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MAP kinase phosphatase-3 (MKP-3) is transcriptionally and post-translationally up-regulated by hCG and modulates cAMP-induced p21 expression in MA-10 Leydig cells

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ABSTRACT

Luteinizing hormone (LH) activates ERK1/2, MAP kinases (MAPKs) necessary for its action on steroidogenesis and cell proliferation, and also induces MAPK phosphatase-1 (MKP-1), which rapidly dephosphorylates nuclear ERK1/2. MKP-3 is a cytoplasmic ERK-phosphatase up-regulated by proliferative stimuli. MKP-3 also dephosphorylates transcription factor FOXO1, promoting its transport to the nucleus. Here we analyzed MKP-3 expression in MA-10 Leydig cells and demonstrated that LH receptor (LHR) activation with human gonadotropin hormone (hCG) and an analog of its second messenger, 8Br-cAMP, up-regulates MKP-3 by transcriptional and post-translational mechanisms. It is known that FOXO1 drives the expression of the cell cycle inhibitor p21. Since the activation of this transcription factor by MKP-3 has been reported, we assessed the effect of shRNA against MKP-3 on p21mRNA levels. 8Br-cAMP increased these levels (2-fold at 2 h) and MKP-3 down-regulation reduced this effect. Our work demonstrates that LH/hCG tightly up-regulates MKP-3 which in turn, dephosphorylates ERK1/2 and drives p21 expression. These events could contribute to counteract hormonal action on cell proliferation.

1. Introduction

Mitogen-activated protein kinases (MAPKs: ERKs, JNKs and p38) exert profound effects on a variety of physiological processes such as proliferation, differentiation and apoptosis (Ashwell, 2006; Raman et al., 2007). MAPKs display maximal enzymatic activity upon phosphorylation on threonine and tyrosine, thus, the magnitude and duration of their activity are linked to the action of phosphatases able to dephosphorylate and inactivate them.

MAPK phosphatases (MKPs) are dual specificity (threonine and tyrosine) phosphatases (DUSPs) involved in MAPK regulation (Boutros et al., 2008; Huang and Tan, 2012; Keyse, 2008). MKP family members differ in their subcellular localization, tissue-specific expression, inducibility by various types of signals, induction kinetics and selectivity for dephosphorylating specific MAPKs. MKP-1 and -3 are archetypes of the MKP family. MKP1 (or DUSP1) is a nucleus-localized phosphatase induced by different growth factors and stress signals while MKP-3 (or DUSP6) is a cytoplasmic enzyme induced by different proliferative stimuli – but not by environmental stress – and is characterized as a highly specific phosphatase for attenuating ERK1/2 signaling (Zhao and Zhang, 2001). Accordingly, enhanced basal ERK1/2 phosphorylation has been described in the heart of MKP-3 deficient mice (Maillet et al., 2008). Therefore, MKPs exhibiting different subcellular localization and induction kinetics lead to strict spatio-temporal control of MAPKs.

It is accepted that MKP substrates are confined specifically to MAPKs. However, at least for a few members of the MKP

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DUSPs, dual specificity phosphatases; FGF, fibroblast growth factor; FOXO1, Forkhead box protein O1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GnRH, gonadotropin releasing hormone; L19, ribosomal L19 protein; LH, luteinizing hormone; LHR, luteinizing hormone receptor; MAPK, mitogen-activated protein kinase; MEK1/2, kinases upstream ERK1/2; MKP, MAPK phosphatase; NES, leucine-rich nuclear export signal; P-ERK1/2, phospho-ERK1/2; RT, reverse transcription; shRNA, short hairpin RNA; StAR protein, Steroidogenic Acute Regulatory protein; STARD1, StAR gene.

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family, growing evidence supports the notion that MKP substrates constitute a broader group of phosphoproteins (Huang and Tan, 2012). Indeed, it has been reported that MKP-3 interacts with and dephosphorylates Forkhead box protein O1 (FOXO1), an event that promotes its nuclear translocation and subsequent activation of key gluconeogenic genes (Jiao et al., 2012; Wu et al., 2010). FOXO1 participates in the regulation of genes related not only to metabolism but also to other processes, like cell cycle arrest, through the induction of the cell cycle inhibitor p21 (Roy et al., 2010). Moreover, FOXO1 negatively regulates ovarian granulose cell proliferation and differentiation induced by FSH (follicle-stimulating hormone) (Park et al., 2005). In addition, in gonadotrope cells, FOXO1 represses basal and GnRH-induced (gonadotropin releasing hormone) transcription of the LH (luteinizing hormone) beta-subunit (Arriola et al., 2012). Therefore, all these processes are potentially modulated by MKP-3.

