Deglycosylation influences the oxidation activity and antigenicity of myeloperoxidase

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KEY WORDS:

SUMMARY AT A GLANCE

This study investigated the influence of glycans on the activity of the myeloperoxidase enzyme (MPO), and the binding capacity of its physiological inhibitor ceruloplasmin and autoimmune antibodies (MPO-ANCA). Deglycosylation of MPO was less antigenic to MPO-ANCA, had less enzymatic activity and did not bind to ceruloplasmin. These data demonstrate the importance of MPO glycosylation for its function and role in autoimmune vasculitis.

ABSTRACT:

Aim: Myeloperoxidase (MPO) is pathogenic in ANCA associated vasculitis. It also acts as bactericidal agent. MPO has five N-linked glycosylation sites on its heavy chains. The effect of glycosylation pattern to the functions of MPO is barely known.

Methods: We used eight glycosidases to remove different glycans on MPO separately. The chlorination activity of MPO, the binding between ceruloplasmin and MPO, and the reversing effect of MPO-ANCA to this binding were measured. Three de-glycosylated MPOs were used to assay the influence of deglycosylation on microbicidal effect of MPO.

Results: Compared with intact MPO, chlorination activity of deglycosylated MPO declined, in which removing of β-galactopyranoside (0.35 ± 0.02 vs. 0.50 ± 0.04, P < 0.001) and α-linked sialic acid (0.35 ± 0.02 vs. 0.50 ± 0.04, P < 0.001) presented the most significance. Deglycosylation reduced the binding capacity between MPO and its physiological inhibitor-ceruloplasmin, with the most significance on the removal of innermost GlcNAc (0.37 ± 0.04 vs. 1.06 ± 0.11, P < 0.001). Binding between MPO and ceruloplasmin was hardly reversed by MPO-ANCA after deglycosylation, especially on the removal of α-linked sialic acid (71.2 ± 5.1% vs. 88.3 ± 1.0%, P = 0.009), chitobiose core (73.6 ± 1.9% vs. 88.3 ± 1.0%, P = 0.001) and GlcNAc (77.9 ± 1.9% vs. 88.3 ± 1.0%, P = 0.002). Removal of innermost GlcNAc, β-galactopyranoside and α-neuraminidase could weaken the bactericidal effect of MPO, especially the removal of α-neuraminidase (P < 0.001).

Conclusions: Deglycosylation decreased oxidation activity of MPO and its binding with ceruloplasmin. Deglycosylation could also decrease the microbicidal effect of MPO, which might contribute to more severe infections and inflammation. Deglycosylated MPO presented less antigenicity to MPO-ANCA, which indicated the contribution of glycans to MPO epitopes.

Kidneys are one of the most common target organs of antineutrophil cytoplasmic autoantibodies (ANCA)-associated systemic vasculitis; the condition may sometimes present as ‘kidney-limited’ vasculitis. Proteinase-3 (PR3) and myeloperoxidase (MPO) are the two major target antigens of ANCA. MPO-ANCA was reported to be more predominant in Chinese patients.1 Both MPO-ANCA complex and MPO itself have been shown to be pathogenic.5–7 Furthermore, linear epitopes on MPO were reported in ANCA-negative vasculitis,8 and occult antibodies against these linear epitopes were blocked in serum by a ceruloplasmin fragment, which further proves the pathogenicity of MPO itself and occurrence of special anti-MPO autoantibodies in ANCA-negative vasculitis.

Myeloperoxidase is also one of the major proteins of the antimicrobial system in mammalian neutrophils.2 It catalyzes the production of hypochlorous acid, a potent reactive oxygen species, from hydrogen peroxide (H2O2) and chloride ions (Cl−).7–9 However, the reactive oxygen species produced by the catalytic activity of MPO has been shown to contribute to tissue damage in certain inflammatory diseases such as, rheumatoid arthritis, atherosclerosis and multiple sclerosis.2 Under physiological conditions, the peroxidase activity of MPO is inhibited by ceruloplasmin, which is a multi-functional copper containing...
protein that oxidizes the highly toxic ferrous ions of MPO to ferric state. Increased levels of ceruloplasmin and its high affinity for binding to MPO has been demonstrated in patients with active MPO-ANCA vasculitis. However, the inhibitory effect of ceruloplasmin on MPO can be disturbed in the presence of MPO-ANCA, which may result in the persistent generation of reactive oxygen by MPO and lead to endothelial insult.

Myeloperoxidase is a glycosylated hemo-enzyme, which is most abundantly present in neutrophils. The mature MPO protein consists of two light and two heavy polypeptide chains with each heavy chain binding to prosthetic heme group. The overall structures of MPO glycan and the biological effects of glycosylation patterns on MPO function are not well characterized.

