One-electron oxidation pathway of peroxynitrite decomposition in human blood plasma: evidence for the formation of protein tryptophan-centred radicals

Donatella PIETRAFORTE and Maurizio MINETTI*

Laboratorio di Biologia Cellulare, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy

Exposure of human blood plasma to peroxynitrite in the presence of 3,5-dibromo-4-nitrosobenzenesulphonic acid (DBNBS) resulted in the trapping of a strongly immobilized nitroxide radical adduct. The adduct was due to protein-centred radicals derived not only from serum albumin but also from other major plasma proteins (fibrinogen, IgG, α_1-antitrypsin and transferrin). Urate significantly protected plasma from the peroxynitrite-induced DBNBS–plasma protein adduct, whereas ascorbate and glutathione were protective at concentrations exceeding those usually found in plasma. Alkylation of plasma −SH groups did not affect the intensity of DBNBS–plasma protein adduct, whereas bicarbonate increased its formation, thus showing a pro-oxidant effect. The DBNBS–plasma protein adduct provided little structural information, but subsequent non-specific-protease treatment resulted in the detection of an isotropic three-line spectrum, indicating the trapping of radicals centred on a tertiary carbon. The nitrogen hyperfine coupling constant of this adduct and its superhyperfine structure were similar to those of DBNBS–tryptophan peptides with the α-amino group of tryptophan linked in the amide bond, consistent with a radical adduct formed at C-3 of the indole ring of tryptophan-containing peptides. DBNBS was unable to trap radicals derived from peroxynitrite-treated tyrosine or tyrosine-containing peptides. Methionine treated with peroxynitrite resulted in the trapping of at least two DBNBS–methionine adducts with hyperfine structures different from that of protease-treated DBNBS–plasma proteins. These results demonstrate that peroxynitrite induced in blood plasma the formation of protein radicals centred on tryptophan residues and underline the relevance of the one-electron oxidation pathway of peroxynitrite decomposition in biological fluids.

INTRODUCTION

Nitric oxide (NO•) is a free-radical messenger molecule synthesized at low constant levels and involved in several physiological processes such as the regulation of blood pressure, platelet adhesion, neutrophil aggregation and synaptic plasticity [1–2]. NO• can, moreover, be synthesized in large amounts in several pathological situations such as inflammatory states and circulatory shock. Under these conditions, NO• may alleviate tissue injury by increasing blood flow through vasodilatation and by inhibiting neutrophil adherence and platelet aggregation [1–3]. Despite these physiological effects, excessive amounts of NO• are detrimental to the organism. This is especially evident in endotoxic shock, where the NO• concentration widely exceeds normal physiological levels.

NO• is not a strong oxidant of most types of organic compound, but secondary reactions with transition metals or reactive oxygen species greatly increase its reactivity. The role of NO• in modulating cellular injury is still unclear, and the toxicity of this radical molecule may largely depend on subsequent reactions with reactive oxygen species [4–6].

The toxicity of NO• is significantly enhanced in the presence of superoxide anion radical ($O_2^{-\bullet}$). These two radicals react at near the diffusion limit ($k = 6.7 \times 10^9$ $M^{-1} \cdot s^{-1}$) [7] to yield the strong oxidant peroxynitrite anion ($ONOO^-$). While the peroxynitrite anion is relatively stable, its protonated form, peroxynitrous acid (HOONO, $pK_a = 6.8$) [4,8], decomposes spontaneously to form nitrates ($t_\frac{1}{2} < 1$ s at pH 7). It has been suggested that the decomposition process proceeds through a rate-limiting isomerization reaction that yields a potent reactive intermediate capable of hydroxylating organic substrates [5,9,10]. Although the reactive intermediate of peroxynitrous acid decomposition is believed to be responsible for most of the oxidative reactions of peroxynitrite, the anion can also oxidize biological substrates such as thiols [4]. In the present paper we use the term 'peroxynitrite' to refer to all the possible forms of peroxynitrite anion and peroxynitrous acid.

In biological systems, peroxynitrite can react with most targets, including proteins [4,11,12], lipids [13], carbohydrates [14] and DNA [15]. This wide range of biological targets makes peroxynitrite a potent tissue-damaging species implicated in several pathological situations [16–19]. In its reaction with proteins, peroxynitrite has been shown to attack preferentially cysteine [4], tyrosine [20], tryptophan [12] and methionine residues [11].

In human blood plasma, peroxynitrite induces the depletion of low-molecular-mass antioxidants, the oxidation of −SH groups, and lipid peroxidation [21]. Moreover, direct ESR and spin-trapping studies with nitrone spin traps revealed the formation of protein thiyl radicals and a radical derived from uric acid [22].

In the present study we examined the oxidative damage produced by peroxynitrite on human blood plasma as detected by the ESR spin-trapping technique using a different spin trap,

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the nitroso-based 3,5-dibromo-4-nitrosobenzenesulphonic acid (DBNBS). This spin trap revealed the formation of a strongly immobilized adduct arising from plasma proteins. Urate significantly protected plasma from peroxynitrite-induced protein oxidations, whereas bicarbonate showed a pro-oxidant effect. The spin-trapping results indicated that the radicals trapped on plasma proteins by DBNBS were centred on tryptophan residues.

MATERIALS AND METHODS

Materials

DBNBS was obtained from OMRF Spin Trap Source (Oklahoma City, OK, U.S.A.). Tryptophan, Gly-Trp, Trp-Gly and Gly-Gly-Trp-Ala were from Serva Feinbiochemia G.m.b.H. (Heidelberg, Germany), and Pronase was from Boehringer-Mannheim G.m.b.H. (Mannheim, Germany). All other reagents, including melittin, fibrinogen type I, IgG, human plasma α_1-antitrypsin, apo-(human transferrin), human serum albumin, ascorbate oxidase, uricase type V from porcine liver, proteinase K, and Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu were purchased from Sigma Biochemicals (St. Louis, MO, U.S.A.).

