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Cadmium effects on ROS production and DNA damage via adrenergic receptors stimulation: Role of Na^+/H^+ exchanger and PKC

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Abstract

The objective of the present study was to elucidate the events that are involved in reactive oxygen species (ROS) production and DNA damage after adrenergic receptors stimulation by cadmium, in relation to cAMP, protein kinase C (PKC) and Na^+/H^+ exchanger (NHE). Cadmium (50 μM) caused increased levels of ROS with a concomitant increase in DNA damage in digestive gland of *Mytilus galloprovincialis*. Either the use of EIPA, a NHE blocker, or calphostin C, the inhibitor of PKC, reduced cadmium effects. Cells treated with α_1-, α_2-, β- and β_1- adrenergic antagonists together with cadmium reversed cadmium alone effects, while the respective adrenergic agonists, phenylephrine and isoprenaline, mimic cadmium effects. Moreover, cadmium caused an increase in the levels of cAMP in digestive gland cells that were reversed after NHE and PKC inhibition as well as in the presence of each type of adrenergic antagonist. The different sensitivity of α_1-, α_2-, β-, β_1- adrenergic receptors on ROS, cAMP production and DNA damage possibly leads to the induction of two signaling pathways that may be interacting or to the presence of a compensatory pathway that acts in concert with the α- and β-adrenergic receptors. In these signaling pathways PKC and NHE play significant role.

Keywords: Cadmium, cAMP, DNA damage, *Mytilus galloprovincialis*, NHE, PKC

Introduction

The marine environment receives a wide variety of chemical and physical agents from natural and anthropogenic contaminant inputs. Heavy metals such as cadmium, represent one of the most widespread and serious forms of environmental contamination. Cadmium concentration in industrial effluents may vary from 0 to 1000 mg/l, whereas that in municipal waste waters is commonly lower than 10 μg/l [1]. In addition, molluscs accumulate large amounts of cadmium in their tissues. Specifically, the bivalve *Pecten novae-zeelandiac*, the oceanic squid *Symplctoteuthis oualaniensis* and oysters has been reported to accumulate 1900–2000 μg/g dry weight in their tissues [2,3].

Many of the pollutants are toxic and cause a series of coordinated physiological reactions in the organisms. Cadmium affects the expression of several genes [4], interferes with mitochondrial function [5] and with calcium and essential metals homeostasis [6,7]. Moreover, there are several studies, which demonstrate that cadmium may be implicated in the induction of oxidative damage and cell death, via the enhancement of intracellular reactive oxygen species (ROS) production and perturbation of antioxidant efficiency, which often prelude the onset of alterations

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like DNA damage, lipid peroxidation and enzyme inhibition [8–14].

To our knowledge, there is no information about the relation between cadmium cytotoxicity and signal cascade induced in isolated digestive gland cells of *Mytilus*. Although there are several reports in marine species concerning the genotoxic effect of heavy metals [15–19], there is limited knowledge about the effect of cadmium on invertebrate cell types [16,19,20]. Adams et al. [21] reported that cadmium can induce signal transduction cascades, such as protein kinase C (PKC), tyrosine kinase and casein kinase II. The involvement of Ca^{+2} in cadmium mediated signaling in the digestive gland cells of *M. galloprovincialis* is also probable since it is reported that cadmium impairs influx of Ca^{+2} in gills of the mussel [22]. Recent studies from our laboratory also showed that cadmium can induce a PKC-mediated signal transduction pathway with a concomitant increase of intracellular pH (pHi) as well as Na^+ influx, which indicate the induction of Na^+/H^+ exchanger (NHE) activity in digestive gland cells of *Mytilus galloprovincialis* [23].

NHE is a membrane system involved in the coupled exchange of Na^+ with H^+ in a variety of eukaryotic cells. This system seems to be a major regulating element of the intracellular pH (pHi) both in vertebrates and invertebrates, [23–26] and cell volume control [27,28]. Furthermore, this ubiquitous, amiloride-sensitive exchanger has been shown to implicate in cell-cycle regulation, cell proliferation, apoptosis and neoplasia [29–33].

It is reported that NHE was activated by cAMP [34]. The actions of cAMP are well known in the regulation of various cellular functions including glycogenolysis [35], cell proliferation, differentiation, and gene induction, through the activation of cAMP-dependent protein kinase A [36]. On the other side, in marine species, little is known concerning the role of cAMP in cadmium- induced signaling pathways. cAMP is reported to be enhanced in mussels tissues collected from polluted sites [37].

ROS include superoxide (O_2^-), singlet oxygen ($\cdot O_2$), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical (HO·) [38,39]. ROS are generated in the mitochondria as a byproduct of the electron transport chain, as well as by non-mitochondrial enzymes such as oxidases or peroxidases [40]. Excess ROS is harmful because of its potent ability to interact with, and modify, a wide range of cellular macromolecules implicated in cytotoxicity and mutagenic damage [38,39]. Conversely, a low level of ROS is necessary in order to maintain cell proliferation and serve as intracellular signaling molecules [41,42].

In the present study, we investigated the possible signal transduction pathways induced by cadmium, in isolated digestive gland cells of *Mytilus galloprovincialis*. It has been reported that cadmium induces ROS production and DNA damage, as well as NHE activity and interacts with adrenergic receptors [23,43]. Since adrenergic receptors are linked to cAMP [44], cAMP to NHE [45–47] and NHE to PKC [23,26,48,49], it was of interest to study ROS production and DNA damage in relation to these parameters, after cadmium effect in isolated digestive gland cells of *M. galloprovincialis*.

