

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

Stefanos Dailianis<sup>1,\*</sup>, Efterpi Patetsini<sup>2</sup> and Martha Kaloyianni<sup>2</sup>

<sup>1</sup>Section of Animal Biology, Department of Biology, Faculty of Sciences, University of Patras, 26 500, Greece and <sup>2</sup>Laboratory of Animal Physiology, Department of Zoology, School of Biology, Aristotle University of Thessaloniki 54124, Greece

\*Author for correspondence (sdailianis@upatras.gr)

Accepted 6 August 2009

### SUMMARY

This study investigated the role of Na<sup>+</sup>/H<sup>+</sup> 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<sup>-1</sup>). 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<sup>-1</sup> cariporide, significantly diminished cadmium effects in each case. Similarly, attenuation of cadmium effects were observed in cells pre-treated with either 11 µmol l<sup>-1</sup> GF-109203X, a potent inhibitor of PKC, 50 nmol l<sup>-1</sup> wortmannin, an inhibitor of PI 3-kinase, 0.01 mmol l<sup>-1</sup> forskolin, an adenylyl cyclase activator, 10 µmol l<sup>-1</sup> DPI, a NADPH oxidase inhibitor, or 10 µmol l<sup>-1</sup> 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<sup>+</sup>/H<sup>+</sup> 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  $\text{Na}^+$  with  $\text{H}^+$  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 ( $\text{pH}_i$ ) (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; Orłowski and Grinstein, 2004; Koliakos et al., 2008). It is regulated mainly by  $\text{pH}_i$  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 ( $\text{O}_2^\bullet$ ) *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  $\text{O}_2^\bullet$  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

Diphenyleiiodonium chloride (DPI), L-NAME, wortmannin, forskolin, bovine serum albumin (BSA),  $\text{NaHB}_4$ , 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 Merck (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 conjugated 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 was 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  $20 \text{ mmol l}^{-1}$  Hepes buffer,  $436 \text{ mmol l}^{-1}$  NaCl,  $10 \text{ mmol l}^{-1}$  KCl,  $53 \text{ mmol l}^{-1}$   $\text{MgSO}_4$ ,  $10 \text{ mmol l}^{-1}$   $\text{CaCl}_2$ .

### 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  $15^\circ\text{C}$ , in order to be acclimatized to laboratory conditions. Then, during the experimental procedure, mussels were fed with approximately 30 mg of dry-microencapsules/mussel (Myspat, Inve Aquaculture<sup>NV</sup>, 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  $200g$  for 15 min and the pellet was resuspended in Leibovitz L-15 medium supplemented with  $350 \text{ mmol l}^{-1}$  NaCl,  $7 \text{ mmol l}^{-1}$  KCl,  $4 \text{ mmol l}^{-1}$   $\text{CaCl}_2$ ,  $8 \text{ mmol l}^{-1}$   $\text{MgSO}_4$ ,  $40 \text{ mmol l}^{-1}$   $\text{MgCl}_2$ , 10% FCS,  $100 \text{ i.u. ml}^{-1}$  penicillin G,  $100 \mu\text{g ml}^{-1}$  streptomycin,  $40 \mu\text{g ml}^{-1}$  gentamycin,  $0.1 \mu\text{g ml}^{-1}$  amphotericin B at pH 7 and osmolarity  $1000 \text{ mosmol l}^{-1}$  (Cao et al., 2003). Cells were kept at  $15^\circ\text{C}$  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  $\text{CdCl}_2$ , at a final concentration of  $5 \mu\text{mol l}^{-1}$ , 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  $0.01 \text{ mmol l}^{-1}$  forskolin, an adenylyl cyclase (AC) activator,  $2 \mu\text{mol l}^{-1}$  cariporide, a NHE inhibitor,  $50 \text{ nmol l}^{-1}$  wortmannin, an inositol phosphate kinase (PI 3-kinase) inhibitor,  $10 \mu\text{mol l}^{-1}$  diphenyleiiodonium chloride (DPI), an inhibitor of

NADPH oxidase,  $10\ \mu\text{mol l}^{-1}$  L-NAME, a nitric oxide synthase inhibitor,  $2\ \mu\text{mol l}^{-1}$  cytochalasin D, an actin polymerization inhibitor, or  $11\ \mu\text{mol l}^{-1}$  GF-109203X, a potent inhibitor of protein kinase C (PKC), and then exposed to  $5\ \mu\text{mol l}^{-1}$  of  $\text{CdCl}_2$  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  $\text{NaHB}_4$  was added. The solution was incubated for 30 min at  $25^\circ\text{C}$ , neutralized with  $2\ \text{mol l}^{-1}$  HCl, followed by overnight dialysis against PS at  $4^\circ\text{C}$  with two buffer changes, dispensed in aliquots, and then stored at  $-80^\circ\text{C}$ .

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  $20\ \mu\text{l}$  EDTA ( $100\ \text{mmol l}^{-1}$ ),  $57\ \mu\text{l}$  ascorbic acid ( $833\ \text{mmol l}^{-1}$ ) and  $2\ \mu\text{l}$  ferrous ammonium sulfate ( $100\ \text{mmol l}^{-1}$ ). The solution was incubated for 90 min at  $37^\circ\text{C}$ , followed by overnight dialysis against PS at  $4^\circ\text{C}$  with two buffer changes, dispensed in aliquots, and then stored at  $-80^\circ\text{C}$ .

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  $5\ \mu\text{g ml}^{-1}$ . 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 ( $5\ \mu\text{g ml}^{-1}$ ).

