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HYDROGEN PEROXIDE-INDUCED CELL AND TISSUE INJURY: Protective Effects of Mn^{2+}

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Abstract—Recent evidence indicates that under in vitro conditions, superoxide anion and hydrogen peroxide (H_2O_2) are unstable in the presence of manganese ion (Mn^{2+}). The current studies show that in the presence of Mn^{2+}, H_2O_2-mediated injury of endothelial cells is greatly attenuated. A source of bicarbonate ion and amino acid is required for Mn^{2+} to exert its protective effects. Injury by phorbol ester-activated neutrophils is also attenuated under the same conditions. EDTA reverses the protective effects. Acute lung injury produced in vivo in rats by intratracheal instillation of glucose–glucose oxidase is almost completely blocked in rats treated with Mn^{2+} and glycine. Conversely, treatment of rats with EDTA, a chelator of Mn^{2+}, markedly accentuates lung injury caused by glucose–glucose oxidase. These data are consistent with the findings of others that Mn^{2+} can facilitate direct oxidation of amino acids with concomitant H_2O_2 disproportionation. This could form the basis of a new therapeutic approach against oxygen radical-mediated tissue injury.

INTRODUCTION

Manganese ion (Mn^{2+}) can participate in a number of reactions that limit the extent of oxygen radical generation. For example, in the presence of pyrophos-

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phate or polyphosphate, inorganic Mn^{2+} catalyzes superoxide anion dismutation (1). Additionally, mitochondrial superoxide dismutase (SOD) is a Mn^{2+}-containing enzyme (2). Under physiological conditions, Mn^{2+} can cause the direct "disproportionation" of hydrogen peroxide (H_2O_2) to molecular oxygen in a reaction involving bicarbonate ion and a carbonate radical (3). In addition, Mn^{2+} can cause bicarbonate ion-dependent amino acid oxidation in the presence of H_2O_2 with concomitant H_2O_2 disproportionation (4, 5). It has been suggested that these reactions may provide protection against injury mediated by toxic oxygen metabolites. To address whether or not Mn^{2+} may function in a protective manner, we have used endothelial cell culture and experimental animal models of lung injury mediated by toxic oxygen products in order to assess protective effects of Mn^{2+} and to delineate the conditions under which protection can be achieved. We show here that Mn^{2+} can protect endothelial cells in culture and whole animals from H_2O_2-mediated injury. Protection requires the concomitant presence of bicarbonate ion and a source of amino acids.

MATERIALS AND METHODS

Endothelial Cells. Rat pulmonary artery endothelial cells were obtained as described previously (6, 7). The isolated cells grew with a "cobblestone" morphology evident at both the light and electron microscopic levels, contained high levels of angiotensin-converting enzyme, and reacted strongly with antibodies to favor VIII.

Neutrophils. Human peripheral blood leukocytes were obtained from heparinized (10 units/ml) venous blood provided by healthy adult volunteers. Neutrophils were separated by dextran sedimentation and density-gradient centrifugation using Ficoll-Hypaque medium (Pharmacia Fine Chemicals; Piscataway, New Jersey). The cells obtained were approximately 98% polymorphonuclear granulocytes. Neutrophils were suspended in HBSS.

Reagents. Manganous chloride, manganous acetate, and manganous sulfate were obtained from Drake Brothers (Menomonee Falls, Wisconsin). Glycine, EDTA, H_2O_2, glucose and glucose oxidase were obtained from Sigma Chemical Co. (St. Louis, Missouri). HBSS was obtained from Gibco (Grand Island, New York).

Cytotoxicity Assay. Cytotoxicity was measured using the release of ^{51}Cr from prelabeled cells as an indicator of injury. This assay has been used extensively by us to measure injury mediated by activated neutrophils or H_2O_2 and has been described in detail (7, 8). In addition to measuring ^{51}Cr release, we also used the inability of endothelial cells to replate after exposure to H_2O_2 as a measure of cytotoxicity. Cells were exposed to H_2O_2 (alone or in the presence of Mn^{2+}, amino acids, etc.) and then harvested from the wells with trypsin and replated in culture medium. Eighteen hours later, the percentage of cells that had reattached was determined. The plating efficiency of the untreated endothelial cells ranged from 85% to 90%. This assay has been described previously (9).

