Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion

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Endothelial cells and activated phagocytes produce both nitric oxide ($^{\cdot}NO$) and superoxide ($O_2^{\cdot-}$), which react to form peroxynitrite. Peroxynitrite has been suggested to be directly cytotoxic and also to decompose into other toxic species. In order to understand the consequences of peroxynitrite generation *in vivo*, we examined its reaction with human blood plasma. Peroxynitrite decreased the total peroxyl-trapping capacity of plasma. In terms of specific antioxidants, addition of peroxynitrite to plasma leads to rapid oxidation of ascorbic acid, uric acid and plasma SH groups. The oxidation of plasma SH groups was enhanced in dialysed plasma and returned to control levels by the addition of physiological levels of bicarbonate. Evidence was found for formation of nitro-adducts to aromatic side chains in plasma proteins by peroxynitrite. Peroxynitrite also leads to depletion of ubiquinol and formation of traces of lipid hydroperoxides in plasma, although α-tocopherol levels were only slightly decreased. Peroxynitrite formation in human body fluids is likely to cause antioxidant depletion and oxidative damage.

INTRODUCTION

Nitric oxide ($^{\cdot}NO$), the endothelium-derived relaxing factor, is synthesized and released by a variety of cells, with diverse biological functions [1]. In the vascular system, $^{\cdot}NO$ can be generated by endothelial cells and probably by neutrophils and macrophages. $^{\cdot}NO$ has diverse anti-inflammatory actions, such as inhibition of platelet aggregation and inhibition of neutrophil adhesion to the endothelium [1].

Endothelial cells and inflammatory cells are able to produce superoxide ($O_2^{\cdot-}$) [2,3], a species which reacts at a rate close to diffusion control with $^{\cdot}NO$ [4]. The reaction of $^{\cdot}NO$ with $O_2^{\cdot-}$ in biological systems was originally discovered by a potentiation of the vascular action of $^{\cdot}NO$ in the presence of antioxidants, especially superoxide dismutase [5]. However, it was recently shown that the reaction product of $^{\cdot}NO$ and $O_2^{\cdot-}$, peroxynitrite, is a potent oxidizing agent and may also serve as a powerful bactericidal and parasiticidal agent [6–14]. In neutral solutions, peroxynitrite rapidly decomposes to a range of products, possibly including nitronium ion (NO_2^+), nitrogen dioxide ($^{\cdot}NO_2$) and hydroxyl radical ($^{\cdot}OH$) [6–15]. $^{\cdot}NO_2$ is a species with well described ability to cause oxidative damage [16–18] and $^{\cdot}OH$ is one of the most reactive free radicals [19,20].

Peroxynitrite might also be an important mediator in oxidative actions originally attributed to other oxygen-derived species. It is capable of oxidizing protein and non-protein SH groups at a rate 1000-fold higher than that of H_2O_2 [9], inactivating α_1-antiproteinase by reaction with methionine [10], inducing lipid peroxidation [11,12] and reacting with aromatic amino acid side chains in proteins to form nitroadducts [13]. Reaction of peroxynitrite with bicarbonate strongly increases luminol chemiluminescence, indicating that this reaction may lead to secondary oxidants [14]. It has been suggested that oxidation of low-density lipoprotein (LDL) in the arterial walls by peroxynitrite may contribute to atherosclerosis [12,21,22].

When both $O_2^{\cdot-}$ and $^{\cdot}NO$ are generated, for example by endothelial cells or neutrophils, peroxynitrite may be generated in the extracellular environment, a system rich in antioxidant defences [23,24]. In order to investigate the consequences of this, we examined the reactions of peroxynitrite with plasma antioxidants. We measured its effect on both the total antioxidant capacity of plasma, using a modified total radical antioxidant parameter (TRAP) assay, and on specific antioxidants such as urate, ascorbate and thiols. Since bicarbonate, which can react with peroxynitrite [14], is present in plasma at high concentrations, we have also investigated the effects of this anion. We found that peroxynitrite causes depletion of important antioxidants and damage to proteins and lipids in plasma.

MATERIALS AND METHODS

Materials

BSA, essentially fatty-acid-free, and human serum albumin (HSA), GSH, dithiothreitol (DTT), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 2,4-dinitrophenylhydrazine (DNPH), 6-amino-2,3-dihydro-1,4-phthalazinedione (isoluminol), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), microperoxidase (type MP-11), DL-α-tocopherol, sodium *cis,cis*-Δ^9,Δ^{12}-octadecatrienoate (sodium linoleate), diethylenetriaminepenta-acetic acid (DTPA), DL-*o*-tyrosine, DL-*m*-tyrosine and DL-*p*-tyrosine, 3-nitro-L-tyrosine, DL-phenylalanine and 4-nitro-L-phenylalanine were purchased from Sigma (St. Louis, MO, U.S.A.). Uric acid, L-ascorbic acid and trichloroacetic acid were obtained from Fisher (Fair Lawn, NJ, U.S.A.). 9-Hydroperoxy-10,12-octadecadienoic acid was obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.), Thiolyte reagent, monobromobimane from Calbiochem

Abbreviations used: ABAP, 2,2′-azo-bis-2-amidinopropane hydrochloride; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DTPA, diethylenetriaminepenta-acetic acid; DTT, dithiothreitol; HSA, human serum albumin; LDL, low-density lipoprotein; TRAP, total radical antioxidant parameter.

