Inactivation of xanthine oxidoreductase is associated with increased joint swelling and nitrotyrosine formation in acute antigen-induced arthritis

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Abstract

Objective

Arthritis is associated with increased articular formation of nitrotyrosine, which may contribute to injury. Nitrotyrosine is formed by nitration of tyrosine by reactive nitrogen species such as peroxynitrite, the formation of which may be enhanced by xanthine oxidoreductase (XOR), since it can generate nitric oxide from nitrite/nitrate, and superoxide during xanthine metabolism. We hypothesized that inactivation of XOR would protect against antigen-induced arthritis (AIA) and decrease nitrotyrosine formation.

Methods

AIA was induced with methylated bovine serum albumin (mBSA) in three groups of Wistar rats: animals fed on (1) tungsten-enriched chow (0.7 g/kg) (TG), which inactivates XOR, (2) standard chow (SG), and (3) rats treated with allopurinol (50 mg/kg/day; p.o.*) (AG). Nitrotyrosine in patella-synovium was quantified by mass spectrometry three weeks after intra-articular (*i.a.*) antigen injection.*

Results

Treatment with tungsten, but not allopurinol, suppressed plasma and articular XOR activity at ≤ 0.9% of normal levels. XOR inactivation was associated with increased knee swelling 24-48 hrs post i.a. *mBSA, compared with controls (mean increase ± SEM of knee diameter from baseline of 3.3 ± 0.5, 2.0 ± 0.3 and 1.9 ± 0.2 mm in TG, SG and AG (n = 14 each group), respectively; $p < 0.05$, TG vs SG, ANOVA). Mean ratio of articular nitrotyrosine-tyrosine (± SEM) was increased in the XOR-inactivated group, compared with controls: 12.3 ± 0.7, 9.6 ± 0.8 and 10.4 ± 0.5 pg/μg in TG, SG and AG, respectively; $p < 0.05$, TG vs SG.*

Conclusion

Contrary to expectation, XOR inactivation was associated with increased joint swelling and articular tyrosine nitration in acute AIA, suggesting a novel, protective role for XOR in inflammatory arthritis.

Key words

Xanthine oxidoreductase, arthritis, reactive nitrogen species, nitrotyrosine, tungsten, allopurinol.

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Introduction

Both nitric oxide (NO) and superoxide ($O_2^{\bullet -}$) are generated in increased amounts in inflamed joints (1-3), but their precise role in pathogenesis remains unclear. NO and $O_2^{\bullet -}$ react rapidly with each other *in vitro* to form peroxynitrite ($ONOO^-$) (4), a highly reactive nitrating and oxidizing agent. $ONOO^-$ induced modification of proteins, DNA and other biomolecules can lead to altered cell function, cell necrosis and apoptosis (5). The nitration of tyrosine to the relatively stable 3-nitrotyrosine (3-NT) is one consequence of $ONOO^-$ exposure *in vitro* (6-8). Other reactive nitrogen species (RNS) can also cause nitration of tyrosine in a peroxidase-dependent manner (9-11), but which of these pathways predominate *in vivo* is unknown. 3-NT is therefore best considered a marker of RNS formation in general (12). $ONOO^-$ can induce expression of cyclooxygenase 2 (COX2) in rheumatoid synoviocytes *in vitro* (13). We and others described the immunolocalisation of 3-NT in the rheumatoid synovium (14,15), suggesting that these reactions occur *in vivo* and contribute to disease pathology.

3-NT formation originates from endogenous NO, generated from arginine by one of the three isoforms of NO synthase (NOS). However, we and others have previously shown that xanthine oxidoreductase (XOR) can also generate NO (16,17) *in vitro*. XOR is expressed in the synovium (18) and best known for its house-keeping role in xanthine oxidation to yield uric acid and $O_2^{\bullet -}$. During xanthine oxidation XOR can also reduce nitrite to NO, suggesting that XOR may be a peroxynitrite synthase (19, 20).

We aimed to test the hypothesis that suppression of XOR activity in inflamed joints decreases nitration of proteins and joint inflammation. This has potentially important implications, since if true, XOR inhibitors, such as allopurinol, may be useful therapeutically in inflammatory arthritis *per se*. In contrast to our expectation we observed that XOR inactivation enhances acute experimental arthritis and nitration of articular proteins.

