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Heat shock triggers MAPK activation and MKP-1 induction in Leydig testicular cells

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Abstract

Testicular function is highly dependent on temperature control. In Leydig testicular cells, the signaling pathway activated by heat stress is poorly defined. Here we describe the molecular events triggered by heat shock (HS, 10 min, 45 °C) in MA-10 cells. HS produced a rapid and transient activation of ERK1/2 and JNK kinases, and also increased MAP kinase phosphatase-1 (MKP-1) protein and mRNA levels. The effect of HS on MKP-1 messenger reached significance at 15 min, peaked (3.5-fold) at 60 min, and was partially dependent on ERK1/2 activity. The temporal profiles of MKP-1 protein levels and MAPKs phospho-dephosphorylation suggest that MKP-1 induction could contribute to ERK1/2 and JNK inactivation after HS. In summary, this study indicates that the response to heat stress in Leydig cells includes the activation of MAPKs related to cell survival (ERK1/2) and death (JNK), and the induction of a MAPK activity inhibitory loop.

Keywords: Leydig cells; Heat shock; MAPKs; MKP-1; HSP72

Heat shock (HS) affects several cellular functions as a consequence of heat-induced modifications in the structure and/or function of key proteins. As such, HS triggers several signaling events which contribute to either cell death or survival, such as the activation of different members of the MAP kinases (MAPKs) family and the well-characterized transcriptional activation of the genes coding for heat shock proteins (HSPs). Thus, the fate of cells exposed to HS depends on the balance between survival and apoptotic mechanisms triggered by this stressor.

MAPKs constitute a family of serine/threonine kinases ubiquitously expressed that play a crucial role in transmitting transmembrane signals required for cell growth, differentiation, and apoptosis [1,2]. They can be classified into at least three subfamilies: extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal protein kinases (JNKs), also known as stress-activated protein kinases (SAPKs), and p38 MAPKs. A common feature of all kind of MAP kinases is the requirement of dual threonine and tyrosine phosphorylation to display maximal activity [2]. Depending on the cellular type, the stressors prompt the phosphorylation of a particular set of MAPKs family members [3–5]. In the case of heat-triggered MAPK activation, ERK phosphorylation was shown to enhance cell resistance to the heat stress [4], while the phosphorylation of JNKs has been reported as an event associated with heat-induced cell death [6].

The duration and magnitude of MAPK activity is linked to the action of specific phosphatases. It is well recognized that the MAP kinase phosphatase (MKPs) family, a dual specificity (threonine/tyrosine phosphatases) group of enzymes, plays an important role in regulating MAPK activity [7]. One member of the MKPs family is MKP-1, a nuclear enzyme rapidly

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induced by growth factors, hormones, and stress signals including HS [8,4], which displays activity towards ERKs, JNKs, and p38 [7]. Thus, MKP1 induction is another potential component of the signaling cascade prompted by HS.

Leydig cells are highly sensitive to heat stress. It is well documented that the main physiological function of Leydig cells, the production of sexual male steroids, is transiently but severely reduced by heat stress [9,10]. However, the signaling pathway activated by HS in Leydig cells has been poorly investigated. Thus, the aim of this study was to analyze the signaling cascade triggered by HS in a Leydig cell line, MA-10 cells. Our study demonstrates that HS prompts the rapid and transient activation of ERKs, JNKs, and also the induction of MKP-1 and HSP72, an inducible HS protein. The effect on MKP-1 protein level mirrors a transcriptional action exerted through a mechanism that involves ERK1/2 activity.

Materials and methods

Reagents. Cell culture supplies were obtained from Gibco-Invitrogen (CA, USA). Plasticware was from Corning-Costar (NY, USA). BSA and sodium orthovanadate were obtained from Sigma Chemicals (MO, USA). BAPTA-AM, PD98059, and A23187 were purchased from Calbiochem–Novabiochem (CA, USA). Electrophoresis reagents and supplies were from Bio-Rad Laboratories (CA, USA). [α-^{32}P]dCTP was from NEN Life Science Products (MA, USA). All other chemicals were commercial products of the highest grade available.

