

Hydroxyl radical generation by rheumatoid blood and knee joint synovial fluid

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Abstract

Objective—To demonstrate directly that highly reactive hydroxyl radicals (OH[•]) can be generated in patients with rheumatoid arthritis and contribute to joint damage, and to examine the ability of blood to cause OH[•] generation.

Methods—The sensitive and specific technique of hydroxylation of aromatic compounds (salicylate and phenylalanine) was used to measure OH[•]. Synovial fluid and blood from patients with active rheumatoid arthritis were aspirated and immediately added to tubes containing salicylate and phenylalanine as detectors of OH[•], or to tubes containing saline as a control. Levels of specific products of attack of OH[•] upon salicylate (2,3- and 2,5-dihydroxybenzoates) and phenylalanine (*ortho*- and *meta*-tyrosines) were measured by high performance liquid chromatography.

Results—Synovial fluid samples aspirated into saline never contained *ortho*- or *meta*-tyrosines or 2,3-dihydroxybenzoate. Of 53 patients examined, synovial fluid and blood from 36 caused formation of *ortho*- and *meta*-tyrosines when aspirated into solutions containing phenylalanine. Repeated sampling from three "positive" patients showed consistent evidence of these hydroxylation products. Similarly, of 22 patients examined, synovial fluid and blood from 18 caused formation of 2,3- and 2,5-dihydroxybenzoates when aspirated into salicylate solutions. Further evidence for the role of OH[•] was provided by inhibition of the hydroxylation by the specific OH[•] scavengers mannitol and sodium formate.

Conclusions—Aspirated knee joint fluids and blood from rheumatoid arthritis patients can generate OH[•], consistent with current views on the importance of this radical as a cytotoxic agent in rheumatoid disease. The ability of body fluids to cause OH[•] formation is not correlated with simple laboratory indices of disease activity, but is reproducible on sequential sampling from the same patients. The mechanism and significance of the phenomenon in rheumatoid arthritis pathology remain to be established.

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There is substantial indirect evidence to support the current view that reactive oxygen species (ROS) such as superoxide radical O₂^{•-},

hydrogen peroxide (H₂O₂), hydroxyl radical (OH[•]), and hypochlorous acid (HOCl), as well as reactive nitrogen species (RNS) such as nitric oxide (NO[•]) and peroxynitrite (ONOO⁻), contribute to tissue injury during chronic inflammation.¹⁻⁷ These species may arise by several mechanisms, including production by neutrophils, chondrocytes, macrophages in the pannus,^{1,5,7} and by the enzyme xanthine oxidase in the synovial membrane through the process of hypoxia reperfusion injury.^{6,8}

ROS and RNS, once formed, are capable of damaging all important biomolecules. Indeed, oxidation of proteins,⁹ lipids,^{10,11} DNA^{12,13} uric acid,¹⁴ polysaccharides,¹⁵ and ascorbic acid^{16,17} has been shown to be increased in rheumatoid arthritis patients. In addition, H₂O₂ dependent inactivation of enzymes in cartilage may lead to inhibition of proteoglycan synthesis,^{18,19} so contributing to cartilage destruction in rheumatoid arthritis by interfering with the repair of proteolytic and oxidative damage.

Elucidation of the precise role of ROS in rheumatoid arthritis has been hampered because it is difficult to measure them *in vivo*.²⁰ However, O₂^{•-}, NO[•], and H₂O₂ cannot damage most biological macromolecules directly, and it is widely thought that they exert deleterious effects by becoming converted into the highly reactive hydroxyl radical, OH[•],^{1,21} which reacts at a diffusion controlled rate with almost all molecules in living cells. Although aromatic hydroxylation data consistent with increased OH[•] generation in rheumatoid arthritis patients have been obtained,^{22,23} the origin of this OH[•] cannot be determined from such whole body measurements. In the present paper we provide direct evidence for the ability of the fluid aspirated from the knee joints of rheumatoid arthritis patients to form OH[•]. In addition, the ability of whole blood to form OH[•] has been demonstrated. This reactive radical was detected using two specific assays: the aromatic hydroxylation of salicylic acid²²⁻²⁴ and of phenylalanine.^{25,26} Aromatic hydroxylation is a trapping assay which involves the reaction of OH[•] with an aromatic "detector" molecule to form specific products, the production of which is diagnostic of the formation of OH[•]. The major products diagnostic for OH[•] from the hydroxylation of salicylate are 2,3- and 2,5-dihydroxybenzoates (2,3-DHB, 2,5-DHB; fig 1), which are measured by high performance liquid chromatography (HPLC). Similarly, the aromatic amino acid L-phenylalanine is attacked by OH[•] to produce three tyrosines (*o*-, *m*-, and *p*-tyrosine; fig 2). Although *p*-tyrosine can be produced physiologically by the activity of a

