Free Rad. Res., Vol. 25, No. 3, pp. 275–283
Reprints available directly from the publisher
Photocopying permitted by license only

Protection Against Peroxynitrite-Dependent Tyrosine Nitration and α_1-Antiproteinase Inactivation by Ascorbic Acid. A Comparison with other Biological Antioxidants

MATTHEW WHITEMAN and BARRY HALLIWELL

Neurodegenerative Disease Research Centre, Pharmacology Group, King's College, Manresa Road, London SW3 6LX, UK

Accepted by Dr V. Darley-Usmar

(Received 31st October 1995; Revised March 5th 1996)

Peroxynitrite, formed by reaction of superoxide and nitric oxide, appears to be an important tissue-damaging species generated at sites of inflammation. In this paper, we compare the abilities of several biological antioxidants to protect against peroxynitrite-dependent inactivation of α_1-antiproteinase, and to inhibit tyrosine nitration upon addition of peroxynitrite. GSH and ascorbate protected efficiently in both systems. Uric acid inhibited tyrosine nitration but not α_1-antiproteinase inactivation. The possibility that ascorbic acid is an important scavenger of reactive nitrogen species *in vivo* is discussed.

Keywords: Peroxynitrite, ascorbate, tyrosine nitration, α_1-antiproteinase, reactive nitrogen species, rheumatoid arthritis, methionine, GSH

INTRODUCTION

It is well-known that various reactive oxygen species such as superoxide radical, hydrogen peroxide, hydroxyl radical, and hypochlorous acid, are formed *in vivo* and contribute to tissue injury in human disease (reviewed in refs. 1–3). Recently, however, there has been considerable interest in reactive *nitrogen* species as mediators of tissue injury (reviewed in refs. 4,5). Although nitric oxide ($NO^{\bullet}$) has many important physiological functions[6], its production in excess may contribute to the pathology of neurodegenerative disease, chronic inflammation, acute respiratory distress syndrome, atherosclerosis and septic shock[4–9].

Part of the toxicity of $NO^{\bullet}$ involves its very fast[10] reaction with superoxide radical ($O_2^{\bullet -}$) to give peroxynitrite[4], $ONOO^-$

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^- \quad (1)$$

Peroxynitrite and its decomposition products induce peroxidation of lipids, oxidize methionine and -SH residues in proteins, deplete antioxidants

Corresponding author: Prof. B. Halliewell.
Present address: Pharmacology Group, King's College, Manresa Road, London SW# 6LX, UK

and cause DNA damage (reviewed in refs. 4,5,11). In particular, addition of peroxynitrite to biological systems leads to nitration of tyrosine residues, and the presence of these has been suggested to be a "marker" of peroxynitrite-dependent damage *in vivo*[4,7,8,12]. Tyrosine nitration can interfere with signal transduction mechanisms involving phosphorylation/dephosphorylation[13]. Peroxynitrite also inactivates α_1-antiproteinase[14], the major inhibitor of serine proteases (such as elastase) in human body fluids[2]. Hence peroxynitrite generation *in vivo* may be able to facilitate both oxidative and proteolytic damage.

Ascorbic acid (vitamin C) is thought to play an important role in protection against reactive oxygen species *in vivo*[15–20]. For example, it can scavenge $O_2^{\bullet-}$ and HOCl, reconvert α-tocopheryl radical to α-tocopherol[17], inhibit lipid-peroxidation catalyzed by haem proteins[20] and protect constituents of the lung lining fluids against inhaled oxidizing air pollutants (reviewed in ref. 21). Ascorbate also reacts with peroxynitrite[22–24] and addition of peroxynitrite to human plasma caused ascorbate depletion[25]. However, it is uncertain whether or not the rate of reaction of $ONOO^-$ with ascorbate is sufficiently high to be of physiological significance[22,24]. The chemistry of peroxynitrite is, however, very complex: different $ONOO^-$-derived species may be responsible for damage to different molecular targets[11]. Hence attempting to deduce the relative biological importance of different antioxidants as $ONOO^-$ scavengers *in vivo* on the basis of published rate constants may give misleading results.

In the present paper, we show that physiological concentrations of ascorbate do appear to be able to scavenge peroxynitrite and/or peroxynitrite-derived species, in that they can protect tyrosine against nitration and α_1-antiproteinase against inactivation upon addition of $ONOO^-$. The protective effect of peroxynitrite was compared with that of methionine and Trolox C and with the known biological antioxidants urate and GSH.

MATERIALS AND METHODS

Reagents

N-succinyl $(ala)_3$ p-nitroanilide (SANA), catalase (type C40), elastase (E0258), α_1-antiproteinase (A9024) and D, L-tyrosine were from Sigma. All other reagents including Analar L-ascorbate were from BDH Chemicals.

