Direct ESR detection or peroxynitrite-induced tyrosine-centred protein radicals in human blood plasma

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Peroxynitrite, the reaction product of $O_2^{\bullet -}$ and $^{\bullet}NO$, is a toxic compound involved in several oxidative processes that modify proteins. The mechanisms of these oxidative reactions are not completely understood. In this study, using direct ESR at 37 °C, we observed that peroxynitrite induced in human blood plasma a long-lived singlet signal at $g = 2.004$ arising from proteins. This signal was not due to a specific plasma protein, because several purified proteins were able to form a peroxynitrite-induced $g = 2.004$ signal, but serum albumin and IgG showed the most intense signals. Hydroxyurea, a tyrosyl radical scavenger, strongly inhibited the signal, and horseradish peroxidase/H_2O_2, a radical-generating system known to induce tyrosyl radicals, induced a similar signal. Furthermore peptides containing a Tyr in the central portion of the molecule were able to form a stable peroxynitrite-dependent $g = 2.004$ signal, whereas peptides in which Tyr was substituted with Gly, Trp or Phe and peptides with Tyr at the N-terminus or near the C-terminus did not form radicals that were stable at 37 °C. We suggest that Tyr residues are at least the major radical sources of the peroxynitrite-dependent $g = 2.004$ signal at 37 °C in plasma or in isolated proteins. Although significantly enhanced by CO_2/bicarbonate, the signal was detectable in whole plasma at relatively high peroxynitrite concentrations (> 2 mM) but, after removal of ascorbate or urate or in dialysed plasma, it was detectable at lower concentrations (100–1000 μM). Our results suggest that the major role of ascorbate and urate is to reduce or 'repair' the radical(s) centred on Tyr residues and not to scavenge peroxynitrite (or nitrosoperoxycarbonate, the oxidant formed in CO_2-containing fluids). This mechanism of inhibition by plasma antioxidants may be a means of preserving the physiological functions of peroxynitrite.

INTRODUCTION

Peroxynitrite (this terms refers to both the anion $ONOO^-$ and the different forms of its conjugate acid ONOOH [1]; the IUPAC-recommended names are: oxoperoxonitrate (1-) and hydrogen oxoperoxonitrate respectively) is a potent oxidant that can be produced in biological systems from the near-diffusion-limited reaction of $O_2^{\bullet -}$ and $^{\bullet}NO$ [2]. Peroxynitrite is capable of oxidizing a variety of biomolecules including proteins [3], lipids [4], carbohydrates [5], DNA [6] and low-molecular-mass antioxidant molecules [7,8].

Peroxynitrite anion reacts directly with Cys and Met residues [3,9], reactions implicated as major mechanisms in the peroxynitrite-mediated inactivation of enzymes. However, the reactivity of peroxynitrite is multifaceted: it is strongly dependent on pH, which regulates the concentrations of the anion and peroxynitrous acid [1]. Once protonated to peroxynitrous acid (pK_a 6.8), this unstable species can oxidize several protein residues including Cys, Tyr, Trp and Met. One of the major modifications of protein Tyr and Trp residues is the nitration of the aromatic rings [10–12].

In human blood plasma, peroxynitrite is able to lower total peroxyl-trapping capacity, deplete low-molecular-mass antioxidants, oxidize thiol groups and induce lipid peroxidation and Tyr nitration [13,14]. The mechanisms responsible for these oxidative reactions are at present under investigation and may involve at least five processes: isomerization to nitrate with the formation of a 'hydroxyl-like' oxidant [15], one-electron oxidation, two-electron oxidation, oxygen transfer and electrophilic nitration [16].

The ESR (or EPR) technique is particularly useful for investigating one-electron oxidation induced by peroxynitrite in complex biological fluids. Direct ESR and spin-trapping studies revealed the formation of peroxynitrite-dependent free-radical intermediates in blood plasma, such as the ascorbyl free radical, protein thiyl radicals and a radical derived from uric acid [17]. Moreover, recent spin-trapping studies with a nitroso spin-trap have shown that, in human blood plasma, peroxynitrite induces the formation of a protein radical(s) centred on Trp residues [18].

Some peroxynitrite-mediated oxidative reactions such as the nitration of Tyr and Trp residues and formation of tryptophanyl radicals are enhanced by CO_2 [16,18–21]. The reaction between peroxynitrite anion and CO_2 (rate constant 3.0×10^4 $M^{-1} \cdot s^{-1}$) is of particular importance in CO_2/bicarbonate-rich extracellular fluids, because it leads to the formation of a postulated highly reactive short-lived secondary oxidant, the nitrosoperoxycarbonate adduct ($ONOO^- + CO_2 \rightarrow ONO_2CO_2^-$) [22]. This oxidant shows a different reactivity from that of peroxynitrite and may thus either increase or protect biological targets from the toxic effects of peroxynitrite [13,19,23].

In this work, we used direct ESR at 37 °C to study peroxynitrite-mediated one-electron oxidation in human blood plasma. After treatment with peroxynitrite, we detected in plasma a broad singlet free radical at $g = 2.004$ arising from proteins. Our results support the hypothesis that these protein radicals are centred on Tyr residues. CO_2/bicarbonate significantly increases

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; HRP, horseradish peroxidase; NEM, *N*-ethylmaleimide; DPPH, α,α'-diphenyl-β-picryl hydroxyl; DBNBS, 3,5-dibromo-4-nitrosobenzenesulphonic acid.

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the formation of $g = 2.004$ radical(s) in plasma, whereas urate and ascorbate produced a relative inhibition, acting mainly as scavengers of tyrosyl radicals.

MATERIALS AND METHODS

Materials

Thymopoietin II fragment (GEQRLDVYVQLYL), Tyr-fibrinopeptide A (YADSGEDFLAEGGGVR), Tyr-somatostatin (YAGCLNFFWLTFTSC), melittin, Pronase, ascorbate oxidase, uricase type V from porcine liver, horseradish peroxidase (HRP) and proteins purified from human plasma (fibrinogen type I, IgG, α_1-antitrypsin, apo-transferrin, serum albumin) were obtained from Sigma Biochemicals (St. Louis, MO, U.S.A.). Peptides MEELQDDYEDMMEENC and FNSVSSYSDSRK-SSGDGPDLC were obtained from Primm s.r.l. (Milan, Italy) and peptides derived from thymopoietin II fragment with Tyr replaced with Gly, Phe or Trp were obtained from Neosystem Laboratories (Strasbourg, France). The spin-trap, 3,5-dibromo-4-nitrosobenzenesulphonate (DBNBS) was obtained from OMRF Spin Trap Source (Oklahoma City, OK, U.S.A.).

Synthesis of peroxynitrite

Peroxynitrite was synthesized by allowing nitrite to react with acidified H_2O_2 as described by Radi et al. [3] and treated with MnO_2 (1 mg/ml; 30 min at 4 °C) to eliminate excess H_2O_2. MnO_2 was removed by centrifugation (for 5 min at 4 °C and 15000 ***g***) and filtration (0.45 μm; Millipore, Molsheim, France). The peroxynitrite solution forms by freeze fractionation (−80 °C) as a yellow top layer, which was retained for further studies. The top layer typically contained 200–500 mM peroxynitrite as determined spectrophotometrically at 302 nm in 1.5 M NaOH ($\epsilon_{302} = 1670\ M^{-1} \cdot cm^{-1}$). The peroxynitrite solution was stored for 1–2 weeks (−20 °C) with negligible changes in its concentration.

