THE PRO-ANTIOXIDANT ROLE OF ZINC SUPPLEMENTATION IN CADMIUM-TREATED CHOROID PLEXUS

A Thesis

by

SAMANTHA DANIELLE FRANCIS STUART

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Alice R.A. Villalobos
Committee Members,	Louise Abbott
	Yanan Tian
Chair of Toxicology Faculty,	Timothy Phillips

May 2014

Major Subject: Toxicology

ABSTRACT

Cadmium (Cd) is a toxic heavy metal with no known physiological function in higher order animals. Previous studies in primary cultures of neonatal rat choroid plexus (CP) epithelial cells indicated Cd induced oxidative stress and stimulated apical choline transport, and suggested zinc (Zn) supplementation might abate both oxidative stress and modulation of transport. The objective of this thesis was to elucidate how Zn, a nutritive mineral normally accumulated by CP, attenuated oxidative stress. I hypothesize that Zn, which can function as a pro-antioxidant, abates Cd-induced oxidative stress either by induction of metallothionein-1 (MT-1) or enhancement of glutathione (GSH) biochemistry. Thus, in primary cultures of neonatal rat CP epithelial cells, I characterized the effects of sub-micromolar Cd and efficacy of Zn supplementation to attenuate Cd-induced cellular and oxidative stress without or with manipulation of GSH synthesis. To characterize the Cd-induced stress response, CP epithelial cells were treated with 0 or 500 nM $CdCl_2$ in serum-free medium (SFM) for 12 h; samples were collected at 3, 6, 9, and 12 h. Induction of heme oxygenase-1 (HO-1), heat-shock protein 70 (HSP70), and metallothionein-1 (MT-1) in Cd-treated cells was compared to time-matched controls by immunoblot and qRT-PCR analyses. Cd induced the catalytic and modifier subunits of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis. To elucidate the effects Zn supplementation in Cd-treated cells depleted of GSH, cells were supplemented for 48 h with 0 or 25 μM $ZnCl_2$ alone or with 100 μM buthionine sulfoximine (BSO), an inhibitor of GCL, before treatment with 0 or 500 nM

$CdCl_2$ ± 100 μM BSO ± 10μM $ZnCl_2$ in SFM for 12 h. By luminescence assay, intracellular GSH and oxidized glutathione (GSSG) concentrations were measured. Cd increased intracellular GSH and GSSG, but markedly decreased GSH:GSSG ratio. Inhibition of GSH synthesis exacerbated Cd-induced stress. However, Zn supplementation attenuated the stress response irrespective of BSO treatment, as per decreased induction of HSP70. These data indicate that CP adapts to low-dose Cd by up-regulation of stress proteins and GSH synthesis. Zinc supplementation also may attenuate Cd-induced cellular and oxidative stress, but cytoprotection is independent of GSH status.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Alice Villalobos, and my committee members, Dr. Louise Abbott and Dr. Yanan Tian, for their guidance and support throughout the course of this research.

Thanks also go to my lab-mates and friends, Robin Young and Mayra Aquino, for all their help during experiments and encouragement.

NOMENCLATURE

BSO	Buthionine-sulfoximine
Cd	Cadmium
CP	Choroid Plexus
GCLC	Glutamate-cysteine ligase, catalytic subunit
GCLM	Glutamate-cysteine ligase, modifier subunit
GSH	Glutathione
GSSG	Oxidized glutathione
HO-1	Hemeoxygenase-1
HSP70	Heat shock protein 70
MT-1	Metallothionein-1
SOD1	Superoxide dismutase-1
Zn	Zinc

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

The choroid plexus (CP) epithelial tissues within each of the four brain ventricles collectively form the barrier between the peripheral blood and cerebrospinal fluid (CSF), *i.e.,* the blood-CSF barrier. These epithelia secrete CSF and regulate fluid/electrolyte balance and composition of CSF, which is continuous with the extracellular fluid that bathes the neurons of the brain. The ability of the central nervous system (CNS) to respond to neurotransmitters is contingent upon the CP maintaining CSF homeostasis[23]. By specific ion channels and carrier- and receptor-mediated transport systems, CP epithelia regulate nutrient availability and metabolic waste and toxicant accumulation in CSF. These tissues metabolize xenobiotics and synthesize and secrete many bioactive proteins into CSF[18]. Blood flow per gram tissue in CP is nearly twice that in the cerebral cortex or liver[13]. Mitochondria comprise up to 15% of CP epithelia volume, continually produce ATP to fuel CSF secretion, active solute transport between blood and CSF, and protein synthesis. Given its high metabolic rate, CP produces large amounts of reactive oxygen species (ROS), and is prone to increased intracellular ROS accumulation and subsequent induction of oxidative stress when exposed to pro-oxidant agents[7,10,14]. CP accumulates trace nutrient minerals, *e.g.,* iron (Fe) and copper (Cu), and contaminant heavy metals, *e.g.,* mercury (Hg) and cadmium (Cd). As such, it is described as a "sink" for both essential and non-essential trace metals in the brain, protecting the CNS from these potential neurotoxins[13,18,23,55]. However, Fe and Cu are Fenton metals that can directly generate free radicals, and non-Fenton metals, such as Cd, can indirectly

increase ROS production and accumulation[37]. Under conditions of cellular stress, the capacity of CP epithelia to transport solute may be up- or down-regulated. In cultured CP epithelia, a low-dose Cd exposure that induced oxidative stress also increased apical uptake of choline, a precursor to the neurotransmitter acetylcholine[15,20]. Nevertheless, mechanisms that facilitate adaptation of CP epithelia to cellular stress induced by pro-oxidants, particularly heavy metals, are not fully understood. The goal of this thesis is to elucidate how CP adapts to cellular stress, as this might compromise the capacity of CP to regulate CSF homeostasis and, in turn, disrupt CNS homeostasis.

Cadmium is a toxic heavy metal with no known physiological function in higher order animals. Similar to Zn and Hg, Cd is not a "true" transition metal; it does not have partially filled *d* and *f* electron shells as transition metals have. Cadmium is found naturally in the earth's crust, is a minor component of Zn ores, and was first discovered as a byproduct of Zn production[21]. Cadmium is also used in electroplating and batteries and provides vibrancy and prevents corrosion in industrial paints and coatings. The metal is accumulated by starchy root plants, mollusks, and grains and thus, is present in many food sources. Cadmium is accumulated by the tobacco plant and is present in high amounts in cigarettes and cigarette smoke[1,11,14,21,34,44]. One cigarette may contain approximately 2 μg of Cd, 100 ng of which is absorbed directly into the systemic circulation[34]. Thus, smokers have much higher blood Cd concentrations and greater body burdens than do nonsmokers, even taking into account second-hand smoke exposure. Inhalation is a direct route of Cd absorption; thus, individuals frequently exposed to second-hand smoke might also have elevated blood Cd concentrations. With a half-life

of 15–30 years, Cd is considered a persistent environmental pollutant that can be bioaccumulated[11]. The Occupational Safety and Health Administration set the eight-hour permissible exposure limit for Cd to 5 $\mu g/m^3$, which is much lower than limits for other toxic metals such as 50 $\mu g/m^3$ for lead. With such a low exposure limit, Cd overexposure can occur even with trace amounts[39]. The best known large-scale Cd exposure occurred in Toyama Prefecture, Japan from 1910–1945. For many years Mitsui Mining and Smelting Co., Ltd. dumped industrial waste containing Cd and other trace metals into the Jinzu River. This water was used to irrigate adjacent rice patties consequently contaminating the rice with heavy metals. The population consuming the rice was chronically exposed to Cd. Termed the "Itai-itai disease" (translated as "ouch-ouch disease"), this Cd-induced toxicity was characterized by severe bone decalcification and fracturing[3]. It was hypothesized that Cd impaired renal Ca reabsorption; this lead to increased urinary Ca excretion and subsequent increased compensatory Ca absorption from bone to maintain plasma Ca levels[51]. However, Cd is also known to disrupt cellular redox balance by initiating pro-oxidant cascades[11]. Cd indirectly increases production and/or accumulation of reactive oxygen species (ROS) by displacing Fenton metals Fe or Cu, inhibiting complex III in the electron transport chain (ETC), and/or depleting glutathione (GSH)[11,37].

Production of ROS and reactive nitrogen species (RNS) is tightly regulated by enzymes such as nitric oxide synthase or NAD(P)H oxidases. When excess ROS accumulate, as a product of electron transport or overstimulation of NAD(P)H, the collective accumulation and subsequent effects of ROS in the cell is termed "oxidative

stress". This stress can have serious effects such as damaging cell structures, lipids, proteins, and DNA[11,24,25].

Although small amounts of ROS/RNS may be beneficial to the cell, maintenance of cellular redox balance is extremely crucial to prevent damage. Glutathione (GSH) is the most abundant intracellular thiol and antioxidant. The importance of GSH is prodigious. It is critical for the detoxification of electrophiles. As such, one of its main actions is to scavenge free radicals, thereby maintaining optimum cellular redox status. GSH, along with glutathione-S-transferases, is a part of a phase II pathway that detoxifies agents by conjugation; these GSH-conjugates then enter the mercapturic acid pathway before being excreted in urine. Through its antioxidant activities, GSH abates oxidative damage by superoxides and hydrogen peroxide. GSH donates electrons to reactive species, forming GSSG, a dimer of oxidized GSH. This dimer can be converted back to GSH by GSSG reductase with the co-enzyme NADH. The intracellular GSH:GSSG ratio at balanced redox state is typically very high; however under conditions of oxidative stress this ratio decreases and therefore, is an index of oxidative stress. In cells, most GSH is located in the cytosol with small amounts in the mitochondria and endoplasmic reticulum[2,11,12,19,25].

Synthesis of GSH is a multi-step process. The first and rate-limiting reaction is the ATP-dependent linkage of a glutamate and a cysteine residue; glutamate-cysteine ligase [GCL] (a.k.a. γ-glutamylcysteine synthetase) catalyzes this reaction and requires either Mg or Mn as a cofactor. The second step, also ATP-dependent, is the addition of a glycine residue onto the glutamyl-cysteine residue; this reaction is catalyzed by

glutathione synthase (GS). However, over-expression of GS does not increase overall GSH production, as does over-expression of GCL[11,12,16,19,25,37]. Redox homeostasis is controlled predominantly by GSH. Both GSH and thioredoxin (TRX) neutralize reactive species and the mixed disulfides formed from this process, primarily GSSG, act as non-specific signaling molecules. Although these are the main antioxidants, there are other compounds, *e.g.,* superoxide dismutases, various amino acids, peptides, and proteins, which in high concentrations can scavenge free radicals[25,49]. When redox balance is disturbed, either by uncontrolled accumulation of ROS/RNS or depletion of antioxidants, the cell must compensate for this stress by other means.

Among the best-known target organs of Cd are the kidneys, liver, and gonads[11,12]. However, similar to hepatic and renal epithelia, the CP also accumulates Cd. *In vivo* chronic, low dose Cd exposure leads to measurable Cd accumulation in CP and notable morphological changes in the epithelia[50]. In the CP epithelium, which naturally produces large amounts of ROS, a pro-oxidant such as Cd could disrupt redox balance and induce oxidative stress. In primary cultures of choroid plexus epithelia low-dose Cd exposure stimulates the apical uptake of choline[52]. Supplementation with N-acetylcysteine (NAC), a precursor to GSH, abated stimulation of uptake, whereas buthionine-sulfoximine, a pharmacological inhibitor of GSH synthesis, enhanced stimulation of uptake by Cd. This suggested GSH is a critical factor in the cellular adaptive response to Cd exposure.

Zinc is among the essential nutrients normally accumulated by CP and is accumulated to levels higher than that by kidney or liver (39 μg/g tissue for CP versus

23-24 μg/g tissue for kidney/liver)[8,54]. Zinc is an essential cofactor or structural stabilizer required for many enzymes, such as glutamate dehydrogenase, superoxide dismutase, and alkaline phosphatase and regulates transcription, cell proliferation, and cellular signaling[24,37,38]. Zinc is also the cofactor for carbonic anhydrase, the enzyme necessary for CSF production by CP. However, Zn is also a "pro-antioxidant" in that it promotes antioxidant activities by several different mechanisms. Zinc protects sulfhydryl groups from attack from free radicals and abridges reactive species formation through antagonism of redox-active metals, such as Fe and Cu[24]. Metallothionein (MT-1), a metal binding protein first discovered for its high affinity for Zn ions, can bind other divalent metals, such as Cd, and is readily induced by both Zn and/or Cd. Metallothionein is critical to regulation of intracellular Zn concentrations and possibly, minimizing Cd cytotoxicity[3,6,48].

Zinc supplementation can abate Cd-induced oxidative stress. In mice injected with Cd without or with Zn for 24 h or 14 d, co-treatment with Zn protected against Cd-induced oxidative stress[5]. Co-treatment with Zn also maintained GSH at levels comparable to those in controls at 24 h and attenuated lipid peroxidation at both 24 h and 14 d. In another study, *Lemna minor* (common duckweed) was treated with Cd without or with Zn[4]. Zinc supplementation decreased lipid and protein oxidation and decreased catalase and peroxidase activities. Omata et al. demonstrated in both neuronal cells and fetal brains Zn deficiency reduced GCLC and GCLM expression, as well as overall GSH production. Although GCLC/M were partially up-regulated after dopamine exposure,

GSH levels did not increase. It was shown that Zn deficiency increased GCLC/M protein and gene expression and impaired Nrf-2, the transcription factor for GCL[38].

The third National Health and Nutritional Examination study (NHANES III) indicated smokers with deficient Zn intake had higher body burdens of Cd and higher risks of COPD than did smokers with adequate Zn intake[34]. Zinc-mediated protection, however, has not been studied in the CP, despite its vulnerability to oxidative stress, marked capacity to accumulate Zn, and importance to homeostasis of the brain milieu. Zinc may protect against oxidative stress in CP epithelia. Protection is not completely characterized, but possible mechanisms include the following. Zinc transporters may mediate cellular uptake of Cd. If this is the case, Zn may directly inhibit and decrease cellular uptake of Cd and therefore, decrease Cd-induced oxidative stress[12,34]. Second, Zn supplementation can induce MT-1 prior to Cd exposure, such that more Cd binds MT-1 and thereby decreasing intracellular free Cd and its capacity to induce oxidative stress[6,24,37]. Lastly, it is possible Zn, which is critical for many enzymatic reactions and antioxidant systems, may have a complimentary role in the antioxidant activities of GSH[38].

The overall goal of this thesis project is to characterize the role of Zn in protection against oxidative stress induced by low-dose Cd exposure in CP.
The three main objectives of this thesis are as follows. The first objective is to characterize the cellular stress response of the CP elicited by Cd exposure. *I hypothesize* that *in vitro* exposure to sub-micromolar concentrations of Cd will induce a cellular stress response characterized by induction of gene and/or protein expression of HSP70,

HO-1, MT-1. The second objective is to determine the role of GSH in the adaptation of CP to Cd-induced oxidative stress. *I hypothesize* the low-dose exposure to Cd will result in a decreased GSH:GSSG ratio, a marker for oxidative stress. While this ratio may be reduced, I also hypothesize that Cd exposure will up-regulate GSH synthesis by inducing gene and/or protein expression of rate-limiting enzymes responsible for mediating glutathione synthesis (GCL) as well as other enzymes responsible for abating oxidative stress (SOD1). The third objective is to determine how Zn supplementation protects against Cd-induced oxidative stress in CP. *I hypothesize* that Zn supplementation will attenuate oxidative stress, not by decreasing Cd accumulation, but by inducing MT-1. Furthermore, I hypothesize Zn supplementation will abate Cd-induced GSH depletion and subsequent changes in regulation of GSH synthesis.

2. MATERIALS AND METHODS

2.1 *Animal care and use*

Time-pregnant rats (Charles River, Roanoke, IL) were held in the Comparative Medicine Program Laboratory Animal Resources and Research (LARR) facility at Texas A&M University with 12-h light/dark cycle. Animals had access to a regular chow diet and water *ad libitum*. Timed-pregnant dams were obtained at approximately gestation day (GD) 16 and littered out between GD 21-22. With approval from the Institutional Animal Care and Use Committee at Texas A&M University (Protocol #2011-128), neonatal rats were collected between post-natal day (PND) 2-5 and choroid plexus (CP) tissues were harvested from the brains of neonatal rats using ethanol-sterilized instruments, and primary cultures of harvest tissues.

