Biometals (2009) 22:753–769
DOI 10.1007/s10534-009-9223-6

Resistance to cadmium as a function of Caco-2 cell differentiation: role of reactive oxygen species in cadmium- but not zinc-induced adaptation mechanisms

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Received: 26 December 2008 / Accepted: 3 March 2009 / Published online: 18 March 2009

Abstract Cadmium (Cd) is a highly toxic metal that enters the food chain. Following oral ingestion, the intestinal epithelium is the first biological barrier crossed by Cd and is also an important target tissue. In the present study, the human intestinal Caco-2 cell line was used to evaluate the impact of a low level of exposure on both undifferentiated and differentiated intestinal cells. As revealed by the LC_{50} values estimated with the 3-[4,5-dimethyl-2-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay, mature Caco-2 cells were more resistant to Cd. However, following a 24-h exposure to non-cytotoxic levels of Cd (10 μM) or zinc (Zn, 100 μM), threefold increases were obtained in the LC_{50} values of 7-day-old cells, whereas increased resistance in 21-day-old cells was observed exclusively with Zn. Induction of MT-IIa and HSP70 mRNAs was higher in undifferentiated cells and an increase in cellular glutathione (GSH) content was observed exclusively in these cell cultures. However, the results obtained with cycloheximide used for inhibiting protein synthesis and with L-buthionine sulfoximine (BSO), which inhibits GSH synthesis, revealed that protein synthesis is not a prerequisite to the development of resistance. The presence of 100 mM 3-amino-1,2,4-triazole (3AT), a catalase inhibitor, prevented Cd-induced but not Zn-induced resistance, as well as sensitized cells to Cd toxicity. These results show for the first time differences in constitutive and acquired resistance to Cd as a function of enterocytic differentiation status and suggest the involvement of different mechanisms for Cd- and Zn-induced adaptation in the intestinal cells. Redox signals may trigger Cd-induced adaptation mechanisms but pro-oxidant conditions would eliminate proliferative intestinal cells capability to develop resistance. This would be critical for Cd- but not Zn-induced mechanisms of resistance since Cd but not Zn may cause oxidative stress.

Keywords Cadmium · Zinc · MT · HSP70 · GSH · Oxidative stress · Tolerance · Intestinal differentiation · Caco-2 cells

Abbreviations

3AT	3-Amino-1,2,4-triazole
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea
BSO	L-Buthionine sulfoximine
CAT	Catalase
CHX	Cycloheximide
DMEM	Dulbecco's modified eagle essential minimum medium
FBS	Fetal Bovine Serum
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
GSSG	Oxidized glutathione
LC_{50}	Lethal concentration 50

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MTT 3-[4,5-Dimethyl-2-thiazol-2-yl]-2,5-diphenyltetrazolium bromide

Introduction

Cadmium (Cd) is a widespread highly toxic metal classified as a type I carcinogen by the International Agency for Research on Cancer (IARC 1993). It is released during the burning of fossil fuels and has many industrial uses (Ayres 1992). Following absorption, Cd accumulates mostly in the kidney and liver, while the kidney is the main target organ (Friberg 1984; Nordberg 1984; Lauwerys et al. 1994). Its biological half-life in humans has been estimated at around 20 years. Plants and vegetables may absorb significant levels of Cd from contaminated soils following atmospheric deposition or phosphate fertilizer use (WHO 1992). Thus, Cd may enter the food chain and food is the main exposure source for non-smokers. The intestinal epithelium is the first protective barrier against Cd toxicity following metal ingestion, and therefore it has mainly been studied in relation to Cd absorption (Cherian 1983; Andersen et al. 1994; Jumarie et al. 1999; Suzuki et al. 2008). However, the intestinal epithelium also represents an important target tissue following oral exposure, but much less information is available about the intestinal toxicity of Cd, especially at low concentrations.

Damage to intestinal epithelium integrity, including increased paracellular permeability, altered cell viability and inflammatory response, have been reported following exposure to Cd (Duizer et al. 1999; Boveri et al. 2004; Berzina et al. 2007). Cd also favors the production of oxygen species leading to oxidative stress (Wang et al. 2004), and induces stress proteins such as MT-IIa, a metal-binding protein, and the heat shock protein HSP70 (Reeves and Rossow 1996; Kiang and Tsokos 1998; Blais et al. 1999). The formation of reactive oxygen species is thought to be mediated mainly by superoxide production in the mitochondria following Cd-induced inhibition of the electron transfer chain (Wang et al. 2004). Other suggested mechanisms include binding to the sulfhydryl groups of specific proteins and the release of iron (Fe) from its binding sites, with the subsequent involvement of Fe in Fenton reactions (Casalino et al. 1997; Souza et al. 2004). These effects could impair the intestinal epithelium's protective role against adverse agents as well as nutrient absorption. In the context of chronic exposure to low levels of Cd, intestinal adaptation mechanisms are critical. Various studies have shown that under these conditions, cells may develop resistance to Cd (Coyle et al. 2000; Urani et al. 2005; Lau et al. 2006), but this phenomenon, as well as the protective effect of zinc (Zn) against Cd toxicity (Reeves and Rossow 1996; Tang et al. 1998) and the exact role of MT-IIa and HSP70 remain to be investigated in intestinal cells. Moreover, to our best knowledge, the intestinal toxic effects of Cd have never been studied as a function of enterocytic differentiation status. The intestinal epithelium has the fastest renewal rate of 4–5 days. Proliferative stem cells located at the bottom of the intestinal crypts give rise to four different cell types: absorptive cells called enterocytes, goblet cells, entero-endocrine cells, and Paneth cells (Traber 1994). Both proliferation and differentiation processes are critical for the adequate renewal and maturation of the intestinal epithelium. As has been shown for Fe (Zöld et al. 2004), undifferentiated and differentiated intestinal cells may not be similarly sensitive to Cd's toxic effects.

The aim of the present study was to investigate Cd toxicity and to obtain insight into the intestinal cells' capability to develop some resistance to Cd as a function of enterocytic differentiation status using the in vitro human intestinal Caco-2 cell line. These cells are unique because they undergo spontaneous enterocytic differentiation once culture dish confluency has been reached. They form a polarized monolayer with tight junctions and express numerous morphological and biochemical features of mature absorptive cells including brush border membrane, hydrolases activities and hexose transporters including GLUT-5 and the Na^+-glucose cotransporter SGLT1 (Pinto et al. 1983; Rousset et al. 1985; Mahraoui et al. 1992; Bissonnette et al. 1996). This cell line has been largely used to study intestinal functions and has been proved to be a relevant in vitro model for pharmacological applications (Meunier et al. 1995). The growth-related differentiation of the Caco-2 cells has been well characterized (Jumarie and Malo 1991; Vachon and Beaulieu 1992). An exponential growth phase is observed during the first week of culture and confluency is generally reached 6-8 days post-seeding. Then, a stationary growth phase is observed with the establishment of tight junctions between adjacent

cells, the development of microvilli at the apical membrane and the expression of a number of hydrolases including sucrase–isomaltase, lactase, aminopeptidase N, dipeptidyl dipeptidase IV and alkaline phosphatase as well as glucose transporters (Jumarie and Malo 1991; Mahraoui et al. 1992; Vachon and Beaulieu 1992). In addition, a dramatic increase in the transepithelial electrical resistance (TEER) has been correlated with the establishment of tight junctions and cell polarity once cell monolayers grown on filters reach confluency (Grasset et al. 1984; Jumarie et al. 1999). The Caco-2 cell monolayers can therefore be used to study both proliferative and differentiated intestinal cells. Our results reveal that differentiated cells are much more resistant to Cd than undifferentiated cells. However, a 24-h pre-exposure to non-cytotoxic levels of Cd or Zn induced resistance to a subsequent exposure to Cd in proliferative cells, whereas acquired resistance was observed in differentiated cells only following exclusive pre-exposure to Zn. We tested the hypothesis that Cd- and Zn-induced protection involves different mechanisms by investigating the role of reactive oxygen species and also studied to what extent upregulation of MT-IIa and HSP70 expression may be responsible for the acquired resistance.

