Protection against peroxynitrite dependent tyrosine nitration and α_1-antiproteinase inactivation by some anti-inflammatory drugs and by the antibiotic tetracycline

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Abstract

***Objective*—To examine in vitro the ability of several drugs to protect against deleterious effects of peroxynitrite, a cytotoxic agent formed by reaction of nitric oxide with superoxide radical, that may be generated in the rheumatoid joint and could cause joint damage.**

***Methods*—The ability of several drugs to protect against such possible toxic actions of peroxynitrite as inactivation of α_1-antiproteinase and nitration of tyrosine was evaluated.**

***Results*—Most non-steroidal anti-inflammatory drugs were moderately (indomethacin, diclofenac, naproxen, tolmetin) or only weakly (sulindac, ibuprofen, aurothioglucose, flurbiprofen, sulphasalazine, salicylate, penicillamine disulphide) effective in preventing tyrosine nitration and α_1-antiproteinase inactivation by peroxynitrite, but 5-aminosalicylate and penicillamine were much more effective, as was the antibiotic tetracycline (but not ampicillin). Phenylbutazone and flufenamic acid protected effectively against tyrosine nitration, but could not be tested in the α_1-antiproteinase system. The analgesic paracetamol was highly protective in both assay systems.**

***Conclusion*—Many drugs used in the treatment of rheumatoid arthritis are unlikely to act by scavenging peroxynitrite. The feasibility of peroxynitrite scavenging as a mechanism of penicillamine, 5-aminosalicylate, and paracetamol action in vivo is discussed.**

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In chronic inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease, there is known to be overproduction of oxygen derived species such as superoxide radical, $O_2^{\bullet -}$.[1] There is also considerable evidence that production of nitric oxide ($NO^{\bullet}$) is increased—for example from measurements of increased nitric oxide synthase activity and demonstration of increased concentrations of nitrite/nitrate in body fluids from patients with RA.[2–4] Nitric oxide reacts with a very high rate constant with $O_2^{\bullet -}$ to give peroxynitrite ($ONOO^-$).[5 6] Peroxynitrite can be directly cytotoxic[6 7] and it may also decompose at physiological pH to give a range of noxious products with reactivities resembling those of hydroxyl radicals ($OH^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), and nitronium ion (NO_2^+).[6–8]

Addition of peroxynitrite to tissues and biological fluids leads to nitration of aromatic amino acid residues, and the presence of these may be a 'marker' of peroxynitrite mediated ($NO^{\bullet}$ dependent) damage in vivo.[6 9–11] Indeed, nitrotyrosine is present in plasma and synovial fluid from patients with RA, but it was not detected in healthy control subjects.[12] Nitration of tyrosine residues on proteins may interfere with cell signal transduction by tyrosine phosphorylation/dephosphorylation.[13] Peroxynitrite also inactivates α_1-antiproteinase, the major inhibitor of serine proteases (such as elastase) in human body fluids,[7 14] and considerable inactivation of α_1-antiproteinase has been shown to occur in the inflamed rheumatoid joint.[15]

RA is treated by the use of a number of drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin, and slower acting 'disease modifying' drugs, such as penicillamine and sulphasalazine, the mechanism of action of which is not always clear.

We have examined the ability of a range of drugs used in the treatment of RA to protect against damage by $ONOO^-$. Two measures of damage that may be relevant to events in vivo were used: ability of the drug to prevent inactivation of α_1-antiproteinase by $ONOO^-$, and ability of the drug to inhibit the nitration of tyrosine by $ONOO^-$.

