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cAMP-activated *Nr4a1* expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells

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ABSTRACT

In Leydig cells, LH and cAMP promote ERK1/2 activation and MAPK phosphatase-1 (MKP-1) induction. MKP-1 up-regulation, which involves post-translational modifications such as ERK1/2-mediated phosphorylation, reduces ERK1/2 phosphorylation as well as Steroidogenic Acute Regulatory (StAR) protein expression and steroidogenesis. As LH- and cAMP-promoted StAR transcription requires the induction of Nur77, product of *Nr4a1* gene, we analyzed the roles of ERK1/2 and MKP-1 in 8Br–cAMP-mediated *Nr4a1* expression in MA-10 Leydig cells. Pharmacological blockade of ERK1/2 activation partially reduced the 8Br–cAMP-mediated increase in both *Nr4a1* messenger levels and promoter activity. MKP-1 knockdown increased 8Br–cAMP-induced promoter activity, while its over-expression produced the opposite effect. It is concluded that *Nr4a1* induction is dependent on ERK1/2 and that MKP-1 negatively regulates this induction. Experiments based on the over-expression of MKP-1 mutated forms revealed that MKP-1 half life is determined by post-translational modifications in ERK-consensus sites, a regulation that modulates the effect of MKP-1 on *Nr4a1* expression.

1. Introduction

Mitogen-activated protein kinases (MAPK), such as ERKs, JNKs and the members of the p38 subgroup, are involved in several processes like proliferation, differentiation, apoptosis and even steroidogenesis activation (Brion et al., 2011; Cagnol and Chambard, 2010; Raman et al., 2007). As MAPK activation is dependent on phosphorylation in specific threonine and tyrosine residues, the magnitude and duration of MAPK activity are linked to the action of phosphatases capable of dephosphorylating and inactivating them. MAPK phosphatases (MKPs) are a family of dual specificity (threonine and tyrosine) protein phosphatases specifically involved in MAPK regulation (Boutros et al., 2008; Caunt and Keyse, 2013). Through their ability to modulate MAPK, MKPs also modulate all MAPK-dependent processes. Moreover, it has been proposed that the stimuli promoting MAPK activation also promote MKP regulation, which leads to the control of MAPK activity in a precise temporal frame.

MKP-1, a member of the MKP family, is a nucleus-localized phosphatase rapidly induced by different types of stimuli such as hormones (Bey et al., 2003), growth factors (Tong and Hamel, 2007) and stress conditions (Gorostizaga et al., 2013). In adrenocortical and Leydig cells, the adrenocorticotropic hormone (ACTH) and the luteinizing hormone (LH), respectively, promote not only ERK1/2 activation but also MKP-1 induction, which contributes to ERK1/2 dephosphorylation (Bey et al., 2003; Brion et al., 2011). We have demonstrated that, in MA-10 Leydig cells, 8Br–cAMP and LH receptor (LHR) activation by human chorionic gonadotropin (hCG) up-regulate MKP-1 by transcriptional and post-translational mechanisms which include MKP-1 phosphorylation mediated by ERK1/2 (Brion et al., 2011). In addition, we have shown that MKP-1 expression

Abbreviations: ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine monophosphate; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LH, luteinizing hormone; MAPK, mitogen-activated protein kinase; MEK, kinases upstream ERK1/2; MKP, MAPK phosphatase; PKA, protein kinase A; PKC, protein kinase C; RT, reverse transcription; SF1, steroidogenic factor 1; shRNA, short hairpin RNA; StAR protein, Steroidogenic Acute Regulatory protein; WT, wild type.

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reduces the effect of 8Br–cAMP and hCG on steroidogenesis (Brion et al., 2011).

It is well documented that S359 and S364 are consensus sites for ERK1/2 phosphorylation within the MKP-1 sequence, and that phosphorylation on these residues impairs MKP-1 proteosomal degradation (Brondello et al., 1999). MKP-1 phosphorylation on sites related with its instability is less widely described, although ERK1/2 activation by serum is known to promote MKP-1 phosphorylation on S296 and S323, two additional ERK1/2 phosphorylation consensus sites, which in turn induces protein ubiquitination and degradation (Lin and Yang, 2006).

The post-translational regulation of MKP-1 in cAMP-stimulated MA-10 Leydig cells is dependent on ERK1/2 activity and leads to an increase in MKP-1 protein levels through a mechanism which might involve, at least in part, S359 and S364 phosphorylation. However, the role played by multi-site ERK phosphorylation in MKP-1 half life in c-AMP-stimulated Leydig cells remains elusive. Moreover, it is unknown whether ERK-mediated MKP-1 phosphorylation is relevant to its actions on steroidogenesis.

