prostate, and hypothalamus in mammals (Nilsson and Gustafsson, 2001). Despite the fact that the HG of reptiles has been considered a potential target for estrogens, the presence of steroid nuclear receptors in this organ remains largely unknown.

In this study, we isolated and sequenced estrogen receptor alpha (ERα) cDNA from the olive ridley HG. The ER cloned from this marine species (*Lepidochelys*) was closely related in nucleotide sequence and peptide domain homology to both the freshwater turtle (*Pseudemys*) and the painted turtle (*Chrysemys*) ERα genes.

2. Materials and methods

2.1. Total RNA extraction and cDNA synthesis

The tissues used in this study were obtained from turtles that were 1-week old (post-hatching) and had been raised at 32 ± 1 °C (female-promoting temperature) for other experimental purposes. The HGs from 10 animals and tissue samples from different organs were removed from the body and were immediately frozen on dry ice before storing at −75 °C. The total cellular RNA was extracted from different turtle tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). The purity and integrity of the RNAs were checked both spectroscopically (at 260/280 nm) and via gel electrophoresis prior to use in experimental assays. Complementary DNA (cDNA) was synthesized from the corresponding RNA (1 μg) using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's guidelines.

2.2. Cloning of ERα by PCR-RACE

A partial ERα cDNA fragment was obtained by RT-PCR using the total RNA obtained from the HG with two degenerate primers: RE35 (sense) 5′-GCTTCAGGCTACCAC/**T**TATGGA/**G**GTC-3′ and RET503 (antisense), 5′-CTCCAT**G**/TCCTTTGTTA/**G**CTCATA/**G**TG-3′. The ERα primers were designed by comparing selected regions of the ER sequences from chicken (NM_205183), rat (NM_012689) and human (NM_000125). After separation on an agarose gel, the PCR products were purified using Centricon-30 columns (Amicon, Beverly, MA) and subjected to sequencing analysis. Subsequently, gene-specific primers (GSP) derived from these sequences were used to generate the 5′- and 3′-end regions. The first strand cDNA synthesis for the RACE reactions was performed using the SMART RACE cDNA amplification kit (Clontech Lab. Inc., CA) and the GSPs: RE220, 5′-AGTCATAAGTGGTGCCTTCTGGGT-3′ (for 5′-RACE) and RE18, 5′-TGCTGCTCGGTTCCGTGTGATGAAT-3′ (for 3′-RACE). The full-length coding sequence of ERα was isolated from RACE-ready cDNA by PCR, using GSPs designed from the 5′- and 3′-RACE fragments. The primers for amplifying ERα were as follows: LER1, 5′-GATATGCCAATTGCTCGCTGCCAT-3′ (forward primer) and LER4, 5′-TGGATTCTCAGAACCTTGTAGGC-3′ (reverse primer). PCR was performed over 30 cycles with a denaturing temperature of 94 °C (1 min), an annealing temperature of 65 °C (1 min), and an extension temperature of 72 °C (3 min); these cycles were followed by a final extension step at 72 °C for 7 min. After the amplification reaction was completed, the purified PCR products were inserted into the TA expression vector pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA) and used to transform the chemically competent TOP10 *Escherichia coli* strain. Positive clones were subjected to restriction-enzyme digestion analysis and DNA sequencing in order to verify the orientation and integrity of the inserts. The nucleotide sequence in the final construct (pDNA3.1-*st*ERα) was determined by automated sequencing using an ABI-PRISM 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA).

2.3. Cell culture and transient transfections

Human embryonic kidney cells (HEK-293) were maintained in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% stripped fetal calf serum (Hyclone, UT, USA), 100 U penicillin/ml, and 100 μg streptomycin/ml. Transient transfections were performed in subconfluent cells (2×10^6 cells/plate) that were maintained in 5% CO_2 at 37 °C. The pDNA3.1-*st*ERα expression vector was transfected into the HEK cells at a concentration of 1.0 μg per plate using Lipofectamine (Invitrogen Co., Carlsbad, CA) and a phenol-red free DMEM according to the supplier's protocol. After 24 h, cells were rinsed with PBS, harvested, and processed for the estradiol binding assays.

2.4. Binding studies

The ligand-binding assays were performed essentially as described previously (Vilchis et al., 1991; Méndez et al., 1999) with only minor modifications. Transfected HEK-293 cells from 10 Petri dishes were pooled and homogenised in ice-cold TEDMG buffer (100 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 0.5 mM dithiothreitol, 10 mM sodium molybdate, and 10% glycerol) to obtain the cellular soluble fraction. The cytosolic fractions were prepared by centrifuging the homogenate at 105,000*g* for 1 h at 4 °C. For saturation analysis, aliquots (50 μl) of the cytosolic fractions (2–3 mg protein/ml) were used in each binding reaction; these aliquots were incubated at 4 °C overnight in the presence of [2,4,6,7,16,17-^{3}H]17β-estradiol (sp.act. 156 Ci/mmol) (Amersham, GE Healthcare Co., UK) at concentrations ranging from 0.25 to 3.0 nM. Non-specific binding was assessed in parallel assays containing a 200-fold excess of unlabeled estradiol. Unbound steroids were removed with dextran-coated charcoal (DCC), and the resulting radioactivity content was determined by liquid scintillation counting. The results were analysed using the Scatchard method (Zivin and Waud, 1982). For the binding specificity studies, cytosolic fractions (50 μl) were incubated with 2.0 nM [^{3}H]-17β-estradiol ([^{3}H]E_2) alone or in the presence of various concentrations (5- to 250-fold) of single competitor, specifically cortisol, progesterone, testosterone, 17β-androstanediol or 17β-estradiol (Steraloids Inc., Wilton, NH, USA). After incubation overnight at 4 °C, the bound and unbound steroid fractions were separated with a solution of DCC. In order to ascertain the specific binding of estrogen in turtle tissues, HG cytosolic preparations (300 μl) were incubated with saturating concentrations of [^{3}H]E_2 (5 nmol/l), layered onto a 15–35% sucrose density gradient, and centrifuged for 2.5 h at 370,000*g*.

2.5. Quantitation of ERα mRNA by Real-Time RT-PCR (RT-qPCR)

The extractions of RNA from frozen tissues (HGs, liver, lung and brain), in addition to single-strand cDNA synthesis, were carried out as described above. Both primers and TaqMan probes (FAM dye-labelled) specific for turtle cDNA sequences were designed by the Assay-by-Design service (Applied Biosystems). Primer sequences for *st*ERα amplification were as follows: RTER 5′-CCAAGCCCCATGG TATTCTTACAA-3′ (forward) and RTERR (reverse) 5′-TGGCTGTGGT GATGGATAAAGG-3′. The primers used to amplify the ribosomal RNA gene of *L. olivacea* (Genebank Accession No. AY390777) were: 5′-CGTGCAAAAGCGAGGATAACATTAT-3′ (forward) and 5′-AGTCTT TAGGGTAGTAAGTGTGATAGTTGAT-3′ (reverse). mRNA expression was analysed using the LightCycler 2.0 from Roche (Mannheim, Germany). The PCR cycling conditions included an initial denaturation