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Effects of cadmium chloride on some mitochondria-related activity and gene expression of human MDA-MB231 breast tumor cells

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ABSTRACT

It was reported that cadmium is able to exert a cytotoxic effect on tumor MDA-MB231 cells, which shows signs of "non-classical" apoptosis and is characterized by drastic changes in gene expression pattern. In this study, we have extended our knowledge of metal-breast cancer cell interactions by analyzing some mitochondria-related aspects of the stress response to $CdCl_2$ at either 5 or 50 μM 24- or 96-h exposure, by cytochemical, conventional PCR and Northern/Western blot techniques. We demonstrated that (i) no modification of the mitochondrial mass was detectable due to $CdCl_2$ exposure; (ii) the respiration activity appeared to be increased after 96-h exposures, while the production of reactive oxygen species was significantly induced, as well; (iii) *hsp60, hsp70, COXII* and *COXIV* expressions were dependent on the duration of Cd exposure; (iv) a different hsp60 protein distribution was observed in mitochondrial and post-mitochondrial extracts and (v) 96-h exposure induced the over-expression of hsc/hsp70 proteins and, conversely, the down-regulation of cytochrome oxidase subunits II and IV. These observations, in addition to providing more information on the cellular and molecular aspects of the interaction between $CdCl_2$ and MDA-MB231 breast tumor cells, contribute to the comprehension of the intracellular molecular mechanisms implicated in the regulation of some mitochondrial proteins.

1. Introduction

Cadmium (Cd), an underground mineral extracted as part of zinc deposits, is broadly used in the industry (steel, plastics and batteries) and, as such, it can be considered a wide-spread industrial and environmental pollutant. Food, drinking water and smoking are at present the main sources of daily exposure of humans to Cd [1, for a review]. It is generally acknowledged that Cd, unlike other metals (Zn, Se and Mg) is not essential for the human body, and is not involved in known enzymatic processes or other biological activities. On the other hand, due to its chemical similarity to Zn, Cd may bind with high affinity the Zn-binding domains of several metalloproteins, affecting in different ways Zn-dependent cellular functions [2]. A number of recent reports have provided increasing information on selected aspects of Cd-dependent regulation of gene expression and signal transduction pathways. Cd ion can enter and accumulate into cells probably through voltage-sensitive calcium channels of the plasma membrane [3].

Evidence exists that, at least in some cytotypes, low concentrations of Cd promote DNA synthesis, enhance the expression of several classes of genes (e.g. *c-fos*, *c-jun*, *c-myc*, *p53*, *metallothioneins*, *glutathione*, *heat shock proteins*, *TIF3*, *TEF-1δ*, *Nur77*), and direct intracellular signaling pathways towards up-regulation of cell proliferation, whilst more elevated concentrations are inhibitory and cytotoxic [4–6]. Even low doses were reported to inhibit proliferation of human cells *in vitro* [7]. The mechanisms underlying such opposite effects are so far poorly understood and, on the other hand, exposure to Cd appears to evoke cytotype-specific responses that involve not only death signalling reactions but also cellular protective reactions against the toxicity, e.g. by inducing the expression of stress-response proteins [8].

Cd is classified in group I of carcinogens by the International Agency of Research on Cancer [9], being active in both the initiation of cancer, by switching-on oncogenes, and in the progression of cancer, by increasing the metastatic potential of existing cancer cells, through mechanisms which are likely to involve multiple factors, such as interference with E-cadherin function, followed by disruption of the junctional complexes of epithelial cell sheets and triggering of β-catenin-mediated activation of oncogenes [10]. On the other hand, some Authors have produced evidence on the apoptosis-promoting nature of Cd including its ability to induce DNA fragmentation and chromatin condensation in kidney, blood and liver cells [11–13]. Although detailed data on the pathway(s) followed are still missing, Cd-induced apoptosis, at least in human myeloid leukaemia HL-60 cells, seems to involve cytochrome c release from mitochondria, that, in turn, may cleave and process caspases thorough the activation of Apaf-1 [14]. In

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addition, a novel type of Cd-induced apoptosis not correlated to DNA fragmentation, up-regulation of reactive oxygen species and *Bcl-2* over-expression, was described by Zhai et al. [15]. Thus, although the carcinogenetic property of Cd is well-documented, in line with such observations, a number of data have appeared demonstrating the Cd-induced tumor suppression when administered at not overtly toxic doses to tumor-cell bearing immune-depleted mice. Cd, in fact, was found to markedly reduce the growth of MSB sarcoma cells [16], plasmacytoma cells [17], Dunn osteosarcoma cells [18] and tumorigenic L6 myoblast cells [19]. In addition, in a liver cancer model system "*in vivo*" Cd was reported to induce tumor-specific necrosis while preserving the normal surrounding tissue [20], whilst it was also proven to inhibit growth and progression of human pulmonary tumor xenografts transplanted into athymic nude mice [21].

As far as breast cancer is concerned, most available data have been focused on the effect of metal administration to the estrogens receptor-positive (ER+) cell line MCF-7, resulting in stimulation of hsp27 synthesis and impairment of p53 function by phosphorylation of a serine residue and conformational changes [22]. Noteworthy, Garcia-Morales et al. [23] described an oestrogen-mimetic effect exerted on ER^+ MCF-7 cells by Cd, which appeared to be able to switch-on the transcription of several oestrogen-inducible genes, like *progesterone receptor*, *cathepsin D* and *pS2*, and to increase cell growth rate; these effects were also observed *in vivo* by Johnson et al. [24].

Some of us [25,26] recently produced some preliminary evidence on the effect of Cd exposure on MDA-MB231 cells, an ER-negative human mammary carcinoma line, thereby expected to be insensitive to the oestradiol-like modulation by the metal. In light of the data obtained, we reported that incubation with $CdCl_2$ for different time lapses did not result in a "ladder"-like electrophoretic pattern of extracted DNA, indicative of the occurrence of oligonucleosomal fragmentation, and that no "flip-flop" of the phosphatydilserine occurred. Interestingly, $CdCl_2$ appeared to exert substantial effects on the expression of genes encoding for apoptosis- and stress response modulators also in this cell line. These results prompted additional studies on the cellular and molecular aspects of the interaction between Cd and MDA-MB231 breast tumor cells.