2.2 *Reagents*

All chemicals were analytical grade and purchased from commercial vendors. The following reagents and kits were most critical to this study and purchased from the respective vendors: cadmium chloride and zinc chloride, Sigma (St. Louis, MO); buthionine sulphoximine, Acros Organics (Morris Plains, NJ); and GSH/GSSG-Glo™ Assay (Promega, Madison, WI).

2.3 *Lateral and fourth choroid plexus tissue harvest*

Lateral and fourth CP tissues were harvested on PND 2-3. Pups were decapitated and brains quickly removed. Brains were placed on filter paper then rinsed with sterile, ice cold PBS. Choroid plexus tissues were removed under a dissecting microscope and

pooled in chilled, sterile collection medium. Tissues were then digested to isolate epithelial cells for preparation of primary cell cultures used for characterization of cellular effects of *in vitro* Cd-exposure.

2.4 *Primary cell culture of choroid plexus epithelial cells*

Epithelial cells were isolated from CP tissues using aseptic techniques. Tissues were left to settle to the bottom of the collection tube before collection medium was removed. 2.5 mL of cold sterile dissociation media (per L H2O: 8.0 g NaCl, 0.20 g KCl, 0.10 g Na_2HPO_4, and 2.38 g HEPES; pH: 7.5) with 10 mg glucose, 10 mg pronase, 40,000 KU DNAse I per 10 mL was added to the collection tube with tissues and incubated for 2.5 min in a 37˚C-shaking water bath (80 shakes/min). The tissue mixture was triturated 10 times with a fire-polished Pasteur pipette. Another 2.5 mL of dissociation medium was added, and the mixture was incubated for 8 min at 37˚C in a shaking water bath (80 shakes/min). The mixture was triturated 10 times before returned to the water bath for 2 min. Then, the mixture was triturated 10 times, and the cell suspension was filtered through a cell strainer (100 µm mesh) with 25-30 mL penicillin-supplemented DMEM-F12 (100 units penicillin per 100 mL DMEM-F12. The filtered cell suspension was centrifuged (650 RPM, 4 min, 4˚C). Supernatant was discarded; the cellular pellet was re-suspended completely in 25 mL DMEM-F12/penicillin and centrifuged again (650 RPM, 3 min, 4˚C). The supernatant was discarded. The cellular pellet was re-suspended completely in 3.0 mL DMEM-F12/penicillin and plated in a 35-mm petri dish, along with 300 µL NuSerum IV and incubated for 3 h. All incubations were at 37˚C in a humidified environment of 95% air/5% CO_2. After incubation all cells

were aspirated from the petri dish and transferred to a sterile 50-mL centrifuge tube. The petri dish was rinsed with DMEM-F12/ penicillin, and these rinses were combined with the initial cell suspension in the collection tube. Cells were centrifuged (650 RPM, 3 min, 4˚C), and supernatant was discarded. Cells were re-suspended with 2000 µL plating medium (D-Val MEM, 10% NuSerum IV, 100 ng/mL PGE1, 10 µM Forskolin, 1.5 µM Triiodothryonine, and 50 ng/mL EGF). Each neonatal rat provides approximately 235,000 cells. Cells were plated at 300,000 cells/cm^2 in sterile polystyrene tissue culture plates or glass tissue culture slides. For cells plated in 12-well plates, total volume was increased by approximately 25% with maintenance media added drop-wise to disperse cells. Seventy-two hours post-plating, the initial plating medium was removed from all cells and replaced with maintenance medium (DMEM-F12, 5% NuSerum IV, 100 ng/mL PGE1, 10 µM Forskolin, 1.5 µM Triiodothryonine, and 50 ng/mL EGF). Medium was replaced every two days until start of experimental treatment. Cells formed fully differentiated, confluent monolayers by post-plating day 5.

2.5 In vitro *treatment of primary cell cultures of choroid plexus and isolated choroid plexus tissues with cadmium, zinc, or buthionine sulphoximine (BSO)*

Primary cultures of CP epithelial cells and intact CP tissues were treated with Cd, Zn, and/or BSO. Solutions of $ZnCl_2$ and $CdCl_2$ were prepared in sterile water weekly and every two weeks respectively. Stock solution of $ZnCl_2$ (approx. 3 mM) was used directly to prepare treatment media, whereas a working stock of $CdCl_2$ (approx. 1.5 mM) was initially prepared in medium that was then used directly to make treatment media. Stock solution of BSO (approx. 71.5 mM in sterile H_2O) was used directly to make

treatment media. Experimental treatment of primary cultures of CP was initiated 5-6 days post-plating. For *in vitro* Zn supplementation, cells were treated with 25 μM $ZnCl_2$ in maintenance medium (DMEM-F12/5% Nu Serum + growth factors) for 48 h before treatment with BSO or Cd. If cells were treated with BSO, they were treated with 100 μM BSO without or with 10 μM $ZnCl_2$ in serum-free media for 12 h after the initial 48-h supplementation period. For these experiments cells were treated with either 250 or 500 nM $CdCl_2$ for 12 h alone or with 10 μM $ZnCl_2$, 100 μM BSO, or both 10 μM $ZnCl_2$ and 100 μM BSO. Intact tissue samples were treated without or with 250-500 nM $CdCl_2$ in serum-free media for 24 h. Intact tissues used for *in vitro* Cd-exposure studies were harvested as stated above, and CP tissues were pooled together in DMEM-F12 with penicillin. One sample was comprised of pooled fourth and lateral CP tissues from 3- 4 animals. Samples were rinsed several times with PBS/Penicillin (approx. 630 mg Penicillin per 100 mL PBS). After rinsing, tissues were placed in sterile 6-well plates with permeable supports in serum free media to equilibrate for 2 h in a humidified environment at 37˚C in 95% air:5% CO_2 before treatment with Cd. Pre-equilibration medium was replaced with 4 mL of fresh serum-free medium without (control) or with 500 nM $CdCl_2$; tissues were incubated at 37˚C in humidified 95% air:5% CO_2 for 24 h.

2.6 *Total glutathione and oxidized glutathione assay*

Changes in intracellular reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations were measured using the GSH/GSSG-Glo™ Assay (Promega, Madison, WI). Following the manufacturer's protocol for adherent cells, cells were plated in flat-bottomed, white-walled 96-well plates (Corning, VWR, Suwanne, GA) and

rinsed 3X with Hank's Buffered Salt solution. Cells were lysed with 50 µL of either Total Glutathione Reagent or Oxidized Glutathione Reagent and rocked vigorously for 5 min. After lysing, 50 µL of Luciferin Generation Reagent was added to each well, and the plate was incubated at room temperature (RT; 30 min). Then 100 µL Luciferin Detection Reagent was added to each well, and the plate was incubated (RT, 45 min) before luminescence was determined by a luminometer (Tecan Infinite M200, Morrisville, NC). To quantify intracellular GSH and GSSG concentrations and calculate GSH/GSSG ratios, relative light units (RLU) of each sample was compared to RLU determined from the set of GSH standards; standards were prepared at 16, 8, 4, 2, 1, 0.50, 0.25, and 0.00 µM GSH. GSSG concentrations were also obtained by the GSH standard curve. Stoichiometrically, two moles of GSH are produced for every one mole of GSSG; therefore, GSSG concentration is calculated by dividing GSH concentration by two.

2.7 *Western blot analysis*

Total protein expression was determined by Western blot analysis. Cells were plated in clear, flat-bottomed 48-well or 96-well plates. Cells were first rinsed once with chilled PBS/0.5% Triton X-100 with a cocktail of phosphatase inhibitors and protease inhibitors, and then lysed on ice with 50 or 25 µL of lysis buffer (50 mM TRIS-HCl pH 6.8, 100 mM DTT, 30% v/v glycerol, 2% w/v SDS, 0.05% v/v Triton X-100, 0.5% w/v bromphenol blue). Samples were collected, heat-denatured, sonicated, and centrifuged (10,000 g, 5 min) before storage in -20˚C. Cellular proteins were electrophoresed by 10% SDS-PAGE; 7 µL of each sample along with colored molecular weight markers

were loaded ino individual wells. Electrophoresis was initiated at 36V; voltage was gradually increased to 140V. After electrophoresis, gels were equilibrated (15 min) in transfer buffer (25 mM Tris base, 192 mM glycine, 10% methanol). Proteins were electroblotted onto polyvinylidene fluoride (PVDF) membrane by tank transfer (0.15 A, 120 min). Membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and 5% or 10% non-fat dry milk (NFDM) for 1 h prior to rocking incubation with primary antibody for 2 h at RT or 18 h at 4˚C. Membranes were then vigorously rinsed with TBS-T (3 x 10 min) before 90-min incubation (RT) with goat derived secondary antibody conjugated with alkaline-phosphatase (AP) or horseradish peroxidase (HRP). Membranes were rinsed vigorously with TBS-T (3 x 10 min) before immunoreactivity detection.

Protein bands were visualized by chromogenic staining with AP substrate 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT; Promega, Madison, WI) or enhanced chemiluminescence (ECL) with horseradish peroxidase (HRP) substrate (Millipore, Bedford, MA). Protein bands were imaged and the intensity of each band was determined by densitometry with an Alpha Innotech gel documentation system with FlurChem HD2 software (ProteinSimple, Santa Clara, CA); intensity of test proteins were normalized to actin protein expression. Blots that were to be probed for a second protein of interest were stripped of primary and secondary antibodies with buffer made from SDS and glycine with a pH of 2. Primary antibodies against actin, hemeoxygenase-1, heat shock protein 70, and NQO-1 (Enzo Life Sciences, Farmingdale, NY) were used at 1:1000 in 10% NFDM/TBS-T; primary antibodies

against GCLC and GCLM (Abcam, Cambridge, MA) were used at 1:500 in 5% NFDM/TBS-T. SOD1 primary antibody (Abcam, Cambridge, MA) was used at 1:1000 in 5% NFDM/TBS-T. Secondary antibodies conjugated with HRP and AP were used at 1:5000 in either 10 or 5% NDFM/TBS-T depending on primary antibody.

2.8 *Extracellular lactase dehydrogenase assay*

Cytotoxicity was evaluated based on extracellular lactate dehydrogenase (LDH) released from non-treated control cells and experimental cells as determined using a commercial kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison WI). Samples were analyzed in triplicate as per manufacturer's protocol. Cells were plated in 48-well plates. Maximum LDH release was determined in non-treated control cells lysed with 0.9% v/v Triton X-100 (45 min, 37˚C). After treatment, 50 µL treatment medium from each control and experimental condition and fresh cell-free DMEM-F12 medium was transferred to individual wells of new 96-well plate. 50 µL Substrate Solution were added to each sample; the mixture was incubated in the dark (10 min, RT). Because samples were assayed in triplicate, there was an intentional 20-sec delay between each set. 50 µL Stop Solution was added to each well, conserving the same time delays. Absorbance was recorded at 490 nm at room temperature using a plate reader (Tecan Infinite M200, Morrisville, NC).

2.9 *Qualitative real-time polymerase chain reaction (qRT-PCR)*

Total RNA was extracted from primary cultures of neonatal CP and from isolated CP tissues with an RNeasy mini kit and a QIAshredder (Qiagen, Valencia, CA). Gene expression was analyzed in cells plated in clear, flat-bottomed 12-well plates. After

treatment, cells were rinsed once with PBS and then gently triturated (approx. 30 s) with 500 µL RLT lysis buffer/1% β-ME. For tissues, after treatment tissues were removed from treatment medium, placed in a 1.7-mL tube with 500 µL PBS and rinsed by gentle inversion. Samples were centrifuged (10,000 rpm, 30 s) before PBS was removed carefully and replaced with 600 µL RLT/1% β-ME in which tissue was gently triturated until moderate homogeneity was achieved. Total RNA was then isolated from tissue lysate or cell lysate by the following procedure. Lysate was transferred into a QiaShredder homogenization column and centrifuged (13,000 rpm, 2 min). Samples were stored at -80˚C until RNA extraction continued at a later time. Samples were then diluted (1:1) with 70% EtOH, loaded onto an RNeasy column, and centrifuged (10,000 rpm, 30 s). The supernatant was discarded. The column was rinsed with 350 µL RW1 buffer and centrifuged (10,000 rpm, 30 s); the supernatant was discarded. Then 80 µL DNase 1:RDD buffer [1:8 dilution] (Qiagen, Valencia, CA) was placed directly onto the center of the column and incubated (15 min, RT). The column was then rinsed with 350 µL RW1 buffer and centrifuged (10,000 rpm, 30 s). The column was transferred to a new 2-mL tube and rinsed twice with 500 µL RPE buffer. Total RNA was eluted with 30 µL nuclease free water and centrifugation (10,000 rpm, 1 min) into a clean 2-mL tube. From each sample, 5 µL was collected for nanospectroscopic determination of absorbance ratios at 260nm and 280nm (A260/A280) and 260nm and 230nm (A260/A230). A260/A280 ratios less than 1.8 indicate protein or phenol contamination; A260/A230 ratios less than 2.0 indicate DNA contamination. After nanospectroscopic analysis, the remaining sample could be stored at -80˚C. From each sample, cDNA was

prepared using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA); the final cDNA concentration was 0.025 μg/μL in a total volume of 40 μL. Primers for rat MT-1, HO-1, HSP70, GCLC, GCLM, and GSTa4 were designed by Primer Express software (PE Applied Biosystems) based on registered GenBank sequences (Table 1). Primers were synthesized by Integrated DNA Technologies (Coralville, IA) and tested using cDNA from samples of untreated primary cultures of neonatal CP. These tests ensured primer-primer dimerization did not occur. Expression of MT-1, HSP70, HO-1, GCLC, GCLM, and GSTa4 genes in each sample of cDNA were analyzed in triplicate by qRT-PCR with SYBR green detection by a MyQ single-color real-time detection system (BioRad, Hercules, CA). Copy number for each test gene, actin, and GAPDH was determined; copy number of each test gene was normalized to the integrated values for actin and GAPDH gene expression. Specifications for qRT-PCR analysis: initial denaturation: 10 min, 95˚C; amplification and quantification (45 cycles): 15 s, 95˚C; 30 s, 60˚C; melt curve: 55˚C-95˚C.

Table 1: Primers used for determination of gene expression in *in vitro* experimental models for choroid plexus; gene name, respective PubMed GeneBank ID number and forward and reverse primer sequences.

Gene	GeneBank ID	Forward Primer (5' – 3')	Reverse Primer (3' – 5')
β-Actin	NM_031144	ATGGTGGGTATGGGTCAG	TACTTCAGGGTCAGGATGC
CAII	NM_019291	ATGACCCTTCCCTACAGC	GAGCCCCAGTGAAAGTGA
GAPDH	NM_017008	ATGACTCTACCCACGGC	ACTCAGCACCAGCATCA
GCLC	NM_012815	GCTTTCTCCTACCTGTTTCTTG	TGGCAGAGTTCAGTTCCG
GCLM	NM_017305	TGTGATGCCACCAGATTTGA	TGGAAACTTGCCTCAGAGAG
GSTa4	NM_001106840	AGAGGCATTTCTTGTTGGCA	CAGGAGCACTGACTTCTTCC
HO-1	NM_012580	ACCCCACAAGTTCAAACAG	CCTCTGGCGAAGAACTCTG
HSP70	NM_153629	ATGGGGGACAAGTCGGA	GTGGGGATGGTGGAGTT
MT-1	NM_138826	CACCGTTGCTCCAGATTCA	CAGCAGCACTGTTCGTCA
SOD1	NM_017050	TTGTGGTGTCAGGACAGATT	CAGTGGTACAGCCTTGTTA

2.10 *Fluorescence immunocytochemistry*

Cells were plated in 4-well glass tissue culture slides. After treatment, cells were washed with PBS and then fixed in 2% formaldehyde/0.1% glutaraldehyde/0.1% Triton-X in PBS (400 μL/well) while rocking (10 min, RT). Cells were rinsed while rocking with PBS (2 x 2 min). Slides then were immersed in chilled acetone (-20˚C, 3 min) before being blocked with 1% BSA/PBS (30 min, RT). Cells were incubated while rocking with primary antibody (1:200 in 1% BSA/PBS; 45 min, RT). Cells were then washed with 1% BSA/PBS (3 x 2 min) and then incubated while rocking with fluorescent secondary antibody (1:200, Alexa Fluor, Molecular Probes-Invitrogen, Eugene, OR) in 1% BSA/PBS (30 min, RT). Cells were washed in 1% BSA/PBS (3 x 10 min) before a coverslip was mounted with SlowFade® Gold Antifade Reagent (Molecular Probes-Invitrogen, Eugene, OR), a liquid mountant that contained the nuclear stain, 4',6-diamidino-2-phenylindole (DAPI). Fixed cells were viewed by an inverted epi-fluorescence microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) fitted with a halogen lamp, mercury illuminator (HBO 100 mercury lamp), 63X oil objective and fluorescein isothiocyanate (FITC)/tetramethylrhodamine isothiocyanate (TRITC)/UV filter set and using AxioCam-MRc digital camera and software (Carl Zeiss, Oberkochen, Germany).

