Osmotic Regulation of MG-132-induced MAP-kinase Phosphatase MKP-1 Expression in H4IIE Rat Hepatoma Cells

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Key Words

Apoptosis • Cell volume • Liver • Proteasome • Proteolysis • Gene expression

Abstract

Background/Aims: Proteasome inhibitors such as MG-132 are considered as potential therapeutical tools in different clinical settings. The dual specificity MAP-kinase phosphatase MKP-1 plays a role in balancing signals mediating cell death or survival. Here the effect of cell hydration on MG-132-induced MKP-1 expression was investigated in H4IIE rat hepatoma cells. **Results:** Hyperosmolarity (405mosmol/l) increased MKP-1 expression by MG-132, which was accompanied by an induction of c-Fos, c-Jun, cJun Ser⁷³ phosphorylation, and AP-1 DNA binding. MKP-1 induction by MG-132 plus hyperosmolarity was sensitive to inhibition of p38^{MAPK} and c-Jun-N-terminal kinases (JNKs) but not extracellular signal-regulated kinases Erk-1/Erk-2, and was accompanied by a decline of MAP-kinase activities. Although hyperosmolarity increased overall protein ubiquitination in presence of MG-132, ubiquitination of MKP-1 was found under normo-, but not hyperosmotic conditions. Hyperosmolarity also

enabled MG-132 to induce poly-ADP-ribose polymerase (PARP) cleavage which was sensitive to inhibition of p38^{MAPK} and JNKs but not Erk-1/Erk-2. PARP cleavage and caspase-3 activation in H4IIE cells treated with hyperosmolarity plus MG-132 was further increased by vanadate, consistent with a contribution of MKP-1 to counterbalance proapoptotic MAP-kinase signals. **Conclusion:** The findings suggest that among other factors cell hydration critically determines the cellular response to proteasome inhibitors.

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Introduction

Proteasomes represent major sites for proteolysis in eukaryotic cells. Proteasome inhibitors such as the peptide aldehyde MG-132 or lactacystin induce differential, cell type- and dose-dependent cellular responses. On the one hand, proteasome inhibitors trigger heat shock protein expression thereby providing tolerance to stressful challenges [1, 2]. On the other, they induce cell death by mechanisms involving changes in MAP-kinase activity [3, 4], inhibition of NF- κ B [5], disruption of the unfolded protein response [6], caspase activation,

and increased CD95 expression at the cell surface [7]. Proteasome inhibitors are under discussion as therapeutic tools for treatment of cancer, protein wasting and inflammatory diseases, lens opacification and vascular restenosis [8-11].

Cell hydration changes play an important role in the regulation of gene expression and metabolism [12] and critically determine the cellular response to different kinds of stress [13, 14]. For example, hepatocellular swelling stimulates anabolic pathways and protects the cells from oxidative stress [15], heat shock [16, 17] and TGF β -induced apoptosis [18]. On the other hand, hepatocellular dehydration leads into a catabolic situation and increases the cellular susceptibility to heat shock [16], oxidative stress [15, 19] and CD95-induced apoptosis [20-22]. The effect of cell hydration on the cellular response to proteasome inhibitors is unknown, but of major interest with regard to potential therapeutic applications. In addition, little is known about the influence of cell hydration on proteasomal activity.

MAP-kinase phosphatases of dual specificity (MKPs) such as MKP-1 dephosphorylate MAP-kinases at the Thr/Tyr residues critical for activation, thereby contributing to the termination of MAP-kinase activity [23, 24]. MKP-1 critically determines the kinetics and thus the cellular outcome of MAP-kinase signaling. Suppression of MKP-1 potentiated apoptosis induced by TNF- α and proteasome inhibitors in rat mesangial and human mammary epithelial cells, respectively, by allowing a sustained c-Jun-N-terminal kinase (JNK) activation [25, 26].

MKP-1 expression levels are regulated at the transcriptional and the (post)-translational level. Proteasome inhibitors including MG-132 accumulated MKP-1 due to both, AP-1-mediated stimulation of MKP-1 gene transcription and stabilization of the MKP-1 protein [4, 27, 28]. Interestingly, a hyperosmotic challenge of H4IIE rat hepatoma cells increased MKP-1 mRNA but not protein expression [17, 29] and even antagonized MKP-1 induction by insulin [30].

Here the effect of hyperosmolarity on MG-132-induced MKP-1 expression was investigated in rat H4IIE hepatoma cells. Hyperosmolarity but not normosmolarity allowed a robust induction of MKP-1 mRNA and protein expression by MG-132, which was mediated by p38^{MAPK} and c-Jun-N-terminal kinases (JNKs) probably due to increased AP-1 DNA binding activity. MKP-1 induction by MG-132 coincided with a vanadate-sensitive downregulation of p38^{MAPK} activity which probably antagonized apoptotic signals under hyperosmotic

conditions. The hyperosmotic sensitization of H4IIE cells to MKP-1 induction by MG-132 may reflect an adaptive process, which prevents decompensation of MAP-kinase activities and excessive cell death.

Materials and Methods

Materials

The antibodies raised against Erk-1/Erk-2 and dual phosphorylated JNK were from Upstate (Charlottesville, VA, USA); the antibodies recognizing the MKP-1 and c-Fos, respectively, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against phospho-p38^{MAPK} was from Promega (Madison, USA) and the anti ubiquitin antibody was from DAKO (Glostrup, Denmark). The antibody recognizing total p38^{MAPK} was from Transduction Laboratories (Heidelberg, Germany). The antibodies recognizing JNK-2, c-Jun, and Ser⁷³-phosphorylated c-Jun were from Cell Signaling (Frankfurt/Main, Germany). Anti PARP antibody was from BD Biosciences Pharmingen (San Diego, CA, USA). PD98059, SB220025, SB202474, MG-132 (benzyloxycarbonyl-leuciny-leuciny-leucinal) and lactacystin were from Calbiochem-Novabiochem GmbH (Darmstadt, Germany). SP600125 was purchased from Biomol (Hamburg, Germany). Cell culture media and fetal calf serum were from Gibco Life Technologies (Gaithersburg, MD). EDTA, sodium vanadate, NP-40, Triton X-100 and 3-methyladenine were obtained from Sigma (Munich, Germany). All other chemicals were from Merck (Darmstadt, Germany). Densitometric analysis was performed with the E.A.S.Y RH system (Herolab, Wiesloch, Germany) and the Scion Image software.

