RHEUMATOLOGY

Rheumatology 2012;51:1796–1803
doi:10.1093/rheumatology/kes193
Advance Access publication 19 July 2012

Original article

Myeloperoxidase and oxidative stress in rheumatoid arthritis

Lisa K. Stamp[1], Irada Khalilova[2], Joanna M. Tarr[3], Revathy Senthilmohan[2], Rufus Turner[2], Richard C. Haigh[3,4], Paul G. Winyard[3] and Anthony J. Kettle[2]

Abstract

Objective. To determine whether MPO contributes to oxidative stress and disease activity in RA and whether it produces hypochlorous acid in SF.

Methods. Plasma and where possible SF were collected from 77 RA patients while 120 healthy controls supplied plasma only. MPO and protein carbonyls were measured by ELISAs. 3-Chlorotyrosine in proteins and allantoin in plasma were measured by mass spectrometry.

Results. Plasma MPO concentrations were significantly higher in patients with RA compared with healthy controls [10.8 ng/ml, inter-quartile range (IQR): 7.2–14.2; $P < 0.05$], but there was no significant difference in plasma MPO protein concentrations between RA patients with high disease activity (HDA; DAS-28 >3.2) and those with low disease activity (LDA; DAS-28 ⩽3.2) (HDA 27.9 ng/ml, 20.2–34.1 *vs* LDA 22.1 ng/ml, 16.9–34.9; $P > 0.05$). There was a significant relationship between plasma MPO and DAS-28 ($r = 0.35$; $P = 0.005$). Plasma protein carbonyls and allantoin were significantly higher in patients with RA compared with the healthy controls. MPO protein was significantly higher in SF compared with plasma (median 624.0 ng/ml, IQR 258.4–2433.0 *vs* 30.2 ng/ml, IQR 25.1–50.9; $P < 0.0001$). The MPO present in SF was mostly active. 3-Chlorotyrosine, a specific biomarker of hypochlorous acid, was present in proteins from SF and related to the concentration of MPO ($r = 0.69$; $P = 0.001$). Protein carbonyls in SF were associated with MPO protein concentration ($r = 0.40$; $P = 0.019$) and 3-chlorotyrosine ($r = 0.66$; $P = 0.003$).

Conclusion. MPO is elevated in patients with RA and promotes oxidative stress through the production of hypochlorous acid.

Key words: RA, neutrophils, MPO, reactive oxygen species.

BASIC SCIENCE

Introduction

RA is a chronic inflammatory disease characterized by synovial and systemic inflammation. The inflamed synovium is infiltrated by neutrophils, macrophages, T cells and B cells, which release a variety of pro-inflammatory mediators [1]. Persistent inflammation results in destruction of cartilage and bone. This occurs through a number of mechanisms, including oxidative and proteolytic breakdown of collagen and proteoglycans [2–5]. Once sequestered within the joint space, neutrophils degranulate and release a variety of potentially harmful enzymes and peptides [1]. They may also undergo a respiratory burst and generate several reactive oxygen species, including superoxide, hydrogen peroxide, hypohalous acids, and possibly hydroxyl radical [2, 6]. Although these destructive oxidants have often been held partly responsible for joint destruction, compelling evidence that they are in fact produced within the synovium is lacking.

The haem enzyme MPO plays a central role in oxidant production by neutrophils. It uses superoxide and hydrogen peroxide to catalyse the generation of anti-bacterial hypochlorous acid and free radicals. In plasma it oxidizes urate to a radical that scavenges both nitric oxide and superoxide [7, 8]. In the latter reaction a hydroperoxide

[1]Department of Medicine, [2]Department of Pathology, University of Otago Christchurch, Christchurch, New Zealand, [3]Peninsula Medical School, University of Exeter and [4]Royal Devon and Exeter Foundation Trust, Exeter, UK.

Submitted 29 February 2012; revised version accepted 7 June 2012.

