Animals and humans rapidly develop respiratory failure and die within a few days when exposed to 100% oxygen. Postmortem examination of the lungs shows histopathologic features characteristic of diffuse alveolar damage, clinically recognized as adult respiratory distress syndrome (ARDS). At the present time, there is no effective therapy available to alter outcomes in ARDS. Importantly, hypomagnesemia also is frequently observed in critically ill patients at risk of developing ARDS. In a model of hyperoxic lung injury, rats were exposed to 100% oxygen for 48, 64, and 96 hr and several experiments were performed. First, changes in the features of bronchoalveolar lavage and in alveolar macrophage function were compared in rats exposed to room air and those exposed to hyperoxia. Second, we studied the effect of hypomagnesemia on the severity of hyperoxic lung injury. Third, we evaluated the pulmonary responses to high-dose and normal-dose Mg therapy in rats exposed to hyperoxia. In all groups, hyperoxia induced significant changes in the total and differential cell counts with increased lipid peroxidation of lavaged cells, enhanced chemiluminescence from alveolar macrophages, and protein leakage into the alveolar spaces. After 48 hr of hyperoxia, oxygen-free radical formation and hydrogen peroxide production by the alveolar macrophage were diminished compared to baseline, implying a toxic effect of hyperoxia on the alveolar macrophages. Overall, hypomagnesemia tended to magnify the degree of hyperoxic lung injury, while high-dose Mg therapy tended to attenuate the effects of hyperoxia. In conclusion, in this animal model of diffuse alveolar damage, alterations in host serum magnesium levels may modulate the degree of lung damage. — Environ Health Perspect 102(Suppl 1):101–105 (1994)

Key words: oxygen toxicity, adult respiratory distress syndrome (ARDS), magnesium, macrophage function, bronchoalveolar lavage, lipid peroxidation, free radicals, chemiluminescence

Introduction

Many clinical conditions, including septic shock and sepsis syndrome, lung contusion, skeletal fractures with fat emboli, bacterial lipopolysaccharide release, acute pancreatitis, gastric aspiration, and inhalation of toxic gases such as nitric oxide and ozone, are associated with acute lung injury or adult respiratory distress syndrome (ARDS). The clinical and histopathologic changes of lung injury produced by these agents are indistinguishable from the changes seen in hyperoxic lung injury. Excessive pulmonary exposure to these oxidant gases results in peroxidative damage to pulmonary membranes with biochemical and metabolic changes in lung tissue (1–10). In humans, the histologic features of these injuries are described as diffuse alveolar damage, and the resultant clinical presentation of hypoxemia, increased physiologic shunt, and diminished pulmonary compliance are associated with such injuries and are known as adult respiratory distress syndrome. In the animal model of acute inhalation injury of interest in this report, exposure of rats to high doses of oxygen for an extended time induces diffuse alveolar damage reflective of the histologic findings of ARDS. Despite recent progress in understanding the pathophysiology of ARDS (11–13), effective therapy remains elusive (1, 2, 13).

Magnesium (Mg) plays an important role in various metabolic processes such as oxidative phosphorylation and cellular enzymatic reactions. Magnesium deficiency (MgL) is frequently observed in intensive care unit patients and can be associated with important and potentially life-threatening adverse reactions, including cardiac arrhythmias and insufficiency, muscle weakness, and seizures (14, 15). On a cellular level, magnesium sulfate (MgSO4) modifies the formation of arachidonic acid (AA) metabolites, important mediators of acute lung injury. Overall, Mg may significantly affect lung structure and mediator reactions. Examples where high-dose magnesium (MgHD) supplementation has been effective include the therapy of eclampsia and pre-eclampsia, tetanus, and severe bronchospasm (16–18). The influence of high or low serum levels of Mg on the histologic effects of hyperoxic lung injury is not known.

We hypothesized that supplemental Mg therapy attenuates diffuse alveolar damage in a rat model of lung injury due to hyperoxia. Our intent was to develop an effective pharmacologic treatment for diffuse alveolar damage, the histologic feature of ARDS. The specific aims were to a) further characterize early biochemical changes of the bronchoalveolar lavage (BAL) and in alveolar macrophage (AM) function due to hyperoxia; b) determine if MgL predisposes rats to hyperoxic lung injury; and c) investigate whether MgHD therapy attenuates hyperoxic lung injury. Changes in pulmonary histopathology in the rat lung receiving MgHD also were evaluated.