Lung Injury. Long-Evans rats were anesthetized with ketamine and infused intratracheally with a combination of glucose and glucose oxidase, mixed in the reaction tube immediately before instillation. Rats were simultaneously given [^{125}I]BSA intravenously and the degree of injury meas-

ured 4 h later as a lung/blood permeability ratio (10). For histological studies, lungs were infused with 10% buffered formalin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

RESULTS

In the first series of experiments, we examined the killing of rat pulmonary artery endothelial cells by H_2O_2 in the presence of Mn^{2+}. As shown in Figure 1, the addition of Mn^{2+} (chloride salt) to the culture medium [Eagle's minimal essential medium containing Earle's balanced salt solution and 200 μg of bovine serum albumin per milliliter (MEM)] inhibited killing by 200 μM H_2O_2 in a concentration-dependent manner. Protection occurred at Mn^{2+} concentrations as low as 10 μM; a 50% inhibition of killing was achieved with the ability of the endothelial cells to attach and spread or to proliferate (Table 1). Although we routinely used a ^{51}Cr-release assay to assess toxicity, we confirmed in a replating assay (9) that the ability of H_2O_2 to induce ^{51}Cr release was associated with inhibition of plating. Likewise, we confirmed that the ability of Mn^{2+} to prevent ^{51}Cr release was associated with protection in the replating assay. In additional experiments, it was found that the chloride, acetate, or sulfate salts of Mn^{2+} were equally protective (not shown). The ability of Mn^{2+} to protect endothelial cells against H_2O_2-mediated injury was overcome in the presence of ethylenediamine tetraacetic acid (EDTA). When added at concentrations of 0.5–2.5 mM, EDTA produced no toxicity by itself and did not inhibit H_2O_2-mediated killing. However, these concentrations of EDTA completely reversed the protective effects of Mn^{2+} (Figure 1). Thus, chelation of Mn^{2+} effectively prevents its participation in the reactions that are responsible for endothelial cell protection.

Additional experiments demonstrated that Mn^{2+} would protect endothelial cells maintained in Medium 199 as well as in MEM but would not protect in either Earle's balanced salt solution (EBSS) or Hanks' balanced salt solution (HBSS). The failure of Mn^{2+} to provide protection in EBSS was overcome when the amino acid mixture of MEM was added to the EBSS. In contrast, addition of the MEM vitamin mixture to EBSS had no effect (Table 2). Table 2 also shows that in the presence of a sufficient amount of Mn^{2+} (0.5 mM), the addition of a single amino acid (glycine) provided the necessary cofactor for protection and that 0.1 mM final concentration was sufficient. A requirement for bicarbonate ion was also suggested from these studies. Substitution of HEPES buffer (40 mM) for the bicarbonate buffer in HBSS resulted in failure to obtain significant protection in the presence of 100 μM Mn^{2+}/1000 μg gly-

