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Evidence for nitric oxide-mediated oxidative damage in chronic inflammation

Nitrotyrosine in serum and synovial fluid from rheumatoid patients

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Abstract

Reaction of nitric oxide (NO•) with superoxide radical generates peroxynitrite, which can decompose to products that nitrate aromatic amino acids. Such nitro-aromatics may be 'markers' of NO•-dependent oxidative damage. Blood serum and synovial fluid from patients with the inflammatory joint disease rheumatoid arthritis contain 3-nitrotyrosine. By contrast, body fluids from normal subjects and patients with osteoarthritis contain no detectable 3-nitrotyrosine; much lower levels were found in serum from patients in the early stages of rheumatoid arthritis. This is evidence that NO• plays a role in joint damage in rheumatoid arthritis.

Key words: Peroxynitrite; Nitric oxide; Nitrotyrosine; Oxidative damage; Rheumatoid arthritis

1. Introduction

The free radical gas nitric oxide (NO•) performs many useful functions when produced in vivo, e.g. by neurones and endothelial cells [1]. However, excess NO• can exert cytotoxic and cytostatic effects [1,2]. These may involve both direct toxicity, e.g. by reaction of NO• with mitochondrial iron-sulphur proteins [3], and the interaction of NO• with other free radicals. Nitric oxide reacts with a very high rate constant with superoxide radical ($O_2^{\bullet -}$) to give peroxynitrite, $ONOO^-$ [4–6]. Peroxynitrite can be directly cytotoxic [7] and it can also decompose to give a range of products, including hydroxyl radicals (OH•) and nitronium ion (NO_2^+) [5–10]. Addition of peroxynitrite to biological fluids leads to nitration of aromatic amino acid residues, and the presence of these may be a 'marker' of peroxynitrite-mediated (i.e. NO•-dependent) damage in vivo [8–11].

In chronic inflammatory diseases such as rheumatoid arthritis (RA) there is excess production of oxygen free radicals, that contributes to tissue damage [12,13]. Nitric oxide has often been suggested to participate in joint damage [2,14–17] and inhibitors of NO• synthetase suppress arthritis in mice [15]. However, there is no direct evidence that NO•-mediated damage occurs in human inflammatory joint disease. If it could be shown to occur, it might provide a route for the development of new therapies.

In the present paper, we show that levels of 3-nitrotyrosine, which can be produced by NO•-dependent oxidative damage [8–11], are elevated in patients with RA. This methodology could also be used to implicate NO•-mediated damage in other human diseases, and to test the ability of NO• synthetase inhibitors to ameliorate such damage.

2. Materials and methods

2.1. Patients

Patients with active RA (criteria of the American Rheumatological Association) or osteoarthritis were examined and the diagnoses confirmed by at least two experienced rheumatologists. Joint aspiration of synovial fluid was performed only when medically necessary. Fluids were centrifuged at 1000 rpm for 10 min to sediment cells and the supernatants analyzed for nitrotyrosines after filtration through Amicon Centrifree micro partition devices at 4,000 × *g* for 1 h at 20°C. In some cases, parallel blood samples were collected and allowed to clot to produce serum, which was treated in the same way.

2.2. Chemicals

3-Nitro-L-tyrosine and other reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

2.3. HPLC analysis of nitro-compounds

HPLC separation of 3-nitro-L-tyrosine was achieved using an HPLC technology Nucleocil 5μ C-18 column (25 cm × 4.6 mm) with a guard column (Hibar from Merck Ltd., Poole, Dorset, UK; with a C-8 cartridge). The eluant was 500 mM KH_2PO_4-H_3PO_4 (pH 3.01) with 10% methanol (v/v) at a flow rate of 1 ml/min through a Polymer Laboratories Ltd. isocratic pump and an Ultraviolet Detector set at 274 nm (sensitivity 0.01). Using this technique the detection limit is 0.2 μM. Identification of the peak was carried out on the basis of the retention time of authentic 3-L-nitrotyrosine, 'spiking' experiments, and comparison of UV and mass spectral (direct probe) analysis, using a KRATOS model MS 890MS with a KRATOS DS90 data system.

