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Estrogen receptor-related receptor γ regulates testicular steroidogenesis through direct and indirect regulation of steroidogenic gene expression

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ABSTRACT

Biosynthesis of testosterone, which mainly occurs in testicular Leydig cells, is controlled by steroidogenic proteins, such as StAR and P450c17. Although estrogen-related receptor gamma (ERRγ), an orphan nuclear receptor, is expressed in the testis, its role is not well understood. In this study, we investigated the expression of ERRγ in Leydig cells and its molecular action on testicular steroidogenesis. ERRγ is expressed in mouse Leydig cells from pre-pubertal stages. ERRγ overexpression in primary Leydig cells elevated the production of testosterone with a marked increase of P450c17 expression at both mRNA and protein levels, albeit decreased expression of StAR. Promoter-reporter analyses showed that ERRγ directly regulated the P450c17 promoter. Further deletion mutant analyses of the P450c17 promoter revealed that ERRγ activated expression of the P450c17 gene by binding to an ERRγ response element within the P450c17 promoter. Meanwhile, ERRγ suppressed cAMP-induced activation of the StAR promoter, which was likely due to ERRγ-mediated inhibition of the transcriptional activity of Nur77, which is induced by cAMP and regulates StAR gene expression in Leydig cells. Interestingly, ERRγ coexpression also decreased the protein level of Nur77, which occurred through proteasomal degradation, suggesting ERRγ-mediated regulation of steroidogenesis at another level. Taken together, these findings suggest that ERRγ regulates testicular steroidogenesis, both directly controlling and indirectly fine-tuning the expression of steroidogenic genes.

1. Introduction

Steroidogenesis, the process of testosterone synthesis, occurs in the adrenal cortex, testis and ovary, and it is controlled by luteinizing hormone (LH), which is synthesized and secreted from the pituitary (Hanukoglu, 1992,Payne and Youngblood, 1995). In the testis, the stimulation of LH from the pituitary increases intracellular cAMP levels in Leydig cells and then results in steroidogenesis by increasing the expression of several steroidogenic genes (Payne and Youngblood, 1995; Habert et al., 2001; Odell et al., 1974). The first step of steroidogenesis is uptake of cholesterols into the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR) (Payne and Youngblood, 1995). Cholesterol within mitochondria is converted to pregnenolone by the cytochrome P450 side chain cleavage complex (P450scc), and pregnenolone is then transported to smooth endoplasmic reticulum (SER) and converted to testosterone through a series of steroidogenic enzymes, including 3β-hydroxysteroid dehydrogenase (3β-HSD) (Rasmussen et al., 2013) and cytochrome P450 17α-hydroxylase (P450c17) (Payne and Youngblood, 1995). Synthesized testosterone plays important roles in male sexual differentiation, reproductive development, and spermatogenesis initiation and maintenance.

Nur77 (also known as NR4A1, NGFI-B, TR3 and NAK-1), an orphan nuclear receptor, is widely expressed in tissues, such as testis, ovary, muscle, thymus, adrenal gland, and brain (Giguere, 1999; Law et al., 1992). As an immediate early response gene, Nur77 is induced by various stimuli, including fatty acids, stress, prostaglandins, growth factors, calcium, inflammatory cytokines,

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peptide hormones, and neurotransmitters (Maxwell and Muscat, 2006). Moreover, previous studies have demonstrated that LH, the regulator of testicular steroidogenesis, also induces Nur77 gene expression in Leydig cells (Song et al., 2001), which in turn regulates the expression of steroidogenic genes, such as StAR (Martin et al., 2008), P450c17 (Zhang and Mellon, 1997), and 3β-HSD type 2 (3β-HSD2) (Martin and Tremblay, 2005), through the Nur77-binding region within the promoter of these genes. Therefore, Nur77 in Leydig cells acts as one of major transcription factors involved in the regulation of steroidogenic genes. Several cytokines, including TNF-α (Hong et al., 2004) and TGF-β1 (Park et al., 2014a,b), as well as hormones, including estrogen (Lee et al., 2012) and thyroid hormone (Park et al., 2014a,b), in Leydig cells regulate LH/cAMP-activated steroidogenesis through the regulation of Nur77 transactivation.

Estrogen receptor-related receptors (ERRs; ERRα, ERRβ and ERRγ) are also orphan nuclear receptors and are named after the sequence homology with estrogen receptors (ER), but their roles are distinctive from ERs (Giguere et al., 1988). ERRs are constitutively activated without binding to natural ligands (Xie et al., 2009), but this constitutive activity can be abolished by their inverse agonists such as GSK5182 (Kim et al., 2016; Joo et al., 2015; Tzameli and Moore, 2001). ERRs bind to DNA as monomers, which recognize estrogen receptor-related receptor response elements (ERREs) with the extended half-site consensus of TnAAGGTCA (Sladek et al., 1997,Johnston et al., 1997,Yang et al., 1996). ERRs also bind to estrogen-response elements (EREs) containing the recognition motif of AGGTCA as monomers, homodimers or heterodimers (Pettersson et al., 1996; Lu et al., 2001). Among ERRs, ERRγ is expressed in various tissues, such as brain, heart, muscle and liver, and it plays roles in energy homeostasis, diabetes and cancers (Huss et al., 2015). However, the expression and role of ERRγ in reproductive organs are yet not fully defined.

