Manganese overload affects p38 MAPK phosphorylation and metalloproteinase activity during sea urchin embryonic development

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Abstract

In the marine environment, manganese represents a potential emerging contaminant, resulting from an increased production of manganese-containing compounds. In earlier reports we found that the exposure of *Paracentrotus lividus* sea urchin embryos to manganese produced phenotypes with no skeleton. In addition, manganese interfered with calcium uptake, perturbed extracellular signal-regulated kinase (ERK) signaling, affected the expression of skeletogenic genes, and caused an increase of the hsc70 and hsc60 protein levels. Here, we extended our studies focusing on the temporal activation of the p38 mitogen-activated protein kinase (p38 MAPK) and the proteolytic activity of metalloproteinases (MMPs). We found that manganese affects the stage-dependent dynamics of p38 MAPK activation and inhibits the total gelatin-auto-cleaving activity of MMPs, with the exclusion of the 90—58 kDa MMPs, whose levels remain high all throughout development. Our findings correlate, for the first time to our knowledge, an altered activation pattern of the p38 MAPK with an aberrant MMP proteolytic activity in the sea urchin embryo.

1. Introduction

Manganese (Mn) is one of the most abundant and widely distributed metals in nature, especially in natural waters, as lakes, streams, rivers, brackish water, oceans and seas. The processes controlling the biogeochemistry of Mn in seawater are complicated and not well understood. In fact, the marine environmental chemistry of Mn is largely governed by pH, oxygen concentration of the solution and redox conditions. Mn is the most important intermediate oxidant between oxygen and organic matter in marine sediments ([Van der Zee and Van Raaphorst, 2004](#)). Free Mn ion transport is regulated by molecular diffusion in the water pores and it follows a concentration gradient (the Mn gradient decreases towards the oxic zone). A reduced dissolved oxygen condition (called hypoxia) causes the rise of the Mn ionic flux, which goes from the sediment to the overlying water, where it reaches concentrations 1000-folds higher than those normally occurring in seawater (up to 22 mg l⁻¹) ([Trefry et al., 1984](#); [Aller, 1994](#)). At present, costal hypoxia is increasing because of man-made alterations of coastal ecosystems and changes in oceanographic conditions due to global warming ([Middelburg and Levin, 2009](#)). While Mn is abundant and widely distributed in nature, it is required only in trace amounts in the organisms, where it guides normal development and body function (for a review see [Pinsino et al., 2012](#)). In the last decades, the massive production of manganese-containing compounds (i.e. metallurgic/chemical/agrochemical products, municipal wastewater discharges, etc) received the attention of the scientific world that considered Mn as a potential emerging contaminant in the environment, especially in the marine milieu ([CICAD 63, 2004; Pinsino et al., 2012](#)). In marine organisms some studies showed that, an Mn overload causes toxicity, although the cause-effect evidence has not been well elucidated ([Regoli et al., 1991; Krång and Rosenqvist, 2006; Oweson et al., 2008; Oweson and Hernroth, 2009](#)). Recently, we took advantage of the amenable model, the Mediterranean sea urchin *Paracentrotus lividus*, to investigate the potential toxicity of Mn on embryonic development, using biological and biochemical approaches. We demonstrated that embryos showed an elevated tolerance/resistance to Mn, as they accumulated high amounts into cells in a time- and concentration-dependent manner without producing lethal effects. Rather, we observed a concentration-dependent increase in the number of morphological abnormalities and in the levels of the hsc70 and hsc60 proteins ([Pinsino et al., 2010](#)). In addition, we found that the exposure to the highest Mn concentration tested...
(61.6 mg l\(^{-1}\) or 1.12 mM) prevents skeleton growth which result in spicule-lacking embryos, interferes with calcium uptake and internalization into cells, and causes remarkable consequences in skeletogenic gene expression and ERK mediated signaling pathway (Pinsino et al., 2011). The MAPK proteins are members of a large group of proteins that allow cells to perceive changes in the intra- and extracellular environment and respond to them appropriately. The MAPK proteins include ERK, JNK, and p38 kinases. In general, MAPK pathways involving ERK stimulate proliferation, growth and differentiation, whereas the p38 kinase has been associated to the survival response or apoptosis/autophagy when strongly activated by stress. The p38 MAPK also plays important roles in tissue regeneration, differentiation, metabolic diseases, cancer, and bone formation (Risco and Cuenda, 2012). For example, results demonstrated that, following TGF-β induction, both ERK and p38 MAPK control differentiation of human osteoblasts (skeletogenic cells) (Lai and Cheng, 2002). Lately it has been demonstrated that p38 MAPK signaling is essential for skeletogenesis and bone homeostasis in mice (Greenblatt et al., 2010; Thouverey and Caverzasio, 2012). However, it has been shown that an increase of p38 MAPK phosphorylation contributed to potentiation of osteoclastogenesis, and arthritic bone loss (Karsdal et al., 2003; Böhm et al., 2013). The activation of MAPK proteins, through the combined activities of the matrix-degrading MMPs, such as MMP2, MMP7 and MMP9, has been observed in osteoarthritic-cartilage-damage human models (Malemud, 2006; Ding et al., 2010). Indeed, a compelling body of evidence has now emerged, implicating MMPs in the process of bone development in mammals (Holmbeck et al., 1999; Malemud, 2006). Taking advantage of these notions, here we decided to extend our previous studies, focusing the attention on the temporal activation of the p38 MAPK and its correlation with the proteolytic activity of MMPs in sea urchin embryos exposed to Mn. Here, we show that Mn overload causes remarkable consequences in the physiological dynamic activation of p38 MAPK and in the proteolytic activities of MMPs. Our results provide new insights into the mechanisms involved in embryo toxicity related to Mn exposure and underlie the role of p38 MAPK and MMPs in the regulation of sea urchin embryonic development.