Peroxynitrite Synthesis[4]

An acidic solution (0.6M HCl) of H_2O_2 (0.7M) was mixed with KNO_2 (0.6M) on ice for one second and the reaction quenched with ice cold NaOH (1.2M). The stock was then frozen overnight (–20°C) and the top layer of the solution collected for the experiment[4]. Concentrations of stock $ONOO^-$ were redetermined before each experiment at 302 nm using a molar absorption coefficient of 1670 cm^{-1} M^{-1}. Concentrations of 250–300 mM were usually obtained.

Measurement of Tyrosine Nitration

A stock concentration (10 mM) of D, L-tyrosine was prepared by dissolving the required amount in 8 ml of water with 250 µl 10% (w/v) KOH followed by 250 µl 5% phosphoric acid with 1.5 ml water. 0.1 ml of tyrosine solution together with 0.1 ml of a solution of the compound to be tested was added to a plastic test tube containing 0.795 ml buffer (500 mM K_2HPO_4-KH_2PO_4 pH 7.4) and incubated in a water bath at 37°C for 15 minutes. After this time peroxynitrite (typically 5 µl) was added to a final concentration of 1 mM, the tubes vortexed for 15 seconds and incubated for a further 15 minutes. The pH was measured after the addition of peroxynitrite and found to be 7.4–7.5.

Measurement of 3-nitrotyrosine was performed essentially as described previously[26] using a Spherisorb 5µm ODS2 C_{18} column (Wellington House, Cheshire, England) with a guard column (Hibar from BDH, Poole, England)

and C_{18} cartridge. The eluent was 500 mM KH_2PO_4-H_3PO_4 pH 3.01, with 20% methanol (v/v) at a flow rate of 1 ml min^{-1} through a Polymer Laboratories pump (Essex Road, Church Stretton, England) and UV detector set at 274 nm. The 3-nitrotyrosine detected was confirmed by spiking with standards. Peak heights of tyrosine and 3-nitrotyrosine were measured and concentrations calculated from a standard curve. The limit of sensitivity was 0.5 μM 3-nitrotyrosine: typical retention time under our experimental conditions was 3.6 min.

Prevention of α_1-Antiproteinase Inactivation

Elastase and α_1-antiproteinase were measured essentially as described in ref. 27 α_1-Antiproteinase was dissolved in phosphate-buffered saline, pH 7.4 (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2.9 mM KH_2PO_4) to a concentration of 4 mg/ml and elastase in the same buffer to 5 mg/ml. The volume of α_1AP needed to inhibit elastase 80–90% (typically 60–70 μl) was added to buffer (500 mM K_2HPO_4-KH_2PO_4 pH 7.4) with or without 0.1 ml compound to be tested to give a volume of 0.945 ml (final α_1AP concentration 0·3 mg/ml) and incubated in a water bath at 37°C for 15 min, when peroxynitrite (typically 5 μl) was added to give a final concentration of 0.5 mM. The sample was vortexed for 10 seconds and incubated for 5 minutes. Then elastase (usually 50 μl) was added followed by 2.0 ml of buffer and the sample incubated at 37°C for a further 15 min. Then 0.1 ml of elastase substrate (SANA) was added and the rate of reaction followed at 410 nm for 30 sec.

RESULTS

In this paper, we compared the ability of various antioxidants to protect against damage by $ONOO^-$ to two molecules, tyrosine and α_1AP. Tyrosine was chosen because its nitration, apparently by $ONOO^-$, has been observed *in vivo*[4,7–9]. α_1AP was selected because it is known to be inactivated in the inflamed rheumatoid joint[28], and the established generation of $ONOO^-$ at this site of inflammation[9] provides a plausible mechanism[13].

Inactivation of α_1-Antiproteinase

The concentration of α_1AP in human plasma is 1.2–1.3 mg/ml, and a lower final concentration of about 0.3 mg/ml was used in our experiments. As expected[14], addition of $ONOO^-$ to α_1-antiproteinase (α_1AP) led to inactivation of the ability of α_1AP to inhibit elastase. Although 12h of incubation was used in the first paper reporting this inactivation[14], we found that reaction was complete within 5 min at physiological pH (Figure 1). The extent of inactivation increased with $ONOO^-$ concentration (Figure 2). A 5 min incubation time with 0.5 mM $ONOO^-$ was selected for further studies. The $ONOO^-$ solution is impure and the possibility that other constituents of the $ONOO^-$ solution were involved in the inactivation was investigated. H_2O_2 was not involved: MnO_2 treatment of the $ONOO^-$ to remove H_2O_2 did not affect its inhibitory capacity, when allowance was made for the fact that the treatment itself decreased $ONOO^-$ concentrations. Catalase (final concentration 10^3 units/ml) had no effect on inactivation of α_1AP by $ONOO^-$. Although incubation of catalase with mM concentrations of $ONOO^-$ did cause a partial inactivation of catalase, the rate of this inactivation would be insignificant under our experimental conditions (data not shown). The effect of other constituents was ruled out by showing that "decomposed $ONOO^-$" had no effect on α_1AP: if the $ONOO^-$ solution was added to the buffer and incubated for 5 min at 37°C before adding α_1AP the resulting "decomposed $ONOO^-$"[4] solution had no effect on α_1AP.

