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Acute thermal stress and various heavy metals induce tissue-specific pro- or anti-apoptotic events *via* the p38-MAPK signal transduction pathway in *Mytilus galloprovincialis* (Lam.)

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Summary

We investigated the effects of various heavy metals such as copper, zinc and cadmium, as well as acute thermal stress, on the signalling mechanisms involved in the protection and/or apoptosis of *Mytilus galloprovincialis* mantle and gill tissues. The results of our studies revealed that mantle and gill tissues differentially respond to the stressful stimuli examined. In the mantle tissue, 1 μmol l^{-1} Cu^{2+} and 50 μmol l^{-1} Zn^{2+} induced a transient p38-MAPK activation, whereas 1 μmol l^{-1} Cd^{2+} induced a biphasic profile of the kinase phosphorylation with maximal values at 15 and 120 min of treatment, respectively. Furthermore, 1 μmol l^{-1} SB203580 abolished the Cu^{2+}-induced kinase phosphorylation. In gills, both Cu^{2+} and Zn^{2+} induced a considerably higher p38-MAPK activation, which remained elevated for at least 60 min, whereas Cd^{2+} induced a maximal kinase activation within 60 min of treatment. Hypothermia (4°C) induced a moderate kinase phosphorylation (maximised at 30 min), whereas hyperthermia (30°C) induced a rapid (within 15 min) p38-MAPK phosphorylation that remained considerably above basal levels for at least 2 h. Our studies on the synergistic effect of hyperthermia and Cu^{2+} revealed that these two stressful stimuli are additive in the mantle tissue, inducing an almost double p38-MAPK activation. Further studies on the involvement of the p38-MAPK signalling pathway in tissue-specific pro- or anti-apoptotic events revealed that identical stressful stimuli possibly lead to apoptotic death *via* the caspase-3 activation in the mantle tissue and to anti-apoptotic events possibly *via* the induction of Hsp70 overexpression in the gill tissue.

Key words: mussel, copper, zinc, cadmium, signalling, apoptosis, hyperthermia, Hsp70.

Introduction

Among marine invertebrates, filter-feeding lamellibranch molluscs, such as mussels, are remarkable for their ability to accumulate high levels of trace metals in their tissues, since these animals have mechanisms of detoxification of heavy metals (Viarengo and Nott, 1993; Hayes and McLellan, 1999; Viarengo et al., 2000). Heavy metals have the potential to act as catalysts in the oxidative deterioration of biological macromolecules and therefore the toxicities associated with these metals may be due, at least in part, to oxidative tissue damage. Mussel gills and digestive gland are the two main target tissues for heavy metal accumulation, which can alter the physiology of respiration and feeding processes (Viarengo et al., 1994). Recent studies have shown that metals such as iron, copper, cadmium, chromium, lead, mercury, nickel and vanadium exhibit the ability to produce reactive oxygen species (ROS), resulting in lipid peroxidation, DNA damage, depletion of sulphydryl groups, altered signal transduction pathways and calcium homeostasis (Stohs and Bagchi, 1995; Galaris and Evangelou, 2002).

On the other hand, marine invertebrates such as *Mytilus* sp. can face and sustain seasonal variations in environmental temperature and, as ectotherms, they respond usually by alterations of their respiration and metabolic rates. As an estimated proportion, 2–3% of the oxygen consumed by aerobic cells is converted to oxygen radicals ($O_2^{\cdot}$) and H_2O_2 (Sohal and Weindruch, 1996), and increasing tissue oxygen consumption will entail elevated rates of ROS production in mitochondria (Boveris and Chance, 1976; Boveris et al., 1976). Higher temperatures are therefore likely to enhance ROS release, thereby increasing the risk of oxidative damage. ROS formation and subsequent damage are balanced by an array of cellular antioxidant defences including various antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, as well as low molecular binding proteins such as metallothioneins that function as radical quenchers and as chain-breaking compounds (Kagi and Shaffer, 1988; Kiningham and Kasarkis, 1998; Viarengo et al., 1999).

The evaluation of oxidative stress and antioxidant balance has been extensively studied in a plethora of cell types, tissues and animals including marine invertebrates (for a review, see Viarengo et al., 2000; Molavi and Mehta, 2004; Vertuani et al., 2004; Warner et al., 2004). Oxidative stress induces various signal transduction pathways that lead to either protection or apoptosis, depending on the cell type. The various signal transduction pathways include the ones involving the mitogen-activated protein kinases (MAPKs). The MAPK superfamily of protein Ser/Thr-kinases is a widely distributed group of enzymes that has been highly conserved through evolution (for reviews, see Graves and Krebs, 1999; Widmann et al., 1999; Kyriakis and Avruch, 2001; Roux and Blenis, 2004). Three subfamilies of the MAPKs have been clearly identified and extensively studied in mammalian experimental models: the extracellularly responsive kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), which are also known as the stress-activated protein kinases (SAPKs), and the p38-MAPKs. The ERK1/2 pathway is primarily responsive to growth factors and mitogens and appears to be involved predominantly in anabolic responses (Widmann et al., 1999; Roux and Blenis, 2004). JNKs and p38-MAPKs are predominantly activated by various stressful stimuli and can be involved in either anti-apoptotic or pro-apoptotic mechanisms, depending on their isoforms and/or cell type (Widmann et al., 1999; Kyriakis and Avruch, 2001; Roux and Blenis, 2004; Wada and Penninger, 2004).

Among the well-established anti-apoptotic proteins, the classic, non-ribosome-binding members of the large heat shock protein (Hsp) family are included. These proteins exist in the cytosol of all eukaryotic cells and are molecular chaperones required for the proper folding and trafficking of many proteins involved in signal transduction pathways (Buchner, 1999; Pearl and Prodromou, 2000). A growing body of evidence suggests that members of the Hsp70 family are either constitutively expressed (Hsc70) or exist as stress-inducible forms (Hsp70) and function by binding and releasing extended polypeptide segments that are expressed by misfolding proteins (Hartl and Hayer-Hartl, 2002; Sreedhar and Csermely, 2004). Although the implication of Hsp70s in the anti-apoptotic molecular mechanisms is well established, the precise signal transduction pathways leading to their induction remain obscure.