Correspondence to: Lisa K. Stamp, Department of Medicine, University of Otago, Christchurch, PO Box 4345, Christchurch 8140, New Zealand. E-mail: lisa.stamp@cdhb.govt.nz

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is formed that ultimately breaks down to allantoin [7, 8]. Hypochlorous acid is the major strong oxidant generated by neutrophils when they kill bacteria [9] and it is also produced at sites of inflammation [10]. It reacts predominantly with methionine and cysteine residues in proteins to disrupt their tertiary structure as well promote intra- and inter-molecular cross-links and inactivate enzymes [11]. Hypochlorous acid also targets lysine residues to form chloramines that break down to protein carbonyls and in the process may release toxic ammonia chloramine (NH_2Cl) [12]. Minor reactions of hypochlorous acid and chloramines result in the chlorination of tyrosine residues to form 3-chlorotyrosine [13]. This halogenated amino acid is a specific biomarker of hypochlorous acid, whereas protein carbonyls are non-specific biomarkers of oxidative stress [14].

RA is a heterogeneous disease, in which MPO may play a role in the pathogenesis, severity and/or outcomes. Indeed, MPO is present at high concentrations in SF of patients with RA. Furthermore, chlorinated IgG has been shown to be proinflammatory [15] and the immunogenic and arthritogenic properties of type II collagen are heightened by chlorination of this protein [16]. MPO could possibly produce hypochlorous acid in the inflamed RA joint, but this has yet to be unequivocally demonstrated. A valid argument against this proposal is the reported finding that most of the enzyme was inactive in SF [17]. This finding suggests that MPO has a limited capacity to produce oxidants in RA.

Importantly with respect to outcomes in RA, MPO is a marker of cardiovascular risk [18]. In apparently healthy individuals, plasma MPO concentrations predict the presence of coronary artery disease as well as future risk of coronary artery disease. Cardiovascular disease (CVD) is recognised as an important cause of death in patients with RA [19]. Women with RA have an increased risk of myocardial infarction (MI) compared with women without RA, even after adjustment for other cardiovascular risk factors (RR 2.0; 95% CI 1.23, 3.29). Traditional cardiovascular risk factors, including body mass index, diabetes, hypertension, hypercholesterolaemia physical activity and family history of early MI, are similar between women with and without RA [20]. Thus RA is associated with an increased incidence of cardiovascular disease, which is not explained by classical cardiovascular risk factors [21]. The link between inflammation and cardiovascular disease is further supported by evidence that disease-modifying anti-rheumatic agents such as MTX, which suppress the inflammatory process in RA, are associated with lower cardiovascular mortality rates in patients with RA [22, 23].

Given the potential of MPO to contribute to both the pathology of RA and the associated complications of CVD, our aims in this study were to determine whether it is active and promotes oxidative stress in SF through the production of hypochlorous acid. In addition, we wished to determine the relationship between inflammatory disease activity of patients with RA and the levels of MPO in plasma and SF.

Methods

Patients

Patients with RA, but without known cardiovascular disease, were recruited from rheumatology departments in Christchurch Hospital, New Zealand and The Royal Devon and Exeter Hospital Trust, UK. All patients fulfilled the American Rheumatism Association 1987 classification criteria for RA [24]. Healthy controls were randomly selected from the Christchurch (NZ) electoral roll. Ethical approval was obtained from the local ethics committees in New Zealand and the UK (Upper South B Regional Ethics Committee, Christchurch, New Zealand; North and East Devon Research Ethics Committee, UK). All patients gave written informed consent.

Standard demographic details were collected including disease duration, RF and CCP status. Disease activity was assessed using a variety of variables including ESR and CRP, swollen joint count (SJC), tender joint count (TJC) and DAS-28. Peripheral blood and where possible SF were collected and processed as described previously [25]. Patients with DAS-28 >3.2 or with joint effusion requiring aspiration were defined as having high disease activity (HDA) and those with DAS-28 $\leqslant 3.2$ low disease activity (LDA).

MPO and protein oxidation assays

The concentration of MPO in plasma and SF was determined by adapting an ELISA [26]. The MPO was initially captured with a monoclonal MPO antibody, and its activity was determined using hydrogen peroxide and Amplex Red. After washing the plate to remove peroxidase reagents and products, the amount of MPO that was bound was determined using a polyclonal MPO antibody. MPO activity and MPO protein were compared with standards of known concentrations, which were determined by measuring the absorbance of MPO at 430 nm (ε 89 000 M/cm/haem) [27].