In steroidogenic cells, the corresponding trophic hormones up-regulate MKPs (Bey et al., 2003; Brion et al., 2011; Casal et al., 2007; Sewer and Waterman, 2003), which play a role in the regulation of steroidogenesis (Bey et al., 2003; Brion et al., 2011; Casal et al., 2007; Sewer and Waterman, 2003). In Leydig cells, LH regulates cell function through a mechanism involving protein kinase A (PKA) activation (Podesta et al., 1976) and PKA-dependent ERK1/2 activation (Hirakawa and Ascoli, 2003). Moreover, Evaul and Hammes demonstrated that, in both mouse tumor Leydig cell lines as well as primary mouse Leydig cells, LH-induced cAMP/PKA leads to epidermal growth factor receptor (EGFR) transactivation, which then activates ERK1/2 (Evaul and Hammes, 2008). LH up-regulates Steroidogenic Acute Regulatory (StAR) protein, which facilitates the access of cholesterol to the inner mitochondrial membrane (Stocco and Clark, 1996), the rate-limiting step of steroid synthesis (Crivello and Jefcoate, 1980). Because ERK activity participates in the expression of the gene encoding for StAR protein (STARD1) (Gyles et al., 2001) and also in StAR protein activation (Poderoso et al., 2008), the down-regulation of steroidogenesis by MKPs is expected. We have demonstrated that the activation of the LH receptor with human Chorionic Gonadotropin (hCG) rapidly induces MKP-1, and that the down-regulation of this enzyme by a specific *short hairpin* RNA (shRNA) increases the hormonal effects on ERK1/2 activity, StAR gene expression and steroidogenesis (Brion et al., 2011). Our results highlight the participation of ERK1/2 in steroidogenesis and are consistent with the conclusions reached by Evaul and Hammes, who postulated that the activation of GFR/ERK1/2 pathway is necessary for early LH-induced steroidogenesis (Evaul and Hammes, 2008).

In addition to the regulation of steroid synthesis, LHR activation increases Leydig cell proliferation. In primary cultures of rat Leydig cells, LHR stimulation induces cell proliferation through an ERK-dependent pathway (Shiraishi and Ascoli, 2007). The same conclusion was drawn using a more physiological model consisting of adult mice carrying a testicular-specific deletion of MEK (kinase upstream ERK1/2), which show Leydig cell hypoplasia and hypergonadotropic hypogonadism, among other abnormalities (Yamashita et al., 2011). Moreover, primary cultures of Leydig cells from these mice exhibit low phospho-ERK1/2 (P-ERK1/2) levels after hCG or cAMP stimulation (Yamashita et al., 2011). Therefore, the regulation of cell proliferation by LH could be an additional target of MKPs.

As LH is a proliferative stimulus for Leydig cells, we hypothesized that LHR activation regulates MKP-3 expression. This phosphatase could promote p21 expression, through dephosphorylation and activation of FOXO1. To test this hypothesis, we examined the effect of hCG on MKP-3 and the impact of MKP-3 down-regulation on p21 expression.

2. Materials and methods

2.1. Materials

Monoclonal antibodies against MKP-3, β-tubulin and FLAG M2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Millipore Corporation (Billerica, MA, USA) and Sigma (St. Louis, MO, USA), respectively. Polyclonal antibodies against P-ERK1/2 and total ERK1/2 were from Cell Signaling Technology, Inc. (Boston, MA, USA). 2-(2-Amino-3-methoxyphenyl) 4H-1-benzopyran-4-one (PD98059), actinomycin D and 8-Bromo-cAMP (8Br-cAMP) were from Sigma (St. Louis, MO, USA) and human chorionic gonadotropin (purified hCG, batch CR-125 of biological potency 11900 IU/mg) was a gift from NIDDK, NIH (Bethesda, MA, USA). All other reagents were of highest quality available.

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, IO, USA), was handled as originally described (Ascoli, 1981). After 24 h of serum starvation, the cells were incubated with hCG, 8Br-cAMP or other agents.

2.3. Plasmid constructs

FLAG-tagged MKP-3 construct (pFLAG-MKP-3) was generated using the p3xFLAG-CMV™-7.1 expression vector. Mouse total RNA was used to obtain total cDNA by reverse transcription. From this sample, a fragment of 1188 kb corresponding to the full-length coding region of MKP-3 (NM_026268.3) was amplified using the following primers: forward, TTGCGGCCGCTATGATAGATACGCTCA-GA (which contains the cleavage site for NotI) and reverse, ACGCGAATGAAGGAATGGGGACAACTC (which contains the cleavage site for EcoRI). This fragment was purified and fused into p3xFLAG-CMV™-7.1 using NotI/EcoRI restriction sites. p3xFLAG-CMV-7.1 containing ERK2 (pFLAG-ERK2) was kindly provided by Melanie Cobb (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX).

A vector for the expression of shRNA against MKP-3 under the control of the polymerase-III H1-RNA promoter (pSUPER.retro-MKP-3) was obtained using the pSUPER.retro vector (OligoEngine, Seatle, WA, USA). A pair of 60-nt annealed DNA oligonucleotides (containing a 17-nucleotide target sequence derived from murine MKP-3 mRNA) was inserted between the *Bgl*II/*Hind*III restriction sites of the pSUPER.retro vector. The set of 60-nt oligos containing this sequence for MKP-3 shRNA is described below: sense, 5′-GATCCCCACGACATTGTTAAGATGAATTCAAGAGATTCATCTTAACAAT GTCGTTTTTTA-3′ and antisense, 5′-AGCTTAAAAAACGACATTGT-TAAGATGAATCTCTTGAATTCATCTTA ACAATGTCGTGGG-3′. Correct in-frame insertions were verified by sequencing.

2.4. 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 manufacturer's instructions (Invitrogen, Life Technologies, Inc.-BRL, Grand Island, NY, USA).

