General and Comparative Endocrinology 238 (2016) 88–95

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Characterization of evolutionary trend in squamate estrogen receptor sensitivity

Ryohei Yatsu [a], Yoshinao Katsu [b], Satomi Kohno [c], Takeshi Mizutani [d], Yukiko Ogino [a,d], Yasuhiko Ohta [e], Jan Myburgh [f], Johannes H. van Wyk [g], Louis J. Guillette Jr. [c], Shinichi Miyagawa [a,d,*], Taisen Iguchi [a,d,*]

[a] Department of Basic Biology, Faculty of Life Science, SOKENDAI (Graduate University for Advanced Studies), 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan
[b] Graduate School of Life Science and Department of Biological Sciences, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan
[c] Department of Obstetrics and Gynecology, Medical University of South Carolina, and Marine Biomedicine and Environmental Science Center, Hollings Marine Laboratory, Charleston, SC 29412, USA
[d] Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan
[e] Department of Veterinary Medicine, Faculty of Agriculture, Tottori University, Koyama, Tottori 680-8553, Japan
[f] Department of Paraclinical Sciences, University of Pretoria, Private Bag 04, Onderstepoort 0110, South Africa
[g] Department of Botany & Zoology, University of Stellenbosch, Stellenbosch 7600, South Africa

ARTICLE INFO

Article history:
Received 20 January 2016
Accepted 8 April 2016
Available online 9 April 2016

Keywords:
Squamates
Estrogen receptor
Estrogen sensitivity
Evolution of estrogen receptor

ABSTRACT

Steroid hormones are a key regulator of reproductive biology in vertebrates, and are largely regulated via nuclear receptor families. Estrogen signaling is regulated by two estrogen receptor (ER) subtypes alpha and beta in the nucleus. In order to understand the role of estrogen in vertebrates, these ER from various species have been isolated and were functionally analyzed using luciferase reporter gene assays. Interestingly, species difference in estrogen sensitivity has been noted in the past, and it was reported that snake ER displayed highest estrogen sensitivity. Here, we isolated additional ER from three lizards: chameleon (*Bradypodion pumilum*), skink (*Plestiodon finitimus*), and gecko (*Gekko japonicus*). We have performed functional characterization of these ERs using reporter gene assay system, and found high estrogen sensitivity in all three species. Furthermore, comparison with results from other tetrapod ER revealed a seemingly uniform gradual pattern of ligand sensitivity evolution. *In silico* 3D homology modeling of the ligand-binding domain revealed structural variation at three sites, helix 2, and juncture between helices 8 and 9, and caudal region of helix 10/11. Docking simulations indicated that predicted ligand-receptor interaction also correlated with the reporter assay results, and overall squamates displayed highest stabilized interactions. The assay system and homology modeling system provides tool for in-depth comparative analysis of estrogen function, and provides insight toward the evolution of ER among vertebrates.

1. Introduction

Steroid hormones operate as powerful endogenous regulators in many of the vertebrate biological processes. Sex steroid hormones, such as progestagens, androgens, and estrogens, play prominent roles in reproductive biology, including development of sexual organs, behavior and reproductive cycles. Estrogens, in particular, are associated with development of female-characteristics in vertebrates, such as ovarian development, oogenesis, and secondary sex characteristics, in addition to metabolic and adipose regulation (Heldring et al., 2007; Hess, 2003; Nilsson et al., 2001). Taken together, study of estrogen action remains one of most crucial component in understanding vertebrate reproductive biology. Currently, much of the insight on vertebrate molecular estrogen mechanism derives from mammalian and aquatic species, such as teleosts (Nelson and Habibi, 2013; Tohyama et al., 2015). However, researches in other vertebrates, namely reptiles, are comparatively lacking.

Endogenous estrogens include 17β-estradiol (E2), estrone (E1), and estriol (E3), and are biosynthesized from androgens via aromatase enzyme in the gonad and the brain. Of these, E2 is widely regarded as the dominant estrogen among vertebrates. In addition to endogenous estrogens, several synthetic compounds with estrogenic activity are also reported; these include pharmaceutical

* Corresponding authors at: Okazaki Institute for Integrative Bioscience, National Institutes for Basic Biology, 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan.
E-mail addresses: ryoheiy@nibb.ac.jp (R. Yatsu), ykatsu@sci.hokudai.ac.jp (Y. Katsu), kohno@musc.edu (S. Kohno), takeshim@nibb.ac.jp (T. Mizutani), ogino@nibb.ac.jp (Y. Ogino), ohta1022@gmail.com (Y. Ohta), jan.myburgh@up.ac.za (J. Myburgh), jhvw@sun.ac.za (J.H. van Wyk), miyagawa@nibb.ac.jp (S. Miyagawa), taisen@nibb.ac.jp (T. Iguchi).

http://dx.doi.org/10.1016/j.ygcen.2016.04.005
0016-6480/

drugs such as diethylstilbestrol (DES) and 17α-ethinylestradiol (EE2). Estrogen signalings are rapidly dispersed internally through circulatory systems, inducing gene expression changes in target cells. They are relayed in target cells via canonical nuclear receptors, estrogen receptors (ERs), for the most part and also via membrane signaling to some extent (Bjornstrom and Sjoberg, 2005). Two main ER subtypes are present: ER alpha (ERα) and ER beta. (ERβ). Similar to other nuclear steroid receptors, these two receptors are structurally composed of six domains, including the DNA-binding domain (DBD), that allows ER binding to DNA at estrogen response element (ERE), and ligand-binding domain (LBD), which forms a ligand-binding pocket for reception of estrogenic and various other compounds (Klinge, 2001). ERα is primarily considered to have higher significance in biological roles than ERβ, and have been associated with multitudes of physiological functions (Couse and Korach, 1999).