Materials and Methods

Chemicals

Ethyl-*N*-isopropyl-amiloride (EIPA), adrenaline (L-epinephrine), calphostin C (from *Cladosporium cladosporioides*), forskolin, isoprenaline-HCl, 3-isobutyl-1-methyl-2,6(1H, 3H)-purinedione (IBMX), metoprolol-HCl, phenylephrine-HCl, prazosin-HCl, propranolol-HCl, superoxide dismutase (SOD), sodium sarcosinate, TRITON X 100, bovine serum alboumin (BSE) and yohimbine-HCl were purchased from Sigma-Aldrich Company Ltd. (USA). Collagenase type CLS IV and Leibovitz L-15 medium were purchased from Biochrom AG (Berlin, Germany). Nitroblue-tetrazolium chloride (NBT) was purchased from AppliChem Inc. (Darmstadt, Germany) and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Eugene (USA, OR). Normal- and low- melting agarose were purchased from Cambrex (Rockland Inc., USA). cAMP [^{3}H] radioimmunoassay kit was purchased from Amersham (Arligton Heights, USA, IL). Cadmium chloride ($CdCl_2$) was purchased from MERCK (Darmstadt, Germany) and bicinchoninic acid protein assay (BCA) was purchased from Pierce (Rockford Inc., USA).

Animals

Mussels were collected from Kalamaria, located at the east side of Thermaikos Gulf (Thessaloniki). The site of mussels sampling is regarded as an ecosystem with low level of cadmium contamination [50] and therefore cadmium does not interfere with the biochemical function of the mussels' tissues. Moreover, results from our laboratory have shown that mussels collected from the same area accumulated cadmium, at a concentration of 27–52 ngr/gr dry weight of digestive gland, which is lower than the minimum levels reported in mussels collected from other sites of Thermaikos Gulf (unpublished results from our laboratory).

Mussels (*M. galloprovincialis)*, 5–6 cm long, were transferred from Kalamaria to the laboratory and maintained in static tanks containing filtered natural sea water (35–40% salinity) for 7 days at 15°C. The seawater was changed every two days. During the adaptation period, animals maintained without food supply. The concentrations of cadmium used in the

present study ranged from 0.05 to 500 μM, the lower concentration being close to that found in Thermaikos Gulf [50,51]. Moreover, concentrations similar to those used in the present study, has been also used in other studies, investigating the effects of cadmium on cell signaling and its ability to induce cytotoxicity [16,19,52,53].

Isolation of digestive gland cells and treatment

Digestive glands from 3–5 animals were cut into pieces and washed with calcium–magnesium–free saline (CMFS; 1100 mOsm, pH 7.3, containing 20 mM HEPES buffer, 500 mM NaCl, 12.5 mM KCl, 5 mM EDTA). Tissues aliquots were minced into small pieces and transferred to a flask containing 15 ml dissociating solution (0.01% collagenase type CLS IV, 175 U/mg) in CMFS, followed by gentle stirring for 60 min at 15°C. After filtration through 250 and 60 μm diameter nylon filters, the procedure as described by Canesi et al. [54] was followed. Cell suspensions maintained in Leibovitz L-15 medium (L-15 medium; supplemented with 350 mM NaCl, 7 mM KCl, 4 mM $CaCl_2$, 8 mM $MgSO_4$ and 40 mM $MgCl_2$) were kept at 15°C for at least 3 h before being used for the experiments. In all cases, isolated cells were incubated for 30 min with cadmium in the presence or absence of various inhibitors or activators as mentioned in the Results Section.

In the dose-response effect of cadmium on DNA damage, concentration higher than 50 μM of cadmium chloride showed to induce significantly high DNA damage, compared to those occurred after treatment with 0.05–5 μM of the metal (results section). In the Dailianis and Kaloyianni [23] study, the use of various concentrations of cadmium also showed that 50 μM of cadmium caused maximum NHE activation. Since one of the goals of the present study is to investigate ROS production and DNA damage in relation to NHE, the concentration of 50 μM of cadmium was used for all the experiments. Furthermore, the same concentration of metal has also been used in other studies on marine organisms, investigating the effect of the metal on cell signaling [16,20,23,52,55].

Viability assay method

Viability tests were carried out for all samples as parallel tests. After the tissue treatment, cell viability was tested using Eosin exclusion and was about 83%. Subsequently, cell viability was tested after incubation with various concentrations of cadmium (0.05–500 μM) and revealed that 98% of the cell remained intact. Concentrations of cadmium higher than 500 μM resulted in a 50% cell death that was also tested by the use of trypan blue exclusion.

Detection of superoxide ions (O_2^-) in isolated digestive gland cells

Superoxide anions (O_2^-) in isolated digestive gland cells were detected intracellularly by a modification of a previously described method [56], with the use of nitroblue tetrazolium (NBT). After isolating the digestive gland cells, the cell suspension was centrifuged at 1000 rpm for 5 min at 4°C. Appropriate amount of cells (approximately 10^6 cells) were incubated in Leibovitz L-15 medium for 30 min with different effectors. When appropriate, cadmium chloride ($CdCl_2$) at 50 μM, phenyleprine-HCl (an α_1-adrenergic agonist) at 1 μM, isoprenaline-HCl (a β-adrenergic agonist) at 1 μM, calphostin C (from *Cladosporium cladosporioides*; a specific inhibitor of protein kinase C) at 20 nM, prazosin-HCl (a peripheral α_1-adrenergic antagonist) at 1 μM, yohimbine-HCl (a α_2- adrenergic antagonist) at 0.1 μM, propranolol-HCl (an antagonist of β-adrenergic receptors) at 10 μM, metoprolol-HCl (a β_1- adrenergic antagonist) at 1 μM and ethyl-*N*-isopropyl-amiloride (EIPA; a selective inhibitor of NHE) at 20 nM were added.