Methods and Results

Changes in Bronchoalveolar Lavage Parameters and Alveolar Macrophage Function due to Hyperoxia

Pulmonary hyperoxic injury was induced in rats (19, 20). Thirty-two male, specific pathogen-free Sprague–Dawley rats (300 ± 25 g) (Hilltop Laboratory Animals Inc., Scottsdale, PA) were housed in an AAALAC-approved animal facility. The rats were divided randomly and exposed to an environment of >95% oxygen (n = 18) or room air (RA) (n = 14). Hyperoxic exposure was conducted by placing four rats into a sealed
plexiglass chamber constructed with partitions to provide an individual compartment for each animal. Oxygen concentrations in the chamber were monitored using an oxygen analyzer (Model 0406C, Instrumentation Lab, Lexington, MA). Animals were provided regular rat chow and water ad libitum. Disruptions in the oxygen-rich environment occurred only during momentary periods of water and food replacement. All rats survived 64 hr of hyperoxia.

Immediately following exposure, rats were sacrificed and the lungs lavaged 10 times with 8 ml of Ca"+- and Mg"+-free phosphate-buffered medium. Approximately 90% of the solution was recovered. The first lavage samples were maintained separate from the others. The cell pellets from the BAL of each rat were combined for analysis of cell counts and functional studies. The first supernatant was used to measure lavage protein and phospholipid (19). We measured

- total and differential cell counts, cell viability, and membrane integrity;
- reduced and oxidized cellular glutathione levels;
- cellular lipid peroxidation;
- H2O2 release from AM;
- chemiluminescence (CL) generated by AM;
- cell spreading and morphology of AM (analysis of cell diameter and perimeter);
- production of oxygen-free radical (FR) species by AM using an electron spin resonance (ESR) technique with a radical spin trap;
- protein and phospholipid content;
- effect of the addition of superoxide dismutase (SOD), catalase, indomethacin, or A63162 to BAL cells to define the contribution of superoxide, H2O2, cyclooxygenase products, or lipoxygenase metabolites to macrophage-generated CL.

The results were expressed as mean ± SE of the separate experiments. Data from the exposed rats were compared to values from controls using a Student’s t-test with significance p<0.05.

Significant changes in lung cells harvested by BAL were observed in the hyperoxic rats (19). Total cell count was 15.8×106 per control rat versus 12.3×106 per hyperoxic-exposed rat, a decrease of 20% following hyperoxic exposure. In the control rats, 86% of the BAL cells were AM, 6% were red blood cells, 5% were neutrophils, and 2% were lymphocytes. In the hyperoxic-exposed animals, 40% were AM, 31% were red blood cells, 12% were neutrophils, and 15% were lymphocytes. AM viability was reduced by 12% (from 92 to 80%) after hyperoxia. Oxidant-induced lung injury resulted in a significant elevation (from 0.29 ± 0.03 – 1.67 ± 0.54 mg/ml) in the protein content of the acellular lavage fluid. In contrast, no significant change in lavage phospholipid content was seen. There was no significant effect on cell spreading, measured as the circumference of AM adhering to cover slips, attributable to hyperoxia.

We studied the alteration in AM function due to oxidant injury by measuring H2O2 release, oxygen-FR production, and CL generation (19). The resting release of H2O2 from AM was unaffected by hyperoxia; however, the ability of AM to secrete H2O2 in response to unopsonized zymosan significantly decreased by 41%. Similarly, the production of oxygen FR by zymosan-stimulated AM decreased 53% after oxygen exposure. Because the decrease in reactive oxygen species far exceeded the decrease in AM viability, cell death cannot account for this decrease in macrophage function.

Resting CL from AM was unchanged, yet oxygen-exposed AM generated 540% more CL in response to zymosan than controls, even though H2O2 on FR production decreases. This discrepancy is not the result of a decrease in cellular antioxidants, since the levels of cellular reduced and oxidized glutathione were unaffected by hyperoxia. This may not be totally unexpected, since upregulation of antioxidant systems has been known to occur after oxidant exposure.

Activation of cyclooxygenase and lipoxygenase products of AA metabolism has been demonstrated in ozone-exposed AM (21). AA metabolites can generate CL (22). To determine if this was the source of the enhanced CL, the BAL cells were treated with SOD, catalase, indomethacin, or A63162 to block superoxide-, H2O2-, cyclooxygenase-, or lipoxygenase-based CL, respectively. Inhibitors of lipoxygenase or cyclooxygenase were not more effective in depressing CL after hyperoxia, while the effectiveness of SOD or catalase did not decrease after oxygen exposure. Therefore, a shift from oxygen-based to arachidonic acid-based reactive species cannot explain the increase in CL after hyperoxia. It is possible that the elevated levels of CL after oxygen exposure resulted from enhanced cellular lipid peroxidation (23). Our data support this, since cellular lipid peroxidation more than doubled as a result of hyperoxia.

