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Cadmium effects on p38/MAPK isoforms in MDA-MB231 breast cancer cells

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Abstract Emerging evidence seems to indicate that the heavy metal cadmium (Cd) is able to regulate gene expression, drastically affecting the pattern of transcriptional activity in normal and pathological eukaryotic cells, also affecting intracellular signalization events. Human p38 is a family of mitogen-activated protein kinases consisting of four isoforms (α, β, γ and δ) which mediate signal transduction cascades controlling several aspects of cell physiology. In this study we examined whether exposure of MDA-MB231 tumor cells from the human breast to Cd may exert some effect on p38 isoform expression and accumulation, as well as on p38 activation. Employing a combination of proliferation tests, conventional and semiquantitative multiplex (SM)-polymerase chain reaction (PCR) and Western blot assays, we report that the treatment of breast cancer cells with 5 μM $CdCl_2$ induces a diversified modulation of the transcription patterns of *p38 isoform* genes and of the accumulation of the related protein products, which are, on the other hand, also affected by α and β isoform functional inactivation induced by SB203580. Our findings suggest the existence of so far unexplored mechanisms of gene regulation in our model system and validate that MDA-MB231 cell line is a suitable in vitro model for further and more detailed studies on the intracellular mechanisms underlying the control of p38 expression, synthesis and activation in mammary tumor cells exposed to different stresses.

Keywords Cadmium · SB203580 · p38 isoforms · p38 activation · Gene expression

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Introduction

The heavy metal cadmium (Cd) is an industrial and environmental pollutant mostly released as an air contaminant from fertilisers and in the form of wastewater. It is not essential for the human body and has no known useful biological functions; its uptake appears to be mediated by the voltage-sensitive Ca^{++} channels of the plasma membrane and, once accumulated within eukaryotic cells, due to its chemical similarity to zinc (Zn) it binds with high affinity to the Zn-binding domains of several metalloproteins, interfering with or preventing Zn-dependent cellular functions from being completed (Lévesque et al. 2008; Beyersmann and Hartwig 2008). The most common targets of Cd toxicity are known to be the renal, skeletal, vascular, nervous and respiratory systems; in addition, it is classified within group I of carcinogens by the International Agency of Research on Cancer (IARC 1993), playing a role in both the initiation of cancer, by activating oncogenes,

and in the progression of cancer. On the other hand, a number of data has demonstrated its ability to promote apoptosis not only in normal cells but also in some tumoral cytotypes (Rana 2008).

Recently, emerging evidence seems to indicate that Cd is capable of regulating gene expression, drastically affecting the pattern of transcriptional activity in both prokaryotic and lower/upper eukaryotic cells, under normal and pathologic conditions (e.g., Bertin and Averbeck 2006, for review). Some of us have recently demonstrated that Cd is able to affect to a different extent the pattern of transcriptional activity, as well as the mitochondria-related activities, of MDA-MB231 adenocarcinoma cells and HB2 immortalized epithelial cells derived from the human breast (Sirchia et al. 2006, 2008; Luparello et al. 2007; Cannino et al. 2008, 2009). In particular, concerning estrogen-receptor-negative and p53-defective MDA-MB231 cells, when exposed to 5 μM $CdCl_2$, corresponding to the median inhibition concentration (IC_{50}) after long-term incubation (96 h) and also to levels encountered in occupational exposure, various prominent changes were found in the expression levels of stress response genes, such as those coding for some heat shock proteins (hsp) and metallothionein isoforms, and of some other genes coding for factors and enzymes involved in the onset of apoptosis.