Materials and methods

Chemicals were obtained from Sigma, Poole, UK, unless stated otherwise and all concentrations are final.

Animals

Male out-bred Wistar rats of weaning age (Charles River, UK) were housed under standard conditions for one week before experimental interventions began. Procedures complied with the Animals (Scientific Procedures) Act 1986, UK. All invasive interventions animals were performed under 4% isoflurane/oxygen (2 l/min) anaesthesia.

Pharmacological inhibition of XOR and induction of antigen-induced arthritis (AIA)

Three groups of animals were studied (Fig. 1): (1) rats treated with tungsten-enriched chow to inactivate XOR by replacing active-centre molybdenum with tungsten (21) ('Tungsten Diet'; TG); (2) animals maintained on standard diet ('Standard Diet'; SG) and (3) mBSA-immunised animals on standard chow, treated with the XOR-inhibitor allopurinol ('+Allopurinol'; AG). AIA was induced as previously described (22). Briefly, from experimental Day 0 (Fig.1) animals (mean weight ± SEM 132±2 gm) were fed for three weeks on tungsten-enriched (sodium tungstate 0.7 g/kg chow; ICN, Basingstoke, UK) or standard chow (SG; SDS, Witham, UK) before subcutaneous (s.c.) immunisation with 500 µg mBSA[as 100 µl of 10 mg mBSA/ml 0.9% saline, mixed 1:1(v/v) with complete Freund's adjuvant (CFA)] on Day 21. Three control animals in each diet group were injected with saline/CFA. This immunisation was repeated one week later (Day 28). One day before intra-articular (i.a.) antigen challenge, a subgroup of the standard diet group was started on allopurinol (50 mg/kg/day, p.o., by gavage). The following day (Day 42), all animals were injected with 500 µg mBSA (as 100 µl of 5mg/ml sterile 0.9% saline) and vehicle via i.a. injection into the right and left knee, respectively. Three weeks later (Day 63) [when a previous study had shown destructive arthritis to be present (22)] all animals

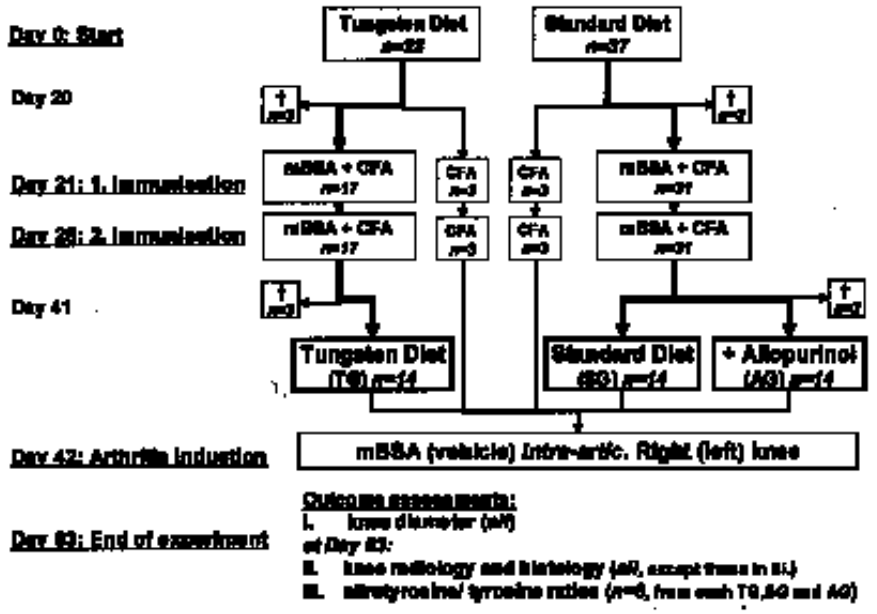

Fig. 1. Experimental design of the antigen-induced arthritis. †, animals sacrificed for measurement of XO activity; mBSA, methylated bovine serum albumin; CFA, complete Freund's adjuvant.

were killed and blood, knee joints or dissected patella with adjacent synovium taken for further analysis.