Treatment of plasma

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 minimize the loss to the gas phase of CO_2 dissolved in plasma, samples (about 0.8 ml) were maintained at 0 °C in closed vials (1 ml) and used within 8 h of collection. Moreover, to estimate the effect of the possible CO_2 loss, control experiments were performed under 5% CO_2. All plasma treatments requiring incubation at 37 °C [ascorbate oxidase, uricase, *N*-ethylmaleimide (NEM) and Pronase], during which presumably a larger loss of CO_2 may occur, were performed in closed vials under 5% CO_2.

Plasma was depleted of ascorbate or urate by incubation (30 min at 37 °C) with ascorbate oxidase (0.04 unit/ml) or uricase (0.1 unit/ml). Plasma thiol groups were alkylated by incubation with 5 mM NEM for 30 min at 37 °C. Proteolysis of plasma proteins was performed by adding Pronase (5 mg/ml) and incubation for 30 min at 37 °C. HRP (50 μM) treatment was performed in plasma diluted to 80% (v/v) in phosphate/diethylenetriaminepenta-acetic acid (DTPA) buffer, pH 7.1. The reaction was started with H_2O_2 (25 mM) and stopped by the addition of catalase (500 μM). α-Tocopherol oxidation was obtained by treating dialysed plasma (80%, v/v) with phosphate buffer/DTPA, pH 7.1, containing 100 μM potassium ferricyanide [24].

To remove low-molecular-mass compounds, plasma was submitted to extensive dialysis (molecular-mass cut-off 10000 Da) against 0.15 M NaCl at 4 °C. To obtain the low-molecular-mass compounds (ultrafiltrate), plasma was centrifuged with Microcon concentrators (molecular-mass cut-off 3000 Da; Amicon Inc., Beverly, MA, U.S.A.) for 15 min at 14000 ***g***. 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.

Treatment of plasma with •NO involved injecting a known volume of an NO-saturated solution (~ 1.9 mM at 25 °C) [25]. This solution was prepared by bubbling 150 mM phosphate buffer, pH 7.1, with argon for 15 min and then with •NO gas (SIO, Pomezia, Italy) for 5 min at room temperature.

Treatment with peroxynitrite

Peroxynitrite was added as a bolus to samples buffered with an equal volume of phosphate buffer, pH 7.1 (150 mM phosphate final concentration), and immediately mixed. To avoid metal-catalysed oxidation of peroxynitrite [26], buffers were treated extensively with Chelex 100 (Bio-Rad, Richmond, CA, U.S.A.) and all samples contained 0.1 mM DTPA. The pH of samples was measured after each incubation and found to change only slightly after the addition of 0.1–5 mM peroxynitrite (pH 7.1–7.5). Decomposed peroxynitrite was obtained by adding peroxynitrite to phosphate buffer/DTPA (pH 7.1) at room temperature for 15 s before the addition of plasma or peptides (reversed order of addition).

ESR spectroscopy

Spectra were measured on a Bruker ECS 106 spectrometer equipped with a variable-temperature accessory (Bruker, Rheinstetten, Germany). 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, inserted into a quartz tube and fixed to the ESR cavity (4108 TMH). Samples were exposed to air or to 5% CO_2 at a temperature of 37 °C using a variable-temperature unit (Bruker; ER4111VT). The dead time of sample preparation and ESR analysis was exactly 2 min after the last addition, except for the decay studies of DBNBS adduct, which started 30 s after the addition of peroxynitrite. To obtain an integrated relative area, all ESR spectra were corrected for baseline drift by a linear function and double-integrated using the software supplied by Bruker (ESP 1600 data system). However, with signals of low intensity, this software did not provide credible values, and we therefore used another method to obtain a relative area from these samples [27]. This second method used a 'peak-picking' function to obtain a relative intensity height of the signal, and the area was estimated by multiplying the intensity height by the square of the measured peak-to-peak linewidth. Comparable relative results for intense signals were provided by the two methods.

RESULTS

Free radicals induced by peroxynitrite in human blood plasma

Figure 1, spectrum A, is the direct ESR spectrum at 37 °C of untreated human plasma; the only radical detectable was that derived from the autoxidation of ascorbic acid in air (ascorbyl radical) [28]. In agreement with previously published results [17], the addition of peroxynitrite increased the intensity of the ascorbyl radical and, at 500 μM, its intensity was increased by about 50% (Figure 1, spectrum B). At higher peroxynitrite concentrations the intensity of the ascorbyl radical decreased and, at 2 mM, it was undetectable (Figure 1, spectrum C), because of depletion of the ascorbate.