2. Materials and methods

2.1. Embryo cultures and manganese exposure

Adult sea urchins (P. lividus) were collected locally, along the North-Western coast of Sicily. Embryos were cultured as described previously (Pinsino et al., 2010). Treatment with manganese (Mn) was carried out by culturing embryos in 1.12 mM MnCl\(_2\) (SIGMA) from fertilization until the pluteus stage.

2.2. SDS-PAGE and immunoblotting

Embryo cultures (2 ml) were collected by centrifugation (1200 rpm, 5 min) at different times of development. Pellets were Dounce-homogenized on ice in about 250 μl of lysis buffer [20 mM Tris, 2 mM ethylenediamine-tetraacetic acid (EDTA), 1% NP-40, 15% glycerol, and 2 mM dithiothreitol (DTT)], supplemented with complete protease inhibitor cocktail (Pinsino et al., 2008). Protein concentration was determined by a Bio-Rad Protein Assay (Bradford method). Total cell lysates (30 μg) from different staged embryos were separated under reducing conditions by electrophoresis
on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (AmershamTM HybondTM-ECL). After blocking for 1 h in 5% dry milk in TBS, replicate membranes were incubated overnight at 4 °C with either one of the following primary antibodies in diluted TBST: phospho-p38 MAP kinase (pT180/pY182) (BD Transduction Laboratories™, 612280) 1:600; p38 MAP Kinase non-activated form (SIGMA, M 8432) 1:1000; Actin 20–33 (SIGMA, A5060) 1:800. After washing three times in TBST, membranes were incubated for 1 h at room temperature with a 1:5000 dilution in TBST of horseradish peroxide-conjugated donkey anti-rabbit IgG (Amersham). Proteins bands were visualized on Hyperfilm-ECL films using the ECL PLUS Western blotting Detection Reagents (Amersham). Protein levels were normalized using actin as internal control. Actin protein bands were detected on the same filters but using alkaline phosphatase-conjugated anti-mouse (1:5000 dilution; Promega) as secondary antibody and the chromogenic substrates NBT/BCIP (SIGMA) as detection. Band intensities of filters were quantified by Quantity One (Bio-Rad) software, version 4.6.6. Results were reported as arbitrary units obtained from the volumetric analysis of bands normalized by comparison to band intensities of actin.

2.3. Gelatin zymography by polyacrylamide gel electrophoresis

Embryo cultures (10 ml) diluted 1:1500, were collected by centrifugation (1200 rpm, 5 min) at different times of development. Pellets were Dounce-homogenized on ice in about 200 μl of TBS lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton X-100) without protease inhibitors for 20 min. Protein concentration was determined by a Bio-Rad Protein Assay (Bradford method). Gelatinase activities were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gelatin zymography. Total cell lysates (5 μg) from different staged embryos were separated by electrophoresis on 10% SDS-PAGE gels, under non-reducing conditions (i.e. absence of heating and reducing agent), by copolymerizing acrylamide and gelatin at a final concentration of 0.5% (w/v). The gels (zymograms) were washed for 30 min in 50 mM Tris and 0.5% Triton X-100 pH 8, and incubated in the Calcium activity Buffer (50 mM Tris—HCL pH 8, 10 mM CaCl2) for 24 h at room temperature. The zymograms were subsequently stained with 0.5% Coomassie Blue G250 (Kodak) for 30 min and destained through the methanol/glacial acetic acid series. Gelatinolytic activity appeared as clear bands against the blue background of Coomassie Brilliant Blue stained gels. Zymogramme band densities were analyzed by Quantity One 4.6.5 software.

