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Cloning and functional characterization of Chondrichthyes, cloudy catshark, *Scyliorhinus torazame* and whale shark, *Rhincodon typus* estrogen receptors

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ABSTRACT

Sex-steroid hormones are essential for normal reproductive activity in both sexes in all vertebrates. Estrogens are required for ovarian differentiation during a critical developmental stage and promote the growth and differentiation of the female reproductive system following puberty. Recent studies have shown that environmental estrogens influence the developing reproductive system as well as gametogenesis, especially in males. To understand the molecular mechanisms of estrogen actions and to evaluate estrogen receptor–ligand interactions in Elasmobranchii, we cloned a single estrogen receptor (ESR) from two shark species, the cloudy catshark (*Scyliorhinus torazame*) and whale shark (*Rhincodon typus*) and used an ERE-luciferase reporter assay system to characterize the interaction of these receptors with steroidal and other environmental estrogens. In the transient transfection ERE-luciferase reporter assay system, both shark ESR proteins displayed estrogen-dependent activation of transcription, and shark ESRs were more sensitive to 17β-estradiol compared with other natural and synthetic estrogens. Further, the environmental chemicals, bisphenol A, nonylphenol, octylphenol and DDT could activate both shark ESRs. The assay system provides a tool for future studies examining the receptor–ligand interactions and estrogen disrupting mechanisms in Elasmobranchii.

1. Introduction

Estrogens play important roles in the reproductive biology of all vertebrates studied to date, including fish. Currently, the majority of the known actions of estrogens are mediated by specific receptors that are localized in the nucleus of target cells. These nuclear estrogen receptors (ESRs) belong to a superfamily of nuclear transcription factors that include all other steroid hormone receptors including those for progestogens, androgens, glucocorticoids, mineralocorticoids, the vitamin D receptor, and the retinoic acid receptor (Blumberg and Evans, 1998). To date, in most vertebrates, two distinct forms of ESR, ESR1 (ERα) and ESR2 (ERβ), have been isolated whereas in teleost fish, one ESR1 and two ESR2 (ESR2a and ESR2b) have been identified. The ESR2b-form appears to be closely related to ESR2a, suggesting a gene duplication event that has occurred within the teleosts (Katsu et al., 2008). Thus, the ancestral condition for the jawed vertebrates (Gnathostomata) is considered to have had two forms of ESR, corresponding to ESR1 and ESR2 (Hawkins et al., 2000). Chondrichthyes are jawed fish with skeletons consisting of cartilage rather than bone. They are divided into two subclasses; Elasmobranchii including sharks, rays and skates, and Holocephali (chimaera). Early study on steroids in Elasmobranchii ovaries identified 17β-estradiol, estrone, and estriol (Wotiz et al., 1960; Chieffi and Lupo di Prisco, 1963; Simpson et al., 1964). Further, Resse and Callard (1991) reported the presence of estradiol-binding protein and characterized its ligand-specificity. However, we were able to identify only one full length of ESR sequence of Chondrichthyes, *Squalus acanthias*, in GenBank.

In vertebrates, 17β-estradiol is the principle estrogen in circulation and appears essential for normal ovarian development and function (Wallace, 1985). Embryonic exposure to inhibitors of aromatase, the enzyme responsible for the conversion of testosterone

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to 17β-estradiol, causes genetic females to become phenotypic males in chicken and at least one species of turtle (Elbrecht and Smith, 1992; Dorizzi et al., 1994). Likewise, embryonic exposure of various fishes, amphibians or reptiles to 17β-estradiol or estrogenic chemicals, pharmaceutical agents or environmental contaminants can induce skewed sex ratios toward females (for reviews, see Crews, 1996; Guillette et al., 1996; Tyler et al., 1998; Iguchi et al., 2001). A number of studies strongly suggest that endogenous 17β-estradiol acts as a natural inducer of ovarian differentiation in non-mammalian vertebrates, including Elasmobranchii (Devlin and Nagahama, 2002; Sinclair et al., 2002; Koob and Callard, 1999). However, the mechanisms of estrogen action on ovarian differentiation have not been determined.

Disruption of the endocrine system has been shown to occur in wild fish populations across the globe (Vos et al., 2000). Although the evidence for endocrine disruption in wild fish is extensive, there are few verifications of a causal relation between the presence of specific endocrine disrupting chemicals (ECDs) and a functional effect (disruption). Several studies have now shown that wild freshwater fish living in rivers heavily contaminated with treated wastewater treatment works effluent have altered reproductive development and function (Hecker et al., 2002; Jobling et al., 2002), and other investigations have shown that estrogens and estrogenic chemicals are present in those effluents (Desbrow et al., 1998; Snyder et al., 2001) and likely involved in these disruptions (Jobling et al., 2006; Katsu et al., 2007b). In marine ecosystem, there are some reports of endocrine disruption in fish (Scott et al., 2006, 2007), and the accumulations of various environmental chemicals with suspected endocrine activity have been reported in sharks (Gelsleichter et al., 2005, 2006; Storelli et al., 2006; Silva et al., 2007; Haraguchi et al., 2009). The mechanisms by which estrogenic chemicals have an effect on sexual development and function in Elasmobranchii, such as shark, however, are still to be determined.

