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Various stressors rapidly activate the p38-MAPK signaling pathway in *Mytilus galloprovincialis* (Lam.)

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Abstract

The stimulation of p38-MAPK signal transduction pathway by various stressful stimuli was investigated in the marine bivalve *M. galloprovincialis*. Oxidative stress (5 μM H_2O_2) induced a biphasic pattern of p38-MAPK phosphorylation with maximal values attained at 15 min (8.1-fold) and 1 h (8.0-fold) of treatment respectively. Furthermore, 1 μM SB203580 abolished the p38-MAPK phosphorylation induced by oxidative stress. Aerial exposure also induced a biphasic pattern of p38-MAPK phosphorylation, with maximal values attained at 1 h (6.8-fold) and 8 h (4.9-fold) respectively. Re-oxygenation following a 15 min of aerial exposure resulted in the progressive dephosphorylation of the kinase. Treatment with 0.5 M sorbitol (in normal seawater) induced the rapid kinase phosphorylation (9.2-fold) and this effect was reversible. Seawater salinities varying between 100–60% had no effect, whereas a salinity of 50% induced a significant p38-MAPK phosphorylation. Furthermore, hypertonicity (120% seawater) resulted in a moderate kinase phosphorylation. All the above results demonstrate for the first time in a marine invertebrate imposed to environmental and other forms of stress as an intact, living organism, that the p38-MAPK pathway is specifically activated by various stressful stimuli which this animal can often face and sustain *in vivo*. (Mol Cell Biochem **260**: 119–127, 2004)

Key words: p38-MAPK, osmotic stress, oxidative stress, *M. galloprovincialis*, mussel, marine bivalve, anoxia, mantle, signal transduction

Abbreviations: MAPK – mitogen-activated protein kinase; ERK – extracellular signal-regulated kinase; JNK – c-Jun N-terminal kinase; p38-MAPK/RK – p38 reactivating kinase; PAGE – polyacrylamide gel electrophoresis; TBS – Tris-buffered saline; DMSO – dimethylsulfoxide; PMSF – phenylmethyl sulfonyl fluoride; DTT – dithiothreitol; ECL – enhanced chemiluminescence; A/R – aerial exposure/reoxygenation

Introduction

Bivalve molluscs such as *Mytilus sp*. are remarkable among marine invertebrates for their highly developed anoxia tolerance and commonly are used as biomarkers in the assessment of environmental quality. Furthermore, these animals are filter-feeding organisms and also capable of concentrating xenobiotics [1]. Therefore, mussels have a number of properties, which make them useful sentinels for environmental pollution. Biochemical and physiological mechanisms of anoxia and salinity tolerance in mussels have been extensively studied [2–4]. The ability of these animals to sustain prolonged periods of anoxia is linked with a coordinated suppression of many metabolic processes including enzymes, protein synthesis, and the movement of ions across membranes. Changes in the phosphorylation state of various en-

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zymes such as regulatory enzymes of glycolysis occur during anoxia and lead to different end-metabolic products [3]. It has been proposed therefore, that reversible protein phosphorylation could be a key mechanism of metabolic reorganization for anaerobiosis. Furthermore, oxidative stress induced by either anoxia or various oxidants such as heavy metals has been shown to result in the activation of antioxidant defense mechanisms, which protect these organisms under such conditions [5, 6]. Recent studies have shown that anoxia induces changes in gene transcription in both, vertebrate [7, 8] and invertebrate tissues [9], indicating that up-regulation of selected genes is important for anoxia survival.

Although the precise mechanisms responsible for triggering gene activation are still unknown, various intracellular pathways that transduce the initial signal to the nucleus may be involved. Among these signaling pathways, the ones including mitogen-activated protein kinases (MAPKs) have been implicated in the regulation of various physiological responses [10, 11]. The basic assembly of MAPK pathways is a three-component module conserved from yeast to humans. This protein kinase cascade comprises of MAPK kinase kinase (also referred to as MEKK), MAPK kinase (also referred to as MEK) and MAPK [12].

Three major subfamilies have been characterized, including the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs) and the p38-MAPKs [10, 11, 13–16]. Although ERKs are mainly involved in mediating anabolic processes such as cell division, growth and differentiation, JNKs and p38-MAPKs are generally associated with cellular response to diverse stresses. Studies on the characterization and function of MAPK modules have been largely conducted using principally mammalian models and to a lesser extent a number of non mammalian experimental systems including *Xenopus laevis*, *Drosophila melanogaster* and *Caenorhabditis elegans* [17], and recently mussel hemocytes or isolated digestive gland cells [18, 19]. Therefore, although these kinases are highly conserved, there is limited information available to indicate their existence and/or function in marine invertebrates. Furthermore, the possible roles of these protein kinases in mediating adaptive organ-specific responses to environmental stresses imposed on intact stress-tolerant living organisms are unknown.