2.4. Statistical analysis

Values obtained from the measurements of band intensities visualized by immunoblotting and zymography were reported as the mean of three and two independent replicate experiments ± SE. Mean values of band intensities were compared using the one-way analysis of variance (ANOVA) test, followed by the multiple comparison test of Tukey. The analyses were performed using OriginPro 7.5 statistical program and level of significance was set to $P \leq 0.05$.

3. Results

3.1. Temporal activation of p38 MAPK during several critical stages of development

Comparison of the primary sequence of human and sea urchin p38 MAPK shows that the phosphorylation site and the surrounding residues are identical in the two species, indicating that any human phospho-specific p38 MAPK antibody is likely to cross-react with sea urchin p38 MAPK (Bradham and McClay, 2006). To obtain quantitative measurements of p38 MAPK phosphorylation during development, immunoblotting analysis was performed in control and Mn-exposed embryos (Fig. 1A). The anti-phospho p38 MAPK antibody used in this work recognized only the phosphorylated (activated) forms of both threonine and tyrosine residues (Thr180/Tyr182) of the protein, detecting a principal protein band with an apparent molecular weight of approximately 40–42 kDa. A dynamic pattern of p38 MAPK activation was observed from the mesenchyme blastula to the pluteus stage in control embryos. Specifically, a strong activation of p38 MAPK occurred at the mesenchyme blastula stage (Fig. 1A, control 15 h). The levels of phospho-p38 MAPK (P-p38 MAPK) decreased when embryos reached the late gastrula stage (Fig. 1, control 21 and 26 h) and increased again at the pluteus stage (Fig. 1A, control 42 h). On the contrary, p38 MAPK was persistently phosphorylated at any stage in Mn-exposed embryos (Fig. 1A, Mn 15, 21, 26, 42 h). No significant differences in the levels of the non-activated (non-phosphorylated) p38 MAPK form were found during development in both controls and Mn-exposed embryos (Fig. 1B). The description of the embryo morphology and developmental stage at any shown time point (for both control and Mn-treatment) are shown in the upper panel of Fig. 1A.

3.2. Proteolytic activity during development

In order to investigate the proteolytic activity of MMPs, a zymographic procedure for the analysis of matrix metalloproteinases was performed in control and Mn-exposed embryos. Using gelatin substrate gel zymography, a number of gelatine cleaving activities was observed in embryos during development (Fig. 2A, control). We found that the proteolytic processing increased in a time-dependent manner, as showed by the increase of the clear bands having different molecular weights (compare 24 h and 42 h controls). At 49 h, the zymogram of control embryos showed a smear of clear bands, indicating an intense gelatin-auto-cleaving activity. Mn exposure did not influence the proteolytic activity at the mesenchyme blastula stage (15 h). Instead, quantitative and qualitative differences were observed in Mn-exposed embryos at gastrula (24 h) to pluteus (42 and 49 h) stages. Specifically, we found a decrease in the total gelatin-auto-cleaving activity of 1.2 ± 0.3 (mean ± SE) at the gastrula stage, and 1.5 ± 0.1 and 3 ± 1 (mean ± SE) at the pluteus stages. The most obvious qualitative difference was found at 49 h, where, in contrast to controls, only a few clear bands were observed having apparent molecular weights of 90–85 kDa and 68–58 kDa.

To assess if P. lividus embryo MMPs were calcium-dependent endopeptidase, as found in other systems (Nagase et al., 2006), total lysates from both control and Mn-exposed embryos were treated in the presence of a divalent metal ion chelator (EDTA) and then separated by electrophoresis on gelatin-SDS-PAGE gels (Fig. 2B). The total proteolytic activity observed at 49 h of development was strongly reduced (4.9 ± 0.9 fold) in control embryos if compared with 49 h controls without EDTA (see Fig. 2A, control), whereas no significant differences were found between Mn-exposed embryos with or without EDTA (compare Mn lanes, 49 h of Fig. 2A and B). Comparing control and Mn-exposed, both after EDTA treatment (see Fig. 2B), we found that in Mn-exposed embryos the levels of 90–85 kDa MMPs were 3 fold higher ($P_{\text{value}}$ 0.04018) and the levels of 68–58 kDa MMPs were 6 fold higher ($P_{\text{value}}$ 0.02345) than controls.

