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Effect of Metals on β -Actin and Total Protein Synthesis in Cultured Human Intestinal Epithelial Cells

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Abstract

As an important structural protein, β -actin is associated with anchoring of tight junctions (TJs) to the cell scaffold. Caco-2 cells, an immortal intestinal epithelial cell line, rely on β -actin to form intact monolayers with high transepithelial electrical resistance in cell culture inserts. We examined the effect of six metals on expression of β -actin mRNA and β -actin synthesis, on total and net production of newly synthesized proteins, on paracellular transport of TJ markers, and on cell viability in confluent monolayers. [3 H]-glycine and [3 H]-tyrosine were used as indicators of newly synthesized proteins in the absence or presence of increasing concentrations of arsenic, cadmium, copper, manganese, mercury and nickel. The monolayers were exposed to 24-hr single exposures as well as continuous daily repeated doses of metals for 48-hr and 96-hr. Results suggest that decreases in newly synthesized proteins, in which β -actin represents about 10%, correlated with 2- to 5-fold higher expression of β -actin mRNA for the higher concentrations of metals. Interestingly, IC_{50} s calculated for each chemical for 24-hr acute and 48- and 96-hr repeated dosing experiments, using the MTT viability assay and paracellular permeability markers, decreased newly synthesized and total proteins to 10% and 40% of control, respectively. Overall, the results indicate that, at equivalent concentrations, the metals affect β -actin mRNA and newly synthesized proteins before cell viability and paracellular permeability are compromised. Consequently the results help in elucidating mechanisms of metal cytotoxicity that lead to understanding the relationship between tight junction integrity, paracellular transport, and cell viability.

Keywords

Caco-2 cells; cell culture; *in vitro* cytotoxicity; paracellular permeability; trans-epithelial electrical resistance; trans-membrane electrical resistance; β -actin

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Conflict of Interest Statement

The authors declare that there is no conflict of interest with the submission or contents of this manuscript and with any private or public affiliations.

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1. Introduction

Widespread exposure to metals in the environmental or occupational setting is associated with long term *in vivo* toxicity. In addition, studies of metal toxicity demonstrate highly selective activation of stress signaling pathways leading to the development of human cancers (Kasprzak *et al.*, 2003;). Alternatively, despite well-recognized carcinogenic potentials of such toxic metals as chromium, nickel and metalloid arsenic, the molecular mechanisms underlying their cell-transforming ability remain poorly understood. Carcinogenic metals are typically weak mutagens and with the exception of chromium, they do not form DNA adducts, which represent a key initiating event in the cancer-inducing activity of organic carcinogens. As described below, the view that elevated production of reactive oxygen species as the main pathway in metal carcinogenicity is not supported with the current understanding of the mutagenic capacity of most metals.

For instance, occupational exposure to nickel (Ni) and arsenic (As) has been shown to increase the incidence of human cancers. Epidemiological studies have implicated Ni(II) compounds as human carcinogens based upon an increased mortality from respiratory tract malignancies in refinery workers chronically exposed to nickel-containing dusts and fumes (Salnikow and Zhitovich, 2008). The proposed mechanism of the Ni-induced tumors centers around changes in DNA methylation and histone acetylation, as well as inactivation or suppression of various DNA repair transcription factors (Karaczyn *et al.*, 2005; Kasprzak *et al.*, 2003; Lee *et al.*, 1995).

Human bioaccumulation of mercury (Hg) due to acute and chronic occupational and environmental exposure has been implicated in neurological and psychological pathologies, as well as renal, immunological, and cardiovascular diseases (Jarup 2003). In fact, a high dietary intake of mercury from consumption of fish is hypothesized to increase the risk of coronary heart disease (Jarup 2003). Interestingly, the toxicity associated with consumption of large amounts of methyl mercury in seafood, with mercury burdens in the range of 9 to 24 µg, is well documented (Balshaw *et al.*, 2007).

Groundwater contamination with high concentrations of As (10 to 100 µg/L) is associated with significantly higher mortality rates for cancers of the bladder, kidney, skin, liver, and colon (Tchounwou *et al.* 2004). A major mechanism by which As exerts its toxic effect is through impairment of cellular respiration and uncoupling of oxidative metabolism. Additionally, *in vitro* studies have linked the carcinogenic effect to the ability of As to induce gene amplification, arrest cells in mitosis, inhibit DNA repair, and stimulates expression of *c-fos* gene in mammalian cells (Huang *et al.*, 2000). Although As carcinogenesis is influenced by cellular metabolism and duration of exposure, the risks for human cancer transpire at various concentrations and environmental levels (Salnikow and Zhitovich, 2008).

Occupational and environmental exposure to cadmium (Cd), as a human carcinogen, is linked to lung, bladder, liver, kidney, stomach, and prostate cancers (Waalkes 2000). Because it is poorly mutagenic at low doses, explanations for Cd carcinogenicity relies on its epigenetic mechanism, namely interference with apoptotic gene expression, inhibition of DNA repair enzymes, and up-regulation of mitogenic signaling (Waalkes 2000).

In vivo, exposure to manganese (Mn)- containing compounds results in neurological and developmental toxicity at even moderate doses, while in mammalian cells, higher doses of Mn cause DNA damage and chromosome mutations. Interestingly, however, it is not regarded as a human carcinogen (Gerber *et al.* 2002).

Finally, interference with normal physiological roles of copper (Cu) has been correlated with human cancers. For instance, increased expression of copper-transporting ATPase genes (Cu-ATPases), which are essential for human cell growth and development, has been observed in some human cancer specimens, and may be associated with tumorigenesis and chemotherapy resistance (Zhang *et al.*, 2009). Recently, Turski and Thiele (2009) have demonstrated that a family of copper-dependent intracellular lysyl oxidase-like proteins (LOXL1-4) interferes with Snail repressor proteins, the latter of which normally regulate the transcription of E-cadherin to maintain cell-cell adhesion. The authors suggest that hyperactivity of these copper-bound cofactors (LOXL2 in particular) diminishes glycogen synthase kinase 3 β -mediated phosphorylation, thereby inhibiting E-cadherin expression, and compromising tight junctions. Clinical human studies have also confirmed the role of elevated LOXL2 expression in highly invasive forms of metastatic breast, colon, and esophageal cancers (Turski & Thiele 2009).

In vivo and *in vitro*, epithelial cells regulate paracellular permeability by forming junctional complexes between the cells. The junctional complexes consist of desmosomes, adherens junctions and tight junctions, the latter of which contribute to the barrier function of the epithelial membrane. Specifically, as a “house-keeping” protein in stationary mammalian cells, β -actin is an integral component in maintaining the integrity of tight junctions (TJs). TJs fasten adjacent epithelial cells and regulate the passage of ions and water between cells (Gonzalez-Mariscal, *et al.*, 2003). As TJs are particularly important in the intestinal lumen and proximal renal tubule, chemicals that interfere with β -actin synthesis can alter TJ integrity and selectively impair absorption and intercellular transport (Fanning *et al.*, 1998).