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(San Diego, CA, U.S.A.), and 2,2′-azo-bis-2-amidinopropane hydrochloride (ABAP) from Polysciences (Warrington, PA, U.S.A.). All h.p.l.c. analytical columns were purchased from Waters (Milford, MA, U.S.A.). Microcon-3 concentrators were obtained from Amicon (Beverly, MA, U.S.A.). Peroxynitrite was synthesized in a quenched-flow reactor as described previously [6,25] and stored at −80 °C for less than 3 weeks. Peroxynitrite was used from the liquid layer formed by freeze fractionation around the crystals, which typically yields concentrations of 200–300 mM. Before each experiment, an aliquot was taken from the top layer and diluted in 1 M NaOH for quantification, by determining A_{302} using ϵ 1670 $M^{-1} \cdot cm^{-1}$. Dilution of peroxynitrite in phosphate buffer at pH 7.4 results in rapid decomposition of peroxynitrite, and generated a solution whose absorbance was used as a blank. All other chemicals used were of the highest purity commercially available.

Incubations with peroxynitrite

Blood was drawn from normolipidaemic volunteers (aged 30–57 years) into heparinized syringes and plasma was obtained after centrifugation for 10 min at 750 *g*. Several (8–10) plasma samples were used in the course of this study from different donors. Aliquots (5 ml) of freshly isolated plasma were incubated in test tubes at 37 °C. After 5 min preincubation, peroxynitrite was added as a drop (2–10 μl) from the stock solution (200–300 mM) and mixed rapidly. Plasma pH was measured after each incubation and found to change only slightly even after addition of peroxynitrite to a final concentration of 1 mM (from 7.9±0.1 to 8.2±0.2). When higher concentrations were used, plasma was buffered, with 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4. At various times after addition of peroxynitrite, samples were drawn from the incubations for immediate analysis of plasma antioxidants, protein SH groups, protein carbonyl groups and lipid hydroperoxides. Because of large variations in antioxidant content among the different plasma samples, antioxidant levels are expressed as a percentage of control samples. Plasma samples were stored at −20 °C after incubation with peroxynitrite for assay of plasma tyrosines and protein fluorescence. All other assays were performed immediately after incubation with peroxynitrite.

Peroxynitrite (0.25–1.0 mM) was also added to solutions of 40 mg/ml BSA or HSA in 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4, in order to investigate protein damage without possible interference with other plasma components. HSA with free thiol was prepared by treating freeze-dried HSA (60 mg/ml) in PBS with 10 mM DTT at 4 °C for 2 h and then dialysing against 2 × 100 vol. of nitrogen-saturated PBS. Free thiol was determined with Ellman's reagent as described [26].

Biochemical assays

Plasma ascorbate and urate levels were measured by h.p.l.c. with electrochemical detection [27]. α-Tocopherol was measured by h.p.l.c. with u.v. detection at 290 nm [28] and ubiquinol-10 and lipid hydroperoxides were measured using the same h.p.l.c. system with chemiluminescence detection after post-column reaction with a mixture of microperoxidase and isoluminol [29,30]. 9-Hydroperoxy-10,12-octadecadienoic acid was used as an external standard for lipid hydroperoxides [30].

Plasma SH groups (which originate predominantly from plasma proteins, especially albumin) were measured as A_{412} after reaction with DTNB, using ϵ 13600 $M^{-1} \cdot cm^{-1}$ [26]. GSH was analysed after pre-column derivatization with monobromobimane using h.p.l.c. with fluorescence detection (excitation λ 394 nm, emission λ 480 nm [31]).

Protein carbonyls were assayed after reaction with dinitrophenylhydrazine DNPH in 2 M HCl [32]. After reaction, protein was precipitated with 10% (w/v) trichloroacetic acid and the pellet was washed once with 10% trichloroacetic acid and three times with ethanol/ethyl acetate (1:1, v/v). The pellets were finally dissolved in 6 M guanidine hydrochloride and the A_{365} was measured. Parallel samples using 2 M HCl without DNPH were used as blanks, and for protein measurements at A_{280} [32].

Tyrosines and 3-nitrotyrosine were measured by h.p.l.c. using a 5 μm-pore-size Spherisorb ODS-2 column (4.6 mm × 250 mm; Alltech, Deerfield, IL, U.S.A.) with A_{274} detection as described previously [15,33]. Plasma samples (400 μl) were centrifuged (15000 rev./min) in Microcon-3 concentrators for 20 min at 4 °C, and 20 μl of the filtrate (usually ~ 70 μl) was injected into the h.p.l.c. system.