Cell culture and experimental treatment of the cells

H4IIE-C3 rat hepatoma cells (A.T.C.C. CRL 1600) were maintained on Cluster six dishes (Greiner bio-one, Frickenhausen, Germany) in DMEM-F12 / 5% CO₂ / 5 mM glucose at 37°C, pH 7.4, supplemented with 10% FCS. When cells had reached about 90% confluency, they were washed with Dulbeccos PBS and the culture was continued in 1.5 ml serum-free medium for an additional 24 h. Extracellular osmolarity was increased by addition of medium of elevated NaCl content, finally leading to hyperosmolarity (405 mosmol/l). In the normosmotic control (305 mosmol/l), the same volume of normosmotic medium was added.

Northern-blot analysis

Total RNA from H4IIE cells was isolated by using guanidinium thiocyanate solution according to the protocol given in [31], using the kit from Qiagen (Hilden, Germany). RNA samples (10 μ g) were electrophoresed in 0.8% agarose/3% formaldehyde and then RNA's were blotted to Hybond-N nylon membranes with 20xSSC (3 M NaCl, 0.3 M sodium citrate). After brief rinsing with water and cross-linking (Hoefer UV-crosslinker 500; Hoefer, San Francisco, CA, U.S.A.), the membranes were observed under UV light to determine RNA integrity and the location of the 28 S and 18 S rRNA bands.

Blots were then subjected to a 3 h prehybridisation at 43°C in 50% deionized formamide in sodium phosphate buffer (0.26 M, pH 7.2) containing 0.25 M NaCl, 1 mM EDTA, 100 µg/ml salmon sperm DNA and 7% SDS. Hybridization was carried out in the same solution with approx. 10⁶ cpm/ml dCTP-labelled random-primed MKP-1 [29] or GAPDH cDNA probes [29]. Membranes were washed three times in 2xSSC/0.1 % SDS for 15 min, twice in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1mM)/0.1% SDS for 10 min and twice in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1mM)/1% SDS for 10 min at 53°C. Blots were then exposed to Kodak AR X-Omat film at -70°C with intensifying screens.

Western Blot analysis and immunoprecipitations

At the end of the experimental treatment, medium was removed from the culture and cells were immediately lysed at 4°C using 10 mM Tris/HCl buffer (pH 7.4) containing 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF and 1 tablet/50 ml of protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The homogenized lysates were centrifuged at 20,000 g at 4°C for 10 min. To perform SDS polyacrylamide gel electrophoresis (PAGE) and Western Blot analysis the supernatant was added to an identical volume of 2x gel loading buffer containing 200 mM dithiothreitol (DTT, pH 6.8). After heating to 95°C for 5 min, proteins were subjected to SDS-PAGE (60 µg protein/lane). Following electrophoresis, gels were equilibrated with transfer buffer (39 mM glycine, 48 mM Tris/HCl, 0.03% SDS, 20% methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia, Freiburg, Germany) or a Trans-Blot Cell (Biorad Laboratories GmbH, Munich, Germany). Blots were blocked overnight in 1-5% bovine serum albumin solubilized in 20 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 and then incubated for 3-4 h with the respective antibody diluted 1:1,000 - 1:20,000. Following washing and incubation for 2 h with horseradish peroxidase-coupled anti-rabbit-IgG antibody or horseradish peroxidase-coupled anti-mouse-IgG antibody (1:10,000-1:20,000, 4°C) the blots were washed again and developed using enhanced chemiluminescent detection (Amersham Biosciences Europe, Freiburg, Germany).

For immunoprecipitation cell lysates containing defined protein amounts (100-200 µg) were incubated with 1-2 µg antibody raised against MKP-1 or ubiquitin, respectively. The immune complexes were collected using protein A-Sepharose (Sigma, Deisenhofen, Germany) and washed three times with a buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM MgCl₂ and 0.5 mM DTT. Then, 1x gel loading buffer was added to the immobilized immune complex. After heating at 95°C for 5 min the samples were centrifuged at 20,000 g and supernatant was subjected to SDS-PAGE.

Preparation of nuclear extracts

At the end of the experimental treatment, medium was removed from the culture and cells were washed with ice cold phosphate-buffered saline. After adding buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 mM sodium vanadate, one tablet/50 ml of the

protease inhibitor cocktail) culture plates were kept on ice for 10 min. Cells were harvested and nuclei were collected by centrifugation for 1 min at 14,000 rpm. The nuclei were resolved in buffer B (20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.2 mM PMSF, 1 mM sodium vanadate, one tablet/50 ml of protease inhibitor cocktail) and samples were maintained for 20 min on ice. After centrifugation (2min, 14,000rpm) supernatants were collected and protein concentration was determined using the assay purchased from BioRad (Munich, Germany). The extracts were used for determination of c-Jun- and c-Fos DNA binding activity.

Determination of DNA binding activity of c-Jun and c-Fos

The EZ-Detect™ Transcription Factor ELISA system for determination of c-Fos and c-Jun DNA binding activity (Pierce Biotechnology, Rockford, IL) basing on 96 well strip plates coated with the consensus duplex for activator protein-1 (AP-1) was used. The assay was performed according to the manufacturers recommendations. Briefly, 50 µl AP-1 binding buffer containing poly (dI•dC) were added into each well. Then 5 µg/10 µl nuclear proteins were added and incubated for 2 h at room temperature with mild agitation. To ensure specificity of the assay in some cases a wild type or a mutant AP-1 competitor duplex (AP-1-W and AP-1-M, respectively) were added before addition of the nuclear proteins. At the end of the incubation period the solid phase was washed three times with 200 µl 1x washing buffer and then incubated for 1 h with 100 µl 1:1000-diluted primary antibody recognizing c-Jun or c-Fos, respectively. After washing three times with 1x washing buffer, 100 µl of an 1:5000-diluted horse radish peroxidase-conjugated secondary antibody was added and the incubation was continued for another 1 h. After washing four times 100 µl chemoluminescence substrate was added and chemoluminescence was monitored using a MLX Micro Plate Luminometer (DYNEX Technologies, Chantilly, USA). Wild Type AP-1 competitor (AP-1-W), 5'-ACACCGCTTGAGTGAGTCAGCCGGAA-3'; Mutant AP-1 competitor (AP-1-M), 5'-ACACCGCTTGAGGAGTCGCCGGAA-3'.

Caspase-3 assay

H4IIE cell lysate containing 50 µg protein was analyzed for caspase-3 activity by the colorimetric assay purchased by R&D systems (Wiesbaden, Germany) according to the manufacturers recommendations. Cleavage of the caspase-3 substrate DEVD-NA was quantified spectrophotometrically at a wavelength of 405 nm after 4 h incubation at 37°C.