Materials and methods

Cell culture

Caco-2 cells were graciously supplied by Dr. A. Zweibaum (Grasset et al. 1984) and were used between passages 201 and 215. The cells were maintained at 37°C in a 5% CO_2 humidified atmosphere in Dulbecco's modified eagle essential minimum medium (DMEM) (GibcoBRL, Grand Island, NY, USA) supplemented with 15% inactivated fetal bovine serum (FBS) (PAA Inc., Montreal, QC, Canada), 0.1 mM non-essential amino acids, 19 mM $NaHCO_3$, and 50,000 U/L-50 mg l^{-1} penicillin–streptomycin (GibcoBRL), pH 7.3. Cultures were passaged by trypsinization (0.05% trypsin–0.53 mM EDTA) every week. Stock cultures were seeded in 75 cm^2 flasks (Corning Inc., Corning, NY, USA) at 2.6×10^4 cells cm^{-2}, while experiments were conducted on cells seeded at a density of 1.3×10^4 cells cm^{-2} in 10-cm and 35-mm diameter culture dishes (Corning Inc.) and 96-well plates (Sarstedt, Nümbrecht, Germany). The culture medium was changed every 2 days and the cells were maintained for 7 and 21 days in order to study early confluent but undifferentiated cell cultures and differentiated cells, respectively, as previously demonstrated (Jumarie and Malo 1991; Jumarie et al. 1999).

MTT activity measurement

The MTT (3-[4,5-dimethyl-2-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma Chemical Co., St. Louis, MO, USA) assay is a colorimetric measurement of MTT reduction to a blue formazan product by mitochondrial dehydrogenases of viable cells (Carmichael et al. 1987). Cells grown for 6 or 20 days on 96-well plates were rinsed twice with FBS-free DMEM prior to incubation for 24 h (referred to as pre-exposure or pretreatment) in the absence or presence of 10 μM Cd, 20 μM Cd, or 100 μM Zn (prepared from 100 to 1,000× sterile stock solutions in ultra pure water) with or without: 1 μM cycloheximide (CHX) (Sigma), 3 mM L-buthionine sulfoximine (BSO) (Sigma), 30 μM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Sigma), 30 μM diamide (Sigma) or 100 mM 3-amino-1,2,4-triazole (3AT) (Sigma). CHX is a protein synthesis inhibitor. BSO, BCNU and 3AT inhibit γ-glutamyl cysteine synthase, glutathione reductase and catalase activities, respectively, whereas diamide oxidizes thiols such as GSH. The concentrations of inhibitors were chosen for their optimal effect, as revealed by concentration-response curves (data not shown). Note that 100 mM 3AT is in the range of concentrations generally used in studies investigating toxicity mechanisms of Cd (Chao and Yang 2001a; Chuang et al. 2003).

After pre-exposure, cells were incubated in FBS-free medium containing increasing concentrations (0–500 μM) of Cd or Zn during another subsequent 24 h (referred to as exposure). Then, MTT was added to each well at a final concentration of 0.5 mg ml^{-1} (1.2 mM). Cells were incubated for 3 h and the medium was changed to DMSO to dissolve the formazan. Optical density was measured at 575 nm using a Tecan SpectraFluor Plus microplate spectrophotometer (Esbe Scientific Industries Inc., Canada). For kinetic studies of resistance induction, the duration of cell pre-exposure to 10 μM Cd was 6, 12, 24 and 48 h, whereas all subsequent exposures to

increasing concentrations of Cd started at the same time, on 7-day-old cell cultures. MTT data are expressed relative to their respective controls measured in pre-exposed cells (for each pretreatment condition) following a subsequent 24-h incubation in FBS-free DMEM alone.

PI exclusion assay and FITC annexin V fluorescence

Six-day-old cells grown on 35-mm diameter culture dishes were maintained for 24 h in FBS-free DMEM with Cd concentrations ranging from 0 to 100 μM. Cells were harvested by trypsinization, collected by centrifugation (200×*g*) and washed three times with PBS prior to fixation with 75% ethanol for 1 h at 4°C (10^6 cells ml^{-1}). Cells were then rinsed twice with PBS and stained with 1 ml propidium iodide (PI, Sigma) (50 μg ml^{-1} with 3.8 mM sodium citrate in PBS) with 50 μl RNaseA (10 μg ml^{-1}) (Worthington Biochemicals, Lake Wood, NJ, USA). In parallel experiments, double staining with PI and fluorescein isothiocyanate-labeled annexin V (FITC annexin V, BD Biosciences, San Jose, CA, USA) was used to discriminate between viable cells (annexin−/PI−), early apoptotic cells (annexin+/PI−), and late apoptotic or dead cells (annexin+/PI+) using a FACScanTM flow cytometer (Becton–Dickinson, Franklin Lake, NJ, USA). For both PI and FITC annexin V, excitation was at 488 nm, whereas emission was read at 530 and 585 nm for FITC annexin V and PI, respectively.

[Methyl-^{3}H] thymidine incorporation assay

Seven-day-old cells grown on 35-mm diameter culture dishes were incubated with 2 ml FBS-free DMEM containing 2 μCi ml^{-1} [methyl-^{3}H] thymidine (specific activity 0.025 μCi $pmol^{-1}$, Amersham, Braunschweig, Germany) in the absence or presence of 10 μM Cd for 24 h at 37°C. Then, the cells were rinsed four times with ice-cold phosphate buffer saline (PBS) and were incubated for 15 min with 1 ml of ice-cold 10% trichloroacetic acid (TCA). Cells were harvested with a rubber policeman and collected by centrifugation at 6,500×*g* for 30 s. The pellets were washed two times with PBS at room temperature, the cells were dissolved in 500 μl 1 N NaOH, and the radioactivity was estimated using a TRI-Carb 2800 TR scintillation counter (Perkin–Elmer, Canada). Total cellular protein was measured according to Bradford (1976) using bovine serum albumin as the calibration standard, and the incorporation levels are expressed as pmol [^{3}H]-thymidine per mg of protein.

^{109}Cd uptake measurements

Six- and 20-day-old cells grown on 35-mm diameter culture dishes were maintained for 24 h in FBS-free DMEM in the absence or presence of 10 μM Cd or 100 μM Zn and were then exposed to 0.5 μM ^{109}Cd (specific activity of 0.3 μCi $nmol^{-1}$, Eckert & Ziegler, Berlin, Germany) in the absence of unlabeled metals for 24 h at 37°C. Then, the cells were rinsed four times with 2 ml of ice-cold stop solution containing 2 mM EDTA as described by Bergeron and Jumarie (2006). The cells were dissolved in 500 ml 1 N NaOH (500 μl) and the radioactivity was estimated using a Cobra II gamma counter (Canberra Packard, Canada). Accumulation levels are expressed as pmol ^{109}Cd per mg of protein.

RT-PCR analyses

The cells were cultured on 10-cm dishes for 6 or 20 days. The cells were then rinsed twice with FBS-free DMEM and incubated for 24 h in the absence or presence of 10 μM Cd or 100 μM Zn. Total RNA was extracted using Trizol® Reagent (Invitrogen Life Technologies, Burlington, ON, Canada) according to the supplier's instructions. The RNA pellets were dissolved in 50 μl water that was treated with 0.1% diethylpyrocarbonate and autoclaved. The purity and concentration of the RNA samples were assessed as described by Bergeron and Jumarie (2006). Reverse transcription was performed using 2 μg RNA in a 20 μl total volume, 1 μM random hexamere pd(N)6 (Amersham Biosciences, UK) and the Omniscript® RT Kit (Qiagen, Mississauga, ON, Canada) according to the supplier's instructions. PCR was conducted with the Taq PCR Core Kit (Qiagen). The sense and antisense primer sequences for *β*-actin were 5′-AAG ATGACCCAGGTGAGTGG and 5′-CAGAGGCGTAC AGGGATAGC (GenBank M10277, bases 1,576–1,901), 5′-GGATCCCAACTGCTCCTG and 5′-CAGCAGCTG CACTTGTCC for MT-IIa (GenBank BT007315, bases 3–179) and 5′-GGCATCGACCTGGGCACCAC and 5′-TCCTTGGTGGCCTGGCGCTG for HSP70 (GenBank

NM005345, bases 219–676). For β-actin and MT-IIa, 40 cycles were used consisting of: denaturation at 94°C for 60 s, annealing at 58°C for 30 s and extension at 72°C for 60 s. For HSP70, 30 cycles were used consisting of: denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 60 s. The resulting PCR products were separated on 2% (w/v) agarose gel with ethidium bromide and visualized under UV trans-illumination using an LKB 2011 Macrovue Fluorescence system controlled by Alphamager™ 2200 software (Alpha Innotech Corporation, San Leandro, CA, USA). MT-IIa and HSP70 mRNAs levels were both normalized to that of β-actin.

