BioMetals (2007) 20:73–81
DOI 10.1007/s10534-006-9016-0

Cadmium and mercury cause an oxidative stress-induced endothelial dysfunction

Matthew B. Wolf[1,*] & John W. Baynes[2]
[1]*Department of Pharmacology, Physiology & Neuroscience, University of South Carolina School of Medicine, Columbia, SC, 29208, USA;* [2]*Developmental Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC, 29208, USA; *Author for correspondence (Tel: +803-733-3241; Fax: +803-733-1523; E-mail: wolf@med.sc.edu)*

Received 1 February 2006; accepted 18 April 2006

Key words: heavy metals, oxidative stress, endothelial dysfunction, endothelial barrier dysfunction, glutathione, ATP, thiol enzymes

Abstract

We investigated the ability of cadmium and mercury ions to cause endothelial dysfunction in bovine pulmonary artery endothelial cell monolayers. Exposure of monolayers for 48 h to metal concentrations greater than 3–5 μM produced profound cytotoxicity (increased lactate dehydrogenase leakage), a permeability barrier failure, depletion of glutathione and ATP and almost complete inhibition of the activity of key thiol enzymes, glucose-6-phosphate dehydrogenase (G6PDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In contrast, metal concentrations less than 1–2 μM induced increases in glutathione and thiol-enzyme activities with minimal changes in LDH leakage, barrier function and ATP content. At shorter incubation times (24 h or less), high concentrations of cadmium caused glutathione induction rather than depletion. Thus, oxidative stress and cytotoxicity induced by lower concentrations of the metal ions stimulate compensatory responses, including increased synthesis of glutathione, which presumably preserved the activity of key thiol enzymes, however these responses were not sustainable at higher metal ion concentrations. We conclude, while high concentrations of heavy metals are cytotoxic, lower concentration induce a compensatory protective response, which may explain threshold effects in metal-ion toxicity.

Introduction

Many heavy metals play important biological roles. The particular importance of iron and copper ions in biological processes is well known (Halliwell & Gutteridge 1999). However, there are other heavy metals, such as cadmium and mercury, which are widely found in our environment, but have no known biological significance, except for their potential toxicity. Humans can be exposed to these metals by contaminated air and water and through food grown in contaminated soil (Järup 2003). Poisoning from these metals causes dysfunction of various organs. Cadmium accumulates in the lungs, liver, bone and kidneys (Stohs & Bagchi 1995; Ercal *et al.* 2001), causing dysfunction of these organs. Cigarette smoke is an important source of cadmium (Ercal *et al.* 2001). Elemental mercury and methyl mercury cause various nervous system disorders and produce nephrotoxicity (Ercal *et al.* 2001).

Redox-active metals, such as iron and copper, undergo redox recycling to produce free radicals. In contrast, cadmium and mercury are redox-inactive metals that challenge antioxidant defenses by binding to thiols in the cell (Stohs & Bagchi

1995; Ercal *et al*. 2001) and induce apoptosis (Pulido & Parrish 2003). Cell toxicity caused by heavy metal ions is attributed to oxidative and nitrosative stress (Stohs & Bagchi 1995; Ercal *et al*. 2001; Pompella *et al*. 2003), defined as an excess of oxidants over antioxidants. Macromolecules in cells are damaged by metal-induced production of oxygen- and nitrogen-containing free radicals (oxidants) and/or metal-induced depletion of the cell's antioxidant defenses. Programmed cell death (apoptosis) or necrotic cell death are usual consequences (Pulido & Parrish 2003). This outcome is also seen in endothelial cells (EC), (Szuster-Ciesielska *et al*. 2000), the focus of this study.

Reduced glutathione (GSH), the major thiol compound in the cell, is an important cytoplasmic antioxidant (Ercal *et al*. 2001; Pompella *et al*. 2003). Besides avidly binding potentially toxic heavy metals ions, other electrophiles and xenobiotics (Halliwell & Gutteridge 1999), it is a coenzyme for glutathione peroxidase, which inactivates hydrogen peroxide (H_2O_2) and lipid peroxides produced from enzymatic or nonenzymatic sources in the cell. In this process, oxidized glutathione (GSSG) is formed. Reduced nicotine adenine dinucleotide phosphate (NADPH), formed in the pentose-shunt pathway, is required for recycling of GSSG to GSH (Halliwell & Gutteridge 1999), thereby maintaining cell redox status. Insufficient NADPH leads to build up of GSSG in cells and its efflux from the cell, resulting in irreversible antioxidant depletion and a consequent oxidant stress. NADPH production depends upon glucose-6-phosphate dehydrogenase (G6PDH), a thiol antioxidant enzyme that is subject to modification during oxidant stress conditions (Ciolino & Levine 1997). Hence, heavy metal-induced inhibition of G6PDH can lead to depletion of both NADPH and GSH, and subsequently to cell death (Tian *et al*. 1999).

