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Hyperthermia-induced Hsp70 and MT20 transcriptional upregulation are mediated by p38-MAPK and JNKs in *Mytilus galloprovincialis* (Lamarck); a pro-survival response

E. Gourgou, I.-K. Aggeli, I. Beis and C. Gaitanaki*

Department of Animal and Human Physiology, School of Biology, University of Athens, Panepistimioupolis, Athens, 157 84, Greece

*Author for correspondence (cgaitan@biol.uoa.gr)

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SUMMARY

In the present study we investigated the signal transduction cascades triggered by acute thermal stress in *Mytilus galloprovincialis* gills. This particular species has been reported to exhibit a significant tolerance to high temperatures; thus, it was intriguing to examine the molecular mechanisms responsible for this extraordinary trait. In particular, exposure to 30°C was found to cause a significant and sustained stimulation of p38-MAPK phosphorylation while the activation profile of JNKs was transient and relatively moderate. We also observed that hyperthermia induced apoptosis as a delayed response, with both MAPK subfamilies rapidly translocating to the nucleus. The phosphorylation of cJun, ATF2 and NFκB was detected next. Using selective inhibitors, phosphorylation of these transcription factors was established to be dependent on p38-MAPK or JNKs. Subsequently, potential changes in gene expression were assessed. In this context, hyperthermia resulted in the transcriptional upregulation of Hsp70 and MT20 genes with a widely known salutary effect, preserving mussel fitness and performance under adverse environmental conditions. Interestingly, p38-MAPK and JNKs were found to mediate the hyperthermia-induced Hsp70 and MT20 upregulation as well as the delayed induction of apoptosis under the interventions studied. Overall this is, to our knowledge, the first time that an insight into the compensatory survival 'programme' initiated in *Mytilus galloprovincialis* gills, contributing to this organism's exceptional tolerance to thermal stress, has been gained. In particular, we provide evidence demonstrating the principal role of p38-MAPK and JNKs in transducing the stress signal *via* mobilization of specific transcription factors and the transcriptional upregulation of cytoprotective genes.

Key words: mussel, thermal stress, signalling pathways.

INTRODUCTION

Organisms respond to changes in their surroundings *via* a series of physiological adaptations. Successful counteraction of the effects of any acute or chronic change in a plethora of parameters determines an organism's survival and biogeographic patterning (Hochachka and Somero, 2002). Ectotherms, especially, apply multiple compensating strategies to ensure homeostasis when facing fluctuations in environmental temperature. Intertidal marine invertebrates in particular, such as mussels, have to cope periodically with perturbations in temperature, desiccation and humidity, with thermal stress constituting one of the most crucial determinants of species latitudinal and vertical distribution, abundance and fitness (Helmuth and Hofmann, 2001). Thus, investigating and clarifying the signal transduction mechanisms responsible for 'sensing' and adjusting to this stressful stimulus is of outstanding interest and importance.

Among *Mytilus* blue mussel congeners, *M. galloprovincialis* (Lamarck 1819) has been reported to be extremely tolerant to high temperatures because it originated in the Mediterranean (Seed, 1992). Several studies have highlighted the key role of behavioural or metabolic adjustments as well as gene expression plasticity strategies, with various mediators contributing to the remarkable stress tolerance of this species. Among the cytoprotective proteins implicated are the heat shock proteins (Hsps) (Snyder et al., 2001; Hamer et al., 2008). Hsps constitute a family of highly conserved proteins which act as chaperones ensuring stabilization and proper folding of newly synthesized proteins at the ribosome and preventing protein aggregation by promoting refolding of denatured proteins (Fink, 1999). One of the numerous classes of Hsps (named according to their apparent molecular mass) identified so far is Hsp70 with its members either constitutively expressed (Hsc70) or existing as stress-inducible forms (Hsp70) (Hartl and Hayer-Hartl, 2002). Hsp70 transcriptional upregulation is elicited primarily by heat (Scharf et al., 1998) but also by other stimuli including osmotic (Tirard et al., 1997) and oxidative stress (Mager et al., 2000) as well as metal ion concentration (Ryan and Hightower, 1994). Another family of proteins known to be induced by adverse environmental conditions (i.e. exposure to increasing metal concentrations and thermal or osmotic stress) is that of metallothioneins (MTs) (Viarengo et al., 1999; Piano et al., 2004; Hamer et al., 2008). MTs are low molecular mass soluble polypeptides with high affinity for IB and IIB metal ions, shown to play a critical role in heavy metal homeostasis as well as oxyradical scavenging (Viarengo et al., 1999; Dondero et al., 2005). They have been identified in organisms as diverse as bacteria and mammals, with two major groups characterized in Mytilidae: MT10 and MT20 (named according to their apparent molecular mass) (Vergani et al., 2007). Given the fundamental role of the molecular mechanisms preserving performance levels in mussels exposed to stressful stimuli, one can deduce that defining the effectors as well as the signalling cascades involved in Hsp70 and MT regulation constitutes a major challenge.

A plethora of studies demonstrate that within the signalling pathways triggered by stress stimuli, mitogen-activated protein kinases (MAPKs) play a central role (reviewed in Kyriakis and Avruch, 1996). MAPKs are highly conserved serine/threonine protein kinases that become activated *via* dual phosphorylation by

MAPK kinases (MKKs). The three best characterized MAPK subfamilies are the extracellular signal regulated kinases (ERKs), cJun N-terminal kinases (JNKs) and p38-MAPK (Bogoyevitch, 2000). Upon activation, MAPKs have been reported to partially translocate to the nucleus where they interact with their target substrates including several transcription factors (Kyriakis and Avruch, 1996; Bogoyevitch, 2000). Thus, MAPKs may regulate gene expression at the transcriptional level, through phosphorylation of members of the cJun and ATF family, NFκB and Elk, favouring the initiation of survival or apoptotic mechanisms (Bogoyevitch, 2000). A classical hallmark of apoptosis is the proteolytic processing of poly(ADP-ribose) polymerase (PARP); this family of enzymes demonstrating poly(ADP-ribosyl)ation activity participate in diverse biological functions including DNA repair and genomic stability (Nagata and Golstein, 1995).

In previous papers, our group has focused on assaying the p38-MAPK phosphorylation profile in samples from mantle tissue of *M. galloprovincialis* specimens exposed to various stressful conditions including anoxia, thermal and oxidative stress, and heavy metals (Gaitanaki et al., 2004; Kefaloyianni et al., 2005). Taking these findings into consideration, we decided to attempt to delineate the signalling pathways activated during acute hyperthermia in the gills of *M. galloprovincialis* specimens. Functional integrity of gills is of great importance as mussels are dependent on this organelle to achieve food intake (by filtering seawater) and the required oxygen supply (Morton, 1983; Wilmer et al., 2005).

Several researchers have so far commented on seasonal or acclimation effects on the thermal stress-induced response or have marked the effects of chronic exposure to thermal stress (Buckley et al., 2001; Anestis et al., 2007; Anestis et al., 2008). Nevertheless, data concerning the molecular responses triggered in these mussels during the daily acute changes that they may endure in their micro-environments are scarce. Therefore, clarification of the molecular mechanisms leading to survival or death of these organisms would be intriguing. In this context, we initially assessed phosphorylation of p38-MAPK and JNKs, and thus activation, by hyperthermia. The rapid activation of these kinases was followed by phosphorylation of their nuclear targets: cJun, ATF2 and NFκB. Given the delayed proteolytic processing of PARP during hyperthermia, indicative of apoptosis, of particular note was the rapid transcriptional upregulation of Hsp70 and MT20 that we observed, as these have been established having a cytoprotective beneficial role. We subsequently used pharmacological inhibitors to determine the potential interactions and cross-talk between all these mediators. To our knowledge, this is the first time that hyperthermia-stimulated phosphorylation of cJun, ATF2 and NFκB as well as fragmentation of PARP have been reported in Mytilidae. The contribution of p38-MAPK and JNKs to the delayed occurrence of apoptosis and the preservation of cell homeostasis was also shown.

