One-electron oxidation pathway of thiols by peroxynitrite in biological fluids: bicarbonate and ascorbate promote the formation of albumin disulphide dimers in human blood plasma

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Recent studies have shown that peroxynitrite oxidizes thiol groups through competing one- and two-electron pathways. The two-electron pathway is mediated by the peroxynitrite anion and prevails quantitatively over the one-electron pathway, which is mediated by peroxynitrous acid or a reactive species derived from it. In CO₂-containing fluids the oxidation of thiols might follow a different mechanism owing to the rapid formation of a different oxidant, the nitrosoperoxycarbonate anion (ONOO₂⁻). Here we present evidence that in blood plasma peroxynitrite induces the formation of a disulphide cross-linked protein identified by immunological (anti-albumin antibodies) and biochemical criteria (peptide mapping) as a dimer of serum albumin. The albumin dimer did not form in plasma devoid of CO₂ and its formation was enhanced by ascorbate. However, analysis of thiol groups showed that reconstituting dialysed plasma with NaHCO₃ protected protein thiols against the

oxidation mediated by peroxynitrite and that the simultaneous presence of ascorbate provided further protection. Ascorbate alone did not protect thiol groups from peroxynitrite-mediated oxidation. ESR spin-trapping studies with *N*-t-butyl- α -phenylnitrone (PBN) revealed that peroxynitrite induced the formation of protein thiyl radicals and their intensity was markedly decreased by plasma dialysis and restored by reconstitution with NaHCO₃. PBN completely inhibited the formation of albumin dimer. Moreover, the addition of iron-diethyldithiocarbamate to plasma demonstrated that peroxynitrite induced the formation of protein *S*-nitrosothiols and/or *S*-nitrothiols. Our results are consistent with the hypothesis that NaHCO₃ favours the one-electron oxidation of thiols by peroxynitrite with formation of thiyl radicals, [•]NO₂, and RSNO_x. Thiyl radicals, in turn, are involved in chain reactions by which thiols are oxidized to disulphides.

INTRODUCTION

Tissue injury in several human diseases is due partly to the formation by activated cells of reactive oxygen and nitrogen species. Recently, however, there has been considerable interest in the possible toxicity of peroxynitrite, the product of the reaction of nitric oxide and the superoxide anion:

The peroxynitrite anion (ONOO⁻) is a relatively stable species capable of oxidizing biological targets such as methionine [1] and thiols [2], through second-order kinetics involving a two-electron transfer. This two-electron oxidation pathway has been implicated as a major mechanism in the peroxynitrite-mediated inactivation of enzymes.

The reactivity of the peroxynitrite anion is, however, strongly dependent on environmental pH: at physiological to acidic pH the anion is in equilibrium with its conjugated acid, peroxynitrous acid (ONOOH, p*K*_a 6.8 at 37 °C). [The IUPAC recommended names for ONOO⁻, ONOOH and [•]NO are: oxoperoxonitrate (1-), hydrogen oxoperoxonitrate and nitrogen monoxide respectively]. In contrast with the relatively stable ONOO⁻, ONOOH is unstable and rapidly decays to nitrate with a half-life of at most 1 s at physiological pH and temperature. During the decay process, peroxynitrous acid can either directly oxidize some substrates by a second-order reaction (direct oxidation

mechanism) or be converted to an activated secondary species derived from it, ^{*}ONOOH [3–5], which oxidizes the substrates through a reaction that is first-order in peroxynitrite and zero-order in the substrate (indirect oxidation mechanism) [6]. The direct oxidation mechanism can take place either by one- or two-electron oxidation pathways, whereas the indirect oxidation mechanism can oxidize the target by a one-electron oxidation pathway or through hydroxylation [1,3,6].

ESR spectroscopy is particularly useful for detecting the formation of radicals through one-electron transfers or hydrogen abstraction processes. The ESR spin-trapping technique applied to the reactions between peroxynitrite and thiols shows the formation of thiyl radicals [7,8]. These findings suggest that at physiological pH the oxidation of thiols by peroxynitrite (the term ‘peroxynitrite’ without other specifications refers to the sum of ONOO⁻, ONOOH and ^{*}ONOOH) occurs, at least partly, through the one-electron oxidation pathway. Recently Quijano et al. [8] suggested that the oxidation of cysteine and glutathione induced by peroxynitrite under physiological conditions occurs throughout competing one- and two-electron oxidation pathways, with the latter induced by ONOO⁻ and prevailing quantitatively over the one-electron oxidation pathway, which is induced by ONOOH and/or ^{*}ONOOH.