Several *in vitro* studies have shown reduced paracellular functionality in the presence of metal exposure. For instance, Rossi *et al.* (1996) demonstrated that Cd, Cu and zinc (Zn) alter Caco-2 cells' ability to transport specific markers across the cell monolayer. Exposure to the metals also resulted in morphological alterations of TJ integrity as demonstrated by changes in the transepithelial electrical resistance (TEER) across the membrane. Similarly, Duizer *et al.* (1999) showed that the decreased epithelial barrier function of intestinal epithelial cells is accompanied by tight junction disruption. They concluded that Cd⁺² causes increased paracellular permeability by disruption of junctional function and structure. Ferruzza *et al.* (2002) present data that suggest different mechanisms are responsible for the changes in TJ permeability produced by Cu and iron exposure in Caco-2 cells. They report that Cu(II) led to an overall reduction in F-actin staining with extensive depolymerization in areas of the monolayer formation on semi-permeable membranes in the absence of cellular loss, whereas Fe(II) treatment produced disorganization of F-actin and decreased staining of the perijunctional actin filaments. Interestingly, no changes in localization of the junctional proteins ZO-1, occludin and E-cadherin were observed after treatment with 100 mM FeSO₄. Natoli *et al.* (2009) showed that acute exposure to 50 μ M of Fe(II)ascorbate in Caco-2 cells resulted in damage to tight junctions. The toxicity was represented by an increase in cell monolayer permeability and by partial delocalization of the tight junction protein, claudin, from the plasma membrane to an intracellular compartment. And, Navarro-Moreno *et al.*, (2009) show that subacute lead intoxication alters renal tubular epithelial cell structure through loss of apical microvilli, decrement of the luminal space and the respective apposition and proximity of apical membranes, and formation of atypical intercellular contacts and adhesion structures. Alternatively, *in vivo* studies show that manganese displays a unique behavior with regard to its toxicity. Although manganese does not represent a significant carcinogenic risk to the population through occupational exposure, the metal causes Parkinson-like symptoms when inhaled even at moderate amounts over longer periods of time (Gerber *et al.*, 2002). However, the metal precipitates DNA damage and chromosome aberrations and large amounts of organic manganese derivatives are embryotoxic in mammals. Interestingly, Prozialeck *et al.* (2006) argue that several proximal

tubule-derived cell lines, commonly used as model systems for *in vitro* toxicity studies, express varied levels of cell adhesion molecules (E-cadherin, N-cadherin, ZO-1, occludin, and claudins-2 and -7), while some cell lines have weak claudin-7 and occludin expression. They conclude that proximal tubule-derived cell lines exhibit characteristics that most closely resemble those of the proximal tubule *in vivo*, and that these cell lines are appropriate models for studying the effects of toxicants on cell–cell junctions and cell adhesion molecules. In a separate review, Prozialeck and Edwards (2007) summarize the effects of a variety of toxic substances, including metals such as Hg and Cd, on gap junctions and cell adhesion molecules in epithelial cell models, both *in vitro* and *in vivo*. Furthermore, they elucidate the role of integrins and connexins in maintaining junctional integrity in the presence of toxicant and metal exposure. Overall, it is well understood, although not frequently justified in explanations of toxicological intercellular mechanisms, that preserving the integrity of gap junctional intercellular communication (GJIC) is essential in maintaining normal cellular development. Thus, the carcinogenic effect of chemical teratogens and tumor-promoting chemicals, inflammatory factors, hormones and growth factors, may be explained in part by interfering with GJIC. In progenitor cells, the chemicals alter proliferation, apoptosis or senescence, while in terminally differentiated cells, these chemicals alter gene expression or apoptosis and possibly de-differentiation. This epigenetic pathway may explain why an “initiated” stem cell would be prevented from terminally differentiating or from dividing asymmetrically in the presence of chemical carcinogens, teratogens, reproductive toxicants and neurotoxicants. That is, the chemicals alter gene expression, in either stem cells, progenitor cells or terminally differentiated cells, by transcriptional, translational or post-translational modifications, thus interfering with intercellular communication (Trosko 2007).

We have studied the human intestinal epithelial cell line, Caco-2, and mortal rat intestinal cells, as *in vitro* models for acute cytotoxicity and paracellular permeability for a variety of cytotoxic compounds (Konsoula & Barile, 2005, 2007). In cell culture, Caco-2 cells form intact monolayers on semi-permeable inserts that yield an organizational arrangement suitable for examining the effects of metal exposure on tight junction integrity. Based on the effects of metals on cell adhesion molecules as noted above, we postulate that monitoring tight junction formation and stability provides a method for detecting subtle changes in membrane integrity as a result of metal exposure. Since β -actin serves as one of the main structural anchors for tight junctions, any disruption in β -actin gene expression or protein synthesis reflects interference with paracellular transport and compromise of the epithelial barrier. To this end, we compared the effects of 24-hr single exposures with 48- and 96-hr repeated, continuous dosing of cultured cells with six metals, while monitoring production of newly synthesized proteins, β -actin synthesis and gene expression, paracellular transport and cell viability.

2. Materials & Methods

Cell Culture

Cell culture supplies were obtained from LIFE technologies (Carlsbad, CA, USA), and from VWR (Bridgeport, NJ, USA). Chemicals (>99.9% purity) were obtained from Sigma Chemical Co., (St. Louis, MO, USA) and from Alfa Products (Ward Hill, MA, USA). Immortal human colon epithelial cells (Caco-2, American Type Culture Collection; Rockville, MD, USA), passage numbers 22–40, were sub-cultured and seeded at 10^4 cells/cm² in 96-well plates or onto 6-well or 12-well plates fitted with BD Falcon polyethylene terephthalate (PET) culture plate inserts (5×10^4 cells/insert). Cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-10), 1% non-essential amino acids (NEAA), 1% glutamine, 50 U/ml penicillin and

50 µg/ml streptomycin in 7.5% CO₂ in air. All metal exposure experiments were performed on confluent monolayers.

Incorporation of radio-labeled precursors into newly synthesized proteins

Confluent monolayers of Caco-2 cells were exposed to increasing concentrations of metals for 24-hr and incubated with [2-³H]-glycine (21 Ci/mmol) or L-[3,5-³H]-tyrosine (51 Ci/mmol) (Amersham BioChemicals, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) in MEM without FBS, to a final concentration of 1.0 µCi/ml. Cells were harvested and radioactivity was measured as previously described (Barile and Cardona 1998; Barnes *et al.*, 1990).

Quantification of mRNA expression for β-actin

Total mRNA was purified using Invitrogen micro-to-midi total RNA purification kit (Carlsbad, CA, USA). Primers for β-actin were obtained from Applied BioSystems (Foster City, CA, USA). Multiplex RT-PCR for β-actin fragment (295 bp) was performed in the same tube as the internal standard (IS; 498 bp) using Invitrogen SuperScript III™ one-step system with Platinum® *Taq* DNA Polymerase, as previously described (18S rRNA was amplified with a primer-competimer combination using QuantumRNA™ Classic IS Kit [Ambion Inc, Austin, TX, USA]) (Gupta *et al.*, 2005; Kebache *et al.*, 2002; Washer *et al.*, 1996). Electrophoresis of PCR products was performed on 1% agarose gels for 1-hr at constant 100v and stained with ethidium bromide. Band intensity was measured relative to IS with the UVP Biospectrum 600 Imaging System® (UVP, Upland, CA, USA). Ratio of the band intensities for β-actin relative to IS was calculated as a semi-quantitative measurement of mRNA synthesized according to mRNA extracted per sample.