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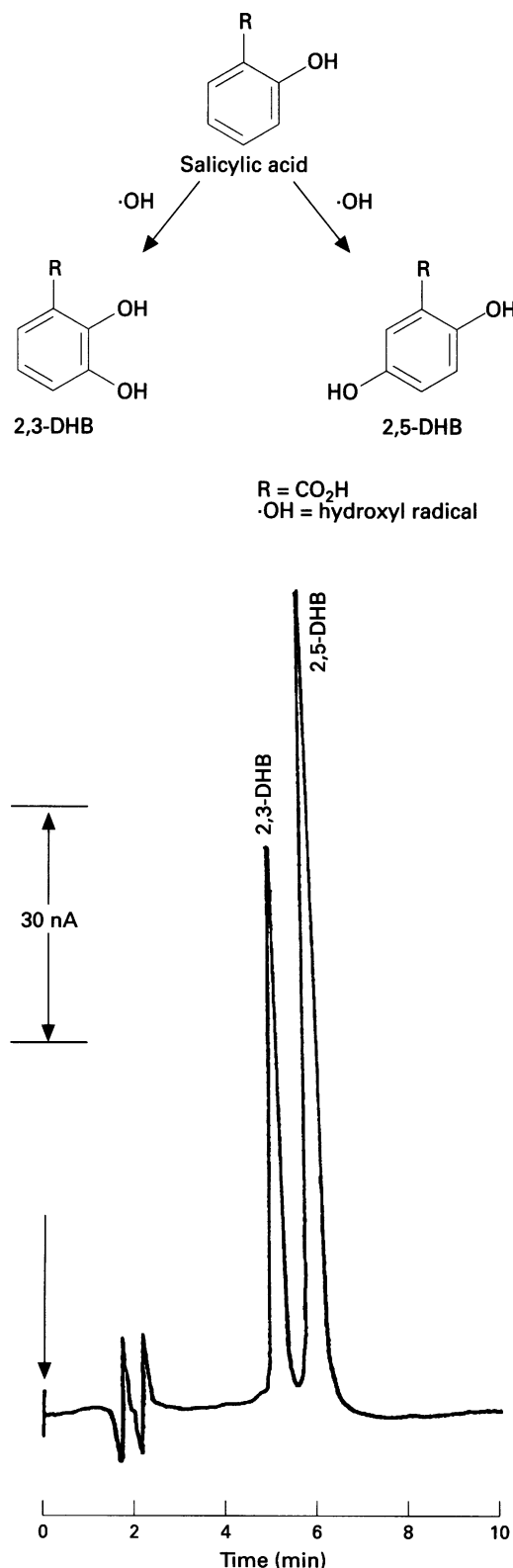


Figure 1 Diagram showing the major products of attack of hydroxyl radicals upon salicylic acid (top) and the HPLC separation achieved following a previously published procedure²³ (bottom).

phenylalanine hydroxylase enzyme, *o*- and *m*-tyrosines do not appear to be enzyme generated products.^{26,27}

Methods

REAGENTS

Salicylic acid, L-phenylalanine, 2,3-DHB and 2,5-DHB, *ortho*-, *meta*-, and *para*-tyrosines, and other reagents were of the highest quality

available from Sigma. Physiological saline was prepared using tablets (Dulbecco "A"), and HPLC grade water was from a Maxima water purification system (ELGA Ltd, High Wycombe, Bucks, UK).

