Myeloperoxidase and oxidation of uric acid in gout: implications for the clinical consequences of hyperuricaemia

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Abstract

Objectives. The aims of this study were to establish whether, in patients with gout, MPO is released from neutrophils and urate is oxidized to allantoin and if these effects are attenuated by allopurinol.

Methods. MPO, urate, allantoin and oxypurinol were measured in plasma from 54 patients with gout and 27 healthy controls. Twenty-three patients had acute gout, 13 of whom were receiving allopurinol, and 31 had intercritical gout, 20 of whom were receiving allopurinol. Ten additional gout patients had samples collected before and after 4 weeks of allopurinol.

Results. Plasma MPO and its specific activity were higher ($P < 0.05$) in patients with acute gout not receiving allopurinol compared with controls. MPO protein in patients’ plasma was related to urate concentration ($r = 0.5, P < 0.001$). Plasma allantoin was higher ($P < 0.001$) in all patient groups compared with controls. In controls and patients not receiving allopurinol, allantoin was associated with plasma urate ($r = 0.62, P < 0.001$) and MPO activity ($r = 0.45, P < 0.002$). When 10 patients were treated with allopurinol, it lowered their plasma urate and allantoin ($P = 0.002$). In all patients receiving allopurinol, plasma allantoin was related to oxypurinol ($r = 0.65, P < 0.0001$). Oxypurinol was a substrate for purified MPO that enhanced the oxidation of urate.

Conclusion. Increased concentrations of urate in gout lead to the release of MPO from neutrophils and the oxidation of urate. Products of MPO and reactive metabolites of urate may contribute to the pathology of gout and hyperuricaemia. At low concentrations, oxypurinol should reduce inflammation, but high concentrations may contribute to oxidative stress.

Key words: gout, myeloperoxidase, cardiovascular disease, urate, allopurinol, oxidative stress.

Introduction

Hyperuricaemia is critical for the development of gout and is also linked to numerous pathologies [1, 2]. Painful attacks of gout occur when crystals of monosodium urate precipitate within joints and promote an acute inflammatory response. Other inflammatory diseases associated with hyperuricaemia include diabetes, hypertension, pre-eclampsia, metabolic syndrome, renal failure and cardiovascular disease (CVD) [3, 4]. Whether urate is a causative factor in these diseases is controversial, but evidence linking hyperuricaemia to their pathology continues to mount.

The pernicious effects of urate are often attributed to its ability to activate the inflammasome [5]. It is also proposed to act as a pro-oxidant when oxidized to its radical [6]. Recently urate was demonstrated to be a physiological substrate for the neutrophil enzyme MPO [7]. This haem enzyme uses hydrogen peroxide ($H_2O_2$) to oxidize chloride to hypochlorous acid ($HOCl$) [8], but under physiological conditions will also convert urate to its urate radical. The urate radical is readily reduced by ascorbate [9], but it also gives rise to several electrophiles including dehydrourate, 5-hydroxyisourate and urate hydroperoxide [7]. These species will react with...
endogenous nucleophilic or break down to the stable biomarker allantoin.

In gout, MPO is likely to be discharged from neutrophils when they are recruited to affected joints and attempt to phagocytose urate crystals. Urate should compete with chloride and be oxidized by MPO. Whether MPO is present in the circulation of patients with gout and oxidizes urate to allantoin is unknown. This is clinically relevant because MPO contributes to cardiovascular risk [10] and both hyperuricaemia and gout are associated with an increased risk of CVD and death [11–14].

Successful long-term management of gout requires sustained reduction of plasma urate to <360 µM [15]. Allopurinol, which inhibits xanthine oxidoreductase, thereby preventing the production of urate [16], is the most commonly used urate-lowering therapy. Allopurinol is rapidly metabolized by aldehyde oxidase to oxyipurinol and H₂O₂ [17]. By virtue of its longer half-life, oxyipurinol exerts most of the inhibitory effects on xanthine oxidoreductase.

We hypothesize that during acute inflammation in gout, MPO will be released from neutrophils and oxidize urate to reactive intermediates that will contribute to the adverse effects of hyperuricaemia. The aims of this study were to determine in patients with gout whether MPO is released from neutrophils and oxidizes urate and if these effects are attenuated by allopurinol.