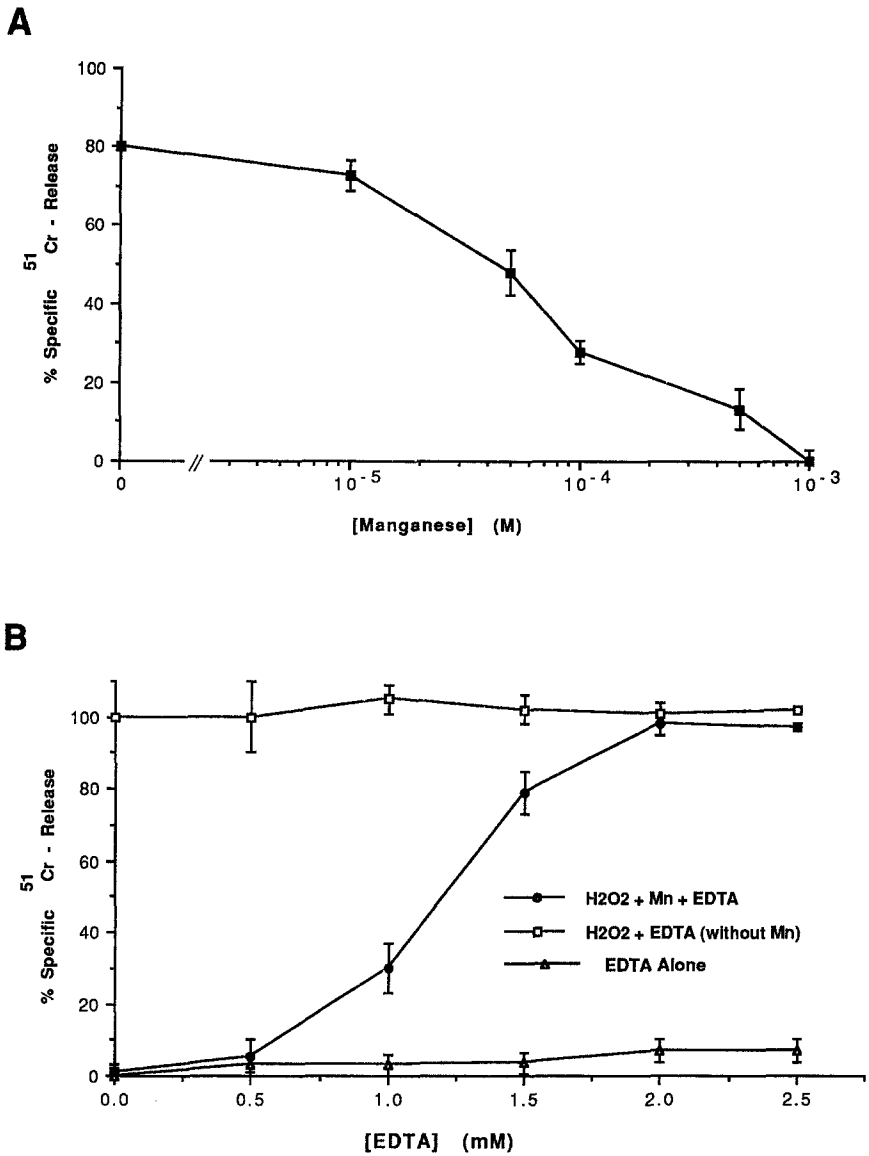

Fig. 1. (A) Inhibition of H_2O_2-mediated killing of endothelial cells by Mn^{2+}. ^{51}Cr-labeled rat pulmonary artery endothelial cells were exposed to 200 μM H_2O_2 in the presence of varying concentrations of Mn^{2+} (manganese chloride) in MEM (pH 7.2–7.4). This buffer contains a full complement of amino acids. ^{51}Cr release was assessed after 4 h of incubation. Values shown represent averages $\pm$ standard deviations based on triplicate samples in a single experiment. The experiment was repeated several times with similar results. (B) EDTA inhibition of the protective effects of Mn^{2+}. ^{51}Cr-labeled endothelial cells were exposed to 200 μM H_2O_2 in the presence of 0.5 mM Mn^{2+} and varying concentrations of EDTA in MEM (pH 7.2–7.4). ^{51}Cr release was assessed after 4 h of incubation. Values shown represent averages $\pm$ standard deviations based on triplicate samples in a single experiment. The experiment was repeated three times with similar results.

Table 1. Proliferation of Rat Pulmonary Artery Endothelial Cells in the Presence of Mn^{2+}[a]

Treatment	Number of cells/well ($\times 10^5$)
None	2.4 ± 0.1
1×10^{-6} M Mn^{2+}	2.2 ± 0.2
5×10^{-6} M Mn^{2+}	2.5 ± 0.1
1×10^{-5} M Mn^{2+}	2.6 ± 0.3
5×10^{-5} M Mn^{2+}	2.5 ± 0.1
1×10^{-4} M Mn^{2+}	2.6 ± 0.2
5×10^{-4} M Mn^{2+}	2.3 ± 0.1
1×10^{-3} M Mn^{2+}	2.3 ± 0.1

[a] Cells were seeded at 1.0×10^5 cells/well into wells of a 24-well dish on day 0 in growth medium (MEM, supplemented with 10% fetal bovine serum). $MnCl_2$ was added at this time. One day later, cells were harvested and counted. Values shown represent means ± standard deviations based on triplicate samples per data point in a single experiment. The experiment was repeated three times with similar results.

cine/ml (5 ± 2% protection vs. 75 ± 5% protection in the presence of bicarbonate buffer).

Protection by Mn^{2+} was not limited to injury by reagent H_2O_2. In previous studies, endothelial cell injury by phorbol ester-activated neutrophils has been shown to be dependent on neutrophil-generated H_2O_2 (6–8). The same concentrations of Mn^{2+} that protected endothelial cells against H_2O_2 also provided significant protection against injury by activated human neutrophils (18 ± 2% protection at 100 μM Mn^{2+} and 69 ± 4% protection at 1000 μM Mn^{2+}).