Synthesis of peroxynitrite

Peroxynitrite was synthesized by reacting nitrite with acidified H_2O_2 as described by Radi et al. [4] and treated with manganese dioxide (30 min, 4 °C) to eliminate excess H_2O_2. Peroxynitrite solution forms a yellow top layer by freeze fractionation. The top layer typically contained 150–200 mM peroxynitrite as determined spectrophotometrically at 302 nm in 1.5 M NaOH ($\epsilon_{302} = 1670$ M$^{-1} \cdot$cm^{-1}). The solution of peroxynitrite was stored for 1–2 weeks (-20 °C) with negligible changes in its concentration ($\leqslant 5 \%$).

Treatment with peroxynitrite

Fresh heparinized human blood was obtained from healthy subjects with their informed consent. Plasma was separated by blood centrifugation for 5 min at 1000 g. To remove low-molecular-mass compounds, plasma was submitted to extensive dialysis (molecular-mass cut-off 10 000) against 0.15 M NaCl at 4 °C. To obtain plasma low-molecular-mass compounds, plasma was ultrafiltered with Microcon concentrators (molecular-mass cut-off 3000; Amicon Inc., Beverly, MA, U.S.A.) for 15 min at 14 000 g. The filtrate (referred to as 'plasma ultrafiltrate') was used for analysis. Reconstitution of plasma with urate was performed by adding a small volume of urate dissolved at 10 mM in 0.1 M NaOH and correcting for the pH change with HCl. Peroxynitrite (0–5 mM) was added as a bolus from the stock solution to aliquots of plasma buffered with an equal volume of phosphate buffer, pH 7.1 (150 mM final phosphate concentration) and immediately mixed. To avoid metal-catalysed reactions [23], buffers were treated extensively with Chelex 100 (Bio-Rad Laboratories, Richmond, CA, U.S.A.), and all samples contained 0.1 mM diethylenetriaminepenta-acetic acid (DTPA). The pH of samples was measured after each incubation and found to change only slightly after the addition of peroxynitrite (pH 7.2–7.5). When peroxynitrite was used at 20 mM, it was added stepwise as four boluses and 1 mM DBNBS was added before each addition. To avoid pH changes due to the alkaline solution of peroxynitrite, an appropriate amount of HCl was added. Decomposed peroxynitrite was obtained by adding peroxynitrite to phosphate buffer/DTPA (pH 7.1) for 5 min at room temperature before the addition of an equal volume of plasma (reversed order of addition).

Thiol alkylation and enzymic treatments of plasma

Thiol groups were chemically modified by incubation with 5 mM N-ethylmaleimide (NEM) for 30 min at 37 °C. Plasma was depleted of ascorbate or urate by incubation for 30 min at 37 °C with ascorbate oxidase (20 munits/ml) or uricase (100 munits/ml). Protease treatment of DBNBS-labelled proteins was performed by adding Pronase (4 mg/ml) and proteinase K (5 mg/ml) to plasma.

HPLC gel-filtration of plasma and protein gel electrophoresis

Plasma was treated with peroxynitrite in the presence of DBNBS and submitted to gel-filtration using a Bio-Sil TSK-125 column (Bio-Rad, Richmond, CA, U.S.A.). The eluted fractions were measured for absorption at 280 nm and for the presence of DBNBS adducts. Moreover, each fraction (10 μl) was dissolved in 4 × loading buffer without -SH reducing agents and the proteins separated by gradient SDS/5–15%-PAGE [24]. Proteins on gels were stained with Coomassie Blue R-250. Stained bands were quantified by densitometric analysis (2400 Gelscan XL laser densitometer; LKB, Bromma, Sweden).

ESR spectroscopy

Spectra were measured on a Bruker ECS 106 spectrometer equipped with a variable-temperature accessory (Bruker Analytische, Rheinstetten, Germany). The DBNBS spin trap was mixed with samples 1 min before the addition of peroxynitrite which, with the exception of decomposed peroxynitrite samples, was always the last addition. The samples were drawn up into a gas-permeable Teflon tube with 0.81 mm internal diameter and 0.05 mm wall thickness (Zeuss Industrial Products, Raritan, NJ, U.S.A.). The Teflon tube was folded four times and inserted into a quartz tube and fixed to the ESR cavity (4108 TMH). Samples were exposed to air at a temperature of 37 °C. Spectra were taken 2 min after the addition of peroxynitrite. To extract an integrated relative area, the ESR signals were double-integrated after baseline correction using the software supplied by Bruker (ESP 1600 data system).

RESULTS

Spin trapping with DBNBS of free radicals induced by peroxynitrite

The addition of peroxynitrite to human plasma in the presence of DBNBS resulted in the detection of an ESR spectrum ($2A_{zz} = 5.96$ mT) characteristic of a highly immobilized nitroxide (Figure 1, spectrum A). Dialysis of DBNBS-labelled plasma resulted in only a slight decrease in the intensity of the ESR spectrum (results not shown), whereas no radicals were detected in the plasma ultrafiltrate (Figure 1, spectrum B; molecular-mass cut-off 3000), suggesting the possible trapping of protein radical(s). When peroxynitrite was added to plasma ultrafiltrate in the presence of DBNBS, a three-line isotropic signal, with a nitrogen hyperfine coupling constant $a_N = 1.27$ mT, was trapped (Figure 1, spectrum C). As suggested by its formation in phosphate buffer treated with peroxynitrite (Figure 1, spectrum D), this DBNBS radical (referred to as 'DBNBS oxidation product') is similar to that first described by Ozawa and Hanaki [25] and subsequently assigned to the radical formed by one-electron oxidation of DBNBS [26].