**Patients and methods**

**Subjects**

Patients with gout as defined by ARA preliminary classification criteria were recruited [18]. Acute gout was defined as the presence of a red, hot, swollen joint typical for gout and intercritical gout was defined as a patient with a confirmed diagnosis of gout with no current inflammatory joint symptoms. Healthy controls were randomly selected from the Christchurch electoral role. Patient demographic and clinical details were collected. Laboratory assessment included plasma urate and oxyipurinol concentrations. Peripheral blood for MPO and allantoin was collected and processed as previously described [19]. Ethical approval was obtained from the Upper South A and B Regional Ethics Committees, New Zealand. Written informed consent was obtained from each patient.

**Laboratory assays**

Urate concentrations in plasma were measured by the Trinder reaction and using HPLC with ultraviolet (UV) light detection [20]. These methods gave comparable results. Plasma oxyipurinol concentrations were measured as previously described [21]. There is currently no validated therapeutic range for plasma oxyipurinol. In healthy volunteers, xanthine oxidase is maximally inhibited (90% inhibition) at a plasma oxyipurinol concentration of 34 µM [22].

Plasma MPO was determined by ELISA as described previously [23]. In this assay, MPO is initially captured with a monoclonal MPO antibody and its activity is determined using H₂O₂ and Amplex Red. Then the amount of MPO that was bound to the plate is determined using a polyclonal MPO antibody. Plasma allantoin was measured by stable isotope dilution mass spectrometry as described elsewhere [24].

The production of HOCl by purified MPO in an in vitro assay was determined by measuring the accumulation of taurine chloramine [10]. Reactions were started by adding 50 µM H₂O₂ to 20 nM MPO in 10 mM phosphate buffer (pH 7.4) containing 140 mM sodium chloride and 5 mM taurine. The reactions were stopped after 4 min by adding 20 µg/ml of catalase and accumulated taurine chloramine was determined. Tyrosine (10 µM), which is a good substrate for compound II of MPO [8], was included with the purines to determine whether they acted by promoting formation of this redox intermediate, which is unable to oxidize chloride. None of the purines reacted directly with taurine chloramine. MPO was obtained from Planta (Vienna, Austria) and had a purity index of 0.82. Oxidation of urate (300 µM) by purified MPO (25 nM) with various concentrations of oxyipurinol in 50 mM phosphate buffer (pH 7.4) was started by adding H₂O₂ (50 µM) and stopped after 30 min by adding catalase (10 µg/ml). Production of allantoin from urate oxidation was measured using stable isotope dilution mass spectrometry as described elsewhere [24]. Xanthine, urate, allopurinol and oxyipurinol were obtained from Sigma (St Louis, MO, USA). All other reagents were of the highest analytical grade available.

**Statistical analysis**

Data are expressed as the median and interquartile range (IQR). The statistical analysis of differences between group medians was carried out using either the Mann–Whitney rank sum test, analysis of variance (ANOVA) on ranks or the Wilcoxon signed rank test. Correlations were tested using Spearman’s rank order correlation. In all cases significance was accepted as $P < 0.05$. Insufficient sample was available to analyse MPO in one patient with acute gout receiving allopurinol.

**Results**

**Patient demographics**

Fifty-four patients with gout and 27 healthy controls of a similar age range and gender mix were recruited. They had peripheral blood samples obtained on a single occasion. The patients were allocated into four groups: acute gout not receiving allopurinol ($n = 10$), acute gout receiving allopurinol ($n = 13$), intercritical gout not receiving allopurinol ($n = 11$) and intercritical gout receiving allopurinol ($n = 20$). An additional 10 patients with gout had peripheral blood samples taken prior to commencing treatment with allopurinol and again after 4 weeks of therapy. No patients were receiving other urate-lowering therapies such as probenecid or febuxostat. Demographic and clinical details are shown in Table 1.

The plasma urate concentrations of patients not on allopurinol with either acute gout [median 552 µM (IQR 496–586), $n = 10$] or intercritical gout [median 530 µM...
Table 1: Demographics and laboratory data of patients