Materials and methods

REAGENTS

N-succinyl $(ala)_3$-*p*-nitroanilide (SANA), catalase (type C40), elastase (E0258), α_1-antiproteinase (A9024), DL-tyrosine, and the anti-inflammatory drugs were from Sigma Chemical Corp, Poole, Dorset, UK. Solutions of sulindac, paracetamol, ibuprofen, diclofenac, ampicillin, and tetracycline were made up in distilled water; those of indomethacin, tolmetin, flurbiprofen, sulphasalazine, sulphapyridine, and piroxicam were made up in water with minimum potassium hydroxide added to ensure solution. Salicylate, 5-aminosalicylate, aurothioglucose, penicillamine, and penicillamine disulphide were dissolved in 500 mmol/l potassium dihydrogen phosphate-dipotassium

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hydrogen phosphate (KH_2PO_4-K_2HPO_4) buffer pH 7·4; phenylbutazone was dissolved in 0·4% (w/v) sodium carbonate. Solutions were made up freshly every day and diluted with distilled water to obtain the required drug concentrations.

PEROXYNITRITE SYNTHESIS[6]

Five millilitres of an acidic solution (0·6 mol/l hydrochloric acid) of hydrogen peroxide (H_2O_2) 0·7 mol/l was mixed with 5 ml of potassium nitrite 0·6 mol/l on ice for one second and the reaction quenched with 5 ml of ice cold sodium hydroxide 1·2 mol/l. The stock was then frozen overnight (–20°C) and the top layer of the solution collected for the experiment. Concentrations of stock $ONOO^-$ were redetermined before each experiment using a molar absorption coefficient of 1670 $(mol/l)^{-1}$ cm^{-1} at 302 nm.[6]

MEASUREMENT OF TYROSINE NITRATION

DL-Tyrosine solutions were made up to a final concentration of 10 mmol/l by dissolving the required amount in 8 ml of water with 250 µl of 10% (w/v) potassium hydroxide followed by 250 µl of 5% phosphoric acid and dilution to 10 ml by the addition of 1·5 ml of water. Tyrosine solution 0·1 ml 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 of buffer (500 mmol/l 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 mmol/l and the tubes were vortexed for 15 seconds and incubated for a further 15 minutes. The pH was measured after the addition of peroxynitrite and found to be between 7·4 and 7·5.

Formation of 3-nitrotyrosine was measured by high performance liquid chromatography (HPLC), rather than spectrophotometrically, to avoid interference as a result of the generation of chromogen from the drugs. Measurement of 3-nitrotyrosine was performed essentially as described previously[8] using a Spherisorb 5 µm ODS2 C_{18} column (25 cm × 4·6 mm) (HPLC Technology, Wellington House, Cheshire, UK) with a guard column C_{18} cartridge (Hibar, BDH, Poole, UK). The eluent was 500 mmol/l K_2HPO_4-KH_2PO_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 Stretten, Shropshire, UK); the ultraviolet detector was set at 274 mm (sensitivity 0·02). 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.

PREVENTION OF α_1-ANTIPROTEINASE INACTIVATION

Elastase and α_1-antiproteinase were measured essentially as described previously.[16] α_1-Antiproteinase was dissolved in phosphate buffered saline, pH 7·4 (140 mmol/l sodium chloride, 2·7 mmol/l potassium chloride, 16 mmol/l disodium hydrogen phosphate, 2·9 mmol/l KH_2PO_4) to a concentration of 4 mg/ml and elastase was dissolved in the same buffer to a final concentration of 5 mg/ml. The volume of α_1-antiproteinase required to inhibit elastase by 80–90% (typically 60–70 µl) was added to buffer (500 mmol/l K_2HPO_4-KH_2PO_4 pH 7·4), with or without 0·1 ml of compound to be tested, to give a volume of 0·945 ml, and incubated in a water bath at 37°C for 15 minutes, when peroxynitrite (typically 5 µl) was added to give a final concentration of 0·5 mmol/l. The sample was vortexed for 10 seconds and incubated for five minutes, elastase (usually 50 µl) was added and the sample further incubated at 37°C for 15 minutes, followed by addition of 2·0 ml of buffer. Then after 15 minutes, 0·1 ml of elastase substrate (SANA) was added and the rate of reaction followed at 410 nm for 30 seconds.