The apparent absence of an isotropic signal arising from the DBNBS oxidation product in peroxynitrite-treated plasma (Figure 1, spectrum A) suggests that the reaction of peroxynitrite
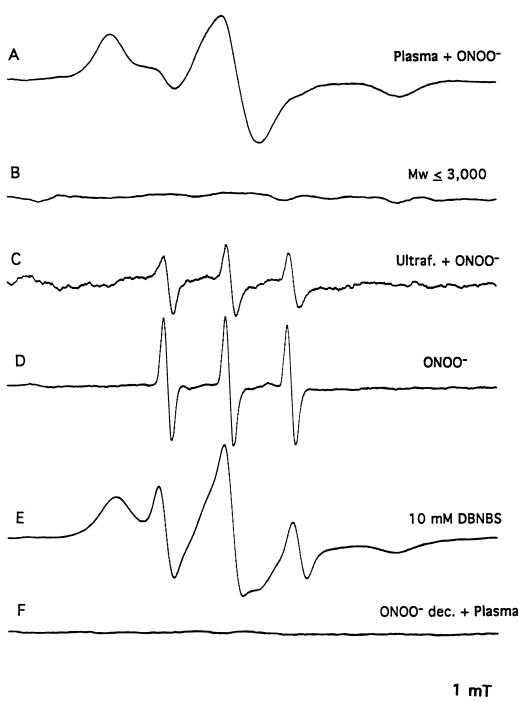
Figure 1   ESR spectra of plasma or plasma ultrafiltrate treated with peroxynitrite in the presence of DBNBS

Spectrum A, peroxynitrite (5 mM) was added to plasma diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1, in the presence of 1 mM DBNBS. Spectrum B, low-molecular-mass filtrate [molecular-mass (= Mw) cut-off 3000] of the solution used in spectrum A. Spectrum C, peroxynitrite (5 mM) was added to plasma ultrafiltrate diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1, in the presence of 1 mM DBNBS (Ultraf. = plasma ultrafiltrate). Spectrum D, peroxynitrite (5 mM) was added to phosphate buffer/DTPA, pH 7.1, in the presence of 1 mM DBNBS. Spectrum E, sample as in spectrum A, except that the concentration of DBNBS was 10 mM. Spectrum F, peroxynitrite (5 mM) was decomposed in phosphate buffer/DTPA, pH 7.1, for 5 min and added to plasma at a dilution of 50% (v/v) in the presence of 1 mM DBNBS (dec. = decomposed). Spectrometer conditions were as follows: modulation amplitude, 0.1 mT; time constant, 164 ms; scan rate, 0.025 mT/s; number of scans, 15; gain 2×10^5; modulation frequency, 100 kHz; microwave frequency, 9.4 GHz; microwave power, 20 mW.

with proteins may precede that with DBNBS. At a higher DBNBS concentration the spectrum of peroxynitrite-treated plasma was the superimposition of a strongly immobilized spectrum and an isotropic spectrum due to the simultaneous presence of the DBNBS oxidation product (Figure 1, spectrum E).

No spectra could be detected if decomposed peroxynitrite was added to plasma (Figure 1, spectrum F), thus ruling out the possibility that radical formation was due to contaminating substances present in the peroxynitrite solution.

Role of ascorbate, urate and thiols

It is presumed that plasma ascorbate, urate and thiols can perform an important protective role in peroxynitrite-induced oxidations [21]. To test the role of these antioxidants, plasma was treated with ascorbate oxidase, uricase or NEM and challenged with increasing concentrations of peroxynitrite.

As shown in Figure 2, the DBNBS–plasma protein adduct was undetectable in plasma at micromolar concentrations of peroxynitrite, but at concentrations higher than 0.5 mM its intensity increased dose-dependently. Treatment with uricase significantly increased the intensity of the DBNBS–plasma protein adduct,
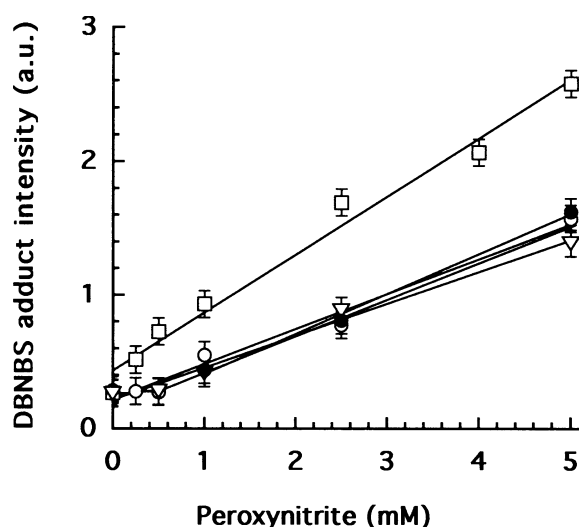

Figure 2   Effect of peroxynitrite concentration on the intensity of DBNBS–plasma protein adduct formed in plasma or in plasma devoid of ascorbate, urate or thiols

Control plasma ($\bigcirc$), plasma pretreated with ascorbate oxidase ($\triangledown$), plasma pretreated with uricase ($\square$) or plasma pretreated with NEM ($\bullet$) was diluted to 50% (v/v) in phosphate buffer/DTPA, pH 7.1, and treated with peroxynitrite in the presence of 1 mM DBNBS. Points represent mean values $\pm$ S.D. ($n = 3$) from a single blood sample; a.u., arbitrary units. In control plasma and in plasma pretreated with ascorbate oxidase, the DBNBS adduct was undetectable at peroxynitrite concentrations $\leqslant 0.5$ mM, and the integrated area was due to the background in the same integration range. Spectrometer conditions were as described in the legend to Figure 1, except that the number of scans was two.

and the minimum concentration of peroxynitrite able to induce the adduct was 250 μM (Figure 2). By contrast, treatment with ascorbate oxidase or alkylation of −SH groups by NEM did not change the intensity of the DBNBS–plasma protein adduct (Figure 2).