We have further demonstrated that MKP-1 induction by cAMP or LH in MA-10 Leydig promotes ERK1/2 dephosphorylation and reduces the actions of these stimuli on ERK1/2-dependent events involved in steroidogenesis. LH regulates Leydig cell function through a mechanism that involves protein kinase A (PKA) activation (Podesta et al., 1976), and PKA-dependent ERK1/2 activation (Hirakawa and Ascoli, 2003). The rate-limiting step in steroid biosynthesis, i.e. the delivery of cholesterol from the outer to the inner mitochondrial membrane, is facilitated by the steroidogenic acute regulatory (StAR) protein (Clark et al., 1994; Stocco and Clark, 1996). LH regulates steroidogenesis through the induction of StAR gene (Clark et al., 1994; Stocco and Clark, 1996) and the activation of StAR protein (Arakane et al., 1997; Poderoso et al., 2008), two events dependent on PKA (Arakane et al., 1997; Clark et al., 1994) and on ERK1/2 activity (Gyles et al., 2001; Poderoso et al., 2008). StAR gene transcriptional regulation requires transcription factors already present in the cell which are activated by post-translational modifications, such as Steroidogenic Factor 1 (SF1) and others which must be de novo synthesized, e.g. the Nur77 (which is encoded by *Nr4a1* gene) (Martin et al., 2008). ERK1/2 is known to participate in LH-/cAMP-induced activation of SF1 by phosphorylation (Gyles et al., 2001); in contrast, its role in LH-/cAMP-induced expression of the *Nr4a1* gene in Leydig cells has been scarcely analyzed, even though ERK1/2 mediates *Nr4a1* induction in other systems (Bliss et al., 2012; Stocco et al., 2002).

By means of MKP-1 over-expression and knock-down experiments, we have shown that MKP-1 reduces the stimulatory effect of 8Br–cAMP and hCG on ERK1/2 phosphorylation, StAR gene and protein expression and steroidogenesis (Brion et al., 2011). Thus, MKP-1 could exert its regulatory action on StAR gene by reducing the expression and/or activity of ERK-dependent transcription factors.

According to these observations, this study explores the role played by ERK1/2 in cAMP-stimulated *Nr4a1* expression in MA-10 Leydig cells and the impact of MKP-1 and its post-translational modifications on this regulation.

2. Materials and methods

2.1. Materials

Monoclonal antibodies against β-tubulin, Nur77 and FLAG M2 were purchased from Millipore Corporation (Billerica, MA, USA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Sigma (St. Louis, MO, USA), respectively; polyclonal antibodies against phospho-ERK1/2 (pERK1/2) and total ERK1/2 were purchased from New England Biolabs, Inc. (Beverly, MA, USA); horseradish peroxidase-conjugated goat-anti-rabbit, goat-anti-mouse secondary antibodies, as well as immuno-blot polyvinylidene fluoride membrane were purchased from Bio-Rad Laboratories (Hercules, CA, USA); 2-(2-amino-3-methoxyphenyl) 4*H*-1-benzopyran-4-one (PD98059) and 8-bromo–cAMP (8Br–cAMP) were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell cultures

The MA-10 cell line, a clonal strain of mouse Leydig tumor cells generously provided by Dr. Mario Ascoli (University of Iowa, College of Medicine, Iowa City, IO, USA), was handled as originally described (Ascoli, 1981). After 24 h of serum starvation, the cells were incubated with 8Br–cAMP or other agents.

2.3. RNA extraction and real-time PCR

Total RNA was extracted using Tri Reagent following the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, OH, USA). Reverse transcription was done using 2 μg of total RNA as previously described (Castilla et al., 2008) and real-time PCR was performed as previously described (Brion et al., 2011) using a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad Laboratories, Hercules, CA, USA). Reactions were carried out using the SYBR Green Master Mix reagent kit (Applied Biosystems, Carlsbad, CA, USA) and the following specific primers: for *Nr4a1*, forward 5′-GGCTTCTTCAAGCGCACAGT-3′ and reverse 5′-GCTGCTTGGGTTTT GAAGGTAG-3′; for GAPDH cDNA, forward 5′-TGCACCACCAACTGC TTAGC-3′ and reverse 5′-GCATGGACTGTGGTCATGAG-3′. Cycling conditions for *Nr4a1*, step 1: 95 °C 10 min, step 2: 95 °C 15 s, step 3: 62.5 °C 1 min (steps 2 and 3 were repeated 40 times). Cycling conditions for GAPDH, step 1: 95 °C 10 min, step 2: 95 °C 15 s, step 3: 60 °C 1 min (steps 2 and 3 were repeated 40 times). Assessment of quantitative differences between samples in the cDNA target was performed as previously described (Brion et al., 2011).

2.4. Plasmid constructs

pGL3-NR4A1 was generously provided by Takashi Yazawa, Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Fukui, Japan (Inaoka et al., 2008). pFLAG-MKP-1, pSUPER.retro.MKP-1 and pFLAG S359A-S364A-MKP-1 were constructed as previously described (Brion et al., 2011). pSUPER.retro.MKP-1 encodes for a shRNA-MKP-1 which efficiently abrogates endogenous MKP-1 expression in MA-Leydig cells (Brion et al., 2011).

The Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was employed to replace serine 296 and 323 with alanine in the pFLAG-MKP-1 sequence to generate the flag-S296A-S323A-MKP-1 double mutant. First, pFLAG-S296A-MKP-1 was obtained using the following oligonucleotides: sense 5′-GAGGC GGAGTATCATCGCCCCGAACTTCAGC-3′ and antisense 5′-GCTGAAGTT CGGGGCGATGATACTCCGCCTC-3′. Then, pFLAG-S296A-MKP-1 was amplified with the specific oligonucleotides to mutate serine 323: sense 5′-CTCTGCTGAAGCTGGGGCCCCTGCCATGGCTGTC-3′ and antisense 5′-GACAGCCATGGCAGGGGCCCCAGCTTCAGCAGAG-3′ (mutated nucleotides are underlined).

2.5. Transfection assays

Cells were seeded the day before transfection, grown up to 80% confluence and transfected during 6 h using Lipofectamine 2000 reagent in Opti-MEM medium according to the manufacturer's instructions (Invitrogen, Life Technologies, Grand Island, NY, USA).