Clinical assessment
Animals were weighed weekly. Mediolateral knee diameters were measured on non-anaesthetised animals with digital callipers (Mitutoyo, Andover, UK) before, 24, 48 hours, 5, 7 and 14 days after i.a. mBSA/vehicle injection. Measurements were undertaken blinded to the intervention group, and recorded to the nearest 0.1 mm.

Delayed-type hypersensitivity (DTH) testing
One week after the second mBSA immunisation (Day 35) , a subgroup of animals (n = 5, from each diet group) were injected intra-dermally into the right and left ear with 2.5 µg mBSA(as 50 µl of 0.5 mg/ml 0.9% saline) and vehicle, respectively. Local skin reaction was assessed 48 hours later for redness and induration.

XOR activity assay
XOR activity was measured in citrated plasma and mechanical homogenates of snap-frozen patella-synovium (in phosphate-buffered saline (PBS) with 10 µg/ml aprotinin and 0.5 nM phenylmethylsulfonylfluoride). The analysis used a sensitive fluorospectrometric method (23), based on the XOR-mediated oxidation of pterin to isoxanthopterin. Briefly, pterin (20 µM) was added to sample of pre-determined optimum dilution in PBS and increase of fluorescence (λ excitation/ emission = 345/390 nm) measured (F4500, Hitachi, UK). Addition of allopurinol (10 µM) verified the enzymatic specificity of the reaction and isoxanthopterin (200 nM) provided a fluorescent standard. Samples were measured in triplicate and XOR activity was calculated and expressed as nmol per min per g sample tissue protein, with protein concentration determined according to Bradford (24).

Quantification of nitrotyrosine
Nitrotyrosine and tyrosine content was measured in patella-synovium by gas chromatography/mass spectrometry (GCMS), as previously described (25). Briefly, snap-frozen tissues were mechanically homogenised in PBS and chloroform-methanol (2:1) on ice. Centrifugation at 9000 g for 15 min yielded a protein precipitate middle layer, which was lyophilized. To avoid artefactual tyrosine nitration associated with the commonly employed acidic hydrolysis, 1-1.5 mg of protein was then hydrolyzed in 4 M sodium hydroxide at 120°C for 16 hrs containing 20 ng $^{13}C_9$-nitrotyrosine and 10 µg D_4-tyrosine as stable isotopic interval standards. Following solid phase extraction, nitrotyrosine and tyrosine was quantified by gas chromatography/negative ion chemical ionization mass spectrometry. Results were expressed as nitrotyrosine/tyrosine [pg/µg].

Radiographic and histological analysis
Knee joints were x-rayed in two planes (Faxitron, Field Emission Ltd., London, UK) and coded radiographs evaluated independently by two experienced investigators (26). Knees were then decalcified, dehydrated and embedded in paraffin. Sagittal sections, stained with haematoxylin-eosin, were examined for inflammatory and destructive changes (27).

Statistical methods
Where appropriate, mean values were analysed by unpaired t-test, or one-way analysis of variance (ANOVA) with Dunnett's *post*-test.

Results

Animals of all three groups thrived well and there were no abnormal macroscopic findings at *post mortem* examination. Three of 5 animals in each diet group demonstrated a positive DTH reaction to mBSA.

Xanthine oxidase activity
The activity of xanthine oxidase in plasma and joint homogenates are shown in Table I. Mean XO activity in plasma or joint homogenates of tungsten-treated (TG) animals was ≤ 0.9% of that of the control group (SG). In the allopurinol-treated (AG) animals mean XO activity in plasma was decreased to 7%, but XOR activity was not significantly different in the patella-synovium compared to controls.

Joint swelling
Mean baseline diameters ± SEM of mBSA-injected knee joints were comparable in the TG, SG and AG groups at 12.3 ± 0.2, 12.5 ± 0.2 and 12.5 ± 0.1 mm, respectively. Twenty-four and 48 hours after *i.a.* mBSA-injection, knee diameters showed a greater increase

Table I. XO activity in rat plasma, and patella-synovium homogenates.