PATIENTS

Patients with rheumatoid arthritis (criteria of the American Rheumatological Association, as revised 1987) had synovial fluid aspirated from the knee joints, using the standard "no touch" technique, only when necessary for therapeutic purposes. The joint was termed "active" by definition only, that is, if an effusion was present requiring aspiration, and not in terms of Ritchie score and related indices. Synovial fluid, once aspirated, was added immediately (1.8 ml) to either physiological saline (0.2 ml) as a control, or to physiological saline (0.2 ml) containing an OH^\cdot trap (5 mM L-phenylalanine [phe] or 5 mM salicylic acid [SAL]), or 5 mM trap plus OH^\cdot scavenger (mannitol or sodium formate) giving a final volume of 2 ml.

Blood samples were also withdrawn from the patients and treated in the same way as synovial fluid. The saline (\pm trap) plus blood was allowed to coagulate and the serum collected. Before analysis both serum and synovial fluid samples were centrifuged at 1500 *g* for 30 min to remove cells and cell debris and the supernatant was collected. Samples were stored at -20°C until HPLC analysis.

PRE- AND POSTEXERCISE STUDY (HYPOXIA-REPERFUSION)

Patients were bed rested for 1 h before collection of their synovial fluid and blood,⁸ then exercised for 15 to 20 min before collecting a further sample. Synovial fluid and serum samples before and after exercise were collected and stored at -20°C until HPLC analysis.

HPLC ANALYSES OF HYDROXYLATED COMPOUNDS

Samples were thawed at room temperature and then filtered through Centrifree micropartition devices (Amicon, Gloucestershire, UK) at 2700 *g* (Heraeus Sepatech centrifuge) for 1 h at 20°C . The ultrafiltrates were then analysed according to published procedures for the dihydroxybenzoates²³ and tyrosines.²⁶ Figure 2B shows a representative chromatogram for tyrosines: chromatograms for dihydroxybenzoates were similar to those already published.^{23,24}

Results

DETECTION OF HYDROXYL RADICAL BY AROMATIC HYDROXYLATION OF PHENYLALANINE

Synovial fluid samples from 53 patients with active rheumatoid arthritis were aspirated into physiological saline (as a control) and solutions assayed for the presence of tyrosines (Tyr). No *o*- and *m*-Tyr were detected, confirming the view that they are not normal metabolic products, that is, not formed by an enzymic pathway in humans. The concentration of *p*-Tyr was 75.2 (SD 30.7) μM ($n = 48$) which was not significantly different from the serum *p*-Tyr concentrations in the same patients [51.5 (21.2) μM , $n = 48$] or from serum values of this amino acid in healthy control subjects [54.4 (21.3) μM , $n = 20$]. The serum and synovial fluid Phe concentrations were less

Table 1 Products of hydroxylation of Phe (*o*-, *m*- and *p*-tyrosines) measured in body fluids of rheumatoid patients and healthy controls added to solutions containing Phe as a detector for hydroxyl radicals

Patients	Tyrosines (μM)						
	In synovial fluid			In serum after addition of whole blood			
	<i>o</i> -	<i>m</i> -	<i>p</i> -	<i>o</i> -	<i>m</i> -	<i>p</i> -	
Healthy controls (n=20)		Not available*		ND	ND	63.3 (23.7) (n=20)	
RA (n=53)	1.6 (2.2) (n=50)	1.5 (2.1) (n=49)	72.5 (29.7) (n=50)	1.8 (2.5) (n=50)	1.8 (2.3) (n=51)	50.5 (20.1) (n=52)	

All values given here are mean (SD) with the number of subjects in brackets. Statistics include zero (ND) values.

ND, not detected.

Levels of *o*-, *m*- and *p*-tyr in serum and SF are not significantly different.

* Synovial fluid was not available from normal controls.

than 100 μM , a level below that needed to trap any OH^\cdot . However, when synovial fluid was aspirated and immediately added to a solution containing a high concentration of Phe (5 mM), *o*- and *m*-Tyr were formed (table 1; fig 2B) but concentrations of *p*-tyrosine were essentially

identical to the saline controls (table 1). A similar phenomenon was seen when blood was added to the Phe solution and tyrosines then measured in the serum after clotting. Blood from healthy human controls never gave any evidence of *o*- or *m*-tyrosine formation.