Human p38 is a family of mitogen-activated protein kinases (MAPK) that, once activated via double phosphorylation of Y and T amino acids present in the T × Y motif of their activation loop in the catalytic domain, mediate signal transduction cascades that are mostly triggered by different stimuli of cellular stress (Torres and Forman 2003). p38/MAPKs are known to be involved in normal cell physiology, controlling chromatin remodelling, DNA methylation and cell transcriptional pattern, as well as regulating cell cycle, its checkpoints and life/death cell decisions; on the other hand, different pathologies are to be ascribed to MAPK dysregulation, such as gastric and colorectal cancer, rheumatoid arthritis, amyotrophic lateral sclerosis and defective myogenesis (Bendotti et al. 2005; Westra and Limburg 2006; Perdiguero et al. 2007; Wang and Fang 2007). Four isoforms of human p38/MAPKs, i.e., α, β, γ and δ, have been described. Among them, the first two have been proven to be expressed in an ubiquitous way, whilst the γ and δ isoforms display tissue-specific expression, the former being most abundant in skeletal muscle whereas the latter in testes, pancreas, kidney and small intestine (Thornton and Rincon 2009). It is also acknowledged that the diverse p38 isoforms show a different affinity for upstream activators and downstream effectors. As far as breast cancer cells are concerned, the only literature data available on p38 isoform functions refers to their differential ability to activate the activator protein-1 (AP-1)-mediated processes, to regulate the production of extracellular enzymes and cell motility, as well as to exert antagonistic effects on Ras transformation and stress response and to control serum withdrawal-induced cyclooxygenase-2 mRNA levels (Jang et al. 2000; Pramanik et al. 2003; Suarez-Cuervo et al. 2004; Qi et al. 2007). Thus, although fragmentary data has appeared in the literature suggesting a complexity of p38 isoform activities, still much remains to be determined in order to have a complete understanding of their specific roles.

The cytokine-suppressive anti-inflammatory drugs (CSAID), such as SB203580, are pyridinylimidazole compounds which selectively inhibit p38/MAPK by competitive binding in the ATP pocket. Noteworthy, a diversified effect was reported for SB203580 on the different p38 isoforms, being equipotent against p38α and p38β whilst ineffective or weakly effective against p38γ, p38δ as well as JNK (with the possible exception of 2β1 isoform) and Erk2 (Lee et al. 2000), thereby constituting a helpful tool for the investigation of the specific involvement of p38 isoforms in biological events.

Increasing evidence has highlighted the ability of Cd to rapidly activate p38/MAPK (e.g., Miguel et al. 2005; Kim et al. 2008); on the other hand, concerning isoform involvement the only literature reports available are about a differential regulation on Cd-induced *hsp70* and *heme oxygenase-1* expression by mouse embryo stem cells and MCF-7 human breast cancer cells, respectively (Nishitai and Matsuoka 2008; Alam et al. 2000).

In light of both the literature data and the results obtained suggesting an active role played by Cd on MDA-MB231 cells, in this study we extended our investigation to examine whether exposure of this cell line to the heavy metal could also exert some effect on p38 isoform expression and accumulation, as well as on p38 activation. Employing a combination of proliferation tests, conventional and semiquantitative multiplex (SM)-polymerase chain reaction (PCR) and

Western blot assays, we report that the treatment of breast cancer cells with 5 μM $CdCl_2$ induces a diversified modulation of the transcription patterns of *p38 isoform* genes and of the accumulation of the related protein products, which are, on the other hand, also affected by α and β isoform functional inactivation induced by SB203580, thereby suggesting the existence of so far unexplored mechanisms of gene regulation in our model system.

Materials and methods

Cell cultures and treatments

The MDA-MB231 breast cancer cell line was routinely grown in RPMI 1640 medium plus 10% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/l amphotericin B (Invitrogen, Carlsbad, CA, USA), at 37°C in a 5% CO_2 atmosphere.

For Cd treatment, MDA-MB231 cells were plated in the presence of 5 μM $CdCl_2$ and grown for different time lapses between 24 and 96 h, as reported by Luparello et al. (2007).

For SB203580 treatment, MDA-MB231 cells were pre-treated with the p38 inhibitor (Sigma, St. Louis, MO, USA) at 20 μM concentration for either 4 h or 30 min before plating in inhibitor-free culture medium as reported by Bobrovskaya et al. (2001) and Birukova et al. (2005), or seeded in SB203580-containing culture medium which was maintained for the whole duration of the experiment, as reported by Zechner et al. (1997).

Co-treatment was performed by seeding cells in 5 μM Cd- and 20 μM inhibitor-containing medium.

Viability and proliferation assays

For the evaluation of cell viability/growth behaviour we submitted trypsinized control and treated MDA-MB231 cells to counting using a Bürker chamber. In parallel, cells were also seeded in 96-well tissue culture plates and submitted to CellTiter 96® AQueous Non-Radioactive Cell Proliferation assay (Promega, Madison, WI, USA); the absorbance of formazan produced by reduction of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] compound was recorded at 490 nm.

RNA extraction and reverse transcription

Isolation of total RNA from trypsinized control and treated MDA-MB231 cells was carried out with the RNAspin Mini RNA isolation kit (GE Healthcare, Little Chalfont, UK), following manufacturer's instructions. The cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen) in the presence of 100 ng random 6-mer primers (Sigma), 50 U RNase inhibitor (Promega) and 0.5 mM each of dNTPs; reverse transcription was carried out for 60 min at 42°C, followed by being treated with 2 U RNase H (USB, Cleveland, OH, USA) for 20 min at 37°C.