In the present study, we investigated if ERRγ, which is expressed in the testis, regulates steroidogenesis, the major function of Leydig cells. ERRγ was expressed in mouse Leydig cells from the prepubertal to adult stage, and it increased the synthesis of testosterone. ERRγ positively regulated P450c17 gene expression through direct binding between the −369 and −135 region of the promoter, whereas it negatively regulated StAR gene expression through inhibition of Nur77 transactivation. Moreover, ERRγ overexpression decreased the stability of Nur77 protein via proteosomal degradation. Taken together, these data suggest that ERRγ regulates the expression of steroidogenic genes, thus controlling and probably fine-tuning testicular steroidogenesis.

2. Materials and methods

2.1. Plasmids and chemicals

The Nur77, SF-1 and LRH-1 mammalian expression vectors and the Sft4-Luc, NurRE-Luc, mouse StAR(-2200/+3)-Luc, mouse P450c17(-1040/+3)-Luc, WT(-447/-399) P450c17-Luc and Mut(-447/-399Δ2) P450c17-Luc reporter plasmids have been described previously (Hong et al., 2004). The deleted P450c17 reporters were constructed by PCR amplification using specific primers containing the Nhe1 restriction site. The PCR products were digested in combination with Nco1 (an internal site in the pGL3 vector) and ligated into a Nhe1/Nco1-digested pGL3 vector. The forward primers [5′-TGCTAGAAGGATCCATAGCGCAGAAGC-3′ (−269/+3) and 5′-TGCTAGCGGACTCAGGCTTGGAGACA -3′ (−135/+3)] were used with a common reverse primer [5′- GAGTTTTCACTGCATACGACG-3′] spanning the pGL3 vector. The P450c17(-269/+3)-Luc point mutant was generated by PCR amplification using the following mutated primer pair: 5′-CAGCCCTTGTGGATTCCAATTCCCTCTCC-3′ and 5′-GGAGAGGGAATTTGGAATCCACAAGGGCTG-3′. All the above amplifications were performed using pfu turbo DNA polymerase (Agilent technologies). The pcDNA3-ERRγ plasmid and adenoviral (Ad) vectors encoding GFP only (Ad-only) and GFP with ERRγ (Ad-ERRγ) have been described previously (Kim et al., 2012).

8Br-cAMP, cycloheximide (CHX), MG132 and chloroquine were purchased from Sigma-Aldrich. GSK5182 was synthesized as previously described and was obtained as a HCl salt dissolved in a sterile-filtered 30% polyethylene glycol (PEG)-400 aqueous solution (Kim et al., 2012).

2.2. Cell culture and purification of primary Leydig cells

The MA-10 mouse Leydig cell line and 293T human embryonic kidney cell line (ATCC CRL-3216) were obtained from Dr. Mario Ascoli (University of Iowa, Iowa) and ATCC (Manassas, VA), respectively. MA-10 cells were maintained in RPMI 1640 medium (containing 25 mM HEPES) supplemented with 15% horse serum and antibiotics (Park et al., 2014a,b). 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. All cells were cultured at 37 °C under an atmosphere of 5% CO2. Purification of primary mouse Leydig cells was performed as previously described (Park et al., 2014a,b). Briefly, testicular cells were dispersed by treating decapsulated mouse testes with collagenase type I (0.25 mg/mL, Sigma-Aldrich). The interstitial cells were prepared by filtrating testicular cells with a 40 μm cell strainer (BD Biosciences, San Jose, CA). Enrichment for Leydig cells was estimated using 3β-HSD immunocytochemistry. The population of Leydig cells was 60%−70% of the total purified cells.

2.3. Radioimmunoassay (RIA)

To measure testosterone levels, serum-free culture medium of primary mouse Leydig cells, which were infected with Ad-only or Ad-ERRγ and treated with cAMP for 48 h, was collected. Medium was separated by centrifugation at 10,000 rpm for 5 min at 4 °C and then stored at −70 °C until testosterone assays. Testosterone concentration was measured by RIA as described previously (Lee et al., 2012). The inter and intra assay coefficient of variation for the testosterone estimation were 8.7% and 9.3%, respectively (Suh et al., 2008).

2.4. Reporter assay

MA-10 and 293T cells were plated in 5% charcoal-stripped serum without antibiotics in 24-well plates in duplicate 24 h before transfection. Each indicated expression vector and reporter plasmid were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two days after transfection, cells were lysed with lysis buffer composed of 0.1% Triton X-100 and 0.2 mM Tris-HCl (pH 8.0). The luciferase and β-galactosidase activities were assayed as previously described (Park et al., 2014a,b). Luciferase activity levels were normalized to lacZ expression.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR) and reverse transcription-polymerase chain reaction (RT-PCR) analysis

qRT-PCR and RT-PCR analyses were performed as described previously (Park et al., 2014a,b). In brief, 2 μg of total RNA from cells was reverse transcribed into cDNA with M-MLV reverse transcriptase (RT) (Promega, Fitchburg, WI). qRT-PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and the SensiMixPlus SYBR Kit (Quantace, London,