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THE LIVER RESPONSE TO IN VIVO HEAT SHOCK INVOLVES THE ACTIVATION OF MAP KINASES AND RAF AND THE TYROSINE PHOSPHORYLATION OF SHC PROTEINS

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We have investigated the mechanisms of signal transduction in the response of liver to heat shock in vivo. By immunoblot experiments we have shown that heat shock decreases the electrophoretic mobility of the 40 and 43 kDa mitogen activated protein kinases (MAPKs) and we have found a significant increase of MAPK activity measured as phosphotransferase capacity of both cytosolic extracts and MAPK immunoprecipitates. To elucidate the signalling pathway which accounts for MAPK activation, we focused our attention on its upstream factors, Raf and Ras. We have shown that, heat shock activates Raf-1 kinase and causes an increase in phosphotyrosine content of the 52 kDa Shc protein accompanied by an increment in the amount of coimmunoprecipitated Grb2. These findings provide the first evidence that the Ras-Raf-MAPK pathway is activated in liver during heat shock in vivo.

The heat shock response is nearly universal and causes to the induction, or enhancement, of the synthesis of so-called heat shock proteins (hsp). Both the cis-acting DNA elements and trans-acting protein factor(s), necessary for the transcriptional activation of heat shock genes, have been identified and the role of molecular chaperones of hsps, in particular of hsp 70, has also been clearly established (1,2). In contrast our understanding of the sensing and signal transduction mechanisms of the heat shock response is rather limited and ill-defined. The increase in cell calcium (3), the rise in cAMP (4), the release of inositol trisphosphate (5), the activation of phospholipase A_2 (PLA_2) (6), that have been reported, suggest that heat shock might initiate transmembrane signalling cascades. More recently it has been shown that heat shock induces mitogen-activated protein kinase (MAPK) activation in several species of mammalian cells in vitro (7), but proved unable to activate the classical MAPK pathway in PC12 and A431 cells, where it triggered a new stress-activated signal transduction pathway (8).

In this report we have studied MAPK activity in an experimental in vivo model of liver hyperthermia, which has been extensively used in our laboratory (9) and we have analysed the possible upstream factors responsible for MAPK activation. Although alternative pathways exist

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(10,11), we directed our investigation towards the major mechanism for MAPK activation which comprises the sequential activation of Ras-Raf-MAPK kinase (MAPKK).

MATERIALS AND METHODS

Animals and treatment. Male Wistar rats were heat shocked by wrapping them in a heating pad under anaesthesia with Formotal (4 mg/100 g body weight). A rectal temperature of 41° C, monitored by a thermocouple, was reached in 10-15 min and was maintained between 41° and 42° C for 30 and 50 min. Animals were monitored at regular intervals for signs of excessive discomfort. The rats were than killed and livers were rapidly excised.

MAP kinase activity. 15 μg of cytosolic proteins, obtained as described (12), were incubated with or without MAPK substrate peptide for 10 min at 30°C in a reaction mixture containing 50 mM Tris pH 7.4, 2 mM EGTA, 40 mM β-glycerophosphate, 12.5 mM $MgCl_2$, 100 μM ATP and 2 μCi [γ-^{32}P] ATP. The reaction was stopped with 5% trichloroacetic acid and 100 μg bovine serum albumin (BSA), after centrifugation aliquots of supernatants were spotted onto P-81 filters and counted in a liquid scintillation counter. The kinase activity was calculated as difference between the ^{32}P incorporation in the presence and the absence of substrate.

MAP kinase immunocomplex kinase assay. 300 μg of cytosolic proteins in 500 μl of buffer (25 mM Tris-HCl pH 8, 0.5 mM EGTA, 137 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM NaF and protease inhibitors) were subjected to immunoprecipitation with 4 μg of anti MAPK R2 antibody (obtained from Upstate Biotechnology Incorporated) or with preimmune rabbit serum for mock precipitation, for 4 h at 4°C, as described (12). Immunoprecipitates were adsorbed to Protein A Sepharose bead, washed extensively and incubated in 100 μl kinase buffer (25 mM Tris-HCl pH 7.4, 1 mM DTT, 25 mM β-glycerophosphate, 10 mM $MgCl_2$, 50 μM ATP, 25 μg of myelin basic protein (MBP), protease inhibitors and 10 μCi of [γ^{32}P] ATP) at 30°C for 10 min in a rotary incubator (12). The beads were spun, aliquots of 20 μl were spotted onto P-81 filters and counted in a liquid-scintillation counter. Mock precipitate counts were subtracted from MAPK immunoprecipitate counts. Aliquots of 20 μl of supernatant were subjected to sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) (13) followed by autoradiography using Kodak X-Omat film.