Results

INACTIVATION OF α_1-ANTIPROTEINASE: PROTECTION BY DRUGS

As expected,[7 14] addition of $ONOO^-$ to α_1-antiproteinase led to inactivation of the ability of α_1-antiproteinase to inhibit elastase. The inactivation was complete within five minutes at pH 7·4 (data not shown).

The extent of inactivation increased with $ONOO^-$ concentration and a five minute incubation time with 0·5 mmol/l $ONOO^-$ was selected for further studies. Investigation of the possibility that other constituents of the $ONOO^-$ solution (such as NO_3^- and H_2O_2, which can be present in $ONOO^-$ preparations[6]) were involved in the inactivation demonstrated that catalase (final concentration 10^3 U/ml) had no effect on inactivation of α_1-antiproteinase by $ONOO^-$, and that, when the $ONOO^-$ solution was added to buffer and incubated for five minutes at 37°C before the addition of α_1-antiproteinase, the resulting 'decomposed $ONOO^-$' solution (which still contains all the other contaminants[6]) had no effect on α_1-antiproteinase (data not shown).

Figure 1 summarises the ability of a range of drugs to protect α_1-antiproteinase against inactivation by $ONOO^-$. There was wide variation in the protective effects seen. Significant protection was observed with penicillamine, paracetamol, indomethacin, 5-aminosalicylate, diclofenac, naproxen, and tolmetin, but flurbiprofen, salicylate, sulphasalazine, sulindac, aurothioglucose, ibuprofen, and penicillamine disulphide were weakly effective. Sulphapyridine had no protective effect: indeed, it seemed to aggravate inactivation of α_1-antiproteinase. We also tested the action of tetracycline, as this antibiotic has previously been found to exert antioxidant activity in vitro,[17] and found it to be a good inhibitor of damage to α_1-antiproteinase by $ONOO^-$, whereas ampicillin was not (fig 1).

Several drugs underwent changes in colour after the addition of $ONOO^-$. Diclofenac and paracetamol developed yellow chromogens.

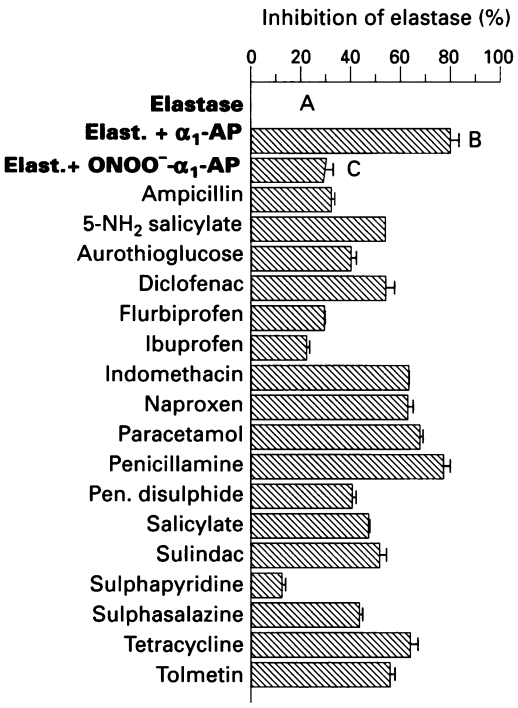

Figure 1 'Screen' of anti-inflammatory drugs (final concentration 0·5 mmol/l) for the ability to protect α_1-antiproteinase against inactivation by peroxynitrite. Bar A represents the activity of elastase (zero inhibition): addition of α_1-antiproteinase inhibited the elastase (bar B), but treatment of the α_1-antiproteinase with peroxynitrite ($ONOO^-$) (final concentration 0·5 mmol/l) decreased this inhibitory effect (bar C). Drugs able to scavenge $ONOO^-$ protect the α_1-antiproteinase and result in less elastase activity. Results are mean, SEM (n = 4). Elast. = Elastase; α_1-AP = α_1-antiproteinase; 5-NH_2 salicylate = 5-aminosalicylate.

