Peroxynitrite-mediated formation of free radicals in human plasma: EPR detection of ascorbyl, albumin-thiyl and uric acid-derived free radicals

Jeannette VÁSQUEZ-VIVAR, Alexandre M. SANTOS, Virginia B. C. JUNQUEIRA and Ohara AUGUSTO*

Department of Biochemistry, Instituto de Química, Universidade de São Paulo, C.P. 26.077, 05599-970, São Paulo, S.P., Brazil

Formation of peroxynitrite by the fast reaction between nitric oxide and superoxide anion may represent a critical control point in cells producing both species, leading to either down-regulation of the physiological effects of superoxide anion and nitric oxide by forming an inert product, nitrate, or to potentiation of their toxic effects by oxidation of nearby molecules by peroxynitrite. (The term peroxynitrite is used to refer to the sum of all possible forms of peroxynitrite anion and peroxynitrous acid unless otherwise specified.) In this report we demonstrate that, in spite of all the antioxidant defences present in human plasma, its interaction with peroxynitrite leads to generation of free radical intermediates such as (i) the ascorbyl radical, detected by direct EPR, (ii) the albumin-thiyl radical, detected by spin-trapping experiments with both *N*-*tert*-butyl-α-phenylnitrone and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and (iii) a uric acid-derived free radical, detected as the DMPO radical adduct in plasma whose thiol groups were previously blocked with 5,5-dithiobis-(2-nitrobenzoic acid). The identity of the latter adduct was confirmed by parallel experiments demonstrating that it is not detectable in plasma pretreated with uricase, whereas it is formed in incubations of peroxynitrite with uric acid. Peroxynitrite-mediated oxidations were also followed by oxygen consumption and ascorbate and plasma-thiol depletion. Our results support the view that peroxynitrite-mediated one-electron oxidation of biomolecules may be an important event in its cytotoxic mechanism. In addition, the data have methodological implications by providing support for the use of EPR methodologies for monitoring both free radical reactions and ascorbate concentrations in biological fluids.

INTRODUCTION

The demonstration that nitric oxide and superoxide anion, which are produced by several cell types, can react with each other in aqueous solution at an almost diffusion-controlled rate to yield peroxynitrite [1] (Figure 1) has opened new possibilities for the understanding of free-radical-dependent damage to biological systems [1–5]. (The term peroxynitrite is used to refer to the sum of all possible forms of peroxynitrite anion and peroxynitrous acid unless otherwise specified.) Indeed, both superoxide anion and nitric oxide are relatively unreactive towards most of the organic molecules, whereas peroxynitrite is a potent oxidant which is able to oxidize a variety of biomolecules including deoxyribose [2], lipids [6], methionine [7,8] and thiols [9].

The mechanisms by which peroxynitrite oxidizes biomolecules have been under active investigation [1–5]. The peroxynitrite anion is relatively stable, but its acid rearranges to form nitrate with a half-life near 1 s at pH 7.0 in the absence of oxidizable substrates (Figure 1). When present, these molecules can be oxidized by one- and two-electron transfer steps [4,5,8,10–14] although the mechanistic details of these processes remain in discussion. In particular, the peroxynitrous acid-mediated oxidations leading to free radical formation could occur either by homolysis of peroxynitrous acid to give the hydroxyl radical, which can diffuse apart to oxidize target molecules, or through an activated isomer, *trans*-peroxynitrous acid, which is able to directly oxidize target molecules to free radical intermediates (Figure 1). At present, the latter mechanism is preferred by most investigators working in the field, although it still needs to be discriminated experimentally [4].

Within the context of oxidative biological damage, an important point to emphasize is that peroxynitrous acid, when triggering free radical production from nearby molecules, also produces nitrogen dioxide (Figure 1), another free radical species which can further propagate peroxynitrite-mediated one-electron oxidations. It is important to emphasize that these processes will occur in the absence of transition-metal ions either from metalloenzymes or from low-molecular-mass complexes which are required for hydroxyl radical generation from superoxide anion and hydrogen peroxide [15]. Considering the low availability of redox-active transition-metal ions in biological fluids [16], which, in addition, contain many antioxidant defences [17–20], it is important to assess the possibility of peroxynitrite mediating free radical reactions in these fluids. Peroxynitrite formation in the extracellular environment by generation of both nitric oxide and superoxide anion by several cell types such as endothelial cells and phagocytes is a likely process [21–23]. Moreover, peroxynitrite induces oxidative damage to human plasma, as has been demonstrated by its effects in decreasing both the total peroxyl-trapping capacity of plasma and the levels of antioxidants such as ascorbic acid, uric acid, plasma-thiol groups and ubiquinol-10 [23]. The latter study, however, which was reported while our work was underway, did not examine the possibility of peroxynitrite mediating one-electron oxidations in plasma.

In this report we demonstrate, by the use of direct- and spin-trapping-EPR techniques, that the interaction of peroxynitrite with human plasma led to generation of free radical intermediates such as ascorbyl, albumin-thiyl and a uric acid-derived free radical. Our studies support the view that peroxynitrite-mediated one-electron oxidation of biomolecules may be an important event in its cytotoxic mechanism. In addition, they have methodological implications by providing further support for the use of EPR methodologies for monitoring both free radical reactions and ascorbate concentrations in biological fluids, by measure-

Abbreviations used: CAT_1, 4-trimethylammonio-2,2,6,6-tetramethylpiperidin-1-oxyl iodide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); PBN, *N*-*tert*-butyl-α-phenylnitrone.

* To whom correspondence should be addressed.