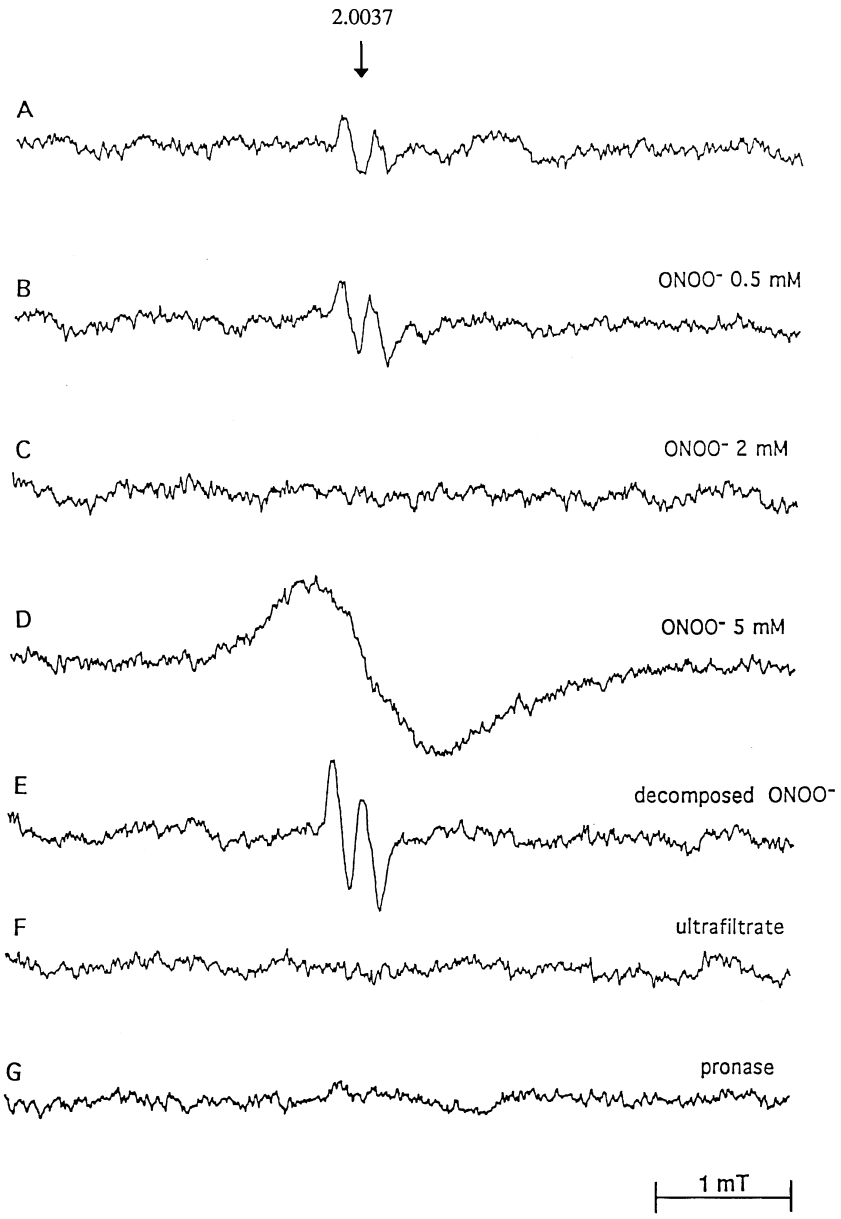

Figure 1 ESR spectra at 37 °C of human blood plasma treated with peroxynitrite

Spectrum A, plasma diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1; spectrum B, sample as for spectrum A after addition of 500 μM peroxynitrite; spectrum C, sample as for spectrum A after addition of 2 mM peroxynitrite; spectrum D, sample as for spectrum A after addition of 5 mM peroxynitrite; spectrum E, peroxynitrite (5 mM) was decomposed in phosphate buffer/DTPA, pH 7.1, for 15 s and then added to plasma; spectrum F, peroxynitrite (5 mM) was added to low-molecular-mass plasma fraction (ultrafiltrate, cut-off 3000 Da) diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1; spectrum G, plasma was maintained under 5% CO_2 and treated with 5 mg/ml Pronase (30 min at 37 °C), diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1, and treated with 5 mM peroxynitrite. Spectrometer conditions were as follows: modulation frequency, 100 kHz; microwave frequency, 9.4 GHz, microwave power, 20 mW; modulation amplitude, 0.1 mT; gain 8×10^5; time constant, 328 ms; sweep time, 84 s. Spectra were corrected for baseline drift by a linear function and represent signal averaging of three scans.

Interestingly, treatment of plasma with 5 mM peroxynitrite induced the formation of a broad singlet free radical(s) with a peak-to-trough of 0.7–1.0 mT (Figure 1, spectrum D). The *g*-value of this signal ($g = 2.0045 \pm 0.0004$) was very close to that of α,α'-diphenyl-β-picryl hydroxyl (DPPH) used as standard ($g = 2.0037$). Taking into consideration the basal levels of the ascorbyl radical in plasma [28], the intensity of the $g = 2.004$ signal could be estimated to be around 5 μM. As shown in Figure 1, spectrum E, this signal was not detected if peroxynitrite decomposed before the addition of plasma. In this sample, the increased intensity of the ascorbyl radical was possibly due to the presence of contaminating oxidants in the decomposed peroxynitrite solution (nitrites and trace amounts of H_2O_2). The $g = 2.004$ signal was probably derived from plasma proteins, because it was undetectable in the low-molecular-mass fraction of plasma (Figure 1, spectrum F) and disappeared after extensive proteolytic treatment (Figure 1, spectrum G).

The $g = 2.004$ signal disappeared within 20–25 min of the addition of peroxynitrite. The decay curve was, however, complex and cannot be fitted with one exponential. Fitted with two straight lines in a semilogarithmic plot, the calculated half-lives of signal decay were 140 s for the fast decay (corrected for the

Table 1 Effects of NEM, CO_2/bicarbonate and hydroxyurea on the intensity of $g = 2.004$ radical(s) induced by peroxynitrite in blood plasma

Plasma, NEM-treated plasma, plasma ultrafiltrate, dialysed plasma or dialysed plasma reconstituted with 25 mM bicarbonate was diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1, before the addition of peroxynitrite (5 mM). Where indicated, samples were maintained under 5% CO_2. Hydroxyurea was added 15 s after the addition of peroxynitrite. a.u., arbitrary units. Points (mean values ± S.D. for the number of determinations shown in parentheses) were analysed statistically by Student's t test. $^*P \leqslant 0.008$ compared with its appropriate control. Spectrometer conditions were as described in the legend to Figure 1.

Samples	$g = 2.004$ peak intensity (a.u.)
Plasma	16.6 ± 3.0 (17)
Plasma, 5% CO_2	20.6 ± 3.7 (9)
NEM-treated plasma, 5% CO_2	19.8 ± 2.0 (3)
Dialysed plasma	8.8 ± 1.4* (3)
Ultrafiltrate	0.0 ± 0.8* (3)
Dialysed plasma + ultrafiltrate	16.9 ± 1.2* (3)
Dialysed plasma + bicarbonate	22.4 ± 2.0* (6)
Dialysed plasma + bicarbonate, 5% CO_2	32.7 ± 1.1* (3)
Dialysed plasma + bicarbonate + hydroxyurea (0.1 mM)	6.8 ± 1.5* (3)
Dialysed plasma + bicarbonate + hydroxyurea (1 mM)	2.8 ± 1.0* (3)

slow decay) and 790 s for the slow decay. It is conceivable that in peroxynitrite-treated plasma more than one radical contributed to the signal or that the same radical species may be present in more than one environment.

The removal of dialysable compounds by exhaustive dialysis decreased (by about 47%) the intensity of the $g = 2.004$ signal (Table 1). This result was unexpected, because the absence of low-molecular-mass antioxidants should increase the oxidative damage by peroxynitrite. Furthermore the radical was undetectable in the low-molecular-mass fraction (ultrafiltrate), but the addition of ultrafiltrate to dialysed plasma restored the signal intensity (Table 1). These results can be explained by the removal, during plasma dialysis, of a pro-oxidant molecule. CO_2 is a likely candidate, since CO_2/bicarbonate is present in blood plasma at high concentrations (bicarbonate is about 25 mM and, in normal plasma, the concentration of CO_2 in equilibrium is about 1.3 mM). It is known that CO_2 can catalyse some peroxynitrite-dependent oxidations [16,19–22]. As shown in Table 1, the intensity of the $g = 2.004$ signal induced by peroxynitrite increased 2.5-fold when dialysed plasma was reconstituted with bicarbonate. However, the CO_2 dissolved in plasma (about 1.3 mM) is in equilibrium with CO_2 in the gas phase (about 5%, v/v) and thus the exposure of samples to air may cause some CO_2 loss. To estimate the possible effects of CO_2 loss in samples exposed to air, control experiments were carried out under 5% CO_2. As shown in Table 1, under 5% CO_2 the treatment with peroxynitrite of plasma or of dialysed plasma reconstituted with bicarbonate produced a slightly increased $g = 2.004$ signal (+24% and +30% respectively).

The $g = 2.004$ signal was not due to Cys residues, because treatment of plasma with the thiol-alkylating agent NEM did not significantly modify the radical yield (Table 1).

