Protection against peroxynitrite dependent tyrosine nitration and α₁-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 α₁-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 α₁-antiproteinase inactivation by peroxynitrite, but 5-aminosalicylate and penicillamine were much more effective, as was the antibiotic tetracycline (but not ampicillin). Phenylbutazone and flu fenamic acid protected effectively against tyrosine nitration, but could not be tested in the α₁-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.

Materials and methods

REAGENTS

Nsuccinyl (ala)₃-p-nitroanilide (SANA), catalase (type C40), elastase (E02588), α₁-antiproteinase (A9024), d,l-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 nitrate 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 KH₂PO₄-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 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 previously⁶ using a Spherisorb 5 µm ODS2 C₁₈ column (25 cm × 4·6 mm) (HPLC Technology, Wellington House, Cheshire, UK) with a guard column C₁₈ cartridge (Hibar, BDH, Poole, UK). The eluent was 500 mmol/l KH₂PO₄-KH₂PO₄ 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, Shropshire, UK); the ultraviolet detector was set at 274 nm (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 α₁-ANTIPROTEINASE INACTIVATION
Elastase and α₁-antiproteinase were measured essentially as described previously.ⁱ⁴ α₁-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 KH₂PO₄) 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 α₁-antiprotease required to inhibit elastase by 80–90% (typically 60–70 µl) was added to buffer (500 mmol/l KH₂PO₄-KH₂PO₄ 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 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 NO⁻ and H₂O₂, which can be present in ONOO⁻ preparations)⁷ were involved in the inactivation demonstrated that catalase (final concentration 10⁻³ 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)⁷ 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, aurothioglucoside, 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,¹⁷ 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. Phenybutazone 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 controls¹⁶ to ensure that the drugs tested had no effect on the assay procedures used to measure α₁-antiprotease—that is, they did not directly inhibit elastase, nor did they interfere with inhibition of elastase by α₁-antiprotease. 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 sufrenamic acid 1-0 mmol/l inhibited the action of α₁-antiprotease; 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-antiprotease with ONOO⁻ had no effect—that is, the drugs could not reactivate α₁-antiprotease 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 α₁-antiprotease 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, sulpyrydine, 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, lung disease, neurodegenerative disorders, and inflammatory bowel disease. 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.
are Results concentrations stated. The concentration = flufenamic acid; *= penicillamine; mean, 0.2 ± 0.4 SEM (n = 5). 3-Aminosalicylate. 0 of peroxynitrite (ONOO-) in paracetamol, against of joint und & this inactivation of (J in occur in mmol/l (n = 15). Phenylbutazone 5-NH2 salicylate Flufenamic acid Sulphapyridine disulphide Pen. = indomethacin; A = paracetamol; toluene; 3-nitro- the nitration of (5-NH2 salicylate), protective with the biological 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 α1-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 al21 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 α1-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 α1-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.12 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-.12 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