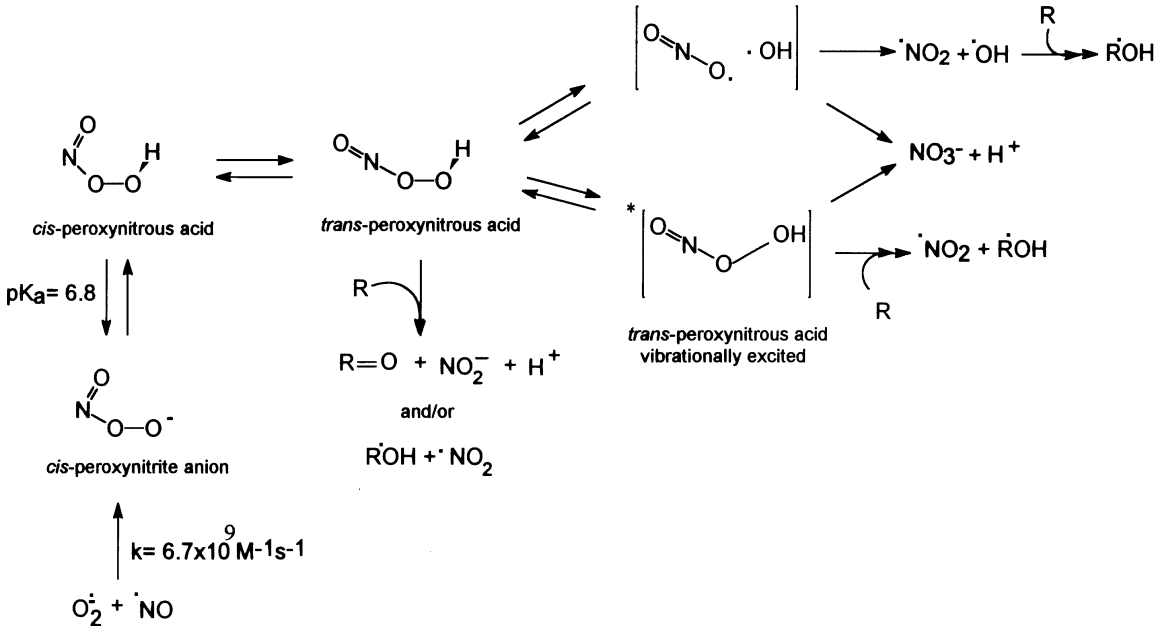

Figure 1 Schematic representation of peroxynitrite formation and decomposition

The fast reaction between superoxide anion and nitric oxide generates the peroxynitrite anion which protonates at physiological pH values to peroxynitrous acid, which in turn decomposes to nitrate and oxidizes target molecules by mechanisms that remains to be fully characterized. A thorough discussion of the proposed mechanisms can be found in ref. [4].

ments of ascorbyl radical levels [24,25] and the rate of nitroxide reduction [26], respectively.

MATERIALS AND METHODS

Materials

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), *N*-*tert*-butyl-α-phenylnitrone (PBN), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and Chelex-100 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CAT_1 (4-trimethylammonio-2,2,6,6-tetramethylpiperidin-1-oxyl iodide) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Uric acid was from Merck (Darmstadt, Germany) and uricase from Boehringer Mannheim (Mannheim, Germany). Purified human albumin was obtained from placental blood haemolysates by a procedure developed by Dr. Joaquin Cabrera-Crespo at the Center of Biotechnology of the Instituto Butantan (São Paulo, Brazil). DMPO was purified by vacuum distillation [14] and PBN was recrystallized from hexane [11]. Peroxynitrite was synthesized in a quenched-flow reactor as described previously [6,9] and kept frozen at -80 °C. Peroxynitrite stock solutions were prepared by dilution with water to keep the pH above 13; their concentrations were determined in 0.1 M NaOH by absorbance at 302 nm ($\epsilon = 1670$ $M^{-1} \cdot cm^{-1}$) [6,9]. All solutions were prepared in distilled water purified with a Millipore Milli-Q system. All glassware and related materials were pretreated with 0.1 M HCl to remove spurious transition-metal ion contamination [27].

Plasma preparation

Plasma was obtained from fresh heparinized blood immediately after venipuncture of healthy adults. Red blood cells and buffy coat were removed by centrifugation at 2000 rev./min (180 ***g***) for 10 min at 4 °C. Aliquots were kept frozen at -80 °C and used within 3 weeks. Separation of low- and high-molecular-mass components of plasma was obtained by ultrafiltration at 4 °C with a Centriprep-10 from Amicon (Beverly, MA, U.S.A.). Depletion of plasma thiol groups was obtained by incubation with equimolar concentrations of DTNB for 10 min at room temperature [28]. Depletion of plasma uric acid was obtained by incubation with uricase (0.1 unit/ml) for 90 min at room temperature [29].

EPR studies

EPR spectra were recorded at room temperature using a Bruker ER 200 D-SRC spectrometer. The plasma samples, with or without the spin traps, were transferred to flat cells immediately after peroxynitrite addition and the spectra recorded. Further additions to the incubation mixtures are described in the text and Figure legends. All incubation mixtures had their pH checked at the end of the experiment; no final pH was higher than 7.8.

Antioxidant levels

Ascorbate concentrations in plasma were systematically determined by monitoring the rate of reduction of 100 μM CAT_1 by EPR as described previously [26]. In summary, 100 μM CAT_1 was added to plasma, the sample was transferred to a flat cell and the disappearance of the central peak of the EPR spectrum of CAT_1 was followed from 1 to approx. 6 min [26]. Initial rates of the nitroxide decay were calculated and a rate constant of 45 $M^{-1} \cdot s^{-1}$ for the reaction between ascorbate and CAT_1 was used to determine ascorbate concentrations before and after peroxynitrite addition [26]. In some plasma samples, ascorbate concentrations were also determined by classical HPLC procedures [30]. Briefly, ascorbate from plasma samples was extracted by cold metaphosphoric acid (10 %, w/v). After centrifugation to remove the precipitated proteins, the supernatant was diluted by mixing with an equal volume of metaphosphoric acid solution (0.8 %, w/v) and filtered through a

0.22 μm-pore-size Millex GV filter. Aliquots (20 μl) were injected on to a Nova-pak C_{18} column and eluted with metaphosphoric acid (0.8 %, w/v) at a flow rate of 0.7 ml/min; elution was followed by UV measurement at 235 nm. Authentic ascorbic acid was used as standard for quantification.

Plasma thiol groups were measured spectrophotometrically after reaction with DTNB. Plasma samples were diluted 30 times with phosphate buffer containing 2 mM EDTA, pH 9.0. DTNB was added at a final concentration of 0.2 mM and absorbance at 412 nm ($\epsilon = 13600\ M^{-1} \cdot cm^{-1}$) was measured after 20 min incubation at room temperature [28].

Oxygen uptake

Oxygen consumption studies were performed using an oxygen monitor (Gilson 5/6 oxygraph) at 25 °C. The saturating oxygen concentration at this temperature, which corresponds to the full response of the electrode, was taken to be 0.24 mM [31].

