Synergistic Effect of Hydrogen Peroxide and Elastase on Elastic Fiber Injury *In Vitro*

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This laboratory has previously shown that hyperoxia enhances airspace enlargement in a hamster model of elastase-induced emphysema. To further understand the mechanism responsible for this finding, the effect of oxidants on elastase activity was studied *in vitro*, using a radiolabeled elastic fiber matrix derived from rat pleural mesothelial cells. Matrix samples were treated with either 0.1%, 1%, 3%, or 10% hydrogen peroxide (H_2O_2) for 1 hr, then incubated with 1.0 μg/ml porcine pancreatic elastase for 2 hrs. Radioactivity released from the matrix was used as a measure of elastolysis. Results indicate that sequential exposure to H_2O_2 and elastase markedly enhanced elastolysis compared to enzyme treatment alone. A 22% increase in elastolysis was seen with 0.1% H_2O_2 (325 vs. 396 cpm; $P < 0.05$), whereas samples pretreated with 1%, 3%, and 10% H_2O_2 showed increases of 53% (274 vs. 420 cpm; $P < 0.05$), 71% (381 vs. 653 cpm; $P < 0.01$), and 38% (322 vs. 443 cpm; $P < 0.01$), respectively. Exposure to various concentrations of H_2O_2 alone (0.1% to 10%) produced only minimal elastolysis (<20 cpm). However, 1% H_2O_2 was capable of degrading peptide-free desmosine and isodesmosine, suggesting that exposure to this oxidant may reduce the stability of the elastic fiber matrix. With regard to lung diseases such as emphysema, H_2O_2 and other oxidants derived from inflammatory cells or the environment could possibly act as priming agents for elastase-mediated breakdown of elastic fibers, resulting in amplification of lung injury. Exp Biol Med 231:107–111, 2006

Key words: elastin; elastase; hydrogen peroxide; oxidants; elastolysis

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Introduction

Since its inception four decades ago, the concept that pulmonary emphysema results primarily from an imbalance between elastases and their inhibitors has undergone considerable advancement (1–3). The putative mechanisms involved in the pathogenesis of the disease now encompass a host of factors that were not apparent when the protease–antiprotease hypothesis was first developed (4). Although increased elastase activity is undoubtedly an important cause of alveolar septal damage, the airspace enlargement that occurs in pulmonary emphysema may actually represent a more generalized, stereotypic response to a number of different types of injury (5).

To address this possibility, our laboratory previously performed a series of experiments involving both the induction and modification of experimental emphysema with agents other than elastases. It was found that exposure to a normally nontoxic concentration of oxygen (60%) enhanced elastase-induced emphysema and also induced airspace enlargement in lungs pretreated with a non-elastolytic enzyme, hyaluronidase (6, 7). These findings support the concept that pulmonary emphysema is a complex, multifactorial disease process, possibly involving synergistic interactions among various enzymes and oxidants.

The current studies further examine the effect of oxidants on elastase activity *in vitro*, using an elastic fiber matrix derived from rat pleural mesothelial cells. In contrast to *in vivo* experiments, this simplified test substrate was particularly useful for determining enzyme–oxidant interactions specifically related to elastic fibers. The results suggest that exposure of these fibers to oxidants significantly increases their susceptibility to elastase injury.

Methods

Preparation of Radiolabeled Matrix. Rat pleural mesothelial cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in 75 cm^2 plastic flasks using Nutrient Mixture Ham's F-12 medium supplemented with 15% fetal bovine serum, 1% glutamine, 20 U/ml streptomycin, and 20 U/ml penicillin G. The

cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells and extracellular matrix were radiolabeled for 6 weeks with [^{14}C]lysine (3.1 μCi per flask). At the end of the labeling period, the cultures were washed with phosphate-buffered saline (PBS), and the cells were lysed with 0.5% sodium deoxycholate and EGTA. Following removal of the cellular material, the matrix was rinsed with PBS and allowed to air dry. The plastic surface containing the radiolabeled matrix was then cut into 2 × 2-cm squares.

Treatment of the Matrix with Elastase. The radiolabeled matrix squares were coated with either 1, 2, or 10 μg/ml porcine pancreatic elastase (Elastin Products, Owensville, MO) in 0.5 ml of 0.1 *M* Tris buffer, pH 8 (or Tris buffer alone to determine background radioactivity) and incubated for 3 hrs at 37°C. The liquid was then removed, combined with a single PBS washing of the matrix, and measured for radioactivity in a liquid scintillation spectrometer. Results were expressed as net counts per minute (cpm) per matrix square after subtraction of background radioactivity released from samples treated with Tris buffer alone.

