Mechanisms of Ageing and Development 150 (2015) 74–82

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

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

Up-regulation of steroid biosynthesis by retinoid signaling: Implications for aging

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ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form 14 July 2015
Accepted 15 August 2015
Available online 21 August 2015

Keywords:
Retinoids
RAR
RXR
cAMP/PKA
StAR expression
Steroid biosynthesis
Steroidogenic cells

ABSTRACT

Retinoids (vitamin A and its derivatives) are critical for a spectrum of developmental and physiological processes, in which steroid hormones also play indispensable roles. The StAR protein predominantly regulates steroid biosynthesis in steroidogenic tissues. We have reported that regulation of retinoid, especially atRA and 9-cis RA, responsive StAR transcription is largely mediated by an LXR–RXR/RAR heterodimeric motif in the mouse StAR promoter. Herein we demonstrate that retinoids are capable of enhancing StAR protein, P-StAR, and steroid production in granulosa, adrenocortical, glial, and epidermal cells. Whereas transient expression of RARα and RXRα enhanced 9-cis RA induced StAR gene transcription, silencing of RXRα with siRNA, decreased StAR and steroid levels. An oligonucleotide probe encompassing an LXR–RXR/RAR motif bound to adrenocortical and epidermal keratinocyte nuclear proteins in EMSAs. ChIP studies revealed association of RARα and RXRα with the StAR proximal promoter. Further studies demonstrated that StAR mRNA levels decreased in diseased and elderly men and women skin tissues and that atRA could restore steroidogenesis in epidermal keratinocytes of aged individuals. These findings provide novel insights into the relevance of retinoid signaling in the up-regulation of steroid biosynthesis in various target tissues, and indicate that retinoid therapy may have important implications in age-related complications and diseases.

1. Introduction

Retinoids, the metabolically active structural derivatives of vitamin A, especially atRA and 9-cis RA, exert a wide range of effects on development, differentiation, reproduction and epidermal homeostasis (Clagett-Dame and Knutson, 2011; Inoue et al., 2012; Jacobs et al., 2006; Vernet et al., 2006). The biological actions of retinoids are principally mediated by the activities of two families of nuclear receptors, the RARs and RXRs, each of which has three subtypes (α, β and γ) with additional isoforms resulting from alternative splicing (Chambon, 2005; Lefebvre et al., 2005). Whereas RARs are activated by both atRA and 9-cis RA, RXRs are induced solely by 9-cis RA. Both RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily of transcription

Abbreviations: RA, retinoic acid; Retinoids, RA and its derivatives; atRA, all-trans RA; RAR, retinoic acid receptor; RXR, retinoid X receptor; LXR, liver X receptor; PKA, protein kinase A; (Bu)2cAMP, dibutyryl adenosine 3′,5′ cyclic monophosphate; StAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450scc; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay; NE, nuclear extract; ChIP, chromatin immunoprecipitation; RT-PCR, reverse transcription polymerase chain reaction.

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http://dx.doi.org/10.1016/j.mad.2015.08.007
0047-6374/

factors that form either hetero- or homo-dimers and bind to a retinoid response element, termed the RARE/RXRE. This element is a direct repeat (DR) of two hexameric half-sites with the consensus sequences 5′-PuG(G/T)TCA-3′, their inverted or everted forms being present in the regulatory region of target genes (Chambon, 2005; Kliewer et al., 1992; Lefebvre et al., 2010; Mangelsdorf et al., 1990). However, RXR–RAR heterodimers are the functional units that transduce the retinoid signal (Chambon, 2005; Lefebvre et al., 2010). RARs and RXRs are expressed in varying amounts in steroidogenic tissues, suggesting they may be independently regulated, respond to discrete ligands, and perform distinct cellular functions (Clagett-Dame and Knutson, 2011; Manna et al., 2014). Of note, mice lacking RARα, RARγ, RXRα, and RXRβ display profound anomalies in gonadal and adrenal functions, including sterility and/or embryonic lethality (Chung et al., 2004; Clagett-Dame and Knutson, 2011; Li and Clagett-Dame, 2009; Mark et al., 2006), underscoring the importance of retinoid signaling in reproduction as well as steroidogenesis. The intramitochondrial transport of cholesterol is the rate-limiting and regulated step in steroid biosynthesis, a process that is primarily mediated by the StAR protein, a rapidly synthesized mitochondrial phosphoprotein whose expression, activation, and removal is influenced by PKA, PKC, and a host of other signaling pathways that produce both acute and chronic effects on steroidogenesis (Clark et al., 1994; Manna and Stocco, 2005; Miller and Bose, 2011; Stocco et al., 2005). At the level of the mitochondria, cytochrome P450scc (CYP11A1) cleaves the side chain of cholesterol to form the first steroid, pregnenolone, which is further converted by a series of enzymes to various steroid hormones in pertinent tissues [reviewed in Refs. (Manna et al., 2009a,b,c; Manna and Stocco, 2005; Miller and Bose, 2011; Stocco et al., 2005)]. Noteworthy, phosphorylation of StAR, especially at Ser194, has been demonstrated to be an indispensable event to obtain the maximal cholesterol transferring activity of StAR for steroid biosynthesis (Jo et al., 2005; Stocco et al., 2005; Manna et al., 2006; Manna et al., 2009a; Manna et al., 2013). In a recent study, we have reported that retinoids, by interacting with an LXR–RXR/RAR motif, synergistically activate cAMP/PKA stimulated StAR expression and steroid biosynthesis in MA-10 mouse Leydig cells (Manna et al., 2014). Additionally, the cooperation/interaction between LXR and RXR/RAR in influencing StAR transcription and steroidogenesis has been shown in gonadal and adrenal cells (Manna et al., 2013; Manna et al., 2014). However, the mechanisms of action of retinoids in the regulation of the steroidogenic response remain elusive. With aging, multifaceted changes in the neuroendocrine system result in a decline in various hormones, including steroids and, thus, many physiological activities. Age-related complications and diseases are also frequently associated with decreases in retinoid metabolism and signaling (Mihaly et al., 2011; Olson and Mello, 2010; Ono and Yamada, 2012). Noteworthy, the systemic administration of retinoids, especially RAs, reverses most reproductive and developmental blocks in vitamin A deficient (VAD) rats/mice, demonstrating that retinoid signaling rescues reproductive defects as well as steroidogenesis in VAD animals (Chung and Wolgemuth, 2004; Clagett-Dame and Knutson, 2011; Mark et al., 2006). The involvement of retinoids in skin physiology/pathophysiology has long been known. Human skin possesses endocrine functions that include the capabilities to synthesize cholesterol and express StAR and also house the functional biochemical apparatus for the synthesis of glucocorticoids and sex steroids de novo or from precursors of systemic origin (Slominski et al., 1996; Cirillo and Prime, 2011; Slominski and Wortsman, 2000; Slominski et al., 2013a; Slominski et al., 2014; Zouboulis, 2009). A question remains as to whether retinoids can reverse the decline in steroid biosynthesis in target tissues and thereby maintain steroid requiring physiological activities that become diminished during aging. The experimental approaches utilized here provide evidence that retinoids up-regulate the steroidogenic response in several different endocrine tissues and that retinoid signaling is able to enhance/restore the age associated decline in steroid biosynthesis in epidermal keratinocytes.