2.11 *Inductively coupled plasma-mass spectrometry (ICP-MS)*

Total accumulation of elemental Cd was measured in cells plated in 12-well plates. At the end of treatment, media were removed and transferred in labeled pre-weighed 15-mL tubes; final weights were recorded (± 0.1 mg). Cells were rinsed twice

with chilled PBS containing 1 mM EDTA and then rinsed once with chilled PBS (no EDTA). The final PBS rinse was removed, and cells within the original culture plate were left to dry at room temperature. Air-dried cells were solubilized in 200 µL concentrated nitric acid (Ultrex grade); (2 h, RT). From each control and treatment well, the entire cell suspension was aspirated and transferred to a pre-weighed 15-mL tube; each well was rinsed with ultra-pure water several times, and each rinse was transferred to the 15-ml tube. Additional water was added to the tube for a final volume of approximately 10 mL (final nitric acid concentration of 2%), and the final weight was recorded (± 0.1 mg). Samples of media removed from the cells and of stock media prepared for treatment were collected but were not diluted. Samples were submitted to Trace Element Research Laboratory (College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX) for analysis of elemental Cd by ICP-MS by Dr. Robert J. Taylor.

2.12 *Statistics*

Results shown are expressed as means ± SE. Control and experimental means from experiments that evaluated time-dependent effects of cadmium on gene expression and protein expression in cultured CP cells were first compared by two-way analysis of variance (ANOVA) or ANOVA with the appropriate post hoc test. If significant difference in the main effect of time was determined, then one-way ANOVA was performed to identify differences across time points within each separate treatment group. If significant difference in the main effect of treatment was determined, then a t-test was performed to compare control and experimental means at each time point to

identify differences between treatment groups. Control and experimental means from experiments that investigated the effects of zinc on cadmium accumulation in cultured cells were compared by one-way ANOVA with a Tukey-Kramer post hoc test. Control and experimental means from experiments that investigated the effects of cadmium with manipulation of glutathione synthesis and zinc supplementation on gene and protein expression and other parameters were compared by one-way ANOVA with a Tukey-Kramer post hoc test. Differences were deemed significant at $P \leq 0.05$.

3. RESULTS

3.1 *Cadmium induced HSP70, HO-1, MT-1, SOD1, GCLC, and GCLM in a time-dependent manner*

To characterize time-dependent induction of specific cellular stress response proteins and genes and cytotoxicity elicited by low dose Cd treatment, primary cultures of CP epithelium were exposed to 500 nM $CdCl_2$ in serum free media for 0-12 h. Cadmium treated cells and time-matched controls were collected at 3, 6, 9, and 12 h after initiation of treatment (Fig. 1). Tracking time-dependent induction of stress proteins, permitted investigation of the progression of cellular stress responses throughout the duration of the exposure, rather than only quantifying the response at a single time point at the end of exposure. Heat-shock protein 70 (HSP70) is a hallmark cellular stress response protein that is induced in response to cellular stress and oxidative stress[28]. Hemeoxygenase-1 (HO-1) is another cellular stress response protein induced under conditions of oxidative stress. However, HO-1 can also be induced by exposure to heavy metals and is directly induced by Cd[46]. Metallothionein-1 (MT-1) is a metal binding protein with a high affinity for divalent metals and known to bind and sequester Cd[6]. It is possible that these proteins are up-regulated by Cd and may therefore, protect against cellular damage or dysfunction. Superoxide dismutase 1 (SOD1) binds to its cofactors Zn and copper, and neutralizes superoxide radicals[17]. Glutathione (GSH) is the most abundant intracellular thiol and antioxidant. Glutamate-cysteine ligase (a.k.a. gamma-glutamylcysteine synthetase) catalyzes the first and rate-limiting step of GSH

synthesis, *i.e.*, ATP-dependent linking of a glutamate and a cysteine residue. Glutamate-cysteine ligase (GCL) has two subunits, the catalytic subunit (GCLC) and the modifier subunit (GCLM). GCLC exhibits all catalytic activity of the enzyme and is subject to feedback inhibition by GSH. GCLM, while not enzymatically active, lowers the Michaelis-Menten constant (K_m) of the enzyme such that the reaction can proceed more efficiently and at lower concentrations of the substrates (*i.e.*, glutamate or cysteine)[11,12,19,25,37]. SOD1, GCLC and GCLM were analyzed to evaluate the Cd-induced stress response, but also identify possible compensatory mechanisms that occur during cellular adaptation to Cd-induced oxidative stress.

RNA extracted from both cells and tissues was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) for HSP70, HO-1, MT-1, SOD1, GCLC, and GCLM gene expression (Fig. 1A, 1B). These values were normalized to β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. At 3 h post-treatment HSP70 induction was approximately 2-fold that of control. Expression increased to approximately 8-fold at 6 h and 9 h before decreasing to 3-fold at 12 h. HO-1 gene expression was induced 5-fold at 3 h as compared to control and progressively increased 12-, 56- and 64-fold at 6 h, 9 h, and 12 h. MT-1 gene expression followed a similar pattern. MT-1 mRNA was induced approximately 4-fold at 3 h and continually increased, reaching 13-, 16- and 54-fold at 6 h, 9 h, and 12 h. This time course was an important tool for this study, because it demonstrated the sensitivity of this model of CP to low-dose Cd exposure. Unlike MT-1 and HO-1, induction of HSP70 gene expression did not continually increase over the duration of Cd exposure. This demonstrated the

ability of CP primary cultures to adapt to low dose Cd exposure and identified proteins in the stress proteome that may function as “first responders” and may be critical to cellular adaptation to prolonged Cd exposure.

To assess induction of oxidative stress and possible adaptive changes in GSH synthesis, changes in gene expression of SOD1, GCLC, and GCLM were also analyzed in response to Cd over the 12-h time course (Fig. 1B). As compared to untreated control cells, SOD1 gene expression increased 1.25-fold at 3 h, 1.8-fold at 6 h and peaked at 9 h at 2.75-fold before declining to 1.5-fold at 12 h. By comparison, Cd induced GCLC and GCLM gene expression more substantially. At 3 h GCLC was induced approximately 2-fold as compared to controls and remained at this level before increasing to 5-fold at 9 h and to 6.5-fold at 12 h. GCLM gene expression was induced as early as 3 h to approximately 2-fold as compared to control and 7-fold at 6 h; gene expression peaked at 9 h with an 11.5-fold increase and remained elevated at 12 h but only 7-fold greater than control.

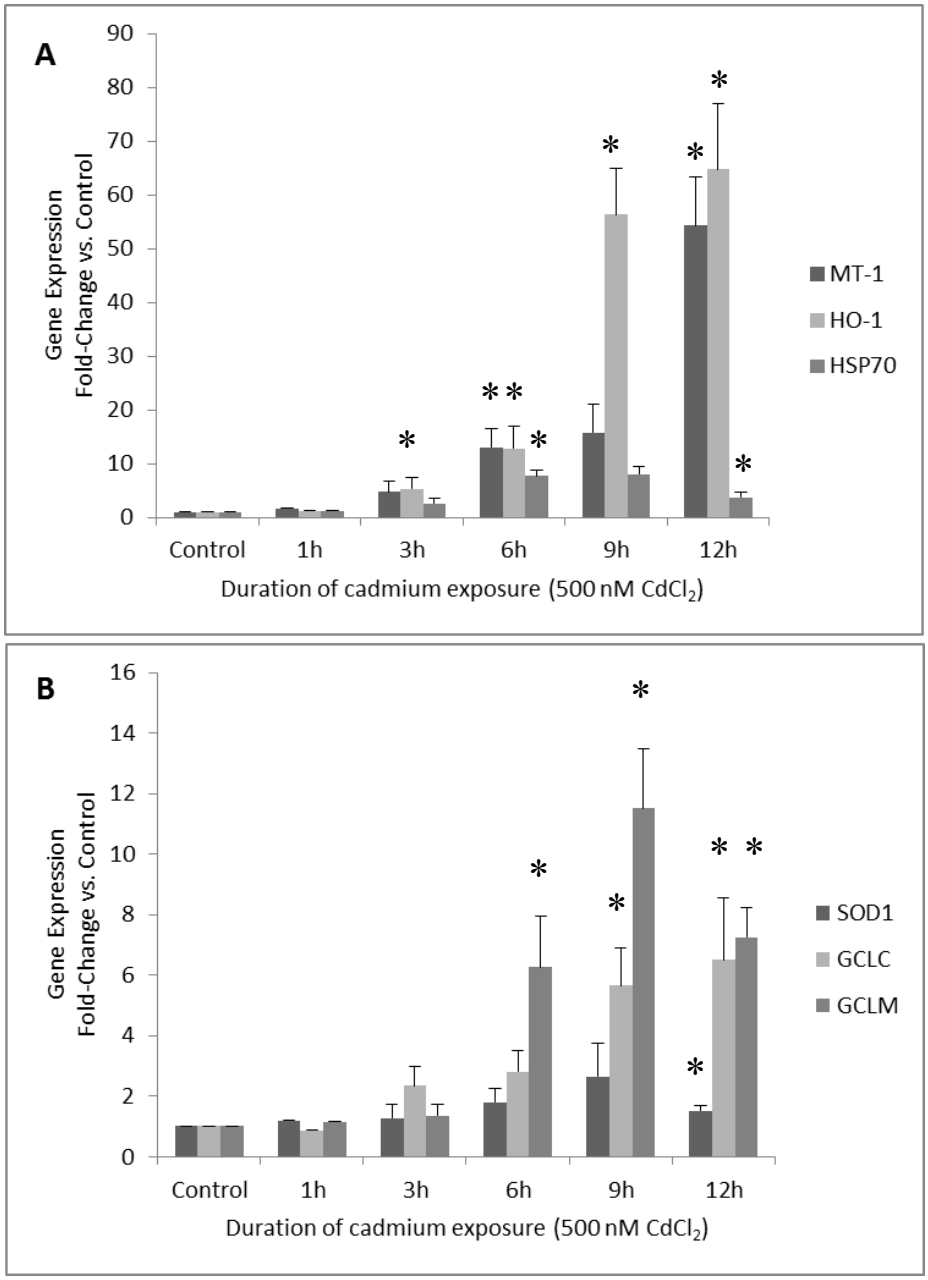
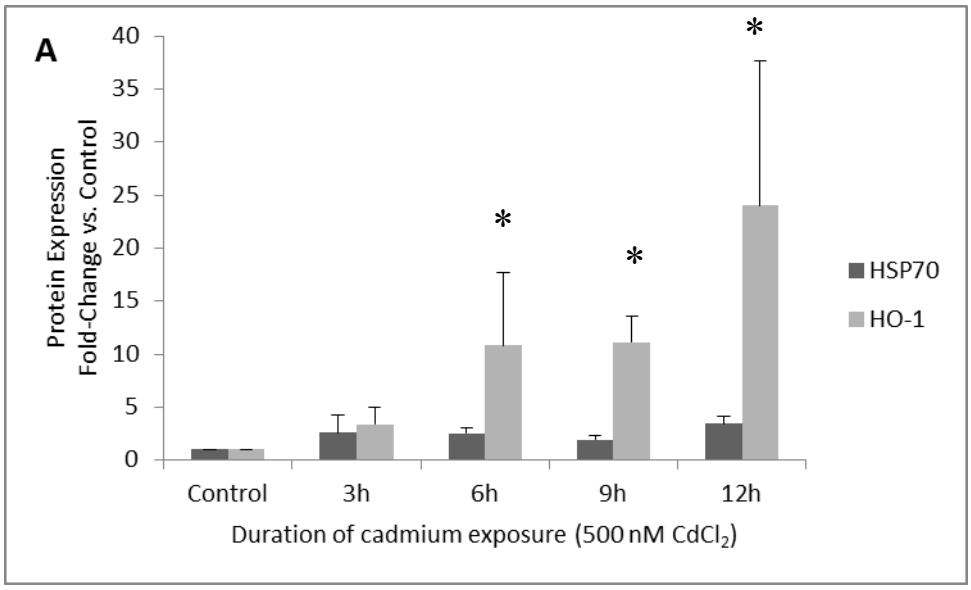


Figure 1. Time-dependent gene expression of heat shock protein-70 (HSP70), heme oxygenase-1 (HO-1), and metallothionein-1 (MT-1), superoxide dismutase-1 (SOD1), and catalytic and modifier subunits of glutamate-cysteine ligase (GCLC, GCLM) during 12-h cadmium exposure in cultured neonatal rat choroid plexus epithelial cells. Cells were treated with 0 or 500 nM $CdCl_2$ in serum-free medium for 12 h. Cd-treated and time-matched control cells were collected at 3, 6, 9, and 12 h. Gene expression of HSP70, HO-1, and MT-1 (**A**) and SOD1, GCLC, and GCLM (**B**) was analyzed by qRT-PCR and normalized to β-actin and GAPDH mRNA. Fold-induction was calculated as the ratio of normalized gene expression in Cd-treated cells to that in time-matched untreated controls; data are mean ± SE. Cadmium induced HSP70, HO-1, and MT-1 in a time-dependent manner. *Significantly different than time-matched controls at $p < 0.05$. (n =5, triplicate measures).

Total protein expression of HSP70, HO-1, SOD1, GCLC, and GCLM were determined by Western blot analysis (Fig. 2). Intensity of the protein bands measured by densitometry was normalized to β-actin expression. Primary cultures of CP epithelial cells were treated with 500 nM $CdCl_2$ for 0-12 h, and protein expression of HSP70, HO-1, SOD1, GCLC, and GCLM were determined and compared to time-matched, untreated control cells. HSP70 protein was induced 2-fold as compared to control at 3 h and remained elevated through 9 h, but further increased to 3.5-fold at 12 h. HO-1 expression was induced 3-fold at 3 h and continued to increase through 12 h; HO-1 increased by 10-, 11- and 24-fold at 6 h, 9 h and 12 h. Determining MT-1 protein expression by immunoblot analysis can be challenging; therefore, MT-1 expression in primary cultures of CP epithelial cells treated with 500 nM Cd for 12 h was assayed by epi-fluorescence immunohistochemistry (Fig. 3). This method demonstrated the differences in MT-1 protein expression in control versus Cd treated cells, but also permitted visualization of intracellular MT-1 localization.