Figures 2 and 3 show that treatment of α_1AP with $ONOO^-$ markedly decreased its ability to inhibit elastase. However, inclusion of ascorbate in the reaction mixtures with α_1AP and $ONOO^-$

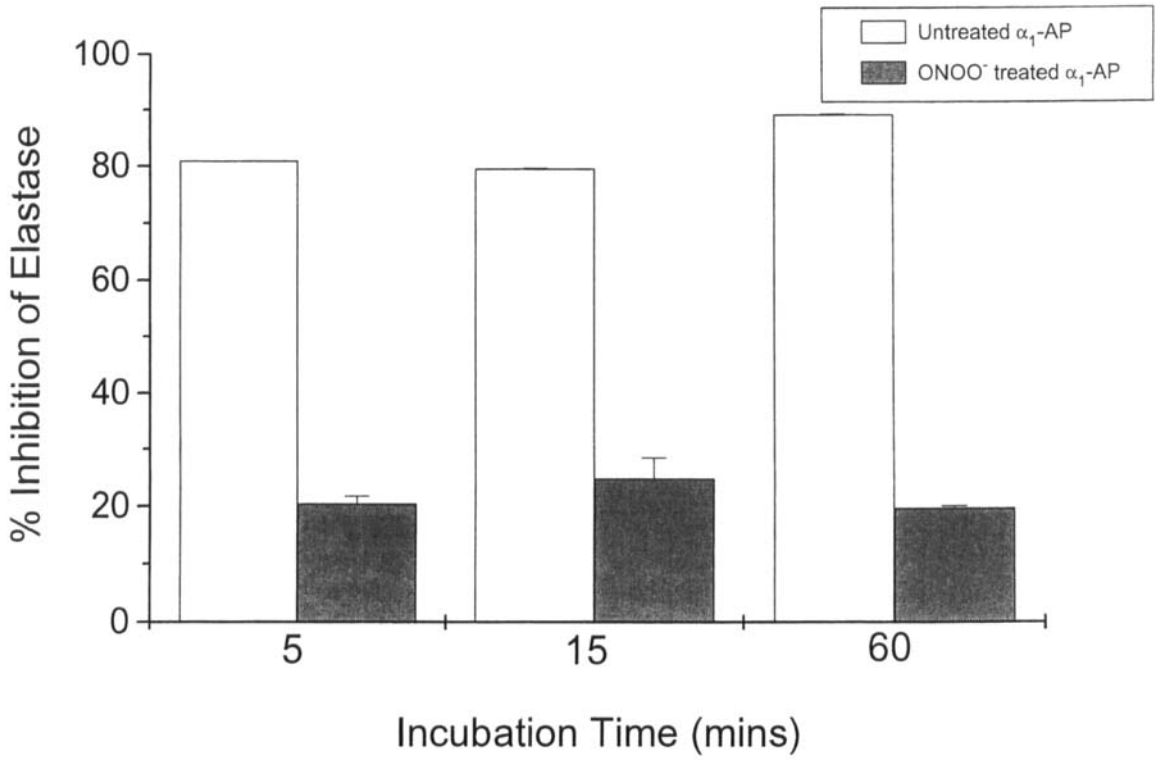

FIGURE 1 Time-Course of Inactivation of α_1-Antiproteinase by Peroxynitrite. α_1-Antiproteinase was incubated with a final concentration of 0.5 mM $ONOO^-$ for the time indicated and residual elastase inhibitory capacity measured as described in Materials and Methods. Results are mean ± SE of 4 or more experiments.

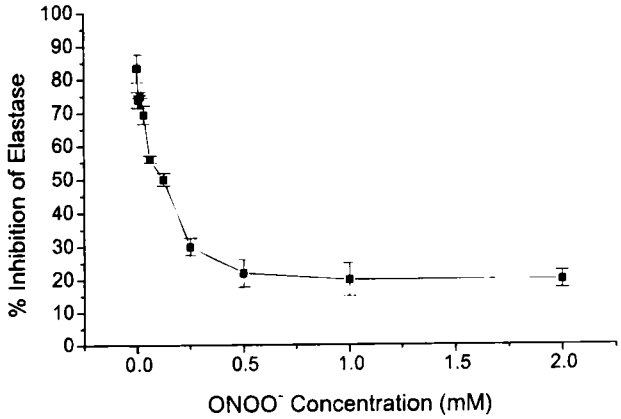

FIGURE 2 Inactivation of α_1-Antiproteinase by Peroxynitrite: Concentration Dependence. α_1-Antiproteinase was incubated with $ONOO^-$ at the final concentration stated for 5 min and residual elastase inhibitory capacity measured as described in Materials and Methods. Data are mean ± SE of 4 or more experiments.

protected α_1AP against inactivation in a concentration-dependent manner (Figure 3). Addition of ascorbate after the 5 min incubation of α_1AP with $ONOO^-$ gave no protective effect. Figure 3 shows that almost complete protection against the effects of 500 μM $ONOO^-$ was given by 300–500 μM ascorbate.