After incubation, the cell suspension was centrifuged at 1000 rpm for 5 min and subsequently the packed cells were resuspended in 300 μl TBS (TRIS/HCl buffer 0.05M, pH 7.6, containing 2% NaCl), with the addition of NBT (1 mg/ml TBS). 300 μl NBT solution, containing superoxide dismutase (SOD 300 U/ml), was added in non- treated cells (control cells). Cells were incubated in the dark for 2 h at 20°C and then centrifuged at 1000 rpm for 3 min, washed twice with TBS, in order to remove extracellular NBT and finally resuspended in 300 μl absolute methanol for 15 min. Then, the cells were spinned at 300*g* and the supernatant was gently discarded. Samples were air-dried for 15 min and dissolved in dimethyl sulphoxide (DMSO) and 2 M potassium hydroxide (KOH). After solubilization for 30 min, the samples were measured spectrophotometrically at 620 nm. Values (% of control value in each experiment) are means ± SD of at least 6 independent experiments.

Detection of ROS production in isolated digestive gland cells

In addition to O_2^- measurement, determination of ROS production, after cadmium exposure was conducted. Quantitative measurement of ROS production in isolated digestive gland cells of *M. galloprovincialis* was performed with the use of 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), according to a previously described method [57]. The fluorescent probe DCFH-DA is sensitive to H_2O_2, hydroxyl radicals as well as to $ONOO^-$. This method is considered as a good indicator of overall changes in

the intracellular redox state (ROS production) of the cell [58–62].

Appropriate amount of cells were incubated in dark for 15 min at 15°C in physiological saline buffer (PS buffer, 1100 mOsm, pH 7.3, containing 20 mM HEPES buffer, 436 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 53 mM $MgSO_4$) with the addition of DCFH-DA dye (5 μg/ml). The isolated cells were then washed twice with the same medium, to remove DCFH-DA and then resuspended in the same medium (10^6 cells/ml). When appropriate, cadmium chloride (50 μM), 20 nM of calphostin C and 20 nM of ethyl-N-isopropyl-amiloride (EIPA) were added and incubated for 30 min. In addition, cells were treated with 0.05 mM IBMX, a non-specific inhibitor of phosphodiesterases that possesses adenosine agonist activity and inhibits cAMP breakdown, and then incubated with either $CdCl_2$ and/or 20 nM EIPA. Similarly, untreated cells and treated with IBMX cells were incubated with 0.01 mM forskolin, a potent activator of adenylate cyclase and biosynthesis of cAMP. After the incubation, cells were washed twice with TBS and then 1 ml of lysis buffer (TBS +1% SDS) was added for 10 min in order to lyse the cells. Finally, 100 μl of cell suspension was diluted in 3 ml distilled water and samples were determined spectrophotometrically.

Fluorescence was measured in a Perkin-Elmer (Connecticut, USA) LS 50B Fluorescence thermostatic spectrometer equipped with a thermostatic holder. DCFH-DA fluorescence was detected at excitation (495 nm) and emission (525 nm) wavelength. The results (are expressed as fluorescence value/mg protein) are means ± SD of at least 6 independent experiments. 100% represents fluorescence value in the absence of cadmium (1254.67 ± 64.82/mg of protein).

cAMP determination

Digestive gland cells were isolated according to the procedure mentioned before and maintained in L-15 medium, containing EDTA, for at least 2 h before the experimental procedure. Approximately 2×10^6 cells/ml were incubated with 50 μM $CdCl_2$ for 30 and 60 min, as well as with 20 nM ethyl-N-isopropyl-amiloride (EIPA), 10 μM propranolol-HCl, 1 μM metoprolol-HCl, a β_1- adrenergic antagonist, 0.1 μM yohimbine-HCl, a α_2- adrenergic antagonist, 1 μM prazosin-HCl, 20 nM calphostin C. All the inhibitors and antagonists were added 15 min before the addition of the metal. Moreover, the effect of 0.05 mM IBMX and 0.01 mM forskolin on cAMP levels was investigated. After each incubation, samples were centrifuged at 1000 rpm for 5 min and resuspended in 4 mM EDTA buffer pH 7.5, boiled for 5 min and thereafter centrifuged for 5 min (16.000*g*, 4°C). The supernatant was used for the determination of cAMP. The concentration of cAMP was measured with the Amersham cAMP [^{3}H] radioimmunoassay kit. The results (expressed as pmol cAMP/mg protein) are means ± SD from at least 4 independent experiments.

Alkaline single-cell gel electrophoresis (Comet) assay

The procedure used follows the method described by Singh et al. [63] with some modifications. Fully frosted microscope slides were coated with 200 μl of 0.5% normal-melting-point-agarose (NMA) in calcium- and magnesium- free phosphate-buffered saline (PBS) and covered with a coverslip. Slides were kept at 4°C in a dark humid box until use. Appropriate amount of cells (approximately 10^6 cells) were treated for 30 min with different concentrations of cadmium chloride (0.05–500 μM) in the dark. When appropriate, cells were treated with 20 nM ethyl-N-isopropyl-amiloride (EIPA), 10 μM propranolol-HCl, 20 nM calphostin C, 1 μM metoprolol-HCl, 0.1 μM yohimbine-HCl, 1 μM prazosin-HCl, that were added before the addition of the metal. Phenylephrine-HCl at different concentrations (1, 50 and 100 μM) was added to cell suspension and incubation for 30 min was followed. In addition, cells were co-incubated with 0.05 mM IBMX and then treated with either 50 μM $CdCl_2$ and/or 20 nM EIPA. Similarly, the effect of 0.01 mM forskolin in IBMX-untreated and IBMX-treated cells was studied.

