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SYSTEMIC IMBALANCE OF ESSENTIAL METALS AND CARDIAC GENE EXPRESSION IN RATS FOLLOWING ACUTE PULMONARY ZINC EXPOSURE

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It was recently demonstrated that particulate matter (PM) containing water-soluble zinc produces cardiac injury following pulmonary exposure. To investigate whether pulmonary zinc exposure produces systemic metal imbalance and direct cardiac effects, male Wistar Kyoto (WKY) rats (12–14 wk age) were intratracheally (IT) instilled with saline or 2 μmol/kg zinc sulfate. Temporal analysis was performed for systemic levels of essential metals (zinc, copper, and selenium), and induction of zinc transporter-2 (ZT-2) and metallothionein-1 (MT-1) mRNA in the lung, heart, and liver. Additionally, cardiac gene expression profile was evaluated using Affymetrix GeneChips (rat 230A) arrays to identify zinc-specific effects. Pulmonary zinc instillation produced an increase in plasma zinc to ~20% at 1 and 4 h postexposure with concomitant decline in the lung levels. At 24 and 48 h postexposure, zinc levels rose significantly (~35%) in the liver. At these time points, plasma and liver levels of copper and selenium also increased significantly, suggesting systemic disturbance in essential metals. Zinc exposure was associated with marked induction of MT-1 and ZT-2 mRNA in lung, heart, and liver, suggesting

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systemic metal sequestration response. Given the functional role of zinc in hundreds of proteins, the gene expression profiles demonstrated changes that are expected based on its physiological role. Zinc exposure produced an increase in expression of kinases and inhibition of expression of phosphatases; up- or downregulation of genes involved in mitochondrial function; changes in calcium regulatory proteins suggestive of elevated intracellular free calcium and increases in sulfotransferases; upregulation of potassium channel genes; and changes in free radical-sensitive proteins. Some of these expression changes are reflective of a direct effect of zinc on myocardium following pulmonary exposure, which may result in impaired mitochondrial respiration, stimulated cell signaling, altered Ca^{2+} homeostasis, and increased transcription of sulfotransferases. Cardiotoxicity may be an outcome of acute zinc toxicosis and occupational exposures to metal fumes containing soluble zinc. Imbalance of systemic metal homeostasis as a result of pulmonary zinc exposure may underlie the cause of extrapulmonary effects.

Although cardiovascular effects of pulmonary exposure to ambient particulate matter (PM) are well recognized (Pope et al., 2002; Weinhold, 2004), the specific causative components have not been identified. It was suggested that combustion-derived bioavailable metals and mobile sources-derived organic components may specifically play a significant role in adverse cardiac outcomes (Magari et al., 2002; Finkelstein et al., 2004; Riediker et al., 2004). Ambient PM contains significant quantities of iron, aluminum, silica, zinc, and copper; the levels of these elements vary depending on the geographical location and nearby industrial operations (Schwar et al., 1988; Tong & Lam, 1998; Harrison & Yin, 2000; Kodavanti et al., 2000, 2005; Saldiva et al., 2002; Banerjee, 2003; Smith et al., 2003).

A thorough review of ambient PM elemental data indicates that zinc is ubiquitously detected in a variety of ambient samples, albeit at varied concentrations (Harrison & Yin, 2000; Kodavanti et al., 2000, 2005; Saldiva et al., 2002; Smith et al., 2003). Because atmospheric zinc is likely derived from power plant emissions (Kodavanti et al., 2003), industrial operations (Dye et al., 2001; Newhook et al., 2003), waste incinerator emissions, and tire/brake wear-off/burning (Adachi & Tainosho, 2004; Councell et al., 2004), metallic concentrations are known to be greater in urban areas (Harrison & Yin, 2000). It is likely that pulmonary exposure to PM-associated zinc produces adverse pulmonary and cardiac effects (Kodavanti et al., 2002, 2003).

Zinc is an essential nutrient required for maintenance of a number of physiological functions, including cell growth, immune maturation, and reproduction, and is known to function as an antioxidant via its induction of metallothioneins (Maret, 2000, 2001, 2004; Henkel & Krebs, 2004). However, there has been a recent growing concern about zinc's potential adverse cardiovascular and neuronal effects. Paradoxically, a role for zinc has been suggested in Alzheimer's disease, as it is known to be accumulated significantly in the affected areas of the brain (Cuajungco & Faget, 2003; Zatta et al., 2003; Fredrickson et al., 2004). Zinc is known to compete for protein binding with other essential metals such as copper and selenium and to produce a systemic metal imbalance that may also alter the functionality of copper- and

selenium-containing proteins (Labbe & Fischer, 1984; Sandstead, 1995; Yamaguchi et al., 1996; Jacob et al., 1998; Klevay, 2000). Studies on zinc homeostasis also suggest its multifaceted effects on mitochondrial respiration (Ye et al., 2001), calcium homeostasis (Hershfinkel et al., 2001; Maret, 2001), sulfur–metal coordination (Maret, 2004), and intracellular signaling (Samet et al., 2003). Zinc is known to inhibit thiolate-dependent phosphatases and thus to activate signal-associated kinases in cells (Samet et al., 1999). A recent in vitro study demonstrated yet additional direct effects of zinc on potassium channel proteins in rodent cardiomyocyte cultures (Graff et al., 2004). While no studies have shown cardiovascular effects of zinc at low ambient concentrations, occupational exposure to zinc fumes was shown to cause pulmonary injury (Blanc et al., 1993; Fine et al., 1997). However, the concentrations and contribution of the water-soluble form of zinc in zinc fumes, and its potential cardiac effects in vivo are less well understood.

PM-associated water-soluble metal ions including zinc are absorbed by the pulmonary vasculature and reach cardiac tissue (Rhoads & Sanders, 1985; Gilmour et al., 2005). Since pulmonary capillary blood carrying soluble and bioavailable PM constituents may reach cardiac tissue prior to mixing with the blood pool or passage through liver or kidney, the high-flow coronary circulation may expose the heart to relatively high concentrations of toxic elements and organics and thus make the heart a target of adverse PM-induced health effects. Indirect cardiac effects of pulmonary PM exposure involving systemic endothelial activation and increased microvascular thrombosis, secondary to pulmonary inflammation (Nemmar et al., 2003), are also hypothesized. Both these hypotheses regarding potential mechanisms of PM-induced cardiac effects are being actively investigated (Kodavanti et al., 2003).