However, extracellular and intracellular fluids contain relatively high concentrations of bicarbonate (approx. 25 mM in blood plasma and 12 mM in intracellular fluids), which would be expected to change the oxidation behaviour of peroxynitrite

Abbreviations used: DETC, diethyldithiocarbamate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTPA, diethylenetriaminepenta-acetic acid; GSNO, S-nitroso-L-glutathione; GSNO₂, S-nitro-L-glutathione; NEM, *N*-ethylmaleimide; PBN, *N*-t-butyl- α -phenylnitrone; RSNO, S-nitrosothiols; RSNO₂, S-nitrothiols; TBS, Tris-buffered saline; Trolox C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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substantially. It has been demonstrated that CO_2 (approx. 1.3 mM in blood plasma, in equilibrium with bicarbonate) is the species that reacts with peroxynitrite and forms a reactive adduct, the nitrosoperoxycarbonate anion [9,10]:

This postulated adduct and/or reactive intermediate(s) derived from it [11] shows oxidative properties similar to, but not identical with, those of peroxynitrite (in this paper reactive intermediate(s) derived from nitrosoperoxycarbonate are referred to as X). CO_2 is a catalyst of the decomposition of peroxynitrite, but the reactive intermediate(s) formed in this catalytic reaction redirect the oxidation pathways of peroxynitrite by increasing or down-modulating the oxidation of some targets [10,11]. These considerations raise the question of the mechanism through which peroxynitrite oxidizes thiols in CO_2 -containing biological systems because in some fluids, such as blood plasma, peroxynitrite reacts with dissolved CO_2 before reacting with plasma thiols [11].

In this study we observed that in blood plasma peroxynitrite induced the formation of a disulphide-mediated protein-protein cross-linking, which was identified as a dimer of serum albumin. The formation of this albumin dimer was not observed in CO_2 -free buffers and the addition of ascorbate increased dimer formation only in CO_2 -containing buffers. Interestingly, the effect of CO_2 and of ascorbate was to decrease the peroxynitrite-mediated oxidation of thiols. Our results are explained by a CO_2 -induced scavenging of ONOO^- and a shift of thiol oxidation towards the one-electron oxidation pathway.

MATERIALS AND METHODS

Materials

Proteins purified from human plasma (α_1 -antitrypsin, α_2 -HS-glycoprotein, apo-transferrin, serum albumin), diethyldithiocarbamic acid sodium salt (DETC), *N*-ethylmaleimide (NEM), trypsin, α -chymotrypsin and the monoclonal anti-(human serum albumin) antibodies were obtained from Sigma Biochemicals (St. Louis, MO, U.S.A.). Ellman's reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analogue of vitamin E (Trolox C), were obtained from Aldrich Chemicals (Milan, Italy). The spin trap *N*-t-butyl- α -phenylnitron (PBN) was obtained from OMRP Spin Trap Source (Oklahoma City, OK, U.S.A) and *S*-nitroso-L-glutathione (GSNO) was from Alexis Corporation (Laufelfingen, Switzerland). NO_2BF_4 was obtained from Fluka (Buchs, Switzerland). *S*-nitro-L-glutathione (GSNO_2) was synthesized by the addition of NO_2BF_4 (final concentration 0.2 M) to 50 mM glutathione dissolved in 0.5 M phosphate, pH 7.1. The NO_2BF_4 was prepared immediately before use as a 2 M solution in 0.1 M HCl. The absorbance at 338 nm was used to estimate the formation of GSNO_2 (ϵ_{338} 980 $\text{M}^{-1}\cdot\text{cm}^{-1}$).

Synthesis of peroxynitrite

Peroxynitrite was synthesized by reacting nitrite with acidified hydrogen peroxide, as described by Radi et al. [2], and treated with manganese dioxide (1 mg/ml, 30 min at 4 °C) to eliminate excess H_2O_2 . Manganese dioxide was removed by centrifugation (for 5 min at 4 °C and 15000 *g*) and filtration (0.45 μm ; Millipore, Molsheim, France). Freeze fractionation (−80 °C) of the peroxynitrite solution formed 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 (ϵ_{302} 1670 $\text{M}^{-1}\cdot\text{cm}^{-1}$). The solution of peroxynitrite

was stored at −20 °C for 1–2 weeks 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 [dissolved CO_2 is in equilibrium with CO_2 in the gas phase, approx. 5% (v/v)], samples (0.8–0.9 ml) were maintained in closed vials (1 ml) and used within 6–8 h of collection. To remove low-molecular-mass compounds, plasma (2–4 ml) was subjected to extensive dialysis (molecular mass cut-off 10000 Da) against 10 litres of 0.15 M NaCl/5 mM phosphate (pH 7.4) at 4 °C. Dialysed plasma reconstituted with NaHCO_3 was maintained in closed vials under air/ CO_2 (19:1). Plasma thiol groups were alkylated by incubation with 5 mM NEM for 30 min at 37 °C in closed vials under air/ CO_2 (19:1). Reconstitution of plasma with urate was performed by adding a small volume of urate (10 mM in 0.1 M NaOH) and correcting for the pH change with HCl.

Gel electrophoresis and Western blot analysis

Peroxynitrite-treated plasma (6 μl) was dissolved in 4 × loading buffer [12], heated in boiling water for 5 min and separated by SDS/PAGE [5–15% (w/v) gel]. Proteins on gels were stained with Coomassie Blue R-250. Stained bands were quantified by densitometric analysis (2400 Gelscan XL Laser Densitometer; LKB, Broma, Sweden). For immunoblotting analysis proteins separated by SDS/PAGE were transferred to nitrocellulose paper (Shleicher and Schuell) at 35 V overnight. Blots were then washed with 0.15 M NaCl/50 mM Tris/HCl (pH 7.4) (Tris-buffered saline, TBS) and incubated overnight with 5% (w/v) non-fat dried milk in TBS. Washed nitrocellulose filters were incubated for 1 h at room temperature with monoclonal anti-albumin antibody (0.9 $\mu\text{g}/\text{ml}$) dissolved in phosphate buffer. After extensive washes in TBS/0.05% (v/v) Tween-20, blots were incubated at room temperature for 1 h with anti-(mouse IgG) horseradish peroxidase-conjugated secondary antibody (1:5000 dilution). Bands were analysed in the presence of H_2O_2 by chemiluminescence (Luminol/Enhancer solution; Pierce, IL, U.S.A.) and exposure of XAR5 film (Kodak, Rochester, NY, U.S.A.).

