Free Rad. Res., Vol. 26, pp. 49–56
Reprints available directly from the publisher
Photocopying permitted by license only

Prevention of Peroxynitrite-Dependent Tyrosine Nitration and Inactivation of α_1-Antiproteinase by Antibiotics

MATTHEW WHITEMAN and BARRY HALLIWELL*

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

Accepted by Prof. J.M.C. Gutteridge

(Received 26 June 1996; In revised form 15 July 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 antibiotics to protect against peroxynitrite-dependent inactivation of α_1-antiproteinase, and to inhibit tyrosine nitration by peroxynitrite, *in vitro*. Tetracycline, minocycline, doxycycline, rifamycin and rifampicin were highly-protective in both assay systems, whereas several other antibiotics tested were not. The possibility that antibiotics could affect tissue injury at sites of inflammation by scavenging peroxynitrite is discussed.

Keywords: Peroxynitrite, antibiotic, tetracycline, rifamycin, nitric oxide

INTRODUCTION

It is well-known that various reactive oxygen species such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and hypochlorous acid (HOCl) are formed *in vivo* and contribute to tissue injury in human disease (reviewed in).[1–3] Indeed, several antibiotics have been suggested to exert protective effects at sites of inflammation by scavenging such species.[4–8] For example, tetracycline, ceftazidime, tobramycin and gentamicin have been reported to be HOCl scavengers[4–5,8] whereas sulbactam and ampicillin[6] scavenge HOCl and $OH^{\cdot}$ and penicillin scavenges H_2O_2.[7] Thus it is possible that beneficial effects of these antibiotics at sites of inflammation can be mediated by mechanisms additional to their antibacterial activity.[4]

Recently, however, there has been considerable interest in reactive *nitrogen* species as mediators of tissue injury (reviewed in).[9–11]) Although nitric oxide ($NO^{\cdot}$) has many important physiological functions, its production in excess may contribute to the pathology of chronic inflammation and septic shock.[12] An important mechanism[11–16] involved in the toxicity of excess $NO^{\cdot}$ is its very fast[17] reaction with $O_2^{\cdot-}$ to give peroxynitrite, $ONOO^-$.

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$$

Peroxynitrite and/or its breakdown products

*Corresponding author.

induce peroxidation of lipids, oxidize methionine and -SH residues in proteins, deplete antioxidants and cause DNA damage.[9,11] 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*.[9,10,13–16,18] Tyrosine nitration can interfere with signal transduction mechanisms involving phosphorylation/dephosphorylation.[19] Peroxynitrite also inactivates α_1-antiproteinase,[20] the major inhibitor of serine proteases (such as elastase) in human body fluids. Hence $ONOO^-$ generation *in vivo* may facilitate not only oxidative but also proteolytic damage to biomolecules.

In a previous paper,[21] we studied the reaction of several drugs with $ONOO^-$ and showed that the antibiotic tetracycline appeared to be able to scavenge $ONOO^-$ and/or peroxynitrite-derived species, in that it could protect tyrosine against nitration, and α_1-antiproteinase against inactivation upon addition of $ONOO^-$. In the present paper, we have extended these studies to several other antibiotics.

MATERIALS AND METHODS

Reagents

All antibiotics and reagents were obtained from Sigma Chemical Corp, Poole, Dorset, UK. Elastase was type E-0258 and α_1-antiproteinase type A9024. Peroxynitrite was synthesized as described in.[9] 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. Antibiotics were made up as solutions in distilled water fresh daily.

Tyrosine Nitration

This was measured by HPLC as described in.[22] Briefly, a Spherisorb 5 μm ODS2 C_{18} column (Wellington House, Cheshire, England) was used with a guard column (Hibar from BDH, Poole, UK) and C_{18} cartridge. The eluant 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, UK) 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.

α1-Antiproteinase Inactivation

This was essentially measured as described in.[23] 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_1-antiproteinase 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_1-antiproteinase concentration about 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 was added and the rate of reaction followed at 410 nm for 30 seconds. Addition of $ONOO^-$ to some antibiotics led to the development of chromogens. Amoxicillin and ceftazidime produced slightly yellow colours. The yellow colours of doxycycline, minocycline and tetracycline deepened after $ONOO^-$ addition with doxycycline and tetracycline producing an orange-brown

colour during the course of the incubation. These chromogens did not affect spectrophotometric or HPLC analysis.