2.5. 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 using the following specific primers: for MKP-3 cDNA, forward, 5′-ATAGATACGCTCAGACCCGTG-3′ and reverse 5′-ATCAGCAGAAGCCGTTCGTT-3′ (Jiao et al., 2012); for p21 cDNA, forward 5′-TTGGAGTCAG GCGCAGATCCACA-3′ and reverse 5′-CGCCATGAGCGCATCGCAATC-3; for GAPDH cDNA, forward 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′-GCATGGACTGTGGTCATGAG-3′. Cycling conditions for p21 were step 1: 95 °C 10 min, step 2: 95 °C 15 s, step 3: 64 °C 1 min, steps 2 and 3 were repeated 40 times. For MKP-3 and 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 in the cDNA target between samples was performed as previously described (Brion et al., 2011).

2.6. Western blot analysis

Proteins were subjected to SDS–PAGE (10%) and electrotransferred onto polyvinylidine fluoride membranes as previously described (Brion et al., 2011). Immunoblotting was performed using the following antibody dilutions: mouse monoclonal anti-flag (1:10,000), mouse monoclonal anti-MKP-3 (1/100), rabbit polyclonal anti-P-ERK1/2 (1:5000), mouse monoclonal anti-β-tubulin (1:5000) or rabbit polyclonal anti total ERK1/2 (1:20000). Bound antibodies were developed by incubation with secondary antibody (goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated) and detected using the enhanced chemiluminescence detection reagent (GE Life Sciences, Princeton, NJ, USA).

2.7. Immunofluorescence and microscopy

MA-10 cells were grown on poly-L-lysine-coated glass coverslips as previously described (Brion et al., 2011) and incubated, following the respective treatments, with anti-MKP-3 or anti-flag, in a humidified chamber for 24 h at 4 °C. Primary antibodies were detected by cy3-conjugated goat anti-(mouse IgG) Ig. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The glass coverslips were mounted in FluorSave reagent (Calbiochem, San Diego, CA) and examined in an epifluorescence microscope (Olympus, Tokyo, Japan). Images of cells were analyzed using ImageJ software. The fluorescence intensity of each cell was evaluated, normalized against the corresponding area and expressed in arbitrary units.

2.8. Statistical analysis

Results are shown as the mean ± SEM. Statistical significance was evaluated using ANOVA followed by Tukey test. Differences were deemed significant when $P < 0.05$.

3. Results

3.1. hCG and 8Br-cAMP activate MKP-3 gene transcription in MA-10 Leydig cells

We first determined the effect of hCG on MKP-3 mRNA levels in MA-10 Leydig cells. As shown in Fig. 1A, hCG produced a transient increase in messenger levels. MKP-3 mRNA levels were significantly raised after 2 h, peaked at 3 h (3-fold) and returned to basal levels at 6 h. An analog of cAMP, 8Br-cAMP, also increased messenger levels in a similar way (Fig. 1B).

Pulse-chase experiments were performed in the presence of actinomycin D in order to evaluate the stability of MKP-3 mRNA from both control and stimulated cells. The rates of decay, assessed by measuring the levels of the messenger at different times after addition of actinomycin D, were similar in samples from non-stimulated and 8Br-cAMP-stimulated cells (Fig. 2A). Moreover, similar results were obtained in hCG-stimulated cells (data not shown). These results suggest that both hCG and 8Br-cAMP raise MKP-3 mRNA levels by increasing gene transcription rather than stabilizing MKP-3 mRNA transcripts. This effect seemed to be dependent on ERK activity, as the MEK inhibitor PD98059 reduced the effect of 8Br-cAMP on MKP-3 mRNA levels (Fig. 2B).

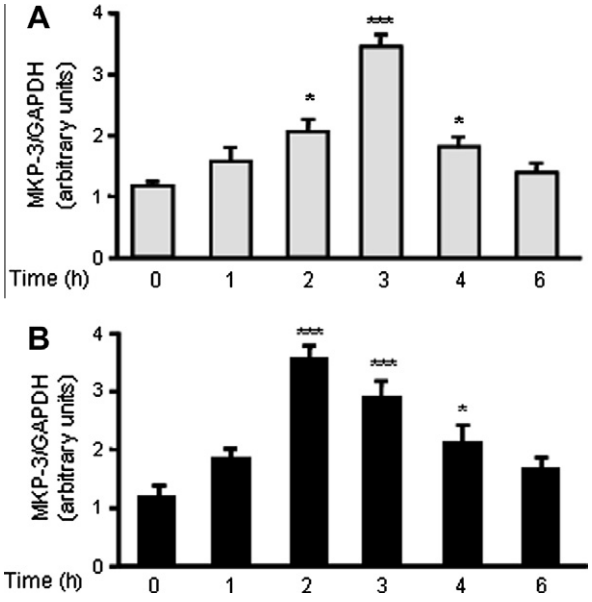

Fig. 1. hCG and 8Br-cAMP increase MKP-3 levels in MA-10 Leydig cells. MA-10 Leydig cells were serum-starved for 24 h and then incubated with 10 ng/ml hCG (A) or 0.5 mM 8Br-cAMPc (B) for the indicated times. Total RNA was isolated and subjected to reverse transcription and Real-time PCR using specific primers for MKP-3 and GAPDH as loading control. Data are expressed in arbitrary units and represent the mean ± SEM of three independent experiments.*, $P < 0.05$ and ***, $P < 0.001$ vs. non-stimulated cells by ANOVA followed by Tukey test.