HPLC gel filtration of plasma proteins

Plasma proteins were subjected to gel filtration in 25 mM NaHCO_3 /150 mM phosphate buffer/0.1 mM diethylene-triaminepenta-acetic acid (DTPA) (pH 7.4) with a Bio-Sil TSK-125 column (Bio-Rad, Richmond, CA, U.S.A.). The eluted fractions were measured for absorbance at 280 nm and treated with 5 mM peroxynitrite. Each fraction (40 μl) was separated by SDS/PAGE [5–15% (w/v) gel] under non-reducing conditions.

Purification of p115 and proteolytic treatments

The p115 protein and purified human serum albumin were electro-eluted from the gel in 25 mM Tris/HCl/192 mM glycine/0.1% SDS (pH 8.5) for 4 h with the 'electro eluter' from Bio-Rad. Eluted proteins were treated with α -chymotrypsin (0.07 mg/ml) or trypsin (0.07 mg/ml) for 30 min at 37 °C. Proteolysis was stopped by the addition of 4 × loading buffer [12] and being heated in boiling water for 5 min. Peptides were separated by SDS/PAGE [10–20% (w/v) gel].

Determination of plasma thiol groups

The reaction of DTNB with thiol groups was used to determine the concentration of plasma thiols [13]. DTNB reacts with protein thiol groups at pH 8.0 to produce a highly coloured anion with a maximum absorbance at 412 nm (ϵ_{412} 13600 M⁻¹·cm⁻¹). Spectra were obtained with a Lambda 17 UV/VIS Spectrophotometer (Perkin Elmer, Norwalk, CT, U.S.A.).

Treatment with peroxynitrite

Peroxynitrite was added as a bolus to samples buffered with an equal volume of phosphate buffer (final phosphate concentration 150 mM) and mixed immediately. The pH of samples was measured after each incubation and found to change only slightly after the addition of peroxynitrite (pH 7.4 ± 0.1). To avoid the metal-catalysed oxidation of peroxynitrite [14], buffers were treated extensively with Chelex 100 (Bio-Rad) and all samples contained 0.1 mM DTPA. 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 (reversed order of addition).

ESR spectroscopy

Spectra were measured on a Bruker ECS 106 spectrometer (Bruker, Rheinstetten, Germany). The samples were drawn into a gas-permeable Teflon tube of 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 at a temperature of 37 °C with a variable-temperature unit (ER4111VT, Bruker). The dead time of sample preparation and ESR analysis was exactly 2 min after the last addition, which was peroxynitrite except in experiments with decomposed peroxynitrite. To extract an integrated relative area, all ESR spectra were corrected for baseline drift by a linear function and double-integrated with the software supplied by Bruker (ESP 1600 data system). The method for quantification of *S*-nitrosothiols (RSNO) was essentially as described by Tsuchiya et al. [15], with the following modifications: the solubilization of iron-DETC (500 µM) was performed by plasma proteins instead of albumin, GSNO (5–50 µM) was used as standard to prepare the calibration curve, and spectra were recorded in air at 37 °C.

RESULTS

Peroxynitrite induces in plasma a disulphide dimer of serum albumin

Figure 1(A) shows an SDS/PAGE analysis under non-reducing conditions of human blood plasma proteins treated with 0–3 mM peroxynitrite. The major change induced by peroxynitrite was an increase in a band with an apparent molecular mass of 115 kDa (hereafter referred to as p115). A band protein with electrophoretic mobility superimposable on p115 was present at low levels in most blood plasma samples analysed; p115 intensity increased when plasma was treated with peroxynitrite at concentrations above 100 µM. Peroxynitrite did not modify the electrophoretic pattern of plasma proteins analysed by SDS/PAGE under reducing conditions (Figure 1B), suggesting the involvement of disulphide-mediated products in the formation of p115. This possibility was strengthened by the observation that pre-treatment of plasma with the thiol-alkylating agent NEM before

Figure 1 Effects of peroxynitrite on the electrophoretic pattern of human blood plasma proteins

(A) Electrophoretic pattern under non-reducing conditions of proteins from plasma diluted to 50% (v/v) with phosphate buffer/DTPA buffer (pH 7.1) and treated with 0, 0.1, 0.5, 1 or 3 mM peroxynitrite or 3 mM decomposed peroxynitrite (lanes 2–7 respectively). Lane 1, molecular mass standards (molecular masses in kDa are shown at the left). (B) Electrophoretic pattern under reducing conditions of plasma proteins treated with 0 or 5 mM peroxynitrite or 5 mM decomposed peroxynitrite (lanes 1–3 respectively). Proteins were separated by SDS/PAGE [5–15% (w/v) gel] and revealed by Coomassie Blue R-250 staining.

peroxynitrite treatment completely inhibited the formation of p115 (results not shown). Decomposed peroxynitrite did not modify the electrophoretic pattern of blood plasma (Figure 1A), excluding the possibility that the formation of p115 was due to contaminating substances present in the peroxynitrite solution or to stable peroxynitrite end products (nitrite and nitrate).