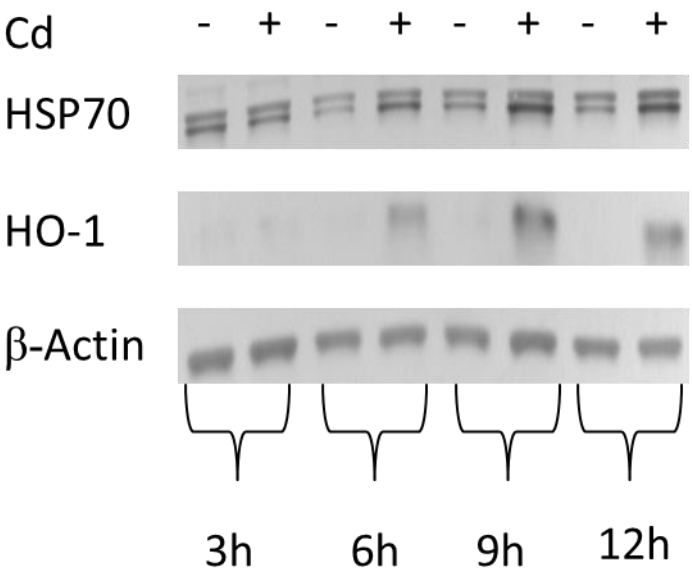


Figure 2. Time-dependent expression of HSP70, HO-1, and MT-1, and SOD1, GCLC and GCLM proteins during 12-h cadmium exposure in cultured neonatal rat choroid plexus epithelial cells. Cells were treated for 12 h with 0 or 500 nM $CdCl_2$ in serum-free medium. Cadmium-treated cells and time-matched control cells were collected at 3, 6, 9, and 12 h. Induction of HSP70 and HO-1 protein expression (**A**) and SOD1, GCLC, and GCLM protein expression (**B**) were assayed by Western blot analysis. Representative blots from a set of 5 separate experiments are shown. Band intensity of each protein of interest was normalized to β-actin expression and summarized in the corresponding graphs; data are expressed as fold-change versus time-matched control. *Significantly different than time-matched controls at $p<0.05$. (n=5; SOD1 was analyzed in one preparation [n=1])

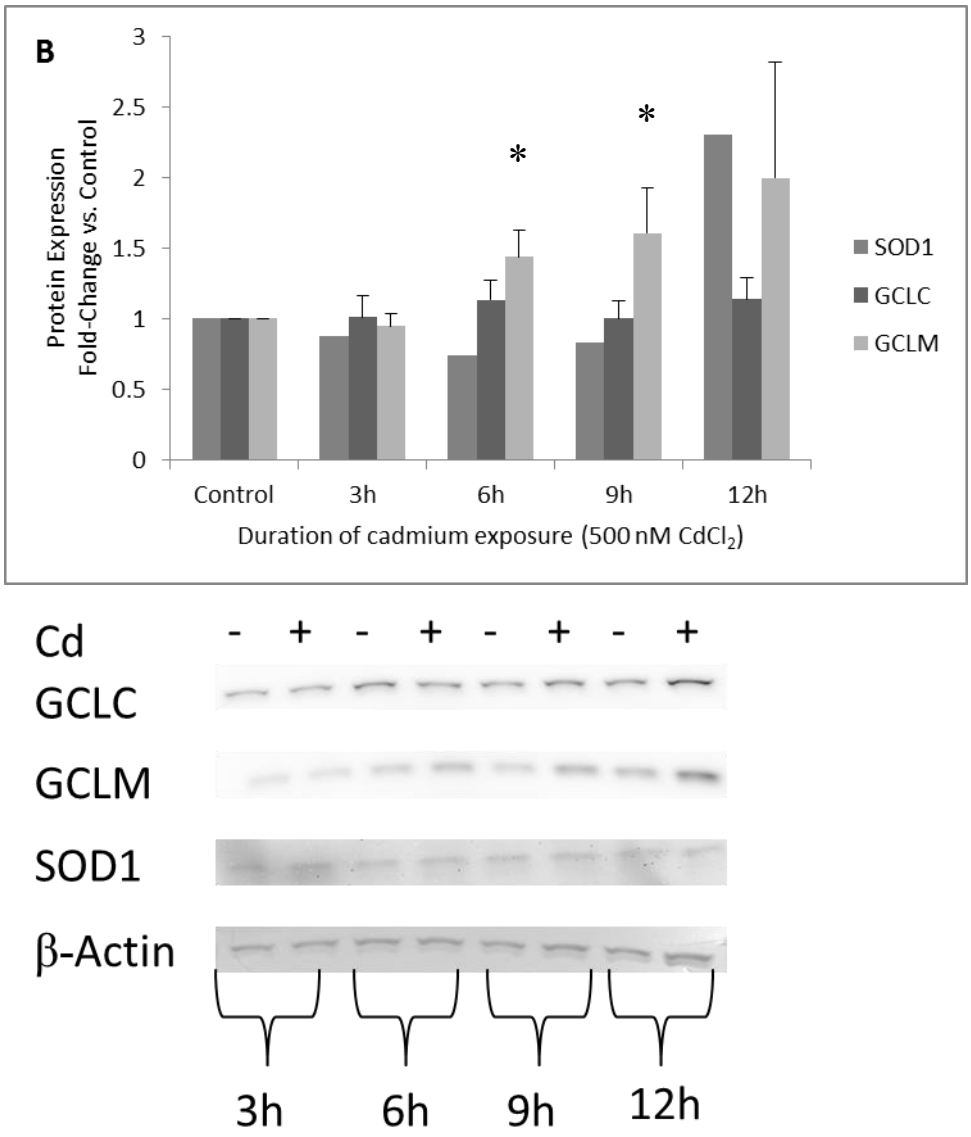


Figure 2. Continued.

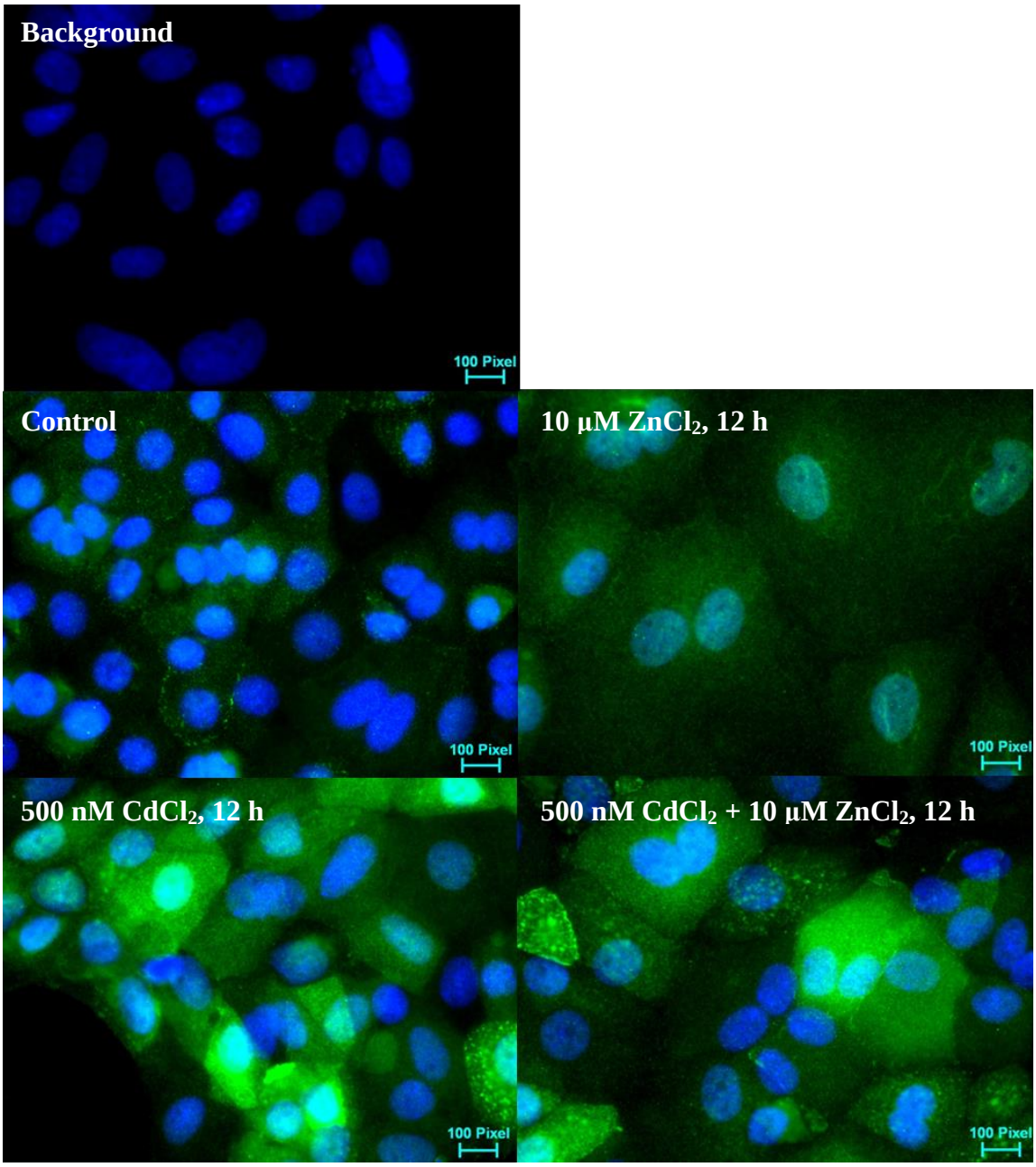


Figure 3. Metallothionein (MT-1) expression in cultured neonatal rat choroid plexus cells supplemented for 48 h with 0 or 25 µM $ZnCl_2$ and treated for 12 h with 500 nM $CdCl_2$ ± 10 µM $ZnCl_2$ in serum-free medium. Fixed cells were probed with mouse monoclonal anti-MT-1 and AlexaFluor-488 secondary antibody; coverslip mounting reagent contained DAPI (nuclear stain; blue). Cells were viewed by inverted epi-fluorescence microscopy (63X oil objective; FITC/UV filters). Cd and Zn markedly induced MT-1 expression (green).

To assess adaptation of CP cells to Cd-induced oxidative stress, SOD1, GCLC, and GCLM proteins were also analyzed (Fig. 2B). Protein expression of SOD1 was comparable to control at 3 h, 6 h, and 9 h before increasing to 2-fold at 12 h (n=1). GCLC protein expression was comparable to that in controls for the duration of the 12-h exposure to Cd ($p>0.50$). GCLM protein was comparable to control expression at 3 h, but was induced 1.5-fold at 6 h and 9 h, and then was induced 2-fold at 12 h.

3.2 *Cadmium induced moderate release of lactate dehydrogenase*

To evaluate general cytotoxicity elicited by Cd exposure, extracellular lactase dehydrogenase (LDH) release was measured by colorimetric analysis at the end of the 12-h exposure to Cd without or with manipulation of GCL activity and/or Zn supplementation (Table 2). Mean LDH release in control cells was 11.39% (±SE 0.76%) of maximal release. Cd alone elicited a moderate but not severe increase in LDH release (14.39%±SE 1.54%; $p>0.19$). Changes in LDH release in Cd treated cells subjected to additional manipulation of GCL activity and/or Zn supplementation will be discussed below.

Table 2. Comparison of extracellular lactate dehydrogenase (LDH) in cultured neonatal rat choroid plexus epithelial cells treated with cadmium without or with manipulation of GSH synthesis and/or zinc supplementation. Cells were supplemented for 48 h in maintenance medium without or with 25 μM $ZnCl_2$ before 12-h treatment in serum-free medium with 500 nM $CdCl_2$ ± 100 μM BSO ± 10 μM $ZnCl_2$ for 12 h. Post-treatment, media was collected from control and experimentally treated cells, and LDH concentration was determined by colorimetric assay. Maximum LDH release was determined in non-treated control cells lysed with 0.9% v/v Triton X-100. Values for LDH were corrected for background absorbance, *i.e.,* cell-free DMEM/F12; LDH release was expressed as a percent of maximal LDH release. LDH release was measured in triplicate in three separate culture preparations. There were no statistical differences in LDH release among conditions or any given experimental condition and control (n = 3; triplicate measures).

Condition	Mean % of Maximal LDH Release	SE
Control	11.39	0.76
Zinc only	10.34	0.20
Cadmium only	14.09	1.54
Zn + Cd	10.49	0.22
BSO only	10.89	0.34
BSO+Zn	10.55	0.85
BSO+Cd	12.85	1.01
BSO+Zn+Cd	10.61	0.29

3.3 *Cadmium induced MT-1, GCLC, and GCLM expression in isolated intact segments of choroid plexus*

To determine whether the effects of Cd observed in primary cultures of CP were reproducible in the intact tissue, analogous *in vitro* Cd exposure experiments were performed using segments of lateral and fourth CP tissues isolated from neonatal rats (Fig. 4). Tissues were exposed to 0 or 500 nM $CdCl_2$ in serum free media for 24 h. Gene expression in control and Cd-treated tissue was analyzed by qRT-PCR; however, copy number of each test gene was normalized to the integrated copy number of actin and GAPDH genes rather than expressed as a fold-induction as compared to control tissues. This quantification more accurately represents the individual variability of tissues isolated from individual animals and depicts the changes characteristic to that tissue sample. HSP70, HO-1 and SOD1 mRNA levels in Cd-treated tissues were similar to those in controls ($p>0.6$). However, mean MT-1 mRNA in Cd-treated tissues was approximately 3.5-fold greater than that in controls ($p<0.05$). GCLC and GCLM mRNA values for Cd treated tissues were 1.5-fold and 2.1-fold greater than the respective means for control tissues ($p<0.04$).

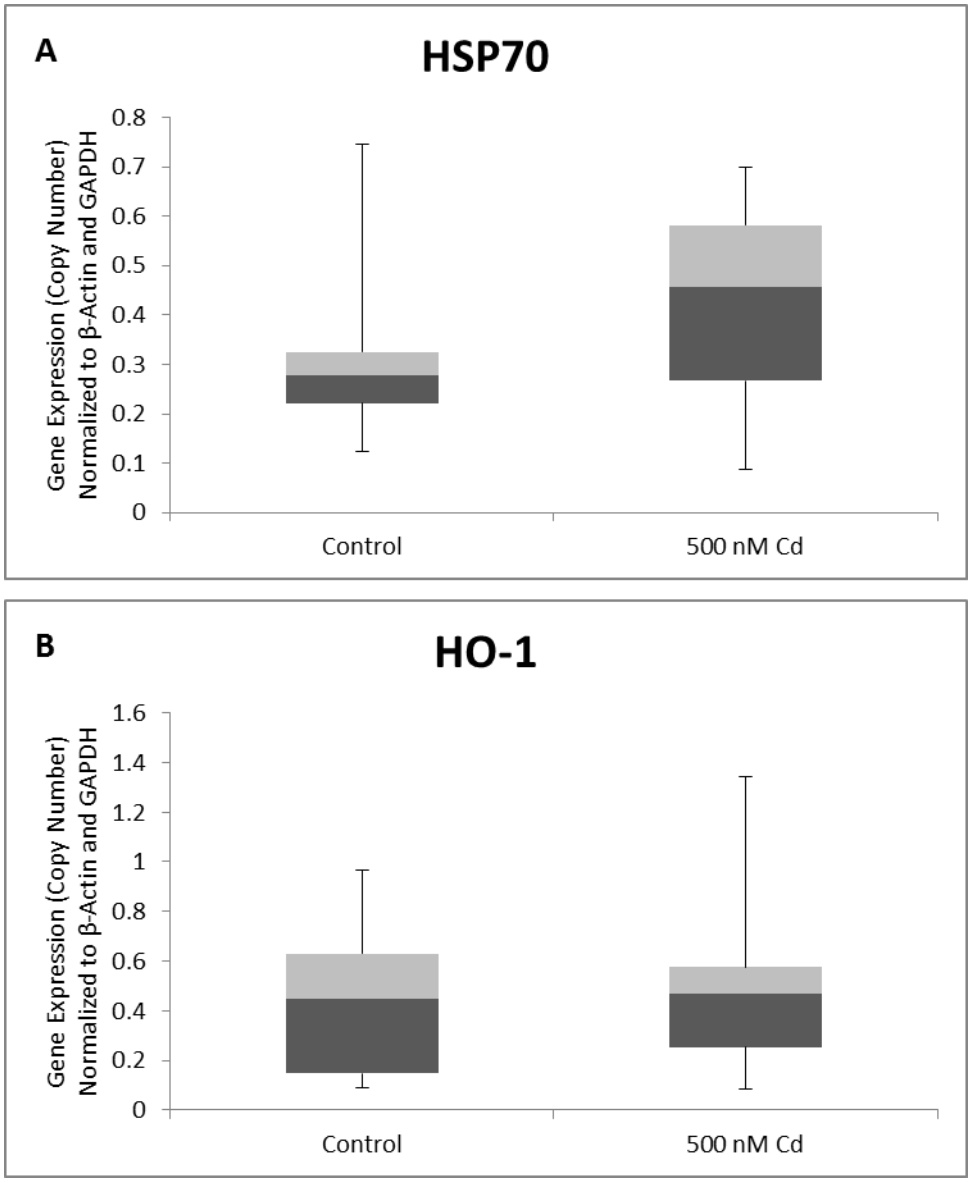


Figure 4. HSP70, HO-1, MT-1, SOD1, GCLC, and GCLM gene expression in isolated neonatal rat choroid plexus (CP) after 24-h cadmium exposure. Lateral and fourth CP tissues harvested and pooled from 3 animals were treated for 24 h in serum-free medium containing 0 or 500 nM $CdCl_2$. Copy number of each test gene was determined by qRT-PCR and normalized to integrated copy number of β-active and GAPDH. For each gene: bar is range; top and bottom of box indicate the 75th and 25th percentiles; interface is mean. *MT-1, GCLC, and GCLM gene expression were greater in Cd-treated tissues vs. non-treated controls ($p<0.05$; n = 6 sets of tissues).

