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MKP-1 mediates glucocorticoid-induced ERK1/2 dephosphorylation and reduction in pancreatic β-cell proliferation in islets from early lactating mothers

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Nicoletti-Carvalho JE, Lellis-Santos C, Yamanaka TS, Nogueira TC, Caperuto LC, Leite AR, Anhê GF, Bordin S. MKP-1 mediates glucocorticoid-induced ERK1/2 dephosphorylation and reduction in pancreatic β-cell proliferation in islets from early lactating mothers. *Am J Physiol Endocrinol Metab* 299: E1006–E1015, 2010. First published September 21, 2010; doi:10.1152/ajpendo.00341.2010.—Maternal pancreatic islets undergo a robust increase of mass and proliferation during pregnancy, which allows a compensation of gestational insulin resistance. Studies have described that this adaptation switches to a low proliferative status after the delivery. The mechanisms underlying this reversal are unknown, but the action of glucocorticoids (GCs) is believed to play an important role because GCs counteract the pregnancy-like effects of PRL on isolated pancreatic islets maintained in cell culture. Here, we demonstrate that ERK1/2 phosphorylation (phospho-ERK1/2) is increased in maternal rat islets isolated on the 19th day of pregnancy. Phospho-ERK1/2 status on the 3rd day after delivery (L3) rapidly turns to values lower than that found in virgin control rats (CTL). MKP-1, a protein phosphatase able to dephosphorylate ERK1/2, is increased in islets from L3 rats. Chromatin immunoprecipitation assay revealed that binding of glucocorticoid receptor (GR) to MKP-1 promoter is also increased in islets from L3 rats. In addition, dexamethasone (DEX) reduced phospho-ERK1/2 and increased MKP-1 expression in RINm5F and MIN-6 cells. Inhibition of transduction with cycloheximide and inhibition of phosphatases with orthovanadate efficiently blocked DEX-induced downregulation of phospho-ERK1/2. In addition, specific knockdown of MKP-1 with siRNA suppressed the downregulation of phospho-ERK1/2 and the reduction of proliferation induced by DEX. Altogether, our results indicate that downregulation of phospho-ERK1/2 is associated with reduction in proliferation found in islets of early lactating mothers. This mechanism is probably mediated by GC-induced MKP-1 expression.

mitogen-activated protein kinase phosphatase-1; extracellular signal-regulated kinase 1/2; dual-specificity phosphatases; pregnancy; lactation

PREGNANCY IS HIGHLIGHTED AS A PHYSIOLOGICAL STATE in which pancreatic β-cells from maternal pancreatic islets undergo a robust mass growth due to proliferation (41). Increasing pancreatic β-cell mass is an adaptive event that allows the maternal organism to meet the insulin demand and compensate peripheral insulin resistance (31). Pregnancy-like changes in pancreatic β-cells, such as an increase in proliferation, can be mimicked by treatment with prolactin (PRL) in cell culture (11). The importance of PRL was further demonstrated in vivo, since pancreatic islets from pregnant PRL receptor (−/+) mice display reduced pancreatic β-cell mass compared with pregnant wild-type mothers (24).

A singular feature of the metabolic adaptation during pregnancy is the rapid reversal that occurs after the delivery, allowing the maternal organism to recover its nonpregnant status (4, 38). Scaglia et al. (38) first demonstrated that maternal pancreatic islets undergo a transient reduction in pancreatic β-cell proliferation as early as the 4th day postpartum. Importantly, this transient reduction in proliferation occurs despite high levels of circulating PRL (27). Glucocorticoids (GCs) are believed to account for this reversal because of its elevated concentration during the end of pregnancy and because this hormone is able to counteract PRL-induced β-cell proliferation in cell culture (44). However, the intracellular mechanism by which this in vivo reversal occurs has not been settled.

The family of serine/threonine kinases known as extracellular signal-regulated kinases (ERKs) belongs to the mitogen-activated protein kinases (MAPKs), a conserved family of enzymes that regulate a large number of physiological processes, including proliferation, differentiation, development, stress responses, and apoptosis (12, 43). The isoforms p44/p42 (ERK1/2) are activated by phosphorylation on threonine and tyrosine residues and are the main MAPKs involved in cell proliferation (33). Because MAPK activities depend primarily on tyrosine/threonine phosphorylation, dephosphorylation of either residue inactivates these enzymes. This task can be accomplished by three distinct families of phosphatases; the major family involved in vertebrate MAPK signaling is comprised by the dual-specificity (Thr/Tyr) phosphatases (DUSPs), also known as MAPK phosphatases (MKPs) (16). MKP-1, a dual-specificity phosphatase known to dephosphorylate and inactivate ERK1/2, is expressed in mouse pancreatic islets and in β-cell line MIN6 (32, 45).

We have demonstrated previously that islets isolated from pregnant rats display increased ERK1/2 activity (1). To study in more detail the involvement of ERK1/2 in postpregnancy β-cell remodeling, we evaluated the GC-dependent regulation of ERK1/2 phosphorylation in early lactating rats and in β-cell lineages. We demonstrate presently that downregulation of ERK1/2 phosphorylation in pancreatic islets from early lactating mothers correlates with the stimulation of MKP-1 by GC. We also present data showing that the induction of MKP-1 expression in pancreatic β-cells in vitro by GC is a mechanism

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underlying the reduction in pancreatic β-cell proliferation and ERK1/2 activity.

MATERIALS AND METHODS

Materials. The reagents, nitrocellulose membrane (0.45 mm), apparatus for SDS-PAGE, and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris, dithiothreitol, Tween-20, glycerol, dexamethasone (DEX), and sodium orthovanadate were obtained from Sigma Chemical (St. Louis, MO). RPMI-1640, Opti-MEM, Lipofectamine 2000, fetal bovine serum (FBS), antibiotics, Trizol reagent, and random primers were from Invitrogen (Carlsbad, CA). Cycloheximide was purchased from Calbiochem (La Jolla, CA). Bromodeoxyurudine (BrdU) Cell Proliferation Kit and EZ Chromatin Immunoprecipitation (ChIP) Kit were from Millipore (Billerica, MA). X-ray-sensitive films and chemicals were from IBF (Rio de Janeiro, Brazil). Antibody against ERK1/2 (06-182) was from Upstate Biotechnology (Lake Placid, NY), and anti-phospho-ERK1/2 Tyr (sc-7383), anti-MKP-1 (sc-370), and anti-GR (M20 sc-1004) antibodies and si-MKP-1 (sc-156118) or scrambled control siRNA (sc-37007) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody (A5316) was from Sigma Chemical. PCR primers were manufactured by Integrated DNA Technologies (Coralville, IA). GoTaq DNA polymerase and ImProm-II reverse transcriptase were from Promega (Madison, WI). Brilliant SYBR Green qPCR MasterMix and plastics were purchased from Agilent-Stratagene (La Jolla, CA). Rat PRL was provided by the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases.

