

Extracellular myeloperoxidase and markers of inflammation in the sepsis syndromes

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Word count: 1,218 (excluding acknowledgements & references)

Funding: The British Heart Foundation

Disclosures: None

INTRODUCTION

The systemic inflammatory response syndrome (SIRS) and its sequelae represent a formidable problem, together constituting the leading cause of morbidity and mortality in critically ill patients [1-3]. SIRS, which is clinically indistinguishable from sepsis, is seen in association with a variety of non-infective insults [4], including surgery necessitating cardiopulmonary-bypass (CPB) [5]. The incidence is insult dependent, with 15-27% [6, 7] of critically ill patients suffering from severe sepsis, and up to 96% of ICU admissions [2, 5] affected by SIRS.

Neutrophils are activated early in the host immune response, and are central to the pathogenesis of sepsis/SIRS [8]. The neutrophil capacity for bacterial killing lacks selectivity, despite stringent regulation; and thereby carries the potential to inflict collateral damage to and destruction of host tissue [9], through generation of pro-oxidant mediators [10].

Myeloperoxidase (MPO) generated hypochlorous acid (HOCl) is the most bactericidal oxidant produced by the neutrophil. HOCl and an array of oxidant molecules generated by MPO/HOCl [10, 11] are implicated in altered cell signalling, growth arrest and tissue damage [12, 13]. Caeruloplasmin (CP) has been shown to have an affinity for MPO; when bound it compromises the enzymatic activity of MPO [14, 15]. CP therefore plays a potentially key role in redox regulation, modulating pro-inflammatory responses related to HOCl under certain defined circumstances [16-19].

Hypothesis

We hypothesised that excess release of extracellular (plasma) MPO, produces a net increase in HOCl production, resulting in redox imbalance in favour of pro-oxidant forces. A sub-optimal anti-oxidant response, specifically CP, places patients at greater risks of developing organ dysfunction. We investigated the following:

Aims

First, using in vitro experiments we explored the relationship between MPO protein and activity, MPO-CP binding, and the effect of the latter on MPO activity. Second, two populations with SIRS (sepsis and post-CPB) were recruited in order to elucidate the relationship between MPO exposure, MPO activity, and CP; to place these within the framework of associated inflammatory mediators; and to demonstrate their relationship to SIRS-induced organ dysfunction.

METHODS

Research ethical approval: Royal Brompton Hospital Research Ethics Committee.

Populations: Severe sepsis, n=44; post-CPB, n=52; healthy controls, n=21; non-cardiac surgical controls, n=8.

Morbidity: Defined within the sequential organ failure assessment score (SOFA) [20].

Biochemical techniques: Spectrophotometry (enzyme kinetics, spectral absorbance); immunoprecipitation; ELISA; radial-immunodiffusion; gel-chromatography; Western blotting; non-denaturing electrophoresis; gas-chromatography–mass-spectroscopy.

RESULTS

Methodology: an assay was developed to determine MPO activity using the chromogen tetramethylbenzidine, with validation by detection of chromogen oxidation product by spectral absorbance, and the absence of activity following immunoprecipitation of MPO from a purified protein solution. Heme-proteins in the plasma peroxidase-pool [21] contribute to measured activity. Plasma from healthy controls and patients with SIRS was therefore subjected to immunoprecipitation of MPO. The MPO fraction, 35% of the measured activity, correlated with total activity (*Figure 1a*). There was no relationship between plasma MPO protein and activity.

MPO activity: was inhibited in protein solution by CP in a dose dependent manner (*Figure 1b & c*); this was not reproducible in plasma or blood. MPO–CP binding affinity was confirmed in a purified protein system by (a) gel-chromatography; (b) immunoprecipitation of MPO/CP by anti-CP/MPO antibody respectively, followed by immuno-detection (ELISA, Western blot); (c) and non-denaturing electrophoresis. Detection of the MPO–CP complex in plasma was inconclusive.

MPO exposure: patients with severe sepsis and those post-CPB were divided into groups based on cumulative MPO exposure over the 72 hour study period. (1) In severe sepsis greater MPO exposure was associated with an increase and/or non-resolution in: leukocyte count, CRP, and IL-6. There was no difference in MPO activity, CP, IL-8, TNF, and IL-10. MPO exposure was associated with non-resolution of organ dysfunction and increased 3-chlorotyrosine (a marker of HOCl-induced oxidative damage [22]) *Figure 2a & b*. By contrast, a relative increase in CP was associated with lower MPO exposure and a decrease in organ dysfunction (1.4 SOFA points). MPO activity was decreased compared to controls, and demonstrated no relationship to MPO or CP protein. (2) Post-CPB, greater MPO exposure was associated with an increase and/or non-resolution

in: leukocyte count, CRP, and IL-6, IL-8, and IL-10; only the former differed between groups. MPO activity and CP decreased significantly, with no difference between groups, nor discernable relationship between indices. There was no difference in organ dysfunction or 3-chlorotyrosine.