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

$200\ \mu\text{l}$  of the diluted standards, samples (each sample contained  $5\ \mu\text{g}$  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  $4^\circ\text{C}$  and then washed three times with  $300\ \mu\text{l}$  PS. Freshly prepared DNPH solution ( $200\ \mu\text{l}$ ) was added, incubated for 45 min at room temperature in the dark, and then washed five times with  $300\ \mu\text{l}$  of PS:ethanol solution (1:1, v/v) and once with  $300\ \mu\text{l}$  of PS.  $260\ \mu\text{l}$  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  $300\ \mu\text{l}$  of PST (PS solution with the addition of 0.1% Tween 20). Anti-DNPH ( $200\ \mu\text{l}$ ) was added and the mixture incubated for 1 h at  $37^\circ\text{C}$  and then washed five times with  $300\ \mu\text{l}$  PST. Secondary antibody ( $200\ \mu\text{l}$  of anti-rabbit HPR-linked IgG in 20 ml blocking solution) was added and the mixture incubated for 1 h at  $37^\circ\text{C}$  followed by five washes with  $300\ \mu\text{l}$  PST.  $200\ \mu\text{l}$  of substrate solution [two tablets TMB and  $4\ \mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$  in 20 ml substrate buffer, (substrate buffer; 1.455 g  $\text{Na}_2\text{HPO}_4$  and 1.91 g citric acid in 150 ml distilled  $\text{H}_2\text{O}$  ( $\text{dH}_2\text{O}$ ), pH 5.

The volume was made up to 200 ml with  $\text{dH}_2\text{O}$  and stored at  $4^\circ\text{C}$ ] was added. After 3–5 min, the reaction was stopped by adding  $100\ \mu\text{l}$   $2\ \text{mol l}^{-1}$  HCl. The absorbance was read at 450 nm. PCC was calculated using a standard curve of BSA. Results (expressed as  $\text{nmol carbonyl mg}^{-1}$  protein after the subtraction of reduced BSA value) are given as means  $\pm$  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  $200\ \text{g}$  for 10 min. The supernatant was discarded and cells (at least  $10^6\ \text{cells ml}^{-1}$ ) were lysed in buffer containing  $50\ \text{mmol l}^{-1}$  Tris-HCl, pH 8,  $0.25\ \text{mol l}^{-1}$  sucrose, 1% (w/v) SDS,  $2\ \text{mmol l}^{-1}$  sodium orthovanadate,  $10\ \text{mmol l}^{-1}$  NaF,  $5\ \text{mmol l}^{-1}$  EDTA,  $5\ \text{mmol l}^{-1}$  NEM (*N*-ethylmaleimide), 0.1% Nonidet-P40, and protease inhibitors ( $1\ \mu\text{g ml}^{-1}$  pepstatin,  $10\ \mu\text{g ml}^{-1}$  leupeptin,  $40\ \mu\text{g ml}^{-1}$  PMSF) for 1 h at  $4^\circ\text{C}$ . After sonication at 50 W for 45 s, whole-cell lysate was centrifuged at  $10,000\ \text{g}$  for 10 min at  $4^\circ\text{C}$ . Supernatant was collected and protein content was determined (Bradford assay and BSA as a standard). At least  $30\ \mu\text{g}$  of protein was boiled for 4 min in  $1\times$  SDS sample buffer ( $0.5\ \text{mol l}^{-1}$  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  $4^\circ\text{C}$ , 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- $\beta$ -actin (as a loading control). Specifically, membranes were blocked overnight at  $4^\circ\text{C}$  with 5% skimmed milk and incubated at room temperature with anti- $\beta$ -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  $\pm$  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 Dunnett 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  $\text{CdCl}_2$  ( $5\ \mu\text{mol l}^{-1}$ ). 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

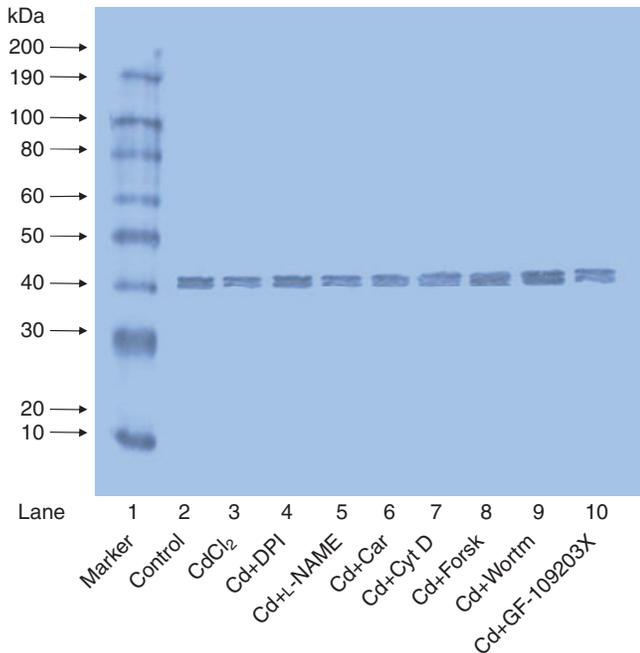


Fig. 1. Western blot using antibodies to actin (one-dimensional SDS 8% polyacrylamide gel electrophoresis), in order to confirm equal loading of protein into the wells. Lane 1, standards; lane 2, control cells; lane 3, Cd-treated cells; lanes 4-10, Cd-treated cells exposed to,  $10 \mu\text{mol l}^{-1}$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