A major question concerns the relevance of these in vitro findings to protection against oxygen radical-mediated injury in vivo. To address this question, lung injury was induced in Long-Evans rats by the intratracheal instillation of glucose–glucose oxidase as described previously (10). Control animals (receiving glucose–glucose oxidase alone) were compared to animals receiving the same reagents and either Mn^{2+} alone, glycine alone, or Mn^{2+} and glycine. For these experiments, rats were pretreated by intraperitoneal injection of 50 mg of manganese acetate and/or 50 mg of glycine 2 h prior to intratracheal instillation of the glucose–glucose oxidase. Preliminary studies had indicated that these concentrations of Mn^{2+} and glycine by themselves produced no detectable lung injury or other signs of toxicity in rats not exposed to glucose–glucose oxidase. Table 3 (experiment A) compares permeability changes in the lungs of rats receiving glucose–glucose oxidase alone and those receiving glucose–glucose oxidase along with Mn^{2+}, glycine, or the combination of the two. It can be seen that either Mn^{2+} alone or glycine alone provided limited protection injury, whereas the two reagents together completely suppressed injury. Figure 2 demonstrates that the reduction in permeability seen in the Mn^{2+} and glycine-treated animals (Table 3) is accompanied by attenuation of the histo-

Table 2. Requirements for Mn^{2+} Protection of Endothelial Cells from H_2O_2-Mediated Killing[a]

Treatment	Specific ^{51}Cr release (%)
Experiment 1	
MEM alone	9 ± 1
MEM + H_2O_2 (200 μM)	50 ± 5
MEM + H_2O_2 + Mn^{2+} (0.2 mM)	13 ± 2
M199 alone	9 ± 2
M199 + H_2O_2 (200 μM)	50 ± 1
M199 + H_2O_2 + Mn^{2+} (0.2 mM)	16 ± 3
Experiment 2	
EBSS alone	8 ± 3
EBSS + H_2O_2 (200 μM)	42 ± 2
EBSS + H_2O_2 + Mn^{2+} (0.5 mM)	45 ± 3
EBSS + AA + H_2O_2 + Mn^{2+}	8 ± 1
EBSS + vitamins + H_2O_2 + Mn^{2+}	48 ± 5
Experiment 3	
HBSS alone	2 ± 1
HBSS + H_2O_2 (200 μM)	63 ± 5
HBSS + H_2O_2 + Mn^{2+} (0.5 mM)	63 ± 1
HBSS + H_2O_2 + 0.1 mM glycine	66 ± 1
HBSS + H_2O_2 + Mn^{2+} + 0.1 mM glycine	58 ± 8
HBSS + H_2O_2 + 1 mM glycine	65 ± 2
HBSS + H_2O_2 + Mn^{2+} + 1 mM glycine	35 ± 1

[a] ^{51}Cr-labeled endothelial cells were treated with the desired reagents and exposed to 200 μM H_2O_2. ^{51}Cr release was assessed in the normal manner. Values shown represent averages ± differences between averages and individual values based on duplicate samples in a single experiment. Experiments 1 and 2 were repeated three times with similar results. Experiment 3 was repeated four times with similar results. EBSS = Earle's balanced salt solution, M199 = Medium 199 with EBSS, MEM = minimal essential medium of Eagle with EBSS, AA = MEM amino acids mixture (used at 1× concentration), vitamins = MEM vitamin mixture (1×), and HBSS = Hanks' balanced salt solution. All solutions were buffered to pH 7.2–7.4 and both M199 and MEM contain full complements of amino acids.

logical features of glucose–glucose oxidase injury. In additional experiments, higher concentrations of glycine were used in the absence of Mn^{2+}. Even at concentrations as high as 100 mg of glycine per rat, protection did not exceed 50% when Mn^{2+} was omitted from the treatment regimen. We also tried using higher Mn^{2+} concentrations, but toxicity limited this. Finally, studies were carried out in which rats were treated with a concentration of glucose–glucose oxidase chosen to produce half-maximal injury. Rats were concomitantly treated with intratracheal EDTA (1 mg/rat). Preliminary experiments showed that this dose of EDTA by itself produced no detectable lung injury or other signs of toxicity. As shown in Table 3 (experiment B), this amount of EDTA significantly enhanced lung injury induced by glucose–glucose oxidase. Thus, treat-