Here, we provide evidence for the expression and activation of p38-MAPK signaling pathway in the mantle of the marine invertebrate *Mytilus galloprovincialis* in response to various forms of stress such as aerial exposure, aerial exposure/re-oxygenation, oxidative stress, hypoosmotic and hyperosmotic stress. These organisms that often face various environmental stresses, may thus prove to be a valuable experimental tool that will provide interesting insights on the function and role of MAPK pathways in important physiological processes.

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 751 84, Sweden). Bradford protein assay reagent was from Bio-Rad (Hercules, CA, USA). Nitrocellulose (0.45 μm) was obtained from Schleicher and Schuell (Keene, NH, USA). P38-MAPK specific inhibitor SB203580 was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA) and a stock solution (10 mM) was prepared in DMSO.

Rabbit polyclonal antibodies specific for total p38-MAPK, as well as for the dually phosphorylated p38-MAPK were obtained from Cell Signalling (Beverly, MA, USA). Anti-histone 1 mouse monoclonal antibody was obtained from BioGenex (CA, USA). Prestained molecular mass markers were from New England Biolabs (Beverly, MA, USA). Biotinylated anti-rabbit and anti-mouse antibodies were from DAKO A/S (DK-2600 Glostrup, Denmark). X-OMAT AR film (13 × 18 cm) was purchased from Eastman Kodak Company (New York, USA).

Animals

Male or female adult mussels (75–80 mm length) *M. galloprovincialis* (Lam.), were obtained from a local dealer and had been collected (from March up to September 2002) in Saronikos gulf, Athens, Greece. All animals were held in re-circulating seawater (18–20°C) at the Laboratory for at least 4 days prior use.

Animal treatments

Animals (3–5 for each group) were equilibrated at 18–20°C in large tanks with re-circulating seawater for at least 4 days. For each treatment animals were transferred into smaller tanks with the proper seawater solution (about 300 ml per animal). Treatment with H_2O_2 (5 μM) in normal seawater induced oxidative stress [20]. In this case, experiments were carried out in the dark in order to minimize H_2O_2 photo-destruction, either in the absence or presence of 1 μM SB203580. Hypoxia or anoxia was induced by exposing the animals in air for increasing time intervals varying from 5 min up to 12 h, at 18°C. Utilizing 0.5 M sorbitol or excess NaCl (17% w/v) in normal seawater induced hyperosmotic stress, while diluting seawater with the convenient volume of de-ionized water induced hypoosmotic stress conditions.

After the end of each treatment, animals were put on ice, mantle was dissected, freeze-clamped between aluminum tongs cooled in liquid nitrogen, pulverized under liquid nitrogen and the powders were stored at –80°C.

Tissue extractions

Mantle powders were homogenized with 3 ml/g of buffer (20 mM Hepes, pH 7.5, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 5 mM dithiothreitol (DTT), 10 mM benzamidine, 200 µM leupeptin, 120 µM pepstatin A, 10 µM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 300 µM 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 M 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 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 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (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 × 5 min) the blots were incubated with horse-radish peroxidase-linked anti-rabbit 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 × 5 min) and the bands were detected using ECL with exposure to X-OMAT AR film. Blots were quantified by laser scanning densitometry.

Subcellular fractionation of phospho-p38-MAPK

Subcellular fractionation was performed by the method described by Takemoto *et al*. [21] with slight modifications [22]. Briefly, frozen tissues were homogenised in 3 volumes of 10 mM HEPES pH 7.9, which contained 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 0.5 mM PMSF, 20 mM β-glycerophosphate, 4 µg/ml aprotinin and 2 µg/ml leupeptin. After incubation on ice for 15 min, homogenates were centrifuged (5,000 g, 10 min, 4°C) and 10% (v/v) Nonidet P40 was added. Samples were vigorously mixed and further incubated on ice for 60 min. Homogenates were centrifuged (1,000 g, 10 min, 4°C) to obtain pellets. Pellets were washed once and re-suspended in a buffer containing 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1.5 mM $MgCl_2$, 20% (v/v) glycerol, 10 mM NaF, 1 mM Na_3VO_4, 0.5 mM PMSF, 0.2 mM DTT, 20 mM β-glycerophosphate, 4 µg/ml aprotinin and 2 µg/ml leupeptin. Tubes were incubated on ice for 60 min and then rocked for 15 min at 4°C. After centrifugation (15,000 g, 10 min, 4°C), the supernatants containing nuclear protein were boiled with 0.33 vol of SDS/PAGE sample buffer.

Statistical evaluations

Western blots shown are representative of at least 3 independent experiments. Each data point represents the mean ± S.E. of samples from at least 3 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. p38-MAPK activation in 'control' animals was set at 1, and the stimulated p38-MAPK activation in treated animals was expressed as fold activation over 'controls'.