Tryptophan fluorescence (excitation λ 290 nm, emission λ 350 nm) was determined in BSA samples after 20-fold dilution [34]. Dityrosine formation was assayed by measuring fluorescence spectra after 10–20-fold dilution of the plasma or albumin in 50 mM phosphate buffer, pH 7.4 [35].

The TRAP value of plasma was measured using the inhibition of the peroxyl-radical-dependent oxidation of luminol [36]. The assay mixture contained air-saturated buffer: 10 mM NaH_2PO_4/Na_2HPO_4, 150 mM NaCl, pH 7.6, 35 mM ABAP, 0.87 mM luminol, 7 mM sodium linoleate and 10 μM DTPA. The assay mixture was pre-incubated for 30 min at 37 °C in the dark prior to the start of the experiment before being dispensed into the sample tubes for the luminometer (LKB model 1251). This resulted in stable signals. Under these conditions, up to 16 samples, each containing 575 μl of assay mixture, could be monitored during one experiment. The chemiluminescence of each sample was measured at 1–2 min intervals over a period of 5–10 min, after which samples of plasma or antioxidant resulted in a temporary inhibition of the signal ['lag phase' (T), as described in [36]]. After a stable and maximal signal had again been achieved, an aliquot of the antioxidant Trolox (5 nmol) was added to act as an internal standard for peroxyl-radical-scavenging activity [37]. The lag phase determined with Trolox was then used to calculate the TRAP, which was expressed as micromolar antioxidant equivalents according to the equation below, in which it is assumed that Trolox is capable of scavenging two peroxyl radicals per molecule in this system [37]:

$$\text{TRAP } (\mu M) = \frac{2(T_{plasma})[\text{Trolox added (nmol)}]}{(T_{Trolox})[\text{plasma added (ml)}]}$$

RESULTS

Effect of peroxynitrite on specific plasma antioxidants

Addition of peroxynitrite (0.25–1.0 mM) to plasma leads to a concentration-dependent decrease in plasma ascorbate and urate levels (Figure 1). In percentage terms, ascorbate was depleted the most, but it must be remembered that urate levels are much higher than those of ascorbate (see legend to Figure 1), and so urate was decreased to the greatest extent in quantitative terms. Figure 2 shows that the lipid-soluble antioxidant ubiquinol-10 was also depleted by peroxynitrite, again in a concentration-dependent manner. Plasma α-tocopherol, however, was only marginally decreased. These effects were observed 5 min after addition of peroxynitrite to plasma. Because peroxynitrite is partially protonated in neutral solution, and the protonated form decomposes very fast, rapid effects are expected to occur. Nevertheless, the more-stable peroxynitrite anion might exert its effects over a longer time period. Moreover, secondary products with a longer lifetime (such as lipid peroxyl radicals) might also

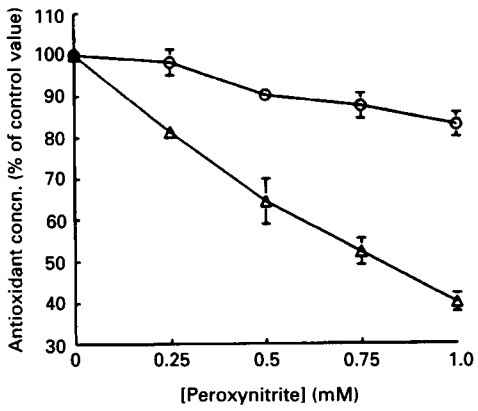

Figure 1 Effect of peroxynitrite on plasma ascorbate (△) and urate (○)

Plasma (5 ml) was incubated for 5 min at 37 °C after addition of peroxynitrite at various concentrations, and subsequently plasma levels of ascorbate and urate were quantified as described in the Materials and methods section. Data (means ± S.E.M. from 3–4 experiments using different plasma samples) are expressed as percentage of values measured in control incubations. Initial plasma concentrations were: 34.0–100.4 μM ascorbate and 154–283 μM urate.

