Metallothionein-induced zinc partitioning exacerbates hyperoxic acute lung injury

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Lee SM, McLaughlin JN, Frederick DR, Zhu L, Thambiyya K, Wasserloos KJ, Kaminski I, Pearce LL, Peterson J, Li J, Latoche JD, Peck Palmer OM, Stolz DB, Fattman CL, Alcorn JF, Oury TD, Angus DC, Pitt BR, Kaynar AM. Metallothionein-induced zinc partitioning exacerbates hyperoxic acute lung injury. Am J Physiol Lung Cell Mol Physiol 304: L350–L360, 2013. First published December 28, 2012; doi:10.1152/ajplung.00243.2012.—Hypozincemia, with hepatic zinc accumulation at the expense of other organs, occurs in infection, inflammation, and aseptic lung injury. Mechanisms underlying zinc partitioning or its impact on extrahepatic organs are unclear. Here we show that the major zinc-binding protein, metallothionein (MT), is critical for zinc transmigration from lung to liver during hyperoxia and preservation of intrapulmonary zinc during hyperoxia is associated with an injury-resistant phenotype in MT-null mice. Particularly, lung-to-liver zinc ratios decreased in wild-type (WT) and increased significantly in MT-null mice breathing 95% oxygen for 72 h. Compared with female adult WT mice, MT-null mice were significantly protected against hyperoxic lung injury induced by reduced inflammation and interstitial edema, fewer necrotic changes to distal airway epithelium, and sustained lung function at 72 h hyperoxia. Lungs of MT-null mice showed decreased levels of immunoreactive LC3, an autophagy marker, compared with WT mice. Analysis of superoxide dismutase (SOD) activity in the lungs revealed similar levels of manganese-SOD activity between strains under normoxia and hyperoxia. Lung extracellular SOD activity decreased significantly in both strains at 72 h of hyperoxia, although there was no difference between strains. Copper-zinc-SOD activity was ~4× higher under normoxic conditions in MT-null compared with WT mice but was not affected in either group by hyperoxia. Collectively the data suggest that genetic deletion of MT-I/II in mice is associated with compensatory increase in copper-zinc-SOD activity, prevention of hyperoxia-induced zinc transmigration from lung to liver, and hyperoxia-resistant phenotype strongly associated with differences in zinc homeostasis during hyperoxic acute lung injury.

zinc; metallothionein; hyperoxia; autophagy; CuZn-SOD; acute lung injury

WHOLE BODY ZINC HOMEOSTASIS is efficiently controlled by a combination of absorption via gastrointestinal tract and excrete-
oxia would not be predicted from experience supporting its antioxidant activity (27) and distinguishes its metal-binding properties from these latter functions (6).

METHODS

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Experiments in WT and MT-null mice were performed to test the hypothesis that MT plays a protective role in hyperoxic acute lung injury. All experimental measurements were made at baseline (0 h) and at 24, 48, and 72 h after initiation of the experiments.

Mice and exposure protocol. Female MT-null mice (129S7/SvEvBrd-Mt1tm1Bri Mt2tm1Bri/J), with their respective matching 129S1/SvImJ wild-type mice (age 6–8 wk), were purchased from the Jackson Laboratory (Bar Harbor, ME). Female mice were chosen because of their increased sensitivity to hyperoxia compared with male mice (50). The mice were randomly assigned to either normoxia (21% O2) or hyperoxia (95% O2). The mice were anesthetized with an intraperitoneal injection of 60 mg/kg pentobarbital sodium (Ovation Pharmaceuticals, Deerfield, IL). A tracheostomy was performed and mice were attached to the ventilator. Following 5 min of stabilization, animals received deep lung inflation to a pressure of 50 cmH2O distending pressure to ensure uniform lung recruitment and low tidal volume. At 0, 24, 48, and 72 h, mice were sacrificed by CO2 asphyxiation and cervical dislocation, and lungs were recovered from mice for histology and immunofluorescence imaging, tissue sections were rehydrated with PBS and counterstained with Alexa Fluor 647-conjugated phalloidin (Life Technologies, Carlsbad, CA). Staining was performed using 5% formaldehyde in 0.1 M PBS, rinsed in PBS, and then blocked with 2% BSA. Tissue sections were then washed once with 0.5% BSA. Staining was accomplished by rabbit anti-LC3 and mouse anti-mouse EC-SOD (1:500) subsequently with secondary Cy3-conjugated goat anti-rabbit antibody (1:1,000) (Jackson ImmunoResearch, West Grove, PA). All tissues were counterstained with Alexa Fluor 647-conjugated phalloidin (1:250) (Invitrogen) and 1% bisbenzimide (ThermoFisher Scientific) to label F-actin and nuclei, respectively. Optical sections (0.4 μm) in z-axis were acquired with an Olympus FluoView 1000 confocal microscope (Olympus, Lehigh Valley, PA). Images were obtained with a ×60 optical lens with ×1.0 digital zoom at same exposure parameters.