RESULTS

Direct EPR studies of plasma ascorbyl radical levels

Examination of plasma samples from healthy Brazilian adults by direct EPR spectroscopy led to detection of basal levels (68 ± 25 nM; $n = 12$) of the ascorbyl radical ($a^H = 0.18$ mT) (Figure 2) which were lower than those reported in a European study (100 ± 30 nM; $n = 20$) [32]. These differences can probably be attributed to different life styles since the two studies employed similar procedures for sample collection and manipulation [32] (see, also, the Materials and methods section). Although it has been long known that freshly collected biological fluids contain EPR-detectable levels of ascorbyl radicals, it has been difficult to establish if the radical is formed during collection and/or sample manipulation. Careful studies, however, are indicating that basal levels of ascorbyl radicals in plasma did not vary much among similar experimental groups of animals [33] or humans [34], suggesting that they reflect the slow oxidations occurring in plasma. In agreement, the steady-state concentration of ascorbyl radical in plasma increases upon oxidative challenges such as those promoted by iron overload [32], paraquat poisoning [33], decomposition of 3-morpholinosydnomine *N*-ethylcarbamide [25], and increases in blood flow in rabbits *in vivo* [33]. Since increases in the steady-state concentration of ascorbyl radical should reflect increases in the rate of the one-electron oxidation of ascorbate [24,25,35], ascorbyl radical levels are being used to monitor free radical reactions both *in vitro* and *in vivo* [24,25,32–34].

Accordingly, peroxynitrite-mediated one-electron oxidations in human plasma could be first demonstrated by the ability of the oxidant to increase the ascorbyl radical levels (Figures 2 and 3). The observed increase was proportional to the added peroxynitrite concentration (from 0.1 to 1.0 mM) and was transient,

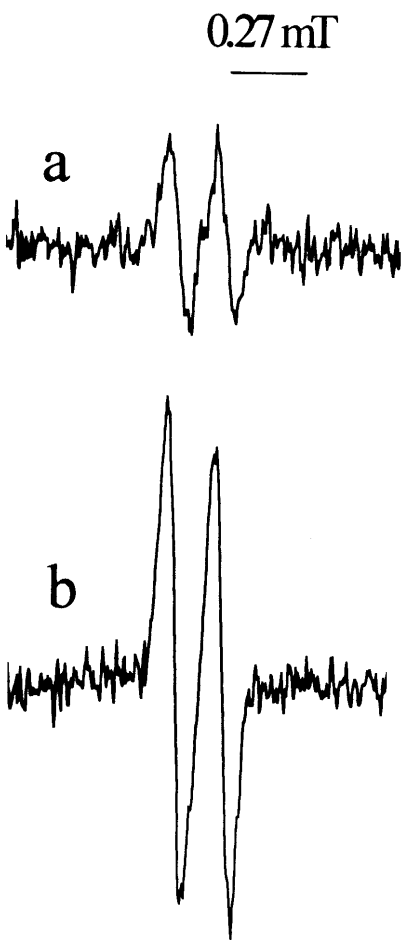

Figure 2 Direct EPR spectra of human plasma

(**a**) Untreated; (**b**) 1 min after the addition of 0.5 mM peroxynitrite. Instrumental conditions: microwave power, 37 mW; modulation amplitude, 0.1 mT; time constant, 1 s; and scan rate, 0.01 mT/s.

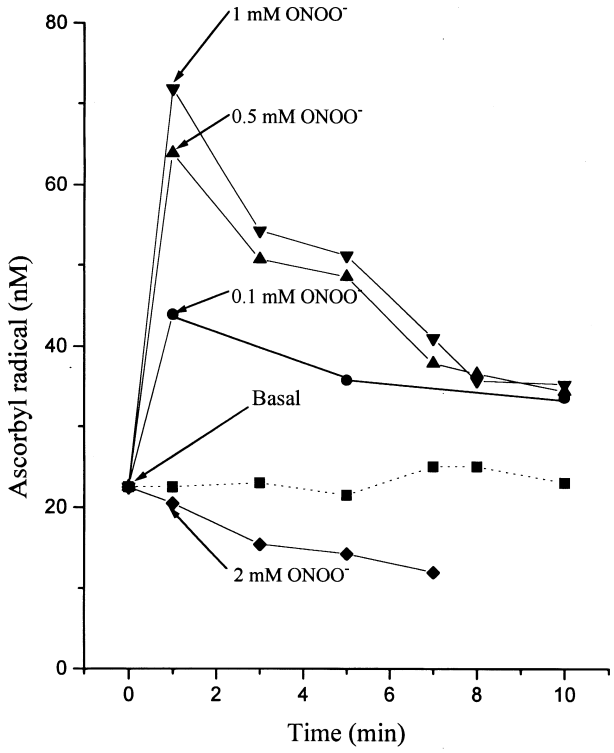

Figure 3 Peroxynitrite-induced changes of plasma ascorbyl radical levels

Representative profile of the changes in plasma ascorbyl radical levels with time during incubation with (solid line) or without (broken line) peroxynitrite at the indicated concentrations. The instrumental conditions are the same as those indicated in Figure 2 with the gain being changed depending on ascorbyl radical concentrations.

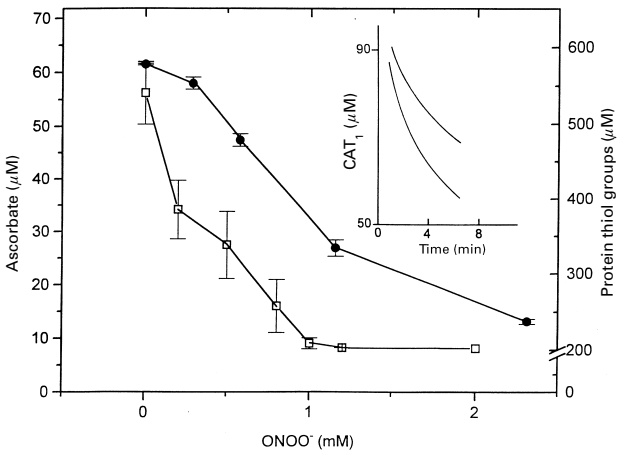

Figure 4 Peroxynitrite-induced depletion of ascorbate and protein-thiol groups in human plasma

The concentrations of ascorbate (□) and protein-thiol (●) were determined after 5 min incubation with peroxynitrite at the indicated concentrations as described in the Materials and methods section; each point represents the mean ± S.D. of at least three independent experiments. The inset displays typical determinations of ascorbate concentrations in plasma before (lower curve) and after (upper curve) 5 min incubation with 0.5 mM peroxynitrite by monitoring the rate of disappearance of the central peak of the nitroxide CAT_1 whose heights were converted into the corresponding nitroxide concentrations.