Eukaryotic cells contain a large number of mitochondria responsible of pyruvate dehydrogenation, Kreb's cycle, and oxidative phosphorylation, the energy-generating processes coupling the oxidation of substrates to the synthesis of ATP. In addition, these organelles are involved in the synthesis of amino acids, nucleotides, and lipids, in ion homeostasis and in cell proliferation, motility and programmed death [27–29]. The number, structure, and functions of mitochondria differ in animal cells and tissues in relation to the energetic needs [30,31], and in response to physiological or environmental alterations [32–34]. Malfunctioning mitochondria are related to aging and to many diseases, including cancer [35–37].

Thus, the present study was designed to obtain information on Cd-dependent control of mitochondria-related intracellular events in the MDA-MB231 cell line; in particular, the effect of metal on mitochondrial mass, respiratory activity, production of reactive oxygen species (ROS), expression of *hsp60, hsc/hsp70* and *cytochrome oxidase* (*COX*) *subunits II* and *IV*.

2. Materials and methods

2.1. Cell cultures and $CdCl_2$ exposures

The MDA-MB231 breast cancer cell line was routinely grown in RPMI 1640 medium plus 10% foetal calf serum (Invitrogen, Carlsbad/CA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/l amphotericin B, at 37 °C in a 5% CO_2 atmosphere. For the exposures, the cells were plated in the presence or absence (as controls) of either 5 or 50 μM $CdCl_2$ (corresponding to 0.56 or 5.6 mg/l of cadmium ions) and grown for either 24- or 96 h, as reported by Luparello et al. [25].

2.2. Cytochemical staining

MDA-MB231 cells were seeded at a concentration of 5×10^4 cm^{-1} on either coverslips or 6-well dishes and grown with or without $CdCl_2$.

For the analysis of mitochondrial mass and activity [38], control and treated cells were incubated with 50 nM MitoTracker® Green and 25 nM MitoTracker® Orange in its reduced form CM-H_2 TMRos (both purchased from Molecular Probes/Invitrogen) in fresh growth medium (RPMI 1640) prewarmed at 37 °C for 15 min. in the dark. In parallel, for the analysis of mitochondrial membrane potential, control and treated cell preparations were also incubated with JC-1 (Molecular Probes/Invitrogen, 250 μg/ml final dilution in saline) for 30 min. in the dark. After washings with pre-warmed medium, cells were observed under an Olympus FV-300 confocal laser-scanning microscope equipped with Argon (488 nm) and Helium/Neon (543 nm) lasers with a PlanApo 60 × 1.40 oil-immersion lens and scanned at 1024 × 1024 pixel resolution. Ten focal plans of 100 different microscopic fields were recorded and images were analyzed, measured and processed by the ImageJ software for semi-quantification. In selected experiments, cells were incubated with 15 μM oligomycin (Sigma) for 5 min. before microscopic observation.

For the analysis of ROS accumulation, control and treated cells were incubated with 5 mM amino phenyl fluorescein (APF) solution (Alexis Biochemicals, Lausen, Switzerland; 2 μl/ml final dilution in saline) for 30 min. in the dark [39], then observed under an Olympus BX50 microscope with 10X objective, equipped with a Cool-SNAP-Pro Colour digital camera (Media Cybernetics, Silver Springs/MD, USA) and images were recorded using Image Pro Plus software (Media Cybernetics). Control images were obtained from cells treated with 100 μM H_2O_2. Fifty different microscopic fields were recorded and images were analyzed, measured and processed by the ImageJ software for semi-quantification.

2.3. RNA extraction and northern analysis

Isolation of total RNA from monolayers of control and treated cells was carried out according to Chomczynsky and Sacchi [40]. For the generation of labeled probes, preparations of cDNA from control MDA-MB231 cells were submitted to PCR in the presence of primers for hsp60, hsp70, COXII, COXIV or 18S (see Table 1). The PCR products were resolved in 1.2% agarose gel, the appropriate bands cut and the DNA purified by GENECLEAN II kit (Q-BIOgene, Morgan Inrvine/CA, USA). DNA labeling with [α-^{32}P] dCTP (specific activity 10 mCi/ml; GE Healthcare, Little Chalfont, UK)

Table 1
Sequence of primers used for PCR amplification

Transcript detected	Oligonucleotides (5′ → 3′)	Predicted product (bp)
Hsp60	ATTCCAGCAATGACCATTGC GAGTTAGAACATGCCACCTC	306
Hsp70	TAACCCCATCATCAGCGGAC GAGCTGAAGCAGAAATGACATAGGA	210
COXII	TTCATGATCACGCCCTCATA TAAAGGATGCGTAGGGATGG	187
COXIV	ATGTTGGCTTCCAGAGCGCTGA CTTCTTCCACTCATTCTTGTCATAG	247
18S	GGACCAGAGGCAAAGCATTTGCC TCAATCTCGGGTGGCTGAACGC	495

was performed using the Random Primed DNA Labeling kit (Roche, Mannheim, Germany). The probes were purified through Sephadex G-50 Quick Spin columns (Roche). Northern analyses were performed according to Castiglia et al. [41] with 1×10^6 cpm/ml of hybridization solution. The filters were hybridized with the labeled probes, then stripped and re-hybridized with 18S probe. Autoradiographs were scanned and ImageJ software was used to semi-quantify the relative abundance of mRNAs after normalization with 18S.

