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The role of signalling molecules on actin glutathionylation and protein carbonylation induced by cadmium in haemocytes of mussel *Mytilus galloprovincialis* (Lmk)

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SUMMARY

This study investigated the role of Na^+/H^+ exchanger (NHE) and signalling molecules, such as cAMP, PKC, PI 3-kinase, and immune defence enzymes, NADPH oxidase and nitric oxide synthase, in the induction of protein glutathionylation and carbonylation in cadmium-treated haemocytes of mussel *Mytilus galloprovincialis*. Glutathionylation was detected by western blot analysis and showed actin as its main target. A significant increase of both actin glutathionylation and protein carbonylation, were observed in haemocytes exposed to micromolar concentration of cadmium chloride (5 μmol l^{-1}). Cadmium seems to cause actin polymerization that may lead to its increased glutathionylation, probably to protect it from cadmium-induced oxidative stress. It is therefore possible that polymerization of actin plays a signalling role in the induction of both glutathionylation and carbonylation processes. NHE seems to play a regulatory role in the induction of oxidative damage and actin glutathionylation, since its inhibition by 2 μmol l^{-1} cariporide, significantly diminished cadmium effects in each case. Similarly, attenuation of cadmium effects were observed in cells pre-treated with either 11 μmol l^{-1} GF-109203X, a potent inhibitor of PKC, 50 nmol l^{-1} wortmannin, an inhibitor of PI 3-kinase, 0.01 mmol l^{-1} forskolin, an adenylyl cyclase activator, 10 μmol l^{-1} DPI, a NADPH oxidase inhibitor, or 10 μmol l^{-1} L-NAME, a nitric oxide synthase inhibitor, suggesting a possible role of PKC, PI 3-kinase and cAMP, as well as NADPH oxidase and nitric oxide synthase in the enhancement of cadmium effects on both actin glutathionylation and protein carbonylation.

Key words: actin, cadmium, cAMP, carbonylation, glutathionylation, haemocytes, NHE, NADPH oxidase, nitric oxide synthase, PI 3-kinase, oxidative stress.

INTRODUCTION

Heavy metals, such as cadmium (Cd), are considered as potent catalysts in the oxidative deterioration of biological molecules and their toxicity may, at least in part, be caused by the production of reactive oxygen species (ROS) and perturbation of anti-oxidant efficiency (Stacey et al., 1980; Winston, 1991; Stohs and Bagchi, 1995; Pourahmad and O'Brien, 2000; Micic et al., 2001; Dailianis et al., 2005; Kaloyianni et al., 2009). In order to maintain their physiological functioning in the presence of heavy metals toxicity cells have developed antioxidant mechanisms. The appropriate redox status of cells is principally decided by the ratio of reduced glutathione (GSH) to oxidized (GSSG) glutathione (Schafer and Buettner, 2001; Filomeni et al., 2002). Glutathionylation arises from the formation of a reversible mixed disulphide between GSH and cysteine-SH groups of proteins, in order to mask sulfhydryl groups until oxidative stress is overcome (Klatt and Lamas, 2000; Shelton et al., 2005; Dalle-Donne et al., 2007).

Another acute effect of oxidative stress that is enhanced after ROS production, is the induction of oxidative damage in proteins with carbonyl groups (Davies and Delsignore, 1987; Stadtman, 1993; Grune et al., 1997; Ghezzi and Bonetto, 2003). Exposure to ROS can cause an irreversible modification of amino acid side chains into aldehyde or ketone groups, a process known as carbonylation, which can lead to protein aggregation, inactivation and degradation (Levine et al., 1990; Costa et al., 2002; Ghezzi and Bonetto, 2003). Indeed, protein carbonylation has been recently reported as a biomarker of oxidative stress in several sentinel species (Almroth et al., 2005; Dowling et al., 2006). Moreover, an increase in carbonyl content of proteins has been detected in haemocytes of mussels exposed to Cd, probably because of antioxidant efficiency depletion (Kaloyianni et al., 2009). Alterations in protein function, either by glutathionylation or protein oxidation through carbonylation, have been identified recently in tissues of mussels (Dalle-Donne et al., 2003a; Dalle-Donne et al., 2003b).

Among the proteins affected by oxidative stress, undergoing both glutathionylation and carbonylation processes is actin. Actin is a key target of glutathionylation in mussels, providing an important means for the cytoskeleton to 'sense' altered redox status (Kirchin et al., 1992; Dalle-Donne et al., 2003a; McDonagh et al., 2005).

Although there are several reports concerning the effects of oxidative stress induced by heavy metals and its role in glutathionylation and carbonylation processes in tissues of mussels (Kirchin et al., 1992; Fagotti et al., 1996; Dalle-Donne et al., 2003a; Dalle-Donne et al., 2003b; Gomez-Mendikute and Cajaraville, 2003; McDonagh et al., 2005), the involvement of signal cascades in the induction of these processes in haemocytes of *Mytilus* after exposure to heavy metals still remains unclear. Cd has been reported to induce signal transduction cascades, such as protein kinase C (PKC), tyrosine kinase and casein kinase II (Adams et al., 2002), and recent studies in our laboratory have shown the induction of a PKC-mediated signal transduction pathway with a concomitant increase of ROS production and Na^+/H^+ exchanger (NHE) stimulation in Cd-treated cells of mussels (Dailianis and Kaloyianni, 2004; Dailianis et al., 2005; Kaloyianni et al., 2005; Kaloyianni et al., 2006). NHE, a ubiquitously