These results suggest that peroxynitrite induced either the aggregation of one or more proteins of lower molecular mass

Figure 2 Treatment with peroxynitrite of plasma proteins separated by HPLC gel filtration

Plasma proteins were separated by gel-filtration chromatography in 25 mM NaHCO₃/150 mM phosphate buffer (pH 7.4). Eluted fractions were treated individually with 5 mM peroxynitrite, separated by SDS/PAGE [5–15% (w/v) gel] and revealed by Coomassie Blue R-250 staining. The formation of p115 is indicated by the arrow.

Figure 3 Comparison of the electrophoretic pattern (A) and Western blot analysis (B) of p115 with human serum albumin

(A) Electrophoretic pattern under non-reducing conditions of plasma proteins (lanes 1 and 2) and serum albumin (lanes 3 and 4) treated with 0 or 5 mM peroxynitrite respectively. Purified p115 protein under non-reducing (lane 5) or reducing conditions (lane 6) is compared with serum albumin under reducing conditions (lane 7). Treatment with peroxynitrite was as described in the legend to Figure 1. Proteins were separated by SDS/PAGE [5–15% (w/v) gel] and revealed by Coomassie Blue R-250 staining. (B) Purified p115, human serum albumin, α_2 -HS-glycoprotein and α_1 -antitrypsin were subjected to SDS/PAGE [5–15% (w/v) gel] under non-reducing conditions and transferred to nitrocellulose filter for immunoblot analysis with anti-albumin antibodies (lanes 1–4 respectively).

through the formation of disulphide cross-linking or the fragmentation of a protein of higher molecular mass containing a disulphide bond. To discriminate between these two possibilities, plasma proteins were separated by HPLC gel filtration and eluted fractions were challenged individually with peroxynitrite. Because bicarbonate is one of the major buffers of plasma and is important for the formation of p115 (see below), 25 mM NaHCO_3 was present in the elution buffer. As shown in Figure 2, SDS/PAGE of eluted fractions treated with peroxynitrite showed the formation of the p115 band in proteins corresponding to the major peak of the chromatographic profile. This result indicates that p115 is not a fragment of a protein with higher molecular mass but is formed by S–S cross-linking from one or more proteins of lower molecular mass. The principal proteins present in this peak were transferrin (90 kDa), serum albumin (68 kDa), α_1 -antitrypsin (52 kDa) and α_2 -HS-glycoprotein (50 kDa). These purified proteins were tested individually with peroxynitrite in a buffer containing NaHCO_3 . Transferrin, α_1 -antitrypsin and α_2 -HS-glycoprotein were unable to form the p115 band (results not shown), whereas serum albumin induced a band with an apparent molecular mass of 115 kDa (Figure 3). These results were expected, at least for transferrin, as this protein is devoid of thiol groups, whereas serum albumin contains a single reduced cysteine residue that has been shown to be accessible to oxidizing agents [16–18]. The p115 was eluted from the gel of peroxynitrite-treated plasma and subjected to SDS/PAGE. As illustrated in Figure 3(A), under reducing conditions purified p115 produced a band with the same electrophoretic mobility as serum albumin. Assuming that p115 can bind Coomassie Blue R-250 to an extent comparable to that of monomeric albumin, we estimated by densitometric analysis that the amount of p115 generated by 1 mM peroxynitrite in plasma diluted to 50% (v/v) was approx. $6.0 \pm 1.0 \mu\text{M}$ (i.e. approx. 2% of serum albumin).

The hypothesis that p115 might be a disulphide dimer of serum albumin was further tested with a monoclonal anti-albumin antibody. Western blot analysis showed a positive reaction with albumin and with p115 eluted from the gel, whereas this antibody did not react with purified α_1 -antitrypsin or α_2 -HS-glycoprotein (Figure 3B). To prove that p115 was a dimer of serum albumin,

Figure 4 Proteolytic treatment of p115 and comparison with human serum albumin

Purified p115 (lane 2) and human serum albumin (lane 3) were subjected to proteolysis with trypsin (lanes 4 and 5) or α -chymotrypsin (lanes 6 and 7) as described in the Materials and methods section. Lane 1, molecular mass standards (molecular masses in kDa are shown at the left). Peptides were separated under reducing conditions by SDS/PAGE [10–20% (w/v) gel] and revealed by Coomassie Blue R-250 staining.

p115 formed in peroxynitrite-treated plasma and serum albumin were eluted from the gel, treated with trypsin or α -chymotrypsin and subjected to SDS/PAGE. As shown in Figure 4, identical peptides were generated by the proteolysis of p115 and serum albumin.