2.4. Preparation of cellular extracts and western analysis

Extracts were prepared as described by Cannino et al. [42,43]. Essentially, pelleted cells were resuspended in homogenization buffer (HB: 0.32 M sucrose; 50 mM sodium phosphate buffer, pH 6.5; 50 mM KCl; 0.15 mM spermine; 0.15 mM spermidine; 2 mM EDTA and 0.15 mM EGTA), containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM benzamidine and 1 mM phenylmethylsulfonylfluoride, all from Sigma), homogenized at 4 °C and centrifuged at 1000×*g* for 10 min. at 4 °C. The supernatant was centrifuged at 12,000×*g* for 20 min. to obtain a post-mitochondrial (PM) supernatant and a mitochondrial (M) pellet, which was resuspended in HB. Aliquots of both fractions were stored in liquid nitrogen until used. Protein concentration was determined according to Bradford [44] using bovine serum albumin standards at concentrations ranging from 1 to 10 mg/ml.

For the western analyses, 15 μg of either PM or M fractions were electrophoresized on denaturing 12.5% polyacrylamide slab gel and blotted onto polyvinylidene fluoride microporous membranes (Immobilon-P, Millipore, USA), in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), pH 11.0, containing 10% methanol, at a constant voltage of 50 V for 30 min at 4 °C. Protein markers (Invitrogen) were run in parallel. After staining with 0.2% ponceau red in trichloroacetic acid (Sigma) and image acquisition, the membranes were reacted with the appropriate anti-human primary antibody, i.e. polyclonal anti–hsp70 (Calbiochem), and monoclonal antibodies: anti–hsp60 (Sigma), anti–hsc70 (Santa Cruz), anti-COXII or anti-COXIV (Molecular Probes). The filters were then reacted with the AP-conjugated anti-mouse/rabbit secondary antibody (Promega, 1:7500 dilution) and treated with NBT-BCIP solution (Sigma). The relative protein amount was calculated with the ImageJ software and the obtained values normalized to the total amount of loaded proteins.

2.5. Statistics

The results are presented as mean value ± SD of at least triplicate experiments. The SD is indicated as vertical bars in the figures. Data were analyzed using software-assisted Mann Whitney Rank

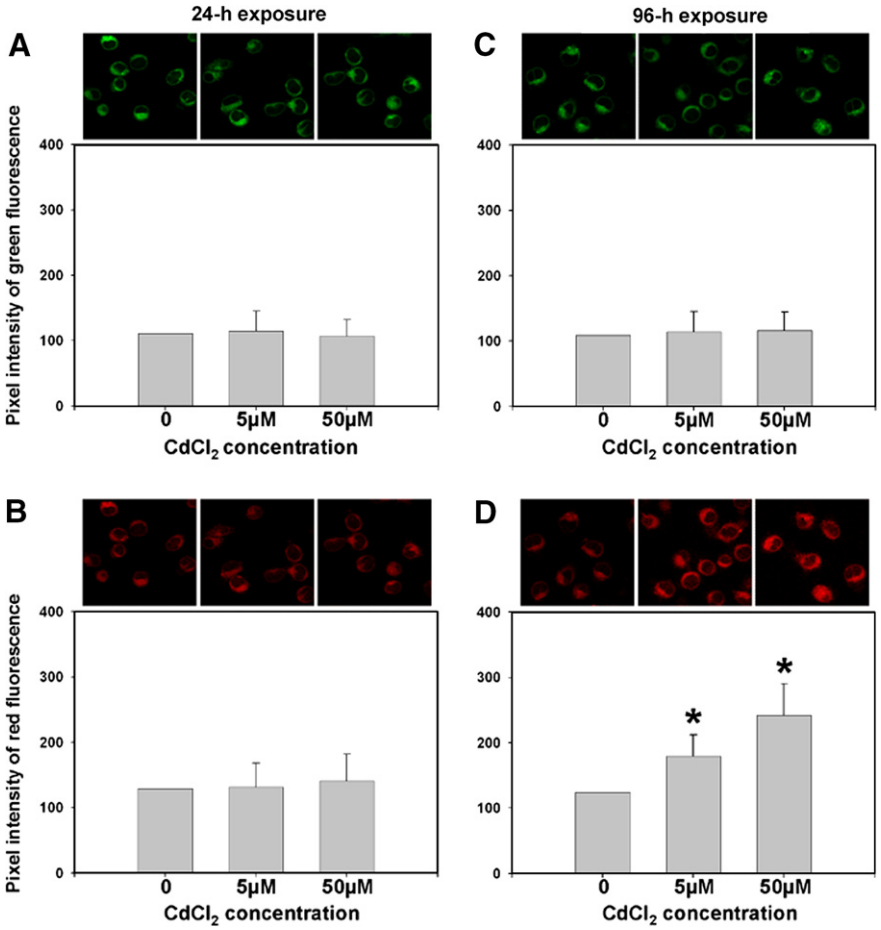

Fig. 1. Mitochondrial mass and respiratory activity of MDA-MB231 cells. (Top) Representative fluorescence micrographs of control (left) 5 μM Cd- (centre) and 50 μM Cd-treated cells (right) stained with MitoTracker Green (A, C) and MitoTracker Orange (B, D) showing that exposure to metal exerted no significant effect on mitochondrial mass ($p = 0.268$) whilst the exposure for 96-h caused a significant increase of respiratory activity ($^{*}p = 0.006$). (Bottom) Histogram displaying the relative pixel intensity of the same 10 focal planes from 100 different fields observed under the confocal laser-scanning microscope. Bars indicate mean values. Standard deviations are indicated.

Sum Test (Sigma Stat v.2.0, SPSS) and $p < 0.05$ was taken as the minimal level of statistical significance.

3. Results

3.1. Mitochondrial mass and respiratory activity

In a first set of experiments, we checked whether the mitochondrial mass and respiratory activity of MDA-MB231 tumor cells were affected by $CdCl_2$ exposure, at the concentrations of 5 and 50 μM for 24-h and 96-h exposures. For this purpose, we incubated control and treated living cells with MitoTracker Green and Orange fluorochromes. MitoTracker™ Green, non-fluorescent in aqueous solutions, becomes fluorescent only when it accumulates in the mitochondrial lipid environment, regardless of membrane potential, thus representing a very useful tool for determining the mitochondrial mass. MitoTracker™ Orange CM-H_2TMRos, the non-fluorescent reduced form of tetramethylrosamine is oxidised by the molecular oxygen within actively respiring cells. It is then sequestered by mitochondria, where it interacts with the thiol groups of peptides and forms an aldehydic mitochondrion-selective probe, fluorescent in the red spectrum, thereby allowing estimating the level of oxidative activity.