Animals and islet isolation. One male and two female rats were housed together for 5 days. The presence of spermatozoa in the vaginal wash indicated day 0 of gestation. Immediately after delivery, the number of pups was adjusted to eight for each lactating mother. Pregnant and lactating rats were euthanized at 19 days postcoitus (P19) and 3 days postpartum (L3). Virgin age-matched rats were used as the control group (CTL). Islets were isolated by collagenase digestion, as described previously (9). All of the experiments involving animals were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation. Our experiments were approved by the Ethics Committee on Animal Use at the Institute of Biomedical Sciences, University of Sao Paulo, Brazil.

Cells, growth conditions, and treatments. Rat RINm5F insulinoma cell line was cultured in RPMI-1640 medium containing 11.1 mM glucose, 10% FBS, and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Early-passage MIN6 cells before passage 20 were also used in this study. MIN6 cells were grown in RPMI-1640 containing 11.1 mM glucose, 10% FBS, and antibiotics, unless otherwise stated.

DEX was diluted in ethanol to a 10-mM stock solution. The same amount of ethanol used in DEX-treated cells was added in control cells. The activity of tyrosine phosphatases was inhibited by cell pretreatment with 1.5 μM sodium orthovanadate for 1 h. General translation blockage was carried out with 40 μg/ml of cycloheximide 1 h prior to DEX treatment. PRL treatment (500 ng/ml) was carried out in RINm5F cells and lasted 3 days in either the absence or presence of DEX (100 nM). When used for Western blot, PRL treatment of RINm5F cells was performed in RPMI medium containing 10% FBS.

Transfection of MIN6 cells with small interference RNA (siRNA) targeted to MKP-1 (si-MKP-1) or scrambled control siRNA were performed as described previously (3, 35). Briefly, cells were washed twice with serum- and glucose-free medium (Opti-MEM) and then incubated with 1 ml of the same medium containing the siRNA (final concentration of 80 nM) previously mixed with 2 μl of Lipofectamine 2000. In parallel, a set of cells were treated with Lipofectamine without siRNA and used as control (CTL). After 7 h, 1 ml of RPMI medium containing 20% FBS and 22.2 mM glucose was added to the culture containing siRNAs and Lipofectamine (final concentration of FBS and glucose was 10% and 11.1 mM, respectively). Thereafter, the cells were allowed to grow for 60 h in RPMI medium (11.1 mM glucose and 10% FBS), which was replaced every 24 h. After this period, cells were treated with DEX for 12 h, unless otherwise stated.

Cell proliferation. Cell proliferation was assessed by measuring BrdU incorporation into DNA using a commercial kit (cat. no. 2750; Millipore), as described previously (35). Briefly, 4×10^4 MIN6 or RINm5F cells were seeded in 96-well plates and allowed to attach overnight in growth medium. MIN6 cells were transfected with si-MKP-1, as described above. Twenty-four hours later, the medium was replaced by low-serum medium (0.1% FBS) containing DEX (100 nM) or not. DEX treatment was carried out for 72 h, and BrdU was added to the medium 4 h prior to the end of the treatment. RINm5F cells were treated for 3 days with DEX (100 nM), PRL (500 ng/ml), or the combination of DEX and PRL in low-serum medium (0.1% FBS). One well of each condition (from both MIN6 and RINm5F cells) was kept without BrdU to be used as background. Next, cells were incubated for 1 h at RT with anti-BrdU antibody and sequentially incubated with goat anti-mouse IgG antibody conjugated with peroxidase. Afterward, cells were washed and incubated with tetramethylbenzidine peroxidase substrate, and absorbance of 450 nm light was acquired in a spectrophotometer. Blank (tetramethylbenzidine peroxidase substrate) was subtracted from all readings and the respective background absorbance was discounted from each condition.

Western blotting. Isolated islets and cultured cells were homogenized in 100 μl of solubilization buffer and processed for protein extraction and Western blot, as described previously (5). After centrifugation, equal amounts of protein were resolved in SDS-PAGE and transferred to nitrocellulose membranes. Detection using specific antibodies, horseradish peroxidase-conjugated secondary antibodies, and luminol solution was performed as described previously (5). Densitometry was performed using the Scion Image software (Scion).

RNA extraction and real-time PCR. Cells and isolated islets were harvested in Trizol reagent (Carlsbad, CA) and processed for total RNA extraction; cDNA synthesis was performed with 2 μg of total RNA. Real-time PCR primers' sequences and amplification conditions are MKP-1 sense 5′-CAAGAGCATCCCTGTGGAGGAC-3′ and antisense 5′-AGGTAAGCAAGGCAGATGGTGG-3′, 56°C; MKP-2 sense 5′-GAAGAAACGGGTGAGGCTGGAG-3′ and antisense 5′-GCTGAAGACGAACTGCGAGGTG-3′, 60°C; MKP-3 sense 5′-TGTCCTGGTGCATTGCTTGG-3′ and antisense 5′-GGTGAAGTAGAGCTGCTGTGCG-3′, 57°C; and RPL37a sense 5′-CAAGAAGGTCGGGATCGTCG-3′ and antisense 5′-ACCAGGCAAGTCTCAGGAGGTG-3′. Relative expression levels were analyzed using the $2^{-\Delta\Delta C_T}$ method with RPL37a as internal control.

ChIP assay. A group of 500 freshly isolated islets were processed using buffers and reagents from the EZ ChIP Kit (cat. no. 17371; Millipore) according to manufacturer's instructions. Briefly, islets were fixed in Hanks' buffer containing 1% formaldehyde for 10 min at room temperature and transferred to lysis buffer. DNA was sheared to fragments of ~200–1,000 bp by applying eight bursts of sonication (40% of power, 10 s each). Samples were diluted in dilution buffer and precleared for 1 h at 4°C with protein A-sepharose (50% slurry) saturated with salmon sperm DNA. An aliquot of 10 μl was collected as "input." The remaining supernatants were submitted to immunoprecipitation with protein A-sepharose saturated with salmon sperm DNA and 2 μg of anti-GR antibody. In parallel, one sample was incubated only with protein A-sepharose to generate the negative control (no-AB). Sepharose pellets were then washed with buffers provided in the kit and treated with elution buffer (1% SDS and 0.1 M $NaHCO_3$). Supernatants were submitted to cross-link reversal and RNAse A treatment. DNA was purified using phenol-chloroform and resuspended in 20 μl of sterile ddH_2O. DNA samples were amplified for detection of the MKP-1 promoter. A 138-bp fragment corresponding to nucleotides −118 to +20 of the rat MKP-1 gene was amplified by real-time PCR. The sequences of the primers were sense 5′-AGCCAGATTAGGATCAGCGAGCAC-3′ and antisense 5′-GAT-

GCCCACCTCCATCACCATG-3′, 57°C. To check the primers' specificity (by estimated product length), an aliquot of the reaction products was resolved in EtBr-agarose gel. GC receptor (GR) binding was calculated after normalization by the input of each sample.