<table>
<thead>
<tr>
<th>Age, mean (range), years</th>
<th>Male, %</th>
<th>NZ European, %</th>
<th>Plasma urate, mean (range), mmol/l</th>
<th>Creatinine, mean (range), mmol/l</th>
<th>Receiving allopurinol, %</th>
<th>Allopurinol dose, mean (range), mg/day</th>
<th>Plasma oxypurinol, mean (range), μmol/l</th>
<th>NSAID, %</th>
<th>Prednisone, %</th>
<th>Colchicine, %</th>
<th>History of ischemic heart disease, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute gout (n = 10)</td>
<td>62.2 (40-91)</td>
<td>70</td>
<td>52.2 (90-91)</td>
<td>29.7</td>
<td>67</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Acute gout + allopurinol (n = 20)</td>
<td>62.9 (40-83)</td>
<td>90.9</td>
<td>59.2 (90-91)</td>
<td>27.7</td>
<td>90.9</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Intercritical gout (n = 11)</td>
<td>59.2 (84-82)</td>
<td>96</td>
<td>59.2 (84-82)</td>
<td>3.8</td>
<td>96</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Intercritical gout + allopurinol (n = 20)</td>
<td>62.2 (40-82)</td>
<td>96</td>
<td>62.2 (40-82)</td>
<td>3.8</td>
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<tr>
<td>Healthy controls (n = 27)</td>
<td>58.1 (39-79)</td>
<td>100</td>
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<td>4.2</td>
<td>100</td>
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Plasma MPO is increased in patients with acute gout

MPO protein and MPO enzyme activity were measured in the plasma of controls and the four patient groups, who were sampled on one occasion, to determine whether gout is associated with systemic increases in this neutrophil-derived protein. Both the activity and concentration of the enzyme were measured because MPO is susceptible to inactivation and is inhibited in plasma by ceruloplasmin [23]. Plasma MPO protein was significantly higher (P < 0.05) in patients with acute gout not receiving allopurinol [median 11.9 mg/ml (IQR 19.6–89.8), n = 10] compared with healthy controls [median 18.4 mg/ml (IQR 14.5–21.5), n = 27] (Fig. 1A), but not in patients with intercritical gout not receiving allopurinol [median 31.2 mg/ml (IQR 13.9–59.7), n = 11]. The enzyme activity of MPO in plasma was significantly higher (P < 0.05) in patients with acute gout not receiving allopurinol [median 37.0 mg/ml (IQR 11.8–77.8), n = 10] compared with healthy controls [median 6.3 mg/ml (IQR 4.3–9.5), n = 27] (Fig. 1B). MPO activity was not significantly elevated in patients with intercritical gout not receiving allopurinol [median 11.6 mg/ml (IQR 5.7–17.4), n = 11]. The specific activity of MPO (MPO activity/MPO protein) was significantly higher in patients with acute gout not on allopurinol compared with controls (Fig. 1C), but not for patients with intercritical gout not on allopurinol. Allopurinol had no significant effects on the levels of MPO protein, MPO activity or the enzyme’s specific activity (Fig. 1A–C).

To determine whether plasma urate affects the release of MPO from neutrophils, we assessed its relationship with plasma MPO protein and activity in all acute and intercritical gout patients irrespective of whether they were receiving allopurinol (Fig. 1D). In these patients, plasma urate concentration was significantly associated with MPO protein (r = 0.50, P = 0.0002, n = 53) and MPO activity (r = 0.29, P = 0.04, n = 53). The effect of urate on MPO activity was more significant (P = 0.005) when patients were split into normouricaemic [median 8.1 mg/ml (IQR 6.6–11.0), n = 23] and hyperuricaemic individuals [median 12.9 mg/ml (IQR 7.9–33.6), n = 30]. Collectively these data suggest that acute gout leads to an increase in plasma MPO that is associated with the concentration of urate. Allopurinol did not appear to affect the concentrations of MPO.

Plasma allantoin is increased in patients with gout

To assess whether urate is oxidized in patients with gout, we measured allantoin in plasma. The concentration of allantoin in plasma of both patients with acute gout not on allopurinol [median 6.4 μM (IQR 4.2–7.98), n = 10] and intercritical gout not on allopurinol [median 4.9 μM (IQR 4.2–5.4), n = 11] was significantly higher (P < 0.05) than that in healthy controls [median 2.6 μM (IQR 1.5–3.8),
The concentration of allantoin was similar in acute patients irrespective of whether they were receiving allopurinol (AG + AP) and patients with intercritical gout off (ICG) and on allopurinol (ICG + AP). Box plots show medians with 25th and 75th percentiles as boundaries. Whiskers are for the 10th and 90th percentiles. Symbols: closed circle indicates an outlier. Groups were compared using analysis of variance on ranks or the Mann–Whitney rank sum test. Asterisk indicates $P < 0.05$ compared with the control. NS: not significant. (D) The relationship between plasma urate and plasma MPO protein in all patients with gout. The association was determined using Spearman’s rank order correlation.

