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Interaction of nitrogen dioxide with human plasma

Antioxidant depletion and oxidative damage

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Nitrogen dioxide ($NO_2^{\bullet}$) is often present in inhaled air and may be generated in vivo from nitric oxide. Exposure of human blood plasma to $NO_2^{\bullet}$ caused rapid losses of ascorbic acid, uric acid and protein thiol groups, as well as lipid peroxidation and depletions of α-tocopherol, bilirubin and ubiquinol-10. No increase in protein carbonyls was detected. Supplementation of plasma with ascorbate decreased the rates of lipid peroxidation, α-tocopherol depletion and loss of uric acid. Uric acid supplementation decreased rates of lipid peroxidation but not the loss of α-tocopherol. We conclude that ascorbic acid, protein –SH groups, uric acid and α-tocopherol may be important agents protecting against $NO_2^{\bullet}$ in vivo. If these antioxidants are depleted, peroxidation of lipids occurs and might contribute to the toxicity of $NO_2^{\bullet}$.

Nitrogen dioxide; Lipid peroxidation; Ascorbic acid; Nitric oxide; Uric acid; Antioxidant

1. INTRODUCTION

Nitrogen dioxide ($NO_2^{\bullet}$) is a powerfully-oxidizing reactive brown gas with established pulmonary toxicity [1–6]. It can be present in homes with unvented heating sources at concentrations up to 2 ppm, in mainstream cigarette smoke (>50 ppm), in smog (up to 1 ppm) and in smoke from other burning organic materials [6–8]. $NO_2^{\bullet}$ might also be formed in vivo by reaction of O_2 with nitric oxide ($NO^{\bullet}$), which is generated (as the 'endothelium-derived relaxing factor') by vascular endothelial cells, and also by other cell types (reviewed in [9]). Nitric oxide might also react with superoxide ($O_2^{\bullet-}$) radical in vivo to give peroxynitrite, which decomposes to yield $NO_2^{\bullet}$ [10]. There is considerable current interest in the toxicity and mutagenicity of nitrogen oxides [1–8,11,12].

$NO_2^{\bullet}$ possesses an unpaired electron and is thus a free radical [13]. For example, $NO_2^{\bullet}$ can stimulate lipid peroxidation, and end-products of peroxidation are present in the lung lining fluids of rats after $NO_2^{\bullet}$ exposure [14–16]. $NO_2^{\bullet}$ can also damage proteins [6] and it is not yet clear which targets of attack by $NO_2^{\bullet}$ are the most important in vivo. Also, human body fluids and tissues contain a wide range of antioxidant defence systems (reviewed in [17,18]), and it seems likely that they might offer some protection against damage by $NO_2^{\bullet}$. Thus any $NO_2^{\bullet}$ formed from $NO^{\bullet}$ (by reaction with O_2) in vivo will presumably react with antioxidants in human extracellular fluids. Inhaled $NO_2^{\bullet}$ probably undergoes considerable interaction with antioxidants and other constituents of lung lining fluids [19]. However, the *relative* importance of different antioxidant defences in protecting against $NO_2^{\bullet}$ has not yet been established, although several authors have suggested that α-tocopherol may be important [20,21].

In the present paper, we have investigated the reaction of $NO_2^{\bullet}$ with antioxidants in freshly-prepared human plasma as a model for both situations [22], especially as methods for measuring antioxidants and oxidative damage in plasma are well-established [17,18,22,23]. We attempted to measure $NO_2^{\bullet}$-induced oxidative damage in several ways: loss of protein –SH groups, inactivation of enzymes, formation of protein carbonyls [24] and formation of lipid hydroperoxides, as measured by a specific HPLC-based chemiluminescence method [23,25].

2. EXPERIMENTAL

2.1. *Materials*

All reagents were of the highest quality available from Sigma. Blood from healthy adult male volunteers was collected into heparinized tubes and centrifuged at 1,000 × *g* and 4°C for 10 min to obtain plasma, which was used immediately.