In order to calculate the total mitochondrial mass, we integrated the values of pixel intensities for the same 10 focal plans from 100 different microscopic fields containing comparable number of cells. The histograms in Fig. 1 (panels A and C) show that $CdCl_2$ concentrations and application regimes we applied did not exert significant changes of mitochondrial mass. When the same microscopic fields (panels B and D) and focal plans were evaluated for the pixel intensity of MitoTracker Orange, whilst no substantial changes in the extent of the respiratory activity were recorded after 24-h exposure, a significant increase of respiratory activity could be observed at long exposure times (96 h).

However, applying MitoTracker Green and Orange staining in the presence of oligomycin, an ATP synthase-inhibiting antibiotic that blocks oxidative phosphorylation, oligomycin treatment of control and Cd-treated MDA-MB231 cells was found to determine the massive switching-off of the fluorescent signal of MitoTracker Orange without affecting that of MitoTracker Green (data not shown).

To detect variations of mitochondrial transmembrane electrical potential after metal exposure, we used the green-fluorescent JC1 probe that is capable of entering selectively into mitochondria, and changes reversibly its emission from green to orange with the increase of membrane potential. The red-to-green ratio of JC1 fluorescence is dependent only on membrane potential and not on other factors that may influence single-component fluorescence signals, such as mitochondrial size, shape and density.

Applying this technique, results of 100 different microscopic fields showed that exposure to the metal exerted no significant effect on the variation of mitochondrial membrane potential (Fig. 2).

3.2. Reactive oxygen species

We also determined whether our $CdCl_2$ application doses and exposure times could induce reactive oxygen species (ROS) pro-

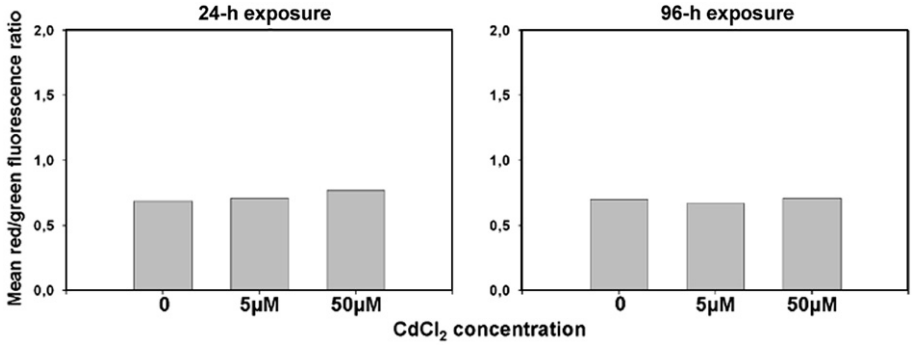

Fig. 2. Evaluation of mitochondrial membrane potential. Pixel intensity ratio of JC1 red/green fluorescence of control and Cd-treated MDA-MB231 cells for 24-h and 96-h. The values represent the average of 100 different microscopic fields showing that exposure to $CdCl_2$ exerted no significant effect on the variation of mitochondrial membrane potential.

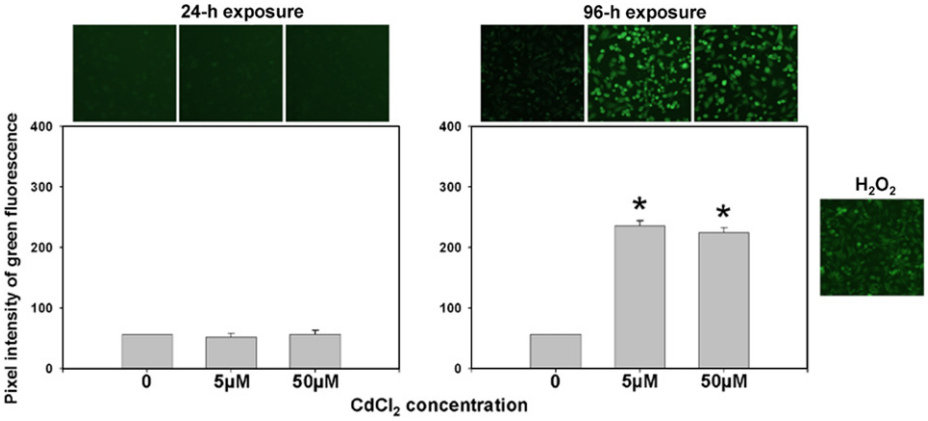

Fig. 3. Production of reactive oxygen species (ROS). (Top) Representative fluorescence micrographs of control and Cd-treated MDA-MB231 cells stained with APF showing that only the exposure for 96-h $CdCl_2$ induced a massive accumulation of ROS ($^*p < 0.001$). (Bottom) Histogram representing the average relative pixel intensity of 50 different microscopic fields. The bars indicate the mean values. Standard deviations are indicated. (Far right) Staining of H_2O_2-treated MDA-MB231 cells taken as a control.