MA-10 Leydig cell culture and heat shock treatment. The MA-10 mouse Leydig tumor cell line was generously provided by Dr. Mario Ascoli, University of Iowa, College of Medicine (Iowa city, IA, USA). Cells were grown in Waymouth's MB752/1 medium supplemented with 1.1 g/L $NaHCO_3$, 20 mM Hepes, 50 μg/ml gentamicin, and 15% horse serum at 36 °C in a humidified atmosphere containing 5% CO_2. Cells were grown in 60 mm dishes up to 60–70% confluence and 24 h prior to the experiments, the medium was replaced by serum-free medium in order to avoid serum-mediated MKP-1 induction. When indicated, the different reagents were added to the incubation medium 30 min (10 μM BAPTA-AM, 10 μM A23187) or 60 min (50 μM PD98059) before HS treatment. The experimental design of heat shock comprised a 10 min incubation in a 45 °C chamber (HS) followed by a second incubation at 37 °C in a humidified atmosphere containing 5% CO_2 for different times (recovery times).

Western blot analysis. After the treatments, cells were washed with phosphate-buffered saline and lysed in a buffer containing 20 mM Tris, pH 7.4, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 mM sodium orthovanadate. Cellular extracts were centrifuged for 10 min at 1500*g* and 4 °C, and proteins from the supernatants were analyzed by Western blot as previously described [8]. Briefly, proteins (40 μg/lane) were resolved by SDS–polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were probed overnight at 4 °C with the appropriate polyclonal antibodies: anti-MKP-1 (Santa Cruz Biotechnology, CA, USA), anti-phosphorylated-ERK1/2, -JNK, and -p38 (Cell Signaling Technology, MA, USA), and anti-HSP72 (Stressgen, Victoria, Canada). After this step, blots were washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad Laboratories). Immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham–Pharmacia Biotech, Bucks, UK) and quantified by densitometry using an image analyzer (Storm Phosphorimager, Molecular Dynamics Scanner, Amersham–Pharmacia Biotech). In order to perform the corresponding loading control, the blots were subsequently stripped and reprobed using anti-β-tubulin (Santa Cruz Biotechnology) or anti-total ERK1/2 (Cell Signaling Technology) antibodies. Results are expressed in arbitrary units representing the intensities of specific signals normalized against the corresponding loading control.

Northern blot analysis. Total RNA was isolated from MA-10 Leydig cells using the Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA samples (20 μg/lane) were electrophoresed in a 1% agarose gel with 2.2 M formaldehyde and blotted onto Hybond-N^+ nylon membranes (Amersham–Pharmacia Biotech) by capillarity. After prehybridization, blots were hybridized overnight at 42 °C using [α-^{32}P]dCTP-radiolabeled cDNA probes. A random priming-labeled 741 bp fragment of mouse MKP-1 cDNA was used for MKP-1 mRNA detection [8]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected as loading control using a PCR-labeled specific probe [8]. Hybridization, washing of the filters, detection of the signals, and other procedures were carried out as previously described [8]. The autoradiograms were quantitated by scanning densitometry and the data were normalized against GAPDH mRNA.

Statistics. Results are shown as means ± SD. Statistical significance was evaluated using ANOVA followed by Tukey test. A value of $P < 0.05$ was considered significant.

Results

Temporal profile of MAPKs activation

Testicular steroidogenesis is particularly sensitive to heat stress. Studies performed using MA-10 Leydig cells have demonstrated that exposure of the cells to 45 °C for 10 min severely and transiently reduced the hormonal stimulation of steroid synthesis [10]. Thus, we first examined the activation kinetics of MAPK in MA-10 cells that were heat-shocked for 10 min at 45 °C. ERK1/2, JNK, and p38 activation was monitored by immunoblot analysis with specific antibodies against the respective phosphorylated forms of the enzymes (P-ERK1/2, P-JNK, and P-p38). As shown in Fig. 1, HS prompted the activation of ERK1/2 and JNK with similar kinetics. By contrast, HS failed to produce a consistent effect on p38 activity (data not shown).

Analysis of MKP-1 and HSP72 protein levels in heat shocked MA-10 cells

In several systems, stimuli which prompt MAPK activation increase also MKP-1 protein levels through a transcriptional effect. We therefore sought to analyze the abundance of MKP-1 protein at various times during the recovery of the cells after HS (Fig. 2A). MKP-1 protein was detected in non-treated cells and HS treatment produced a transient increase of MKP-1 protein levels that reached a maximum at 30 min (2.5–3-fold).