Generation of p115 depends on NaHCO_3 and is enhanced by ascorbate

To investigate the role of CO_2 and that of low-molecular-mass antioxidants, blood plasma was dialysed, treated with peroxynitrite and analysed by SDS/PAGE. The ratio between the area of p115 and that of transferrin in Coomassie Blue-stained gel was used to compare different samples. To obtain relative quantitative data and to keep differences between individual plasma samples to a minimum, data were expressed as percentages of the control

Table 1 Effect of peroxynitrite, NaHCO_3 and plasma antioxidants on the intensity of p115

Plasma (PI), dialysed plasma (D PI) or dialysed plasma reconstituted with 25 mM NaHCO_3 (D PI/ NaHCO_3) was diluted to 50% (v/v) with phosphate buffer/DTPA (pH 7.1) before the addition of peroxynitrite; proteins were separated by SDS/PAGE [5–15% (w/v) gel]. The intensity of p115 was evaluated by the ratio between the area of p115 and that of transferrin and expressed as a percentage of its control (plasma treated with decomposed peroxynitrite). Abbreviation: AH, ascorbic acid. Results are means $\pm$ S.D. for three different samples.

Sample	p115 intensity (% of control)
PI	97 $\pm$ 21
PI + 0.1 mM ONOO^-	159 $\pm$ 50
PI + 0.5 mM ONOO^-	310 $\pm$ 12
PI + 1.0 mM ONOO^-	490 $\pm$ 44
PI + 3.0 mM ONOO^-	467 $\pm$ 44
PI + 3.0 mM ONOO^- (decomposed)	100 $\pm$ 17
PI + 3.0 mM ONOO^- + 0.5 mM glutathione	572 $\pm$ 25
PI + 3.0 mM ONOO^- + 0.6 mM bilirubin	627 $\pm$ 27
PI + 3.0 mM ONOO^- + 30 mM PBN	101 $\pm$ 18
D PI	97 $\pm$ 15
D PI + 5 mM ONOO^-	98 $\pm$ 19
D PI/ NaHCO_3 + 5 mM ONOO^-	148 $\pm$ 6
D PI/ NaHCO_3 + 5 mM ONOO^- (decomposed)	100 $\pm$ 18
D PI/ NaHCO_3 + 5 mM ONOO^- + 50 μM AH	173 $\pm$ 9
D PI/ NaHCO_3 + 5 mM ONOO^- + 0.5 mM AH	492 $\pm$ 53
D PI/ NaHCO_3 + 5 mM ONOO^- + 1 mM AH	1291 $\pm$ 15
D PI + 5 mM ONOO^- + 0.5 mM AH	97 $\pm$ 25

plasma (treated with decomposed peroxynitrite). The addition of up to 5 mM peroxynitrite to dialysed plasma did not increase the intensity of p115 as it did in whole plasma. The intensity of p115 increased if dialysed plasma was reconstituted with NaHCO_3 (Table 1) before the peroxynitrite treatment. However, in these samples the intensity of p115 was low when compared with that of whole plasma treated with the same concentration of peroxynitrite (Table 1). This finding suggests that other plasma components might be involved in its formation.

The intensity of peroxynitrite-dependent p115 was not affected by the addition of urate (100–1000 μM), Trolox C (5–500 μM), glutathione (100–1000 μM) or bilirubin (100–600 μM) to NaHCO_3 /dialysed plasma (results not shown). In contrast, when ascorbate was added to NaHCO_3 /dialysed plasma the intensity of p115 increased significantly. Densitometric analyses showed that the effect of ascorbate was dose-dependent between 50 and 1000 μM (Table 1). It is noteworthy that dialysed plasma reconstituted with ascorbate but without the addition of NaHCO_3 did not increase the intensity of p115 after peroxynitrite treatment (Table 1). As expected, when the antioxidants tested in this study were added to plasma not treated with peroxynitrite they were unable to modify the p115 intensity (results not shown).

Although glutathione and bilirubin were inactive in NaHCO_3 /dialysed plasma, when they were added to whole plasma treated with peroxynitrite the p115 intensity increased (Table 1).

Peroxynitrite induces the formation of thiol radicals of plasma proteins

Previously published ESR studies have shown that, in blood plasma, peroxynitrite induces the formation of protein thiol radicals [7]. To clarify the role of CO_2 in peroxynitrite-dependent thiol oxidation in plasma we investigated this topic further with the nitron spin trap PBN. This spin trap does not produce adducts with peroxynitrite or with uric acid [7] and the ESR spectrum therefore contains only a strongly immobilized spec-

Figure 5 ESR spectra of plasma treated with peroxynitrite in the presence of PBN

Spectrum a, peroxynitrite (1 mM) added to plasma diluted to 50% (v/v) with phosphate buffer/DTPA buffer (pH 7.1) in the presence of 30 mM PBN; spectrum b, as in spectrum a, except that 1 mM peroxynitrite was decomposed before the addition of plasma-PBN; spectrum c, as in spectrum a, but with dialysed plasma; spectrum d, as in spectrum c, but with dialysed plasma containing 25 mM NaHCO_3 ; spectrum e, as in spectrum c, but with dialysed plasma pretreated with NEM. Spectrometer conditions were as follows: modulation amplitude, 0.1 mT; time constant, 328 ms; scan rate, 0.12 mT/s; number of scans, 2; gain, 2.5×10^5 ; modulation frequency, 100 kHz; microwave frequency, 9.4 GHz; microwave power, 20 mW.

trum as shown in Figure 5, spectrum a. This signal depends mostly on the PBN–thiyl radical adduct of serum albumin [7]. The formation of this adduct depends on peroxynitrite and not on one of its decomposition products or contaminants because the adduct did not form with decomposed peroxynitrite (Figure 5, spectrum b). The small doublet detected in plasma treated with decomposed peroxynitrite was the ascorbyl radical [19].