Recently it was reported that long-term episodic exposure to combustion particle samples containing water-leachable zinc produced myocardial injury in the Wistar Kyoto rat (Kodavanti et al., 2003). Furthermore, studies demonstrated that acute bolus intratracheal instillation of zinc sulfate produced procoagulant effects on the heart, effects that were associated with acute lung injury/inflammation and an increase in systemic levels of zinc (Gilmour et al., 2005). However, because the procoagulant effect and resulting systemic endothelial activation may occur via pulmonary inflammation induced by pulmonary zinc exposure, it is likely that those cardiac effects of zinc may be indirect. This work is an extension of a previous report on zinc-induced coagulation effects (Gilmour et al., 2005), now focusing on identification of possible direct effects of pulmonary-delivered zinc on the heart by analysis of gene expression profile. This is the first report to show that pulmonary zinc exposure, likely in an occupational scenario, can have profound systemic effects and changes in cardiac gene expression. Data on zinc-induced systemic imbalance of essential metals such as zinc, copper, and selenium, along with metallothionein and zinc transporter expressions in the lung, heart, and liver, are also reported.

MATERIALS AND METHODS

Animals

Twelve- to 14-wk-old male WKY rats weighing between 280 and 340 g were used in all experiments (Charles River, Raleigh, NC). Animals were housed in an isolated animal room in the AAALAC-approved animal facility (21 ± 1°C, 50 ± 5% relative humidity, 12-h light–dark cycle) and allowed free access to standard 5001 Purina rat chow (Brentwood, MO) and water.

Intratracheal Instillation

Rats in each group were randomized by body weight into saline control ($n = 4$ for each time point) and zinc sulfate ($ZnSO_4$) exposure ($n = 6$ for each time point) groups. The $ZnSO_4$ (Sigma Chemical Co., St Louis, MO) was dissolved in pyrogen-free sterile saline and instilled intratracheally (IT) at a dose of 2 μmol/kg, in a volume of 1 ml/kg, under halothane anesthesia (Costa et al., 1986); control rats received 1 ml/kg sterile saline. Although the high concentration of zinc used in this study was perhaps >1000-fold than what may be encountered environmentally, occupational exposures associated with zinc fume fever may occur at rather high zinc oxide concentrations (Barceloux, 1999). However, it is not known whether the lung injury is produced by insoluble particulate zinc oxide or a water-soluble fraction of zinc in the fumes. The objective for this study was to develop a pulmonary exposure model that would induce changes in systemic levels of zinc and demonstrable cardiac changes. Since systemic zinc levels are high, it was necessary to use high zinc concentrations such that changes in tissues can be detectable using nonisotope zinc sulfate. Responses were determined at 1, 4, 24, and 48 h following IT instillation.

Necropsy, Sample Collection, and Analysis

At the designated time points, rats were weighed and anesthetized with an overdose of sodium pentobarbital (50–100 mg/kg, ip). The blood was collected in blood collection tubes containing heparin for analysis of blood and plasma levels of zinc, copper, and selenium. The heart, liver, kidney and lung were removed, blotted dry, and weighed; portions of the left ventricle, liver, kidney and lung lobes were snap-frozen in liquid nitrogen and retained for metal and mRNA analysis.

RNA Isolation

Total lung, liver, and heart RNA was isolated from tissues snap-frozen in liquid nitrogen by homogenization in TriReagent (Sigma, St Louis, MO) and dissolved in 50 μl diethylpyrocarbonate (DEPC)-treated water. RNA was DNase treated (Promega, Madison, WI) and reverse transcribed to cDNA with the application of a GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). Total heart RNA was also used for expression analysis using Affymetrix gene chips.

Microarray Analysis

Microarray data was collected at Expression Analysis, Inc. (www.expressionanalysis.com; Durham, NC), using the GeneChip rat 230A array (Affymetrix Inc., Santa Clara, CA) containing 15923 probe sets with known genes including ESTs. The probe sets were selected from sequences derived from GenBank, dbEST, and RefSeq. The sequence clusters were created from the UniGene database (Build 107, June 2002).

Before cRNA production, the quality and quantity of each RNA sample were assessed using a 2100 BioAnalyzer (Agilent Technologies, Inc., Palo Alto, CA). The target cRNA was prepared and hybridized according to the Affymetrix Technical Manual. Total RNA (10 μg) was converted into cDNA using reverse transcriptase (Invitrogen) and a modified oligo(dT) 24 primer that contains T7 promoter sequences (GenSet). After first strand synthesis, residual RNA was degraded by the addition of RNaseH and a double-stranded cDNA molecule was generated using DNA Polymerase I and DNA Ligase. The cDNA was then purified and concentrated using phenol:chloroform extraction followed by ethanol precipitation. The cDNA products were incubated with T7 RNA polymerase and biotinylated ribonucleotides using an in vitro transcription kit (Enzo Diagnostics Inc., Farmingdale, NA). One-half of the cRNA products were purified using an RNeasy column (Qiagen Inc., Valencia, CA) and quantified with a spectrophotometer. The cRNA target (20 μg) was incubated at 94°C for 35 min in fragmentation buffer (Tris, MgOAc, KOAc). The fragmented cRNA was diluted in hybridization buffer (MES, NaCl, ethylenediamine tetraacetic acid [EDTA], Tween 20, herring sperm DNA, acetylated bovine serum albumin [BSA]) containing biotin-labeled oligoB2 and eukaryotic hybridization controls (Affymetrix). The hybridization cocktail was denatured at 99°C for 5 min, incubated at 45°C for 5 min, and then injected into a GeneChip cartridge. The GeneChip array was incubated at 42°C for at least 16 h in a rotating oven at 60 rpm. GeneChips were washed with a series of nonstringent (25°C) and stringent (50°C) solutions containing variable amounts of MES, Tween 20, and SSPE. The microarrays were then stained with streptavidin phycoerythrin and the fluorescent signal was amplified using a biotinylated antibody solution. Fluorescent images were detected in a GeneChip scanner 3000, and expression data were extracted using MicroArray Suite 5.0 software (Affymetrix). All GeneChips were scaled to a median intensity setting of 500.