Of course, there may be other molecules that can react with $ONOO^-$ *in vivo*, especially thiols and urate[4,11,26]. However, Figure 4 shows that urate had no protective effect against α_1AP inactivation, and GSH was generally less protective than ascorbate except at the highest concentration tested (1 mM). Data for Trolox are included for comparison.

Inactivation of α_1AP by $ONOO^-$ is thought to involve attack on methionine residues[14]. Consistent with this, addition of methionine to the reaction mixtures gave excellent protection against α_1AP inactivation (Figure 4).

Inhibition of Tyrosine Nitration

When the amino acid tyrosine is exposed to $ONOO^-$ at pH 7.4, 3-nitrotyrosine is formed[4,12,29]. Figure 5 shows that ascorbate was able to decrease 3-nitrotyrosine formation, in a concentration-dependent manner. Concentrations of 500 μM ascorbate or higher were able to prevent nitration almost completely. GSH and Trolox were approximately equally protective with ascorbate, whereas methionine was less effective and uric acid (at low concentrations) was more effective than ascorbate. Tyrosine could also protect α_1AP against inactivation to some extent (Figure 6).

DISCUSSION

Peroxynitrite generation *in vivo* is being implicated in a wide range of human diseases, including atherosclerosis[8], lung disease[7], neurodegenerative disorders[5] and chronic inflammation[9,30]. Hence agents able to protect against $ONOO^-$-dependent damage may be physiologically important and perhaps therapeutically useful. Ascorbate is present in human blood plasma at concentrations in the 30–100 μM range (higher in lung lining, cerebrospinal and seminal fluids)[15,16,18,19,21] and intracellular ascorbate concentrations, especially in neutrophils, may be in the millimolar range[16,31]. Hence the *in vitro* protective effects of ascorbate

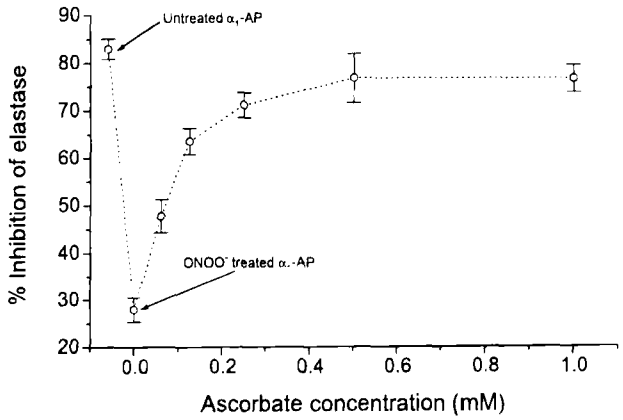

FIGURE 3 Prevention of Peroxynitrite-Dependent α_1-Antiproteinase Inactivation by Ascorbate. α_1-Antiproteinase was incubated with 0.5 mM $ONOO^-$ and the final ascorbate concentration stated for 5 min. Residual elastase inhibitory capacity was then measured as described in Materials and Methods. Data are mean ± SE of 4 or more experiments. Control experiments showed that ascorbate had no direct effect on elastase (run experiment without α_1AP added) or on the ability of α_1AP to inhibit elastase (run experiment without $ONOO^-$), nor could ascorbate reactivate α_1AP after it had been inhibited by $ONOO^-$ (add ascorbate after 5 min incubation of α_1AP with $ONOO^-$).