There was a significant correlation between the concentrations of allantoin and urate in the plasma of controls and gout patients not treated with allopurinol ($r = 0.62$, $P < 0.001$, $n = 48$) (Fig. 2B). In this group there was also a significant correlation between the concentrations of allantoin and MPO activity in plasma ($r = 0.45$, $P < 0.002$, $n = 48$) (Fig. 2C). There was a weaker correlation of MPO protein with allantoin ($r = 0.31$, $P < 0.035$, $n = 48$). Patients treated with allopurinol were excluded from these analyses because of its positive effect on allantoin in the intercritical group. These data indicate that urate is oxidized in patients with gout and that oxidation is associated with higher concentrations of urate and MPO.

(A) Allantoin was measured in plasma from controls and patients with gout and analysed as described in Fig. 1. Closed circle indicates an outlier. Allantoin was compared with (B) plasma urate or (C) plasma MPO activity. Filled and empty dots are for controls and patients, respectively. Correlations were determined using Spearman’s rank order correlation. NS: not significant.

Effects of allopurinol on the oxidation of urate

A notable feature of the data on plasma allantoin was that even though allopurinol lowered urate concentrations in patients with gout, it did not lower the concentration of allantoin. In fact, the plasma concentration of allantoin was highest in patients with intercritical gout treated with allopurinol (Fig. 2A). To directly assess the effect of allopurinol on allantoin and MPO levels, we selected 10 additional patients with gout who were commenced on allopurinol and looked for changes in urate, allantoin and MPO after 4 weeks of therapy. In a paired analysis, allopurinol was found to lower circulating urate ($P = 0.002$) (Fig. 3A) as well as the plasma concentration of allantoin ($P = 0.002$) (Fig. 3B). It had no significant effect on concentrations of MPO protein or MPO activity (data not shown).

These data were in conflict with those in Fig. 2A for the effect of allopurinol in patients with intercritical gout. Therefore we assessed how the concentration of oxypurinol affected the plasma concentration of allantoin in all patients receiving allopurinol. As shown in Fig. 3C, there was a strong association between plasma oxypurinol concentrations and allantoin ($r = 0.65$, $P < 0.0001$, $n = 42$).

Allopurinol and oxypurinol are analogues of hypoxanthine and xanthine and are potential targets for oxidation by reactive oxygen species and peroxidases. Therefore, to understand how oxypurinol might affect the oxidation of
urate, we determined the effects of these purines on the in vitro activity of purified MPO by assessing whether they interfere with its production of HOCl (Fig. 4A). Oxypurinol inhibited MPO by 50% (IC$_{50}$) at 6.8 μM (Fig. 4A), while xanthine had an IC 50 of 2.8 μM (not shown). Allopurinol and hypoxanthine had no effect (data not shown).

Inhibition by oxypurinol reached a maximum of only 75% and was less effective in the presence of tyrosine [8]. These results suggest that oxypurinol is a substrate for the redox intermediates of MPO. To test this possibility we determined how oxypurinol affected the oxidation of urate by MPO. Adding oxypurinol to a system containing MPO, urate and H$_2$O$_2$ enhanced the production of allantoin (Fig. 4B). Importantly, allantoin was not formed in the absence of urate. Oxypurinol also enhanced the conversion of urate to allantoin by MPO in a dose-dependent manner (Fig. 4C). These results indicate that oxypurinol is a substrate for MPO that can promote the oxidation of urate.

**Discussion**

Neutrophils swarm into the joints of patients with gout to phagocytose MSU crystals [2] and in the process they generate numerous cytokines and release enzymes into the extracellular milieu [26], MPO is the most abundant enzyme discharged by neutrophils [8]. Therefore it is surprising that until now there have been no reports on how MPO contributes to the pathology of gout. Our identification of elevated MPO in the circulation of patients with acute gout and its association with increased oxidation of urate strongly suggest that it will participate in the sequelae of gout, including the predisposition to CVD.

Our results indicate that during an attack of gout neutrophils are stimulated and release MPO. The significant correlation between the concentrations of urate and MPO in plasma is in accord with the established findings that MSU crystals activate the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome, which promotes an influx of neutrophils to the site of...
Oxidative metabolism of urate

Urate is produced by xanthine oxidoreductase (XOR) from xanthine and in the presence of oxygen can form hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^-$) [16].