After incubation, 20 μl of cell suspension was added to 80 μl of 0.5% low-melting-point-agarose (LMPA, at 35°C) in physiological saline and layered over the NMA layer. After agarose polymerization for 10 min on metal tray over ice, a final layer of 80 μl of LMPA 0.5% was added. Following agarose solidification, the coverslips were removed and slides lowered into freshly prepared lysing solution (10% DMSO, 1% TRITON X-100, 2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 1% sodium sarcosinate, pH 10) for at least 1 h at 4°C in the dark. Slides were rinsed in distilled water, placed on horizontal gel electrophoresis tray and covered with freshly prepared electrophoresis buffer (0.075 M NaOH, 1 mM EDTA, pH > 12) for 20 min to allow the DNA to unwind. Electrophoresis was carried out at 25 V, 300 mA for 10 min. Slides were then removed, placed on a staining tray and covered with neutralizing solution (0.4 M TRIS, pH 7.5) for 5 min. This procedure was repeated three times. Slides were drained and 50 μl of filtered ethidium bromide (20 μg/ml) was added with a coverslip overlaid. Slides were placed in a humid dark box at 4°C until analysis (within 48 h).

The presence of comets was examined in cells from the digestive gland cell suspension using a fluorescent microscope, Zeiss axovert inverted fluorescent microscope × 200 magnification and WANG epi-fluorescence microsope (WANG BioMedical, The Netherlands). All slides were coded and the whole

slide was randomly scanned. At least 250 cells per slide were analyzed. Cells from 4 experiments were pooled together and categorized for the amount of DNA damage. Comets on each slide were scored visually as belonging to one of five predefined classes according to tail intensity and were given a value of 0, 1, 2, 3, or 4 (from undamaged, 0, to maximally damaged, 4). The percentage of DNA in tail was also estimated using an image analysis system (Kinetic analysis, UK) connected to a computer with suitable programme [64].

Protein content determination

Protein content was determined using bicinchoninic acid (BCA) protein assay reagent protocol with Bovine Serum Albumin (BSA) as standard [65].

Statistical analysis

For the determination of superoxide ions, ROS production and cAMP concentration, statistical analysis was carried out using Instat 2 Software (Graphpad Instat), Dunnet's test. The minimal level of significance chosen was $P < 0.01$. Tukey test (one way ANOVA, $P < 0.01$) was used for the comparison of the grade of damage between control and exposed cells.

Results

Effect of cadmium on ROS production in isolated digestive gland cells

Superoxide anions (O_2^-) production was primarily chosen to be measured in isolated digestive gland cells, after treatment with cadmium, since these anions take part in all other reactions that lead to the production of H_2O_2, OH^-, $OH\cdot$ and HNO_2^- radicals [66]. Moreover, changes in the intracellular redox state of the cells (ROS production) were also measured.

Treatment of isolated digestive gland cells of *Mytilus galloprovincialis* for 30 min with 50 μM cadmium chloride, caused a significant increase in superoxide ions (O_2^-) production, compared to control cells (Table I). According to our results, cells treated with the antioxidant enzyme SOD, (control + SOD cells), did not show any significant activity compared to non treated with SOD cells (control cells), which demonstrate minimum levels of O_2^- in the cells at physiological level. Effect of cadmium (50 μM) also caused a significant increase in ROS production in isolated digestive gland cells of *M. galloprovincialis* (Figure 1).

According to the study of Dailianis and Kaloyianni [23], the activity of NHE was found to increase after cadmium treatment via the induction of a signal transduction pathway mediated by PKC in digestive gland cells of *M. galloprovincialis*. In order to investigate whether NHE and PKC are involved in cadmium induction of ROS production in isolated digestive gland cells we tested the effect of inhibitors of both NHE and PKC on ROS production. Treatment of the cells with 50 μM of cadmium together with the inhibitor of NHE, EIPA (20 nM), revealed a

Table I. Superoxide anion ($\cdot O_2^-$) production in isolated digestive gland cells of *Mytilus galloprovincialis* after treatment with cadmium and different effectors.

	% of control value
Control	100.0 ± 16.3
Control & SOD	95.3 ± 21
$CdCl_2$ 50 μM	164.0 ± 6[a,b]
$CdCl_2$ 50 μM + EIPA 20 nM	108.7 ± 6[c]
$CdCl_2$ 50 μM + calphostin C 20 nM	85.7 ± 3[c]
$CdCl_2$ 50 μM + propranolol 10 μM	91.0 ± 15[c]
$CdCl_2$ 50 μM + metoprolol 1 μM	94.0 ± 8[c]
$CdCl_2$ 50 μM + prazosin 1 μM	85.0 ± 3[c]
$CdCl_2$ 50 μM + yohimbine 0.1 μM	80.0 ± 9.6[c]
Phenylephrine 1 μM	214.0 ± 44[a,b]
Isoprenaline 1 μM	187.0 ± 48[a,b]

Cells were treated for 30 min with cadmium 50 μM or cadmium together with EIPA, or calphostin C, or propranolol, or metoprolol, or cadmium together with or prazosin, or with yohimbine, or phenylephrine or isoprenaline. Values labelled with [a] indicate significant difference with the control value. Values labelled with [b] indicate significant difference with the control + SOD value. Values labelled with [c] indicate significant difference with the value obtained in cells treated with cadmium (Dunnet's test, $P < 0.01$).