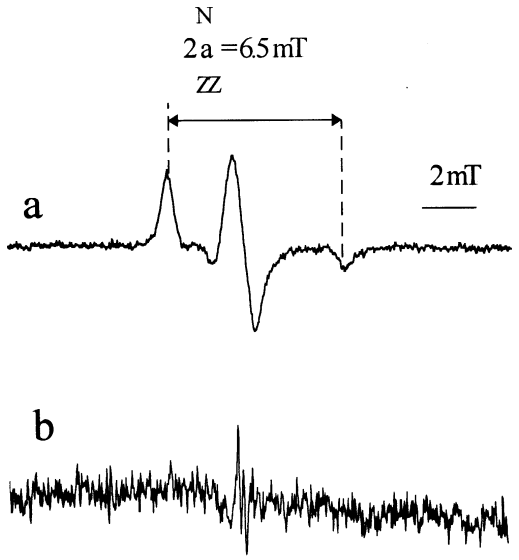

Figure 5 EPR spectra of the PBN–radical adduct obtained during the interaction of peroxynitrite with human plasma

The spectra were obtained after a 1 min incubation of human plasma at room temperature with 50 mM PBN and (**a**) 0.5 mM peroxynitrite, or (**b**) 0.5 mM peroxynitrite preincubated at a ten times higher concentration for 5 min in phosphate buffer, pH 7.4. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.25 mT; time constant, 0.5 s; scan rate 0.1 mT/s.

stabilizing after about 10 min at values a little higher than the basal level which remained unchanged in the absence of exogenously added oxidant (Figure 3). The profile displayed in Figure 3 was always observed, although the concentrations of peroxynitrite required to maximally increase or to start depleting basal ascorbyl radical levels (Figure 3) varied with the initial concentration of the parent ascorbate in plasma, and hence with the donor. Such behaviour is to be expected since steady-state concentrations of the ascorbyl radical are dependent on the rate of ascorbate oxidation and, hence on its concentration [35]. In the case of the plasma used to obtain Figure 3, addition of 2 mM peroxynitrite decreased ascorbyl radical basal levels and depleted ascorbate concentrations to about 5 μM, as attested by parallel determination of ascorbate levels (Figure 4). Consequently, oxidation of these low ascorbate levels in peroxynitrite-treated plasma are not enough to maintain steady-state ascorbyl radical concentrations close to the basal ones (Figure 3).

Since peroxynitrite decomposition at pH 7.4 should last only a few seconds [2–5], it is important to note that ascorbyl radical levels keep changing for several minutes before stabilizing (Figure 3). This result suggests that peroxynitrite decomposition in human plasma triggers free radical reactions which continue through the generated free radicals even after peroxynitrite decomposition is over, although the major effects are observed within the lifetime of the oxidant (Figure 3). In agreement, previous studies by Van der Vliet et al. [23] have demonstrated a time-dependent increase in the oxidation of ascorbate and ubiquinol-10 present in human plasma treated with peroxynitrite. Also in agreement with the latter studies, which have reported that plasma α-tocopherol levels are only slightly decreased upon the addition of peroxynitrite [25], we were unable to demonstrate the formation of the one-electron oxidation product of vitamin E (see for instance, Figure 2), the α-tocopheroxyl radical, which would be stable enough to be detected by direct EPR spectroscopy [14].

EPR-spin-trapping experiments

In order to investigate whether, in addition to the ascorbyl radicals, other free radicals were formed upon the addition of peroxynitrite to human plasma, we performed spin-trapping experiments in the presence of both PBN and DMPO. Incubation of human plasma with 0.5 mM peroxynitrite in the presence of 50 mM PBN led to the detection of an anisotropic EPR spectrum ($2a^N_{zz} = 6.5$ mT) (Figure 5A) almost identical to the one previously described for the PBN–albumin-thiyl radical adduct obtained during the peroxynitrite-mediated oxidation of BSA [11]. Consequently, the EPR spectrum shown in Figure 5(A) should correspond to the PBN–thiyl radical adduct of human albumin. Oxidation of albumin to the thiyl radical in plasma depends on peroxynitrite and not on one of its stable decomposition products, since no radical adduct could be detected in experiments where 10 mM peroxynitrite was preincubated for 5 min in phosphate buffer to allow its complete decomposition before being diluted 10-fold with human plasma (reverse addition) (Figure 5B).

Trapping of the human albumin-thiyl radical in incubations of peroxynitrite with plasma was confirmed by spin-trapping experiments in the presence of DMPO. Incubation of either purified human albumin (Figure 6A) or human plasma (Figure 6B) with peroxynitrite in the presence of DMPO also rendered a strongly immobilized EPR spectrum as the main component [11]. As expected for the DMPO–albumin-thiyl radical adduct, the immobilized adduct was decreased by pretreatment of human plasma with the sulphydryl blocking agent DTNB (Figure 6C). Under the latter experimental condition, mobile DMPO adducts which were barely visible in untreated plasma (Figure 6B) became evident. One of these radical adducts was easily identified by its hyperfine splitting constants ($a^N = 1.49$ mT; $a^H = 1.49$ mT) (Figure 6C) as the DMPO–hydroxyl radical adduct [36].