3-Chlorotyrosine in proteins was determined by hydrolysing proteins in SF with methanesulphonic acid and measuring the liberated amino acid along with tyrosine by using gas chromatography with stable isotope dilution mass spectrometry [10]. Allantoin in plasma was measured by using hydrophilic interaction chromatography followed by stable isotope dilution mass spectrometry as described elsewhere [8, 28].

Protein carbonyls were determined by ELISA supplied by Zentech, Dunedin, New Zealand. Proteins in supernatants were reacted with 2,4-dinitrophenylhydrazine, then absorbed onto wells in an ELISA plate and probed with an antibody raised against protein conjugated 2,4-dinitrophenylhydrazine. The biotin-conjugated primary antibody was then reacted with streptavidin-biotinylated horseradish peroxidase for quantification [29].

Statistical analysis

Data were expressed as the median and inter-quartile range (IQR). The statistical analysis of differences between group medians was carried out using the

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Mann–Whitney rank sum test. Correlations were tested using the Spearman rank order correlation method.

Results

Patient demographics

Seventy-seven patients were recruited, of which 49 (63.6%) had HDA disease (DAS-28 >3.2). Patient demographics for the whole group and those with HDA and LDA disease are outlined in Table 1. Of the 49 patients with HDA disease, 26 (53.1%) had paired plasma and SF samples.

MPO is increased in patients with RA compared with healthy controls

Plasma MPO protein was significantly higher in patients with RA compared with healthy controls (10.8 ng/ml, IQR 7.2–14.2; $P < 0.05$) (Fig. 1). However, there was no significant difference in plasma MPO protein concentrations between RA patients with HDA or LDA [HDA 27.9 ng/ml (20.2–34.1 ng/ml) *vs* LDA 22.1 ng/ml (16.9–34.9 ng/ml); $P > 0.05$] (Fig. 1A). The median specific activity of MPO (i.e. the ratio of MPO activity to MPO protein) was 61% (IQR 29–80%; $n = 28$) in healthy controls, 45% (IQR 26–57%; $n = 26$) in patients with LDA RA and 39% (IQR 25–65%; $n = 29$) in RA patients with HDA (Fig. 1B). There was no significant difference between these groups ($P = 0.14$), but the values indicated that the specific activity of detectable MPO in plasma is highly variable. Values of MPO protein <10 ng/ml were excluded from this calculation to avoid erroneously over-estimating the specific activity.

Relationship between disease activity and MPO plasma concentration

There were statistically significant correlations between plasma MPO protein concentrations and SJC ($r = 0.27$; $P = 0.023$), TJC ($r = 0.38$; $P < 0.001$) and CRP ($r = 0.31$; $P = 0.009$) but not ESR ($r = 0.23$; $P = 0.06$). There was also a statistically significant correlation between DAS-28 and plasma MPO protein ($r = 0.35$; $P = 0.005$) (Fig. 2).

MPO is significantly higher in SF compared with plasma of patients with RA

MPO protein was also detected in SF from patients with RA (Fig. 3A). It was ~20-fold higher in SF [median 624.0 ng/ml (IQR 258.4–2433.0)] than in plasma [30.2 ng/ml (IQR 25.1–50.9)]. Using paired SF and plasma samples ($n = 26$), the difference was found to be highly significant ($P < 0.0001$). In general, the MPO protein present in SF retained most of its activity (Fig. 3B). The levels of MPO in SF did not correlate with disease activity.