Results

As the first step in the present study, oxidative stress as exemplified by direct exposure of specimens to 5 µM H_2O_2 in normal seawater was examined. Phosphorylation of p38-MAPK was assessed by immunoblotting, using a polyclonal antibody specific for the dually phosphorylated (hence activated) form of the kinase. Animal exposure to this stressor induced a biphasic phosphorylation pattern of the kinase in *M. galloprovincialis* mantle. In particular, a rapid maximal phosphorylation of the kinase (approximately 8.1 ± 1.0-fold relative to controls, $p < 0.01$) was observed at 15 min, while a second maximum (approximately 8.0 ± 0.6-fold relative to controls, $p < 0.001$) was detected at 60 min (Figs 1A, top panel and 1B). Furthermore, the selective p38-MAPK inhibitor SB203580 even at a concentration of 1 µM abolished the phosphorylation of the kinase induced by 5 µM H_2O_2 (Figs 2A, top panel and 2B). Equivalent protein loading was confirmed by probing identical samples with an antibody recognizing total p38-MAPK levels (Figs 1A and 2A, bottom panels).

In Fig. 3 the effect of increasing time periods of aerial exposure on the p38-MAPK phosphorylation is shown. For these experiments, animals (4–5 per group) were exposed to air for various time intervals varying from 15 min up to 12 h. The results of these experiments clearly showed that aerial

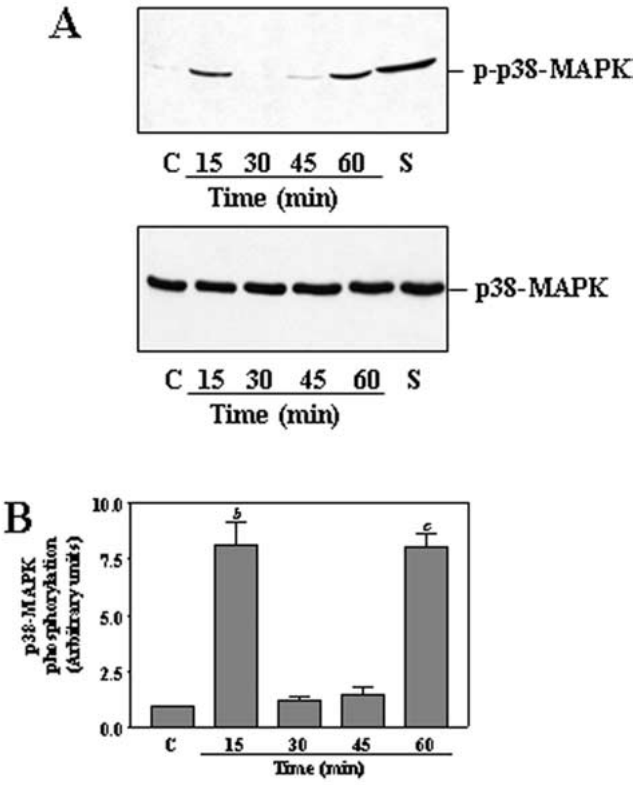

Fig. 1. Time course of p38-MAPK phosphorylation in the mantle of *M. galloprovincialis*, in response to oxidative stress. (A, top panel): phospho p38-MAPK was detected in extracts (100 μg of protein) from the mantle of control animals (C), animals subjected to hyperosmotic stress with 0.5 M sorbitol for 15 min (S), or animals treated with 5 μM H_2O_2 for increasing time periods (varying from 15 up to 60 min). (A, bottom panel): immunoblot of identical samples for total p38-MAPK levels were included as a control for protein loading (B): Densitometric analysis of phospho p38-MAPK bands by laser scanning. Results are means ± S.E. for 4 independent experiments performed with similar findings. Western blots shown are representative of four independent experiments. $^{c}p < 0.001$ vs. control value; $^{b}p < 0.01$ vs. control value.