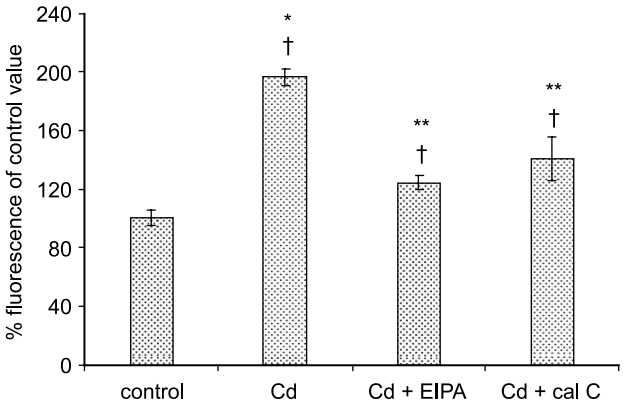

Figure 1. Detection of ROS production in isolated digestive gland cells of *M. galloprovincialis* after treatment with cadmium and various effectors. Cells were treated for 30 min with cadmium 50 μM alone or cadmium 50 μM + 20 nM EIPA, or cadmium 50 μM + 20 nM calphostin C. Values (fluorescence is measured as % of control value) are means ± SD of at least 6 independent experiments. 100% represents fluorescence measured in control cells (1254.67 ± 65/mg of protein). In each experiment, the tissue of four animals was used. * indicate significant difference between control value and that observed after cadmium treatment; ** indicate significant difference between control value and that observed after cadmium + effector treatment; † indicates significant difference between the value obtained after cadmium treatment with that obtained after cadmium + effector treatment (Dunnet's test, $P < 0.01$).

significant decrease in O_2^- and ROS production, compared to the value observed after treatment with cadmium alone (Table I, Figure 1). Similarly, treatment of the cells with the metal together with the inhibitor of PKC, calphostin C (20 nM), which is a well-known inhibitor of both novel PKC (PKC-δ, -ε, -θ and -η) and conventional PKC (PKC-α and -β) isoforms [67–69], showed significant decrease both in O_2^- and ROS (Figure 1, Table I). The effect of each inhibitor alone did not show any significant change in both O_2^- and ROS production, compared to control cells (data not shown).

The effect of cadmium on O_2^- production was counteracted either by propranolol (10 μM), an antagonist of β- adrenergic receptors or metoprolol (1 μM), a β_1-adrenergic antagonist (Table I). The same results were observed after treatment of the cells with cadmium together either with prazosin (1 μM), an antagonist of α_1- adrenergic receptors, or with yohimbine (0.1 μM), a α_2- adrenergic antagonist (Table I), while treatment with each antagonist alone did not show any significant changes on ROS production (data not shown). Our results show the significant role of adrenergic receptors in the transduction pathway induced by cadmium. In support of that, treatment of cells for 30 min with phenylephrine (1 μM), a α_1- adrenergic agonist, showed significant elevation of O_2^- production, even higher to that observed after cadmium treatment (Dunnet's test, $P < 0.01$, Table I). Furthermore, treatment with isoprenaline (1 μM), a β- adrenergic agonist, also induced a significant rise in O_2^- production (Table I).

DNA damage in isolated digestive cells, after cadmium exposure

In order to investigate the genotoxic effects of cadmium on isolated digestive gland cells of *M. galloprovincialis,* the Comet assay method (alkaline single cell electrophoresis assay) was used. Control cells of digestive gland of *M. galloprovincialis* demonstrated similar levels of single-strand breaks (SSBs) compared to other reports in invertebrates (Table II), [70–72].

The effect of various concentrations of cadmium on DNA damage showed that the concentrations at 50 and 500 μM of cadmium induced significantly higher DNA damage in comparison to the lower (0.05 and 5 μM) concentrations used (Figure 2). To investigate whether the metal induced effect on the disturbance of DNA integrity was related to NHE, the inhibitor of the exchanger (EIPA) was used. It is noteworthy that treatment of cells with EIPA (20 nM) together with cadmium resulted in a significant reverse of cadmium effect on DNA integrity (Table II). Therefore, our results show a protective effect of EIPA on cadmium treated cells.

The possible link of NHE with the PKC-dependent signaling pathway induced by cadmium [23], lead us to investigate whether cadmium effect on DNA damage is due to PKC activation. Cells treated with calphostin C (20 nM), a specific inhibitor of PKC, together with cadmium showed significant decrease in DNA damage compared to that observed in cells treated with cadmium alone (Table III), which indicates a possible link of the PKC-dependent transduction pathway with the induction of DNA damage in isolated digestive gland cells after cadmium exposure.

Cells treated with the β- adrenergic antagonist, propranolol (10 μM) or the β_1- adrenergic antagonist, metoprolol (1 μM) or the α_2- adrenergic antagonist, yohimbine (0.1 μM) or the α_1-adrenergic antagonist, prazosin (1 μM) together with the metal, showed a significant decrease in DNA damage compared to that obtained in cells treated with the metal alone (Table III). The effects of agonists on specific physiological responses further confirm the results obtained by the use of the antagonists. In consistent with that, incubation with α_1- agonist, phenylephrine, showed a dose-response increase in DNA damage (Table IV). It is noteworthy that EIPA as well as the α_1- and α_2- adrenergic antagonists (prazosin and yohimbine) efficiency in reversing cadmium effect on DNA integrity was higher than

Table II. Results of the single cell gel electrophoresis (comet assay) expressed in terms of the percentage of DNA in the comet tail (% DNA in tail) of isolated digestive gland cells suspensions of *Mytilus galloprovincialis*.

	% DNA in tail	
	Untreated with IBMX cells	Cells pre-treated with IBMX 0.05 mM
Control	$2.86 \pm 1.1^{a,b,e}$	25.10 ± 1^{f}
$CdCl_2$ 50 μM	$29.82 \pm 5.7^{b,c,d}$	27.43 ± 1.6^{g}
$CdCl_2$ 50 μM + EIPA 20 nM	$8.21 \pm 2.3^{c,e}$	$12.96 \pm 1.9^{f,g}$
Forskolin 0.01 mM	$14.50 \pm 4.8^{a,d}$	19.90 ± 6.0

Cells, incubated for 30 min either in physiological saline buffer (PS), or in PS + IBMX (an inhibitor of cAMP breakdown) were treated with cadmium or cadmium + EIPA or forskolin (a potent activator of adenylate cyclase and the biosynthesis of cAMP). Values are means ± SD of at least 4 independent experiments. In each experiment, the tissue of four animals was used and 250 cells per incubation per slide were analyzed. Values, in each column, that share the same letter indicate statistically significant difference from each other (Tukey test, $P < 0.01$).