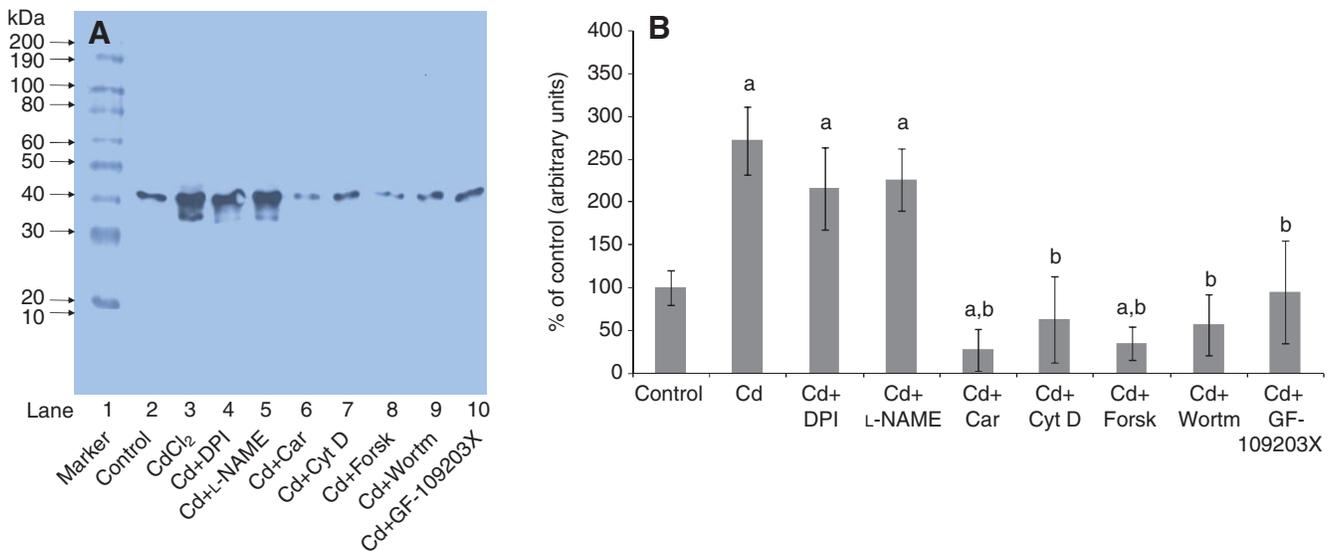


Fig. 2. (A) Detection of actin glutathionylation by western blotting (one-dimensional SDS 8% polyacrylamide gel electrophoresis) using anti-GSH, in haemocytes of mussels. Cells were exposed to  $5 \mu\text{mol l}^{-1}$  Cd alone (lane 3) or to Cd with either  $10 \mu\text{mol l}^{-1}$  DPI (lane 4),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

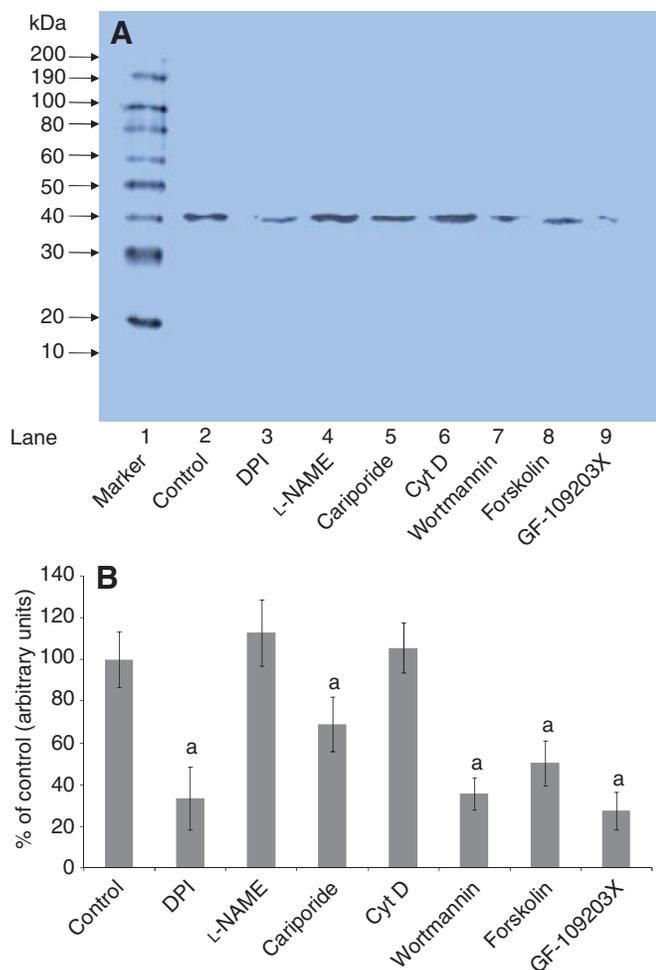


Fig. 3. (A) Detection of actin glutathionylation by western blotting (one-dimensional SDS 8% polyacrylamide gel electrophoresis) using anti-GSH, in control cells (lane 2) and cells treated with each agonist and antagonist in the absence of Cd (lanes 3–9). Specifically, cells were treated with either  $10 \mu\text{mol l}^{-1}$  DPI (lane 3),  $10 \mu\text{mol l}^{-1}$  L-NAME (lane 4),  $2 \mu\text{mol l}^{-1}$  cariporide (lane 5),  $2 \mu\text{mol l}^{-1}$  cytochalasin D (lane 6),  $50 \text{ nmol l}^{-1}$  wortmannin (lane 7),  $0.01 \text{ mmol l}^{-1}$  forskolin (lane 8) or  $11 \mu\text{mol l}^{-1}$  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  $\pm$  s.d. from four independent experiments and expressed as a percentage of control band optical density (arbitrary units). <sup>a</sup>Significantly different from control cells (Dunnett test,  $P < 0.01$ ).

addition, inhibition of actin polymerization, in cells pre-treated with  $2 \mu\text{mol l}^{-1}$  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  $5 \mu\text{mol l}^{-1}$  Cd had significantly increased protein carbonyl content, relative to untreated haemocytes. Cells pre-treated either with  $0.01 \text{ mmol l}^{-1}$  forskolin or  $50 \text{ nmol l}^{-1}$  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