On the other hand, the caspase-mediated apoptotic death induced by diverse stressful conditions is well established in a plethora of mammalian cell types (for a review, see Bredesen et al., 2004; Jiang and Wang, 2004; Philchenkov, 2004). Various experimental approaches and studies have established that oxidative stress can lead to apoptotic cell death, possibly *via* cytochrome *c* release and the activation of various caspases (for a review, see Jiang and Wang, 2004). On the contrary, very little is known on the precise molecular mechanisms induced by environmental stress in lower vertebrates and marine invertebrates that lead to apoptotic cell death.

In a previous paper, we had described the expression and activation of the p38-MAPK signalling pathway in *Mytilus galloprovincialis* mantle tissue in response to diverse forms of stress such as anoxia, anoxia/re-oxygenation, oxidative stress and osmotic stress (Gaitanaki et al., 2004). In the present paper, we describe the effects of acute thermal stress and indirect oxidative stress induced by various trace metals on the p38-MAPK signal transduction pathway and its possible involvement in anti-apoptotic or apoptotic mechanisms, depending on the cell type examined. Furthermore, we examined the synergistic effects of thermal stress and trace metal accumulation on the p38-MAPK signalling pathway in both mantle tissue and gills of *M. galloprovincialis*.

Materials and methods

Materials

All chemicals were of the highest grade available and purchased from Sigma Chemical Co. (St Louis, MO, USA). The enhanced chemiluminescence (ECL) kit was from Amersham International (Uppsala, Sweden). Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene, NH, USA). The p38-MAPK selective inhibitor SB203580 was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA), and a stock solution (10 mmol l^{-1}) was prepared in DMSO.

Rabbit polyclonal antibodies specific for total p38-MAPK, as well as for the dually phosphorylated form of p38-MAPK, and for total Hsp70 were obtained from Cell Signalling Technology (Beverly, MA, USA). A rabbit monoclonal antibody specific for caspase-3 (#9665) that detects the endogenous levels of full-length (35 kDa) and large active fragments (17/19 kDa) of caspase-3 resulting from cleavage at Asp 175 was also purchased from Cell Signalling Technology. Prestained molecular mass markers were from New England Biolabs (Beverly, MA, USA). Biotinylated anti-rabbit antibody was from DAKO A/S (DK-2600 Glostrup, Denmark). X-OMAT AR film (13×18 cm) was purchased from Eastman Kodak Company (New York, NY, USA).

Animals

Male or female adult mussels (75–80 mm length) *Mytilus galloprovincialis* (Lam.) were obtained from a local dealer and had been collected (from March up to September) in Saronikos gulf, Athens, Greece. All animals were held in re-circulating seawater (15–18°C) at the laboratory for at least 4 days prior use.

Animal treatments

Specimens (4–6 for each group) were equilibrated at 15°C in large tanks with aerated re-circulating seawater for at least 4 days. For each treatment, animals were transferred into smaller tanks with the proper seawater solution (~300 ml per animal). Lowering the temperature down to 4°C or increasing it up to 30°C for increasing time intervals (5–120 min) induced hypothermia or hyperthermia, respectively. For the experiments with heavy metals, either $CuCl_2$ (1 μmol l^{-1}), $ZnCl_2$ (50 μmol l^{-1}) or $CdCl_2$ (1 μmol l^{-1}) were added to a

convenient volume of normal seawater and animals were incubated in these solutions for increasing time intervals, varying between 15 and 120 min. In other experiments, specimens were exposed to 1 μmol l^{-1} $CuCl_2$ (in normal seawater) either in the presence or absence of the p38-MAPK selective inhibitor SB203580 (1 μmol l^{-1}). Control experiments had shown that neither DMSO solvent nor SB203580 (1 μmol l^{-1}) alone induced any increase in p38-MAPK phosphorylation levels.

At the end of each treatment, animals were put on ice and mantle and gill tissues were dissected, freeze-clamped between aluminium tongs cooled in liquid nitrogen, pulverised under liquid nitrogen and the powders stored at –80°C.

Tissue extractions

Mantle or gill tissue powders were homogenised with 3 ml g^{-1} of buffer [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) sodium dodecyl sulphate (SDS), 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue]. Protein concentrations were determined using the BioRad 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, the membranes were incubated with the appropriate antibody according to the manufacturer's instructions. After washing in TBS-T (3×10 min), the blots were incubated with horseradish peroxidase-linked anti-rabbit immunoglobulin G (IgG) antibodies [1:5000 dilution in TBS-T containing 1% (w/v) non-fat milk powder; 1 h; room temperature]. The blots were washed again in TBS-T (3×10 min) and the bands were detected using ECL with exposure to X-OMAT AR film. Blots were quantified by laser scanning densitometry.

DNA laddering

Extraction of high-molecular-mass DNA from the mantle and gill tissues of *M. galloprovincialis* specimens was performed according to the method described by Winnepenninckx et al. (1993) with slight modifications. Briefly, frozen tissue was powdered under liquid nitrogen and homogenised with 3 ml g^{-1} of preheated (60°C) CTAB buffer [100 mmol l^{-1} Tris-HCl, pH 8.0, 1.4 mol l^{-1} NaCl, 0.2% (v/v) β-mercaptoethanol, 2% (w/v) hexadecyltrimethylammonium bromide-CTAB, 20 mmol l^{-1} EDTA]. To each extract, proteinase K (0.1 mg ml^{-1}) was added and after incubation at 60°C for at least 30 min, the suspension was extracted with an equal volume of phenol:chlorophorm:isoamyl alcohol (24:24:1) and centrifuged at 7700 ***g*** for 10 min at room temperature. The aqueous phase was transferred to a new tube, extraction and centrifugation steps were repeated and an equal volume of chlorophorm:isoamyl alcohol (24:1) was added to the final aqueous phase. After centrifugation (7700 ***g***, 10 min), RNAase (30 μg ml^{-1}) was added to the aqueous phase, samples were incubated at 37°C for 30 min and to each sample approximately two-thirds volume of 2-propanol was added, in order to precipitate the DNA. This was accomplished by incubating the solution overnight at room temperature. The DNA was finally precipitated by centrifugation (7700 ***g***, 10 min), washed in 76% (v/v) ethanol/10 mmol l^{-1} ammonium acetate for at least 30 min and recovered by centrifugation (7700 ***g***, 10 min). The samples were left to dry in the air and the DNA was dissolved in an appropriate volume of TE buffer (10 mmol l^{-1} Tris-HCl, pH 7.5, 0.1 mmol l^{-1} EDTA).