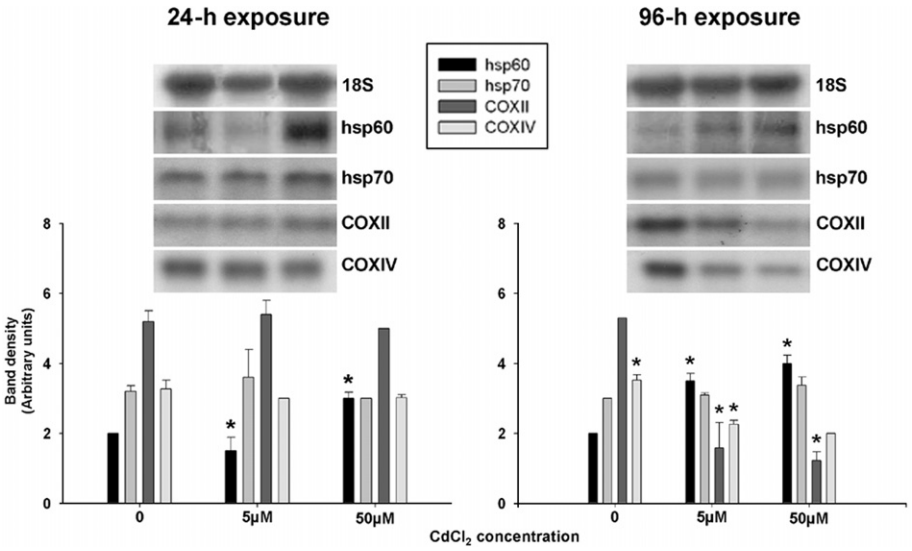

Fig. 4. *Hsp60* mRNA expression levels. (Top) Northern blots of total RNA from control and Cd-treated MDA-MB231 cells hybridized with hsp60, hsp70, COXII, COXIV and 18S probes. (Bottom) Histogram showing the average normalized expression levels of the mRNAs after densitometric analysis of triplicate blots. *Bars* indicate mean values for each group of samples. SDs are also indicated. The amount of hsp60 mRNA increased in all the treated samples, except for that obtained after incubation with 5 μM $CdCl_2$ for 24-h; on the other hand, the amount of hsp70 mRNA remained invariant, whilst the amount of COXII and COXIV mRNAs showed no change after 24-h exposure and a decrease after 96-h exposure ($^{*}p \leqslant 0.05$).

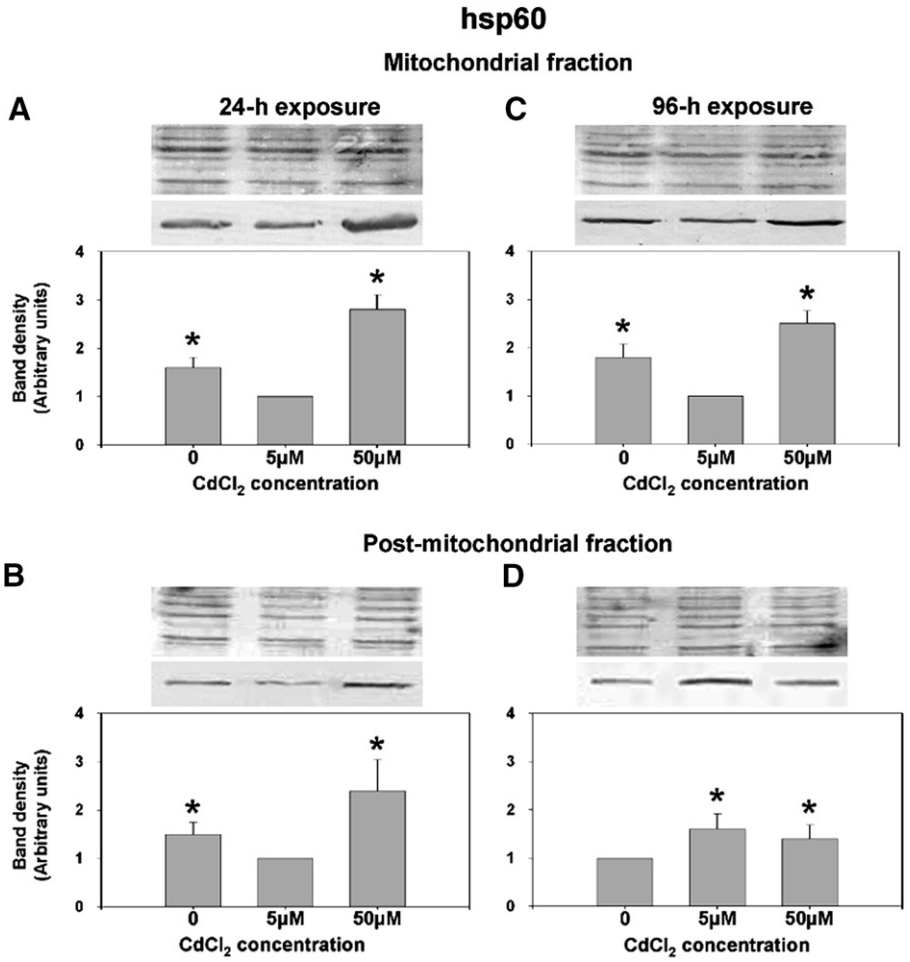

Fig. 5. Hsp60 protein levels. Western blot of mitochondrial (A, C) or post-mitochondrial (B, D) protein fractions, stained with Ponceau Red (top) or incubated with anti-hsp60 antibody (bottom). The intensity of each band was measured by densitometry and normalized to that of the total amount of loaded proteins. The histograms show the accumulation levels of hsp60 of six independent experiments after $CdCl_2$ exposure. The amount of hsp60 in M fraction decreased after incubation with 5 μM $CdCl_2$ for 24- or 96-h; whilst it increased at both exposure times when $CdCl_2$ concentration was raised up to 50 μM. In the PM fraction, the protein amount decreased after 24-h exposures with 5 μM $CdCl_2$ although increased in the other experimental conditions. Bars indicate mean values of six independent experiments for each group of samples. SDs are also indicated ($^{*}p \leqslant 0.05$).

duction. As shown in Fig. 3, APF-staining of cells clearly revealed that, whilst no substantial ROS production was detectable in treated cells after 24-h of incubation with $CdCl_2$, metal exposure for 96-h triggered fluorescence emission, indicative of massive ROS production, to an extent similar to that of H_2O_2-treated cells, utilized as controls.

Cumulatively, the data obtained from the first group of experiments indicated that mitochondria appear to be a target of $CdCl_2$ after prolonged (96-h) exposure; although no modification of the mitochondrial mass was detectable after all treatments.

Interestingly, cellular respiratory activity appeared to be increased and a significant induction of reactive oxygen species could be concomitantly revealed.