Enzymatic activity measurements

Catalase (CAT) and glutathione peroxidase (GPx) activities were estimated on 7-day-old undifferentiated control cells according to the methods used by Dorval and Hontela (2003). CAT activity was measured by the decrease in sample absorbance at 240 nm ($\varepsilon = 0.04$ $mM^{-1}cm^{-1}$) resulting from H_2O_2 decomposition, and GPx activity was estimated by the decrease in absorbance at 340 nm ($\varepsilon = 6.22$ $mM^{-1}cm^{-1}$) resulting from NADPH oxidation. CAT activity was also estimated on cells pre-exposed for 24 h (from day 6 to day 7 in culture) to 0, 1, 5, 10, 15 or 20 µM Cd in FBS-free DMEM. Measurements were conducted in 3 ml PBS buffer containing 13.4 mM H_2O_2 and 150 µl of cellular lysate. GPx activity was measured in 1.7 ml 50 mM Tris–phosphate buffer (pH 7.6) containing 0.1 mM EDTA, 2.5 mM GSH, 1.7 units glutathione reductase, 0.12 mM NADPH, 2 mM cumene hydroperoxide, and 100 µl cellular lysate. Absorbance was measured using a Beckman DU®650 spectrophotometer (Beckman Coulter, Canada).

Thiol content measurements

Six- and 20-day-old cells grown on 35-mm diameter culture dishes were rinsed twice with FBS-free DMEM and then maintained for 24 h in the absence or presence of 10 µM Cd. Cellular thiol content was determined using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) according to the method of Anderson (1985). Briefly, the cells were washed and harvested in 10 mM Hepes–phosphate buffer (pH 7.3). One ml of cellular lysate was incubated with 4 ml DTNB 0.5 mM for 5 min at room temperature. Absorbance was measured at 410 nm, and total cellular thiol content was estimated using GSH as the calibration standard. Thiol content is expressed relative to that of protein.

Statistical and data analyses

Unless otherwise specified, results are mean ± SD estimated on three independent cell cultures, each time in three to five replicates. Cellular viability as a function of increasing concentrations of Cd was analyzed according to the following concentration-response equation:

$$y = y_{\min} + \frac{y_{\max} - y_{\min}}{1 + 10^{(\log LC_{50} - X) \times \text{Hillslope}}}$$

where $Y_{\max}$ and $Y_{\min}$ are the maximal and minimal ratio of cell viability, respectively, and LC_{50} is the concentration of Cd for which a cell viability ratio of 0.5 is observed. The errors associated with the LC_{50} values given in the text represent the standard errors of regression (SER).

Statistical analyses were performed by one-way ANOVA with Tukey–Kramer multiple comparison tests. Stress protein levels were compared using the unpaired Student's *t*-test. Nonlinear regression and statistical analysis were performed using Prism and Instat 4 software (GraphPad Software, San Diego, CA, USA). Statistical significance was assessed at $P \leq 0.05$.

Results

Metal-induced resistance as a function of days in culture

Following a 24-h exposure to increasing concentrations of Cd added to the FBS-free medium, a typical sigmoid concentration-response curve was observed for both 7- and 21-day-old cell cultures (Fig. 1a ,b). Flow cytometry analyses revealed that necrosis was responsible for cellular death without any significant Cd-induced apoptosis (data not shown). It is noteworthy that LC_{50} (Cd concentration for which cell viability is half the control value) was almost threefold higher in 21-day-old control cells compared

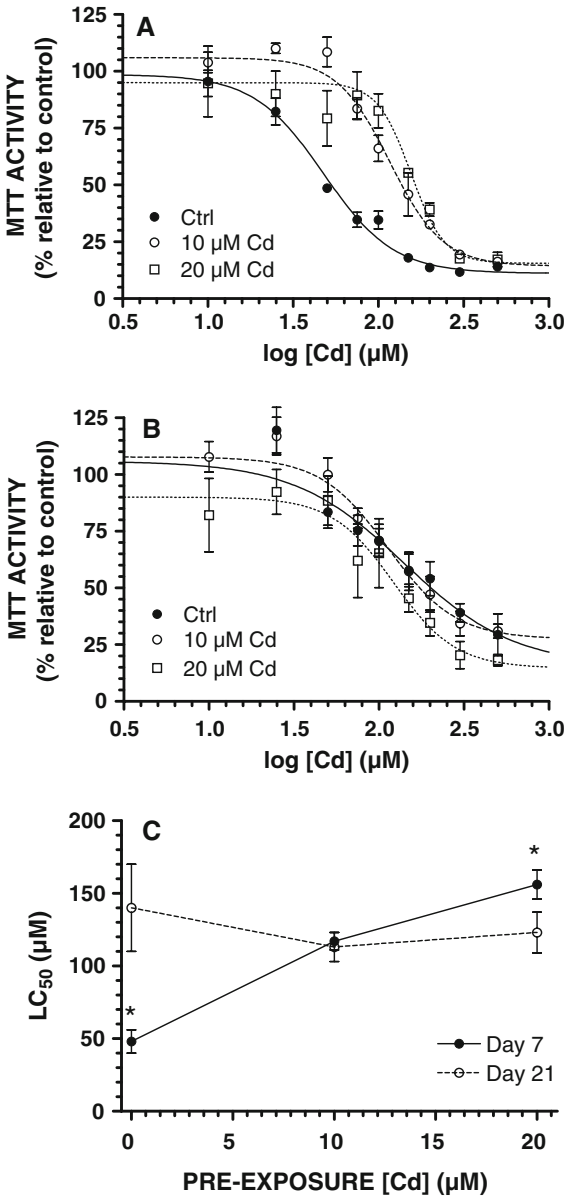

Fig. 1 Effect of pre-exposure to 10 or 20 µM Cd on MTT activity concentration-response curve as a function of increasing concentrations of Cd for **a** undifferentiated 7-day-old cells, **b** differentiated 21-day-old cells. **c** LC_{50} values as a function of increasing pre-exposure concentrations of Cd. Cells were cultured in the presence of FBS for 6 or 20 days, and were then pre-exposed to 10 or 20 µM Cd for 24 h before a subsequent 24-h exposure to increasing concentrations of Cd in the culture medium. Pre-exposure and exposure were both performed in the absence of FBS, and *Ctrl* refers to FBS-free conditions exclusively. Data shown are means ± SD estimated on three independent cell cultures. * Significant differences compared to the corresponding conditions in 21-day-old cells

to 7-day-old control cells ($LC_{50} = 140 \pm 30$ vs. 48 ± 8 µM), showing that differentiated cultures are much more tolerant than undifferentiated cells. However, following a 24-h pre-exposure to 10 µM Cd, which led to a maximal 5% cell mortality, 7-day-old cells became resistant to Cd similar to 21-day-old cells ($LC_{50} = 117 \pm 6$ vs. 140 ± 30 µM), whereas the resistance of differentiated cells remained the same. Increasing the Cd concentration to 20 µM slightly enhanced the acquired resistance to Cd in 7-day-old cells but led to significantly higher cell mortality. Thus, 10 µM was the Cd concentration chosen for the subsequent experiments (Fig. 1c). Cell viability recorded by PI exclusion and flow cytometry analyses gave similar results compared to MTT measurements: a near twofold increase was obtained in the LC_{50} value estimated for 7-day-old cells following a 24-h pre-exposure to 10 µM Cd (Fig. 2). Therefore, MTT activity measurement is adequate for estimating cell viability under our experimental conditions. Kinetic studies revealed that optimal induction of resistance occurs between 12 and 24 h (Fig. 3). Consequently, 24 h was chosen for cell pre-exposure in subsequent experiments. Note that a

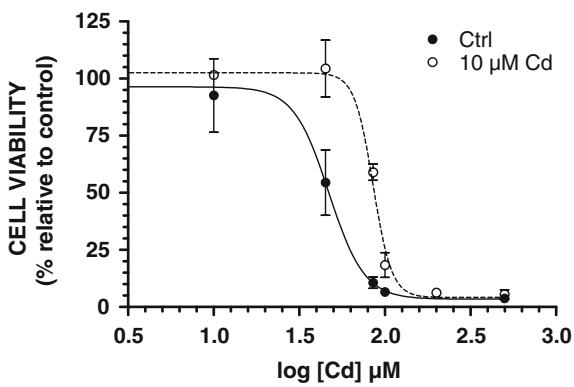

Fig. 2 Effect of pre-exposure to Cd on cell viability concentration-response curve as a function of increasing concentrations of Cd for undifferentiated 7-day-old cell cultures. Cell viability was measured by PI exclusion assay and FITC annexin V fluorescence as described in "Materials and methods". Cells were cultured in the presence of FBS for 6 days, and were then pre-exposed to 10 µM Cd for 24 h before a subsequent 24-h exposure to increasing concentrations of Cd in the culture medium. Pre-exposure and exposure were both performed in the absence of FBS, and *Ctrl* refers to FBS-free conditions exclusively. Data shown are means ± SD estimated on two independent cell cultures

significant decrease in LC_{50} was observed following a 6-h incubation with FBS-free culture medium, but this effect was no longer observed after 48 h.