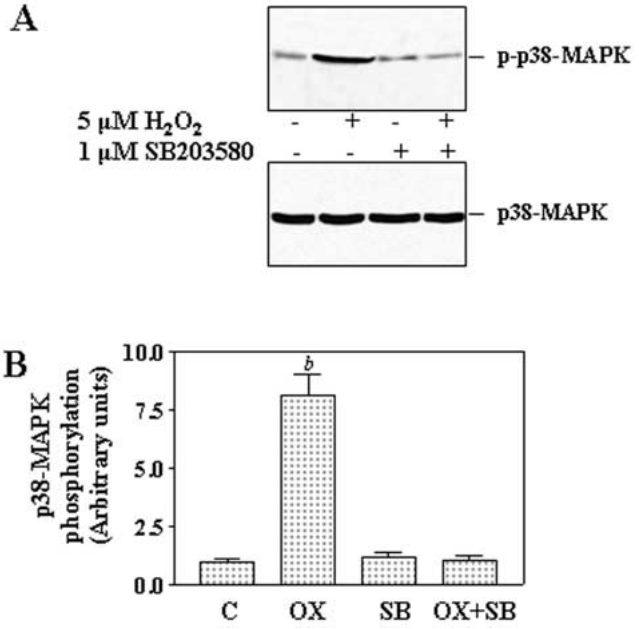

Fig. 2. Effect of SB203580 on the p38-MAPK phosphorylation induced by oxidative stress. (A, top panel): phospho p38-MAPK was detected in extracts (100 μg of protein) from the mantle of control animals, animals treated with 5 μM H_2O_2 for 15 min either in the absence or presence of 1 μM SB203580. (A, bottom panel): immunoblot of identical samples for total p38-MAPK levels were included as a control for protein loading. (B): Densitometric analysis of phospho p38-MAPK bands by laser scanning. Results are means ± S.E. for 4 independent experiments performed with similar findings. Western blots shown are representative of four independent experiments. $^{b}p < 0.01$ vs. control value. C, control; OX, oxidative stress.

exposure induced a biphasic phosphorylation pattern of p38-MAPK in *M. galloprovincialis* mantle. In particular, a rapid maximal phosphorylation of the kinase (approximately 6.8 ± 0.5-fold relative to controls, $p < 0.001$) was observed at 1 h, while a second maximum (approximately 4.9 ± 0.2-fold relative to controls, $p < 0.001$) was detected at 8 h of aerial exposure. p38-MAPK reached control values at 12 h of exposure to air (Figs 3A, top panel and 3B). The phosphorylation state of p38-MAPK also increased upon re-oxygenation following a 15 min of aerial exposure. In particular, aerial exposure/re-oxygenation induced phosphorylation of the kinase was further increased at 5 min (approximately 4.2 ± 0.1 vs. 3.1 ± 0.2-fold, $p < 0.01$, for 15 min aerial exposure and 5 min re-oxygenation, respectively), with a progressive decline thereafter, reaching control values at 60 min (Figs 3C, top panel and 3D). Bottom panels in Figs 3A and 3C show that there were no changes in the total cellular pools of p38-MAPK and therefore provide a control for protein loading under these conditions.

To determine the time course of the effect of extracellular osmolarity on the phosphorylation of *M. galloprovincialis* mantle p38-MAPK, specimens were subjected to various either hypertonic or hypotonic solutions for time periods varying from 15 min up to 2 h. The phosphorylation state of p38-MAPK was assessed by immunoblotting with an antibody specific for the dually phosphorylated (activated) form of the kinase. Sorbitol (0.5 M), the most potent activator of p38-MAPK in all cell types examined to date, induced a rapid increase in p38-MAPK phosphorylation (Figs 4A and 4B) which was comparable to the one induced in the amphibian heart [23]. The time course of p38-MAPK phosphorylation by 0.5 M sorbitol showed that phosphorylation was maximal within 15 min (approximately 9.2 ± 2.1-fold relative to control values, $p < 0.05$) and remained considerably elevated for at least 60 min (Figs 4A and 4B).

Following a 15 min exposure of the animals to this hypertonic solution, we examined the effect of recovery to normal seawater (for 5 min up to 2 h). Our results clearly showed