3.3. Mitochondrial gene expression

Cells can react to both physical and chemical environmental insults by promoting the heat shock response. The next set of assays was designed to examine, at a molecular level, the effect exerted by $CdCl_2$ on the organelles of MDA-MB231 cells, in particular the expression levels of two mitochondrial heat shock proteins coded by nuclear genome, i.e. hsp60 and hsp70, whose induction is an event largely shared by mammalian cells submitted to heat shock or chemical stresses. We also evaluated the effects exerted by the metal on the expression of two subunits of the cytochrome oxidase complex (COX), the terminal component of the mitochondrial respiratory chain, i.e. the subunits II (encoded by the mitochondrial genome) and IV (encoded by a nuclear gene).

We studied *hsp60*, *hsp70*, *COXII* and *COXIV* expression levels. Equal amounts of total RNA, purified from cells after different $CdCl_2$ exposures, were analyzed by Northern blot assays. As shown in the panel of Fig. 4, 24-h exposures with 5 μM $CdCl_2$ were able to promote the down-regulation of *hsp60*, whereas different degrees of up-regulation could be observed after 24-h exposures with 50 μM or 96-h exposures with both 5 and 50 μM $CdCl_2$. The exposures to the metal did not appear to modify *hsp70* expression. On the other hand 24-h exposure to the metal did not change *COXII* and *COXIV* expression, while 96-h exposure induced the decrease of both messengers.

3.4. Mitochondrial protein expression

We then analyzed the effect of $CdCl_2$ exposure on the accumulation of hsp60 protein product by Western blots. As shown in Fig. 5, hsp60 was recognized by the specific antibody in both M and PM protein extracts of control and Cd-treated MDA-MB231 cells. In particular, when the cells were incubated with the metal at 5 μM $CdCl_2$ for 24- or 96-h, the amount of hsp60 in M fraction decreased; vice versa, when $CdCl_2$ applied at a dose of 50 μM, the level of accumulated protein increased at both exposure times (panels A and C). In the PM fraction, the protein amount decreased after 24-h exposures with 5 μM $CdCl_2$ although increased at the other experimental conditions (panels B and D).

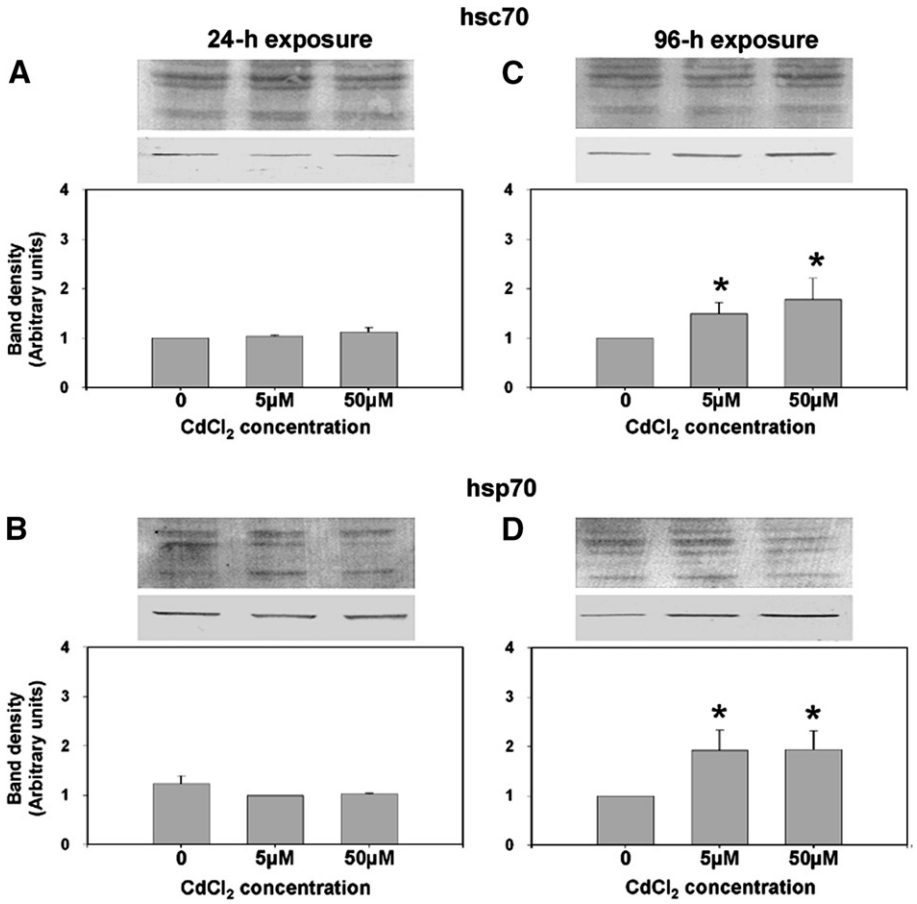

Fig. 6. Hsc70/hsp70 protein levels. Western blot of post-mitochondrial protein fractions, stained with Ponceau Red (top) or incubated with either anti-hsc70 (A, C) or hsp70 (B, D) antibodies (bottom). The histograms show the average normalized accumulation levels of hsc70 and hsp70 of six independent experiments after $CdCl_2$ exposure. The amount of hsp70 and hsc70 after 24-h exposure did not change, while it increased after 96-h exposure. Data are represented as means ± SD and significance was tested at $^{*}p \leqslant 0.05$.