MATERIALS AND METHODS

Materials

All chemicals used were purchased from Sigma Chemical Co. (St Louis, MO, USA) and were of the highest grade available. The enhanced chemiluminescense (ECL) kit was from Amersham International (Uppsala, Sweden); the Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA); nitrocellulose (0.45 μm) was from Schleicher & Schuell (Keene, NH, USA). The selective inhibitors SB203580 (#559389) and SP600125 (#4201119) were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Antibodies specific for the phosphorylated forms of p38-MAPK (#9211), JNKs (#9251), p65 NFκB (#3037), cJun (#9261) and ATF2 (#9221) as well as for total protein levels of PARP (#9542) were obtained from Cell Signaling Technology (Beverly, MA, USA). The anti-actin was from Sigma-Aldrich (St Louis, MO, USA) while anti-histone H1 was from Neomarkers (Fremont, CA, USA). HRP-conjugated anti-rabbit (#P0448) and anti-mouse (#P0447) antibodies were from DAKO A/S (DK-2600, Glostrup, Denmark). Prestained molecular mass markers were obtained from New England Biolabs (Beverly, MA, USA). XO-MAT AR film (13 cm×18 cm) was from Eastman Kodak Company (New York, NY, USA). TRIzol® reagent (#15596-026) and Reverse Transcriptase M-MLV (#28025-013), as well as primers for the detection of Hsp70, metallothionein 10 and 20, and β-actin were from Invitrogen Life Technologies (Carlsbad, CA, USA). Taq polymerase (#101025) was from Bioron GmbH (Ludwigshafen, Germany).

Animals

Adult mussels (65–75 mm) of *M. galloprovincialis* (Lamarck) were obtained from a mussel culture farm in the area of Megalo Pefko in the Saronikos gulf, south of Athens, Greece. Animals were collected during spring (from February to March) when the average seawater temperature is approximately 16–17°C and were held in appropriate laboratory aquaria with aerated recirculating natural seawater at 15°C (salinity ~35‰) for at least a week prior to use.

Animal treatments

The untreated (control) animals were kept at 15°C, in agreement with water temperature in the culture farm and other research studies reporting that the average temperature of surface waters in the collection area is in the range 15–17°C. As for thermal stress, during the low tide in summer, mussels come across temperature conditions that may be unusual but are not at all unlikely. Thus, in order to examine the effect of hyperthermia on the various signal transduction mechanisms triggered by these interventions, mussels (N=4–6 for each group) were transferred into aquaria with natural seawater at a temperature of 30°C (acute hyperthermia) for increasing time intervals (30 min to 8 h). In other experiments, mussels were exposed to hyperthermia for the times indicated in the presence of the selective p38-MAPK inhibitor SB203580 (1 μmol l^{-1}) or the selective JNKs inhibitor SP600125 (1 μmol l^{-1}). Both pharmacological inhibitors used were diluted in DMSO. Control experiments with DMSO or the inhibitors alone were also performed (see figures).

Each experiment was performed at least 4 times. At the end of each experiment, gill tissue was carefully dissected, freeze-clamped between aluminium tongs, cooled in liquid nitrogen and pulverized under liquid nitrogen. Tissue powder was stored at –80°C.

Preparation of whole extracts

Gill tissue powder was homogenized with 3 ml g^{-1} of buffer A [20 mmol l^{-1} Hepes, pH 7.5, 20 mmol l^{-1} β-glycerophosphate, 20 mmol l^{-1} NaF, 2 mmol l^{-1} EDTA, 0.2 mmol l^{-1} Na_3VO_4, 5 mmol l^{-1} dithiothreitol (DTT), 10 mmol l^{-1} benzamidine, 200 μmol l^{-1} leupeptin, 120 μmol l^{-1} pepstatin A, 10 μmol l^{-1} trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 300 μmol l^{-1} phenyl methyl sulfonyl fluoride (PMSF), 0.5% (v/v) Triton X-100] and extracted on ice for 30 min. The samples were centrifuged (10,000 ***g***, 5 min, 4°C) and the supernatants were boiled with 0.33 volumes of SDS-PAGE sample buffer [0.33 mol l^{-1} Tris/HCl, pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) Bromophenol blue]. Protein concentrations were determined using the Bio-Rad Bradford assay.

Subcellular fractionation: preparation of nuclear extracts

Gill tissue powder was homogenized in 3 ml g^{-1} of buffer B [10 mmol l^{-1} Hepes pH 7.9, 10 mmol l^{-1} KCl, 0.1 mmol l^{-1} EGTA, 0.1 mmol l^{-1} EDTA, 1.5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} NaF, 1 mmol l^{-1} Na_3VO_4, 0.5 mmol l^{-1} PMSF, 20 mmol l^{-1} β-glycerophosphate, 4 μg ml^{-1} aprotinin and 2 μg ml^{-1} leupeptin]. After incubation on ice for 15 min, homogenates were centrifuged (5000 ***g***, 10 min, 4°C) and 10% (v/v) Nonidet P40 was added to the supernatants collected. Subsequently, samples were vigorously mixed and further incubated on ice for 10 min. Homogenates were then centrifuged (1000 ***g***, 10 min, 4°C) to obtain pellets. Pellets were washed once and re-suspended in a buffer containing: 20 mmol l^{-1} Hepes pH 7.9, 400 mmol l^{-1} NaCl, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA, 1.5 mmol l^{-1} $MgCl_2$, 20% (v/v) glycerol, 10 mmol l^{-1} NaF, 1 mmol l^{-1} Na_3VO_4, 0.5 mmol l^{-1} PMSF, 0.2 mmol l^{-1} DTT, 20 mmol l^{-1} β-glycerophosphate, 4 μg ml^{-1} aprotinin and 2 μg ml^{-1} leupeptin. After incubation on ice for 60 min and centrifugation (15,000 ***g***, 10 min, 4°C), the supernatants containing nuclear protein were boiled with 0.33 volumes of SDS/PAGE sample buffer. Protein concentrations were once more determined using the Bio-Rad Bradford assay.

SDS-PAGE and immunoblot analysis

Proteins were separated by SDS-PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Membranes were then incubated in TBS-T (20 mmol l^{-1} Tris-HCl, pH 7.5, 137 mmol l^{-1} NaCl, 0.05% (v/v) Tween 20] containing 5% (w/v) non-fat milk powder for 30 min at room temperature. Subsequently, membranes were incubated overnight with the appropriate antibody, according to the manufacturer's instructions. After being washed in TBS-T (3×10 min), blots were incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG antibodies [1:5000 dilution in TBS-T containing 1% (w/v) non-fat milk powder, 1 h at room temperature]. Blots were again washed in TBS-T (3×10 min), and bands were detected using enhanced chemiluminescence with exposure to XO-MAT AR film and were quantified by laser scanning densitometry (Gel Analyzer v.1.0). Equal protein loading was verified by probing identical samples with an anti-actin (whole extracts) or an anti-histone H1 (nuclear extracts) antibody.

RNA extraction, cDNA synthesis and ratiometric reverse transcription PCR (RT-PCR)

Expression of endogenous Hsp70, MT10 and MT20 was determined by ratiometric reverse transcription of total RNA followed by PCR analysis. Total RNA was extracted from gill tissue using Trizol, according to the manufacturer's instructions. RNA concentration and quality were assessed by UV spectroscopy (calculating the absorbance ratio 260 nm/280 nm) and electrophoresis using a 1.2% (w/v) agarose gel. For cDNA synthesis, 1 μg of total RNA was denatured in the presence of 5 pmol oligo-dT primer in a reaction volume of 13.5 μl at 65°C for 5 min. Reverse transcription was performed with M-MLV Reverse Transcriptase, first strand buffer (Promega, Madison, WI, USA), DTT (Promega) and deoxynucleotide triphosphates (dNTPs; Promega). The first strand reaction was incubated at 37°C for 1 h. Termination of the reaction was achieved by inactivation of the reverse transcriptase at 65°C for 5 min. The PCR reaction was performed in a 25 μl final volume mixture, containing 2 μl cDNA template, 1.5 μmol l^{-1} $MgCl_2$, 0.5 μl dNTPs, 1.5 U of Taq DNA polymerase and the appropriate pair of primers. The primers used are presented in Table 1. All primers were based on the respective sequences (accession numbers in GenBank).