**Relationship between Hypomagnesemia and Acute Hyperoxic Injury**

Pulmonary hyperoxic injury was induced in 26 rats by exposure to 100% oxygen for 48 hr. MgL (serum Mg <0.7 mEq/l) was induced in 16 rats by a Mg-deficient (Mg = 40 mcg/g) diet (TD 90278, Wayne Laboratory Diets, Madison WI) given for 3 weeks. Eight rats were exposed to 100% O2 for 48 hr and eight served as controls on RA. In addition, five rats on a normal diet (Mg = 400 mcg/g) were exposed to 100% O2 for 48 hr and five served as controls on RA. All animals survived 48 hr of hyperoxia. Serum Mg levels were monitored at the start and at the time of sacrifice. BAL assessment was carried out at the time of sacrifice (24).

The mean serum Mg level in the MgL diet group was 0.6 mEq/l (range 0.4–0.7 mEq/l) compared to a mean of 1.7 mEq/l (range 1.1–2.1 mEq/l) in the group of rats ingesting a regular diet.

Results from RA-exposed rats with normal serum Mg level were compared to RA-exposed rats with MgL serum level. BAL comparison showed no significant changes in total or differential cell count, protein content, CL, or cell viability.

BAL features of hyperoxic and RA rats with normal serum Mg level were compared. Statistically significant (p<0.05) changes in the differential cell count (increased RBC and lymphocytes) occurred after hyperoxia. In addition, elevated protein and CL were noted in the hyperoxic rats.

BAL features of hyperoxic rats with MgL and normal Mg level were compared. Hypomagnesemia significantly increased protein levels in the BAL of hyperoxic rats. Compared to hyperoxic normal Mg rats, AM and total cells were decreased by 48 and 42%, respectively, in the hyperoxic MgL group. The increase in CL from the RA baseline was also maximal, 530% at rest and 350% postzmysomal stimulation, in the hyperoxic MgL group.

**High-dose MgSO4 and a Hyperoxia Model**

Two studies were undertaken using this model. First, we addressed the effect of MgSO4 loading in a rat model of oxygen toxicity. Second, to show the interaction between MgSO4 and arachidonate, an in vitro experiment was performed mixing varying concentrations of MgSO4 with arachidonate.

Thirty-four rats weighing 400±15 g were divided into three groups. Eighteen rats in the control group (C) received no MgSO4 supplementation. Sixteen rats were further divided into two groups and given MgSO4 subcutaneously in a dosing regimen aimed at maintaining plasma Mg levels between 4 and 6 mEq/l. Eight rats received
low-dose (LD) MgSO\(_4\) (1.6–3.6 mEq/day) supplementation and eight received high-dose (HD) MgSO\(_4\) (7.2–9.6 mEq/day). The total dose varied from 18 to 24 mEq/kg/day. Each rat had a catheter placed in the femoral artery to allow for sampling of blood for the measurement of plasma Mg levels. Rats were then exposed to 100% O\(_2\); after death the lungs were removed for histopathologic examination and a quantitative lung injury score was made based on the severity of damage. Each specimen was graded from 0 to 4 on a severity grading scale: 0 = none, 1 = minimal, 2 = mild, 3 = moderate, or 4 = severe lung damage. The lung damage scores were expressed as the mean ± SEM and compared by analysis of variance. The results were considered significantly different at \(p < 0.05\). Duncan’s multiple range test was also used to identify differences among groups. For the \textit{in vitro} experiment, a solution of sodium arachidonate in a concentration of 2.0 mEq/l was mixed with MgSO\(_4\) in concentrations of 0.5, 1.0, and 2.0 mEq/l. Turbidity was assessed by visual inspection.

In rats receiving the LD Mg supplementation, the mean plasma peak Mg level (4.5 mEq/l) was in the desired range (4–6 mEq/l), but the mean plasma trough level (1.6 mEq/l) fell far below the desired range. In this group, the doses of Mg supplementation were small and infrequent, delivered every 8 to 12 hr. The mean plasma peak Mg (5.3 mEq/l) and trough (3.5 mEq/l) levels in the HD group were in the desirable range.