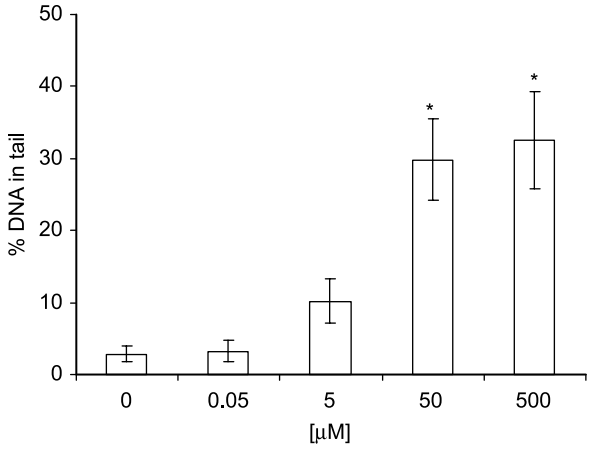

Figure 2. The effect of different concentrations of cadmium on DNA damage of isolated digestive gland cells of *M. galloprovincialis*. Values are means ± SD of at least 4 independent experiments. In each experiment, the tissue of four animals was used and 250 cells per incubation per slide were analyzed. * indicate significant difference between control value and that observed after cadmium treatment; (Tukey test, $P < 0.01$).

that of β- and β_1- adrenergic antagonists (Table III). Treatment of the cells with each antagonist alone, showed similar range of DNA damage to those occurred in control cells (data not shown).

cAMP concentration in isolated digestive gland cells, after exposure to cadmium

Since cAMP activates NHE [34,47] it was of interest to measure cAMP levels, after treatment of the cells with cadmium. Treatment of isolated digestive gland cells of *M. galloprovincialis* with 50 μM of cadmium chloride for 60 min caused a significant increase in cAMP levels. Specifically, the maximum increase in cAMP was observed after incubation for 30 min (19 ± 3.43 pmol cAMP/mg protein) followed by a decrease at 60 min of treatment (14.18 ± 1.31 pmol cAMP/mg protein), in relation to control cells (7.35 ± 1.24 pmol cAMP/mg protein). During the exposure period no significant changes in the cAMP levels were observed in control cells (Table V).

In addition, treatment of the cells with cadmium (50 μM) together with α_1-, or α_2- or β-, or β_1-adrenergic antagonists, showed a significant decrease in cAMP, compared to levels of the nucleotide occurred in cells treated with cadmium alone (Table V). The β- and β_1- adrenergic antagonists seem to be more effective than the α_1- and α_2- in reversing cadmium effect on cAMP content. Moreover, in the presence of each antagonist alone, no significant difference in the levels of cAMP was observed compared to control cells (data not shown).

Furthermore, treatment of cells with cadmium, in the presence of the selective amiloride-type inhibitor

Table III. Results of the single cell gel electrophoresis (comet assay) expressed in terms of the percentage of DNA in the comet tail (% DNA in tail) of isolated digestive gland cells suspensions of *Mytilus galloprovincialis*.

	% DNA in tail
Control	2.86 ± 1.1
$CdCl_2$ 50 μM	29.82 ± 5.6[a]
$CdCl_2$ 50 μM + EIPA 20 nM	8.21 ± 2.3[b]
$CdCl_2$ 50 μM + Calphostin C 20 nM	14.02 ± 3.8[a,b]
$CdCl_2$ 50 μM + Propranolol 10 μM	13.29 ± 1.0[a,b]
$CdCl_2$ 50 μM + Metoprolol 1 μM	12.57 ± 3.4[a,b]
$CdCl_2$ 50 μM + Yohimbine 0.1 μM	8.04 ± 1.1[b]
$CdCl_2$ 50 μM + Prazosin 1 μM	7.35 ± 1.0[b]

Cells were exposed for 30 min to cadmium alone, or cadmium + EIPA, or cadmium + calphostin C, or cadmium + yohimbine, or cadmium + metoprolol, or cadmium + propranolol, or cadmium + prazosin. Values labelled with [a] indicate significant difference with the control value. Values labelled with [b] indicate significant difference with the value obtained in cells treated with cadmium (Tukey test, $P < 0.01$).

Table IV. Results of the single cell gel electrophoresis (comet assay) expressed in terms of the percentage of DNA in the comet tail (% DNA in tail) of isolated digestive gland cells suspensions of *Mytilus galloprovincialis*.

	% DNA in tail
Control	2.86 ± 1.1
Phenylephrine 1 μM	13.79 ± 1.9[a]
Phenylephrine 50 μM	14.78 ± 2.5[a]
Phenylephrine 100 μM	25.38 ± 1.0[a]

Cells were exposed for 30 min to different concentration of phenylephrine (PE). Values are means ± SD of at least 4 independent experiments. In each experiment, the tissue of four animals was used and 250 cells per incubation per slide were analyzed. Values labelled with [a] indicate significant difference with the control value (Tukey test, $P < 0.01$).