Statistical analysis. Results are presented as means ± SE. Comparisons were performed by using unpaired Student's *t*-test or one-way ANOVA, followed by Tukey-Kramer post hoc testing when appropriate (INStat; GraphPad Software, San Diego, CA). *P* values <0.05 indicate a significant difference.

RESULTS

ERK1/2 phosphorylation and MKP expression in pancreatic islet postpregnancy. Protein content of ERK1/2 was not altered in pancreatic islets from P19 and L3 rats (Fig. 1*A*). On the other hand, ERK1/2 tyrosine phosphorylation (phospho-ERK1/2) was increased in islets from P19 rats (1.92 ± 0.10-fold for P19 compared with CTL). The higher level of phospho-ERK1/2 was temporally limited to pregnancy. In pancreatic islets from L3 rats, phospho-ERK1/2 was lower than in CTL rats (0.58 ± 0.10-fold; Fig. 1*B*).

MKP-1, MKP-2, and MKP-3 expression were evaluated in islets from CTL and L3 rats. MKP-1 mRNA content was significantly augmented (1.17 ± 0.05-fold of CTL; Fig. 1*C*), whereas MKP-2 was diminished (0.70 ± 0.09-fold of CTL; Fig. 1*D*). MKP-3 did not change in pancreatic islets from L3 rats (Fig. 1*E*).

GCs are known to stimulate MKP-1 expression through direct activation of the GR (14). We next demonstrate that in vivo binding of GR to MKP-1 promoter is increased in islets from L3 rats (2.51 ± 0.43-fold of CTL islets; Fig. 1*F*). Increased GR binding to MKP-1 promoter correlates with increased MKP-1 protein content in islets from L3 rats (1.50 ± 0.13-fold of CTL islets; Fig. 1*G*).

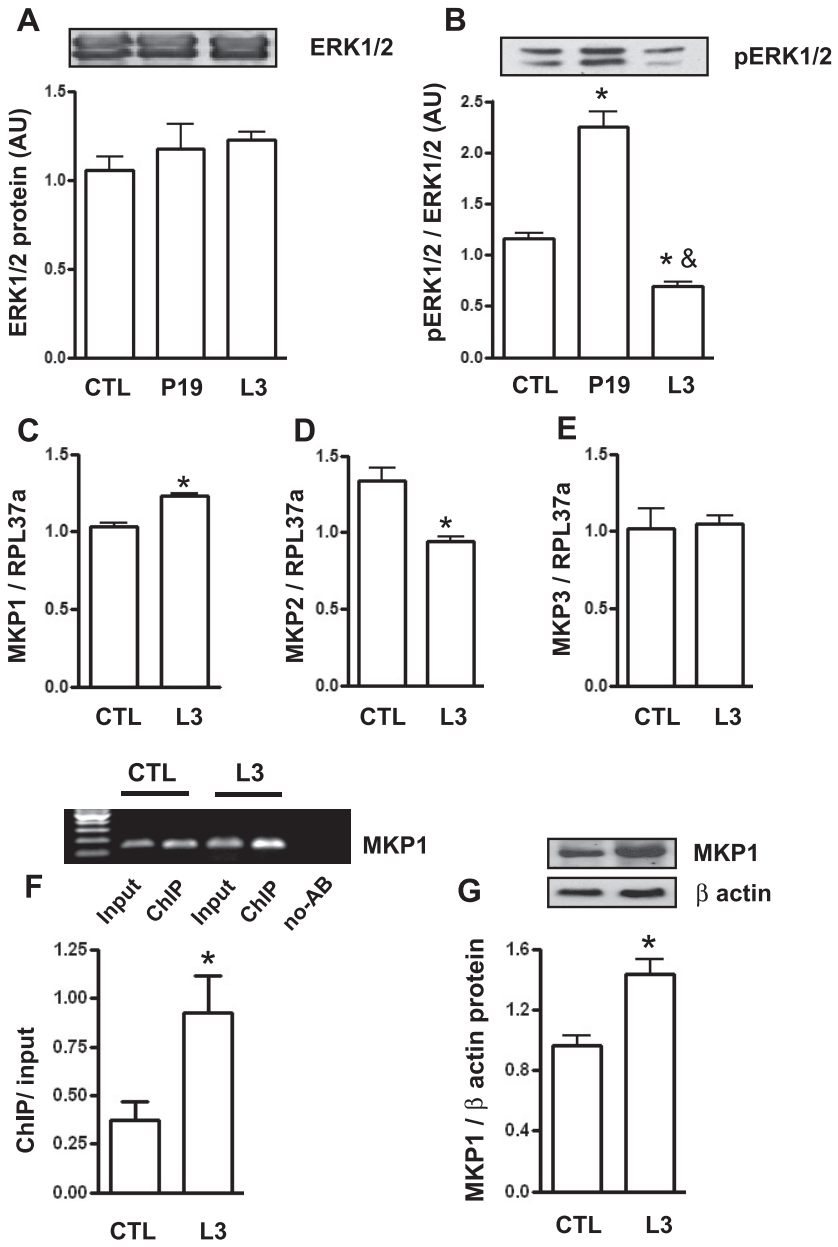

Fig. 1. Relationship between ERK1/2 tyrosin (Tyr) phosphorylation and MAPK phosphatase (MKP)-1 expression in pancreatic islets isolated from pregnant and lactating rats. Pancreatic islets were isolated from virgin [control (CTL)] rats and rats on the 19th day of pregnancy (P19) and on the 3rd day of lactation (L3). Isolated islets were processed for protein extraction and Western blot detection of ERK1/2 (*A*) and Tyr-phosphorylated ERK1/2 (pERK1/2; *B*), with the latter expressed as the ratio between pERK and total ERK1/2 content. Islets were also processed for RNA extraction and used for cDNA synthesis and real-time PCR analysis of MKP-1 (*C*), MKP-2 (*D*), and MKP-3 (*E*) mRNA content. Binding of glucocorticoid receptor (GR) to MKP-1 gene was evaluated by the amplification of MKP-1 promoter region from DNA immunoprecipitated [chromatin immunoprecipitation (ChIP)] with anti-GRα antibody (see MATERIALS AND METHODS for details). DNA was also amplified from total DNA (input) from each sample and from a negative control (no-AB). *F*: PCR products from each sample were normalized by the respective input. *G*: extracted proteins were also used for Western blot detection of MKP-1 and β-actin, with the latter used as inner control. Data are shown as means ± SE. *$P < 0.05$ vs. CTL; &$P < 0.05$ vs. P19 (n = 3–10 for Western blot, 4–8 for RT-PCR, and 3 for ChIP assay). AU, arbitrary units.