It is well recognized that Zn may protect against Cd toxicity (Reeves and Rossow 1996; Tang et al. 1998), thus cell pre-exposure to Zn was also studied. From the concentration-response curve obtained in 7-day-old cells (Fig. 4a), 100 μM was the selected as the Zn concentration because of the maximal 5% loss in cell viability (as for 10 μM Cd). As shown in Fig. 4b, as was observed with 10 μM Cd, a 24-h pre-exposure to 100 μM Zn also enhanced cell resistance to Cd with a threefold increase in the LC_{50} values. However, contrary to Cd, cell pre-exposure to Zn still increased the resistance to Cd in 21-day-old cultures (twofold) (Fig. 4c). Note that the MTT activity of 21-day-old cells remained unaffected by Zn up to 500 μM (data not shown).

Cd effects on cell cycle progression

Some studies have reported that Cd may affect cell cycle progression (Chao and Yang 2001b; Yang et al. 2004a), and we therefore studied whether an acquired resistance to Cd may modify this effect. Cd increased

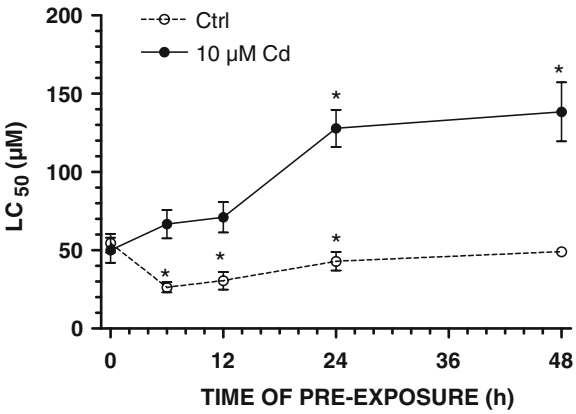

Fig. 3 Effect of time of pre-incubation with 10 μM Cd on LC_{50} values estimated with MTT activity in undifferentiated cells in response to a subsequent exposure to Cd. Cells were cultured in the presence of FBS for 6 days and were then incubated with 10 μM Cd for 6, 12, 24 or 48 h before a subsequent 24-h exposure to increasing concentrations of Cd in the culture medium. Pre-incubation and exposure were both performed in the absence of FBS. Data shown are mean ± SD estimated on four to five replicates of the same cell culture. * Significant differences compared to control value estimated following a total 48-h incubation in the absence of FBS

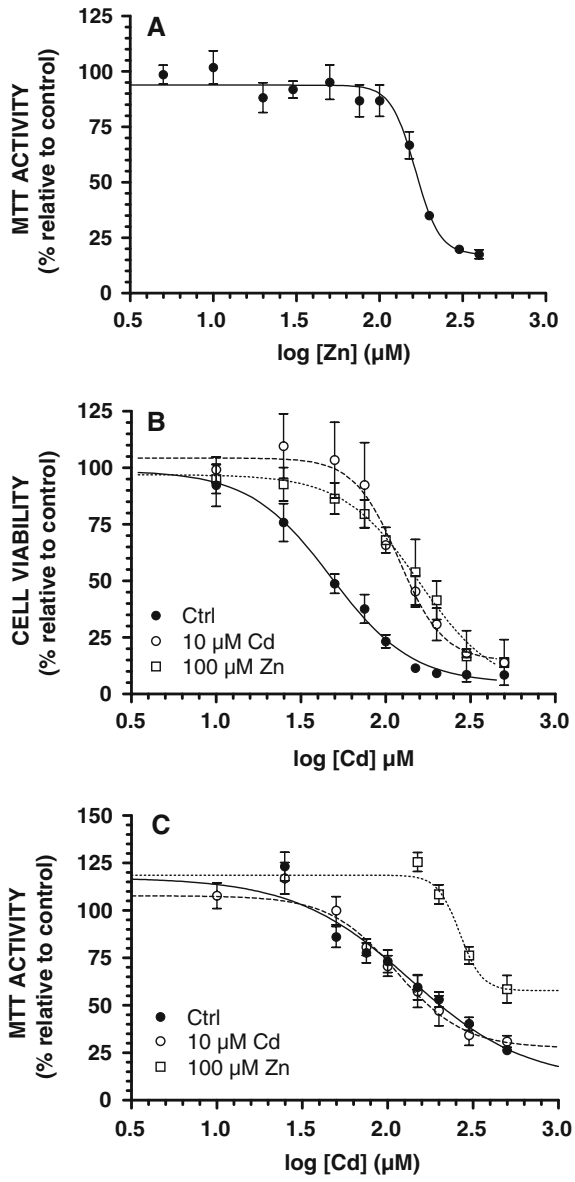

Fig. 4 **a** MTT activity concentration-response curve as a function of increasing concentrations of Zn for undifferentiated cells. Cells were cultured in the presence of FBS for 6 days and were then exposed for 24 h to increasing concentrations of Zn in the absence of FBS. Effect of a pre-exposure to Zn on MTT activity concentration-response curve as a function of increasing concentrations of Cd for, **b** 7-day-old, and **c** 21-day-old cell cultures. Cells were cultured in the presence of FBS for 6 or 20 days, and were then pre-exposed to 10 μM Cd or 100 μM Zn for 24 h before a subsequent 24-h exposure to increasing concentrations of Cd in the culture medium. Pre-exposure and exposure were both performed in the absence of FBS, and *Ctrl* refers to FBS-free conditions exclusively. Data shown are means ± SD estimated on two to three independent cell cultures

the proportion of the cell population in the S phase with a concomitant decrease in G2/M, in a concentration-dependent manner for exposure levels above 10 μM (Fig. 5, Ctrl in a, b). It is noteworthy that 10 μM Cd increased the cell distribution in G2/M, with this effect being more pronounced in cells pre-exposed to either 10 μM Cd or 100 μM Zn with a subsequent 24-h incubation in FBS-free medium. However, under these conditions, the cellular distribution pattern as a function of Cd concentration remained the same with a significant increase in the S phase and a concomitant decrease in G2/M at 80 μM Cd (Fig. 5b). Incorporation of [^{3}H]-thymidine has been estimated in order to better characterize the effect of cell incubation with 10 μM Cd. As shown in Fig. 5c, a 24-h incubation with 10 μM Cd in the FBS-free medium did not modify cellular uptake of [^{3}H]-thymidine.

Resistance and levels of cellular accumulation

Because metal toxicity is closely related to cellular accumulation, Cd uptake was measured under similar control or pre-exposure conditions. As shown in Fig. 6, pre-exposure to 10 μM Cd did not modify the 24-h cellular accumulation of ^{109}Cd, whereas pre-exposure to 100 μM Zn led to a significant 13% reduction in ^{109}Cd in both 7- and 21-day-old cells. Thus it is unlikely that Cd- or Zn-induced resistance leading to a 2.4- and 3-fold increase in cell viability, respectively, may be exclusively related to lower levels of cellular accumulation. Similarly, there is no correlation between variation in resistance and metal accumulation levels in 7- and 21-day-old control cells (a 12% higher uptake level was estimated for the latter).