Transmission electron microscopy. Specimens were fixed in cold 2.5% glutaraldehyde in 0.1 M PBS, rinsed in PBS, postfixed in 1% osmium tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol, and finally embedded in Epon. Semithin sections (300 nm) were cut on a Reichert Ultracut (Reichert, Depew, NY), stained with 0.5% toluidine blue and examined under the light microscope. Ultrathin sections (65 nm) were stained with uranyl acetate and Reynolds’s lead citrate and examined on JEOL 1011 transmission electron microscope (JEOL, Tokyo, Japan).

Measurement of SOD activity. The lungs were homogenized in 3 ml of 50 mM potassium phosphate with 0.3 M potassium bromide, pH 7.4. SOD specific activity was measured in the EC-SOD as well as the CuZn- and Mn-SOD fractions using a xanthine/xanthine oxidase system to generate superoxide anion and measuring the rate of reduction of acetylated cytochrome c as described (10, 56). SOD activity was determined in a 100-μl assay mixture containing 1 mM acetylated cytochrome c, 1 mM xanthine, and 1 mM EDTA in 50 mM potassium phosphate buffer, pH 7.8, at 25°C. Xanthine oxidase was added to give a rate of reduction of cytochrome c of ~0.0075 absorbance units/min (±10%). The absorbance was measured at 550 nm with a Spectramax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA). SOD activities were calculated from assays in which there was an inhibition of cytochrome c reduction of between 40 and 50%. Although all samples initially were analyzed at the same volume, the volume of sample was then adjusted repeatedly until the level of inhibition fell within the defined range. One unit of SOD activity was defined as the amount of SOD that gives 50% inhibition of the rate of cytochrome c reduction. An SOD standard curve was plotted with various dilutions of a CuZn-SOD standard at 40 McCord-Fridovich units/ml. One unit in the assay was equivalent to 0.027 McCord-Fridovich units/ml based on the standard curve. The total SOD (before DCC or cyanide treatment), Mn-SOD (after diethyldithiocarbamate or cyanide treatment), and CuZn-SOD activities (the difference between total SOD and Mn-SOD measurements) were determined (7, 36). EC-SOD was separated from intracellular CuZn- and Mn-SOD by concanavalin A-Sepharose chromatography as described (11, 43). EC-SOD activity analysis was performed on the eluates as described above for other SOD isoforms (9).

Measurement of zinc concentration. We used zinc-free HBSS to flush lungs and liver free of blood. Electrothermal atomic absorption spectrometry and inductively coupled plasma mass spectrometry (ICP-MS) were used to measure lung and liver zinc content. We initially measured a representative group of lung and liver tissues using atomic absorption, and because of low levels of zinc in the hyperoxic lung tissues we adapted ICP-MS owing to its improved sensitivity (45). The lung-to-liver zinc ratios were similar using both methods. Lung and liver zinc contents are reported from individual animals as matched ratios (Fig. 2).