Conventional and semi-quantitative "multiplex" polymerase chain reaction

Conventional PCR analysis was performed using 2.5 μM of appropriate sense and antisense primers (see Table 1) obtained from Invitrogen, 1 U RedTaq DNA polymerase (Sigma)/μl, 200 μM each of dNTPs,

Table 1 Sequence of primers used for PCR amplification

Transcript detected	Oligonucleotides (5′→3′)	References
p38α	AACCTGTCTCCAGTGGGCTCT	Hale et al. (1999)
	CGTAACCCCGTTTTTGTGTCA	
p38β	CACCCAGCCCTGAGGTTCT	Hale et al. (1999)
	AGATGCTGCTCAGGTCCTTCT	
p38γ	ACATGAAGGGCCTCCCCG	Uddin et al. (2004)
	TCTCCTTGGAGACCCTGG	
p38δ	TGCTCGGCCATCGACAA	Hale et al. (1999)
	TGGCGAAGATCTCGGACTGA	
18S	GGACCAGAGGCAAAGCATTTGCC	Luparello and Sirchia (2005)
	TCAATCTCGGGTGGCTGAACGC	

and 1 µl of the cDNA template obtained from total RNA. The thermal cycle used was a denaturation step of 94°C for 3 min, followed by 45 cycles of 94°C for 30 s, the appropriate annealing temperature for 30 s, and 72°C for 30 s. A final extension of the product was performed for 5 min at 72°C. PCR products were analysed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. For SM-PCR we followed a published protocol (e.g., Luparello and Sirchia 2005; Sirchia et al. 2008; Luparello et al. 2007) in which the species of interest is co-amplified with 18S cDNA. The intensities of the band of interest, normalized for those of 18S, were plotted as a function of cycle number and exponential regression equations fitted to the curves and were used to calculate the number of cycles necessary to reach a normalized intensity threshold value = 1 for each sample. The relative difference in abundance between two samples was taken as 2^n where n is the difference between the numbers of cycles required by the samples to reach the threshold. PCR products from triplicate experiments were visualized by 2% agarose electrophoresis in the presence of ethidium bromide and, when required, band intensity evaluated with SigmaScan software (SPSS).

Protein extraction, electrophoresis and immunoblot

Trypsinized control and treated cells were homogenized in a lysis buffer (7 M urea, 2% Chaps, 1% immobilized pH gradient (IPG) buffer, and 10 mM DTT) containing protease inhibitors (complete, Mini, EDTA-free protease inhibition cocktail, Roche). Protein concentrations were evaluated using Bradford method, and 30 µg of samples analyzed by 10% acrylamide SDS–PAGE. The molecular masses were evaluated by comparison with a set of standard proteins (PageRulerTM Prestained Protein Ladder, Fermentas). The electrophoretic separation was performed for 1.5 h at 100 V. After the run, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham) using miniVE Blotter apparatus (Amersham Pharmacia Biotech), in blotting buffer (24 mM Tris, 1 M glycine, 20% methanol), at 100 mA for 12 h. Filters were pre-incubated for 2 h in a blocking solution made of TBS-T (Tris-buffered saline, 0.05% Tween 20) plus 5% non-fat dried milk, washed once in TBS-T, and then incubated for 1 h with anti-actin, rabbit polyclonal antibody (1:250 final dilution, Sigma) and overnight with the following antibodies diluted 1:600 in TBS-T: anti p38 MAPK (pT180/pY182) phospho-specific mouse monoclonal antibody (BD Bioscience, Franklin Lakes, NJ, USA), anti-p38α MAP kinase rabbit polyclonal antibody, anti-p38β MAP kinase rabbit monoclonal antibody, anti-p38γ MAP kinase rabbit polyclonal antibody, and anti-p38δ MAP kinase rabbit polyclonal antibody (all from Cell Signaling Inc., Danvers, MA, USA). After removal of non-specific complexes by three washes in TBS-T and two washes in TBS, the filters were incubated for 1 h with the alkaline phosphatase-conjugated secondary antibody, diluted 1:7,500 in TBS. After removal of unspecific complexes by several washings with TBS, the filters were stained with BCIP/NBT (Sigma). The colorimetric signals were analyzed by ImageJ software, and then normalized with respect to the correspondent values obtained with anti-actin antibody reaction.