The samples were diluted with water (1:200) and the degree of purity was determined by measuring the absorbance at 260 and 280 nm. Subsequently, 5 μg of DNA from each sample were loaded onto an agarose gel (1.2% w/v), along with loading buffer [0.25% (w/v) bromophenol blue, 30% (v/v) glycerol]. Electrophoresis in running buffer (445 mmol l^{-1} Tris-HCl, pH 8.0, 445 mmol l^{-1} boric acid, 10 mmol l^{-1} EDTA) was performed at 70 V for approximately 4 h, the gel was treated with 4 μg ml^{-1} ethidium bromide, observed and photographed (Fluorchem 8800; Alpha Innotech, San Leandro, CA, USA).

Activation of caspase-3

Mantle or gill tissue powders were homogenised with CHAPS buffer (1:1 w/v), which contained 50 mmol l^{-1} Hepes, pH 6.5, 2 mmol l^{-1} EDTA, 0.1% (w/v) CHAPS, 20 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A, 10 μg ml^{-1} aprotinin, 5 mmol l^{-1} DTT, 1 mmol l^{-1} PMSF. Following the homogenisation with a micro-pestle, samples were frozen (–80°C) and thawed, twice. The homogenates were then centrifuged (14 000 ***g***, 4°C, 20 min) and the supernatants were boiled with 0.33 volumes of SDS–PAGE sample buffer. Protein concentrations were determined using the BioRad Bradford assay. Protein samples were separated by SDS–PAGE on 15% (w/v) acrylamide, 0.411% (w/v) bisacrylamide slab gels and processed for western blotting by the use of a specific antibody raised against caspase-3, according to the manufacturer's instructions.

Statistical evaluations

Western blots shown are representative of at least four independent experiments. Each data point represents the mean ± S.E.M. of samples from at least four separate specimens treated with the respective conditions. Comparisons between

control and treatments were performed using Student's unpaired *t*-test. A value of $P<0.05$ was considered to be statistically significant. MAPK or caspase-3 activation and Hsp70 levels in 'control' animals were set at 1, and the stimulated MAPK phosphorylation or caspase-3 activation and Hsp70 accumulation in treated animals was expressed as -fold activation over controls.

Results

In a previous paper, we had shown that oxidative stress, as exemplified by exogenous H_2O_2 (5 μmol l^{-1}), induced a strong activation of the p38-MAPK signalling pathway in *M. galloprovincialis* mantle tissue (Gaitanaki et al., 2004). This response was found to follow a time-dependent profile, exerting a biphasic maximum, at 15 min (8.1-fold relative to control value) and 60 min (8.0-fold above basal level) of treatment, respectively.

As the first step in the present study, we made an effort to examine the effects of copper-induced oxidative stress, at a relatively low concentration (1 μmol l^{-1}), on p38-MAPK phosphorylation levels. To this end, specimens (4–6 per group) were incubated in small tanks of artificial seawater (300 ml $animal^{-1}$) containing 1 μmol l^{-1} $CuCl_2$ for time periods varying from 15 min up to 2 h. The phosphorylation state of p38-MAPK was assessed by immunoblotting using antibodies specific for the dually phosphorylated (hence activated) form of the kinase. The results of this study revealed that 1 μmol l^{-1} $CuCl_2$ induced a strong phosphorylation of the kinase in the mantle tissue. In particular, this trace metal induced a rapid (within 15 min) activation, reaching maximal values within 30 min of

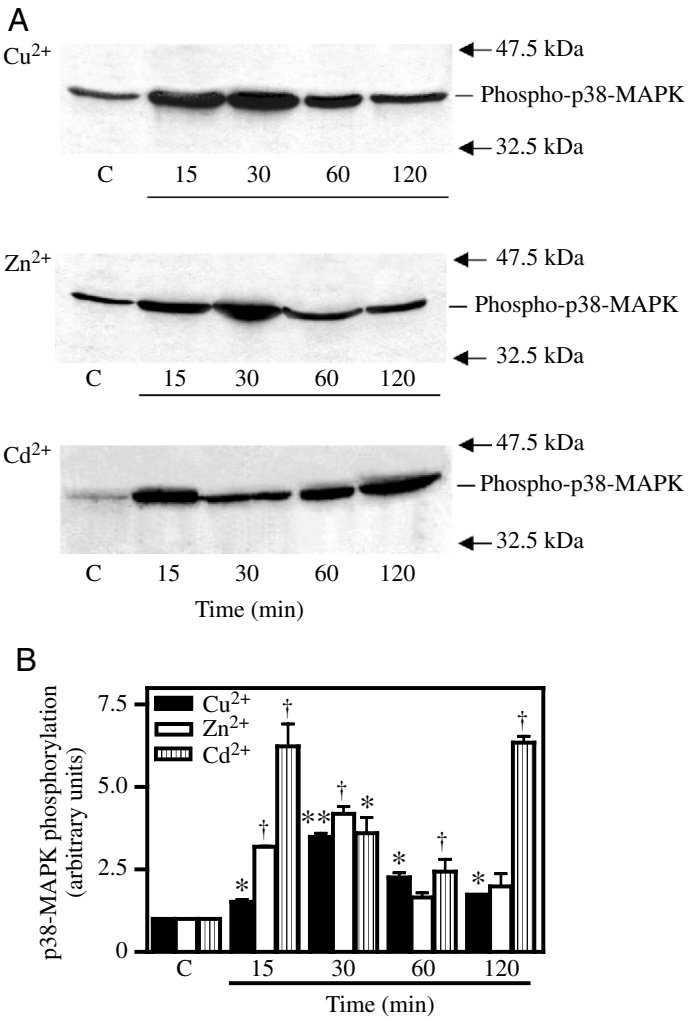


Fig. 1. Effect of various heavy metals on the p38-MAPK phosphorylation in *M. galloprovincialis* mantle tissue. (A) Phospho-p38-MAPK was detected in extracts (100 μg of protein) from control animals (C) or animals treated with either 1 μmol l^{-1} $CuCl_2$ (top panel), 50 μmol l^{-1} $ZnCl_2$ (middle panel) or 1 μmol l^{-1} $CdCl_2$ (bottom panel) for the times indicated. Western blots shown are representative of four to six independent experiments. The molecular mass markers (kDa) are shown to the right of the panel. (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± S.E.M. for four to six independent experiments performed with similar findings. †$P<0.001$, *$P<0.05$, **$P<0.01$ *versus* control value.