Real-Time Quantitative PCR

One-step reverse-transcription polymerase chain reaction (RT-PCR) was carried out using the platinum quantitative RT-PCR ThermoScript one-step system (Invitrogen, Carlsbad, CA), following the protocol recommended for the kit. All reactions were run in duplicate using 1 μg total RNA. Reactions were multiplex, that is, containing one pair of target gene primers and one pair of endogenous control gene (β-actin) primers in each sample well. Real-time PCR was conducted on an ABI Prism 7900 HT sequence detection system

(Applied Biosystems, Foster City, CA). RT-PCR conditions consisted of reverse transcription for 20 min at 60°C and inactivation of reverse transcriptase activity for 5 min at 95°C. There were 40 amplification cycles at 95°C for 15s followed by 60°C for 45s. Primer sequences were: (1) MT-1, forward 5′-CTGACTGC-CTTCTTGTCGCTTACA-3′ and MT-1 reverse 5′-CTCTTCTTGCAGGAGGTG-CATTTG-3′, (2) ZT-2 forward, 5′-GAGCTGCCTTCATTCATGTGGTTG-3′ and ZT-2 reverse 5′-CACGTACTTGTACTCGGGCTTGGG-3′. TaqMan probe sequences were MT-1, 5′-FAM-GCCTCCAGATTCACCAGATCTCGG-3′, and ZT-2, 5-FAMTGGGC GTCCTGGTGGCAGCCTATA-3′, where FAM is 6-carboxyfluorescein. Primers for β-actin were Lux primers: forward 5′-CGGTTGGCCTTAGGGTTCA-3′ and 5′-GGTTGGCCTTAGGGTTCAGA-3′ and reverse 5′-CACGCCACTTTCTACAAT-GAGCTGCG*6*G-3′, where *6* represents the position of a Joe fluorescent label. Data were analyzed using ABI sequence detection system software, version 2.1. For each plate run, the cycle threshold (cT) was set to about one order of magnitude above background fluorescence, and cT values were obtained for each sample. In each case, cT of the target gene was normalized to changes in actin cT to account for variability in starting RNA amount. Expression for each exposure condition was quantified relative to expression of the corresponding saline control group.

Statistical Analysis

The data for metals and PCR were analyzed statistically by a two-way analysis of variance (ANOVA), with treatment as one factor and time as the other, using SigmaStat software, version 3.0 (SPSS, Inc., Chicago). For PCR data, delta CT values were used prior to their conversion to fold change. In the case of significant interaction, step-down ANOVAs were used to test for main effects of zinc with time. Pairwise comparisons between groups were made using Fisher's least significant difference (LSD) test. The accepted level of significance was $p < .05$.

Statistical analysis of Affymetrix gene array data was performed differently. Data from the Expression Analysis file include normalized density values from 4 control animals (2 at 4 h and 2 at 24 h postexposure) and 8 zinc-exposed animals (4 at 4 h and 4 at 24 h postexposure). The values were $\log_2$-transformed and plotted by slide and treatment against the treatment group mean log signals. The normalization appeared to be sufficient. There was more scatter in the lower intensity levels, but much of this disappeared when non-present probe sets were removed from the graph. There were 15,923 probe sets, 15,866 after removing the Affymetrix controls. For this analysis, a probe set was considered to be "present" if the detection call was "P" for 2/2 chips of at least one saline group or 3/4 chips of at least 1 zinc group. A total of 8045 probe sets was defined to be present. Each of the 8045 probe sets was analyzed with a one-way analysis of variance. Where the p value from the F test was $<.05$, the probe set was considered to be statistically significant, and 1409 probe sets reached this level of significance. For each of these, four pairwise t-tests were calculated within the analysis of variance to determine whether there were

differences in treatment effect within each time point or differences in time effect within each treatment. The signal intensity means and standard errors for each treatment by hour group from the analysis of variance were calculated. The estimated fold change and p value for each pairwise difference were also calculated. The 1409 probe sets were further divided into groups according to effect type using a two-way factorial analysis of variance, which tested for any effect of treatment, time, or any interaction of the two. Where there was a significant or near significant interaction ($p < .1$), the probe set was included in the effect type = interaction group. If the interaction had $p \geq .1$, the interaction was removed from the analysis and a main effects model was used. From this, probe sets were assigned to treatment effects (treatment, $p < .05$), time (h, $p < .05$), or treatment × time (both $p < .05$). There were seven probe sets that did not reach the criteria for any of these four groups and were placed in a different category. The mean table and fold-change files were sorted by the effect type and then by probe set ID.

RESULTS

Zinc, Copper, and Selenium Changes in Plasma and Tissues

Pulmonary exposure to water-soluble zinc sulfate may produce systemic metal imbalance. As reported earlier (Gilmour et al., 2005), the levels of total zinc increased significantly in lung tissue at 1 h following instillation of zinc sulfate but decreased to normal background levels by 24 and 48 h postexposure (Figure 1). Zn returning to baseline was associated with a significant increase in lung copper. However, lung selenium levels did not change.

Plasma levels of zinc increased at 1 and 4 h postexposure to nearly 20% above control but returned to baseline by 24 to 48 h (Figure 1). At these times, however, copper and selenium levels increased significantly. There were no changes in cardiac zinc, and selenium levels at any times. At 48 h post zinc exposure, however, there was a significant increase in the level of copper in the heart.

The total zinc concentration in the liver increased significantly at 24 h, and remained elevated at 48 h postexposure (Figure 1). Mass calculation of zinc in liver at 24 or 48 h postexposure revealed accumulation of 5–6 times more zinc than total instilled lung dose. Interestingly, this increase in liver zinc was also associated with marked rise in copper and selenium, suggesting that these essential metals were pooled from other tissue sites and sequestered in the liver. With kidney being the organ involved in clearance of metals, zinc, copper, and selenium accumulated in the kidney at the later time point of 48 h (data not shown).

Gene Expression Changes in the Heart From Pulmonary Zinc Exposure

Because zinc is an integral component of thousands of proteins and a modulator of cell functions, it was anticipated that the cardiac gene expression