Statistics

Data is presented as mean ± s.e.m. of triplicate experiments; a software-assisted one-way ANOVA was performed (SigmaStat v.2.0, SPSS, Chicago, IL, USA) and $P < 0.05$ was taken as the minimal level of statistical significance between treated and control samples.

Results

$CdCl_2$/SB203580 co-treatment reverses the decrease in the number of MDA-MB231 cells promoted by single treatments

Preliminarily to the evaluation of *p38* expression and accumulation/activation of the protein product, in a first set of experiments we checked whether treatment with the p38 inhibitor SB203580, either alone or in combination with 5 µM $CdCl_2$, could somehow affect the biological behaviour of MDA-MB231 cells, by checking cell viability and proliferative behaviour. In particular, we tested the effect of different durations of exposure to SB203580, i.e., a pre-treatment for a limited time lapse, or the continuous presence of the inhibitor for the whole duration of the experiment, by performing parallel cell counting and MTS

colorimetric test on control and treated cells, the latter evaluating the activity of mitochondrial dehydrogenases thereby providing information deriving from the state of cell metabolic activity. No statistically significant difference was recorded in our model system when SB203580 exposure was limited to pre-treatment (data not shown), whilst the continuous presence of SB203580 in the culture medium produced effects in some single and combined treatments; thus, for all the subsequent analyses we chose to maintain cells in the presence of the inhibitor until the end of the assays. Specifically, as shown in Fig. 1, cumulative results from cell number evaluation indicated that no treatment exhibited significant difference with respect to control when observed within 72 h of incubation. Conversely, after 96 h $CdCl_2$ promoted a decrement of the number of cells down to about 60%, as expected from previous data (Luparello et al. 2007; Sirchia et al. 2008), but, although single SB203580 treatment resulted ineffective in producing any change, to our surprise, $CdCl_2$/SB203580 co-treatment induced a consistent increase of cell number (approx. +50% and +100% vs. untreated and Cd-treated cells, respectively). Interestingly, despite the absence of difference the quantity of cells obtained after direct counting of the control and SB203580-treated cells, when cell metabolism was monitored by

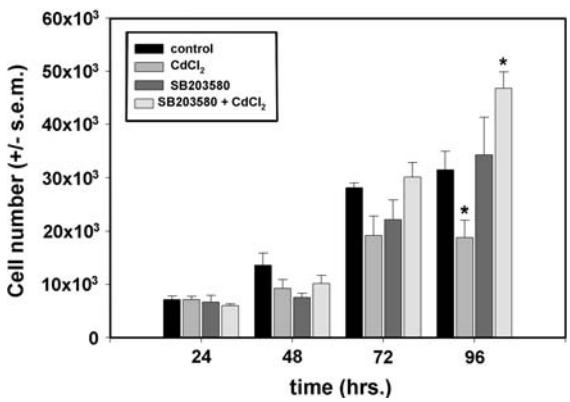

Fig. 1 Histogram showing the number of MDA-MB231 cells in the control and treated conditions, evaluated at different times of culture. Cells were plated in 24-well plates at the concentration of 2.5×10^4 cells/well in culture medium, either un-supplemented or supplemented with 5 μM $CdCl_2$, 20 μM SB203580, or both, and grown for 24, 48, 72 or 96 h. At the end of the incubation, cells were trypsinized and counted in a Bürker chamber. Data is presented as mean ± s.e.m. of triplicate experiments ($n = 3$). * $P < 0.05$ (treated vs. control cells)

MTS assay, i.e., monitoring formazan accumulation, an increase of absorbance could be detected at 96 h of incubation with SB203580 (approx. +22% vs. untreated cells; not shown), thereby suggesting that inactivation of p38 α/β, although producing no significant effect on MDA-MB231 cell viability and growth, could in some way ameliorate their rate of mitochondrial metabolism, as also reported in other model systems (e.g., Sumida et al. 2005; Dey and Cederbaum 2007).

On the other hand, by analogy with cell count data, the amount of formazan generated decreased in Cd-treated and increased in co-treated MDA-MB231 cells when compared to control cells (approx. −15% and +15%, respectively; not shown).

Only SB203580 treatment and co-treatment induce an increased activation of p38/MAPK

In order to reveal the extent of p38/MAPK activation in either control, SB203580-, Cd- or co-treated MDA-MB231 cells, Western blot assays were performed in the presence of anti-bi-phosphorylated p38 antibodies. As shown in the histograms in Fig. 2, we found a statistically significant increase of p38/MAPK activation with respect to controls only when cells were either treated with SB203580 (approx. +0.8-folds) or co-treated (approx. +1.5-folds).