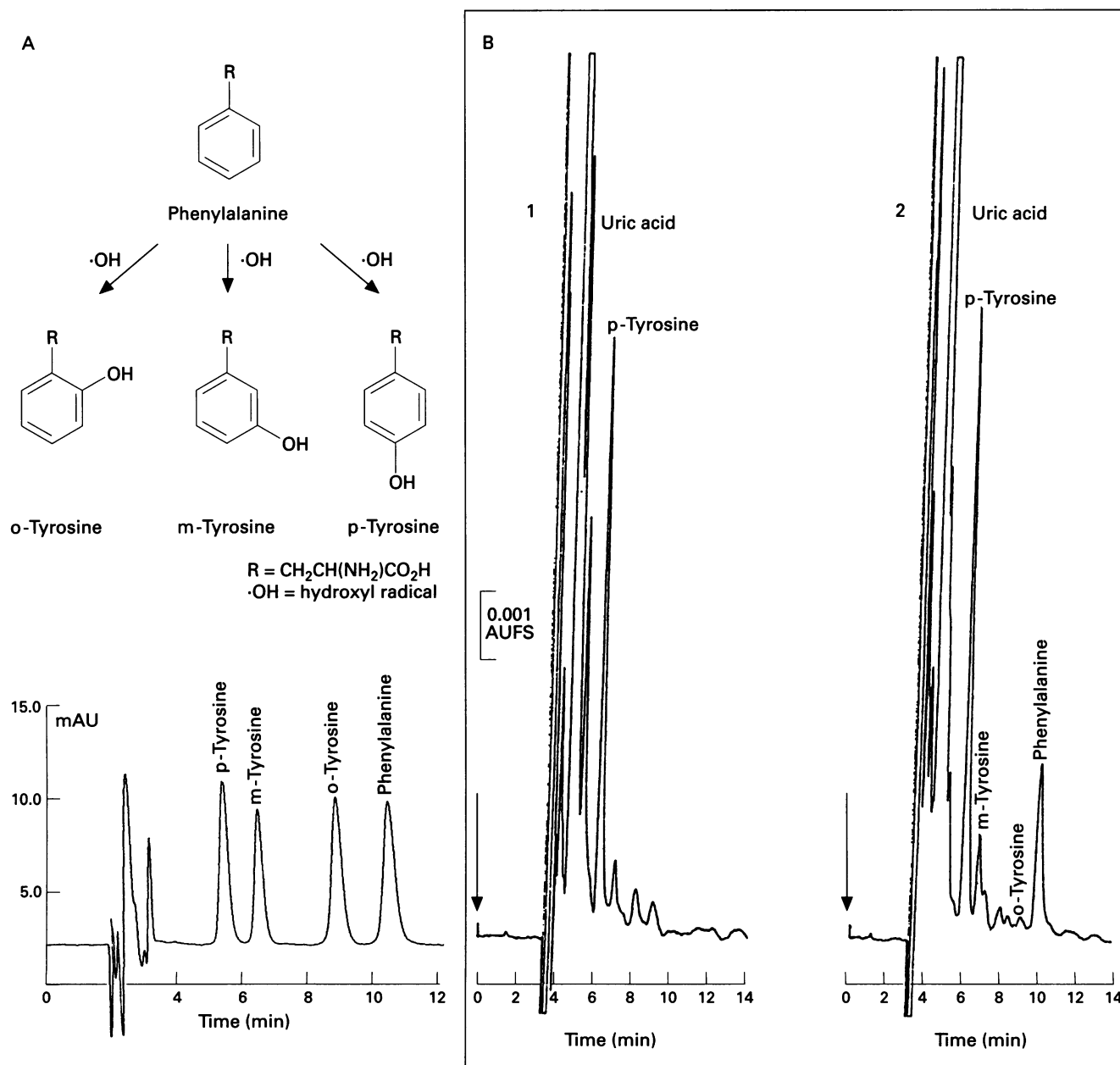


Figure 2 (A) Diagram showing the products of attack of hydroxyl radical upon phenylalanine (top) and the HPLC separation achieved following a previously published procedure²⁶ (bottom). (B) A representative chromatogram showing HPLC analysis of tyrosines after addition of synovial fluid to phenylalanine (2) or to saline (1).