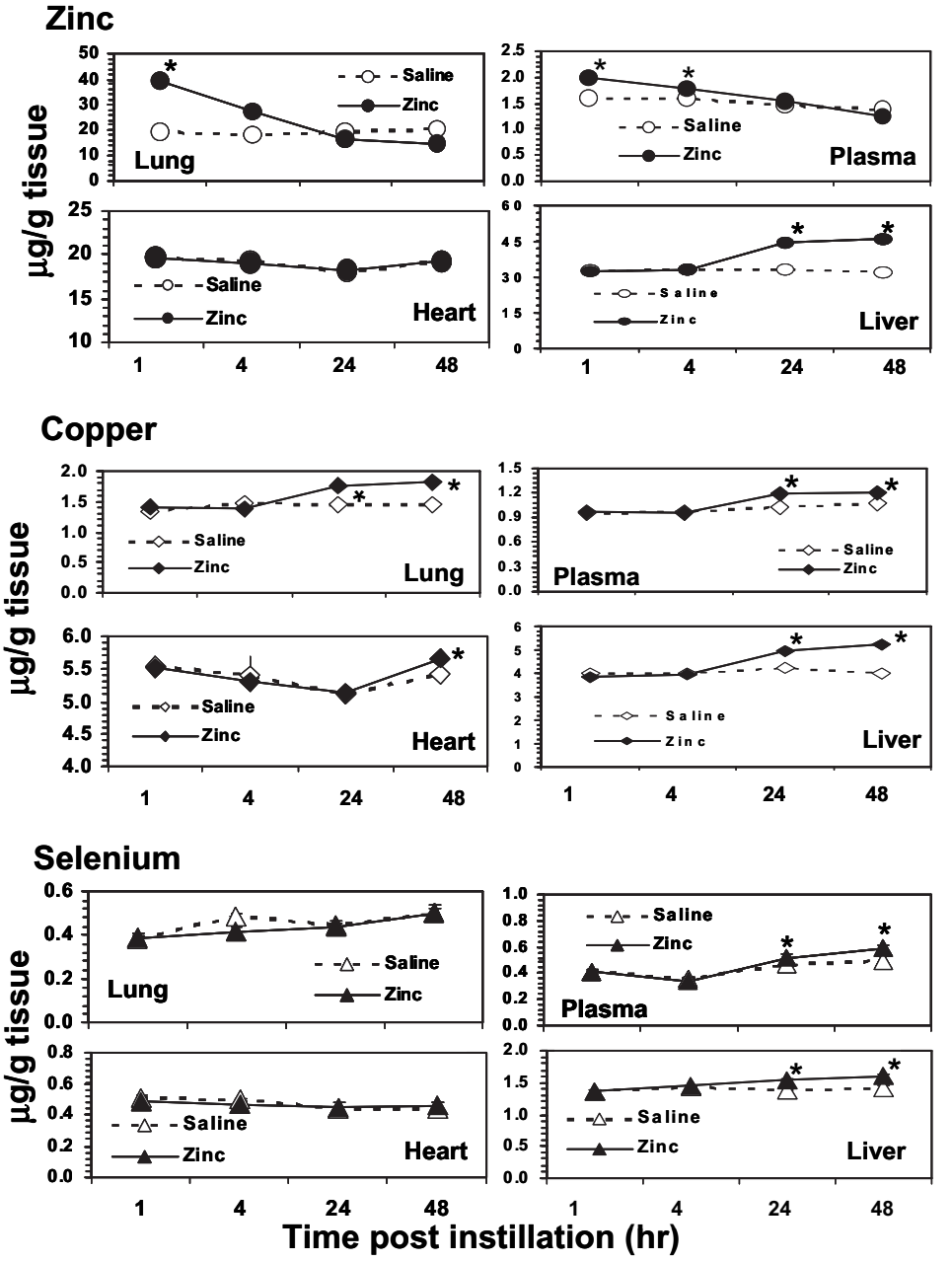

FIGURE 1. Temporal changes in the levels of zinc, copper, and selenium in the lung, plasma, heart, and liver following intratracheal instillation of zinc. At various time points following pulmonary zinc instillation, metals were analyzed in the right accessory lung lobe, plasma, left ventricle, and liver using ICP-MS. Changes reflect mean ± SE of four control and six exposed rats. Note that the error bars are within the size of symbol in most cases. Asterisk indicates significant difference at $p < .05$ relative to time-matched control. A part of the figure with data on zinc is reprinted with permission from Gilmour et al. (2005).

pattern would reflect known biological activities of zinc in the cell. To determine what impact pulmonary zinc exposure exerted on cardiac gene expression, which may indicate potential direct zinc effects on the heart, the gene expression pattern was determined using Affymetrix rat 230A GeneChips. After rigorous statistical analysis, the genes that changed at a p value of $<.05$ were carefully sorted out into biologically functional groups listed in Tables 1–6. Overall, gene expression changes were more pronounced at 24 h following zinc instillation than at 4 h, except for changes in kinases and phosphatases. Liver metal concentrations increased significantly at this time point, while all pulmonary zinc was cleared.

TABLE 1. Expression of Genes Involved in Cell Signaling (Kinases, Phosphatases, and Transcription Factors) in the Heart Following Pulmonary Zinc Exposure

	Zinc/saline—4 h		Zinc/Saline—24 h	
Gene description	Fold change	p Value	Fold change	p Value
Kinases				
Serine threonine kinase pim 3	2.14	.00	2.09	.00
FMS-like tyrosine kinase 1	2.00	.03	1.88	.05
Eukaryotic elongation factor-2 kinase	2.00	.03	2.19	.02
PX serine/threonine kinase	1.3	.07	1.63	.01
Mitogen activated protein kinase kinase kinase 1	0.74	.01	0.75	.01
Phosphatases				
MAP-kinase phosphatase (cpg 21)	0.56	.00	0.68	.03
Protein tyrosine phosphatase, non receptor type	0.87	.22	0.66	.00
Protein tyrosine phosphatase receptor type D	1.54	.03	1.71	.01
Protein phosphatase 1B, magnesium dependent	0.79	.20	1.57	.03
Transcription factors				
GATA binding protein 4	0.76	.02	0.79	.03
GATA binding protein 6	1.36	.03	1.71	.00
CCAAT/enhancer binding protein (C/EBP) beta	1.67	.00	1.42	.02
CCAAT/enhancer binding protein (C/EBP) delta	1.55	.10	2.56	.00
Regulator of G-protein signaling 19	0.69	.04	0.67	.03
Kinase insert domain protein receptor	0.84	.11	0.76	.02
Kruppel-like factor 15	1.63	.01	1.59	.01
Kruppel-like factor 9	1.49	.00	1.66	.00
Transcription factor MTSG 1	0.99	.96	2.16	.00
Other				
Protein kinase inhibitor, alpha	0.91	.48	1.71	.00
Cycline-dependent kinase inhibitor 1A	1.71	.02	2.74	.00
Nuclear receptor subfamily 1, group D, member 2	0.96	.85	2.26	.00
Phospholipase C, beta 4	1.03	.73	1.44	.00
Phospholipase A2, group IVA (cytosolic, calcium-dependent)	1.15	.11	1.76	.00

Note. Genes significantly differing from saline exposure and related to signaling based on literature search are listed. Saline values include 4 pooled controls (2 at 4 h and 2 at 24 h postinstillation) and 4 zinc-exposed rats at each time point. Values indicate mean fold change over saline and p value based on statistical analysis.