Inhibition of ERK1/2 phosphorylation by GCs in RINm5F cells correlates with the upregulation of MKP-1. To further demonstrate the role of GCs in the regulation of MKP in pancreatic β-cells, we next evaluated MKP's expression in RINm5F cells treated with DEX. RNA levels in these cells followed the same pattern of that found in islets from L3 rats. Cells treated with DEX displayed an increase of MKP-1 (1.33 ± 0.03-fold; Fig. 2*A*) and a decrease of MKP-2 (0.70 ± 0.08-fold; Fig. 2*B*) compared with vehicle-treated cells. Also, MKP-3 expression did not change after DEX treatment (Fig. 2*C*). Because MKP-1 was consistently augmented in both islets from L3 rats and DEX-treated RINm5F cells, we performed the next experiments to explore the putative role of MKP-1 expression and ERK1/2 activity relationship in β-cells. Thus, the kinetics of phospho-ERK1/2 levels and MKP-1 expression in RINm5F under DEX treatment was evaluated. DEX induced a rapid and transient increase in phospho-ERK1/2, followed by a gradual decrease after 2 h of treatment (0.67 ± 0.25- to 0.35 ± 0.45-fold of CTL for 2- and 8-h treatment, respectively; Fig. 2*D*). Neither ERK1/2 nor β-actin contents were changed by DEX. MKP-1 protein content drastically increased after short-term treatment, which was maintained throughout the treatment period (12.81 ± 0.45- to 10.00 ± 0.32-fold of CTL for 0.5- and 8-h treatment, respectively; Fig. 2*E*).

In the next set of experiments, we evaluated the concentration of DEX that causes both a decrease in phospho-ERK1/2 and an increase in MKP-1 protein content. Figure 3 shows that 50 nM DEX was sufficient to inhibit phospho-ERK1/2 (0.48 ± 0.11-fold compared with vehicle alone; Fig. 3*A*) and stimulate MKP-1 expression (6.24 ± 0.58-fold of CTL; Fig. 3*B*).

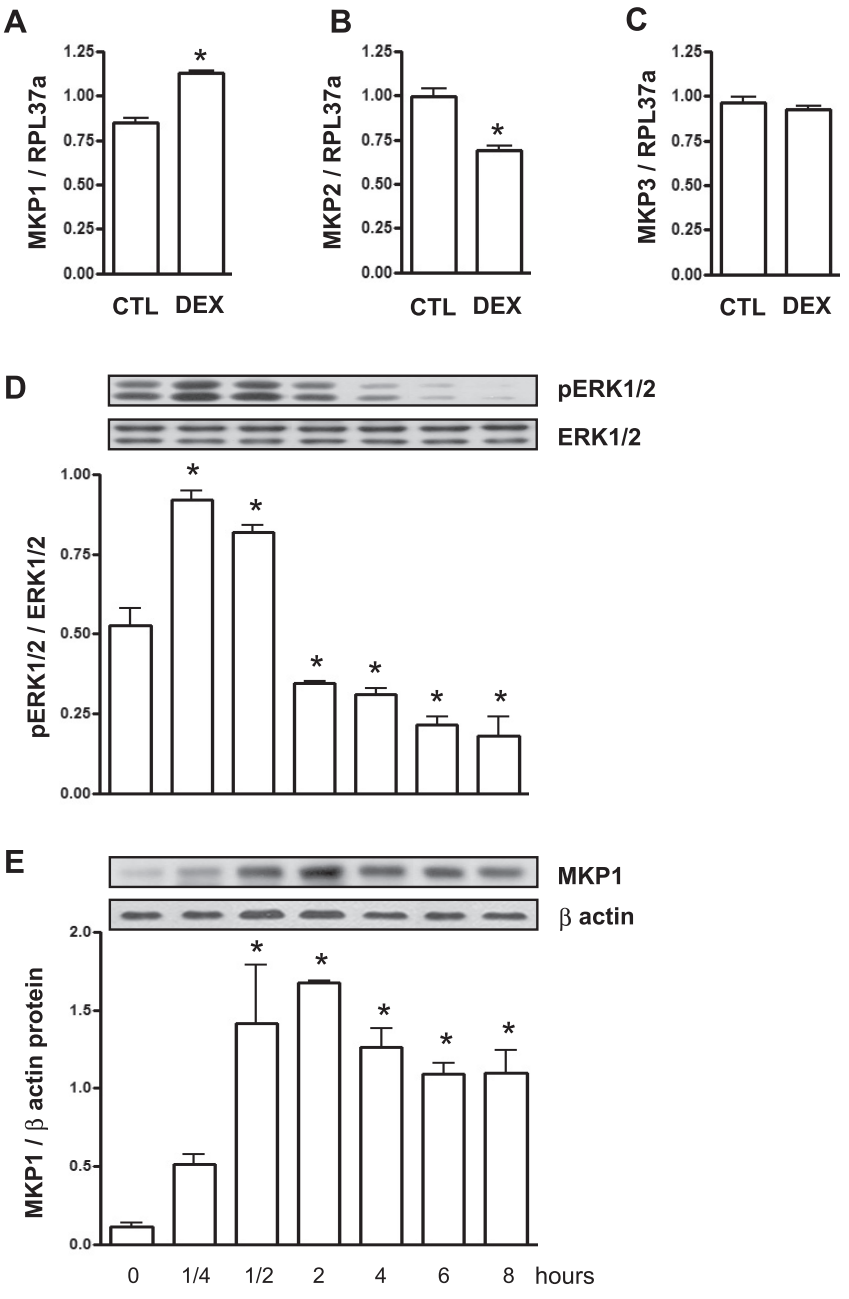

Fig. 2. Time course of ERK1/2 dephosphorylation and MKP-1 expression induced by dexamethasone (DEX) in RINm5F cells. RINm5F cells were treated with 100 nM DEX for 12 h and then processed for RNA extraction and cDNA synthesis; CTL cells were treated with vehicle. Samples were used for real-time PCR analysis of MKP-1 (*A*), MKP-2 (*B*), and MKP-3 (*C*). Another set of cells was subjected to DEX treatment during intervals that ranged from 15 min to 8 h. These cells were used for protein extraction and Western blot detection of total ERK1/2, pERK1/2 (*D*), and MKP-1 and β-actin content (*E*). Results are expressed as MKP/RPL37a ratio for MKP mRNA content, pERK1/2/ERK1/2 ratio for ERK1/2 phosphorylation, and MKP-1/β-actin for MKP-1 expression. Data are shown as means ± SE. *$P < 0.05$ vs. CTL (n = 2–4 for RT-PCR and 3 for Western blot).