expressed integral membrane protein involved in the coupled exchange of Na^+ with H^+ in a variety of eukaryotic cells, is considered of vital importance, since it has been suggested to play important roles in cytoskeletal reorganization, cell migration (Reshkin et al., 2000; McHardy et al., 2004; Paradiso et al., 2004; Konstantinidis et al., 2009), regulation of intracellular pH (pH_i) (Moolenaar et al., 1983; Bianchini and Pouyssegur, 1994), cell volume control (Cala, 1983a; Cala, 1983b) and other cell activities such as cell adhesion, proliferation and apoptosis (Tominaga and Barber, 1998; Khaled et al., 2001; Moor et al., 2001; Avkiran and Haworth, 2003; Orlowski and Grinstein, 2004; Koliakos et al., 2008). It is regulated mainly by pH_i levels and a wide variety of hormones activating protein kinases, such as protein kinase C (PKC) and cAMP/PKA (Sauvage et al., 2000; Pederson et al., 2002). It has also been reported in a recent study that NHE is stimulated under oxidative stress in digestive gland and gill cells of mussels (Dailianis and Kaloyianni, 2004; Koutsogiannaki et al., 2006).

Another signalling molecule studied is phosphoinositide 3-kinase (PI 3-kinase), a key signalling enzyme that responds to growth factors, cytokines and environmental agents (Cantley, 2002). PI 3-kinase activation supports various cell functions, such as growth, migration and survival *via* the activation of Akt/protein kinase B, which in turn triggers cytoprotective events (Shimamura et al., 2003). In mussels, PI 3-kinase appears to be involved in the regulation of phagocytosis (Garcia-Garcia et al., 2008). Recently, Barthel et al. (Barthel et al., 2007) reported that ROS enhancement could regulate the activation of the PI 3-kinase–Akt signalling pathway. To our knowledge, involvement of the PI 3-kinase–Akt cascade and ROS in the signalling pathways leading to glutathionylation and protein carbonylation in mussels has not yet been demonstrated.

Haemocytes of mussels play an important role in several functions, such as wound and shell repair, and internal defence (Cheng, 1981). In cell-mediated immune responses, phagocytosis by circulating haemocytes is the main defence against foreign materials, such as heavy metals (Cheng and Sullivan, 1984; Cheng, 1988; Pipe et al., 1999). Production of superoxide anions (O_2•) *via* NADPH oxidase, as well as nitric oxide generation (NO), *via* nitric oxide synthase activation, was reported to be associated with phagocytic ability in haemocytes of mussels (Padgett and Pruet, 1992; Salman-Tabcheh et al., 1995; Winston et al., 1996; Carballal et al., 1997; Dailianis, 2009). Although various effectors have been shown to provoke O_2• and NO generation (for more details see Noel et al., 1993; Ottaviani et al., 1993; Franchini et al., 1995a; Winston et al., 1996; Gourdon et al., 2001; Tafalla et al., 2002; Novas et al., 2004), little is known about the involvement of NADPH oxidase and NO synthase in the processes of glutathionylation and protein carbonylation in haemocytes of mussels exposed to foreign substances, such as cadmium.

Our data show for the first time the involvement of NHE, NADPH oxidase and NO synthase, as well as the signalling molecules cAMP, PKC and PI 3-kinase in the processes of both actin glutathionylation and protein carbonylation, induced by Cd in haemocytes of the mussel *Mytilus galloprovincialis* (Lmk). Moreover, inhibition of actin polymerization seemed to maintain redox balance within the cells, thus verifying the regulatory role of the actin cytoskeletal function in cellular homeostasis.

MATERIALS AND METHODS

Chemicals and reagents

Diphenyleneiodonium chloride (DPI), L-NAME, wortmannin, forskolin, bovine serum albumin (BSA), $NaHB_4$, 2.4-dinitrophenylhydrazine, 3,3′,5,5′ tetramethylbenzidine (TMB) tablets, anti-DNP, GF-109203X and anti-actin antibodies were purchased from Sigma Chemical Co. (St Louis, MO, USA); cariporide was kindly provided by Aventis (Frankfurt, Germany). Ascorbic acid, hydrogen peroxide (30%) and Tween 20 were purchased from Merk (Darmstadt, Germany), ferrous ammonium sulfate and cytochalasin D (cyt D) from Fluka (Buchs, Switzerland), fetal calf serum (FCS), penicillin G, streptomycin, gentamycin, amphotericin B from Applichem (Darmstadt, Germany), primary mouse monoclonal antibody for glutathione (anti-GSH) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), anti-mouse IgG-peroxidase conjucated antibody from H&L, Upstate Cell Signaling (Hertfordshire, UK), flat ELISA plates from Greiner bio-one (Solingen, Germany). For signal detection, the ECL detection (Cell Signaling, Hertfordshire, UK) was used. Leibovitz L-15 medium were purchased from Biochrom A.G (Berlin, Germany). All the reagents used were reagent grade and prepared in double-distilled water. PS (physiological saline) solution (1100 mOsm, pH 7.3) contained 20 mmol l^{-1} Hepes buffer, 436 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 53 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} $CaCl_2$.

Animals and biological material

Mussels (5–6 cm long) were collected from Kalamaria, on the east side of Thermaikos Gulf (Thessaloniki), transferred to the laboratory and maintained without feeding in static tanks containing filtered natural sea water (35–40‰ salinity) for 7 days at 15°C, in order to be acclimatised to laboratory conditions. Then, during the experimental procedure, mussels were fed with approximately 30 mg of dry-microencapsules/mussel (Myspat, Inve Aquaculture^{NV}, Belgium).