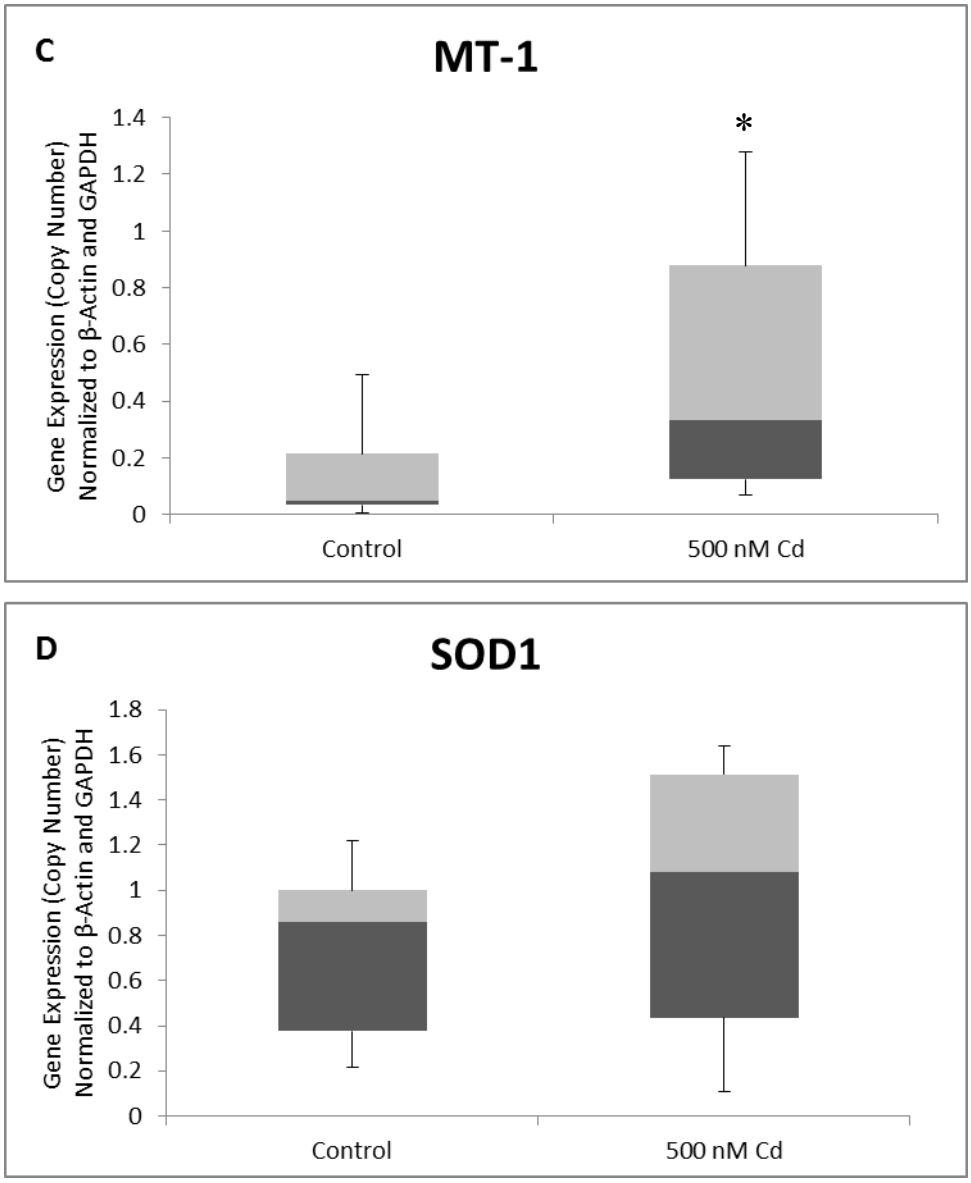


Figure 4. Continued.

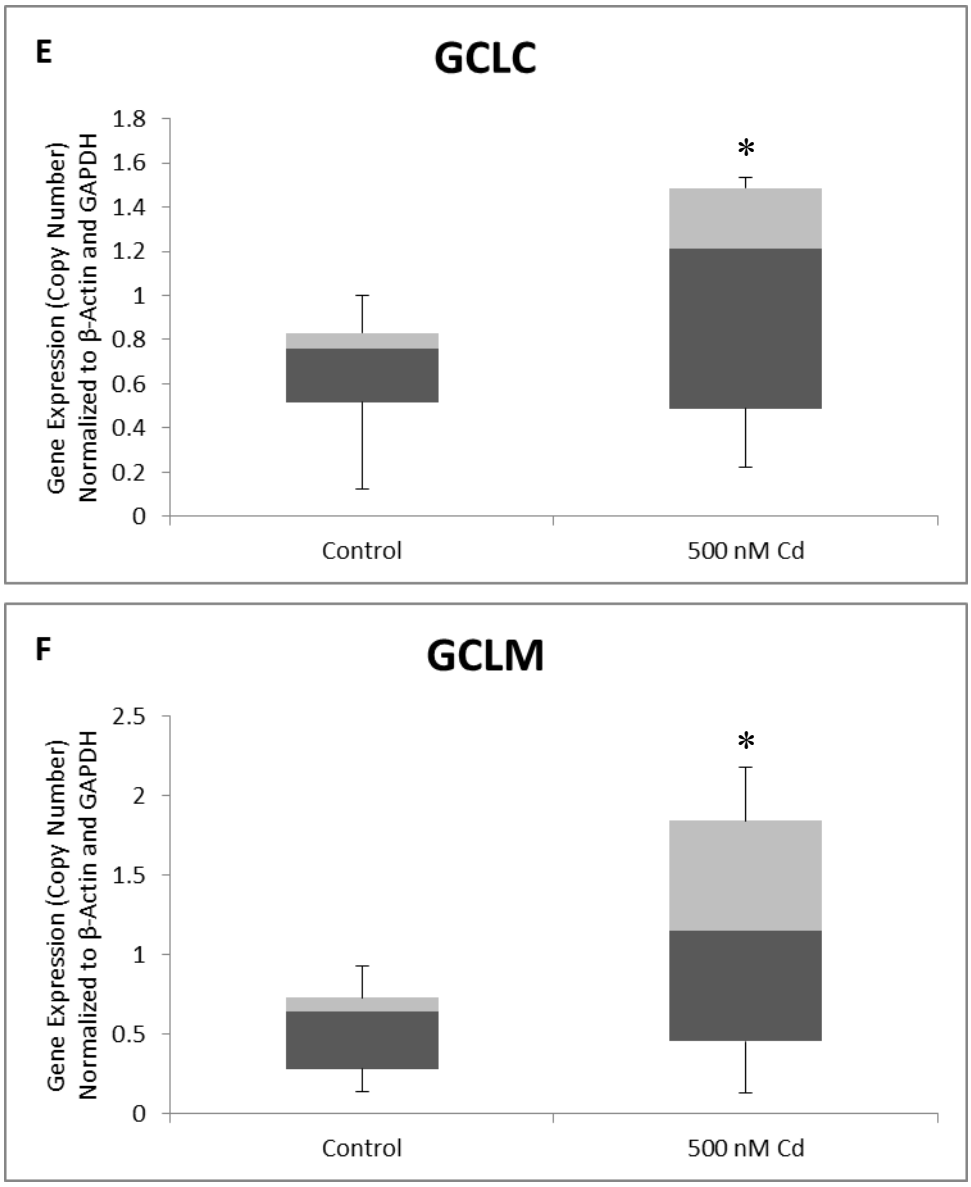


Figure 4. Continued.

3.4 *Cultured choroid plexus accumulated cadmium and zinc supplementation was without effect*

One possible and direct mechanism by which Zn supplementation might abate Cd-induced oxidative stress would be inhibition of Cd transport into the cell. Such inhibition would reduce the effective intracellular concentration of Cd. To determine whether Zn supplementation reduced Cd uptake, cells were pre-supplemented with 25 μM $ZnCl_2$ in maintenance medium for 48 h before 12-h treatment with 500 nM $CdCl_2$ without or with 10 μM $ZnCl_2$, and cellular Cd was measured by ICP-MS. Total accumulation of elemental Cd was normalized to total cell protein. Whereas the background level of Cd in non-treated control cells (No Cd/No Zn) was 1.02 ± SE 0.047 ng/mg protein, total Cd accumulation in cells incubated with the metal was 265.79 ± SE 11.79 ng/mg protein ($p<0.0001$). In cells Zn supplemented before and during Cd treatment, mean total cellular Cd was 243.72 ± SE 8.62 ng/mg protein ($p>0.54$; Zn versus Cd+Zn); in contrast, in cells supplemented with Zn without Cd, total Cd accumulation was comparable to non-treated controls (1.14 ± SE 0.05 ng/mg protein; $p>0.9$). These data indicated that a decrease in total Cd accumulation was not a predominant mechanism of Zn protection.

3.5 *Cadmium exposure altered GSH availability and zinc abated the induced stress response*

Glutathione (GSH) is a predominant intracellular scavenger of free radicals and reactive molecules. Under conditions of oxidative stress GSH donates electrons to reactive species, forming GSSG. This dimer can then be converted back to GSH by

GSSG reductase[2,11,12,19,25]. As determined above, Cd exposure markedly induced gene and protein expression of GCL, the rate-limiting enzyme in GSH synthesis. Thus, it was plausible to assume that intracellular GSH levels also might be increased. The first goal was to determine the role of GSH in the adaptation of CP to Cd-induced oxidative stress by characterizing how Cd exposure alone altered intracellular GSH. The CP is unique in that it naturally accumulates heavy metals, *e.g.,* Cd, and nutrient minerals. CP accumulates the mineral Zn, which is said to have "pro-antioxidant" properties. Others have shown that Zn can abate Cd-induced oxidative stress when co-administered with Cd[4,5,38]. Thus, the second goal was to determine whether Zn might have similar protective effects in CP cells. To also test whether Zn abated Cd-induced stress in this model, and whether GSH availability was critical to such abatement, CP primary cultures were supplemented with Zn before and during treatment with Cd and/or buthionine sulfoximine (BSO). BSO pharmacologically inhibits GSH synthesis by selectively inhibiting GCL, the rate-limiting enzyme in GSH synthesis. Thus, BSO was used to assess cellular responses to Cd-induced stress in the absence of GSH and therefore, evaluate the role of GSH in cellular adaptation to Cd. Based on the premise that GSH is responsible for the alleviation of Cd-induced oxidative stress, it was expected that BSO treatment would exacerbate the effect of Cd alone and/or interfere with potential protection by Zn. CP primary cultures were supplemented without or with 25 μM $ZnCl_2$ in maintenance media (DMEM-F12/5% NuSerum/growth factors) for 48 h. Cells were then pre-treated in serum free media without or with 100 μM BSO ± 10 μM $ZnCl_2$. Finally, cells were treated with 250 nM or 500 nM $CdCl_2$ without or with 100 μM

BSO±10 μM $ZnCl_2$ for 12 h. To assess the capacity of Cd to induce oxidative stress and alter intracellular GSH, the GSH/GSSG-Glo™ luminescence assay was used to measure changes in intracellular GSH concentrations and oxidative conversion of GSH to GSSG. From these values the ratio of GSH to GSSG was stoichiometrically calculated and used as an index of oxidative stress (Fig. 5). As compared to untreated controls, Cd treatment alone increased total intracellular GSH approximately 2-fold and increased GSSG approximately 10-fold. Along with the substantial increase in GSSG, the GSH:GSSG ratio markedly decreased, indicating an induction of oxidative stress. The mean GSH:GSSG ratio in control cells was 279, whereas that in Cd-treated cells was 69 (Table 3). These data established that Cd up-regulated GSH synthesis, while also increasing GSH oxidation. These data were also consistent with the observed increases in gene and protein expression of GCLC and GCLM.

Zinc supplementation abated Cd-induced increases in intracellular GSH and GSSG. GSH concentrations in cells supplemented and treated with Zn alone were comparable to controls ($p>0.9$). In cells supplemented with Zn and then co-treated with Cd and Zn, GSH concentrations increased by 1.5-fold as compared to controls. However, GSH concentrations in Cd-treated cells also supplemented with Zn (Zn+Cd: 6.8 ± 1.4 μM) were less than those in cells treated with Cd alone (Cd: 8.1 ± 1.5 μM). BSO treatment alone decreased GSH by 90% as compared to control cells ($p<0.05$), and co-treatment with Cd and BSO, further decreased GSH. This established the efficacy of BSO to diminish GSH synthesis and thereby greatly decrease intracellular GSH. To determine whether zinc's pro-antioxidant effects would persist despite diminished GSH

availability, cells were treated with Zn and BSO without or with Cd. In cells treated with only Zn and BSO, GSH concentrations were comparable those in cells treated with BSO alone. Interestingly, GSH concentration in cells treated with Zn, BSO, and Cd was comparable to that in cells treated with BSO alone ($p>0.9$). However, GSH concentration in cells treated with BSO and Cd was approximately half that of cells treated with BSO alone. Similar trends were observed in GSSG concentration. Cadmium alone increased GSSG by 2.6-fold as compared to controls, but Zn was without effect. In cells co-treated with Zn and Cd, GSSG increased 1.8-fold as compared to controls, but this was still less than that in cells treated with only Cd. In the presence of BSO, there were no differences in GSSG concentrations regardless of co-treatment with Cd and/or Zn. Each treatment (BSO, BSO+Zn, BSO+Cd, and BSO+Zn+Cd) had GSSG concentrations comparable to those in cells treated with Zn and Cd without BSO. This suggested that even under conditions of impaired GSH synthesis, the remaining fraction of GSH could be dimerized/oxidized to GSSG to scavenge reactive molecules.

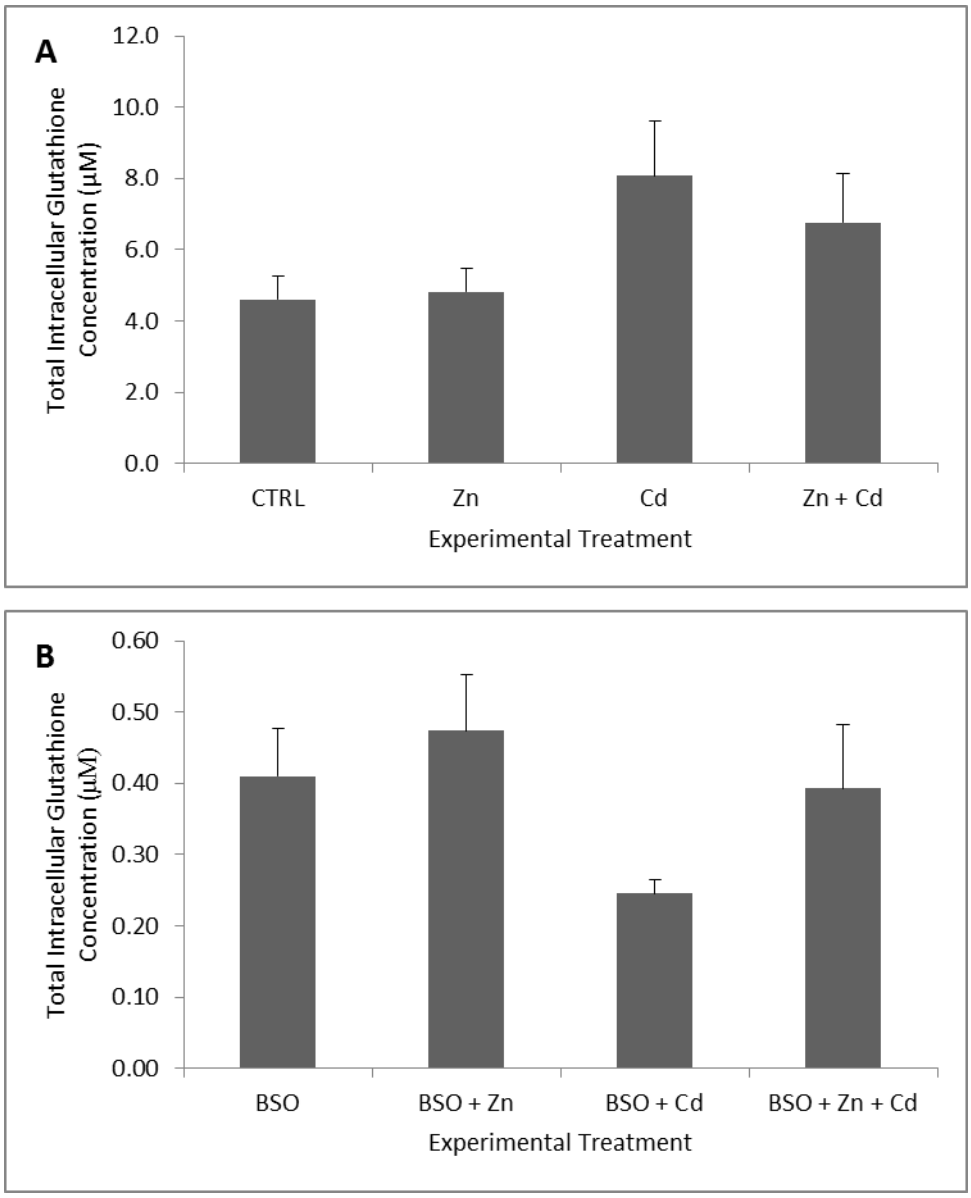


Figure 5. Intracellular concentrations of glutathione (GSH) and oxidized GSH (GSSG) in neonatal rat choroid plexus epithelial cells treated with cadmium without or with manipulation of GSH synthesis and/or zinc supplementation. Cadmium-treated cells were supplemented for 48 h without or with 25 µM $ZnCl_2$ in maintenance medium then treated without or with 100 µM buthionine sulfoximine (BSO) ± 10 µM $ZnCl_2$ in serum-free medium; where indicated 10 µM $ZnCl_2$ was present during Cd treatment. Total GSH (**A, B**) and GSSG (**C**) concentrations were determined by luminescence assay, and GSH/GSSG ratios (Table 3) were subsequently calculated. Cadmium exposure increased GSH and GSSG concentrations, and BSO effectively knocked-down GSH synthesis. (n=5)