TABLE 2. Expression of Genes Involved in Respiration/Mitochondrial Function in the Heart Following Pulmonary Zinc Exposure

	Zinc/saline—4 h		Zinc/saline—24 h	
Gene description	Fold change	*p* Value	Fold change	*p* Value
Acetylcoenzyme A carboxylase beta	1.33	.03	1.33	.03
Uncoupling protein 3	1.74	.01	1.44	.04
6-Phosphofructo-2 kinase/fructose-2, 6-bisphosphatase 2	0.49	.00	0.44	.00
Cytochrome *c* oxidase subunit VIIIa	0.90	.36	0.76	.03
Phosphofructokinase, liver, B-type	0.86	.28	0.78	.09
Enzymatic glycosylation-regulating gene	1.32	.16	1.48	.05
Cytochrome *c* oxidase, subunit XVII assembly protein homolog	0.93	.03	0.80	.01
Cytochrome P-450, subfamily 4B, polypeptide 1	1.62	.00	1.77	.00
Pyruvate dehydrogenase phosphatase isoenzyme 2	0.70	.05	0.69	.05
P-450 (cytochrome) oxidoreductase	1.58	.00	1.20	.13
Pyruvate dehydrogenase kinase 2	0.94	.44	1.21	.03
Acylcoenzyme A dehydrogenase, very long chain	0.98	.81	1.30	.00
Diacylglycerol *O*-acyltransferase 1	1.13	.21	1.48	.00
Pyruvate dehydrogenase phosphatase isoenzyme 1	1.02	.83	1.40	.01
Pyruvate dehydrogenase kinase 4	2.36	.00	4.15	.00
Acylcoenzyme A dehydrogenase, short/branched chain	0.74	.39	3.13	.01
Isocitrate dehydrogenase 1	0.96	.78	1.56	.01
Citrate synthase	1.03	.74	1.42	.01
ADP-ribosyltransferase 1	0.94	.19	0.81	.00
Phosphoribosyl pyrophosphate synthetase-associated protein 2	1.02	.86	1.53	.01
ADP-ribosyltransferase 3	0.96	.35	1.41	.00
Rattus norvegicus similar to uridine phosphorylase, mRNA	1.70	.02	0.89	.54
3-Hydroxy-3-methylglutaryl-coenzyme A reductase	0.86	.38	0.47	.00
Hexokinase 1	1.12	.22	0.75	.01
Carnitine palmitoyltransferase 1, liver	1.37	.01	1.76	.00
ATP-binding cassette, subfamily G (WHITE), member 1	0.86	.28	1.93	.00
ATPase, Ca^{2+}-sequestering	0.88	.37	1.38	.05
ATPase, Ca^{2+}-transporting, cardiac muscle, slow twitch 2	1.21	.17	0.72	.03

Note. Genes significantly differing from saline exposure and related to mitochondrial respiration and function based on literature search are listed. Saline values include 4 pooled controls (2 at 4 h and 2 at 24 h postinstillation) and 4 zinc-exposed rats at each time point. Values indicate mean fold change over saline and *p* value based on statistical analysis.

Zinc produced marked induction of mRNA for kinases at both 4 and 24 h postexposure and was associated with inhibition of MAP-kinase phosphatase and protein tyrosine phosphatase gene expression (Table 1). While zinc induces cell signaling and affects the phosphorylation state of kinases via inhibition of phosphatases (Samet et al., 1999), this effect of zinc on mRNA expression may represent secondary feedback control mechanisms. Changes

TABLE 3. Expression of Ion Channel and Calcium Regulatory Genes in the Heart Following Pulmonary Zinc Exposure

	Zinc/saline—4 h		Zinc/saline—24 h	
Gene description	Fold change	*p* Value	Fold change	*p* Value
Potassium/sodium and other channels				
Potassium voltage-gated channel, KQT-like subfamily, member 1	1.21	.14	1.47	.01
Potassium inwardly rectifying channel, subfamily J, member 8	1.14	.12	1.37	.00
Potassium voltage-gated channel, shaker-related subfamily, member 5	2.36	.00	1.83	.02
Potassium inwardly-rectifying channel, subfamily J, member 5	1.21	.33	1.99	.01
Sodium channel, voltage-gated, type 1, alpha polypeptide	0.88	.36	1.54	.01
Solute carrier family 20 (phosphate transporter), member 1	0.99	.93	0.73	.00
Aquaporin 1	1.66	.02	1.67	.02
Ca^{2+} homeostasis				
Calcium channel, voltage-dependent, alpha 1C subunit	1.09	.48	1.52	.01
Cathepsin L	1.40	.07	1.67	.01
Voltage-gated calcium channel	0.89	.46	0.63	.01
Calpactin 1 heavy chain	0.87	.22	0.70	.00
Calponin 3, acidic	0.87	.15	0.71	.00
Ca^{2+}-dependent activator protein	1.00	.97	1.56	.00
Calmodulin 1	1.01	.96	0.64	.00
Calmodulin 3	0.66	.00	0.90	.23
ATPase, Ca^{2+}-sequestering	0.88	.37	1.38	.05
Calcium binding protein p22	1.10	.32	1.68	.00
ATPase, Ca^{2+}-transporting, cardiac muscle, slow twitch 2	1.21	.18	0.72	.03

Note. Genes significantly differing from saline exposure and related to ion channel and calcium regulation based on literature search are listed. Saline values include 4 pooled controls (2 at 4 h and 2 at 24 h postinstillation) and 4 zinc-exposed rats at each time point. Values indicate mean fold change over saline and *p* value based on statistical analysis.

in transcription factors include increased expression of GATA binding protein 6 with decreased expression of GATA binding protein 4. CCAAT/enhancer binding protein and Kruppel-like factors expressions were increased. Transcription factor MTSG-1 expression rose at 24 h post zinc exposure. Increases in phospholipases and kinase inhibitors were also apparent.

A number of gene expression changes related to mitochondrial function were suggestive of the potential impact of pulmonary zinc on respiration and mitochondria (Table 2). A number of enzymes involved in tricarboxylic acid cycle were induced markedly, while there was some degree of inhibition of

TABLE 4. Expression of Transferases and Metabolism Genes in the Heart Following Pulmonary Zinc Exposure

	Zinc/saline—4 h		Zinc/saline—24 h	
Gene description	Fold change	*p* Value	Fold change	*p* Value
Glutamine synthetase 1	1.61	.04	2.06	.01
Xanthine dehydrogenase	1.54	.01	1.45	.02
Sulfotransferase family 1A, phenol-preferring, member 1	4.28	.00	4.94	.00
Rattus norvegicus similar to microsomal glutathione ***S***-transferase 2	1.54	.04	1.66	.02
Enzymatic glycosylation-regulating gene	1.32	.16	1.48	.05
Sulfatase FP	1.77	.00	2.06	.00
Mercaptopyruvate sulfotransferase	0.97	.80	0.71	.01
Protein disulfide isomerase-related protein	0.97	.80	0.57	.00

Note. Genes significantly differing from saline exposure and those related to transferases and metabolism based on literature search are listed. Saline values include 4 pooled controls (2 at 4 h and 2 at 24 h postinstillation) and 4 zinc-exposed rats at each time point. Values indicate mean fold change over saline and *p* value based on statistical analysis.