	Tungsten Diet	Standard Diet	Allopurinol
Plasma			
Day 20	0.3 ± 0.4	172 ± 16 *	NA
Day 41	ND	219 ± 5	NA
Day 63	ND	162 ± 11 †	12 ± 2
Patella-Synovium			
Day 63	1.1 ± 3.1	127 ± 22 β	160 ±17

Values are mean XO activity (± SEM), as nmol x min-1 x g tissue protein-1, measured by spectro-fluorimetric pterin assay. Sample sizes were n = 3 animals for each group, except for plasma of day 21 (*n* = 6). Left and right patella-synovium samples were analysed separately. Each sample was measured in triplicate. Experimental days were: Day 20, after 3 weeks on experimental diet and prior to s.c. mBSA immunisation; Day 41, prior to *i.a.* mBSAinjection; Day 63, three weeks after *i.a.* injection. ND, not detectable; NA, not applicable. * $p < 0.001$ vs Tungsten Diet (unpaired t-test); † $p < 0.001$ vs Allopurinol (unpaired t-test) and β $p < 0.001$ vs Tungsten Diet (ANOVAwith Dunnett's *post*-test).

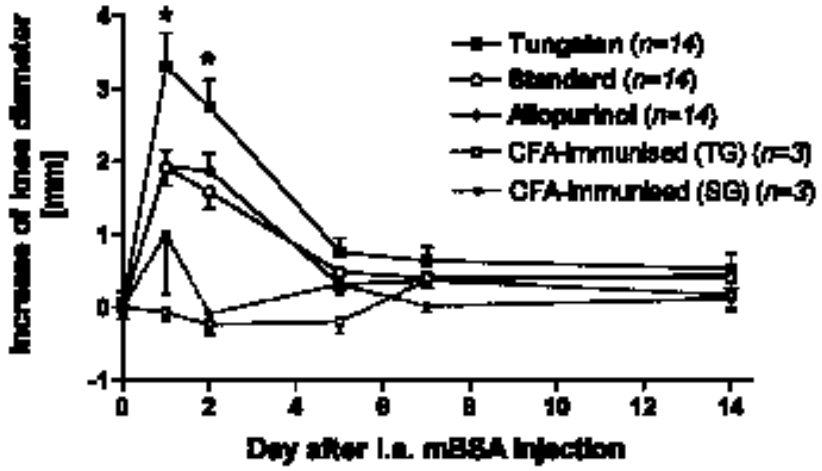

Fig. 2. Mean change of transverse diameter of mBSA-injected knees from baseline (*i.e.* prior to *i.a.* mBSA) over time. Bars represent SEM. * $p < 0.05$ vs . standard diet group (ANOVA with Dunnett's post-test).

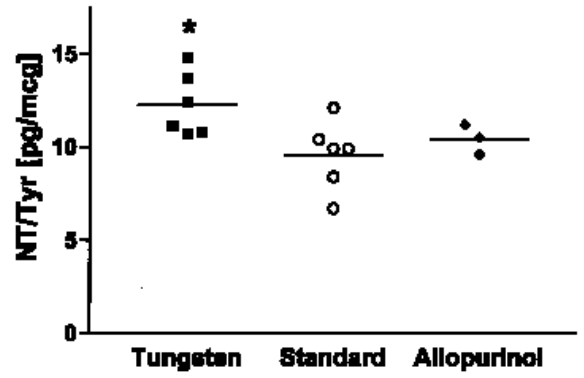

Fig. 3. Nitrotyrosine-tyrosine ratios of patella-synovium homogenates (3 weeks post *i.a.* mBSA injection). Bars represent mean values. * $p < 0.05$ for difference of means between tungsten- vs. standard-diet group (ANOVA with Dunnett's post-test).

from baseline in the tungsten- compared with the standard-diet group (n = 14, each group): mean difference (95%-confidence interval) were 1.3 (0.3, 2.4) mm and 1.2 (0.2, 2.1) mm, respectively ($p < 0.05$; ANOVA with Dunnett's post-test) (Fig. 2). There was no significant increase in knee diameter in the CFA-immunised animals.

Nitrotyrosine/tyrosine content

Mean nitrotyrosine-tyrosine ratios (± SEM) of right patella-synovium homogenates of day 21 were significantly higher at 12.3 ± 0.7 pg/µg in the tungsten group (n = 6) compared with controls (9.6 ± 0.8 pg/µg) (*n* = 6), and were unchanged at 10.4 ± 0.5 pg/µg in the allopurinol group (*n* = 3) ($p < 0.05$, TG vs SG; ANOVA with Dunnett's *post*-test) (Fig. 3).

