Osmotic regulation of insulin-induced mitogen-activated protein kinase phosphatase (MKP-1) expression in H4IIE rat hepatoma cells

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A contribution of intracellular dehydration to insulin resistance has been established in human subjects and in different experimental systems. Here the effect of hyperosmolarity (405 mosmol/l) on insulin-induced mitogen-activated protein (MAP) kinase phosphatase (MKP)-1 expression was studied in H4IIE rat hepatoma cells. Insulin induces robust MKP-1 expression which correlates with a vanadate-sensitive decay of extracellular-signal-regulated kinase (Erk-1/Erk-2) activity. Hyperosmolarity delays MKP-1 accumulation by insulin and this corresponds to impaired MKP-1 synthesis, whereas MKP-1 degradation remains unaffected by hyperosmolarity. Rapamycin, which inhibits signalling downstream from the mammalian target of rapamycin (mTOR) and a peptide inhibiting protein kinase C (PKC) ζ/λ abolish insulin-induced MKP-1 protein but not mRNA expression, suggesting the involvement of the p70 ribosomal S6 protein kinase (p70S6-kinase) and/or the eukaryotic initiation factor 4E-binding proteins (4E-BPs) as well as atypical PKCs in MKP-1 translation. Hyperosmolarity induces sustained suppression of p70S6-kinase and 4E-BP1 hyperphosphorylation by insulin, whereas insulin-induced tyrosine phosphorylation of the insulin receptor (IR) β subunit and the IR substrates IRS1 and IRS2, recruitment of the phosphoinositide 3-kinase (PI 3-kinase) regulatory subunit p85 to the receptor substrates as well as PI 3-kinase activation, and Ser-473 phosphorylation of protein kinase B and Thr-410/403 phosphorylation of PKC ζ/λ are largely unaffected under hyperosmotic conditions. The hyperosmotic impairment of both, MKP-1 expression and p70S6-kinase hyperphosphorylation by insulin is insensitive to K_2CrO_4, calyculin A and vanadate, and inhibition of the Erk-1/Erk-2 and p38 pathways. The suppression of MKP-1 may further contribute to insulin resistance under dehydrating conditions by allowing unbalanced MAP kinase activation.

Key words: cell volume, insulin resistance, liver, protein synthesis, rapamycin.

INTRODUCTION

Insulin exerts numerous effects on hepatic function. It contributes to anabolic metabolism for example by stimulating glycogen, protein and lipid synthesis and simultaneously inhibiting glycogenolysis, proteolysis, lipolysis and gluconeogenesis, thereby antagonizing the action of glucagon and catecholamines [1]. In addition, insulin supports liver regeneration and protects liver cells from apoptosis [2].

Progress has been made in identifying major signalling components mediating the pleiotropic insulin effects (reviewed e.g. in [3,4]). Binding of insulin to its receptor induces tyrosine phosphorylation of the insulin receptor (IR) β subunit and of IR substrates (IRSs) such as IRS1 and IRS2. Recruitment of the phosphoinositide 3-kinase (PI 3-kinase) regulatory subunit p85 to the IRS proteins activates PI 3-kinase, which on the one hand mediates activation of the atypical protein kinase Cs (PKCs) ζ/λ and of protein kinase B (PKB) on the other. PKB directly phosphorylates the mammalian target of rapamycin (mTOR), from which signalling branches to the p70 ribosomal S6 protein kinase (p70S6-kinase) and to the eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4E-BP1), respectively. Ras/Raf-dependent activation of the mitogen-activated protein (MAP) kinases extracellular-signal-regulated kinase (Erk)-1 and Erk-2 by insulin is triggered by the recruitment of the Grb2/SOS complex to IRS proteins. In addition, the $p38^{MAPK}$ responds to insulin in some cell systems including H4IIE rat hepatoma cells [5], hepatocytes [6] and perfused rat liver [7].

In recent decades evidence has accumulated that hyperosmotic dehydration of insulin target tissues triggers a catabolic state and contributes to insulin resistance (reviewed in [8,9]). Thus, correction of systemic hyperosmolarity of severe diabetics improves the patient's sensitivity to low doses of therapeutically applied insulin [10] and, conversely, artificial systemic hyperosmolarity induces insulin resistance in healthy subjects [11]. Hyperosmolarity impairs insulin-induced glucose uptake, glycogen synthesis and lipogenesis in 3T3 L1 adipocytes [12] and inhibits insulin-induced K^+ retention, cell swelling and proteolysis inhibition in perfused rat liver [9]. The interaction between hyperosmotic and insulin signalling has not yet been fully understood at the molecular level.

MAP kinase phosphatases (MKPs) of dual specificity, such as MKP-1, dephosphorylate MAP kinases at Thr/Tyr residues critical for activation, thereby contributing to down-regulation of MAP kinase activity [13]. MKP-1 participates in the determination of the kinetics and thus the cellular outcome of MAP kinase signalling (e.g. proliferation versus differentiation [14], survival versus apoptosis [15]). In vascular smooth muscle cells p38 activity was shown to mediate insulin resistance by hyperglycaemia and hyperinsulinaemia [16] and this is associated with a non-responsiveness of MKP-1 expression to insulin [17], suggesting a role of MKP-1 also in the maintenance of insulin

Abbreviations used: CHX, cycloheximide; DTT, dithiothreitol; eIF-4E, eukaryotic initiation factor 4E; 4E-BP; eIF-4E-binding protein; Erk, extracellular-signal-regulated kinase; IR, insulin receptor; IRS, IR substrate; MAP, mitogen-activated protein; MKP, MAP kinase phosphatase; m^7GTP–Sepharose, 7-methyl-GTP–Sepharose; mTOR, mammalian target of rapamycin; NP-40, Nonidet P-40; p70S6-kinase, p70 ribosomal S6 protein kinase; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; TCA, trichloroacetic acid.

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sensitivity. On the other hand one can imagine that MKP-1 antagonizes MAP kinase activation by insulin and thereby contributes to insulin resistance.

Hyperosmolarity induces expression of the MKP-1 mRNA in H4IIE cells and it was initially speculated that hyperosmotic MKP-1 expression could account for the reduced potential of insulin to activate Erk-1 and Erk-2 in hyperosmotically challenged H4IIE cells [18,19]. However, MKP-1 mRNA induced by hyperosmolarity is not translated into the protein, although hyperosmolarity amplifies MKP-1 expression in heat-shocked H4IIE cells [20]. Whether insulin induces MKP-1 expression in liver cells and how MKP-1 induction by insulin would be osmotically regulated is currently unknown.

Here the induction of MKP-1 by insulin and its osmotic regulation was studied in H4IIE cells. Insulin stimulates a robust expression of the MKP-1, which correlates with a vanadate-sensitive termination of Erk-1/Erk-2 activity induced by insulin. Hyperosmolarity interferes with MKP-1 synthesis but is without effect on MKP-1 degradation. Insulin-induced hyperphosphorylation of p70S6-kinase and 4E-BP1 were suppressed under hyperosmotic conditions, whereas only minor effects were found at the levels of PI 3-kinase, PKC ζ/λ and PKB, suggesting that hyperosmolarity interferes with insulin signalling downstream from mTOR. Due to the rapamycin sensitivity of MKP-1 protein but not mRNA induction by insulin it is concluded that hyperosmotic impairment of signalling downstream from mTOR may account for the delay in MKP-1 translation. Insulin sensitizers such as K_2CrO_4, calyculin A and vanadate as well as inhibition of p38 cannot compensate for the hyperosmotic impairment of insulin-induced MKP-1 expression and p70S6-kinase phosphorylation, suggesting that hyperosmotic dehydration is sensed directly by mTOR-related signalling components.