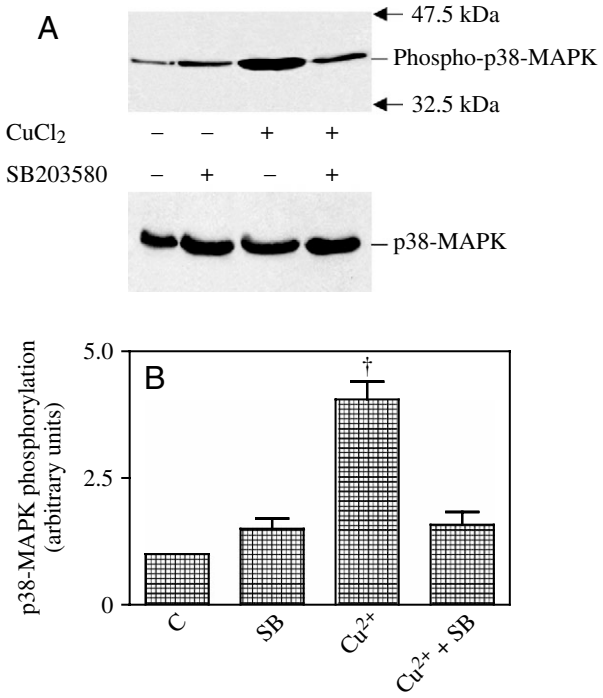


Fig. 2. Effect of SB203580 on the mantle tissue p38-MAPK activation by 1 μmol l^{-1} $CuCl_2$. SB203580 (1 μmol l^{-1}) was added to normal seawater and, after a 15 min equilibration period of the animals, it was present throughout the experiment. Phosphorylated (A, top panel) and total (A, bottom panel) p38-MAPK levels were assayed in mantle tissue extracts (100 μg of protein) from control animals, as well as from animals treated with 1 μmol l^{-1} $CuCl_2$ in the absence or presence of the inhibitor. Western blots shown are representative of four to six independent experiments performed with similar findings. The molecular mass markers (kDa) are shown to the right of the panel. (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± S.E.M. for four independent experiments. †$P<0.001$ *versus* control value.

treatment (3.49±0.11 relative to controls; $P<0.01$), with a progressive decline thereafter (Fig. 1).

Furthermore, the p38-MAPK selective inhibitor SB203580, even at a concentration of 1 μmol l^{-1}, abolished the kinase phosphorylation induced by 1 μmol l^{-1} $CuCl_2$, revealing that the p38-MAPK signalling pathway is specifically activated by the trace metal examined (Fig. 2). The bottom panel in Fig. 2A shows that there were no changes in the total cellular pools of p38-MAPK and therefore provides a control for equal protein loading under these conditions.

From the other trace metals tested, Zn^{2+} at a higher concentration (50 μmol l^{-1}) induced a rapid (within 15 min) strong activation of p38-MAPK (3.19±0.02-fold relative to control values; $P<0.001$), reaching maximal values within 30 min (4.19±0.20-fold relative to control values; $P<0.001$), with a progressive decline thereafter, reaching control values within 60 min of treatment (Fig. 1). On the contrary, animal exposure to 1 μmol l^{-1} Cd^{2+} induced a biphasic phosphorylation pattern of p38-MAPK in *M. galloprovincialis* mantle tissue. In particular, a rapid maximal phosphorylation of the kinase (6.23±0.68-fold relative to controls; $P<0.001$) was observed at 15 min, while a second maximum (6.89±0.20-fold relative to controls; $P<0.001$) was detected at 120 min of treatment (Fig. 1).

The effects of the trace metals mentioned above on the gill p38-MAPK phosphorylation state were also examined. The results of this study showed that, in this tissue, the phosphorylation (hence activation) of p38-MAPK followed a qualitatively and quantitatively different profile. In particular, 1 μmol l^{-1} $CuCl_2$ induced a strong, rapid phosphorylation, reaching maximum values (10.53±0.20-fold, relative to control values; $P<0.001$) within 15 min, which remained elevated for up to 60 min of treatment, with an apparent decline, although still significantly higher than basal level, for up to 2 h of treatment (Fig. 3). The maximal p38-MAPK activation obtained was comparable with that induced by 5 μmol l^{-1} H_2O_2 in *M. galloprovincialis* mantle tissue (Gaitanaki et al., 2004).

Of the other trace metals examined, 50 μmol l^{-1} Zn^{2+} induced a rapid, strong activation of p38-MAPK (9.04±0.02-fold relative to control values; $P<0.001$), reaching maximal values within 30 min (13.23±0.08-fold relative to control values; $P<0.001$), and remained significantly higher than basal level for at least 60 min (Fig. 3). On the other hand, 1 μmol l^{-1} Cd^{2+} induced a rapid p38-MAPK phosphorylation within 15 min (6.67±0.49-fold relative to control values; $P<0.001$), reaching maximal values at 60 min of treatment (8.63±0.47-fold relative to control values, $P<0.001$), and remaining elevated for at least 2 h (8.72±0.87-fold above basal level; $P<0.001$; Fig. 3).

Cold stress as well as heat stress activated mantle tissue p38-MAPK in a time-dependent manner. Hypothermia (4°C) induced a relatively moderate phosphorylation of the kinase, with a maximum value attained within 30 min (1.76±0.13-fold, relative to control animals maintained at 15°C; $P<0.05$), whereas at 60 or 120 min of treatment the kinase phosphorylation levels did not differ significantly from the control values (1.63±0.34-fold or 1.44±0.23-fold, relative to control animals maintained at 15°C; $P>0.05$; Fig. 4A,B). Hyperthermia (30°C) also induced a rapid (within 15 min) p38-MAPK phosphorylation (2.18±0.07-fold, relative to control animals maintained at 15°C; $P<0.05$), reaching maximal values at 30 min (2.71±0.02-fold above basal level; $P<0.01$) but remaining considerably above basal values for at least 120 min (1.88±0.27-fold relative to control, $P<0.05$; Fig. 4A,B).

In order to examine the synergistic effect of thermal stress and indirect oxidative stress as exemplified by 1 μmol l^{-1} $CuCl_2$, animals were subjected to hyperthermia (30°C) for 30 min either in the presence or absence of this trace metal.

