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OXIDATIVE DAMAGE TO HUMAN PLASMA PROTEINS BY OZONE

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Exposure of human plasma to ozone produces oxidative protein damage, measured as protein carbonyl formation. Isolated human albumin or creatine phosphokinase are oxidized much faster than are total proteins. Consideration must be given to proteins as targets of oxidative injury by ozone *in vivo*.

KEY WORDS: Ozone, free radical, protein carbonyl, albumin, creatine kinase.

1. INTRODUCTION

The respiratory tract is constantly exposed to oxygen-derived species generated by reactions involving inhaled pollutant gases. For example ozone (O_3), an important component of photochemical air pollution, can directly oxidize several biological molecules[1-3] and, in addition, it reacts slowly with water at physiological pH to yield highly-reactive hydroxyl radicals.[4] Thus it is widely believed that at least some of the deleterious effects of O_3 involve oxidative damage to the respiratory tract.[1,2]

The first biological fluids that come into contact with inhaled oxidant gases are the respiratory tract lining fluids (RTLFs). Some information is available about the antioxidant ability of these fluids[5-7] but the problems of sampling them (by the techniques of respiratory tract lavage) have greatly hindered elucidation of their precise chemical composition, since lavage produces considerable and variable dilution of RTLFs, and some constituents may be oxidized during the lavage and preparation procedures themselves.[8,9] By contrast, the antioxidant defenses of human plasma have been well characterized:[10] uric acid appears to be the major antioxidant that scavenges O_3 when plasma is exposed to this gas.[11] Indeed, in many ways plasma seems to be an acceptable model system in which to study events that are relevant to reactions occurring in RTLFs.[10-12]

Almost all previous studies of biological damage by O_3 have concentrated upon damage to lipids.[1,2,13] However, oxidative stress can damage many other molecules, including proteins and DNA.[14-17] Indeed, damage to proteins and DNA may often be more important than damage to lipids in oxidative stress situations *in vivo*.[16,18-21]

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Attack of reactive oxygen species upon proteins can damage several amino acid residues, including histidine, tryptophan, cysteine, proline, tyrosine, methionine, arginine and lysine.[22,23] Oxidative damage to several of these amino acid residues (arginine, proline, lysine, histidine) as well as to the peptide backbone of proteins can lead to the formation of carbonyl products.[23] Indeed measurement of "protein carbonyls" is a generally-accepted and sensitive assay for oxidative damage to proteins.[18–20,23] In the present paper, we have used this assay to measure oxidative damage to isolated proteins, and proteins in human plasma, exposed to O_3.

2. MATERIALS AND METHODS

2.1. *Plasma.*

Blood was drawn from healthy normolipidaemic male volunteers (age range 30–56) into heparinized tubes and centrifuged to obtain fresh plasma. 5 ml aliquots were placed in open plastic Falcon dishes 31 mm inside diameter, 12 mm deep contained within a closed humidified container (a Teflon jar, 4″ inside diameter, 2″ deep) at 37°C and exposed to a constantly monitored and maintained level of 16 ppm O_3 in 5% CO_2/95% air (using Teflon inlet and outlet ports) in a purpose-built apparatus (fully described in [24]). Control plasma was exposed to an identical gas-stream in an adjacent chamber, but without the O_3. 16 ppm was chosen to impose a high oxidative stress upon the system, so as to accelerate for ease of measurement the changes that take place, since we are using a much lower surface/volume ratio than would exist in the lung *in vivo*. Measurement of the concentrations of plasma electrolytes (Na^+, K^+, Cl^-, HCO_3^-) showed that, as expected,[24] even 6 h of exposure caused no evaporation of water from the plasma, nor did its pH change.

2.1.1. *Carbonyl Assay.* Carbonyls were determined by a modification of the procedure described in[25] using dinitrophenylhydrazine (DNPH). After the DNPH reaction, proteins were precipitated with 20% (w/v) trichloroacetic acid (TCA) (1:1 volume) and washed once with 10% (w/v) TCA and three times with an ethanol-ethyl acetate mixture (1:1). Finally, the precipitates were dissolved in 6M guanidine- HCl solution and the peak absorbance in the range of 400–320 nm was determined by spectral scanning. Protein contents were determined using HCl-treated parallel samples, washed and dissolved in Gn–HCl, using a BSA-standard curve in Gn–HCl and reading absorbance at 280 nm.

2.1.2. *Creatine Kinase.* This was measured by a colorimetric assay based on the reaction of phosphocreatine with ADP to form creatine, measured by formation of a chromogen in the presence of alpha-naphthol and diacetyl (Sigma kit 520).

2.1.3. *Other Enzymes.* Lactate dehydrogenase, aspartate aminotransferase, and gammaglutamyl transferase were measured by standard autoanalyzer methods at UC Davis Medical Center, Sacramento CA 95817, USA.

2.1.4. *Reagents.* Except for highly-purified human serum albumin certified for intravenous infusion (donated by Cutter Laboratories, Berkeley, CA, USA), all reagents were of the highest quality available from Sigma Chemical Corp. Creatine kinase was the rabbit muscle enzyme type C3755.

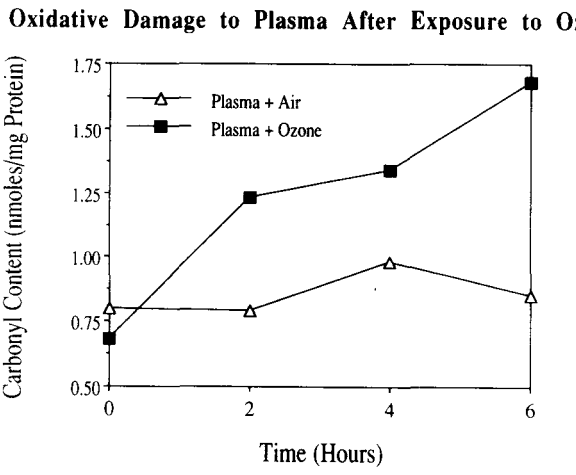

FIGURE 1 Oxidative damage to plasma after exposure to O_3. A representative experiment is shown.