Quantification of β-actin synthesis and total protein

Synthesis of total β-actin protein was performed using the Pathscan® Total β-actin Sandwich ELISA kit (Cell Signaling Technology, Inc.; www.cellsignal.com). The method is based on a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of β-actin. Reactions are measured by reading absorbance at 450 nm on the BioTek FL600® fluorescence/absorbance plate reader (BioTek Instruments Inc., Winooski, VT, USA).

Total protein was determined using the Pierce BCA (bicinchoninic acid) protein assay reagent kit (Thermo Scientific, Rockford, IL, USA). Absorbance of final protein product was read at 562 nm.

MTT cell viability assay

Median effect analysis of cell viability for metal cytotoxicity was performed as previously described (Konsoula and Barile, 2005). Briefly, cells were grown to confluence in 96-well plates and exposed in the absence or presence of increasing concentrations of metals, at 37°C in an atmosphere of 7.5% CO₂ in air. Viability was evaluated with the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide) assay (cell counts were simultaneously performed using a hemocytometer). Absorbance was read at 550 nm using the BioTek FL600® fluorescence/absorbance plate reader and viability was expressed as a percentage of the control group. Determinations from each group-concentration represent the mean of six separate wells (wells with media only served as blanks) and each experiment was repeated at least three times. For continuous dosing assessment, confluent monolayers of Caco-2 cells were incubated with increasing concentrations of each chemical for 48- and 96-hr. Fresh medium (with chemical) was replaced in each well daily until the end of the experiment.

Trans-membrane electrical resistance (TMER) measurements

Caco-2 cells were seeded onto 6- or 12-well plates fitted with PET culture plate inserts. DMEM, supplemented as above, was added to the apical and basolateral chambers and replenished three times a week. Cultures were confluent at 7-days, but maximum resistance values (between 750–1000 $\Omega\text{-cm}^2$) were achieved in intact monolayers after 15-days. Trans-membrane specific electrical resistance (TMER) was measured as previously described (Calabro *et al.*, 2008) using the Millicell-ERS® resistance system (Millipore) before and after 24-, 48- and 96-hr incubation with test chemicals. Blanks (inserts without cells containing media and chemical) were used to determine baseline values, and are expressed as percent TMER of untreated control groups.

Paracellular permeability (PP) assay

Cells were seeded onto 6- or 12-well plates fitted with PET culture plate inserts, as described above for TMER experiments, and incubated with test chemicals for 48- and 96-h. New medium with test chemicals was introduced each day until the end of the assay. Low- and high-molecular weight indicators were added to the wells in the last 90 min of incubation. D-[1- ^3H]-mannitol ($^3\text{H-D-M}$; 20 Ci/mmol; 9:1 in ethanol; mw=182) has low lipophilicity and lucifer yellow (LY, mw=450) is more hydrophobic; however, both permeate via the paracellular route. To each apical chamber of an insert, 150 μl of DMEM-10 was added plus HEPES, mannitol (0.1% w/v), and [^3H]-D-M, to a final concentration of 1.0 $\mu\text{Ci/ml}$ (ethanol solvent was evaporated before application; Konsoula and Barile, 2005). Simultaneously, DMEM without radioactivity was dispensed into the basolateral chamber. At the end of the incubation period, passage of [^3H]-D-M through the membrane was determined by dissolving an aliquot of the basolateral medium in Budget-solve® and measuring the *cpm* by liquid scintillation (MicroBeta TriLux scintillation counter, Perkin-Elmer Analytical Sciences, Shelton CT, USA). Inserts without cells (blanks) and control cultures (minus chemical) were monitored simultaneously. Samples were also taken from the apical solutions to measure the initial donor concentrations. Background radioactivity was determined using DMEM, and *dpm* were calculated based on the instrument's counting efficiency for [^3H] (~45%).

Lucifer yellow was used at concentrations of 1mg/ml in DMEM and applied to the apical chamber. At the end of the exposure/incubation period, the basolateral medium was collected and the concentration of LY was determined by measuring fluorescence intensity (BioTek FL600 fluorescence/absorbance microplate reader). Inserts without cells (blanks) were also monitored simultaneously. Excitation and emission wavelengths for LY are 430nm and 540nm, respectively. As with the PP assay, fluorescence values for treatment groups were compared to control cell cultures. Relative cell permeability is expressed as a percent of untreated control groups.

Statistical analysis

IC_{50}s were extrapolated from concentration-effect curves using linear regression analysis (Figure 1a–f). Calculation of IC_{50} value for each chemical is based on a minimum of 3 experiments performed at separate times, 6 to 8 groups (concentrations) per chemical (including control and blank), and 6 separate measurements (samples) per group, ($N = 3$; sample number of measurements per experiment = 144). When the IC_{50} was not bracketed in the initial dosage range used for the chemical, the experiments were repeated and the concentrations were adjusted as necessary.

For statistical significance ($P < 0.05$), the coefficient of determination (R^2), slope (m), and t -statistic (two-tailed paired Students' t -test with the more stringent equal variances assumption) were calculated (Microsoft Excel®). All experiments were repeated at least

three times. Since the data generated from the β -actin synthesis experiments were determined to be of high precision and cost, these repetitions were performed only twice (Table 3). Values in all figures and tables are expressed as percentage of untreated controls, unless otherwise noted.

3. Results

Table 1 shows MTT IC_{50} data generated from exposure of Caco-2 cells to 24-hr single dosing and 48- and 96-hr repeated dosing with metals. IC_{50} s were calculated based on concentration-effect curves (Figures 1a–f). All experiments were performed until the regression (R^2) for the percent of control vs. log of concentration was greater than 0.90 for at least 6 concentrations including control. Comparison of 24-, 48-, and 96-hr IC_{50} values were not significantly different from each other (paired Students' t -test). However, comparisons using one-way ANOVA, slopes (m) and regressions for the lines were significantly different (*). Using these statistical indicators, IC_{50} values for 48- and 96-hr repeat dosing were lower than 24-hr data, suggesting that cells were more sensitive to 48- and 96-hr exposures. [It is important to note that in all assays, the steeper slopes of the lines suggest that the 24-h assays (y -values), in general, require higher doses than 48- or 96-hr assays (x -values) for determination of cell viability [in the calculation of slope on log scale, when m is greater than 1.0, as in Table 1, the line is shifted to the left and the y -values (24-hr) are higher than corresponding x -values (48- and 96-hr)(Konsoula & Barile, 2005)]. It is not unreasonable to conclude therefore that, at equivalent doses, cell viability is affected to a greater extent with prolonged, repeated exposures.