Table 2 Biochemical variables and tyrosines measured in biological fluids of three rheumatoid patients examined more than once

Subject	Date seen	Biochemical variable		Tyrosines - μM					
				In synovial fluid			In serum		
		ESR mm h ⁻¹	CRP mg l ⁻¹	<i>o</i> -	<i>m</i> -	<i>p</i> -	<i>o</i> -	<i>m</i> -	<i>p</i> -
Patient 1	April '92	53.0	7.92	2.0	2.0	78.0	2.0	1.0	73.0
	May '92	76.0	5.66	1.0	2.0	61.0	1.0	2.0	54.0
	June '94	Not available	Not available	2.7	1.6	108.0	3.0	2.0	110.0
Patient 2	April '92	56.0	2.52	4.0	1.0	84.0	2.0	1.0	65.0
	June '93	80.0	Not available	0.5	0.5	84.0	0.2	0.3	65.0
Patient 3	June '92	100.0	9.17	0.9	3.5	66.5	1.0	2.5	60.0
	Nov '92	86.0	3.61	1.0	2.5	60.0	2.6	0.8	39.0

Note, all other patients were sampled only once in this study.

Not every rheumatoid arthritis patient examined produced fluids that caused conversion of Phe to products diagnostic of OH[•] attack. Of the 53 patients examined, 17 did not (these negative values were included in calculating the mean and standard deviations in table 1). No significant correlation was found between laboratory indices of inflammation [erythrocyte sedimentation rate (ESR); C reactive protein in serum or synovial fluid] and whether or not the fluids showed evidence of OH[•]. ESR values ranged from 6 to 100 mm/h [mean 58.7 (25.9), mm/h, n = 39]; C reactive protein estimations in serum ranged from 0.1 to 89.8 mg litre⁻¹ [mean 15.4 (24.9) mg litre⁻¹ (n = 28)].

REPRODUCIBILITY OF THE DETECTION OF OH[•] FROM PATIENTS USING PHENYLALANINE

During the course of this study we were able to obtain samples from three patients at different times. Table 2 shows the results. Even though C reactive protein, ESR, and clinical indices of disease activity were different at each time of sampling, OH[•] was always detected.

EFFECT OF HYPOXIA-REOXYGENATION

It has been suggested that exercise of the inflamed rheumatoid joint creates a hypoxia-reperfusion phenomenon that induces and exacerbates free radical damage.⁸ The generation of *o*- and *m*-tyrosines was therefore investigated under conditions of hypoxia and hypoxia-reoxygenation. The levels of *p*-tyrosine from rheumatoid arthritis fluids collected in saline as a control were not significantly different: synovial fluid before hypoxia-reoxygenation = 75.2 (30.7) μM , n = 48; after hypoxia-reoxygenation = 72.2 (40.2) μM , n = 22; serum before hypoxia-reoxygenation = 51.5 (21.2) μM , n = 49; after hypoxia-reoxygenation = 43.5 (22.4) μM , n = 23. *Ortho*- and *meta*-tyrosines were never observed.

Table 3 shows the results of measurements on the same fluids collected into 5 mM phenylalanine. There was a tendency for levels of

Table 3 Products of hydroxylation of Phe (*o*-, *m*-, *p*-tyrosines) measured in body fluids (collected in 5 mM Phe) of rheumatoid patients following *in vivo* hypoxia-reoxygenation

RA fluid	<i>o</i> -Tyr- μM	<i>m</i> -Tyr- μM	<i>p</i> -Tyr- μM
SF _{PRE}	1.6 (2.2) (n=50)	1.5 (2.1) (n=49)	72.5 (29.7) (n=50)
SF _{POST}	2.5 (3.7) (n=27)	2.0 (2.8) (n=28)	74.6 (64.6) (n=27)
S _{PRE}	1.8 (2.5) (n=50)	1.8 (2.3) (n=51)	50.5 (20.1) (n=52)
S _{POST}	2.4 (3.2) (n=27)	3.2 (4.1) (n=26)	63.3 (23.7) (n=20)

All values are mean (SD) including ND as a zero value. SF, synovial fluid; S, serum.

o- and *m*-Tyr to increase, but this was not statistically significant. Levels of *p*-Tyr were similar.