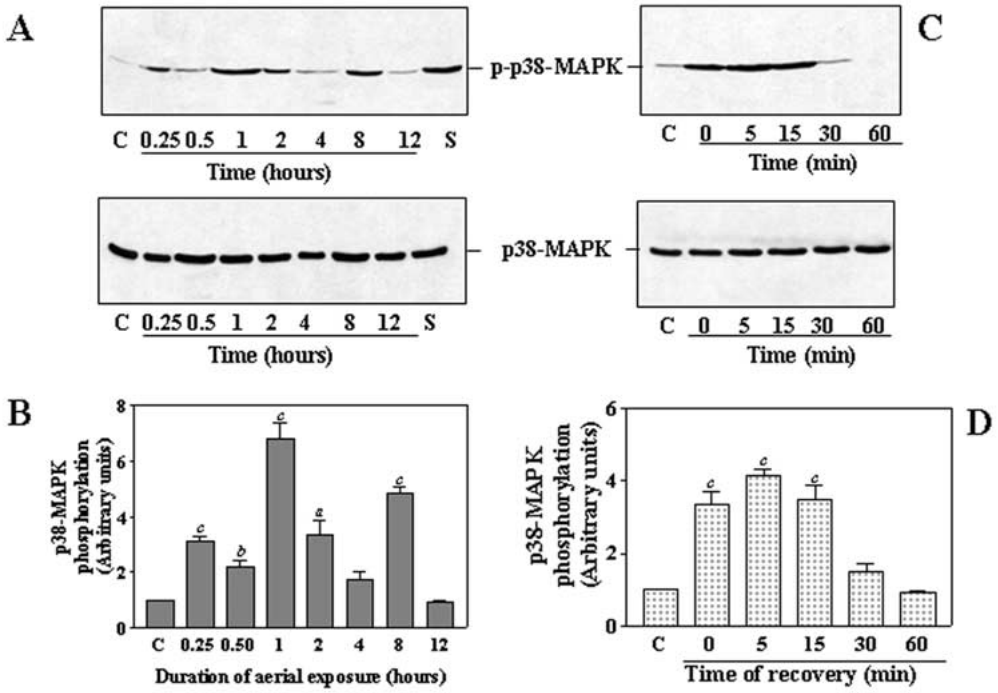

Fig. 3. Time course of p38-MAPK phosphorylation in *M. galloprovincialis* mantle, in response to aerial exposure or aerial exposure/re-oxygenation. (A, top panel): phospho p38-MAPK was detected in extracts (100 μg of protein) from the mantle of control animals (C), animals subjected to hyperosmotic stress with 0.5 M sorbitol for 15 min (S), or animals subjected to anoxia for the times indicated; (C, top panel): phospho p38-MAPK was also detected in extracts (100 μg of protein) from the mantle of animals subjected to aerial exposure for 15 min followed by re-oxygenation for the times indicated. (A, C bottom panels): immunoblots of identical samples for total p38-MAPK levels were included as a control for protein loading (B and D): Densitometric analysis of phospho p38-MAPK bands by laser scanning. Results are means ± S.E. for 5 independent experiments performed with similar findings. Western blots shown are representative of 5 independent experiments. $^{c}p < 0.001$ vs. control value, $^{b}p < 0.01$ vs. control value; $^{a}p < 0.05$ vs. control value.

that p38-MAPK phosphorylation by 0.5 M sorbitol was reversible (Figs 4C and 4D). Interestingly, a 5 min recovery period with normal seawater resulted in an approximately 70% decrease of the kinase phosphorylation levels (~ 2.9 ± 0.5-fold relative to control values, $p < 0.05$), with its phosphorylation showing a progressive decline that reached control values after a period of 120 min (Figs 4C and 4D). Equivalent protein loading was confirmed by probing identical samples with an antibody recognizing the total p38-MAPK levels (Data not shown).

To examine the effects of hyposmoticity or hyperosmoticity on the p38-MAPK phosphorylation we subjected specimens (for 30 min) to seawater of decreasing or increasing salinity with a range varying between 50–120% of normal. The results of these experiments showed that hyposmotic solutions with a salinity as low as 60% of normal seawater induced no significant phosphorylation of p38-MAPK (Figs 5A, top panel and 5B). However, a 50% salinity of normal induced a considerable kinase phosphorylation, comparable with the one induced by 0.5 M sorbitol (Figs 5A, top panel and 5B). The time course of *M. galloprovincialis* p38-MAPK response to 50% of normal seawater was also tested. The results of this study showed that p38-MAPK phosphorylation levels maximized within 30 min (approximately 7.3 ± 0.8-fold relative to controls, $p < 0.01$) and remained elevated for at least 60 min, reaching control values at 120 min (Figs 5C, top panel and 5D). On the contrary, hypertonicity (120% seawater) had a slight only effect whatsoever on the phosphorylation of p38-MAPK (Figs 5A, top panel and 5B). Bottom panels in Figs 5A and 5C show that there were no changes in the total cellular pool of p38-MAPK and therefore provide a control for protein loading under these conditions.

It is well known that p38-MAPK is involved in the phosphorylation of substrates localized not only in the cytoplasm but in the nucleus as well. Thus, it was of interest to examine the subcellular localization of phosphorylated p38-MAPK modulated by aerial exposure, aerial exposure/re-oxygenation or hyperosmotic stress. For this purpose, extracts of mantle from specimens subjected to various forms of stress were fractionated. Purity and protein homogeneity of the nuclear fractions were confirmed by immunodetection of the nuclear marker protein histone 1 (Fig. 6B).

Results in Fig. 6A demonstrate that phospho p38-MAPK is present in both, cytosolic and nuclear fractions. The stressful stimuli tested induced a strong phosphorylation of the cytosolic p38-MAPK and a moderate phosphorylation of the