DISCUSSION

In the validated plasma MPO activity assay, the activity due to MPO and the total measured activity correlated well. The proportion of measured activity attributable to MPO was similar in health and SIRS, despite differing plasma concentrations of MPO protein. This may reflect a functional capacity of MPO, supporting a role during normal physiological states [23-29]. CP inhibited MPO activity in solution but not plasma; possibly due to the presence of endogenous CP, dysfunctional exogenous CP [30], or plasma factors. CP binding affinity to MPO was confirmed in protein solution, with MPO–CP complex visualisation for the first time.

In the SIRS populations studied, MPO was associated with an increase of inflammatory indices. Greater plasma MPO appears to be detrimental in terms of non-resolution of organ dysfunction and CRP in severe sepsis, but exerts no impact on inflammatory indices or morbidity post-CPB. MPO activity was decreased in both populations, despite leukocyte response and elevated MPO protein. Post-CPB the activity did not return to pre-operative levels, a phenomenon not previously documented.

CP appears to neither to modulate MPO activity in SIRS post-CPB, nor in severe sepsis. Thus, despite *in vitro* data confirming the ability of CP to inhibit MPO activity, no such evidence could be demonstrated in patient plasma. The data presented here did not support a role for the modulation of MPO by CP within 72 hours of onset of the syndromes studied. A rise in CP was however associated with a decrease in MPO protein in sepsis, perhaps, in conjunction with its binding affinity, conferring a regulatory role of the former in the elimination of extracellular MPO protein.

The reduced enzymatic capacity of MPO during SIRS may be due to diminished functional capacity of MPO (e.g. molecular damage) [31-34]; reduction of substrate or co-factor availability [35]; or inhibition [14, 15, 33, 36-38]. MPO activity may be subject to regulation. However, the evidence of chlorination indicates that this population may also be either more susceptible to, or subject to, elevated HOCl-induced oxidative damage [39]. This paradox possibly relates to substrate availability [40], a mechanism that would support the increased production of HOCl.

The data highlight further evidence for a regulatory role of MPO activity. MPO activity levels on resolution of organ dysfunction and systemic inflammation are similar to, and indeed return to those levels seen pre-operatively and in health. The ‘*excess*’ MPO detectable in plasma is enzymatically dysfunctional, retaining an intact epitope detectable immunologically. Increased release of MPO into plasma may be physiological, compensating for the limited enzymatic lifespan and associated molecular damage sustained. The mechanism of MPO clearance remains undetermined. Dysfunctional MPO protein may thus remain in plasma for a prolonged time period until cleared.

CONCLUSION

The relationship between plasma MPO and its measured activity remains to be further elucidated. What seems clear from the data presented here is (i) plasma MPO protein is not representative of activity in severe sepsis or post-CPB; and (ii) that maintenance of a constant activity, or return to a ‘*normal*’ activity following resolution of disease process seems to be desirable.

ACKNOWLEDGEMENTS

This research was generously funded by the British Heart Foundation through Clinical Research Fellowship. NSM conducted all experiments over a three year period with PhD supervision by Dr. G.J. Quinlan and Professor T.W. Evans. The Royal Marsden Hospital and Dr. M.B. Hacking for access to surgical control patients. The staff from the Unit of Critical Care research group and the Royal Brompton Hospital.

FIGURES

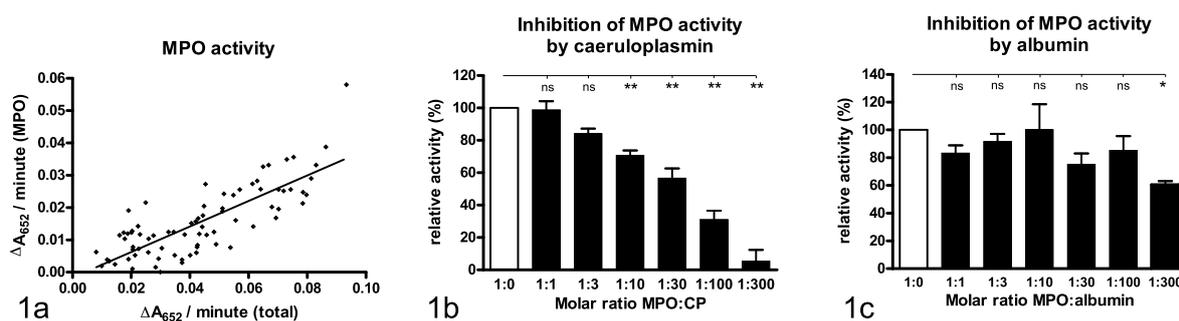


Figure 1

A Myeloperoxidase activity of plasma supernatant post immunoprecipitation: total versus fraction due to MPO

MPO activity, measured as $\Delta A_{652} \text{min}^{-1}$, total versus fraction due to MPO; $n=80$. MPO fraction = total $\Delta A_{652} \text{min}^{-1}$ – residual $\Delta A_{652} \text{min}^{-1}$ post IP). There was a significant correlation between total activity and activity due to MPO, correlation coefficient 0.80, r^2 0.64; $p < 0.0001$ (Pearson correlation test).