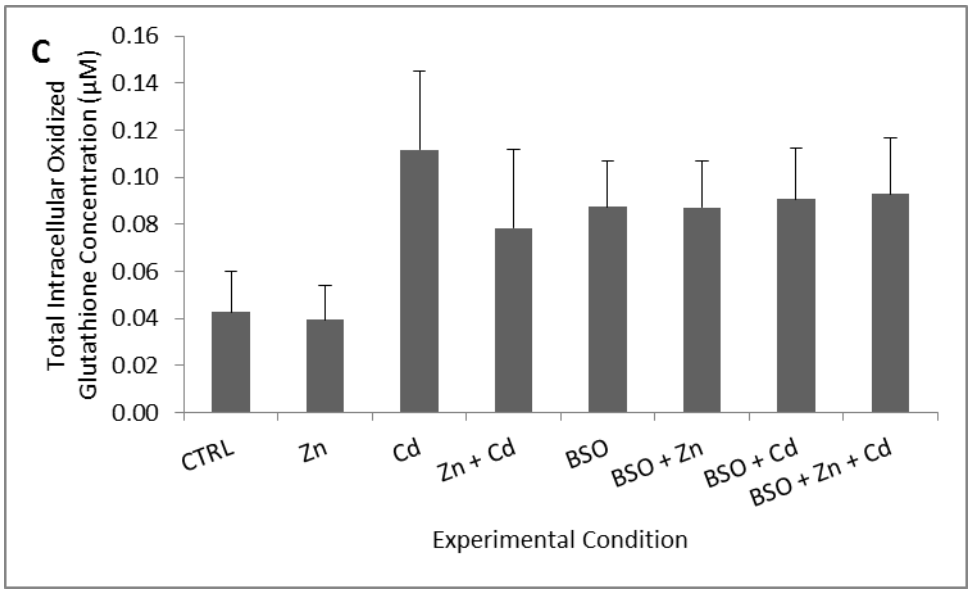


Figure 5. Continued.

Table 3. Ratios of intracellular glutathione (GSH) and oxidized GSH (GSSG) concentrations in neonatal rat choroid plexus epithelial cells treated with cadmium without or with manipulation of GSH synthesis and/or zinc supplementation. These ratios were calculated from total GSH and GSSG concentrations determined for the experimental data presented in Figure 5. Cadmium-treated cells were supplemented for 48 h without or with 25 μM $ZnCl_2$ in maintenance medium then treated without or with 100 μM buthionine sulfoximine (BSO) ± 10 μM $ZnCl_2$; where indicated 10 μM $ZnCl_2$ was present during Cd treatment. Cadmium exposure decreased GSH/GSSG and BSO effectively knocked-down GSH synthesis resulting in markedly reduced GSH/GSSG ratios. (n=5)

Condition	GSH/GSSG Ratio	SE
Control	279.76	99.92
Zn alone	337.75	135.63
Cd alone	69.59	13.94
Zn + Cd	184.13	96.76
BSO alone	4.68	1.89
BSO + Zn	5.57	2.32
BSO + Cd	3.98	1.68
BSO + Zn + Cd	4.21	1.78

3.6 *Cadmium exposure induced HSP70, HO-1, MT-1, SOD1, GCLC, and GCLM and zinc attenuated the stress response*

As reported above, when CP primary cultures were exposed to low-doses of Cd, HSP70, HO-1, and MT-1, and GSH synthesis enzymes, GCLC and GCLM, were markedly induced. Cadmium also elicited moderate cytotoxicity as indicated by modest increases in LDH. The GSH/GSSG-Glo™ assay also established that BSO effectively 'knocked-down' GSH synthesis. To further assess the role of GSH in both the adaptation of CP to Cd-induced cellular and/or oxidative stress and the capacity of Zn to attenuate this stress, I examined possible compensatory changes in expression of stress proteins and GCL catalytic and modifier subunits in cells treated with Cd without or with BSO and Zn supplementation. Cells were treated for 12 h with 500 nM $CdCl_2$ alone or with prior and combined supplementation with Zn and/or BSO; then expression of HSP70, HO-1, MT-1, SOD1, GCLC, and GCLM were analyzed by qRT-PCR and/or Western blotting (Fig. 6).

To determine whether the 48-h Zn supplementation might up-regulate baseline expression of the various cytoprotective genes prior to start of Cd treatment, gene expression was also compared in cells supplemented for 48-h in maintenance medium without (control) or with 25 μM $ZnCl_2$ (Fig. 6A). There were no significant differences in HSP70, HO-1, SOD1, GCLC, or GCLM gene expression in 48-h Zn supplemented cells as compared to non-supplemented cells ($p>0.10$). However, MT-1 gene expression was significantly induced 13-fold in 48-h Zn supplemented cells ($p<0.05$). In contrast, as compared to control, 12-h Cd exposure induced HSP70, HO-1 and MT-1 genes by

1.5-fold, 11-fold and 16-fold, respectively (Fig. 6B; $p<0.03$), but also induced GCLC and GCLM genes by approximately 3-fold and 6-fold respectively (Fig. 6C; $p<0.03$). In cells subjected to 48-h Zn-supplementation with an additional 12-h treatment with only Zn, there were no marked differences in gene expression of cellular and oxidative stress response proteins (*i.e.,* HSP70, HO-1, SOD1, GCLC, and GCLM) as compared to untreated cells ($p>0.28$); however, MT-1 gene expression was induced 2-fold (Fig. 6B, 6C; $p<0.05$). In cells supplemented with Zn and then co-treated with Cd and Zn, gene expression of the stress response proteins was decreased as compared to cells treated with Cd alone. Gene expression of MT-1, HO-1, GCLC, and GCLM in cells treated with Zn and Cd was approximately half that of cells treated with Cd alone; however, HSP70 gene expression in cells co-treated with Cd and Zn was comparable to that in cells treated with Cd alone (Fig. 6B; $p>0.19$).

In the presence of BSO, the pharmacological inhibitor of GSH synthesis, gene expression of HSP70, HO-1 and MT-1 was comparable to untreated controls ($p>0.20$), but as anticipated GCLC and GCLM expression was induced 2- and 1.5-fold respectively as compared to untreated cells ($p<0.20$). BSO exacerbated the Cd-induced stress response. Co-treatment with Cd and BSO induced HO-1 gene expression 12-fold as compared to control (versus 11-fold for Cd alone), but increased HSP70 gene expression 10-fold as compared to control. (Cd alone increased Hsp70 mRNA by 1.47-fold as compared to controls.) As compared to controls, Cd alone induced MT-1 gene expression by 16-fold, whereas co-treatment with Cd and BSO induced MT-1 by 74-fold. Co-treatment with BSO also enhanced induction of gene expression of both GCLC

and GCLM in Cd-treated cells. Whereas treatment with Cd alone induced GCLC gene expression by 3-fold, co-treatment with Cd plus BSO induced GCLC gene expression by 11-fold. Similarly, treatment with Cd alone induced GCLM gene expression 6-fold, but co-treatment of Cd plus BSO, GCLM gene expression 17-fold.

To determine whether the pro-antioxidant effects of Zn were dependent on GSH availability, cells were also treated without or with Cd in the presence of 100 μM BSO ± 10 μM $ZnCl_2$. With the exception of MT-1, co-treatment with BSO and Zn did not alter expression of HSP70, HO-1, SOD1, GCLC, or GCLM genes as compared treatment with BSO alone. In cells co-treated with BSO and Zn, MT-1 gene expression was approximately 3-fold higher than in cells treated with BSO alone. Zinc supplementation also markedly attenuated gene expression of the stress proteome in cells co-treated with Cd plus BSO. As compared to controls, co-treatment with Cd plus BSO induced MT-1 by 74-fold and HSP70 by 10-fold, whereas co-treatment with Cd plus BSO in zinc-supplemented cells induced MT-1 by only 30-fold and HSP70 by only 2-fold. Zinc supplementation also attenuated GCLC and GCLM gene expression in cells co-treated with Cd and BSO by 34% and 67%, respectively (Fig. 6C). Nonetheless, HO-1 gene expression in cells co-treated with Cd, BSO, and Zn did not differ from that of cells only co-treated with Cd and BSO ($p>0.66$). In this capacity, the protective effect of zinc persisted under conditions of diminished GSH availability.

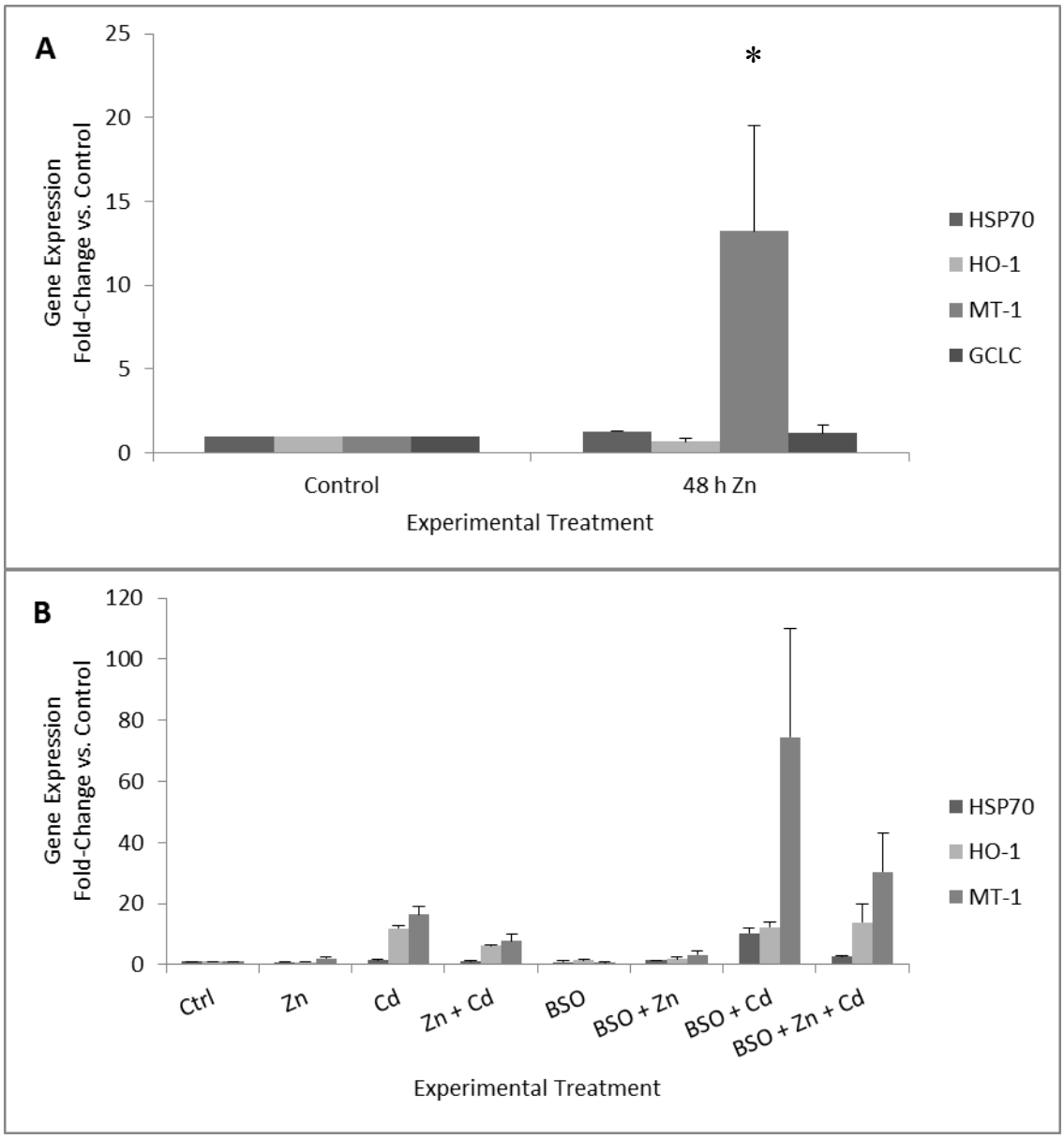


Figure 6. Gene expression of HSP70, HO-1, MT-1, SOD1, GCLC and GCLM in cultured neonatal rat choroid plexus epithelial cells treated with cadmium without or with manipulation of GSH synthesis and/or zinc supplementation. Cells were supplemented for 48 h in maintenance medium without or with 25 µM $ZnCl_2$ to determine baseline status of cells prior to Cd-treatment (**A**). *Significantly different than non-treated controls ($p<0.05$). GCLM and SOD1 were not induced after 48-h Zn supplementation and therefore are not depicted on the graph. CP cells were supplemented for 48 h in maintenance medium without or with 25 µM $ZnCl_2$ before 12-h treatment without or with 500 nM $CdCl_2$ ± 100 µM BSO ± 10 µM $ZnCl_2$. Gene expression of HSP70, HO-1, and MT-1 (**B**) and SOD1, GCLC, and GCLM (**C**) were analyzed by qRT-PCR and normalized to β-actin and GAPDH mRNA. Fold-induction was calculated as the ratio of normalized gene expression in Cd-exposed cells to that in control cells incubated in Cd-free medium without BSO or zinc. Inhibition of GCL with BSO further enhanced gene expression of both cellular and oxidative stress proteins. (n=4, triplicate measures)

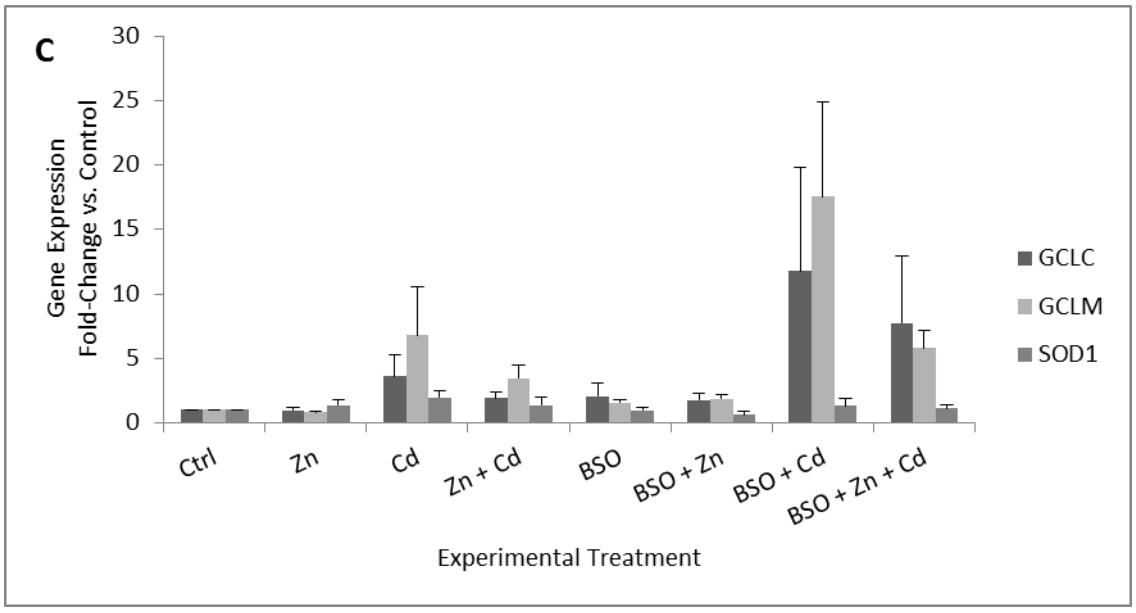


Figure 6. Continued.