Evidence for oxidative stress in RA

Protein carbonyls were measured as a non-specific marker of oxidative stress and were present in both plasma and SF. Plasma protein carbonyls were measured in 120 healthy controls [median age 67.5 years (IQR 57.2–76) and 72.5% male] and compared with those in patients with RA. They were significantly higher in patients with RA than in healthy controls (13.0 pmol/mg protein,

Table 1 Demographics of patients with active RA and inactive RA

	All RA patients ($n = 77$)	HDA RA (DAS-28 >3.2) ($n = 49$)	LDA RA (DAS-28 ⩽3.2) ($n = 28$)
Age, years	54.7 (22–82)	54.5 (23–82)	54.9 (23–74)
Female, %	71.4	73.5	67.9
TJC28	3.9 (0–27)	5.8 (0–27)	0.5 (0–2)
SJC28	4.0 (0–24)	6.1 (0–24)	0.36 (0–4)
DAS-28 (ESR)	3.81 (0.07–7.66)	4.9 (2.8–7.66)	1.84 (0.07–3.03)
ESR, mm/h	29.6 (1–99)	39.1 (2–99)	9.8 (1–32)
CRP, mg/l	26.2 (0–206)	38.9 (0–206)	5 (0–22)
RF positive, *n* (%)	47/71 (66.2)	25/43 (58.1)	22/28 (78.6)
CCP positive, *n* (%)	37/45 (82.2)	17/22 (77.3)	20/23 (86.9)
Erosive disease, *n* (%)	46/76 (60.5)	29/28 (60.4)	17/28 (60.7)
MTX, *n* (%)	41 (53.2)	22 (44.9)	19 (67.9)
Salazopyrin, *n* (%)	15 (19.5)	8 (16.3)	7 (25)
HCQ, *n* (%)	14 (18.2)	9 (18.4)	5 (17.9)
Adalimumab, *n* (%)	8 (10.4)	6 (12.2)	2 (7.1)
LEF, *n* (%)	14 (18.2)	8 (16.3)	6 (21.4)
Prednisone, *n* (%)	20 (26)	15 (30.6)	5 (17.9)
AZA, *n* (%)	13 (16.9)	12 (24.5)	1 (3.6)
Rituximab, *n* (%)	1 (1.3)	1 (2.0)	0
IM Gold, *n* (%)	1 (1.3)	0	1 (3.6)
NSAID, *n* (%)	32 (41.6)	24 (49)	8 (28.6)

Data are expressed as mean (range) unless otherwise stated.

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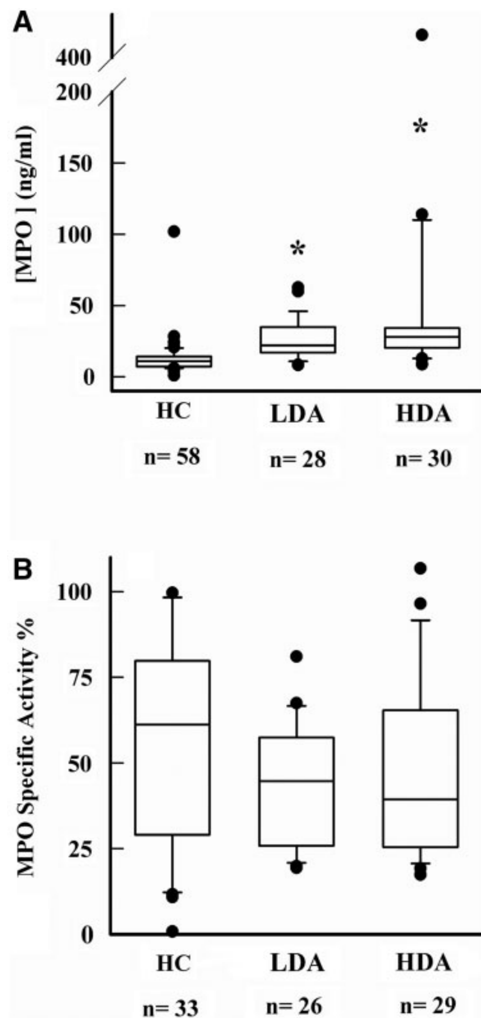
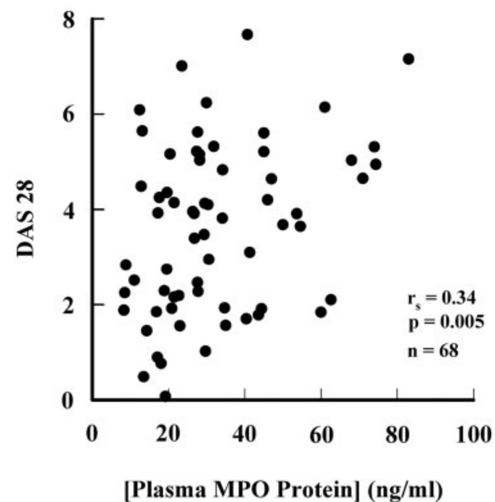
Fig. 1 MPO protein and specific activity in plasma.