Electrothermal atomic absorption spectrometry. Dried liver and lung tissues (~0.3 g) were digested in concentrated 67–70% nitric acid (Fisher, Fair Lawn, NJ) in Teflon digestion vessels. Tissue-free blanks were similarly prepared. Samples and blanks were digested (200°C, 800 psi, 15 min) in CEM MarsXpress microwave system
(CEM, Matthews, NC). Digested tissues were transferred to polypropylene volumetric flasks and reconstituted to 50 ml with 2% HNO₃, and trace metal acid was diluted in twice-deionized H₂O. All plasticware was prewashed with 2% HNO₃ and dried in HEPA-filtered laminar-flow cabinet configured for trace metal work (AirClean Systems, Raleigh, NC). Zinc was analyzed by graphite-furnace atomic absorption spectroscopy (AAnalyst 800, PerkinElmer, Waltham, MA) employing Zeeman background correction with magnesium nitrate (Sigma-Aldrich) [99.999% Mg(NO₃)₂·6H₂O] as matrix modifier. Calibration curves of zinc (Zn²⁺) in 2% HNO₃ were prepared from 99.999% zinc shot (Sigma-Aldrich) and 1,000 mg/l commercial standard SPEX CertiPrep (Spex, Metuchen, NJ). All experiments were measured in triplicate.

Inductively coupled plasma mass spectrometry. Tissue samples were dried at 55°C for 48 h prior to analysis. A 250 mg aliquot of dried tissue was digested for 6 h in 30% nitric acid and analyzed for zinc by ICP-MS. All data were normalized on a dry weight basis not to bias the results as a result of different degrees of lung or liver injury and edema. At least 10% of each analytical batch consisted of quality control samples. Analytical accuracy was assessed with reagent blank, initial and continuing calibration verification standards, and standard reference material. Analytical precision was assessed with sample duplicates.

Measurement of ALT specific activity. Measurement of serum alanine transaminase (ALT) specific activity was performed by monitoring the rate of NADH oxidation in a coupled reaction including lactate dehydrogenase as an indirect marker of liver injury in serum samples. The reaction includes transamination of l-alanine to pyruvate through ALT-mediated catalysis. Pyruvate is then converted to lactate by lactate dehydrogenase, during which NADH is oxidized to NAD⁺. The oxidation of NADH to NAD⁺ is accompanied by decreased absorbance at 340 nm. ALT specific activity is rate limiting and therefore oxidation is directly proportional to the ALT specific activity, presented as units per liter (Cayman Chemical, Ann Arbor, MI).

Data analysis. Data are expressed as means ± SD. A one-way ANOVA and post hoc comparisons were performed. A P value of < 0.05 was considered significant. The values of the P-V loop areas were compared by use of paired two-tailed t-tests.

RESULTS

Effect of hyperoxia on pulmonary and hepatic levels of MT and zinc. Intrapulmonary immunoreactive MT was detectable at low levels in control WT mice (Fig. 1A) but was readily apparent in liver of one of two mice breathing room air as assessed by Western blot (Fig. 1B). Our results are consistent with published reports demonstrating exposure of WT mice to hyperoxia for 72 h was associated with a notable increase in MT in lung and liver (19, 28, 46, 48, 64). As expected, MT immunoreactivity was not observed in either the lung or liver of MT-null mice, regardless of exposure, confirming that MT-I and -II (and not MT-III or MT-IV) are major pulmonary and hepatic isoforms of MT. We then measured total zinc in lung and liver tissues from normoxia- and hyperoxia-exposed WT and MT-null mice by atomic absorption spectroscopy (n = 4 to 8) and ICP-MS (n = 3 to 4). The absolute data within each organ normalized to dry weight were similar between the two assays. 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Pulmonary static compliance at 48 and 72 h of hyperoxia significantly decreased in WT but was not affected in MT-null mice (Fig. 3A). Hyperoxia significantly impaired lung hysteresis in WT mice and the increase in P-V loop area was significantly greater in WT than MT-null mice at 48 and 72 h hyperoxia (Fig. 3B) (3). Tissue damping and elastance (Fig. 3C, D) were similarly significantly increased in WT mice during hyperoxia but not affected in MT-null mice. These latter two measurements are closely related to tissue resistance and lung stiffening, respectively. Collectively, changes in lung mechanics are consistent with decreased sensitivity to hyperoxia in MT-null mice.