RESULTS

We compared the ability of various antibiotics to protect against damage by $ONOO^-$ using two different molecular targets, tyrosine and α_1-antiproteinase. Tyrosine was chosen because its nitration, apparently due to $ONOO^-$, has been observed *in vivo* at sites of inflammation.[13–16] α_1-Antiproteinase was selected because it is known to be inactivated at sites of chronic inflammation,[24] and the generation of $ONOO^-$ provides a plausible mechanism to account for this.[20]

Inactivation of α1-Antiproteinase and Protection by Antibiotics

As expected from previous reports,[20–22] addition of $ONOO^-$ to α_1-antiproteinase (α_1-AP) led to inactivation of the ability of α_1-AP to inhibit elastase. On the basis of previous experiments,[22] a 5 min incubation time with 0.5 mM $ONOO^-$ was selected. Figure 1, column B, shows that α_1-AP is

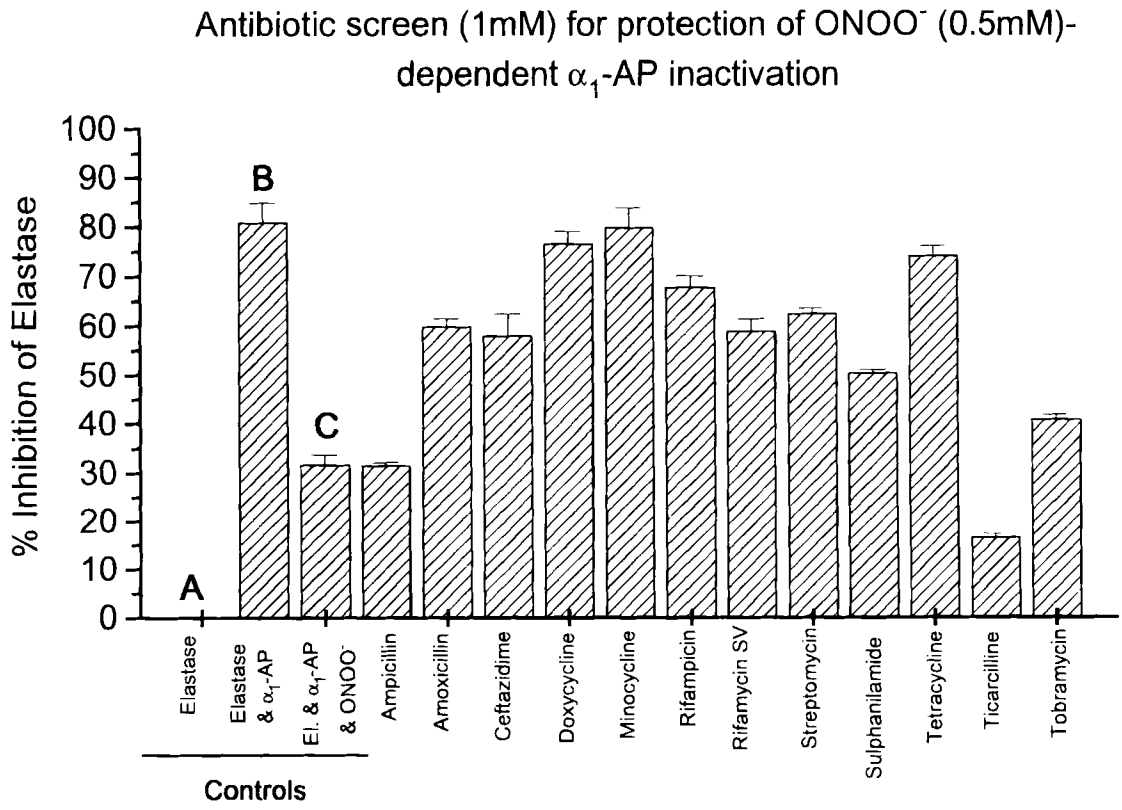

FIGURE 1 A "screen" of antibiotics for the ability to protect α_1-AP against inactivation by peroxynitrite. Column A represents the activity of elastase (i.e. zero inhibition). Addition of α_1-AP inhibits the elastase (column B) but treatment of the α_1-AP with $ONOO^-$ decreases this inhibitory effect (column C). Antibiotics able to scavenge $ONOO^-$ protect the α_1-AP and result in less elastase activity. $ONOO^-$ was present at a final concentration of 0.5 mM and antibiotics at 1.0 mM. Results are mean ± SEM (n = 4). Incubations with $ONOO^-$ were for 5 minutes at 37°C. El—elastase. Control experiments showed that antibiotics had no direct effect on the activity of elastase itself or on the ability of α_1-antiproteinase to inhibit elastase. If α_1-antiproteinase was incubated with $ONOO^-$ before adding antibiotics, subsequent addition of them did not reverse the inactivation.

