Chemico-Biological Interactions 206 (2013) 47–54

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Modulation of albumin-induced endoplasmic reticulum stress in renal proximal tubule cells by upregulation of mapk phosphatase-1

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ARTICLE INFO

Article history:
Received 8 April 2013
Received in revised form 18 July 2013
Accepted 16 August 2013
Available online 29 August 2013

Keywords:
Albumin
OK cells
ERK1/2
MKPs
Endoplasmic reticulum stress

ABSTRACT

High amounts of albumin in urine cause tubulointerstitial damage that leads to a rapid deterioration of the renal function. Albumin exerts its injurious effects on renal cells through a process named endoplasmic reticulum (ER) stress due to the accumulation of unfolded proteins in the ER lumen. In addition, albumin promotes phosphorylation and consequent activation of MAPKs such as ERK1/2. Since ERK1/2 activation promoted by albumin is a transient event, the aims of the present work were to identify the phosphatase involved in their dephosphorylation in albumin-exposed cells and to analyze the putative regulation of this phosphatase by albumin. We also sought to determine the role played by the phospho/dephosphorylation of ERK1/2 in the cellular response to albumin-induced ER stress. MAP kinase phosphatase-1, MKP-1, is a nuclear enzyme involved in rapid MAPK dephosphorylation. Here we present evidence supporting the notion that this phosphatase is responsible for ERK1/2 dephosphorylation after albumin exposure in OK cells. Moreover, we demonstrate that exposure of OK cells to albumin transiently increases MKP-1 protein levels. The increase was evident after 15 min of exposure, peaked at 1 h (6-fold) and declined thereafter. In cells overexpressing flag-MKP-1, albumin caused the accumulation of this chimera, promoting MKP-1 stabilization by a posttranslational mechanism. Albumin also promoted a transient increase in MKP-1 mRNA levels (3-fold at 1 h) through the activation of gene transcription. In addition, we also show that albumin increased mRNA levels of GRP78, a key marker of ER stress, through an ERK-dependent pathway. In line with this finding, our studies demonstrate that flag-MKP-1 overexpression blunted albumin-induced GRP78 upregulation. Thus, our work demonstrates that albumin overload not only triggers MAPK activation but also tightly upregulates MKP-1 expression, which might modulate ER stress response to albumin overload.

1. Introduction

Whenever excreted into urine in high amounts, albumin is responsible for tubulointerstitial damage [1], causing a rapid deterioration of the renal function that often leads to severe kidney disease [2,3]. Albumin, which is the most abundant component of plasma proteins, exerts its injurious effects on renal cells through a process named endoplasmic reticulum (ER) stress due to the accumulation of unfolded and/or misfolded proteins in the ER lumen [4]. ER stress produces the activation of stress-response signaling pathways which allow cells to respond to perturbations and recover homeostasis [5]. These pathways are collectively named unfolded protein response (UPR). One major pro-survival mechanism prompted by the UPR is mediated by chaperone glucose-regulated protein 78 (GRP78). GRP78 plays an essential role in counteracting the apoptotic potential of ER stress by multiple mechanisms, such as binding to the unfolded proteins to alleviate ER stress conditions [6]. Accordingly, it has been established that albumin overload induces the expression of GRP78 in mouse proximal tubular cells [7].

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; GRP78, glucose-regulated protein 78; ERKs, extracellular signal-regulated kinases; JNKs, c-Jun NH2-terminal protein kinases; SAPKs, stress-activated protein kinases; MAPKKs, MAPK kinases; MKP, MAPK phosphatase; BSA, Free fatty acid albumin; Act D, actinomycin D; CHX, cycloheximide; PD98059, 2-amino-3-methoxyphenyl)4H-1-benzopyran-4-one; SP600125, Anthra[1-9-cd]pyrazol-6(2H); OK, Opossum kidney.

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http://dx.doi.org/10.1016/j.cbi.2013.08.009

It is documented that albumin interacts with renal tubular cells through an unspecific receptor involved in its internalization [8] and promotes ER stress. It has been reported that the albumin reabsorbed by proximal tubular cells triggers a signal transduction pathway that includes ROS generation and the subsequent activation of the tyrosine kinase Src and the serine/threonine kinase mTOR (the mammalian target of rapamycin), and that these events play a central role in ER stress promoted by albumin [9].

This albumin-induced ER stress could involve MAPK family members. Indeed, in several systems the induction of ER stress is linked to the activation of MAPKs [10–12]. Moreover, albumin overload is known to promote the activation of MAPKs in renal tubular cells [13,14]. MAPKs play a crucial role in signal transduction pathways related to cell growth, differentiation and apoptosis and are classified in three subgroups: extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal protein kinases (JNKs) or stress-activated protein kinases (SAPKs), and p38 MAPKs [15]. MAPKs are phosphorylated in threonine and tyrosine residues by specific kinases named MAPK kinases (MAPKKs), such as MEK1 and MEK2 [15]. After dual phosphorylation, MAPKs acquire maximal activity and translocate to the nucleus, where they phosphorylate transcription factors driving the expression of MAPK-dependent genes. While JNK and p38 activation promote signaling pathways related to apoptosis [16], the activation of the MEK/ERK pathway is a common cause for cell resistance to death [15]. It has been demonstrated that the inhibition of the MEK/ERK pathway sensitizes cells to ER stress-induced apoptosis, and that this is associated with the downregulation of GRP78 expression and blockage of its induction by the UPR in colorectal cancer cells [17].