Characterization of the $g = 2.004$ signal induced by peroxynitrite in human blood plasma

The $g = 2.004$ signal was not due to a specific plasma protein, because several purified human plasma proteins treated separately with peroxynitrite in the presence of CO_2/bicarbonate were able to form a broad singlet signal at $g = 2.004$. Figure 2 shows the spectra of albumin, IgG, α_1-antitrypsin, apo-transferrin and fibrinogen (type I) treated with peroxynitrite at their normal plasma concentrations. Serum albumin and IgG showed the most intense signals, suggesting a substantial contribution by these proteins to the radical detected in plasma.

Line shape and g value of the radical(s) shown in Figure 1, spectrum D, suggest the formation of protein-centred organic radical(s) [29]. Analysis of the literature revealed that long-lived organic protein radicals that are stable at room temperature with $g = 2.004$ could be tyrosyl [30–32] or tryptophanyl radicals [33].

In a previous study [18], we showed that peroxynitrite added to plasma in the presence of the spin-trap DBNBS induced the formation of an immobilized adduct assigned to Trp-centred protein radicals. To test if tryptophanyl radical(s) were involved in the $g = 2.004$ signal, we measured the decay kinetics of the radical trapped by DBNBS by adding the spin-trap (1 mM) immediately after (1–10 s) or before peroxynitrite (time = 0) and determining the DBNBS adduct intensity. The DBNBS adduct in peroxynitrite-treated plasma is a three-line immobilized signal [18], and thus, to avoid the possible interference of $g = 2.004$ signal, the intensity of DBNBS adduct was measured on the low-field line (at $g = 2.021$), where there is no overlapping of the $g = 2.004$ signal. The measured lifetime of tryptophanyl radicals was significantly shorter than that of the $g = 2.004$ signal (4.0 ± 1.2 and 1320 ± 120 s respectively), suggesting the involvement of a different radical species. If DBNBS was added to plasma 5 s after peroxynitrite, only the $g = 2.004$ signal was observed thus clearly showing that DBNBS was unable to trap the $g = 2.004$ radical(s). When DBNBS was added before peroxynitrite it was difficult to establish the presence of $g = 2.004$ radical(s) because of signal overlapping and the low intensity of the $g = 2.004$ signal.

The hypothesis that the $g = 2.004$ signal was not due to tryptophanyl radicals was further strengthened by the observation that peroxynitrite-treated melittin, a 26-amino-acid peptide containing only one Trp and no Cys, Met or Tyr residues, formed an intense DBNBS–protein adduct [18], but no $g = 2.004$ signal for this peptide after treatment with peroxynitrite was detected by direct ESR at 37 °C.

To characterize the protein residue(s) involved in the $g = 2.004$ signal, we used hydroxyurea as a scavenger of tyrosyl radicals [32] and HRP/H_2O_2 as a tyrosyl-radical-generating system [34,35]. To avoid the possibility of a direct scavenging effect of hydroxyurea on peroxynitrite or on nitrosoperoxycarbonate, the drug was added 15 s after peroxynitrite. After this time, peroxynitrite is completely decomposed (in the presence of 10 mM bicarbonate, the half-life of peroxynitrite at pH 7 is < 0.1 s [16]), but the $g = 2.004$ signal is still present because of its relatively long lifetime (20–25 min). As shown in Table 1, hydroxyurea (0.1–1 mM) added after peroxynitrite reduced the intensity of the $g = 2.004$ signal by 70–90%.

In contrast with peroxynitrite, which produces a burst of radicals during its short lifetime, HRP/H_2O_2 produces free radicals continuously during its enzymic catalysis. To compare the two radical-generating systems, HRP/H_2O_2 was allowed to react with dialysed plasma for different times and the reaction was stopped by the addition of catalase before spectra recording. When plasma was incubated for 2 min with HRP/H_2O_2 before catalase addition, the 37 °C direct ESR spectrum (Figure 3, spectrum A) was consistent with the formation of α-tocopheroxyl radical [25]. To confirm the spectrum assignment, we oxidized plasma with ferricyanide, which is known to induce the formation of the α-tocopheroxyl radical [25]. The spectrum obtained by

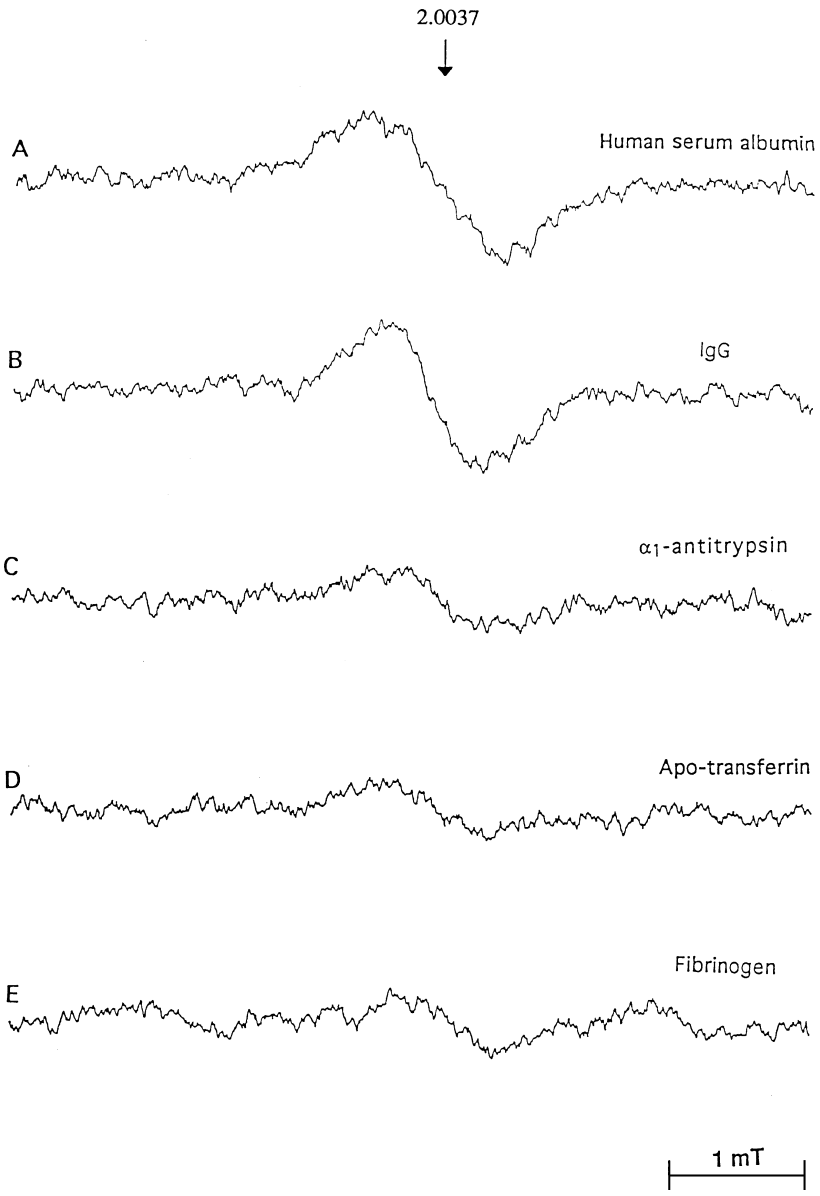

Figure 2 ESR spectra at 37 °C of purified human plasma proteins treated with peroxynitrite