Upregulation of MT-IIa and HSP70 mRNAs

In order to estimate to what extent MT and/or HSP synthesis may be responsible for the acquired resistance to Cd in 7-day-old cell cultures, MT-IIa and HSP70 expressions were studied by RT-PCR (Fig. 7a). Densitometry analysis of RT-PCR products showed that a 24-h exposure to 10 μM Cd or 100 μM Zn increased to a similar extent (2.5 to 3 times) the MT-IIa mRNA in Caco-2 cells with higher levels of induction in undifferentiated cells (Fig. 7b). An increase in HSP70 mRNA level was also observed in 7-day-old cells exposed to either Cd or Zn, whereas a change in differentiated cells was not obvious (Fig. 7c).

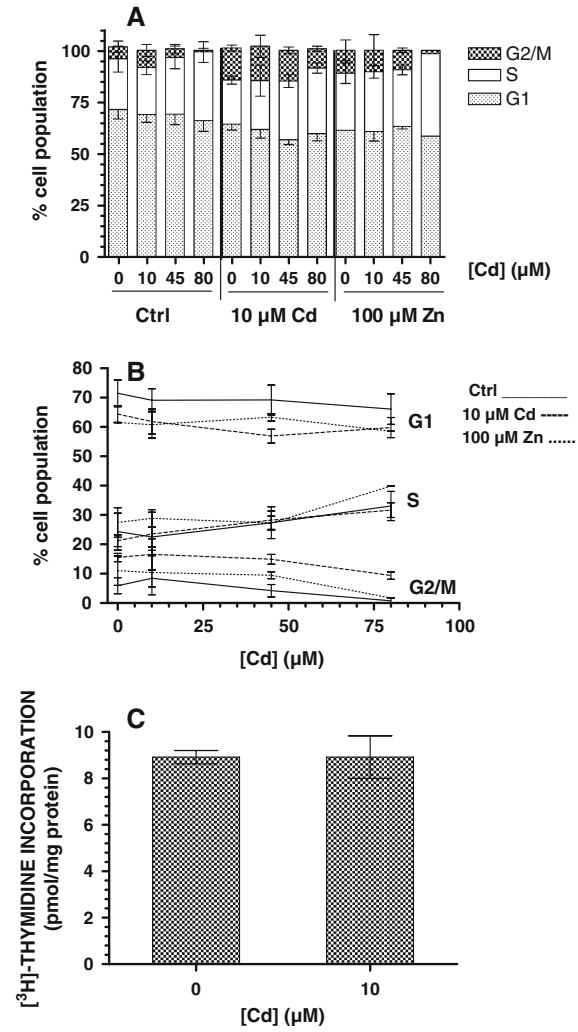

Fig. 5 **a**,**b** Effect of pre-exposure to Cd or Zn on cell cycle progression for undifferentiated cell cultures. Cell cycle was studied with PI fluorescence and FACS can analyses as described in "Materials and methods". Cells were cultured in the presence of FBS for 6 days, and were then pre-exposed to Ctrl FBS-free condition, 10 μM Cd, or 100 μM Zn for 24 h before a subsequent 24-h exposure to 0, 10, 45, 80 μM Cd in the culture medium. Pre-exposure and exposure were both performed in the absence of FBS. Data shown are means ± SD estimated on two independent cell cultures. **c** [^{3}H]-thymidine incorporation in undifferentiated cells. Cells were cultured in the presence of FBS for 6 days and were then incubated with 2 μCi ml^{-1} [methyl-^{3}H] in FBS-free DMEM in the presence or absence of 10 μM Cd. Data shown are means ± SD estimated on three replicates of the same cell cultures

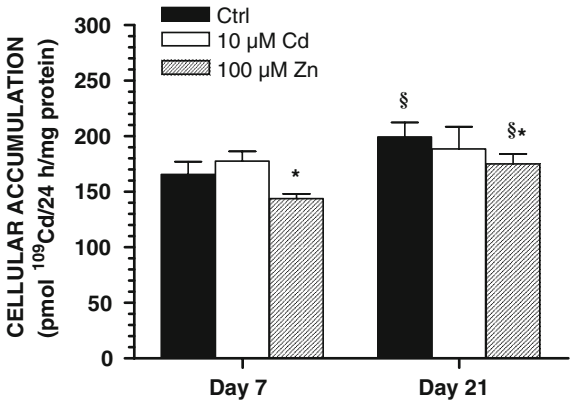

Fig. 6 Effect of pre-exposure to Cd or Zn on the cellular accumulation of ^{109}Cd in undifferentiated and differentiated cells. Cells were cultured in the presence of FBS for 6 or 20 days, were then exposed to 10 μM Cd or 100 μM Zn for 24 h before a subsequent 24-h incubation with 0.5 μM ^{109}Cd in the absence of unlabeled metals. Exposure and uptake were both performed in the absence of FBS, and *Ctrl* refers to FBS-free conditions exclusively. Values are means ± SD estimated on five replicates of the same cell culture, and data shown are representative of three independent cell cultures. *, § Significant differences compared to the respective control value and the corresponding conditions in 7-day-old cells, respectively

CHX, known to inhibit protein synthesis, was used to evaluate the role of newly synthesized proteins in adaptation mechanisms (Fig. 8). Pre-exposure to 1 μM CHX alone significantly sensitized proliferative cells to Cd toxicity (LC_{50} = 49.4 ± 5.1 μM vs. 26.8 ± 3.4 for control and CHX-pretreated cells, respectively), but did not prevent Cd- or Zn-induced resistance. For both control and CHX-pretreated cells, pre-exposure to 10 μM Cd or 100 μM Zn led to a similar twofold and threefold increase, respectively, in the LC_{50} values estimated for a subsequent exposure to Cd.

Cellular thiol content and glutathione synthesis

The role of intracellular thiols and GSH in acquired resistance to Cd was also investigated. Measurement of intracellular reduced thiols revealed an almost twofold higher thiol content in 21-day-old control cells than in 7-day-old control cells (Fig. 9a). Interestingly, a 24-h exposure to 10 μM Cd led to a significant 65% increase in the cellular reduced thiol content in undifferentiated but not differentiated cells. The oxidation of GSH in addition to the inhibition of glutathione reductase in the presence of 30 μM BCNU and 30 μM diamide had no significant

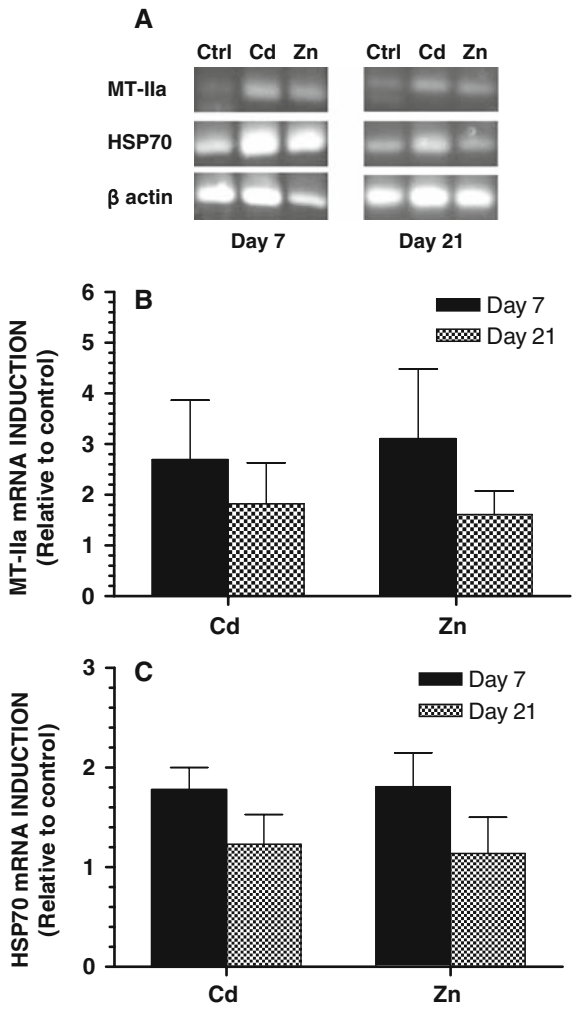

Fig. 7 Effect of exposure to Cd or Zn on MT-IIa and HSP70 mRNAs levels in 7-day-old undifferentiated and 21-day-old differentiated cells, as measured with RT-PCR, showing **a** qualitative results on 2% agarose gel and **b** densitometry analyses of mRNA inductions. Cells were cultured in the presence of FBS for 6 and 20 days and were then exposed for 24 h to 10 μM Cd or 100 μM Zn in the absence of FBS. Data shown are means ± SD estimated on three independent cell cultures

effect on cell sensitivity to Cd or acquired resistance (Fig. 9b).