Another important thiol enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is involved in ATP production from carbohydrate precursors during glycolysis. Like G6PDH, it is subject to modification by oxidative stress (Ciolino & Levine 1997). Inhibition of this enzyme and consequent ATP depletion produces cytotoxicity and apoptosis (Leist *et al*. 1997; Terminella *et al*. 2002).

The vascular endothelium is very sensitive to oxidative stress. Since a major function of endothelium is to form a barrier to movement of material between blood and tissues, a consequence of oxidative stress is a barrier dysfunction (Lum & Roebuck 2001). The result *in vivo* would be an increased movement of water, small solutes and proteins into tissues, tissue edema and loss of organ function. Edema may have severe effects, for example on lung and cardiac function (pulmonary edema and congestive heart failure), but also results in slow removal of toxic products from the extracellular space. Hence, a possible result of heavy-metal poisoning is edema formation and organ-system dysfunction (Nolan & Shaikh 1986), although this outcome is not well documented.

Both cadmium and mercury cause damage to EC (Nolan & Shaikh 1986; Szuster-Ciesielska *et al*. 2000). However, the evidence for a metal-induced barrier dysfunction is sparse. We suggested (Hui *et al*. 2001) previously that these heavy metals might cause such a dysfunction. The mechanism proposed was an oxidative stress caused by alteration of glutathione and/or ATP metabolism. We now hypothesize that these metals could cause oxidative stress resulting in a general EC dysfunction, including an increase in endothelial permeability. Our present aim is to determine if these metals cause such dysfunction and investigate the mechanism. This study was conducted on an *in vitro*-model of the vascular endothelium, the bovine pulmonary artery EC (BPAEC) monolayer. We have previously used this model system to study EC dysfunction resulting from oxidative stress induced by cancer chemotherapeutic agents (McAmis *et al*. 2003; Wolf & Baynes 2006) and other agents (Hui *et al*. 2001). In the latter study, we hypothesized that unknown heavy metals contained in phosphate buffers might produce a barrier dysfunction. We found that this dysfunction could be inhibited by various factors, such as metal chelators and the antioxidant, *N*-acetylcysteine (NAC). Hence, in the present study, we examine biologically relevant antioxidant responses to heavy metal ions and also study kinetic and dose–response curves for these responses.

Materials and methods

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated.

Cell culture

Procedures for cell culture were as described previously (Hui *et al.* 2001). BPAEC (passages 6–13) were grown to confluence (3–5 d) in DMEM (Cellgro) with 10% FBS (Atlanta Biologic) on Corning-Costar Transwells (TW, 0.33 cm^2 surface area of membrane inserts). Cells were also grown on 12-well plates and 60 or 100 mm dishes for other measurements. Before experiments, the monolayers were washed with MEM, containing 15 mM HEPES and then incubated in HEPES–MEM along with the various agents used for periods up to 48 h.

Barrier-function measurement

After discarding the protein-containing medium from the TWs, various concentrations of $CdCl_2$ or $HgCl_2$ were added to the medium bathing the cells (top well inserts) and to the bottom wells of the TWs (wells of a 24-well plate). After exposure of the monolayers to these agents for up to 48 h, they were washed off and FITC–BSA (Molecular Probes, Eugene, OR) in HEPES–MEM, was added to each top well insert and HEPES–MEM to the corresponding bottom wells in a clean 24-well plate. At time zero, the top well inserts, containing EC bathed in medium with FITC–BSA were transferred to the top of bottom wells containing only HEPES–MEM in order to measure the rate of transport of FITC–BSA through the monolayer. At 1.5 h, the upper and lower solutions were analyzed for FITC fluorescence (excitation at 485 nm; emission at 535 nm) in a Tecan SPECTRAfluor microplate reader. FITC–BSA diffusional permeability, a measure of barrier function, was determined from the fluorescence ratio (lower solution fluorescence divided by upper solution fluorescence) as described previously (McAmis *et al.* 2003).

Enzyme and metabolite assays

Cellular GSH concentration was measured by the monochlorobimane (MCB) fluorometric method (Kamencik *et al.* 2000). G6PDH, GAPDH and LDH activities in EC or cell culture medium were measured using NAD(P)H linked assays, as previously described (McAmis *et al.* 2003). ATP was determined in EC grown on 12-well plates using the firefly luciferase assay (Molecular probes), as previously described (Wolf & Baynes 2006).