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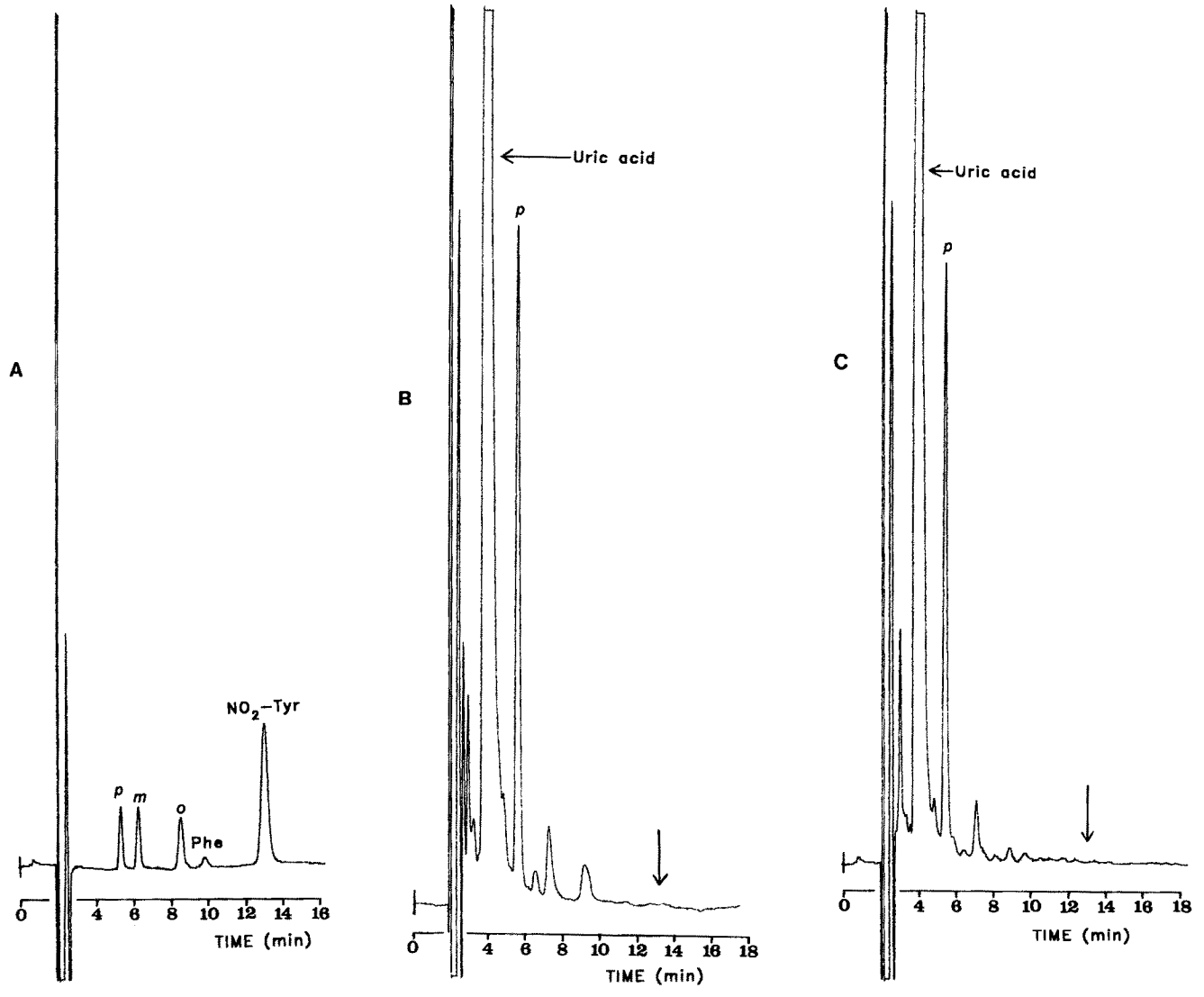

Fig. 1. HPLC separation of (A) a standard aqueous mixture of *o*-tyrosine (*o*-Tyr, 5 μM), *m*-tyrosine (*m*-Tyr, 5 μM), *p*-tyrosine (*p*-Tyr, 5 μM), L-phenylalanine (Phe, 2.5 mM) and 3-nitro-L-tyrosine (NO_2- Tyr, 5 μM). (B) Serum from a healthy volunteer. (C) Synovial fluid from an osteoarthritic patient (the chromatogram of the serum sample was almost identical and again no peak due to nitrotyrosine was detected; the position of the expected peak is marked with an arrow). (D) Synovial fluid from a rheumatoid patient showing the presence of 3-nitrotyrosine (0.5 μM). (E) Synovial fluid from the same rheumatoid patient as in (D) was 'spiked' with an equal volume of a mixture of standards (from A above), giving an effective 1 : 1 dilution.