Treatment of the Matrix with H_2O_2. The radiolabeled matrix squares were coated with 0.5 ml of either 0.1%, 1%, 3%, 10%, or 20% H_2O_2 in PBS (or PBS alone to determine background radioactivity) and incubated for 1 hr at 37°C. The liquid was then removed, combined with 2 PBS washings of the matrix, and measured for radioactivity in a liquid scintillation spectrometer. Results were expressed as net cpm per matrix square after background subtraction.

Treatment of the Matrix with H_2O_2 Followed by Elastase. The radiolabeled matrix squares were coated with 0.5 ml of either 0.1%, 1%, 3%, or 10% H_2O_2 in PBS (or PBS alone as a control) and incubated for 1 hr at 37°C. Following removal of the liquid, the squares were washed twice with PBS, then coated with 0.5 ml of 1 μg/ml porcine pancreatic elastase in 0.1 *M* Tris buffer, pH 8 (or 0.5 ml Tris buffer alone to determine background radioactivity) and incubated for 2 hrs at 37°C. The fluid was then removed, combined with a single PBS washing of the matrix, and measured for radioactivity in a liquid scintillation spectrometer. Results were expressed as net cpm per matrix square after background subtraction.

Treatment of the Matrix with Elastase Followed by H_2O_2. The radiolabeled matrix squares were coated with 0.5 ml of either 100 ng/ml or 1 μg/ml porcine pancreatic elastase in 0.1 *M* Tris buffer, pH 8 (or Tris buffer alone as a control) and incubated for 1 hr at 37°C. Following removal of the liquid, the squares were washed twice with PBS, then coated with 0.5 ml of 3% H_2O_2 in PBS (or PBS alone to determine background radioactivity) and incubated for 2 hrs at 37°C. The fluid was then removed, combined with a single PBS washing of the matrix, and measured for radioactivity in a liquid scintillation spectrometer. Results were expressed as net cpm per matrix square after background subtraction.

Treatment of the Matrix Concurrently with Elastase and H_2O_2. The radiolabeled matrix squares were coated with 0.5 ml of a mixture of 1 μg/ml porcine pancreatic elastase and 3% H_2O_2 in 0.1 *M* Tris buffer, pH 8 (or Tris buffer alone to determine background radioactivity), and incubated for 2 hrs at 37°C. Controls were treated with elastase alone. The liquid was then removed, combined with a single PBS washing of the matrix, and measured for radioactivity in a liquid scintillation spectrometer. Results were expressed as net cpm per matrix square after background subtraction.

Treatment of Desmosine and Isodesmosine with H_2O_2. Purified desmosine and isodesmosine (Elastin Products) were separately incubated in a 1% solution of H_2O_2 in PBS (or PBS alone as a control) for either 1 or 2 hrs at 37°C. The samples were then frozen, lyophilized, and subjected to high-performance liquid chromatography followed by electrospray ionization mass spectrometry to quantify desmosine and isodesmosine, as previously described (8). The results were expressed as a percentage change in total desmosine or isodesmosine compared to controls.

Data Analysis. The two-sample *t* test was used to determine statistical significance between two treatment groups. When more than two groups were compared, the Newman-Keuls multiple-comparisons test was performed. Pearson's test was used to determine correlations between two variables. *P* values less than 0.05 were considered to be significant.

Results

Release of Radioactivity as a Measure of Elastolysis. Cell-free radiolabeled matrix was prepared from cultures of rat pleural mesothelial cells that have previously been shown to predominantly synthesize elastin (9, 10). The matrix was treated with increasing concentrations of elastase to determine the relationship between elastolysis and release of radioactivity. As shown in Figure 1, there was a positive correlation between these two parameters ($r = 0.97$; $P < 0.05$). The nonlinear nature of the plot most likely reflects exhaustion of the substrate with the highest concentration of elastase (10 μg/ml).

H_2O_2-Induced Elastolysis. Treatment of the matrix with 0.1% to 10% H_2O_2 for 1 hr resulted in only minimal release of radioactivity. Less than 20 cpm was measured at each concentration (Fig. 2). Even with 20% H_2O_2, only 71 cpm was released from the matrix.