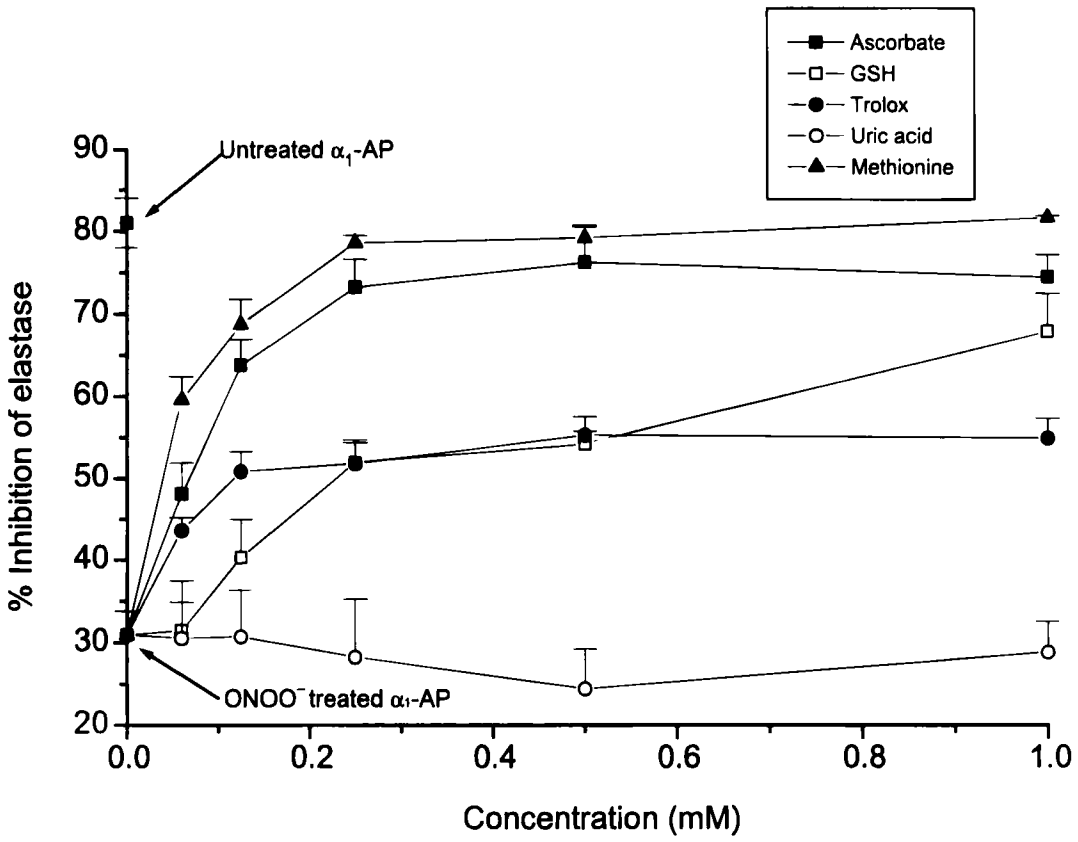

FIGURE 4 Prevention of Peroxynitrite-Dependent α_1-Antiproteinase Inactivation by Ascorbate: A Comparison with GSH, Trolox, Uric Acid and Methionine. Reaction conditions and controls are as specified in the legend to Figure 3: none of the above compounds had any effect on the assay procedures. Concentrations given on the X-axis are the final concentrations in the reaction mixtures.

against $ONOO^-$-dependent tyrosine nitration and α_1AP inactivation reported in this paper could be feasible *in vivo*. Ascorbate has also been shown to inhibit tyrosine nitration by reactive nitrogen species present in cigarette smoke[32]. Whether ascorbate is actually an important $ONOO^-$ scavenger *in vivo* will obviously depend upon several factors, including the concentration of ascorbate in relation to other potential scavengers of $ONOO^-$, such as thiols and urate. For example, GSH is present at millimolar levels intracellularly, whereas concentrations in human body fluids are in the low micromolar range, except in lower respiratory tract lining fluid (~100–400 μM)[23]. GSH was approximately equally protective to ascorbate against tyrosine nitration, but less so against α_1AP inactivation. Urate, present in human plasma at 150–300 μM concentrations, was very protective against tyrosine nitration but could not protect against α_1AP inactivation.

Our data indicate that ascorbate appears to be a fairly-good overall protector against $ONOO^-$-dependent damage, comparable to GSH. Our data also illustrate the complexities of $ONOO^-$ chemistry, as discussed in[11]. Whereas inactivation of α_1AP might be due to attack of $ONOO^-$ itself upon methionine within the protein, as illustrated by the high degree of protection by added methionine, tyrosine nitration is a complex reaction and antioxidants could act not only by scavenging