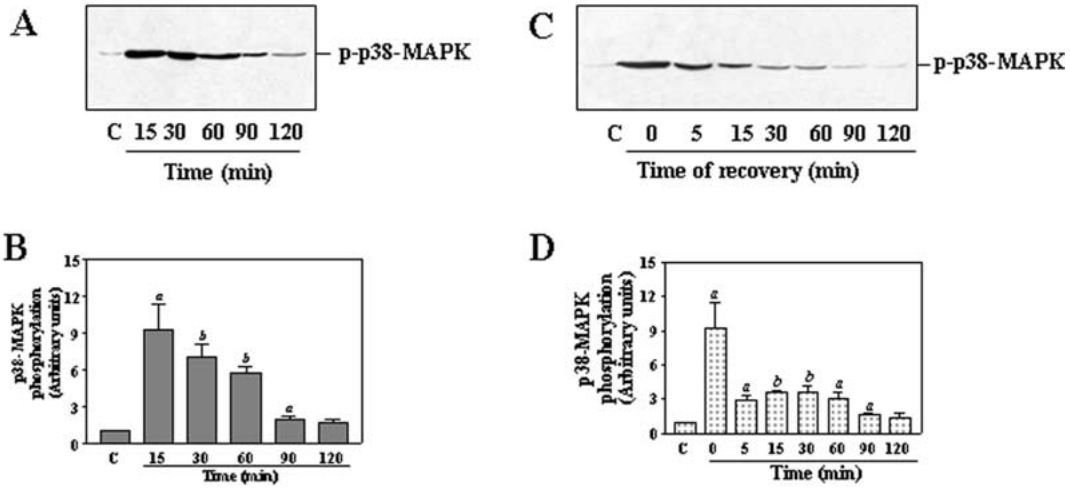

Fig. 4. Time course of p38-MAPK phosphorylation in the mantle of *M. galloprovincialis*, in response 0.5 M sorbitol. Phospho p38-MAPK was detected in extracts (100 µg of protein) from the mantle of animals treated with 0.5 M sorbitol for 15 up to 120 min (A), or for 15 min followed by recovery to normal seawater for the indicated time periods (C). (B), (D): Densitometric analysis of phospho p38-MAPK bands by laser scanning. Results are means ± S.E. for 3 independent experiments performed with similar results. Western blots shown are representative of 3 independent experiments. $^{b}p < 0.01$ vs. control value; $^{a}p < 0.05$ vs. control value.

nuclear respective kinase. In particular, in the cytosolic fraction, all stressful conditions examined resulted in a considerable phosphorylation of p38-MAPK (approximately 5.5 ± 0.3, 6.8 ± 0.3, and 7.6 ± 0.4-fold relative to control values, $p < 0.01$, for aerial exposure, aerial exposure/re-oxygenation or 0.5 M sorbitol, respectively). These phosphorylation lev-