Unexpectedly, the removal of dialysable antioxidants by exhaustive dialysis of plasma decreased (by about 40%) the intensity of DBNBS–plasma protein adduct (Figure 3). One possible explanation of this result is that a pro-oxidant molecule present in plasma is removed during dialysis. Bicarbonate, which is present in plasma at high concentrations (about 25 mM), is known to increase some oxidative reactions of peroxynitrite [27–29]. As shown in Figure 3, when dialysed plasma was reconstituted with bicarbonate and treated with peroxynitrite, the intensity of DBNBS–plasma protein adduct increased 2-fold. The intensity of the DBNBS–plasma protein adduct in dialysed plasma reconstituted with bicarbonate was higher (145%) than that of control plasma.

The role of urate, ascorbate and thiols in peroxynitrite-treated plasma was further investigated in dialysed plasma reconstituted with bicarbonate. As shown in Figure 3, urate at the physiological concentration range (160–450 μM) significantly reduced the DBNBS adduct intensity, whereas ascorbate and glutathione inhibited the intensity of DBNBS adduct at concentrations exceeding those usually found in plasma (physiological concentrations of ascorbate and reduced glutathione in plasma are 30–70 and 5–10 μM respectively). Moreover, ascorbate and glutathione at 1 mM decreased the intensity of the DBNS adduct by reducing the nitroxide radical. At 1 mM concentration, the scavenging activity of these antioxidants was in the order urate = ascorbate > glutathione (Figure 3).
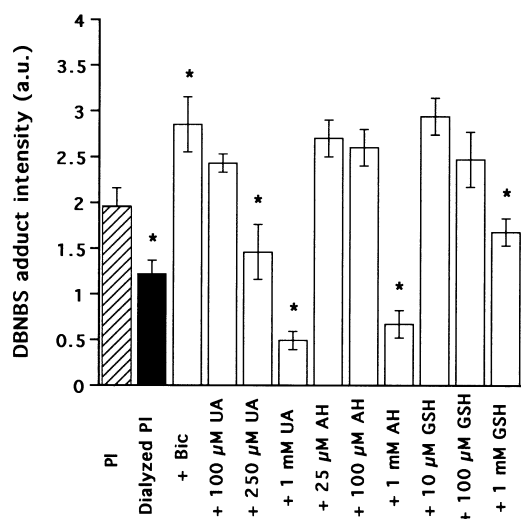
Figure 3   Effects of reconstitution of dialysed plasma (Pl) with bicarbonate (Bic), urate (UA), ascorbate (AH) and glutathione (GSH) on the intensity of DBNBS adduct induced by peroxynitrite

Peroxynitrite (5 mM) was added to plasma (hatched bar) or to plasma exhaustively dialysed against 0.15 M NaCl (solid bar) or to dialysed plasma reconstituted with bicarbonate alone or with bicarbonate and the antioxidants indicated (open bars). Samples were diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1, in the presence of 1 mM DBNBS. The final concentration of bicarbonate was 12.5 mM. Points represent mean values $\pm$ S.D. for four different blood samples and analysed statistically by means of Student's t test. *$P < 0.01$ versus its appropriate control. a.u., arbitrary units. Spectrometer conditions were as described in the legend to Figure 1, except that the number of scans was two.
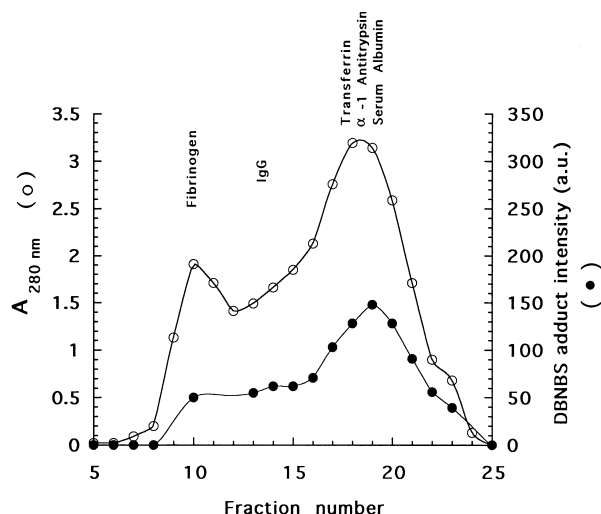

Figure 4   Separation by HPLC gel-filtration of proteins from plasma treated with peroxynitrite in the presence of DBNBS

Plasma treated as in Figure 1, spectrum A, was separated by gel-filtration chromatography in 10 mM phosphate buffer, pH 7.4. Eluted fractions were analysed by SDS/PAGE and by the DBNBS-adduct content. a.u., arbitrary units. Spectrometer conditions were as described in the legend to Figure 1.

Characterization of the DBNBS adduct

To investigate the plasma proteins involved in the formation of the DBNBS adduct, plasma was treated with peroxynitrite in the presence of DBNBS and then submitted to HPLC gel-filtration.
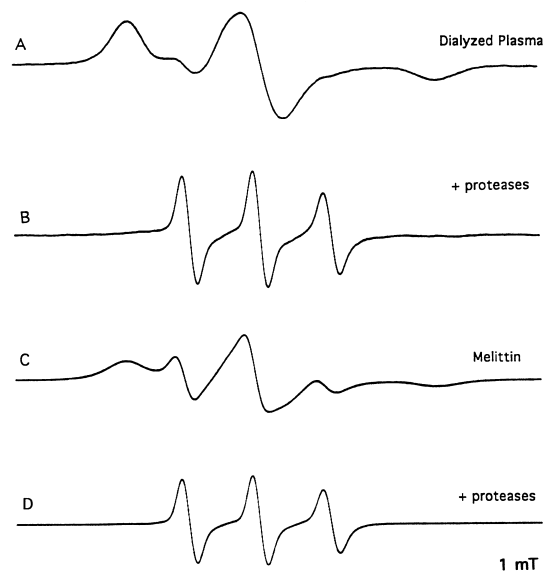
Figure 5   Effect of protease treatment on DBNBS–protein adducts induced by peroxynitrite