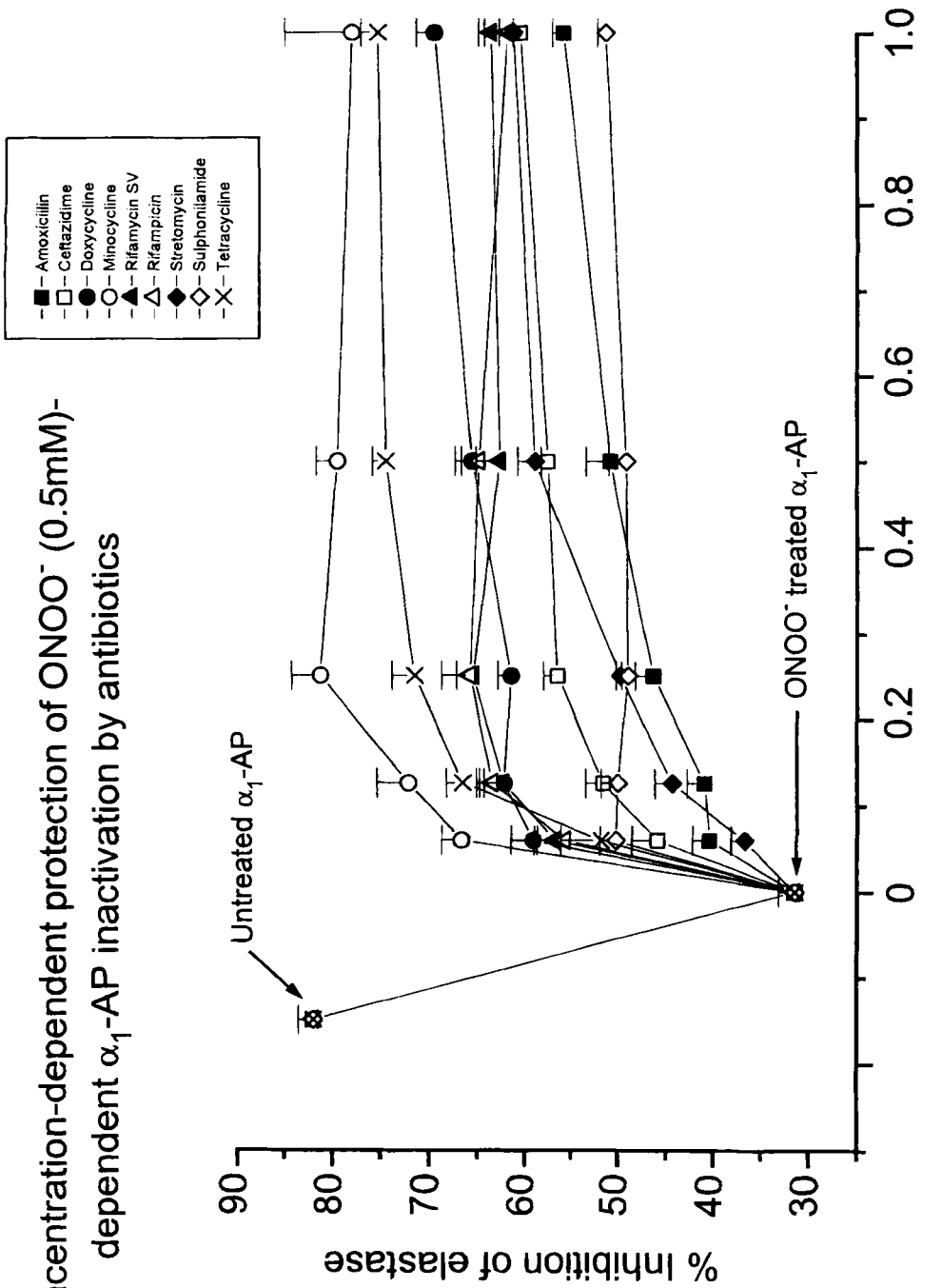

FIGURE 2 Protection by antibiotics of α_1-AP against inactivation by peroxynitrite: concentration-dependence. Experiments were performed as described in the legend to Figure 1, except that antibiotic was added to give the final concentration stated whilst [$ONOO^-$] was kept at 0.5 mM. Results are mean ± SEM (n = 4).

a powerful inhibitor of the enzyme elastase, causing ≥80% of inhibition under our experimental conditions. Treatment of α_1-AP with $ONOO^-$ caused a severe reduction in its elastase inhibitory capacity (column C). The effect of including various antibiotics in the reaction mixture was examined: a concentration greater than that of $ONOO^-$ was used for an initial "screen" of effectiveness (Figure 1). Some antibiotics had very little action (e.g. ampicillin, ticarcilline) but others were highly protective. Figure 2 shows the concentration-dependence of the protective action of the most effective antibiotics. Concentrations of tetracycline, doxycycline and minocycline less than one-fifth those of $ONOO^-$ showed substantial protective effects. Rifampicin and rifamycin were also significantly protective at low concentrations but could not achieve complete protection of α_1-AP at higher concentrations.

Inhibition of Peroxynitrite-Dependent Tyrosine Nitration by Antibiotics

When the amino acid tyrosine is exposed to $ONOO^-$ at pH 7.4, 3-nitrotyrosine is formed.[9,18,25] Figure 3 shows a screen of the ability of antibiotics to inhibit nitration of tyrosine. Ampicillin, amoxicillin, ceftazidime, streptomycin, ticarcilline and tobramycin were poorly protective, whereas tetracycline, doxycycline, minocycline, rifamycin SV, and rifampicin inhibited nitration markedly. Figure 4 shows the concentration-dependence of these effects. Again, concentrations of minocycline, doxycycline, rifampicin, rifamycin SV and