Raf-1 immunocomplex kinase assay. 500 μg of proteins of liver lysates in RIPA buffer (10 mM sodium phosphate pH7.00; 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 0.1 % SDS, 1% Nonidet P40, 1% aprotinin, 50 mM NaF, 0.2 mM sodium orthovanadate, 0.1% β-mercaptoethanol and 1mM PMSF) (14) were precleared by incubation with 5 μl of normal rabbit serum and 50 μl of 10% protein A Sepharose in RIPA for 1 hour at 4°C. Supernatants were incubated with 1 μg of rabbit antibody anti Raf-1 (C-12, obtained from Santa Cruz Biotechnology) for 2 hours at 4°C in a rotary incubator. Samples were then adsorbed to protein A Sepharose and spun through a 500 μl cushion of 10 % sucrose in RIPA. Immunocomplexes were than extensively washed as described (14). The immunoprecipitates were incubated for 45 min at 30°C in 50 μl of a reaction mixture containing 20 mM Pipes pH 7, 100 mM $MgCl_2$, 20 μg/ml aprotinin, 10 μg of histone H1 and 0.1 mCi [γ-^{32}P]ATP (15). Kinase reactions were stopped with electrophoresis buffer (13) and 20 μl were analysed by 15% SDS-PAGE. Gels were stained with Coomassie Blue, dried and exposed to Kodak X-Omat film for autoradiography. Stained histone H1 was excised from gels and counted in a liquid scintillation counter.

Immunoprecipitation of Shc proteins. 1 mg of proteins of lysates in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate and protease inhibitors, were incubated with 3 μg of polyclonal anti Shc antibody (obtained from Upstate Biotechnology Incorporated) for 5 h at 4° C and adsorbed to protein A Sepharose (16). Immunoprecipitates were extensively washed with lysis buffer, resuspended in Laemmli's (13) buffer and analysed by Western blot with anti Shc antibodies, with monoclonal antiphosphotyrosine antibodies (clone 4G10) horseradish peroxidase (HRP) conjugated (obtained from Upstate Biotechnology Inc.) and with monoclonal anti Grb2 antibodies (obtained from Tansduction Laboratories).

Immunoblot analysis. Proteins of cytosol for MAPK, proteins of lysates obtained with boiling lysis buffer (containing 63 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 5% βmercaptoethanol, 100 mM DTT, 100 mM orthovanadate, 0.1 mM sodium molybdate, 100 μg/ml leupeptin and 100 μg/ml aprotinin) for Raf-1, and the different immunoprecipitates were subjected to SDS-PAGE (13) and transferred to polyvinylidene difluoride membranes using a Bio-Rad Trans-Blot semidry cell (17). The membranes were blocked with 5% BSA in 10 mM Tris-HCl pH 7.4, 150 mM NaCl then probed with different antibodies as indicated in figure legends. Primary antibodies were detected using antispecies secondary antibodies HRP conjugated. To reprobe a blot with a different antibody, the membrane was incubated in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) for 30 min at 50°C and rebloked in buffer containing 5% BSA. Immunoreactive proteins were made visible by ECL method. Densitometric analyses were performed by LKB Ultroscan laser densitometer.

Other procedures. The amount of protein was estimated by the Bradford's method (18)using BSA as standard. Significance of differences was tested by Student's t test.

RESULTS

The possible effects of heat shock were assessed by comparison with normal rats, since the animals injected with the anaesthetic were always within the normal range.

Immunoblot analysis of MAP kinase. First of all we examined the electrophoretic behaviour of MAPK proteins using immunoblotting experiments. In agreement with our previous observations (12), immunoblot analysis of liver proteins from control rats, probed with anti MAPK R2 antibodies (which recognise the products of erk1-erk2 and mpk genes, U.B.I.), detected three isoenzymes of 40, 43 and 44 kDa molecular masses (Fig. 1 a). After 30 min of heat shock a part (~40% by densitometric analysis), of 40 kDa MAPK showed a decrease of the electrophoretic mobility. The shift was more evident after 50 min of hyperthermia when it involved the most of the protein. The thermal stress also affected the behaviour of the 43 and 44 kDa bands. The amount of the 44 kDa protein increased concomitant with a decrease in the 43 kDa protein. We used a specific anti-Erk1 antibody to clarify this point. In Western blotting experiments of anti-MAPK R2 immunoprecipitates from control and 50 min treated animals, the anti Erk1 antibodies only recognised the 43 kDa protein in control samples, but detected both the 43 kDa protein and a minor band at 44 kDa region in treated sample (1 a). We conclude that heat shock involves the rat 43 kDa MAPK, Erk1, which increases in molecular weight migrating in the region of the 44 kDa band.

