Protection against peroxynitrite dependent tyrosine nitration and \(\alpha_1\)-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 \(\alpha_1\)-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, fenamic acid) protective in preventing tyrosine nitration and \(\alpha_1\)-antiproteinase inactivation by peroxynitrite, but 5-aminosalicylate and penicillamine were much more effective, as was the antibiotic tetracycline (but not ampicillin). Phenylbutazone and fluromenamic acid protected effectively against tyrosine nitration, but could not be tested in the \(\alpha_1\)-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.


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, \(O_2^-\). There is also considerable evidence that production of nitric oxide (NO) 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. \(O_2^-\) reacts with a very high rate constant with \(O_2^-\) to give peroxynitrite (ONOO\(^-\)). Peroxynitrite can be directly cytotoxic and it may also decompose at physiological pH to give a range of noxious products with reactivities resembling those of hydroxyl radicals (\(OH^+\)), nitrogen dioxide (\(NO_2^-\)), and nitronium ion (\(NO_2^+\)).

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\(^-\) dependent) damage in vivo. Indeed, nitrotyrosine is present in plasma and synovial fluid from patients with RA, but it was not detected in healthy control subjects. Nitration of tyrosine residues on proteins may interfere with cell signal transduction by tyrosine phosphorylation/dephosphorylation. Peroxynitrite also inactivates \(\alpha_1\)-antiproteinase, the major inhibitor of serine proteases (such as elastase) in human body fluids, and considerable inactivation of \(\alpha_1\)-antiproteinase has been shown to occur in the inflamed rheumatoid joint.

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 \(\alpha_1\)-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), \(\alpha_1\)-antiproteinase (A9024), \(\alpha_1\)-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...
hydrogen phosphate (KH₂PO₄-K₂HPO₄) 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**

Five millilitres of an acidic solution (0-6 mol/l hydrochloric acid) of hydrogen peroxide (H₂O₂) 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 determined 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% (v/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 K₂HPO₄·KH₂PO₄ 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 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 α₁-Antiproteinase: Protection by Drugs**

As expected,⁷ ¹⁴ addition of ONOO⁻ to α₁-antiproteinase led to inactivation of the ability of α₁-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 NO₃⁻ and H₂O₂, which can be present in ONOO⁻ preparations)⁸ were involved in the inactivation demonstrated that catalase (final concentration 10³ U/ml) had no effect on inactivation of α₁-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 α₁-antiproteinase, the resulting 'decomposed ONOO⁻' solution (which still contains all the other contaminants)⁹ had no effect on α₁-antiproteinase (data not shown).

Figure 1 summarises the ability of a range of drugs to protect α₁-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 α₁-antiproteinase. We also tested the action of tetracycline, as this antibiotic has previously been found to exert antioxidant activity in vitro,¹⁰ and found it to be a good inhibitor of damage to α₁-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.
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.

All experiments were accompanied by controls to ensure that the drugs tested had no effect on the assay procedures used to measure α₁-antiproteinase—that is, they did not directly inhibit elastase, nor did they interfere with inhibition of elastase by α₁-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 fufенен酸 acid 1-0 mmol/l inhibited the action of α₁-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 α₁-antiproteinase with ONOO⁻ had no effect—that is, the drugs could not reactivate α₁-antiproteinase after it had been inactivated by ONOO⁻.

Figure 2 shows the concentration dependence of the protective action of these 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 α₁-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 Salicylate, tolmelin, sulphasalazine, salicylate, 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-amino-salicylate, 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,21 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
Results are stated. The concentration of 3-nitrotyrosine (3-NO₂ tyrosine) by drugs

\* = flufenamic acid; I = phenylbutazone; \( \Delta \) = indomethacin; \( \odot \) = paracetamol;  
\( \bullet \) = flufenamic acid; \( \odot \) = phenylbutazone; \( \Delta \) = paracetamol;  
\( \Delta \) = penicillamine; \( \text{SEM} \) = 5-aminosalicylate.

Figure 3  Prevention of peroxynitrite dependent tyrosine nitration to 3-nitrotyrosine (3-NO₂ tyrosine) by drugs present in the reaction mixtures at 1 mmol/l. 3-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-NH₂ 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 \( \alpha \)-antiproteinase is known to occur in RA, 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, but the action of sulphasalazine in RA is unlikely to be the result of ONOO⁻ scavenging, as neither sulphasalazine nor sulphasalazine 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 \( \alpha \)-antiproteinase system). The disulfide 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 al²² have also shown that penicillamine reacts with ONOO⁻. The reaction presumably involves the -SH group, as the disulfide was poorly effective. Although, in general, drugs good at inhibiting tyrosine nitration are also good at protecting \( \alpha \)-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.

It was interesting to note that the analgesic paracetamol was also very effective in preventing tyrosine nitration and \( \alpha \)-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.²² 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⁻.²⁰ 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.

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Ability of drugs to protect against peroxynitrite


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