MATERIALS AND METHODS

Materials

The antibodies raised against Erk-1/Erk-2, IRβ, IRS1, IRS2, phosphotyrosine (clone 4G10), p85, phospho-Akt1/PKBα (Ser-473) and eIF-4E were from Upstate Biotechnology (Charlottesville, VA, U.S.A.); the antibody recognizing MKP-1 was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies raised against p70S6-kinase and Akt1/PKBα were obtained from Transduction Laboratories (Heidelberg, Germany). Antibodies against phospho-PKC ζ/λ (Thr-410/403), phospho-p70S6-kinase (Thr-421/Ser-424), phospho-p70S6-kinase (Thr-389) and 4E-BP-1 were obtained from Cell Signalling (Frankfurt am Main, Germany). The antibody against phospho-p38 was obtained from Promega (Madison, WI, U.S.A.). PD98059, SB220025, SB202474, wortmannin, LY294002, rapamycin and calyculin A were from Calbiochem-Novabiochem GmbH (Darmstadt, Germany). Gö6850 was a gift from Gödecke AG (Freiburg, Germany). Cell-culture media and fetal calf serum were from Gibco Life Technologies (Gaithersburg, MD, U.S.A.). K_2CrO_4, EDTA, sodium vanadate, Nonidet P-40 (NP-40) and Triton X-100 were obtained from Sigma (Munich, Germany). A PKCζ-inhibitory pseudosubstrate, myristoyl-SIYRRGARRWRKL, in which the N-terminal myristoylation allows for membrane permeability, was synthesized by Dr Ralf Hoffmann (Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-University, Düsseldorf, Germany). ATP was from Boehringer (Mannheim, Germany); [γ-^{32}P]ATP and 7-methyl-GTP–Sepharose (m^{7}GTP–Sepharose) were from Amersham Biosciences Europe GmbH (Freiburg, Germany). All other chemicals were from Merck (Darmstadt, Germany). Densitometric analysis was performed with the E.A.S.Y. RH system (Herolab, Wiesloch, Germany).

Cell culture and experimental treatment of the cells

H4IIE-C3 rat hepatoma cells (ATCC CRL 1600) were maintained on Cluster six dishes (Costar) in Dulbecco's modified Eagle's medium/Ham's F12/5 % CO_2/5 mM glucose at 37 °C, pH 7.4, supplemented with 10 % fetal calf serum. When cells had reached 90 % confluency, they were washed with Dulbecco's PBS and the culture was continued in 1.5 ml of serum-free medium for an additional 24 h. Extracellular osmolarity was increased by dilution of the media with medium of elevated NaCl content, leading to hyperosmolarity (405 mosmol/l). In the normo-osmotic control (305 mosmol/l), the same volume of normo-osmotic medium was added.

^{35}S-Labelling of proteins

For ^{35}S-labelling of proteins serum-starved cells were exposed to insulin or not in the presence of Tran^{35}S-Label™ (20 μCi/ml; Amersham Biosciences Europe GmbH). After another 2.5 (experiments shown in Figure 4, see below) or 3 h (experiments documented in Table 1, see below) medium was removed and cells were washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 8.7 mM Na_2HPO_4 and 1.5 mM NaH_2PO_4) and then lysed in 100 μl of 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 one tablet/50 ml of protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein precipitation was performed with 1 ml of 1 M trichloroacetic acid (TCA) and subsequent centrifugation. The precipitate was washed three times by resuspension and centrifugation, with 1 M TCA in each washing step. The pellet obtained after the last washing step was assayed for ^{35}S radioactivity using Liquid Scintillation Analyser 2000 CA (United Technologies Packard, Zürich, Switzerland). Incorporation of ^{35}S into TCA-precipitable material was taken as a measure of overall protein synthesis.

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. The homogenized lysates were centrifuged at 20000 ***g*** at 4 °C for 10 min. To perform SDS/PAGE and Western blot analysis the supernatant was added to an identical volume of 2 × gel-loading buffer containing 200 mM dithiothreitol (DTT), pH 6.8. After heating to 95 °C for 5 min, the proteins were subjected to SDS/PAGE (60 μg of protein/lane; 8, 10 or 12 % gels). Following electrophoresis, gels were equilibrated with transfer buffer (39 mM glycine, 48 mM Tris/HCl, 0.03 % SDS and 20 % methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Amersham Biosciences Europe GmbH) or a Trans-Blot Cell (Bio-Rad Laboratories GmbH, Munich, Germany). Blots were blocked overnight in 1–5 % BSA 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 1:1000–1:20000. Following washing and incubation for 2 h with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG antibody (1:10000–1:20000; 4 °C) the blots were washed again and developed using

enhanced chemiluminescence detection (Amersham Biosciences Europe GmbH).

For immunoprecipitation cell lysates containing defined amounts of protein (0.5–1 mg) were incubated with 2 μg of antibody raised against IRβ, IRS1 or IRS2, respectively. The immune complexes were collected using Protein A–Sepharose (Sigma, Deisenhofen, Germany) and washed five times with a buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM DTT. Then, 1 × gel-loading buffer was added to the immobilized immune complex. After heating at 95 °C for 5 min the samples were centrifuged at 20000 ***g*** and the supernatant was subjected to SDS/PAGE.

Immune-complex kinase assays

The Erk-1/Erk-2 and PI 3-kinase assays were performed as described previously [21,22]. Briefly, aliquots of the cell lysate containing 100 μg of protein were incubated with 1.5 μg of the polyclonal antibody against Erk-1/Erk-2 or the p85 subunit of the PI 3-kinase for 2 h at 4 °C. Immune complexes were collected by using Protein A–Sepharose 4B. In the case of Erk-1/Erk-2 immune complexes were washed six times with kinase buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM DTT) and incubated with 1 mg/ml myelin basic protein, 25 μM ATP and 5 μCi of [γ-^{32}P]ATP for 30 min at 37 °C. The activity of Erk-1/Erk-2 was monitored by autoradiography after SDS/PAGE (12.5 % gel). In the case of PI 3-kinase immune complexes were washed three times with 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM DTT, twice with a buffer containing 0.5 mM LiCl and 100 mM sodium vanadate in 0.1 mM Tris/HCl (pH 7.5) and twice with a buffer containing 10 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA (pH 7.5) and 200 μM sodium vanadate. Then, 30 μl of assay buffer (50 mM Hepes, pH 7.5, 200 mM ATP, 50 mM $MgCl_2$ and 1 mM EGTA) supplemented with 20 μg of sonicated PtdIns and 8 μCi of [γ-^{32}P]ATP was added to the immune complex. The reactions were allowed to proceed at 35 °C for 30 min and were stopped by the addition of 100 μl of 1 M HCl to each sample. Lipid extracts were obtained by the addition of 180 μl of chloroform/methanol (1:1, v/v), intense mixing of the probes, and centrifugation for phase separation. Aliquots of 15 μl of lower organic phase were spotted on to TLC plates (Merck). The plates were developed using chloroform/methanol/4 M NH_4OH (9:7:2, by vol.). The activity of PI 3-kinase was monitored by autoradiography.

4E-BP1 binding to m^{7}GTP–Sepharose

The assay was performed as described [23]. Briefly, cell extracts were incubated with m^{7}GTP–Sepharose at 4 °C over night. After centrifugation the sediment was washed three times with 10 mM Tris/HCl buffer (pH 7.4) containing 1 % Triton X-100, 0.5 % NP-40, 150 mM NaCl, 1 mM EDTA, 20 mM NaF and one tablet/50 ml of protease inhibitor cocktail. Then, 1 × gel-loading buffer was added to the immobilized proteins. After heating at 95 °C for 5 min the samples were centrifuged at 20000 ***g*** and the supernatant was subjected to SDS/PAGE. The proteins bound to m^{7}GTP–Sepharose were analysed by Western blot using antibodies raised against 4E-BP1 and eIF-4E.

Northern blot analysis

Total RNA from H4IIE cells was isolated by using guanidinium thiocyanate solution according to the protocol given in [24], using a kit from Qiagen (Hilden, Germany). RNA samples (10 μg) were electrophoresed in 0.8 % agarose/3 % formaldehyde and then RNAs were blotted on to Hybond-N nylon membranes with 20 × SSC (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 and 18 S rRNA bands. Blots were then subjected to a 3 h prehybridization 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. 10^6 c.p.m./ml dCTP-labelled random-primed MKP-1 or glyceraldehyde-3-phosphate dehydrogenase ('GAPDH') cDNA probes [18]. Membranes were washed three times in 2 × SSC/0.1 % SDS for 15 min, twice in 25 mM sodium phosphate buffer, pH 7.2/1 mM EDTA/0.1 % SDS for 10 min and twice in 25 mM sodium phosphate buffer, pH 7.2/1 mM EDTA/1 % SDS for 10 min at 53 °C. Blots were then exposed to Kodak AR X-Omat film at −70 °C with intensifying screens. Densitometry was performed with the E.A.S.Y. RH system (Herolab).