3.2. cAMP promotes MKP-3 protein accumulation in MA-10 Leydig cells

The expression and subcellular localization of MKP-3 in control and stimulated cells were analyzed by immunofluorescence microscopy. MKP-3 was barely detected in both the cytoplasm and nucleus of unstimulated cells (Fig. 3). Stimulation with 8Br-cAMP increased the intensity of MKP-3 signal in both compartments and in a time-dependent manner (Fig. 3).

3.3. MKP-3 is post-translationally regulated by hCG in MA-10 Leydig cells

Next, we evaluated whether hCG regulates MKP-3 expression by a post-translational mechanism. For this purpose, MA-10 Leydig cells were transiently transfected with pFLAG-MKP-3, stimulated with hCG for different times and then, flag-tagged MKP-3 protein levels evaluated by Western blot using an anti-flag antibody. Since flag-tagged MKP-3 protein expression is driven by a constitutive promoter, differences between MKP-3 protein levels from stimulated and non-stimulated cells are attributed to a post-translational action of the stimuli. As shown in Fig. 4A, hCG stimulation provoked a transient increase in the amount of flag-MKP-3 protein. This amount was significantly higher than in control cells after 1 h of stimulation, remained elevated for 4 h (3-fold) and declined

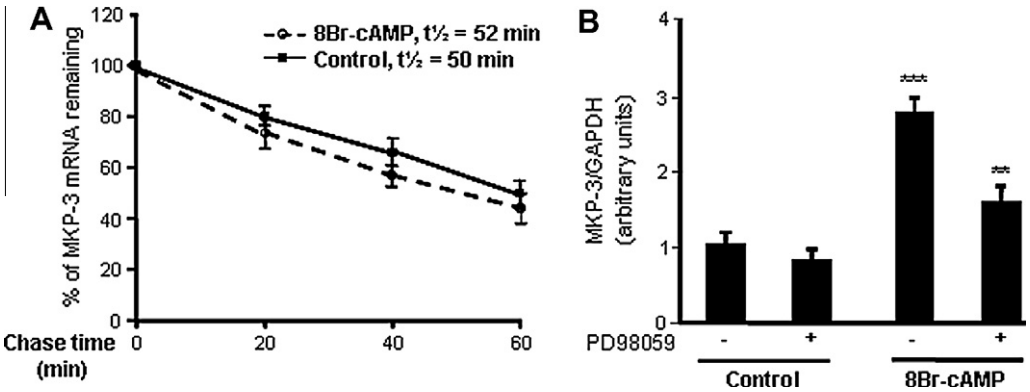

Fig. 2. cAMP increases MKP-3 gene transcription by a mechanism partially dependent on ERK1/2. MA-10 cells were serum-starved for 24 h and then treated as follows: (A), incubated with or without 0.5 mM 8Br-cAMP for 2 h and then incubated with actinomycin D for different times (30 min of actinomycin = chase time 0); (B), incubated with or without 50 μM PD98059 for 40 min and then stimulated with 0.5 mM 8Br-cAMP for 2 h. Total RNA was isolated and subjected to reverse transcription and Real-time PCR using specific primers for MKP-3 and GAPDH as loading control. (A): Normalized levels of MKP-3 mRNA after actinomycin D addition are expressed as a percentage of those at time 0. (B): Data are expressed in arbitrary units and represent the mean ± SEM of three independent experiments. ***, $P < 0.001$ vs. non-stimulated cells, **, $P < 0.01$ vs. stimulated cells incubated with PD98059, by ANOVA followed by Tukey test.