Effect of H_2O_2 on Elastase-Induced Elastolysis. Pretreatment with H_2O_2 significantly enhanced elastase-mediated release of radioactivity (Fig. 3). A 22% increase in elastolysis was seen with 0.1% H_2O_2 (325 vs. 396 cpm; $P < 0.05$), whereas samples pretreated with 1% and 3% H_2O_2 showed increases of 53% (274 vs. 420 cpm; $P < 0.05$) and 71% (381 vs. 653 cpm; $P < 0.01$), respectively. Interestingly, samples exposed to 10% H_2O_2 showed a

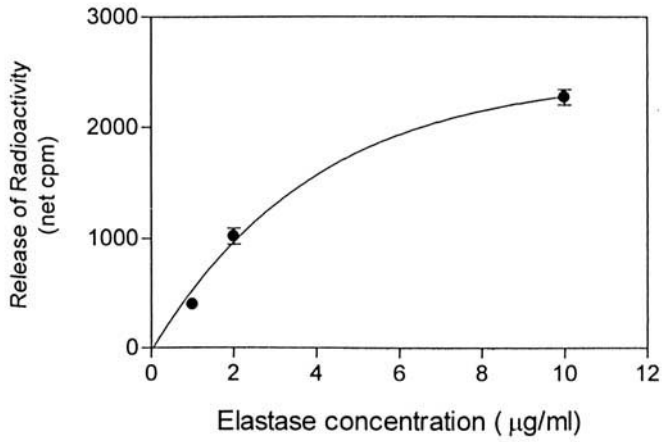

Figure 1. The radiolabeled matrix was treated with increasing concentrations of elastase to determine the relationship between elastolysis and release of radioactivity. There was a positive correlation between these two parameters ($r = 0.97$; $P < 0.05$). The nonlinear nature of the plot most likely reflects exhaustion of the substrate with the highest concentration of elastase (10 μg/ml). T-bars denote SEM (N ≥ 3 for each group).

smaller increase in elastase-induced elastolysis (38%) than those receiving 3% H_2O_2 (322 vs. 443 cpm; $P < 0.01$). This loss of activity may possibly reflect impaired enzyme–substrate interactions as a result of more extensive changes in the matrix with the higher concentration of H_2O_2.

Effect of Elastase on H_2O_2-Induced Elastolysis. Pretreatment with elastase enhanced H_2O_2-mediated release of radioactivity (Fig. 4). Matrix exposed to 1 μg/ml of elastase before treatment with 3% H_2O_2 showed a significant increase in elastolysis (53.1 vs. 6.6 cpm; $P < 0.001$), whereas samples pretreated with 100 ng/ml of elastase only showed a minimal increase (11.4 vs. 6.6 cpm; $P > 0.05$).

Effect of Concurrent Treatment with Elastase and H_2O_2. As shown in Figure 5, concurrent treatment of the matrix with 1 μg/ml elastase and 3% H_2O_2 resulted in a

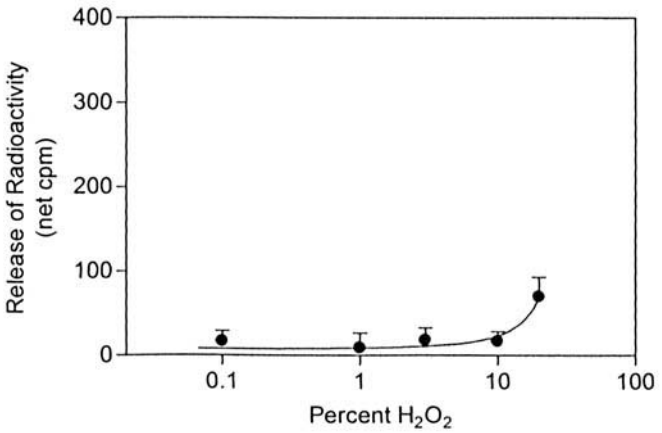

Figure 2. Treatment of the matrix with concentrations of H_2O_2 ranging from 0.1% to 10% resulted in minimal elastolysis. Even with 20% H_2O_2, the amount of radioactivity released from the matrix was only 71 cpm. T-bars denote SEM (N ≥ 5 for each group).

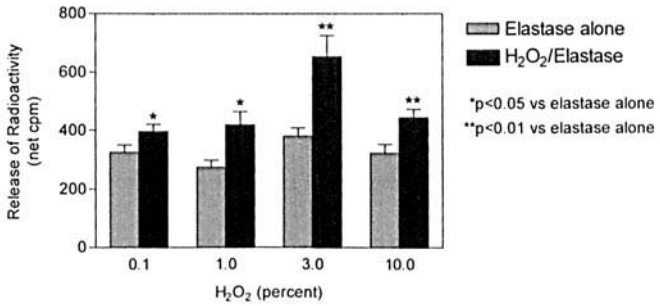

Figure 3. Pretreatment of matrix with H_2O_2 enhanced elastase-mediated release of radioactivity. Samples exposed to 10% H_2O_2 showed a reduction in elastase-induced elastolysis relative to those receiving 3% H_2O_2. This decrease may possibly reflect impaired enzyme–substrate interactions because of more extensive changes in the matrix with a higher concentration of H_2O_2. T-bars denote SEM (N ≥ 5 for each group).