Table 1. RT-PCR primer sequences

Gene	Accession no.	Primer sequences (5′–3′)	Amplicon size (bp)
Hsp70	AF172607	F: TTGACTTGGGTGGTGGAACT R: GGCTACAGCTTCATCAGGG	517
MT10	AY566248	F: TATCAAGATCACTGTGACTACTA R: GAATAAGTTCGGTATATGTCTT	285
MT20	AY566247	F: TTAAGCAGACCAATTCAAATA R: CACGTTAACGTTGTGGCTG	272
β-Actin	AF157491	F: GCACTTCCTCACGCTATCGTC R: TGTCCATCTGGCAGTTCGTAGC	217

F, forward primer; R, reverse primer.

After a 10 s denaturation at 94°C, PCR was carried out as follows: 94°C for 30 s, 59°C for 30 s and 72°C for 30 s (30 cycles) for Hsp70, and 94°C for 30 s, 65°C for 30 s and 72°C for 30 s (33 cycles) for β-actin, with a final extension at 72°C for 4 min. For MTs, initial denaturation at 94°C lasted 4 min and PCR was carried out as follows: 94°C for 15 s, 60°C for 15 s and 68°C for 2 min (27 cycles) for MT10 and 94°C for 15 s, 60°C for 15 s and 68°C for 2 min (36 cycles) for MT20. cDNA samples derived from gill tissue of 'control' (untreated) and treated animals were always amplified simultaneously. Equal volume aliquots from each PCR product were separated on a 2% (w/v) agarose gel and stained with ethidium bromide (0.1 mg l^{-1}). PCR product band intensity (Hsp70 517 bp, MT10 285 bp, MT20 272 bp, β-actin 217 bp) was determined using Gel Analyzer (v. 1.0) and all values were normalized for the amount of β-actin mRNA.

Statistical evaluations

Western blot and PCR images shown are representative of at least four independent experiments. Each data point represents the mean ± s.e. of samples from at least six separate specimens, treated under the respective conditions, in at least four independent experiments. One-way analysis of variance (ANOVA) was used to evaluate the effect of high temperature over time, and a statistical difference was accepted when $P<0.05$. Appropriate *post-hoc* tests (Bonferroni) were performed for group comparisons.

RESULTS

Effect of hyperthermia on phosphorylation levels of p38-MAPK and JNKs

The survival of marine intertidal invertebrates largely depends on their ability to counteract the acute changes in their physical surroundings. Therefore, the effect of acute hyperthermia on the survival of *M. galloprovincialis* was initially assessed by exposing mussels to hyperthermia (30°C) for increasing time intervals varying from 8 h to 1 week. Our results (data not shown) revealed that 95±3.6% of the animals exposed to high temperature died within 24 h, as estimated by failure to close their shells in response to external stimuli. These findings prompted us to investigate the short time profile of the effect of hyperthermic stress. The time points chosen in the present study are also in line with the results reported by our group in previous studies, using this particular experimental model (Kefaloyianni et al., 2005). All samples assayed came from mussel gill tissue as gills have been documented to be thermally labile (Hofmann and Somero, 1996).

Given the established activation of members of the MAPK superfamily in mussels, as well as in other experimental models, by multiple forms of environmental stress (Bogoyevitch, 2000; Kefaloyianni et al., 2005; Gaitanaki et al., 2008), phosphorylation

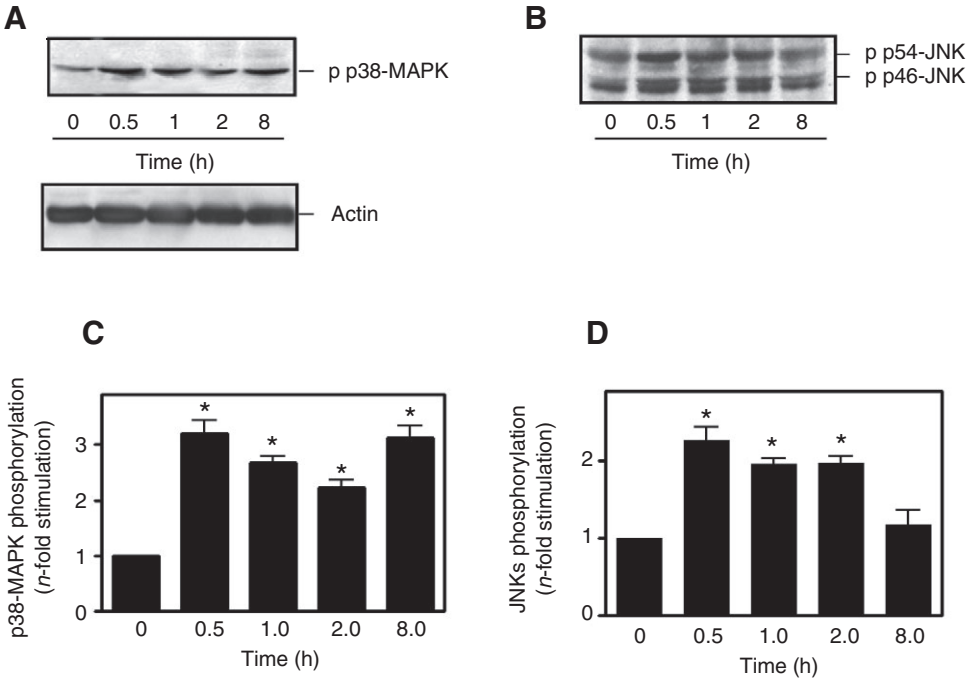

Fig. 1. Time-dependent phosphorylation profile of p38-MAPK and JNKs in samples from gills of *Mytilus galloprovincialis* specimens subjected to hyperthermia (30°C). Tissue extracts (50 μg) from control animals and animals exposed to hyperthermia (30°C) for the times indicated were subjected to SDS-PAGE and immunoblotted with antibodies for phosphorylated p38-MAPK (A, top panel) and phosphorylated JNKs (B). Equal protein loading was confirmed by blotting identical samples with an anti-actin specific antibody (A, bottom panel). Bands were quantified by laser scanning densitometry (C, phospho-p38-MAPK and D, phospho-JNKs). Blots shown are representative of four independent experiments and results are means ± s.e. for four independent experiments. **P*<0.01 compared with control animals (kept at 15°C).

levels of p38-MAPK and JNKs, and thus activation levels, were initially monitored in gill tissue samples. As illustrated in Fig. 1, p38-MAPK phosphorylation levels induced by exposure of mussels to 30°C were maximal within 30 min (3.2±0.23-fold relative to control animals) and remained elevated for at least 8 h (3.11±0.22-fold relative to control animals) (Fig. 1A, top panel, C). JNKs exhibited a relatively moderate but similar time profile, with their phosphorylation levels remaining statistically significant for at least 2 h (1.97±0.1-fold relative to control animals), having attained maximal values within 30 min (2.27±0.18-fold relative to control animals) (Fig. 1B,D). Equal protein loading was verified by immunoblotting identical samples with a specific anti-actin antibody (Fig. 1A, bottom panel).