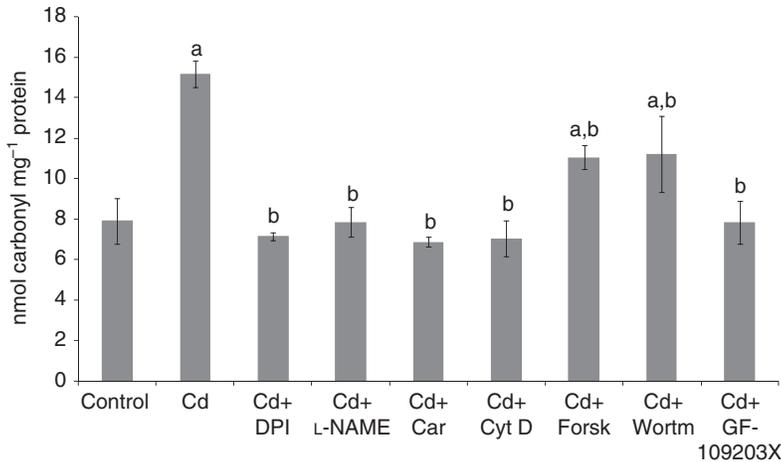


Fig. 4. Detection of protein carbonyl content in haemocytes of mussels. Cells were exposed to  $5\mu\text{mol l}^{-1}$   $\text{CdCl}_2$  alone or together with either  $10\mu\text{mol l}^{-1}$  DPI,  $10\mu\text{mol l}^{-1}$  L-NAME,  $2\mu\text{mol l}^{-1}$  cariporide,  $2\mu\text{mol l}^{-1}$  cytochalasin D,  $0.01\text{ mmol l}^{-1}$  forskolin,  $50\text{ nmol l}^{-1}$  wortmannin or  $11\mu\text{mol l}^{-1}$  GF-109203X. Results (expressed as  $\text{nmol carbonyl mg}^{-1}$  protein) are means  $\pm$  s.d. from three independent experiments. In each experiment, measurements on treated cells were carried out in triplicate. <sup>a</sup>Significantly different from control cells; <sup>b</sup>significantly different from cells treated only with Cd (Dunnett 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).

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 (Hershinkel 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; Torrelles 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 ( $\text{O}_2\bullet$ ) 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;

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

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

Cells were exposed to each agonist or antagonist alone, without the addition of cadmium.

Values are means  $\pm$  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 Dunnett test,  $P<0.01$ .

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 sulphhydryl 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.