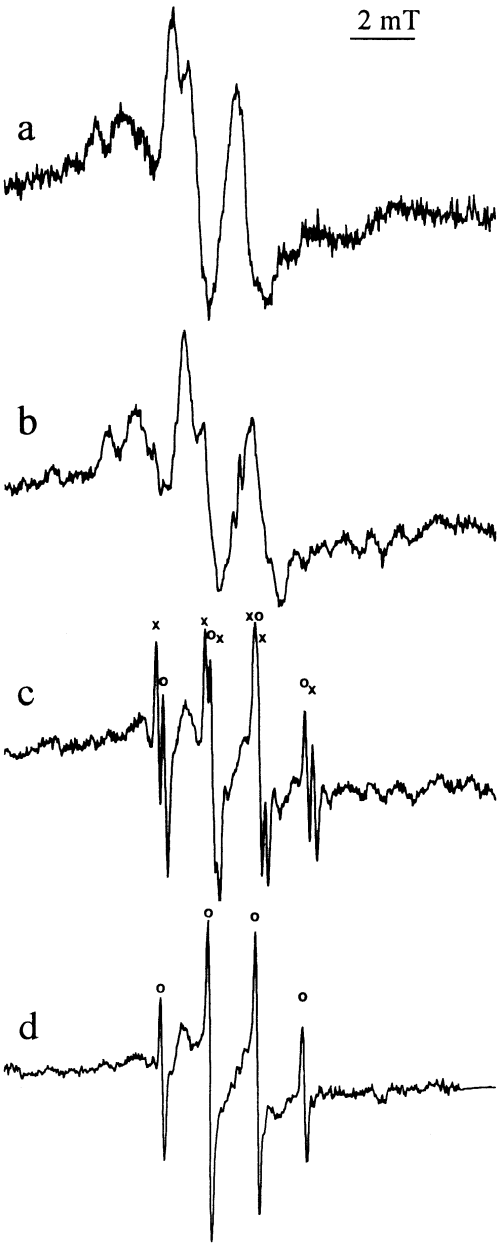

Figure 6 EPR-spectra of the DMPO–radical adducts obtained during the interaction of peroxynitrite with human plasma and purified human serum albumin

The spectra were obtained 1 min after the addition of peroxynitrite to incubation mixtures containing 0.1 M DMPO with: (**a**) purified human serum albumin (1.0 mM -SH) in 0.1 M phosphate buffer, pH 7.5, and (**b**) human plasma (0.58 mM -SH). Peroxynitrite concentrations were 1.0 and 0.5 mM for (**a**) and (**b**) respectively. (**c**) This is the same as (**b**) but using human plasma pretreated with 0.4 mM DTNB; (**d**) is the same as (**b**) but using human plasma pretreated with both uricase (0.1 units/ml) and 0.4 mM DTNB. The composite EPR spectra of (**c**) and (**d**) are labelled to show their mobile components: DMPO–hydroxyl radical adduct (○), DMPO–urate-derived radical adduct (×). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.25 mT; time constant, 0.5 s; scan rate, 0.1 mT/s.

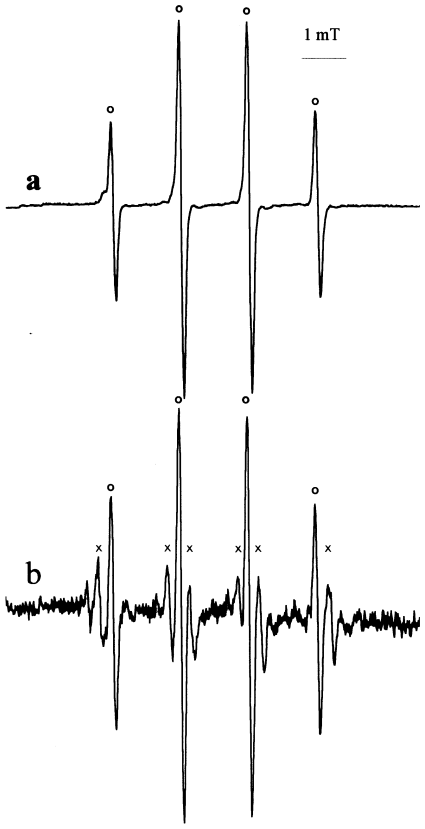

Figure 7 EPR spectra of DMPO–radical adducts obtained during peroxynitrite decomposition in the presence of bicarbonate and uric acid

The spectra were obtained 1 min after the addition of 0.5 mM peroxynitrite to incubations containing 0.1 M DMPO and (**a**) bicarbonate (1.0 M) plus GSH (1 mM), pH 7.5; (**b**) uric acid (0.5 mM) in 0.1 M phosphate buffer, pH 7.5. The EPR spectra are labelled to show their components: DMPO–hydroxyl radical adduct (○) and DMPO–urate radical adduct (×). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.1 mT; time constant, 0.2 s; scan rate, 0.05 mT/s; gain 6.3×10^4 for (**a**) and 1.25×10^6 for (**b**).

Formation of the DMPO–hydroxyl radical adduct during the decomposition of peroxynitrite, particularly in the presence of thiol groups which protect DMPO adducts from decay to EPR-silent products promoted by peroxynitrite [14], has been described before [10] and could result from either the trapping of the hydroxyl radical formed during *trans*-peroxynitrous acid homolysis or the direct oxidation of DMPO by the *trans*-peroxynitrous acid (Figure 1) (see, also, the Introduction). It should be noted that in oxidizing albumin-thiols with DTNB this reagent becomes reduced and thus can provide the thiols to permit DMPO adduct detection [14].

The source of the other mobile DMPO adduct was more difficult to determine. Its hyperfine splitting constants ($a^N = 1.5$ mT; $a^H = 1.9$ mT) are close to those of the DMPO carbon dioxide anion radical [36], suggesting that it could result from protein fragmentation as recently demonstrated in the case of transition-metal ion-catalysed fragmentation of protein hydroperoxides [37]. This possibility, however, was excluded because the adduct was not detectable in incubations containing either purified human albumin (Figure 6A) or human plasma depleted of low-molecular-mass components, even in the presence of DTNB (results not shown). In contrast, a weak signal of the adduct could be observed in the low-molecular-mass component fraction of human plasma. Consequently, the source of this DMPO adduct should be a low-molecular-mass component. Two of these components which are present in high concentrations in plasma, bicarbonate and uric acid [20,23], were

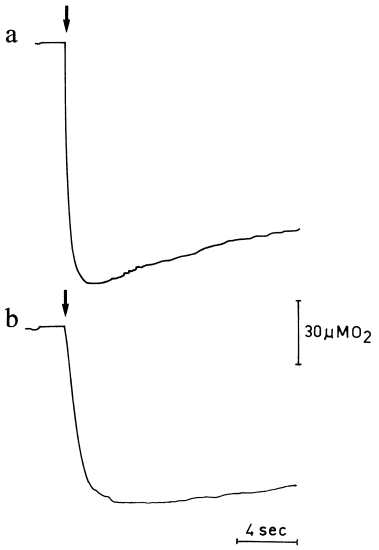

Figure 8 Peroxynitrite-induced oxygen consumption by human plasma and purified human serum albumin