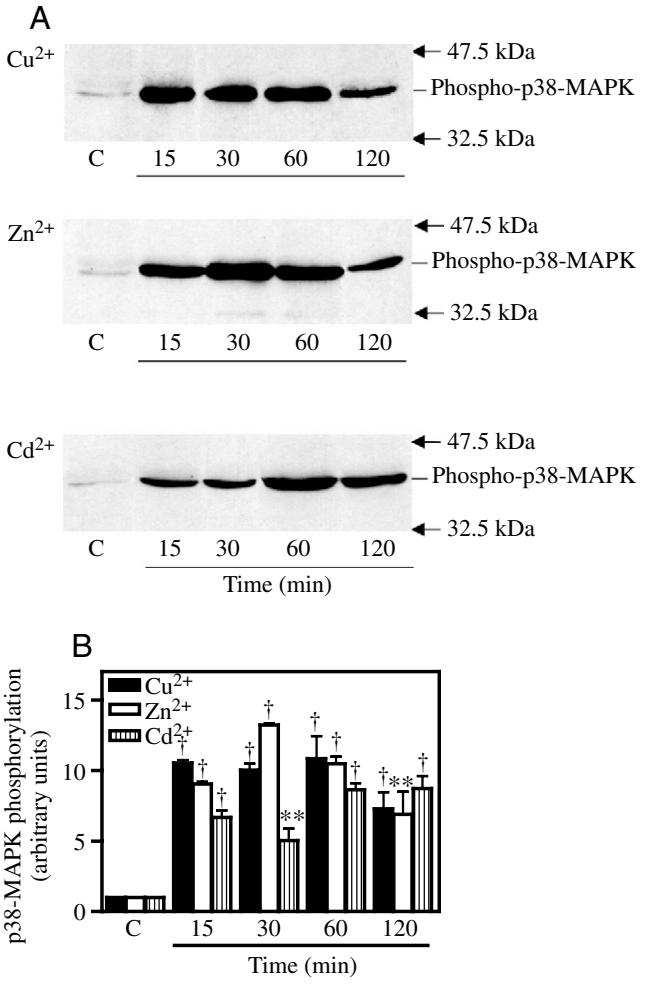
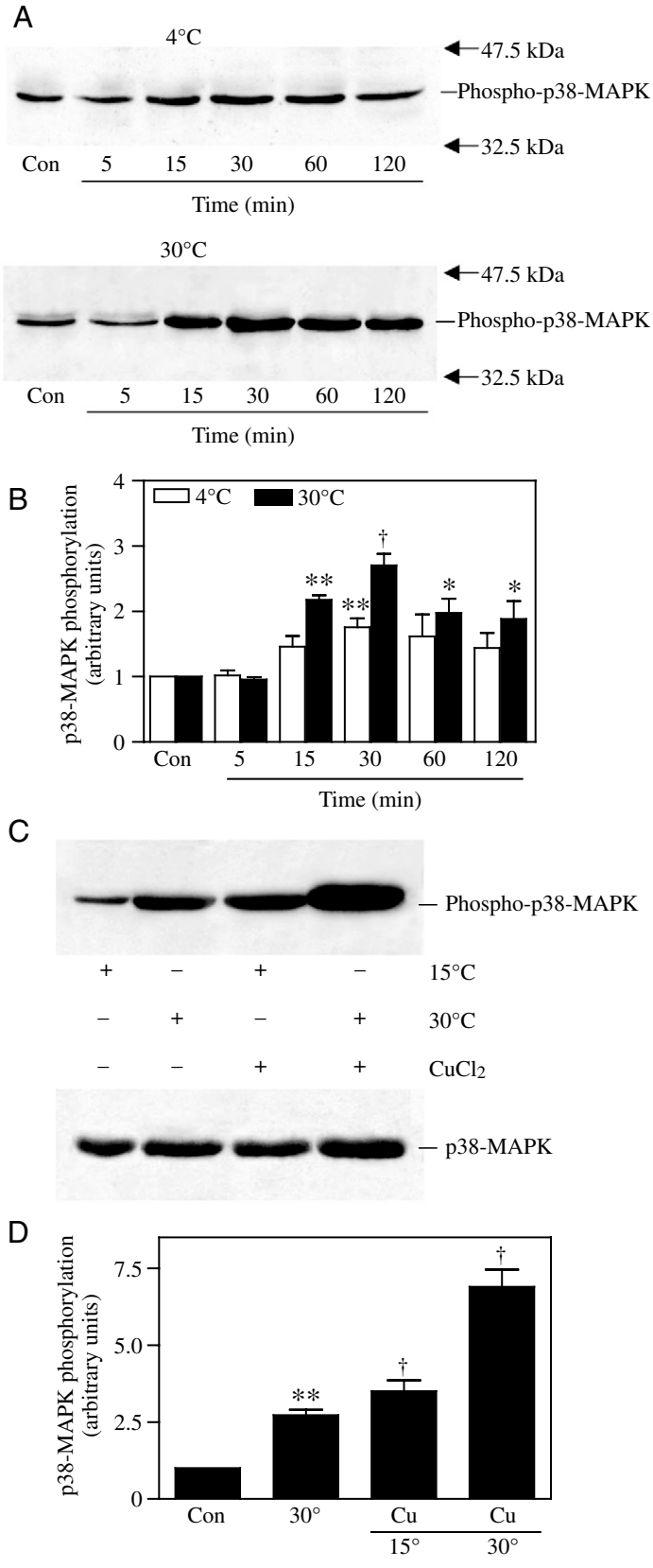


Fig. 3. Effect of various heavy metals on the p38-MAPK phosphorylation in *M. galloprovincialis* gill tissue. (A) Phospho-p38-MAPK was detected in extracts (100 μg of protein) from control animals (C) or animals treated with either 1 μmol l^{-1} $CuCl_2$ (top panel), 50 μmol l^{-1} $ZnCl_2$ (middle panel) or 1 μmol l^{-1} $CdCl_2$ (bottom panel) for the times indicated. Western blots shown are representative of four to six independent experiments. The molecular mass markers (kDa) are shown to the right of the panel. (B) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± S.E.M. for four to six independent experiments performed with similar findings. †$P<0.001$, **$P<0.01$ *versus* control value.

The results obtained clearly showed that p38-MAPK phosphorylation levels induced by Cu^{2+} were almost double when combined with hyperthermia alone (Fig. 4C,D). The bottom panel in Fig. 4C shows that there were no changes in the total cellular pools of p38-MAPK and therefore provides a control for equal protein loading under these conditions.