Hyperthermia stimulates nuclear translocation of p38-MAPK and JNKs and delayed apoptosis as evidenced by PARP fragmentation

MAPKs have previously been reported to partially translocate to the nucleus upon activation, where they interact with their substrates and exert their actions (Widmann et al., 1999). Therefore, we decided to examine the subcellular distribution pattern of phosphorylated p38-MAPK and JNKs by assaying their presence in the nuclear compartment, indicative of the potential interactions of these kinases under the interventions studied. Interestingly, enhanced immunoreactivity for both kinase subfamilies was observed in nuclear extracts from gill tissue samples of mussels exposed to 30°C for 30 min, indicating the rapid partial translocation of p38-MAPK

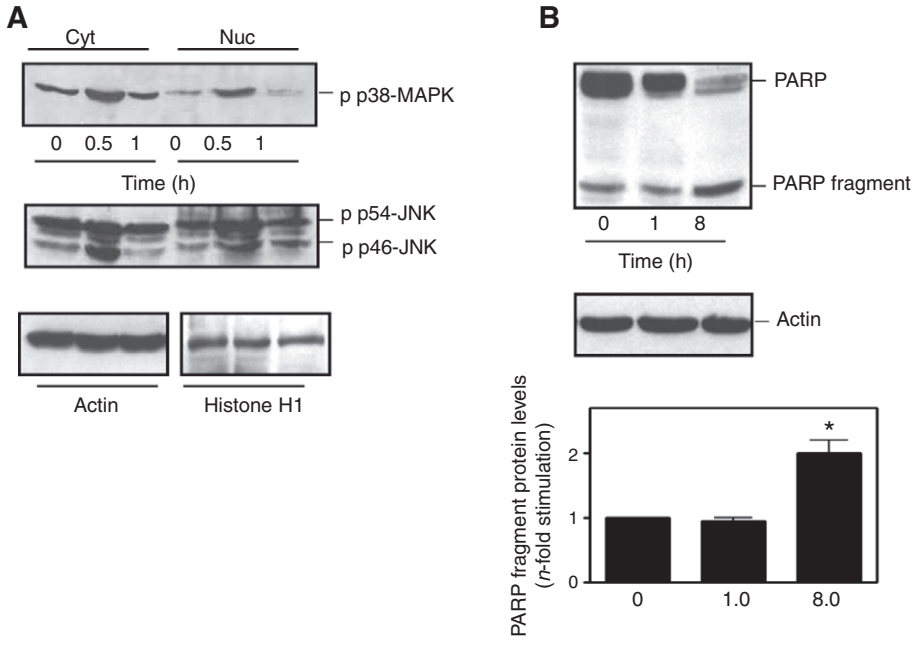

Fig. 2. Hyperthermia-induced subcellular distribution of phosphorylated p38-MAPK and JNKs; profile of PARP cleavage. (A) Cytoplasmic (Cyt) or nuclear (Nuc) fractions of tissue extracts (30 μg) from control animals and animals exposed to hyperthermia (30°C) for the times indicated were subjected to SDS-PAGE and immunoblotted with antibodies for phosphorylated p38-MAPK (top panel) and phosphorylated JNKs (middle panel). Equal protein loading was confirmed by blotting identical samples with antibodies against actin for the cytoplasmic fractions (bottom left panel) and histone H1 for the nuclear fractions (bottom right panel). (B) Proteolytic processing of PARP was assessed by immunoblotting samples of tissue extracts (50 μg) from control animals and animals exposed to hyperthermia (30°C) for the times indicated (top panel) and quantification of PARP fragment was performed by laser scanning densitometry (graph). The membrane was re-probed with an anti-actin antibody so as to verify equal protein loading (bottom panel). Blots and results shown are representative of at least four independent experiments. Results are means ± s.e. for at least four independent experiments. **P*<0.01 compared with control animals (kept at 15°C).

as well as JNKs to the nucleus (Fig. 2A, top and middle panel, respectively). Purity and equal protein loading of both cytoplasmic and nuclear fractions were confirmed by immunoblotting identical samples against actin and the nuclear marker protein histone H1, respectively (Fig. 2A, bottom panel).

In order to evaluate the biological impact of acute hyperthermia, PARP proteolysis was examined, a marker routinely used to monitor apoptotic cell death (Strasser et al., 2000). Thus, 8 h exposure to hyperthermia was observed to markedly increase the intensity of the generated PARP fragment (Fig. 2B, top panel and graph). No PARP fragmentation was detected at earlier time points. Equal protein loading was verified by reprobing the membrane with a specific anti-actin antibody (Fig. 2B, bottom panel).

Hyperthermia stimulates phosphorylation of several transcription factors; involvement of p38-MAPK and JNKs

Given a number of transcription factors have been demonstrated to constitute downstream nuclear substrate targets of MAPKs (Kyriakis and Avruch, 1996), we next decided to assess the phosphorylation levels of three transcription factors which constitute well known MAPK targets: cJun, ATF2 and NFκB (Gupta et al., 1995; Bogoyevitch, 2000; Buschmann et al., 2000). Notably, all three factors were found to be phosphorylated in nuclear extracts from gill tissue of animals exposed to hyperthermia. In particular, cJun and ATF2 phosphorylation levels were maximal within 30 min (3.56±0.23-fold and 2.67±0.17-fold, relative to control animals, respectively), while NFκB exhibited a more delayed transactivation profile with maximal phosphorylation values attained 60 min after the onset of the stimulation (4.77±0.14-fold, relative to control animals) (Fig. 3A–C). Equal protein loading was verified by immunoblotting identical samples with a specific anti-histone H1 antibody (Fig. 3D).

Given the complete lack of data surrounding the mechanisms transducing the hyperthermic signal in mussels, we next tried to decipher this by using selective pharmacological inhibitors of p38-MAPK (SB203580 1 μmol l^{-1}) and JNKs (SP600125 1 μmol l^{-1}), as these kinases were found to be activated under the conditions studied (Fig. 1). The efficiency of the selective inhibitors was tested and verified as shown in Fig. 4. In particular, SB203580 was confirmed to ablate hyperthermia-induced p38-MAPK phosphorylation (Fig. 4A,C) while SP600125 was found to abolish hyperthermia-induced cJun phosphorylation (Fig. 4B,D). Equal protein loading of the samples was confirmed by immunoblotting identical samples against actin and histone H1, respectively (Fig. 4A,B, bottom panels). Subsequently, we observed that the hyperthermia-induced phosphorylation of ATF2 was JNK dependent as it was abolished in the presence of SP600125 (Fig. 5A), while JNKs were not found to mediate NFκB transactivation (Fig. 5B). In order to evaluate any secondary effects of the solvent used (DMSO) to dilute the aforementioned inhibitors, the effect of DMSO on the phosphorylation levels of p38-MAPK, cJun, ATF2 and NFκB was also assessed and found to be null (Fig. 5C). Equal protein loading of the samples was once again confirmed by immunoblotting identical samples against actin and histone H1 (Fig. 5C). Hyperthermia-induced cJun phosphorylation levels were found to be unaffected by SB203580 (Fig. 6A). On the other hand, both ATF2 phosphorylation and NFκB transactivation were abolished, verifying their dependence on the p38-MAPK pathway (Fig. 6B,C). Equal protein loading was once more confirmed by immunoblotting identical samples with a specific anti-histone H1 antibody (Fig. 6D).

Effect of hyperthermia on Hsp70, MT10 and MT20 mRNA levels; involvement of p38-MAPK and JNK signalling pathways

In view of the delayed occurrence of apoptosis, as evidenced by the aforementioned PARP fragmentation shown in Fig. 2B and the fact that mussels of the *M. galloprovincialis* species are known to endure quite harsh environmental conditions (Seed, 1992), an effort was made to look into the mechanisms involved in the extraordinary tolerance they exhibit. To this end, we decided to examine the possible hyperthermia-induced transcriptional upregulation of Hsp70 and of two metallothioneins (MT10 and MT20), which have previously been reported to exert a cytoprotective function in invertebrates exposed to adverse conditions (Hamer et al., 2008).