Statistics

Statistically significant differences between data sets or from controls were determined with a Student's '*t*' test or ANOVA (Instat software, GraphPad, San Diego, CA) at a probability level of <0.05. Smooth curves were fit to concentration- or activity-response data and exponential curves to MCB fluorescence data using the SigmaPlot computer program (Systat, Richmond, CA). The fits were weighted by the inverse of the square of the standard error (SE).

Results

To gain insight into the cytotoxicity caused by heavy metals, we first looked at the changes in extracellular LDH leakage from BPAEC monolayers at various incubation times. Sets of experiments were carried out for exposures to 1 and 10 μM cadmium for up to 48 h. As shown in Table 1, cytotoxicity increased with both incubation time and cadmium concentration. However, there were no significant differences between the data for 1 μM cadmium-exposed and control monolayers (ANOVA or linear regression). On the basis of the data in Table 1, we selected 48 h as the standard incubation time for further experiments and

Table 1. Cadmium-induced BPAEC monolayer LDH leakage for various incubation times

Incubation time (h)	Cadmium concentration (μM)		
	Control	1	10
1	0.38[a] ± 0.08	0.44[a] ± 0.04	
	0.23[b] ± 0.02		0.50[b] ± 0.08
12	0.35 ± 0.06	0.32 ± 0.07	
	0.27 ± 0.02		0.48 ± 0.09
24	0.71 ± 0.09	0.77 ± 0.07	
	0.51 ± 0.02		1.59[c] ± 0.14
48	1.19 ± 0.06	1.65 ± 0.31	
	0.86 ± 0.05		4.54[c] ± 0.29

[a]Control and 1 μM cadmium-induced LDH leakages (U/mg protein), respectively, upper rows. [b]Control and 10 μM cadmium-induced LDH leakage, respectively, lower rows. $N = 4$ or 7 for 1 or 10 μM cadmium and respective controls. [c]Significantly different from control, $p < 0.001$.

10 μM as the maximum metal concentration examined. Figure 1A shows that the LDH leakage curves were similar for a 48-h exposure to either cadmium or mercury. Significant increases from control (*) occurred at concentrations above about 1 μM, whereas maximum values occurred at concentrations >4–5 μM.

Figure 1B shows the changes in monolayer permeability to FITC–BSA induced by the metals. These curves roughly paralled the LDH leakage curves. However, the transition from minimum to maximum effect took place over a narrower concentration range for mercury, compared to cadmium. These data are indicative of a severe cell injury and loss of barrier function. Not shown was that 10 μM cadmium also induced a significant permeability increase above control, 4.13 ± 0.42, $N = 3$, $p < 0.05$, for 24 h of incubation. However, no significant increase was observed with only 4 h of incubation.

At lower concentrations, both metals induced significant increases (*) in GSH (Figure 2A) for 48 h incubation time. Higher metal ion concentrations (greater than 2–3 μM) led to GSH depletion, in concert with the sharp increase in LDH release and loss of barrier function. Although it appears that mercury induced greater increases in GSH than did cadmium, the data points at 0.5 and 1 μM concentrations for each metal were not significantly different. Figure 2B shows that the changes in G6PDH activity were similar to those for GSH. Again, the apparent differences in the curves at these concentrations were not significant.

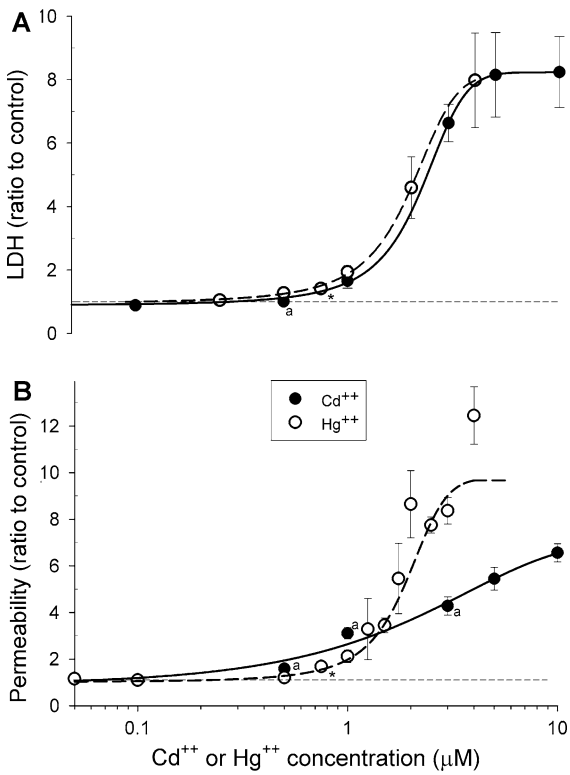

Figure 1. Effects of 48 h incubations on BPAEC monolayers of cadmium (solid circles and line) and mercury (open circles and dashed line) ions on LDH leakage (A) and FITC–BSA permeability (B). Shown are mean values and standard error (SE) bars for the changes relative to control (dashed lines at 1.0). For LDH experiments, $N = 7$ for each metal. For permeability experiments, $N = 5$–11 for cadmium and 5–18 for mercury. Sigmoidal curves were fit to the data using a weighting of the inverse of SE squared for each data point. Significant differences ($p < 0.05$) from control for LDH and permeability are at metal concentrations equal to and above those denoted by (*). Significant differences between the changes induced by the two metals (a) are shown at the data points where the same concentrations were used.