It is widely known that the p38-MAPK signalling pathway may be either pro-apoptotic or anti-apoptotic depending on the kinase isoforms present in different cell types (reviewed in Wada and Penninger, 2004). To examine the possible involvement of this signalling pathway in pro-apoptotic events in mantle or gill tissues under the conditions tested, we made an effort to study the genomic DNA fragmentation pattern in selected tissue samples. To this end, mantle and gill tissue samples from animals treated with trace metals such as Cu^{2+} (1 μmol l^{-1}, for 30 min) and Zn^{2+} (50 μmol l^{-1}, for 30 min) were used to isolate and examine the genomic DNA fragmentation. The selected heavy metal concentrations as well as the incubation time periods were identical to the ones inducing maximum activation of p38-MAPK. The results obtained revealed that mantle and gill tissues responded to these stressful conditions quite differently. In particular, in the mantle tissue, both Cu^{2+} (1 μmol l^{-1}) and Zn^{2+} (50 μmol l^{-1}) induced a rapid, extensive increase of DNA strand breakage levels (Fig. 5A, left panel). Interestingly, the p38-MAPK selective inhibitor SB203580 at a concentration of 1 μmol l^{-1} abolished the DNA fragmentation induced by 1 μmol l^{-1} Cu^{2+}, confirming that p38-MAPK is involved in a pro-apoptotic signalling pathway under such conditions in the *M. galloprovincialis* mantle tissue. By contrast, in gills, these two heavy metals, as well as Cd^{2+} (1 μmol l^{-1}, for 15 min), induced no genomic DNA fragmentation, confirming that in this tissue p38-MAPK is involved in an anti-apoptotic signalling pathway under such stressful conditions (Fig. 5A, right panel).

As another pro-apoptotic marker, activation of caspase-3 in identical samples from both mantle and gill tissues was examined. The results of these experiments clearly showed that in the mantle tissue, Cu^{2+}, Zn^{2+} and Cd^{2+} induced a strong activation of caspase-3. In particular, a significant accumulation of active caspase-3 fragments with a

Fig. 4. Time course of the effect of thermal stress and synergistic effect of Cu^{2+} combined with hyperthermia (30°C) upon p38-MAPK phosphorylation in *M. galloprovincialis* mantle tissue. Phosphorylated p38-MAPK was detected in extracts (100 μg of protein) from control animals maintained at 15°C (Con) or animals maintained at 4°C (A, top panel) or 30°C (A, bottom panel) for the indicated times. The molecular mass markers (kDa) are shown to the right of the panel. (C) Phosphorylated p38-MAPK (top panel) or total p38-MAPK (bottom panel) levels were detected in extracts (100 μg of protein) from control animals maintained at 15°C and animals maintained at 30°C for 30 min, either in the absence or presence of 1 μmol l^{-1} $CuCl_2$. Western blots shown are representative of four independent experiments. (B,D) Densitometric analysis of phospho-p38-MAPK bands by laser scanning. Results are means ± S.E.M. for four independent experiments performed with similar results. $^{\dagger}P<0.001$, $^{*}P<0.05$, $^{**}P<0.01$ *versus* control value.

molecular mass of 17–19 kDa was observed in the presence of all three heavy metals tested (Fig. 5B, top panel). Furthermore, the p38-MAPK selective inhibitor SB203580 (1 μmol l^{-1}) abolished the activation of caspase-3 induced by Cu^{2+}, a result reconfirming the pro-apoptotic p38-MAPK signalling pathway in this tissue (Fig. 5B, top panel). On the contrary, in gills, no activation of this pro-apoptotic marker was observed under identical conditions (Fig. 5B, bottom panel), reconfirming the results of DNA fragmentation mentioned above.

In parallel, the effect of these stressful conditions on the accumulation of Hsp70 in the mantle and gill tissues was tested. The results of these experiments revealed that in gills, Cu^{2+} or hyperthermia (30°C) induced a significant increase of Hsp70 levels (5.14±0.45-fold and 4.21±0.50-fold relative to control values for Cu^{2+} and hyperthermia, respectively), whereas 1 μmol l^{-1} SB203580 abolished the Hsp70 accumulation induced by 1 μmol l^{-1} Cu^{2+}, a result reconfirming the involvement of p38-MAPK in an anti-apoptotic signalling pathway in this tissue (Fig. 6). Interestingly, the combined effect of Cu^{2+} and hyperthermia did not result in an additive Hsp70 accumulation (Fig. 6A,B). Respective experiments on mantle tissue extracts, from animals subjected to identical stresses, showed that in this tissue there is no apparent increase in Hsp70 levels (data not shown), a result consistent with those obtained above for DNA fragmentation and caspase-3 activation (Fig. 5), indicating the induction of a possible pro-apoptotic signalling pathway.