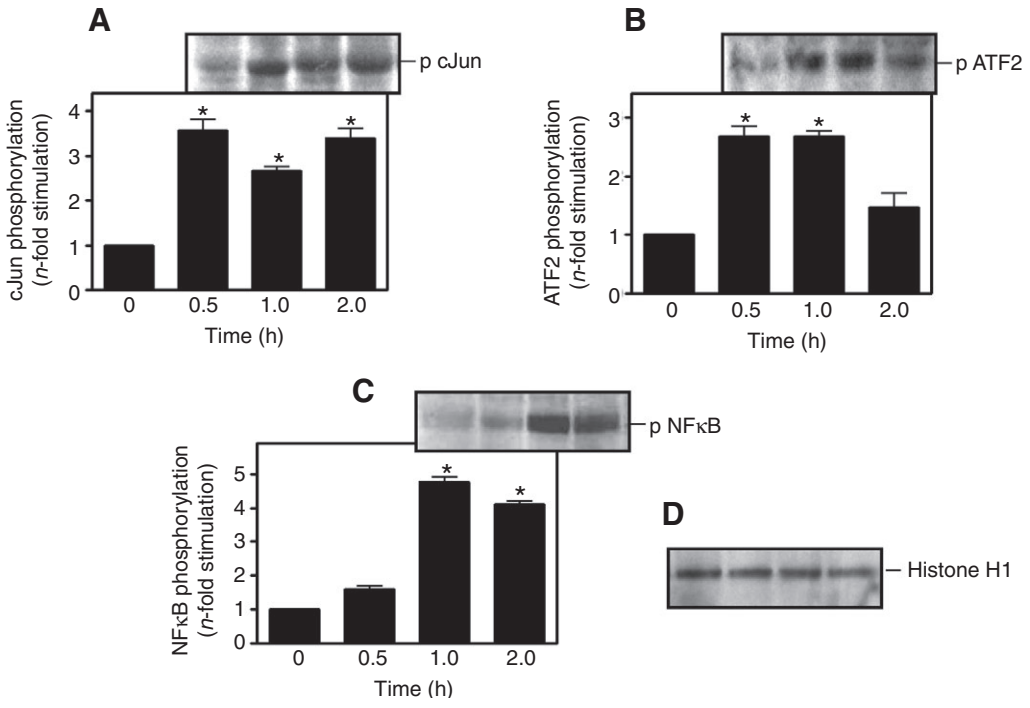

Fig. 3. Time-dependent phosphorylation profile of cJun, ATF2 and NFκB in nuclear extracts from gills of *M. galloprovincialis* specimens subjected to hyperthermia (30°C). Tissue extracts (30 μg) from control animals and animals exposed to hyperthermia (30°C) for the times indicated were subjected to SDS-PAGE and immunoblotted with antibodies for phosphorylated cJun (A), phosphorylated ATF2 (B) and phosphorylated NFκB (C). Equal protein loading was confirmed by blotting identical samples with an anti-histone H1 specific antibody (D). Bands were quantified by laser scanning densitometry (A–C, respective graphs). Blots and results shown are representative of at least four independent experiments. Results are means ± s.e. for at least four independent experiments. *P<0.01 compared with control animals (kept at 15°C).

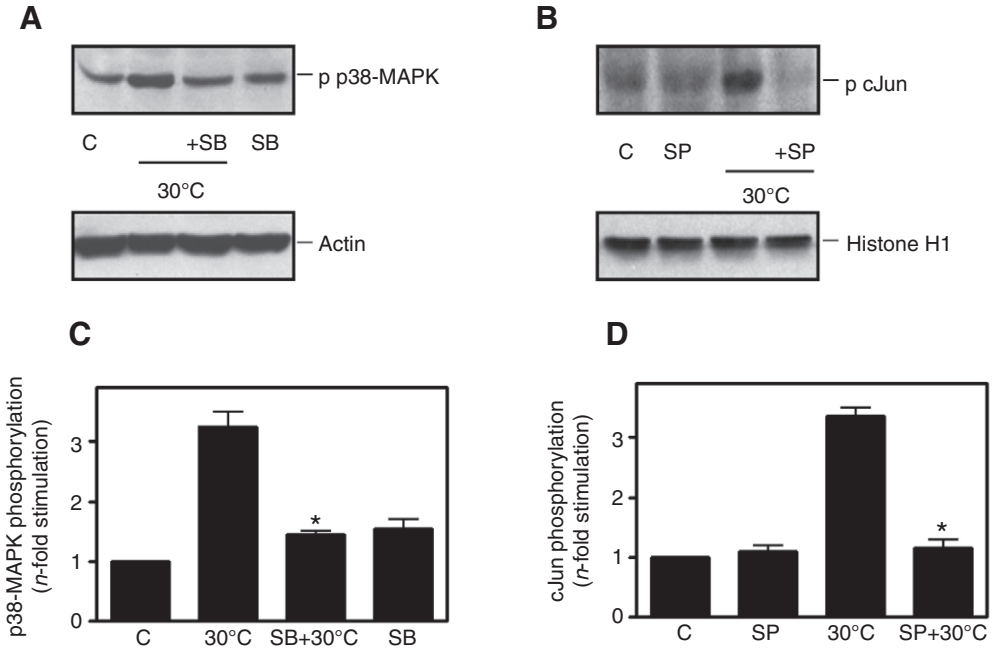

Fig. 4. Effect of the selective pharmacological inhibitors used. (A) Inhibitory effect of SB203580 on hyperthermia-induced phosphorylation levels of p38-MAPK (top panel) was assessed in samples of gill tissue extracts (50 μg) from control animals (15°C) and animals exposed to hyperthermia (30°C) for 30 min. Equal protein loading was confirmed by blotting identical samples with an anti-actin specific antibody (bottom panel). (B) Inhibitory effect of SP600125 on hyperthermia-induced phosphorylation levels of cJun (top panel) was assessed in the nuclear fractions from gill tissue extracts (30 μg) from control animals (15°C) and animals exposed to hyperthermia (30°C) for 30 min. Equal protein loading was confirmed by blotting identical samples with an anti-histone H1 specific antibody (bottom panel). Bands were quantified by laser scanning densitometry (C, phospho-p38-MAPK and D, phospho-cJun). Blots and results shown are representative of four independent experiments. Results are means ± s.e. for four independent experiments. *P<0.01 compared with identically treated animals in the absence of the respective inhibitor.

Indeed, we found Hsp70 transcript levels to be rapidly stimulated, attaining maximal values at 2 h (3.08±0.18-fold, relative to control animals), and remaining elevated for at least 8 h (1.96±0.05-fold, relative to control animals) (Fig. 7A,C). As far as MTs are concerned, there was no fluctuation detected in MT10 mRNA levels (Fig. 7B, top panel) while MT20 mRNA levels were maximized at 1 h (3.23±0.14-fold, relative to control animals), and remained elevated for at least 2 h (2.67±0.03-fold, relative to control animals) (Fig. 7B, bottom panel, D). β-Actin mRNA levels in identical samples were also assayed (Fig. 7A, bottom panel).

In the light of the aforementioned results, implicating p38-MAPK and JNKs in the hyperthermia-stimulated cellular response in mussels (Figs 5 and 6), we attempted to evaluate their potential role in the transcriptional upregulation of Hsp70 and MT20. To this end, selective pharmacological inhibitors were once more used. SB203580 was found to completely block hyperthermia-induced transcriptional upregulation of both Hsp70 (Fig. 8A) and MT20 (Fig. 8B) while the inhibitory effect of SP600125 was significant but partial (Fig. 8A,B). β-Actin mRNA levels in identical samples were also assayed (Fig. 8C). Subsequently, so as to evaluate any effects of the solvent used (DMSO) to dilute the aforementioned inhibitors, the effect of DMSO on Hsp70, MT20 and β-actin mRNA levels was also assessed and found to be insignificant (Fig. 8D). Furthermore, in an effort to examine the effect of these inhibitors on the proteolytic cleavage of PARP and hence on apoptosis, we found PARP fragment protein levels to be considerably enhanced in the presence of SB203580 and to a smaller but significant extent by SP600125 (Fig. 8E, left upper panel). Therefore, when hindering the activity of p38-MAPK and JNKs, exposure to hyperthermia for as long as 1 h was observed to trigger apoptosis, as evidenced by PARP cleavage. The nature of the second band detected is still under investigation but it is most likely to represent another fragment of PARP (PARP fragment I). Any interference of DMSO should be excluded as the solvent alone did not induce PARP fragmentation (Fig. 8E, right upper panel). Equal protein loading was confirmed by immunoblotting identical samples with a specific anti-actin antibody (Fig. 8E, bottom panels).

DISCUSSION

Thermal stress constitutes one of the most crucial factors in determining the distribution pattern of ectothermic organisms, particularly invertebrates of the intertidal zone. Nevertheless, the molecular pathways by which fluctuations in the temperature regime of an environmental niche are 'sensed' and transformed into physiological performance remain obscure. Thus, the major scope of the present study was to gain a better insight into the interplay between the signal transduction mechanisms triggered by acute hyperthermia in the mussel *M. galloprovincialis*. This submerged bivalve species lives in relatively shallow waters of the intertidal zone, routinely facing acute changes of temperature that can often reach extreme values, especially during summer. Therefore, one can deduce that it constitutes an ideal experimental model for investigation of the molecular mechanisms transducing the thermal stress signal.