Measurement of proteasome activity

The proteasome activity was determined as described [32]. The cells were lysed in 200 µl lysis buffer containing 10 mM HEPES pH 7.5, 24 mM KCl, 10 mM MgCl₂ and 1 mM dithiothreitol by repeating freezing and thawing cycles. The remaining non-lysed cells, membranes and nuclei were removed by centrifugation at 14,000 g for 10 min at 4°C. The supernatant was incubated in a buffer consisting of 225 mM Tris (pH 7.8), 7.5 mM MgAc₂, 7.5 mM MgCl₂, 45 mM KCl and 1 mM DTT. The

Fig. 1. Osmosensitivity of MG-132-induced MKP-1 expression: time course. H4IIE cells were incubated under normo- or hyperosmotic conditions (305 and 405mosmol/l) in presence or absence of MG-132 (25 μ M) for the time periods indicated. At the end of the incubations cells were lysed. Total RNA was analyzed for the presence of MKP-1 mRNA by Northern Blot analysis using the cDNA probe for MKP-1 [29]. Proteins were analyzed for the presence of MKP-1 by Western Blot using an antibody recognizing MKP-1. In addition to MKP-1 the antibody recognizes a band above which only occasionally responds to MG-132 and which is of unknown identity. Representative blots of at least four independent experiments are shown. MKP-1 mRNA (ratio MKP-1 mRNA to GAPDH mRNA) or protein expression levels were expressed as arbitrary units. (A) Time course of MKP-1 mRNA expression induced by MG-132 under normo- or hyperosmotic conditions, respectively. (B) Time course of MKP-1 mRNA expression under normo- and hyperosmotic conditions in absence of MG-132. (C) Time course of MKP-1 protein expression induced by MG-132 under normo- and hyperosmotic conditions, respectively. *, significant increase in MKP-1 expression in presence of hyperosmotic medium compared with the MKP-1 expression in presence of normosmotic medium at the corresponding time point ($p \leq 0.05$).

fluoropeptide substrate suc-LLVY-MCA was used at a concentration of 200 μ M to measure the chymotrypsin-like activity of the proteasome. After 30min of incubation at 37°C the fluorescence of the reaction mixture was immediately measured using a fluorescence reader (360nm excitation/485nm emission) using free MCA as standards.

Analysis of results

Results from n independent experiments are expressed as means $\pm$ SEM. Results were compared using the Student's t -test: $p < 0.05$ was considered statistically significant.

Results

Osmosensitivity of MKP-1 induction by proteasome inhibitors

H4IIE cells were exposed to normo- or hyperosmotic medium (305 and 405mosmol/l, respectively) in presence or absence of the peptide aldehyde MG-132 (25 μ M). As shown in Figure 1A, under normosmotic conditions induction of MKP-1-mRNA expression by MG-132 became detectable after 3h and was maximal (7.3 ± 1.7 -fold, $n=4$) after 6h. Thereafter MKP-1 mRNA levels remained stable for at least 24h (8.3 ± 2.3 -fold, $n=4$, after 24h). Hyperosmolarity allowed

Table: Proteasome inhibition by MG-132 and lactacystin in H4IIE cells. Cells were exposed for 2h to normo- or hyperosmotic medium (305 or 405mosmol/l, respectively) in presence or absence of MG-132 (25 or 50μM) or lactacystin (5 or 10μM). At the end of the incubations cells were washed and scraped off from the culture plate. After centrifugation the cell pellet was used for assaying proteasome activity as described in Material and Methods. Activities were related to that found under the normoosmotic control condition (305mosmol/l, in absence of MG-132 or lactacystin, respectively) which was set to 1. The table summarizes data from 6 independent experiments.

	-	25μM MG-132	50μM MG-132	5μM lactacystin	10μM lactacystin
305mosmol/l	1.0	0.38±0.04	0.36±0.04	0.19±0.02	0.17±0.01
405mosmol/l	1.03±0.12	0.39±0.04	0.32±0.02	0.21±0.03	0.17±0.03

MKP-1 mRNA expression by MG-132 already after 1h and MKP-1 mRNA after 6, 9 and 24h (15.9 ± 3.8 -, 25.7 ± 7.0 - and 52.2 ± 16.0 -fold, $n=4$) was significantly increased compared to expression induced by MG-132 under normoosmotic conditions. Corresponding to earlier findings [17, 29, 30, 33] hyperosmolarity *per se* induced a transient expression of MKP-1 mRNA, which was maximal after about 6h and returned towards basal levels within 24h (Figure 1B). Hyperosmotic induction of MKP-1 mRNA expression after 6, 9 and 24h was 4.4 ± 1.6 -fold, 3.4 ± 1.9 -fold and 1.7 ± 1.2 -fold ($n=4$) and was small compared with that induced by coexposure of the cells to hyperosmolarity and MG-132.

MG-132-induced MKP-1 protein expression became detectable after 6h and was stable up to 24h (Figure 1C). Although hyperosmolarity *per se* induced MKP-1 mRNA expression in H4IIE cells (this paper, Figure 1B, [17, 29, 30, 33]), MKP-1 protein remained absent as shown previously [17, 30]. However, hyperosmolarity strongly increased MKP-1 protein expression induced by MG-132 (Figure 1C).

As shown in Figure 2A the induction of MKP-1 mRNA by MG-132 was dose- and osmolarity-dependent. 25μM MG-132 after 2h induced under hyper- but not under normoosmotic conditions a significant MKP-1 mRNA expression (3.1 ± 0.4 -fold ($p < 0.05$) vs. 1.3 ± 0.2 -fold ($p > 0.05$), $n=3$, lanes 3 and 4). Under hyperosmotic conditions, 50μM MG-132 induced a 7.5 ± 1.3 -fold ($n=3$) MKP-1 mRNA expression, which differed significantly ($p < 0.05$) from that induced by 25μM MG-132 (compare lanes 4 and 6). Similar findings were obtained at the protein level (Figure 2B, lanes 3-6). In order to examine whether the dose- and osmolarity-dependence of MKP-1 mRNA expression relates to a gradual inhibition of the proteasome by MG-132, proteasome activities were measured (Table 1). MG-132 potently inhibited the proteasome in H4IIE cells. Although there was a tendency to increased proteasome inhibition by 50μM MG-132 compared to that found in response to 25μM MG-132, this observation did not reach significance.

Fig. 2. Osmosensitive MKP-1 expression by MG-132 and lactacystin. H4IIE cells were exposed to MG-132 (25 or 50μM), lactacystin (5 or 10μM) and 3-methyladenine (3-MA, 10 mM) for 2h under normo- and hyperosmotic conditions (305 and 405mosmol/l). MKP-1 mRNA (A) and protein (B) were analyzed as described in the legend to Figure 1. Representatives of three experiments are shown.