## REFERENCES

- Adams, T. K., Saydam, N., Steiner, F., Schaffner, W. and Freedman, J. H. (2002). Activation of gene expression by metal-responsive signal transduction pathways. *Environ. Health Perspect.* **110**, 813-817.
- Alamdari, H. D., Kostidou, E., Paletas, K., Sarigianni, M., Konstas, G. P. A., Karaperidou, A. and Koliakos, G. (2005). High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein. *Free Rad. Biol. Med.* **39**, 1362-1367.
- Almroth, C. B., Sturve, J., Berglund, A. and Förlin, L. (2005). Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. *Aquat. Toxicol.* **73**, 171-180.
- Arumugam, M., Romestand, B., Torrelles, J. and Roch, P. (2000). In vitro production of superoxide and nitric oxide (as nitrite and nitrate) by *Mytilus galloprovincialis* haemocytes upon incubation with PMA or laminarin or during yeast phagocytosis. *Eur. J. Cell Biol.* **79**, 513-519.
- Avkiran, M. and Haworth, R. (2003). Regulatory effects of G protein-coupled receptors on cardiac sarcolemmal Na<sup>+</sup>/H<sup>+</sup> exchanger activity: signaling and significance. *Cardiovasc. Res.* **57**, 942-952.
- Baggiolini, M., Ruch, W. and Cooper, P. H. (1986). Measurement of hydrogen peroxide production by phagocytes using homovanillic acid and horseradish peroxidase. *Meth. Enzymol.* **132**, 395-400.
- Baldini, P. M., De Vito, P., Vismara, D., Bagni, C., Zalfa, F., Minieri, M. and Nardo, P. Di. (2005). Atrial natriuretic peptide effects on intracellular pH changes and ROS production in HEPG2 cells: Role of p38 MAPK and phospholipase D. *Cell Physiol. Biochem.* **15**, 77-88.
- Bao, S. and Knoell, D. L. (2006). Zinc modulates airway epithelium susceptibility to death receptor-mediated apoptosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* **290**, L433-L441.
- Barthel, A., Ostrakhovitch, E. A., Walter, P. L., Kampkötter, A. and Klotz, L. O. (2007). Stimulation of phosphoinositide 3-kinase/Akt signaling by copper and zinc ions: mechanisms and consequences. *Arch. Biochem. Biophys.* **463**, 175-182.
- Bianchini, L. and Pouyssegur, J. (1994). Molecular structure and regulation of vertebrate Na<sup>+</sup>/H<sup>+</sup> exchangers. *J. Exp. Biol.* **196**, 337-345.
- Cala, P. M. (1983a). Cell volume regulation by Amphiuma red blood cells. The role of Ca<sup>2+</sup> as a modulator of alkali metal/H<sup>+</sup> exchange. *J. Gen. Physiol.* **82**, 761-784.
- Cala, P. M. (1983b). Volume regulation by red blood cells: Mechanisms of ion transport between cell and mechanisms. *Mol. Physiol.* **4**, 33-52.
- Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657.
- Cao, A., Mercado, L., Ramos-Martinez, J. I. and Barcia, R. (2003). Primary cultures of haemocytes from *Mytilus galloprovincialis* Lmk: Expression of IL-2R $\alpha$ . *Aquaculture* **216**, 1-8.
- Carballal, M. J., López, C., Azevedo, C. and Villalba, A. (1997). Enzymes involved in defence functions of hemocytes of mussel *Mytilus galloprovincialis*. *J. Invert. Pathol.* **70**, 96-105.
- Cheng, T. C. (1981). Bivalves. In *Invertebrates Blood Cells* (ed. N. A. Ratcliffe and A. F. Rowley), pp. 233-300. London: Academic Press.
- Cheng, T. C. (1988). In vivo effects of heavy metals on cellular defence mechanisms of *Crassostrea virginica* phagocytic and endocytotic indices. *J. Invertebr. Pathol.* **51**, 215-220.
- Cheng, T. C. and Sullivan, J. T. (1984). Effects of heavy metals on phagocytosis by molluscan hemocytes. *Mar. Environ. Res.* **14**, 305-315.
- Conte, A. and Ottaviani, E. (1995). Nitric oxide synthase activity in molluscan hemocytes. *FEBS Lett.* **365**, 120-124.
- Coogan, T. P., Bare, R. M. and Waalkes, M. P. (1992). Cadmium-induced DNA strand damage in cultured liver cells: Reduction in cadmium genotoxicity following zinc pre-treatment. *Toxicol. Appl. Pharmacol.* **113**, 227-233.
- Costa, V. M., Amorim, M. A., Quintanilha, A. and Moradas-Ferreira, P. (2002). Hydrogen peroxide-induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae*: the involvement of the oxidative stress response regulators Yap1 and Skn7. *Free Radic. Biol. Med.* **33**, 1507-1515.
- Dailianis, S. (2009). Production of superoxides and nitric oxide generation in haemocytes of mussel *Mytilus galloprovincialis* (Lmk.) after exposure to cadmium: a possible involvement of Na<sup>+</sup>/H<sup>+</sup> exchanger in the induction of cadmium toxic effects. *Fish Shellfish Immunol.* **27**, 446-453.
- Dailianis, S. and Kaloyianni, M. (2004). Cadmium induces both pyruvate kinase and Na<sup>+</sup>/H<sup>+</sup> exchanger activity through protein kinase C mediated signal transduction, in isolated digestive gland cells of *Mytilus galloprovincialis* (L.). *J. Exp. Biol.* **207**, 1665-1674.
- Dailianis, S., Piperakis, S. M. and Kaloyianni, M. (2005). Cadmium effects on ROS production and DNA damage via adrenergic receptors stimulation: role of Na<sup>+</sup>/H<sup>+</sup> exchanger and PKC. *Free Radic. Res.* **39**, 1059-1070.
- Dalle-Donne, I., Rossi, R., Milzani, A., Di Simplicio, P. and Colombo, R. (2001). The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free Rad. Biol. Med.* **31**, 1624-1632.
- Dalle-Donne, I., Rossi, R., Giustarini, D., Colombo, R. and Milzani, A. (2003a). Actin S-glutathionylation: evidence against a thio-disulphide exchange mechanism. *Free Rad. Biol. Med.* **35**, 1185-1193.
- Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A. and Colombo, R. (2003b). Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **329**, 23-38.
- Dalle-Donne, I., Scaloni, A., Giustarini, D., Cavarra, E., Tell, G., Lungarella, G., Colombo, R., Rossi, R. and Milzani, A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. *Mass Spectrom. Rev.* **24**, 55-99.
- Dalle-Donne, I., Rossi, R., Giustarini, D., Colombo, R. and Milzani, A. (2007). S-glutathionylation in protein redox regulation. *Free Radic. Biol. Med.* **43**, 883-898.
- Davies, K. J. A. and Delsignore, M. E. (1987). Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* **262**, 9908-9913.
- Davies, S. M., Poljak, A., Duncan, M. W., Smythe, G. A. and Murphy, M. P. (2001). Measurements of protein carbonyls, ortho- and meta-tyrosine and oxidative phosphorylation complex activity in mitochondria from young and old rats. *Free Radic. Biol. Med.* **31**, 181-190.
- Dowling, V., Pascal, C., Hoarau, P. C., Romeo, M., O'Halloran, J., van Pelt, F., O'Brien, N. and Sheehan, D. (2006). Protein carbonylation and heat shock response in *Ruditapes decussatus* following p,p'-dichlorodiphenyldichloroethylene (DDE) exposure: A proteomic approach reveals that DDE causes oxidative stress. *Aquat. Toxicol.* **77**, 11-18.
- England, K. and Cotter, T. (2004). Identification of carbonylated proteins by MALDI-TOF mass spectroscopy reveals susceptibility of ER. *Biochem. Biophys. Res. Commun.* **320**, 123-130.
- Eom, S. J., Kim, E. Y., Lee, J. E., Kang, H. J., Shim, J., Kim, S. U., Gwag, B. J. and Choi, E. J. (2001). Zn<sup>2+</sup> induces stimulation of the c-Jun N-terminal kinase signaling pathway through phosphoinositide 3-Kinase. *Mol. Pharmacol.* **59**, 981-986.
- Fagotti, A., Di Rosa, I., Simoncelli, F., Pipe, R. K., Panara, F. and Pascolini, R. (1996). The effects of copper on actin and fibronectin organization in *Mytilus galloprovincialis* haemocytes. *Dev. Comp. Immunol.* **20**, 383-391.
- Fang, F. C. (1997). Mechanisms of nitric oxide-related antimicrobial activity. *J. Clin. Invest.* **99**, 2818-2825.
- Filomeni, G., Rotilio, G. and Ciriolo, M. R. (2002). Cell signalling and the glutathione redox system. *Biochem. Pharmacol.* **64**, 1057-1064.
- Forman, H. J., Fukuto, J. M. and Torres, M. (2004). Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am. J. Physiol. Cell Physiol.* **287**, C246-C256.
- Franchini, A., Fontanili, P. and Ottaviani, E. (1995a). Invertebrates immunocytes: Relationship between phagocytosis and nitric oxide production. *Comp. Biochem. Physiol.* **110B**, 403-407.
- Franchini, A., Conte, A. and Ottaviani, E. (1995b). Nitric oxide: an ancestral immunocyte effector molecule. *Adv. Neuroimmunol.* **5**, 265-278.
- Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I., Salmons, M., Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E. et al. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc. Natl. Acad. Sci. USA* **99**, 3505-3510.
- García-García, E. (2005). Diversity in phagocytic signaling: a story of greed, sharing, and exploitation. In *Molecular mechanisms of phagocytosis* (ed. C., Rosales), pp. 1-22. Georgetown, TX: Landes Biosciences/Eurekah.com and Springer Science+Business Media.
- García-García, E. and Rosales, C. (2002). Signal transduction during Fc receptors-mediated phagocytosis. *L. Leukoc. Biol.* **72**, 1092-1108.
- García-García, E., Prado-Álvarez, M., Novoa, B., Figueras, A. and Rosales, C. (2008). Immune responses of mussel hemocyte subpopulations are differentially