Thermal stress in the form of hyperthermia has been shown to stimulate the phosphorylation and thus activation of multiple mitogen-activated protein kinases (MAPKs), a superfamily of protein kinases (Kyriakis and Avruch, 1996). Among the MAPK subfamilies, JNKs and p38-MAPK are established as being the kinases principally responsive to stress, transducing extracellular

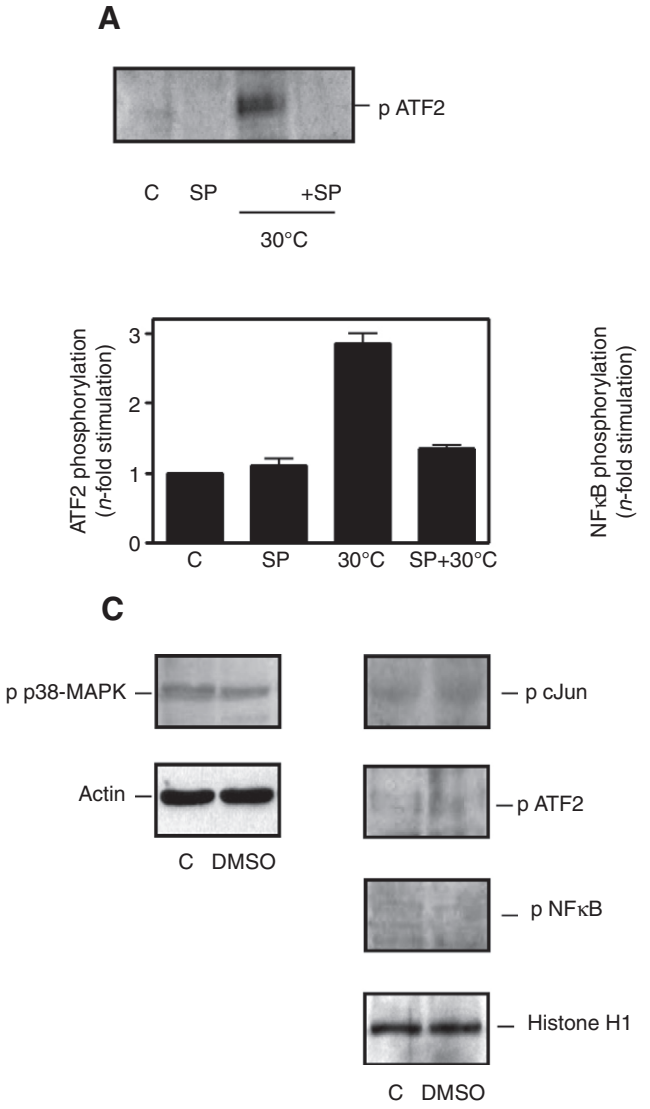

Fig. 5. JNKs mediate hyperthermia-induced ATF2 phosphorylation. (A) The effect of SP600125 on hyperthermia-induced phosphorylation levels of ATF2 was assessed in nuclear fractions from gill tissue extracts (30 μg) from control animals (15°C) and animals exposed to hyperthermia (30°C) for 30 min. Bands were quantified by laser scanning densitometry (graph). (B) The effect of SP600125 on hyperthermia-induced phosphorylation levels of NFκB was assessed in nuclear fractions from gill tissue extracts (30 μg) from control animals (15°C) and animals exposed to hyperthermia (30°C) for 60 min. Bands were quantified by laser scanning densitometry (graph). (C) The effect of DMSO on p38-MAPK, cJun, ATF2 and NFκB phosphorylation levels was assessed in extracts (whole or nuclear fractions) from gill tissue (30 μg) from control animals (C) and animals exposed to 30°C in the presence of DMSO alone (DMSO). Equal protein loading was confirmed by blotting identical samples with an anti-actin or anti-histone H1 specific antibody. Blots and results shown are representative of four independent experiments. Results are means ± s.e. for four independent experiments. **P*<0.01 compared with identically treated animals in the absence of the respective inhibitor.

stimuli to the cytoplasm or nucleus, where these kinases interact with their respective substrates, eliciting cellular responses (Bogoyevitch, 2000). Accordingly, we have found p38-MAPK to exhibit a considerable and sustained phosphorylation profile in samples from gill tissue of mussels exposed to 30°C, while JNKs were shown to have a moderate and transient activation (Fig. 1). The presence and activation of p38-MAPK and JNKs has been demonstrated before in mussel studies either in haemocytes during the immune response to a bacterial challenge (Canesi et al., 2002; Canesi et al., 2005) or in mantle and gill tissue from *M. galloprovincialis* species exposed to diverse forms of stress (Gaitanaki et al., 2004; Kefaloyianni et al., 2005). The sensitivity of gills to thermal stress could justify the more profound effect of hyperthermia on p38-MAPK phosphorylation compared with the kinase profile in mantle reported previously by our group (Kefaloyianni et al., 2005). What is more, the observed sustained activation of p38-MAPK could underscore the critical role of this kinase under the interventions investigated. Such a hypothesis may be justified as activation of p38-MAPK has been shown to exert a beneficial role in various experimental models exposed to stressful conditions (Lavoie et al., 1995; Clerk et al., 1998) as well as in the isolated perfused amphibian heart subjected to hyperthermia (Gaitanaki et al., 2008). Nevertheless, no data are available so far regarding the potential effect of p38-MAPK on the modulation of gene expression in mussels. Notably, this is the first time that JNKs have been shown to be transiently responsive to short-term hyperthermia, since a study by Anestis and colleagues commented on the effect of various temperatures on the phosphorylation profile of a single JNK isoform (p46) during long-term acclimation (Anestis et al., 2007).

The detection of the hyperthermia-induced rapid translocation of activated p38-MAPK and JNKs to the nuclear compartment illustrated in Fig. 2A may be indicative of the interaction of the kinases with their nuclear targets, potentially including several transcription factors, leading to modification of gene expression. Indeed, Adler and colleagues have marked heat shock as a potent phosphorylation inducer of JNKs and of their established nuclear substrate, cJun, in 3T3-4A mouse fibroblast cell line (Adler et al., 1995).

Subsequently, in an effort to evaluate the biological impact of hyperthermia in mussels and with heat stress known to favour apoptosis (Gaitanaki et al., 2008; Bellmann et al., 2009), we assessed the occurrence of apoptosis. Detection of cleaved fragments of PARP by immunoblotting 8 h after the onset of hyperthermia (Fig. 2B) substantiated the delayed initiation of the apoptotic process, which also explains the high mortality rate for mussels observed after exposure to 30°C for 24 h. Increased temperature may severely