3. RESULTS

Freshly-prepared human plasma was exposed to 16 ppm O_3 at 37°C. This high level of O_3 was used to accelerate the changes observed, since the surface/volume ratio of our system is much lower than that present in the respiratory tract.[11] Freshly-prepared plasma was found to contain 0.6–1.0 nmol/mg of protein carbonyls, and this value did not vary significantly with age in the subjects studied. No increase in carbonyl content was detected when plasma was incubated under air at 37°C for up to 6 h (Figure 1).

Exposure of plasma to O_3 (under controlled conditions of temperature, pH, CO_2 and humidity[24]) caused a gradual increase in protein carbonyl formation (Figure 1). Addition of mannitol (a hydroxyl radical scavenger) to a final concentration of 100 mM did not inhibit carbonyl formation.

Several plasma enzymes were assayed in all our O_3-exposed samples. Even 6 h of exposure to O_3 produced less than 10% loss of the activities of lactate dehydrogenase, alkaline phosphatase, gammaglutamyl transferase or aspartate transaminase.

Another important target for oxidative damage, plasma lipoproteins, were monitored for oxidative damage by shifts in electrophoretic mobility of the different plasma lipoprotein species.[27] These shifts were assessed by agarose gel electrophoresis perfomed as described in.[26] Exposure of plasma to ozone resulted in no discernible changes in either the staining intensity of the bands or electrophoretic mobility of any of the lipoprotein species visualized by lipoprotein electrophoresis.[26]

About 20% of plasma creatine kinase (CK) was lost after 4 h O_3 exposure. However, exposure of isolated CK to O_3 produced a rapid loss of activity (Table I).

These data suggest that the bulk of carbonyl formation is due to oxidation of proteins other than lipoproteins and enzymes. For example, preferential reaction of O_3 with albumin (a protein that may act as an antioxidant by being a "sacrificial target" of oxidative attack[28,29]) might protect the other proteins present. Hence studies on the reaction of O_3 with albumin were performed. Isolated commercial human plasma albumin was found to already contain significant amounts of protein carbonyls

TABLE I
Action of Ozone upon Isolated Creatine Phosphokinase. A representative experiment is shown 10 mg/ml of enzyme protein at pH 7.4 was exposed to O_3 as described in the Materials and Methods section and aliquots withdrawn for assay of enzyme activity at the times stated. Air exposed controls showed no loss of activity

Time of exposure (h)	% Of Initial Activity
0	100
1	57
2	32
3	7
4	1

(3–5 nmol/mg protein). Exposure to O_3 produced a rapid further increase in carbonyls (Figure 2). Addition of EDTA and desferrioxamine (to inhibit any oxidative modification dependent on traces of copper[30] or iron[31] ions contaminating the protein) had no effect (Figure 2), nor did reduced glutathione (GSH). Mannitol (100 mM) slightly decreased carbonyl formation (Figure 2).

4. DISCUSSION

Our data suggest that oxidative damage to proteins in human body fluids may occur as a result of exposure to O_3. The two isolated proteins that we studied showed much greater damage than did proteins within plasma. Thus ozonation of plasma caused only a slow loss of creatine kinase activity, whereas the isolated enzyme was almost

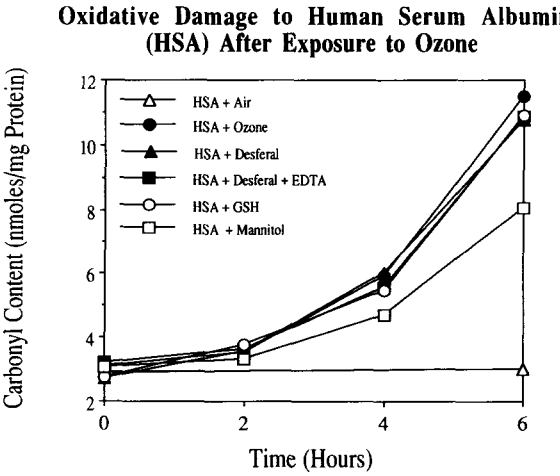

FIGURE 2 Oxidative damage to human serum albumin after exposure to O_3. A representative experiment is shown. Human serum albumin (70 mg/ml) was exposed to O_3 as described in the Materials and Methods section. Desferrioxamine, EDTA and GSH were added to final concentrations of 1 mM, mannitol to 100 mM. All reaction mixtures were exposed to ozone except the one listed first.

completely inactivated under the same conditions. Isolated albumin was also damaged faster than albumin in plasma. These differences are presumably due to the extensive antioxidant ability of plasma.[10] The hydroxyl radical scavenger mannitol had no effect on protein carbonyl formation in plasma, and only a small effect with isolated albumin. This suggests that formation of $^{\cdot}OH$ radicals from O_3 is not the mechanism of oxidative protein modification. It is more likely that O_3 oxidizes proteins directly: O_3 is known to attack histidine and tyrosine residues in proteins.[32,33]

The total antioxidant ability of RTLFs is probably less than that of blood plasma, and so proteins may be an important target of damage by inhaled O_3 in the respiratory tract, especially as albumin levels are low.[9] For example, surfactant protein A may be a target of injury by O_3.[34] Consideration of oxidative damage to proteins as well as to lipids is necessary in understanding the mechanisms of O_3 toxicity and in designing suitable protective agents.

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