- regulated by enzymes of the PI 3-K, PKC, and ERK kinase families. *Dev. Comp. Immunol.* **32**, 637-653.
- Ghezzi, P.** (2005). Regulation of protein function by glutathionylation. *Free Rad. Res.* **39**, 573-580.
- Ghezzi, P. and Bonetto, V.** (2003). Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* **3**, 1145-1153.
- Goldstein, B. J., Mahadev, K., Wu, X., Zhu, L. and Motoshima, H.** (2005). Role of insulin-induced reactive oxygen species in the insulin signaling pathway. *Antioxid. Redox Signal.* **7**, 1021-1031.
- Gómez-Mendikute, A. and Cajaraville, M. P.** (2003). Comparative effects of cadmium, copper, paraquat and benzo[a]pyrene on the actin cytoskeleton and production of reactive oxygen species (ROS) in mussel haemocytes. *Toxicol. In Vitro* **17**, 539-546.
- Gourdon, I., Guerin, M.-C., Torreilles, J. and Roch, P.** (2001). Nitric oxide generation by haemocytes of the mussel *Mytilus galloprovincialis*. *Nitric Oxide: Biol. Chem.* **5**, 1-6.
- Grune, T., Reinheckel, T. and Davies, K. J. A.** (1997). Degradation of oxidized proteins in mammalian cells. *FASEB J.* **11**, 526-534.
- Haynes, V., Effering, S. L., Squires, R. J., Traaseth, N., Solien, J., Ettl, A. and Giulivi, C.** (2003). Mitochondrial nitric-oxide synthase: role in pathophysiology. *IUBMB Life* **55**, 599-603.
- HCMR, Oceanography Institute** (1997). Monitoring of the quality of the marine environment of the gulf of Thessaloniki through 1997. Final technical report, pp. 1-250 (In Greek), www.hcmr.gr.
- Hershinkel, M., Moran, A., Grossman, N. and Sekler, I.** (2001). A zinc-sensing receptor triggers the release of intracellular  $Ca^{2+}$  and regulates ion transport. *Proc. Natl. Acad. Sci. USA* **98**, 11749-11754.
- Humphries, K. M., Juliano, C. and Taylor, S. S.** (2002). Regulation of cAMP-dependent protein kinase activity by glutathionylation. *J. Biol. Chem.* **277**, 43505-43511.
- Kaloyianni, M., Stamioti, R. and Dailianis, S.** (2005). Zinc and 17 $\beta$ -estradiol induce modifications in  $Na^+/H^+$  exchanger and pyruvate kinase activity through protein kinase C in isolated mantle/gonad cells of *Mytilus galloprovincialis*. *Comp. Biochem. Physiol. C* **141**, 257-266.
- Kaloyianni, M., Ragia, V., Tzeranaki, I. and Dailianis, S.** (2006). The influence of Zn on signaling pathways and attachment of *Mytilus galloprovincialis* haemocytes to extracellular matrix proteins. *Comp. Biochem. Physiol. C* **144**, 93-100.
- Kaloyianni, M., Dailianis, S., Chrisikopoulou, E., Zannou, A., Koutsogiannaki, S., Alamdari, D. H., Koliakos, G. and Dimitriadis, V. K.** (2009). Oxidative effects of inorganic and organic contaminants on haemolymph of mussels. *Comp. Biochem. Physiol. C* **149**, 631-639.
- Khaled, A. R., Moor, A. N., Li, A., Kim, K., Ferris, D. K., Muegge, K., Fisher, R. J., Fliegel, L. and Durum, S. K.** (2001). Trophic factor withdrawal: p38 mitogen-activated protein kinase activates NHE1, which induces intracellular alkalinization. *Mol. Cell Biol.* **21**, 7545-7557.
- Kim, S., Jung, Y., Kim, D., Koh, H. and Chung, J.** (2000). Extracellular zinc activates p70 S6 kinase through the phosphatidylinositol 3-kinase signaling pathway. *J. Biol. Chem.* **275**, 25979-25984.
- Kirchin, M. A., Moore, M. N., Dean, R. T. and Winston, G. W.** (1992). The role of oxyradicals in intracellular proteolysis and toxicity in mussels. *Mar. Environ. Res.* **34**, 315-320.
- Klatt, P. and Lamas, S.** (2000). Regulation of protein function by S-glutathionylation in response to oxidative and nitrosative stress. *Eur. J. Biochem.* **267**, 4928-4944.
- Klebanoff, S. J.** (1988). Phagocyte cells: Products of oxygen metabolism. In *Inflammation Basic Principles and Clinical Correlates* (ed. J. I. Gallin, I. M. Goldstein and R. Snyderman), pp. 391-344. New York: Raven Press Ltd.
- Koliakos, G., Paletas, K. and Kaloyianni, M.** (2008). NHE-1: a molecular target for signaling and cell matrix interactions. *Connect. Tissue Res.* **49**, 157-161.
- Konishi, H., Fujiyoshi, T., Fukui, Y., Matsuzaki, H., Yamamoto, T., Ono, Y., Andjelkovic, M., Hemmings, B. A. and Kikkawa, U.** (1999). Activation of protein kinase B induced by  $H_2O_2$  and heat shock through distinct mechanisms dependent and independent of phosphatidylinositol 3-kinase. *J. Biochem.* **126**, 1136-1143.
- Konstantinidis, D., Paletas, K., Koliakos, G. and Kaloyianni, M.** (2009). The ambiguous role of the  $Na^+/H^+$  exchanger isoform 1 (NHE1) in leptin-induced oxidative stress in human monocytes. *Cell Stress and Chaperones* (doi:10.1007/s12192-009-0110-4).
- Koutsogiannaki, S., Evangelinos, N., Koliakos, G. and Kaloyianni, M.** (2006). Cytotoxic mechanisms of  $Zn^{2+}$  and  $Cd^{2+}$  involve  $Na^+/H^+$  exchanger (NHE) activation by ROS. *Aquat. Toxicol.* **78**, 315-324.
- Kwon, J., Lee, S.-R., Yang, K.-S., Ahn, Y., Kim, Y. J., Stadtman, E. R. and Rhee, S. G.** (2004). Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proc. Natl. Acad. Sci. USA* **101**, 16419-16424.
- Laemmli, U. K.** (1972). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I. and Lenz, A.** (1990). Determination of carbonyl content in oxidatively modified proteins. *Method. Enzymol.* **186**, 464-478.
- Mahadev, K., Wu, X., Zilbering, A., Zhu, L., Lawrence, J. T. R. and Goldstein, B. J.** (2001). Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes. *J. Biol. Chem.* **276**, 48662-48669.
- McDonagh, B. and Sheehan, D.** (2007). Effect of oxidative effects on protein thiols in the blue mussel *Mytilus edulis*: Proteomic identification of target proteins. *Proteomics* **7**, 3395-3403.
- McDonagh, B., Tyther, R. and Sheehan, D.** (2005). Carbonylation and glutathionylation of proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and western blotting: Actin as a target for oxidative stress. *Aquat. Toxicol.* **73**, 315-326.
- McHardy, L., Sinotte, R., Troussard, A., Sheldon, C., Church, J., Williams, D. E., Andersen, R. J., Dedhar, S., Roberge, M. and Roskelley, C. D.** (2004). The tumor invasion inhibitor dihydromotuporamine C activates Rho, remodels stress fibers and focal adhesions, and stimulates sodium/proton exchange. *Cancer Res.* **64**, 1468-1474.