Peroxynitrite (5 mM) was added to: 300 μM serum albumin (spectrum A); 45 μM IgG (spectrum B); 40 μM α_1-antitrypsin (spectrum C); 10 μM apo-transferrin (spectrum D); or 4.5 μM fibrinogen (spectrum E). Proteins were dissolved in phosphate buffer/DTPA/12.5 mM bicarbonate, pH 7.1. Final concentrations of proteins were comparable with those of plasma diluted to 50% (v/v). Spectrometer conditions and baseline correction were as described in the legend to Figure 1 except that the number of scans was two, modulation amplitude was 0.26 mT, gain was 4×10^5 and sweep time was 42 s.

ferricyanide was, however, slightly different (Figure 3, spectrum B) and suggests the presence in the HRP/H_2O_2 spectrum of other radical(s) in the central portion of the spectrum. The increase in the incubation time between dialysed plasma and HRP/H_2O_2 produced a time-dependent decrease in the intensity of the α-tocopheroxyl radical. When plasma was incubated for 15 min with HRP/H_2O_2 before catalase addition, the α-tocopheroxyl radical disappeared almost completely, because of depletion of plasma α-tocopherol, and the spectrum showed only the presence of a broad singlet signal at $g = 2.0044$ with a peak-to-trough of 1.1 mT (Figure 3, spectrum C). The formation of these radicals by HRP/H_2O_2 was completely dependent on the presence of plasma (Figure 3, spectrum D).

Free radicals induced by peroxynitrite in synthetic peptides

The inhibition by hydroxyurea of the peroxynitrite-dependent $g = 2.004$ signal as well as the similarity to the signal induced in plasma by HRP/H_2O_2 are no proof but suggest that the $g = 2.004$ signal may be a radical centred on Tyr residues.

To explore this possibility, we performed ESR studies with synthetic peptides exposed to oxidative reactions with peroxynitrite in CO_2/bicarbonate-containing phosphate buffer. We observed that a 13-amino-acid peptide derived from thymopoietin II (the domain 29–41, GEQRLDVYVQLYL, containing the thymopoietin active site [36]) treated with 10 mM peroxynitrite was able to induce a $g = 2.004$ signal (Figure 4, spectrum A) stable at 37 °C and similar to that observed in plasma. This

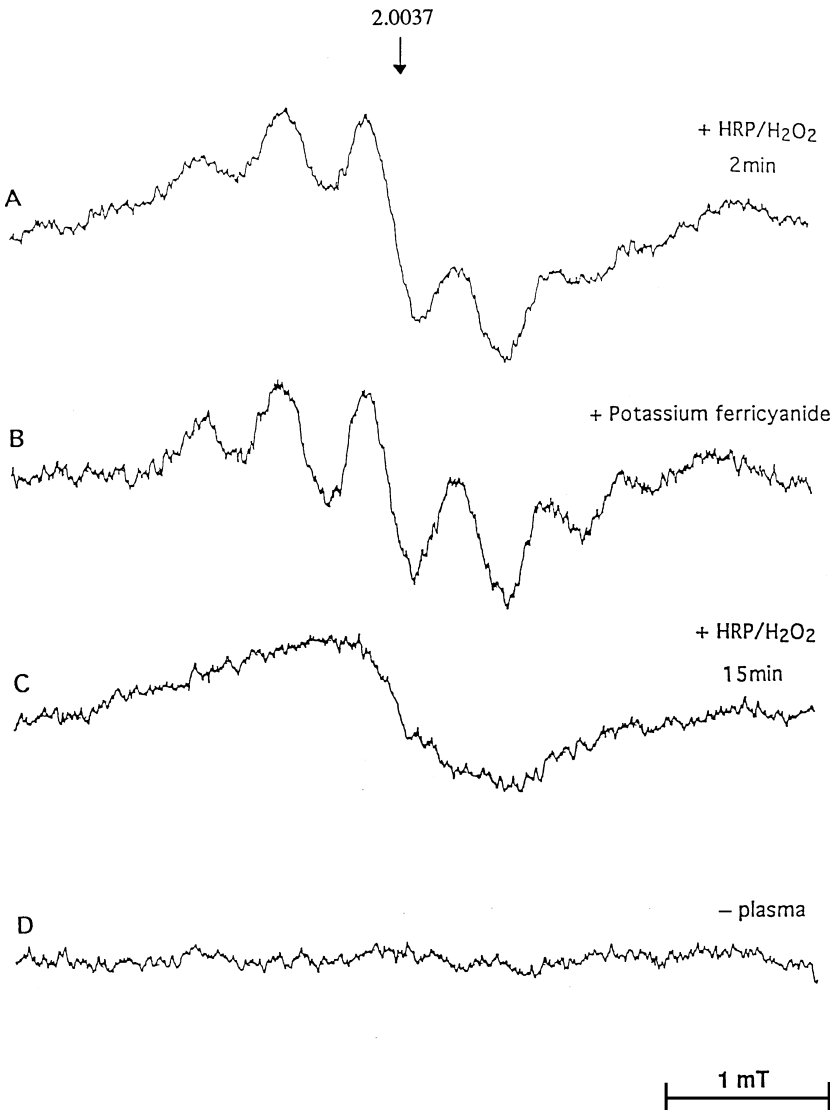

Figure 3 ESR spectra at 37 °C of dialysed plasma treated with HRP/H_2O_2

Spectrum A, 50 μM HRP and 25 mM H_2O_2 were added to dialysed plasma diluted to 80% (v/v) with phosphate buffer/DTPA, pH 7.1, and the reaction was stopped after 2 min by the addition of catalase (500 μM). Spectrum B, potassium ferricyanide (100 μM) was added to dialysed plasma diluted to 80% (v/v) with phosphate buffer/DTPA, pH 7.1. Spectrum C, sample as in spectrum A except that the reaction was stopped with catalase after 15 min. Spectrum D, sample as in spectrum A, but without dialysed plasma. Spectrometer conditions and baseline correction were as described in the legend to Figure 1 except that the number of scans was six in spectrum B.

peptide contains two Tyr residues (Y8Y12), and notably substitution of Tyr with two Gly (G8G12) or two Trp (W8W12) residues completely eliminated the $g = 2.004$ signal (Figure 4, spectra B and C). No radical was detectable if the thymopoietin II peptide was added after peroxynitrite decomposed (Figure 4, spectrum D). Two other peptides containing a central Tyr residue (MEELQDDYEDMMEENC and FNSVSSYSDSRK-SSGDGPDLC) were also able to induce a $g = 2.004$ signal (spectra not shown).

The position of the Tyr residue was also important, since peptides of 15 and 16 amino acids with Tyr at the N-terminus (YAGCLNFFWLTFTSC and YADSGEDFLAEGGGVR) were unable to form a radical that was stable at 37 °C when treated with peroxynitrite (spectra not shown). Moreover, only the Tyr in position 8 of thymopoietin II peptide was responsible for the formation of the $g = 2.004$ signal. Substitution of Tyr-12 with Phe (Y8F12) did not significantly affect the signal intensity, whereas peptides containing only one Tyr in position 12 (F8Y12) or two Phe residues (F8F12) did not form the $g = 2.004$ radical (spectra not shown).