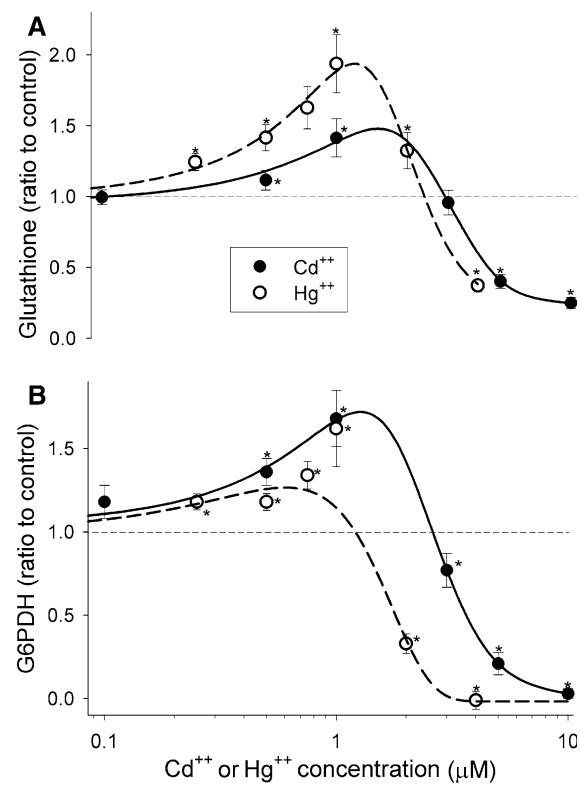

Figure 2. Effect of the two metals on glutathione concentration (A) and G6PDH activity (B). $N = 7$ for each metal. Both metals produced significant increases from control in each quantity at lower concentrations, but significant inhibition at higher concentrations (*). There were no significant differences between the responses produced by either metal.

Mercury may have induced a change from increased to decreased G6PDH activity at a lower concentration compared to cadmium, which could account for the apparently steeper fall in GSH due to mercury. The qualitative similarities between the two sets of curves suggest that metal-induced changes in G6PDH activity play a role in the changes in GSH concentration.

One of the adaptive responses to cadmium-induced oxidative stress is generation of GSH (Stohs & Bagchi 1995; Ercal *et al.* 2001). We examined this phenomenon by determining changes in GSH during incubation of cells for 4–48 h in the presence of 1 and 10 μM cadmium (Figure 3). It is clear that BPAEC cultured in MEM (in the absence of plasma) lose GSH gradually, however incubation of the monolayers with either 1 or 10 μM cadmium for 4 h produced no changes in GSH, compared to controls. In contrast, incubation for longer times produced significant increases (*) in cellular GSH. For 1 μM cadmium, this effect was observed at incubation times out to 48 h. Hence, the cells appeared to respond to stress at low metal concentration by increased synthesis of GSH. Cadmium, at 10 μM concentration, induced a greater increase in GSH for 24 h, but then a steep fall to just above control by 48 h, indicating that the EC were unable to compensate for the stress produced by high metal concentrations for extended periods.

Both metals induced profound ATP depletion (Figure 4A). It appears that the depletion induced by mercury occurred at lower concentrations compared to that by cadmium, which could account for the steeper permeability increase induced by mercury (Figure 1B). However, the data points at 0.5 and 1 μM concentrations of the metals were not significantly different. Changes in GAPDH activity induced by both metals were also similar (Figure 4B). There was a small activity increase at lower concentrations, with inhibition at higher concentrations. The general similarities