Spectrum A, four boluses of peroxynitrite (20 mM final concn.) were added to dialysed plasma reconstituted with bicarbonate and diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1. DBNBS (1 mM) was added before each peroxynitrite addition. Spectrum B, an aliquot of the solution used to obtain spectrum A after the addition of proteases (4 mg/ml Pronase + 5 mg/ml proteinase K) and incubation for 30 min at 37 °C. Spectrum C, peroxynitrite (5 mM) was added to 0.8 mM melittin in phosphate buffer/DTPA, pH 7.1, in the presence of 1 mM DBNBS. Spectrum D, an aliquot of the solution used to obtain spectrum C after the addition of proteases (4 mg/ml Pronase + 5 mg/ml proteinase K) and incubation for 30 min at 37 °C. Spectrometer conditions were as described in the legend to Figure 1 except that the number of scans was two; gains: spectrum A, 2×10^5; spectrum B, 1×10^5; spectrum C, 8×10^4; spectrum D, 2×10^4.

Each fraction was analysed for protein absorption at 280 nm, for the intensity of the DBNBS adduct, and submitted to SDS/PAGE. As shown in Figure 4, the DBNBS adduct was detected mainly in the protein peak containing serum albumin, α_1-antitrypsin and transferrin as major components (not separated by HPLC gel-filtration), but DBNBS-labelled proteins appeared also in fractions containing fibrinogen and IgG as major components. As judged by SDS/PAGE, fibrinogen accounted for about 68% of proteins in fraction 10 and IgG for about 65% of those in fraction 14. Moreover, the DBNBS adduct was found in fractions 10–13, which did not contain albumin, α_1-antitrypsin or transferrin.

From these results we infer that the DBNBS–plasma protein adduct is not specific to a single protein, adducts being formed with several major plasma proteins. To support this hypothesis, purified fibrinogen, IgG, α_1-antitrypsin, apo-transferrin and serum albumin (purity by SDS/PAGE $\geqslant 95\%$) were treated with 5 mM peroxynitrite in the presence of DBNBS. All these proteins, tested separately at their plasma concentrations, produced a strongly immobilized spectrum of DBNBS with signal intensities in the order: serum albumin > IgG > α_1-antitrypsin > apo-transferrin > fibrinogen (spectra not shown).

Identification of the amino acid residue trapped by DBNBS

Recent studies [30] have shown that the high degree of rotational anisotropy observed in DBNBS/whole-protein spectra can be removed by proteolytic digestion. To obtain a high-intensity signal, dialysed plasma was reconstituted with bicarbonate and
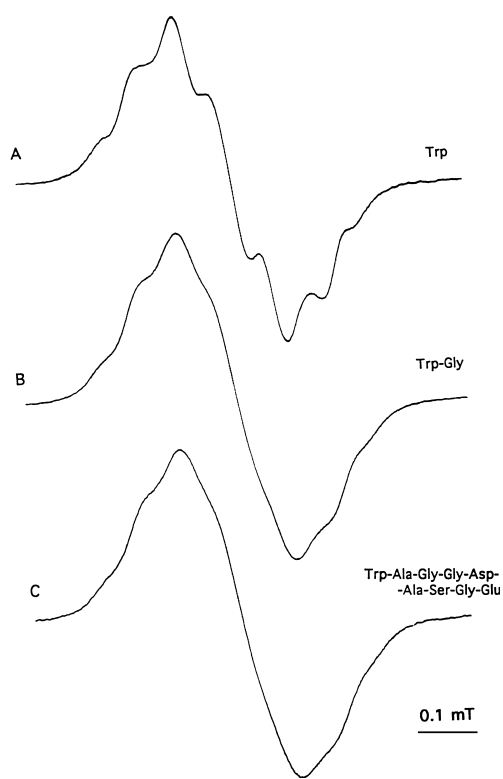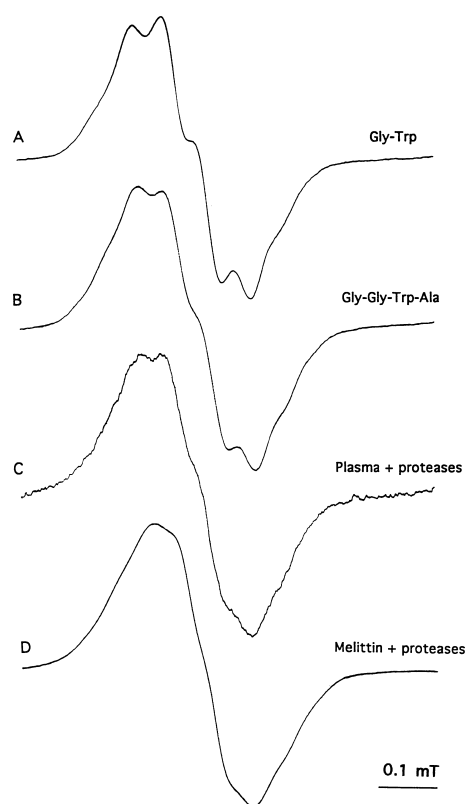
Figure 6   High-resolution scans of the middle line of the spectra of DBNBS adducts with tryptophan and tryptophan-containing peptides

H_2O_2 (0.1 mM) was added to a solution of tryptophan or tryptophan-containing peptides (10 mM), metmyoglobin (25 μM) and DTPA (0.1 mM) in the presence of 10 mM DBNBS. Spectrum A, Trp; spectrum B, Trp-Gly; spectrum C, Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu. Spectrometer conditions were: modulation frequency, 100 kHz; microwave frequency, 9.4 GHz; microwave power, 20 mW; modulation amplitude, 0.01 mT; time constant, 655 ms; scan rate, 0.005 mT/s; number of scans, one; gain, 1 $\times$ 10^5.