Table V. Intracellular cAMP concentration in isolated digestive gland cells of *Mytilus galloprovincialis*.

	cAMP content (pmol cAMP/mg protein)
Control	7.35 ± 1.24
Control + IBMX 0.05 mM	15.83 ± 2.30[a,b]
Control + forskolin 0.01 mM	15.20 ± 1.67[a,b]
$CdCl_2$ 50 μM	19.00 ± 3.43[a]
$CdCl_2$ 50 μM + EIPA 20 nM	4.70 ± 0.94[b]
$CdCl_2$ 50 μM + prazosin 1 μM	13.08 ± 1.00[a,b]
$CdCl_2$ 50 μM + yohimbine 0.1 μM	9.85 ± 1.51[b]
$CdCl_2$ 50 μM + propranolol 10 μM	2.67 ± 0.69[a,b]
$CdCl_2$ 50 μM + metoprolol 1 μM	4.73 ± 1.03[b]
$CdCl_2$ 50 μM + calphostin C 20 nM	10.98 ± 1.71[a,b]

Cells were incubated for 30 min with IBMX or forskolin as well as cadmium alone, or cadmium + EIPA, or cadmium + prazosin, or cadmium + yohimbine, or cadmium + metoprolol, or cadmium + calphostin C, or cadmium + propranolol. The results (pmol cAMP/mg protein) are means ± SDs of at least 4 experiments. In each experiment, the tissue of 8 animals was used. Values labelled with [a] indicate significant difference with the control value. Values labelled with [b] indicate significant difference with the value obtained in cells treated with cadmium (Dunnet's test, $P < 0.01$).

of the exchanger (EIPA 20 nM) significantly diminished cadmium effects on cAMP (Table V). The presence of the inhibitor alone did not change the levels of cAMP, compared to control cells (data not shown). It is noteworthy the calphostin C effect on cAMP, in cells treated with cadmium, that partially counteracted cadmium effect (Table V).

ROS production, cAMP content and DNA damage in pre-treated with IBMX cells:

In order to investigate, whether cAMP is implicated in the induction of DNA damage, we determined ROS production and DNA damage in cells pre-treated either with forskolin or with a non-specific inhibitor of phosphodiesterases, IBMX. As it was expected, cells treated either with forskolin or with IBMX maintained high levels of cAMP in relation to control (Table V).

The presence of cadmium (50 μM) resulted to a significant increase in ROS production in both cell groups (Table VI). It is noteworthy that EIPA reversed cadmium effects by causing a 68% inhibition of ROS production in cells treated to maintain high cAMP levels and a 37% inhibition in untreated cells (Table VI).

Our results showed that the cells treated to maintain high cAMP levels resulted to higher DNA damage, in relation to untreated cells (Table II). However, the presence of cadmium seems to induce similar levels of DNA damage in both cells groups. The addition of NHE inhibitor, (EIPA 20 nM) in both cases, partly counteracted the effect of cadmium on DNA damage (Table II).

Discussion

Our results indicate that cadmium at micromolar concentrations (50 μM) caused a significant rise in ROS production as well as an increase in DNA damage in isolated digestive gland of mussel *M. galloprovincialis*. This agrees with previous studies, which showed that cadmium can cause DNA damage at cytotoxical concentrations similar to those used in the present study [16,19,52,53]. The concomitant increase in DNA damage together with the induction of ROS production, as our results showed, indicates the possible association of these two events. In accordance with our results, Zhong et al. [73] reported that cadmium may also be implicated in the induction of DNA single-strand-breaks SSBs via the production of free radicals and Walker et al. [74] showed that DNA fragmentation was associated with intracellular accumulation of ROS under glutamate-mediated GSH depletion.

Treatment of cells with EIPA 20 nM, a selective inhibitor of NHE, together with the metal, resulted to a significant decrease in O_2^- and ROS production, compared to cells treated with the metal alone (Figure 1, Table I). Similarly, a rapid decrease in DNA SSBs was observed in cells treated with the inhibitor of NHE together with cadmium, compared to the increased levels of SSBs observed in cells treated with cadmium alone (Tables II and III). Therefore, EIPA and consequently inhibition of NHE seems to exert a protective effect both on ROS production and on DNA damage. In addition, cells that maintained high levels of cAMP were possibly more efficient in keeping low levels of ROS, especially when the NHE was inhibited (Table VI). Perhaps, this exchanger merely affects the processes leading to the actual endpoints being measured. Therefore, it seems that both ROS production and DNA damage that are induced by cadmium, are possibly mediated through the NHE in isolated digestive gland cells of *M. galloprovincialis*. To our knowledge, it is reported for the first time, the relation of the NHE with cellular responses, such as oxidative stress and induction of genotoxicity in cells of aquatic animals, after exposure to toxicants such as cadmium.

Inhibition of PKC activity with a specific inhibitor, calphostin C, significantly diminished the effects of cadmium on ROS production and DNA damage (Table III, Figure 1). Our results demonstrate that either ROS regulate PKC activity and DNA damage or PKC induces ROS production and DNA damage after cadmium exposure of isolated digestive gland cells of *M. galloprovincialis*. In agreement with our results, it has been suggested that the PKC activator, PMA, induced ROS production (and specifically superoxide and nitric oxide) through NADPH oxidase and nitric oxide synthase pathways [75]. Similarly, Higuchi and Matsukawa [76] reported in C6 glioma

Table VI. Detection of ROS production in isolated digestive gland cells suspensions of *Mytilus galloprovincialis*.