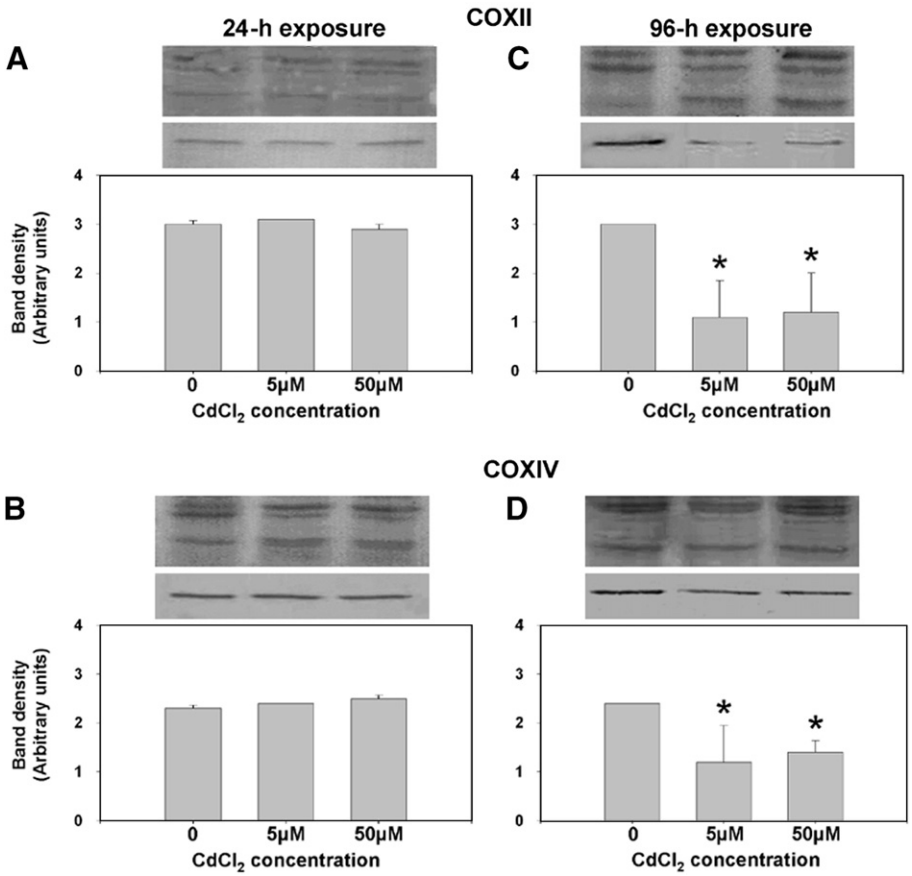

Fig. 7. COXII and COXIV protein levels. Western blot of mitochondrial protein fractions stained with Ponceau Red (top) or incubated with either anti-COXII (A, C) or -COXIV (B, D) antibodies (bottom). The histograms show the average normalized accumulation levels of COXII and COXIV of six independent experiments in the mitochondrial fraction after $CdCl_2$ exposure. The amount of COXII and COXIV remained constant after 24-h exposure, while it appeared to decrease after 96-h exposure. Data are represented as means ± SD and significance was tested at $^{*}p \leqslant 0.05$.

To exclude that the presence of the Hsp60 protein in the PM extracts was due to contamination with broken mitochondria, parallel blots were tested with antibodies directed against some mitochondrial proteins like COXIII and MnSOD (data not shown).

We also evaluated the effects exerted by the heavy metal on the degree of accumulation of both hsp70 and hsc70 proteins in the PM fraction. As shown in Fig. 6, the 24-h exposure did not cause any change in the amount of hsp70 and hsc70 present in the protein extract (panels A and B), while the 96-h exposure induced a drastic increase of both stress response proteins (panels C and D).

In the last set of experiments, we analyzed the effect of $CdCl_2$ on the accumulation of COXIV and COXII proteins in the mitochondrial fraction. As shown in the panel of Fig. 7 both COXII and COXIV were recognized by the specific antisera only in M extracts from control and Cd-treated cells. In addition, we found that after 24-h exposures the levels of COXII and COXIV amounts remained constant (panels A and B), while they appeared to decrease after longer incubations (96-h) (panels C and D).

4. Discussion

It is widely acknowledged that Cd is able to exert multiple effects on biological systems, both cytotoxic, which frequently lead to the production of specific stress response proteins thereby often determining the switching-on of apoptotic mechanisms, and carcinogenetic, responsible of neoplastic cell transformation (e.g. [4,8,11]. The complexity and diversity of the molecular events underlying cell–metal interactions are responsible of the data fragmentation and poor knowledge of the intracellular pathways involved; nonetheless, significant indications are surely emerging from the evaluation of peculiar aspects of cell metabolism (i.e. gene expression and protein accumulation/traffic) and of intracellular oxidative state. Mitochondria are among the key intracellular targets for different stressors including Cd, but the mechanisms of metal-induced mitochondrial damage are not fully understood [45].

In the present study, we incubated ER^{-} MDA-MB231 neoplastic cells, derived from human breast epithelium, to incubation with 5 or 50 μM $CdCl_2$ (similar to levels encountered in occupational or acute exposure, respectively) for 24- or 96-h time lapses. The viability/proliferative response of these cells in the presence of $CdCl_2$ at different concentration have been already evaluated [25]. Here, we specifically focused our analysis on features related to the organelle function and to the expression, biosynthesis and accumulation of selected mitochondrial components.

4.1. Mitochondria-related activity

In mammals, the regulation of mitochondrial biogenesis requires the coordinated expression of nuclear and mitochondrial genomes [46] and is influenced by external factors, such as nutrients, hormones, temperature, exercise, or by hypoxia and aging [47]. In our model, we have observed that the mitochondrial mass remained constant under every experimental condition utilized,

thus demonstrating that $CdCl_2$ was not capable to influence mitochondrial proliferation. Interestingly, the 96-h exposure induced a significant increase of respiratory activity even in the absence of variations of mitochondrial membrane potential. This result suggests that $CdCl_2$ induced an increased rate of respiration when accumulated in MDA-MB231 cells. In different tissues, in fact, Cd was proven to affect the activity of complexes II and III of the electron transfer chain, also depleting glutathione and other free radical scavengers, thereby generating respiratory dysfunctions and production of ROS, and, in some cases, inducing some of the known variants of apoptosis death [48–50]. An equivalent oxidative damage was also reported recently for Cd-intoxicated plant cells indicating that such Cd-dependent effect is shared by different model systems [51].