Table 1
3-Nitrotyrosine in body fluids from rheumatoid patients and controls

Patients	[3-Nitrotyrosine] (μM)			
	In serum		In synovial fluid	
	Range	Mean ± S.D.	Range	Mean ± S.D.
Active rheumatoid arthritis (n = 22)	*0.0–1.2	0.49 ± 0.27	0.1–1.2	0.49 ± 0.26
Osteoarthritis (n = 3)	0	0	0	0
Early rheumatoid arthritis (n = 6)	0.0–0.5	0.18 ± 0.07	Not available	
Healthy controls (n = 6)	0	0	Not available**	

*Only one RA patient showed a zero value. All differences between active RA and osteoarthritis, early RA or healthy controls were highly significant ($P < 0.02$). All subjects gave informed consent for samples to be taken and used in research. **It is not ethically acceptable to take synovial fluid from normal controls.

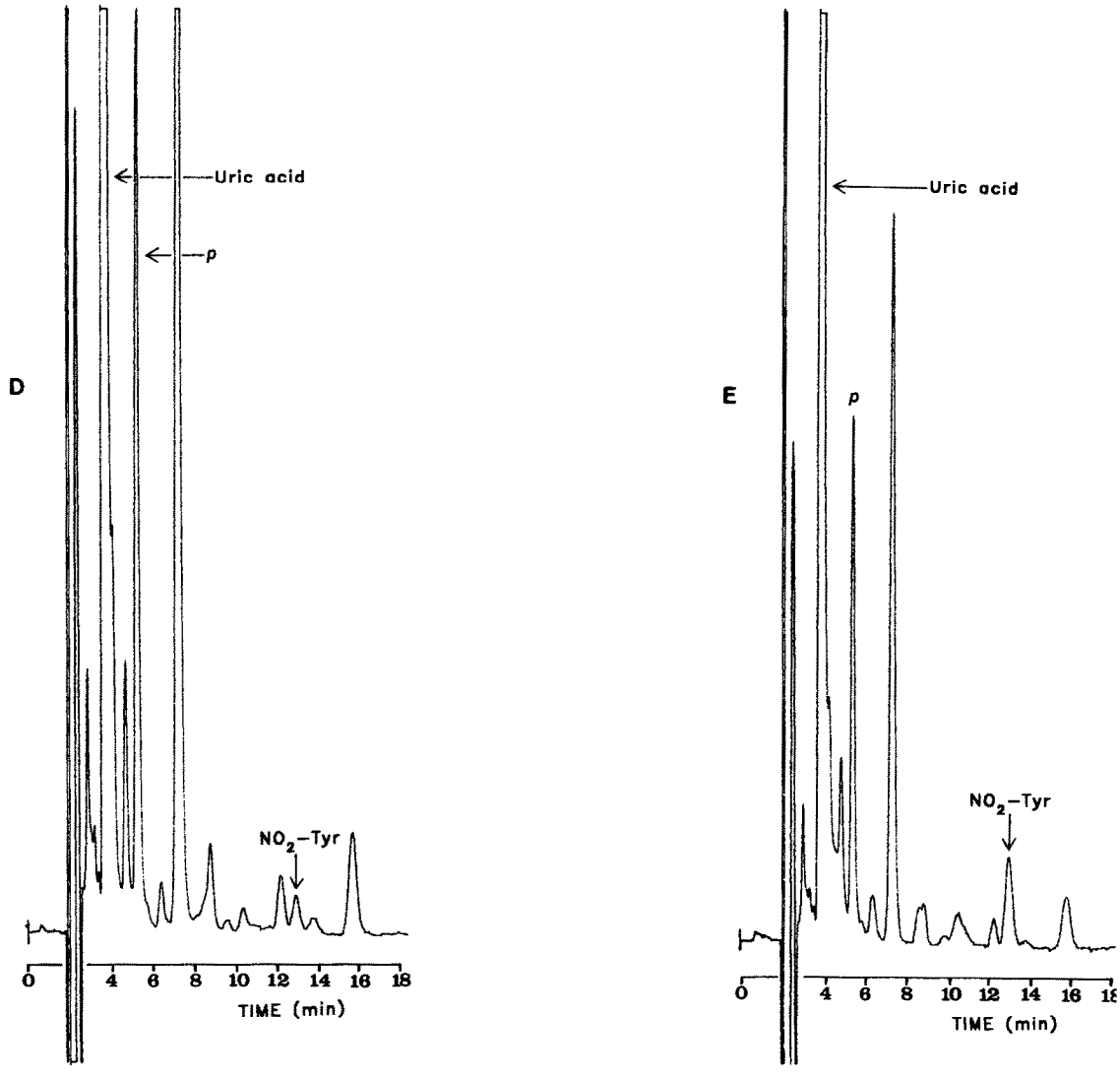

Fig. 1. (*Continued*).

3. Results

3.1. 3-Nitrotyrosine in body fluids from patients with rheumatoid arthritis (RA)

Peroxynitrite-dependent damage in human body fluids nitrates aromatic amino acid residues. In order to investigate the occurrence of such damage in patients with active RA, we looked for the presence of 3-nitrotyrosine in blood serum and in synovial fluid (aspirated from inflamed knee joints) using HPLC.

Fig. 1D shows a representative HPLC chromatogram of synovial fluid from a patient with active RA. A well-defined peak at the retention time of 3-nitrotyrosine can be seen. The identity of this peak was confirmed by three methods. First, authentic 3-nitrotyrosine added to the samples co-eluted exactly with this peak (Fig. 1E) and the height of this peak increased at detector wavelengths of 260 nm and at 420 nm, as expected for 3-nitrotyrosine. Second, if this fraction was collected, its UV spectrum was identical to that of 3-nitrotyrosine showing a maximum at 260 nm (HPLC eluent, pH 3.01) and at 420 nm (pH 12.0). Third, the collected fraction was freeze-dried and analyzed for 3-nitrotyrosine