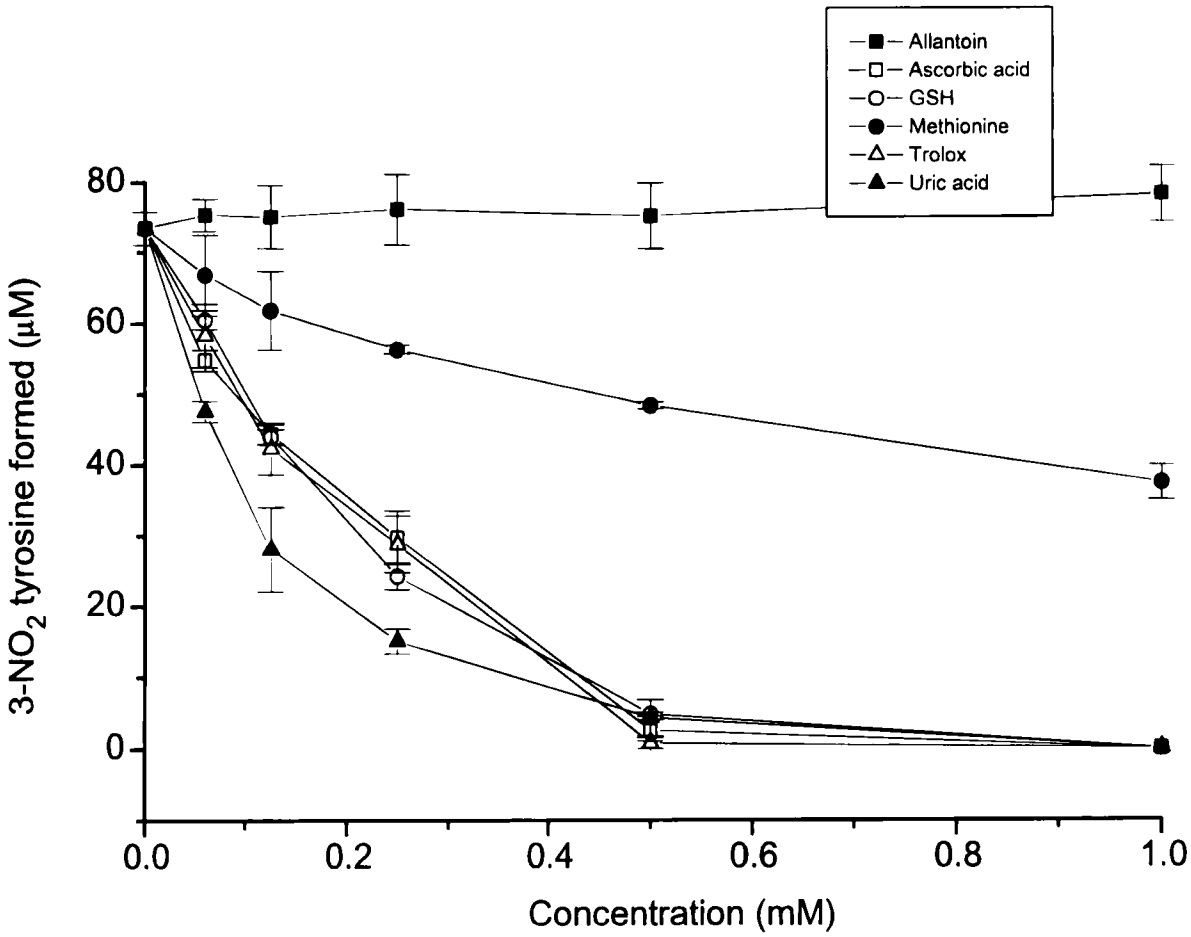

FIGURE 5 Effect of Ascorbate, Allantoin, GSH, Methionine, Trolox, GSSG and Uric Acid on Tyrosine Nitration by Peroxynitrite. DL-Tyrosine (1 mM) was incubated with $ONOO^-$ (1 mM) for 15 min at 37°C. Where indicated, antioxidants were present in the reaction mixtures at the final concentrations indicated. Data are mean ± SE of 4 or more experiments. None of the antioxidants tested interfered with HPLC analysis of 3-nitrotyrosine.

$ONOO^-$ but also by reacting with tyrosine intermediates or nitrating species such as NO_2^+ or $NO_2^{\cdot}$[29,32]. Indeed, methionine was relatively less protective to tyrosine than it was to α_1AP, implying a different reaction mechanism. One striking discrepancy is the effect of urate: highly-protective against tyrosine nitration but not against α_1AP inactivation. A similar situation has been observed in studies of $OH^{\bullet}$-mediated damage to proteins (including α_1AP). Urate reacts rapidly with $OH^{\bullet}$ but failed to protect, apparently because the resulting urate-derived radicals could also damage the proteins[34,35]. It is unlikely that $ONOO^-$ decomposition produces much $OH^{\bullet}$, but it is possible that urate radical arising by other mechanisms could inactivate α_1AP[35]. Allantoin, a major product of oxidative damage to urate[36] had no effect on tyrosine nitration (Figure 5) or α_1AP inactivation (data not shown).

Our observations may be particularly relevant in patients with rheumatoid arthritis, in whom 3-nitrotyrosine formation has been demonstrated[9], as has inactivation of α_1AP in the synovial fluid[28]. Levels of ascorbate in RA patients are sub-normal[37,38], perhaps because it is being consumed by $ONOO^-$ and other oxidiz-