Haemolymph collection and handling

Haemolymph from 10 mussels was extracted from the posterior adductor muscle with a sterile 1 ml syringe, with an 18G1/2′ needle, containing 0.1 ml of physiological saline buffer (PS). In order to collect haemocytes, haemolymph was centrifuged at 200 ***g*** for 15 min and the pellet was resuspended in Leibovitz L-15 medium supplemented with 350 mmol l^{-1} NaCl, 7 mmol l^{-1} KCl, 4 mmol l^{-1} $CaCl_2$, 8 mmol l^{-1} $MgSO_4$, 40 mmol l^{-1} $MgCl_2$, 10% FCS, 100 i.u. ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, 40 μg ml^{-1} gentamycin, 0.1 μg ml^{-1} amphotericin B at pH 7 and osmolarity 1000 mosmol l^{-1} (Cao et al., 2003). Cells were kept at 15°C for at least 3 h before being used for the experiments. Throughout this period, cell viability was assayed by the Eosin exclusion test and the cells were counted in a Neubauer hemocytometer. The cells showed about 90% viability before the beginning of the experimental procedures.

Experimental and exposure procedure

Haemocytes were exposed for 3 h to $CdCl_2$, at a final concentration of 5 μmol l^{-1}, in the presence or the absence of different agonists or antagonists of signal transduction pathways. The Cd concentration used was within the range of Cd levels found in Thermaikos Gulf (HCMR, 1997). Moreover, similar concentrations of Cd have been used in other studies, investigating the effects of the metal on cell signalling and its ability to induce cellular toxic effects (Coogan et al., 1992; Misra et al., 1998; Olabarrieta et al., 2001; Pruski and Dixon, 2002; Dailianis and Kaloyianni, 2004; Dailianis et al., 2005; Dailianis, 2009). Specifically, haemocytes were pre-incubated for 20 min with either 0.01 mmol l^{-1} forskolin, an adenylyl cyclase (AC) activator, 2 μmol l^{-1} cariporide, a NHE inhibitor, 50 nmol l^{-1} wortmannin, an inositol phosphate kinase (PI 3-kinase) inhibitor, 10 μmol l^{-1} diphenyleneiodonium chloride (DPI), an inhibitor of

NADPH oxidase, 10 μmol l^{-1} L-NAME, a nitric oxide synthase inhibitor, 2 μmol l^{-1} cytochalasin D, an actin polymerization inhibitor, or 11 μmol l^{-1} GF-109203X, a potent inhibitor of protein kinase C (PKC), and then exposed to 5 μmol l^{-1} of $CdCl_2$ for 3 h. In parallel, Cd-free haemocytes were treated with each agonist or antagonist alone (Cd-free-treated cells). Routinely, after the exposure of cells to each chemical, cell viability was assayed by an Eosin exclusion test and revealed that almost 95% of cells remained intact in all cases.

ELISA (enzyme linked immunosorbent assay) for measuring carbonyl groups in haemocyte proteins

For the quantitative estimation of protein carbonylation, a recently established method in haemocytes of mussels was used (Kaloyianni et al., 2009). This method is a modification of the experimental procedure reported by Alamdari et al. (Alamdari et al., 2005) that used 2.4-dinitrophenylhydrazine (DNPH) derivatisation, after attachment of the protein to a polystyrene plate.

Reduced and oxidized forms of bovine serum albumin (BSA) were used in order to determine carbonyl proteins. For the reduced form of BSA, 1 g of BSA was dissolved in 100 ml of PS buffer, and then 0.1 g of $NaHB_4$ was added. The solution was incubated for 30 min at 25°C, neutralized with 2 mol l^{-1} HCl, followed by overnight dialysis against PS at 4°C with two buffer changes, dispensed in aliquots, and then stored at –80°C.

Oxidized BSA was prepared using a modification of a previously described method (Davies et al., 2001). Specifically, 50 mg of BSA was dissolved in 1 ml of PS with the addition of 20 μl EDTA (100 mmol l^{-1}), 57 μl ascorbic acid (833 mmol l^{-1}) and 2 μl ferrous ammonium sulfate (100 mmol l^{-1}). The solution was incubated for 90 min at 37°C, followed by overnight dialysis against PS at 4°C with two buffer changes, dispensed in aliquots, and then stored at –80°C.

The protein concentration of the oxidized and the reduced BSA were measured using the Bradford reagent, and then the protein concentration was adjusted with PS to 5 μg ml^{-1}. Samples for the standard curve were prepared by mixing oxidized and reduced BSA in differing proportions ranging from 0 to 100%, while maintaining a constant total protein concentration (5 μg ml^{-1}).

After the end of the incubation period, cells were centrifuged at 200 ***g*** for 10 min. The cell pellet was dissolved in 1 ml PS (at least 10^6 cells ml^{-1}), lysed by sonication and centrifuged at 10,000 ***g*** for 10 min. Finally, the supernatant of the cell lysate was stored at –80°C and used to measure the protein carbonyl groups.

200 μl of the diluted standards, samples (each sample contained 5 μg protein, as modified after protein determination) or PS without protein (blank) were placed into the wells; each was assayed in triplicate. The plate was incubated overnight at 4°C and then washed three times with 300 μl PS. Freshly prepared DNPH solution (200 μl) was added, incubated for 45 min at room temperature in the dark, and then washed five times with 300 μl of PS:ethanol solution (1:1, v/v) and once with 300 μl of PS. 260 μl of blocking solution (2.5 g non-fat milk, diluted in 50 ml of PS) was added and the mixture incubated for 1.5 h at room temperature and then washed three times with 300 μl of PST (PS solution with the addition of 0.1% Tween 20). Anti-DNPH (200 μl) was added and the mixture incubated for 1 h at 37°C and then washed five times with 300 μl PST. Secondary antibody (200 μl of anti-rabbit HPR-linked IgG in 20 ml blocking solution) was added and the mixture incubated for 1 h at 37°C followed by five washes with 300 μl PST. 200 μl of substrate solution [two tablets TMB and 4 μl of 30% H_2O_2 in 20 ml substrate buffer, (substrate buffer; 1.455 g Na_2HPO_4 and 1.91 g citric acid in 150 ml distilled H_2O (dH_2O), pH 5. The volume was made up to 200 ml with dH_2O and stored at 4°C)] was added. After 3–5 min, the reaction was stopped by adding 100 μl 2 mol l^{-1} HCl. The absorbance was read at 450 nm. PCC was calculated using a standard curve of BSA. Results (expressed as nmol carbonyl mg^{-1} protein after the subtraction of reduced BSA value) are given as means ± s.d. from three independent experiments. In each experiment, measurement of each sample was carried out in triplicate.