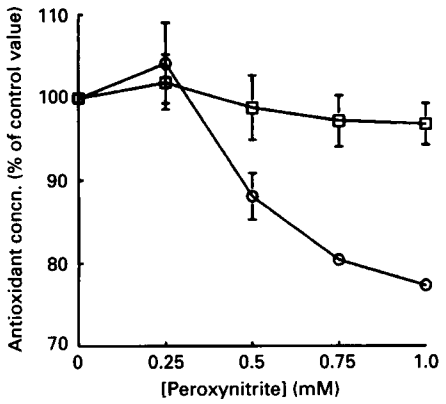

Figure 2 Effect of peroxynitrite on the lipid-soluble plasma antioxidants α-tocopherol (□) and ubiquinol-10 (○)

Plasma (5 ml) was incubated with various concentrations of peroxynitrite for 5 min at 37 °C, and subsequently α-tocopherol and ubiquinol-10 were measured as described in the Materials and methods section. Data (means ± S.E.M. from 3–4 experiments using different plasma samples) are expressed as percentage of values measured in control incubations.

contribute to the effects. Hence, we also investigated the various constituents 20 and 60 min after addition of peroxynitrite, and found that the losses of ascorbate and ubiquinol-10 increased significantly from 5 to 20 and 60 min after peroxynitrite addition (Table 1). However, the major effects were observed within the first 5 min of incubation. No further changes with time were observed for urate or α-tocopherol.

Because the rapid decomposition of peroxynitrite when added to plasma could conceivably limit its reaction with biological targets, we compared the addition of peroxynitrite as a single bolus with addition of the same amount in smaller aliquots (resulting in a final concentration of 0.1 mM) every 30 s. As demonstrated in Table 1, this increased oxidation of ascorbate and ubiquinol-10 slightly, but did not markedly affect other results.

Effects of peroxynitrite on the total peroxyl-radical-scavenging capacity of plasma

In order to assess the effect of peroxynitrite on the total peroxyl-radical-scavenging capacity of plasma, the TRAP value was determined before and after peroxynitrite treatment. The capacity of a sample of plasma to scavenge peroxyl radicals (the TRAP value) depends not only on the concentrations of the antioxidants

Table 2 TRAP values of some plasma antioxidants

TRAP values for the compounds shown were determined as described in [36]. Data are means ± S.D. (*n*). The value reported is an operational indication of the number of peroxyl radicals scavenged by each molecule of antioxidant.

Compound	TRAP (μM)/compound concn. (μM)
Urate	2.50 ± 0.25 (6)
Ascorbate	1.49 ± 0.23 (4)
α-Tocopherol	2.25 ± 0.13 (7)
SH as GSH	0.29 ± 0.02 (10)
SH as reduced HSA	0.26 ± 0.03 (3)

Table 1 Concentration- and time-dependent effect of peroxynitrite on plasma constituents

Plasma was incubated for 5, 20 or 60 min at 37 °C after addition of peroxynitrite at the indicated concentrations. Parameters were measured as described in the Materials and methods section. Antioxidant levels in peroxynitrite-treated plasma were expressed in relation to the levels in time-corresponding control incubations (without peroxynitrite). Control levels of ascorbate decreased by 8 ± 7% and ubiquinol-10 (Ub-10) levels decreased by 29 ± 8% after 60 min incubation. Urate, α-tocopherol (α-Toc) and plasma SH levels did not change significantly in control incubations. Peroxynitrite was added either as one bolus or as repeated smaller doses (e.g. 5 × 0.1 mM reflects 5 additions of a dose resulting in a final concentration of 0.1 mM every 30 s). The data represent means ± S.E.M., $n = 3$–4.

	Antioxidant remaining (%)				
Addition of peroxynitrite	Ascorbate	Urate	α-Toc	Ub-10	Plasma SH
0.5 mM, 5 min	64.4 ± 9.4	90.2 ± 2.1	98.8 ± 6.7	88.1 ± 4.9	91.2 ± 5.3
5 × 0.1 mM, 5 min	58.3 ± 5.1	93.6 ± 4.2	98.0 ± 6.7	77.8 ± 6.5	86.7 ± 7.1
1 mM, 5 min	40.1 ± 4.0	82.9 ± 5.0	96.8 ± 4.3	77.3 ± 1.0	78.6 ± 4.8
10 × 0.1 mM, 5 min	30.4 ± 4.8	84.1 ± 10.9	91.6 ± 4.3	50.2 ± 10.2	69.6 ± 7.1
1 mM, 20 min	34.2 ± 2.7	81.6 ± 4.8	91.1 ± 5.2	64.1 ± 7.9	76.2 ± 12.6
1 mM, 60 min	25.1 ± 1.0	80.1 ± 4.4	93.3 ± 3.4	59.1 ± 9.5	70.3 ± 1.7

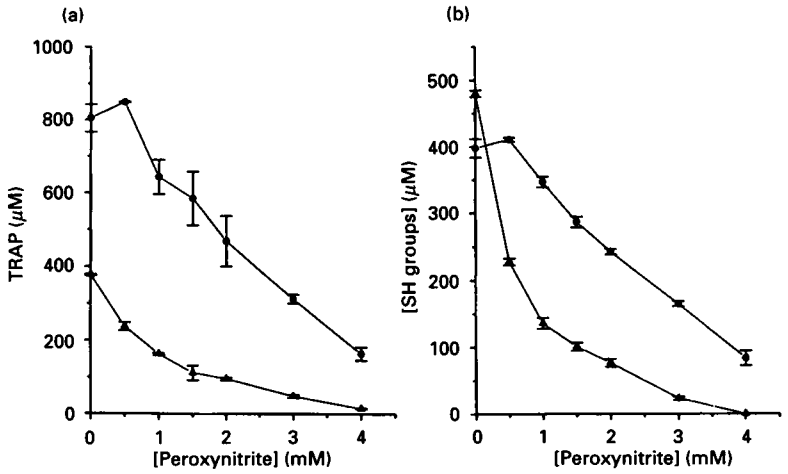

Figure 3 Treatment of dialysed and normal plasma with peroxynitrite and measurements of (a) TRAP value and (b) thiol content