TABLE 5. Expression of Structural Genes in the Heart Following Pulmonary Zinc Exposure

	Zinc/saline—4 h		Zinc/saline—24 h	
Gene description	Fold change	*p* Value	Fold change	*p* Value
Myosin heavy chain, polypeptide 7	1.74	.01	2.13	.00
Rattus norvegicus similar to myosine light chain alkali, smooth-muscle isoform (MLC3SM) (LOC297831), mRNA	0.87	.05	0.70	.00
Tropomyosin 4	0.92	.23	0.62	.00
Actin alpha cardiac 1	0.96	.71	0.68	.01
Actin, beta	0.90	.50	0.53	.00

Note. Genes significantly differing from saline exposure and those encoding for structural proteins are listed. Saline values include 4 pooled controls (2 at 4 h and 2 at 24 h postinstillation) and 4 zinc-exposed rats at each time point. Values indicate mean fold change over saline and *p* value based on statistical analysis.

components of electron transport proteins. This effect of zinc is suggestive of an impact on mitochondria and ATP-generating pathways.

Zinc is known to induce changes in expression of ion channels in isolated cardiomyocytes in vitro (Graff et al., 2004) and to alter Ca^{2+} homeostasis of cells (Hershfinkel et al., 2001; Maret, 2001). Gene expression pattern analysis indicated increased expression of potassium channel genes at both 4- and 24-h time points (Table 3). In contrast, a solute carrier family phosphate transporter expression was inhibited, and expression of aquaporin was induced. Similarly, the expression of Ca^{2+} channel proteins was changed, with some

TABLE 6. Expression of Genes Encoding for Proteins Involved in Injury and Repair in the Heart Following Pulmonary Zinc Exposure

	Zinc/saline—4 h		Zinc/saline—24 h	
Gene description	Fold change	*p* Value	Fold change	*p* Value
HIF-1-responsive RTP801	3.97	.00	2.67	.00
Heme oxygenase 1	0.94	.65	1.56	.01
Heat-shock 70-kD protein 5	0.87	.08	0.69	.00
Heat-shock protein 86	0.84	.04	0.64	.00
BCL2/adenovirus E1B 19 kDa-interacting protein 3, nuclear gene for mitochondrial protein	1.10	.48	1.54	.01
Bcl2-like 1	2.37	.00	1.68	.04
Tissue inhibitor of metalloproteinase 3	1.24	.26	2.41	.00
Gap junction membrane channel protein alpha 1	0.59	.00	0.74	.02
Epoxide hydrolase 1	1.04	.71	1.71	.00
Endothelin receptor type B	0.75	.02	0.81	.08
Cyclin D2	0.70	.01	0.61	.00
Cyclin D1	0.52	.00	0.37	.00
Fibroblast growth factor 1	1.10	.41	1.67	.00
Thrombomodulin	0.61	.00	0.78	.08
Vascular endothelial growth factor C	0.52	.00	0.87	.32
Cd36 Antigen	1.23	.30	2.02	.01

Note. Genes significantly differing from saline exposure and those encoding for proteins involved in injury and repair are listed. Saline values include 4-pooled controls (2 at 4 h and 2 at 24 h postinstillation) and 4 zinc-exposed rats at each time point. Values indicate mean fold change over saline and *p* value based on statistical analysis.

channels showing increased and some decreased mRNA expression. Increased or decreased expressions were also noted for Ca^{2+}-binding proteins, with a decrease in Ca^{2+}-transporting ATPase expression, which may suggest potential dysregulation of Ca^{2+} homeostasis by different mechanisms.

Based on the role of zinc as a mediator of protein sulfur–metal exchange (Maret, 2004), a group of genes related to transferase activities was separated (Table 4). Zinc produced more than fourfold increased expression of sulfotransferase family 1A at both time points; however, the expression of mercaptopyruvate sulfotransferase was decreased at 24 h. Expression of glutamine synthetase was increased, along with expression of sulfatase FP and microsomal glutathione *S*-transferase-like gene.

There were very few changes in gene expression related to structural components following zinc exposure (Table 5). Myosin heavy chain expression was increased, whereas a gene similar to myosin, light chain smooth muscle isoform, was inhibited following zinc exposure. Expression of tropomyosin and both alpha- and beta-actin genes were reduced at the 24-h time point.

Genes that have important biological functions during injury and that were not necessarily grouped based on their functional properties are listed in Table 6. Expression of HIF-1-responsive RTP801 was increased nearly fourfold.

Expression of genes sensitive to oxidative stress, such as heme oxygenase 1 and epoxide hydrolase 1, were increased, whereas expression of heat-shock proteins was inhibited. Two genes likely involved in apoptosis were increased significantly. Gap junction membrane channel protein alpha 1 was inhibited, supportive of its role in cell-to-cell communication. Genes involved in vascular function were changed; endothelin receptor protein mRNA and vascular endothelial growth factor C were inhibited. Expressions of cd36 and fibroblast growth factor 1 were induced. Surprisingly, expression of cyclin D1 and D2 were inhibited following zinc exposure.

Zinc is known to induce metallothionein-1 (MT-1) mRNA expression. Studies were undertaken to determine mRNA expression of MT-1 and also zinc transporter-2 (ZT-2), not only in the heart but also in the lung and liver, to understand the kinetics relative to changes in essential metals. Immediately following 1 h of zinc exposure, more than fivefold increase in MT-1 mRNA expression occurred in the lung, and at 4 h it was 24-fold over the saline control. At 24 and 48 h the expression declined from its peak at 4 h, but still remained significantly elevated over control (Figure 2). Cardiac MT-1 mRNA doubled from saline at 1 and 4 h and was twofold higher at 24 h and onefold at 48 h. The temporal pattern of MT-1 mRNA expression in the liver differed from that of the lung and heart. Liver mRNA expression, although increased at 1 and 4 h, peaked (nearly 15-fold over control) at 24 h and remained at that level at 48 h following pulmonary zinc exposure, which correlated with increases in zinc, copper, and selenium in the liver.

Unlike MT-1, ZT-2 mRNA expression increased to a lesser degree in all three tissues (Figure 2). ZT-2 mRNA increased by threefold at 1 and 4 h postexposure in the lung, but this increase was less remarkable at 24 and 48 h. Cardiac ZT-2 mRNA expression doubled at 24 h postexposure but was not changed significantly at any other time points. An increase occurred in liver ZT-2 mRNA expression at 4 h but not at any other time points, suggesting that ZT-2 may play a distinct and unique role in maintaining zinc homeostasis in different tissues.