Analysis of results

Results from n independent experiments are expressed as means ± S.E.M. Results were compared using the Student's t test: $P < 0.05$ was considered statistically significant.

RESULTS

Insulin-induced MKP-1 expression in H4IIE cells

Insulin (100 nM) induces MKP-1 mRNA and protein expression in H4IIE cells. MKP-1 mRNA was maximal after 0.5 h (2.3 ± 0.4-fold, $n = 3$; Figure 1A) and MKP-1 protein was maximal after 3 h (5.1 ± 1.3-fold, $n = 5$; Figure 1B).

MKP-1 may be involved in termination of insulin-induced MAP kinase activation. As reported previously, early MAP kinase inactivation depends on protein phosphatase 2A-type Ser/Thr phosphatases and is insensitive to the protein tyrosine phosphatase inhibitor vanadate, whereas later inactivation due to MKP-1 expression can be antagonized by vanadate [25]. Insulin in H4IIE cells strongly activates Erk-1/Erk-2 (11.03 ± 0.78-fold after 5 min) and Erk-1/Erk-2 activity declines progressively between 5 and 180 min (Figure 1C). Presence of vanadate between 30 and 60 min of insulin exposure is without influence on Erk-1/Erk-2 activity at the 60 min time point, whereas vanadate present between 60 and 90 min prevents further inactivation of Erk-1/Erk-2 during this time period (Figure 1C). This correlates well with the appearance of MKP-1 between 60 and 120 min (Figure 2A) and would be consistent with insulin-induced MKP-1 expression to be involved in termination of the Erk-1/Erk-2 response to insulin.

Hyperosmolarity impairs insulin-induced MKP-1 expression

The interaction between insulin and hyperosmolarity was examined at the levels of MKP-1 protein and mRNA expression. Therefore H4IIE cells were treated hyperosmotically (405 mosmol/l) or were maintained under the normo-osmotic control condition (305 mosmol/l) for 1 h. Then insulin (100 nM) was added to the medium without change of the respective osmolarity and incubations were continued for different time periods. Figure 2(A) shows that hyperosmolarity delays the induction of the MKP-1 protein in response to insulin. The ratio between MKP-1 expression levels under hyperosmotic conditions/MKP-1 expression levels under normo-osmotic conditions

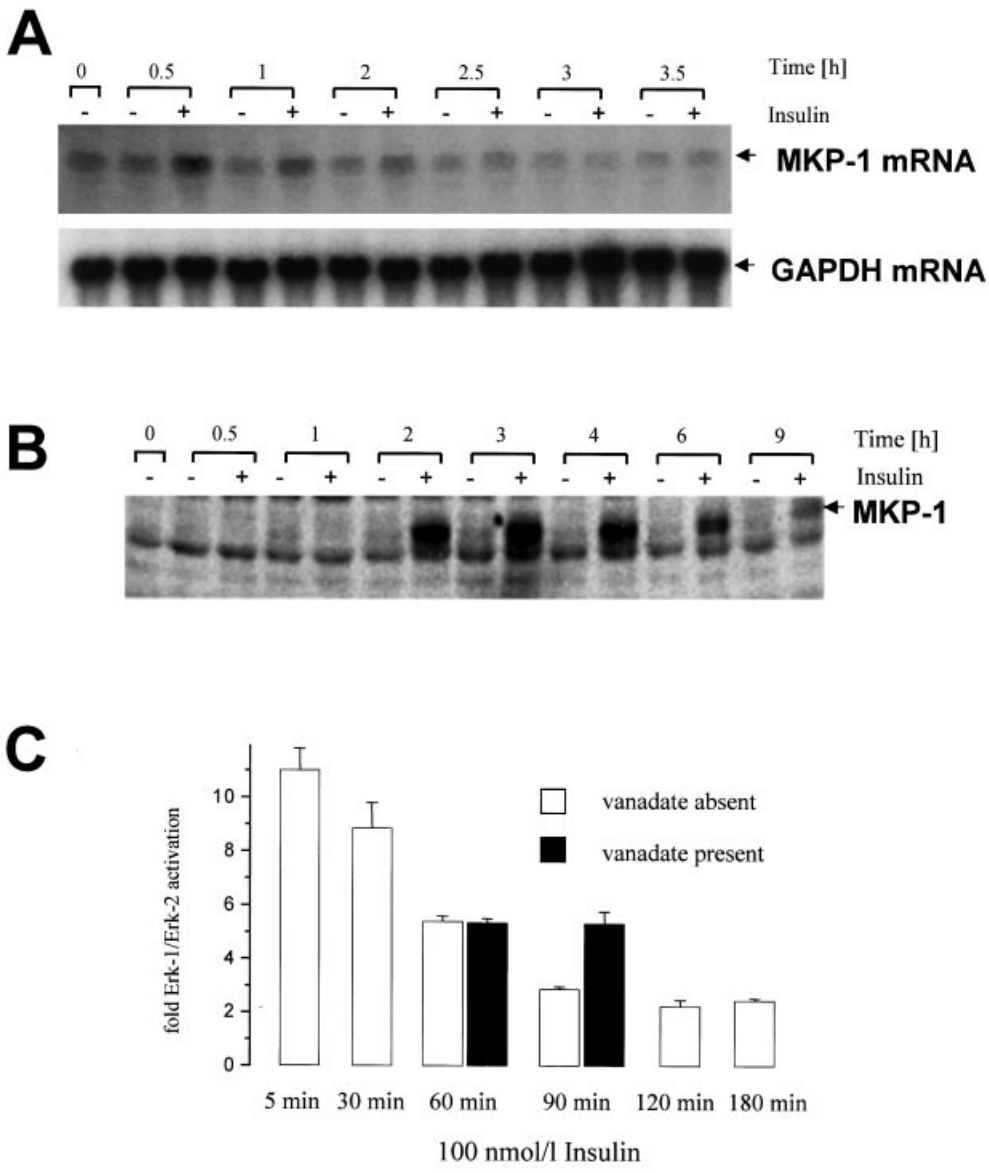

Figure 1 Insulin-induced MKP-1 expression and Erk-1/Erk-2 inactivation in H4IIE cells

H4IIE cells were treated with 100 nM insulin for the time periods indicated or were maintained without insulin. At the end of the incubations cells were lysed. Total RNA was analysed for the presence of MKP-1 mRNA by Northern blot analysis using the cDNA probe for MKP-1 [18]. Proteins were analysed for the presence of MKP-1 by Western blot using an antibody recognizing MKP-1. Erk-1/Erk-2 activity was measured by an immune-complex assay using myelin basic protein as a substrate. Representative blots of at least three independent experiments are shown. (**A**) Time course of MKP-1 mRNA expression. (**B**) Time course of MKP-1 protein expression. (**C**) Time course of Erk-1/Erk-2 activity in the presence of insulin. In the case of 60 and 90 min incubation with insulin vanadate (500 μM) was present during the last 30 min (black bars). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

increases from 0.28 ± 0.05 after 1.5 h to 0.73 ± 0.08 ($n = 5$–7, $P < 0.05$) after 3.5 h of insulin treatment. Similar results were obtained with insulin administration after a 5 h hyperosmotic pretreatment (results not shown). Corresponding to earlier findings [20], MKP-1 protein is not produced by hyperosmolarity in the absence of insulin (Figure 2A).

However, hyperosmolarity induces MKP-1 mRNA by itself ([18,19] and Figure 2B, lanes 8 and 12), and the presence of insulin does not further increase MKP-1 mRNA expression (Figure 2B, compare lanes 8 and 10, 1.96 ± 0.17 versus 1.68 ± 0.65-fold induction; lines 12 and 14, 2.58 ± 0.64 versus 2.89 ± 0.8-fold induction; $n = 6$). MKP-1 mRNA accumulation by insulin under normo-osmotic conditions was 2.09 ± 0.47-fold after 0.5 h and 2.01 ± 0.48-fold after 1 h (Figure 2B, lanes 5 and 9) and thus reaches levels similar to that present under hyperosmotic conditions.