(**A**) Plasma MPO concentration in RA patients with high or low disease activity compared with healthy controls (HC). *$P < 0.05$ cf. control MPO protein. (**B**) Specific activity of MPO in plasma samples.

Fig. 2 Relationship between DAS-28 and MPO in patients with RA.



IQR 0–50; $P < 0.05$) (Fig. 4A). However, there was no significant difference between RA patients with HDA and those with LDA (51.0 pmol/mg protein; IQR 25.1–70.1 *vs* 37.7 pmol/mg protein, 21.1–60.2; $P > 0.05$) (Fig. 4A). Plasma protein carbonyls were not correlated with plasma MPO concentrations or disease activity.

Allantoin, a biomarker of urate oxidation [7, 8], was elevated in plasma from patients compared with controls (2.0 μM, IQR 1.4–3.6; $P < 0.001$). There was no significant difference between concentrations in patients with HDA (3.5 μM, IQR 3.0–5.6 μM) and LDA (3.7 μM, IQR 2.9–5.3 μM). Allantoin concentrations were not correlated with MPO or protein carbonyls in plasma.

Protein carbonyls were significantly higher in SF of patients with RA (47.0 pmol/mg protein, IQR 36–59.5; $P < 0.05$) than in plasma from healthy controls but were not elevated compared with those in plasma from patients with LDA or HDA (Fig. 4A). In SF, protein carbonyls were associated with MPO concentration (Fig. 4B) ($r = 0.40$, $P = 0.019$, $n = 34$). They were not associated with markers of disease activity.

Evidence of hypochlorous acid production and oxidative stress in RA

3-Chlorotyrosine, the specific biomarker of hypochlorous acid, was detected in proteins from SF using stable isotope dilution mass spectrometry (40.5 μmol/mol tyrosine, IQR 37.0–58.0, $n = 18$). In accord with a previous study [10], detection of 3-chlorotyrosine in proteins was validated by the findings that it co-eluted with an authentic standard, had the correct molecular mass and fragmentation pattern, and its stable isotopes were present in the expected 3 : 1 ratio for a chlorine-containing molecule. Levels of 3-chlorotyrosine were related to the concentration of MPO ($r = 0.69$, $P = 0.001$, $n = 18$) (Fig. 5A). Also, they were strongly correlated with levels of protein carbonyls ($r = 0.66$, $P = 0.003$, $n = 18$) (Fig. 5B). This chlorinated amino acid could not be detected in plasma proteins.

Discussion

Reactive oxygen species are often implicated in the pathology of RA [30, 31], yet until now there has been a paucity of solid data to establish which oxidants are actually produced in the inflamed joint. Detection of 3-chlorotyrosine confirms that hypochlorous acid is produced in SF and reacts with proteins. There is no other known biological reaction that produces 3-chlorotyrosine, and MPO is the only human enzyme capable of generating hypochlorous acid [14]. Hence, the strong correlation between 3-chlorotyrosine and the levels of MPO indicates that this enzyme catalyses the production of hypochlorous acid in SF. Furthermore, the association of protein