Because MAPK activity depends on phosphorylation processes, the magnitude and duration of this activity are linked to phosphatases capable of promoting MAPK dephosphorylation. The MAPK phosphatase (MKP) family comprises dual specificity enzymes that promote MAPK inactivation [18]. MKP-1 is a short-lived nuclear enzyme – product of an early gene – which is rapidly induced by a wide range of stimuli [19,20]. Due to its cellular localization, MKP-1 is responsible for nuclear MAPK inactivation. Consequently, this phosphatase controls the activity and/or expression of MAPK-dependent transcription factors and, ultimately, gene transcription [20]. MKP-1 expression is tightly regulated by a broad array of stimuli like hormones [21–25], drugs [26] and physical stress like heat shock [27] and UV radiation [28]. Moreover, this regulation is exerted through several mechanisms including the increase in gene transcription, as was documented in adrenocortical cells [22,29] and in Leydig cells [21] under stimulation with the corresponding trophic hormone. Other mechanisms include the stabilization of MKP-1 mRNA and the increase in its translational activity [30]. In addition, the regulation of MKP-1 by post-translational modifications has been described in several systems. In particular, different works have described MKP-1 stabilization mediated by ERK1/2 phosphorylation [21,31], although enhanced MKP-1 degradation after ERK-mediated phosphorylation has also been reported [32].

Although albumin overload has been shown to promote ERK1/2 and JNK1/2 activation in renal tubular cells [13,14] and MAPK activity has been linked to ER stress-induced apoptosis in a colorectal model of cancer cells exposed to albumin [17], information about MAPK regulation of albumin-induced ER stress is scarce. Proximal tubule cells are among the first to be exposed to high protein concentrations. This tubular segment is responsible for tubular reabsorption mechanisms but it is also sensitive to the cytotoxic effects of albumin. A tight regulatory mechanism is therefore crucial for an appropriate handling of filtered proteins. The fact that activation of MAPKs by albumin is transient [13,14] points to the involvement of a dephosphorylation event operating in the cellular response to the noxious effects of albumin. The dephosphorylation of ERK after albumin exposure could therefore modulate the response to ER stress and, consequently, the aim of the present work was to identify the phosphatase involved in MAPK dephosphorylation in albumin-exposed cells and to determine whether this phosphatase is regulated by albumin overload. Our results demonstrate that albumin rapidly induces MKP-1, which promotes ERK1/2 dephosphorylation and also modulates the expression of GRP78 and ER stress-induced MAPK-regulated gene expression in OK cells.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA) (Cat # A8806, fatty acid free and low endotoxin lyophilized powder), LPS (lipopolysaccharides), okadaic acid, sodium orthovanadate, actinomycin D (Act D), cycloheximide (CHX), PD98059 (PD) and SP600125 (SP) were obtained from Sigma (St. Louis, MO, USA). Cell culture supplies were obtained from Life Technologies Inc. BRL (Carlsbad, CA, USA). Plasticware was purchased from Corning-Costar (Corning, NY, USA). Specific polyclonal antibody against MKP-1 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against phospho-ERK1/2 (pERK1/2) and total ERK1/2 (ERK1/2) were from New England Biolabs Inc. (Beverly, MA, USA). Electrophoresis supplies, polyvinylidene difluoride membrane and secondary antibody (horseradish peroxidase-conjugated goat antibody) were from Bio-Rad Laboratories Inc. (Hercules, CA, USA). The Bio-Lumina kit for enhanced chemiluminescence was provided by Kalium Technology (Bernal, Buenos Aires, Argentina). All other chemicals were commercial products of the highest grade available.

2.2. Cell culture

OK is a cell line derived from Opposum (*Didelphis virginiana)* kidney proximal tubular cells. OK cells were maintained in low glucose Dulbeccós modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated bovine fetal serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin and maintained in a humidified atmosphere containing 5% CO_2 as already described [33]. Cells were arrested early in the G1 phase of the cell cycle by transferring the cultures in the logarithmic phase of growth to serum-free medium for 24 h. This medium was then replaced with fresh medium, where cells were maintained during 2 h for stabilization to be later incubated with or without BSA as stated in the legend of the corresponding figures. When indicated, different reagents were preincubated for the following times, before BSA addition: 5 μg/ml Act D or 2 μg/ml CHX, 30 min; 50 μM PD, 40 min; 20 μM SP, 1 h; 100 nM okadaic acid (OA) plus 10 μM pervanadate (PV), 15 min. PV was prepared from orthovanadate as previously described [34]. After treatments, total RNA or cell lysates were obtained.

2.3. Plasmids and transfections

The FLAG-tagged MKP-1 expression vector (pFLAG-MKP-1) was generated from pRc/CMV-MKP-1 expression vector, as already described [21]. This vector contains murine MKP-1 cDNA cloned in *Eco*RI/*Xba*I sites of p3xFLAG-CMV-7.1. OK cells were seeded the day before transfection, grown up to 80% confluence, then transfected during 16 h using Lipofectamine 2000 reagent in Opti-MEM medium according to the manufacturer's instructions and finally

submitted to serum starvation. Transfections were carried out in 6-well plates using 2 μg of the p3xFLAG-CMV-7.1.MKP-1 vector or the p3xFLAG-CMV-7.1 empty vector as control. Then, experiments were carried out as indicated.