Electrophoresis and western blotting

After the end of the exposure period, cells were centrifuged at 200 ***g*** for 10 min. The supernatant was discarded and cells (at least 10^6 cells ml^{-1}) were lysed in buffer containing 50 mmol l^{-1} Tris-HCl, pH 8, 0.25 mol l^{-1} sucrose, 1% (w/v) SDS, 2 mmol l^{-1} sodium orthovanadate, 10 mmol l^{-1} NaF, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} NEM (*N*-ethylmaleimide), 0.1% Nonidet-P40, and protease inhibitors (1 μg ml^{-1} pepstatin, 10 μg ml^{-1} leupeptin, 40 μg ml^{-1} PMSF) for 1 h at 4°C. After sonication at 50 W for 45 s, whole-cell lysate was centrifuged at 10,000 ***g*** for 10 min at 4°C. Supernatant was collected and protein content was determined (Bradford assay and BSA as a standard). At least 30 μg of protein was boiled for 4 min in 1× SDS sample buffer (0.5 mol l^{-1} Tris-base, pH 6.8, containing 20% SDS, 0.25% Bromophenol Blue, 75% glycerol) and the samples were resolved by 8% SDS-polyacrylamide gel electrophoresis (Laemmli, 1972). The proteins were transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and stained with anti-GSH (1:1000) overnight at 4°C, followed by anti-mouse IgG-peroxidase conjugated antibody (1:10,000) for 1 h at room temperature. In parallel, for the evaluation of equal protein loading into the wells, membranes were incubated with anti-β-actin (as a loading control). Specifically, membranes were blocked overnight at 4°C with 5% skimmed milk and incubated at room temperature with anti-β-actin (1:3000). Finally, membranes were washed and incubated with secondary antibody (1:5000). For signal detection, the ECL detection was used. Optical density in each gel track was estimated with Gell Pro (Cybernetics, Inc., Bethesda, MD, USA) software analysis and results are expressed as the percentage of control total optical density (arbitrary units).

Statistical analysis

All data are presented as means ± standard deviations of four independent experiments for glutathionylation, and three independent experiments, measured in triplicate in each case, for protein carbonyl content measurement. For the statistical analysis of two means, a Dunnet test, using Graphpad Instat 3 software was performed. Significance was established at $P<0.01$.

RESULTS

Glutathionylation of actin in haemocytes of mussels exposed to cadmium

Since actin is considered as the main target of glutathionylation in mussels, we tried to investigate whether actin glutathionylation occurred after treatment of cells with micromolar concentration of $CdCl_2$ (5 μmol l^{-1}). In addition, we investigated whether signalling molecules, such as NHE, adenylyl cyclase, PI 3-kinase, PKC, as well as proteins, such as NADPH oxidase and NO synthase, and actin polymerization could be involved in the process of glutathionylation in haemocytes pre-treated with different antagonists and/or agonists, with or without the addition of the metal (Cd-exposed and Cd-free cells, respectively).

In all cases, there was an equal loading of protein into the wells, as measured by the Bradford assay and confirmed electrophoretically

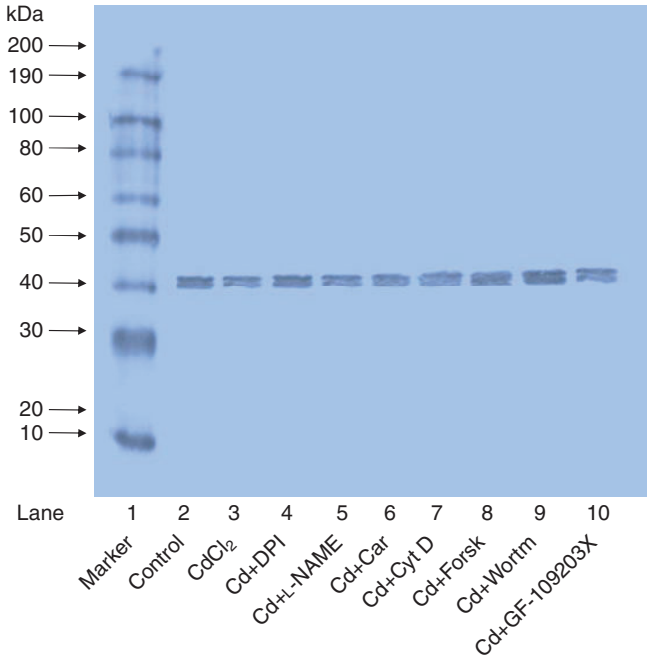

Fig. 1. Western blot using antibodies to actin (one-dimensional SDS 8% polyacrylamide gel electrophoresis), in order to confirm equal loading of protein into the wells. Lane 1, standards; lane 2, control cells; lane 3, Cd-treated cells; lanes 4-10, Cd-treated cells exposed to, 10 μmol l^{-1} DPI (lane 4), 10 μmol l^{-1} L-NAME (lane 5), 2 μmol l^{-1} cariporide (lane 6), 2 μmol l^{-1} cytochalasin D (lane 7), 0.01 mmol l^{-1} forskolin (lane 8), 50 nmol l^{-1} wortmannin (lane 9), or 11 μmol l^{-1} GF-109203X (lane 10). (A representative blot is shown.)

with the use of actin antibodies (Fig. 1). Protein glutathionylation in haemocytes of mussels was detected by western blotting, with the use of commercially available antibodies to GSH. The western blots showed that actin was the main target of glutathionylation, since Cd-treated cells showed significantly increased levels of cysteine-SH groups in a single-protein at 42 kDa, previously identified as actin (Fig. 2A,B).