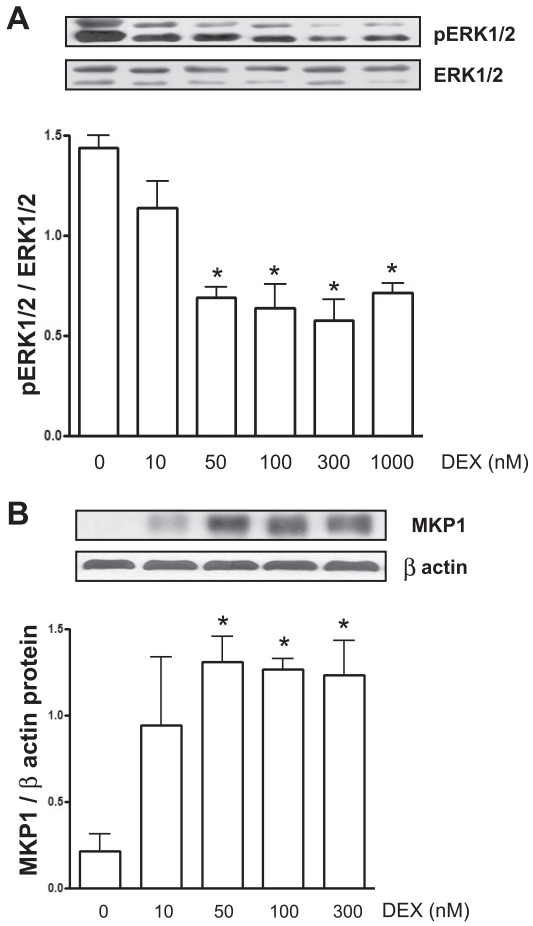

Fig. 3. Dose-response curve of ERK1/2 dephosphorylation and MKP-1 expression to DEX in RINm5F cells. RINm5F cells were treated with 10, 50, 100, 300, and 3,000 nM DEX for 2 h. Afterward, cells were processed for protein extraction and Western blot detection of total ERK1/2, pERK1/2 (*A*), and MKP-1 and β-actin content (*B*). Results are expressed as pERK1/2/ERK1/2 ratio for ERK1/2 phosphorylation and MKP-1/β-actin for MKP-1 expression. Data are shown as means ± SE. *$P < 0.05$ vs. CTL ($n = 2$–5).

It was previously postulated that DEX at the concentration of 100 nM is equivalent to the maternal endogenous GCs at the end of pregnancy (44). To mimic the physiological milieu of pregnancy/lactation transition, the following experiments were performed with 100 nM DEX.

ERK1/2 inactivation by DEX depends on the expression of a phosphatase. To determine whether DEX-dependent ERK1/2 dephosphorylation was mediated by the upregulation of a phosphatase, we used two distinct protocols: RINm5F cells treated with *1*) the inhibitor of protein synthesis cycloheximide and *2*) the inhibitor of protein tyrosine phosphatase sodium orthovanadate. DEX-mediated ERK1/2 dephosphorylation (0.52 ± 0.19-fold of CTL cells) was prevented by treatment with cycloheximide (Fig. 4*A*). Orthovanadate produced similar results, thus suppressing DEX-induced decrease in phospho-ERK1/2. When orthovanadate was added to the medium, with or without DEX, phospho-ERK1/2 was higher than that found in CTL (5.06 ± 0.14- and 5.19 ± 0.26-fold of CTL cells; Fig. 4*B*). This result further indicates that basal phospho-ERK1/2 is maintained by a constitutive phosphatase activity that is stimulated by DEX.

As previously shown herein, MKP-1 protein content was significantly increased by DEX treatment (8.46 ± 0.36-fold of vehicle-treated cells). The presence of cycloheximide in the culture medium abrogated DEX-induced MKP-1 expression, which suggests that ERK1/2 inactivation is secondary to the increased MKP-1 expression (Fig. 4*C*). In addition, cell treatment with orthovanadate did not change MKP-1 content. In these cells, DEX increased MKP-1 content (3.10 ± 0.33-fold of vehicle-treated cells), even in the presence of orthovanadate (2.85 ± 0.49-fold of orthovanadate-treated cells) (Fig. 4*D*).

ERK1/2 inactivation by DEX depends on the stimulation of MKP-1 expression. Before the evaluation of the effects of MKP-1 knockdown with siRNA (si-MKP-1), we brought about again some key experiments using the mouse insulinoma cell lineage MIN6, which is known to express MKP-1 (45). Figure 5 shows that the effects of DEX in RINm5F cells were reproduced in MIN6 cells. Phospho-ERK1/2 was reduced 0.58 ± 0.17-fold (Fig. 5*A*), whereas MKP-1 protein content increased 3.52 ± 0.33-fold compared with vehicle-treated cells (Fig. 5*B*).

Transfection of MIN6 cells with siRNA targeted to MKP-1 led to the reduction of almost 60% of MKP-1 content, even in the presence of DEX (Fig. 5*D*). β-Actin protein content was not affected by the transfection (Fig. 5*C*). In MIN6 cells, phospho-ERK1/2 was for orthovanadate (Fig. 5*E*) and si-MKP-1 (Fig. 5*F*) 3.02 ± 0.07- and 2.62 ± 0.38-fold, respectively, higher than DEX-treated cells. The combination of DEX with either orthovanadate or si-MKP-1 did not result in any decrease of phospho-ERK1/2. Thus, the values of phospho-ERK1/2 obtained by the combination of DEX and orthovanadate or DEX and si-MKP-1 were the same as orthovanadate and si-MKP-1 alone, respectively.

Involvement of MKP-1 expression, DEX, and PRL on β-cell proliferation. In parallel with phospho-ERK 1/2, DEX treatment reduced MIN6 cell proliferation (0.48 ± 0.12-fold of vehicle-treated cells). MKP-1 knockdown experiments revealed that the reduction of MKP-1 protein content by itself did not increase the proliferation rate of MIN6 cells. However, MKP-1 knockdown abolished the reduction in proliferation induced by DEX in such a way that proliferation rate of cells transfected with si-MKP-1 and treated with DEX was not different from si-MKP-1 cells (Fig. 6*A*). We have also measured β-cell proliferation under PRL and DEX treatment. In Fig. 6*B*, we show that PRL increased cell proliferation (1.21 ± 0.06-fold of CTL). DEX reduced β-cell proliferation in either the absence or presence of PRL (0.79 ± 0.09- and 0.81 ± 0.06-fold of CTL, respectively). In parallel with the increased β-cell proliferation, PRL induced an increase in phospho-ERK1/2 (1.31 ± 0.1-fold of CTL; Fig. 6*C*). DEX reduced phospho-ERK1/2 irrespective of the presence of PRL (0.54 ± 0.15-fold of CTL).