2.4. RNA extraction and semiquantitative RT-PCR

Total RNA was isolated from OK cells using Tri-Reagent (MRC Carlsbad, CA) according to the manufacturer's instructions. MKP-1 cDNA was obtained by RT-PCR from OK cells, using random primers for reverse transcription (RT) and specific primers for polymerase chain reaction (PCR), as already described [21]. In order to design suitable primers to isolate MKP-1 cDNA from OK cells, we used the MKP-1 sequence (NCBI Reference Sequence: XM_001380535.2) of a closely related species, *Monodelphis* sp. These primers were: forward, 5́-ATGGAAATCTGCGCCCTG-3́ and reverse, 5-TCAGCAGCTTGG GGAAGT-3. The cDNA obtained (1081 pb) was sequenced and turned out to be highly homologous (97%) to *Monodelphis* sp. Moreover, this sequence was observed to share 82% and 81% gene sequence identity with human and mouse genes, respectively (data not shown). Based on the sequence of the isolated cDNA, we designed the primers to perform semiquantitative PCR analysis. These primers were: forward, 5́-AGGAGGATACGAAGCCTTCT-3́ and reverse, 5-GCTTCTGCTGAACAGTGAG-3́. The isolated fragment had the expected size (577 bp). GRP78 and GAPDH cDNA from OK cells were also obtained using data from *Monodelphis* sp (GAPDH cDNA NCBI Reference Sequence: XM_001364697, GRP78 NCBI Reference Sequence: XM_001365677). Primer sequences were as follows: GAPDH forward 5-TGTGGAGTCCACTGGCGTG-3, reverse 5-TACTCCT TGGTGGCCATGTA-3 and GRP78 forward 5-GGTGATGCTGC-CAAAAACCA-3 and reverse 5-TGATGTTGAGAGGACAAGGC-3. The fragments obtained had the expected size for GAPDH (723 bp) and GRP78 (663 bp) and were homologous to *Monodelphis* sp cDNA 96% and 97%, respectively. Cycling conditions for MKP-1 were step 1: 95 °C 5 min, step 2: 95 °C 45 s, step 3: 59 °C 30 s, step 4: 72 °C 1 min; steps 2 and 3 were repeated 24 times. Conditions for GRP78 were step 1: 95 °C 5 min, step 2: 95 °C 30 s, step 3: 53 °C 30 s, step 4: 72 °C 1 min; steps 2 and 3 were repeated 26 times. Conditions for GAPDH were step 1: 95 °C 5 min, step 2: 95 °C 30 s, step 3: 51 °C 1 min, step 4: 72 °C 1 min; steps 2 and 3 were repeated 26 times. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification. PCR products were resolved on a 1.5% (wt/vol) agarose gel containing ethidium bromide. Gel images were obtained with a digital camera (Kodak Easy Share Z712 IS) and quantitated using the Gel-Pro-analyzer software (Media Cybernetics, LP, Silverspring. MD, USA) for windows. PCR results for each sample were normalized using GAPDH mRNA as an internal control.

Sequence data for MKP-1, GAPDH and GRP78 from OK cells (*D. virginiana*) were submitted to Genebank and are available at Nucleotide as MKP-1, KC862383; GAPDH, KC862381.1; GRP78, KC862382.1).

2.5. Western blot analysis

After the appropriate treatments, OK cells were washed with PBS and scraped into a buffer containing 20 mM Tris (pH 7.4), 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM NaCl, 50 mM NaF, 40 mM glycerol-phosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 mM sodium orthovanadate. The suspension was centrifuged at 1500*g* at 4 °C for 10 min. Pellet was discarded and supernatant (total lysate) was subjected to Western blot analysis. Equal amounts of protein from total lysates (15–30 μg) were separated by 10% SDS–PAGE, as described by Laemmli (22) and transferred onto polyvinylidene difluoride membranes according to the procedure described by Towbin et al. (23). Immunoblotting was performed using the following antibody dilutions: mouse monoclonal anti-flag (1:10,000), rabbit polyclonal anti-MKP-1 (1:1000), rabbit polyclonal anti-P-ERK1/2 (1:1000), mouse monoclonal anti-β-tubulin (1:4000) or rabbit polyclonal anti total ERK1/2 (1:10,000). Bound antibodies were developed by incubation with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse). Immunoreactive bands were detected using an enhanced chemiluminescence kit (Bio-Lumina).