Expression of p38α, β, γ and δ by MDA-MB231 cells is switched-on under all the experimental conditions but to different levels

In light of both previously published (Luparello et al. 2007; Cannino et al. 2008; Sirchia et al. 2008) and present data demonstrating that treatment with 5 μM $CdCl_2$ for 96 h actually induced transcriptional alterations in MDA-MB231 cells and that SB203580 was in some way effective in modifying cell proliferative/metabolic behaviour, in a subsequent set of experiments we wanted to test whether modifications in the expression levels of genes coding for the isoforms of p38 MAPK could occur following the different cell treatments. To this purpose, cDNA preparations from either Cd-, SB203580- or co-treated MDA-MB231 cells and from the untreated counterpart were submitted to conventional- and SM-PCR in the presence of primers specific for the cDNAs of *p38α*, *β*, *γ* and *δ*. The panel in Fig. 3 representatives three independent

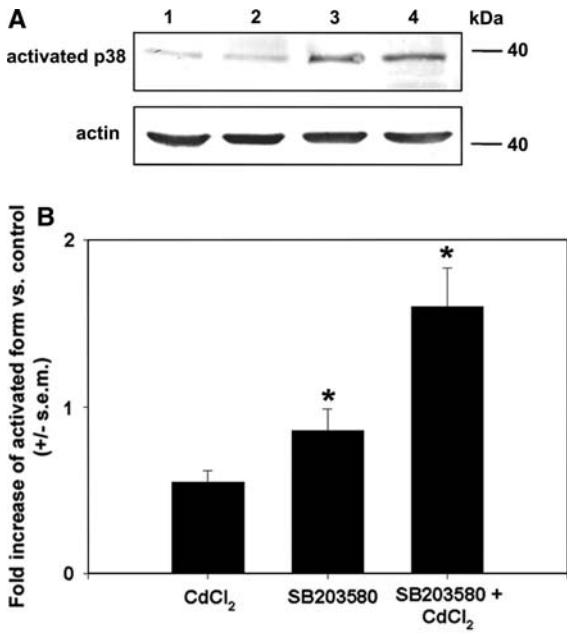

Fig. 2 **a** Representative Western immunodetection of the activated form of p38 in lysates of control (*1*), Cd-treated (*2*), SB203580-treated (*3*) and co-treated MDA-MB231 cells (*4*). Immunoreaction with anti-actin antibody was performed to check protein loading. **b** Histogram showing the changes of the activation levels of p38/MAPK in MDA-MB231 submitted to the different treatments, as resulted from immunoblot assays. Cell lysates were submitted to 10% acrylamide SDS–PAGE, blotted onto nitrocellulose membranes and the bands visualized by immunoreaction with the primary and secondary antibodies and staining with BCIP/NBT. Data in the histogram is presented as mean ± s.e.m. of triplicate experiments ($n = 3$). * $P < 0.05$

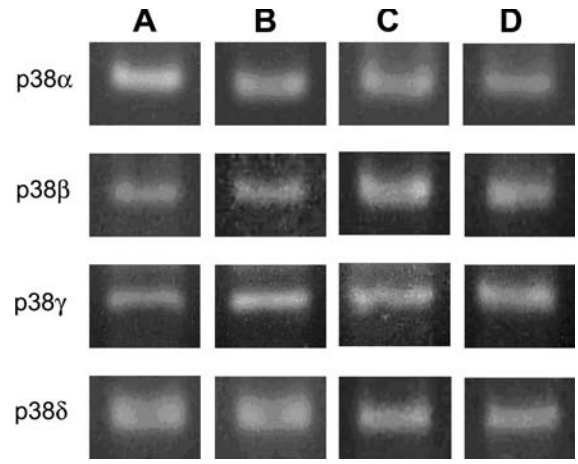

Fig. 3 PCR analysis of expression of genes coding for *p38 isoforms* by control (**a**), Cd-treated (**b**), SB203580-treated (**c**) or co-treated MDA-MB231 cells (**d**). Total RNA was extracted from monolayers of cells grown for 96 h, reverse transcribed and amplified in the presence of the specific primers. The amplification products were analyzed by 2% agarose electrophoresis and ethidium bromide stain

experiments, and shows that in non-quantitative conventional PCR assays a positive signal was found for all the isoforms tested, indicating that such gene expressions are switched-on in all the experimental conditions under study.