Table 2 summarizes the IC_{50} data generated from TMER studies from exposure of Caco-2 cells to 24-hr single dosing and 48- and 96-hr repeated dosing with metals. In contrast to the MTT assay results, there were no significant differences between acute and repeated dosing experiments. Only the slopes and regression values for 48- vs. 96-hr plots ($m = 1.7$, $R^2 = 0.96$) were significantly different. Consequently, transmembrane electrical resistance is not significantly affected over a 24- to 96-hr dosage range. As noted above, therefore, although there were no significant differences among TMER assays (with one exception), the flatter slopes of the lines on log scale reflect a shift to the right (Table 2: $m = 0.18$ and 0.31 , for 24- vs. 48-hr and 24- vs. 96-hr, respectively), suggesting that the 24-hr single concentrations (y -values) are lower than corresponding 48- or 96-hr repeated concentrations (x -values) [when m , or $\Delta y/\Delta x$, is less than 1.0, the data line is shifted to the right, thus x -values are higher than corresponding y -values (Konsoula and Barile, 2005)]. Thus the data indicate that repeated 48- or 96 hr dosing does not affect TMER as significantly as cell viability, and confirms our previous conclusions that cell viability is disrupted before PP is compromised (Konsoula and Barile 2005; Calabro *et al.*, 2008).

Figures 2a through 2f summarize the results of [3H]-glycine incorporation into net production of newly synthesized proteins in the absence or presence of six metals. A significant reduction ($> 50\%$) of radioactivity was detected with all metals at concentrations of 10^{-5} - to 10^0 -times their mean IC_{50} TMER concentrations. It is important to note that 5 metals and arsenic demonstrated greater than 95% or 70% reduction of glycine incorporation, respectively, at the IC_{50} values determined with the TMER assay for the corresponding metal, although significant differences from control were detected at even lower concentrations.

Figures 3a through 3f summarize the results of [3H]-tyrosine incorporation into net production of newly synthesized proteins in the presence of six metals. As with [3H]-glycine incorporation, a significant reduction ($> 50\%$) of radioactivity was determined for [3H]-tyrosine with all metals at concentrations 10^{-5} - to 10^0 -times their mean IC_{50} TMER

concentrations. In general, the decreases parallel those seen with [^3H]-glycine; 5 metals and arsenic demonstrated greater than 95% or 18% reduction of tyrosine incorporation, respectively, at the IC_{50} value determined with the TMER assay for the corresponding metal.

Figures 4a through 4f illustrate the amplification of β -actin gene transcripts from extracted mRNA expressed from cell cultures during 24-hr exposures to increasing concentrations of metals. Image analysis of band intensities was used to calculate semi-quantitative determinations of β -actin/IS ratios (control ratios were empirically adjusted so that β -actin/IS ratio would approximate 1.0 upon application to gels, and sample aliquots that were applied to each well were identical). More importantly β -actin expression, relative to IS, is based on the same quantity of mRNA starting material extracted from treated cultures, rather than on the number of cells—i.e. although total amount of mRNA extracted decreases in proportion to cell numbers in the treated cultures, the expression of β -actin/IS increases per unit of mRNA isolated.

All metals demonstrated a 1.5 to 3.0-fold increase in β -actin mRNA expression (relative to IS) at concentrations comparable to those showing decreases in newly synthesized proteins. Almost none of the doses used demonstrated gene expression significantly below control values (*; only Figure 4c, copper, is below 1.0 and significantly different from control). Thus, as cell numbers and newly synthesized proteins decrease in the presence of trace metals, synthesis of mRNA for β -actin rises, (standardized to control mRNA synthesis).

Figures 5, 6 and 7 illustrate the representative data obtained from exposure of Caco-2 cells to metals using passive paracellular permeability (PP) markers. The concentrations are based on the most effective IC_{50} s determined experimentally for either the MTT (Table 1) or TMER (Table 2) studies as well as the upper and lower limits (data not shown). Analysis of the data indicates that paracellular transport of [^3H]-D-mannitol, as a tight junction marker, more closely parallels the fall in TMER than LY, indicating that transport of [^3H]-D-mannitol to the basolateral surface correlates better with a decrease in resistance. This correlation, and the concentrations used, confirms our previous acute cytotoxicity results with these metals and other chemicals using a variety of PP markers (Konsoula & Barile, 2005). In only a few instances, such as with cadmium (Figure 5A) and manganese (Figure 6A,B), is LY transported at a greater rate than [^3H]-D-mannitol, as TMER decreases. These results are probably associated with effect of Cd or Mn on non-linear transport of low molecular weight molecules, both of which are known to interfere with passage through Ca^{+2} channels.

Table 3 summarizes and compares the results of experiments performed at 24-hr exposures with six metals at single concentrations represented by the 24-hr IC_{50} for TMER experiments. Values are systematically compared by percent of control for: cell viability, (MTT assay), newly synthesized proteins (^3H -glycine and ^3H -tyrosine incorporation), mRNA β -actin gene expression and total β -actin synthesis (PCR data and ELISA assay, respectively), cell proliferation (cell counts), total protein synthesis, and amount of protein synthesized per unit cell (per 10^4 cells). Each data point represents the mean of at least 3 experiments incorporating 6 concentrations per metal and involving 6 replications per concentration (wells per group-concentration) [experiments for β -actin, cell counts, and total protein were performed twice]. Close analysis reveals that: 1. except for Cd, MTT values are lower than the concentrations necessary to reduce TMER by 50% (TMER IC_{50} concentrations), suggesting that cell viability decreases before PP is compromised. This occurs most dramatically for nickel—i.e. 8.4 mM lowers TMER by 50% but decreases cell viability to 16% of control (Table 3). This data confirms our previous results for several diverse compounds with both Caco-2 cells (Konsoula and Barile, 2005) and mouse

embryonic stem cells (Calabro et al. 2008); 2. with only one exception (arsenic), percent of newly synthesized proteins, synthesis of total protein and β -actin protein (Figure 8), and cell counts, are dramatically decreased from the TMER IC_{50} and from MTT values. This indicates that concentrations of metals needed to reduce all protein synthesis, including proteins involved in cell adhesion, are significantly lower than those required for both PP and cell viability. Gene expression for β -actin mRNA, however, is increased several fold. Interestingly, since total protein synthesis and cell counts are lowered at the same rate, the amounts of protein synthesized by remaining viable cells are identical (approximately 18 to 28 μ g per 10^4 cells). The data for β -actin synthesis is also graphically presented in Figure 8.

4. Discussion

Preliminary concentration-effect studies were performed for each metal, to determine the concentration at which paracellular permeability (PP), as measured by transmembrane electrical resistance (TMER), would be inhibited by 50% (IC_{50} TMER). This concentration was then used as a benchmark for subsequent concentration-effect studies to assess the consequence of metals on cell viability, as measured by MTT absorbance, and net protein production, as measured by incorporation of isotope into acid-insoluble precipitate. Results of the MTT assay and the labeling experiments with 3H -glycine and 3H -tyrosine (Figures 1 through 3) clearly demonstrate that the concentration-effect curves for the two isotopes are identical; i.e. with the exception of As, the concentrations used to decrease TMER by 50% for each metal significantly inhibited incorporation of isotopes into newly synthesized proteins to less than 5% of control. This allows us to safely conclude that, at equivalent concentrations of metals, net protein production is significantly affected before PP is disturbed.