MAP kinase activity. We estimated the MAPK activity as the phosphotransferase capacity of crude cytosolic extracts towards a specific substrate, namely a synthetic nonapeptide containing aminoacids 95-98 of bovine MBP sequence, which includes the phosphorylation site (Thr 97) for MAPK (19). MAPK activity was detectable in control animals and showed a significant increase after heat shock. In five experiments 30 ± 4, 62 ± 10 and 75 ± 10 pmoles of ^{32}P were incorporated into the peptide/mg protein/10 min by control, 30 and 50 min heat shocked liver.

To validate these results, MAPK activity was studied after immunoprecipitation of the enzyme. By this procedure the effect of heat shock proved greater than seen in the kinase assay in crude

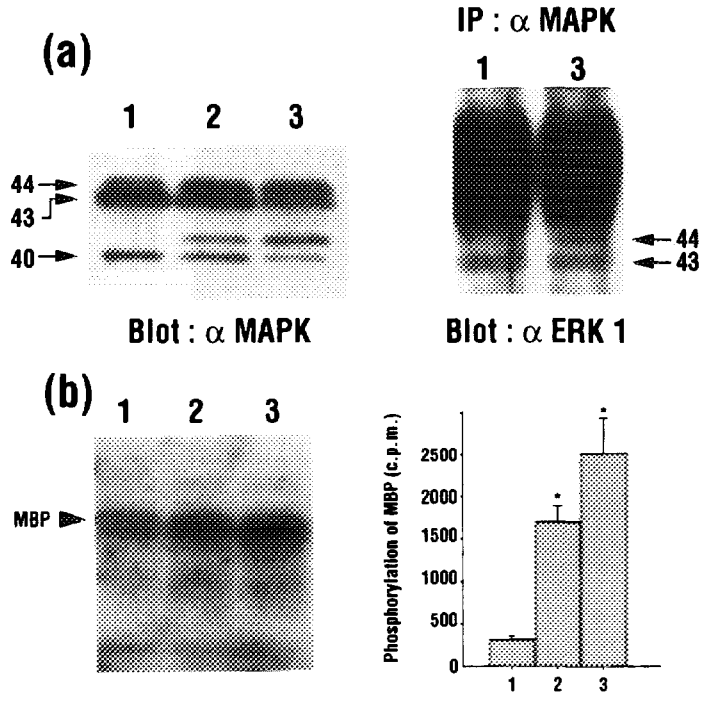

Fig. 1. Effect of heat shock on MAP kinases.

(a) on the left, 80 μg of cytosolic proteins were analysed by immunoblot with anti-MAPK antibodies (α MAPK); on the right, immunprecipitates obtained with αMAPK were analysed by immunoblot with anti-Erk1 antibody (α Erk1). Immunoreactive bands were detected by anti rabbit IgG HRP conjugated and the ECL method. The arrows indicate 40, 43, 44 kDa proteins.

(b) on the left, autoradiography of SDS-PAGE of 20 μl of MAPK immunocomplex kinase assay; on the right: cpm of ^{32}P incorporated into MBP (means ± S.E.M. of four separate experiments. * Significantly different from control, $p<0.01$) calculated as described in Materials and Methods section.

Similar results were obtained in four separate experiments. 1: control rats; 2,3: rats heat stressed for 30 and 50 min.

extracts: the ^{32}P incorporated into MBP was 5 and 8 times higher than the control after 30 and 50 min of hyperthermic treatment respectively (Fig. 1 b).