Due to the hyperosmotic delay of insulin-induced MKP-1 accumulation and the absence of MKP-1 protein expression in the presence of hyperosmolarity, in spite of pronounced accumulation of the transcript, it seems conceivable that hyperosmolarity affects MKP-1 protein expression. Hyperosmolarity could impair insulin-induced MKP-1 expression by accelerating MKP-1 degradation or suppressing MKP-1 synthesis. To examine whether hyperosmolarity accelerates MKP-1 degradation, MKP-1 was induced by a 3 h exposure to insulin. Then medium was removed and the cells were further incubated in normo- or

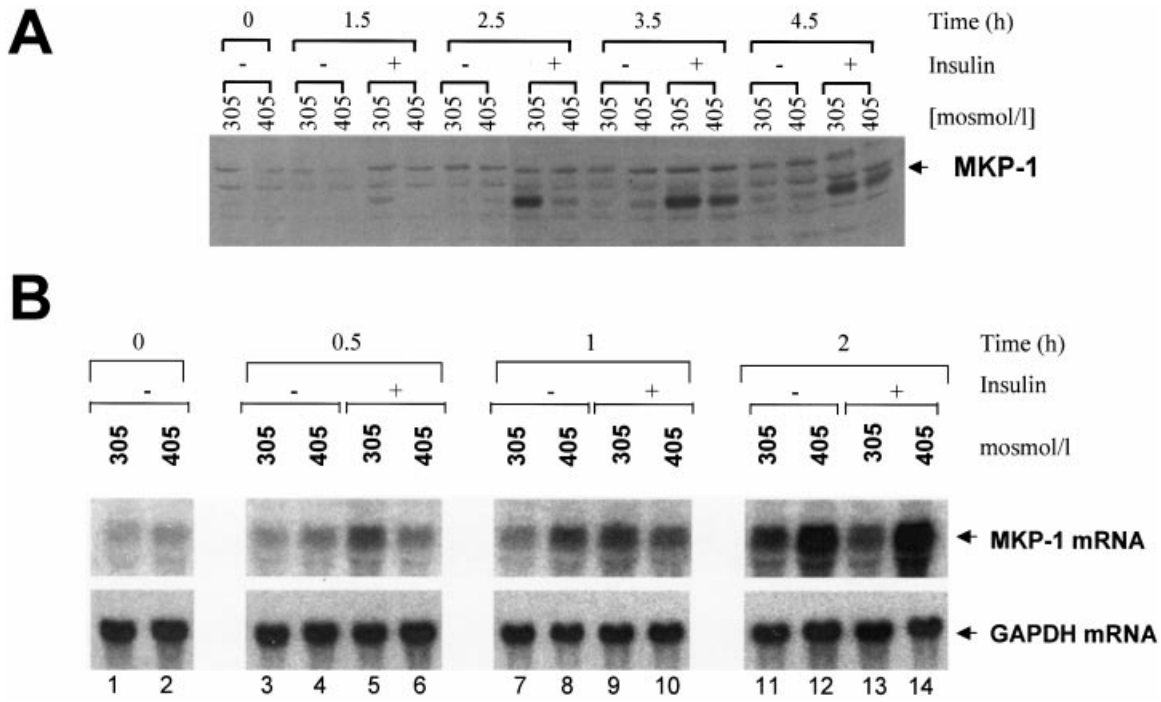

Figure 2 Hyperosmolarity interferes with insulin-induced MKP-1 expression

H4IIE cells were incubated with normo- or hyperosmotic medium (305 and 405 mosmol/l, respectively) for 1 h. Then the incubation was continued in the presence of 100 nM insulin for the times indicated. Representatives of three experiments are given. (**A**) Hyperosmotic modulation of insulin-induced MKP-1 expression. Protein extracts were analysed for the presence of MKP-1 protein by Western blot. (**B**) Hyperosmotic modulation of insulin-induced MKP-1 mRNA expression. Total RNA preparations were analysed by Northern blot for the expression of MKP-1 mRNA. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

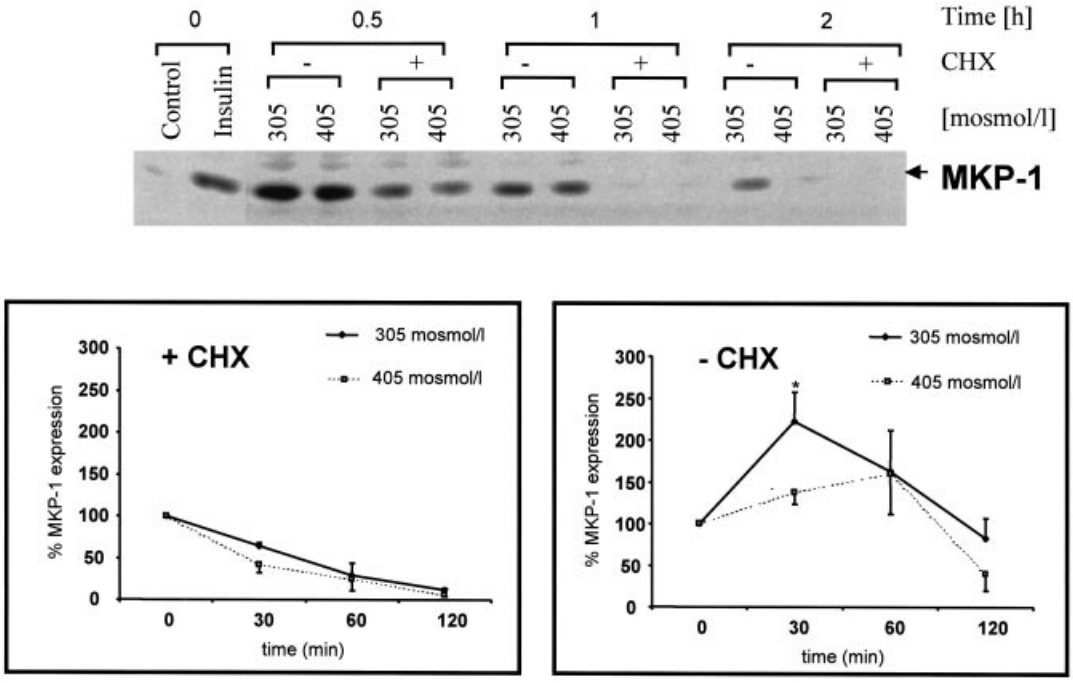

Figure 3 Effect of hyperosmolarity on proteolytic degradation of MKP-1

To induce MKP-1 protein expression, H4IIE cells were stimulated with 100 nM insulin for 3 h. Then medium was exchanged and the cells were further incubated in normo- or hyperosmotic medium in the absence or presence of CHX for the times indicated. The expression levels of MKP-1 protein were monitored by Western blot analysis. Representatives of at least three independent experiments are given. For quantification, densitometry was performed and MKP-1 expression levels after the 3 h incubation with insulin were set to 100%. $^*P < 0.05$, significant increase in MKP-1 expression compared with the expression levels at the zero time point.