Myeloperoxidase (MPO) uses $H_2O_2$ to oxidize urate to the urate radical and to produce hypochlorous acid (HOCl) from chloride [7, 8]. Urate is also susceptible to one-electron oxidation by reactive oxygen species (ROS). The urate radical reacts rapidly with superoxide to form urate hydroperoxide or is converted to 5-hydroxyisourate. These electrophilic products can either react or decay to allantoin. Aldehyde oxidase (AO) oxidizes allopurinol to oxypurinol, which inactivates xanthine oxidoreductase. Oxypurinol is oxidized to a radical that promotes the oxidation of urate. Arrows with dashed lines indicate pathways with multiple stages between reactants and products. Urate is toxic because it can crystalize and activate the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome or is oxidized to the reactive species shown in italics. Allantoin is a stable biomarker of oxidative stress.

From our results it is apparent that allopurinol has contrasting effects on oxidative stress depending on the oxypurinol concentration. Low concentrations of oxypurinol lowered allantoin. This effect was lost with increasing plasma concentrations of oxypurinol, to the point where it increased formation of allantoin. At low concentrations, oxypurinol should act by decreasing inflammation through its urate-lowering action. However, high concentrations of oxypurinol could promote oxidation of urate via two pathways (see Fig. 5). First, allopurinol is chiefly metabolized to oxypurinol by aldehyde oxidase and in the process produces superoxide and $H_2O_2$ [17, 38, 39]. These oxidants may evoke endogenous scavengers and contribute to oxidation of urate by spawning several reactive oxidants. Secondly, our data show that oxypurinol is a good substrate for the redox intermediates of MPO and gives rise to a radical that is capable of oxidizing urate. Consequently, when oxypurinol is metabolized by MPO, related peroxidases or one-electron oxidants, urate will be oxidized and there will be an increase in the production of allantoin.

Increased MPO and allantoin in patients with acute gout point to a potential mechanism for the increase in CVD observed in patients with gout and asymptomatic hyperuricaemia [40]. MPO is an emerging player in the promotion and progression of CVD [41, 42]. Once released from neutrophils, it sticks to the vascular endothelium, where it generates HOCl that damages endothelial cells. It also oxidizes low-density lipoprotein (LDL) to a form readily taken up by macrophages, as well as impairing the reverse cholesterol transport function of high-density lipoprotein (HDL) by selectively nitrating and chlorinating tyrosine residues [43]. In addition to these oxidative reactions, MPO also scavenges nitric oxide by producing free radicals, including the urate radical [7, 44]. Depleting nitric oxide, a potent vasodilator, may lead to hypertension and endothelial cell dysfunction [44]. Enhanced oxidation of urate in patients with gout will also result in formation of cytotoxic urate hydroperoxide and 5-hydroxyisourate [7]. These reactions of MPO-derived oxidants and metabolites of urate link MPO and urate to CVD. In support of this proposal, MPO is also elevated in RA [45] and promotes increased oxidation of HDL, especially in those patients...
with CVD [45]. Further long-term clinical studies are required to determine the association between MPO and CVD in patients with gout.

Successful long-term management of gout requires a sustained reduction of plasma urate to <360 μM and potentially <300 μM if tophi are present. Achieving this target is associated with improved clinical outcomes [46–48]. It is plausible that achieving the target will also have a beneficial effect on cardiovascular outcomes in patients with gout by lowering inflammation and associated oxidative stress. However, the target concentration of urate is more likely to be obtained with a plasma oxypurinol concentration between 100 and 150 μM [21]. These concentrations of oxypurinol tilt the antioxidant/oxidant balance to a pro-oxidant position by increasing the production of reactive oxygen species, as observed by the elevation in plasma allantoin. Whether this effect is clinically relevant will require long-term clinical trials to ascertain, but it raises the issue of how long patients need to maintain the target plasma urate concentration once attacks of gout and tophi have abated. A two-phase approach to treatment has been suggested whereby the lower urate target must be achieved in the first phase but once attacks of gout and tophi have resolved a higher target may be acceptable [49]. This may have benefits if lower doses of allopurinol, and hence lower plasma oxypurinol concentrations, could be used in the second phase to minimize oxidative stress.

In conclusion, we have found that elevated MPO and urate oxidation are associated with gout. In future work valuable mechanistic insights into the link between hyperuricaemia and inflammatory diseases may become apparent when probing the involvement of MPO-dependent oxidative stress, especially that related to oxidation products of urate.

**Rheumatology key messages**

- Circulating MPO is elevated during acute gout.
- High urate in gout is associated with increased oxidative stress.

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