The yellow colour of tetracycline deepened to orange/yellow after the addition of $ONOO^-$ and became yellow/brown during the incubation. 5-Aminosalicylate developed a purple/pink colour and purple precipitate after $ONOO^-$ addition, and this darkened to a purple/brown colour during the incubation. Phenylbutazone produced a slightly cloudy solution after the addition of $ONOO^-$. None of these colour changes interfered with spectrophotometric measurement of elastase activity.

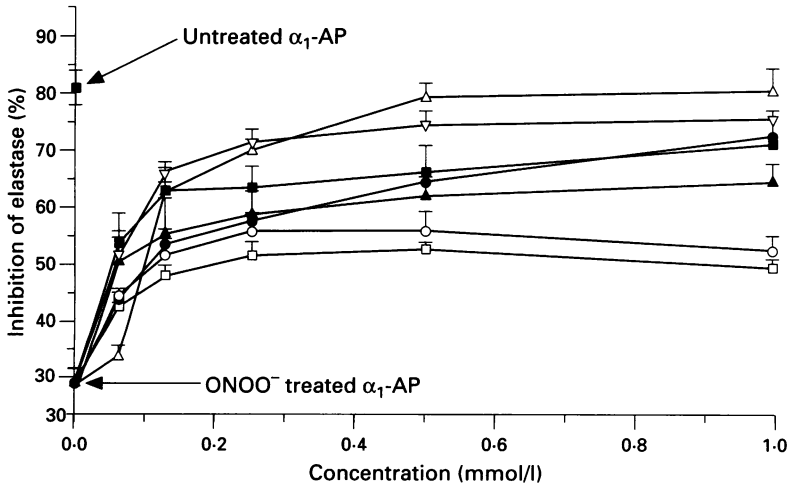

Figure 2 Concentration dependence of the protection given by drugs (each added to give the final concentration stated) against the inactivation of α_1-antiproteinase by peroxynitrite. The concentration of peroxynitrite ($ONOO^-$) was kept at 0·5 mmol/l. Results are mean, SEM (n = 4). △ = Penicillamine; ▽ = tetracycline; ● = naproxen; ■ = 5-aminosalicylate; ▲ = paracetamol; ○ = indomethacin; □= diclofenac.

All experiments were accompanied by controls[16] to ensure that the drugs tested had no effect on the assay procedures used to measure α_1-antiproteinase—that is, they did not directly inhibit elastase, nor did they interfere with inhibition of elastase by α_1-antiproteinase. These controls were negative for the drugs listed here, except that naproxen inhibited elastase activity slightly: this was corrected for using appropriate controls. Piroxicam 1·0 mmol/l and phenylbutazone 1·0 mmol/l markedly inhibited elastase, while flufenamic acid 1·0 mmol/l inhibited the action of α_1-antiproteinase; data for these drugs are not presented. Addition of the drugs listed in figure 1 to the reaction mixtures after five minutes of incubation of α_1-antiproteinase with $ONOO^-$ had no effect—that is, the drugs could not reactivate α_1-antiproteinase after it had been inactivated by $ONOO^-$.

Figure 2 shows the concentration dependence of the protective action of those drugs shown to be most effective in the 'screen' (fig 1). Most protective drugs were active at concentrations of 100 μmol/l—much less than the concentration of $ONOO^-$ used (0·5 mmol/l). However, even at 1 mmol/l concentrations, most drugs were unable to protect α_1-antiproteinase completely: the most effective were penicillamine and tetracycline.

NITRATION OF TYROSINE BY $ONOO^-$: PREVENTION BY DRUGS

None of the drugs tested coeluted with nitrotyrosine on HPLC or otherwise interfered with the HPLC analysis.