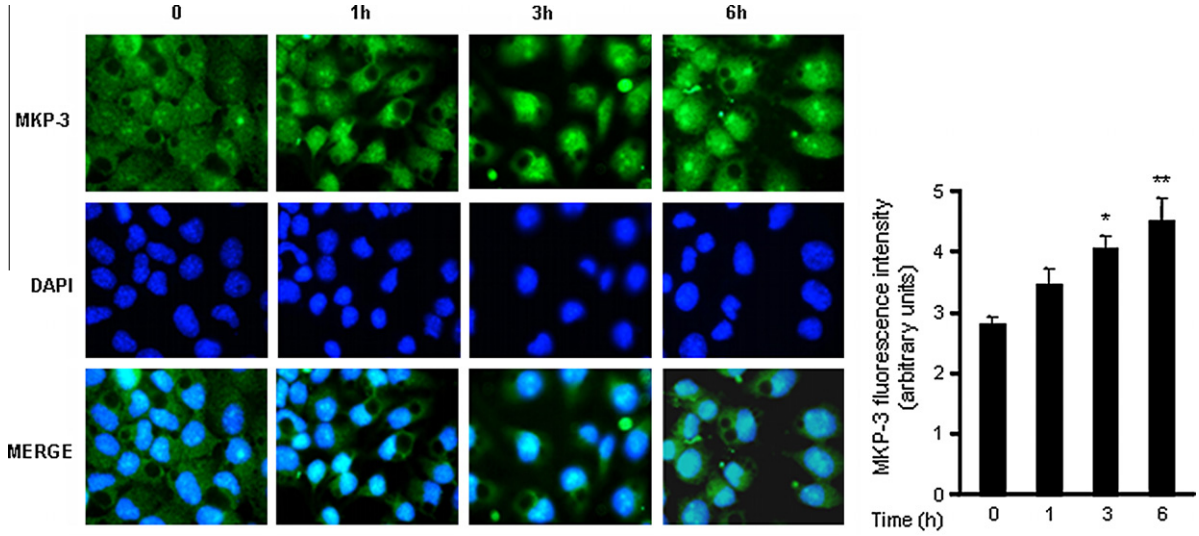

Fig. 3. MKP-3 protein is accumulated in both cytoplasm and nucleus in 8Br-cAMP-stimulated cells. Cells were grown on coverslips, serum-starved for 24 h and stimulated for the indicated times with 0.5 mM 8Br-cAMP, fixed, stained with an antibody against MKP-3 (*green*) and DAPI (*blue*) and then subjected to immunofluorescence microscopy (*left panel*). The fluorescence intensity of each cell was evaluated (80–100 cells per treatment), normalized against the corresponding area and expressed in arbitrary units and represent the mean ± SEM from three separate experiments *(right panel)*.

thereafter (Fig. 4A). 8Br-cAMP-induced the accumulation of the recombinant protein in a similar way (Fig. 4B). In addition, 8Br-cAMP induced stimulation had no effect on the accumulation of other flag-tagged proteins such as flag-ERK2, which indicates that the effect of hCG/cAMP on flag-MKP-3 is specific for the MKP-3 moiety of the chimera (Fig. 4C). Taken together, our results suggest that, in stimulated cells, flag-MKP-3 accumulation is probably due to a post-translational stabilization of the protein mediated by PKA.

We also analyzed the levels of MKP-3 by Western blot after 2 h of 8Br-cAMP stimulation, when the maximal increase in flag-MKP-3 accumulation was observed. In 8Br-cAMP-treated cells, MKP-3 was detected as a much stronger signal than in control cells (Fig. 4D).

3.4. MKP-3 modulates P-ERK1/2 levels in 8Br-cAMP-stimulated cells

Next, we analyzed whether MKP-3 expression modified 8Br-cAMP-stimulated P-ERK1/2 levels. As observed in Fig. 5A, pFLAG-MKP-3-transfected cells showed lower P-ERK1/2 levels when compared to mock-transfected cells. These results demonstrate that MKP-3 recognizes P-ERK1/2 as a substrate in Leydig cells. On the other hand, down-regulation of MKP-3 by shRNA increased P-ERK1/2 levels (Fig. 5B). It is worth pointing out that this shRNA was efficient in downregulating MKP-3 mRNA and flag-MKP-3 (Fig. 5C).

3.5. cAMP increases p21 mRNA levels in an MKP-3-dependent manner

We also aimed to determine the effect of cAMP on p21 expression and the putative participation of MKP-3 in this regulation. For this, p21 mRNA levels were evaluated by Real-time PCR. A time-course analysis of p21 mRNA levels showed a transient accumulation of this messenger in 8Br-cAMP-treated cells. The effect was statistically significant, reached a maximum (2-fold increase) at 2 h and returned to basal levels after 6 h (Fig. 6A).

Next, we tested the effect of shRNA-silencing of MKP-3 expression on p21 mRNA levels in both 8Br-cAMP-stimulated (0.5 mM, 2 h) and non-stimulated cells. As shown in Fig. 6B, 8Br-cAMP stimulation increased the levels of p21 mRNA in mock-transfected cells, while down-regulation of MKP-3 reduced this effect (Fig. 6B).