2. Materials and methods

2.1. Cells, plasmids, transfections, and luciferase assays

Mouse granulosa KK-1 (Manna et al., 2009b; Manna et al., 2013), human astroglial A172 (Davis and Syapin, 2004), adrenocortical H295R (Clark et al., 1995; Tu et al., 2014) and epidermal keratinocyte HaCaT (Boukamp et al., 1988; Slominski et al., 2013a) cells were respectively cultured in DMEM/F12 medium (Invitrogen Life Technologies, Inc., Grand Island, NY) plus 10% FBS, DMEM/F12 with 1% ITS plus, 2.5% NuSerum, DMEM/F12 with 10% FBS and 1% nonessential amino acids, and F-12K medium with 10% HS, containing antibiotics.

The 5-flanking −254/−1 bp region of the mouse StAR promoter was synthesized using a PCR based cloning strategy and inserted into the *Xho*I and *Hind*III sites of the pGL3 basic vector (Promega, Madison, WI) that contains firefly luciferase as a reporter gene (Manna et al., 2002; Manna et al., 2013; Manna et al., 2014). The −254/−1 bp StAR segment was used for generating mutations in the LXR–RXR/RAR heterodimeric motif 5′-**TGACCC**CTGC**TTTCCC**C-3′ (−200/−185 bp region in the mouse StAR promoter, Wt–LXR–RXR/RAR) using the Quikchange site directed mutagenesis kit (Stratagene, La Jolla, CA) (Manna et al., 2014). The sense strand of the oligonucleotide sequence used in mutating the LXR–RXR/RAR site was 5′-CCGTGA**att**CTGCTT**gat**CTATATG-3′ (Mut–LXR–RXR/RAR; mutated bases in lowercase boldface letters) and the mutation was verified by *Eco*RI and *Sau*3A1. The pRL-SV40 plasmid containing the Renilla luciferase gene driven by SV40 promoter was obtained from Promega. Expression plasmids for RARα and RXRα isoforms have been previously described (Manna et al., 2014). All plasmids were confirmed by either restriction edonuclease digestion or sequencing on a PE Biosystems 310 Genetic Analyzer (Perkin-Elmer, Boston, MA).

For transfection studies, different cell types were cultured in either 6- or 12-well plates to ∼70% confluence and were transfected using Lipofectamine 2000 transfection reagent (Invitrogen), under optimized conditions (Manna et al., 2011; Manna et al., 2013; Manna et al., 2014). Transfection efficiency was normalized by co-transfecting 10–20 ng pRL-SV40 vector. The amount of DNA used in transfections was equalized with an empty expression vector.

Luciferase activity in the cell lysates was determined by the Dual-luciferase reporter assay (Promega) system (Manna and Stocco, 2007; Manna et al., 2009a; Manna et al., 2014). Following treatments, cells were washed with 0.01 M PBS and 250 μl of the reporter lysis buffer was added to the cells. Cellular debris was pelleted by centrifugation at 12,000 × g for 10 min at 4 °C, and the supernatant was measured for relative light units (luciferase/Renilla) using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA).

2.2. Immunoblotting

Immunoblotting studies were carried out using total cellular protein (Manna et al., 2011; Manna et al., 2013; Manna et al., 2014). Briefly, equal amounts of protein were loaded onto 10–12% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were electrophoretically transferred onto Immuno-Blot PVDF membranes, which were probed with the specific antibodies (Abs) that recognize StAR (Bose et al., 1999), P-StAR [Ser194; (Manna et al., 2006)], RARα, RXRα (Cell Signaling Technology,