Glutathionylation of actin in haemocytes of mussels exposed to different agonists and antagonists of signal transduction pathways, in the presence of cadmium

According to our results, haemocytes of mussels exposed to 5 μmol l^{-1} of Cd for 3 h, had significantly higher levels of actin glutathionylation, compared with levels observed in untreated (control) samples (Fig. 2A,B). Inhibition of either NADPH oxidase or NO synthase, in haemocytes pre-treated with 10 μmol l^{-1} DPI and L-NAME respectively, did not show any significant attenuation of Cd effects on actin glutathionylation (Fig. 2A,B), whereas each inhibitor alone did not seem to enhance actin glutathionylation in Cd-free cells (cells treated with each antagonist, without the addition of the metal), compared with levels of glutathionylation occurred in control cells (Fig. 3A,B). However, the effect of Cd on actin glutathionylation was significantly diminished in cells pre-treated with 2 μmol l^{-1} cariporide, an inhibitor of NHE (Fig. 2A,B), whereas in Cd-free cells treated only with the inhibitor, a significant decrease of actin glutathionylation was observed, compared with levels of glutathionylation observed in control cells (Fig. 3A,B). Similarly, Cd-exposed and Cd-free cells, pre-treated for 20 min with 0.01 mmol l^{-1} forskolin, an adenylyl cyclase activator, showed lower levels of glutathionylation, compared with levels observed either in cells exposed to Cd alone or in unexposed (control) cells (Fig. 2A,B, Fig. 3A,B). Actin glutathionylation detected in haemocytes, pre-treated with either 50 nmol l^{-1} wortmannin, a PI 3-kinase inhibitor, or 11 μmol l^{-1} GF-109023X, a potent inhibitor of protein kinase C, before the addition of the metal, was significantly lower compared with levels of glutathionylation observed in cells treated with the metal alone (Fig. 2A,B). Inhibition of either PI 3-kinase or PKC in Cd-free cells, did not enhance the levels of actin glutathionylation, compared with levels observed in control cells (Fig. 3A,B). In

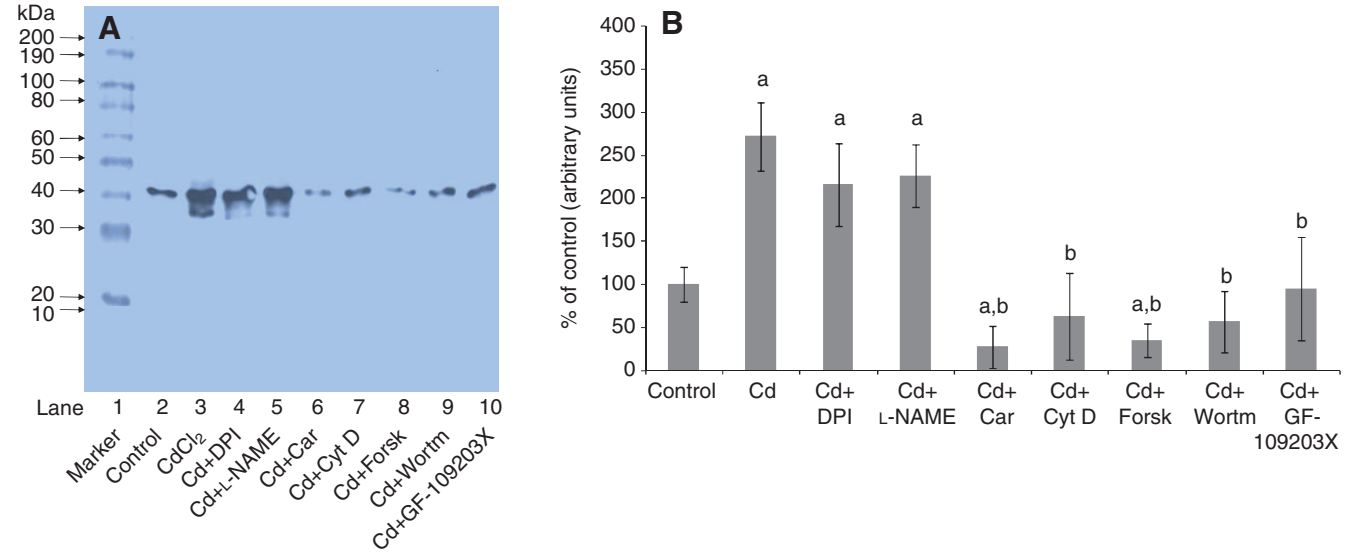

Fig. 2. (A) Detection of actin glutathionylation by western blotting (one-dimensional SDS 8% polyacrylamide gel electrophoresis) using anti-GSH, in haemocytes of mussels. Cells were exposed to 5 μmol l^{-1} $CdCl_2$ alone (lane 3) or to Cd with either 10 μmol l^{-1} DPI (lane 4), 10 μmol l^{-1} L-NAME (lane 5), 2 μmol l^{-1} cariporide (lane 6), 2 μmol l^{-1}cytochalasin D (lane 7), 0.01 mmol l^{-1} forskolin (lane 8), 50 nmol l^{-1} wortmannin (lane 9) or 11 μmol l^{-1} GF-109203X (lane 10). (A representative blot is shown.) (B) Glutathionylation of actin in haemocytes treated with Cd alone or Cd together with either 10 μmol l^{-1} DPI, 10 μmol l^{-1} L-NAME, 2 μmol l^{-1} cariporide, 2 μmol l^{-1} cytochalasin D, 0.01 mmol l^{-1} forskolin, 50 nmol l^{-1} wortmannin or 11 μmol l^{-1} GF-109203X. Results are means ± s.d. from four independent experiments and expressed as a percentage of control total band optical density (arbitrary units). [a]Significantly different from control cells; [b]significantly different from cells treated only with Cd (Dunnet test, $P<0.01$).