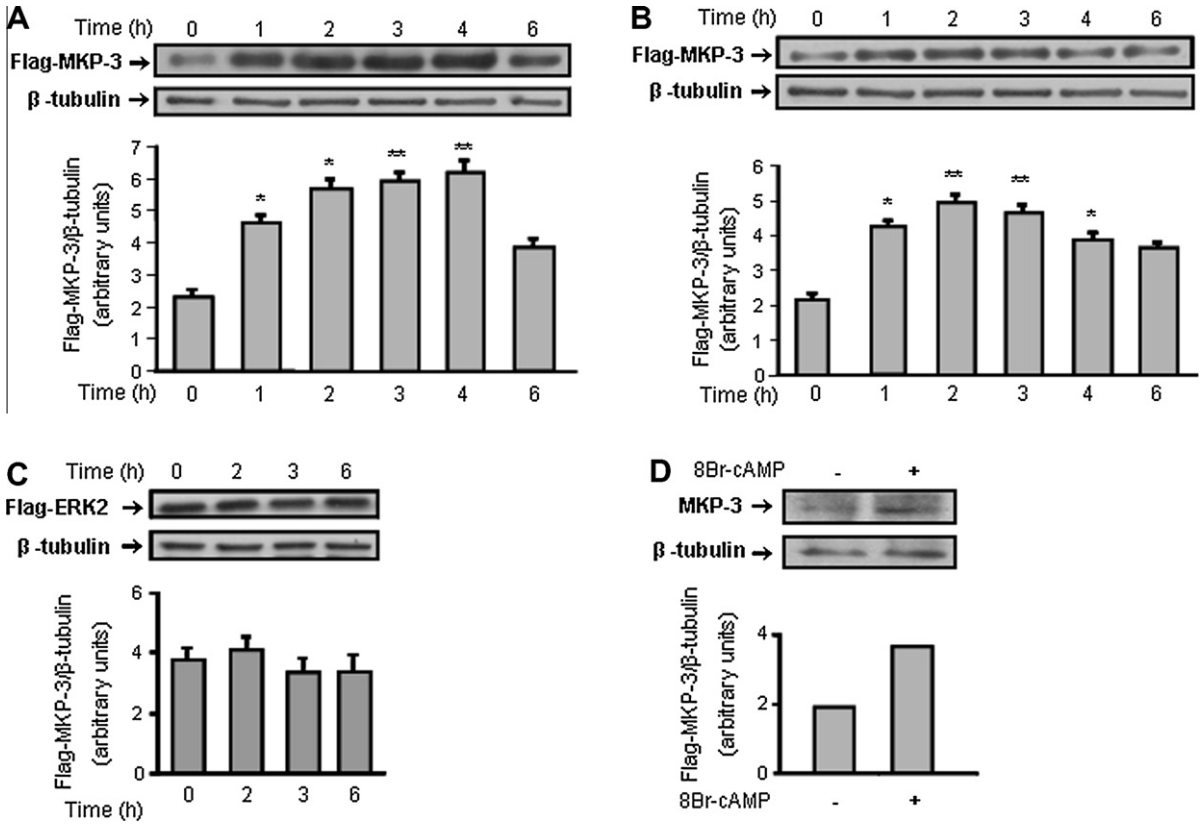

Fig. 4. hCG and 8Br-cAMP up-regulate MKP-3 by a post-translational mechanism: MA-10 cells were transiently transfected with pFLAG-MKP-3 expression vector (A and B) or pFLAG-ERK2 expression vector (C). After 24 h, cells were serum-starved for 24 h and incubated with 10 ng/ml hCG (A) or 0.5 mM 8Br-cAMP (B and C) for the indicated times. Cells were then lysed and flag-MKP-3 (A and B) or flag-ERK2 (C) levels analyzed by Western blot using an anti-flag antibody. (D), MA-10 cells were serum-starved for 24 h and incubated with 0.5 mM 8Br-AMPc for 2 h. Cells were then lysed and MKP-3 levels analyzed by Western blot using an anti-MKP-3 antibody (D). Membranes were then stripped and β-tubulin was detected as loading control. Each panel shows representative immunoblots (*upper panels*). Integrated optical density of each specific band was quantitated and the values were normalized against β-tubulin abundance. Data are expressed in arbitrary units and represent the mean ± SEM of three independent experiments (*lower panels*). *, $P < 0.05$ **, $P < 0.01$ and ***, $P < 0.001$ vs. non-stimulated cells by ANOVA followed by Tukey test.

4. Discussion

The present study provides evidence, for the first time, that LHR activation up-regulates MKP-3 expression by transcriptional and post-translational mechanisms in MA-10 Leydig cells. Our results also indicate that MKP-3 induction could contribute to modulate LH action on Leydig cell proliferation through the up-regulation of the cell cycle inhibitor p21, as 8Br-cAMP provokes p21 mRNA accumulation and the down-regulation of MKP-3 abrogates this effect.

MKP-3 messenger is accumulated in hCG- or 8Br-cAMP-stimulated cells with similar temporal profiles. Hypoxia-induced increments in MKP-3 mRNA due to ERK-dependent messenger stabilization has been described (Bermudez et al., 2011). However, in our system, hCG/8Br-cAMP up-regulate MKP-3 mRNA without changes in messenger half life. Therefore, we conclude that the effects of hCG and of cAMP on MKP-3 mRNA accumulation are exerted at a transcriptional level and involve the MEK-ERK pathway. The presence of a conserved binding site for the Ets (E twenty-six) family of transcriptional regulators (which functions as a target for ERK signaling) has been found within the MKP-3 gene promoter region (Ekerot et al., 2008). Indeed, regulation of MKP-3 expression by fibroblast growth factor (FGF) through binding of Ets2 to the promoter region of the gene has been demonstrated (Ekerot et al., 2008). Even though our results agree with these findings, it should be mentioned that PKA-driven events, both ERK-dependent and -independent, act together to promote the increase in MKP-3 mRNA levels triggered by hCG.

Regarding the post-translational regulation of MKP-3, our studies suggest that, in hCG- or 8Br-cAMP-treated cells, this protein undergoes a post-translational modification that provokes its accumulation. In MA-10 Leydig cells, LH/hCG leads to ERK activation via PKA (Hirakawa and Ascoli, 2003). Then, the accumulation of flag-MKP-3 detected in hCG- or 8Br-cAMP-stimulated cells could be explained by an ERK-dependent modification that increases protein stability. ERK-mediated phosphorylation of MKP-3 at two different serine residues has been demonstrated in fibroblast exposed to serum, although this modification reduced MKP-3 stability due to enhanced proteasomal degradation of the protein (Marchetti et al., 2005). In view of those results, it seems unlikely that hCG or 8Br-cAMP will cause the phosphorylation of MKP-3 at the same residues reported by Marchetti and coworkers in serum-stimulated fibroblasts. Noteworthy, an aminoacid sequence analysis of MKP-3 shows the presence of several consensus sites for phosphorylation by different kinases, as well as for other post-translational modifications. Based on the results presented here, our current efforts aim to determine the post-translational modification responsible for hCG/8Br-cAMP-induced MKP-3 stabilization.