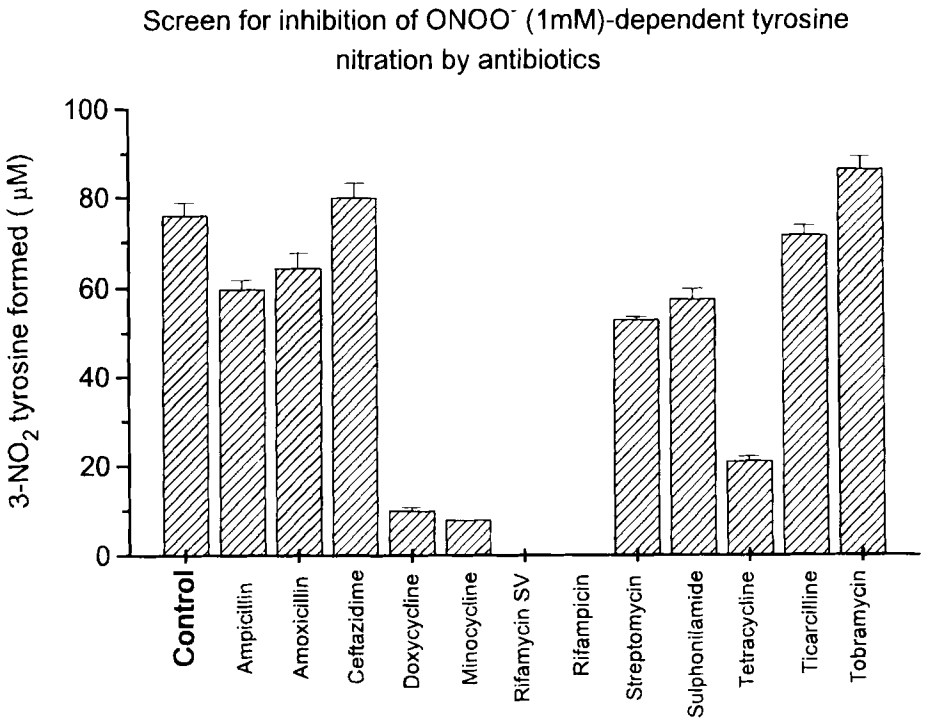

FIGURE 3 Prevention of peroxynitrite-dependent tyrosine nitration by antibiotics. DL-Tyrosine (1 mM) was incubated with $ONOO^-$ (1 mM) for 15 minutes at 37°C. Where indicated, drugs were also present in the reaction mixtures at 1 mM. Data are mean ± SEM (n = 4). None of the antibiotics tested interfered with the HPLC analysis of 3-nitrotyrosine. Rifamycin and rifampicin inhibited nitration completely.