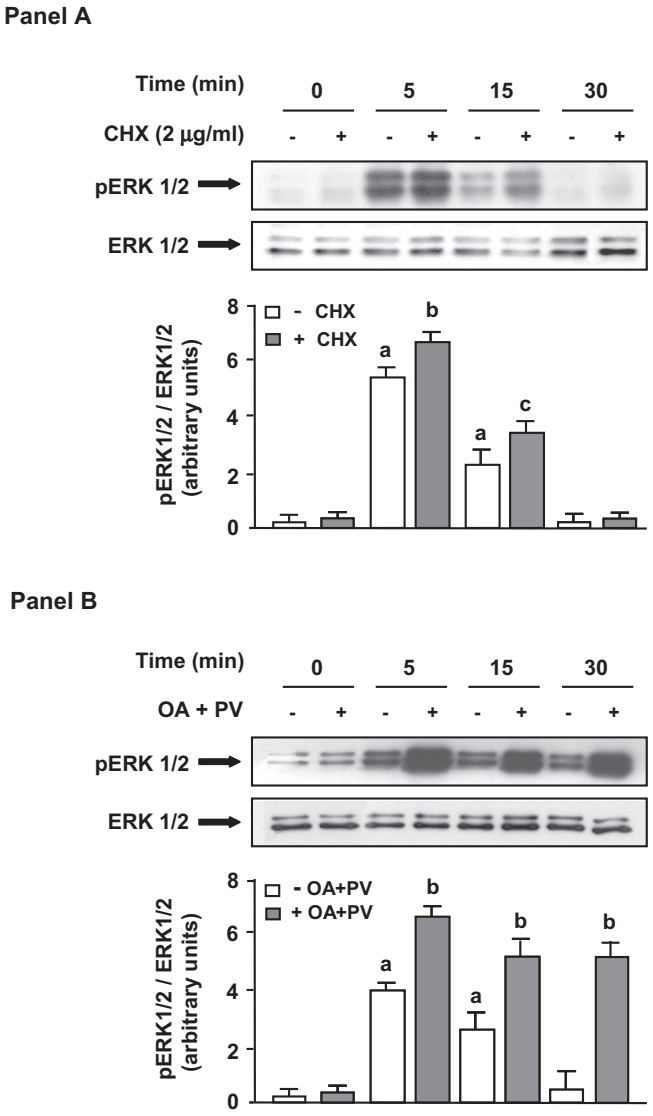

Fig. 1. Effect of CHX and phosphatase inhibitors upon BSA-promoted phosphorylation of ERK 1/2. OK Cells were treated as described in Section 2 and incubated with BSA (20 mg/ml) for different times, in the presence or absence of CHX (2 μg/ml) (Panel A) or the phosphatase inhibitors okadaic acid (OA, 100 nM) and sodium pervanadate (PV, 10 μM) (Panel B) which were added 30 min prior to BSA. Total cell lysates were analyzed by Western blot using antibodies against the phosphorylated forms of ERK1/2 (pERK1/2). Specific bands were detected by chemiluminescence. The membranes were stripped and total ERK1/2 were detected using specific antibody, as loading control. The intensity of specific bands was quantified and normalized against loading control and values were expressed in arbitrary units. The figure shows a representative Western blot (top) and quantification ($n = 3$) (bottom), expressed as mean ± SEM. Panel A: (a), $P < 0.001$ vs. time 0 min in the absence of CHX; (b) and (c), $P < 0.001$ and $P < 0.01$, respectively vs. the corresponding times in the absence of CHX. Panel B: (a), $P < 0.001$ vs. time 0 min in the absence of OA+PV and (b), $P < 0.001$ vs. the corresponding times in the absence of OA+PV.

2.6. Immunofluorescence and microscopy

OK cells were grown on coverslips, subjected to treatments and then incubated with an anti-MKP-1 antibody in a humidified chamber for 24 h at 4 °C. Primary antibodies were detected by cy3-conjugated goat anti-mouse IgG. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The glass coverslips were mounted in FluorSave reagent (Calbiochem, San Diego, CA, USA) and observed in an epifluorescence microscope (Olympus, Tokyo, Japan).

2.7. 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

We analyzed the phospho-ERK1/2 levels reached in OK cells exposed to BSA (20 mg/ml) for different times, in the presence or absence of protein synthesis inhibitor cycloheximide (CHX, 2 μg/ml). Western blot analysis using specific antibodies against the phosphorylated forms of these kinases showed that BSA promotes the transient phosphorylation of ERK1/2 (Fig. 1A), as previously described [7,13] and that CHX significantly increases this effect (Fig. 1A). In addition, the presence of cell-permeable phosphatase inhibitors in the culture media extend and increase phospho-ERK levels reached by BSA-exposure (Fig. 1B), suggesting that the decrease in phospho-ERK levels after the stimulation is due to the presence of an active enzyme.

The fact that the inhibition of protein synthesis increases BSA-induced phospho-ERK1/2 levels suggests that a short-lived phosphatase is involved in MAPK dephosphorylation. Since MKP-1