The thymopoietin II peptide generated the $g = 2.004$ signal after treatment with HRP/H_2O_2 (spectrum not shown).

Role of ascorbate, urate, thiols and $^{\bullet}NO$

Several investigations have demonstrated that ascorbate, urate and thiols can perform in blood plasma an important protective role against peroxynitrite-induced oxidative damage [14,17,18]. To examine the role of these antioxidants, plasma was treated with uricase or ascorbate oxidase and challenged with increasing concentrations of peroxynitrite. As shown in Figure 5, these enzymic treatments decreased the minimum concentration of

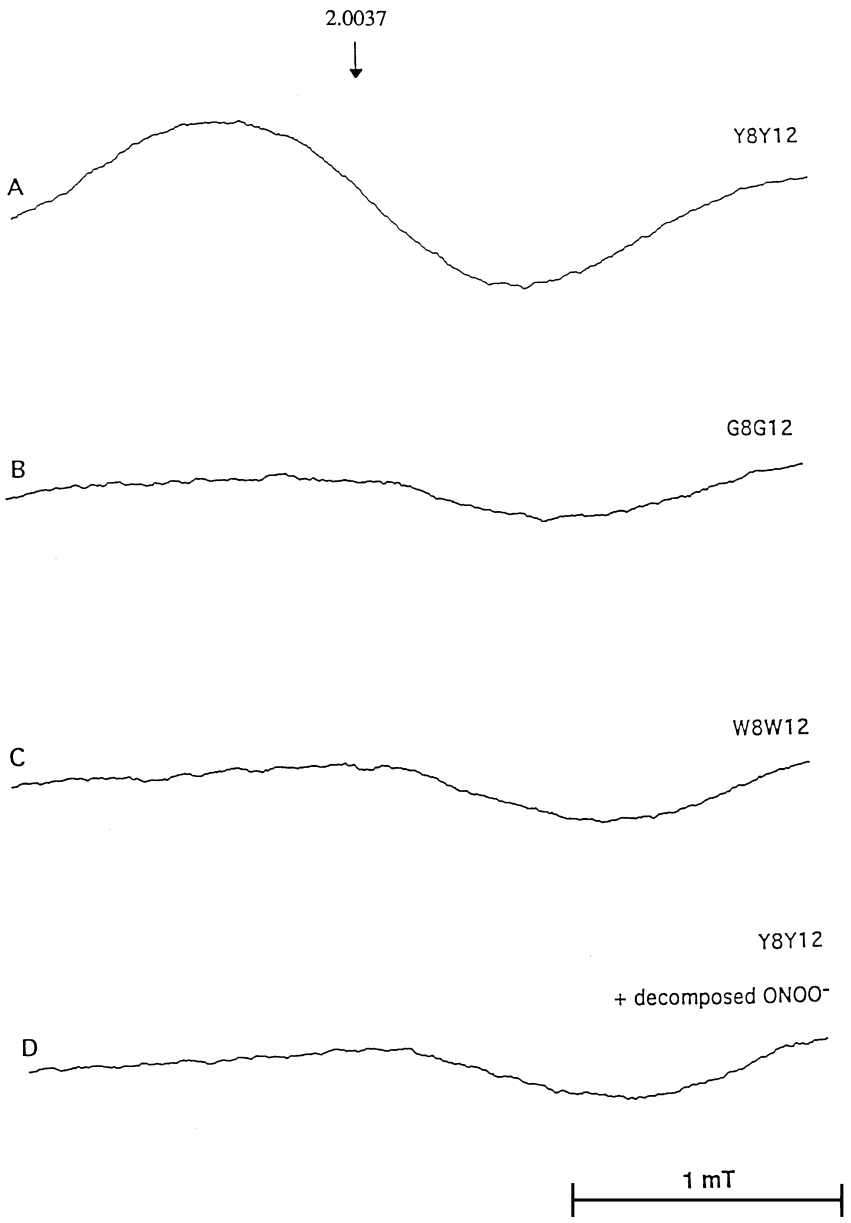

Figure 4 ESR spectra at 37 °C of peptides related to thymopoietin II (fragment 29–41) treated with peroxynitrite

Spectrum A, peroxynitrite (10 mM) was added to 1 mM thymopoietin II peptide (Y8Y12). Spectrum B, peroxynitrite (10 mM) was added to 1 mM thymopoietin II peptide with Tyr residues substituted with Gly residues (G8G12). Spectrum C, peroxynitrite (10 mM) was added to 1 mM thymopoietin II peptide with Tyr residues substituted with Trp residues (W8W12). Spectrum D, peroxynitrite (10 mM) was decomposed in phosphate buffer for 15 s before the addition of 1 mM thymopoietin II peptide (Y8Y12). All samples contained phosphate buffer/DTPA/12.5 mM bicarbonate, pH 7.1. Spectrometer conditions and baseline correction were as described in the legend to Figure 1 except that the number of scans was 15, modulation amplitude was 0.7 mT, gain was 2×10^5 and sweep time was 21 s.

peroxynitrite able to induce the $g = 2.004$ signal, but, at 5 mM peroxynitrite, did not significantly increase the radical(s) yield.

The role of urate and ascorbate was further investigated in plasma devoid of low-molecular-mass antioxidants by dialysis. As shown in Figure 6, in dialysed plasma reconstituted with CO_2/bicarbonate the $g = 2.004$ signal was observed at peroxynitrite concentrations as low as 100 μM. The most evident effects of the addition of urate and ascorbate were to increase the minimum concentration of peroxynitrite able to induce the $g =$ 2.004 signal, whereas, at 5 mM peroxynitrite, these antioxidants did not significantly decrease the radical(s) yield (Figure 6).

Antioxidants can be classified into at least two broad classes: (i) preventive antioxidants, which are able to scavenge the primary oxidant molecule (in this case peroxynitrite or nitrosoperoxycarbonate); (ii) chain-breaking antioxidants, which are able to inhibit free-radical propagation reactions (in this case through the scavenging or 'repair' of the oxidized protein). At the normal plasma concentration, ascorbate, urate and low-molecular-mass thiols cannot react appreciably with peroxy-

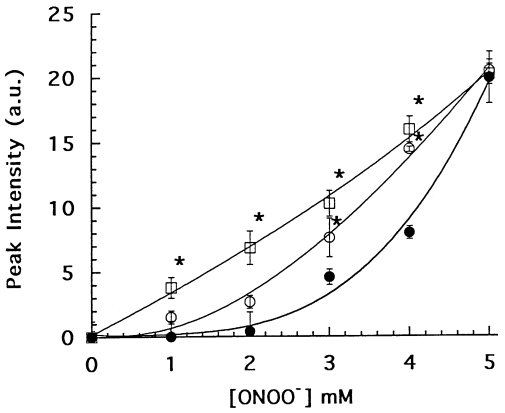

Figure 5 Effects of ascorbate oxidase and uricase treatment on the intensity of $g = 2.004$ radical(s) induced by peroxynitrite in plasma

Peroxynitrite (0–5 mM) was added to plasma diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1, and maintained under 5% CO_2. ●, Untreated plasma; ○, ascorbate oxidase-treated plasma; □, uricase-treated plasma. Plasma was depleted of ascorbate or urate by incubation (30 min under 5% CO_2 at 37 °C) with ascorbate oxidase (0.04 unit/ml) or uricase (0.1 unit/ml) respectively. a.u., arbitrary units. Points represent mean values ± S.D. for three different samples and analysed statistically by Student's *t* test. $^*P \leqslant 0.05$. Spectrometer conditions were as described in the legend to Figure 1.