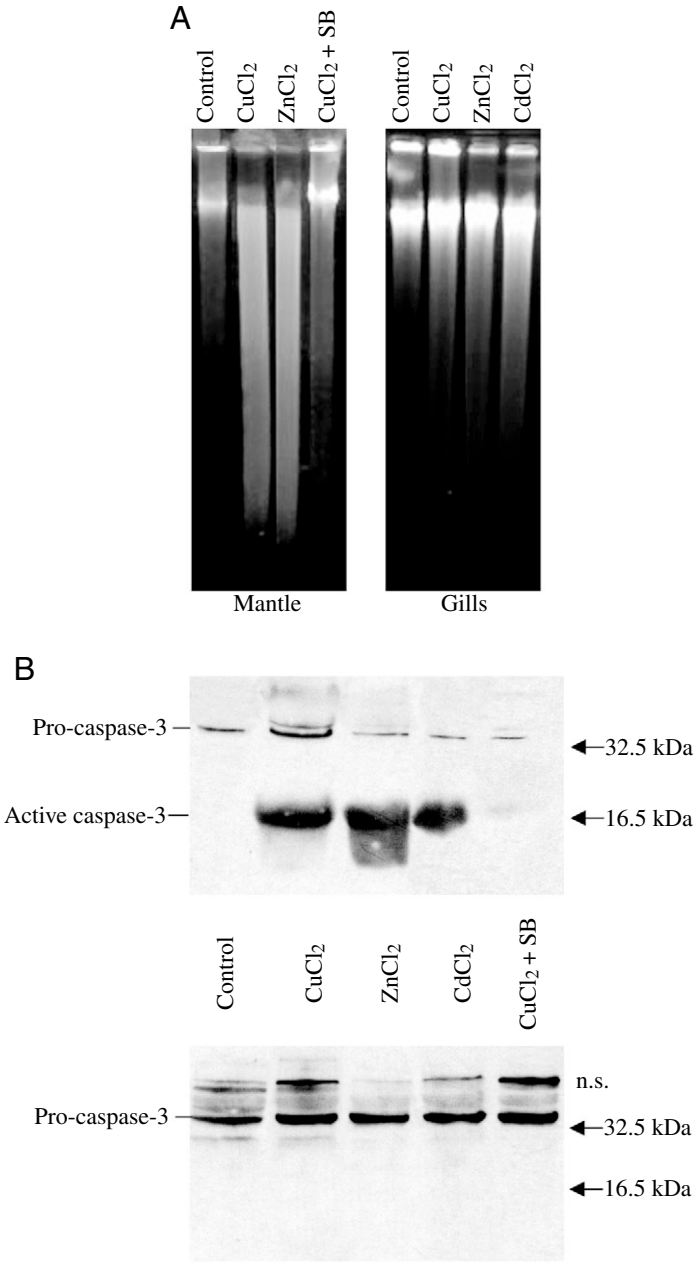


Fig. 5. DNA fragmentation and caspase-3 activation in the mantle and gill tissues from *M. galloprovincialis* specimens treated with various heavy metals. (A) (Left panel) DNA fragmentation induced by 1 μmol l^{-1} $CuCl_2$ for 30 min, in the absence or presence of 1 μmol l^{-1} SB203580, or 50 μmol l^{-1} $ZnCl_2$ for 30 min in the mantle tissue. (Right panel) DNA fragmentation induced by 1 μmol l^{-1} $CuCl_2$ for 30 min, 50 μmol l^{-1} $ZnCl_2$ for 30 min or 1 μmol l^{-1} $CdCl_2$ for 60 min in the gill tissue. Gels shown are representative of three independent experiments performed with similar results. (B) Specimens (four animals per group) were incubated in normal seawater (controls) or treated with either 1 μmol l^{-1} $CuCl_2$ in the absence or presence of 1 μmol l^{-1} SB203580, 50 μmol l^{-1} $ZnCl_2$ or 1 μmol l^{-1} $CdCl_2$ (for 30, 30 or 60 min, for each heavy metal, respectively). Endogenous full-length pro-caspase-3 and large active fragments of caspase-3 were detected using a specific rabbit monoclonal antibody in extracts (100 μg of protein) from mantle (top panel) and gill (bottom panel) tissues. Western blots shown are representative of four independent experiments performed with similar results. n.s., non specific.

Discussion

Marine bivalves constitute an intriguing candidate experimental system for studying the effects of environmental stress on multiple aspects of physiology at the cellular and molecular level. These organisms are able to survive in a wide range of oxygen concentrations, ranging from anoxic to high levels of dissolved oxygen, and variations in this ability have been proposed as an index of environmental stress. It is also widely known that free radicals produced in animal tissues during re-oxygenation following an oxygen deprivation can induce oxidative stress. Furthermore, various trace metals are capable of becoming involved in processes leading to oxidative stress in molluscs, *via* the Fenton reaction, which can result in the production of oxyradicals. Therefore, oxyradicals can be highly toxic to aquatic organisms, often resulting in lipid peroxidation in membranes, altered pyridine nucleotide redox status and DNA damage (Lemaire and Livingstone, 1993; Canesi et al., 1998; Lopez-Barea and Pueyo, 1998; Frenzilli et al., 2001). However, the signal transduction pathways involved in defence mechanisms remain obscure.

Among the various signal transduction pathways involved in the responses to environmental stress, MAPKs have been

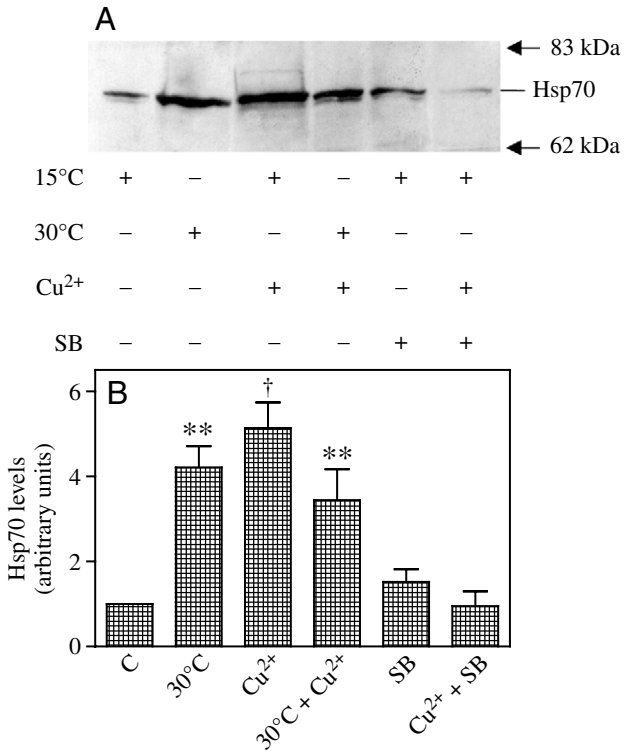


Fig. 6. Induction of Hsp70 expression by thermal stress and/or 1 μmol l^{-1} $CuCl_2$ in *M. galloprovincialis* gill tissue. (A) Hsp70 was detected in extracts (100 μg of protein) from gill tissue of animals incubated at 30°C for 30 min either in the absence or presence of 1 μmol l^{-1} $CuCl_2$ or at 15°C with 1 μmol l^{-1} $CuCl_2$ in the absence or presence of 1 μmol l^{-1} SB203580 for 30 min. (B) Densitometric analysis of Hsp70 bands by laser scanning. Results are means ± S.E.M. for four independent experiments performed with similar results. The molecular mass markers (kDa) are shown to the right of the panel. †P<0.001, **P<0.01 *versus* control value.

shown to play a significant role (Schaeffer and Weber, 1999; Widmann et al., 1999; Kyriakis and Avruch, 2001). In particular, p38-MAPK has been characterised as the principal stress-kinase responsive to fluctuations in ambient osmolality and temperature (Zhang and Cohen, 1996; Kultz and Burg, 1998; Gon, 1998).

In the present study, we investigated the response of the p38-MAPK signalling pathway to acute thermal stress and various heavy metals such as Cu^{2+}, Zn^{2+} and Cd^{2+}, as well as its possible implication in either anti-apoptotic or pro-apoptotic events. Our results show, for the first time, that identical stressful conditions exemplified *in vivo* at the whole-animal level lead to different tissue-specific events.