EFFECT OF CENTRIFUGATION

If synovial fluid was centrifuged (1500 *g* for 30 min) to remove cells before it was added to 5 mM Phe, the formation of *o*- and *m*-tyrosines was no longer observed (table 4).

CONFIRMATION OF HYDROXYL RADICAL FORMATION BY USING A DIFFERENT DETECTOR MOLECULE, THE AROMATIC HYDROXYLATION OF SALICYLATE

We attempted to trap OH[•] from the fluids of 22 rheumatoid arthritis patients using 5 mM salicylic acid as an alternative trap and the results are shown in table 5. When synovial fluid was collected in saline, no 2,3-DHB was detected and 0.018 (0.03) μM (n = 12) of 2,5-DHB found (these statistics include "not detected" levels taken as zero). This may be because traces of 2,5-DHB can come from the diet, for example plant materials and ingested drugs containing aspirin or salicylate (five patients were taking 75-100 mg aspirin daily). For comparison blood added to solutions containing saline gave serum that contained no detectable 2,3-DHB and 0.024 (0.051) μM (n = 16) of 2,5 DHB. When synovial fluid was aspirated into salicylate (5 mM), 2,3- and 2,5-DHB were formed (table 5). These 22 patients had also been tested using Phe as a trap, and again the same 18 showed evidence of OH[•] generation (*o*- and *m*-Tyr formation; see above).

FURTHER EVIDENCE FOR HYDROXYL RADICAL FORMATION: THE EFFECT OF SCAVENGERS

Mannitol and formate are specific scavengers of OH[•].²⁸ Their inclusion in the sampling tubes with salicylate caused inhibition of the formation of 2,3- and 2,5-DHB (table 5), as would be expected if generation of these products is due to OH[•].

Table 4 Synovial fluid collected in 5 mM Phe, freshly aspirated or first centrifuged to remove the cells. Data from two representative patients are shown

Subject	Fluid	Tyrosines - μM		
		<i>o</i> -	<i>m</i> -	<i>p</i> -
Patient 1	SF	1.0	0.5	55.5
	Spun SF	ND	ND	46.0
Patient 2	SF	3.5	1.0	127.5
	Spun SF	ND	ND	97.5

SF, synovial fluid.

Table 5 Products of hydroxylation of salicylate (2,3- and 2,5-dihydroxybenzoate) measured in body fluids of rheumatoid patients. The effects of OH[•] scavengers (mannitol and formate). Values are mean (SD)

Fluid	2,3-DHB μM	2,5-DHB μM
SF	0.38 (0.69) (n=12)	0.42 (0.61) (n=13)
SF + mannitol (100 mM)	0.16 (0.08) (n=6)	0.21 (0.08) (n=6)
SF + formate (100 mM)	0.03 (n=1)	0.15 (n=1)
S	0.11 (n=1)	0.16 (n=1)
S + mannitol (100 mM)	0.31 (0.21) (n=16)	0.79 (0.96) (n=16)
S + formate (100 mM)	0.13 (0.15) (n=11)	0.53 (0.71) (n=11)
	0.07 (0.03) (n=5)	0.19 (0.14) (n=5)

SF, synovial fluid; S, serum (obtained after addition of blood to salicylate solution, then allowing to clot).

Discussion

The results in the present paper show that aspirated knee joint fluid from patients with active rheumatoid arthritis can make the highly damaging OH[•] radical, as demonstrated using specific techniques of aromatic hydroxylation of two different detector molecules and the effects of OH[•] scavengers. The ability of synovial fluid to make OH[•] has long been suspected,¹⁻³ for example on the basis of detection of hydroxylation products of salicylate in vivo,^{22,23} but is here shown directly. The OH[•] could be formed by at least three mechanisms.