Hyperosmolarity did neither affect proteasome activity, nor proteasome inhibition by MG-132.

Similar results were obtained with lactacystin (Figure 2, Table 1), a more specific proteasome inhibitor structurally different from MG-132 [9]. 3-Methyladenine, which inhibits autophagic proteolysis [34], was ineffective to increase MKP-1 expression under normo- and hyperosmotic conditions, respectively (Figure 2).

It is concluded, that the hyperosmotic increase of MKP-1 induction by MG-132 and lactacystin can not be attributed to an osmosensitive modulation of proteasomal activity. This does not exclude a role of proteasomal inhibition for MKP-1 induction by the proteasome inhibitors.

Fig. 3. Hyperosmotic increase in c-Jun and c-Fos expression and DNA binding activity in presence of MG-132. H4IIE cells were exposed to MG-132 (25 μ M) under normo- or hyperosmotic conditions (305 and 405 mosmol/l). (A) Time course. Protein extracts were analyzed for the presence of c-Fos, c-Jun, [Ser(P)⁷³]c-Jun and GAPDH. (B) DNA binding activity of c-Jun and c-Fos. After 6h medium was removed and nuclear protein extracts were prepared. Binding of nuclear c-Fos and c-Jun to an immobilized double stranded AP-1 consensus oligonucleotide was assayed as described in Materials and Methods. For control of specificity some determinations were performed in presence of soluble wild type and mutant AP-1 competitor duplex (AP-1-W and AP-1-M, respectively).

Hyperosmotic increase of MG-132-induced expression and DNA binding activity of c-Fos and c-Jun

Activity of AP-1 transcription factor complex may be critical for MKP-1 induction by proteasome inhibitors [28, 35]. Figure 3A shows that MG-132 (25 μ M) strongly induces expression of c-Fos under hyperosmotic but not under normoosmotic conditions. Hyperosmolarity per se had almost no effect on c-Fos expression. Likewise, hyperosmolarity strongly increased MG-132-induced c-Jun expression but by itself was almost without effect on c-Jun expression. Ser⁷³ phosphorylation of c-Jun is important for c-Jun-dependent transcriptional activity [36]. As shown in Figure 3A, hyperosmolarity per se hardly affected levels of Ser⁷³-phosphorylated c-Jun, whereas accumulation of Ser⁷³-phosphorylated c-Jun by MG-132 was increased under hyperosmotic conditions.

DNA binding activity of c-Fos and c-Jun was measured by exposing immobilized AP-1 competitor double-stranded oligonucleotide to nuclear extracts of H4IIE cells and subsequent detection of c-Jun and c-Fos specifically bound to the oligonucleotide. As shown in Figure 3B (upper bar chart), MG-132 at a concentration of 25 μ M slightly but not significantly ($p=0.09$) induced c-Jun binding to the AP-1 consensus oligonucleotide under normoosmotic conditions. However, c-Jun binding activity was strongly increased by coexposure of the cells to MG-132 and hyperosmolarity. Hyperosmolarity by itself was without effect on DNA-binding activity of c-Jun. Likewise, c-Fos binding to the AP-1 consensus oligonucleotide was neither induced by MG-132, nor by hyperosmolarity itself. However, MG-132 plus hyperosmolarity strongly increased c-Fos binding to the AP-1 competitor duplex (Figure 3B, lower bar chart). For control of binding specificity, soluble wild type or mutant AP-1 competitor duplex was added to the nuclear

protein extracts. Wild type but not the mutant AP-1 consensus oligonucleotide competes with c-Fos and

Fig. 4. Osmosensitivity of MG-132-induced MAP-kinase activation. H4IIE cells were exposed to MG-132 (25 μ M) under normo- or hyperosmotic conditions (305 and 405 mosmol/l) for the time periods indicated. Protein extracts were analyzed by Western Blot using antibodies recognizing dual (Thr/Tyr) phosphorylated Erk-1/Erk-2, p38^{MAPK}, and JNK-2 and total Erk-1/Erk-2, p38^{MAPK} and JNK-2, respectively. Representatives of 3 independent experiments are shown. Quantification was performed densitometrically. Dual phosphorylation of Erk-2, p38^{MAPK} and JNK-2 under the respective control condition (305 mosmol/l, MG-132 absent) was set to one and phosphorylation observed in presence of hyperosmolarity and/or MG-132 is given as a fraction thereof. Changes in phosphorylation were normalized to expression of the respective total protein. Symbols: ^a, significant MAP-kinase phosphorylation compared to that found under the respective control condition; ^b, MAP-kinase phosphorylation in presence of MG-132 plus hyperosmolarity significant different from that found in presence of hyperosmolarity alone. ^c, MAP-kinase phosphorylation after 6h significantly different from that found after 3h under the corresponding condition.

c-Jun binding to the immobilized oligonucleotide (Figure 3B), underlining the specificity of the assay.

The data suggest that moderate hyperosmolarity sensitizes H4IIE cells to MG-132-induced stimulation of AP-1 DNA binding by increasing induction of c-Jun and c-Fos and Ser⁷³ phosphorylation of c-Jun. This may contribute to the hyperosmotic increase of MKP-1 expression by MG-132.

Hyperosmotic modulation of MAP-kinase activation by MG-132

MAP-kinases mediate induction of c-Fos and c-Jun in response to different stimuli [36]. In H4IIE cells hyperosmolarity (405 mosmol/l) activated MAP-kinases of the Erk-, JNK- and p38-type. Hyperosmotic MAP-kinase activation was maximal at about 60-80min and then declined toward basal levels [29, 33]. Coexposure of H4IIE cells to MG-132 and hyperosmolarity for 1h activated p38^{MAPK} and JNK-2 beyond the levels found in presence of hyperosmolarity alone. Under normosmotic conditions MG-132 hardly affected MAP-kinase activities. (Figure 4, lanes 2-5). After 3h hyperosmolarity increased Erk-1/2-, p38^{MAPK}- and JNK-activation by MG-132, whereas phosphorylation of these kinases by hyperosmolarity alone already declined to near basal levels (Figure 4, lanes 6-9) After 6h exposure of the cells to hyperosmolarity plus MG-132 MAP-kinase activation was similar (Erk-1/2, JNK-2) or even lower (p38^{MAPK})

compared to that induced by MG-132 under normosmotic conditions (Figure 4, lanes 10-13). This downregulation of MAP-kinase activities coincided with a pronounced MKP-1 expression (Figure 1C).