Glycine and tyrosine are both non-essential amino acids and, like other amino acids in mammalian cells, these intracellular constituents are derived from glycolytic side reactions. Their biochemical similarities, however, diverge here. For instance, their participation in glycolytic reactions differ significantly—serine is a major source of glycine via a reversible reaction catalyzed by serine hydroxymethyltransferase, while tyrosine is produced from the essential amino acid phenylalanine in a single step catalyzed by phenylalanine hydroxylase. Also, glycine is more highly represented in β -actin (10%) than tyrosine (5%), and is more frequently found in all proteins (7.2%) than tyrosine (3.2%) [only leucine and alanine are more frequently detected in proteins than glycine, 9.0% and 8.3%, respectively] (Hennessey *et al.*, 1993; Nakajima-Iijina *et al.*, 1985). Glycine and tyrosine have different energy requirements for hydropathy—i.e. the tendency to prefer a hydrophobic environment (0.67 kJ/mol vs. 0.08 kJ/mol, respectively). Moreover, the amino acids have different energy requirements for biosynthesis—glycine requires 12 moles of ATP for biosynthesis whereas tyrosine requires 62 moles of ATP. Finally, the pK_a values for the α -amino groups for glycine and tyrosine differ significantly at 25 °C (9.8 vs. 9.2, respectively), considering that the range of pK_a values for all 20 free amino acids are narrow (from 8.7 for asparagine, to 10.7 for cysteine) (Horton *et al.*). Thus, such differences in biochemical features between the amino acids would predict different results for any set of parallel studies, particularly concerning incorporation into nascent proteins. The close agreement between the results with radioactive glycine and tyrosine, however, with the exception of arsenic, indicates that the decreases in amounts of label incorporated into acid-insoluble material are most likely due to decreases in net protein production, rather than to changes in pool size or availability (uptake). Moreover, monitoring the quick turnover of proteins in the presence of metals is best determined by the percentage change between experimental and control groups, but may also involve the minor contribution of protein degradation over the 24-hr time span, the data for which is not pertinent for the scope of this study.

In our studies, incorporation of glycine and tyrosine in cell cultures reflects a decrease of newly synthesized proteins at concentrations of metals significantly lower than those affecting TMER and cell viability. Amino acid incorporation into acid-insoluble material was reduced by greater than 90% from controls at concentrations equivalent to the calculated TMER IC₅₀ concentrations. Total protein synthesis, using the BCA assay (Table 3), did not decrease as dramatically as with the radio-labeling experiments, but in fact, the former paralleled the cell count data. Consequently, micrograms of total protein produced per unit cell did not change from control at the IC₅₀ concentrations. Total synthesis for β -actin protein, however, decreased significantly, at a rate lower than total protein production, and more closely mimics the radioactive experiments. Interestingly, gene expression for β -actin mRNA, however, is increased several fold. We conclude, therefore, that the metals at concentrations that affect PP are not specific for total proteins but appear to target TJ proteins such as β -actin, and simultaneously trigger mRNA synthesis for the latter.

This data confirms our previous results with Caco-2 cells (Konsoula and Barile, 2005) and mouse embryonic stem cells (Calabro *et al.* 2008) that cell viability decreases before PP is compromised. More importantly, nascent protein production, total protein production at confluence, and specifically, β -actin protein, are affected by metal exposure before TJ integrity or cell viability are compromised. This also implies that concentrations of metals needed to reduce all protein synthesis, including proteins involved in cell adhesion, are significantly lower than those required for both PP and cell viability. Subsequent data is necessary to prove that there is a disconnection between transcription and post-translational modification of TJ proteins, where in fact there may be an upstream signaling pathway stimulated by the alteration in cell adhesion molecules.

Related studies have demonstrated enhanced cellular defense mechanisms in the presence of metal exposure. For instance, Bae *et al.* (2001) reported that cytotoxicity switches from synergistic to antagonistic when keratinocytes are exposed to increasing concentrations of metal mixtures, particularly with expression of metallothioneins. In an attempt to understand the complex biological effects of cadmium, Koizumi and Yamada (2003) show that cellular protection and anti-oxidant genes, such as those associated with metallothioneins and heat shock proteins, were induced in the presence of non-lethal Cd toxicity. Bertin and Averbeck (2006) review the suppression of protein production and cellular proliferation in mammalian cells with Cd, while modulating corresponding gene expression and signal transduction. Recent studies indicate that cell adhesion molecules associated with TJs, such as occludin and claudins, are primary targets for metal-induced renal injury (Prozialeck and Edwards, 2007). Furthermore, Pb and Hg disrupt heme synthesis and microtubules, respectively, the latter of which interferes with developmental processes (Clarkson, 1987). And, metals, such as As, Cd, Hg, and Pb, induce oxidative stress conditions, triggering cellular anti-oxidant mechanisms in an attempt to abate metal toxicity (Ercal *et al.*, 2001).

Although the Caco-2 cell model has historically been instrumental for absorption studies, limited reports have investigated the relationship between *in vitro* paracellular permeability and cytotoxicity. For instance, Laparra *et al.* (2005) have shown that inorganic As (As[III]) alters TJ permeability in Caco-2 cells. Chao *et al.* (1998) reported that long-chain acylcarnitines increase intestinal absorption of small hydrophilic compounds through the paracellular pathway by dilating paracellular spaces in Caco-2 monolayers. By calculating the ratio between IC₅₀ and EC₅₀ values, Liu *et al.* (1999) developed a potency index for novel absorption enhancers, which can be used to predict the effective concentration at which transepithelial electrical resistance (TEER) drops to 50%. Duizer *et al.* (1999) detected a decrease in TEER with 5- to 30- μ M CdCl₂ exposure in Caco-2 cells, with a corresponding disruption of the paracellular barrier, yet without a significant loss of viability. Boveri *et al.* (2004) report that CdCl₂ toxicity is detected in Caco-2 cells at very

low concentrations using LDH release and HSP70 as toxicological endpoints. Okada *et al.* (2000) compared changes in TEER in Caco-2 cell monolayers with cell viability for marine toxins using the LDH release assay. They detected that a decrease in TEER, associated with increased transepithelial permeability, preceded LDH release.