2.2. *Exposure to $NO_2^{\bullet}$*

Plasma was diluted 80:20 with 0.5 M KH_2PO_4/K_2HPO_4 buffer, pH

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7.4, to maintain pH during exposures to $NO_2^{\bullet}$. A correction has been made for this dilution factor in the results presented. 3 ml aliquots were placed into 6 ml Falcon plates (3 cm diameter) in a controlled-environment exposure chamber (Primate Center, UC Davis) in which a steady concentration of 14 ppm of NO_2 was maintained. $NO_2^{\bullet}$ was generated by bubbling nitrogen through the liquid dimer, N_2O_4, held at 0°C [26]. The $NO_2^{\bullet}$ (in N_2) was conveyed to the mixing inlet of the chamber through stainless-steel lines. Flow was automatically controlled with a mass flow controller (Tylan General, Torrance, CA; Model FC-2900KZ). Chamber NO_2 concentration was monitored with a chemiluminescent oxide of nitrogen analyzer (Dasibi Corp., Glendale, CA; Model 2108). The monitor was calibrated by gas-phase titration of ozone with nitric oxide [27] and periodically checked against nitric oxide span gas of known concentration. Humidity was 40–60% and temperature 25–27°C. Plasma aliquots were also incubated for the same time periods in an identical chamber containing filtered air. At the end of the exposure, the plates were removed, the volume of plasma diluted back to 3 ml (if necessary) with distilled water and aliquots taken for the various assays.

2.3. *Assays of antioxidants*

Ascorbic acid, uric acid and lipid hydroperoxides were measured by HPLC [25], as was α-tocopherol [28]. Plasma sulfhydryl (–SH) groups were determined using Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoate), DTNB. Fifty μl of plasma was mixed with 1.0 ml of 0.1 M Tris buffer, containing 10 mM EDTA, pH 8.2. Any absorbance at 412 nm contributed by the plasma alone was subtracted from that obtained from the same sample 20 min after addition of 40 μl of 10 mM DTNB. A blank containing DTNB only was also included, and –SH concentration was calculated using a molar extinction coefficient of 13,600 $M^{-1} \cdot cm^{-1}$ at 412 nm. Protein carbonyls were measured spectrophotometrically by a modification [29] of the method in [24]. Total bilirubin was measured by using a Beckman Synchron CX4 autoanalyzer, as were the activities of lactate dehydrogenase, aspartate aminotransferase, γ-glutamyltransferase and creatine kinase.

In some experiments, the effect of $NO_2^{\bullet}$ exposure upon the electrophoretic migration of plasma lipoproteins was examined by agarose gel electrophoresis of $NO_2^{\bullet}$-exposed or air-exposed plasma, using Beckman Paragon Lipid gels run according to the manufacturer's recommendations. Gels were stained with fat 7B stain.

3. RESULTS

Freshly-prepared human plasma was exposed to 14 ppm $NO_2^{\bullet}$ for various times. The value of 14 ppm may seem high in relation to the concentrations of $NO_2^{\bullet}$ reported in polluted air (usually <1 ppm), but it was used because the surface:volume ratio in our Falcon plates is far lower than the enormous ratio found in the respiratory tract. In addition, such high $NO_2^{\bullet}$ concentrations are found in mainstream cigarette smoke and smoke from other burning organic materials [6–8] and might also be achieved in the vicinity of activated phagocytes [11,12]. We buffered the plasma against pH changes by adding a small amount of concentrated phosphate buffer.

Exposure of human plasma to $NO_2^{\bullet}$ produced a rapid loss of ascorbic acid, uric acid and protein –SH groups. In percentage terms, ascorbic acid was always lost at the greatest rate, with uric acid being lost slightly more slowly (reproducible in 8 different experiments utilizing plasma from 5 different donors). Fig. 1 shows a representative experiment. However, one must also consider the fact that the concentration of ascorbic acid in plasma is much lower than that of uric acid or protein –SH [17]. For example, after 1 h exposure to 14 ppm $NO_2^{\bullet}$, the following losses of antioxidants were observed (mean ± SD for 5 different plasma samples): ascorbate 49.9 ± 15.8 μM, uric acid 118 ± 41 μM and protein –SH groups 76 ± 48 μM. These results are corrected for the slow losses observed in the air-exposed controls (Fig. 1). Hence all three antioxidants appear to make a significant contribution to scavenging $NO_2^{\bullet}$, but uric acid seems to be quantitatively the most important.