Fig. 5. Pharmacological characterization of MG-132-induced expression of [Ser(P)⁷³]c-Jun, c-Fos and MKP-1. H4IIE cells were exposed to MG-132 (25μM) under normo- or hyperosmotic conditions (305 and 405 mosmol/l) without further treatment (lanes 1-4, 17-20) or following a 30 min preincubation with PD98059 (20μM, lanes 5-8), SB220025 (20μM, lanes 9-12), SB202474 (20μM, lanes 13-16), or SP600125 (50μM, lanes 21-24). After 6 h, cells were lysed. Protein extracts were analysed by Western Blots for expression of [Ser(P)⁷³]c-Jun, c-Fos, MKP-1 and GAPDH. Representatives of 3-5 independent experiments are given.

Pharmacological characterization of MKP-1 induction by MG-132

In order to further characterize osmosensitive MKP-1 expression by MG-132, the effect of different MAP-kinase inhibitors was examined at the levels of [Ser(P)⁷³]c-Jun, c-Fos, and MKP-1 expression (Figure 5). PD98059, which blocks the Erk-1/Erk-2 pathway at the level of MEK-1/MEK-2 immediately upstream of Erk-1/Erk-2 [37], was largely without effect on MG-132-induced [Ser(P)⁷³]c-Jun and MKP-1 expression but impaired induction of c-Fos (lanes 5-8). SB220025, a p38^{MAPK} inhibitor [38], reduced [Ser(P)⁷³]c-Jun and completely abolished c-Fos and MKP-1 expression by MG-132 (lanes 9-12), whereas the inactive analogue SB202474 was largely without effect (lanes 13-16). SP600125, a JNK inhibitor [39], prevented induction of [Ser(P)⁷³]c-Jun and MKP-1 expression whereas c-Fos expression was not affected by the inhibitor.

The findings suggest that Erk-type MAP-kinases and p38^{MAPK} mediate c-Fos induction, whereas p38^{MAPK} and JNKs are involved in [Ser(P)⁷³]c-Jun expression by MG-132. Activation of c-Jun but not c-Fos may be essential for MKP-1 induction by MG-132.

Proteolysis inhibition contributes to MG-132-induced MKP-1 expression

MKP-1 underlies proteasomal degradation which can be prevented by MG-132 [40]. In order to examine a potential osmosensitivity of MKP-1 stability, MKP-1 expression was induced by exposure of H4IIE cells to 25μM MG-132 under hyperosmotic conditions for 6h (Figure 6A, lane 1). Then, medium was exchanged and cells were exposed to the protein synthesis inhibitor cycloheximide (CHX) under normo- or hyperosmotic conditions in presence or absence of MG-132 for the time periods indicated. As shown in Figure 6 MKP-1 protein levels remained stable in presence of MG-132, irrespective of the ambient osmolarity. This is consistent with the finding that MG-132 inhibits the proteasome equally under normo- and hyperosmotic conditions (Table 1). A slight attenuation of MKP-1 degradation was observed under hyperosmotic conditions in absence of MG-132 at the 60 min time point.

In order to address a potential osmosensitivity of MKP-1 ubiquitination, H4IIE cells were incubated with normo- or hyperosmotic medium in presence or absence of MG-132 (25μM). As shown in Figure 7A,

hyperosmolarity increases overall accumulation of ubiquitinated proteins in presence of MG-132. On the other hand, MG-132-induced ubiquitination of MKP-1 was observed under normo- but not hyperosmotic conditions (Figure 7B). This would be consistent with some attenuation of MKP-1 degradation under hyperosmotic conditions as suggested by the experiments presented in Figure 6B.

Functional impact of MKP-1 induction by MG-132 plus hyperosmolarity

MKP-1 antagonizes apoptosis induction by proteasome inhibitors [26]. Therefore it seems well conceivable that the hyperosmotic increase of MKP-1 expression antagonizes proapoptotic MAP-kinase signals and thereby confers protection of H4IIE cells challenged with MG-132 under hyperosmotic conditions.

p38^{MAPK} was most effectively inactivated after a 6h coexposure of H4IIE cells to MG-132 and hyperosmolarity (Figure 4). In order to address a potential contribution of MKP-1 to inactivation of p38^{MAPK}, H4IIE cells were incubated for 6h with MG-132 (25μM) under normo- or hyperosmotic conditions without additional treatment or in presence of vanadate, which inhibits tyrosine phosphatases including MKP-1 [41, 42]. Vanadate (25μM) most strongly increased p38^{MAPK} activity in H4IIE cells coexposed to hyperosmolarity plus MG-132 (Figure 8, compare lanes 4 and 8), whereas vanadate had weaker effects on p38^{MAPK} under normoosmotic conditions (MG-132 present or not, compare lanes 1 and 5, 3 and 7) and in presence of hyperosmolarity alone (compare lanes 2 and 6). This would be consistent with a contribution of MKP-1 to suppression of p38^{MAPK} activity in H4IIE cells coexposed to MG-132 and hyperosmolarity.

Caspase-3 activity and formation of the 85 kDa poly-ADP-ribose polymerase (PARP) fragment were taken to indicate apoptosis induction. As shown in Figure 9A, exposure of H4IIE cells to 25μM MG-132 under hyperosmotic but not under normoosmotic conditions induced caspase-3 activity, which was further increased in presence of vanadate. MG-132 induced formation of the 85 kDa PARP cleavage product (Figure 9B, lanes 3 and 4). Vanadate increased MG-132-induced PARP cleavage under hyper- but not normoosmotic conditions (compare lanes 4 and 8, 3 and 7). This would be consistent with a contribution of MKP-1 to antagonize apoptosis in H4IIE cells challenged with MG-132 under hyperosmotic conditions.

Fig. 6. Effect of MG-132 and hyperosmolarity on MKP-1 proteolysis. In order to induce MKP-1 expression, H4IIE cells were treated with 25μM MG-132 under hyperosmotic conditions for 6h. Then medium was removed and cells were further incubated in presence of cycloheximide (CHX, 5μg/ml) in presence or absence of MG-132 for the indicated time periods. Protein extracts were analyzed for MKP-1 expression by Western Blot. MKP-1 expression levels were quantified by densitometry. MKP-1 expression at the zero time point was set to 1 (n=5). *, MKP-1 level significantly (p<0.05) different from the MKP-1 level at the zero time point; §, MKP-1 level in presence of MG-132 differs significantly (p<0.05) from that found in absence of MG-132 at the corresponding time point.