As illustrated in Figure 5, spectrum c, the removal by dialysis of low-molecular-mass compounds from plasma decreased the intensity of the PBN adduct by 78%, a result that is explained if a pro-oxidant molecule was removed during dialysis. Reconstitution of plasma with NaHCO_3 markedly increased the intensity of PBN adduct (Figure 5, spectrum d) by approx. 3.5-fold, demonstrating that this compound significantly increased thiol radical formation. As proof of the involvement of thiol radicals, we found that pretreatment of plasma with NEM inhibited the formation of PBN adduct completely (Figure 5, spectrum e). Thiyl radicals are probably involved in the process of albumin dimer formation, as addition of PBN to plasma inhibited the peroxynitrite-dependent formation of p115 (Table 1). The singlet radical evident at $g = 2.004$ in spectrum e of

Figure 6 Effect of peroxynitrite treatment on thiol (-SH) content of dialysed human blood plasma

Dialysed plasma was diluted to 50% (v/v) with phosphate buffer/DTPA (pH 7.1) and treated with peroxynitrite. Symbols: ○, no addition; ●, dialysed plasma reconstituted with ascorbate (final concentration 100 μ M); ■, dialysed plasma reconstituted with NaHCO₃ (final concentration 12.5 mM); □, dialysed plasma reconstituted with ascorbate and NaHCO₃ (final concentrations 100 μ M and 12.5 mM). Thiol content referred to undiluted plasma was measured by the reaction with DTNB at 412 nm, subtracting the absorbance of DTNB in phosphate buffer and that of a plasma sample treated in the same way but without DTNB. Points are means $\pm$ S.D. ($n = 4$).

Figure 5 is not a spin-trapped radical but has been assigned to peroxynitrite-dependent endogenous protein tyrosyl radicals [20].

In spin-trapping studies the effect of ascorbate is not easy to interpret, because of its known ability to reduce nitroxides. However, 50 μ M ascorbate added to dialysed plasma reconstituted with NaHCO₃ and treated with peroxynitrite did not significantly modify the intensity of PBN adduct (results not shown). As expected, higher concentrations of ascorbate decreased the adduct intensity.

Oxidation of plasma thiols by peroxynitrite is inhibited by NaHCO₃ and ascorbate

As reported previously [21], the oxidation of thiols by peroxynitrite in dialysed plasma was partly protected by NaHCO₃. We investigated the effect of NaHCO₃ and ascorbate on protein thiol oxidation mediated by peroxynitrite in dialysed plasma. As shown in Figure 6, the reconstitution of dialysed plasma with NaHCO₃ significantly protected SH groups from peroxynitrite-mediated oxidation. The concentration of peroxynitrite able to oxidize 50% of the SH (EC₅₀) was increased approx. 2.7-fold (from 0.3 to 0.8 mM peroxynitrite). The addition of ascorbate to dialysed plasma did not affect the peroxynitrite-mediated oxidation of SH groups, whereas, in the presence of NaHCO₃, ascorbate further inhibited peroxynitrite-mediated SH oxidation as shown by the EC₅₀, which increased to 1.5 mM peroxynitrite (Figure 6).

Formation of RSNO_x by peroxynitrite

It has been suggested that RSNO and/or *S*-nitrothiols (RSNO₂) are possible oxidation products of the reaction of peroxynitrite with thiols [22–24]. To detect the formation of stable RSNO_x in dialysed plasma, we added iron-DETC 2 min after the treatment

Figure 7 ESR spectra of peroxynitrite-treated dialysed plasma after addition of iron-DETC

Spectrum a, dialysed plasma diluted to 50% (v/v) with phosphate buffer/DTPA (pH 7.1) and treated with 5 mM peroxynitrite and 500 μ M iron-DETC; spectrum b, as in spectrum a, except that peroxynitrite was decomposed before the addition of dialysed plasma; spectrum c, as in spectrum a, but with dialysed plasma containing 25 mM NaHCO₃; spectrum d, as in spectrum c, but with 100 μ M ascorbate; spectrum e, GSNO (30 μ M) added to dialysed plasma diluted to 50% (v/v) with phosphate buffer/DTPA (pH 7.1) and treated with 500 μ M iron-DETC; spectrum f, GSNO₂ (30 μ M) added to dialysed plasma diluted to 50% (v/v) with phosphate buffer/DTPA (pH 7.1) and treated with 500 μ M iron-DETC. Iron-DETC was added 2 min after the treatment of plasma with peroxynitrite or after the addition of GSNO. Spectrometer conditions were as described in the legend to Figure 5 except that the number of scans was 5 and the gain was 3.2×10^5 .