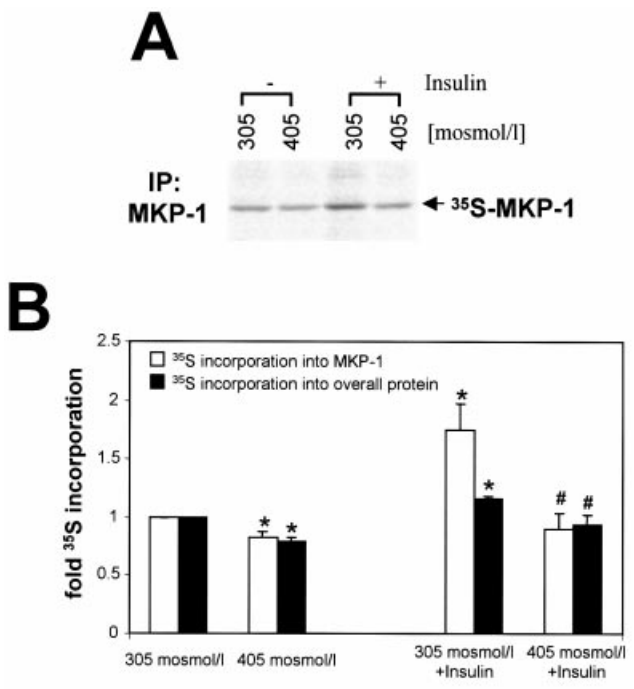

Figure 4 Effect of hyperosmolarity on MKP-1 synthesis in H4IIE cells

Representatives of four independent experiments are given. After a 1 h normo- or hyperosmotic pretreatment H4IIE cells were incubated with 100 nM insulin in the presence of 20 μCi/ml Tran^{35}S-Label^{TM} for another 2.5 h. Then cells were lysed. (**A**) The MKP-1 protein was immunoprecipitated from the protein extracts and the immune complex was subjected to SDS/PAGE. Incorporation of ^{35}S into MKP-1 was visualized by autoradiography. (**B**) MKP-1 synthesis was quantified by densitometric analysis of the autoradiogram. In addition, overall protein synthesis was considered by counting ^{35}S radioactivity in TCA-precipitable material. $^{*}P < 0.05$, significant difference from the respective normo-osmotic control; $\#P < 0.05$, significantly different to the respective '305 + insulin' condition.

hyperosmotic medium in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). As shown in Figure 3, hyperosmolarity was without significant effect on the decline of MKP-1 protein levels in the presence of CHX. Thus proteolytic degradation of MKP-1 may not account for the hyperosmotic attenuation of insulin-induced MKP-1 expression in H4IIE cells. In the absence of CHX and insulin MKP-1 levels increased significantly to $222 \pm 36\,\%$ ($n = 3$) at 0.5 h under normo-osmotic conditions, whereas the increase in MKP-1 accumulation was lower ($138 \pm 10\,\%$, $n = 3$) under hyperosmotic conditions (Figure 3). In view of the insensitivity of MKP-1 degradation to hyperosmolarity in the presence of CHX this suggests hyperosmotic impairment of MKP-1 synthesis.

To further examine osmotic regulation at the level of MKP-1 synthesis, ^{35}S incorporation into MKP-1 protein was measured. H4IIE cells were challenged hyperosmotically or were exposed to the normo-osmotic control medium for 1 h. Then the cells were stimulated with insulin in the presence of 20 μCi/ml Tran^{35}S-Label^{TM} for a period of 2.5 h. At this point of time MKP-1 expression is suppressed under hyperosmotic conditions (Figure 2A). Figure 4 shows that insulin induces ^{35}S incorporation into MKP-1 under normo-osmotic conditions, which greatly exceeds the induction of overall protein synthesis by insulin. Hyperosmolarity itself was ineffective in incorporating ^{35}S into MKP-1 and largely suppressed the ^{35}S labelling of MKP-1 induced by insulin. Likewise, stimulation of overall protein synthesis by insulin was suppressed under hyperosmotic conditions (Figure 4B).

Hyperosmotic modulation of insulin signalling in H4IIE cells

The hyperosmotic attenuation of MKP-1 induction by insulin may relate to modulation of early insulin signals. As shown in Figure 5(A) a 1 h hyperosmotic pretreatment does not induce Tyr phosphorylation of IRβ, IRS1 and IRS2 and association of p85 with IRS1 and IRS2, respectively. Further, insulin-induced Tyr phosphorylation of the IRβ, IRS1 and IRS2 as well as p85 association with IRS1 and IRS2 are virtually unaffected by a 1 h hyperosmotic pretreatment, although hyperosmolarity slightly reduces IRS2 electrophoretic mobility.

Hyperosmolarity in H4IIE cells stimulates activation of the MAP kinases Erk-1/Erk-2 and p38, which is maximal after about 2 h and then declines towards basal levels within 4–5 h [18,19]. Insulin-induced MAP kinase activation added to MAP kinase activation induced by a 1 h hyperosmotic pretreatment (Figure 5B). Extension of hyperosmotic pretreatment to 5 h attenuated insulin-induced Erk-1/Erk-2 activation, whereas p38 phosphorylation was still increased under hyperosmotic conditions (Figure 5B).

Hyperosmolarity (1 h) was largely without effect on insulin-induced activation of PI 3-kinase and Thr-410/403 phosphorylation of PKC ζ/λ, whereas Ser-473 phosphorylation of PKB in response to insulin was somewhat delayed under hyperosmotic conditions (Figure 5C). After prolonged insulin treatment PKB (Ser-473) phosphorylation reached similar levels in hyper- and normo-osmotically treated cells (Figure 5C; see also Figure 7A, below). However, insulin-induced hyperphosphorylation of p70S6-kinase as visualized by antibodies recognizing p70S6-kinase phosphorylated on Thr-389 and Thr-421/Ser-424, and by upward shifts of p70S6-kinase in electrophoretic mobility, appeared to be suppressed continuously under hyperosmotic conditions (Figure 5C; see also Figure 7A, below).

Insulin-induced phosphorylation of 4E-BP1 antagonizes binding to the eukaryotic initiation factor eIF-4E, thereby allowing translation of a specific subset of mRNAs [4]. Phosphorylation states of 4E-BP1 were visualized by shifts in electrophoretic mobility and by monitoring of 4E-BP1 binding to eIF-4E, the latter isolated using m^{7}GTP–Sepharose. Hyperosmolarity in the absence of insulin increased electrophoretic 4E-BP1 mobility and significantly ($P < 0.05$) increased binding of 4E-BP1 to eIF-4E (Figure 5C). In addition, hyperosmolarity interfered with the insulin-induced phosphorylation of 4E-BP1 (Figure 5C).

The data indicate that insulin signalling downstream of mTOR was diminished under hyperosmotic conditions and it seems possible that this was related to the delay of MKP-1 expression by insulin under hyperosmotic conditions.

Pharmacological characterization of insulin-induced MKP-1 expression

To identify specific signalling events involved in MKP-1 induction by insulin, MKP-1 mRNA and protein expression and ^{35}S incorporation into overall protein were examined by pharmacological means (Figure 6, Table 1). PD98059, which blocks the Erk-1/Erk-2 pathway at the level of MAP kinase kinases immediately upstream of Erk-1/Erk-2 [26] and completely abolishes insulin-induced Erk-1/Erk-2 activation in H4IIE cells [23] (Figure 6A, lanes 1–3), significantly impaired induction of MKP-1 mRNA and protein by insulin (Figure 6B, Table 1). In addition, stimulation of overall protein synthesis by insulin was significantly affected by PD98059 (Table 1). The p38 inhibitor SB220025 [27], but not its inactive analogue SB202474, prevented