Fig. 7. Osmosensitivity of MG-132-induced protein ubiquitination. H4IIE cells were exposed to MG-132 (25μM) for 6h under normo- or hyperosmotic conditions (305 and 405 mosmol/l). (A) Overall ubiquitination of proteins. After the indicated time periods cells were lysed and protein extracts were analyzed for presence of ubiquitinated proteins by probing Western blots with an antibody recognizing ubiquitin. (B) MG-132-induced accumulation of ubiquitinated MKP-1. Immunoprecipitates obtained with an anti MKP-1 antibody were analyzed in Western Blot for the presence of ubiquitin (lanes 9-12) and MKP-1 (lanes 5-8) respectively. In addition, immunoprecipitates obtained with an anti ubiquitin antibody were analyzed in Western Blot for the presence of MKP-1 (lanes 13-16). The non-ubiquitinated protein precipitated by the MKP-1 antibody exhibits an electrophoretic mobility identical to the protein recognized by the MKP-1 antibody in Western blot analysis of total protein lysates (arrow, lanes 1-8). Recognition of ubiquitinated MKP-1 by the MKP-1 antibody on the membrane may be limited. Representatives of 3 independent experiments are shown.

In order to estimate a potential involvement of MAP-kinases in MG-132-induced PARP cleavage, H4IIE cells were exposed to MG-132 (25μM) for 6h under normo- or hyperosmotic conditions in presence or absence of PD98059 (20μM), SB220025 and its inactive analogue SB202474 (each 20μM) and SP600125 (50μM). As shown in Figure 10A, MG-132 under hyperosmotic conditions increased formation of the 85 kDa PARP cleavage product (lanes 2, 4, and 18, 20) but was largely without effect under normoosmotic conditions (lanes 1, 3, and 17, 19). Hyperosmolarity per se slightly induced formation

of the 85 kDa PARP fragment (lanes 1, 2 and 17, 18), which was increased to some extent in presence of the MAP-kinase inhibitors and the inactive SB202474 (compare lane 2 with lanes 6, 10, 14, and lane 18 with lane 22). Taking this into account unravelled a significant net reduction of “MG-132 plus hyperosmolarity”-induced PARP cleavage by SB220025 and SP600125 but not by PD98059 and SB202474, respectively. It is suggested that under hyperosmotic conditions p38^{MAPK} and JNKs but not Erk-1/Erk-2 contribute to MG-132-induced apoptosis.

Fig. 8. Effect of vanadate on p38^{MAPK} activation induced by MG-132. H4IIE cells were exposed to MG-132 (25 μ M) under normo- or hyperosmotic conditions (305 and 405mosmol/l) for 6h. In some experiments vanadate (25 μ M) was present as indicated. Protein extracts were analyzed by Western Blot for presence of dual phosphorylated p38^{MAPK}, p38^{MAPK} and MKP-1. Representatives of 3 independent experiments are given.

Discussion

This study shows that hyperosmotic dehydration sensitizes H4IIE cells to induction of MKP-1 by the proteasome inhibitor MG-132. In addition, activation of MAP-kinases and AP-1 DNA binding activity by MG-132 were increased by hyperosmolarity.

AP-1 transcriptional activity is important for MKP-1 induction by different stimuli including MG-132 [28, 35]. As shown here, hyperosmolarity in H4IIE cells amplified MG-132-induced p38^{MAPK} and JNK activation, which was accompanied by increased expression, Ser⁷³ phosphorylation, and DNA binding of c-Jun (Figures 3 and 4). The pharmacological analysis unravelled that p38^{MAPK} and JNKs, but not Erk-type MAP-kinases may play a role in c-Jun activation and MKP-1 induction (Figure 5). Hyperosmolarity also increased MG-132-induced c-Fos expression and DNA binding but this was probably not involved in upregulation of MKP-1 (Figures 3 and 5), consistent with an earlier report that c-Jun but not c-Fos was required for AP-1-mediated MKP-1 expression by MG-132 in rat mesangial cells [28]. Probably, in addition to c-Jun, the activating transcription factor ATF-2 is involved in AP-1-mediated MKP-1 induction [43]. It is suggested that hyperosmolarity promotes MKP-1 expression by MG-132 at the transcriptional level. Hyperosmolarity in H4IIE cells also

Fig. 9. MG-132-induced PARP cleavage and caspase-3 activation: sensitivity to hyperosmolarity and vanadate. H4IIE cells were exposed to MG-132 (25 μ M) under normo- or hyperosmotic conditions (305 and 405 mosmol/l) for 24h. In some experiments vanadate (25 μ M) was present as indicated. (A) Protein extracts were analyzed for caspase-3 activity *, caspase-3 activity is significantly ($p < 0.05$) increased compared to that under control condition (normosmotic incubation); ^s, caspase-3 activity is significantly increased compared to that found in cells coexposed to MG-132 and hyperosmolarity in absence of vanadate. (B) Protein extracts were analyzed by Western Blots for presence of the 85 kDa PARP fragment. A representative of 4 independent experiments are shown.

increased MKP-1 induction by heat shock [17] but delayed the MKP-1 response to insulin [30], suggesting different types of cross talk, depending on the MKP-1-inducing stimulus.

Hyperosmolarity increased the MG-132-induced accumulation of ubiquitinated proteins (Figure 7A), which reflects an overall increase of proteasomal proteolysis under hyperosmotic conditions [44]. However, MG-132-induced accumulation of ubiquitinated MKP-1 was reduced by hyperosmolarity (Figure 7B) and hyperosmolarity even stabilized MKP-1 to some extent after removal of MG-132 (Figure 6), suggesting MKP-1

Fig. 10. Involvement of MAP-kinases in MG-132-induced PARP cleavage. H4IIE cells were exposed to MG-132 (25 μ M) under normo- or hyperosmotic conditions (305 and 405 mosmol/l) for 6 h without further treatment (lanes 1-4, 17-20) or following a 30 min preincubation with PD98059 (20 μ M, lanes 5-8), SB220025 (20 μ M, lanes 9-12), SB202474 (20 μ M, lanes 13-16) and SP600125 (50 μ M, lanes 21-24). Protein extracts were analyzed for PARP cleavage by Western Blot. (A) A representative of 3 independent experiments is given. (B) Levels of the 85 kDa PARP cleavage product in presence of MG-132 plus hyperosmolarity were analyzed densitometrically and normalized to the levels found in presence of hyperosmolarity alone. *: level of the 85 kDa PARP fragment significantly ($p < 0.05$) different from that found under the control condition (hyperosmotic incubation).

ubiquitination and proteasomal degradation to be antagonized under hyperosmotic conditions. No osmosensitivity of MKP-1 stability was observed after induction of the protein by insulin [30], suggesting that the specific signaling background plays another role for the determination of MKP-1 stability.