Cell viability was then measured in the presence of GSH synthesis inhibitor. Pre-exposure to 3 mM BSO alone significantly sensitized cells to Cd toxicity (LC_{50} = 48.5 ± 5.2 vs. 14.8 ± 1.6 μM for control and BSO-pretreated cells, respectively) but did not prevent Cd-induced resistance (LC_{50} = 14.8 ± 1.6 vs. 24.9 ± 1.6 μM for cells pre-exposed to BSO and to

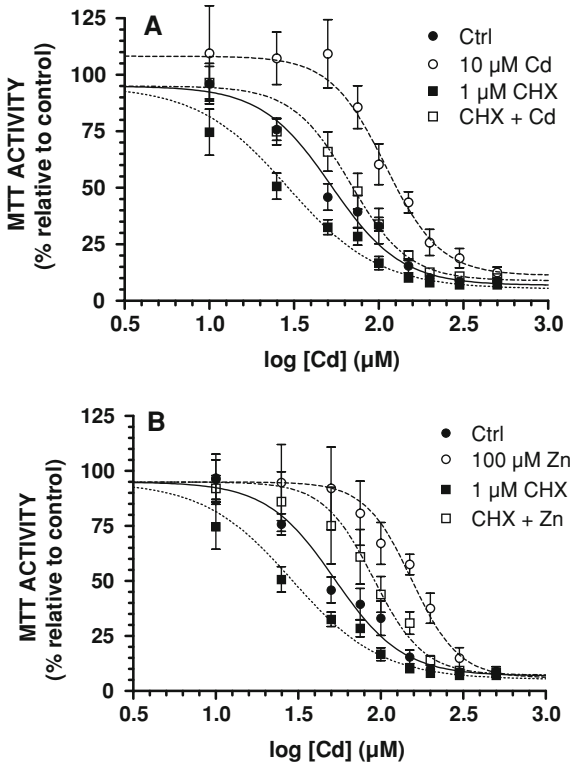

Fig. 8 Effect of pre-exposure to 1 μM CHX and 10 μM Cd (**a**) or 100 μM Zn (**b**) on MTT activity in response to a subsequent exposure to Cd in undifferentiated cells. Cells were cultured in the presence of FBS for 6 days and were then pretreated under various experimental conditions before exposure to increasing concentrations of Cd in the culture medium. Pretreatment and exposure were both 24 h in the absence of FBS. Points are means ± SD estimated on three independent cell cultures

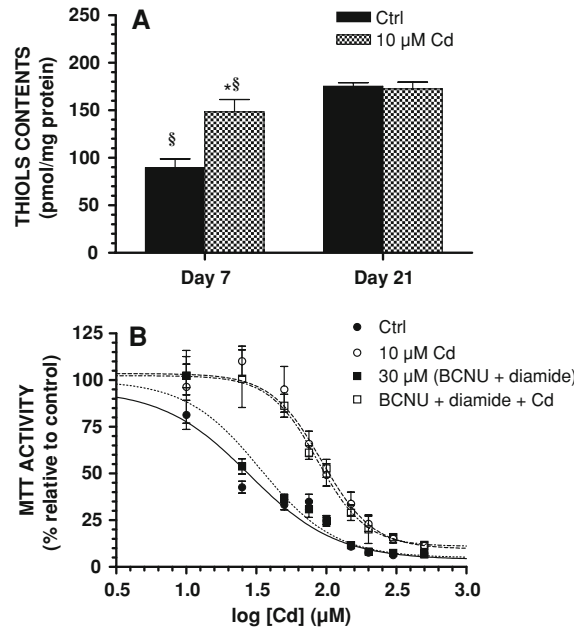

Fig. 9 **a** Effect of exposure to Cd on intracellular reduced thiol contents in undifferentiated and differentiated cells. Cells were cultured in the presence of FBS for 6 and 20 days and were then exposed to 10 μM Cd in the absence of FBS for 24 h. Data are means ± SD of three replicates of the same subculture. *, § Significant differences compared to the respective Ctrl conditions or the corresponding condition in 21-day-old cells, respectively. **b** Effect of pre-exposure to 30 μM diamide, 30 μM BCNU and 10 μM Cd on MTT activity in response to a subsequent exposure to Cd in undifferentiated cells. Cells were cultured in the presence of FBS for 6 days and were then pretreated under various experimental conditions before exposure to increasing concentrations of Cd in the culture medium. Pretreatment and exposure were both 24 h in the absence of FBS. Data are means ± SD estimated on three independent experiments

BSO + Cd, respectively) (Fig. 10a). In this case, the Cd-induced increases in LC_{50} values (1.6- to 1.9-fold) were similar in control and BSO-pretreated cells. Similar results were obtained with Zn-induced resistance but with the following differences: unexpectedly, induced resistance was much higher in cells pre-exposed to BSO alone compared to control cells, and therefore, cell sensitization by BSO was completely alleviated by Zn (Fig. 10b). In contrast, cells pre-exposed to BSO + Cd still remained more sensitive to a subsequent exposure to Cd than control cells.

Catalase and glutathione peroxidase activities

To obtain further insights into the possible involvement of oxidative stress in Cd-induced mechanisms of acquired resistance, antioxidant enzyme activities were estimated in addition to cellular viability following CAT inhibition. CAT activity was about 150 times higher than GPx activity in 7-day-old control cells (Fig. 11a). A 24-h exposure to increasing concentrations of Cd led to a small but significant decrease in CAT activity with a 22% inhibition at 20 μM Cd (Fig. 11b). Contrary to what has been observed in cell pretreatment with BSO, the 94% inhibition of CAT in the presence of 100 mM 3AT (data not shown) did not modify cell sensitivity to Cd toxicity, but prevented Cd-induced resistance and clearly sensitized the cells exposed to both 3AT and 10 μM Cd (Fig. 12a). However, contrary to Cd, pre-exposure to both 100 mM 3AT and 100 μM Zn did

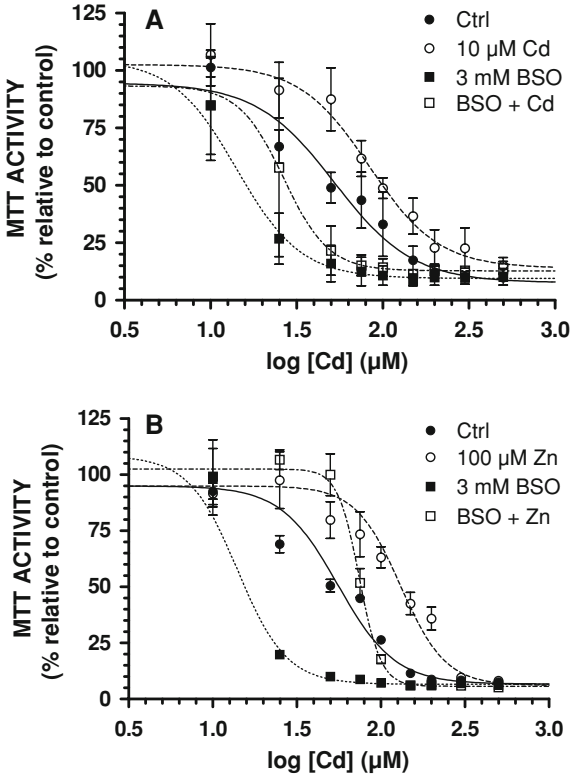

Fig. 10 Effect of pre-exposure to 3 mM BSO and 10 µM Cd (**a**) or 100 µM Zn (**b**) on MTT activity in response to a subsequent exposure to Cd in undifferentiated cells. Cells were cultured in the presence of FBS for 6 days and were then pretreated under various experimental conditions before exposure to increasing concentrations of Cd. Pretreatment and exposure were both 24 h in the absence of FBS. Points are means ± SD estimated on three independent cell cultures