Thus, the current study follows our initial findings that investigated the relationship between PP and cytotoxicity in Caco-2 and other mammalian cells (Konsoula & Barile, 2005; Schmidt *et al.*, 2004). We report that the decreased epithelial barrier function of intestinal epithelial cells is accompanied by TJ disruption due, in part, to specific alterations in β -actin synthesis, and that passive paracellular transport of TJ markers correlates well with IC₅₀s determined with the TMER measurements. Since the concentrations of metals required to decrease protein synthesis were significantly lower than IC₅₀s for MTT and PP measurements, and the β -actin gene was up-regulated for the same concentrations, we conclude that synthesis and mRNA expression for β -actin is altered before cell viability and TJ integrity are compromised. The mechanism suggests that metal toxicity independently alters transcriptional and post-translational pathways over short periods of time and at relatively low concentrations, and may account for initiation or promotion of the carcinogenic pathway. Thus the study furthers our understanding of how metals contribute to the carcinogenic process by interfering with TJ integrity.

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Abbreviations

³H-D-M	[³ H]-D-mannitol
As	arsenic
Cd	cadmium
Cu	copper
Hg	mercury
Mn	manganese
Ni	nickel
PP	paracellular permeability
TEER	transepithelial electrical resistance
TJ	tight junction
TMER	trans-membrane electrical resistance

References

- Bae DS, Gennings C, Carter W, Yang RHS, Campain AJA. Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes. *Toxicological Sciences* 2001;63:132–142. [PubMed: 11509753]
- Balshaw S, Edwards J, Daughtry B, Ross K. Mercury in seafood: mechanisms of accumulation and consequences for consumer health. *Rev Environ Health* 2007;22:91–113. [PubMed: 17894202]

- Barile FA, Cardona M. Acute cytotoxicity testing with cultured human lung and dermal cells. In vitro Cell Developmental Biology 1998;34:631–635.
- Barnes Y, Houser S, Barile FA. Temporal effects of ethanol on growth, thymidine uptake, protein and collagen production in human foetal lung fibroblasts. Toxicology in Vitro 1990;4:1–7. [PubMed: 20702277]
- Bertin G, Averbeck D. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences. Biochimie 2006;88:1549–1559. [PubMed: 17070979]
- Boveri M, Pazos P, Gennari A, Casado J, et al. Comparison of the sensitivity of different toxicological endpoints in Caco-2 cells after cadmium chloride treatment. Archives of Toxicology 2004;78:201–206. [PubMed: 14661071]
- Calabro AR, Konsoula R, Barile FA. Evaluation of *in vitro* cytotoxicity and paracellular permeability of intact monolayers with mouse embryonic stem cells. Toxicology in Vitro 2008;22:1273–1284. [PubMed: 18468840]
- Chao AC, Taylor MT, Daddona PE, Broughall M, Fix JA. Molecular weight-dependent paracellular transport of fluorescent model compounds induced by palmitoylcarnitine chloride across the human intestinal epithelial cell line Caco-2. J Drug Targeting 1998;6:37–43.
- Clarkson TW. Metal toxicity in the central nervous system. Environmental Health Perspectives 1987;75:59–64. [PubMed: 3319566]
- Duizer E, Gilde AJ, Versantvourt CH, Groton JP. Effects of Cadmium Chloride on the Paracellular Barrier Function of Intestinal Epithelial Cell Lines. Toxicology Applied Pharmacology 1999;155:117–126.
- Ercal N, Gurer-Orhan H, Aykin-Burns N. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Current Topics Medicinal Chemistry 2001;1:529–539.
- Fanning AS, Jameson BJ, Jesaitis A, Anderson JM. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J Biological Chemistry 1998;273:29745–29753.
- Ferruzza S, Scacchi M, Scarino ML, Sambuy Y. Iron and copper alter tight junction permeability in human intestinal Caco-2 cells by distinct mechanisms. Toxicology in Vitro 2002;16:399–404. [PubMed: 12110278]
- Gerber GB, Léonard A, Hantson P. Carcinogenicity, mutagenicity and teratogenicity of manganese compounds. Crit Rev Oncol Hematol 2002;42:25–34. [PubMed: 11923066]
- Gonzalez-Mariscal L, Betanzos P, Nava P, Jaramillo BE. Tight junction proteins. Prog Biophys Mol Bio 2003;81:1–44. [PubMed: 12475568]
- Gupta N, Miyauchi S, Martindal RG, Herdman AV, Podolsky R, et al. Up-regulation of the amino acid transporter ATB⁰⁺(SLC6A14) in colorectal cancer and metastasis in humans. Biochim Biophys Acta 2005;1741:215–223. [PubMed: 15905073]
- Hennessey E, Drummond D, Sparrow J. Molecular genetics of actin function. Biochem J 1993;282:657–671. [PubMed: 8489492]
- Horton HR.; Moran LA.; Ochs RS.; Rawn JD.; Scrimgeour KG. Principles of Biochemistry. 3. Vol. Chapter 17. Prentice Hall; NJ, USA: 2005. Amino Acid Metabolism.
- Huang SC, Huang C-YF, Lee TC. Induction of mitosis-mediated apoptosis by sodium arsenite in HeLa S3 cells. Biochem Pharmacol 2000;60:771–780. [PubMed: 10930531]
- Jarup L. Hazards of heavy metal contamination. Br Med Bull 2003;68:167–182. [PubMed: 14757716]
- Karaczyn AA, Golebiowski F, Kasprzak KS. Truncation, deamidation, and oxidation of histone H2B in cells cultured with nickel (II). Chem Res Toxicol 2005;18:1934–1942. [PubMed: 16359184]
- Kasprzak KS, Sunderman FW, Salnikow K. Nickel carcinogenesis. Mut Res/Fund Mol Mech Muta 2003;533:67–97.
- Kebache S, Zuo D, Chevet E, Larose L. Modulation of protein translation by Nck-1. Proc Natl Acad Sci USA 2002;99:5406–5411. [PubMed: 11959995]
- Konsoula R, Barile FA. Correlation of *in vitro* cytotoxicity with paracellular permeability in mortal rat intestinal cells. J Pharmacological Toxicological Methods 2007;55:176–183.