Peroxynitrite was added at the indicated points to: (**a**) human plasma (0.578 mM total -SH); (**b**) purified human serum albumin (0.850 mM total-SH) in 0.1 M phosphate buffer, pH 7.5, to a final concentration of 1 mM.

incubated in phosphate buffer with DMPO and several concentrations of peroxynitrite in the presence and absence of GSH [10,13,14]. None of the tested conditions resulted in the detection of bicarbonate-derived DMPO adducts, whereas a uric acid-derived DMPO adduct could be detected, as shown in the representative spectra displayed in Figure 7. The latter adduct has the same hyperfine splitting constants ($a^N = 1.52$ mT; $a^H = 1.94$ mT) (Figure 7B) as those observed in plasma (Figure 6C), identifying uric acid as the source of the plasma adduct. In agreement, treatment of plasma with uricase (0.1 units/ml) [29] and DTNB before peroxynitrite addition led to the disappearance of the uric acid-derived DMPO radical adduct (Figure 6D).

Oxygen consumption studies

Although the uric acid-derived free radical was not fully characterized (see the Discussion), its peroxynitrite-mediated formation in human plasma was also shown by oxygen uptake studies, which demonstrated that addition of peroxynitrite to plasma triggers oxygen consumption (0.2 μM oxygen/μM plasma-thiol) which is higher than that obtained by peroxynitrite addition to purified human albumin (0.07 μM oxygen/μM albumin-thiol) (Figure 8). This indicates that oxygen is consumed by other radicals in addition to the albumin-thiyl radical in plasma. Oxygen uptake by the uric acid-derived free radical was confirmed by control experiments, demonstrating that incubations of uric acid (0.5 mM) with peroxynitrite (0.5 mM) in phosphate buffer, pH 7.5, consumed 100 μM oxygen (results not shown).

It is important to note that, in working with purified human albumin, a careful selection of the protein sample should be made since purified human albumin from several commercial sources yielded poor and variable results in both spin-trapping and oxygen consumption experiments. In general, the yields of formed thiyl radical adducts and of consumed oxygen upon peroxynitrite treatment were much lower than those obtained with either commercial bovine albumin [11] or total human plasma for about the same protein thiol content. This suggests that human albumin denatures during isolation from human plasma, assuming a conformation which impairs thiol oxidation by peroxynitrite. The human albumin sample used in the experiments shown here was freshly isolated and rendered about the same yield of DMPO–albumin-thiyl adduct as that obtained in plasma but still consumed less oxygen per thiol group, suggesting that other free radicals which consume oxygen are being formed in plasma (Figure 8).

Table 1 Comparison of ascorbate levels in human plasma measured by HPLC and EPR methods

Plasma samples were obtained from healthy adult donors. Measurements of ascorbate levels by HPLC and EPR methods were performed as described in the Materials and methods section. The HPLC and EPR data represent mean ± S.D. for at least four determinations unless specified. The obtained values by both methods were not significantly different. $P < 0.05$.

	Ascorbate (μM)	
Donor	HPLC	EPR
A	40.95 ± 1.21	36.64 ± 3.03
B	33.39 ± 1.28	37.54 ($n = 2$)
C	75.56 ± 2.12	64.58 ± 8.85
D	64.24 ± 2.43	66.33 ± 9.17
E	53.66 ± 3.07	47.45 ± 7.12

Thiol and ascorbate depletion

Depletion of the various human plasma constituents by peroxynitrite has been described before [23] and we show here (Figure 4) only the results corresponding to those antioxidants which were oxidized to fully characterized free radicals, ascorbate (Figure 2) and plasma thiol (mostly, albumin-thiol) (Figures 5 and 6). Our results (Figure 4) are in close agreement with those previously reported by Van der Vliet et al. [23] and are worth mentioning since we measured ascorbate levels by following the rate of CAT_1 disappearance by EPR as proposed by Mehlhorn (Figure 4, inset). The agreement between our results and those reported previously [23] provides an important validation of the use of CAT_1 reduction to measure ascorbate levels in biological fluids [26], a methodology which is much less time-consuming than the usually employed HPLC procedures. Further validation of the EPR methodology was obtained by comparing the values of ascorbate concentrations determined in five human plasma samples by both EPR and classical HPLC procedures. The values obtained by both methodologies are in excellent agreement as shown in Table 1.

It is important to note that addition of 1 mM peroxynitrite to plasma caused depletion of 244 μM thiol groups (Figure 4) and induced consumption of 120 μM oxygen (Figure 8). This comparison, taken together with the fact that other formed radicals such as the uric acid-derived free radical should be consuming oxygen (see above), suggests that peroxynitrite is oxidizing plasma thiols by both one- and two-electron processes [4,9], since only the former should result in oxygen uptake.

DISCUSSION

Formation of peroxynitrite by the fast reaction between nitric oxide and superoxide anion may represent a critical control point in cells producing both species, leading to either down-regulation of the physiological effects of superoxide anion and nitric oxide

by forming an inert product, nitrate, or to potentiation of their toxic effects through oxidation of nearby molecules by peroxynitrite (Figure 1). The predominance of each of these [4,5,23,38] or other [39–41] pathways will be dependent on both the fluxes of peroxynitrite achieved and the environment in which they are generated. In this report we demonstrate that peroxynitrite concentrations of 0.1–1.0 mM added to human plasma led to the formation of free radicals such as ascorbyl (Figures 2 and 3), albumin-thiyl (Figures 5 and 6) and a uric acid-derived free radical (Figures 6 and 7). Both one- and two-electron oxidations of plasma-thiol groups by peroxynitrite are suggested by our results comparing thiol depletion (Figure 4) with oxygen consumption (Figure 8). The demonstration that peroxynitrite-mediated one-electron oxidation of biomolecules can occur in extracellular fluids, however, is the main point of our results. The biological significance of these findings could be questioned due to the high concentrations of peroxynitrite employed. The short half-life of this oxidant under physiological pH values, however, implies that a bolus of 1 mM peroxynitrite will be equivalent to an exposure of only 26 $\mu M \cdot min^{-1}$ as can be estimated by dividing the initial peroxynitrite concentration by its rate of decomposition at 37 °C, pH 7.4, which is equal to 0.64 s^{-1} [42]. In addition, formation of peroxynitrite at rates of 0.1 $nmol \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ has been reported in the case of activated murine macrophages [43] and human neutrophils [44], making it possible that local concentrations as high as 0.5–1.0 $mM \cdot min^{-1}$ may be achieved under certain pathological conditions [6,23,42–44].