Figure 3 summarises the ability of the various anti-inflammatory drugs to inhibit the nitration of tyrosine to 3-nitrotyrosine by exposure to $ONOO^-$ at pH 7·4.[6-11 18 19] Salicylate, tolmetin, sulphasalazine, sulphapyridine, ampicillin, ibuprofen, sulindac, penicillamine disulphide, naproxen, aurothioglucose, and flurbiprofen were fairly weak inhibitors, whereas paracetamol, flufenamic acid, piroxicam, diclofenac, tetracycline, 5-aminosalicylate, phenylbutazone, penicillamine, and indomethacin were more effective. Indeed, paracetamol, 5-aminosalicylate, and penicillamine prevented nitration completely at a concentration equimolar to that of $ONOO^-$. Figure 4 shows the concentration dependence of inhibition by the drugs found to be most effective in the first screen (fig 3). Effects were seen at concentrations of 100–200 μmol/l, compared with 1 mmol/l $ONOO^-$.

Discussion

Peroxynitrite generation in vivo has been implicated in a wide range of human diseases, including atherosclerosis,[20] lung disease,[10] neurodegenerative disorders,[4] and inflammatory bowel disease.[21] Agents able to protect against $ONOO^-$ dependent damage should therefore be therapeutically useful. The reported presence of nitrotyrosine in serum and synovial fluid from patients with RA,[12] combined with the information that synovium from RA joints immunostains with antibodies

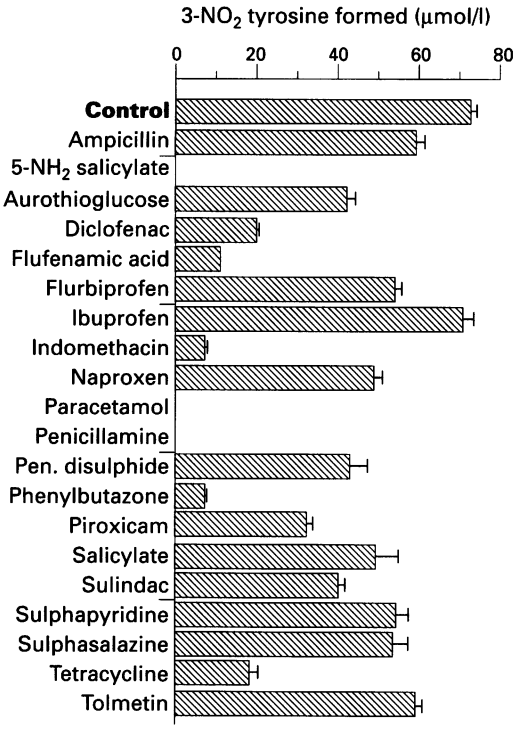

Figure 3 Prevention of peroxynitrite dependent tyrosine nitration to 3-nitrotyrosine (3-NO_2 tyrosine) by drugs present in the reaction mixtures at 1 mmol/l. DL-Tyrosine 1 mmol/l was incubated with peroxynitrite ($ONOO^-$) 1 mmol/l for 15 minutes at 37°C. Data are mean, SEM (n = 4). 5-Aminosalicylate (5-NH_2 salicylate), paracetamol, and penicillamine inhibited nitration completely at the 1 mmol/l concentration.

directed against proteins containing nitrotyrosine (J Beckman, personal communication) is good evidence that $ONOO^-$ is generated in the rheumatoid joint in vivo. As inactivation of α_1-antiproteinase is known to occur in RA,[15] it is relevant to examine the ability of various compounds to protect against this process. Most NSAIDs, which are generally understood to decrease pain and swelling in RA but not inhibit the progression of joint damage, protected only moderately against inactivation, suggesting that $ONOO^-$ scavenging is unlikely to contribute to their therapeutic actions at the plasma concentrations achieved during routine treatment. Sulphasalazine did not protect well, but its metabolite 5-aminosalicylate was very protective. Aminosalicylate might therefore be capable of scavenging $ONOO^-$ when sulphasalazine is used to treat inflammatory bowel diseases,[21] but the action of sulphasalazine in RA is unlikely to be the result of $ONOO^-$ scavenging, as neither sulphasalazine nor sulphapyridine exerted significant protective effects. In contrast, penicillamine was a powerful protective agent.