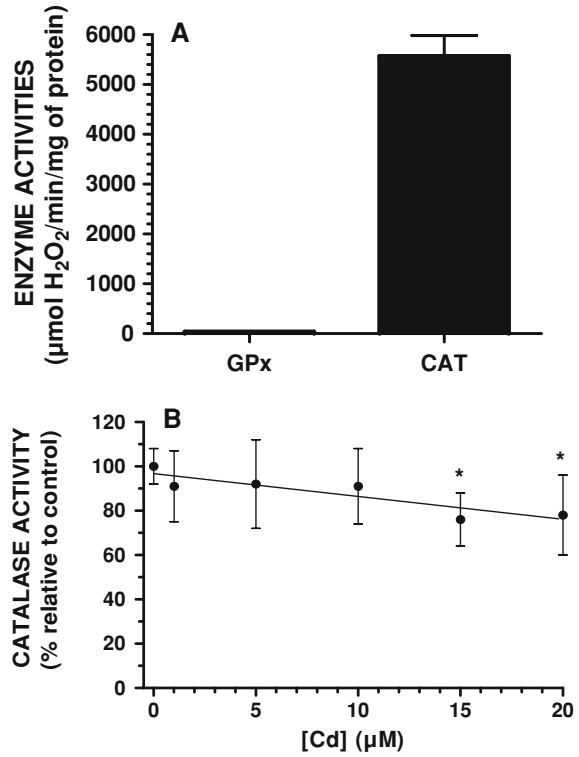

Fig. 11 **a** Basal CAT and GPx activities in 7-day-old undifferentiated cells measured as rate of decrease of hydrogen peroxide (*CAT*) and of cumene hydroperoxide (*GPx*) as described in the "Materials and methods". Data shown are means ± SD estimated on three replicates of the same subculture. **b** Effect of exposure to Cd on CAT activity in undifferentiated cells. Cells were cultured in the presence of FBS for 6 days and were exposed to 1, 5, 10, 15, or 20 µM Cd for 24 h in the absence of FBS. Points are means ± SD estimated on three independent cell cultures. Linear regression ($r^2 = 0.80$). * Significant differences compared to control activity values estimated at 0 µM Cd

not sensitize cells to Cd, and Zn-induced resistance still occurred in the presence of 3AT (Fig. 12b).

Discussion

Constitutive and acquired resistance to Cd vary with the differentiation status

The results of this study reveal variation in basal (constitutive) resistance to Cd as a function of enterocytic differentiation status. Indeed, 21-day-old Caco-2 cells are more resistant to Cd than 7-day-old cell cultures: a threefold higher LC_{50} value was estimated in the former (Fig. 1). Similar results have been obtained by other investigators for iron toxicity in preconfluent and postconfluent Caco-2 cell cultures (Zöld et al. 2004), whereas no evidence could be observed in Zn toxicity as a function of days in culture (Zöld et al. 2003). Higher basal resistance in 21-day-old cells is not related to a lower uptake level since comparable cellular accumulations were measured at both day 7 and day 21 of culture (Fig. 6). Also, higher levels of MT-IIa or HSP70 were not responsible for the higher resistance of differentiated cells since MT-IIa and HSP70 mRNAs levels were only 60% of those of undifferentiated cells (data not shown). However, higher activity levels of antioxidant enzymes, notably CAT, GPx and glutathione reductase, shown by other investigators in 21-day-old Caco-2 cells, may be responsible for the higher resistance to Cd (Baker and Baker 1992). Cd and Fe,

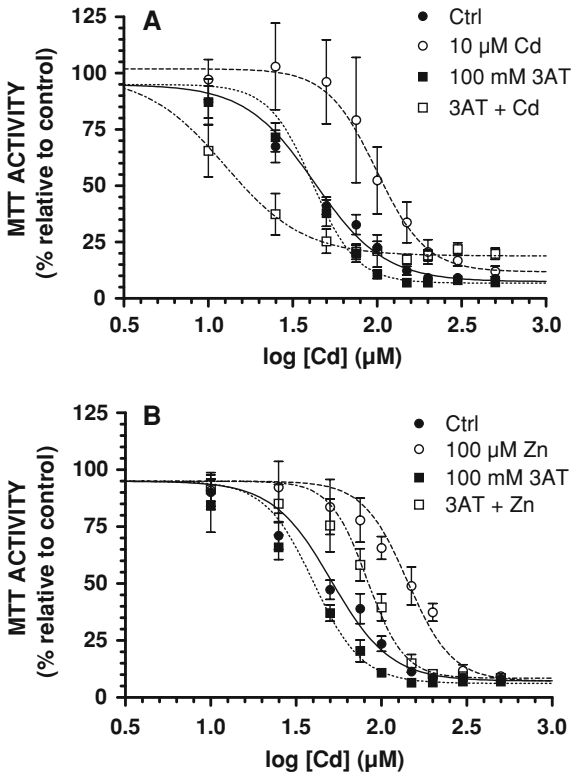

Fig. 12 Effect of pre-exposure to 100 mM 3AT and 10 µM Cd (**a**) or 100 µM Zn (**b**) on MTT activity in response to a subsequent exposure to Cd in undifferentiated cells. Cells were cultured in the presence of FBS for 6 days and were then pretreated under various experimental conditions before exposure to increasing concentrations of Cd in the culture medium. Pretreatment and exposure were both 24 h in the absence of FBS. Points are means ± SD estimated on three independent cell cultures

but not Zn, are both known to induce oxidative stress, although Cd does not directly participate in redox reactions (for review see Beyersmann and Hartwig 2008).

The LC_{50} value of about 140 µM estimated in differentiated cells following a 24-h exposure to Cd suggests that Caco-2 cells are moderately resistant compared to other cell types. An LC_{50} value of 5 µM was estimated in the human bronchial epithelial cell line BEAS-2B following a 24-h exposure (Schmid et al. 2007), whereas 18 µM was obtained in osteoblast-like MG63 cells (Lévesque et al. 2008). For liver cells, LC_{50}s of 500 µM and 70 µM were obtained following a 3-h exposure in the human HepG2 cell line and in Reuber H-35 rat hepatoma cells, respectively (Yang et al. 2004b; Yang et al. 1995). In contrast, renal LLC-PK1 cells were found to be much more resistant since an LC_{50} around 50 mM was estimated (Olabarrieta et al. 2001). Although the culture media differ, all these studies have been performed using serum-free media. To our knowledge, there is no LC_{50} value published for Cd in Caco-2 cells, but from FACScan analyses with PI, an LC_{50} of about 75 µM can be estimated at 24 h (Boveri et al. 2004). Also a 13-fold increase in LDH leakage was measured by Blais et al. (1999) following a 24-h exposure to 24 µM Cd.

For the first time, our results also show differences between undifferentiated and differentiated intestinal cells in their capability to acquire resistance. A 24-h pre-exposure to a non-cytotoxic concentration of 10 µM Cd induced resistance to a subsequent exposure to Cd in 7- but not 21-day-old Caco-2 cells, even though the latter are more resistant to Cd (Fig. 1). Note that 20 µM Cd, which led to similar 5% cell mortality in 21-day-old cells compared to 10 µM Cd in 7-day-old cells, also failed to induce resistance in differentiated cultures. Now, why can't additional resistance to Cd be induced in differentiated cells, which are already more resistant? Two hypotheses can be suggested: (1) mechanisms of acquired resistance are lost during enterocytic differentiation or; (2) resistance by itself involves saturable mechanisms still maximally expressed in differentiated control cells. Discrimination between the two hypotheses requires further investigation, but as for the constitutive resistance to Cd, the acquired one is clearly not related to lower levels of cellular accumulation (Fig. 6). Interestingly, Zn-induced resistance following cell incubation with 100 µM Zn, which also led to 5% cell mortality, was comparable to that obtained in response to exposure to Cd (Fig. 4). However, contrary to what has been observed with Cd, Zn-induced resistance still occurred in 21-day-old cells. These results suggest the involvement of different mechanisms in Cd- and Zn-induced resistance to Cd; the Zn-mediated adaptation would not be lost during Caco-2 cell differentiation.