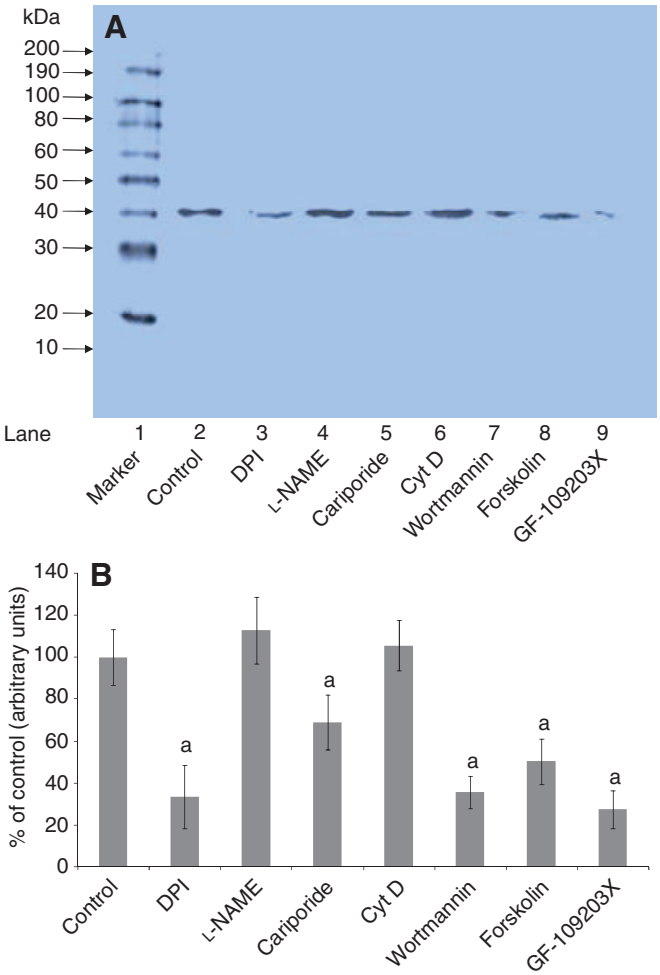

Fig. 3. (A) Detection of actin glutathionylation by western blotting (one-dimensional SDS 8% polyacrylamide gel electrophoresis) using anti-GSH, in control cells (lane 2) and cells treated with each agonist and antagonist in the absence of Cd (lanes 3–9). Specifically, cells were treated with either 10 μmol l^{-1} DPI (lane 3), 10 μmol l^{-1} L-NAME (lane 4), 2 μmol l^{-1} cariporide (lane 5), 2 μmol l^{-1} cytochalasin D (lane 6), 50 nmol l^{-1} wortmannin (lane 7), 0.01 mmol l^{-1} forskolin (lane 8) or 11 μmol l^{-1} GF-109203X (lane 9). (A representative blot is shown.) (B) Glutathionylation of actin in cells treated with each antagonist and/or agonist, in the absence of Cd. Results are means ± s.d. from four independent experiments and expressed as a percentage of control band optical density (arbitrary units). [a]Significantly different from control cells (Dunnet test, $P<0.01$).

addition, inhibition of actin polymerization, in cells pre-treated with 2 μmol l^{-1} cytochalasin D, prior to Cd exposure, revealed significant decrease of actin glutathionylation, at levels similar to those obtained both in control cells and Cd-free cells (Fig. 2A,B, Fig. 3A,B).

Protein carbonyl content in haemocytes of mussels

Protein carbonyl content in haemocytes of mussels was significantly different in control and Cd-treated cells (Fig. 4). Specifically, haemocytes of mussels exposed to 5 μmol l^{-1} Cd had significantly increased protein carbonyl content, relative to untreated haemocytes. Cells pre-treated either with 0.01 mmol l^{-1} forskolin or 50 nmol l^{-1} wortmannin, had significantly decreased protein carbonyl content, compared with cells treated only with Cd, but significantly higher levels than control cells, at least at the concentrations of these chemicals used in the present study (Fig. 4). However, inhibition of NHE, actin polymerization, NADPH oxidase, NO synthase or PKC, significantly diminished the effects of cadmium on carbonylation of proteins (Fig. 4). Cd-free haemocytes treated with each agonist or antagonist alone, did not show any significant differences, compared with untreated (control) cells (Table 1).

DISCUSSION

Oxidative stress is a situation in which cellular homeostasis is altered because of excessive production of ROS and/or impairment of cellular antioxidant defences. Cadmium (Cd) is a non-redox metal that causes enhancement of oxidative stress in mussels possibly not *via* its participation in Fenton reactions, but through the induction of ROS production within cells (Stohs and Bagchi, 1995; Dailianis et al., 2005; Koutsogiannaki et al., 2006).