Exposure of plasma to $NO_2^{\bullet}$ for 1 h led to the formation of lipid hydroperoxides and loss of α-tocopherol (Fig. 1D). Lipid peroxidation and loss of α-tocopherol accelerated after 3 h exposure, when ascorbate and uric acid had been almost completely lost. Cholesterol ester hydroperoxides were the major species formed (mean ± SD, 2.9 ± 1.8 μM for 3 different plasma samples after 6 h exposure to $NO_2^{\bullet}$), whereas levels of phospholipid hydroperoxides in the same experiments were only 0.03–0.18 μM. There was also loss of ubiquinol, another lipid-soluble antioxidant [30,31], in $NO_2^{\bullet}$-exposed plasma. For example, whereas 6 h of exposure to $NO_2^{\bullet}$ depleted α-tocopherol in human plasma from 40.6 ± 5.7 μM (mean ± SD, 5 different plasma samples) to 20.5 ± 4.0 μM, ubiquinol was completely lost (0.95 ± 0.05 μM to zero; $n = 3$).

The starting concentrations of ascorbic acid in plasma samples from different donors varied from 34 μM to 227 μM (the latter in a subject consuming vitamin C supplements). Inspection of the data suggested that the initial rate of uric acid depletion, but not that of protein –SH, was slower in plasmas containing the higher ascorbate concentrations. The effect of adding additional ascorbate to plasma upon the rates of oxidative damage and antioxidant depletion was therefore examined. Fig. 1 shows a representative experiment using 1 mM added ascorbate. Added ascorbate (1 mM) was oxidized fast by $NO_2^{\bullet}$, but some remained even after 5 h exposure (Fig. 1A). This ascorbate supplementation greatly slowed the $NO_2^{\bullet}$-induced loss of uric acid (Fig. 1C); it also reproducibly slowed the accumulation of lipid hydroperoxides and the loss of α-tocopherol (Fig. 1D). By contrast, ascorbate addition had no significant effect upon the loss of protein –SH groups (Fig. 1B). Lower ascorbate concentrations (0.2–0.5 mM) were also protective, but to a lesser extent (data not shown). The effects of supplementing plasma with uric acid and GSH were also examined. GSH, an antioxidant known to be present in lining fluids of the lower respiratory tract [32,33], did not inhibit lipid peroxidation or α-tocopherol depletion, even when added at 1 mM final concentrations. By contrast, adding an additional 0.5 mM uric acid to plasma decreased the concentration of total lipid hydroperoxides after exposure to $NO_2^{\bullet}$ for 6 h by a mean of 55% in two experiments. However,