	Untreated with IBMX cells	Cells pre-treated with IBMX 0.05 mM
Control	$1254.67 \pm 65^{a,b}$	$963.90 \pm 112^{f,g}$
$CdCl_2$ 50 μM	$2466.54 \pm 147^{a,c,d}$	$2141.90 \pm 66^{h,i,f}$
$CdCl_2$ 50 μM + EIPA 20 nM	$1553.09 \pm 75^{b,c,e}$	$688.18 \pm 89^{j,h,g}$
Forskolin 0.01 mM	$1134.46 \pm 68^{d,e}$	$1013.92 \pm 105^{j,i}$

Cells, incubated for 30 min either in physiological saline buffer (PS) or in PS + IBMX, were treated with cadmium alone, or cadmium + EIPA, or forskolin. Values (fluorescence value/mg protein) are means ± SD of at least 6 independent experiments. In each experiment, the tissue of four animals was used. Values, in each column, that share the same letter indicate statistically significant difference from each other (Dunnet's test, $P < 0.01$).

cells that ROS generated endogenously, may trigger DNA fragmentation through lipid peroxidation and PKC activation. Moreover, Nemoto et al. [77] and Klann et al. [78] reported that endogenous production of ROS appeared to contribute to the modulation of PKC. Since NHE activation is mediated by PKC activity [23,26,49,79], we could suggest that cadmium may induce the formation of free radicals due to/or as a result of the activation of PKC with the involvement of NHE in isolated digestive gland cells of *M. galloprovincialis*. In agreement with that, Baldini et al. [80], Sand et al. [81] and Rothstein et al. [82] reported that ROS production is through NHE1-dependent pathway.

Cadmium-induced ROS formation has been suggested to be due to inhibition of a number of enzymes in charge of maintenance of cellular redox state, as GSH peroxidase, catalase, superoxide dismutase and also due to decrease of cellular glutathione content (which is known to scavenge intracellular ROS by a direct reaction or via the GSH peroxidase/GSH system), [13]. The decrease in antioxidant enzymes activities caused by cadmium, together with the generation of radicals that are produced during normal metabolism, may explain the increase in DNA damage in cells. Another possible mechanism, which explains the increase in ROS caused by cadmium is the displacement of iron and copper from various intracellular sites (cytoplasmic and membrane proteins), thus increasing the concentration of ionic copper and iron, which can then cause oxidative stress through Fenton reaction. In addition, cadmium-induced cytotoxicity occurs as a result of mitochondrial ROS formation (due to decline of mitochondrial membrane potential) independently of cytosolic ROS formation due to redox cycling [11].

Previous data demonstrated the close relationship of the NHE with cAMP-PKA-dependent signal transduction pathway [34]. In addition, Borgese et al. [34] showed that trout red cell NHE responded to both PKA and PKC activators. Since inhibition of both NHE and PKC results to decreased production of cAMP (Table V), we could suggest an important role of PKC and NHE in the induction of cAMP signaling pathway.

Treatment of isolated digestive gland cells with the α_1- adrenergic agonist, phenylephrine, resulted to a significant increase in superoxide anions (O_2^-) which was reversed by treatment of the cells with antagonists of α_1- adrenergic receptors. In consistence with our results, Sand et al. [81] has reported that α_1-adrenoreceptors stimulation induces ROS production (via NADH/NADPH oxidase activation) through NHE stimulation. Incubation of the cells with the α_2- and β-, β_1- adrenergic receptors antagonists resulted in reversal of cadmium effect on O_2^- production (Table I). Similarly, DNA SSBs were reversed after propranolol, or metoprolol, or prazosin, or yohimbine treatment of digestive gland cells (Table III). Thus, the results show that the interaction of adrenergic receptors with cadmium probably affects ROS production and subsequently induces DNA damage.

Our results showed that there is a differential effect of cadmium interaction with β-, β_1- and α_1-, α_2-adrenergic receptors on DNA damage and cAMP levels. DNA damage seems to be induced mainly after cadmium interaction with α_1- and α_2- adrenergic receptors, while cAMP levels are mainly induced after cadmium interaction with β- and β_1- adrenergic receptors. Interestingly, α- adrenergic receptors are more refractory to reversal by inhibition, while blockade of β- adrenergic receptors caused a decrease in cAMP levels to values that are lower than the untreated control. These receptor interaction differences reflect possible variations in drug efficacy, or show the presence of a compensatory pathway that acts in concert with α- and β- adrenergic receptors to mediate cadmium effects on DNA. These differential effects on DNA damage and cAMP levels may also be critical for assessing the potency of cadmium effects on mussel physiology.

Cadmium via its interaction with α_1-, α_2-, β- and β_1-adrenergic receptors causes increase in cAMP levels, ROS production and DNA damage with the involvement of PKC and NHE. Specifically, stimulus from the interaction of cadmium with the receptors either pass through PKC affecting NHE, or pHi changes caused by NHE activation induces PKC stimulation. Since inhibition of both PKC and NHE leads to a decrease of ROS production and DNA damage, it seems reasonable to suggest that cadmium exerts its action through PKC and NHE. The different sensitivity of α_1-, α_2-, β-, β_1- adrenergic receptors on cAMP, ROS production and DNA damage possibly leads to the induction of two signaling pathways that may be interacting or to the presence of a compensatory pathway that acts in concert with the α- and β-adrenergic receptors. Interestingly, Faurskov and Bjerregaard [83] showed that cadmium interacts with a cation-sensing receptor, belonging to the superfamily of G-protein coupled receptors, resulting in phospholipase C activation which may lead to stimulation of PKC in epithelia cells.

In conclusion, cAMP, PKC and NHE could play an important role in the induction of DNA damage via or independently of ROS production. Our results indicate that cadmium can cause an increase in DNA damage, via the induction of ROS production and PKC (ROS-dependent pathway). On the other hand, in cells treated with forskolin (high cAMP content), cadmium seems to induce DNA damage without the induction of ROS production. It seems that when cAMP levels are maintained high, DNA damage occurs with the participation of a ROS-independent pathway. Inhibition of NHE seems to

exert a protective effect on ROS production and DNA damage. Thus, our results highlight the important regulatory role of NHE that may contribute to the fine-tuning of several intracellular signal transduction pathways in digestive gland cells of *M. galloprovincialis*. Further work is needed in order to clarify the signaling mechanism induced by heavy metal in marine organisms' tissues.

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