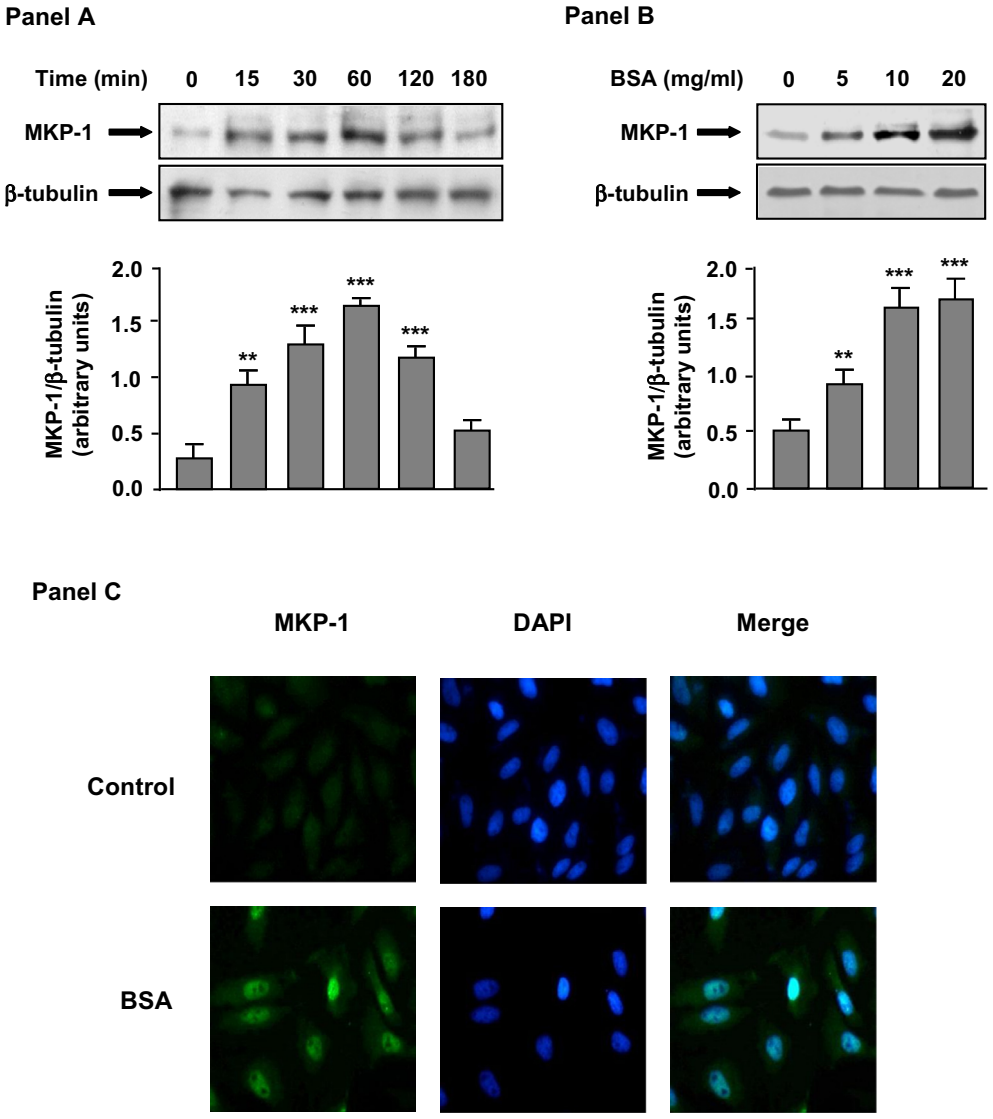

Fig. 2. Effect of BSA on MKP-1 protein levels. Panel A and B: OK cells were incubated with different concentrations of BSA for the indicated times. Total cell lysates were analyzed by Western blot using an anti-MKP-1 antibody. Specific bands were detected by chemiluminescence. The membranes were stripped and β-tubulin was detected as loading control. The intensity of specific bands was quantified and normalized against loading control and values expressed in arbitrary units. The figure shows a representative Western blot (top) and quantification (bottom). Data are mean ± SEM ($n = 3$). Panel A: $^{***}P < 0.001$ and $^{**}P < 0.01$ vs. time 0 min. Panel B: $^{***}P < 0.001$ and $^{**}P < 0.01$ vs. 0 mg/ml of BSA. Panel C: Cells were cultured on coverslips and then incubated with BSA for 1 h. After treatment, the cells were fixed and successively incubated with an anti-MKP-1 antibody, cy2-conjugated secondary antibody (green signal) and DAPI (blue signal), for visualization of MKP-1 and the nuclei respectively. Samples were analyzed by fluorescence microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

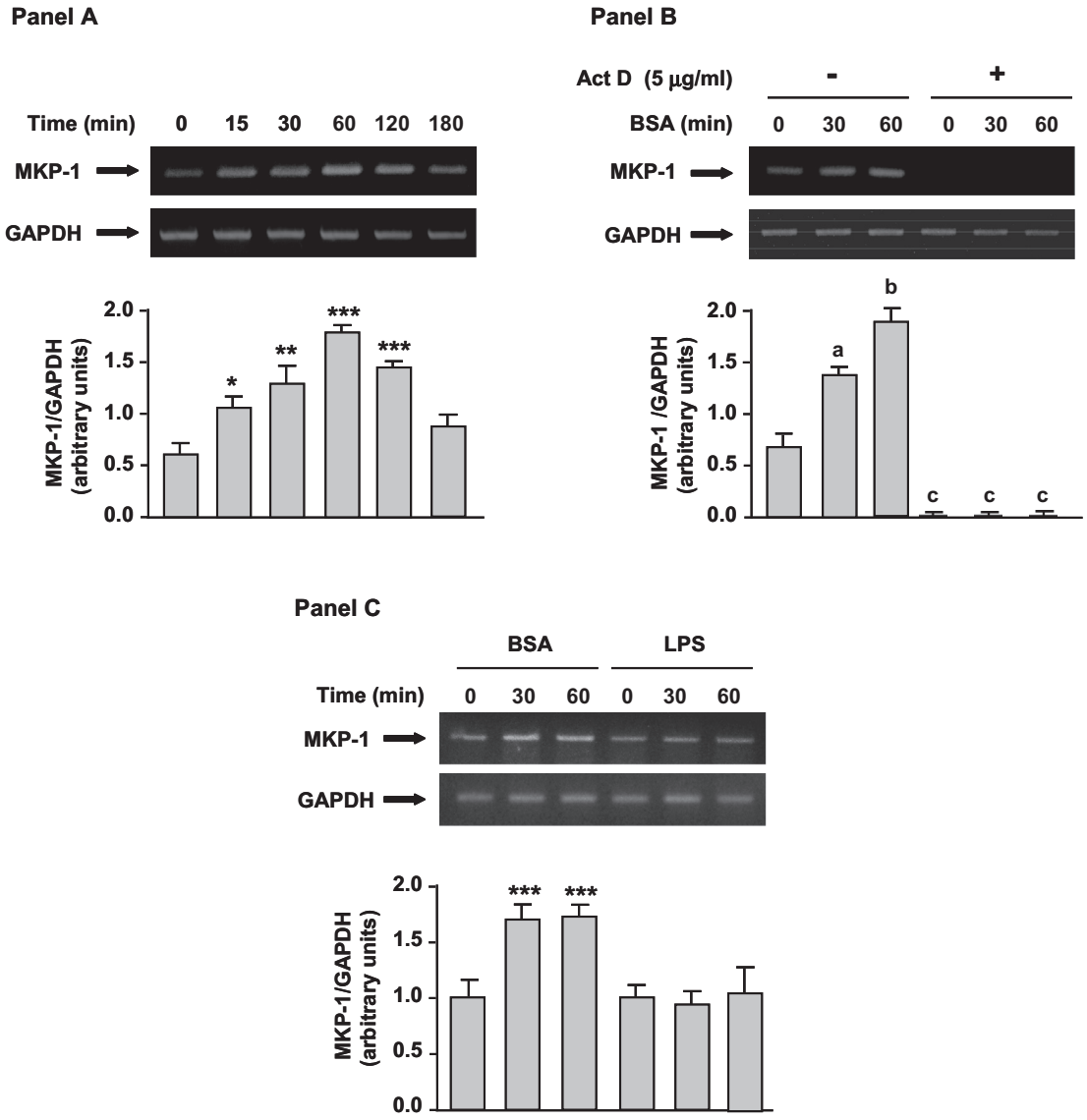

Fig. 3. Effect of BSA on MKP-1 mRNA levels. OK cells were incubated for the indicated times with 20 mg/ml BSA (Panel A), BSA alone or in the presence of Act D (5 µg/ml) added 30 min before the addition of BSA (Panel B) and with BSA or 50 ng/ml LPS (Panel C). Then, total RNA was isolated and subjected to semiquantitative RT-PCR assays using specific oligonucleotides for MKP-1 and for GAPDH (as loading control), designed as described in Materials and Methods. PCR products were resolved on agarose gels with ethidium bromide. The optical density of each band was quantified and MKP-1 mRNA values were normalized against GAPDH mRNA abundance and expressed in arbitrary units. The figure shows a representative gel (top) and quantification, expressed as the mean ± SEM ($n = 3$) (bottom). Panel A: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ vs. time 0 min. Panel B: (a), $P < 0.001$ and (b), $P < 0.01$ vs. time 0; (c), $P < 0.001$ vs. the corresponding time in the absence of Act D. Panel C: $^{***}P < 0.001$ vs. time 0 min.