In this study, we isolated cDNA clones encoding shark orthologs of ESR. The transactivation function of shark ESR was subsequently determined by expressing these cDNAs in transiently transfected HEK293 cells which were then used to determine ligand-specificity of shark ESR with natural, synthetic and environmental estrogens. The extensive global distribution of sharks in tropical to polar aquatic ecosystems and their capability to tolerate highly contaminated environments make these animals an interesting biological model for assessing endocrine disruptors in a wide range of aquatic environments.

2. Methods

2.1. Animals

Cloudy catshark, *Scyliorhinus torazame*, were purchased from a local supplier. Whale shark (*Rhincodon typus*) tissues were obtained for research purposes from the Georgia Aquarium's Correll Center for Aquatic Animal Health. All experiments in this study involving sharks were carried out under the guidelines specified by the Institutional Animal Care and Use Committee at the Gergia Aquarium's Correll Center and Hokkaido University.

2.2. Chemical reagents

17β-Estradiol (E2), estrone (E1), estriol (E3), ethinylestradiol (EE2), and diethylstilbestrol (DES) were purchased from Sigma–Aldrich Corp. (St. Louis, MO). Bisphenol A (BPA) was purchased from Nacalai Tesque (Kyoto, Japan). The ERα selective agonist, propyl pyrazole triol (PPT) and ERβ-selective agonist, diarylpropionitrile (DPN) were obtained from Tocris Bioscience (Ellisville, MO). 4-Nonylphenol (NP) and 4-*tert*-octylphenol (OP) were purchased from Tokyo Kasei (Tokyo, Japan). Purity of BPA, NP, and OP were over 99%. DDT and its metabolites were purchased from Chem Service (West Chester, PA). Purity of *p*,*p*′-DDD, *p*,*p*′-DDE, *o*,*p*′-DDE, and *p*,*p*′-DDT were 99%, and *o*,*p*′-DDD and *o*,*p*′-DDT were 99.5%. All chemicals were dissolved in dimethylsulfoxide (DMSO). The concentration of DMSO in the culture medium did not exceed 0.1%.

2.3. Molecular cloning of estrogen receptors

Two conserved amino acid regions in the DNA-binding domain (GYHYGVW) and the ligand-binding domain (NKGM/IEH) of vertebrate ESRs were selected for the design of degenerate oligonucleotides. The second PCR using the first PCR amplicon, and nested primers that were selected in the DNA-binding domain (CEGCKAF) and the ligand-binding domain (NKGM/IEH). As a template for PCR, the first-strand cDNA was synthesized using total RNA isolated from liver. The amplified DNA fragments were subcloned with TA-cloning plasmids pCR2.1-TOPO (Invitrogen, Carlsbad, CA). The 5′ and 3′ ends of ER cDNAs were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE cDNA Amplification kit (BD Biosciences Clontech., Palo Alto, CA). Sequencing was performed using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and analyzed on the ABI PRISM 377 automatic sequencer (Applied Biosystems).

2.4. Database and sequence analysis

All sequences generated were searched for similarity using Blastn and Blastp at web servers of the National Center of Biotechnology Information (NCBI). Phylogenetic analysis was demonstrated with the amino acid sequences for estrogen receptor (ESR) from selected vertebrates and invertebrates as shown in Supplementary Table S1. The regions from immediately outside of the DNA-binding domain through the ligand-binding domain of the sequences were trimmed according to the Conserved Domains Database in NCBI. The trimmed sequences were aligned using the PRANK program, a probabilistic multiple alignment program, which is good at insertions and deletions (Löytynoja and Goldman, 2008). The aligned sequences were used for the estimation of phylogenetic trees by the maximum likelihood analysis with the PhyML 3.0 program with JTT substitution model, NNI (Nearest Neighbor Interchange) and SH-like aLRT (approximately Likelihood-Ratio Test) branch supports (Guindon and Gascual, 2003). The estimated tree was edited on MEGA4 (Tamura et al., 2007). The aligned sequences were also used for the rate shift analysis (Knudsen et al., 2003).

2.5. Construction of plasmid vectors

The full-coding region of shark ESR was amplified by PCR with KOD DNA polymerase (TOYOBO Biochemicals, Osaka, Japan). The PCR product was gel-purified and ligated into the pcDNA3.1 vector (Invitrogen). An estrogen-regulated reporter vector containing four estrogen-responsive elements (4×ERE), named pGL3–4×EREtkLuc was constructed as described previously (Katsu et al., 2006).

2.6. Transactivation assays

To examine ligand (e.g., environmental estrogen) interactions with the cloned estrogen receptors, HEK293 cells were seeded in 24-well plates at 5×10^4 cells/well in phenol-red free Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 10% charcoal/dextran treated fetal bovine serum (Hyclone, South