- Konsoula R, Barile FA. Correlation of *in vitro* cytotoxicity with paracellular permeability in Caco-2 cells. *Toxicology in Vitro* 2005;19:675–684. [PubMed: 15896555]
- Koizumi S, Yamada H. DNA microarray analysis of altered gene expression in cadmium exposed human cells. *J Occup Health* 2003;45:331–334. [PubMed: 14676411]
- Laparra JM, Velez D, Barbera R, Montoro R, Farre R. An approach to As(III) and As(V) bioavailability studies with Caco-2 cells. *Toxicology in Vitro* 2005;19:1071–1078. [PubMed: 16023827]
- Lee YW, Klein CB, Kargacin B, Salnikow K, Kitahara J, et al. Carcinogenic nickel silences gene expression by chromatin condensation and DNA methylation: a new model for epigenetic carcinogens. *Mol Cell Biol* 1995;15:2547–2557. [PubMed: 7537850]
- Liu DZ, LeCluyse EL, Thakker DR. Dodecylphosphocholine-mediated enhancement of paracellular permeability and cytotoxicity in Caco-2 cell monolayers. *J Pharmaceutical Sciences* 1999;88:1161–1168.
- Nakajima-Iijima S, Hameda H, Reddy P, Kakunaga T. Molecular structure of the human cytoplasmic β -actin gene: interspecies homology of sequences in the introns. *Pro Natl Acad Sci USA* 1985;82:6133–6137.
- Natoli M, Felsani A, Ferruzza S, Sambuy Y, Canali R, Scarino ML. Mechanisms of defence from Fe(II) toxicity in human intestinal Caco-2 cells. *Toxicology in Vitro* 2009;23:1510–1515. [PubMed: 19540330]
- Navarro-Moreno LG, Quintanar-Escorza MA, González S, Mondragón R, Cerbón-Solorzáno J, Valdés J, Calderón-Salinas JV. Effects of lead intoxication on intercellular junctions and biochemical alterations of the renal proximal tubule cells. *Toxicology in Vitro* 2009;23:1298–1304. [PubMed: 19619637]
- Okada T, Narai A, Matsunaga S, Fusetani N, Shimizu M. Assessment of the marine toxins by monitoring the integrity of human intestinal Caco-2 cell monolayers. *Toxicology in Vitro* 2000;14:219–226. [PubMed: 10806372]
- Prozialeck WC, Edwards JR. Cell adhesion molecules in chemically-induced renal injury. *Pharmacology and Therapeutics* 2007;114:74–93. [PubMed: 17316817]
- Prozialeck WC, Edwards JR, Lamar PC, Smith CS. Epithelial barrier characteristics and expression of cell adhesion molecules in proximal tubule-derived cell lines commonly used for *in vitro* toxicity studies. *Toxicology in Vitro* 2006;20:942–953. [PubMed: 16387471]
- Rossi A, Poverini R, Di Lullo G, Modesti A, Modica A, Scarino ML. Heavy metal toxicity following apical and basolateral exposure in the human intestinal cell line caco-2. *Toxicology in Vitro* 1996;10:27–36. [PubMed: 20650179]
- Salnikow K, Zhitovich A. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic and chromium. *Chem Res Toxicol* 2008;21:28–44. [PubMed: 17970581]
- Schmidt CM, Cheng CN, Marino A, Konsoula R, Barile FA. Hormesis effect of trace metals on cultured normal and immortal human mammary cells. *Toxicology Industrial Health* 2004;20:1070–1082.
- Tchounwou PB, Centeno JA, Patlolla AK. Arsenic toxicity, mutagenesis, and carcinogenesis-a health risk assessment and management approach. *Mol Cell Biochem* 2004;255:47–55. [PubMed: 14971645]
- Trosko JE. Gap junctional intercellular communication as a biological “Rosetta Stone” in understanding, in a systems biological manner, stem cell behavior, mechanisms of epigenetic toxicology, chemoprevention and chemotherapy. *J Membrane Biol* 2007;218:93–100. [PubMed: 17960321]
- Turski ML, Thiele DJ. New roles for copper metabolism in cell proliferation, signaling, and disease. *J Biol Chem* 2009;284:717–721. [PubMed: 18757361]
- Waalkes M. Cadmium carcinogenesis in review. *J Inorg Biochem* 2000;79:241–244. [PubMed: 10830873]
- Washer LP, Law D, Boland CR, Horon I, Feinberg A. Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation. *Pro Natl Acad Sci USA* 1996;93:10366–10370.

Zhang Y, Li M, Yao Q, Chen C. Roles and mechanisms of copper transporting ATPases in cancer pathogenesis. *Med Sci Monit* 2009;15:1–5.

Figure 1.

Concentration-effect graphs of confluent monolayers of Caco-2 cells exposed to increasing concentrations of: A. cadmium, B. copper, C. nickel, D. manganese, E. mercury, F. arsenic, for 24-hr single dose exposures and 48- and 96-hr repeated dose exposure. Cells were processed for the MTT assay as described in Materials & Methods. Results are expressed as percent of control (no metal exposure). Each bar represents the mean $\pm$ SE of six replicates from one of at least 3 representative experiments.

Figure 2.

Incorporation of [2-³H]-glycine by confluent monolayers of Caco-2 cells exposed to increasing concentrations of: A. Cd, B. Ni, C. Cu, D. Mn, E. Hg, and F. As, for 24-hr. Cells were harvested and radioactivity was measured as described in Materials & Methods. Logarithmic concentrations are based on IC₅₀s determined with the TMER assay. Results are expressed as a percent of the control cells (no metal exposure). Each bar represents the mean $\pm$ SE of six samples. Each experiment was repeated at least three times. * = significantly different from control group, P<0.05.

Figure 3.

Incorporation of L-[3,5-³H]-tyrosine by confluent monolayers of Caco-2 cells exposed to increasing concentrations of: A. Cd, B. Ni, C. Cu, D. Mn, E. Hg, and F. As, for 24-hr. Cells were harvested and radioactivity was measured as described in Materials & Methods. Logarithmic concentrations are based on IC₅₀s determined with the TMER assay (Table 2). Results are expressed as a percent of the control (no metal exposure). Each bar represents the mean $\pm$ SE of six samples. Each experiment was repeated at least three times. * = significantly different from control group, P < 0.05.

Figure 4.

Expression of gene transcripts for internal standard (IS) and β -actin for confluent monolayers of Caco-2 cells exposed to increasing concentrations of: A. Cd, B. Ni, C. Cu, D. Mn, E. Hg, and F. As, for 24-hr. Cells were harvested, mRNA was extracted, and RT-PCR amplification of transcripts was performed as described in Materials & Methods. Logarithmic concentrations are based on IC_{50} s determined with either the TMER assay (Table 2). Bar graphs represent the ratio of β -actin/IS as relative band intensities; the expected PCR product for β -actin is 295bp and for the 18S rRNA IS is 498 bp, relative to the DNA ladder (not shown). Amount of PCR product applied to each well was determined empirically for the control cells to attain a final ratio of approximately 1.0 (dotted line). Each bar represents the mean $\pm$ SE of three to six samples and each experiment was repeated at least three times.

Figure 5.

Effect of: A. Cd 48-hr, B. Cd 96-hr, C. Cu 48-hr, and D. Cu 96-hr, on paracellular transport in Caco-2 cells. Concentrations correspond to IC_{50} s generated with either the MTT or TMER studies. Scale for Percent of Control Response for PP markers (bars) are on left axis; for TMER measurements (dashed-dotted line), scale is on the right axis. Control values are set at 100% (bars) or based on 1000 $\Omega\text{-cm}^2$ (TMER). LY = lucifer yellow, $[^3\text{H}]\text{-D-M}$ = $[^3\text{H}]\text{-D-mannitol}$, TMER = transmonolayer electrical resistance.

Figure 6.

Effect of: A. Mn 48-hr, B. Mn 96-hr, C. Ni 48-hr, and D. Ni 96-hr, on paracellular transport in Caco-2 cells. Concentrations correspond to IC_{50} s generated in the MTT studies. Scale for Percent of Control Response for PP markers (bars) are on left axis; for TMER measurements (dashed-dotted line), scale is on the right axis. Control values are set at 100% (bars) or based on 1000 $\Omega\text{-cm}^2$ (TMER). LY = lucifer yellow, $[^3\text{H}]\text{-D-M} = [^3\text{H}]\text{-D-mannitol}$, TMER = transmonolayer electrical resistance.