(1) In the presence of "catalytic" iron ions, O₂^{•-} and H₂O₂ may be converted to OH[•] by a metal ion catalysed Haber-Weiss reaction.²¹ The necessary iron could arise from H₂O₂ dependent degradation of haemoglobin,^{29,30} perhaps released by traumatic microbleeding in the joint. Another source could be O₂^{•-} dependent release of iron from synovial fluid ferritin.³¹

(2) The interaction of NO[•] and O₂^{•-} forms the cytotoxic product peroxynitrite, ONOO⁻. At physiological pH, ONOO⁻ converts to its protonated form — peroxynitrous acid, ONOOH — which decays to generate multiple toxic products, believed to include nitronium ion (NO₂⁺), nitrogen dioxide radical (NO₂[•]), and a species resembling OH[•].³²⁻³⁵ Production of not only O₂^{•-} but also NO[•] appears to be increased in rheumatoid arthritis patients, measured in terms of increased nitrite levels in serum and synovial fluid from these patients.⁷ There is also an increase in nitrotyrosine levels, consistent with ONOO⁻ formation in vivo.³⁶

(3) Activated neutrophils secrete the enzyme myeloperoxidase which uses H₂O₂ produced from O₂^{•-} to generate the powerful anti-bacterial agent hypochlorous acid, HOCl. In a pathway apparently independent of metal ions, HOCl reacts with O₂^{•-} to generate OH[•].³⁷ A second key point from our results is the ability of whole blood to cause OH[•] formation in some rheumatoid arthritis patients, but not in others or in normal controls. Plasma from healthy subjects is known not to be capable of catalysing OH[•] formation, and "catalytic" iron ions are not present in plasma even in rheumatoid arthritis patients.⁴³ Indeed, prior centrifugation of the blood or allowing it to clot and then adding serum or plasma to salicylate or phenylalanine produced no detectable OH[•]. The same was true if synovial fluid was spun before addition (table 4). These data suggest that cells are involved in the OH[•] generation,

perhaps primed/activated neutrophils, which may occur not only in synovial fluid but also in the systemic circulation in rheumatoid arthritis, given the extensive overproduction of various cytokines.⁴⁵ However, this conclusion is tentative, since it is possible that during the centrifugation time the fluids lose their ability to generate OH[•]. There was no significant correlation between white cell count and ability of blood to cause OH[•] formation (data not shown).

A third key point from our results is the variability in OH[•] formation between different rheumatoid arthritis patients, and it seems that factors other than the extent of ongoing inflammation affect the levels of OH[•] generation. Factors that could account for this include variations in the availability of iron catalytic for OH[•] generation in synovial fluid,³⁸ variations in endogenous antioxidant defences,³⁹ and the effects of drug treatment.⁴⁰ Some drugs may decrease oxidative damage (reviewed in ¹) whereas others may actually increase it, despite being anti-inflammatory overall.⁴⁰⁻⁴² These points will be explored in future studies.

The techniques of aromatic hydroxylation used in our study are well established. Thus, for example, salicylic acid has been used to show raised levels of 2,3- and 2,5-DHB in rheumatoid arthritis patients taking aspirin,^{22,23} and phenylalanine has been used to trap OH[•] produced in isolated cells,²⁵ in blood from some premature babies,²⁶ and from reperfused heart in situ.²⁷

It has been proposed that, in the early stages of inflammation, exercise promotes cycles of hypoxia-reoxygenation which can lead to the production of oxidising species that cause further damage to the joint.⁸ Our results are consistent with this concept in that they suggest a rise in OH[•] production, although statistical significance was not achieved.

In conclusion, we have shown that synovial fluid and blood from certain rheumatoid arthritis patients have the ability to generate OH[•]. It is likely that this highly reactive radical species contributes to injury at sites of chronic inflammation and may also contribute to systemic damage, although its precise pathological role remains to be ascertained.

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