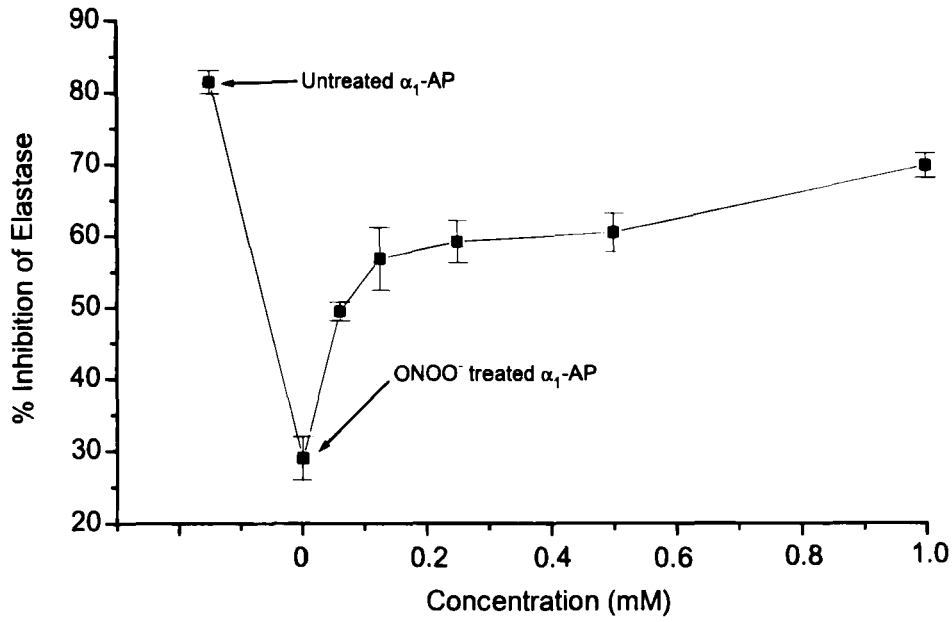

FIGURE 6 Effect of tyrosine on inactivation of α_1-Antiproteinase by Peroxynitrite. Reaction conditions and controls are as specified in the legends to figures 3 and 4. Concentrations on the X-axis are the final concentrations of tyrosine in the reaction mixture.

ing species (such as HOCl[39]) in the inflamed joints. Synovial fluid has little, if any GSH and so the low ascorbate levels in RA may predispose to α_1AP inactivation and resultant proteolytic damage in the inflamed joints.

Acknowledgements

We thank the Arthritis and Rheumatism Council (UK) for research support, including a research studentship for MW. We are grateful to Carroll Cross, Albert van der Vliet, and Jason Eiserich for helpful comments on the manuscript and to Pat Evans for advice on the assay of α_1AP.

References

[1] B. Halliwell and J. M. C. Gutteridge, (1989) *Free Radicals in Biology and Medicine*, 2nd edn. Clarendon Press, Oxford.

[2] S. J. Weiss,(1989) Tissue destruction by neutrophils. *New England Journal of Medicine*, **320**, 365–376.

[3] B. Halliwell, C. E. Cross and J. M. C. Gutteridge, (1992) Free radicals, antioxidants and human disease. Where are we now? *Journal of Laboratory and Clinical Medicine*, **119**, 598–620.

[4] J. S. Beckman, J. Chen, H. Ischiropoulos and J. P. Crow, (1994) Oxidative chemistry of peroxynitrite. *Methods in Enzymology*, **233**, 229–240.

[5] V. Darley-Usmar, H. Wiseman and B. Halliwell, (1995) Nitric oxide and oxygen radicals: a question of balance. *FEBS Letters*, **369**, 131–135.

[6] S. Moncada and A. Higgs, (1993) The L-arginine-nitric oxide pathway. *New England Journal of Medicine*, **329**, 2002–2011.

[7] I. Y. Haddad, G. Pataki, P. Hu, C. Galliani, J. S. Beckman, and S. Matalon (1994) Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *Journal of Clinical Investigation*, **94**, 2407–2413.

[8] J. S. Beckman, Y. Zu Ye, G. Anderson, J. Chen, M. A. Accavitti, M. M. Tarpey and C. R. White, (1994) Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biological Chemistry Hoppe Seyler*, **375**, 81–88.

[9] H. Kaur and B. Halliwell, (1994) Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. *FEBS Letters*, **350**, 9–12.

[10] R. E. Huie, and S. Padmaja, (1993) The reaction of NO with superoxide. *Free Radical Research Communications*, **18**, 195–199.

[11] W. A. Pryor, and G. L. Squadrito, (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *American Journal of Physiology*, **268**, L699–L722.

[12] H. Ischiropoulos, L. Zhu, J. Chen, M. Tsai, J. C. Martin, C. D. Smith and J. S. Beckman, (1992) Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Archives of Biochemistry and Biophysics*, **298**, 431–437.

[13] S. B. Kong, M. B. Yim, E. R. Stadtman and P. B. Chock, (1995) Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism by nitrating the tyrosine residue: tyrosine kinase fails to phosphorylate nitrated tyrosine. *FASEB Journal*, **9**, A1303 (abstract).

[14] J. J. Moreno and W. A. Pryor, (1992) Inactivation of α_1-proteinase inhibitor by peroxynitrite. *Chemical Research in Toxicology*, **5**, 425–431.

[15] B. Frei, L. England and B. N. Ames, (1989) Ascorbate is an outstanding antioxidant in human blood plasma. *Proceedings of the National Academy of Sciences of the USA*, **86**, 6377–6381.

[16] A. Bendich, L. J. Machlin, O. Scandurra, G. W. Burton and D. D. M. Wayner, (1986) The antioxidant role of vitamin C. *Advances in Free Radical Biology and Medicine*, **2**, 419–444.

[17] G. R. Buettner, (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol and ascorbate. *Archives of Biochemistry and Biophysics*, **300**, 535–543.

[18] R. C. Rose and A. M. Bode, (1993) Biology of free radical scavengers: an evaluation of ascorbate. *FASEB Journal*, **7**, 1135–1142.

[19] B. Halliwell, (1990) How to characterize a biological antioxidant. *Free Radical Research Communications*, **9**, 1–32.

[20] C. Rice-Evans, G. Okunade and R. Khan, (1989) The suppression of iron release from activated myoglobin by physiological electron donors and by desferrioxamine. *Free Radical Research Communications*, **7**, 45–54.