Although cell shrinkage is obligatory in apoptosis [45], the apoptotic potential of hyperosmotic shrinkage depends e.g. on the efficacy of volume regulatory mechanisms and the intracellular composition of osmolytes and heat shock proteins [13, 14]. In rat hepatocytes hyperosmolarity targeted CD95 from intracellular pools to the plasma membrane. Despite formation of the death-inducing signaling complex and activation of caspases 3 and 8, hyperosmotically treated hepatocytes did not execute apoptosis, however, hyperosmolarity potently increased

CD95 ligand-induced apoptosis [20-22]. Similarly, hyperosmolarity sensitized HeLa cells to apoptosis induction by TNF- α [46]. In H4IIE cells hyperosmolarity only slightly affected caspase-3 activity and PARP cleavage but enabled MG-132 to potently activate caspase-3 and PARP cleavage, which was counteracted by a vanadate-sensitive tyrosine phosphatase activity (this paper, Figure 9).

Erk-1/Erk-2 [4, 27, 47], JNKs [25, 48-50] and p38^{MAPK} [49, 51] mediate apoptosis or survival, depending on the experimental setup. PARP cleavage in H4IIE cells coexposed to hyperosmolarity and MG-132 was antagonized by inhibition of p38^{MAPK} and JNK but not Erk-type MAP-kinases (Figure 10), suggesting that the hyperosmotic amplification of p38^{MAPK} and JNK activation by MG-132 not only promotes MKP-1 expression but

also apoptosis induction. MKP-1 expression coincided with a vanadate-sensitive suppression of p38^{MAPK} activity. It is suggested that MKP-1 antagonized proapoptotic signaling within a feed back inhibitory loop which may be part of an adaptation mechanism allowing survival of H4IIE cells treated with MG-132 under hyperosmotic conditions. This view is supported by the recent finding that MKP-1 antagonizes apoptosis induced by proteasome inhibitors in human mammary epithelial cells [26].

As shown earlier, cellular effects of proteasome inhibitors depend on dosage and cell type under investigation [1-5, 7]. This study suggests that cell hydration changes potentially modulate the cellular response to proteasome inhibitors at the levels of signal transduction and gene expression. This may switch the outcome from a non-toxic or even protective one to injury and apoptosis. Because cell hydration is not only determined by ambient osmolarity, but also by hormones, nutrient supply and oxidative stress [12], multiple factors may modulate the therapeutic efficacy of proteasome inhibitors in the *in vivo* situation.

Abbreviations

AP-1 (activator protein 1); caspase (cysteine-aspartate protease); DMEM (Dulbecco's modified Eagle's medium); EDTA (ethyleneglycol-*bis*(β -aminoethyl)-N,N,N',N'-tetraacetic acid); Erk (extracellular signal-regulated kinase); GAPDH (glyceraldehyde 3-phosphate dehydrogenase); JNK (c-Jun-N-terminal kinase); MAP (mitogen-activated protein kinase); MKP (MAP-kinase phosphatase); NF (nuclear factor); PARP (poly-ADP ribose polymerase); TNF (tumor necrosis factor); TGF (tissue growth factor).

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References

- Kim D, Kim SH, Li GC: Proteasome inhibitors MG132 and lactacystin hyperphosphorylate HSF1 and induce hsp70 and hsp27 expression. *Biochem Biophys Res Commun* 1999;254:264-8.
- Bush KT, Goldberg AL, Nigam SK: Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. *J Biol Chem* 1997;272:9086-92.
- Meriin AB, Gabai VL, Yaglom J, Shifrin VI, Sherman MY: Proteasome inhibitors activate stress kinases and induce Hsp72. Diverse effects on apoptosis. *J Biol Chem* 1998;273:6373-9.
- Orlowski RZ, Small GW, Shi YY: Evidence that inhibition of p44/42 mitogen-activated protein kinase signaling is a factor in proteasome inhibitor-mediated apoptosis. *J Biol Chem* 2002;277:27864-71.
- Fujihara S, Ward C, Dransfield I, Hay RT, Uings IJ, Hayes B, Farrow SN, Haslett C, Rossi AG: Inhibition of nuclear factor-kappaB activation unmasks the ability of TNF-alpha to induce human eosinophil apoptosis. *Eur J Immunol* 2002;32:457-66.
- Lee AH, Iwakoshi NN, Anderson KC, Glimcher LH: Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. *Proc Natl Acad Sci USA* 2003;100:9946-51.
- Lu M, Kitson RP, Xue Y, Goldfarb RH: Activation of multiple caspases and modification of cell surface Fas (CD95) in proteasome inhibitor-induced apoptosis of rat natural killer cells. *J Cell Biochem* 2003;88:482-92.
- Awasthi N, Wagner BJ: Interferon- γ induces apoptosis of lens α TN4-1 cells and proteasome inhibition has an antiapoptotic effect. *Invest Ophthalmol Vis Sci* 2004;45:222-9.
- Lee DH, Goldberg AL: Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 1998;8:397-403.
- Tawa-NE J, Odessey R, Goldberg AL: Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. *J Clin Invest* 1997;100:197-203.
- Meiners S, Laule M, Rother W, Guenther C, Prauka I, Muschick P, Baumann G, Kloetzel PM, Stangl K: Ubiquitin-proteasome pathway as a new target for the prevention of restenosis. *Circulation* 2002;105:483-9.
- Lang F, Busch GL, Ritter M, Völkl H, Waldegger S, Gulbins E, Häussinger D: Functional significance of cell volume regulatory mechanisms. *Physiol Rev* 1998;78:247-306.
- Lang F, Ritter M, Gamper N, Huber S, Fillon S, Tanneur V, Lepple-Wienhues A, Szabo I, Gulbins E: Cell volume in the regulation of cell proliferation and apoptotic cell death. *Cell Physiol Biochem* 2000;10:417-28.
- Schliess F, Häussinger D: The cellular hydration state: role in apoptosis and proliferation. *Signal Transduction-Receptors, Mediators and Genes* 2005; DOI:10.1002/sita.200400046.
- Saha N, Stoll B, Lang F, Häussinger D: Effect of anisotonic cell-volume modulation on glutathione-S- conjugate release, t-butylhydroperoxide metabolism and the pentose-phosphate shunt in perfused rat liver. *Eur J Biochem* 1992;209:437-44.
- Kurz AK, Schliess F, Häussinger D: Osmotic regulation of the heat shock response in primary rat hepatocytes. *Hepatology* 1998;28:774-81.
- Schliess F, Wiese S, Häussinger D: Osmotic regulation of the heat shock response in H4IIE rat hepatoma cells. *FASEB J* 1999;13:1557-64.