Figure 7.

Effect of: A. As 48-hr, B. As 96-hr, C. Hg 48-hr, and D. Hg 96-hr, on paracellular transport in Caco-2 cells. Concentrations correspond to IC_{50} s generated with either the MTT or TMER studies. Scale for Percent of Control Response for PP markers (bars) are on left axis; for TMER measurements (dashed-dotted line), scale is on the right axis. Control values are set at 100% (bars) or based on 1000 $\Omega\text{-cm}^2$ (for TMER). LY = lucifer yellow, [3H]-D-M = [3H]-D-mannitol, TMER = transmonolayer electrical resistance.

Figure 8.

Effect of metals on confluent monolayers of Caco-2 cells exposed to 2 concentrations for 24-hr. Cells were harvested and β -actin synthesis was quantified as described in Materials & Methods. Concentrations are based on IC₅₀ and 10⁻² X IC₅₀ for each metal as previously determined with the TMER assays (Tables 2 and 3). Results are expressed as percent of the control cells (Con, no metal exposure). Each bar represents the mean $\pm$ SE of six samples; each experiments were repeated twice. * = significantly different from control group, P<0.05.

Table 1

IC₅₀ (mmol/L) for Caco-2 cells using MTT assay: 24-hr single dosing and 48- and 96-hr repeated dosing exposures.

Slope (*m*) and regression (*R*²) calculations for treatment groups are noted below. All plots were analyzed for one-way ANOVA (*p*<0.001); SEM of percent values are shown in Table 3;

Metals	MTT 24-hr	MTT 48-hr	MTT 96-hr
arsenic trioxide	0.068	0.0131	0.0096
cadmium chloride	0.109	0.041	0.034
cupric sulfate	1.566	0.670	0.439
manganese chloride	9.6	8.186	0.356
mercury chloride	0.019	0.333	0.242
nickel chloride	0.968	0.820	0.778

IC ₅₀ Data	R ²	m
24-hr (y) vs. 48-hr (x)	0.99*	1.2
24-hr (y) vs. 96-hr (x)	0.97*	2.7
48-hr (y) vs. 96-hr (x)	0.98*	2.3

* Significant at *p*<0.001.

Table 2

IC₅₀ (mmol/L) for Caco-2 cells using TMER measurements: 24-hr single dosing and 48- and 96-hr repeated dosing exposures.

Slope (*m*) and regression (*R*²) calculations for treatment groups are noted below. All plots were analyzed for one-way ANOVA (*p*<0.001); SEM of percent values are shown in Table 3;

Metals	TMER 24-hr	TMER 48-hr	TMER 96-hr
arsenic trioxide	0.13	0.0051	0.0045
cadmium chloride	0.049	0.030	0.022
cupric sulfate	1.8	0.578	0.473
manganese chloride	3.082	8.740	5.204
mercury chloride	0.09	0.181	1.192
nickel chloride	8.4	0.673	0.757

IC ₅₀ Data	R ²	m
24-hr (y) vs. 48-hr (x)	0.04	0.18
24-hr (y) vs. 96-hr (x)	0.04	0.31
48-hr (y) vs. 96-hr (x)	0.96*	1.7

* Significant at *p*<0.001.

Table 3

Summary of effects of 24-hr single dose exposures to 6 metals on Caco-2 cells.

Groups ± metals	IC ₅₀ TMER (mM)	MTT	GLY	TYR	β-actin mRNA ^a	β-actin protein production ^b	Cell count ^b	Total protein synthesis ^{b,c}	μg total protein/ 10 ⁴ cells ^b
control group	0	100 ± 1.2	100 ± 2.3	100 ± 2.0	100 ± 5.2	100 ± 2.8 100 ± 1.6	100 ± 3.8 100 ± 2.0	100 ± 3.0 100 ± 2.1	23.5 ± 3.2 21.6 ± 2.1
cadmium chloride	0.05	55.6 ± 4.2	2.4 ± 1.1	3.0 ± 9.5	107.8 ± 1.0	11.5 ± 0.2 4.3 ± 0.2	40.1 ± 0.8 40.6 ± 2.7	40.0 ± 1.6 42.2 ± 1.3	22.4 ± 1.0 25.2 ± 2.1
nickel chloride	8.4	16.0 ± 6.5	1.4 ± 3.7	2.5 ± 2.7	159.8 ± 1.7	5.4 ± 0.3 2.8 ± 0.2	33.9 ± 2.1 30.4 ± 0.9	37.9 ± 0.5 26.6 ± 1.1	22.4 ± 1.2 23.7 ± 1.0
cupric sulfate	1.8	44.0 ± 5.0	1.8 ± 3.1	0.2 ± 1.5	110.9 ± 2.0	10.4 ± 0.3 15.7 ± 0.2	37.2 ± 1.8 35.5 ± 1.3	49.5 ± 0.5 47.6 ± 0.4	28.1 ± 1.3 24.7 ± 0.9
manganese chloride	3.1	43.7 ± 1.6	1.8 ± 2.3	1.9 ± 1.1	250.0 ± 3.9	6.5 ± 0.2 13.9 ± 0.3	26.1 ± 1.3 28.4 ± 1.8	23.8 ± 2.8 34.9 ± 0.3	21.7 ± 2.0 18.4 ± 1.1
mercury chloride	0.09	45.0 ± 3.4	2.7 ± 5.1	1.3 ± 2.4	307.3 ± 4.1	8.5 ± 0.2 8.9 ± 0.2	42.2 ± 2.0 46.9 ± 1.9	34.1 ± 1.0 30.1 ± 2.3	20.3 ± 1.5 22.1 ± 2.1
arsenic trioxide	0.13	47.8 ± 8.9	31.3 ± 5.1	85.1 ± 0.8	177.4 ± 3.3	25.9 ± 0.9 23.1 ± 0.5	56.3 ± 1.9 62.3 ± 1.4	59.8 ± 4.5 65.8 ± 1.1	25.9 ± 3.2 23.3 ± 1.3

All values are expressed as **percent of control** group in the absence or presence of metal, except for **IC₅₀ TMER** concentrations (mM, column 2). Values represent effect measured at one concentration, i.e. the IC₅₀ concentration determined for TMER in paracellular permeability assays. All groups were analyzed for one-way ANOVA ($p < 0.001$) and Student's t -test (paired 2-sample for means assuming equal variances). Statistical analysis reveals that all values are significantly different from control groups ($P < 0.001$) except for **μg total protein per 10⁴ cells**.

^a = represents ratio of β-actin mRNA expression in the presence or absence of metal, as compared to IS;

^b = each value represents mean of separate experiments, 6 replications per data point/experiment;

^c = from BCA assay for total proteins;

Abbreviations: GLY = [³H]glycine incorporation into acid-insoluble precipitate; IS = internal standard; TYR = [³H]tyrosine incorporation into acid-insoluble precipitate.