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# Degradation of soluble collagen by ozone or hydroxyl radicals

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Collagen exposed to ozone or hydroxyl radicals was degraded in a time- and dose-dependent manner. This degradation was inhibited by free radical scavengers. Furthermore, lower levels of these oxidants did not degrade the molecule, but caused it to become susceptible to proteolytic degradation. We suggest an alternative mechanism by which oxygen-derived free radicals participate in the destruction of extracellular matrix observed during acute lung injury by oxidant gas, in addition to the commonly accepted proteinase–antiproteinase theory of lung injury.

*Collagen* *Inflammation* *Oxygen-derived free radical* *Ozone*

## 1. INTRODUCTION

The mechanism of acute oxidant gas injury to the lung is not yet understood. It is known that $\alpha_1$-antiproteinase, a major inhibitor of inflammatory cell proteinases, can be inactivated by oxidation [1–3]. Cigarette smoke contains various oxidizing agents which inactivate $\alpha_1$-antiproteinase [4,5]. It has been suggested, therefore, that nonfunctional $\alpha_1$-antiproteinase in the lungs of smokers fails to protect the lung from proteinases released from inflammatory cells, and this may lead to excess proteolytic degradation of extracellular matrix, as is observed in emphysema [1,2]. In contrast, a recent study reported no significant difference in the mean functional $\alpha_1$-antiproteinase levels in the lavage fluids of smokers and nonsmokers [6]. Instead of finding nonfunctional $\alpha_1$-antiproteinase, the investigators found a 5-fold increase in the number of neutrophils and alveolar macrophages in the lungs of smokers.

We report that oxygen-derived free radicals can (i) directly degrade soluble collagen and (ii) at low levels can potentiate the destructive role of proteinases by increasing the susceptibility of collagen to degradation by proteinases. The mechanism of oxidant gas injury to the lung may share with inflammation both nonenzymatic (oxidant) and enzymatic (inflammatory proteinase) damage to connective tissues.

In order to separate the direct effects of oxidants on collagen from the effects of inflammatory cell proteinases, we examined the effects of oxygen-derived free radicals on purified type I collagen and its biosynthetic precursor, procollagen. We considered it important to test the effects of highly reactive oxygen species on procollagen because it is believed that structurally intact procollagen secreted from the cell is essential for collagen fibers to align and polymerize correctly in the extracellular matrix. This is especially critical in tissues such as lung and joint synovia in which there is more than one type of collagen [7,8]. Our data demonstrate a direct degradation of soluble collagen by these oxidant species. In addition, intact procollagen which has been damaged or modified, but not degraded, by exposure to ozone shows a marked susceptibility to proteolytic degradation by trypsin. This proteinase is used as a probe to determine whether the triple helix of collagen is intact [9]. On the basis of

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these data we propose that oxygen-derived free radicals may provide an alternative as well as a synergistic pathway to that of inflammatory proteinases in the destruction of extracellular matrix components.

## 2. MATERIALS AND METHODS

Type I collagen labeled with [$^{14}$C]proline was prepared from embryonic chick tendons by the ultrafiltration method of [10]. Type I chick procollagen labeled with [$^{14}$C]proline was prepared according to the method of [11]. Radioisotopes used in this study, [$^{14}$C]proline and $^{14}$C-labelled amino acid mixture, were purchased from New England Nuclear, Boston, MA. The resin Agarose A 5 m (200–400 mesh) was a product of Bio-Rad. Mannitol, superoxide dismutase, catalase and diethylenetriamine pentaacetic acid (Detapac) were purchased from Sigma, St. Louis, MO. Hydrogen peroxide, used freshly, was obtained as a 30% solution from Fisher Chemical, Springfield, NJ. Hydroxyl radicals were generated from Fenton's reagent, which involves the ferrous catalyzed reduction of $H_2O_2$: $Fe^{2+}$ (Detapac) + $H_2O_2 \longrightarrow Fe^{3+}$ (Detapac) + $\cdot OH$ + $OH^-$. Ozone was generated from oxygen by a silent electric arc discharge apparatus maintained in a properly vented hood. The ozone concentration was measured in parts per million (ppm) by the neutral buffered iodide method.