Effect of heat shock treatment on Raf. At first we examined the effect of hyperthermia on electrophoretic mobility of the enzyme by immunoblotting experiments (Fig. 2 a). In total liver extracts from control animals a polyclonal antibody anti-Raf-1 (C-12) detected a 74 kDa protein as reported for c-Raf-1 (20). 30 min of heat shock resulted in a decreased electrophoretic mobility of Raf-1 which was more evident at 50 min. of treatment, suggesting an increase in the phosphorylation of Raf-1. To measure Raf-1 kinase activity, total liver proteins from control and heat shocked rats were subjected to immunoprecipitation with anti c-Raf-1 antibodies followed by kinase activity toward histone H1 (15) (Fig. 1b). Evaluating the histone H1 phosphorylation by counting the protein excised from the gels, we found that heat shock significantly increased the liver Raf-1 activity, which after 30 and 50 minutes of hyperthermia was approximately twice

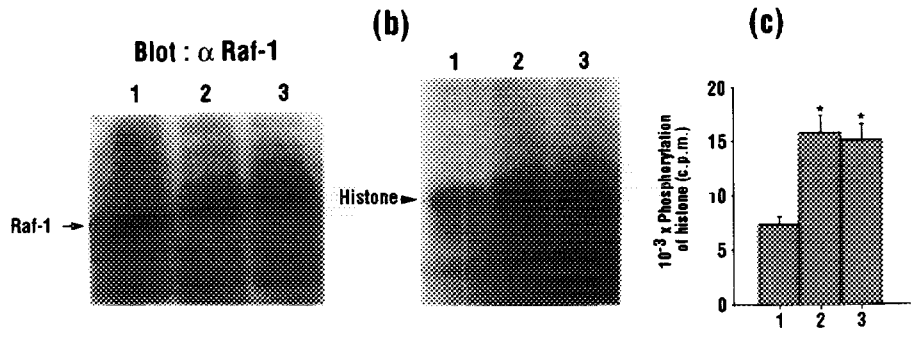

Fig. 2. Effect of heat shock on Raf-1 kinase.

(a) immunoblot analysis with anti Raf-1 antibodies (α Raf-1) of liver extract proteins. Immunoreactive proteins were detected by anti rabbit IgG HRP-conjugated and the ECL method.

(b) autoradiography of SDS-PAGE of 20 µl of Raf-1 immunocomplex kinase assay.

(c) cpm of ^{32}P incorporated into histone excised from gel (means ± SEM of three separate experiments) * Significantly different from control ($P<0.01$).

Similar results were obtained in three separate experiments. 1: control rats, 2,3: rats heat stressed for 30 and 50 minutes.

the controls (Fig. 2 c). Since Raf-1 kinase activity may depend on tyrosine phosphorylation (15,21,22), we examined the phosphotyrosine content of the Raf-1 immunoprecipitates. Phosphotyrosine signal was never detectable in the region of the 74 kDa protein (not shown).

Shc proteins involvement. Considering that Raf kinase represents an effector of Ras (23), which in turn may be activated as a consequence of tyrosine phosphorylation of Shc proteins, we examined the tyrosine content of liver Shc proteins after heat shock, in the light of the reported induction of tyrosine kinase activity by thermal stress (24). Shc proteins are good substrates for receptor (25) and non receptor tyrosine kinases (16) and phosphotyrosine residues of Shc act as recognition sites for binding of Grb2-Sos complex (26). These interactions lead to Ras activation by triggering the GDP-GTP exchange reaction (27).

Liver lysates from control, 30 min and 50 min heat shocked rats were immunoprecipitated with a polyclonal antibody against Shc proteins and the immunoprecipitates were analysed by Western blotting (Fig. 3). Anti Shc antibody detected the 66, 52 and 46 kDa isoforms of Shc proteins in control rat livers and heat stress did not modify their amount (Fig. 3 a). The same blots, after stripping of anti Shc antibody, were probed with antiphosphotyrosine antibodies (Fig. 3 b). Tyrosine phosphorylation never involved the 66 and 46 kDa isoforms of Shc proteins. In control animals the 52 kDa Shc protein showed a basal level of phosphotyrosine content which doubled after 30 and 50 min of thermal stress, as estimated by densitometric analysis. Finally, to study if heat shock leads to the association of the Shc proteins with Grb2, essential for Ras activation, the Shc immunoprecipitates were immunoblotted with anti Grb2 antibody (Fig. 3 c). Shc-Grb2 association occurred in control animals, but, after 30 and 50 minutes of heat shock, the amount of Grb2 immunoprecipitated with Shc proteins was twice the normal values.