meets this requirement, we analyzed MKP-1 protein levels in OK cells treated with BSA (20 mg/ml) for different times. Western blot analysis showed that MKP-1 is barely detected in control cells and that exposure to albumin increases protein levels. This increase in MKP-1 levels was evident after 15 min of exposure, peaked at 1 h (6-fold) and declined thereafter (Fig. 2A). Moreover, the effect of BSA on MKP-1 protein levels was concentration-dependent (Fig. 2B). Also, an increased expression of MKP-1 was detected by immunocytochemistry in the nuclei of OK cells exposed to BSA for 1 h when compared to control cells (Fig. 2C).

Next we analyzed the effect of BSA on MKP-1 mRNA levels. As shown in Fig. 3A, BSA treatment produced a transient, albeit time-dependent, increase in MKP-1 mRNA levels. This effect was statistically significant already after 15 min, reached a maximum 3-fold increase at 60 min, and returned to basal levels after 3 h (Fig. 3A). Inhibition of RNA transcription by Act D abolished the effect of BSA on MKP-1 mRNA levels (Fig. 3B), which suggests that BSA promotes MKP-1 gene transcription activation. Since BSA could contain endotoxins (<0.1 ng/mg BSA, according to manufacturer) able to induce MKPs, we also analyzed the levels of MKP-1 in cells exposed to LPS in a concentration higher than those that could be expected by contamination of BSA (50 ng/ml). As shown in Fig. 3C, MKP-1 mRNA levels were increased after 15 and 30 min of BSA exposure while LPS was ineffective.

Several reports demonstrate that posttranslational modifications increase MKP-1 half life [21,31]. Therefore, the upregulation of MKP-1 protein by albumin can be produced not only by the activation of MKP-1 gene transcription but also by posttranslational modifications that lead to MKP-1 accumulation. To test whether albumin upregulates MKP-1 protein levels by posttranslational modifications, cells were transiently transfected for the overexpression of recombinant flag-MKP-1 protein. Because the expression of flag-MKP-1 is driven by a constitutively active promoter, monitoring recombinant protein levels, by Western blot using an antibody against flag, is a useful approach to assess posttranslational effects of BSA on protein stability, regardless of its action on MKP-1 gene expression.

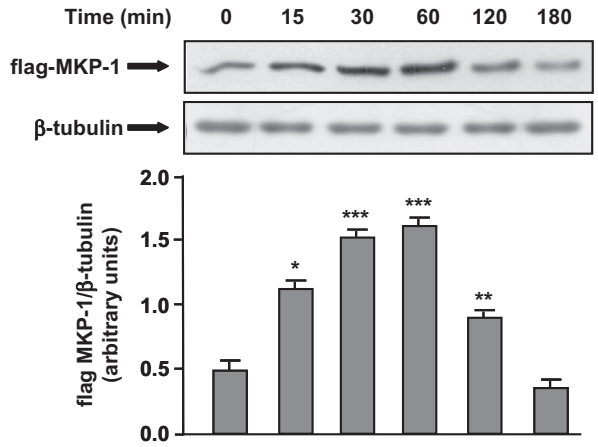

Fig. 4. Postranslational regulacion of flag-MKP-1 protein levels by BSA. OK cells were transfected with p3xFLAG-CMV-7.1.MKP-1 (pFLAG-MKP-1) for the expression of flag-MKP-1 protein, and incubated with BSA for the indicated times. Total cell lysates were analyzed by Western blot using an anti-flag antibody. Specific bands were detected by chemiluminescence. The membranes were stripped and β-tubulin was detected as a loading control. The intensity of specific bands was quantified and normalized against loading control and values were expressed in arbitrary units. The figure shows a representative Western blot (top) and the quantification, expressed as mean ± SEM ($n = 3$), (bottom). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ vs. time 0 min.

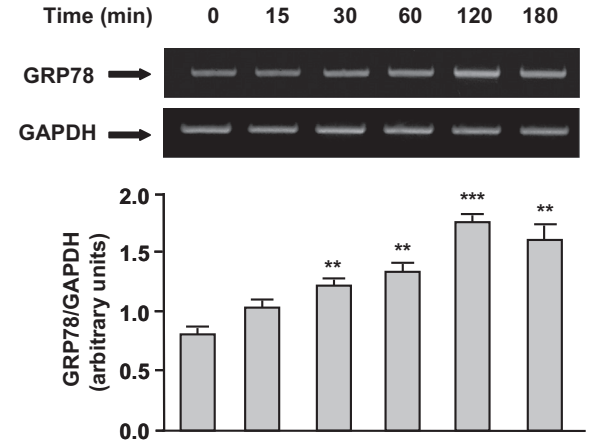

Fig. 5. Effect of BSA on the mRNA levels of GRP78. Cells were incubated with BSA for the indicated times and then, RNA was isolated and subjected to semiquantitative RT-PCR assays using specific primers for GRP78 and GAPDH (loading control). PCR products were resolved on agarose gels with ethidium bromide. The optical density of each band was quantified and GRP78 mRNA values were normalized against GAPDH abundance and expressed in arbitrary units. The figure shows a representative gels (top) and the quantification expressed as mean ± SEM ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$ vs. 0 min.