Samples (5–10 ml) of human plasma were used as prepared (●) or dialysed (▲) against 2 × 500 ml of PBS and then buffered with 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4. Peroxynitrite was added to give the final concentration indicated and, after incubation for 20 min at room temperature, samples were placed on ice and the TRAP value and thiol content were determined as described in the Materials and methods section. The data represent the means ± S.D. of three independent determinations using the same samples of plasma.

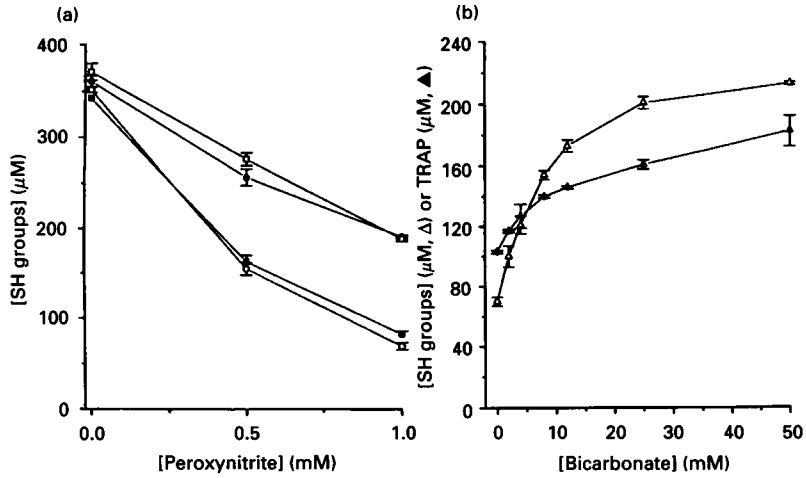

Figure 4 Effect of ascorbate, urate and bicarbonate on the depletion of thiols in dialysed plasma

Samples (5–10 ml) of human plasma were used as prepared or dialysed against 2 × 500 ml of PBS and then buffered with 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4. (**a**) Peroxynitrite was added at the final concentrations shown to dialysed plasma (○), dialysed plasma + 25 mM bicarbonate (□), dialysed plasma + 500 μM urate + 100 μM ascorbate (■) and undialysed plasma (●). (**b**) Peroxynitrite was added to samples of dialysed plasma at a final concentration of 1 mM in the presence of the concentrations of bicarbonate indicated, after which the TRAP value (▲) and thiol content (△) were determined. The data represent means ± S.D. of three independent determinations using the same sample of plasma.

but also on their relative radical-trapping efficiencies [36,37]. The apparent value for the latter depends on the assay conditions, but in the ABAP/luminol system that we used to measure the TRAP value of whole plasma [36], it is determined by measuring the slope of the (linear) relationship between the TRAP value and the nominal concentration of any particular antioxidant. The soluble α-tocopherol derivative Trolox [37] is used as a standard, and it is assumed that it scavenges 2 peroxyl radicals per molecule. In Table 2 we report the values for a range of antioxidants found in plasma determined from the TRAP assay using the inhibitions of the peroxyl-radical-dependent oxidation of luminol [36,37]. Our values are in good agreement with those of Wayner et al. [37], who determined TRAP in plasma using the inhibition of the oxygen-dependent peroxidation of linoleic acid.

The mixing of plasma with increasing concentrations of peroxynitrite results in a progressive depletion of the TRAP value, indicating that the ability of the plasma to scavenge peroxyl radicals has diminished (Figure 3a). After dialysis of the plasma to remove low-molecular-mass soluble antioxidants such as ascorbate and urate, peroxynitrite was more effective in depleting TRAP (Figure 3a), suggesting that one or more dialysable component(s) in plasma react with peroxynitrite, consistent with the results in Table 1.