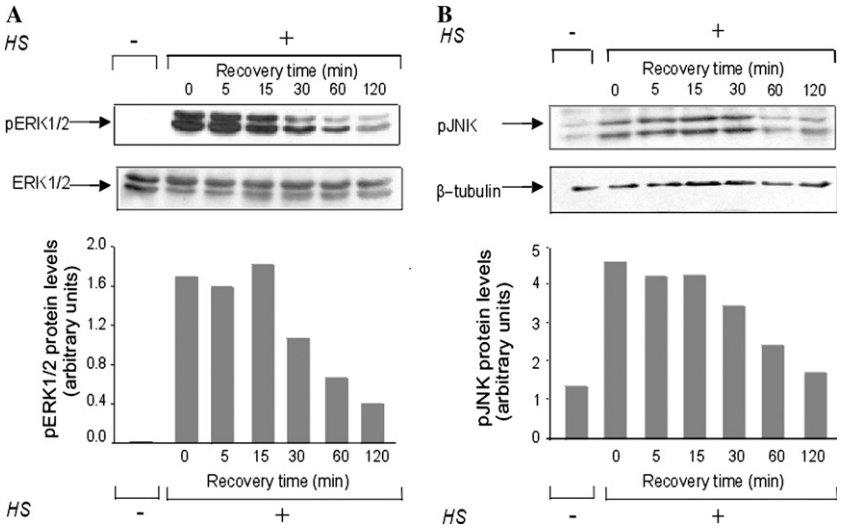

Fig. 1. Effect of HS on MAPKs activity. MA-10 Leydig cells were cultured under normal conditions or heat shocked (HS) and allowed to recover for the indicated times. Samples were prepared as indicated in Materials and methods, and the activation of ERK1/2 (A) or JNK (B) was analyzed by Western blot with anti-phosphorylated-ERK1/2 or -JNK antibodies. Shown are representative Western blots and the corresponding scanning densitometry quantitations of the films, normalized against total ERK1/2 (A) or β-tubulin (B) of three independent experiments.

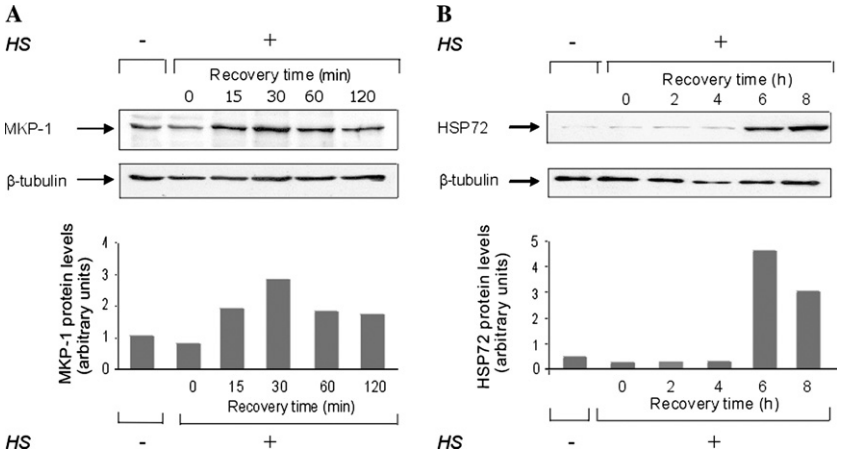

Fig. 2. Effect of HS on MKP-1 and HSP72 protein levels. MA-10 Leydig cells were cultured under normal conditions or heat shocked (HS) and allowed to recover for the indicated times. Samples were prepared as indicated in Materials and methods, and cellular proteins were analyzed by Western blot in order to determine the cellular content of MKP-1 (A) and HSP72 (B). Shown are representative Western blots and the corresponding scanning densitometry quantitations of the films, normalized against β-tubulin of three independent experiments.

Another component of the cellular response to heat stress is the induction of HS proteins, molecular chaperones which accumulate following heat shock or other stress, and that are crucial for cell survival after injury. Thus, the kinetics of the heat-mediated induction of HSP72, a major inducible HS protein, was evaluated. As shown in Fig. 2B, exposure of the cells to HS resulted in a 6–9-fold increase in HSP72 levels after 6 h of injury as detected by Western blot analysis.