15% reduction in elastolysis compared to samples treated with elastase alone (263 vs. 311 cpm), but the difference between the groups was not statistically significant. This finding suggests that H_2O_2 may impair enzyme activity when given concurrently with elastase.

Effect of H_2O_2 on Desmosine and Isodesmosine. Exposure to 1% H_2O_2 resulted in degradation of peptide-free desmosine and isodesmosine, as reflected by a reduction in measurable amounts of these compounds using electrospray ionization mass spectrometry (Fig. 6). There was a positive correlation between exposure time and percentage decrease of each cross-linking amino acid ($P < 0.05$).

Discussion

In terms of understanding the pathogenesis of pulmonary emphysema, the use of papain to experimentally induce airspace enlargement represented an initial breakthrough (11). Introduction of the papain model had added significance because it came at a time when the role of α_1-antiproteinase in pulmonary emphysema was just being understood (4). The two findings emphasized the importance of proteolysis as a cause of the disease and led to the hypothesis that an imbalance between lung proteases and their inhibitors was responsible for the airspace enlargement that characterizes pulmonary emphysema.

The proteinase–antiproteinase concept served to focus research on the role of elastases, with the hope that inhibiting the activity of these enzymes would prevent lung injury. However, if pulmonary emphysema represents a more general response of the lung to a variety of insults (with elastases playing a variable role), then enzyme inhibition may have only limited efficacy, and other potential forms of treatment may be required.

As an alternative source of alveolar injury, oxidants have been gaining importance in recent years. A number of studies have shown that exposure to various oxidants can cause inflammation and destruction of lung tissue (12–17). Among the postulated mechanisms of action for oxidants

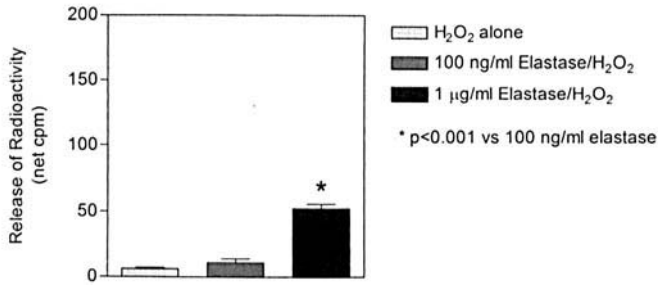

Figure 4. Pretreatment of matrix with elastase enhanced H_2O_2-mediated release of radioactivity. Compared to untreated samples, those exposed to 1 μg/ml of elastase before treatment with 3% H_2O_2 showed a significant increase in elastolysis, whereas matrix pretreated with 100 ng/ml of elastase only showed a minimal increase. T-bars denote SEM (N ≥ 5 for each group).

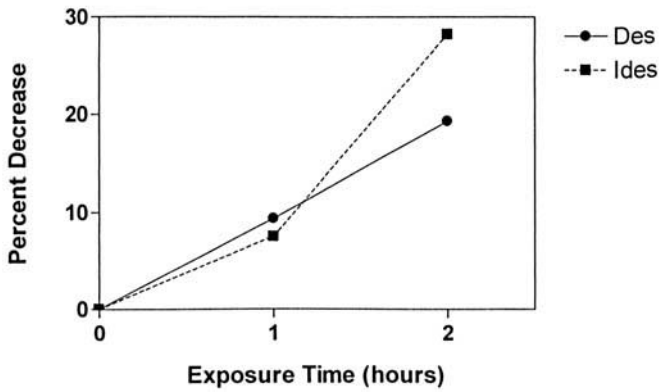

Figure 6. Exposure to 1% H_2O_2 resulted in degradation of peptide-free desmosine and isodesmosine. There was a positive correlation between exposure time and percentage decrease of each cross-linking amino acid ($P < 0.05$). The loss of desmosine and isodesmosine in the extracellular matrix could facilitate enzyme-mediated breakdown of elastic fibers (N ≥ 2 for each group).

are the generation of free radicals. These short-lived, highly reactive molecules can damage cell membranes and cause the release of cytokines that recruit inflammatory cells to the lung. Although oxidants may also cause direct injury to the extracellular matrix (18, 19), exposure to H_2O_2 *per se* did not produce significant elastolysis in the current studies. Instead, pretreatment of the matrix with H_2O_2 potentiated the effects of elastase.