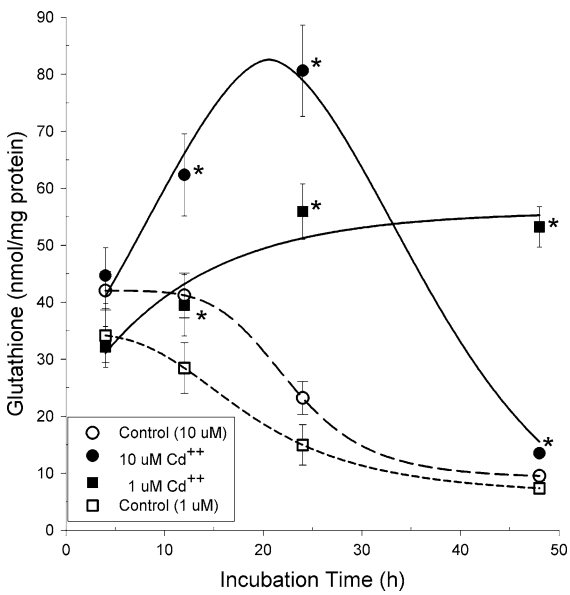

Figure 3. Effect of incubation time on the changes in glutathione induced by 1 μM (solid squares and line, $N = 4$) and 10 μM (solid circles and line, $N = 7$) cadmium compared to controls (open squares and circles and dashed lines, respectively). Control values decreased with incubation time. Cadmium (1 μM) induced significant (*) increases in glutathione production at all incubation times beyond 4 h. Cadmium (10 μM) also induced significant increases at these times, but by 48 h, the increases were only slightly greater than control.

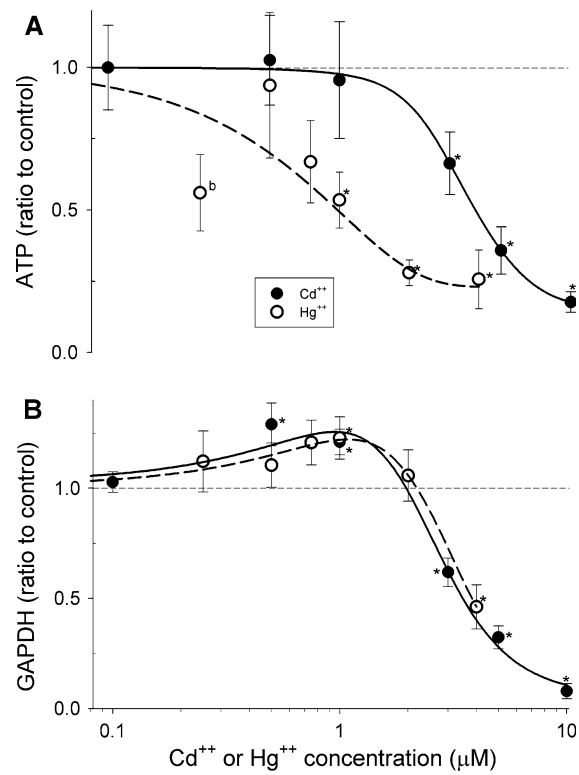

Figure 4. Effect of cadmium and mercury on ATP content (A) and GAPDH activity (B). $N = 7$ for cadmium and $N = 4$ for mercury. Both metals caused ATP depletion at higher concentrations (*), but there were no significant differences between the curves. Both metals caused slight increases in GAPDH activity at lower concentrations, but there were no significant differences between the responses produced by either metal.

between the changes in GAPDH activity and ATP content at higher metal concentrations suggests that metal-induced GAPDH inhibition plays a role in ATP depletion in EC. However, other factors, such as actions of the two metals on mitochondrial ATP production pathways, may also explain the quantitative differences in ATP depletion induced by the two metals.

We investigated the effects of factors suggested previously (Hui *et al*. 2001) as inhibiting the barrier dysfunction caused by these metals (Table 2). The damaging effects on the endothelial barrier function by 10 μM cadmium (Figure 1B) were significantly inhibited by 10 mM NAC and totally inhibited by10% serum, similar to their effects on the phosphate buffer-induced barrier dysfunction (Hui *et al*. 2001). Another antioxidant, α-lipoic acid, also effectively inhibited the cadmium-induced barrier dysfunction. These effects may reflect some intracellular antioxidant actions, but the major effect is probably attributable to direct metal ion reactions with the thiol compounds or albumin in the medium, possibly enhanced by sequestration or chelation of the metals by albumin, and lipoic acid.

Discussion

The ability of mercury, cadmium and other divalent metal ions to cause toxicity to cells and organs has been known for some time (Valee & Ulmer 1972). The cytotoxicity is caused, in part, by their strong affinity for ligands such as phosphates, cysteinyl and histidyl side chains of proteins, pteridines and porphyrins (Valee & Ulmer 1972).

Table 2. Effect of various factors on cadmium-induced permeability changes

Factor	FITC–BSA permeability (ratio to control)
NAC	3.37[a] ± 0.54
10 mM	(N = 3)
Serum	1.92 ± 0.39
10%	(N = 4)
α-lipoic acid	2.07 ± 0.38
1 mM	(N = 4)

[a]Significantly different ($p < 0.05$) from control and from 10 μM cadmium response (Figure 1B).