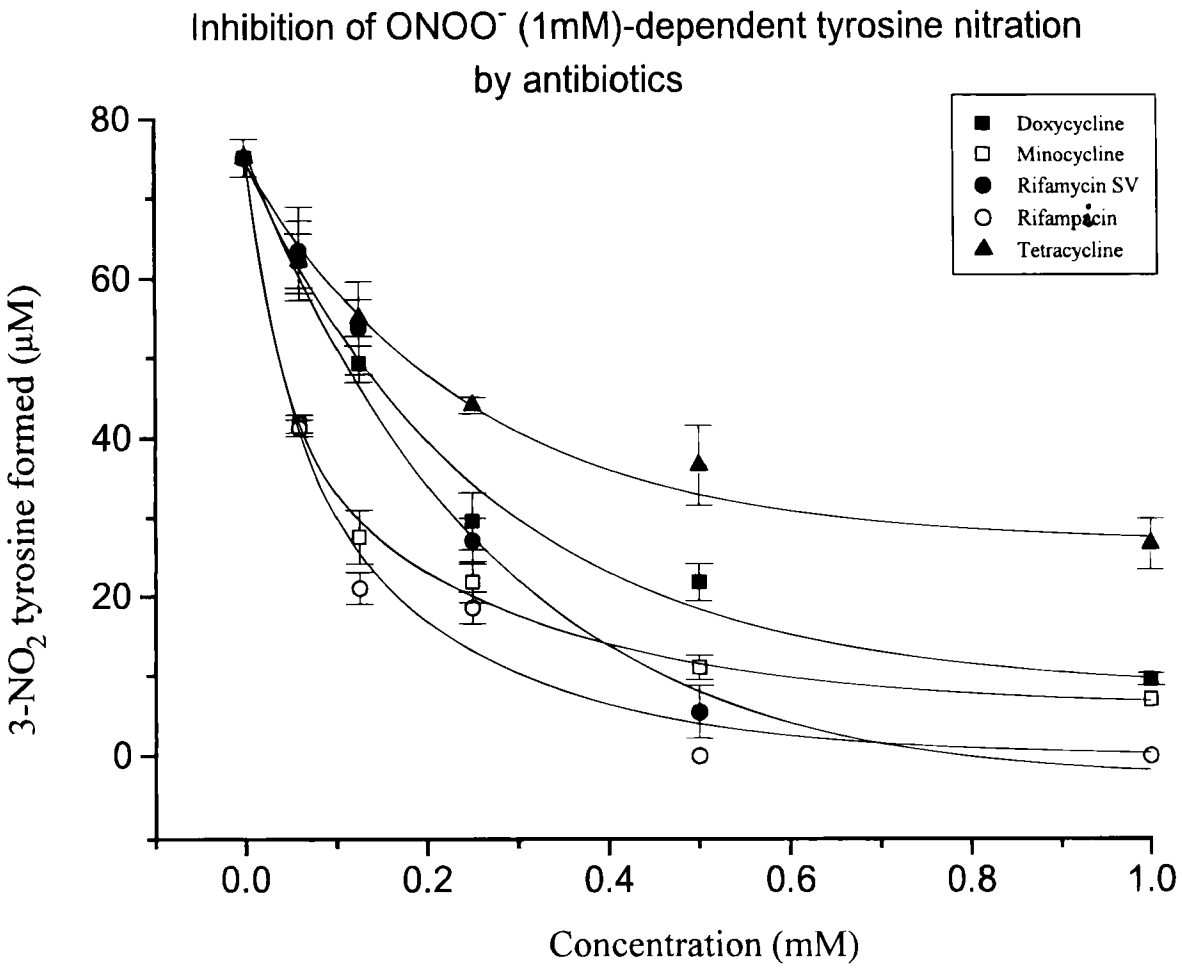

FIGURE 4 Prevention by antibiotics of peroxynitrite-dependent tyrosine nitration: concentration-dependence experiments were carried out as described in the legend to Figure 3, except that antibiotics added to give the final concentration stated whilst [$ONOO^-$] was kept at 1 mM. Results are mean ± SEM (n = 4).

tetracycline less than one-fifth those of $ONOO^-$ exerted substantial protective effects.

DISCUSSION

Peroxynitrite generation *in vivo* has been implicated in a wide range of human diseases, including atherosclerosis,[14] lung disease,[13] neurodegeneration,[11] rheumatoid arthritis[15] and inflammatory bowel disease.[16] Hence agents able to protect against $ONOO^-$-dependent damage may be useful. It has already been suggested that antibiotics might exert protective effects additional to antibacterial action, such as scavenging of reactive oxygen species[4–8] and inhibition of metalloproteinases[26] or phospholipases.[27] Our data suggest that certain antibiotics might also be able to prevent damage by $ONOO^-$, which is known to be a cytotoxic agent. Particularly-effective antibiotics are tetracycline and related drugs (doxycycline, minocycline) as well as rifampicin and rifamycin. At concentrations much less than those of $ONOO^-$, they are able to exert significant protection against α_1-AP inactivation and tyrosine

nitration, two potentially-important damaging consequences of $ONOO^-$ generation *in vivo*. Steady-state concentrations of $ONOO^-$ *in vivo* are low (sub-micromolar) because of its very high reactivity, so if we extrapolate to these low concentrations, it seems reasonable to suggest that levels of antibiotics obtained *in vivo* during their normal therapeutic use, often in the 5–10 μM range,[28] might be able to react with $ONOO^-$. This will need to be established by direct experimentation, however, since $ONOO^-$ can react with many biomolecules.