Radiographic and histological analysis

Radiological and histological analysis did not show chronic inflammatory and/or destructive joint disease in any group (*n* = 8, for each TG, SG and AG).

Discussion

We hypothesized that XOR contributes to the formation of RNS and nitration of articular proteins and that inhibition of XOR would ameliorate the course of experimental arthritis. In contrast, inactivation of articular XOR activity by tungsten was associated with a greater increase of mean knee swelling during acute antigen-induced arthritis (AIA) compared to standard-fed controls. Furthermore, tungsten-treated animals showed increased nitrotyrosine formation in arthritic joints compared with controls, as measured by a highly sensitive gas chromatographic-mass spectrometric method. This suggests that XOR inactivation enhanced joint inflammation early during the course of AIA. Although the effect is relatively modest, this is the first study to indicate that XOR may have a protective effect in immune complex-mediated disease, and supplements our previous observation that suggests a beneficial role for XOR in innate immune responses (19). We are unaware of other published studies of tungsten-induced XOR inactivation in AIA. Using dietary tungsten in adjuvant arthritis in Lewis rats, our group has previously observed a reduction in radiographic erosion and bone demineralization scores in tungsten-treated animals compared to controls, suggesting that XOR contributes to joint damage (28). Pathogenetic differences between animal models may account for these contrasting results. Acute AIA is a localised, non-destructive, immune complex disease of a few days duration (29). T-cell hypersensitivity and intra-articular antigen-retention are required for a chronic, destructive arthritis to develop. In contrast, adjuvant arthritis is a destructive, T-cell mediated disease from the outset (30). XOR could thus have divergent effects

on acute synovial inflammation and chronic destructive arthritis. Indeed, distinct anti-inflammatory activity is increasingly recognised as an integral part of classical pro-inflammatory enzymes, such as iNOS (31, 32) and COX2 (33) and may hold clues to explain the progression of joint damage despite inactive synovial inflammation seen in rheumatoid arthritis (RA) (34, 35). Our findings may also partly explain why acute gout arthritis is exacerbated by XOR inhibition with allopurinol.

How may XOR limit protein nitration and acute joint inflammation? Uric acid, the final oxidation product of XOR-mediated purine metabolism, is a potent inhibitor of peroxynitrite-induced tyrosine nitration under physiological conditions *in vitro* (36). Endogenous uric acid inhibits protein tyrosine nitration in rat heart homogenates (37). Administration of uric acid reduced tissue damage in experimental autoimmune encephalitis, an animal model of multiple sclerosis, (38) and zymosan-induced rat knee arthritis (39). Since oxidative effects of $ONOO^-$ seem unaffected (36) or even enhanced by uric acid (40), it is suggested that protein tyrosine nitration itself is an important mediator of tissue inflammation and damage (41).

This study has limitations. Although increased 3-NT concentration is good indirect evidence that increased inflammation occurred, we lack histological data of enhanced acute inflammation in the XOR-inactivated animals. This is due to the fact that study design and power considerations were aimed at the detection of a difference in the development of chronic destructive arthritis, as the outcome most relevant in comparison to RA. Although the majority of animals developed delayed-type hypersensitivity to mBSA, they failed to progress to a chronic destructive arthritis, contrasting our earlier experience with the same AIA induction protocol (22). Subtle strain variation in our outbred Wistar rats may account for this observation. Secondly, tungsten is not a specific XOR inhibitor, but will also inhibit the two other molybdenum-enzymes known to exist in rat and man, *i.e.* sulfite oxidase (SO) and aldehyde oxidase (AO) (42). To our knowledge SO and AO have no reported relevance to inflammatory arthritis. Finally, sample sizes, especially for 3-NT measurements in the allopurinol group, were small. This was partly due to sample size limits of the GCMS assay and, based on the better XOR inactivation by tungsten, samples were prioritized accordingly.

In conclusion, we have shown that inactivation of XOR by dietary tungsten was associated with increased acute joint swelling and increased nitration of articular proteins, indicating increased joint inflammation during acute antigen-induced arthritis. This suggests that XOR may have a novel protective role in immune complex-mediated arthritis. Further studies are required to confirm these findings histologically and clarify the relevance of uric acid and allopurinol in this model.

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