It has been demonstrated that MKP-3 shuttles between the nucleus and cytoplasm. However, the export process predominates under steady-state conditions, which results in a largely cytoplasmic localization for MKP-3 (Karlsson et al., 2004). In our studies, endogenous MKP-3, as well as the flag-tagged version of the protein (data not shown), is induced by 8Br-cAMP and remains elevated and equally distributed between the nucleus and the cytoplasm up to 6 h of stimulation as demonstrated by fluores-

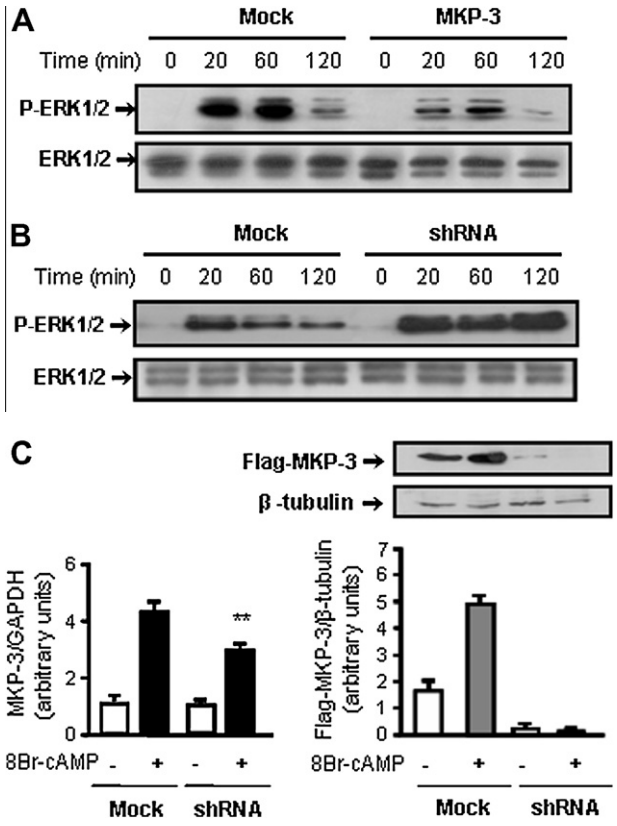

Fig. 5. MKP-3 down-regulation increases P-ERK1/2 levels reached by 8Br-cAMP stimulation: A and B, cells were transfected with pFLAG-MKP-3 for the over-expression of flag-MKP-3 (MKP-3) or the empty plasmid (Mock) (A) or pSUPER.retro-MKP-3 for the expression of shRNA against MKP-3 (shRNA) or the empty plasmid (Mock) (B). Cells were incubated with 0.5 mM 8Br-cAMPc for the indicated times. Then cells were lysed and P-ERK1/2 analyzed by Western blot using an anti-P-ERK antibody (A and B). Membranes were then stripped and total ERK1/2 detected as loading control. The figures show representative Western blot of P-ERK1/2 levels (*upper panels*) and total ERK1/2 (*lower panels*). Each experiment was performed at least three times with similar results. (C), cells were transfected with pSUPER.retro-MKP-3 for the expression of shRNA against MKP-3 (shRNA) or the empty plasmid (Mock) and incubated with 0.5 mM 8Br-cAMPc for the indicated times. Total RNA was isolated and subjected to reverse transcription and Real-time PCR using specific primers for MKP-3 and GAPDH as loading control (*left panel*) or cells were then lysed and flag-MKP-3 levels analyzed by Western blot using an anti-flag antibody (*right panel*).

cence microscopy using a specific monoclonal antibody. The nuclear export of a group of proteins is mediated by transport receptors that bind to specific sequences in cargo proteins. Interestingly, one of those sequences is a leucine-rich nuclear export signal (NES) that is present in the amino terminus of MKP-3. This NES sequence is not only necessary for the nuclear export of MKP-3 but also for its binding to ERK2 and the export of this kinase from the nucleus (Karlsson et al., 2004). Therefore, the abundance of both MKP-3 and the transport receptors present in each cell type, along with the binding affinity between these proteins may determine the efficiency of nuclear export of MKP-3, which would explain the presence of MKP-3 in the nucleus of MA-10 Leydig cells.

A relevant question about MKP-3 expression in Leydig cells is the significance of its induction under LHR stimulation. Given that MKP-3 binds to ERK2 regardless of its phosphorylation state (Camps et al., 1998), it has been proposed that MKP-3 can access the nucleus, bind to ERK2 there to facilitate the translocation of inactive ERK2 to the cytoplasm (Karlsson et al., 2004). Whether the induction of MKP-3 by hCG/8Br-cAMP contributes to the re-localization of ERK2 in the cytoplasm after stimulation of Leydig cells deserves further studies. The present results show that shRNA-silencing of MKP-3 results in higher levels of P-ERK1/2 after 8Br-cAMP stimulation. We have previously demonstrated that the same stimulus rapidly induces MKP-1, which is accumulated in the nucleus and contributes to the early P-ERK1/2 dephosphorylation (Brion et al., 2011). In addition, we have demonstrated that 8Br-cAMP-induced MKP-1 down-regulates StAR expression (Brion et al., 2011). Therefore, the main role of MKP-3 in our system may involve its activity in the cytoplasm in connection with an LH-regulated function different from the acute regulation of steroid synthesis. For this reason we have focused on the study of cell-cycle regulator p21. The expression of p21 is dependent on FOXO1 activation (Roy et al., 2010), which requires its dephosphorylation by MKP-3 for its subsequent translocation to the nucleus where it can regulate gene expression (Jiao et al., 2012; Wu et al., 2010). Our results show, for the first time, that LHR activation increases p21 mRNA levels and that this effect is down-regulated by MKP-3. Although the mechanism involved in the regulation of p21 expression by MKP-3 is yet to be elucidated, LH/hCG could trigger ERK1/2 activation and Leydig cell proliferation and, at later stage, induce MKP-3. The phosphatase may, in turn, down-regulate both processes through ERK1/2 dephosphorylation and p21 induction, thus acting as a negative regulator of cell proliferation. Fig. 7