Penicillamine and phenylbutazone were also powerfully protective against nitration of tyrosine by peroxynitrite (phenylbutazone could not be tested in the α_1-antiproteinase system). The disulphide form of penicillamine is the one usually administered to patients, but it is believed to be converted to the dithiol form in vivo. Althaus *et al*[22] have also shown that penicillamine reacts with $ONOO^-$. The reaction presumably involves the -SH group, as the disulphide was poorly effective. Although, in general, drugs good at inhibiting tyrosine nitration are also good at protecting α_1-antiproteinase, there is no absolute comparability between the two assays. This is probably because nitration of tyrosine is a complex reaction sequence; inhibitory compounds can act not only by scavenging $ONOO^-$, but also by quenching the $ONOO^-$ derived nitrating species, the tyrosine radical intermediates in the nitration pathway, or both.[18]

It was interesting to note that the analgesic paracetamol was also very effective in preventing tyrosine nitration and α_1-antiproteinase activation by $ONOO^-$, as was the antibiotic tetracycline (whereas ampicillin had little effect). This further supports our previous argument that not all the biological actions of tetracycline are necessarily attributable to its antibacterial action.[17] Our data show that a possible mechanism of action of certain anti-inflammatory drugs is the scavenging of $ONOO^-$, especially as drugs such as penicillamine were effective at very low drug/$ONOO^-$ molar ratios.

Our studies were, of course, in vitro and do not prove that such mechanisms of action occur in vivo. Many constituents of human body fluids, such as albumin and ascorbate, also react with $ONOO^-$.[19] We are currently searching for specific end products of the reaction of $ONOO^-$ with the most effective drugs: the ability to demonstrate such end products in patients with RA would provide direct evidence for the reaction of these drugs with $ONOO^-$ in vivo. The findings of the present study have shown us which drugs are worth further examination in this context.

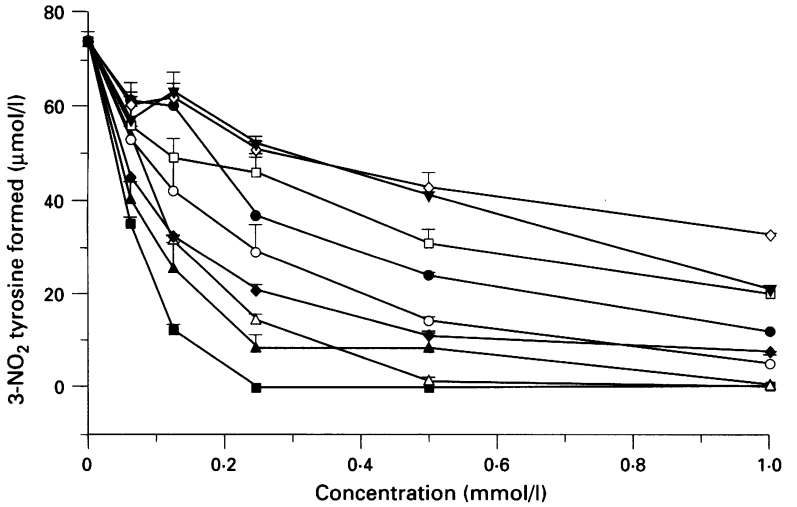

Figure 4 Concentration dependent prevention of peroxynitrite dependent tyrosine nitration to 3-nitrotyrosine (3-NO_2 tyrosine) by drugs present in the reaction mixtures to the final concentrations stated. The concentration of peroxynitrite ($ONOO^-$) was kept at 1 mmol/l. Results are mean, SEM (n = 4). ◇ = Piroxicam; ▼ = tetracycline; □ = diclofenac; ● = flufenamic acid; ◆ = phenylbutazone; ○ = indomethacin; ▲ = paracetamol; △ = penicillamine; ■ = 5-aminosalicylate.

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