Although Cd may induce apoptosis in vitro (Chuang et al. 2000; Lag et al. 2005), loss of Caco-2 cell viability was exclusively related to necrosis in our study without evidence for any significant apoptosis. According to what has been reported by others (Misra et al. 2003), the increase in the S phase could suggest that Cd may stimulate DNA synthesis

(Fig. 5a, b). However, for Cd concentrations above 10 μM, a concomitant decrease in cell distribution in G2/M (not G1) was observed with the increase in the S phase, suggesting that Cd may block S to G2/M transition. On the other hand, exposure to 10 μM Cd increased cell distribution in G2/M without any apparent increase in the S phase and no stimulation of DNA synthesis (Fig. 5c), suggesting that lower levels of Cd would instead block cell cycle progression at G2/M. Note that most studies showing Cd-induced DNA synthesis reported this effect at a very low level of exposure (0.1–1 μM), which was not used in our study (Von Zglinicki et al. 1992; Misra et al. 2003). At higher concentrations, Cd inhibits DNA synthesis (Cao et al. 2007) and may also cause G2/M cell cycle arrest (Chao and Yang 2001b; Yang et al. 2004a; Cao et al. 2007). One should keep in mind that 7-day-old Caco-2 cell cultures are almost confluent, and therefore no longer in an exponential growth phase. However, even under these conditions, Cd could maintain the capacity for cell cycle modification.

Upregulation of stress proteins is not responsible for acquired resistance

The level of MT expression has been correlated with intracellular Cd content in Caco-2 cells (Blais et al. 1999), and MT-IIa may protect against Cd toxicity and oxidative stress (Hawse et al. 2006). Similarly, HSP70 expression is induced in the presence of Cd or under oxidative stress conditions (Kiang and Tsokos 1998; Souza et al. 2004). This heat shock protein protects against oxidative stress caused by hydrogen peroxide (Spitz et al. 1987) but its protective effect against Cd toxicity remains to be clarified (Urani et al. 2005). Although higher constitutive levels of MT-IIa and HSP70 mRNAs were measured in 7-day-old cells, higher levels of induction were obtained in these same cell cultures (Fig. 7). This could be explained by the possible higher sensitivity to oxidative stress of undifferentiated cells because of lower levels of antioxidant enzyme activities (Baker and Baker 1992). The levels of induction were comparable to what has been observed by Shimoda et al. (2001) in HepG2 cells following a 24-h exposure to either 5 μM Cd or 100 μM Zn, but much lower compared to the inductions measured by Urani et al. (2005) in the same cells. In agreement with our results, the induction of MT (I and II) was much higher than that of HSP70 (Urani et al. 2005). Gene expression of both MT-IIa and HSP70 are induced within the first hours following exposure to Cd (or Zn), with maximal expression being reached as soon as 6 h in various human cells (Murata et al. 1999; Souza et al. 2004). Although the kinetics of MT-IIa and HSP70 induction were not investigated in our study, the huge increases in LC_{50} values occurring between 12 and 24 h (Fig. 3) suggest that these stress proteins are not entirely responsible for the acquired resistance. This conclusion is further supported by the fact that significant increases in both stress protein mRNAs were also observed for differentiated cells, although they did not show increased resistance to Cd (Fig. 7). In agreement with these observations, studies have shown that cells may maintain resistance to Cd seven passages or 15 days following the end of exposure to Cd, although cellular MT levels returned to the control values (Croute et al. 2000; Lau et al. 2006). Furthermore, pre-exposure with 1 μM CHX sensitized proliferative cells to Cd, clearly demonstrating that some protein synthesis is crucial for basal resistance to Cd (Fg. 8). However, cell treatment with CHX did not prevent Cd- or Zn-induced resistance: similar increases in LC_{50} values were obtained whether or not the cells were pretreated with CHX. This suggests that even though MTs and HSPs certainly protect the cells against Cd toxicity, they are seemingly not the main factor responsible for the observed acquired resistance.

Redox signaling could trigger Cd-induced but not Zn-induced resistance

Low concentrations of Cd, leading to the production of low levels of free radicals, have been shown to increase intracellular content of GSH, whereas higher Cd concentrations may decrease it (Almazan et al. 2000; Elbekai and El-Kadi 2005). Following a 24-h exposure to 10 μM Cd, an increase in the levels of reduced thiol-containing proteins and/or peptides, presumably MTs and GSH, was observed in 7- but not 21-day-old Caco-2 cells (Fig. 9a), supporting the hypothesis that 10 μM Cd induces a mild oxidative stress in undifferentiated cells, but probably not in differentiated cultures. The higher constitutive levels of total reduced thiols measured in 21-day-old cell cultures may represent additional protection for the

differentiated cells. Actually, increased intracellular levels of GSH may be a marker of Cd-induced oxidative stress (Waisberg et al. 2003). The role of GSH in basal and induced resistance was then further investigated using diamide and BCNU, which oxidizes GSH and inhibits glutathione reductase, respectively. Pre-exposure to a mixture of 30 μM diamide and BCNU has been shown to markedly decrease the intracellular GSH:GSSG ratio, even though it was not as efficient as BSO in lowering intracellular GSH levels in Caco-2 cells (Noda et al. 2001). The same pre-exposure conditions did not modify Caco-2 cell sensitivity to Cd and did not inhibit the induction of resistance (Fig. 9b). Similar results were obtained with higher diamide and BCNU concentrations (data not shown). The possible importance of newly synthesized GSH was studied using BSO, an inhibitor of γ-glutamyl cysteine synthase, which catalyses the rate-limiting step during GSH synthesis. Pre-exposure to 3 mM BSO did not prevent Cd- or Zn-induced resistance: similar increases in the LC_{50} values were observed following pre-exposure to 10 μM Cd, whether or not the cells were pretreated with BSO (Fig. 10). Surprisingly, for Zn-induced resistance, the relative increase in LC_{50} was even higher in BSO-pretreated cells. On the other hand, as did CHX, BSO highly sensitized cells to Cd toxicity. These results show the importance of newly synthesized GSH as protection against Cd toxicity, but also reveal that GSH synthesis is not a prerequisite to acquired resistance to Cd.

Our results clearly show that CAT, not GPx, is the main enzyme for hydrogen peroxide detoxification in undifferentiated Caco-2 cells (Fig. 11). Similar results were obtained by Wijeratne et al. (2005), showing CAT and GPx activities in the range of mmol min^{-1} and μmol min^{-1}, respectively, in confluent Caco-2 cell cultures. A 24-h exposure to 10 μM Cd led to a small 9% decrease in CAT activity (Fig. 11). It seems unlikely that this decreased activity would generate oxidative stress, but a slight change in the cell redox state may be sufficient to trigger adaptation mechanisms: the involvement of higher levels of hydrogen peroxide cannot be ruled out. Indeed, it has been shown that hydrogen peroxide accumulates in cells exposed to Cd (Schützendübel et al. 2001), and it has been suggested that hydrogen peroxide is the main ROS involved in Cd toxicity (Yang et al. 1997). Therefore, the impact of CAT inhibition was studied using 3AT. Pre-exposure to 100 mM 3AT did not sensitize cells to Cd toxicity, but co-exposure with 10 μM Cd completely prevented the induction of resistance as well as sensitized cells significantly to a subsequent exposure to Cd (Fig. 12). In contrast, Zn-induced resistance was insensitive to the presence of 3AT. These results suggests that a redox signal may trigger Cd-induced resistance, but the cell's capacity to manage redox imbalance is critical for the capability to develop resistance. Oxidative stress resulting from CAT inhibition would impair this ability. The fact that Cd but not Zn may lead to oxidative stress is likely responsible for the differences observed in Cd- versus Zn-induced resistance as a function of differentiation status.

Conclusion

Our results demonstrate the importance of stress proteins and GSH in the protection against Cd toxicity in intestinal cells. Higher constitutive protection against Cd-induced oxidative stress may explain, at least in part, the higher basal resistance observed in differentiated compared to undifferentiated Caco-2 cells. However, upregulation of either MT-IIa or HSP70 is not a prerequisite to the development of resistance, which does not necessarily involve protein synthesis. Different mechanisms would be involved in Zn- and Cd-induced resistance to Cd. A slight change in the cell redox state would trigger Cd- but not Zn-mediated adaptation mechanisms. However, oxidative stress may eliminate a cell's capability to develop resistance in response to Cd. Also, Cd-induced resistance was no longer observed in differentiated cells, whereas Zn-induced adaptation mechanisms were as efficient in differentiated and undifferentiated cells. Considering the rapid renewal of the intestinal epithelium, adaptation during chronic oral exposure is critical and may protect against Cd toxicity. The differences observed in Cd- versus Zn-induced resistance as a function of differentiation status clearly deserves further investigation.

Acknowledgments The authors thank Denis Flipo for technical assistance. This research was supported by NSERC, the Natural Sciences and Engineering Research Council of Canada.

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