Table 3. Modulation of Glucose–Glucose Oxidase-Mediated Lung Injury

Treatment	Permeability index
Experiment A[a]	
Buffer alone (saline)	0.30 ± 0.09
Glucose–glucose oxidase	1.16 ± 0.20
Glucose–glucose oxidase + glycine	0.88 ± 0.15
Glucose–glucose oxidase + Mn^{2+}	0.90 ± 0.19
Glucose–glucose oxidase + glycine + Mn^{2+}	0.29 ± 0.03
Experiment B[b]	
Buffer alone (saline)	0.35 ± 0.06
EDTA	0.36 ± 0.15
Glucose–glucose oxidase	0.68 ± 0.31
Glucose–glucose oxidase + EDTA	2.56 ± 0.81

[a] Lung injury was induced in fully anesthetized Long-Evans rats by intratracheal instillation of glucose oxidase (100 units) and 2 mg of glucose mixed in the reaction tube immediately before instillation. Some animals were given 50 mg of manganese acetate, 50 mg of glycine, or 50 mg of manganese acetate and glycine by intraperitoneal injection 2 h prior to instillation of glucose–glucose oxidase. Lung injury was assessed using changes in the permeability index 4 h later as a measure of injury. Values shown represent averages ± standard deviations based on groups of rats with seven to eight rats per group.

[b] Submaximal lung injury was induced in fully anesthetized Long-Evans rats by intratracheal instillation of glucose oxidase (75 units) and 2 mg of glucose mixed in the reaction tube immediately before instillation. Some animals were given 1 mg of EDTA alone by intratracheal instillationi or 1 mg of EDTA added along with the glucose–glucose oxidase. Lung injury was assessed using changes in the permeability index 4 h later as a measure of injury. Values shown represent averages ± standard deviations based on groups of rats with eight to nine rats per group.

ment with a Mn^{2+} chelator potentiates oxidant-mediated lung injury. It should be noted that EDTA could have a number of effects (independent of Mn^{2+} chelation) that potentiate lung injury. If a significant amount of Ca^{2+} and Mg^{2+} were removed, for example, cell–cell junctures would be weakened and allow for increased permeability following exposure to H_2O_2. Additionally, EDTA can chelate iron, resulting in enhanced hydroxyl radical production from H_2O_2 in the Fenton reaction (11). Our data cannot rule out these possibilities.

DISCUSSION

Taken together, these data demonstrate that Mn^{2+} can bring about potent suppression of endothelial cell injury resulting from exposure to H_2O_2 in vitro and can also suppress H_2O_2-induced lung injury in vivo. Since we are able to suppress localized tissue injury (in the lung) by systemic treatment with Mn^{2+} and glycine, these data provide the basis for a possible new approach to inter-

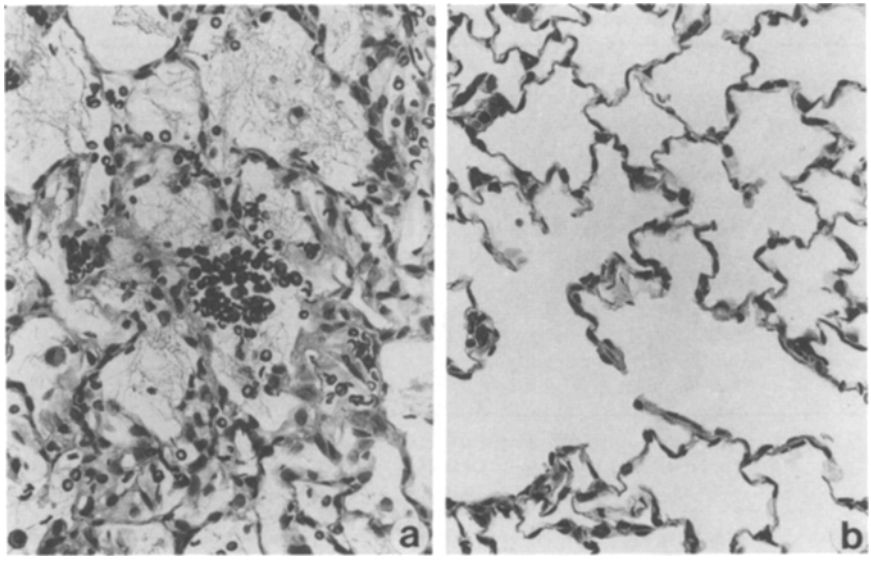

Fig. 2. Lungs from animals exposed to 100 units of glucose oxidase and 2 mg of glucose (frame a) and lungs from animals pretreated for 2 h by intraperitoneal injection of 50 mg of manganese acetate and 50 mg of glycine and then exposed to the same amounts of glucose and glucose oxidase (frame b). There is intraalveolar hemorrhage and extensive fibrin deposition in the lungs of unprotected animals. In the protected animals, aside from vascular congestion, the alveolar spaces are clear of red cells and fibrin. (hematoxylin and eosin, × 230).