Figure 7   Comparison of high-resolution scans of the middle line from DBNBS–tryptophan peptide adducts and protease-treated DBNBS–protein adducts

Spectrum A, H_2O_2 (0.1 mM) was added to a solution of Gly-Trp (10 mM), metmyoglobin (25 μM) and DTPA (0.1 mM) in the presence of 10 mM DBNBS. Spectrum B, H_2O_2 (0.1 mM) was added to a solution of Gly-Gly-Trp-Ala (10 mM), metmyoglobin (25 μM) and DTPA (0.1 mM) in the presence of 10 mM DBNBS. Spectrum C, plasma treated with peroxynitrite in the presence of DBNBS and digested with proteases as described in the legend to Figure 5. Spectrum D, melittin treated with peroxynitrite in the presence of DBNBS and digested with proteases as described in the legend to Figure 5. Spectrometer conditions were as described in the legend to Figure 6 except that number of scans was 15 in spectra C and D; gain: spectrum C, 4 $\times$ 10^5; spectrum D, 8 $\times$ 10^4.

treated with four boluses of 5 mM peroxynitrite (20 mM final concentration) in the presence of DBNBS (Figure 5, spectrum A). Treatment of this product with unspecific proteases resulted in the detection of a nearly isotropic three-line spectrum with a_N = 1.35 mT (Figure 5, spectrum B). The a_N value of this adduct is larger (by about 0.08 mT) than that of the DBNBS oxidation product (Figure 1, spectrum D) and is, therefore, due to a different species. This interpretation was supported by the observation that the sample containing the DBNBS–plasma protein adduct exhaustively dialysed before protease treatment showed an adduct with an identical a_N value.

Hiramoto et al. [31] showed that DBNBS can bind plasma components to form a spontaneous 'ene' addition through a non-radical pathway. To investigate the contribution of this pathway, a control experiment was performed and 1 mM DBNBS-treated plasma was submitted to exhaustive dialysis before the addition of 5 mM peroxynitrite. A very small immobilized nitroxide signal was detected in this sample (peak intensity $\sim$ 6% of that in Figure 1, spectrum A), showing that the non-radical formation of DBNBS adduct is only a minor pathway.

The DBNBS spectrum of protease-treated plasma showed no additional hyperfine structure, thus suggesting the possible trapping of tertiary carbon radical(s). Analysis of the literature suggested that the indole group of tryptophan may be a plausible

candidate. Gunther et al. [32] showed that, in the reaction between free tryptophan and metmyoglobin/H_2O_2, DBNBS can trap a tertiary carbon radical of tryptophan with an a_N value close to that which we detected in protease-treated DBNBS–protein adducts. With this radical-generating system we observed that tryptophan-containing peptides with free α-amino groups of tryptophan showed an a_N (1.38 mT for Trp-Gly and Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu) slightly larger then that of peptides with the α-amino group of tryptophan linked in the amide bond (e.g. a_N = 1.35 mT for Gly-Trp and Gly-Gly-Trp-Ala). These last-named peptides showed an a_N value identical with that of protease-treated DBNBS–plasma proteins.

Melittin, a 26-amino-acid peptide containing only one tryptophan residue and no cysteine, methionine or tyrosine residues, treated with peroxynitrite in the presence of DBNBS gave the partially immobilized spectrum shown in Figure 5, spectrum C. Treatment with proteases of the DBNBS–melittin adduct resulted in a nearly isotropic three-line spectrum (Figure 5, spectrum D) with an a_N (1.35 mT) identical with that of protease-treated DBNBS-plasma proteins.

Further proof that DBNBS trapped tryptophan radicals in
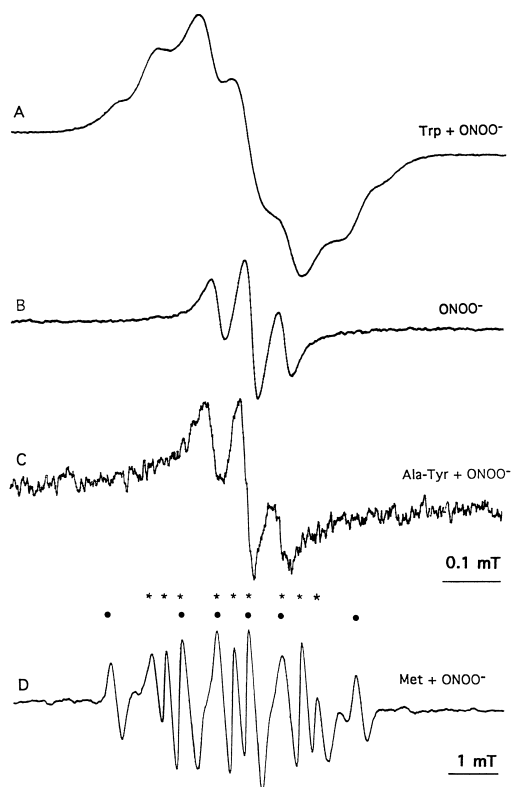
Figure 8 ESR spectra of DBNBS adducts obtained by treatment of tryptophan, Ala-Tyr and methionine with peroxynitrite