with peroxynitrite. This spin trap is able to induce the release of $\cdot$ NO from NO donors and to form a paramagnetic complex, $[\text{Fe}^{2+}(\text{NO})(\text{DETC})_2]$, characterized by a three-line ESR spectrum [$a_N = 12.7$ G (1 G = 10^4 T); $g_{\text{iso}} = 2.04$] [25]. As shown in Figure 7, spectrum a, dialysed plasma treated with iron-DETC after peroxynitrite resulted in the trapping of $[\text{Fe}^{2+}(\text{NO})(\text{DETC})_2]$. The reaction was specific to peroxynitrite, as shown by a control experiment with decomposed peroxynitrite (Figure 7, spectrum b). The addition of NaHCO₃ to dialysed plasma increased the radical intensity by approx. 50% (Figure 7, spectrum c), whereas the addition of ascorbate to NaHCO₃/dialysed plasma did not further change the radical intensity (Figure 7, spectrum d). Spectra e and f in Figure 7 show the results obtained by trapping $\cdot$ NO from 30 μ M GSNO or GSNO₂ added to plasma with iron-DETC. Both these NO donors produced radicals of comparable intensities, thus showing that this trapping method does not discriminate between RSNO and RSNO₂. Using GSNO as standard and assuming that RSNO_x from plasma proteins are trapped by iron-DETC with comparable efficiency, we estimated that the amounts of stable RSNO_x induced by 1–5 mM peroxynitrite were 4.9–14.4 μ M (approx. 3–8% of the SH groups of dialysed plasma diluted to 50%). The effect of NaHCO₃ was

to increase the concentration of RSNO_x induced by 1–5 mM peroxynitrite to 8.8–28.8 μM respectively.

DISCUSSION

Peroxynitrite oxidizes thiols through two competing pathways: two-electron oxidation mediated by ONOO^- , and one-electron oxidation mediated by ONOOH and/or $^*\text{ONOOH}$. The two-electron oxidation pathway leads to the formation of sulphenic acid (RSOH), which with cysteine and glutathione reacts with another reduced thiol to form disulphides. For the thiol group of serum albumin, the postulated sulphenic acid induced by ONOO^- is further oxidized by two-electron transfers to sulphinic (RSO_2^-) or sulphonic (RSO_3^-) acids [2].

We found that, in plasma, peroxynitrite induced the formation of a p115 disulphide protein identified as the dimer of serum albumin. The involvement of this protein was proved by immunological (anti-albumin antibody) and biochemical (peptide mapping) criteria. As reported by Radi et al. [2], the oxidation of purified serum albumin by ONOO^- does not result in disulphide-mediated dimerization of serum albumin. We found that plasma freed of CO_2 by dialysis and treated with up to 5 mM peroxynitrite did not form electrophoretically detectable amounts of albumin dimers, notwithstanding the massive oxidation of thiols. The formation of an albumin dimer was a characteristic of CO_2 -containing samples (whole plasma, dialysed plasma reconstituted with NaHCO_3 , and purified serum albumin in NaHCO_3 /buffers). ESR spin-trapping studies of peroxynitrite-treated dialysed

plasma showed low levels of PBN–thiyl radical adduct in CO_2 -free buffers and a strong increase in the signal on the addition of NaHCO_3 . Moreover, PBN added to plasma completely inhibited the formation of albumin dimers. These results indicate a relationship between albumin dimer formation and the production of thiyl radicals, suggesting that thiyl radicals are critical intermediates in the process of dimer formation.

Although NaHCO_3 supported the formation of albumin dimers in peroxynitrite-treated plasma, it afforded significant protection for thiols by scavenging ONOO^- and thus inhibiting the two-electron oxidation pathway. The protection of thiols by NaHCO_3 is, however, incomplete because thiols react with the reactive intermediate(s) derived from the reaction of peroxynitrite with CO_2 . These postulated reactive species are not exactly known, but might be ONOOCO_2^- and/or X, one or more reactive intermediates derived from it [10,11,26] (Scheme 1, eqn. 1).

By analogy with peroxynitrite, it is likely that the oxidation of thiols mediated by peroxynitrite in CO_2 -containing fluids occurs through competing one- and two-electron pathways, but the present results support the hypothesis that the one-electron oxidation pathway of peroxynitrite might be promoted. The one-electron oxidation pathway would give the thiyl radical ($\text{RS}^\bullet$), $^*\text{NO}_2$ and HCO_3^- (Scheme 1, eqn. 1). Previous ESR studies showed that peroxynitrite induces the formation of tryptophanyl [27], tyrosyl [20] and cysteinyl [7] radicals in proteins and, notably, the addition of NaHCO_3 significantly increases the yield of all these radicals. These protein radicals could be either direct

Scheme 1 One-electron oxidation of thiols by peroxynitrite in CO_2 -containing solutions

X represents all the possible reactive intermediates derived from ONOOCO_2^- (see [11,26]). Rate constants ($\text{M}^{-1} \cdot \text{s}^{-1}$) are for glutathione, except k_2 , which is for cysteine at pH 9.2 [33]; k_1 is from [11]; k_3 is from [32]; k_{4b} is from [26]; k_{5a} , k_{5b} , k_6 and k_8 are from [37]; k_7 is from [34].

one-electron oxidation products of nitrosoperoxycarbonate and/or X or the products of inter/intra-molecular transfer of the radical [28]. However, the general effect of CO_2 is to direct the oxidations of peroxynitrite towards the one-electron pathway.