DISCUSSION

It is well accepted that most of the changes comprising maternal pancreatic β-cell adaptation to pregnancy, including increased proliferation, result primarily from placental lactogen and PRL action (1, 24). The contribution of gestational hormones on β-cell adaptation to pregnancy is highlighted by the demonstration that, even when the increased demand for insulin is compensated by exogenous insulin, changes in islet function characteristic of gestation still occur (36). However,

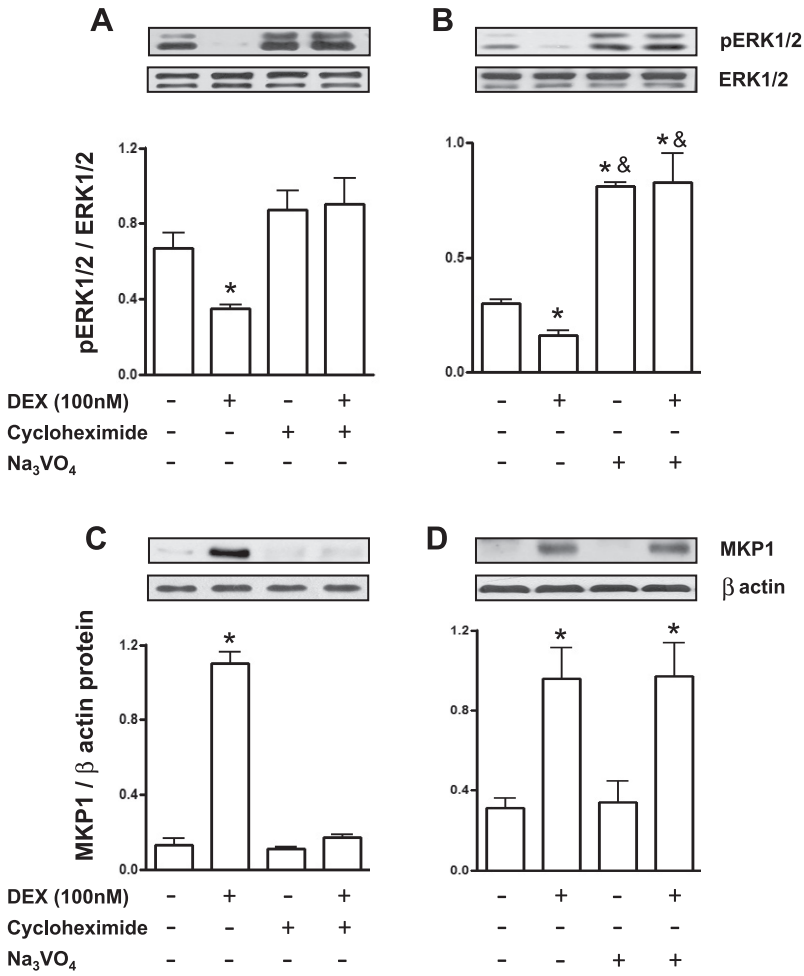

Fig. 4. Effects of cycloeheximide and orthovanadate on DEX-dependent ERK1/2 dephosphorylation and MKP-1 expression. RINm5F cells were pretreated with 1.5 μM orthovanadate (Na_2VO_4) or 40 μg/ml cycloheximide 1 h prior to DEX treatment (100 nM for 2 h). CTL cells were treated with vehicle. Cells were processed for protein extraction and Western blot detection of ERK1/2, pERK1/2, MKP-1, and β-actin (inner control). Variations of pERK1/2 and total ERK1/2 content (representative blots), as well as pERK1/2/ERK1/2 ratio (graphs), induced by DEX were calculated under cycloheximide (*A*) and Na_2VO_4 (*B*) pretreatment. Membranes were also probed for detection of MKP-1 and β-actin. *C* and *D*: representative blots of MKP-1 and β-actin and MKP-1/β-actin ratio of cycloheximide- and Na_2VO_4-treated cells, respectively, are shown. Data are expressed as means ± SE. *$P < 0.05$ vs. CT; $n = 2–4$ for RT-PCR and 3 for Western blot.

the increases in both insulin secretion and β-cell proliferation are time-limited events that resume the nonpregnant features just after the delivery (5, 38). It is likely that the reduction of intracellular calcium stores and SERCA2 expression in pancreatic islets mediate the decrease of maternal insulin secretion just after delivery (5, 25). However, the precise mechanism by which the pancreatic β-cell switches from a high to a low proliferative status is much less comprehended. Our results show that the dynamics of ERK1/2 phosphorylation are in temporal accordance with the pattern of maternal β-cell proliferation during the peripartum, as described by Scaglia et al. (38). This temporal coincidence suggests a basic role of ERK1/2 in this adaptation and prompted us to investigate the mechanism involved in ERK1/2 dephosphorylation at the postpartum.

ERK1/2 signaling activation is a bona fide requirement for cell proliferation (33). In islets and β-cell lines, extracellular signals such as GLP-1 (8), islet neogenesis-associated protein (7), IGF-I (21), and PRL (2) are able to acutely induce ERK1/2 phosphorylation. Because all of these signals are strong mitogenic factors, it is likely that increased ERK1/2 phosphorylation mediates β-cell growth during pregnancy.

In contrast to its activation, ERK1/2 is negatively regulated by DUSP, also known as MKP (19). In osteoblasts and smooth muscle cells, increased MKP-1 expression has been shown to target ERK1/2 dephosphorylation (18, 23, 30). Accordingly, our study demonstrates that ERK1/2 dephosphorylation found in islets of L3 rats correlates with increased MKP-1 expression.

Data accumulated thus far suggest that endogenous GCs oppose the overall actions of PRL on pancreatic β-cell function, because the simultaneous treatment of isolated islets or β-cell lines with PRL and DEX resembles the postpregnancy β-cell phenotype. It was already settled that DEX reverses PRL-induced β-cell gain of function by inhibiting insulin secretion and cell proliferation while increasing apoptosis (39, 44). Accordingly, as shown herein, DEX reduced both ERK1/2 phosphorylation and RINm5F cell proliferation, even in the presence of PRL.

GCs are known to reduce proliferation in a variety of cells, including smooth muscle (20), lymphoid (40), bone (23), and pancreatic β-cells (6). As such, GCs can modulate the activity and/or expression of various kinases and phosphatases, thus affecting the proliferative action of mitogenic signals. It has been shown that GC antiproliferative action correlates with increased MKP expression (18). Moreover, the inhibition of ERK1/2 phosphorylation by GC depends on the induction of MKP-1 expression (23). Corroborating the hypothesis that GCs favor the reduction of ERK1/2 phosphorylation in maternal pancreatic β-cell after the delivery, we found that the direct binding of GR to MKP-1 promoter was increased in islets from L3 rats. To our knowledge, this is the first report showing a direct binding of GR to a MKP-1 promoter in pancreatic islets.