## 3. RESULTS

Fig.1 represents the chromatography of control and ozone-treated collagen on an agarose A 5 m column eluted with 0.1 M Tris buffer containing 0.1% sodium dodecyl sulfate (SDS), pH 7.5. In addition to the degraded material, higher molecular mass species also appeared (fig.1). This material

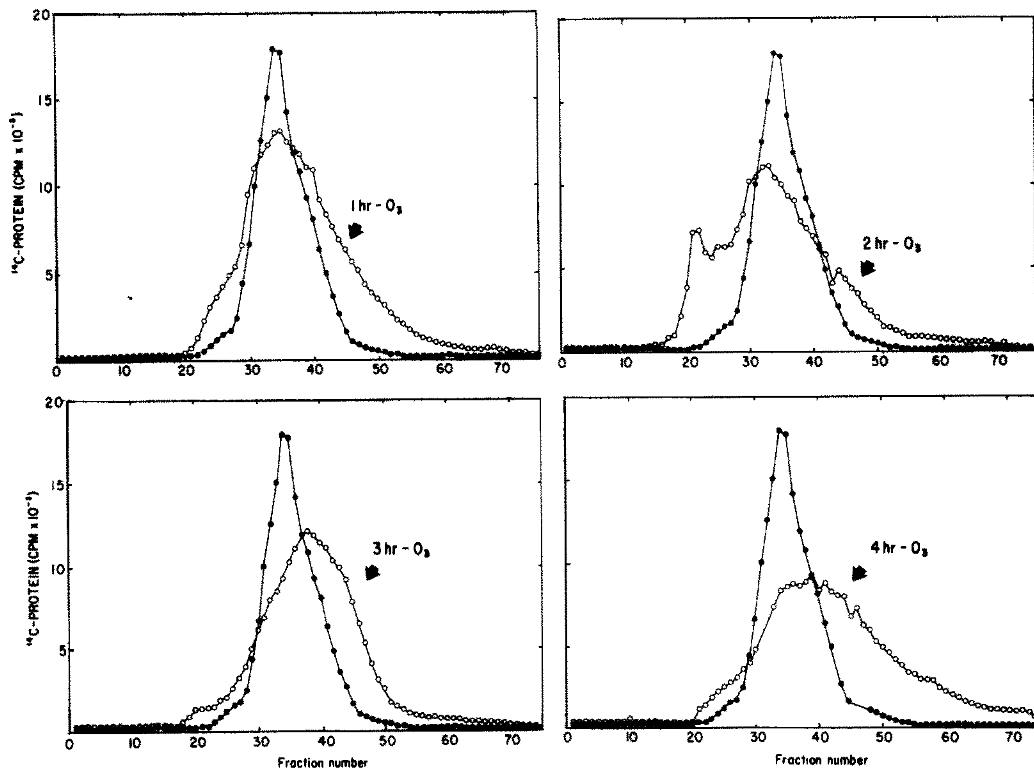


Fig.1. Embryonic chick tendon collagen (100 $\mu$g/ml), labeled with [$^{14}$C]proline was dissolved in 0.1 M ammonium acetate, pH 5.0, and exposed to an atmosphere of 10 ppm ozone (53.5 $\mu$mol/h) for 1–4 h. Collagen samples were then chromotographed on a 1.5 × 50 cm A 5 m agarose column in a 0.1 M Tris, 0.1% SDS buffer (pH 7.5) at room temperature. ●—●, air-exposed samples (controls); ○—○, $O_3$-treated samples.

elutes from the gel filtration column with a molecular mass greater than that of the control (fig.1, panel 2), but is also degraded with increased exposure time to oxygen-derived free radicals [12] (fig.1, panel 4). Since ozone ($O_3$) has been shown to crosslink proteins, [13] this high-molecular mass material may represent crosslinked collagen. We are currently investigating the composition and origin of these collagen species.