**Morphological differences between WT and MT-null mice at 72 h hyperoxia.** After 72 h of hyperoxia, histopathological changes were apparent in WT but not MT-null mice as determined by light (Fig. 4A) and transmission electron microscopy (Fig. 4B). Lungs of hyperoxic WT mice showed increased inflammatory cells and apparent interstitial edema compared with normoxic controls. Conversely, there was little evidence of increased inflammatory cells or edema in MT-null mice after 72 h of hyperoxia (Fig. 4). At the cellular level, lungs of hyperoxic WT mice showed damage of type I and II epithelial cells including presence of immature autophagosomes and necrotic changes with nuclear disintegration (Fig. 4Bb), whereas ultrastructure of distal epithelium appeared well preserved at 72 h of hyperoxia in MT-null mice (Fig. 4Bd). To confirm our observation of autophagy, lungs were analyzed by immunofluorescence staining for the autophagic protein LC3 (40, 57), LC3 signal showed a diffuse pattern in normoxic WT and MT-null mice (Fig. 5, Aa and Ac). Hyperoxia exposure resulted in a punctate staining pattern for LC3 indicating the induction of autophagy in these cells in WT mice (Fig. 5Ab). However, autophagy was much less pronounced in hyperoxia-treated MT-null mice (Fig. 5Ad). To further confirm our microscopic assessments, lung tissue conversion of LC3-I to LC3-II was quantified in whole lung tissue by Western blot microscopic assessments, lung tissue conversion of LC3-I to LC3-II was quantified in whole lung tissue by Western blot analysis (Fig. 5, B and C). In WT mice, LC3-II protein increased significantly at 72 h of hyperoxia compared with the normoxic state. In contrast, there were no significant changes in LC3-II protein at 72 h of hyperoxia in MT-null mice. Collectively, these data suggest that hyperoxia induces a significantly greater degree of lung injury in WT mice compared with MT-null mice, through autophagic and necrotic cell death pathways.

**Superoxide dismutase in hyperoxic WT and MT-null mice.** The various forms of the zinc-containing antioxidant enzyme SOD have long been considered important in the context of hyperoxic lung injury (44, 63). Initial measurements of total lung SOD activity were made and a significant decrease in total SOD activity was observed in hyperoxic WT mice only (Fig. 6A). The activity of the non-zinc-binding isofrom, Mn-SOD, was similar in both groups and was not significantly affected by

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**Fig. 2.** Both wild-type (WT) and MT-null mice had similar weight loss following hyperoxia exposure, but the WT mice had more pronounced lung injury. A: both WT and MT-null mice lost weight, and the weight loss was statistically significant as early as 48 h, but there was no difference in the rate of weight loss between WT and MT-null mice (*P < 0.05). At baseline both WT (n = 10) and MT-null (n = 6) mice had similar total cell counts in the bronchoalveolar lavage (BAL) and percent neutrophils (C). Although the total cell count did not change between the WT (n = 6) and MT-null (n = 6) mice by 48 h of hyperoxia, the differential count of neutrophils was significantly higher in WT mice compared with MT-null mice (*P < 0.05). The lung injury was also corroborated by the measurement of BAL protein concentration (D). At 48 and 72 h, the WT had significantly higher BAL protein concentrations than the MT-null mice (*P < 0.05). At 72 h, the BAL protein concentration was also significantly elevated within the groups (*P < 0.05).
hypoxia in either WT or MT-null mice (data not shown). Zinc-dependent SOD activity was then measured (Fig. 6B) and similar changes of SOD activity were noted to occur in response to hyperoxia (e.g., decrease in WT and no change in MT-null mice) (Fig. 6A). This prompted further investigation of direct measurements of the two forms of zinc-containing SODs: EC-SOD and CuZn-SOD. Hyperoxia caused a decrease in immunoreactive EC-SOD (Fig. 6, C and D) and EC-SOD activity (Fig. 7A) in both WT and MT-null mice. In contrast, immunoreactive CuZn-SOD activity was not affected by hyperoxia in either WT or MT-null mice (Fig. 7B). CuZn-SOD activity, however, was approximately 4× greater in lungs of MT-null mice than WT mice at control and at 72 h of hyperoxia. Collectively these data suggest that EC-SOD is sensitive to hyperoxia regardless of intrapulmonary zinc or MT status consistent with prior studies (44), whereas CuZn-SOD levels are elevated in MT-null mice, perhaps as compensation for effects of MT ablation or alterations in zinc availability.