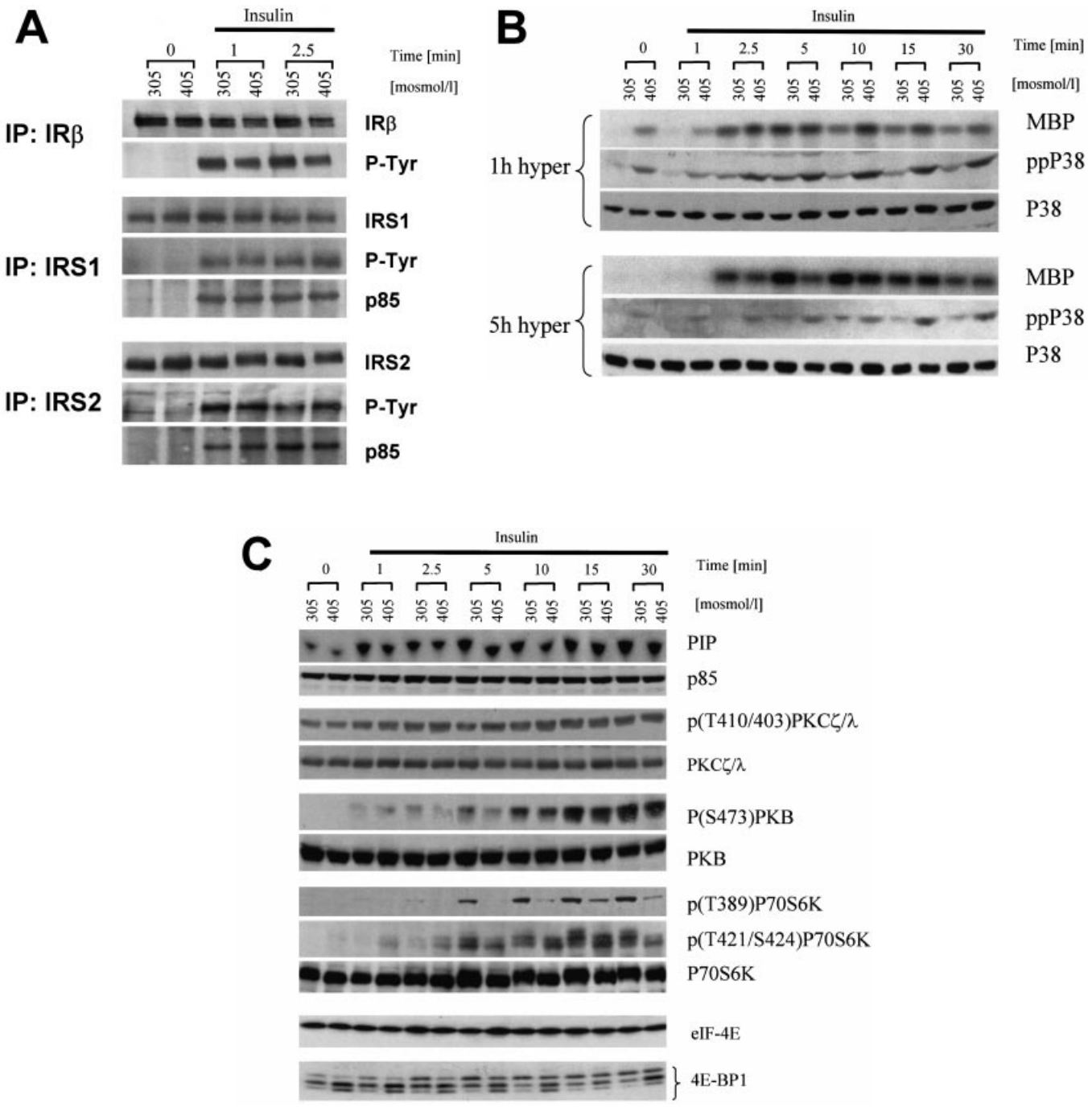

Figure 5 Hyperosmotic modulation of insulin signalling in H4IIE cells

H4IIE cells were exposed to hyperosmolarity (405 mosmol/l) or to the normo-osmotic control medium (305 mosmol/l) for 1 h if not stated otherwise. Then the incubation was continued in presence of 100 nM insulin for the times indicated. Representatives of at least three independent experiments are shown. (**A**) Hyperosmolarity does not affect insulin-induced Tyr phosphorylation of IRβ, IRS1 and IRS2 as well as p85 association with IRS1 and IRS2. Immunoprecipitated (IP) IRβ, IRS1 and IRS2 were analysed in Western blots with antibodies recognizing phosphotyrosine or the respective immunoprecipitated protein. IRS1 and IRS2 immunoprecipitates were checked in Western blots for the presence of the p85 regulatory subunit of the PI 3-kinase. (**B**) Hyperosmotic modulation of insulin-induced Erk-1/Erk-2 activation and dual phosphorylation of p38 (ppP38). Protein extracts were analysed for Erk-1/Erk-2 activity by an immune-complex assay. Dual phosphorylation of the p38 and p38 expression were detected in Western blot with antibodies recognizing phosphorylated p38 and total p38, respectively. MBP, myelin basic protein. (**C**) Hyperosmotic modulation of insulin-induced activation of PI 3-kinase-dependent signals. Protein extracts were analysed for PI 3-kinase activity by an immune complex assay, whereas p85 expression was determined in Western blot. Changes in phosphorylation of PKC ζ/λ, PKB, p70S6-kinase and 4E-BP1 were visualized in Western blots using phospho-specific antibodies. Expression levels of PKCζ/λ, PKB and p70S6-kinase were determined using antibodies recognizing the respective total protein. 4E-BP1 and eIF-4E precipitated by m^7GTP–Sepharose were assayed by Western blot. PIP, PtdIns*P*.