Effects of HS on MKP-1 mRNA levels

Next, we also examined the effect of HS on MKP-1 mRNA abundance by Northern blot. As observed in Fig. 3, HS produced a significant increase in MKP-1 mRNA levels as early as 15 min. The effect was maximal (3.5-fold) after 60 min of HS and declined thereafter.

In order to gain insight into the molecular basis underlying MKP-1 gene transcription in response to HS in MA-10 Leydig cells we employed a pharmacological approach. For this purpose, MKP-1 mRNA levels were evaluated in control or heat-shocked cells harvested after 60 min (recovery time of maximal induction) in the presence or absence of appropriate inhibitors.

To test the putative role of ERK1/2 pathway in the transcriptional induction of MKP-1, the effects of PD98059-compound that inhibits the upstream ERK1/2

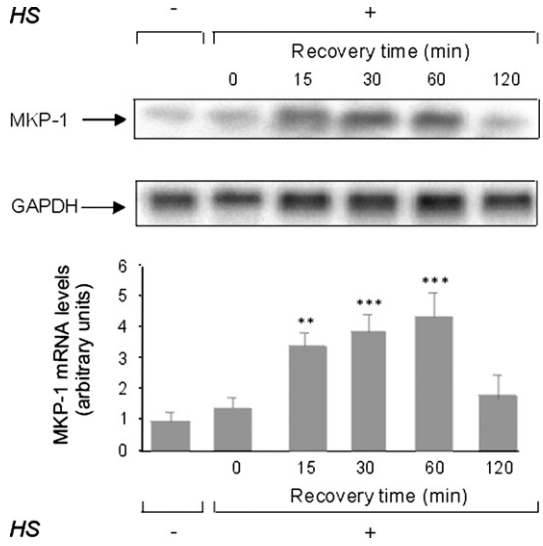

Fig. 3. Time-course of HS induction of MKP-1 mRNA levels. MA-10 Leydig cells were cultured under normal conditions or heat shocked (HS) and allowed to recover for the indicated times. Samples were prepared as indicated in Materials and methods, and total RNA was analyzed by Northern blot in order to determine MKP-1 mRNA levels. The figure shows one representative Northern blot of three independent experiments. Autoradiograms were quantitated by scanning densitometry and the data were normalized against GADPH mRNA. Values presented in the lower part of the figure are expressed in arbitrary units and represent means ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control cells (HS−).

kinase (MEK1/2)- on MKP-1 messenger accumulation were examined. Northern blot analysis revealed that this compound abrogated the effect of HS on MKP-1 messenger levels (Fig. 4A), indicating the involvement of MEK1/2–ERK1/2 activities in the regulation of MKP-1 mRNA levels.

Several reports underline the essential role played by Ca^{2+} in the regulation of MKP-1 expression [12,13]. Therefore, we assessed the role of Ca^{2+} in the heat-mediated regulation of MKP-1 gene expression by studying the effects of the Ca^{2+} ionophore A23187 and the cell permeant Ca^{2+} chelator BAPTA-AM on MKP-1 mRNA. A23187 strongly increased HS-mediated MKP-1 induction. However, that response had a similar magnitude to the one produced by the ionophore itself (Fig. 4B). On the other hand, BAPTA-AM notably reduced HS-mediated MKP-1 induction (Fig. 4C). These results demonstrate that Ca^{2+} is necessary and sufficient to trigger MKP-1 induction in MA-10 cells, but they do not allow us to establish whether or not HS action involves an increase in intracellular Ca^{2+}.

Discussion

This study describes molecular events triggered by heat stress (10 min, 45 °C) in MA-10 Leydig cells such as the rapid activation of MAP kinases and also two more delayed events such as MKP-1 and HSP72 induction.

Regarding HS-mediated MAP kinase activation, HS triggered the phosphorylation of ERK1/2 and JNK to a similar extent. In our experimental conditions, heat shock failed to produce a consistent effect on p38 activity in MA-10 cells.