- Micic, M., Bihari, N., Labura, Z., Muller, W. E. G. and Batel, R.** (2001). Induction of apoptosis in the blue mussel *Mytilus galloprovincialis* by tri-n-butyltin chloride. *Aquat. Toxicol.* **55**, 61-73.
- Misra, R. R., Smith, G. T. and Waalkes, M. P.** (1998). Evaluation of the direct genotoxic potential of cadmium in four different rodent cell lines. *Toxicology* **126**, 103-114.
- Moolenaar, W. H., Sien, T. R. Y., Van de Saag, P. T. and De Laat, S. W.** (1983).  $Na^+/H^+$  exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature* **304**, 645-648.
- Moor, A., Gan, X., Karmazyn, M. and Fliegel, L.** (2001). Protein kinase mediated regulation of the  $Na^+/H^+$  exchanger isoform 1 (NHE1) in ischemic and ischemic/reperfused rat heart. *J. Biol. Chem.* **27**, 16113-16122.
- Noel, D., Bachere, E. and Mialhe, E.** (1993). Phagocytosis associated chemiluminescence of hemocytes in *Mytilus edulis* (Bivalvia). *Develop. Comp. Immunol.* **17**, 483-493.
- Novas, A., Cao, A., Barcia, R. and Ramos-Martinez, J. I.** (2004). Nitric oxide release by haemocytes of the mussel *Mytilus galloprovincialis* Lmk was provoked by interleukin-2 but not lipopolysaccharide. *Int. J. Biochem. Cell Biol.* **36**, 390-394.
- Olabarrieta, I., L'Azou, B., Yuric, S., Cambar, J. and Cajaraville, M. P.** (2001). In vitro effects of cadmium on two different animal cell models. *Toxicol. In Vitro* **15**, 511-517.
- Orlowski, J. and Grinstein, S.** (2004). Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflug. Archiv.* **447**, 549-565.
- Ostrakhovitch, E. A., Lordnejad, M. R., Schliess, F., Sies, H. and Klotz, L. O.** (2002). Copper ions strongly activate the phosphoinositide-3-kinase/Akt pathway independent of the generation of reactive oxygen species. *Arch. Biochem. Biophys.* **397**, 232-239.
- Ottaviani, E., Paemen, L. R., Cadet, P. and Stefano, G. B.** (1993). Evidence for nitric oxide production and utilization as a bacteriocidal agent by invertebrate immunocytes. *Eur. J. Pharmacol.* **248**, 319-324.
- Padgett, E. L. and Pruet, S. B.** (1992). Evaluation of nitrite production by human monocyte-derived macrophages. *Biochem. Biophys. Res. Commun.* **186**, 775-781.
- Paradiso, A., Cardone, R. A., Bellizzi, A., Bagorda, A., Guerra, L., Tommasino, M., Casavola, V. and Reshkin, S. J.** (2004). The  $Na^+/H^+$  exchanger-1 induces cytoskeletal changes involving reciprocal RhoA and Rac1 signaling, resulting in motility and invasion in MDA-MB-435 cells. *Breast Cancer Res.* **6**, R616-R628.
- Pederson, S. F., Varming, C., Christensen, S. T. and Hoffmann, E. K.** (2002). Mechanisms of activation of NHE by cell shrinkage and by calyculin A in *Ehrlich ascites* tumor cells. *J. Membr. Biol.* **189**, 67-81.
- Persad, S., Elimban, V., Kaila, J. and Dhalla, N. S.** (1997). Biphasic alterations in cardiac beta-adrenoceptor signal transduction mechanism due to oxyradicals. *J. Pharmacol. Exp. Ther.* **282**, 1623-1631.
- Persad, S., Panagia, V. and Dhalla, N. S.** (1998). Role of  $H_2O_2$  in changing  $\beta$ -adrenoceptor and adenylyl cyclase in ischemia-reperfused hearts. *Mol. Cell. Biochem.* **186**, 99-106.
- Pipe, R. K., Coles, J. A., Carissan, F. M. M. and Ramanathan, K.** (1999). Copper induced immunomodulation in the marine mussel *Mytilus edulis*. *Aquat. Toxicol.* **46**, 43-54.
- Pourahmad, J. and O'Brien, P. J.** (2000). A comparison of hepatocyte cytotoxic mechanisms for  $Cu^{2+}$  and  $Cd^{2+}$ . *Toxicology* **143**, 263-273.
- Pruski, A. M. and Dixon, D. R.** (2002). Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of *Mytilus edulis* L. *Aquat. Toxicol.* **57**, 127-137.
- Putney, L. K., Denker, S. P. and Barber, D. L.** (2002). The changing face of the  $Na^+/H^+$  exchanger, NHE1: Structure, regulation, and cellular actions. *Ann. Rev. Pharmacol. Toxicol.* **42**, 527-552.
- Rabek, J. P., Boylston, W. H. and Papaconstantinou, J.** (2003). Carbonylation of ER chaperone proteins in aged mouse liver. *Biochem. Biophys. Res. Commun.* **305**, 566-572.
- Reshkin, S. J., Bellizzi, A., Albarani, V., Guerra, L., Tommasino, M., Paradiso, A. and Casavola, V.** (2000). Phosphoinositide 3-kinase is involved in the tumor-specific activation of human breast cancer cell  $Na^+/H^+$  exchange, motility, and invasion induced by serum deprivation. *J. Biol. Chem.* **275**, 5361-5369.
- Rothstein, E. C., Byron, K. L., Reed, R. E., Fliegel, L. and Lucchesi, P. A.** (2002).  $H_2O_2$ -induced  $Ca^{2+}$  overload in NRVM involves ERK1/2 MAP kinases: Role for an NHE-1-dependent pathway. *Am. J. Physiol. Heart Circ. Physiol.* **283**, H598-H605.
- Salman-Tabcheh, S., Guerin, M.-C. and Torreilles, J.** (1995). Nitration of tyrosyl-residues from extra and intracellular proteins in human whole blood. *Free Rad. Biol. Med.* **19**, 695-698.
- Sand, C., Peters, S. L., Pfaffendorf, M. and van Zwieten, P. A.** (2003). The influence of endogenously generated reactive oxygen species on the inotropic and chronotropic effects of adrenoreceptor and ET-receptor stimulation. *Naunyn Schmiedeberg's Arch. Pharmacol.* **367**, 635-639.
- Sauvage, M., Maziere, P., Fathallah, H. and Giraud, F.** (2000). Insulin stimulates NHE1 activity by sequential activation of phosphatidylinositol 3-kinase and protein kinase C zeta in human erythrocytes. *Eur. J. Biochem.* **267**, 955-962.
- Schafer, F. Q. and Buettner, G. R.** (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **30**, 1191-1212.
- Shelton, M. D., Chock, P. B. and Mielay, J. J.** (2005). Glutaredoxin: role in reversible protein S-glutathionylation and regulation of redox signal transduction and protein translocation. *Antioxid. Redox Signal.* **7**, 348-366.
- Shimamura, H., Terada, Y., Okado, T., Tanaka, H., Inoshita, S. and Sasaki, S.** (2003). The PI3-kinase-Akt pathway promotes mesangial cell survival and inhibits apoptosis in vivo via NF $\kappa$ B and Bad. *J. Am. Soc. Nephrol.* **14**, 1427-1434.