B Myeloperoxidase activity: inhibition by caeruloplasmin

Mean \pm SEM of relative MPO activity. MPO $0.2 \mu\text{g}$ incubated alone and with CP in molar ratios of 1:1 to 1:300 for 30 minutes at room temperature ($n=5$). MPO activity determined using 3, 5, 3', 5'-tetramethylbenzidine substrate, expressed as activity relative to MPO $0.2 \mu\text{g}$ incubated without CP. There was a significant decrease in activity with increasing ratio of CP; $p < 0.0001$ (one-way ANOVA, Dunnett's post test).

C Myeloperoxidase activity: inhibition by albumin

Mean \pm SEM of relative MPO activity. MPO $0.5 \mu\text{g}$ incubated alone and with albumin in molar ratios of 1:1 to 1:300 for 30 minutes at room temperature ($n=4$). MPO activity determined using 3, 5, 3', 5'-tetramethylbenzidine substrate, expressed as activity relative to MPO $0.5 \mu\text{g}$ incubated without albumin. There was no significant decrease in activity with increasing ratio of albumin; $p=0.0609$ (one-way ANOVA, Dunnett's post test).

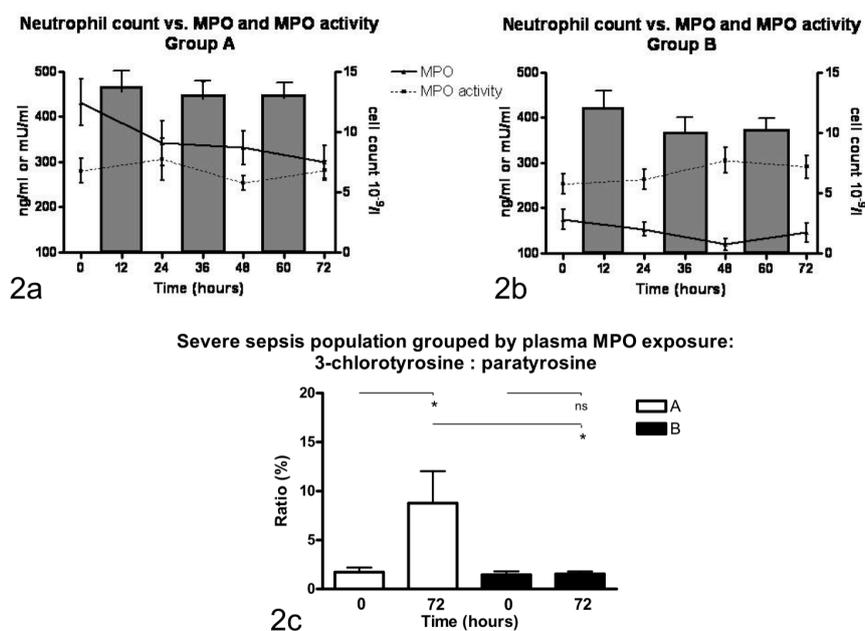


Figure 2

A, B Relationship of neutrophil count to plasma myeloperoxidase protein and activity over time: MPO grouping

Mean \pm SEM of neutrophil count, plasma MPO and plasma MPO activity for group A – higher ($n=20$), group B – lower ($n=20$) plasma MPO AUC during the 72 hour study period. Group A had a greater neutrophil response ($p=0.051$) and MPO concentration ($p<0.0001$) than group B (two-way ANOVA, Bonferroni post test). This achieved similar measured plasma MPO activity between groups.

C Plasma 3-chlorotyrosine: MPO grouping

Mean \pm SEM of plasma 3-chlorotyrosine (expressed as ratio of parent compound paratyrosine) for: group A: time 0 ($n=7$), time 72 ($n=7$); and group B time 0 ($n=6$), time 72 ($n=6$). There was a significant difference between group A time 0 and time 72 ($p=0.030$), and group A time 72 and group B time 72 ($p=0.016$) plasma 3-chlorotyrosine, but not between time point 0 group A versus group B ($p=ns$), or between group B ($p=ns$); Mann-Whitney.

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