[21] C. E. Cross, A. van der Vliet and C. A. O'Neill, (1994) Oxidants, antioxidants and respiratory tract lining fluids. *Environmental Health Perspectives*, **102**, (suppl 10), 185–191.

[22] D. Bartlett, D. F. Church, P. L. Bounds and W. H. Koppenol, (1995) The kinetics of the oxidation of L-ascorbic acid by peroxynitrite. *Free Radical Biology and Medicine*, **18**, 85–92.

[23] X. Shi, Y. Rojanasakul, P. Gannett, K. Liu, Y. Mao, L. N. Daniel, N. Ahmed and U. Saffiotti, (1994) Generation of thiyl and ascorbyl radicals in the reaction of peroxynitrite with thiols and ascorbate at physiological pH. *Journal of Inorganic Biochemistry*, **56**, 77–86.

[24] G. L. Squadrito, X. Jin and W. A. Pryor, (1995) Stopped-flow kinetic study of the reaction of ascorbic acid with peroxynitrite. *Archives of Biochemistry and Biophysics*, **322**, 53–59.

[25] A. van der Vliet, D. Smith, C. A. O'Neill, H. Kaur, V. Darley-Usmar, C. E. Cross, and B. Halliwell (1994) Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion. *Biochemical Journal*, **303**, 295–301.

[26] A. van der Vliet, C. A. O'Neill, B. Halliwell, C. E. Cross and H. Kaur, (1994) Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite. *FEBS Letters*, **339**, 89–92.

[27] P. J. Evans, R. Cecchini and B. Halliwell, (1992) Oxidative damage to lipids and α_1-antiproteinase by phenylbutazone in the presence of haem proteins. Protection by ascorbic acid. *Biochemical Pharmacology*, **44**, 981–984.

[28] K. Chidwick, P. G. Winyard, Z. Zhang, A. J. Farrell and D. R. Blake, (1991) Inactivation of the elastase inhibitory capacity of α_1-antitrypsin in fresh samples of synovial fluid from patients with rheumatoid arthritis. *Annals of Rheumatic Disease*, **50**, 915–916.

[29] A. van der Vliet, J. P. Eiserich, C. A. O'Neill, B. Halliwell, and C. E. Cross, (1995) Tyrosine modification by reactive nitrogen species. A closer look. *Archives of Biochemistry and Biophysics*, **319**, 341–349.

[30] D. Rachmilewitz, J. S. Stamler, F. Karmeli, M. E. Mullins, D. J. Singel, J. Loscalzo, R. J. Xavier and D. K. Podelsky, (1993) Peroxynitrite-induced rat colitis—a new model of colonic inflammation. *Gastroenterology*, **105**, 1681–1688.

[31] P. Bergsten, G. Amitai, J. Kehrl, K. R. Dhariwal, H. G. Klein and M. Levine, (1990) Millimolar concentrations of ascorbic acid in purified human mononuclear leukocytes. *Journal of Biological Chemistry*, **265**, 2584–2587.

[32] J. P. Eiserich, V. Vossen, C. A. O'Neill, B. Halliwell, C. E. Cross and van der Vliet, A. (1994) Molecular mechanisms of damage by excess nitrogen oxides: nitration of tyrosine by gas-phase cigarette smoke. *FEBS Letters*, **353**, 53–56.

[33] B. Halliwell, M. L. Hu, S. Louie, T. R. Duvall, B. R., Tarkington, P. Motchnik and C. R. Cross, (1992) Interaction of nitrogen dioxide with human plasma. Antioxidant depletion and oxidative damage. *FEBS Letters*, **313**, 62–66.

[34] K. J. Kittridge, and R. L. Willson, (1984) Uric acid substantially enhances the free radical-induced inactivation of alcohol dehydrogenase. *FEBS Letters*, **170**, 162–164.

[35] O. I. Aruoma, and B. Halliwell, (1989) Inactivation of alpha-1-antiproteinase by hydroxyl radicals. The effect of uric acid. *FEBS Letters*, **244**, 76–80.

[36] H. Kaur, and B. Halliwell, (1990) Action of biologically-relevant oxidizing species upon uric acid. Identification of uric acid oxidation products. *Chemico-Biological Interactions*, **73**, 235–247.

[37] D. R. Blake, N. D. Hall, D. A. Treby, B. Halliwell and J. M. C. Gutteridge, (1981) Protection against superoxide and hydrogen peroxide in synovial fluid from rheumatoid patients. *Clinical Science*, **61**, 483–486.

[38] J. Lunec, and D. R. Blake, (1985) The determination of dehydroascorbic acid and ascorbic acid in the serum and synovial fluid of patients with rheumatoid arthritis. *Free Radical Research Communications*, **1**, 31–39.

[39] B. Halliwell, M. Wasil and M. Grootveld, (1987) Biologically-significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by ascorbic acid. Implications for antioxidant protection in the inflamed rheumatoid joint. *FEBS Letters*, **213**, 15–18.