MKP-1 is an immediate-early gene product that is induced in response to both mitogenic and stress stimuli. In steroidogenic systems, we have demonstrated the transcriptional activation of MKP-1 gene in Y1 adreno-

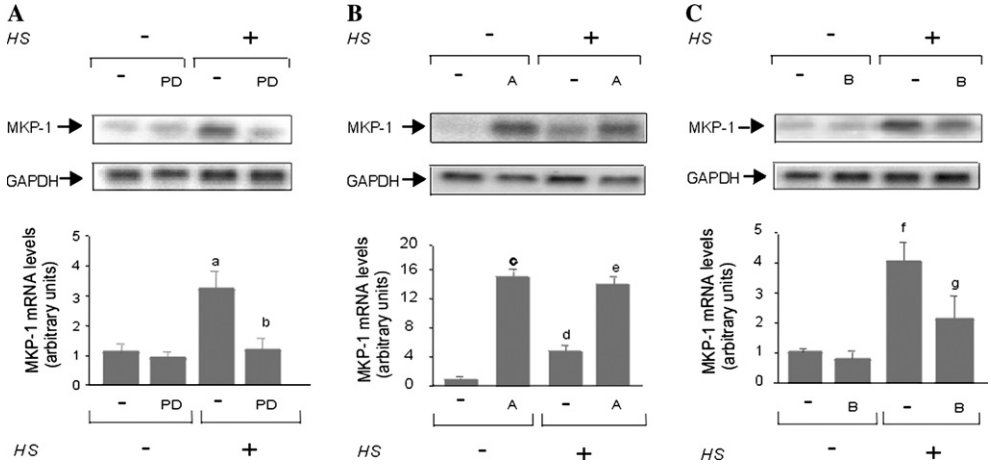

Fig. 4. Role of MEK-ERK1/2 activity and intracellular Ca^{2+} on HS-mediated MKP-1 induction. MA-10 Leydig cells were preincubated in the presence or absence of 50 μM PD 98059 (A), 10 μM A23187 (B) or 10 μM BAPTA (C), cultured under normal conditions or heat shocked (HS), and allowed to recover for 1 h. Samples were prepared as indicated in Materials and methods, and total RNA was analyzed by Northern blot in order to determine MKP-1 mRNA levels. Shown are representative autoradiograms of those obtained in three independent experiments. The autoradiograms were quantitated by scanning densitometry, data were normalized against GADPH mRNA and presented as histograms. Data are expressed as mean arbitrary units ± SD of three independent experiments. (a) $P < 0.01$ vs. HS− alone; (b) $P < 0.01$ vs. HS+; (c) $P < 0.001$ vs. HS−; (d) $P < 0.05$ vs. HS− alone; (e) $P < 0.001$ vs. HS+; (f) $P < 0.01$ vs. HS− alone; and (g) $P < 0.05$ vs. HS+.

cortical cells by ACTH [8]. Moreover, a role of MKP-1 in the expression of steroidogenic enzymes was also demonstrated in human adrenocortical cells [14,15]. Here we demonstrate that the response of MA-10 Leydig cells to HS also includes MKP-1 induction. We detected a significant increase of MKP-1 mRNA levels in heat-shocked cells that were left to recover for a period of 15 min. Moreover, this increase in mRNA level was followed by an increase in the levels of MKP-1 protein at 30 min.

The mouse MKP-1 promoter contains multiple binding sites for different transcription factors [16]. In accordance, a broad spectrum of MKP-1 inducers is well documented [11,17,18]. The present data indicate that MKP-1 gene induction by HS in MA-10 cells is strongly dependent on ERK1/2 activity. Moreover, we demonstrated that in this cell line Ca^{2+} plays an obligatory role in MKP-1 induction by HS.

It is known that in mammalian cells HSPs expression is mainly regulated by heat shock factor-1 (HSF1) [19], a transcription factor which activity is regulated by phosphorylation. In fact, ERK1/2 and JNK phosphorylate HSF-1 and suppress its transcriptional activity promoting its dispersion from the sites of transcription during recovery from heat shock [20,21]. In this context, it its possible to speculate that MKP-1 could avoid HSF-1 phosphorylation by MAPKs and thus, prolong HSF-1 action in the nucleus. Here we demonstrated that another component of the signaling cascade prompted by HS in MA-10 cells is the induction of HSP72. Since the induction of HSP72 occurs after MAPK activation and MKP-1 induction, a relationship between these molecular processes seems plausible. The study of such relationship is to be addressed in the near future.

In summary, in this report we showed that Leydig cells respond to heat stress with rapid events, such as MAPKs activation (related to cell survival-ERK activation and death-JNK activation), intermediate responses (MKP-1 induction), and with more delayed actions like the induction of HSP72.

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