DISCUSSION

While pulmonary air pollution exposures may result in translocation of particle-associated water-soluble zinc to cardiac tissue at very low concentrations but in a chronic manner, occupational exposures to zinc fumes occur at high and potentially toxic levels. Accidental exposures resulting in zinc fume fever were associated with severe pulmonary inflammation (Blanc et al., 1993); however, the cardiac effects are not well studied. It is also not clear how much zinc is water-soluble in zinc fume particulates that are predominantly zinc oxide. In the recently published companion paper, we have shown that pulmonary exposure to high-dose zinc sulfate produced extensive pulmonary injury and inflammation and increased expression of tissue factor, plasminogen activator inhibitor-1, and thrombomodulin genes in the heart (Gilmour

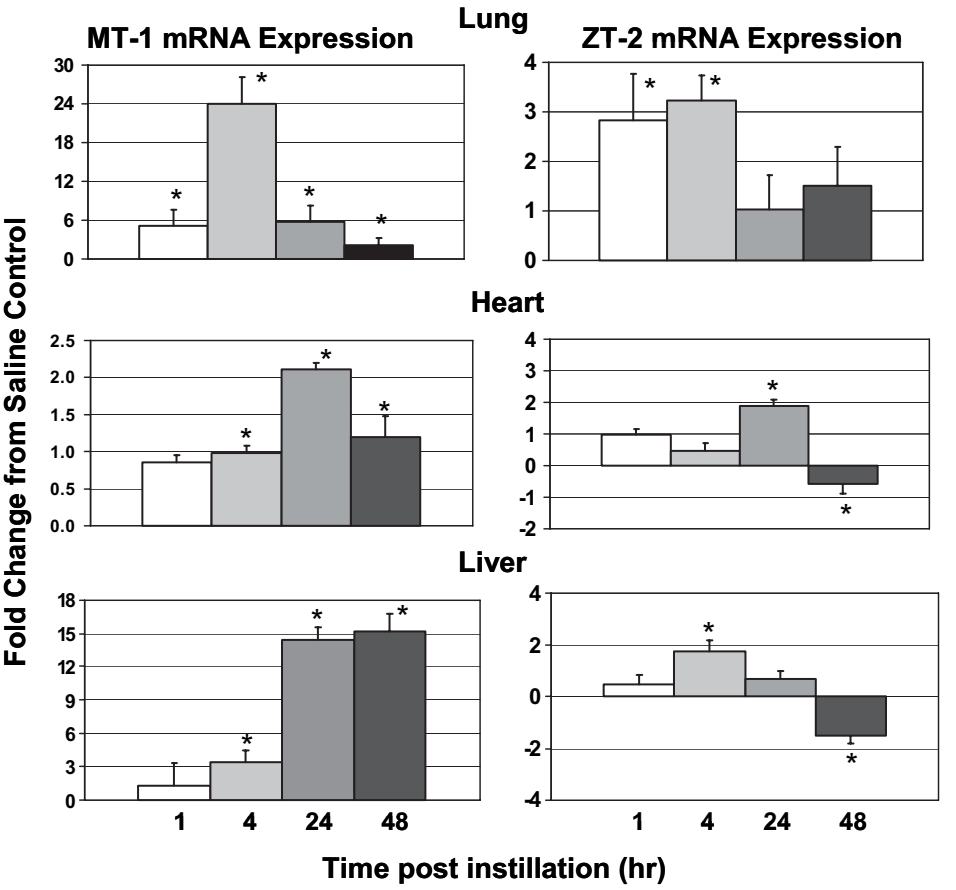

FIGURE 2. Time course of lung, heart, and liver metallothionein-1 (MT-1) and zinc transporter-2 (ZT-2) gene expression following pulmonary exposure to zinc. At various time points following pulmonary zinc instillation, MT-1 and ZT-2 gene expressions were analyzed using real-time PCR and fold increases in expression values were calculated from delta cT. Changes reflect mean ± SE of four control and six exposed rats. Asterisk indicates significant at $p < .05$ relative to time-matched control.

et al., 2005). However, these effects may be secondary to pulmonary inflammation. The current data demonstrated zinc-induced systemic imbalance of essential metals, along with upregulation of MT-1 and ZT-2 in the lung, heart and liver. While circulating levels of zinc were increased at 1 h after zinc exposure, those of copper and selenium were increased at 24 and 48 h. Changes in circulating zinc, copper, and selenium were associated with accumulation of these metals in the liver at 24- and 48-h time points. Temporality in MT-1 mRNA expression in the lung, liver, and heart is consistent with a disturbance in systemic metal homeostasis. Affymetrix gene expression analysis of cardiac tissue demonstrated changes in gene clusters involving mitochondria, Ca^{2+}

homeostasis, cell signaling, potassium/calcium and sodium channels, and transferases, some of which are suggestive of a direct cardiac effect of zinc.

Inhalation of zinc oxide fumes was associated with acute pulmonary inflammation and cytokine expression in the lung and plasma (Blanc et al., 1993; Fine et al., 1997). Pulmonary exposure to zinc sulfate produced acute pulmonary inflammation and injury (Gilmour et al., 2005). This recent study suggested that the cardiac proinflammatory and procoagulative effects induced by zinc may be the result of an acute and persistent pulmonary inflammatory response (Gilmour et al., 2005). The present study focused on essential metal homeostasis and cardiac gene expression changes, which are suggestive of direct effects of zinc on the heart and may also be the result of systemic imbalance of zinc, copper, and selenium.

As previously noted (Gilmour et al., 2005), the accumulation in the liver of 5.4 times the amount of instilled zinc suggests removal of zinc from other tissue sites, which may be due to an overwhelming stimulation of metal sequestration as an acute-phase response of liver following systemic elevation of zinc, and pulmonary inflammation. In an isolated heart model, free zinc in cells was shown to preferentially displace copper from its protein binding sites and therefore to produce copper translocation (Powell et al., 1999). This systemic response may profoundly affect copper and selenium homeostasis. Consequently, present studies showed that plasma levels of copper and selenium were significantly elevated at 24 and 48 h post zinc exposure and metallothionein gene expression was markedly increased. Surprisingly, this rise was associated with accumulation of copper and selenium in the liver to a significant extent, along with marked increases in MT-1 mRNA expression. This zinc, copper, and selenium accumulation in liver, likely bound to metallothioneins, may result in metal sequestration from bone and other tissues such as heart (Yamaguchi et al., 1996). Although heart tissue did not show gross metal level changes, induction of MT-1 and ZT-2 is suggestive of a significant disturbance in essential metal homeostasis.

The storage, mobilization, and detoxification of metals, including zinc, are primarily facilitated by metallothioneins, induced by the free zinc that competes for the binding of transition metals from other metal-containing proteins (Tapiero & Tew, 2003). Mobilization of zinc from other tissue macromolecules may contribute to toxicity by altering the functions of important proteins and enzymes. Induction of MT-1 not only in the lung and liver but also in the heart following pulmonary zinc exposure emphasizes zinc-induced alterations in the heart despite the lack of significant accumulation. A relatively small effect on MT-1 expression in the heart might, however, suggest that metallothioneins are not the primary storage mechanism for handling extra zinc in the heart. Instead, cardiac tissue might have mechanisms to remove extra zinc encountered in cells, and thus, zinc accumulation does not occur but physiological mechanisms to handle extra zinc are upregulated. Metallothionein was found to be upregulated in the heart in response to tumor necrosis factor (TNF) and interleukin (IL)-6 (Nath et al., 2000) and in the lung from inhalation of zinc

oxide (Cosma et al., 1992). Marked upregulation of MT-1 expression in the liver at later time points following pulmonary zinc exposure together with increased tissue zinc, copper, and selenium levels is reflective of a disturbance in metal homeostasis.