Effect of peroxynitrite on protein thiols

The thiols in plasma, particularly the cysteine residues on serum albumin, make a significant contribution to the peroxyl-radical-

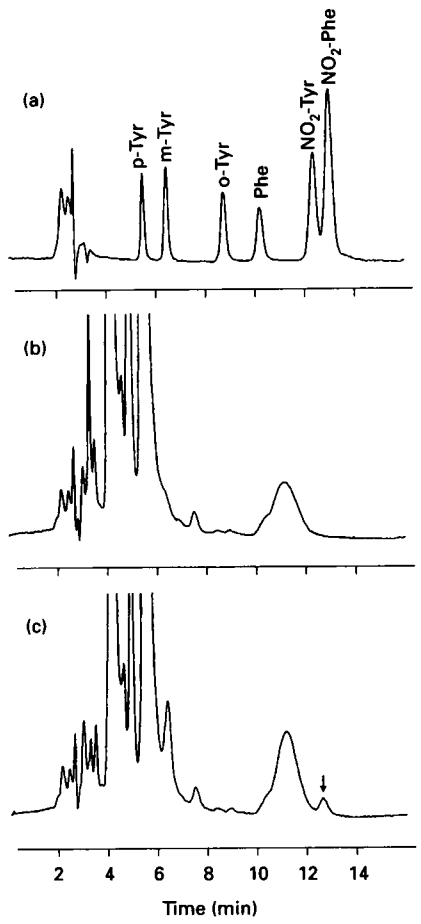

Figure 5 Formation of 3-nitrotyrosine in human plasma by peroxynitrite

Plasma was supplemented with 1 mM DL-tyrosine and subsequently treated with peroxynitrite as described in the Materials and methods section. Samples were analysed for 3-nitrotyrosine by h.p.l.c. as described [15]. The Figures show typical chromatograms of (**a**) a mixture of *p*-tyrosine, *m*-tyrosine, *o*-tyrosine, phenylalanine, 3-nitrotyrosine and 4-nitrophenylalanine, (**b**) plasma supplemented with 1 mM *p*-tyrosine, and (**c**) the same plasma sample after incubation with 1 mM peroxynitrite, showing formation of 3-nitrotyrosine (see the arrow).

scavenging capacity of plasma [37]. Addition of peroxynitrite to plasma causes loss of plasma SH groups (Figure 3b). Decreases in the number of plasma SH groups by peroxynitrite were found to be slightly more pronounced at longer incubation periods (Table 1), although the major effect was observed after 5 min. Similarly, repeated smaller doses of peroxynitrite induced a slightly larger loss of plasma SH groups than one large single dose.

Under the same conditions used to measure the depletion of TRAP in normal and dialysed plasma, the free thiol content was measured (Figure 3b). The SH depletion is linear with peroxynitrite concentration in control plasma but becomes exponential after dialysis. One possible explanation for this result is that the urate and ascorbate spare the albumin thiol from reaction with peroxynitrite. The plasma thiols are present at a high concentration (400–500 μM) and it should be expected, therefore, that depletion of TRAP is strongly associated with loss of thiols; indeed, this result is evident from a comparison of Figures 3(a) and 3(b).

In order to determine if urate and ascorbate can protect thiol groups from oxidation by peroxynitrite, they were added in combination to dialysed samples of plasma and the thiol content measured after addition of peroxynitrite (Figure 4a). However, there was little or no effect of plasma antioxidants on the depletion of plasma thiol by peroxynitrite. Because peroxynitrite has been reported to react with bicarbonate [14], which is present in high amounts of plasma (25 mM), the effect of bicarbonate on peroxynitrite-induced loss of protein SH groups was studied. To test the effect of bicarbonate, which was removed from plasma by dialysis, it was added back to the dialysed plasma and the thiols remaining after exposure to peroxynitrite were determined. Interestingly, this resulted in a slowing of the concentration-dependent depletion of thiols by peroxynitrite to the same rate found with the same samples of undialysed plasma (Figure 4a). The concentration dependence of this effect of bicarbonate on both the thiol content and TRAP value was explored using 1 mM peroxynitrite (Figure 4b). It is clear that as well as protecting the free thiol from oxidation, plasma concentrations of bicarbonate maintain the ability of the sample to scavenge peroxyl radicals.

Incubation of BSA (40 mg/ml in 50 mM KH_2PO_4/K_2HPO_4 buffer) with peroxynitrite (up to 1 mM) caused a rapid dose-dependent loss of protein SH groups. This loss appeared unrelated to metal-ion-catalysed reactions, as addition of 1 mM EDTA did not significantly alter plasma SH loss by peroxynitrite. When 25 mM bicarbonate was present in the buffer, the loss of protein SH groups in BSA caused by 1 mM peroxynitrite was comparable with the loss observed in plasma, confirming the findings with dialysed plasma. Similar effects of bicarbonate were found with HSA or GSH (results not shown).

The reversibility of the reaction of peroxynitrite with thiols was determined by reacting peroxynitrite with GSH in 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.4, and treating the reaction mixture with a 10-fold excess of DTT for 2 h. When GSH was present in excess over peroxynitrite, oxidation of GSH was completely reversible by DTT, suggesting that peroxynitrite oxidizes GSH to the disulphide. However, when peroxynitrite was added in excess over GSH (1 mM peroxynitrite added to 100 μM GSH), resulting in complete oxidation of GSH, only $\sim 20\%$ of the GSH was recovered after DTT treatment, indicating that, in this case, GSH is further oxidized by peroxynitrite, for example to sulphenic acid.