Circulating levels of alanine transaminase in hyperoxic WT and MT-null mice. The serum ALT specific activity was assessed in normoxic and hyperoxic WT and MT-null mice as a marker of liver injury. The ALT specific activity was similar in between the genotypes in both normoxia and hyperoxia (Fig. 7C).

DISCUSSION

The present study demonstrates for the first time that translocation of zinc from lung to liver in hyperoxic mice is critically dependent on the presence of functional MT (Fig. 1C) (28, 47). Importantly, the lack of zinc translocation in MT-null mice was associated with a resistant phenotype to hyperoxia (Figs. 2–5), underscoring the potential importance of zinc homeostasis in affecting the course of hyperoxic acute lung injury (29). As others have shown, MT is readily induced in lung and liver during hyperoxia (Fig. 1) (28, 64). In contrast to emerging literature suggesting MT as part of cellular defense against partially reduced oxygen and nitrogen species, including our own work in hyperoxic cultured pulmonary endothelium, the role of MT in zinc partitioning seems more critical and detrimental than its antioxidant potential in vivo (6, 35, 49).

Intrapulmonary and intrahepatic MT and zinc in hyperoxia. MT emerged as a hyperoxia-sensitive gene in salient studies of Veness-Meehan et al. (64) using DNA subtraction hybridization techniques in lungs of hyperoxic rabbits. In situ hybridization localized MT mRNA to chondrocytes, fibroblasts, and type II epithelial cells in hyperoxic mouse lung (48). Microarray analyses confirmed elevations in MT expression during hyperoxia in mouse lungs (46). Hypoxia-induction of MT mRNA was significantly greater in various sensitive strains of mice, underscoring the utility of this stress gene as a biomarker of lung injury (19). Interestingly, hyperoxia also increased MT gene expression in other tissues such as the retina and liver (28, 71). Of the many zinc-binding proteins in tissues, MT has always assumed an important function as a zinc buffer primarily because of its abundance upon induction by hyperoxia and zinc-binding stoichiometry (7 mol zinc/mol MT) (35). Thus we would predict that under many conditions zinc availability may change with respect to MT levels; this was suggested by Levy et al. (28) and shown more decisively with MT-null mice in the present study (Fig. 1). It is unclear whether our observations regarding hyperoxia-induced changes in intrahepatic zinc and MT in hyperoxia have any corollary to hepatic zinc accumulation and hypozincemia associated with infection or inflam-
In the case of the latter, critical cytokines such as IL-1β (32) and IL-6 (34) affect expression of zinc transporters (e.g., Zip14/Slc39a14), resulting in increased intrahepatic zinc accumulation and thus providing zinc for acute-phase protein synthesis, regulation of gluconeogenesis, and/or control of microbial growth (34). It is noteworthy that MT is required for zinc accumulation in liver after IL-6 infusion (6) and endotoxin-induced inflammation (47). The former observation of MT-dependent hepatic zinc accumulation was not apparent in cultured hepatocytes underscoring the important differences between isolated cells and intact animals (6). Recently, similar conclusions about homeostasis, transmigration, and compartmentalization of zinc from lung to liver were reached by use of an inflammatory liver injury model secondary to HIV-1 transgene expression in intact rats (20).