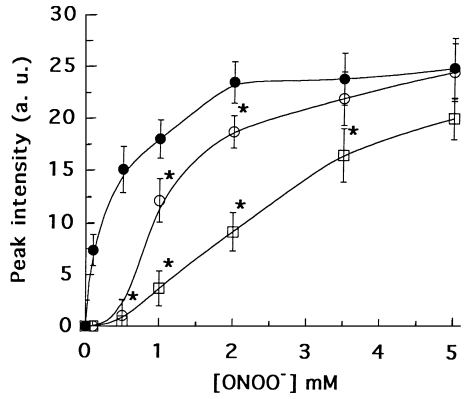

Figure 6 Effects of ascorbate and urate on the intensity of $g = 2.004$ radical(s) induced by peroxynitrite in dialysed plasma reconstituted with bicarbonate

Peroxynitrite (0–5 mM) was added to dialysed plasma reconstituted with bicarbonate and diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1. ●, Without antioxidants; ○, with 25 μM ascorbate; □, with 150 μM urate. Final concentration of bicarbonate was 12.5 mM. a.u., arbitrary units. Points represent mean values ± S.D. for three different samples and analysed statistically by Student's *t* test. $^*P \leqslant 0.04$. Spectrometer conditions were as described in the legend to Figure 1.

nitrite, because the CO_2 present in this fluid rapidly reacts with peroxynitrite leading to the formation of a postulated strong secondary oxidant, the nitrosoperoxycarbonate adduct [14,21]. This species, however, does not react efficiently with ascorbate, urate or thiols, and thus, in physiological buffers, the major effect of CO_2/bicarbonate addition is the protection of the antioxidant. We found that peroxynitrite-dependent oxidation of urate, ascorbate and glutathione in phosphate buffer, pH 7.1, was significantly protected by the addition of CO_2/bicarbonate (D. Pietraforte and M. Minetti, unpublished work). Therefore a direct reaction between these antioxidants and nitrosoperoxycarbonate in plasma will occur to only a minor extent and their

Table 2 Effects of ascorbate, urate, glutathione, cysteine and $^{\bullet}$NO on the intensity of $g = 2.004$ radical(s) induced by peroxynitrite in dialysed blood plasma

Dialysed plasma was reconstituted with 25 mM bicarbonate, diluted to 50% (v/v) with phosphate buffer/DTPA, pH 7.1, and treated with peroxynitrite (1 mM). The antioxidant was added to plasma either before or 15 s after the addition of peroxynitrite. a.u., arbitrary units. Points represent mean values ± S.D. for three different samples and analysed statistically by Student's *t* test. $^*P \leqslant 0.02$ and $^{**}P \leqslant 0.005$ compared with control. Spectrometer conditions were as described in the legend to Figure 1.

Additions	Time after $ONOO^-$(s)	$g = 2.004$ peak intensity (a.u.)
None		19.0 ± 3.0
Ascorbate (25 μM)	0	12.1 ± 1.5*
Ascorbate (25 μM)	15	0.2 ± 0.5**
Glutathione (10 μM)	0	18.5 ± 1.1
Glutathione (100 μM)	0	12.1 ± 1.5*
Cysteine (10 μM)	0	16.3 ± 2.0
Cysteine (100 μM)	0	6.6 ± 2.5**
Cysteine (100 μM)	15	7.0 ± 1.0*
Urate (150 μM)	0	3.7 ± 1.7**
Urate (150 μM)	15	8.7 ± 2.1*
$^{\bullet}$NO (50 μM)	15	12.6 ± 1.0*
$^{\bullet}$NO (100 μM)	15	8.1 ± 1.5**

protective mechanism may be that of chain-breaking antioxidants, a hypothesis compatible with the shape of the inhibition curves shown in Figures 5 and 6.

To examine further the inhibition of plasma antioxidants, we take advantage of the relative stability of $g = 2.004$ radical(s) and the short lifetime of peroxynitrite. In Table 2 there is a comparison of the effects of ascorbate, urate and thiols added to dialysed plasma reconstituted with CO_2/bicarbonate and treated with 1 mM peroxynitrite. The antioxidants were added either before or 15 s after peroxynitrite. The inhibitory effect of ascorbate was stronger if added after peroxynitrite, thus suggesting that its protective mechanism was only through the reduction of $g = 2.004$ radical(s). The spectra of peroxynitrite-treated plasma in which ascorbate was added 15 s after peroxynitrite did not show the presence of $g = 2.004$ signal, but showed only the ascorbyl radical. When ascorbate is added before peroxynitrite, its participation in other 'repair' reactions can explain the decreased inhibitory effect.

Cysteine and glutathione decreased the intensity of the $g = 2.004$ signal at 100 μM, but at the concentration usually found in plasma (about 5–10 μM [37]) did not significantly affect radical yield. However, the effect of Cys at 100 μM was comparable if added before or after peroxynitrite (Table 2), suggesting that its protective mechanism was only or mainly through the reduction of $g = 2.004$ radical(s).

Urate was more effective in the inhibition of the $g = 2.004$ signal if added before peroxynitrite, although approx. 57% of the total urate inhibition appears to be due to a direct reduction of $g = 2.004$ radical(s) (Table 2). The stronger inhibition observed with urate added before peroxynitrite may be due to both radical(s) reduction and scavenging of peroxynitrite (or nitrosoperoxycarbonate), but accurate kinetic studies are needed to support this interpretation.

$^{\bullet}$NO is a very efficient scavenger of tyrosyl radical in ribonucleotide reductase and of tyrosyl radical generated from free

Tyr [38,39]. As shown in Table 2, $^{\bullet}NO$ (50–100 μM) added 15 s after peroxynitrite efficiently scavenged the $g = 2.004$ radical(s).

DISCUSSION

Treatment of human blood plasma with peroxynitrite induces the oxidation of several protein residues including Cys, Trp and Tyr [13,14,17,18]. The observed formation of dityrosine strongly suggests that peroxynitrite is able to induce tyrosyl radicals [11,12].

Using the direct ESR technique at 37 °C, we found that peroxynitrite induced a singlet radical signal centred at $g = 2.004$ arising from plasma proteins. Although several amino acid radicals, including those generated from Cys, Trp and Tyr, have been detected directly or indirectly by ESR [17,18,40–43], only the protein tyrosyl and tryptophanyl radicals have been shown to form around $g = 2.004$ a singlet (tyrosyl radicals) or a doublet (tyrosyl and tryptophanyl radicals) detectable by direct ESR at room temperature [30–33].