In our previous study involving patients with ANCA-associated vasculitis, the MPO-ANCA positive sera recognized linear peptides in the N-terminal part of MPO heavy chain, especially H1279. We further found that the major epitopes in this peptide were located at H4 and H11. Each of these two regions contain one N-linked glycosylation site in each MPO heavy chain, which are occupied by either complex or high mannose glycan structures. The overall structures of MPO glycan and the biological effects of glycosylation patterns on MPO function are not well characterized.

We hypothesized that the glycosylation of MPO may influence its antigenicity as well as its functions as a peroxidase.

Oxidation and antimicrobial activity are two major functions of MPO under physiological conditions, and ceruloplasmin prevents overactivation of MPO. In ANCA-associated vasculitis, the inhibitory effect of ceruloplasmin on MPO is disturbed because of MPO-ANCA. Therefore, we conducted a study to investigate the influence of glycans on the chlorination activity and microbicidal effect of MPO, on the binding capacity of ceruloplasmin to MPO, and the effect of MPO-ANCA on this binding. The objective was to explore the influence of glycosylation patterns on MPO functions and the potential role in the pathogenesis of ANCA associated vasculitis.

METHODS

Deglycosylation of MPO by glycosidases

Human leukocyte myeloperoxidase (Calbiochem, Billerica, MA, USA) was deglycosylated according to the respective standard protocols of Peptide-N-Glycosidase F (PNGase-F) kit (New England Biolabs, Swich, MA, USA), α-neuraminidase (Roche, Mannheim, Germany), α1,6 fucosidase (Sigma, St Louis, MO, USA), β-galactosidase (Sigma, St Louis, MO, USA), β-mannosidase (Sigma, St Louis, MO, USA), α1–2,3 mannosidase kit (New England Biolabs, Swich, MA, USA), α1,6 mannosidase kit (New England Biolabs, Swich, MA, USA) and Endo H kit (New England Biolabs, Swich, MA, USA), respectively.

To remove the link between the innermost N-acetylglucosamine (GlcNAc) and asparagine residues from MPO, 10 μL human MPO (1 mg/mL) was incubated overnight at 37°C with 2 μL PNGase-F, 2 μL 10× G7 reaction buffer (500 mM sodium phosphate, pH 7.5), and 6 μL protease inhibitor diluent (Calbiochem, Billerica, MA, USA). To remove α2,3, α2,6 and α2,8-linked sialic acid from the N-glycan of MPO, 10 μL human MPO (1 mg/mL) were incubated overnight at 37°C with 6.25 μL α-neuraminidase, 33.75 μL reaction buffer (50 mM sodium citrate, pH 5). To remove branched terminal fucose from the N-glycan of MPO, 10 μL human MPO (1 mg/mL) were incubated overnight at 37°C with 2 μL α1,6 fucosidase, 3 μL reaction buffer (50 mM sodium phosphate, pH 5). To remove β-galactopyranosidase from MPO, 10 μL human MPO (1 mg/mL) were incubated overnight at 37°C with 2.5 μL β-galactosidase (diluted to 1 U/mL with deionized water), 37.5 μL reaction buffer (100 mM sodium phosphate, 30 mM magnesium chloride, pH 7.3).

To remove β-linked mannose residues from MPO, 10 μL human MPO (1 mg/mL) were incubated overnight at 37°C with 5 μL β-mannosidase (diluted to 1 U/mL with deionized water), 35 μL reaction buffer (50 mM sodium citrate, pH 4). To remove α1–2 and α1–3 linked D-mannopyranosyl residues from MPO, 8 μL human MPO (1 mg/mL) were incubated overnight at 37°C with 1 μL α1–2,3 mannosidase, 1 μL 10 × G6 reaction buffer (50 mM sodium acetate, 5 mM CaCl₂, pH 5.5).

To remove unbranched α1, 6 mannopyranosyl from MPO, 8 μL human MPO (1 mg/mL) were incubated overnight at 37°C with 1 μL α1, 6 mannosidase, 1 μL 10 × G2 reaction buffer (50 mM sodium citrate, pH 4.5). To remove chitobiose core, 8 μL human MPO (1 mg/mL) were incubated overnight at 37°C with 1 μL Endo H, 1 μL 10 × G5 reaction buffer (500 mM sodium citrate, pH 5.5).

The glycans removed from MPO are shown in Figure 1 according to the classical structure of high-mannose and complex N-linked glycoproteins.

Influence of various glycans on the chlorination activity of MPO

Myeloperoxidase (4 μg/mL) was diluted in 0.01 M phosphate buffered saline (PBS) with Cl⁻ concentration of 137 mmol/L and then coated on polystyrene microtire plates (Nunc Immunoplate, Roskilde, Denmark) at 37°C for 1 h. After three washes with PBS, the plates were incubated with 30% H₂O₂ and 0.4 g/L o-Phenylenediamine (Sigma, St Louis, MO, USA) in PBS. The absorbance value was measured at 450 nm every 2 min. The same procedure was repeated four times and the mean level at each time-point obtained. Different kinds of deglycosylated MPO were coated on the plates and the same experiments were performed to measure the influence of various glycans on the chlorination activity of MPO.