4. Discussion

Manganese is considered an emerging contaminant because it is a perceived or real threat to the human health and the
environment, especially the marine environment (for a review see Pinsino et al., 2012). While, in adult mammals Mn excess is renowned for its role in neurotoxicity (Takeda, 2003), effects of Mn exposure on embryonic development have only recently begun to be explored (Colomaina et al., 1996; Torrente et al., 2002). Moreover, very little information is available about marine model systems. Mn dissolved in seawater is bio-concentrated significantly more at lower trophic levels than at higher, so marine invertebrates accumulate an elevated amount of Mn into tissues (CICAD 63, 2004). In fact, the reported Bio Concentration Factor (BCF) values range between 100 and 600 for fish and 10,000 and 40,000 for marine invertebrates (ATSDR, 2008). In marine organisms Mn uptake significantly increases with temperature increase and pH decrease (CICAD 63, 2004; Rouleau et al., 1996). Crustaceans and molluscs are the most Mn-sensitive invertebrates, followed by arthropods and echinoderms (Peters et al., 2010). Interestingly, Mn is found at the highest concentrations in the calcified parts of these organisms. Field studies have shown that during growth, their calcified structures incorporate Mn in direct proportion to its concentration in the seawater (Baden and Eriksson, 2006). For example, high levels of Mn (50 fold higher than those measured in the controls from non Mn-impacted areas) have been found in the calcareous matrix of the species Nephrops norvegicus and Balanus amphitrite (Baden and Eriksson, 2006).

In one of our previous studies we investigated the biological effects of exposure to increasing concentrations of Mn on P. lividus embryo development (from 1.0 to 61.6 mg l$^{-1}$) according to classical toxicological criteria: concentration- and time-dependent responses, analysis of impact on development, Mn accumulation (Pinsino et al., 2010). We found that P. lividus embryos accumulate high amounts of Mn in a concentration- and time-dependent manner. Its accumulation was also directly correlated with a concentration-dependent increase in the number of morphological abnormalities (Pinsino et al., 2010). As the highest Mn concentration used (61.6 mg l$^{-1}$), severely inhibited skeleton formation in totality of the embryos (100%), we extended our studies concerning the effects of Mn toxicity on sea urchin development at the molecular (Pinsino et al., 2011) and biochemical level (this study). In the sea urchin embryo, the development of the endoskeleton is an essential step for constructing the framework of the “body”; the absence of embryonic skeleton greatly influences the fitness of the resulting larvae reducing their feeding capability. Thus, abnormal larvae are not able to undergo metamorphosis and, consequently, this impacts the sea urchin reproductive success and the integrity of the ecosystem where they live. The importance of the sea urchins as pivotal components of sub-tidal marine ecology is well known (Hereu et al., 2005). The high concentration of Mn used in this study (61.6 mg l$^{-1}$) is about three times higher than the highest Mn concentration found in nature under hypoxic/anoxic condition (18–24 mg l$^{-1}$) (Treffry et al., 1984; Aller, 1994). Hypoxic areas are marine dead zones in the world’s oceans which can happen for example in the fjords coastlines or closed seas (such as Black, Baltic and Mediterranean Seas), where the water turnover, that should increase the oxygen content, is very slow or not present (Middelburg and Levin, 2009).

In order to improve our knowledge regarding the toxicological mechanisms occurring upon Mn exposure during sea urchin development, we tracked the temporal activation (phosphorylation) of the p38 MAPK. We found that the dynamic pattern of p38 MAPK activation from mesenchyme blastula to pluteus stage was affected by Mn exposure, namely the steady-stable activation from mesenchyme blastula to pluteus. Firstly, this finding was consistent with the notion that growth factors, such as TGF-β and FGF, stimulate both ERK and p38 MAPK phosphorylation in mammal skeletogenic cells (Lai and Cheng, 2002; Shimoaka et al., 2002). Secondly, our result was also in agreement with recent reports highlighting the finding that in glial cells Mn exposure causes the prolonged MAPK activation, including p38 MAPK phosphorylation (Crittenden and Filipov, 2008, 2011). A possible explanation for the long-lasting p38 MAPK activation caused by Mn exposure could be a decreased activity of the MAP kinase phosphatase (MKP), responsible for the de-activating (de-phosphorylating) p38 MAPK. It is worth reminding us that MKPs selectively inactivate MAPKs by de-phosphorylation of the regulatory Thr and Tyr residues, and that MKPs are directly or indirectly responsive to the calcium ion content or oscillations (Scimeca et al., 1997; Boutros et al., 2008).