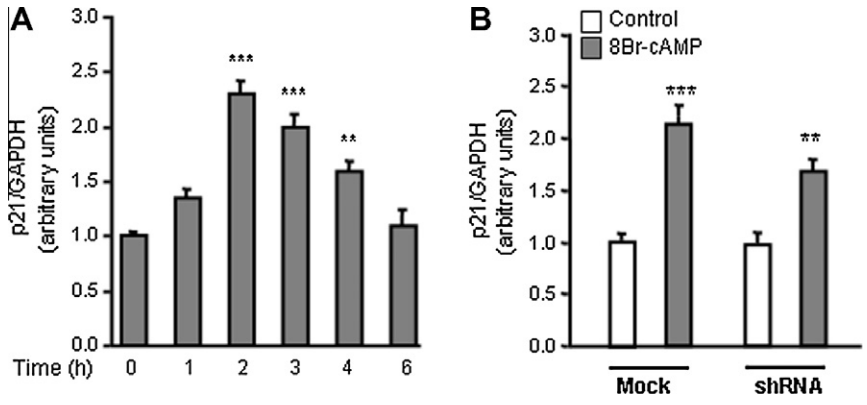

Fig. 6. 8Br-cAMP promotes p21 mRNA levels increase by a MKP-3 dependent mechanism. Cells were incubated with 0.5 mM 8Br-cAMPc for the indicated times (A). Cells were transfected with the vector pSUPER.retro-MKP-3 to express the shRNA against MKP-3 (shRNA) or the empty vector (Mock) and incubated with 0.5 mM 8Br-cAMPc for 2 h (B). Total RNA was isolated and subjected to reverse transcription and Real-time PCR using specific primers for p21 and GAPDH as loading control. The levels of p21 mRNA were normalized against the corresponding GAPDH mRNA levels. Data represent the mean ± SEM of three independent experiments. (A): **, $p < 0.01$ and ***, $p < 0.001$ vs. non-stimulated cells; (B): *** $P < 0.001$ vs. mock-transfected non-stimulated cells, ** $P < 0.01$ vs. mock-transfected stimulated cells with 8Br-cAMP, by ANOVA followed by Tukey test.

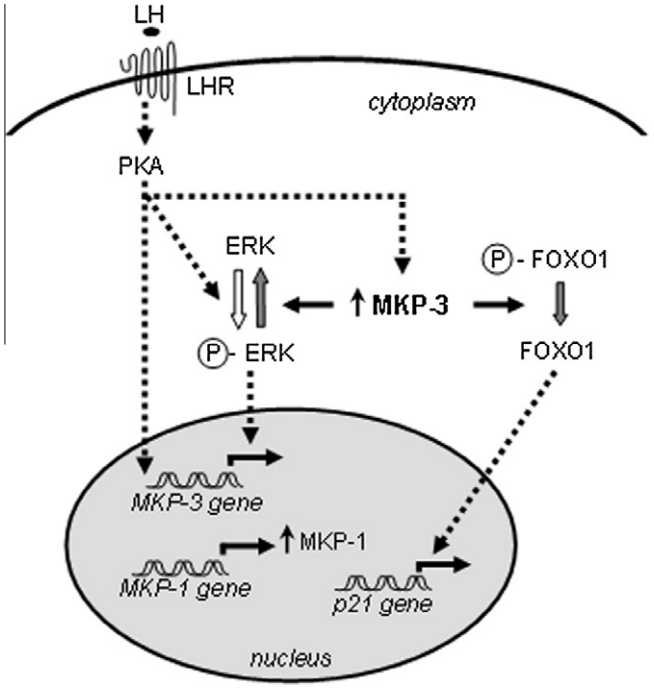

Fig. 7. Proposed model for the role of MKP-3 in the action of LH on Leydig cells. Binding of LH to its receptor triggers the activation of PKA, which leads to the rapid phosphorylation of ERK1/2. Activated ERK (P-ERK) translocates to the nucleus where, together with PKA, rapidly promotes MKP-1 expression. MKP-1 remains in the nucleus where dephosphorylates P-ERK. PKA and P-ERK also trigger MKP-3 expression. MKP-3 accumulates in the cytoplasm where dephosphorylates P-ERK and FOXO1, which translocates to the nucleus and drives p21 gene expression. Direct effects are indicated by *solid lines*, whereas indirect effects are indicated as *dotted lines*.

shows a cartoon depicting the regulation of MKP-3 by LH and its role in p21 expression.

In summary, we described here that hCG and 8Br-cAMP up-regulate MKP-3 expression acting at both transcriptional and post-translational levels. We propose that LH regulation of MKP-3 expression could play a double role in Leydig cells: (1) the re-localization of ERK upon cell stimulation via cytosolic dephosphorylation of the kinase and (2) attenuation of the hormonal effect on cell proliferation through p21 induction.

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