Hence, a large number of biochemical processes are affected: enzymes are inhibited, nucleic acid conformation is changed, and oxidative phosphorylation is affected. Effects of heavy metals on endothelial barrier function have been noted previously. Mercury, at 10μM concentration, caused a significant increase in low molecular weight fluorescein leakage in a blood–brain barrier model using transformed EC (Toimela *et al*. 2004). A review (Nolan & Shaikh 1986) summarized earlier studies and suggested that the vascular endothelium was a primary target for cadmium and that the result was an increase in vascular permeability in a number of tissues. Stohs and Bagchi (1995) were among the first to show that these metals, at high concentration, cause oxidative damage, including lipid peroxidation, DNA damage and depletion of sulfhydryls. Szuster-Ciesielska *et al*. (2000) also observed that cadmium induced increases in the reactive oxygen species (ROS), superoxide and H_2O_2, with subsequent apoptotic and necrotic cell death in a bovine aortic EC (BAEC) system. These authors suggested that cadmium stimulated ROS production, however it is equally likely that ROS concentrations increased because cadmium reduced antioxidant defenses (Stohs & Bagchi 1995; Ercal *et al*. 2001).

Our studies confirm that both cadmium and mercury ions can induce an oxidative stress through depletion of GSH and inactivation of thiol enzymes, and that the increase in oxidative damage leads to severe EC dysfunction, including disruption of the endothelial barrier. We found that metal-induced increases in permeability (Figure 1B) paralleled the cytotoxicity (LDH leakage; Figure 1A). A previous study (Kaji *et al*. 1992) suggested that cadmium-induced BAEC detachment from culture plates also paralleled LDH activity in the medium. Thus, it is likely that some of the permeability increases in the present study were due to EC detachment, although this was only apparent at the very highest metal concentrations by light microscopy (not shown). We conclude that, for both metals, the cytotoxicity is the result of GSH depletion and thiol protein modification, but that at low doses of the metal ions, there is a significant compensatory antioxidant response, characterized by an increase in GSH concentration and an increase, possibly an induction, of the thiol enzymes, G6PDH and GAPDH.

We hypothesized previously that decreases in GSH and ATP concentration, either together or independently, caused cell toxicity and resulting barrier dysfunction. In the present studies, we found that both of these metabolites were depleted (Figures 2A and 4A) at the metal concentrations that also caused the most severe LDH leakages (Figure 1A) and permeability increases (Figure 1B), but that GSH concentrations were increased at lower metal concentrations (Figure 2A). GSH induction is one of the many ways cells have to combat an oxidant stress. GSH is known (Pompella *et al*. 2003) to bind heavy metal ions in addition to a wide range of organic electrophilic agents and drugs. However, this ability is limited, as was shown in this study for higher metal concentrations and in other studies with various cell types (Stohs & Bagchi 1995). We have shown (McAmis *et al*. 2003; Wolf & Baynes 2006) previously that glutathione depletion by drugs, such as buthionine sulfoximine, also leads to EC barrier dysfunction.

There is also substantial evidence that depletion of ATP causes an EC barrier dysfunction (Lum & Roebuck 2001). In previous studies (McAmis *et al*. 2003; Wolf & Baynes 2006), we found that the anticancer quinones depleted ATP and caused a barrier dysfunction. In other studies, mercury caused ATP depletion along with barrier dysfunction (Toimela *et al*. 2004) and cadmium caused ATP depletion and increased permeability in renal epithelial cells (Stinson *et al*. 2003). These changes may have just been simultaneous occurrences, but the sulfhydryl reagent, iodoacetate, depletes ATP prior to the appearance of a barrier dysfunction in EC (Wilson *et al*. 1990). In the present study, cadmium and mercury also altered energy metabolism in EC (Figure 4A) through effects on GAPDH.

In previous work we showed (Hui *et al*. 2001) that traces of heavy metals in phosphate buffers were able to induce increased permeability of BPAEC monolayers and that this effect could be blocked by EDTA. Although we were unable to identify the specific metal involved, we investigated the effects of cadmium and mercury, as well as copper, iron, lead, lithium, manganese, nickel, tin, and zinc. However, none of the latter could increase permeability at concentrations $<50\ \mu$M. Although we have not examined the full spectrum of metal ions in phosphate buffer, cadmium and mercury illustrate the ability of these metals to bind avidly to sulfhydryl groups on biologically important molecules and threaten the viability of