The cDNA preparations were then submitted to triplicate SM-PCR for semi-quantitative evaluation of the expression levels of p38/MAPKs. As shown in Fig. 4, we found that incubation of MDA-MB231 cells with $CdCl_2$ induced the up-regulation of *p38γ* (approx. +7-folds), and the down-regulation of *p38α* and *β* (approx. −8 and −4-folds, respectively) with respect to the control; the treatment with SB203580, on the other hand, was active in decreasing the expression levels of *p38γ* (approx. −2.5-folds) and, more prominently, *p38β* (approx. −16-folds). Co-treatment reduced the level of down-regulation of *p38α* and -*β* down to approx. −2- and −3.5-folds vs. the control, respectively, being the only condition capable to exert an effect, in particular up-regulatory, on *p38δ* gene expression (approx. +3-folds).

Accumulation of p38*β* and *γ* protein products in MDA-MB231 cells is differently regulated by the diverse treatments

In light of the observed changes induced on p38/MAPK isoform at a transcriptional levels, in a following set of experiments we examined whether Cd-, SB203580- and co-treatment-induced any change in p38/MAPK isoforms at protein accumulation levels in tumor cells by Western blot analysis with the specific antibodies. As shown in the panels in Fig. 5, in opposition to what we found for the corresponding mRNA, the amount of p38*β* protein product increased after exposure to Cd and, more prominently, SB203580 (approx. +1.6- and +2.2-folds, respectively); on the other hand, after co-treatment no significant difference to the control was found. Again in contrast to mRNA data, incubation with Cd resulted also in a slight but reproducible decrease of the accumulation of the protein product of p38*γ* isoform (approx. −0.4-folds). No treatment, at least under the conditions used in our study, was able to induce any change in the accumulation of the p38α and *δ* protein

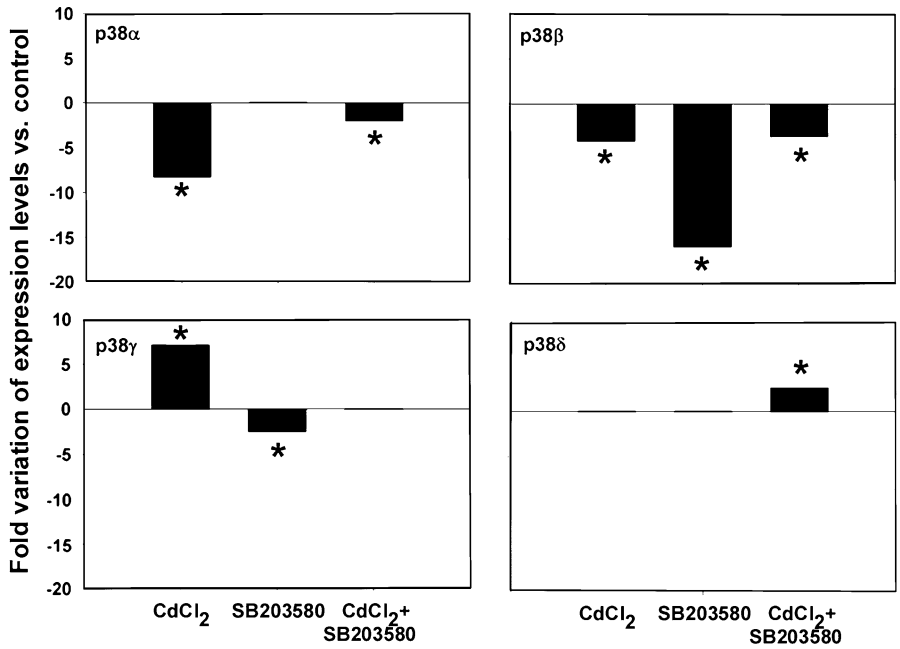

Fig. 4 Histograms showing changes of the expression levels of *p38 isoform* genes in MDA-MB231 submitted to the different treatments, as resulted from SM-PCR assays. Total RNA was extracted from monolayers of cells grown for 96 h, reverse transcribed, and the cDNAs of interest were co-amplified with cDNA from 18S rRNA over a range of cycles followed by 2% agarose electrophoresis and ethidium bromide stain. The intensities of the bands of interest, evaluated with SigmaScan software, were normalized for those of 18S and plotted as a function of cycle number, and exponential regression equations fitted to the curves, and were used to calculate the number of cycles necessary to reach a normalized intensity threshold value of 1 for each sample. The relative difference in abundance between two samples was taken as 2^n where n is the difference between the numbers of cycles required by the samples to reach the threshold. Data are presented as mean of triplicate experiments with no significant variation between each assay. * $P < 0.05$

product, although the co-treatment (for both isoforms) and, more prominently, the exposure to Cd (for α isoform) were previously found to exert a restraining effect at gene expression level.