Recent crystallographic X-ray analysis of serum albumin showed that the only reduced cysteine (Cys-34) is located near the surface of the molecule partly protected from the solvent [29] and, probably for this reason, dimers are formed mainly under denaturing conditions [30,31]. Thus to form the disulphide dimer of albumin it is necessary to expose Cys-34 to the solvent. The conformation of albumin necessary to expose Cys-34 could be that of the thiolate anion (RS^-). Assuming for Cys-34 of albumin a pK_a of 7.8–8.0 [2], at pH 7.5 approx. 30 % of the thiol might be in the form of RS^- [32]. Moreover, Cys-34 residue could be exposed to the solvent in peroxynitrite-treated albumin for the formation of thiyl radical and/or for the formation of oxidized polar thiol intermediates. In fact, the simultaneous generation of $\text{RS}^\bullet$ and $\bullet\text{NO}_2$ might be expected to generate a complex series of reactions (see Scheme 1). The $\bullet\text{NO}_2$ can react with thiolate anions to give additional $\text{RS}^\bullet$ (eqn. 2) [33], whereas the $\text{RS}^\bullet$ can (1) dimerize to disulphides (RSSR) [32]; (2) react with $\bullet\text{NO}_2$ to give RSNO_2 , a relatively stable intermediate [23,24]; (3) react with RS^- yielding the disulphide radical anion ($\text{RSSR}^{\bullet-}$); (4) react with oxygen to form the peroxy radical ($\text{RSOO}^\bullet$); and (5) react with RSNO to form RSSR and $\bullet\text{NO}$ [34] (see eqns. 3–7 respectively in Scheme 1). The feasibility of the reactions in eqns. 4 and 7 is supported by our finding that RSNO_x are stable end products in peroxynitrite-treated plasma. The pathway leading to the formation of RSNO_x from peroxynitrite is not completely understood [24,35] and might involve a direct nitration mechanism [36], but our findings that NaHCO_3 increased the formation of both $\text{RS}^\bullet$ and RSNO_x open the possibility that the one-electron oxidation pathway is also involved (eqns. 1 and 4). The formation of RSNO_x is believed to be an important pathway of peroxynitrite decay because these compounds are responsible for its NO-like effects [22–24].

As shown in eqns. (4)–(8) in Scheme 1, RSNO_2 , $\text{RSSR}^{\bullet-}$, $\text{RSOO}^\bullet$ and RSNO do not inhibit the formation of RSSR because, depending on the availabilities of RS^- , O_2 , RSH or $\text{RS}^\bullet$, these intermediates are involved in the formation of RSSR or in the regeneration of $\text{RS}^\bullet$, also through the intermediate formation of $\text{O}_2^{\bullet-}$ [37]. Rate constants of reactions in Scheme 1 have been measured for low-molecular-mass thiols and are unknown for thiols of plasma protein; it is therefore unclear which reaction prevails over the others in plasma. However, it is clear that a relatively small amount of $\text{RS}^\bullet$ could lead to significant formation of RSSR .

The addition of ascorbate to NaHCO_3 /dialysed plasma decreased the total oxidation of SH groups induced by peroxynitrite, thus showing that this antioxidant performs a protective role for thiols. However, under these conditions ascorbate significantly increased the formation of albumin dimers. A plausible explanation for the effect of ascorbate on albumin dimer formation might be that it scavenges a species that, by reacting with $\text{RS}^\bullet$ or RS^- , lowers the disulphide yield. This postulated species is not peroxynitrite [11] or sulphenic–sulphinic–sulphonic acids, as shown by the inability of ascorbate to protect thiols in dialysed CO_2 -free plasma, but might be one of the intermediate(s) produced in the decay process. For example, it has been suggested that bicarbonate radical might be formed in the reaction [10], and this radical can react with cysteine residues with a significant rate constant ($4.6 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for free cysteine [38]). In conclusion, ascorbate in CO_2 -containing fluids protects thiols from peroxynitrite-mediated oxidation and directs thiol oxidation partly towards the formation of

disulphides, but characterization of its mechanism requires further investigation. All the other antioxidants tested (urate, glutathione, Trolox C, bilirubin) were unable to increase the formation of albumin dimers in dialysed plasma but, interestingly, glutathione and bilirubin added to whole plasma increased dimer formation. The effect of these antioxidants in whole plasma might be that of sparing ascorbate.

The implications of peroxynitrite-induced albumin dimers in plasma are not clear, because it is unknown whether the dimer can be formed under real physiological conditions and whether cross-linking modifies albumin functions. However, it should be noted that blood possesses several ‘repair’ mechanisms that can reduce disulphides, whereas sulphinic and/or sulphonic acids probably cannot be reduced, so that the function of directing thiol oxidation, at least in part, towards disulphides could be considered a protective effect of bicarbonate and ascorbate in physiological fluids.

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