fering with tissue injury mediated by toxic oxygen metabolites. In past studies, exogenous enzymes such as superoxide dismutase or catalase (12–14), agents that act as oxygen radical scavengers, or ferrous iron chelators (6–8) have been employed. Effectiveness of the enzymes, toxicity of the iron chelators, and difficulty in achieving adequate blood levels with scavengers have limited the application of these agents. Additional experiments with other in vivo models of acute inflammation will be necessary to determine how effective (and under what conditions) treatment with combinations of Mn^{2+} and amino acids might be. Additional studies also will be necessary to determine the minimum concentrations of Mn^{2+} required for protection and to determine if these concentrations can be tolerated. Consistent with the data presented here, other investigators have shown that high concentrations of Mn^{2+} are not cytotoxic for cells in culture (15, 16). Interestingly, Mn^{2+} can potentiate injury due to irradiation (16) or to high concentrations of dopamine (15). In both situations, enhanced injury is thought to reflect a role for Mn^{2+} in free radical generation. The situation is different here, where a free radical-generating system is already

in place and Mn^{2+} functions (in conjunction with amino acids and bicarbonate ion) to "shunt" the free radical away from critical substrates. Furthermore, consistent with our findings, others have shown that high concentrations of Mn^{2+} can be tolerated by experimental animals, even during long-term exposure (17–19). However, systemic treatments have been shown to result in reproductive failure (19) and to decrease the concentration of free amino acids in the liver (18) and brain (17). Whether these toxic effects would prevent use of Mn^{2+} in cases of acute lung injury is not known at present and more work will need to be done. It should be noted in this regard, however, that factors such as pH, bicarbonate ion concentration, and amino acid availability are all critical determinants in the reactions involving Mn^{2+} (4). Thus, under the appropriate conditions, much lower concentrations of Mn^{2+} than used here maybe beneficial.

With regard to protection of endothelial cells, in vitro, the effectiveness of Mn^{2+} depends on a source of amino acids and the presence of bicarbonate ion in the buffer. Thus, these data are consistent with the suggestion that of the several reactions that Mn^{2+} can participate in (1–5), it is the direct oxidation of amino acids with concomitant disproportionation of H_2O_2 as defined by Stadtman and coworkers (3–5) that is responsible for protection. According to their mechanism, Mn^{2+} forms a coordinate complex with both the carboxyl and amino moieties of the amino acid along with bicarbonate ion and H_2O_2, resulting in the liberation of a proton. The Mn^{2+} is then oxidized by H_2O_2 to form Mn^{3+}, OH · and OH^-, which in concert with the proton accounts for the net release of water. Because the OH · is formed in close proximity to the amino acid moiety of the complex, it abstracts a hydrogen atom from the amino acid to form a carbon-centered amino acid radical. This radical promptly reduces the Mn^{3+} to regenerate the Mn^{2+} and form the imino acid derivative, which spontaneously hydrolyzes to form an alpha-keto acid and NH_3 (4). Whether the same reactions are responsible for removal to H_2O_2 generated in vivo and for protection of animals against injury by glucose–glucose oxidase is not known for certain. However, the fact that either glycine or Mn^{2+} alone provides partial protection, while the two combined provide almost complete protection, is at least consistent with the mechanism deduced from in vitro studies. Additional studies will be needed to fully characterized the events occurring in vivo following treatment with Mn^{2+} and/or glycine.

Mn^{2+} may be involved in normal antioxidant barrier function. While the concentrations of Mn^{2+}, bicarbonate ion, and amino acids in plasma are probably too low to effect much protection, local variations in tissue concentrations might facilitate elimination of H_2O_2 from tissue sites under normal conditions. The fact that intratracheal instillation of EDTA along with glucose–glucose oxidase significantly enhanced the toxicity of this combination of reagents supports the possibility of a role for Mn^{2+} in the normal antioxidant barrier.

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