**MT and hyperoxic acute lung injury.** MT maintains metal ion homeostasis and cellular defense against high or toxic levels of essential and nonessential metals (23). The abundance of cysteine residues (~30 mol%) suggested the ability of MT to quench superoxide anions (61), hydroxyl radicals (1), and...
partially reduced nitrogen species (70) and set the stage for MT as an antioxidant (27). Support for its antioxidant properties in hyperoxic lung injury emerged from studies by Hart et al. (15), who showed cross-resistance of cadmium-treated rats to heavy metal and hyperoxic toxicity. Further proof awaited the availability of genetically modified mice since cadmium is a promiscuous agent and assigning such cross-resistance to MT alone was ambiguous. Nonetheless, we noted that MT overexpression after either cadmium treatment or direct gene transfer in cultured pulmonary endothelial cells reduced their sensitivity to oxidant injury including hyperoxia (49). With the development of MT-null mice in two laboratories (38, 39), several investigators observed that MT-null mice were sensitive to acute lung injury due to endotoxin (54), ozone (17), and nickel (65). In light of these experiences in which MT-null mice were sensitive to pathophysiological conditions of excess partially reduced oxygen or nitrogen species, we hypothesized that MT-null mice would be sensitive to hyperoxia. Our data clearly show that MT-null mice were resistant to hyperoxia including functional (Figs. 2 and 3) and structural (Fig. 4) outcomes. We pursued early effects before any lethality was noted in the adult females of either strain and it is unclear whether these phenotypic differences between strains would persist after longer hyperoxic exposure. Although we did not design a priori experiment for mortality, our observation suggests that by 96 h, hyperoxia caused mortality in all of the WT (6/6) and half of the MT-null mice (3/6). The earliest detectable physiological change in lung mechanics was a decrease in lung compliance by 48 h (Fig. 3A). Overall zinc-dependent SOD activity was then measured, and similar changes of SOD activity were noted to occur in response to hyperoxia with a decrease in SOD activity in WT (n = 5) and no change in MT-null mice (n = 5) (#,*P < 0.05) (B). There was similar degree of loss of EC-SOD fluorescence staining following hyperoxia in both WT and MT-null mice (C). This was corroborated with the whole lung Western blot analysis, and the decrease was statistically significant for both WT (n = 5) and MT-null (n = 5) mice (#P < 0.05) (D and E).
sensitive WT mice in our study, the relatively greater stress than noted in the MT-null mice activated autophagy as revealed by immunohistochemistry (Fig. 5, Aa and Ac) and Western blot analysis of LC3-II (Fig. 5B). As Tanaka et al. (57) suggest, cross talk between autophagy and apoptosis is known to exist, and this cross talk may convert a potentially protective mechanism (e.g., autophagy) under hyperoxia to a contributor of sensitivity toward acute lung injury in WT mouse.

Nachman-Clewner et al. (42) have examined effects of hyperoxia on extrapulmonary tissues for longer periods of time. Similar to what is observed in our model, they noted slight protection in MT-null mice with respect to degeneration of central retinal photoreceptors. These authors did not pursue zinc partitioning during intermittent (3×/wk) exposure (3 h) to 3 atmospheres of 100% oxygen for up to 5 wk. Nonetheless, it is apparent that the role of MT in hyperoxic injury in intact animals, including target (lung, retina) and nontarget (liver), is complex. The observed important differences between cell culture and intact animals (49), considerations about compensatory and/or coexisting pathways, and interactions of MT and essential metals may preclude definitive conclusions on mechanisms and pathways involved in MT as an antioxidant.