According to the results of the present study, oxidative stress induced by Cd at micromolar concentrations, enhances both glutathionylation and protein carbonylation in haemocytes of the mussel *M. galloprovincialis*. The increased levels of carbonylation in haemocytes of Cd-exposed mussels are in accordance with previously published studies, reporting the carbonylation of proteins in tissues of mussels exposed to heavy metals such as zinc, copper and Cd (Kirchin et al., 1992; Kaloyianni et al., 2009). Actin is considered to be one of the main proteins to be carbonylated in tissues of mussels (McDonagh et al., 2005; McDonagh and Sheehan, 2007). Nevertheless, the method of carbonyl content detection used in the present study, does not exclude the carbonylation of other proteins, apart from actin, since there is evidence that actin is not the only redox-target protein that may be carbonylated in cells (see Van Der Vlies et al., 2002; Rabek et al., 2003; England and Cotter, 2004; Winterbourn, 2008). In addition, actin is the main target of glutathionylation in tissues of mussels (McDonagh et al., 2005) and other animals (Klatt and Lamas, 2000; Dalle-Donne et al., 2001; Fratelli et al., 2002).

Actin seems to be involved in the processes of glutathionylation and carbonylation in haemocytes of mussels. Actin glutathionylation occurs even in the absence of exogenous oxidative stress (Dalle-Donne et al., 2003a; Dalle-Donne et al., 2005). According to the results of the present study, inhibition of actin polymerization seemed to maintain glutathionylation levels at a level similar to those observed in control cells. This fact seems to be in accordance with previously published studies, revealing that under normal cellular conditions, a portion of G-actin is glutathionylated, thus inhibiting actin polymerization into F-actin (Dalle-Donne et al., 2003a; Dalle-Donne et al., 2003b). Further enhancement of oxidative stress, caused by oxidants such as Cd, could cause a range of reversible and irreversible modification of proteins such as actin. Specifically, cells exposed to Cd showed increased levels of both actin glutathionylation and protein carbonylation, probably with the involvement of actin, since inhibition of its polymerization significantly attenuated the effects of Cd in each case. Since glutathionylation and carbonylation of actin are considered as an early mechanism of protection against stress (Fagotti et al., 1996; Forman et al., 2004; Ghezzi, 2005; McDonagh et al., 2005; Shelton et al., 2005), we could hypothesize that under oxidative stress, there is a rearrangement of the actin cytoskeleton in order for cells to mask critical proteins, such as actin, against irreversible oxidative damage. Polymerization of actin occurring after Cd exposure could act as a signal, further enhancing the glutathionylation process in order to protect cytoskeletal function and therefore cells against oxidative stress. However, polymerization of actin could be a critical signal for the induction of protein carbonylation within cells, as a result of redox balance disturbance. Despite the fact that actin is considered to play a regulatory role both in cytoskeletal function

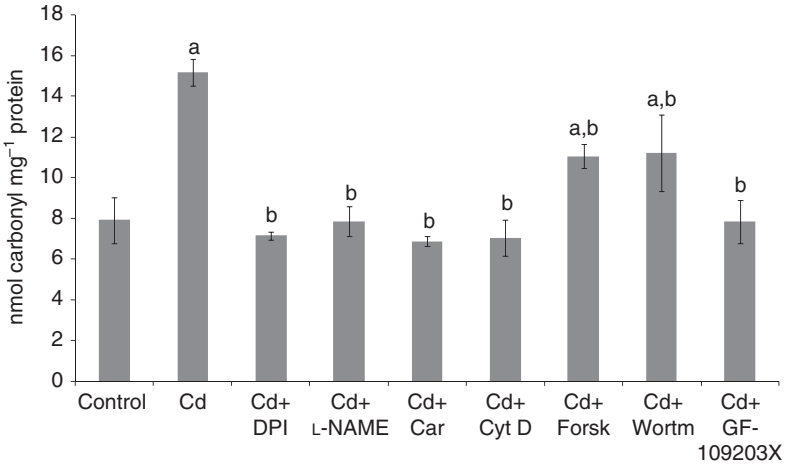

Fig. 4. Detection of protein carbonyl content in haemocytes of mussels. Cells were exposed to 5 μmol l^{-1} $CdCl_2$ alone or together with either 10 μmol l^{-1} DPI, 10 μmol l^{-1} L-NAME, 2 μmol l^{-1} cariporide, 2 μmol l^{-1} cytochalasin D, 0.01 mmol l^{-1} forskolin, 50 nmol l^{-1} wortmannin or 11 μmol l^{-1} GF-109203X. Results (expressed as nmol carbonyl mg^{-1} protein) are means ± s.d. from three independent experiments. In each experiment, measurements on treated cells were carried out in triplicate. [a]Significantly different from control cells; [b]significantly different from cells treated only with Cd (Dunnet test, $P<0.01$).

and cellular homeostasis (Ghezzi, 2005; McDonagh et al., 2005) further research is needed in order to verify the role of actin polymerization under oxidative stress induced by oxidants such as Cd.

There are a lot of studies, reporting that oxidative stress can cause a range of reversible (glutathionylation) and irreversible (carbonylation) modifications of protein amino acid side chains (Ghezzi and Bonetto, 2003), which affect the response of numerous cell signalling pathways (Tan et al., 1995; Tan et al., 2001; Persad et al., 1997; Persad et al., 1998; Ward et al., 2000; Mahadev et al., 2001; Humphries et al., 2002; Kwon et al., 2004; Goldstein et al., 2005). Our results showed that actin glutathionylation and protein carbonylation in haemocytes exposed to Cd seems to enhance a signalling cascade with the involvement of both cAMP and PKC. Specifically, actin glutathionylation and carbonylation of proteins seems to involve PKC activation, whereas activation of adenylyl cyclase seemed to attenuate the effects of Cd both on actin glutathionylation and protein carbonylation. The later is in accordance with the results of Dailianis et al. (Dailianis et al., 2005) who reported that cells treated to maintain high levels of cAMP were more efficient in keeping low levels of ROS, compared with control cells. Subsequently, high levels of cAMP could contribute to a decrease of protein carbonylation and actin glutathionylation. Moreover, it has been reported that there is a crosstalk between cAMP- and PKC-dependent activation of NHE, under oxidative stress induced by Cd (Dailianis et al., 2005; Dailianis, 2009).