The chemistry of tissue damage by $ONOO^-$ is extremely complex.[11,33] Whereas inactivation of α_1-AP is probably due to attack of $ONOO^-$ itself upon essential methionine residues within the protein,[20] tyrosine nitration may be mediated by an excited-state *trans* isomer of ONOOH as well by as decomposition products such as NO_2^+ and $NO_2^{\cdot}$.[11,18,25] Hence there is not necessarily a complete correlation between the ability of an antibiotic to protect in these different assay systems.[22] Tetracycline, minocycline, doxycycline, rifamycin and rifampicin were significantly protective in *both* assay systems, supporting the view that protection against damage by $ONOO^-$ might be a significant mechanism of action *in vivo*. However, it must not be forgotten that tetracyclines and rifamycins can interact with transition metal ions to promote oxidative damage[29–31] and that $ONOO^-$ can displace copper ions from caeruloplasmin.[32] It should never be assumed that biological effects of an antibiotic necessarily involve actions upon infecting bacteria.

Acknowledgements

We are grateful to the Arthritis and Rheumatism Council for research support, including the provision of a studentship for Matthew Whiteman.

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] M. Wasil, B. Halliwell and C. P. Moorhouse (1987). Scavenging of hypochlorous acid by tetracycline, rifampicin and some other antibiotics: a possible antioxidant action of rifampicin and tetracycline? *Biochemical Pharmacology*, **37**, 775–778.

[5] A. Cantin and D. E. Woods (1993). Protection by antibiotics against myeloperoxidase-dependent cytotoxicity to lung epithelial cells in vitro, *Journal of Clinical Investigation*, **91**, 38–45.