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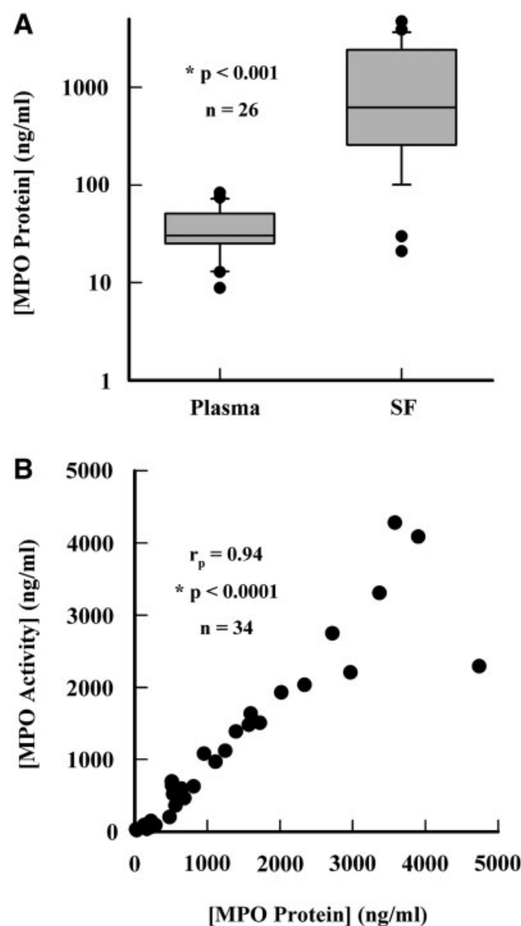
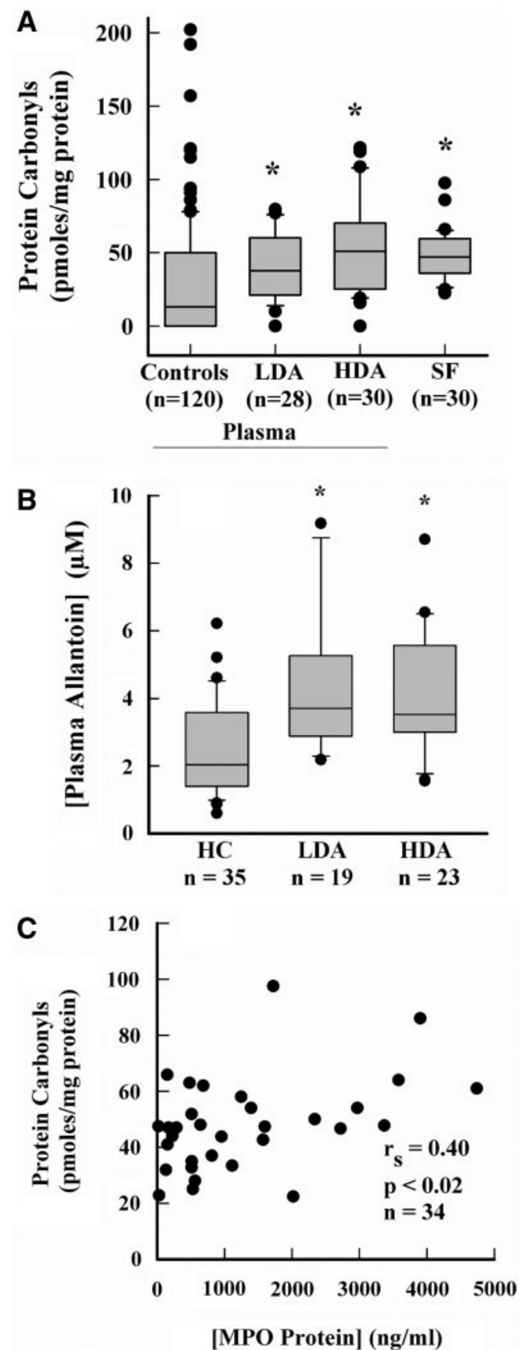
FIG. 3 SF and plasma MPO concentrations from patients with RA.



(**A**) MPO protein was measured in paired SF and plasma samples from 26 patients, revealing higher MPO protein concentrations in SF compared with plasma. (**B**) Relationship between MPO protein and activity in SF from patients with RA.

FIG. 4 Biomarkers of oxidative stress in RA.