- 18 Michalke M, Cariers A, Schliess F, Häussinger D: Hypoosmolarity influences the activity of transcription factor NF-kappa B in rat H4IIE hepatoma cells. *FEBS Lett* 2000;465:64-8.
- 19 Wettstein M, Häussinger D: Cytoprotection by the osmolytes betaine and taurine in ischemia-reoxygenation injury in the perfused rat liver. *Hepatology* 1997;26:1560-6.
- 20 Reinehr RM, Graf D, Fischer R, Schliess F, Häussinger D: Hyperosmolarity triggers CD95 membrane trafficking and sensitizes rat hepatocytes towards CD95L-induced apoptosis. *Hepatology* 2002;36:602-14.
- 21 Reinehr RM, Schliess F, Häussinger D: Hyperosmolarity and CD95L trigger CD95/EGF receptor association and tyrosine phosphorylation of CD95 as prerequisites for CD95 membrane trafficking and DISC formation. *FASEB J* 2003;17:731-3.
- 22 Reinehr RM, Becker S, Höngen A, Häussinger D: The Src family kinase Yes triggers hyperosmotic activation of the epidermal growth factor receptor and CD95. *J Biol Chem* 2004;279:23977-87.
- 23 Keyse SM: An emerging family of dual specificity MAP-kinase phosphatases. *Biochim Biophys Acta* 1995;1265:152-60.
- 24 Camps M, Nichols A, Arkinstall S: Dual specificity phosphatases: a gene family for control of MAP-kinase function. *FASEB J* 2000;14:6-16.
- 25 Guo YL, Kang B, Williamson JR: Inhibition of the expression of mitogen-activated protein phosphatase-1 potentiates apoptosis induced by tumor necrosis factor- α in rat mesangial cells. *J Biol Chem* 1998;273:10362-6.
- 26 Small GW, Shi YY, Edmund NA, Somasundaram S, Moore DT, Orlowski RZ: Evidence that mitogen-activated protein kinase phosphatase-1 induction by proteasome inhibitors plays an antiapoptotic role. *Mol Pharmacol* 2004;66:1478-90.
- 27 Lin YW, Chuang SM, Yang JL: ERK1/2 achieves sustained activation by stimulating MAPK phosphatase-1 degradation via the ubiquitin-proteasome pathway. *J Biol Chem* 2003;278:21534-41.
- 28 Nakayama K, Furusu A, Xu Q, Konta T, Kitamura M: Unexpected transcriptional induction of monocyte chemoattractant protein 1 by proteasome inhibition: involvement of the c-Jun N-terminal kinase-activator protein 1 pathway. *J Immunol* 2001;167:1145-50.
- 29 Schliess F, Heinrich S, Häussinger D: Hyperosmotic induction of the mitogen-activated protein kinase phosphatase MKP-1 in H4IIE rat hepatoma cells. *Arch Biochem Biophys* 1998;351:35-40.
- 30 Lornejad-Schäfer MR, Schäfer C, Graf D, Häussinger D, Schliess F: Osmotic regulation of insulin-induced mitogen-activated protein kinase phosphatase (MKP-1) expression in H4IIE rat hepatoma cells. *Biochem J* 2003;371:609-19.
- 31 Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
- 32 Mehlhase J, Gieche J, Ullrich O, Sitte N, Grune T: LPS-induced protein oxidation and proteolysis in BV-2 microglial cells. *IUBMB Life* 2000;50:331-5.
- 33 Wiese S, Schliess F, Häussinger D: Osmotic regulation of MAP-kinase activities and gene expression in H4IIE rat hepatoma cells. *Biol Chem* 1998;379:667-71.
- 34 Shigemitsu K, Tsujishita Y, Hara K, Nanahoshi M, Avruch J, Yonezawa K: Regulation of translational effectors by amino acid and mammalian target of rapamycin signaling pathways. Possible involvement of autophagy in cultured hepatoma cells. *J Biol Chem* 1999;274:1058-65.
- 35 Kwak SP, Hakes DJ, Martell KJ, Dixon JE: Isolation and characterization of a human dual specificity protein-tyrosine phosphatase gene. *J Biol Chem* 1994;269:3596-604.
- 36 Karin M, Liu Zg, Zandi E: AP-1 function and regulation. *Curr Opin Cell Biol* 1997;9:240-6.
- 37 Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR: PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J Biol Chem* 1995;270:27489-94.
- 38 Jackson JR, Bolognese B, Hillegass L, Kassis S, Adams J, Griswold DE, Winkler JD: Pharmacological effects of SB 220025, a selective inhibitor of P38 mitogen-activated protein kinase, in angiogenesis and chronic inflammatory disease models. *J Pharmacol Exp Ther* 1998;284:687-92.
- 39 Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW: SP600125, an anthranyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci USA* 2001;98:13681-6.
- 40 Brondello JM, Pouyssegur J, McKenzie FR: Reduced MAP-kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. *Science* 1999;286:2514-7.
- 41 Gordon JA: Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol* 1991;201:477-82.
- 42 Alessi DR, Gomez N, Moorhead C, Lewis T, Keyse SM, Cohen P: Inactivation of p42 MAP-kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr Biol* 1995;5:283-95.
- 43 Erhardt A, Hassan M, Heintges T, Häussinger D: Hepatitis C virus core protein induces cell proliferation and activates ERK, JNK, and p38 MAP-kinases together with the MAP kinase phosphatase MKP-1 in a HepG2 Tet-Off cell line. *Virology* 2002;292:272-84.
- 44 Kornitzer D, Ciechanover A: Modes of regulation of ubiquitin-mediated protein degradation. *J Cell Physiol* 2000;182:1-11.
- 45 Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y: Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci USA* 2000;97:9487-92.
- 46 Franco DL, Nojek IM, Molinero L, Coso OA, Costas MA: Osmotic stress sensitizes naturally resistant cells to TNF- α -induced apoptosis. *Cell Death Differ* 2002;9:1090-8.
- 47 Wang X, Martindale JL, Holbrook NJ: Requirement for ERK activation in cisplatin-induced apoptosis. *J Biol Chem* 2000;275:39435-43.
- 48 Guo YL, Baysal K, Kang B, Yang LJ, Williamson JR: Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor- α in rat mesangial cells. *J Biol Chem* 1998;273:4027-34.
- 49 Roulston A, Reinhard C, Amiri P, Williams LT: Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor α . *J Biol Chem* 1998;273:10232-9.
- 50 Schwabe RF, Bradham CA, Uehara T, Hatano E, Bennett BL, Schoonhoven R, Brenner DA: c-Jun-N-terminal kinase drives cyclin D1 expression and proliferation during liver regeneration. *Hepatology* 2003;37:824-32.
- 51 Nemoto S, Xiang J, Huang S, Lin A: Induction of apoptosis by SB202190 through inhibition of p38 β mitogen-activated protein kinase. *J Biol Chem* 1998;273:16415-20.