Concerning ROS production by mitochondria [4,8], our results showed that only the 96-h exposure with $CdCl_2$ induced a massive accumulation of these reactive species, even in the absence of changes of transmembrane potential. Noteworthy, in marine invertebrates Cd-triggered apoptosis can take place in the absence of significant alterations of mitochondrial membrane permeability [52]. On the other hand, the prolonged production of ROS can cause the disruption of the mitochondrial electron transport and strongly interfere with mitochondrial permeability transition pores, as reported in other model systems [48,50]. Interestingly, Rip-1, member of a kinase family acting as sensor of cellular stress and crucial regulator of cell viability [53], whose gene expression is prominently switched-on after $CdCl_2$ exposure of MDA-MB231 cells [25], is known to be involved in ROS formation by activation of cytosolic phospholipase A2 and/or enhanced electron flow through the mitochondrial electron transport chain [54]. It remains to be elucidated whether *Rip-1* up-regulation is responsible of ROS accumulation in MDA-MB231 following $CdCl_2$ exposure. Moreover, Dorta et al. [55], proposed a sequence of events accounting for Cd-induced mitochondrial impairment in normal rat liver mitochondria, beginning with an apparent interaction of the metal with specific protein thiols in the mitochondrial membrane, resulting in the onset of mitochondrial permeability transition (MPT) and in the dissipation of the transmembrane electrical potential.

4.2. Mitochondrial gene expression

Some of us demonstrated that $CdCl_2$ administration to tumor and immortalized breast cells generated a very different pattern of gene expression [25]; dealing with MDA-MB231 cells, in particular the metal also promoted a massive transcriptional activation of *Dap kinase* and of six different *caspases*, whilst down-regulated *grp78* and *Bcl-2*. In the present work, we have expanded the list of Cd-responsive genes by demonstrating that selected concentrations and duration times of $CdCl_2$ exposure are active in modifying *hsp60* expression levels; in particular, 24-h exposition to 5 μM $CdCl_2$ resulted in *hsp60* down-regulation, whereas up-regulation was attained by increasing metal concentration up to 50 μM for both exposure periods. The induction of *hsp60* would be expected to provide assistance to the cell in the refolding and processing of damaged proteins or, as recent findings also suggest in the regulation of programmed cell death [56]. The over-expression of hsp chaperones can depend on Cd-promoted denaturation and oxidation of proteins, and is probably linked to the increase of oxidative stress. Although hsp60 is mostly localized in the mitochondrial matrix, it has also been found in other subcellular localizations including the ER, cell surface, and unidentified vesicles and cytoplasmic granules [57] and also found to be over-expressed and localized in the cytoplasm of cancer cells [58]. In the current study, we report the presence of hsp60 in the cytoplasm of MDA-MB231 tumor cells and demonstrate that $CdCl_2$ exposure modifies the cytosolic level of the protein. In particular, we revealed a decrease of the protein amount in PM fractions after 24-h exposure to 5 μM $CdCl_2$, in contrast to the other experimental conditions where the content of hsp60 was found to be increased. In the light of the data obtained from 96-h exposure to 5 μM $CdCl_2$, demonstrating the increase of *hsp60* expression level, the decrease of protein amount in the mitochondria and, conversely, its increase in the PM fraction, we can tentatively hypothesize an impairing effect on both biosynthetic and import processes, resulting in the accumulation of the chaperonine in the cytoplasm. The mechanism by which hsp60 is retained or sequestered in the cytoplasm is not known yet, and warrants further studies.

In response to environmental stresses, the gene coding for the inducible 70 kDa heat shock protein (hsp70) is rapidly activated. Hsp70 prevents the incorrect folding and assembly of peptides, avoids the transport of misfolded or degraded proteins, preparing them to elimination, and inhibits the induction of apoptosis [59]. Hsp70 functions as part of multi-component chaperone machinery in living cells, and its activity is modulated by different sets of cofactors [60]. In A549 human pulmonary cells line, the hsp72 protein was over-expressed under stressing conditions, following short-term exposure (2–6 h) to Cd doses higher than 50 μM, or doses as low as 0.1–1 μM for exposures lasting longer than 48 h, thereby being considered an early biomarker of short-term exposure [61]. Here, we analyzed the expression levels of inducible hsp70 and also of hsc70, its constitutive form, and we found that in MDA-MB231 cells, only the 96-h exposure induced the over-expression of the two proteins. Moreover, we noticed that the relative concentration of hsp70 mRNA remained constant under every experimental conditions. It is known that the control of the oxidative phosphorylation mainly relies on the terminal component of the respiratory chain, i.e. the cytochrome c oxidase complex (COX), which catalyzes the transfer of electrons from cytochrome c to molecular oxygen. So far, the effect of $CdCl_2$ on the expression of the genes coding for COX subunits has been poorly investigated, except for a single paper suggesting that in some mollusc species *COXI* gene is up-regulated after metal contamination [62]. We therefore studied the expression of some subunits of this complex in our experimental model system and we found that the levels of COXII and COXIV mRNAs and proteins remained constant after 24-h exposure, while they decreased after longer incubations (96-h). It is noteworthy that the lack of physiologic amounts of some COX subunits did not influence the mitochondrial energetic rate, however, it must be taken into account that in all tissues studied, the enzyme content was in a large excess compared with what is required for state 3 respiration [63]. Hypothetically, the reduction of the amount of COX subunits could be related to ROS production since mitochondrial proteins are often among the first targets of ROS attack in cells because of their immediate proximity to the ROS-generating sources [64].

Taken together, our results suggest that mitochondria can be considered as a target of $CdCl_2$ after exposure of ER^- MDA-MB231 cell line for periods as long as 96-h. In addition the present data provide additional indications that this cell line can be considered a suitable *in vitro* model for further studies of the intracellular molecular mechanisms of death response induced by Cd intoxication in human tumor cells.

5. Abbreviations

Cd	cadmium
ER^-	estrogens receptor-negative
ROS	reactive oxygen species
HB	homogenization buffer
EGTA	ethylene glycol tetraacetic acid
CAPS	cyclohexylamino-1-propanesulfonic acid
MPT	mitochondrial permeability transition

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