- Sonoda, Y., Watanabe, S., Matsumoto, Y., Aizu-Yokota, E. and Kasahara, T.** (1999). FAK is the upstream signal protein of the phosphatidylinositol 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis of a human glioblastoma cell line. *J. Biol. Chem.* **274**, 10566-10570.
- Stacey, N. H., Cantilena, L. R., Jr and Klaassen, C. D.** (1980). Cadmium toxicity and lipid peroxidation in isolated rat hepatocytes. *Toxicol. Appl. Pharmacol.* **53**, 470-480.
- Stadtman, E. R.** (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* **62**, 797-821.
- Stohs, S. J. and Bagchi, D.** (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.* **18**, 321-336.
- Tafalla, C., Novoa, B. and Figueras, A.** (2002). Production of nitric oxide by mussel (*Mytilus galloprovincialis*) hemocytes and effect of exogenous nitric oxide on phagocytic functions. *Comp. Biochem. Physiol. B* **132**, 423-431.
- Tan, C. M., Xenoyannis, S. and Feldman, R. D.** (1995). Oxidant stress enhances adenylyl cyclase activation. *Circ. Res.* **77**, 710-717.
- Tan, C. M., Kelvin, D. J., Litchfield, D. W., Ferguson, S. S. G. and Feldman, R. D.** (2001). Tyrosine kinase-mediated serine phosphorylation of adenylyl cyclase. *Biochemistry* **40**, 1702-1709.
- Thomas, D. D., Espey, M. G., Vitek, M. P., Miranda, K. M. and Wink, D. A.** (2002). Protein nitration is mediated by heme and free metals through Fenton-type chemistry: An alternative to the NO/O<sub>2</sub><sup>-</sup> reaction. *Proc. Natl. Acad. Sci. USA* **99**, 12691-12696.
- Tominaga, T. and Barber, D. L.** (1998). Na-H exchange acts downstream of RhoA to regulate integrin-induced cell adhesion and spreading. *Mol. Biol. Cell* **9**, 2287-2303.
- Torreilles, J., Guerin, M.-C. and Roch, P.** (1996). Espèces oxygénées réactives et système de défense des bivalves marins. *C. R. Acad. Sci. Paris* **318**, 209-218.
- Van Der Vlies, D., Pap, E. H. W., Post, J. A., Celis, J. E. and Wirtz, K. W. A.** (2002). Endoplasmic reticulum proteins of normal human dermal fibroblasts are the major targets for oxidative stress induced by hydrogen peroxide. *Biochem. J.* **366**, 825-830.
- Ward, N. E., Stewart, J. R., Ioannides, C. G. and O'Brian, C. A.** (2000). Oxidant-induced S-glutathiolation inactivates protein kinase C- $\alpha$  (PKC- $\alpha$ ): A potential mechanism of PKC isozyme regulation. *Biochemistry* **39**, 10319-10329.
- Winston, G. W.** (1991). Oxidants and antioxidants in aquatic animals. *Comp. Biochem. Physiol. C* **100**, 173-176.
- Winston, G. W., Moore, M. N., Kirchin, M. A. and Soverchia, C.** (1996). Production of reactive oxygen species by haemocytes from the marine mussel, *Mytilus edulis*: lysosomal localization and effect of xenobiotics. *Comp. Biochem. Physiol. C* **113**, 221-229.
- Winterbourn, C. C.** (2008). Reconciling the chemistry and biology of reactive oxygen species. *Nature Chem. Biol.* **4**, 278-286.
- Wong, J. M. and Billiar, T. R.** (1995). Regulation and function of inducible nitric oxide synthase during sepsis and acute inflammation. *Adv. Pharmacol.* **34**, 155-170.