Discussion

It is now widely accepted that Cd intervenes in the biological behaviour of numerous cytotypes by stimulating the stress response and/or the switching-on of apoptotic mechanisms, and also being a powerful promoter of neoplastic transformation. The complexity and diversity of the molecular events underlying cell-metal interactions are responsible for the data fragmentation and poor knowledge of the intracellular pathways involved in each experimental situation; nevertheless, significant indications are surely emerging from the evaluation of gene expression and protein accumulation patterns in the different cell model systems exposed to Cd. Concerning breast cancer cells, it is acknowledged that in estrogen-receptor positive cells, such as MCF-7, the metal acts as an estrogen-mimetic compound, thereby promoting cell proliferation and inducing the expression of hormone-dependent genes (e.g., Garcia-Morales et al. 1994). On the other hand, we have already demonstrated that exposure of p53- and estrogen-receptor-negative MDA-MB231 tumor cells to $CdCl_2$ also causes a number of biological consequences, such as drastic changes in the expression level of several genes and in some mitochondrial activities including mitochondrial protein import (Sirchia et al. 2006, 2008; Luparello et al. 2007; Cannino et al. 2008).

To extend our knowledge on the effects of Cd-breast tumor cell interactions, here we have submitted MDA-MB231 cells to exposure to $CdCl_2$ at a concentration corresponding to the IC_{50} at 96 h with and without the addition of a p38α/β inhibitor and analysed the activation rate via quantitation of the

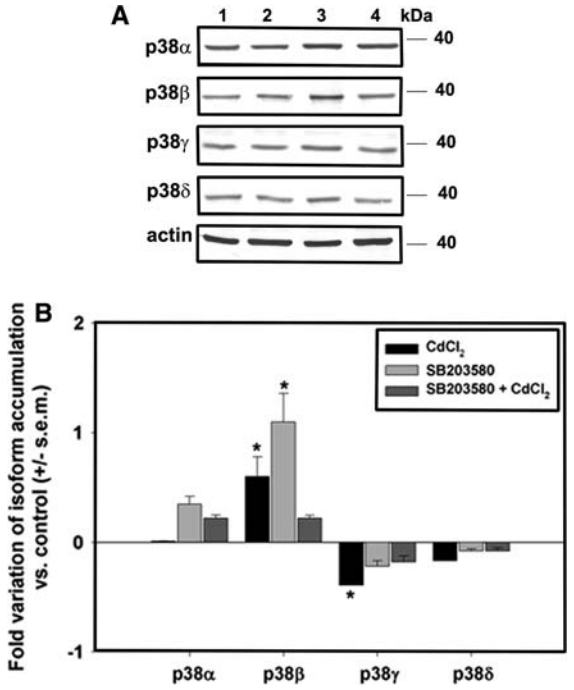

Fig. 5 **a** Representative Western immunodetection of the different p38 isoforms in lysates of control (*1*), Cd-treated (*2*), SB203580-treated (*3*) and co-treated MDA-MB231 cells (*4*). Immunoreaction with anti-actin antibody was performed to check protein loading. **b** Histogram showing the changes of the accumulation levels of the different p38 isoforms in MDA-MB231 submitted to the different treatments, as a result of immunoblot assays. Cell lysates were submitted to 10% acrylamide SDS–PAGE, blotted onto nitrocellulose membrane and the bands visualized by immunoreaction with the primary and secondary antibodies and staining with BCIP/NBT. Data in the histogram are presented as mean ± s.e.m. of triplicate experiments ($n = 3$). * $P < 0.05$

phosphorylated form of p38/MAPK as well as the expression levels of the four p38 isoforms and the accumulation of the related protein products. The data obtained indicates that only incubations with SB203580 or SB203580/$CdCl_2$ are able to augment the extent of activation of the intracellular pool of p38/MAPK, indicating that the absence of active α and/or β isoforms is a positive signal to increase the extent of phosphorylation of the isoforms. On the other hand, all the treatments performed in the present study affected the expression/accumulation of the isoforms, although in diversified ways; moreover, in some cases the regulation at gene expression and protein production levels appeared opposite, thereby suggesting the occurrence of control feedbacks which still have to be determined.