Global cardiac gene expression profile was analyzed in control and zinc-instilled rats in vivo to determine whether the gene expression changes are similar to known zinc-induced effects in cell cultures in vitro. There were numerous changes in the expression patterns for which genes were grouped into different functional categories. Cell signaling by metals, including zinc, was induced by inhibition of phosphatases and subsequent activation of kinases (Eom et al., 2001; Samet et al., 1999, 2003; Kindermann et al., 2005). It is not clear whether zinc also works at the transcriptional level to inhibit phosphatases and induces expression of kinases, but this appears to be the case in our study. These changes were associated with increased expression of protein kinase inhibitors. Thus, zinc, in addition to affecting phosphorylation events, induced mRNA changes, suggesting transcriptional regulation, possibly secondary to its effects on cells signaling (Maret, 2001; Samet et al., 2003; Kindermann et al., 2005). Zinc may induce downstream genes, including CCAAT/enhancer binding proteins, via metal responsive transcription factor (Tong et al., 2005).

Zinc, being an important functional component of hundreds of proteins, also has a profound effect on mitochondrial respiration. Metallothionein-bound zinc is known to translocate to mitochondria and to inhibit respiration in rat liver but not heart (Ye et al., 2001). However, the present study showed that pulmonary zinc exposure produced a variety of changes in mitochondria protein gene expressions suggestive of oxidative stress. Pyruvate dehydrogenase kinases mRNA was induced over fourfold. Increased activation of this enzyme with protein kinase A is known to increase free radical formation in isolated rat heart mitochondria (Raha et al., 2002), suggesting that there may be increased free radical formation in cardiac mitochondria following in vivo pulmonary zinc exposure. Zinc is also known to interact with bc1 complex and to inhibit mitochondrial aconitase activity in vitro (Larusso et al., 1991; Link & van Jagow, 1995; Costello et al., 1997), consistent with its potential role in mitochondrial oxidative stress. Inhibition of cytochrome *c* subunit mRNA expression and activation of uncoupling protein 3 correlate with increased free radical formation (Abdallah & Samman, 1993; Brad et al., 2004). Based on acute pulmonary zinc-mediated alterations of mitochondrial proteins and possible oxidative stress, the impact of chronic environmentally relevant exposure levels on mitochondrial respiration needs to be further examined.

Extracellular zinc may compete with other cations and metal ions and affect ion channel proteins, resulting in conductance abnormalities. Recently it was reported that zinc at higher than physiological levels induces mRNA expression of several potassium channels in rat cardiomyocytes (Ravindran et al., 1991; Graff et al., 2004). This may suggest that potassium channel activity was

increased via transcriptional activation. This effect was much more pronounced at 4 h postexposure as opposed to 24 h, when circulating zinc concentrations were higher. This is suggestive of a direct effect of pulmonary zinc on myocytes. However, similar changes are likely during ischemic preconditioning (Riksen et al., 2004), which may also occur as a result of pulmonary injury/inflammation. Thus, activation of potassium channels may suggest ischemic preconditioning (Riksen et al., 2004; Pollosello & Mabezaa, 2004).

The significant effect on mRNA for calcium regulatory proteins, especially at the 24-h time point, was suggestive of disturbance in intracellular Ca^{2+} homeostasis. Changes in extracellular zinc concentration participate in modulating ion transport (Maret, 2001); however, the mechanisms are less well understood. It was shown that G-protein-coupled receptors may sense changes in extracellular zinc concentration and subsequently trigger a release of intracellular Ca^{2+} (Hershfinkel et al., 2001). Inhibition of mRNA for calmodulin 1 and Ca transporting ATPase and an increase in voltage-dependent Ca^{2+} channel protein expression may indicate an overall increase in intracellular Ca^{2+} that can affect a variety of cellular processes.

The ligands associated with catalytic zinc ions in hundreds of enzymes are nitrogen and oxygen with histidine and glutamate/aspartate (Pearce et al., 2000; Maret, 2004). Zinc is also associated with cysteine and histidine ligands in nearly a thousand zinc finger proteins (Maret, 2004). Zinc–sulfur bonds participate in protein–protein and protein–nucleic acid interactions and maintain redox-active coordination environment for the redox-inactive zinc ion (Maret, 2000). It is not clear how zinc pulmonary exposure may have induced sulfotransferase and sulfatase; however, one can speculate that either excess zinc or its deficiency may exert a wide spectrum of effects on a variety of proteins and their catalytic activities.

The expression of hypoxia-inducible genes is upregulated by hypoxia-inducible factor-1 (HIF-1). Metals such as cobalt, nickel, and zinc, which can substitute for iron in ferroprotein, induce HIF-1 protein (Chun et al., 2001). Pulmonary zinc exposure produced a significant induction of cardiac HIF-1 responsive genes at both time points, such as induction of heme oxygenase-1, suggesting hypoxemia response as a result of acute pulmonary injury reported in previous study (Gilmour et al., 2005). Surprisingly, heat-shock proteins expressions were inhibited in general while Bcl-2 expression was increased. One can presume that some of these effects of zinc on the heart may be direct and some effects may result from pulmonary injury/inflammation.

Although at high concentration, pulmonary zinc exerted a profound effect on cardiac genes governing normal cardiac function by affecting mitochondrial energetics, ion transport regulation, Ca^{2+} homeostasis, and cell signaling. Environmental exposures are unlikely to occur in such a high concentration; however, cardiac complications following zinc exposures may be significant (Evangelou & Kalfakakou, 1993; Klevay et al., 1994) as balance in essential metals concentration is vital for protein functioning. Chronic low-level pulmonary exposure to zinc-containing ambient particles might result in significant cardiac exposure, as pulmonary capillary blood will encounter cardiac tissue

first, prior to its dilution in the bloodstream and subsequent systemic distribution/liver sequestration. Therefore, the consequences of low-level pulmonary metal exposure on heart may be significant.

In this study, data demonstrated that pulmonary zinc exposure results in systemic imbalance in zinc, copper, and selenium, associated with induction of metallothioneins in lung, heart, and liver. Temporal analysis of levels of these metals suggests that the liver may be sequestering these metals from other body organs, including heart, as no demonstrable increase occurred of any metals in the heart despite an elevation in MT-1 expression. Concomitantly, the cardiac gene expression profile depicted a variety of changes reflective of direct and indirect effects of zinc, which may disturb cell signaling, mitochondrial respiration, Ca^{2+} homeostasis, and ion channel functions.

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