Functionality of nuclear receptors, such as ER, can be comprehensively assessed by various methods, such as *in silico* molecular simulations, and more commonly, with *in vitro* luciferase reporter gene assay system (Katsu et al., 2004; Kohno et al., 2008; Tohyama et al., 2015). ER sensitivity to various administrations of estrogenic compounds has been previously investigated, and quantified in the form of effective concentration, EC_{50} values. In current nuclear receptor studies, EC_{50}s are instrumental in evaluating the functionality, chemical sensitivity, and evolution of ERs in many organisms. Cross-species comparative analyses from wide spectrum of vertebrates, including reptiles, revealed species difference in ERα sensitivity in response to various ligands. Snakes displayed the lowest recorded EC_{50} values in all studied vertebrates, followed by other reptile and avian species (Katsu et al., 2008a, 2010a; Naidoo et al., 2008). The biological implication of such species difference is yet to be elucidated, and lowered EC_{50} values may indicate a reptile-specific evolutionary trend.

Here, we report additional detail on for three more reptiles, Cape dwarf chameleon (*Bradypodion pumilum*), Japanese skink (*Plestiodon finitimus*), and Japanese gecko (*Gekko japonicus*), and provide more comprehensive description of ERα in reptiles, focusing particularly on squamates. In order to fully characterize the evolutionary trend in reptile ER sensitivity, we have isolated and performed *in vitro* luciferase reporter assay on these lizard ERs. Interestingly, a potential evolutionary trend was observed as ERα from squamates were found to be highly sensitive to estrogen compared to other vertebrate clades. Furthermore, *in silico* modeling of tetrapod ERαs was utilized to analyze the structural changes that may have lead to species differences in ER sensitivity.

2. Materials and methods

2.1. Animals and chemical reagents

The Japanese skink (*Plestiodon finitimus*) and the Japanese gecko (*Gekko japonicus*) were collected in the field in Okazaki, Aichi, Japan. Cape dwarf chameleon (*Bradypodion pumilum*) tissues were obtained from South Africa. All estrogenic compounds used in the study (E2, E1, E3, EE2, and DES) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The chemicals were dissolved in dimethylsulfoxide (DMSO) for use in reporter assay, and did not exceed 0.1% concentration in the culture medium.

2.2. Molecular cloning of estrogen receptors

Total RNA was extracted from ovary, and liver tissues, using RNeasy kit (Qiagen, Valencia, CA, USA) for chameleon and skink, and from tail tissue using ISOGEN reagent (Nippon Gene, Toyama, Japan) for gecko. Full coding region of the estrogen receptor from chameleon and skink was determined by standard procedure using SmartRACE kit (Takara, Ohtsu, Japan), and GeneRacer kit (Life Technologies, Carlsbad, CA, USA) for gecko. Full-length ERs of each species were cloned with KOD+ polymerase (Toyobo, Osaka, Japan). Primer information is reported in Supplementary Table S1. The amplified full-length ER cDNA products were then subcloned into pcDNA3.1 vector (Life Technologies). Estrogen regulated reporter vector with four estrogen-responsive elements (pGL3-4xERE) was also constructed as described previously (Katsu et al., 2004, 2006).

2.3. Sequence analysis

Multiple sequence alignment and amino acid similarity was calculated for various vertebrate ER homologues using CLUSTAL OEMGA (Goujon et al., 2010; Sievers et al., 2011). Phylogenetic relationships of ER were then examined using predicted ER amino acid sequences from current study and previously reported sequences from GenBank database, summarized in Supplementary Table S2. Phylogenetic tree was constructed based on ER conserved sites, which include the DNA binding domain (DBD), hinge region, and the ligand binding domain (LBD), with all the alignment gap sites eliminated using the maximum-likelihood methods with Jones-Taylor-Thornton model using MEGA 6 software (Tamura et al., 2013). The statistical confidence was then computed by bootstrap method with 1000 replications.

2.4. Transactivation assays

HEK293 cells (DS Pharma Biomedical, Osaka, Japan) were transfected with reporter vectors and pcDNA3.1-ER constructs, and estrogen-induced transcriptional activity was recorded using reporter gene assay in three technical replicates per each dosage concentration as previously described (Katsu et al., 2008a, 2008b, 2010b). Luciferase activity was recorded with Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI, USA) under manufacturer's protocol, using GLOMAX 20/20 Luminometer (Promega).

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (Version 5.0b; GraphPad Software, Inc., San Diego, CA, USA). Dosage-response curve was constructed from normalized transactivation assay results, using three parametric non-linear regression fit, and EC_{50} values were subsequently calculated. For correlation analysis, average EC_{50} values were used for species with multiple previous data. Linear regression analysis was performed and graphed with 95% confidence interval of the best-fit line.

2.6. In silico modeling

Computational 3D homology modeling of ERα-LBD, and subsequent analyses were performed using Molecular Operating Environment (MOE; Chemical Computing Group, Montreal, Quebec, Canada), using protocol previously described (Tohyama et al., 2015). The homology models were constructed using the crystal structure of human ERα-LBD with E2 (1A52 in Protein Data Bank), and optimized using AMBER12:EHT force field (Labute, 2008; Tanenbaum et al., 1998). Protonate three-dimensional program was used to prepare the model, and the protonation state of running buffer was adjusted to pH 7.0, same as the luciferase transactivation assays environment. Structural homologies between the constructed models were analyzed using Protein Consensus program. Ligand binding pockets were identified using MOE Alpha Site Finder. Most stable interaction potential between E2 and LBD models were predicted based on lowest calculated U-total value