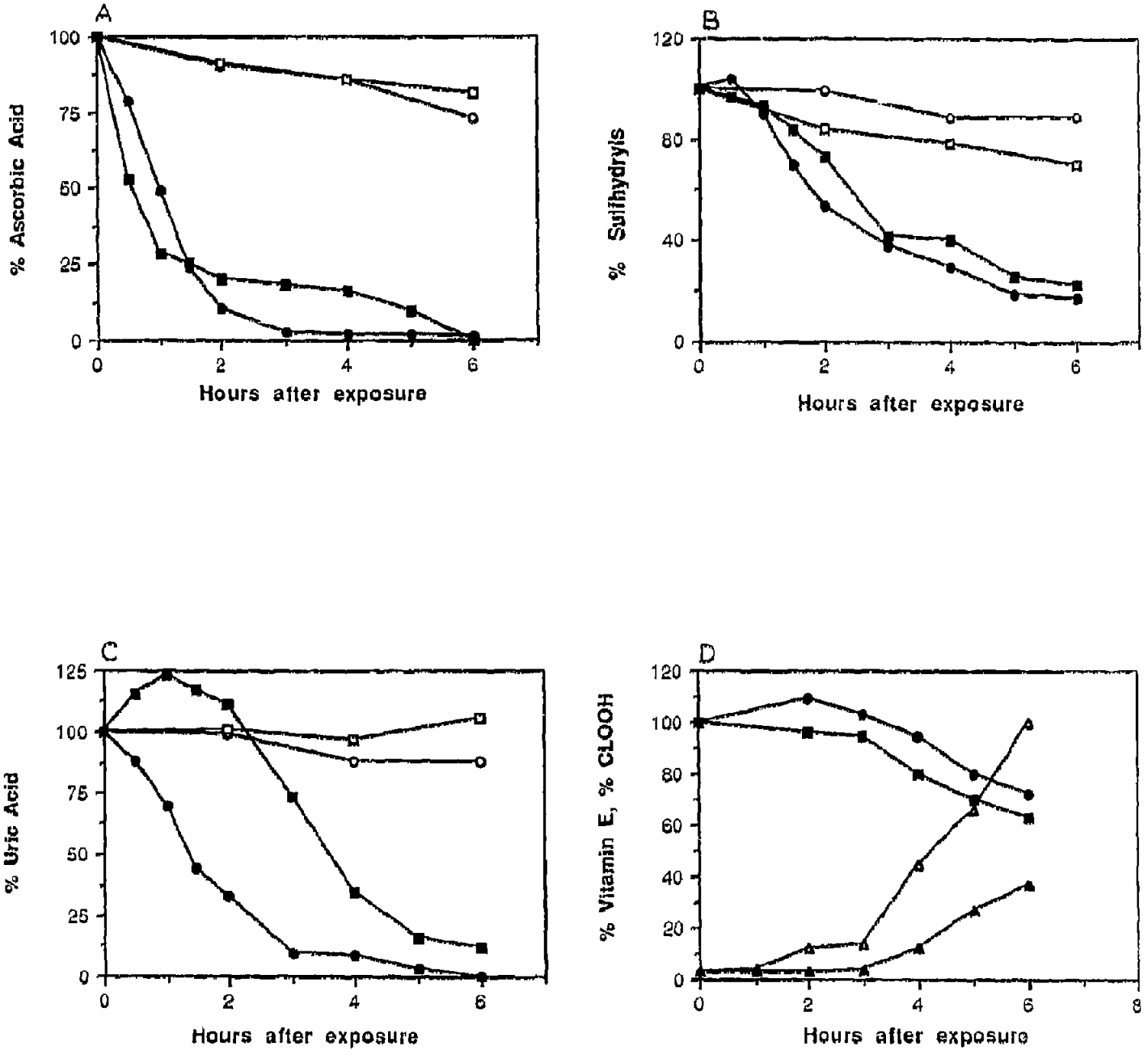

Fig. 1. Effects of exposing plasma to $NO_2^\bullet$. A representative experiment is shown in which all measurements were made on the same plasma samples. Each data point is the mean of at least duplicate determinations that differed by <10%. (A) Loss of ascorbic acid. (●) $NO_2^\bullet$; (■) $NO_2^\bullet$, plasma supplemented with an additional 1 mM ascorbic acid; (○) air-exposed control; (□) air control + ascorbate. (B) Sulfhydryl groups. (●) $NO_2^\bullet$; (■) $NO_2^\bullet$ + 1 mM ascorbate; (○) air control; (□) air + ascorbate. (C) Uric acid. (●) $NO_2^\bullet$; (■) $NO_2^\bullet$ + 1 mM ascorbate; (○) air control; (□) air + ascorbate. (D) Upper lines: α-tocopherol. (■) $NO_2^\bullet$-exposed; (●) $NO_2^\bullet$, plasma supplemented with an additional 1 mM ascorbic acid. Lower lines: cholesterol ester hydroperoxide (concentration at 6 h $NO_2^\bullet$ exposure denoted as 100%); (△) $NO_2^\bullet$-exposed; (▲) $NO_2^\bullet$ + 1 mM ascorbate. There was no significant formation of lipid hydroperoxide or depletion of α-tocopherol in air-exposed controls.

uric acid did not significantly affect the loss of α-tocopherol.