(**A**) Plasma protein carbonyls in healthy controls and RA patients with high and low disease activity. Also shown are protein carbonyls in SF from patients with RA. (**B**) Plasma allantoin concentrations in healthy controls and RA patients with HDA and LDA. (**C**) Correlation of protein carbonyls with MPO in SF of patients with RA. *$P < 0.05$ compared with healthy controls.

carbonyls with both MPO and 3-chlorotyrosine suggests that hypochlorous acid is a major driver of the oxidative damage that occurs to proteins in the inflamed joint. This finding has important implications for RA because hypochlorous acid is the strongest two-electron oxidant produced *in vivo*. It is much more reactive than either hydrogen peroxide or peroxynitrite [32]. It readily oxidizes cysteine and methionine residues as well as cross-linking and fragmenting proteins [33], inactivating α_1-antiprotease inhibitor [11] and adversely affecting the functions of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [34]. Given its extreme and diverse reactivity, it is likely that hypochlorous acid contributes to the tissue

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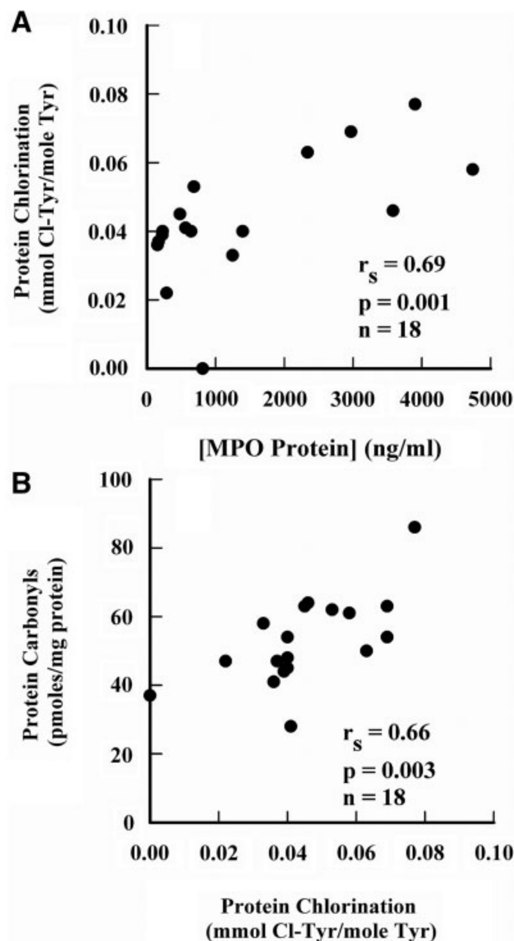
Fig. 5 Protein chlorination in SF.



The association of protein chlorination with (**A**) MPO and (**B**) protein carbonyls in SF from patients with RA.

damage that occurs in patients with RA. Thus, targeting specific inhibitors, such as 2-thioxanthines, against MPO would be expected to lower oxidative stress within the inflamed joint [35].

In contrast to a previous report [17], we found that the MPO present in most SF samples had retained a large proportion of its activity and therefore was capable of producing hypochlorous acid. In plasma, however, MPO activity was highly variable and on average was ∼50% active. MPO may have been inactivated by the oxidants it generates or inhibited by plasma proteins such as ceruloplasmin [36]. The reason for the low MPO activity in plasma requires further investigation. This result suggests that MPO has a limited capacity to generate oxidants as it will eventually lose much of its activity. Inactivation of MPO may not have been apparent in SF because it was present at enormous concentrations compared with plasma and was likely to be continually replenished by activated neutrophils present within the inflamed joint.

In agreement with previous studies of patients with inflammatory joint disease [37–39], we found that patients with RA have increased plasma MPO concentrations. We also showed that plasma MPO concentrations are significantly correlated with disease activity. This is in contrast to a recent study that found no correlation between plasma MPO concentrations and DAS-28 score [39]. This may reflect a greater range of DAS-28-ESR scores in our study compared with the earlier work. In this work, the better correlation of disease activity with plasma MPO rather than with MPO in SF may reflect the systemic inflammatory state of patients with RA [40]. Alternatively, this finding may have arisen because plasma concentrations are more likely to reflect cumulative release of the enzyme from neutrophils and its accumulation due to its adherence to the endothelium [41]. In contrast, the MPO measured in SF is most likely representative of that recently released from activated cells.