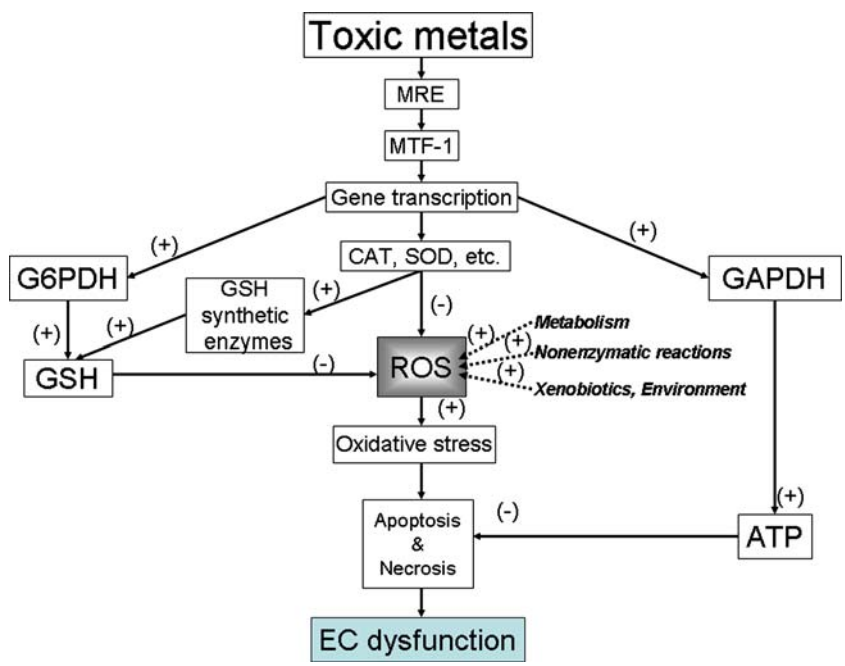

Figure 5. Mechanistic model of how low concentrations of toxic metal ions induce protection against both oxidative stress and EC dysfunction. (+) Denotes stimulation and (−) denotes inhibition caused by individual components.

the organism. At the same time, strong protective response at low metal ion concentration would explain threshold responses in metal ion toxicity, i.e. low doses of metals may enhance protective mechanisms, while high doses lead gradually to cytotoxicity (Kroes *et al*. 2004).

Mechanistically, we propose the model shown in Figure 5 to explain the findings in this study and to serve as a basis for future investigation. Normal metabolic oxidative phosphorylation processes, nonenzymatic reactions, xenobiotics and environmental factors produce ROS that are kept in bounds by antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), and by the ability of GSH to neutralize H_2O_2 and lipid peroxides. When these antioxidant defenses are compromised, ROS increase, leading to an oxidant stress to cells, cell death and dysfunction. An adequate supply of ATP is required to maintain normal function. At low metal concentrations, metal response elements (MRE) and other sensors, signal transcription factors, metal transcription factor-1, MTF-1, for example, and others, (Murata *et al*. 1999; Ercal *et al*. 2001; Giedroc *et al*. 2001; García-Fernández *et al*. 2002; Zhang *et al*. 2003) up-regulate the expression of SOD, CAT, enzymes involved in glutathione biosynthesis and other antioxidant enzymes. Together, these actions enhance antioxidant defenses. The present results extend earlier observations by demonstrating induction of protection against lower concentrations of metal ions on major biosynthetic enzymes. Not shown in the diagram is that higher concentrations of metal ions inhibit these enzymes, deplete GSH and ATP, thus overwhelming antioxidant defenses. ROS accumulation results in toxicity-induced dysfunction in BPAEC monolayers. Our results suggest that similar mechanisms may serve to protect lung tissue, in general, against environmental insults from metal ions.

Acknowledgements

We thank Bill McAmis for his excellent assistance in performing the experiments and the various assays in the study. Thanks to Megan Titus for performing some of the early studies with cadmium and to Rebecca Leeper for her efforts in performing early studies with mercury.

References

Ciolino HP, Levine RL. 1997 Modification of proteins in endothelial cell death during oxidative stress. *Free Radic Biol Med* **22**, 1277–1282.

Ercal N, Gurer-Orhan H, Aykin-Burns N. 2001 Toxic metals and oxidative stress Part I: Mechanisms involved in metal induced oxidative damage. *Curr Top Med Chem* **1**, 529–539.

García-Fernández AJ, Bayoumi AE, Pérez-Pertejo Y, Motas M, Reguera RM, Ordóñez C, Balaña-Fouce R, Ordoñez D. 2002 Alterations of the glutathion-redox balance induced by metals in CHO-K1 cells. *Comp Biochem Physiol* **132**, 365–373.

Giedroc DP, Chen X, Apuy JL. 2001 Metal respone element (MRE)-binding transcription factor-1 (MTF-1): structure, function and regulation. *Antioxid Redox Signal* **3**, 577–596.