Spectrum A, high-resolution scan of the middle line of the DBNBS adduct obtained by treatment of 1 mM tryptophan with 5 mM peroxynitrite in the presence of 10 mM DBNBS. Spectrum B, high-resolution scan of the middle line of the DBNBS adduct obtained by treatment of 1 mM DBNBS with 5 mM peroxynitrite. Spectrum C, high-resolution scan of the middle line of DBNBS adduct obtained by treatment of 1 mM Ala-Tyr with 5 mM peroxynitrite in the presence of 10 mM DBNBS. Spectrum D, low-resolution scan of the whole ESR spectrum of DBNBS adducts obtained by treatment of 10 mM methionine with 5 mM peroxynitrite in the presence of 10 mM DBNBS. Key to symbols above the spectrum: ●, DBNBS adduct with β-hydrogen hyperfine splitting (a_N = 1.46 mT; a_H = 2.19 mT); *, DBNBS adduct with β-nitrogen hyperfine splitting (a_N = 1.41 mT; $a_{N\beta}$ = 0.3 mT). All the amino acids were dissolved in phosphate buffer/DTPA, pH 7.1. Spectrometer conditions for spectra A–C were as described in the legend to Figure 6, except for the number of scans, which was five for spectrum B and seven for spectrum C; the gain in spectrum C was 1×10^6. Spectrometer conditions for spectrum D were as described in the legend to Figure 1, except that the gain was 1×10^5 and number of scans six.

plasma treated with peroxynitrite was obtained by analysis of the superhyperfine structure of the central line of the DBNBS adducts. As previously reported [32], by using a smaller modulation amplitude it is possible to investigate the superhyperfine structure of the DBNBS adduct due to the small hyperfine coupling constants from the nuclei in β to the tertiary carbon atom.

Figure 6, spectrum A, shows the superhyperfine structure of the central line of the DBNBS adduct obtained by treatment of free tryptophan with metmyoglobin/H_2O_2. Substitution of tryptophan with Trp-Gly (Figure 6, spectrum B) or with Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu (Figure 6, spectrum C) resulted in a superhyperfine structure similar to that of free tryptophan, although the linewidth and the line-broadening increased in parallel with the increase in the molecular mass of radical adducts. In contrast, peptides with the α-amino group of tryptophan linked in the amide bond, such as Gly-Trp (Figure 7, spectrum A) or the tetrapeptide Gly-Gly-Trp-Ala (Figure 7,

spectrum B), showed a superhyperfine structure of DBNBS adducts slightly different from that of free tryptophan. These differences in the superhyperfine structure of DBNBS–tryptophan and DBNBS–tryptophan-containing peptides agree with those previously documented using different peptides [32].

The superhyperfine structure of the central line of protease-treated DBNBS–plasma protein adduct (Figure 7, spectrum C) is similar to those in Figure 7, spectra A and B, although an evident line-broadening was observed. The superhyperfine structure of the protease-treated DBNBS–melittin adduct (Figure 7, spectrum D) was also similar to that of Figure 7, spectra A, B and C.

Treatment of tryptophan or tryptophan-containing peptides with peroxynitrite in the presence of DBNBS also resulted in the trapping of DBNBS-tryptophan adducts. As illustrated in Figure 8, spectrum A, tryptophan treated with peroxynitrite in the presence of DBNBS resulted in the trapping of DBNBS adducts with a superhyperfine structure similar to that of DBNBS–tryptophan obtained by metmyoglobin/H_2O_2. However, we observed that by changing the ratio between DBNBS concentration and that of tryptophan derivatives, the ESR spectra also revealed the presence of the radical due to DBNBS oxidation product. The superhyperfine structure of DBNBS oxidation product is a triplet [25] arising from DBNBS *meta*-protons with a_H (2) = 0.06 mT (Figure 8, spectrum B). Although at the DBNBS/tryptophan ratio used in Figure 8, spectrum A, the contribution of the DBNBS oxidation product was negligible, the presence of this radical explained the differences observed between the spectra of DBNBS–tryptophan adducts obtained by metmyoglobin/H_2O_2 and those obtained by peroxynitrite.

Tyrosine and methionine are other easily oxidizable sites in proteins, and peroxynitrite has been reported to induce dityrosine formation [12,33] and oxidation of methionine residues [11,34]. Dityrosine formation in peroxynitrite-treated proteins indicates that tyrosyl radicals (which can dimerize to dityrosine) are likely to be formed. However, DBNBS was unable to trap tyrosyl radicals, since analysis of the superhyperfine structure of the central isotropic line of spectra obtained by peroxynitrite treatment of Ala-Tyr revealed only the presence of the DBNBS oxidation product (Figure 8, spectrum C).Methionine treated with peroxynitrite in the presence of DBNBS showed an 11-line spectrum (Figure 8, spectrum D). Presumably this spectrum is due to the trapping of at least two DBNBS adducts: one of six lines, suggesting the presence of a β-hydrogen (a_H = 2.19 mT; a_N = 1.46 mT) and the other of nine lines tentatively assigned to a tertiary carbon radical with β-nitrogen hyperfine splitting (a_N = 1.41 mT; $a_{N\beta}$ = 0.3 mT). The latter adduct may result from a methionine radical centred on the α-carbon of the amino acid.

DISCUSSION

Several cell types, including not only those involved in immunological defences but also vascular endothelial cells, neurons and smooth-muscle cells, can generate NO˙ and $O_2^{-˙}$ simultaneously. The very fast reaction between these two radicals probably leads to the formation, *in vivo*, of peroxynitrite. Peroxynitrite-releasing cells were possibly evolved to down-regulate the physiological functions of NO˙ and $O_2^{-˙}$ and also to increase their cytotoxic effects. In contrast with its parent radicals, peroxynitrite is a strong and extremely versatile oxidant of several biological targets [8]. The detailed mechanisms of these reactions are currently under examination, but recent results have shown that the one-electron oxidation pathway may be an important event in its cytotoxic mechanism [8,22,29,34].