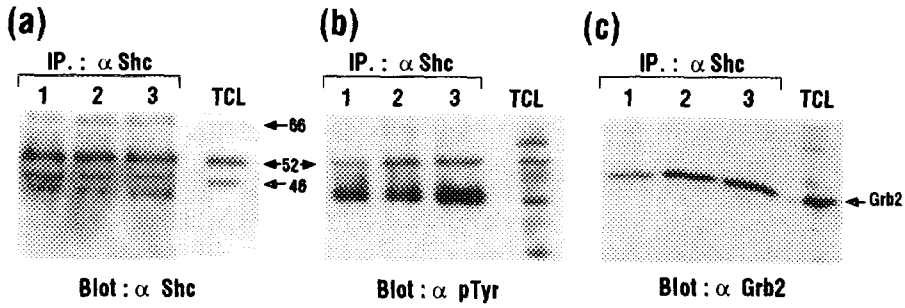

Fig. 3. Effect of heat shock treatment on Shc tyrosine phosphorylation and on coimmunoprecipitation of Grb2

Immunoprecipitates obtained with anti-Shc antibody (α Shc) were analysed by Immunoblot with (a), α Shc, (b), anti phosphotyrosine antibodies (α pTyr), (c), anti Grb2 antibodies (α Grb2). Immunoreactive proteins were detected by antispecies secondary antibodies HRP conjugated and the ECL method. The arrows indicate 46, 52 and 66 kDa isoforms of Shc proteins. The protein present under 46 kDa Shc proteins aspecifically interacts with protein A Sepharose bead. Comparable results were obtained in three separate experiments. Lane 1: control rats, lanes 2,3 rats heat stressed for 30 and 50 min, TCL: total cellular lysate.

DISCUSSION

In this report we provide the first demonstration that heat shock stimulates the MAPK activity in living animal involving the 40 kDa and 43 kDa isoforms. The effect is already evident after 30 minutes of hyperthermia and increases with time.

Some events, instrumental in the mounting of heat shock response, could be conceived as dependent on increased MAPK activity. The transactivating protein that starts the transcription of heat shock genes, HSF, undergoes phosphorylation that is necessary for the attainment of maximal transcriptional activity (28). Looking at the aminoacid sequence of cloned human HSF1 (29) we noted, in the transactivation C-terminal region of the molecule, two optimal phosphorylation consensus sites for MAPKs, namely the extended consensus sequence Pro-X-Ser/Thr-Pro (30). A second function of MAPK in the regulation of hsp gene transcription could be the release of paused RNA polymerase II, which is arrested in the promoter of hsp genes, shortly after initiation of transcription. The C-terminal domain of the largest subunit of the eukaryotic RNA polymerase II is a good substrate for MAPK (30) and the heat shock induces its phosphorylation in HELA cells (31).

Two other well identified substrates of MAPK are the cytoplasmic PLA_2 and the transcription factor Elk-1. Phosphorylation by MAPKs stimulates the activity of PLA_2 and the release of free arachidonic acid (32): the latter events, accompanied by increased prostaglandin synthesis, have been observed in different mammalian cells subjected to heat shock (5,6). Phosphorylation of TCF/Elk-1 by MAPK increases the transcription of c-fos (33), after both in vivo (9) and in vitro heat shock (34).

All these facts suggest that the MAPK activity could be involved in different aspects of the stress response.

A second point relevant to our results is the mechanism of MAPK stimulation upon heat shock. The MAPK is activated by the specific threonine-tyrosine directed kinase, MAPKK, which in turn may be phosphorylated and activated by Raf-1 (10,11). Here we present evidence that heat shock causes an increase in Raf-1 kinase activity which is not mediated by direct Raf-1 tyrosine phosphorylation. On the contrary we have identified that a suitable target for heat shock-induced protein tyrosine kinase activity is a member of the family of Shc proteins. We found an increase in tyrosine phosphorylation of the p52 Shc protein which is accompanied by an increase in the amount of coimmunoprecipitated Grb2: this association has been demonstrated to lead to an increase of the GTP loading onto Ras (26,27), through the activity of the nucleotide exchange factor Son of sevenless, SOS, costitutively associated with Grb2 (27).

Taken together, our results suggest that the Ras-Raf-MAPK pathway is activated in rat liver during heat shock in vivo, similar to what happens during the cellular stress induced by ultraviolet irradiation which triggers the Ras-Raf-MAPK pathway by Src tyrosine kinase activation (35). The similarity could be explained by a state of oxidative stress that occurs under both circumstances (35-37), however the MAPK activation observed in response to treatment of the cells with oxidants does not involve the Shc proteins (38).

It has been demonstrated that in vivo hyperthermia causes the increase of circulating IL-1 (39,40), which, as many other cytokines (41,42), shares a signalling pathway involving the Ras cascade. Therefore we hypothesise that the activation of MAPKs in rat liver by thermal stress is a secondary effect of heat shock, which may be mediated by an autocrine or paracrine or endocrine mechanism. Studies are in progress to obtain findings about this hypothesis.

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