Thus, we studied the effect of BSA on the accumulation of MKP-1 and demonstrated that flag-MKP-1 levels increase after 15 min of exposure to BSA, reaching a maximum after 1 h of stimulation and decreasing to control levels thereafter (Fig. 4).

Next we tested the effect of BSA upon GRP78 expression, an ER stress marker. As expected, BSA promoted an increase in GRP78 mRNA levels, which reached a maximum at 2 h (Fig. 5). To test the role played by ERK1/2 in the increase in GRP78 mRNA mediated by BSA, cells were exposed to compounds that inhibit MAPK activation and then incubated with BSA. MEK kinase inhibition by PD98059, which impairs ERK activation, abrogated the increase in GRP78 mRNA by BSA, whereas JNK inhibitor SP600125 had no effect on GRP78 upregulation by BSA (Fig 6A). Since these findings suggest that ERK1/2 are involved in GRP78 expression induced by BSA, we also tested the effect of MKP-1 on the expression of this messenger. As shown in Fig. 6B, albumin did not increase GRP78 mRNA levels in OK cells overexpressing MKP-1 (Flag-MKP-1) (Fig 6B).

4. Discussion

The present work describes the effect of BSA on MKP-1 expression in OK cells. MKP-1 is a phosphatase involved in the inactivation of different MAPKs, such as ERK1/2, JNK1/2 and p38. Our study demonstrates that exposure of OK cells to BSA promotes a rapid increase in MKP-1 through a mechanism that includes both the activation of MKP-1 gene transcription and the stabilization of MKP-1 protein by a posttranslational modification. We also demonstrate that BSA increases mRNA levels of GRP78, a key marker of ER stress, through an ERK-dependent pathway. In line with this finding, we also demonstrate that MKP-1 overexpression prevented BSA-induced GRP78 upregulation. We then postulate that MKP-1 might participate in the regulation of the ER stress response to an albumin overload.

It is well recognized that albuminuria is an adverse prognostic feature in patients with renal disease. Dysfunction of albumin reabsorption in the proximal tubule, due to reduced expression of the receptor that mediates its endocytosis, that is megalin, may explain the microalbuminuria in early-stage diabetes. Meanwhile, massive non-selective proteinuria is ascribed to various disorders of the glomerular filtration barrier, including podocyte detachment, glomerular basement membrane rupture, membranous nephropathy and others [35]. Therefore, patients with albuminuria frequently develop tubulointerstitial damage and fibrosis and, ultimately, suffer end-stage renal failure [36]. Although albumin has been considered an inert molecule whose main functions are contributing to oncotic pressure and carrying several compounds, growing evidence supports an action of albumin on the cells with which it makes contact [37,38]. In the kidney, albumin stimulates proximal tubule hypertrophy and hyperplasia [39], while *in vivo* experiments have shown that proteinuria induces both proliferation and apoptosis of proximal tubular epithelial cells [1]. Accordingly, albumin has been demonstrated to promote MAPK activation [13,14], although the corresponding signaling cascade triggered by this stimulus is still scarcely documented. The present work demonstrates that albumin triggers not only ERK1/2 activation but also the induction of MKP-1, a phosphatase able to dephosphorylate and, consequently, inactivate MAPKs [18].

The rapid increase in MKP-1 mRNA levels by BSA was abolished by Act D, which indicates that the effect is due to the activation of gene transcription. The increase in MKP-1 mRNA levels by other stimuli, such as adrenocorticotropin [22], luteinizing hormone (LH) [21], and heat shock [27] showed a similar temporal pattern and was also dependent on gene transcription. BSA also induced MKP-1 protein levels in a similar way, as a significant increase was detected after 15 min. MKP-1 protein sequence reveals several putative sites for different posttranslational modifications, among which ERK-dependent phosphorylation is the one most thoroughly analyzed [21,31]. S359 and S364, conserved sites in different species, are the major sites for ERK1/2 phosphorylation and the ones responsible for a decrease in human MKP-1 proteolysis [31]. Moreover, S359 and S364 are also involved in 8Br-cAMP-induced, ERK-mediated MKP-1 stabilization in mouse MA-10 Leydig cells [21]. Analysis of the MKP-1 cDNA fragment obtained from OK cells shows that it shares 97% gene sequence identity with *Monodelphis* sp. Moreover, cDNA analysis reveals that MKP-1 from *Opposum* shares 82 and 81% gene sequence identity with human and mouse