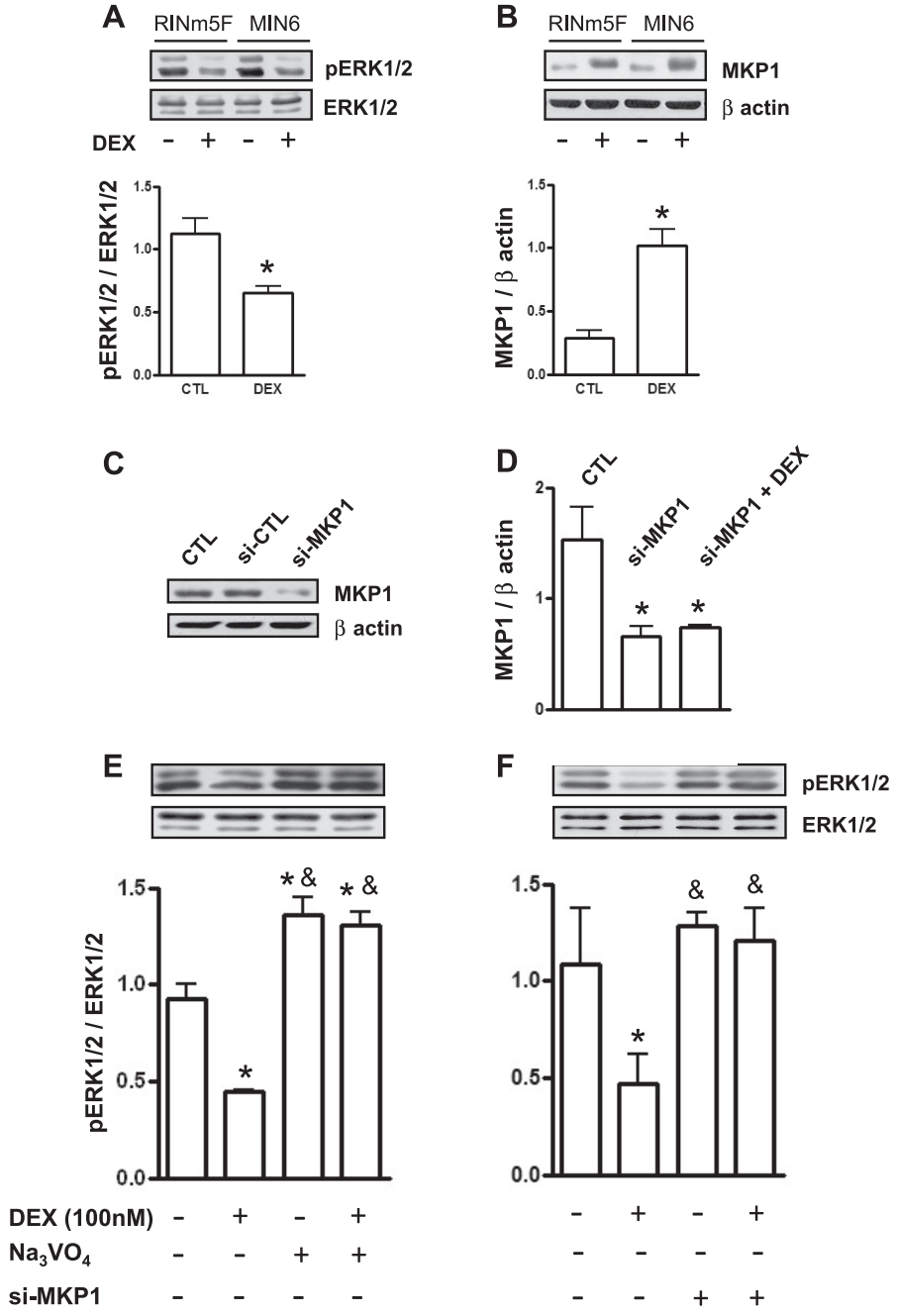

Fig. 5. Effects of MKP-1 knockdown on DEX-dependent ERK1/2 dephosphorylation and MKP-1 expression in MIN6 cells. RINm5F and MIN6 cells were treated with DEX (100 nM for 6 h). CTL cells were treated with vehicle. Cells were processed for protein extraction and Western blot detection of total ERK1/2, pERK1/2 (*A*), and MKP-1 and β-actin content (*B*). Results are expressed as pERK1/2/ERK1/2 ratio for ERK1/2 phosphorylation and MKP-1/β-actin for MKP-1 expression. MIN6 cells were transfected with siRNA targeted to MKP-1 (si-MKP-1) or a scrambled control (si-CTL). In parallel, a set of cells was treated only with transfection reagents. After transfection, these cells were subjected to protein extraction and Western blot detection of MKP-1 and β-actin content (*C*); the results are expressed as MKP-1/β-actin (*D*). MIN6 cells were treated with 1.5 μM orthovanadate (Na_2VO_4; *E*) or transfected with si-MKP-1 (*F*) prior to treatment with DEX (100 nM for 2 h). Next, cells were subjected to protein extraction and Western blot detection of total ERK1/2 and pERK1/2; results are expressed as pERK1/2/ERK1/2 ratio. Data are expressed as means ± SE. $^*P < 0.05$ vs. CTL; $\&P < 0.05$ vs. DEX ($n = 4–8$).

Importantly, GC-mediated MKP-1 expression has already been demonstrated to result in a direct binding of the GR to GC-responsive elements within the MKP-1 promoter in other cell types (15, 26). In accordance, the use of RU-486, an antagonist of GR, inhibited DEX-induced MKP-1 expression (26).

To strengthen the interplay among GC, MKP-1, ERK1/2 activity, and β-cell proliferation, we next moved on to a series of experiments using RINm5F and MIN6 cells. Our data show that MKP-1 expression correlates with DEX-induced ERK1/2 dephosphorylation, reaching maximal effect after 2-h treatment. Also, the repression of ERK1/2 activation and the increase in MKP-1 content induced by DEX are fully prevented by the treatment with the protein synthesis inhibitor cyclohexemide. This result shows that the effect of GC on MKP-1 regulation in pancreatic β-cell may be mediated exclusively by transcriptional regulation rather than posttranscriptional events. On the other hand, DEX-induced increase in MKP-1 content in RBL-2H3 cells was demonstrated to be mediated by both transcriptional and posttranscriptional regulation (26). Our results showing that the reduction of ERK1/2 phosphorylation mediated by DEX in pancreatic β-cell was inhibited by sodium orthovanatade, an unspecific phosphatase inhibitor, further suggested a crucial role for the increase in MKP-1 in this regulation.

We also observed a transient increase in ERK1/2 phosphorylation, peaking at 30 min of DEX stimulation. This transitory effect was followed by a significant decrease in ERK1/2 phosphorylation, reaching levels as low as one-half of basal