SOD, MT, and zinc in hyperoxic acute lung injury. In light of these latter possibilities and in the case of hyperoxic lung injury, classical oxidative stress model, various forms of SODs that are 1) important in response to hyperoxia (62, 63) and 2) zinc dependent (CuZn- and EC-SOD) may be indirectly or directly affected by alterations in intrapulmonary zinc homeostasis (8, 16, 51, 58). Zinc-dependent SOD activity was decreased after 72 h hyperoxia (Fig. 6) in WT but not MT-null mice. There were no significant differences between strains nor effects of hyperoxia on Mn-SOD. Additional studies were performed to evaluate the effects of hyperoxia and zinc on EC-SOD and CuZn-SOD activities. EC-SOD expression and activity (Figs. 6, 7) were significantly decreased at 72 h of hyperoxia in WT and MT-null mice consistent with previous studies (44). Overexpression of EC-SOD (2, 12) or aerosolized administration of recombinant EC-SOD protein (69) protects mouse lungs against hyperoxia, conversely targeted or inducible ablation of EC-SOD sensitizes mice to hyperoxia (4). Nonetheless, observation that EC-SOD was decreased in both sensitive and resistant strains of mice suggests that EC-SOD per se is sensitive to hyperoxic oxidative damage but is not sufficient to account for the observed phenotypic differences. CuZn-SOD activity was much more abundant than EC-SOD activity in both WT and MT-null mice and importantly there was a 3–4× increase in CuZn-SOD activity in both normoxia and hyperoxia in MT-null mice vs. WT mice (Fig. 7), whereas CuZn-SOD activity was not affected by hyperoxia in either strain. The difference in baseline CuZn-SOD activity between the two strains suggests that MT-null mice compensate, in part, by an elevation in this enzyme. Interestingly, Ghoshal et al. (14) noted compensatory increases in MT-I and -II expression by an elevation in this enzyme. Interestingly, Ghoshal et al. (14) noted compensatory increases in MT-I and -II expression in liver of CuZn-SOD-null mice. The ability of MT to substitute for CuZn-SOD in yeast deleted in the latter underscores the ability of these two proteins to functionally compensate for each other (55). Previous reports using cells from different strains of mice in which MT was genetically ablated did not show baseline differences of this magnitude (27), suggesting important contributions of genetic strain differences in CuZn-SOD activity and its impact on response to hyperoxia. We (33)
and others (25) have examined the subtleties of metal ion delivery to CuZn-SOD and apo-SOD and MT can indeed deliver metals (Cu or zinc) to increase activity of CuZn-SOD or apo-CuZn-SOD, in vitro. It is tempting to attribute the resistance of MT-null mice to hyperoxia due to a compensatory increase in CuZn-SOD. However, forced overexpression of CuZn-SOD had very little protective effect in mature mice at normal altitude (66). Thus an alternative explanation for elevated CuZn-SOD activity is that without MT-dependent partitioning of zinc to liver in hyperoxia (Figs. 1 and 2) there is an increased availability of zinc to maintain its activity (52) or that zinc per se (or other zinc-dependent pathways) contributed to hyperoxia-resistant phenotype of MT-null mice.

**Hyperoxia and liver injury.** The literature suggests lung-liver communication under oxidative stress. Hyperoxia increases liver injury initiated by agents undergoing oxidative biotransformation and decreases injury due to galactosamine, LPS, and others undergoing reduction (37). In addition to reactive oxygen species, hyperoxia increased liver 5-lipooxygenase and cyclooxygenase-2 levels in newborn mice after 14 days, possibly playing a role in lipid mediators of acute lung injury (53). Liver redox state, glutathione reductase, and cytochrome P-450 1A1/1A2 levels were increased at 72 h of hyperoxia (53). Liver redox state, glutathione reductase, and cytochrome P-450 1A1/1A2 peak at 48 h and decrease back to baseline by 60 h (41, 68). The decline by 60 h could be due to downregulation or degradation of these enzymes. In another model of reactive oxygen species (ROS)-mediated alcoholic liver injury, murine liver injury was prevented with zinc supplementation in both WT and MT-null mice through inhibition of accumulation of ROS and restore alveolar epithelial and macrophage function (21). Although the above studies suggest a link between lungen and liver under hyperoxic condition and a favorable role for zinc in the prevention of liver injury due to various stressors, the models usually took weeks to months to establish compared with our model of days. This may explain the lack of liver injury in our acute experiments. In this regard, the lack of changes in liver enzymes during hyperoxia in either genotype are reminiscent of the lack of histological changes of either inflammation, fibrosis, or cholestasis in livers of newborn mice at 72 h of hyperoxia (53).

In conclusion, this study shows that zinc partitioning from lung to liver during hyperoxia is dependent on MT. In contrast to WT mice, MT-null mice increase intrapulmonary zinc levels during hyperoxia, and this is associated with a resistant phenotype to hyperoxia. The protective mechanisms are unclear but may involve either compensated elevations in CuZn-SOD in MT-null mice or activation of other zinc-dependent pathways that inhibit early aspects of hyperoxic acute lung injury. Recently, several groups have noted that zinc deficiency sensitizes experimental animals to acute lung injury (21, 24, 59). Although these models all involved carefully calibrated zinc deficiencies (and successful reversal with zinc repletion), it does open the possibility that zinc supplementation can be rationally used as a therapeutic modality for acute lung injury.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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