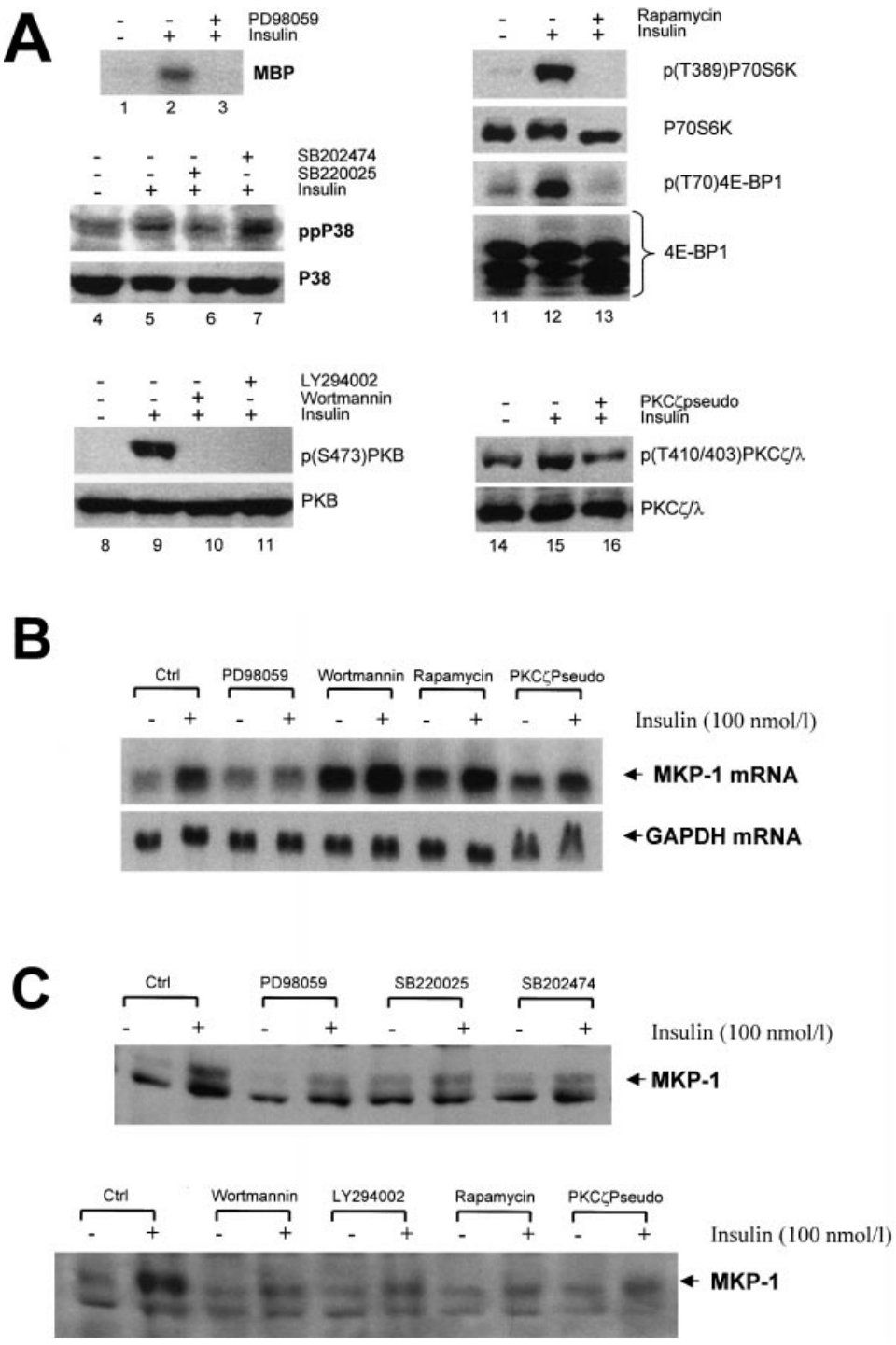

Figure 6 Pharmacological characterization of insulin-induced MKP-1 expression

H4IIE cells remained untreated or were exposed to 100 nM insulin (10 min for analysis of kinase activities, 0.5 h for mRNA and 3 h for protein analysis) without further treatment or following a 30 min preincubation with PD98059 (20 μM), wortmannin (500 nM), LY294002 (50 μM) or rapamycin (500 nM) or a 2 h pretreatment with the myristoylated PKCζ pseudosubstrate SIYRRGARRWRKL (50 μM; PKCζpseudo), as indicated. Protein extracts were analysed for Erk-1/Erk-2 by an immune-complex assay using myelin basic protein (MBP) as a substrate. In addition, dual phosphorylation of p38 (ppP38), Ser-473 phosphorylation of PKB, Thr-389 and Thr-421/Ser-424 phosphorylation of p70S6-kinase, Thr-70 phosphorylation of 4E-BP-1 and Thr-410/403 phosphorylation of PKCζ/λ were determined in Western blots using phospho-specific antibodies. Overall expression of these proteins was determined by Western blots using antibodies recognizing the respective total protein. The presence of MKP-1 was examined by Western blot using an antibody recognizing MKP-1. Total RNA was analysed for the presence of MKP-1 mRNA by Northern blot analysis using the cDNA probe for MKP-1 [18]. (**A**) Effectiveness of the respective inhibitors. (**B**) Pharmacological characterization of insulin-induced MKP-1 mRNA expression. (**C**) Pharmacological characterization of insulin-induced MKP-1 protein expression. Representatives from at least three independent experiments are given. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 Pharmacological characterization of insulin-induced accumulation of MKP-1 mRNA and protein and incorporation of ^{35}S into overall protein

Insulin (100 nM) was added to the H4IIE cells or not. Exposure was continued for 0.5 h (MKP-1 mRNA analysis) or 3 h (analysis of MKP-1 protein and incorporation of ^{35}S into overall protein). For determination of the latter, in addition 20 μCi/ml Tran^{35}S-Label^{TM} was present. Cells remained without further pretreatment or were pretreated for 30 min with PD98059 (20 μM), SB220025 (10 μM), SB202474 (20 μM), wortmannin (500 nM), LY294002 (50 μM) or rapamycin (500 nM) or for 2 h with PKCζ pseudosubstrate (50 μM). Lysates were prepared and analysed for the presence of MKP-1 mRNA and protein as described in the legend to Figure 5. Incorporation of ^{35}S into TCA-precipitable material was taken as a measure of overall protein synthesis. Fold induction of MKP-1 mRNA and protein expession and ^{35}S incorporation are shown relative to the respective condition in the absence of insulin. For quantification of MKP-1 mRNA and protein induction Northern and Western blots were analysed densitometrically. For quantification of protein synthesis ^{35}S radioactivity within the TCA-precipitable material was counted. Controls with DMSO (vehicle) did not show any significant effect on induction of MKP-1 or ^{35}S incorporation into overall protein. This evaluation is based on $n \geqslant 5$ independent experiments. n.d., not determined.

	Fold induction		
	MKP-1 mRNA	MKP-1 protein	^{35}S incorporation
Insulin (100 nM)	2.98 ± 0.16*	4.56 ± 0.38*	1.86 ± 0.27*
+ PD98059 (20 μM)	1.3 ± 0.2†	2.4 ± 0.23*†	1.12 ± 0.03*†
+ SB220025 (10 μM)	n.d.	2.77 ± 0.43*†	1.79 ± 0.08*
+ SB202474 (20 μM)	n.d.	2.8 ± 0.26*†	1.3 ± 0.15
+ Wortmannin (500 nM)	1.62 ± 0.2*†	1.56 ± 0.24*†	1.2 ± 0.09
+ LY294002 (50 μM)	n.d.	2.83 ± 0.39*†	1.19 ± 0.07*†
+ Rapamycin (500 nM)	2.45 ± 0.26*	2.03 ± 0.11*†	1.53 ± 0.05*
+ PKCζ pseudosubstrate (50 μM)	2.64 ± 0.33*	0.93 ± 0.19†	1.97 ± 0.09*

* $P < 0.05$, significant induction of MKP-1 mRNA, protein or overall protein synthesis, by insulin under the respective condition.

† $P < 0.05$, fold induction significantly different from that found to be induced by insulin in the absence of any inhibitor.

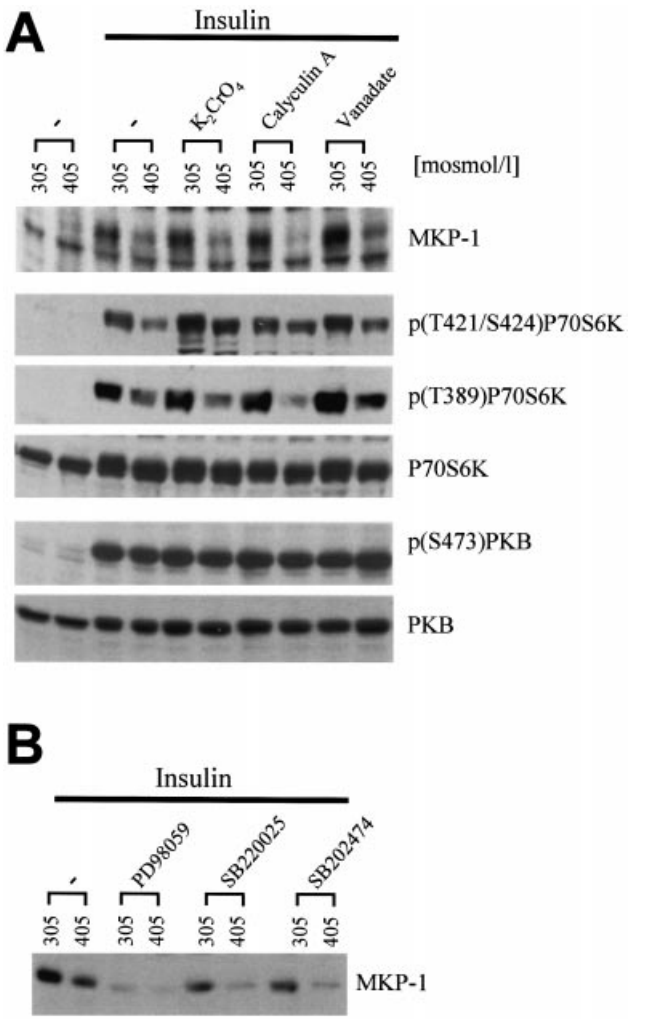

Figure 7 Effect of insulin sensitizers and MAP kinase inhibition on hyperosmotic impairment of MKP-1 expression

K_2CrO_4, calyculin A and vanadate (**A**) and PD98059 and SB220025 (**B**) do not restore MKP-1 induction by insulin under hyperosmotic conditions. H4IIE cells were incubated with normo- or hyper-osmotic medium (305 and 405 mosmol/l, respectively) for 1 h without further additives or in presence of K_2CrO_4 (10 μM), calyculin A (5 nM), vanadate (500 μM), PD98059 (20 μM), SB220025 (10 μM) or SB202474 (20 μM) as indicated. Then insulin was added for another 2.5 h, and cells were lysed and analysed in Western blots for the presence of MKP-1 and phosphorylated p70S6-kinase and PKB. In addition, overall expression of p70S6-kinase and PKB were monitored. Representatives of three experiments are given.

insulin-induced phosphorylation of p38 (Figure 6A, lanes 4–7). According to [28] this suggests p38 autophosphorylation to be involved in activation of p38 by insulin. SB220025 and SB202474 tend to induce MKP-1 protein expression by themselves. MKP-1 induction by insulin was apparently reduced in the presence of both compounds (Figure 6C, Table 1) and therefore is probably not related to impairment of p38 signalling. Both SB220025 and SB202474 did not significantly affect induction of overall protein synthesis by insulin (Table 1). It is suggested that activation of the MAP kinases Erk-1/Erk-2 by insulin mediates MKP-1 expression at the transcriptional level. This is probably part of a feedback-inhibitory loop contributing to termination of MAP kinase activity (see Figure 1B).