Halliwell B, Gutteridge JMC. 1999 Free Radicals in Biology and Medicine. Oxford: Oxford University Press.

Hui YY, McAmis WC, Baynes JW, Schaeffer RC Jr, Wolf MB. 2001 Effect of advanced glycation end products on oxidative stress in endothelial cells in culture: a warning on the use of cells studied in serum-free media. *Diabetologia* **44**, 1310–1317.

Järup L. 2003 Hazards of heavy metal contamination. *Brit Med Bull* **68**, 167–182.

Kaji T, Mishima A, Yamamoto C, Sakamoto M, Koizumi F. 1992 Effect of cadmium on the monolayer maintenance of vascular endothelial cells in culture. *Toxicology* **71**, 267–276.

Kamencik H, Lyon AL, Paterson PG, Juurlink HJ. 2000 Monochlorobimane fluorometric method to measure tissue glutathione. *Analyt Biochem* **286**, 35–37.

Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, Schilter B, Schlatter J, van Schothorst F, Vos JG, Wurtzen G. 2004 Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. *Food Chem Toxicol* **42**, 65–83.

Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P. 1997 Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* **185**, 1481–1486.

Lum H, Roebuck KA. 2001 Oxidant stress and endothelial dysfunction. *Am J Physiol* **280**, C719–C741.

McAmis WC, Schaeffer RC Jr, Baynes JW, Wolf MB. 2003 Menadione causes endothelial barrier failure by a direct effect on intracellular thiols, independent of reactive oxidant production. *Biochim Biophys Acta* **1641**, 43–53.

Murata M, Gong P, Suzuki K, Koizumi S. 1999 Differential metal response and regulation of heavy metal-inducible genes. *J Cell Physiol* **180**, 105–113.

Nolan CV, Shaikh ZA. 1986 The vascular endothelium as a target tissue in acute cadmium toxicity. *Life Sci* **39**, 1403–1409.

Pompella A, Visvikis A, Paolicchi A, DeTata V, Casini AF. 2003 The changing faces of glutathione, a cellular protagonist. *Biochem Pharmacol* **66**, 1499–1503.

Pulido MD, Parrish AR. 2003 Metal-induced apoptosis: mechanisms. *Mut Res* **533**, 227–241.

Stinson LJ, Darmon AJ, Dagnino L, D'Souza SJA. 2003 Delayed apoptosis post-cadmium injury in renal proximal tubule epithelial cells. *Am J Nephrol* **23**, 27–37.

Stohs SJ, Bagchi D. 1995 Oxidative mechanisms in the toxicity of heavy metals. *Free Radic Biol Med* **18**, 321–336.

Szuster-Ciesielska A, Stachura A, Slotwinska M, Kaminska T, Sniezko R, Paduch R, Abramczyk D, Filar J, Kandefer-Szerszen M. 2000 The inhibitory effect of zinc on

cadmium-induced cell apoptosis and reactive oxygen species (ROS) production in cell cultures. *Toxicology* **145**, 159–171.

Terminella C, Tollefson K, Kroczynski J, Pelli J, Cutaia M. 2002 Inhibition of apoptosis in pulmonary artery endothelial cells by altered pH, mitochondrial function and ATP supply. *Am J Physiol Lung Cell Mol Physiol* **283**, L1291–L1302.

Tian W-N, Braunstein LD, Apse K, Pang J, Rose M, Tian X, Stanton RC. 1999 Importance of glucose-6-phosphate dehydrogenase activity in cell death. *Am J Physiol* **276**, C1121–C1131.

Toimela T, Mäenpää H, Manerström M, Tähti H. 2004 Development of an in vitro blood–brain barrier model – cytotoxicity of mercury and aluminum. *Toxicol Appl Pharmacol* **195**, 73–82.

Valee BL, Ulmer DD. 1972 Biochemical effects of mercury, cadmium and lead. *Ann Rev Biochem* **41**, 91–128.

Wilson J, Winter M, Shasby DM. 1990 Oxidants, ATP depletion, and endothelial permeability to macromolecules. *Blood* **76**, 2578–2582.

Wolf MB, Baynes JW. 2006 The anti-cancer drug, doxorubicin, causes oxidative stress-induced endothelial dysfunction. *Biochim Biophys Acta* **1760**, 267–271.

Zhang B, Georgiev O, Hagmann M, Günes C, Cramer M, Faller P, Vasák M, Schaffner W. 2003 Activity of metal-responsive transcription factor 1 by toxic heavy metals and H2O2 in vitro is modulated by metallothionein. *Mol Cell Biol* **23**, 8471–8485.