Peroxynitrite-mediated formation of free radicals will become an important cytotoxic event, however, only if the initially formed free radicals are reactive and capable of attacking other molecules to propagate the damage. In plasma such propagation is indicated by peroxynitrite-induced oxygen consumption (Figure 8) and lipid oxidation [23]. Which radicals propagate the damage, however, is more difficult to determine. For instance, the many constituents of plasma make it difficult to assess the sequence of events following peroxynitrite addition. Considering the usual concentrations of albumin-thiol (approx. 400 μM), ascorbate (approx. 80 μM) and uric acid (approx. 500 μM) [20], and the known rate constants of the reaction of peroxynitrite with thiols (1200–5900 $M^{-1} \cdot s^{-1}$) [3,9] and with ascorbate (200 $M^{-1} \cdot s^{-1}$ at pH 5.0) [12], it is expected that the albumin-thiol group will be oxidized first. The resulting albumin-thiyl radical reacts with oxygen (Figure 7) and the formed peroxyl radical should oxidize ascorbate to the ascorbyl radical [11,24,25]; the latter can also be formed by repair of the uric acid-derived radical [45]. Such a sequence of events explains the described inability of both ascorbate and uric acid to protect thiol depletion from dialysed plasma [23], as well as the increased yield of the uric acid-derived free radical after blocking the albumin-thiol group (Figure 6). Also, it provides some indications of the radicals that may propagate peroxynitrite-mediated damage.

The ascorbyl radical is excluded since it is unreactive, decaying mostly by dismutation [24,25,35]. In contrast, the nitrogen dioxide resulting from peroxynitrite-mediated oxidations (Figure 1) is reactive, including towards plasma constituents [46], and should propagate peroxynitrite-initiated damage. The uric acid-derived free radical reported here remains to be fully characterized and studied with regard to its reactivity. The EPR characteristics of the detected DMPO-adduct (Figures 6D and 7) are more consistent with an electron-deficient carbon-centred radical [36] than with the nitrogen-centred free radical detected by continuous flow direct-EPR spectroscopy of incubations of uric acid with either permanganate or horseradish peroxidase–hydrogen peroxide systems [45]. Formation of a carbon-centred radical of uric acid has been proposed before in hydroxyl radical-generating systems [47,48], although no structural information has been provided. It is important to emphasize, however, that the latter radical has been shown to inactivate both alcohol dehydrogenase [47] and α_1-antiproteinase [48], properties which make it a likely species for propagating peroxynitrite-mediated damage. Another possible candidate is the albumin-thiyl radical, since although little is still known about the toxicology of protein-thiyl radicals [5,11], low-molecular-mass thiyl radicals are usually reactive species which can initiate free radical chain reactions [49,50]. For instance, the albumin-thiyl radical has been shown to transfer its free electron to other protein amino acids, forming carbon-centred radicals [51] which are reactive species and able to propagate free radical reactions. Consequently, although the biological fate of the radicals generated by peroxynitrite in plasma remains to be thoroughly studied, it is clear that localized formation of large flows of peroxynitrite may lead to antioxidant depletion [23] and formation of antioxidant-derived free radicals such as the albumin-thiyl and the uric acid-derived free radical which can attack other plasma components propagating the deleterious effects of peroxynitrite. The possible role of other secondary radical species such as the bicarbonate free radical [23,52] is not excluded but was not emphasized since these species were not detected under the experimental conditions employed (Figures 5–7).

In conclusion, our results support the view that peroxynitrite-mediated one-electron oxidation of biomolecules may be an important event in its cytotoxic mechanism [4,5,8,10–14,53] since it may occur under physiologically relevant environments such as human plasma. By extension, the role of peroxynitrite as a key intermediate in free radical-dependent damage to biological systems is substantiated, although much work is needed to determine the precise contribution of the oxidant to the development of several pathological conditions such as immunocomplex-stimulated pulmonary oedema [54], heart ischaemia–reperfusion injury [55], and atherogenesis [56,57].

This work was supported by grants from Fundação de Amparo à pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico and Financiadora de Estudos e Projetos.

REFERENCES

1 Huie, R. E. and Padmaja, S. (1993) Free Radical Res. Commun. **18**, 195–199
2 Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. and Freeman, B. A. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1620–1624
3 Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H. and Beckman, J. S. (1992) Chem. Res. Toxicol. **5**, 834–842
4 Pryor, W. A. and Squadrito, G. L. (1995) Am. J. Physiol. L699–L722
5 Augusto, O. and Radi, R. (1995) in Biothiols in Health and Disease (Packer, L. and Cadenas, E., eds.), pp. 83–116, Marcel Dekker, New York
6 Radi, R., Beckman, J. S., Bush, K. M. and Freeman, B. A. (1991) Arch. Biochem. Biophys. **288**, 481–487
7 Moreno, J. J. and Pryor, W. A. (1992) Chem. Res. Toxicol. **5**, 425–431
8 Pryor, W. A., Jin, X. and Squadrito, G. L. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 11173–11177
9 Radi, R., Beckman, J. S., Bush, K. M. and Freeman, B. A. (1991) J. Biol. Chem. **266**, 4244–4250
10 Augusto, O., Gatti, R. M. and Radi, R. (1994) Arch. Biochem. Biophys. **310**, 118–125
11 Gatti, R. M., Radi, R. and Augusto, O. (1994) FEBS Lett. **348**, 287–290
12 Bartlett, D., Church, D. F., Bounds, P. L. and Koppenol, W. H. (1995) Free Radical Biol. Med. **18**, 85–92
13 Denicola, A., Souza, J. M., Gatti, R. M., Augusto, O. and Radi, R. (1995) Free Radical Biol. Med. **19**, 11–19
14 Augusto, O., Radi, R., Gatti, R. M. and Vasquez-Vivar, J. (1996) in Methods in Enzymol., in the press
15 Halliwell, B. and Gutteridge, J. M. C. (1992) FEBS Lett. **307**, 108–112
16 Gutteridge, J. M. C., Rowley, D. A. and Halliwell, B. (1982) Biochem. J. **206**, 605–609