Fenton's reagent produces hydroxyl radicals by ferrous catalyzed reduction of peroxide [14]. Procollagen was exposed to Fenton's reagent and then examined by polyacrylamide slab gel electrophoresis in SDS to determine its molecular mass. The results are shown in fig.2, which is a photograph of a 6% polyacrylamide gel in SDS of intact and treated procollagen. Lane 1 is intact purified procollagen. Lanes 2 and 3 represent procollagen treated with 2.5 mM Fenton's reagent for 2 h at room temperature. The procollagen in these lanes is degraded to peptides smaller than 40 kDa (exclusion molecular mass of gel). Although a portion of the procollagen was degraded to small peptides, procollagen exposed to Fenton's reagent (lanes 4 and 7) had a slight difference in mobility compared with the starting material. This may have been due to modification of amino acid residues such as increased hydroxylation of prolyl residues [15] or other oxidative modifications. The hydroxylation of prolyl residues in collagen affects its mobility in polyacrylamide gel electrophoresis in SDS [16]. When procollagen was examined by agarose gel chromatography in SDS under conditions similar to those described in fig.1, it was shown to be degraded to randomly sized peptides, some eluting at the final column volume with tritiated $H_2O$ (not shown). Catalase which destroys $H_2O_2$, and several hydroxyl radical scavengers, protected procollagen from degradation by Fenton's reagent (fig.2).

It is known that ultraviolet light irradiation causes random scission of the collagen polypeptide chains, and decreases the stability of the triple-helical collagen conformation [17]. Damaged collagen molecules which are not perfectly triple helical become susceptible to tissue proteinases [9,18]. We hypothesized that low doses and short exposure to oxidants would damage soluble collagen so that the molecule would become susceptible to proteinases. We exposed procollagen to 0.25 ppm ozone for 3, 15 and 60 min. The samples were subjected to polyacrylamide slab gel electrophoresis in SDS and the results are shown in fig.3. Lane 2 shows that exposure to 0.25 ppm $O_3$ for 3 min does not in itself degrade collagen but after ozone exposure all of the collagen is susceptible to degradation by trypsin (lane 3). Controls indicated that inactivated trypsin was unable to degrade $O_3$-treated collagen. In-as-much as $O_3$ exposure

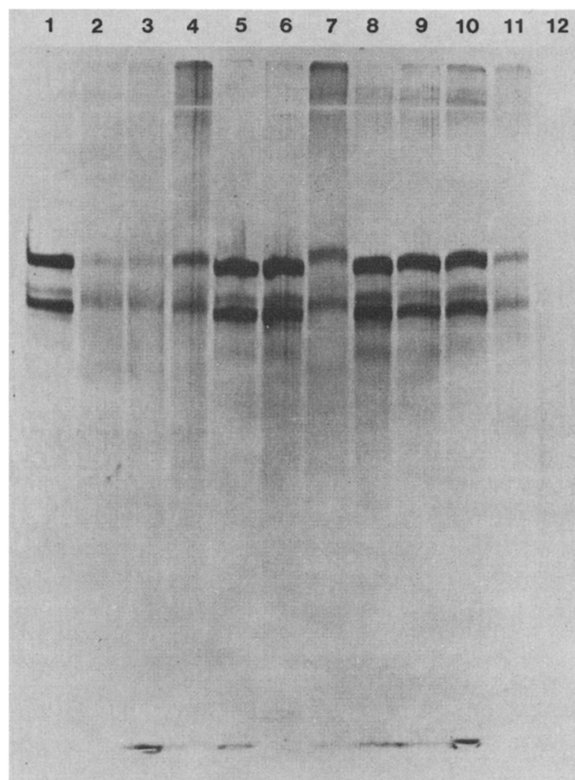


Fig.2. Fluorogram of polyacrylamide gel electrophoresis in SDS (6% acrylamide) of procollagen labeled with [$^{14}$C]proline exposed to Fenton's reagent for 2 h at room temperature. Lane 1, type I procollagen control; 2, procollagen + 2.5 mM Fenton's reagent; 3, duplicate of lane 2; 4, procollagen + 2.5 mM Fenton's reagent + 5 mM mannitol; 5, procollagen + 2.5 mM Fenton's reagent + 5% ethanol; 6, procollagen + 2.5 mM Fenton's reagent + 100 $\mu$g/ml catalase; 7, procollagen + 2.5 mM Fenton's reagent + 100 $\mu$g/ml superoxide dismutase; 8, procollagen + 0.125 mM Fenton's reagent; 9, procollagen + 0.25 mM Fenton's reagent; 10, procollagen + 0.5 mM Fenton's reagent; 11, procollagen + 1.25 mM Fenton's reagent; 12, procollagen + 2.5 mM Fenton's reagent.