Expression of respective stress response proteins, GCLC, and GCLM in Cd treated cells co-treated with BSO and supplemented with zinc was consistent with changes in gene expression (Fig. 7). As compared to untreated controls, Cd treatment alone induced HSP70 protein expression approximately 2.5-fold ($p<0.05$). However, zinc supplementation with extended zinc treatment did not induce HSP70 ($p>0.28$), nor did co-treatment with Cd alter HSP70 induction as compared to Cd alone (Fig. 7A; $p>0.88$). Similarly, BSO treatment alone did not markedly alter HSP70 expression as compared to controls ($p>0.28$), but BSO enhanced HSP70 induction in Cd-treated cells to 4-fold greater than in untreated controls ($p<0.05$). In both cells co-treated with zinc plus BSO, and cells treated with zinc, BSO, and Cd, HSP70 protein expression was comparable to that in cells treated with BSO only ($p>0.66$).

As compared to control, Cd alone induced HO-1 protein 8-fold, but zinc supplementation plus Cd induced HO-1 protein by 6-fold. Zinc supplementation and extended zinc treatment did not alter HO-1 protein expression ($p>0.9$). Whereas BSO alone induced HO-1 protein by approximately 2.5-fold as compared to non-treated controls, co-treatment with BSO plus Cd plus enhanced HO-1 induction 13-fold as compared to non-treated controls. Zinc supplementation did not alter HO-1 expression in BSO-treated cells, and only slightly attenuated HO-1 induction in cells co-treated with BSO plus Cd (7-fold increase as compared to controls).

As compared to non-treated controls, GCLC protein expression was induced in cells co-treated with BSO and Cd by 1.6 fold; other treatments were without effect. Similarly, BSO and Cd co-treatment increased GCLM protein expression by 2-fold as

compared to controls; all other experimental treatments did not markedly alter GCLM protein expression. Protein expression of SOD1 was comparable to controls. These data demonstrate the importance of GSH in the ability of this model to adapt to the stress induced by low-dose Cd exposure.

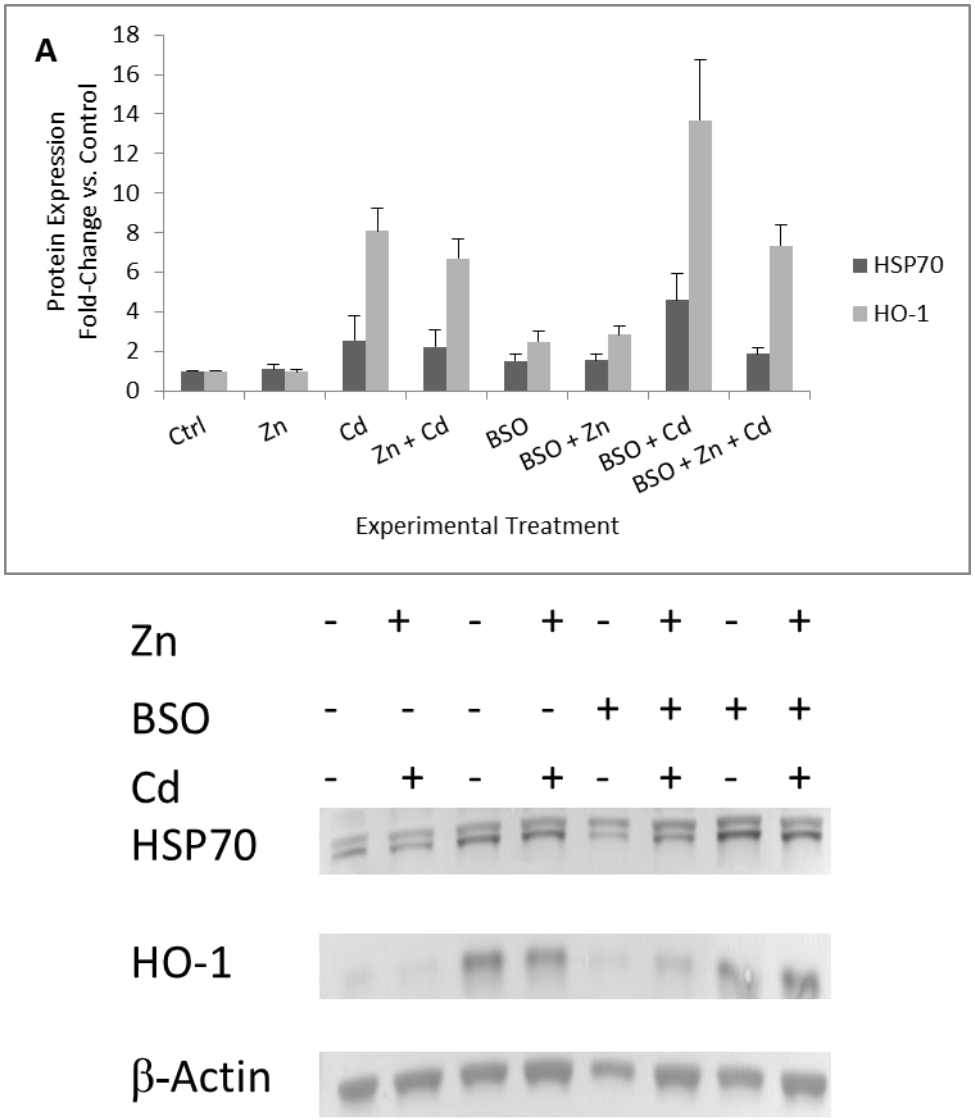
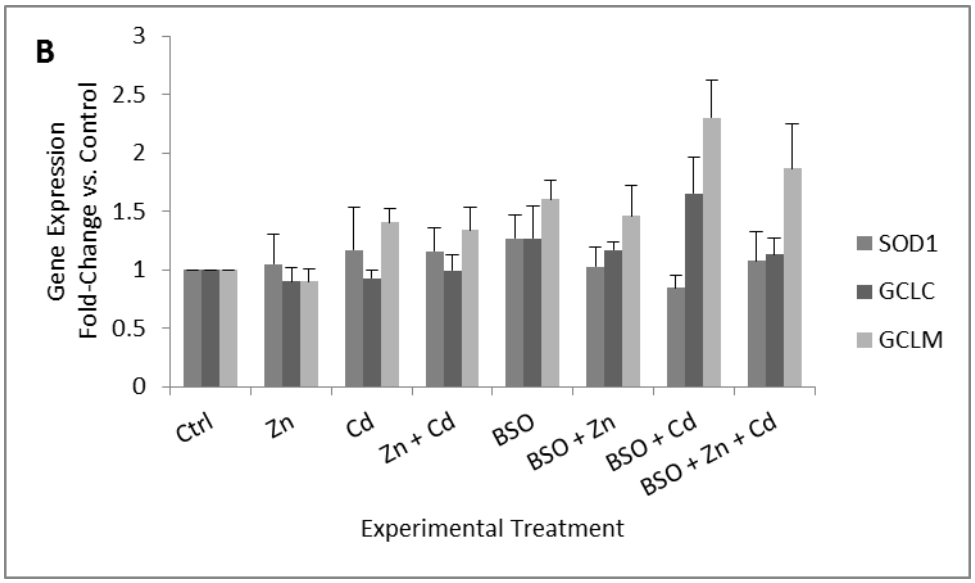


Figure 7. Expression of HSP70, HO-1, SOD1, GCLC, and GCLM proteins in cadmium-treated cultured choroid plexus epithelial cells without or with manipulation of GSH synthesis and/or zinc supplementation. Cells were supplemented for 48 h in maintenance medium without or with 25 μM $ZnCl_2$ before 12-h treatment in serum-free medium with 500 nM $CdCl_2$ ± 100 μM BSO ± 10 μM $ZnCl_2$ for 12 h. Protein expression was determined by Western blot analysis; HSP70 and HO-1 proteins **(A)**; SOD1, GCLC, and GCLM proteins **(B).** Fold-induction was calculated as the ratio of normalized protein expression in Cd-exposed cells to that in control cells incubated in Cd-free medium without BSO or Zn. Inhibition of GCL with BSO further enhanced expression of both cellular and oxidative stress proteins. (n=5)



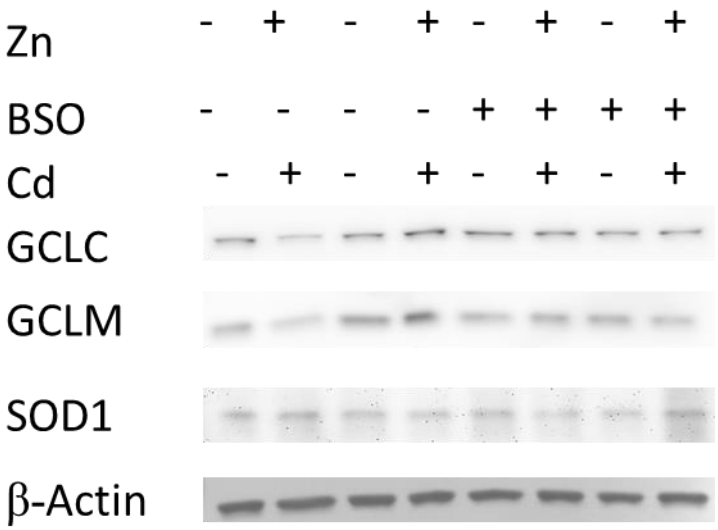


Figure 7. Continued.

4. DISCUSSION

I have demonstrated that low-dose Cd exposure induces a cellular stress response as indicated by increases in expression of heat shock protein 70 (HSP70), hemeoxygenase-1 (HO-1), and metallothionein (MT-1) in primary cultures of neonatal choroid plexus (CP). Based on cellular lactate dehydrogenase (LDH) release data, these treatments, although enough to elicit a stress response, are not so severe that the cell cannot adapt. Previous research demonstrated the anti-oxidant activities of MT-1 and specifically its role in alleviating Cd-induced stress[24,37]. Cucu and colleagues exposed renal tubular cells to concentrations of $CdCl_2$ ranging from 0-1000 nM for 5 h and assayed gene expression of MT-1 and HO-1[9]. Gene expression of MT-1 and HO-1 was induced approximately 100-fold and 3-fold respectively. Interestingly, that study also demonstrated the ability of long-term low-dose Cd exposure (24-72 h) to shift the Bax/Bcl2 ratio to a slightly more pro-apoptotic state in accordance with decreases in MT-1 and HO-1 gene expression. This small change after prolonged Cd treatment also is consistent with my cytotoxicity data in that cells are capable of adapting to lower doses of Cd for a longer duration. MT-1 may protect against Cd-induced lethality by binding and sequestering Cd in the cytosol, thus keeping Cd from disrupting other cellular organelles; however, it does not reduce the absorption or distribution of Cd[29]. It has been suggested that Cd-induced transcription of HSP70 is due to activation of the transcription factor heat shock factor 1 and its binding to the heat shock element within the promoter region of HSP70. Koizumi demonstrated marked increases in HO-1 and

HSP70 in HeLa cells exposed to Cd[28]. However, those studies employed Cd concentrations much greater than those used in this study (0-300 μM $CdSO_4$ versus 500 nM $CdCl_2$). In the present study the inductions of HSP70, HO-1, and MT-1 are indices of cells stress and here I have established their importance to the adaptation of CP to low-dose Cd exposure.

Low-dose Cd exposure increased both intracellular glutathione (GSH) and oxidized glutathione (GSSG) concentrations, but also decreased the GSH/GSSG ratio, an indication of oxidative stress. These changes were consistent with the observed marked inductions of the catalytic and modifier subunits of glutamate cysteine ligase (GCLC and GCLM), the rate-limiting enzyme responsible for GSH synthesis. In the presence of BSO, a pharmacological inhibitor of GSH synthesis, the stress response was exacerbated in Cd-treated CP cells. Total GSH levels were increased by approximately 130-300% in rat lung fibroblasts exposed to 6 μM $CdCl_2$ for 1-6 h[53]. Shukla also demonstrated an increase in total GSH in rats exposed to aerosolized Cd[41]. There are, however, many studies that demonstrate a decrease in total GSH levels in response to much higher Cd concentrations. In a study in which rats were exposed to Cd via drinking water (approximately a 1 mM solution), both the levels of GSH in the liver and the GSH/GSSG ratio were significantly decreased, whereas GSSG levels were significantly increased[22]. GCLC expression is readily induced in response to oxidative stress and/or depletion of intracellular GSH[33,41,42,53]. Although not studied as extensively as GCLC, GCLM is also readily induced, if not more so than GCLC, in response to oxidative stress. Solis showed that GCLM expression resulted in a maximal induction of 10-fold

as compared to control after an 8-h exposure to 50 μM tert-butylhydroquinone (tBHQ), which induces mitochondrial oxidative stress, whereas GCLC was only induced 2-fold[43]. Solis and colleagues also found that GCLC and GCLM response is mostly transcriptional which is also consistent with my findings. The efficacy of BSO to deplete intracellular GSH has also been demonstrated in other studies[27,30,32]. Lizotte reported that osteoblastic cells co-treated with Cd and BSO had an LD_{50} of approximately 2.5 μM as compared to the LD_{50} of 6 μM in cells treated with Cd alone[30]. Likewise, Kalariya demonstrated that in human lens epithelial cells (HLECs) exposed to 15 μM Cd, BSO co-treatment reduced cell viability by approximately 50%, whereas Cd treatment alone only decreased viability by 35%[27].

I have demonstrated that Cd can induce cellular and oxidative stress and have shown that Zn supplementation before and during Cd exposure attenuated the cellular and oxidative stress response. After the initial 48 h of Zn supplementation, expression of MT-1 is markedly induced; however, GCLC and GCLM expression is greatly diminished. This lack of GSH synthesis points to a more balanced cellular redox state. Jihen and colleagues demonstrated that rats exposed to both Cd and Zn via drinking water had significantly higher liver GSH levels and GSH/GSSG ratios than did rats exposed to Cd only; this co-treatment also resulted in significantly lower GSSG levels[22]. Interestingly, in my experiments I also demonstrated that Zn supplementation attenuated Cd-induced stress even in the absence of GSH. In my studies I also have demonstrated that Zn supplementation before and during co-treatment with Cd and BSO resulted in markedly attenuated expression of HSP70, HO-1, MT-1, GCLC, and GCLM as

compared to cells co-treated with Cd and BSO without Zn. Tang demonstrated the ability of Zn to protect against Cd-induced nephrotoxicity. Using MT-1 null mice, they showed that Zn supplementation before Cd treatment resulted in less Cd-MT conjugation and renal uptake (accumulation) of Cd and Cd-MT and thus decreased nephrotoxicity. In their study, however, they demonstrated that this effect was only possible with sufficient GSH concentrations. In the absence of GSH (presence of BSO) Zn supplementation did not impart the same protective effects[48]. It is important to emphasize that their study was performed in MT-1 null mice. My hypothesis is that Zn protects against Cd-induced stress through up-regulation of MT-1. I have demonstrated that Zn still reduces Cd-induced stress even in conditions of GSH depletion (*i.e.,* BSO treatment) and that Cd alone can induce oxidative stress. I propose that Zn protection is due to the higher levels of MT-1 at the start of Cd exposure. Thus, based on this hypothesis it follows that MT-1 null mice that are also deficient in GSH would not be protected from Cd-induced stress by Zn supplementation. This is because, biochemically, all cells must have a complement of antioxidant defenses, not just one element, to maintain life and evolve. An alternative hypothesis was that Zn attenuated Cd-induced stress by decreasing the amount of Cd transported into the cell via competitive inhibition of transport. I have demonstrated by ICP-MS that Zn supplementation does not markedly alter Cd uptake. However, it is possible that Zn supplementation could change the intracellular distribution and/or compartmentation of Cd, which I did not investigate here. Furthermore, even in the presence of Zn, Cd treatment still induces HO-1. Cadmium treatment has been shown to induce the nuclear export of transcription factors Bach1 and

Bach2. Bach1 heterodimers specifically repress HO-1 transcription under normal physiological conditions and therefore, the nuclear export as a result of stress and/or injury abolishes this suppression. Cadmium treatment also induces the translocation of Nrf2 into the nucleus. Nrf2 then activates HO-1 transcription[46]. Thus, the fact that Cd treatment, with Zn supplementation before and during, still up-regulates HO-1 is another indication that a marked decrease of intracellular Cd was not a primary mechanism of Zn protection. Another hypothesis was that Zn supplementation enhanced GSH biochemistry leading to a more effective abatement of Cd-induced oxidative stress. I have demonstrated that Zn supplementation alone (48 h) actually decreases expression GCLM and has no effect on GCLC expression or GSH concentrations. Upon extended treatment (48 h + 12 h), Zn supplementation has no effect on GCLC and GCLM expression or GSH concentrations. Therefore, I have demonstrated that, of my three hypotheses regarding Zn protection, the most plausible and validated is that Zn supplementation protects against Cd-induced stress by inducing MT-1 and therefore leading to a decreased concentration of total 'free' Cd within the cell.