17 Frei, B., Stocker, R. and Ames, B. N. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9748–9752
18 Ames, B. N., Cathcart, R., Schwiers, E. and Hochstein, P. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 6858–6862
19 Halliwell, B. and Gutteridge, J. M. C. (1990) Arch. Biochem. Biophys. **280**, 1–8
20 Wayner, D. D. M., Burton, G. W., Ingold, K. U., Barclay, L. R. C. and Locke, S. J. (1987) Biochim. Biophys. Acta **924**, 408–419
21 Oury, T. D., Piantadosi, C. A. and Crapo, J. (1993) J. Biol. Chem. **268**, 15394–15398
22 Laurindo, F. R. M., Pedro, M. A., da Luz, P. and Augusto, O. (1996) Ciência e Cultura, in the press
23 Van der Vliet, A., Smith, D., O'Neill, C. A., Kaur, H., Darley-Usmar, V., Cross, C. E. and Halliwell, B. (1994) Biochem. J. **303**, 295–301
24 Buettner, G. R. and Jurkiewicz, B. A. (1993) Free Radical Biol. Med. **14**, 49–55
25 Pedro, M. A., Gatti, R. M. and Augusto, O. (1993) Química Nova **16**, 370–372
26 Mehlhorn, R. J. (1991) J. Biol. Chem. **266**, 2724–2731
27 Buettner, G. R. (1980) Free Radical Biol. Med. **10**, 5–9
28 Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 70–77
29 Schultz, A. L. (1988) in Química Clínica (Kaplan, L. A. and Pesce, A. J., eds.), pp. 1491–1497, Panamericana Editora Medica, Buenos Aires
30 Hatch, L. L. and Sevanian, A. (1984) Anal. Biochem. **138**, 324–328
31 Robinson, J. and Cooper, J. M. (1970) Anal. Biochem. **33**, 390–399
32 Minetti, M., Forte, T., Soriani, M., Quaresima, V., Menditto, A. and Ferrari, M. (1992) Biochem. J. **282**, 459–465
33 Laurindo, F. R. M., Pedro, M. A., Barbeiro, H. V., Pileggi, F., Carvalho, M. H. C., Augusto, O. and da Luz, P. L. (1994) Circ. Res. **74**, 700–709
34 Minakata, K., Suzuki, O., Saito, S. and Harada, N. (1993) Arch. Toxicol. **67**, 126–130
35 Roginsky, V. A. and Stegman, H. B. (1994) Free Radical Biol. Med. **17**, 93–103
36 Buettner, G. R. (1987) Free Radical Biol. Med. **3**, 259–303
37 Davies, M. J., Fu, S. and Dean, R. T. (1995) Biochem. J. **305**, 643–649
38 Gatti, R. M., Augusto, O., Kwee, J. K. and Giorgio, S. (1995) Redox Report **1**, 261–265
39 Moro, M. A., Darley-Usmar, V. M., Goodwin, D. A., Read, N. G., Zamora-Pino, R., Feelish, M. and Moncada, S. (1994) Proc. Natl Acad. Sci. U.S.A. **91**, 6702–6706
40 Villa, L. M., Salas, E., Darley-Usmar, V. M., Radomski, M. W. and Moncada, S. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 12383–12387
41 Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M. and Freeman, B. A. (1994) J. Biol. Chem. **269**, 26066–26075
42 Zhu, L., Gunn, C. and Beckman, J. S. (1992) Arch. Biochem. Biophys. **298**, 452–457
43 Ischiropoulos, H., Zhu, L. and Beckman, J. S. (1992) Arch. Biochem. Biophys. **298**, 446–451
44 Carreras, M. C., Pargament, G. A., Catz, S. D., Poderoso, J. J. and Boveris, A. (1994) FEBS Lett. **341**, 65–68
45 Maples, K. R. and Mason, R. P. (1988) J. Biol. Chem. **263**, 1709–1712
46 Halliwell, B., Hu, M.-L., Louie, S., Duvall, T. R., Tarkington, P. M. and Cross, C. E. (1992) FEBS Lett. **313**, 62–66
47 Kittridge, K. J. and Willson, R. L. (1984) FEBS Lett. **170**, 162–164
48 Auroma, O. I. and Halliwell, B. (1989) FEBS Lett. **244**, 76–80
49 Mason, R. P. and Rao, D. N. R. (1990) in Methods in Enzymology (Packer, L. and Glazer, A. N., eds.), vol. 186, Part B, pp. 319–329, Academic Press, New York
50 Asmus, K.-D. (1990) in Methods in Enzymology (Packer, L. and Glazer, A. N., eds.), vol. 186, Part B, pp. 169–180, Academic Press, New York
51 Davies, M. J., Gilbert, B. C. and Haywood, R. M. (1993) Free Radical Res. Commun. **18**, 353–367
52 Radi, R., Cosgrove, T. P., Beckman, J. S. and Freeman, B. A. (1993) Biochem. J. **290**, 51–57
53 Van der Vliet, A., O'Neill, C. A.,. Halliwell, B., Cross, C. E. and Kaur, H. (1994) FEBS Lett. **339**, 89–92
54 Mulligan, M. S., Hevel, J. M., Marletta, M. A. and Ward, P. A. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6338–6342
55 Mahteis, G., Sherman, M. P., Buckberg, G. D., Haybron, D. M., Young, H. H. and Ignarro, L. (1992) Am. J. Physiol. **262**, H616–H620
56 White, R. C., Brock, T. A., Chang, L. Y., Crapo, J. D., Briscoe, P., Ku, D., Bradley, W. A., Gianturco, S. H., Gore, J., Freeman, B. A. and Tarpey, M. M. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 1044–1048
57 Beckman, J. S., Ye, Y. Z., Anderson, P., Chen, J., Accavitti, M. A., Tarpey, M. M. and White, C. R. (1994) Biol. Chem. Hoppe-Seyler **375**, 81–88

Received 17 July 1995/27 October 1995; accepted 8 November 1995