Although peroxynitrite added to blood plasma is known to induce both thiyl [17] and tryptophanyl [18] protein radicals, their involvement in the $g = 2.004$ signal can be excluded by the following considerations. First, the thiol-alkylating agent NEM was unable to decrease the intensity of the $g = 2.004$ signal and most thiyl radicals have a different g value ($g = 2.01$) [44]. Secondly, tryptophanyl radicals induced by peroxynitrite in plasma proteins could be trapped by DBNBS and decayed in 3–5 s, whereas the $g = 2.004$ signal showed a life time of 20–25 min and was not trapped by DBNBS. Thirdly, tryptophanyl radicals formed by treating Trp-containing peptides with peroxynitrite were not sufficiently stable to be detectable by direct ESR at 37 °C. These results together with our previously published data [18] suggest that peroxynitrite induced in plasma at least two different radical species: (i) the relatively short-lived tryptophanyl radical(s); and (ii) the long-lived $g = 2.004$ radical(s) detected by direct ESR. It should be noted that all direct ESR spectra at 37 °C of this study were recorded 2 min after the addition of peroxynitrite, that is when the tryptophanyl radicals completely decayed.

Tryptophanyl radicals, however, could be intermediates in the formation of the $g = 2.004$ radical(s); charge transfers from tryptophanyl radicals to tyrosine residues in peptides are favourable processes [45]. However, the finding that alkylation of thiol groups did not affect the $g = 2.004$ radical(s) intensity suggests that charge transfer from thiyl to tyrosine residues is an unlikely process in peroxynitrite-treated plasma proteins.

The hypothesis that the $g = 2.004$ signal is centred on protein Tyr residues is based on the following experimental findings: (i) the $g = 2.004$ signal was inhibited by the tyrosyl scavenger hydroxyurea; (ii) a signal with similar stability and g value was induced in plasma by the known tyrosyl-radical-generating system HRP/H_2O_2; (iii) only synthetic peptides containing an internal Tyr residue generated a $g = 2.004$ signal stable at 37 °C. Nevertheless, since the $g = 2.004$ region of the ESR spectra is particularly crowded, we cannot exclude the possibility that other as yet unidentified radicals can also be responsible for the signal. We suggest, however, that Tyr residues are at least the major radical sources of the peroxynitrite-dependent $g = 2.004$ signal.

This conclusion is further supported by previous studies showing that the stability of tyrosyl radicals can be greatly enhanced by protein structure. The tyrosyl radical of the free amino acid is very short lived and is only detectable by ESR using fast flow [46]. In contrast, tyrosyl radicals produced by protein oxidation are stable at room temperature [47] and in some enzymes are extremely stable [32]. The stability of tyrosyl radicals in proteins may depend on a hidden or a more exposed position of Tyr residues [48]. According to this hypothesis, we found that proteolysis of plasma proteins eliminated the $g = 2.004$ signal, possibly by exposing Tyr residues and thus decreasing the stability of tyrosyl radicals. Moreover, we found that, when treated with peroxynitrite, 13-amino-acid peptides with Tyr in position 8 were able to form a radical sufficiently stable to be detectable by direct ESR at 37 °C, whereas the same peptide with a Tyr near the C-terminus or other peptides of even higher molecular mass (14 or 16-amino acids) but with Tyr as the first amino acid did not form a stable radical.

To our knowledge this is the first report showing that peroxynitrite induces a $g = 2.004$ signal assigned to a radical centred on protein Tyr residues. This study may be of help in the characterization of similar radicals detected in haemoproteins or in tissues by direct ESR at room temperature [29,42,49].

Peroxynitrite-induced tyrosyl radicals are either one-electron oxidation products of peroxynitrite itself or radicals induced by secondary oxidants produced by the reaction of peroxynitrite with other plasma components. CO_2 is an example of a plasma component that can substantially enhance some peroxynitrite-dependent oxidative reactions [16,19–21]. The possibility exists that other as yet unknown plasma components could also increase peroxynitrite-dependent tyrosyl radical formation.

The mechanism of CO_2-catalysed oxidation of Tyr by peroxynitrite has recently been elucidated. The reaction of peroxynitrite anion with CO_2 leads to the formation of a nitrosoperoxycarbonate adduct, which, in its activated form [16,20,21], is more reactive in Tyr oxidation than peroxynitrite itself. The products of Tyr oxidation are the tyrosyl and $^{\bullet}NO_2$ radicals, which can further react together to form 3-nitrotyrosine. The nitration of Tyr, however, may also proceed through a non-radical mechanism as proposed by others [14]. Two tyrosyl radicals can also react together leading to the formation of 3,3′-dityrosine, a product that is less probable in proteins because of steric hindrance. We found that the addition of CO_2/bicarbonate enhanced several one-electron oxidation reactions of peroxynitrite, leading to increased formation of thiyl [50], tryptophanyl [18] and tyrosyl radicals (this work). Since peroxynitrite can be either a one-electron or a two-electron oxidant [9] and the two pathways can compete, one function of CO_2 could be to direct the oxidation pathways of peroxynitrite towards the one-electron oxidation. Moreover, dissolved CO_2 is in equilibrium with CO_2 in the gas phase ($\sim 5\%$, v/v) and thus air-exposed samples may lose some CO_2 to the gas phase. Indeed, we found that, in air-exposed samples, the effect of CO_2 loss on tyrosyl radical yield is relatively small ($+30\%$) compared with the total effect of CO_2/bicarbonate ($+270\%$).

It has previously been demonstrated that tyrosyl radicals play essential roles in mediating catalytic transformations in a number of different enzymes [41] and in haemoprotein-mediated oxidation [42,47,51]. Interestingly, $^{\bullet}NO$ was a very efficient scavenger of protein tyrosyl radicals, and peroxynitrite can promote the formation of tyrosyl radicals in proteins. These observations suggest the interesting possibility that NO and peroxynitrite at physiological concentrations may regulate enzymic catalysis of specific cellular targets. At high peroxynitrite concentrations, however, the oxidative damage may be prevalent, resulting in changes in protein structure and conformation, and loss of function.

The $g = 2.004$ signal could be detected by direct ESR at 37 °C in plasma only at concentrations of peroxynitrite higher than 2 mM. Although activated human neutrophils can generate peroxynitrite concentrations of up to 0.2 nmol/min per 10^6 cells [52],

it is unlikely that *in vivo*, even under strong activation of phagocytic cells, peroxynitrite concentrations as high as 2–3 mM could be achieved. However, our results show that protein Tyr residues can intercept both peroxynitrite and nitrosoperoxycarbonate, whereas low-molecular-mass antioxidants were unable (ascorbate and thiols) or showed a limited ability (urate) to react directly with these oxidants, consistent with results reported previously by others [13,14,21]. Low-molecular-mass antioxidants, however, were able to scavenge the tyrosyl radicals (chain-breaking antioxidants), thus decreasing the oxidation of protein tyrosine residues. These results imply that at peroxynitrite concentrations of 2 mM and below, protein tyrosyl radicals are formed in plasma, although their subsequent reduction by antioxidants prevents radical detection by direct ESR at 37 °C. In plasma devoid of low-molecular-mass antioxidants and reconstituted with CO_2/bicarbonate, we observed that tyrosyl radicals were detectable at peroxynitrite concentrations as low as 100 μM, a concentration that could conceivably be achieved *in vivo*. This mechanism of inhibition by antioxidants in plasma may be a means of preserving the physiological functions of peroxynitrite, for example its cytotoxic activity or other as yet unknown functions.

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