Interestingly, both p38 and ERK MAPKs are implicated in mediating osteoarthritic-cartilage damage by up-regulating MMPs that degrade the ECM (Malemud, 2004; Ding et al., 2010). MMPs play an integral role in the skeletal transformations during long bone maturation (Malemud, 2006). The genome of the purple sea urchin Strongylocentrotus purpuratus contains at least 26 predicted MMP genes, 8 of which seem to be expressed in the skeletogenic cells of the embryo (Angerer et al., 2006). The phylogenetic evaluation indicates that it is not possible to assign homology among the vertebrate MMPs and the sea urchin MMPs, as they appear to
The MMPs activity detected in Mn-exposed embryos, in the presence or absence of EDTA, was very similar, suggesting that the proteolytic cascade inhibition could be due to a cellular calcium deficiency, since Mn is competing with calcium uptake, as demonstrated previously (Pinsino et al., 2011, 2012). In control embryos, this proteolytic cascade (i.e. auto-cleavage of a pro-enzyme which generates active forms with different molecular weights triggering a proteolytic cascade) increased in a time-dependent manner during development. This auto-cleavage activity was inhibited by EDTA, a versatile chelating agent of divalent ions with high affinity for calcium, known to prevent the endogenous auto-cleavage of calcium-dependent MMPs in echinoderms (Ranganathan et al., 2004; Lamash and Dolmatov, 2013). The seawater contains calcium ions at a high concentration (10 mM), thus we believe that, in analogy with what described in other sea urchin species (Quigley et al., 1993; Mayne and Robinson, 1998; Ranganathan et al., 2004), calcium ions play a key role in the regulation of most P. lividus MMPs. Instead, the strong activity of MMPs having the apparent molecular weights of 90–85 kDa and 68–58 kDa found in Mn-exposed embryos after EDTA treatment, suggests that these species could be Ca-independent. Another hypothesis could postulate persistent MMPs dimerization events or their association with matrix components.

Our results are in agreement with the few reports describing Mn effects on metalloprotease activity in bacteria and human cells. Specifically, in Curtobacterium luteum it has been demonstrated that Mn ions stimulate the enzymatic activity of a metalloprotease of 115 kDa (Kuddus and Ramteke, 2008). In agreement, in normal human keratinocytes it was found that Mn causes an increase of the secreted MMP2 and MMP9 (Chebassier et al., 2004).

As mentioned above, a steady-stable activation of the p38 MAPK was found in Mn-exposed embryos during development, in addition to an aberrant MMPs proteolytic activity. A fascinating hypothesis to link the results obtained in this report together would suggest a critical role of the p38 MAPK pathway in the MMP proteolytic activity occurring during sea urchin embryogenesis. Future studies in this direction are needed to clarify this hypothesis. Finally, we propose a speculative model on the possible mechanism by which Mn overload, interfering with calcium uptake, can affect p38 MAPK phosphorylation and metalloproteinase activity in skeletogenic cells, by blocking the induction of MAP kinase phosphatase function (Fig. 3).

5. Conclusions

Recently, Mn contamination has become a global problem, but information on its toxic effects on marine organisms is still insufficient. In addition, ocean acidification increases Mn availability in the water column, thus increasing the risk for the health of organisms (Sunda et al., 1983; for a review see Pinsino et al., 2012). For example, Mn increased availability could result in an additive factor to ocean acidification that has been already demonstrated to have a negative impact on calcification of some species, including the sea urchins (Byrne, 2012; Catarino et al., 2012). The promotion of P. lividus sea urchin embryo as a good model to study the effects of Mn exposure will expand our knowledge on Mn toxicity at the biochemical level, highlighting the mechanisms of action, probably in common to all marine organisms bearing calcified structures.

In mammals, the transcriptional regulation of ECM macromolecules and MMP activity operating during skeletal development involves specialized intracellular signaling pathways controlled by the activity of MAPKs (Malemud, 2004). In turn, these intracellular signaling pathways are regulated by the availability of intracellular calcium ions. Here, our results provide some intriguing new findings concerning the effects of Mn overload during embryonic development, and emphasize the role played by p38 MAPK and MMPs in sea urchin embryo skeleton development. To the best of our knowledge, this is the first report describing MMP activity during development in the Mediterranean species P. lividus.

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References