Table 1. Detection of protein carbonyl content in haemocytes of mussels

Agonist/antagonist treatment	nmol carbonyl mg^{-1} protein
Control	7.936±1.12
2 μmol l^{-1} Cariporide	7.737±1.5
2 μmol l^{-1} Cytochalasin D	7.717±1.3
0.01 mmol l^{-1} Forskolin	7.799±1.4
50 nmol l^{-1} Wortmannin	8.128±0.9
11 μmol l^{-1} GF-109203X	7.689±1.2
10 μmol l^{-1} L-NAME	10.059±1.3
10 μmol l^{-1} DPI	7.991±1.5

Cells were exposed to each agonist or antagonist alone, without the addition of cadmium.
Values are means ± s.d. of triplicate samples from three independent experiments.
No significant difference was obtained between control cells and cells treated with each agonist and antagonist as determined by Dunnet test, $P<0.01$.

According to the results of the present study, NHE, a plasma membrane anchoring protein for the actin cytoskeleton (Putney et al., 2002), seems to play a regulatory role in the induction of actin glutathionylation, as well as protein carbonylation in haemocytes of mussels. Previously published studies have shown the activation of NHE by heavy metals in HT 29 cells (Hershfinkel et al., 2001) as well as in haemocytes, digestive gland, gills and mantle/gonad cells of mussels (Dailianis and Kaloyianni, 2004; Kaloyianni et al., 2005; Kaloyianni et al., 2006). In addition, it has been reported that activation of NHE enhances ROS production, after exposure to micromolar concentrations of Cd (Dailianis et al., 2005; Koutsogiannaki et al., 2006). Therefore, it seems that under oxidative stress induced by Cd, actin glutathionylation and protein carbonylation are possibly mediated through NHE activity (Paradiso et al., 2004). It is possible, therefore, that Cd could cause NHE activation, possibly through actin glutathionylation, which would lead to the induction of oxidative damage of proteins. Thus, NHE possibly has a regulatory role in the induction of reversible or irreversible modifications of proteins in cells faced with oxidative stress. To our knowledge, this is the first report of the involvement of NHE with cellular responses, such as glutathionylation and carbonylation, after exposure to heavy metals in cells of mussels. Therefore, ROS, cAMP, PKC and NHE may be involved in the processes of protein carbonylation and actin glutathionylation after exposure to Cd in haemocytes of mussels. This statement is in accordance with previously published studies, indicating that ROS production could be induced by a NHE-dependent pathway (Rothstein et al., 2002; Sand et al., 2003; Baldini et al., 2005). However, further research is needed in order for this to be verified.

There is evidence that the signalling molecules PKC and PI 3-kinase are involved in the regulation of phagocytosis, possibly through activation of NADPH oxidase and NO synthase (Baggiolini et al., 1986; Klebanoff, 1988; Torreilles et al., 1996; Thomas et al., 2002; Garcia-Garcia and Rosales, 2002; Garcia-Garcia, 2005; Haynes et al., 2003). The involvement of PKA in NO synthesis has been also suggested (Novas et al., 2004). NADPH oxidase, as well as nitric oxide synthase activation, enhance the production of oxygen radicals, such as superoxides (O_2•) and nitric oxide (NO), thus representing the main enzymes of the immune response of haemocytes against foreign substances (Noel et al., 1993; Conte and Ottaviani, 1995; Franchini et al., 1995a; Franchini et al., 1995b; Winston et al., 1996; Carballal et al., 1997; Arumugam et al., 2000;

Gourdon et al., 2001). NO is of great importance, protecting the cells from oxidant injury and scavenging radical species (Wong and Billiar, 1995; Fang, 1997). Our results show that PI 3-kinase, NADPH oxidase and NO synthase seemed to be involved both in actin glutathionylation and protein carbonylation in haemocytes exposed to Cd. Activation of PI 3-kinase by Cd has also been reported in previous studies (Kim et al., 2000; Eom et al., 2001; Ostrakhovitch et al., 2002; Bao and Knoell, 2006). Since, PI 3-kinase has been proposed as a survival signal in mild oxidative stress (Konishi et al., 1999; Sonoda et al., 1999; Shimamura et al., 2003), we could hypothesize that NADPH oxidase and NO synthase activation may be a result of PKC and PI 3-kinase activation after Cd exposure in haemocytes of mussels.

In conclusion, Cd can cause transient oxidative stress resulting in actin glutathionylation and protein carbonylation in haemocytes of mussels. Although oxidative stress induced by ROS enhancement after exposure to Cd could lead to irreversible oxidative damage of proteins, the formation of a reversible mixed disulphide between GSH and cysteine-SH groups in proteins such as actin could be a protective strategy to mask sulfhydryl groups until oxidative stress is overcome.

Our results support previous evidence that NHE is a regulator of intracellular signal transduction pathways, thus participating in strategies adopted by cells for maintaining their intracellular homeostasis and function. This is also the first report of the involvement of NADPH oxidase and NO oxidase and signalling molecules, such as PKC, cAMP and PI 3-kinase, in the induction of both glutathionylation and protein carbonylation in Cd-treated haemocytes of mussels. Further research is needed in order to clarify the role of these molecules in the above processes.

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