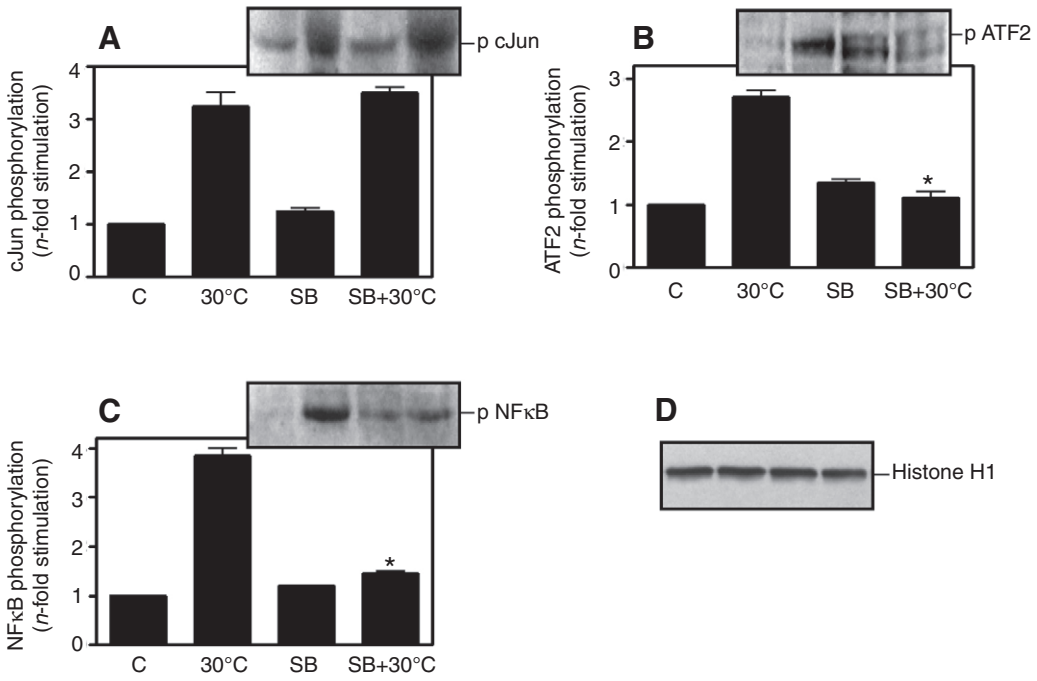

Fig. 6. Hyperthermia-induced ATF2 and NFκB phosphorylation is p38-MAPK mediated. The effect of SB203580 on hyperthermia-induced phosphorylation levels of cJun (A), ATF2 (B) and NFκB (C) was assessed in nuclear fractions from gill tissue extracts (30 μg) from control animals (15°C) and animals exposed to hyperthermia (30°C) for 30 min (cJun, ATF2) or 60 min (NFκB). Equal protein loading was confirmed by blotting identical samples with an anti-histone H1 specific antibody (D). Bands were quantified by laser scanning densitometry (respective graphs). Blots and results shown are representative of at least four independent experiments. Results are means ± s.e. for at least four independent experiments. **P*<0.01 compared with identically treated animals in the absence of the respective inhibitor.

compromise an organism's survival by principally compromising the functionality of its biochemical machinery. This is, to our knowledge, the first time that PARP proteolytic processing has been shown to take place in Mytilidae. Next, we decided to probe into the mechanisms that contribute to the initial preservation of homeostasis during the early stages of mussel exposure to hyperthermia. To this end, phosphorylation levels of a number of transcription factors that could serve as a link between hyperthermia-activated MAPKs and the observed physiological outcome were examined. In agreement with a report by Kyriakis and Avruch establishing JNK-dependent phosphorylation of the cJun transcription factor (Kyriakis and Avruch, 1996), cJun phosphorylation was observed under the interventions studied (Fig. 3A). What is more, in accordance with studies noting the induction of ATF2 and NFκB by heat shock (Chen et al., 1997; Pogliaghi et al., 2001), immunoblot analysis revealed the rapid phosphorylation of ATF2 and the relatively pronounced transactivation of NFκB (Fig. 3B,C) in the present study.

Interestingly, phosphorylation of cJun and ATF2 was found to be JNK dependent (Fig. 4B, Fig. 5A) with ATF2 and NFκB phosphorylation found to be p38-MAPK dependent (Fig. 6B,C), being abolished by the respective kinase inhibitors. These findings correlate well with a study by Nahas and colleagues (Nahas et al., 1996) reporting p38-MAPK to be involved in ATF2 phosphorylation and another by McGee and colleagues (McGee et al., 2002) underlining the involvement of JNKs in both ATF2 and cJun phosphorylation.

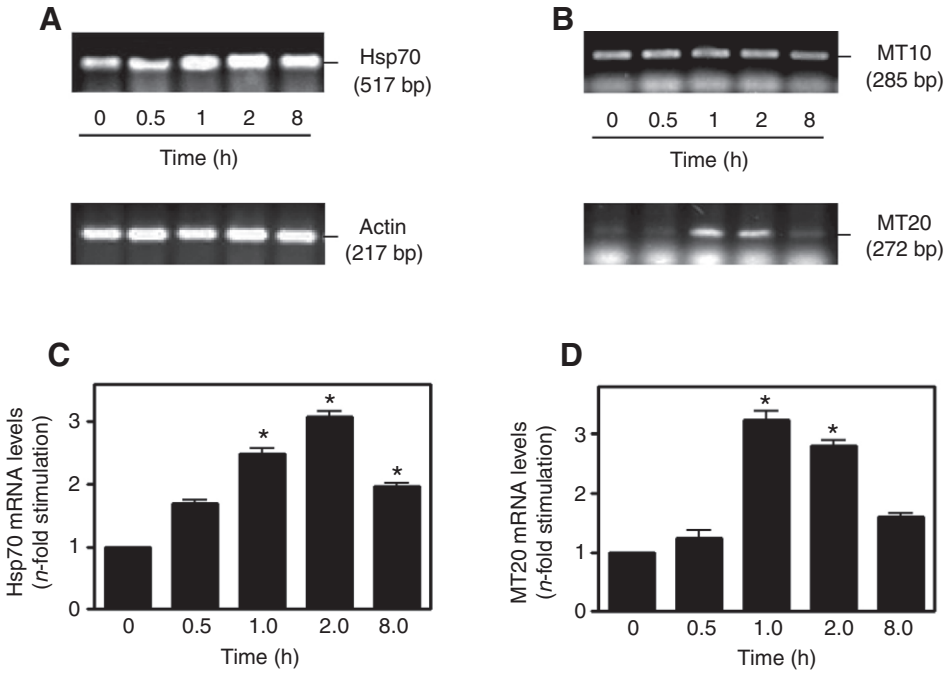

Fig. 7. Time-dependent profile of Hsp70, MT10 and MT20 mRNA upregulation by hyperthermia. RNA was extracted from gill tissue of *M. galloprovincialis* specimens exposed to hyperthermia (30°C) for the times indicated or left untreated (control animals, 15°C). Expression of Hsp70 (A, top panel), MT10 (B, top panel), MT20 (B, bottom panel) and actin (A, bottom panel) mRNA was analysed by ratiometric RT-PCR. PCR product band size is indicated to the right of the panels. After densitometric analysis of the PCR products, results were normalized for actin and the data are presented as fold stimulation (C,D). Results are means ± s.e. for four independent experiments. **P*<0.01 compared with control values.