by mass spectroscopy using direct probe analysis. Characteristic ions of fragmentation of authentic 3-nitrotyrosine observed in this fraction were $m^+_{\bullet}$ the molecular ion = *m/z* 226, $m^+_{\bullet}$ + 1 the molecular ion plus one = *m/z* 227 and $m^+_{\bullet}$ −COOH and NO_2 = *m/z* 135.

3-Nitrotyrosine was detected in both synovial fluid and, at comparable concentrations, in blood plasma, from 21 patients with established active RA (Table 1).

3.2. Control subjects

As controls for these studies, we used three groups of patients. First, we studied patients with osteoarthritis, a largely non-inflammatory joint disease. In no case was

any 3-nitrotyrosine detected in serum or synovial fluid (Table 1). Fig. 1C shows a representative HPLC chromatogram. Second, patients in the early stages of RA, without severe joint inflammation, were examined. Synovial fluid was not available from these patients. However, plasma contained much lower concentrations of 3-nitrotyrosine than those observed in patients with active RA (Table 1). Third, serum collected from healthy volunteers ($n = 6$) showed no detectable levels of 3-nitrotyrosine (Table 1 and Fig. 1B).

4. Discussion

The analyses presented here are highly indicative of the presence of 3-nitrotyrosine in body fluids from patients with RA. It is not present in control subjects, and present only at much lower levels in patients in the early stages of RA. Our data provide direct evidence supporting earlier suggestions that $NO^{\bullet}$ may be a mediator of joint damage in chronic inflammatory joint disease [13–17]. Measurement of 3-nitrotyrosine (and possibly of other nitro-aromatic compounds) may be a useful way of demonstrating $NO^{\bullet}$-mediated pathology and testing the effectiveness of therapeutic agents in preventing such damage.

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