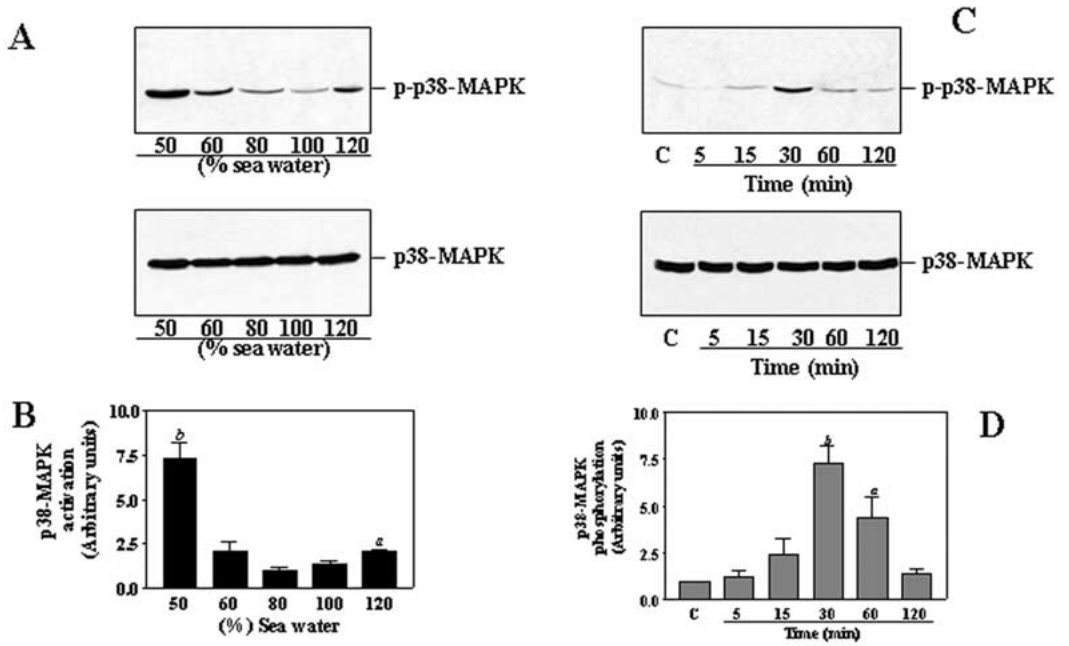

Fig. 5. Effect of salinity on the p38-MAPK phosphorylation in the mantle of *M. galloprovincialis*. (A, top panel): Phospho p38-MAPK was detected in extracts (100 µg of protein) from the mantle of control animals (100% seawater) or animals subjected to various hypotonic (varying from 50 up to 80% of sea water) or hypertonic (120% seawater) environments for 30 min. (C, top panel): phospho p38-MAPK was also detected in extracts (100 µg of protein) from the mantle of control animals (C) or animals treated with 50% seawater for increasing time periods (varying from 5 up to 120 min). (A, C, bottom panels): Identical samples were also assessed for total (phosphorylation state independent) p38-MAPK. (B, D): Densitometric analysis of phospho p38-MAPK bands by laser scanning. Results are means ± S.E. for 3 independent experiments performed with similar results. Western blots shown are representative of 3 independent experiments. $^{b}p < 0.01$ vs. control value; $^{a}p < 0.05$ vs. control value.

els were comparable with the ones observed in whole tissue extracts from specimens subjected to the respective forms of stress described above. In parallel, the same stressful conditions also induced a moderate phosphorylation of the kinase in the nuclear fraction (approximately 2.0 ± 0.1-fold relative to control values, $p < 0.05$).

Discussion

Activation of the MAPK signaling pathways has been extensively investigated in numerous cell types (reviewed in [11–17]). In particular, p38-MAPK has been characterized as the principal stress-kinase responsive to fluctuations in ambient osmolality and temperature [24, 25]. In this study, we investigated the response of p38-MAPK to various forms of environmental and other stresses in the *M. galloprovincialis* mantle. The model system we studied allows the elucidation of another diverse role of p38-MAPK in organisms with a different physiology compared to that of vertebrates.

Among marine bivalves the mussel *Mytilus* represents an organism that often faces various stressful environmental conditions, which lead to oxidative stress. Many recent studies *in vitro* and *in vivo* have shown that oxidative stress induces the activation of various antioxidant enzymes and metallothioneins [1, 5, 6, 26–28]. However, the signaling pathways implicated in this adaptation remain obscure. Here, we have examined the effect of oxidative stress induced by a relatively low H_2O_2 concentration (5 μM) on the principal stress- activated member of MAPKs, the p38-MAPK in *M. galloprovincialis* mantle. Our results clearly showed that oxidative stress induces a strong, rapid (within 15 min) phosphorylation of the kinase and a second maximum phosphorylation at 60 min of treatment (Fig. 1), and that the selective p38-MAPK inhibitor SB203580 (1 μM) abolishes this activation (Fig. 2). The biphasic phosphorylation pattern of the kinase observed may be explained by the possibility that this specific kinase can be re-activated by its either upstream or downstream substrates [10, 17]. Furthermore, the phosphorylated p38-MAPK could in turn stimulate MAPKAPK2 activity and increase the phosphorylation of the small HSP27 [29]. This particular signaling pathway may have an important cytoprotective function in this organism and will be further investigated.

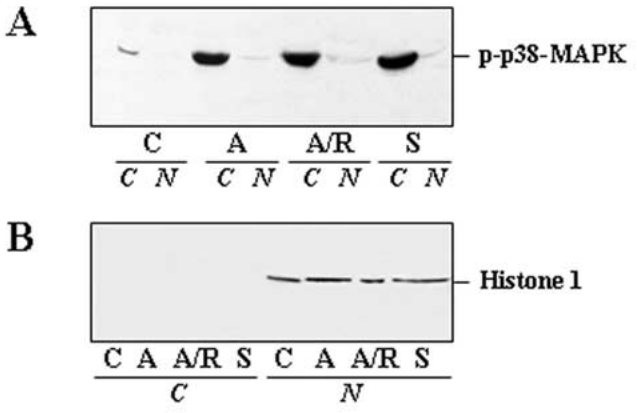

Fig. 6. Subcellular localization of phospho p38-MAPK during environmental or other forms of stress. (A) Subcellular fractions were prepared from the mantle of control animals (C), animals subjected to aerial exposure for 15 min (A), animals subjected to re-oxygenation for 5 min following a 15 min aerial exposure (A/R) or 0.5 M sorbitol (S) for 15 min. The cytoplasmic (C) or nuclear (N) fractions were subjected to immunoblotting with a polyclonal antibody specific for the dually phosphorylated p38-MAPK. (B) Densities of the nuclear marker protein histone 1 are also shown. In this case, proteins were separated by SDS-PAGE on 15% (w/v) acrylamide, 0.332% (w/v) bisacrylamide slab gels and immunoblotted using a mouse monoclonal antibody specific for this nuclear protein marker. The experiment was repeated with similar results.