The carbonyl assay, which measures oxidative damage to several amino acid residues in proteins [24], was used to look for $NO_2^\bullet$-induced protein damage involving amino acid residues other than –SH. Reaction of $NO_2^\bullet$ with plasma led to the formation of a pale yellow-orange chromogen. When allowance was made for this, no increase in protein carbonyls was detected even after exposure of plasma to $NO_2^\bullet$ for 6 h. Similarly, this 6 h $NO_2^\bullet$ exposure produced no changes in the electrophoretic mobility of plasma low-density lipoproteins (LDL) when compared with air-exposed controls, nor did it change the activities of the enzymes lactate dehydrogenase, aspartate aminotransferase and gammaglutamyl transferase in plasma. Creatine kinase activity was slightly but reproducibly decreased (by 16 ± 5%, n=3) by 6 h of exposure to $NO_2^\bullet$. This exposure also

decreased total plasma bilirubin from 0.8 ± 0.4 mg/dl (n=3) to 0.3 ± 0.2 mg/dl.

4. DISCUSSION

In the present paper, we have studied the reaction of $NO_2^•$ with human plasma to gain information about the possible fates of $NO_2^•$ generated in vivo (e.g. from endogenous $NO^•$ [10]) and of $NO_2^•$ present in polluted inhaled air. Ascorbic acid, protein −SH groups and especially uric acid all appear to be important scavengers of $NO_2^•$, being rapidly depleted when plasma is exposed to this gas. Addition of extra ascorbate to plasma slowed loss of uric acid. Indeed, 1 mM ascorbate appeared to reproducibly increase plasma uric acid concentrations in the early stages of $NO_2^•$ exposure (by 11 ± 14%, n=5, after 30 min), for an unknown reason. This prevention of uric acid loss may occur because ascorbic acid is oxidized faster than uric acid (Fig. 1) and so 'spares' uric acid. An additional reason may be that reaction of $NO_2^•$ (a free radical) with uric acid produces a uric acid radical that can be re-converted to uric acid by ascorbate: such a 'recycling' of uric acid radicals has been demonstrated in vitro [34,35].

It is frequently suggested that $NO_2^•$ toxicity involves lipid peroxidation [1,5,6,14,15,21,36]. Our data are consistent with this view, in that exposure of plasma to 14 ppm $NO_2^•$ led to peroxidation of lipids and depletion of the lipid-soluble antioxidants ubiquinol-10 and α-tocopherol. Supplementing plasma with ascorbic acid slowed the loss of α-tocopherol. Ascorbate could act by preferentially scavenging $NO_2^•$, so protecting the lipid. Ascorbate also recycles α-tocopherol radicals back to α-tocopherol. Adding additional uric acid also slowed lipid peroxidation, perhaps by preferentially scavenging $NO_2^•$, but it did not preserve α-tocopherol. By contrast, apart from the rapid loss of protein −SH groups, little evidence for $NO_2^•$-induced oxidative damage to proteins was provided by measurements of enzyme inactivation or protein carbonyl formation.

Although we observed $NO_2^•$-induced lipid peroxidation in plasma under our experimental conditions, there were no changes in the electrophoretic mobility of LDL, despite the fact that peroxidation of LDL frequently causes such changes [37]. This could mean that insufficient peroxides were formed in the LDL, or that the peroxides were not decomposed into aldehydes that could modify apoprotein B and change the electrophoretic mobility of the LDL [37]. Further work is needed to investigate these possibilities.

It is interesting to compare the effects of $NO_2^•$ on plasma with those exerted by another common air-pollutant, the non-radical ozone (O_3). Uric acid, ascorbic acid and protein −SH are again important scavengers of O_3 in plasma [22]. Uric acid has been suggested to play a particularly-important scavenging role against O_3 in the human upper respiratory tract [22,38]: our data suggest that it may also be protective against $NO_2^•$. However, exposure to O_3 produced little lipid peroxidation in plasma in our previous experiments, whereas significant protein damage was detected by the carbonyl assay [22,39]. Hence, inhaled O_3 and $NO_2^•$ may attack different molecular targets in vivo. Perhaps this is relevant to suggestions that inhalation of mixtures of these gases produces synergistic biological damage [40].

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