The pulmonary histology showed Mg dose-dependent changes of diffuse alveolar damage (20). Prolonged hyperoxic exposure resulted in extensive morphologic lung damage (Figure 1) in the C group (mean score 3.38) and considerably less damage in the HD group (mean score 1.57) (Figure 2). The histopathology score for the HD group indicates better preservation of lung compared to the C group (\(p = 0.0008\)). The LD group had a mean score of 2.75, between HD and C. This intermediate value in the group receiving intermediate doses of Mg supplementation validates our approach and adds significance to the pathology scoring.

In an accompanying \textit{in vitro} study, a very opaque solution formed with the 2 mEq/l MgSO\(_4\) solution was added. No turbidity was seen with 0.5 mEq/l solution.

**Discussion**

These investigations describe changes in the BAL cellular and supernatant response, as well as histologic alterations resulting from hyperoxic exposure of rats. Hyperoxia resulted in an increase in the number of neutrophils, red blood cells, and lymphocytes. The amount of protein in the alveolar space was also increased. In contrast, the number of recoverable alveolar macrophages decreased. Histologically, these features correlated well with the histologic features of diffuse alveolar damage. Tsan et al. \(25\) reported similar shifts in lavage cell differential counts after a 55-hr exposure of rats to 100% oxygen. Likewise, rats exposed to 1 to 2 ppm ozone for 4 to 8 hr showed an increased number of lavagable neutrophils and a decreased number of AM \((26,27)\). In these reports, as well as in our own work, the decreased AM yield is likely the result of a toxic gas

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**Figure 1.** Histopathology of control Mg hyperoxic lungs. High-power photomicrograph of the lung showing features of diffuse alveolar damage. These include inflammatory cells in the interstitial and alveolar spaces, sloughed alveolar epithelial cells, and hyaline membranes.

**Figure 2.** Histopathology of high-dose Mg hyperoxic lungs. High-power photomicrograph of the lung showing greatly preserved lung parenchyma with a minimal number of inflammatory cells, negligible amounts of blood in the alveolar space, and only occasional sloughed alveolar epithelial cells.
Effect. The increase in lavagable neutrophils, red blood cells, and the elevated amounts of alveolar protein reflects the loss of the integrity of the pulmonary blood/air barrier. Protein and cellular changes suggest capillary leak due to oxidant-induced lung injury (diffuse alveolar damage).

A significant decline in zymosan-induced production of H₂O₂ and oxygen radicals by pulmonary AM suggests a depression in AM function due to hyperoxia. Similarly, declines in superoxide release from pulmonary phagocytes have been reported following both in vitro and in vivo exposure to ozone as well as in vivo exposure to nitrogen dioxide (26–29). Production of reactive oxygen species plays a role in the antibacterial activity of AM. Exposure to oxidants has been shown to increase the susceptibility to inhaled infectious agents (30).

Hyperoxia, regardless of Mg level, caused an increased CL at rest and with zymosan stimulation compared to the RA group. This was most evident in the MgL hyperoxic group (p<0.05). In contrast, CL from the pulmonary AM was not significantly affected by hypomagnesemia in normoxic rats. We have previously shown that the abnormal CL is due to enhanced lipid peroxidation and indicates oxidant-induced injury (19). Banks et al. (10) reported elevated levels of CL from both resting and stimulated AM after in vitro exposure to ozone, while Esterline et al. (26) found elevated CL from TPA- or zymosan-stimulated phagocytes after in vivo exposure to ozone. Therefore, our data suggest that hypomagnesemia predisposes rats to an early acute hyperoxic lung disease.

In this model of oxygen toxicity, HD Mg therapy (18–24 mEq/kg/day of MgSO₄), which achieved plasma levels of 4 to 6 mEq/l, reduced lung injury. Although we have not identified the mechanism of action of Mg in this setting, literature review and the briefly described in vitro experiment suggest that Mg has the potential to affect AA metabolism. This in vitro experiment simply shows binding between arachidonate and Mg. The reaction between sodium oleate and differing concentrations of MgSO₄ results in the dose-dependent formation of a water-insoluble complex of Mg oleate. If Mg binds to arachidonate, this would lead to decreased levels of AA metabolites, products well described to cause lung injury. At high concentrations, Mg alters the metabolism of long chain fatty acids. On a cellular level, Mg could alter phagocyte and vascular endothelium function. Additional experiments are necessary to understand the mechanism of Mg on the cellular level.

In summary, hypomagnesemia predisposed animals to hyperoxic lung injury, while hypermagnesemia attenuated hyperoxic lung injury in our animal model. Alteration in BAL parameters, AM function, and pulmonary histology provided a very consistent relationship between the degree of lung injury due to hyperoxia and amelioration of this injury with Mg supplementation.

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