Further studies will be required to assess which isoforms are phosphorylated in response to each specific treatment and to define a precise model for the specific molecular mechanisms involving p38/MAPKs through which Cd accomplishes its cellular effects; however, the data we present allows the following comments.

The incubation with only SB203580 was found to induce an increase of dehydrogenase activity and the down-regulation of one of the two inactivated p38 isoforms, i.e., p38β, which, on the other hand underwent higher accumulation at protein level, whereas no effect was exerted on the other inhibited isoform, i.e., p38α. This suggests the existence of a positive transcriptional regulatory mechanism requiring the presence of active α and/or β isoform and adds a new example to the list of the effects which have been reported as a consequence to p38α/β inactivation by SB203580 treatment. The inhibitor, in fact, was already proven to stabilize cyclooxygenase-2 mRNA, up-regulate endoplasmic reticulum protein 29 (*ERp29*) and *VEGF*, down-regulate urokinase, and decrease both basal and TGFβ1-triggered metalloprotease-9 activity by this cell line, and to negatively affect its in vivo invasiveness in a mouse model of bone metastasis (Jang et al. 2000; Suarez-Cuervo et al. 2004; Liu and Mueller 2006; Bambang et al. 2009).

Interestingly, Cd treatment was active in modifying the expression levels of *p38*α, β and γ whilst only for the last isoform a variation in the extent of protein accumulation was recorded. To our knowledge, this is the first reported evidence that Cd affects the levels of transcription and of protein synthesis in some *p38* isoforms. It is known that expression of *p38*γ in MDA-MB231 cells is switched-on by Ras, and that p38γ protein is one of the effectors of Ras which is known to play a critical role relating to breast cancer malignancy, i.e., stimulating Matrigel cell invasion by the same cell line (Qi et al. 2006). Preliminary results obtained by some of us (Luparello et al., in press) have demonstrated that Cd-treated MDA-MB231 cells display a massive decrease of their invasive potential in Matrigel; thus, a hypothesis which requires further validation could be that Cd realizes the observed anti-invasive role via p38γ depletion. On the other hand, in light of the massive changes in gene expression levels and mitochondrial activities triggered in these cells by the concentration of the metal and the duration of the incubation chosen

(Sirchia et al. 2006, 2008; Luparello et al. 2007; Cannino et al. 2008), an involvement, at least partial, in the under-production of p38γ in such cellular events, although still unproven, cannot be excluded.

When the SB203580/$CdCl_2$ co-treatment was performed, the presence of the inhibitor was capable of reversing the anti-proliferative effect of the metal on MDA-MB231 cells, also determining an increase of cell number with respect to untreated controls. This is a clear indication that Cd-triggered inhibitory effects on cell growth, and conceivably other intracellular changes related to Cd toxicity, require that p38α and/or β are in the activated state and capable to interact, in turn, with their downstream targets. Interestingly, in line with our results, SB203580 was demonstrated to function as a pro-survival factor counteracting the lethal effect exerted by both aplidin and asiatic acid on MDA-MB231 cells (Cuadrado et al. 2003; Hsu et al. 2005). Concerning p38 isoforms, in the case of co-treatment the amount of p38β returned to control levels and this was coupled to the transcriptional switching-on of *p38*δ gene. Noticeably, MDA-MB468 and MCF-7 breast cancer cells p38β and -δ were shown to, respectively, promote and inhibit AP-1 transcription factor, whose activity is linked to the regulation of many target genes implicated in cell proliferation and cell cycle progression (Pramanik et al. 2003). On the basis that the net response in cells expressing all p38 isoforms is ruled by integrations of the positive (p38β) and the negative (p38δ) AP-1 regulatory signalling, co-treatment could therefore interfere with the latter one via unbalancing this ratio towards activation of AP-1-dependent pathways.

In conclusion, we have presented an analysis in the gene and protein expression levels of the effect of Cd exposure on p38/MAPKs in a human breast cancer cell line, with the presence of, or not, inactivation of α and β isoforms. Although much remains to be determined at isoform transcriptional and activation levels, the diverse responses to the different treatments reported here strongly suggest that the various isoforms of p38 are subjected to complex molecular mechanisms of gene regulation and validate that the MDA-MB231 cell line is a suitable in vitro model for further and more detailed studies on the intracellular mechanisms underlying the control of p38 expression, synthesis and activation in mammary tumor cells exposed to different stresses.

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