Our results demonstrate that the spin-trapping technique can be used to detect protein-centred radicals induced by peroxy-

nitrite in human plasma. These radicals are either one-electron oxidation products of peroxynitrite or radicals induced by secondary oxidants produced by the reaction of peroxynitrite with other plasma components. Bicarbonate is an example of this latter mechanism. The reaction of peroxynitrite with bicarbonate is sufficiently fast (rate constant 3×10^4 $M^{-1} \cdot s^{-1}$) [35] to precede that with other biological targets [29]. It has been suggested that, in the presence of CO_2, peroxynitrite forms highly reactive oxidants such as the nitrosoperoxycarbonate anion adduct, $O=N-OOCO_2^-$ [27] and/or its rearranging product nitro-carbonate anion, $O_2N-OCO_2^-$ [29]. In the present study we observed an increased formation of protein radicals induced by peroxynitrite in the presence of bicarbonate. A similar pro-oxidant effect of bicarbonate was observed on thiyl radicals of plasma proteins induced by peroxynitrite [36].

In spite of the prooxidant effect of bicarbonate in plasma, the DBNBS adduct of proteins was detected only at high peroxynitrite concentrations ($\geqslant 1$ mM). It is unclear whether these concentrations of peroxynitrite can be achieved in vivo (see, e.g., a discussion of this aspect by Van der Vliet et al. [21]), but it is conceivable that, under pathological conditions, the pCO_2 increase and the reduction of antioxidant defences may significantly decrease the cytotoxic concentration of peroxynitrite. We observed that the removal of urate from plasma reduced the minimum concentration of peroxynitrite able to induce the DBNBS adduct to 250 μM.

As detected by spin-trapping with DBNBS, peroxynitrite was found to oxidize all the major plasma proteins. Serum albumin, which is considered an important sink for radicals in plasma, was one of the major targets of peroxynitrite but, notwithstanding its high plasma concentration, it was unable to protect the other proteins completely. The apparent lack of a preferential protein target emphasizes the unspecific toxic properties of peroxynitrite.

Previous spin-trapping data obtained with nitrone spin traps showed that peroxynitrite added to serum albumin [37] or to blood plasma [22] induced the formation of immobilized protein-radical adducts. These adducts have been assigned to a thiyl radical, as demonstrated by their complete inhibition after treatment with thiol-specific reagents. In contrast, the intensity of the DBNBS adduct produced by peroxynitrite in plasma was unaffected by NEM, thus excluding the trapping of cysteine residues.

The similarity between the spectrum obtained by protease treatment of DBNBS–plasma proteins and that of DBNBS–tryptophan peptides strongly suggests that peroxynitrite induced the formation of radicals localized on a tertiary carbon of protein tryptophan residues. The a_N value and the superhyperfine structure of the protease-treated DBNBS–plasma protein adducts were identical with, or similar to, those of DBNBS–tryptophan peptide adducts and, in particular, were identical with those of peptides with the α-amino group of tryptophan linked in the amide bond. The involvement of tryptophan residues was further supported by the experiments with melittin (a peptide containing only one tryptophan residue and no cysteine, methionine or tyrosine residues), which, when treated with peroxynitrite, showed, after proteolysis, a DBNBS adduct very similar to that of protease-treated DBNBS–plasma proteins.

Previous studies [32] have demonstrated that DBNBS–tryptophan adducts can be successfully simulated using contributions from the nitrogen atom of the indole group, three nonequivalent protons and the two meta-protons of DBNBS, thus proving the involvement of a radical formed at C-3 of the indole ring. The similarity of the superhyperfine structure of protease-treated DBNBS–proteins with that of DBNBS–tryptophan-containing peptides strongly supports the hypothesis that peroxy-

nitrite in blood plasma forms a radical centred on the same site. The minor differences observed in the spectra can be accounted for by linewidth increase and line-broadening due to both the steric effects of additional amino acids and the molecular inhomogeneity produced by protease treatment of plasma proteins containing multiple tryptophan sites.

Our finding that DBNBS trapped, as major radical species, radicals formed on tryptophan residues suggests that this amino acid may be a critical site of peroxynitrite-induced damage. In agreement with this hypothesis, Alvarez et al. [38] recently showed that peroxynitrite was able to induce the nitration of free tryptophan, a reaction that, with nitrotyrosine, may be a 'footprint' of peroxynitrite production in vivo. Interestingly, nitration of tryptophan could occur through the intermediacy of tryptophan radicals [38].

The possible involvement of tyrosine residues, another probably oxidizable tertiary carbon atom in proteins (at C-4 of the phenol ring), was excluded because tyrosine is absent in melittin and DBNBS was unable to trap peroxynitrite-induced tyrosyl radicals.

Methionine is also oxidized by peroxynitrite by one-electron or two-electron oxidation pathways [11,34], but its involvement in the DBNBS–plasma protein adduct was excluded by results obtained with melittin, a peptide lacking methionine, and by the observation that the hyperfine structure characteristic of DBNBS–methionine adducts (a 11-line spectrum) was not observed in protease-treated DBNBS–plasma proteins. Methionine treated with peroxynitrite resulted in the trapping of DBNBS adducts with hyperfine structure, suggesting the presence of both β-hydrogen and β-nitrogen hyperfine couplings.

The peroxynitrite-mediated formation of tryptophan radicals will probably have biological implications on protein function and may be a means of propagating peroxynitrite-initiated damage through radical chain reactions capable of inducing further oxidative processes [39]. The reaction of tryptophan radicals with molecular oxygen to form peroxyl radicals [32,40] and the transfer of the oxidative equivalent to other tryptophan or tyrosine residues [41] are likely reactions in protein-rich aerobic biological environments. Such sequences of events underline the possible biological relevance of the one-electron oxidation pathway of peroxynitrite decomposition in tissues.

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