It is well known that *M. galloprovincialis* is remarkable among bivalves for its highly developed anoxia tolerance. Therefore, we made an effort to examine the effect of aerial exposure or aerial exposure/re-oxygenation on the p38-MAPK phosphorylation in *M. galloprovincialis* mantle. The results of these experiments showed that oxygen deprivation induces a strong phosphorylation of the kinase with a biphasic profile in respect to duration of this limitation (Fig. 3). The immediate strong kinase phosphorylation induced by oxygen limitation, may reflect preparatory responses that may be key to triggering protective changes in gene expression or metabolic re-organization that is needed for long-term anoxia survival. The biphasic pattern of the kinase phosphorylation could be explained by the fact that the energy metabolism in this tissue is suppressed during anoxia possibly via the reversible phosphorylation of key regulatory glycolytic enzymes leading to different metabolic end products [2–4]. Furthermore, re-oxygenation following a 15 min of aerial exposure resulted in a further increased phosphorylation of the kinase at 5 min, with a progressive dephosphorylation thereafter, reaching control values within 60 min (Figs 3C and 3D). Similar activation of the p38-MAPK signaling pathway by ischaemia or ischaemia/reperfusion has been reported for the mammalian heart [11, 30]. On the contrary, it has been reported the lack of p38-MAPK responses to anoxia by either anoxia or freeze tolerant vertebrates [23, 31–33].

Sorbitol (0.5 M) is the most potent activator of p38-MAPK in all cell types examined to date [10, 16]. According to numerous previous reports, hyperosmotic stress induced by 0.5 M sorbitol, also induced a significant phosphorylation of *M. galloprovincialis* mantle p38-MAPK, a result indicative of this conservative signaling pathway from yeast to humans. The time course of the kinase phosphorylation by sorbitol observed, clearly showed that this effect is reversible (Figs 4C and 4D). Furthermore, in animals treated with sorbitol for

15 min followed by the removal of the agent from their environment a progressive decline of p38-MAPK phosphorylation was observed, reaching control values within 2 hours of recovery (Figs 4C and 4D). The above results are consistent with our previous reports concerning the activation of the kinase by 0.5 sorbitol in the amphibian heart [23, 34].

Bivalves being remarkable for their ability to face and survive in extremely varying salinities of their environment [2, 3], we further examined the effect of hyperosmolality and hyposmolality of the seawater on the p38-MAPK phosphorylation. The results of these experiments showed that seawater salinities varying between 100–60% have no significant effect on the p38-MAPK phosphorylation, whereas a slightly lower salinity of 50% results in the strong phosphorylation of the kinase (Figs 5A and 5B). On the other hand, hyperosmolality induced by the use of 120% seawater, resulted in a moderate but statistically significant ($p < 0.05$, relative to control value) phosphorylation of the kinase (Figs 5A and 5B). Furthermore, since 0.5 M sorbitol increases the seawater osmolality by ~ 50%, the responses of *M. galloprovincialis* mantle p38-MAPK to both, hyposmotic (50%) or hyperosmotic (150%) solutions obtained, support the suggestion that these responses are qualitatively similar, but they follow a different time-dependent manner. Our results on the p38-MAPK phosphorylation by hyposmolality are in contrast to various respective reports concerning either mammalian or amphibian tissues [34, 35]. Resent studies probed the role of MAPKs in osmosensory signaling pathways in gills of the fish *Fundulus heteroclitus* [36]. In this experimental model, the activity of all three MAPKs increased significantly during hyperosmotic stress and oppositely decreased under hyposmotic stress. The different p38-MAPK responses of *M. galloprovincialis* to a variety of salinities obtained may represent the physiological responses which these bivalves can face *in vivo* and possess a solute adaptation strategy capable of allowing the organism to survive as euryoxic [2, 37].

One of the characteristic features of all phosphorylated MAPKs in vertebrate tissues is their localization in both the cytoplasm and the nucleus, where they interact with their substrates, phosphorylating and activating other upstream or downstream protein kinases, cytoskeletal proteins, transcription factors etc. [11]. The biochemical analysis we performed demonstrates that stress elicits a strong increase in the cytosolic and a moderate one in the nuclear fraction of phosphorylated p38-MAPK which is associated with the presence of these proteins in both compartments (Fig. 6). While the nuclear translocation of MAPKs is generally thought to be associated with their activation, Mizukami *et al.* [38] have demonstrated that in rat hearts subjected to ischaemia/reperfusion, JNK1 translocates while inactive, from the cytoplasm to the nucleus, where it is then activated. Our results support the suggestion that in *M. galloprovincialis* mantle, p38-MAPK substrates are located in the cytoplasmic rather than in the nuclear compartment. This location could possibly represent the kinase interaction with its certain generally suggested substrates.

In conclusion, the present study demonstrates that certain environmental stressful stimuli activate p38-MAPK signaling pathway in the mantle of *M. galloprovincialis*, presenting detailed information on the magnitude and timing of these responses, as well as its subcellular localization pattern, a promising approach reported in this field for the first time at the whole organism level. The functional roles of the kinase studied in this physiological setting remain to be established, as the ultimate biological effects of its activation may depend on the duration and extent of the latter.

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