As the first step in the present investigation, we examined the effect of various heavy metals at sub-lethal concentrations on the p38-MAPK signalling pathway in both mantle and gill tissues of *M. galloprovincialis*. The results of our studies revealed that both 1 μmol l^{-1} Cu^{2+} and 50 μmol l^{-1} Zn^{2+} induced a strong and transient phosphorylation (hence activation) of p38-MAPK in the mantle tissue. Comparison of the results obtained using these two trace metals clearly showed that mantle tissue p38-MAPK is more sensitive to Cu^{2+} than to Zn^{2+} (Fig. 1), a result supported by previous studies suggesting that the former induces oxidative stress *via* the Fenton reaction (for a review, see Galaris and Evangelou, 2002). On the contrary, 1 μmol l^{-1} Cd^{2+} induced a biphasic time-dependent kinase phosphorylation profile (Fig. 1), a result that may be explained by the possibility that this specific kinase can be re-activated by either its upstream or downstream substrates (Widmann et al., 1999; Kyriakis and Avruch, 2001).

Furthermore, the selective p38-MAPK inhibitor SB203580 (1 μmol l^{-1}) was found to abolish this kinase phosphorylation induced by 1 μmol l^{-1} Cu^{2+}, confirming that this signalling pathway is specifically induced by the stressful condition examined (Fig. 2). In addition, our results support the suggestion that in *M. galloprovincialis* mantle tissue either the α or the β_1 p38-MAPK isoform exists, since only these two are selectively inhibited by SB203580 (Goedert et al., 1997; Kumar et al., 1997). Although several investigators propose that pyridinylimidazoles inhibit the enzyme activity rather than the phosphorylation of p38-MAPK (Lee et al., 1994; Cuenda et al., 1995), we have clearly demonstrated that SB203580 inhibits stimulus-induced phosphorylation of p38-MAPK. Recent studies support our finding, suggesting that this inhibition may be due to its binding to the inactive form of the kinase, resulting in a significant reduction of the kinase activation rate (Frantz et al., 1998; Aggeli et al., 2001; Moro et al., 2005).

Examination of the effects of the trace metals mentioned above on the gill p38-MAPK activation showed qualitatively and quantitatively a quite different time-dependent profile. In particular, 1 μmol l^{-1} Cu^{2+} and 50 μmol l^{-1} Zn^{2+} induced a strong and prolonged p38-MAPK activation for 60 min, whereas 1 μmol l^{-1} Cd^{2+} induced a strong, rapid activation of the kinase that remained elevated significantly above basal levels for at least 2 h (Fig. 3). The differential p38-MAPK activation by these trace metals in mantle and gill tissues may reflect their different physiological function.

Furthermore, we have found that hyperthermia (30°C) induced the p38-MAPK activation in a rapid (maximum at 30 min), sustained (over 120 min) and considerable way, whereas the effect of hypothermia was not so intense (Fig. 4). The moderate response of p38-MAPK to hypothermia in this tissue could be attributed to the fact that these organisms are routinely subjected to hypothermic stress and have consequently developed multiple adaptive responses in order to preserve their function under analogous conditions (Sheehan and Power, 1999; Lesser and Kruse, 2004). On the other hand, the pleiotropic effects of heat are likely to lead to the activation of multiple protein kinases, including p38-MAPK, which may then regulate stress response, apoptosis or may facilitate the repair of damaged proteins and other cellular components (Woessmann et al., 1999). However, the detailed regulation of heat shock response through activation of these signalling pathways remains to be determined.

A growing body of evidence suggests that the p38-MAPK signalling pathway is involved in a variety of complicated cellular

responses. It has been shown that p38-MAPK cascade promotes either cell death (Sarkar et al., 2002; Porras et al., 2004) or cell survival (Liu et al., 2001; Park et al., 2002), depending on the cell type and the kinase isoforms activated by various stressful stimuli (Kyriakis and Avruch, 2001; Roux and Blennis, 2004; Wada and Penninger, 2004). In the present study, we made an effort to examine the effect of selected stressful conditions that induce strong p38-MAPK phosphorylation on the caspase-3 activation, genomic DNA fragmentation and Hsp70 induction in *M. galloprovincialis* mantle and gill tissues. The results of our studies clearly demonstrate that mantle tissue responses to the heavy metals mentioned above are quite different compared with gill tissue responses to identical stresses. In particular, we showed for the first time that these trace metals induce a pro-apoptotic event in the mantle tissue, possibly *via* the p38-MAPK signalling pathway, since the p38-MAPK selective inhibitor abolishes both the caspase-3 activation and the DNA fragmentation induced by such stimuli (Fig. 5).

On the contrary, identical *in vivo* stimuli seem to lead to an anti-apoptotic event in gills, *via* this signalling pathway, a result that is strongly supported by the absence of DNA fragmentation and/or caspace-3 activation (Fig. 5) but by the induction of Hsp70 levels (Fig. 6). Our results are in accordance with the previously described induction of metallothioneins and various antioxidant enzymes such as superoxide dismutase and catalase by Fe in *M. galloprovincialis* digestive gland (Viarengo et al., 1999; Cavaletto et al., 2002).

It has also been previously reported that differences in the accumulation of Hsp70 and other stress proteins might be useful in identifying tissues that are particularly vulnerable to damage by a specific stressor (Sanders et al., 1994). Our results are in accordance with those described by Chapple et al. (1997), who also reported that hyperthermia induces in gills the largest increase in Hsp70 levels, compared with the mantle and the adductor muscle of *Mytilus edulis*.

Mussels have been the subject of several stress protein studies and represent the organisms for which elevated levels of stress proteins have been established at natural environmental temperatures (Hofman and Somero, 1995). Members of the Hsp70 family function as chaperones, including aiding assembly, proper folding and the intracellular transport of proteins, thereby helping to protect cells from thermal or other stress-induced damage (Morimoto et al., 1990; Gupta and Golding, 1993). According to recent studies, Hsp70s antagonise the apoptosis-inducing factor, and therefore these family members can function as potent endogenous modulators of the apoptotic cell death (Garrido et al., 2001; Zhang et al., 2002; Takayama et al., 2003).

In conclusion, the results of the present study demonstrate that, in *Mytilus galloprovincialis*, diverse stimuli induce either pro-apoptotic or anti-apoptotic events *via* the p38-MAPK signalling pathway depending on the cell type examined. The differential tissue-specific responses may reflect the presence of different p38-MAPK isoforms and/or the different physiology of these tissues at the cellular and molecular level.

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