Although the concentrations of H_2O_2 used in the these experiments (30 m*M* and above) exceed those usually observed under physiological conditions, recent studies of the release of oxidants by neutrophils indicate that it occurs in directed bursts that could involve concentrations far in excess of what is recorded in the pericellular environment (20). In the absence of myeloperoxidase, levels of H_2O_2 within neutrophil phagosomes might conceivably reach as high as 100 m*M* (21).

However, such concentrations of H_2O_2 may not be necessary to potentiate elastolysis *in vivo*. Because injury to elastic fibers may be cumulative, chronic exposure to lower levels of H_2O_2 *in vivo* may produce changes similar to those seen in the current studies. Furthermore, the damaging effects of lower concentrations of H_2O_2 might be enhanced *in vivo* by the presence of metal ions that facilitate the conversion of H_2O_2 to highly reactive hydroxyl radicals (22, 23). The potential role of these ions in amplifying H_2O_2-mediated elastic fiber injury needs further investigation.

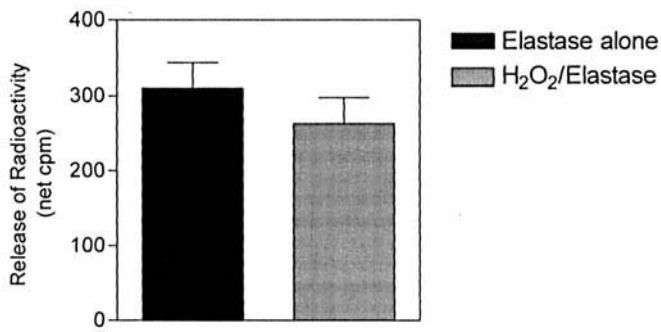

Figure 5. Concurrent treatment of the matrix with 1 μg/ml elastase and 3% H_2O_2 resulted in a 15% reduction in elastolysis compared to samples treated with elastase alone, but the difference between the groups was not statistically significant. This finding suggests that H_2O_2 may impair enzyme activity when given concurrently with elastase. T-bars denote SEM (N = 10 for each group).

As suggested by the current studies, treatment of the matrix with H_2O_2 may adversely affect the desmosine and isodesmosine cross-links of elastin. Other investigators have shown that H_2O_2 can convert these cross-links to less stable intermediates that might weaken the structure of elastin (24). Thus, H_2O_2 may act as a priming agent for enzyme-mediated elastolysis.

This concept is supported by an earlier investigation that similarly determined the combined effects of H_2O_2 and elastase on a radiolabeled matrix preparation (25). In that study, a much lower concentration of H_2O_2 than that used in the current experiments significantly increased elastase-induced elastolysis. This disparity may be at least partly a result of differences in the composition of the labeled matrix. Whereas the matrix employed in the earlier work was derived from 3-week-old cultures, that used in current study was grown for several months. Consequently, the latter preparation was presumably much denser and less susceptible to the effects of H_2O_2.

Collagen fibers also appear to be relatively resistant to H_2O_2. *In vitro* studies indicate that pretreatment with H_2O_2 does not increase enzymatic degradation of this matrix component (26). A significant enhancement of protease-induced collagenolysis was observed only when myeloperoxidase was added to H_2O_2. As with elastic fibers, resistance to H_2O_2 may be related to the density of the matrix.

The seemingly paradoxic finding that 10% H_2O_2 was less effective in promoting elastase-induced elastolysis than the 3% H_2O_2 may possibly be explained by a further loss of elastic fiber tethering with the higher concentration, permitting hydrophobic portions of elastin to contract and become less accessible to elastase.

Reversing the order of treatment did not yield the same

degree of synergy between elastase and H_2O_2, and concurrent treatment with elastase and H_2O_2 did not produce any synergistic effect at all. These results suggest that the temporal relationship of the two agents is important in determining overall elastolysis.

Because elastin-derived peptides attract neutrophils and induce them to release oxidants (27), potential treatments for pulmonary emphysema should include strategies aimed at maintaining the integrity of elastic fibers. With regard to this therapeutic approach, our laboratory has previously demonstrated that the binding of hyaluronan (HA) to elastic fibers significantly decreases elastolysis induced by various elastases (9, 28, 29). HA was also shown to significantly reduce airspace enlargement and elastic fiber breakdown in mice exposed to cigarette smoke (30).

In contrast to currently proposed treatments for pulmonary emphysema, such as elastase inhibitors, the use of agents designed to directly prevent elastic fiber injury might protect the lung from a greater variety of insults, including oxidants and enzymes other than elastases. The generally slow progression of pulmonary emphysema suggests that even a small reduction in the rate of elastic fiber degradation could significantly delay the worst features of the disease, eliminating them from the lives of most patients.

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