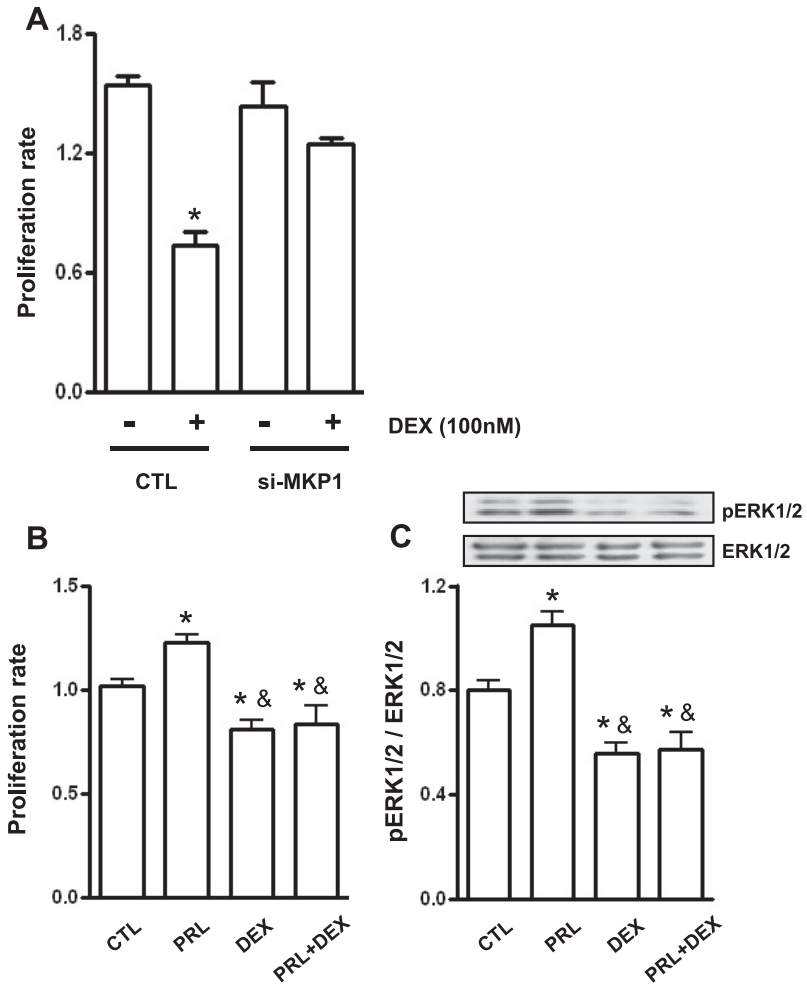

Fig. 6. Effects of MKP-1 knockdown, DEX, and prolactin (PRL) on pancreatic β-cell proliferation. MIN6 cells were transfected with siRNA targeted to MKP-1 (si-MKP-1) or treated only with transfection reagent. The next day, at the beginning of transfection, medium was changed, and cells were treated with DEX (100 nM) or vehicle for 72 h. RINm5F cells were also treated for 72 h with DEX (100 nM), PRL (500 ng/ml), or the combination of both. Treatments were carried out in medium with 0.1% FBS. After treatments, MIN6 (*A*) and RINm5F (*B*) cells were processed for bromodeoxyurudine detection incorporation into newly synthesized DNA. Results are expressed as proliferation rate (which was defined as the absorbance of cells at each condition subtracted from their respective background). RINm5F cells were also processed for Western blot detection of ERK1/2 and pERK1/2; results are expressed as pERK1/2/ERK1/2 ratio. Data are shown as means ± SE. *$P < 0.001$ vs. CTL, si-MKP-1, and si-MKP-1 + DEX; &$P < 0.05$ vs. PRL (n = 6–18 for proliferation and 8–10 for Western blot).

values. We do not know if this event has any physiological relevance in β-cells chronically exposed to GCs. Interestingly, DEX enhanced ERK1/2 phosphorylation at times up to 20 min in the kidney cell line HK-2, which was correlated to a protective effect against kidney injury, probably by a mechanism independent of transcriptional activity (29). Of note, rapid stimulation of MKP-1 and MKP-3 expression was described to be mediated by ERK1/2 activation, resulting in a negative feedback over this signaling pathway (17, 40). Therefore, it is possible that the rapid and transient stimulation of ERK1/2 phosphorylation by DEX found by us (15 min after the beginning of treatment) in RINm5F cells would play a role in the induction of MKP-1 (30 min after the beginning of treatment) and then in the long-term ERK1/2 dephosphorylation. Transposing this interpretation to our in vivo results, it is possible that the increase in ERK1/2 phosphorylation found in islets of pregnant rats contributes to the increased MKP-1 expression and decreased ERK1/2 phosphorylation in islets of early lactating mothers.

Because ERK1/2 phosphorylation is induced by a plethora of signals, the participation of other factors in the increased phospho-ERK1/2 in late pregnancy must not be ruled out. It is well known that circulating levels of lipids increase in the last third of gestation (22). In addressing this issue, Brelje et al. (10) have demonstrated that palmitate (0.4 mM), combined or not with PRL, increases proliferation of neonatal islets cultured for 4 and 8 days. On the basis of these results, the authors suggested that fatty acids may also contribute to the large increase in β-cell mass. Indeed, it has been shown recently that palmitate (0.5 mM) increases ERK1/2 phosphorylation in MIN6N8 cells chronically exposed (48 h) to the fatty acid (28).

We next used siRNA, attempting to specifically inhibit the rise in MKP-1 content induced by DEX. Knockdown of MKP-1 resulted in the blockade of DEX-induced ERK1/2 dephosphorylation, pointing out the role of this phosphatase in DEX modulation of ERK1/2 signaling in the pancreatic β-cell. In a recent review, the several isoforms of MKP already described (i.e., MKP-1, MKP-2, MKP-3, and MKPx) have been related to preferential targets among the MAPK proteins (37). In this sense, MKP-1 is generically linked to a preferential dephosphorylation of p38 and JNK but is also able to dephosphorylate ERK1/2. However, MKP-2, MKP-3, and MKPx show high affinity for ERK1/2 and less for p38 and JNK (37). Despite this general preference for its substrate, MKP phosphatase activity seems to be highly cell type and context specific. For instance, MKP-1 inactivated ERK2, JNK2, and p38 equally, and MKP-2 was unable to inactivate p38 in HeLa, NIH3T3, and COS cells (13). Also, the ability of DEX in inhibit IL-1- and granulocyte macrophage colony-stimulating factor-induced ERK1/2 phosphorylation in human bronchial epithelial cells was highly dependent on induction of MKP-1 expression (34). Therefore, we hypothesize that increased

MKP-1 determines reduced ERK1/2 phosphorylation in pancreatic islets from L3 rats and in pancreatic β-cells treated with DEX. Our findings showing increased MKP2 and unaltered MKP3 in both of these situations further corroborate this proposition.

The functional relevance of our findings is evidenced by the experiments measuring incorporation of BrdU to DNA as an estimative of cell proliferation. We found that DEX-induced reduction in proliferation of MIN6 cells was completely abolished by MKP-1 knockdown. In accordance with these results, DEX was already demonstrated to reduce osteoblast proliferation secondary to induction of MKP-1 and reduction of ERK1/2 phosphorylation (18, 23).

Altogether, the new findings presented herein can be summarized as follows. First, the temporal changes of ERK1/2 phosphorylation in pancreatic islets from rats at the end of pregnancy and at the beginning of lactation coincide with changes in MKP-1 expression. The increase in MKP-1 probably results from GC action, because GR binding to MKP-1 promoter is increased in islets from L3 rats. Second, experiments with β-cell lines show that DEX-induced dephosphorylation of ERK1/2 and reduced proliferation rate are highly dependent on the induction of MKP-1 expression.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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