Both wortmannin and LY294002, which inhibit PI 3-kinase by different mechanisms, effectively prevented insulin-induced phosphorylation of PKB at Ser-473 (Figure 6A, lanes 8–11). Wortmannin partly inhibited insulin-induced MKP-1 mRNA expression (Figure 6B, Table 1). Both wortmannin and LY294002 effectively antagonized the synthesis of MKP-1 protein in response to insulin (Figure 6C, Table 1) and both compounds (wortmannin, $P = 0.08$; LY294002, $P < 0.05$) affected induction of overall protein synthesis by insulin (Table 1).

Rapamycin, a highly specific inhibitor of mTOR-dependent signalling [29], blocks insulin-induced hyperphosphorylation of p70S6-kinase and 4E-BP1 in H4IIE cells [23,30] (Figure 6A, lanes 11–13). Rapamycin was without effect on insulin-induced MKP-1 mRNA expression but effectively antagonized synthesis of MKP-1 protein (Figure 6, Table 1). Rapamycin did not significantly ($P = 0.34$) affect induction of overall protein synthesis by insulin (Table 1). This indicates that inhibition of MKP-1 expression by rapamycin was not an indirect effect related to a general down-regulation of protein synthesis.

The myristoylated PKCζ pseudosubstrate SIYRRGARRW-RKL [31] prevented an increase in PKCζ/λ phosphorylation at Thr-410/403 in response to insulin (Figure 6A, lanes 14–16). Like rapamycin, the PKCζ pseudosubstrate inhibited MKP-1 protein but not mRNA induction by insulin (Figures 6B and 6C, Table 1). The PKCζ pseudosubstrate did not affect stimulation of overall protein synthesis by insulin (Table 1), suggesting some specificity with regard to its inhibitory effect on MKP-1 protein synthesis.

These studies suggest that in H4IIE cells PI 3-kinase, atypical PKCs ζ/λ and rapamycin-sensitive signalling via mTOR contribute to MKP-1 expression by insulin largely at the protein level. Whereas the contribution of PI 3-kinase may in part relate

to a general stimulation of protein synthesis by insulin, the data suggest a more selective involvement of mTOR and PKCζ/λ in MKP-1 protein synthesis.

The influence of potential insulin sensitizers on insulin-induced MKP-1 expression, p70S6-kinase and PKB phosphorylation was examined (Figure 7A). Neither K_2CrO_4, nor the protein phosphatase 2A-type Ser/Thr-phosphatase inhibitor calyculin A and the tyrosine phosphatase inhibitor vanadate improved MKP-1 induction by insulin under hyperosmotic conditions. None of these compounds compensated for the hyperosmotic impairment of p70S6-kinase phosphorylation by insulin. Also PD98059 and SB220025 were ineffective in allowing MKP-1 expression by insulin under hyperosmotic conditions, suggesting that Erk-1/Erk-2 and p38 did not account for the hyperosmotic suppression of MKP-1 expression (Figure 7B).

DISCUSSION

There is a close relationship between liver cell hydration and insulin sensitivity (reviewed in [32,33]). In perfused rat liver, insulin produces a PI 3-kinase-dependent K^+ retention and cell swelling, which essentially contributes to MAP kinase activation and proteolysis inhibition [9]. On the other hand, hyperosmotic dehydration blocks insulin-induced K^+ retention, cell swelling and proteolysis inhibition in this model [9]. In H4IIE rat hepatoma cells hyperosmolarity reduces the sensitivity of the cells with regard to Erk-1/Erk-2 activation by insulin [18]. Here we give an example of insulin-induced gene expression to be modulated by hyperosmolarity, i.e. the hyperosmotic delay of MKP-1 expression by insulin.

MKP-1 expression is decreased and Erk-1/Erk-2 activity is increased in H4IIE cells stimulated with insulin after a 1 h hyperosmotic preincubation (Figures 2A and 5B), suggesting that Erk-1/Erk-2 activity is not limiting for MKP-1 expression by insulin under hyperosmotic conditions. On the other hand, hyperosmolarity modulates insulin-induced expression of MKP-1 and phosphorylation of p70S6-kinase and 4E-BP1 in the same direction (Figures 2A and 5C), suggesting that inhibition of mTOR-mediated signalling could account for the hyperosmotic suppression of MKP-1 expression by insulin. MKP-1 protein but not mRNA induction by insulin is blocked by rapamycin (Figure 6), indicating that the hyperosmotic restriction of insulin-induced p70S6-kinase and 4E-BP1 phosphorylation may affect MKP-1 expression at the protein level by modulating synthesis and/or degradation. Although hyperosmolarity stimulates autophagy in liver cells [34], degradation of MKP-1 is not accelerated (Figure 3), probably due to proteasomal degradation of MKP-1 [35]. However, translation of MKP-1 mRNA is affected, as concluded from insulin-induced ^{35}S incorporation into MKP-1, which is impaired by hyperosmolarity (Figure 3). Hyperosmolarity not only delays insulin-induced synthesis of MKP-1 (this study) but in addition completely blocks the translation of MKP-1 mRNA, which accumulates in H4IIE cells under hyperosmotic conditions in the absence of any additional stimulus (Figure 2) [18–20]. As shown here (Figure 5C), hyperosmolarity in the absence of insulin suppresses 4E-BP1 phosphorylation and release from eIF-4E below basal levels. This further supports the view that hyperosmotic impairment of signals downstream from mTOR interferes with MKP-1 mRNA translation. That insulin partly overcomes the hyperosmotic effects on 4E-BP1 (Figure 5C) may account for the delayed onset of MKP-1 translation under hyperosmotic conditions in the presence of the hormone.

Rapamycin completely abolishes insulin-induced translation of MKP-1 mRNA but is without significant effect on general protein synthesis stimulated by insulin (Figure 6C, Table 1). Similar results were obtained with the myeloid progenitor cell line 32D [36] and it was estimated that about 90% of insulin-induced protein synthesis represents a general stimulation of mRNA translation, whereas 10% is a preferential translation of a small subset of growth-regulated mRNAs, which is sensitive to rapamycin [36] and which probably includes MKP-1 (this study). Thus although hyperosmolarity affects insulin-induced MKP-1 synthesis and overall protein synthesis in parallel, the underlying mechanisms apparently differ. It should be noted that hyperosmolarity does not interfere with MKP-1 synthesis in general. For example, hyperosmolarity amplifies the MKP-1 response to heat shock in H4IIE cells [20], further underlining some specificity of the hyperosmotic impairment of insulin-induced MKP-1 synthesis. It remains to be proven to what extent hyperosmolarity interferes with expression of other proteins known to be translated in a rapamycin-sensitive manner.

Some features of hyperosmotic interference with signalling downstream from PI 3-kinase depend on the experimental system under investigation. In 3T3 L1 adipocytes hyperosmolarity (600 mM sorbitol) prevents phosphorylation of PKB by insulin and this is antagonized by calyculin A [12]. In monkey kidney CV1 cells hyperosmolarity (400 mM sorbitol) impairs p70S6-kinase activation in a calyculin A-sensitive manner, whereas PKB phosphorylation was not affected in this system [37]. The hyperosmotic impairment of insulin-induced p70S6-kinase phosphorylation in H4IIE cells (this study) was insensitive to K_2CrO_4, vanadate, calyculin A (Figure 7) and okadaic acid (results not shown), suggesting that hyperosmotic activation of phosphatases is probably not involved. mTOR was recently suggested to sense ATP and glutamine [38,39] and one is tempted to speculate that mTOR-related components could sense changes in intracellular hydration either directly or indirectly by detecting a hyperosmotic decrease in ATP/AMP. In line with this would be the finding that hyperosmolarity activates the AMP-activated protein kinase in a rat liver endothelial cell line (clone 9), which is indicative of small changes in energy charge [40].

Originally MKP-1 was hypothesized to account for insulin resistance in hyperosmotically challenged H4IIE cells [18] but the data presented here show that insulin resistance by hyperosmolarity is recognizable at the level of MKP-1 expression. Suppression of MKP-1 may further promote insulin resistance under dehydrating conditions by allowing an unbalanced elevation of MAP kinases, which is characteristic for some insulin-resistant states [41]. Further studies, including MKPs different from MKP-1, are required to settle this issue.

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