5. CONCLUSIONS AND PERSPECTIVES

The choroid plexus (CP) is not only critical to the regulation of fluid, electrolyte, and nutrient balance in the brain, but it is also responsible for metabolizing and/or clearing xenobiotics, thus protecting the brain[18,23]. Similar to other epithelial tissues, such as the lung, kidney, or liver, the CP has a substantially high metabolic rate and is therefore prone to accumulation of reactive species (*e.g.,* ROS)[7,10,14,17]. The CP naturally accumulates trace metals, both toxic (*e.g.,* Cd, As, Hg) and essential (*e.g.,* Zn, Cu)[13,18,45,55]. Zinc, a nutritive mineral accumulated in high amounts in the CP, is said to be a 'pro-antioxidant'[37]. In this capacity, the accumulation of Zn may serve more to protect CP and regulate its function rather than to solely protect the brain. The World Health Organization estimates that up to one third of the world population is Zn deficient. Zinc deficiency is also the fifth leading risk factor for death and disease in developing nations. It is likely that supplementation with Zn implemented to improve Zn status can alleviate these morbidity and mortality rates[36]. Many populations around the world are exposed every day to Cd through the environment and food. Several areas in countries such as China, Japan, and Belgium have high levels of Cd in the soil. These same populations might also be Zn deficient, therefore, these populations are at a greater risk for Cd-induced disease states[26,37]. Thus, in this study I used Cd, a toxic metal naturally accumulated by CP, to induce cellular and oxidative stress in primary cultures of neonatal rat CP. Furthermore, I tested the hypothesis that Zn supplementation before and during this exposure may help attenuate the induced stress response.

The data herein indicate that low-dose Cd exposure elicited cellular and oxidative stress responses in primary cultures of CP. Exposure to sub-micromolar doses of Cd for 12 h markedly induced of HSP70, HO-1, and MT-1 expression. Increases in expression of GCLC and GCLM, the subunits of the rate-limiting enzyme in GSH synthesis, as well as increases in total intracellular GSH and GSSG concentrations, indicated an induction of oxidative stress. The collective up-regulation of these endogenous cytoprotective factors also demonstrates the adaptive response to the induced stress. By pharmacological manipulation of GSH synthesis with BSO, I exacerbated the Cd-induced stress response. Supplementation with Zn had pro-antioxidant effects and attenuated the Cd-induced stress response, even in the absence of GSH. Based on these data, I conclude that low-dose Cd exposure induces a cellular and oxidative stress response in CP and that, under conditions of sufficient Zn status, this tissue may more effectively respond and adapt to this stress. These data are novel in that, to my knowledge, no studies investigating the pro-antioxidant role of Zn in the abatement of Cd-induced stress in CP have been published. These studies have also provided new insight on the possible protective role of Zn accumulation in this critical brain-blood barrier.

REFERENCES

1. Agency for Toxic Substances and Disease Registry. (2012). *Toxicological profile for cadmium*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.
2. Anderson, M., Underwood, M., Bridges, R.J., Meister, A. "Glutathione metabolism at the blood-cerebrospinal fluid barrier." *FASEB Journal,* 3 (1989): 2527-2531.
3. Baba, H., Tsuneyama, K., Yazaki, M., Nagata, K., Minamisaka, T., Tsuda, T., Nomoto, K., Hayashi, S., Miwa, S., Nakajima, T., Nakanishi, Y., Aoshima, K., Imura, J. "The liver in itai-itai disease (chronic cadmium poisoning): pathological features and metallothionein expression." *Modern Pathology* 26.9 (2013): 1228-234
4. Balen, B., Tkalec, M., Sikic, S., Tolic, S., Cvjetko, P., Pavlica, M. "Biochemical responses of *Lemna minor* experimentally exposed to cadmium and zinc." *Ecotoxicology,* 20.4 (2011):815-826.
5. Bernotiene, R., Ivanoviene, L., Sadauskiene, I., Liekis, A., & Ivanov, L. "Influence of cadmium ions on the antioxidant status and lipid peroxidation in mouse liver: Protective effects of zinc and selenite ions." *Trace Elements and Electrolytes*, 29.2 (2012): 137-142.

6. Blindauer, C. A., Leszczyszyn, O.I. “Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more.” *Natural Product Reports,* 27 (2009): 720-741.
7. Bressler, J. P., Olivi, L., Cheong, J.H., Kim, Y., Maerten, A., Bannon, D. "Metal transporters in intestine and brain: Their involvement in metal-associated neurotoxicities." *Human & Experimental Toxicology* 26.3 (2007): 221-29.
8. Chen, R.W., Vasey, E.J., Whanger, P.D. "Accumulation and depletion of zinc in rat liver and kidney metallothionens." *Journal of Nutrition* 107.5 (1977): 805-813.
9. Cucu, D., D’Haese, P.C., De Beuf, A., Verhulst, A. "Low doses of cadmium chloride and methallothionein-1-bound cadmium display different accumulation kinetics and induce different genes in cells of the human nephron." *Nephron Extra* 1.1 (2011): 24-37.
10. Cserr, H. F. “Physiology of the choroid plexus.” *Physiological Reviews,* 51.2 (1971): 273-307.
11. Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Nair, A.R., Munters, E., Artois, T.J., Nawrot, T., Vangronsveld, J., Smeets, K. "Cadmium stress: An oxidative challenge." *BioMetals* 23.5 (2010): 927-40.
12. Dalton, T. P., He, L., Wang, B., Miller, M.L., Jin, L., Stringer, K.F., Chang, X., Baxter, C.S., Nebert, D.W. “Identification of mouse SLC39A8 as the transporter

responsible for cadmium-induced toxicity in the testis." *Proceedings of the National Academy of Sciences,* 102.9 (2005): 3401-3406.

13. Davson, H., Segal, M.B. *Physiology of the CSF and blood-brain barriers*. Boca Raton, FL: CRC Press, 1996. 1-24.
14. El-Maraghy, Shohda A., Gad, M.Z., Fahim, A.T., Hamdy, M.A. "Effect of cadmium and aluminum intake on the antioxidant status and lipid peroxidation in rat tissues." *Journal of Biochemical and Molecular Toxicology* 15.4 (2001): 207-14.
15. Ennis, S. R., Keep, R.F. "The effects of cerebral ischemia on the rat choroid plexus." *Journal of Cerebral Blood Flow & Metabolism,* 26 (2006) 675-683.
16. Franklin, C. C., Backos, D.S., Mohar, I., White, C.C., Forman, H.J., Kavanagh, T.J. "Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase." *Molecular Aspects of Medicine*, 30 (2009), 86-98.
17. Fukui, S., Ookawara, T., Nawashiro, H., Suzuki, K., Shima, K. "Post-ischemic transcriptional and translational responses of EC-SOD in mouse brain and serum." *Free Radical Biology & Medicine,* 32.3 (2002): 289-298.
18. Ghersi-Egea, J.F., Strazielle, N. "Brain drug delivery, drug metabolism, and multidrug resistance at the choroid plexus." *Microscopy Research and Technique* 52.1 (2001): 83-88.
19. Ghersi-Egea, J.F., Strazielle, N., Murat, A., Jouvet, A., Buenerd, A., Belin, M.F. "Brain protection at the blood–cerebrospinal fluid interface involves a

glutathione-dependent metabolic barrier mechanism." *Journal of Cerebral Blood Flow & Metabolism,* 26 (2006): 1165-1175.

20. Girardin, F. "Membrane transporter proteins: A challenge for CNS drug development." *Dialogues in Clinical Neuroscience,* 8 (2006): 311-321.
21. Hermann, C. S. "Noch ein schreiben über das neue metall." *Annalen Der Physik,* 59.5 (1818): 113-116.
22. Jihen E.H., Sfar S., Hammouda F., Sfar M.T., and Kerkeni A. "Interrelationships between cadmium, zinc and antioxidants in the liver of the rat exposed orally to relatively high doses of cadmium and zinc." *Ecotoxicology and Environmental Safety* 74.7 (2011): 2099-104.
23. Johanson, C.E., Stopa, E.G., McMillan, P.N. "The blood-cerebrospinal fluid barrier: Structure and functional significance." *The blood-brain and other neural barriers: Reviews and protocols*. 686th ed. New York, NY: Humana Press, 2011. 101-31.
24. Jomova, Klaudia, and Marian Valko. "Advances in metal-induced oxidative stress and human disease." *Toxicology* 283 (2011): 65-87.
25. Lu, S.C. Regulation of glutathione synthesis. *Molecular Aspects of Medicine,* 30 (2009): 42-59.
26. Kah, M., Levy, L., Brown, C. "Potential for effects of land contamination on human health. 1. The case of cadmium." *Journal of Toxicology and Environmental Health, Part B* 15.5 (2012): 348-63.

27. Kalariya N.M., Nair B., Kalariya D.K., Wills N.K., Kuijk F.JG.M.V. “Cadmium-induced induction of cell death in human lens epithelial cells: implications to smoking associated cataractogenesis.” *Toxicology Letters* 198 (2010):56–62

28. Koizumi, S., Gong, P., Suzuki, K., Murata, M. "Cadmium-responsive element of the human heme oxygenase-1 gene mediates heat shock factor 1-dependent transcriptional activation." *Journal of Biological Chemistry* 282.12 (2007): 8715-723.

29. Liu, J., Liu, Y., Habeebu, S.S., Klaassen, C.D. "Susceptibility of MT-null mice to chronic CdCl-induced nephrotoxicity indicates that renal injury is not mediated by the CdMT complex." *Toxicological Sciences* 46.1 (1998): 197-203.

30. Lizotte, J., Abed, E., Signo, C. “Expression of macrophage migration inhibitory factor by osteoblastic cells: protection against cadmium toxicity,” *Toxicology Letters* 215. 3 (2012): 167–173

31. Maret, W., Sandstead, J. "Zinc requirements and the risks and benefits of zinc supplementation." *Journal of Trace Elements in Medicine and Biology* 20.1 (2006): 3-18.

32. Min, K., Tanaka, N., Horie, T., Kawano, H., Tetsuchikawahara, N., Onosaka, S. "Metallothionein-enriched hepatocytes are resistant to ferric nitriloacetate toxicity during conditions of glutathione depletion." *Toxicology Letters* 158.2 (2005): 108-15.

33. Mohanpuria, P., Rana, N.K., Kudav, S.Y. "Cadmium induced oxidative stress influence on glutathione metabolic genes of *Camellia sinensis* (L.) O. Kuntze."*Environmental Toxicology* 22.4 (2007): 368-74.

34. Napolitano, J. R., Liu, M.J., Bao, S., Crawford, M., Nana-Sinkam, P., Cormet-Boyaka, M., Knoell, D.L. "Cadmium-mediated toxicity of lung epithelia is enhanced through NF-kB-mediated transcriptional activation of the human zinc transporter ZIP8." *American Journal of Physiology: Lung Cellular and Molecular Physiology,* 302 (2012): L909-L918.

35. Nilsson, C. "Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system." *Brain Research Reviews,* 17 (1992): 109-138.

36. Nriagu J. "Zinc deficiency in human health." (2007) [http:/ / www.extranet.elsevier.com/ homepage_about/ mrwd/ nvrn/ Zinc%20Deficiency%20in%20Humans.pdf]

37. Nzengue, Y., Candéias, S.M., Sauvaigo, S., Douki, T., Favier, A., Rachidi, W., Guiraud, P. "The toxicity redox mechanisms of cadmium alone or together with copper and zinc homeostasis alteration: Its redox biomarkers." *Journal of Trace Elements in Medicine and Biology* 25.3 (2011): 171-80.

38. Omata, Y., Salvador, G. A., Supasai, S., Keenan, A. H., Oteiza, P. I. "Decreased zinc availability affects glutathione metabolism in neuronal cells and in the developing brain." *Toxicological Sciences,* 133.1 (2013): 90-100.

39. "Safety and Health Topics Cadmium." Occupational Safety and Health Administration. US Department of Labor. Web. 23 Apr. 2012. <http://www.osha.gov/SLTC/cadmium/index.html>.

40. Segal, M. B. “The choroid plexuses and the barriers between the blood and the cerebrospinal fluid.” *Cellular and Molecular Neurobiology,* 20 (2000): 183-196.

41. Shukla, G.S., Chiu, J.F., Hart, B.A. “Enhanced expression of pulmonary γ-glutamylcysteine synthetase heavy subunit in rats exposed to cadmium aerosols.” *Toxicology and Applied Pharmacology* 163 (2000): 249-259.

42. Shukla, G.S., Shukla, R.J., Potts, M., Hart, B.A., Chiu, J.F. “Cadmium-mediated oxidative stress in alveolar epithelial cells induces the expression of γ-glutamylcysteine synthetase catalytic subunit and glutathione s-transferase α and π isoforms: Potential role of activator protein-1.” *Cell Biology and Toxicology* 16 (2000): 347-362.

43. Solis, W.A., Dalton, T.P., Dieter, M.Z., Freshwater, S., Harrer, J.M., He, L., Shertzer, H.G., Nebert, D.W. "Glutamate–cysteine ligase modifier subunit: Mouse *Gclm* gene structure and regulation by agents that cause oxidative stress." *Biochemical Pharmacology* 63.9 (2002): 1739-754.

44. Stenstrom, T. "Cadmium availability to wheat: A study with radioactive tracers under field conditions." *AMBIO* 3.2 (1974): 87-90.

45. Strazielle, N., Ghersi-Egea, J. "Demonstration of a coupled metabolism-efflux process at the choroid plexus as a mechanism of brain protection toward xenobiotics." *The Journal of Neuroscience* 19.15 (1999): 6275-289.

46. Suzuki, H., Tashiro, S., Sun, J., Doi, H., Satomi, S., Igarashi, K. "Cadmium induces nuclear export of Bach1, a transcriptional repressor of heme oxygenase-1 gene." *Journal of Biological Chemistry* 278.49 (2003): 49246-49253.

47. Szewczyk, B., Kubera, M., Nowak, G. "The role of zinc in neurodegenerative inflammatory pathways in depression." *Progress in Neuropsychopharmacology & Biological Psychiatry,* 35.3 (2011): 693-701.

48. Tang, W., Sadovic, S., Shaikh, Z.A. "Nephrotoxicity of cadmium-metallothionein: Protection by zinc and role of glutathione." *Toxicology and Applied Pharmacology* 151.2 (1998): 276-82.

49. Valko, M., Leibfritz, D., Moncola, J., Croninc, M. T. D., Mazura, M., Telserd, J. "Free radicals and antioxidants in normal physiological functions and human disease." *The International Journal of Biochemistry & Cell Biology*, 39.1 (2007): 44-84.

50. Valois, A. A., Webster, W. S. "The choroid plexus as a target site for cadmium toxicity following chronic exposure in the adult mouse: An ultrastructural study." *Toxicology,* 55.1-2 (1989): 193-205.

51. Van Kerkhove, E., Pennemans, V., Swennen, Q. "Cadmium and transport of ions and substances across cell membranes and epithelia." *BioMetals* 23.5 (2010): 823-55.

52. Young, R.K., Villalobos, A.R. "Stress-induced stimulation of choline transport in cultured choroid plexus epithelium exposed to low concentrations of cadmium."

American Journal of Physiology Regulatory, Integrative, and Comparative Physiology 306.5 (2014): R291-303

53. Zhao, Y., Chen, L., Gao, S., Toselli, P., Stone, P., Li, W. "The critical role of the cellular thiol homeostasis in cadmium perturbation of the lung extracellular matrix." *Toxicology* 267.1-3 (2010): 60-69.
54. Zheng, W., Chodobski, A. *The blood-cerebrospinal fluid barrier*. Boca Raton, FL: Taylor & Francis, 2005. 222-226
55. Zheng, W., Perry, D.F., Nelson, D.L., Aposhian, H.V. “Choroid plexus protects cerebrospinal fluid against toxic metals.” *FASEB Journal,* 5 (1991): 2188-2193.