Protein carbonyls were present in plasma at similar levels to those in SF yet there was no detectable 3-chlorotyrosine in plasma. Others have suggested that because 3-chlorotyrosine is susceptible to oxidation by peroxynitrite and hypochlorous acid, its levels may be underestimated at sites of chronic inflammation [42]. Previously, however, it was demonstrated that 3-chlorotyrosine should be present in plasma proteins if hypochlorous acid was solely responsible for producing protein carbonyls at the levels we detected [26]. Consequently, we must conclude that hypochlorous acid was unlikely to be responsible for producing protein carbonyls in plasma proteins. This does not rule out the involvement of MPO in promoting oxidative stress via production of other oxidants such as hypothiocyanous acid, free radicals and urate hydroperoxide [7, 8]. Indeed, allantoin, the oxidation product of urate, was elevated in the plasma of patients with RA, which confirms that these patients had increased levels of oxidative stress. It is not possible to determine whether allantoin was derived from the direct oxidation of urate by MPO [7, 8] or via other oxidants. The lack of correlation between MPO and allantoin should not be used to exclude this enzyme because the turnover of soluble allantoin is expected to be very different from that of MPO, which is mostly adhered to the endothelium [41].

MPO and hypochlorous acid may also contribute to the complications of RA. RA is associated with an increase in mortality, particularly in those with more severe disease and extra-articular manifestations [43]. This increase in mortality is largely due to an increase in cardiovascular deaths, with a recent meta-analysis indicating that cardiovascular mortality is increased by ∼50% in RA patients compared with the general population [44]. This increase in cardiovascular disease and mortality does not appear to be due to an increase in traditional cardiovascular risk factors such as family history, smoking, hypertension and hyperlipidaemia. The quest for biomarkers that can predict disease and/or treatment outcomes is a key research focus in RA. However, to date no reliable soluble biomarkers have been identified.

MPO has been identified as another marker of CVD risk. Within the vascular system, MPO may contribute to the pathogenesis of CVD as well as to acute cardiovascular

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events. MPO converts LDL into a form which promotes foam cell formation within atherosclerotic plaques. It may also promote formation of a dysfunctional form of HDL, which has a reduced ability to promote cellular efflux of cholesterol [34]. The involvement of MPO in the pathogenesis of CVD is supported by evidence that elevated MPO concentrations are an independent risk factor for future cardiovascular events in healthy individuals [45]. MPO and metalloproteinases contribute to atherosclerotic plaque rupture by degrading the collagen layer of the atheroma [46].

Our findings that MPO is elevated in RA and contributes to oxidative stress in these patients provide a potential mechanism for the increase in CVD observed in patients with RA. Interestingly, treatment with infliximab has been shown to reduce disease activity and reduce plasma MPO concentrations [38]. Whether MPO is an independent predictor of CVD in patients with RA has not been formally examined. Large prospective clinical trials will be required to determine whether MPO is a reliable biomarker for the prediction of CVD in RA, where MPO may be increased by the systemic inflammatory process, and to determine whether suppression of inflammation by DMARDs is associated with reduction of MPO and CVD risk.

Rheumatology key messages

- MPO is associated with oxidative stress in RA.
- Hypochlorous acid is produced in the inflamed joints of patients with RA.

Acknowledgements

L.K.S. co-designed the study and co-wrote the manuscript. I.K. did the immunoassays and co-ordinated other assays. J.T. co-performed parts of the research, revised the manuscript and approved the final version. R.S. measured 3-chlorotyrosine in proteins. R.C.H. selected patients according to ARA criteria, organised sample collection, revised the manuscript and approved the final version. P.G.W. co-designed the research and co-wrote the manuscript. A.J.K. co-designed the research and co-wrote the manuscript. J.M.T. was supported by a grant from the Peninsula Medical School, Exeter, to P.G.W.

Funding: This study was funded in part by the Health Research Council of New Zealand.

Disclosure statement: A.J.K. has received financial support from AstraZeneca to investigate mechanisms of inhibition of myeloperoxidase. All other authors have declared no conflicts of interest.

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