Effects on other amino acids

Oxidative modification of proteins often leads to the formation of protein carbonyls [32]. Peroxynitrite was found to react with proteins to form a chromogen, which caused an increased absorbance in the control measurements for protein carbonyls. When corrections were made for this, no significant formation of protein carbonyls could be detected after addition of up to 1 mM of peroxynitrite to plasma or to 40 mg/ml BSA in 50 mM KH_2PO_4/K_2HPO_4 buffer. The chromogen formed in plasma or albumin upon peroxynitrite addition is likely to be a nitration product of tyrosine or phenylalanine residues in proteins [13,15]. Consistent with this, spectral analysis of HSA or BSA treated with peroxynitrite showed that the absorbance is reproducibly increased between 300 and 450 nm compared with untreated albumin. At peroxynitrite concentrations higher than 1 mM, a distinct absorbance peak ($\lambda_{max.}$ 429 nm, which shifted to 356 nm on acidification) became visible, characteristic of the presence of nitrotyrosine [13–15].

Plasma levels of free tyrosine (40–70 μM) were only marginally decreased by 1 mM peroxynitrite (to $93.2 \pm 3.3\%$; mean $\pm$ S.D., $n = 4$) and h.p.l.c. analysis did not show any detectable formation of nitro-adducts. When plasma was supplemented with 1 mM

tyrosine prior to peroxynitrite addition, trace amounts of 3-nitrotyrosine were detected (0.7 ± 0.4 μM; $n = 3$). Figure 5 shows a typical chromatogram demonstrating the formation of 3-nitrotyrosine in tyrosine-supplemented plasma after peroxynitrite addition.

We have observed formation of dityrosine upon treatment of tyrosine solutions with peroxynitrite [15], but this was not observed in whole plasma or in BSA. Other possible targets for peroxynitrite include tryptophan residues in proteins. However, tryptophan fluorescence in BSA was not significantly affected after incubation with 1 mM peroxynitrite.

Effects on plasma lipids

Low levels of cholesteryl ester hydroperoxides were detected after addition of 1 mM peroxynitrite to plasma (control values 0.6 ± 1.0 nM, 5 min after peroxynitrite addition 4.2 ± 2.3 nM; $n = 4$; $p < 0.05$ using Student's unpaired t test), indicating that peroxynitrite induces oxidation of plasma lipids. A similar increase in cholesteryl ester hydroperoxides was observed after 10 sequential additions of 0.1 mM peroxynitrite (to 3.7 ± 0.8 nM, $n = 3$).

DISCUSSION

Endothelial cells and phagocytes can release both $^{\bullet}NO$ and $O_2^{\bullet -}$. These radicals can react at an almost diffusion-controlled rate to form peroxynitrite [4]. Peroxynitrite and products derived from it could serve as a powerful oxidizing system, causing tissue damage [6–14].

Peroxynitrite has a pK_a of 6.8 at 37 °C [9], and hence less than 20% of it will be protonated at pH 7.4. However, the protonated form of peroxynitrite is very unstable (half-life < 1 s), and decomposes to give various products, possibly including $^{\bullet}OH$, $^{\bullet}NO_2$ and NO_2^+, all highly reactive species. The peroxynitrite anion itself can also oxidize biological components [9,10].

When peroxynitrite is generated extracellularly, it may react with a vast array of biomolecules. In this study, addition of peroxynitrite to plasma depleted total peroxyl-radical-scavenging activity, and led to oxidation of the water-soluble antioxidants ascorbic acid, uric acid and plasma SH groups. Despite the fact that antioxidants were not completely depleted, trace amounts of lipid hydroperoxides were detected after addition of peroxynitrite, indicating oxidation of lipids. Initiation of lipid peroxidation is also suggested by the fall in ubiquinol-10, which disappears in plasma lipoproteins subjected to oxidative stress much faster than does α-tocopherol [38–40]. This peroxidation may well involve plasma LDL, peroxidation of which contributes to atherosclerosis [12,21,22]. Moreover, indirect evidence for nitration of tyrosine residues in proteins was observed, as increased absorbance between 350 and 450 nm in plasma or albumin after addition of peroxynitrite. Free 3-nitrotyrosine was also detected after peroxynitrite treatment of plasma supplemented with 1 mM tyrosine. Oxidative protein damage was not observed as protein carbonyls or as altered tryptophan fluorescence.