[6] M. R. Gunther, J. Mao and M. S. Cohen (1993). Oxidant-scavenging activities of ampicillin and sulbactam and their effects on neutrophil functions, *Antimicrobial Agents and Chemotherapy*, **37**, 950–956.

[7] C. Lagercrantz (1992). Formation of aminoxyl radicals in the reaction between penicillins and hydrogen peroxide, *Free Radical Biology and Medicine*, **13**, 455–457.

[8] M. Mathy-Hartert, G. Deby-Dupont, C. Deby, L. Jadoul, A. Vandenberghe and M. Larry (1995). Cytotoxicity towards human endothelial cells, induced by neutrophil myeloperoxidase: protection by ceftazidime, *Mediators of Inflammation*, **4**, 437–443.

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

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

[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*, L699–L722.

[12] S. Moncada and A. Higgs (1993). The L-arginine-nitric oxide pathway, *New England Journal of Medicine*, **329**, 2002–2012.

[13] 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.

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

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

[16] 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.

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

[18] H. Ischiropoulos, L. Zhu, J. Chen, M. Tsai, J. C. Martin, C. D. Smith and J. S. Beckman (1992). Peroxynitrite-mediated tyrosine nitration catalysed by superoxide

dismutase, *Archives of Biochemistry and Biophysics*, **298**, 431–437.

[19] 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*, **AI303**, (abstract).

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

[21] M. Whiteman, H. Kaur and B. Halliwell (1996). Protection against peroxynitrite dependent tyrosine nitration and α_1-antiproteinase inactivation by some anti-inflammatory drugs and by the antibiotic tetracycline, *Annals of Rheumatic Disease*, **55**, 383–387.

[22] M. Whiteman and B. Halliwell (1996). Protection against peroxynitrite-dependent tyrosine nitration and α_1-antiproteinase inactivation by ascorbic acid. A comparison with other biological antioxidants, *Free Radical Research* **55**, 383–387.

[23] 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.

[24] 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.

[25] 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.

[26] R. A. Greenwald, S. A. Moak, N. S. Ramamurthy and L. M. Golub (1992). Tetracyclines suppress matrix metalloproteinase activity in adjuvant arthritis and in combination with flurbiprofen, ameliorate bone damage, *Journal of Rheumatology*, **19**, 927–938.

[27] W. Pruzanski, R. A. Greenwald, I. P. Street, F. Laliberte, E. Stefanski and P. Vadas (1992). Inhibition of enzymatic activity of phospholipases A_2 by minocycline and doxycycline, *Biochemical Pharmacology*, **44**, 1165–1170.

[28] L. P. Yu Jr., G. N. Smith, K. A. Hasty and K. D. Brandt (1991). Doxycycline inhibits type XI collagenolytic activity of extracts from human osteoarthritic cartilage and of gelatinase, *Journal of Rheumatology*, **18**, 1450–1452.

[29] G. J. Quinlan and J. M. C. Gutteridge (1991). DNA base damage by β-lactam, tetracycline, bacitracin and rifamycin antibacterial antibiotics, *Biochemical Pharmacology*, **42**, 1595–1599.

[30] G. J. Quinlan and J. M. C. Gutteridge (1989). Bacitracin and a bacitracin-zinc complex damage DNA and carbohydrate in the presence of iron and copper salts, *Free Radical Research Communications*, **7**, 37–44.

[31] C. Buschfort and I. Witte (1994). Induction and mechanism of DNA single- and double-strand breaks by tetracycline/Cu (II) in the absence of light, *Carcinogenesis*, **15**, 2927–2930.

[32] J. A. Swain, V. Darley-Usmar and J. M. C. Gutteridge (1994). Peroxynitrite releases copper from caeruloplasmin: implications for atherosclerosis, *FEBS Letters*, **342**, 49–52.

[33] R. M. Uppu, G. L. Squadrito and W. A. Pryor (1996). Accelerations of peroxynitrite oxidations by carbon dioxide, *Archives of Biochemistry and Biophysics*, **327**, 335–343.