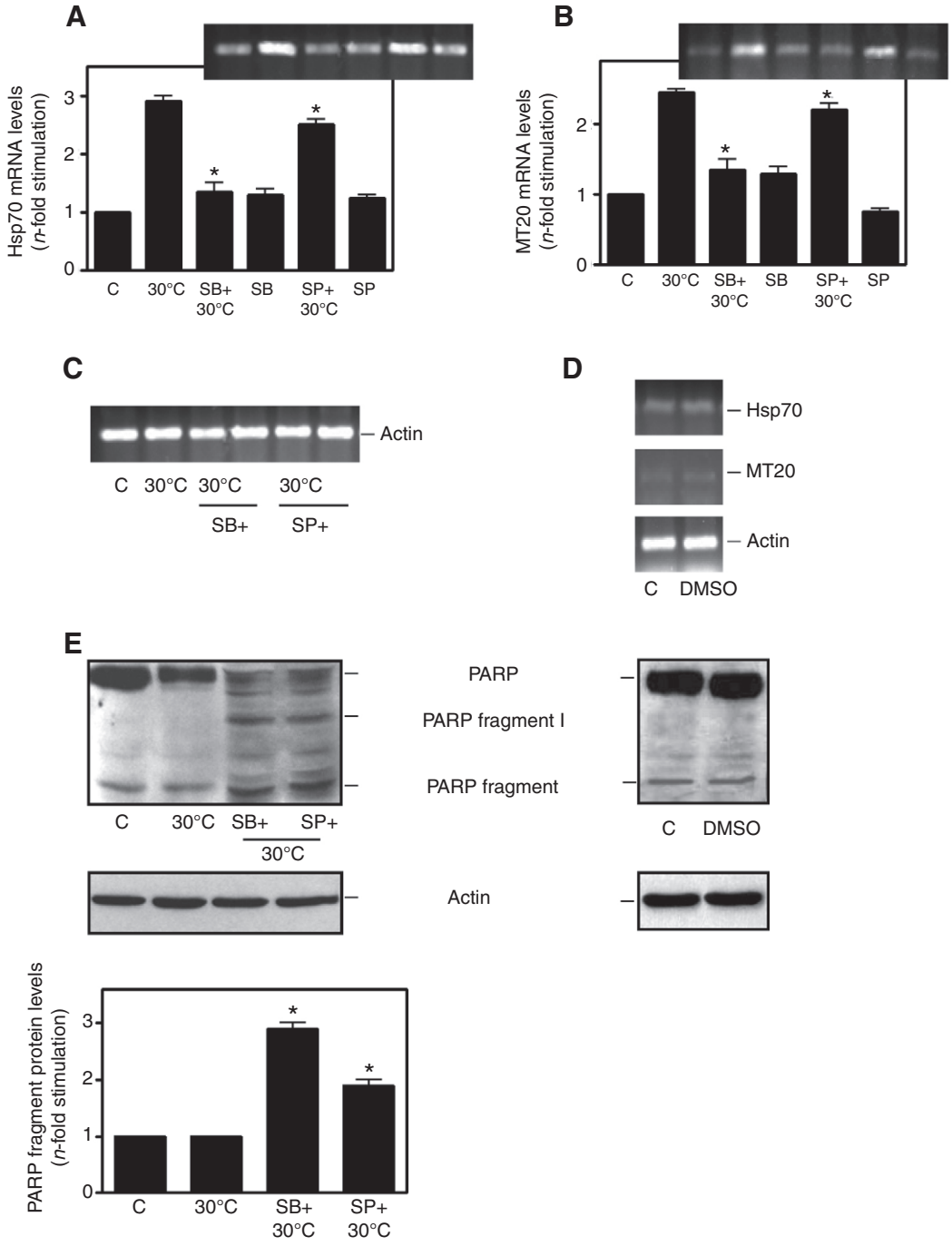

Fig. 8. p38-MAPK and JNKs mediate hyperthermia-induced Hsp70 and MT20 mRNA upregulation and prevent PARP proteolytic processing. RNA was extracted from gill tissue of *M. galloprovincialis* specimens exposed to hyperthermia (30°C) for 1 h in the presence or absence of SB203580 and SP600125, or left untreated (control animals, 15°C). Expression of Hsp70 (A), MT20 (B) and actin (C) mRNA was analysed by ratiometric RT-PCR. (D) The effect of DMSO alone on the mRNA levels of these genes was also assessed in samples from gill tissue of *M. galloprovincialis* specimens exposed to hyperthermia (30°C). After densitometric analysis of the PCR products, results were normalized for actin and the data are presented as fold stimulation (respective graphs). Results are means ± s.e. for four independent experiments. (E) Proteolytic processing of PARP was assessed by immunoblotting samples of tissue extracts (50 μg) from control animals (15°C), animals exposed to hyperthermia (30°C) for 1 h in the presence or absence of SB203580 and SP600125 (left upper panel) and animals exposed to hyperthermia (30°C) in the presence or absence of DMSO (right upper panel). Quantification of PARP fragment was performed by laser scanning densitometry (graph). The membranes were re-probed with an anti-actin antibody so as to verify equal protein loading (bottom panels). Blots and results shown are representative of four independent experiments. **P*<0.01 compared with identically treated animals in the absence of the respective inhibitor.

One can therefore attribute the observed cellular response to these diverse effector interactions possibly promoting particular changes in gene transcription. Thus, we next looked for any modification of the expression of genes known to preserve mussel fitness and performance under adverse conditions. Our hypothesis was verified as a particularly sustained stimulation of Hsp70 and a more transient stimulation of MT20 transcript levels was observed (Fig. 7). The participation of Hsp70 in the cellular responses to stress in *M. galloprovincialis* has been documented in several previous studies (Snyder et al., 2001; Hamer et al., 2004; Malagoli et al., 2004; Franzellitti and Fabbri, 2005) and Hsp70 mRNA and metallothioneins content were found to be stimulated in gills from the bivalve *Ostrea edulis* after exposure to heat (Piano et al., 2004). Evidently, Hsp induction has long been demonstrated to constitute a ubiquitous mechanism which compensates for stressful conditions including osmotic perturbations, hypoxia and heavy metal exposure (Snyder et al., 2001), with different animals exhibiting diverse sensitivity thresholds and tissue-specific expression patterns (Hofmann, 1999). In accordance with our findings, apart from Hsps which are principally triggered and mobilized so as to preserve cell homeostasis under such conditions (Hamer et al., 2008), MTs have also been shown to exert a cytoprotective role in many phyla against conditions that may compromise protein tertiary structure, including heat, metal exposure and UV irradiation (Serafim et al., 2002). Furthermore, correlating with the results reported by Raspor and colleagues (Raspor et al., 2004) as well as by Dondero and colleagues (Dondero et al., 2005) showing MT10 to be the housekeeping isoform of mussel MTs and MT20 to be the inducible one, we also observed no change in MT10

transcript levels during hyperthermia, while MT20 exhibited a transient stimulation (Fig. 7B,D).

Interestingly, hyperthermia-induced Hsp70 upregulation was found to be mediated by p38-MAPK, with JNKs also involved to a certain degree (Fig. 8A), which was also the case for MT20 regulation (Fig. 8B). The p38-MAPK pathway has been shown before to regulate Hsp70 expression, at the protein level, in samples from gill tissue from *M. galloprovincialis* specimens exposed to high copper concentrations (Kefaloyianni et al., 2005). In that particular study, a cytoprotective role was attributed to Hsp70, substantiated by the lack of any apoptotic markers in gills. Recently, Banerjee and colleagues have highlighted p38-MAPK involvement in heat stress-induced Hsp70 upregulation in V76 lung fibroblasts (Banerjee et al., 2009), with other studies also corroborating the effect of p38-MAPK on Hsp70 upregulation by multiple stimuli in diverse experimental models (Rafiee et al., 2006; Ko et al., 2008). The contribution of JNKs to Hsp70 stimulation has also been documented previously in agreement with our results (Rafiee et al., 2006). As far as regulation of MTs is concerned, numerous studies have implicated p38-MAPK or JNKs, primarily in mammalian cell lines (Yeh and Yen, 2005; Williams et al., 2007). Furthermore, NFκB along with cJun and ATF2 have also been shown to participate in the signalling cascades resulting in stimulation of MTs by diverse stimuli (Koo et al., 2002; Peng et al., 2007). In addition to this, our inhibitor studies have established the central role of p38-MAPK and JNKs in the survival of cells in the early stages of mussel exposure to hyperthermia, with PARP fragmentation occurring in the presence of their respective inhibitors (Fig. 8E, upper left panel). PARP fragments originate as cleavage products from poly-(ADP-ribose) polymerase, a DNA repairing enzyme that is catalytically activated by DNA strand breaks and has a negative regulatory role during apoptosis. PARP is one of several 'death substrates' and is cleaved to an 85 kDa fragment by caspase-3 in mammals (Nagata and Golstein, 1995). However, given this is the first time that cleaved fragments of PARP have been detected by immunoblotting in a mussel tissue, further studies are required in order to decipher and fully characterize the nature of the second band (PARP fragment I) which may be a result of incomplete PARP proteolysis or may originate from the activity of a so far unknown effector. Overall, controversy surrounds the participation of p38-MAPK and JNKs in the determination of cell fate by preserving homeostasis or favouring apoptosis (Bogoyevitch, 2000; Davis, 2000; Aoki et al., 2002; Dougherty et al., 2002). Evidently, their beneficial or detrimental impact is stimulus and tissue specific.

Collectively, our novel findings highlight the signalling cascades transducing the heat stress signal to the transcriptosome of *M. galloprovincialis*, stimulating expression of Hsp70 and MT20. The salutary role of these genes is well documented. Nevertheless, while considerable progress has been made, further studies are required to elucidate and determine the multiple roles of these effector molecules in the mechanisms regulating these cytoprotective genes, at an eco-physiological level, particularly in the perspective of global climatic changes. Elucidating these mechanisms may provide further useful information regarding the use of mussels as biomarkers in monitoring studies of marine environments, based on their mode of life and behaviour.

LIST OF ABBREVIATIONS

DMSO	dimethyl sulphoxide
DTT	ditheiothreitol
ECL	enhanced chemiluminescence
ERK	extracellular signal regulated kinase
Hsp	heat shock protein
JNK	cJun NH_2 terminal kinase
MAPK	mitogen-activated protein kinase
MT	metallothionein
NFκB	nuclear factor-κB
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PMSF	phenyl methyl sulphonyl fluoride
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SAPK	stress activated protein kinase
TBS	Tris-buffered saline

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