The concentrations of peroxynitrite used in this study (0.25–1.0 mM) may appear to be very high. However, the short half-life of this species at physiological pH (1–2 s) results in much lower effective exposures. For example, it has been calculated that 100 μM peroxynitrite is only equivalent to a steady-state concentration of 2.8 μM for 1 min [9]. The fact that repeated small doses of peroxynitrite resulted in similar oxidative damage compared with one large dose (Table 1) also indicates that it is not the absolute concentration but rather the effective exposure (concentration × exposure time) that determines the toxicity. Although the concentrations of peroxynitrite which may be achieved *in vivo* are currently unknown, it has been shown that activated murine macrophages may generate up to 0.11 nmol·$(10^6$ cells$)^{-1}$·min^{-1}, which may result in local concentrations of as much as 0.5–1.0 mM·min^{-1} [41]. Recently, stimulated human neutrophils were reported to produce similar amounts of peroxynitrite [42].

Possible mechanisms

Peroxynitrite could oxidize plasma constituents by multiple mechanisms. First, the protonated form, peroxynitrous acid, decomposes rapidly to form a range of products, probably including $^{\bullet}OH$ and $^{\bullet}NO_2$ [6,15]. Second, peroxynitrite itself has powerful oxidizing capacity (e.g. against methionine or cysteine) [9,10]. Third, metal ions might catalyse heterolytic scission of the O–O bond in peroxynitrite, resulting in the formation of a NO_2^+-like species, which is a powerful nitrating agent [19,43].

The lipid peroxidation caused by peroxynitrite might involve $^{\bullet}OH$ [19] and/or $^{\bullet}NO_2$ [16,18]. Although only low levels of lipid hydroperoxides were generated, peroxidation was seen even when substantial plasma antioxidant activity was still present. Thus peroxynitrite generated in, for example, an endothelial/neutrophil interface might cause significant lipid peroxidation, which could be an important mechanism of phagocyte-induced endothelial injury. Treatment of isolated LDLs with peroxynitrite has been found to induce rapid peroxidation and α-tocopherol depletion [12,21]. In this study, addition of peroxynitrite to plasma only marginally affected plasma α-tocopherol levels, possibly because it was recycled by ascorbic acid and/or ubiquinol-10 (which were significantly decreased, but not completely depleted) [38–40]. The mechanism of reaction of peroxynitrite with ascorbate or urate is currently unknown, but it seems likely that at physiological pH, when both ascorbate and urate are present as anions, both antioxidants react with decomposition products of peroxynitrous acid (such as $^{\bullet}OH$ or $^{\bullet}NO_2$), rather than with the peroxynitrite anion.

The peroxynitrite anion appears to react directly with SH groups [9,23]. Reaction of peroxynitrite with an excess of GSH was reversed by DTT addition. However, when peroxynitrite was added in excess over GSH, further oxidation (e.g. to sulphenic acids) probably occurs.

Peroxynitrite has also been demonstrated to react with bicarbonate [14], in a reaction that leads to increased luminol chemiluminescence, and this might affect oxidation of other targets in plasma. As shown in this study, peroxynitrite-induced protein SH oxidation in plasma or in isolated albumin is decreased in the presence of bicarbonate. This has also been demonstrated to inhibit the bactericidal activity of peroxynitrite [7]. Our data indicate that reaction of peroxynitrite with bicarbonate yields a product less reactive to SH groups. The fact that ascorbate and urate did not significantly attenuate thiol oxidation in plasma by peroxynitrite also suggests that the peroxynitrite anion does not react at competitive rates with ascorbate and urate.

Nitration of (protein-associated) tyrosine by peroxynitrite has been postulated to occur via a metal-catalysed formation of a NO_2^+-like intermediate [13,43]. However, the availability of transition metal ions to catalyse free-radical reactions in plasma is virtually zero [19], although it was recently demonstrated that peroxynitrite is able to release copper from caeruloplasmin [44]. It is also conceivable that peroxynitrous acid, upon decompo-

sition or isomerization, is able to abstract a hydrogen from tyrosine, which then recombines with the simultaneously generated $^{\cdot}NO_2$, similar to nitrotyrosine formation by $^{\cdot}NO_2$ alone [17,45]. Recently, evidence for nitration of protein-associated tyrosine was also demonstrated after treatment of surfactant protein A with peroxynitrite [46]. Using polyclonal and monoclonal antibodies, nitrotyrosine was recently found to be present in human atherosclerotic lesions [47].

Conclusions

Peroxynitrite has been demonstrated to react with many biomolecules. When peroxynitrite is added to plasma, rapid losses of plasma ascorbic acid, uric acid and SH groups, important antioxidant defences in plasma [23,48], are observed. In relative terms, ascorbate is affected to the largest extent. Nevertheless, despite the presence of multiple plasma antioxidants, peroxynitrite is still able to oxidize lipids and nitrate protein tyrosine residues, suggesting that even the powerful plasma antioxidant systems are not capable of fully preventing reaction of peroxynitrite with other constituents. Generation of large amounts of peroxynitrite in localized regions, for example when phagocytes adhere to endothelium or in atherosclerotic lesions, could therefore cause significant oxidative damage.

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