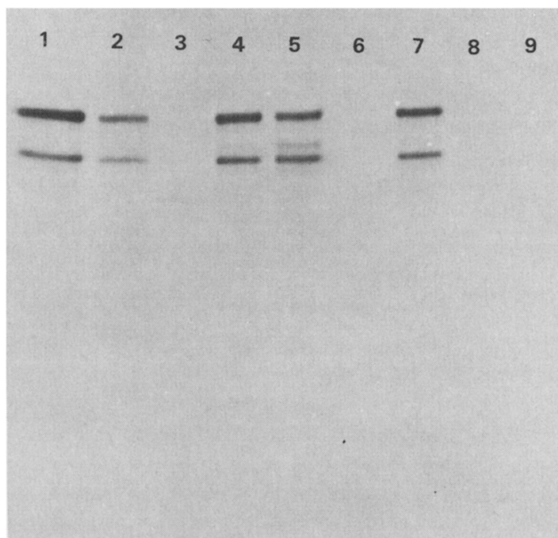


Fig.3. Fluorogram of polyacrylamide (6%) gel electrophoresis in SDS of [$^{14}$C]proline-procollagen exposed to an atmosphere of 0.25 ppm $O_3$ (66 nmol $O_3$/h) for varying times. Lane 1, procollagen exposed to air for 3 min; 2, procollagen exposed to $O_3$ for 3 min; 3, procollagen exposed to $O_3$ for 3 min and then treated with 75 $\mu$g/ml trypsin for 60 min [31]; 4, procollagen exposed to air for 15 min; 5, procollagen exposed to $O_3$ for 15 min; 6, procollagen exposed to $O_3$ for 15 min and then treated with 75 $\mu$g trypsin for 60 min [31]; 7, procollagen exposed to air for 60 min; 8, procollagen exposed to $O_3$ for 60 min; 9, procollagen exposed to $O_3$ for 60 min and then treated with 75 $\mu$g/ml trypsin for 60 min [31]. At the end of trypsin exposure the samples were incubated with 400 $\mu$g/ml soybean trypsin inhibitor to inactivate the trypsin. The samples were then treated with SDS for electrophoresis.

alone (lane 2) leaves what appears to be intact polypeptide chains (shown in lane 2), the data suggest that the collagen is modified in such a way as to render the triple-helical conformation unstable. It should be noted that the allowable USA primary air quality standard for ozone is 0.12 ppm for 1 h. Recorded levels in southern California have approached 1 ppm.

## 4. DISCUSSION

There is considerable evidence that oxygen derived free radicals produced by inflammatory cells [19–21], toxic environmental pollutants [22] and hyperoxia [23] are involved in direct cytotoxic damage and tissue injury [24–28]. In the lung, oxidant species have been implicated in vivo in the etiology of immune complex induced alveolitis and alveolar-capillary 'leak' [29–31], in emphysema [32–35] and in interstitial lung fibrosis [36–38]. In the synovium, oxidant species produced by inflammatory cells have been implicated in destructive joint disease [39,40].

Physiological damage to the extracellular matrix of tissues in vivo is almost always accompanied by inflammation. It is well documented that inflammatory cells recruited to a site of injury release elastase and other proteinases capable of destroying connective tissues such as collagen [41]. Inflammatory cells also release highly reactive and destructive oxidant species. Recently, it was reported that large amounts of long-lived oxidative species, probably *N*-chloroamines, were generated by neutrophils and were capable of reacting with peptides [42]. Oxidant species produced during hyperoxia have been shown to stimulate alveolar macrophages to produce a potent chemotaxin for neutropils [43]. These investigators have reported also that neutrophil influx to the lung occurred shortly before the onset of lung injury in animals exposed to hyperoxia [44]. This injury was blocked by catalase ($H_2O_2$ scavenger) and by dimethylthiourea ($H_2O_2$) and/or $\cdot$OH scavenger) [44].

Authors in [45] have recently reported that superoxide anions can degrade microfibrillar collagen. We have established that oxidants can directly degrade soluble collagen, and at low levels can modify collagen, making it susceptible to proteolytic degradation. The destruction of extracellular matrix components, including collagen and elastin, generates peptides which are known to be chemotactic for inflammatory cells and fibroblasts [46–50]. Such an effect of oxygen-derived free radicals may be responsible for the initial recruitment of inflammatory cells to the site of injury. This may be the triggering step in the pathogenesis of many diseases such as emphysema, lung fibrosis and arthritis, as the inflammatory cells perpetuate the inflammation and recruit fibroblasts which then synthesize and deposit excess collagen fibers in the postinflammatory repair phase of fibrotic diseases.

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