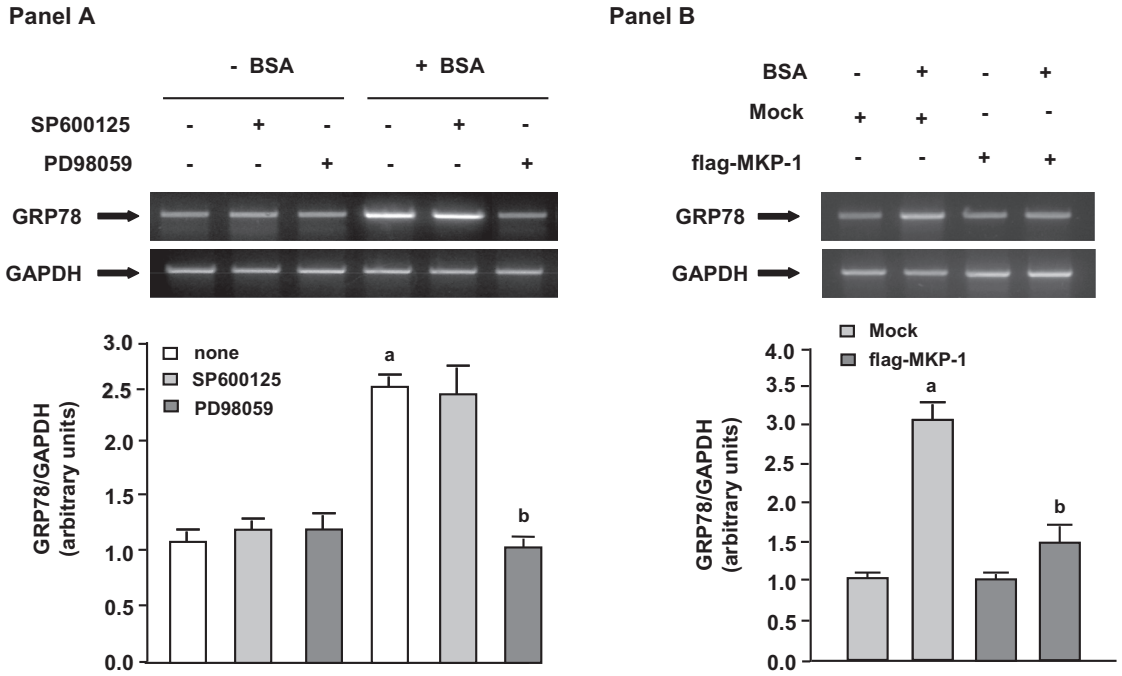

Fig. 6. Effect of JNK 1/2 and ERK 1/2 activity on GRP78 mRNA levels. Cells were incubated with 20 μM SP600125 or 50 μM PD98059 or without inhibitors (None) and then incubated in the absence (Control) or presence of BSA (Panel A); or cells were transfected with pFLAG-MKP-1 (flag-MKP-1) or the empty vector (Mock) and then incubated with BSA (Panel B). After 2 h of treatment, total RNA was isolated and subjected to semiquantitative RT-PCR assays using specific primers for GRP78 and GAPDH designed as described in Materials and Methods. GAPDH cDNA was used as loading control. PCR products were resolved on agarose gels with ethidium bromide. The optical density of each band was quantified and GRP78 mRNA values were normalized against GAPDH abundance and expressed in arbitrary units. The figure shows representative gels (top) and quantification (bottom) expressed as the mean ± SEM ($n = 3$). Panel A: (a), $P < 0.001$ vs. control in the absence of inhibitors and (b) $P < 0.001$ vs. BSA without PD98059. Panel B: (a), $P < 0.001$ vs. mock transfected cells incubated without BSA and (b), $P < 0.001$ vs. mock transfected cells incubated with BSA.

genes, respectively. This strongly suggest that the consensus sites for ERK-phosphorylation that are responsible for MKP-1 stabilization in mouse an human could also be present in MKP-1 from OK cells and, ultimately, be involved in BSA-induced MKP-1 stabilization.

In addition to S359 and S364, MKP-1 protein presents other ERK consensus sites whose phosphorylation seems to affect protein stability *in vivo* in different ways [21,32]. In our study we made use of a flag-MKP-1 construct driven by a constitutive promoter that allows us to study whether BSA-induced protein accumulation is due to an increase in its half life caused by a posttranslational modification. Although we cannot yet establish the nature of this modification, phosphorylation by ERK is a likely candidate. Moreover, although the flag-MKP-1 chimera contains the murine MKP-1, the fact that the ERK-phosphorylation consensus sites are conserved among several species, argue in favor of ERK-phosphorylation and stabilization of MKP-1 by BSA in OK cells. Then, we conclude that BSA-mediated MKP-1 protein increase is tightly upregulated by, at least, two different mechanisms: activation of gene transcription and protein stabilization.

MKP-1 is described as a nuclear protein, although it lacks a classical nuclear targeting sequence [20]. In line with this, we detected MKP-1 in the nucleus of BSA-treated OK cells. Due to its nuclear localization, it has been proposed that MKP-1 has an important role as a regulator of MAPK-dependent nuclear events, such as gene transcription. Indeed, we have demonstrated that, in Leydig cells, LH hormone rapidly triggers ERK1/2 activation and the ERK-dependent expression of STAR, a key steroidogenic gene. After a short period, LH also induces MKP-1, which turns off the expression of this gene [21]. Here we focused our attention on a gene potentially modulated by MAPK/MKP signaling, GRP78, a widely known marker of ER stress. First, we determined that induction of GRP78 by BSA is dependent on ERK activity. In line with this finding, we demonstrated that the effect of BSA on GRP78 induction is significantly reduced in OK cells overexpressing flag-MKP-1, a catalytically active chimera [21]. Then, we conclude that MKP-1 induction by BSA contributes to attenuate its effect on OK cells, particularly those effects dependent on MAPKs.

Here we have analyzed the effect of MKP-1 only on GRP78, a gene which is induced by BSA through an ERK-dependent mechanism. However, it should be mentioned that BSA also activates JNK1/2 [7], which suggests the participation of these kinases in protein expression or activation as a response to BSA. Consequently, MKP-1 could also participate in the regulation of JNK-dependent processes triggered by BSA and, potentially, in all BSA-mediated processes in which MAPK are involved. In this context, it is important to mention that the action of MKP-1 on JNK1/2 mediates cell survival in a model of ER stress. Indeed, it has been demonstrated that tunicamycin promotes JNK1/2 transient activation and MKP-1 accumulation as a result of its phosphorylation [40]. In turn, MKP-1 promotes JNK1/2 dephosphorylation, which results in cell survival [40].

In summary, we demonstrated that albumin, a molecule largely considered inert, not only promotes ERK1/2, but also tightly upregulates MKP-1, which is involved in – at least – ERK1/2 dephosphorylation. Our study also shows that MKP-1 is a potential modulator of the events associated with ER stress induced by BSA in OK cells and, consequently, a putative target of pharmacological approaches to overcome albumin-induced tubular cell damage.

Acknowledgments

This work was supported by Grants from Universidad de Buenos Aires to CP and Consejo Nacional de Investigaciones Científicas y Técnicas to CP and CFM.

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