Microbicidal functions of deglycosylated MPOs on ATCC 6538

We used Staphylococcus aureus ATCC 6538 to assess the microbicidal functions of deglycosylated MPOs after the removal of
innermost GlcNAc, β-galactosidase, and α-neuraminidase. The bacteria were cultured overnight (about 16 h) in Luria Broth (LB) at 37°C, centrifuged at 1962 g for 15 min and resuspended with normal saline. The colony forming units (cfu) was estimated by measuring OD600 of bacteria suspension. About 10⁸ cfu/mL ATCC6538 was incubated with 2 units/mL of different patterns of MPOs at 37°C for 15 min. Then 0.8 mM H₂O₂ and 1 mM potassium iodide (KI) were added to the reaction system. After incubation at 37°C for 30 min, the mixtures were inoculated in LB medium and incubated overnight at 37°C. The microbicidal actions were assessed by measuring OD600 of bacterial suspensions; each assay was repeated four times.¹⁶

Influence of various glycans on the binding capacity of ceruloplasmin to MPO

Myeloperoxidase (3 μg/mL) was diluted in coating buffer (0.05 M carbonate buffer, pH 9.6) and coated on polystyrene microtitre plates (Nunc Immunoplate, Roskilde, Denmark) at 37°C for 1 h. Human ceruloplasmin (Sigma, St Louis, MO, USA) diluted to a range of physical concentrations (0–640 μg/mL) with PBS containing 0.1% Tween-20 (PBST) was added and incubated at 37°C for 2 h. After repeated washing with PBST, goat anti-ceruloplasmin sera (Sigma, St Louis, MO, USA) diluted at 1:1,000, and alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma, St Louis, MO, USA) at 1:20,000 were added subsequently. P-nitrophenylphosphate (pNPP) was used in substrate buffer. The absorbance value was measured at 405 nm. All incubations were 100 μL/well at 37°C for 1 h, with three washes by PBST between steps. Antigen-free wells were included for each sample. The same procedures were repeated four times. Different kinds of deglycosylated MPO were coated on the plates to measure the influence of various glycans on the binding capacity of MPO to ceruloplasmin.

Purification of MPO-ANCA

Myeloperoxidase-anti-neutrophil cytoplasmic antibodies immunoglobulin G (IgG) was prepared from two patients with active MPO-ANCA positive vasculitis. Informed consent was obtained from each patient prior to collection of blood samples. The research was in compliance with the declaration of Helsinki and approved by the ethics committee at the Peking University First Hospital.

Myeloperoxidase was purified from human polymorphonuclear neutrophils isolated from buffy coats as described previously.¹⁷,¹⁸ Purified MPO (1.5 mg) was coupled to 1.5 mL cyanogen bromide-activated Sepharose 4B gel (0.7 g, Amersham Pharmacia, Uppsala, Sweden) with 0.1 M sodium bicarbonate (NaHCO₃) and 0.5 M sodium chloride (NaCl; pH 8.3) as coupling buffer at room temperature for 2 h, and blocked with 0.2 M glycine (pH 8.0) at room temperature for 2 h.

Total IgGs were purified using a High-Trap-protein G column on an AKTA-FPLC system (GE Biosciences, South San Francisco, CA, USA), with 0.01 M PBS (pH 7.4) as starting buffer and 0.1 M glycine (pH 2.7) as eluting buffer, at a flow rate of 1 mL/min at room temperature. Total IgG was eluted and neutralized to pH 7.0 by 2 M Tris-HCl (pH 9.0) immediately, and dialyzed overnight against PBS. MPO-ANCA IgG was purified from total IgG, with MPO-affinity chromatography and the same method mentioned above.
**Influence of various glycans on the inhibitory effect of MPO-ANCA on the binding between deglycosylated MPO and ceruloplasmin**

ELISA assay was performed as described previously with minor modifications. MPO (2 μg/mL) was diluted in coating buffer (0.05 M carbonate buffer, pH 9.6) and then coated on polystyrene microtitre plates (Nunc Immunoplate, Roskilde, Denmark) at 37°C for 1 h. Affinity-purified antibodies to intact MPO (MPO-ANCA) (100 μg/mL) were diluted with 1% bovine serum albumin (BSA) in PBST and incubated at 37°C for 1 h. Then human ceruloplasmin (100 μg/mL) was added and incubated for 1 h. Finally, the binding was measured as described above. The reversing effect of MPO-ANCA was presented as the percentage of the decline of MPO and ceruloplasmin binding after addition of ANCA:

\[
\%\text{of reversal} = \frac{\text{OD (MPO + ceruloplasmin)} - \text{OD (MPO-ANCA)}}{\text{OD (MPO + ceruloplasmin)}} \times 100\%
\]

as compared to control. The reversing effect of MPO-ANCA was presented as the percentage of the decline of MPO and ceruloplasmin binding after addition of ANCA:

\[
\frac{\text{OD (MPO + ceruloplasmin)} - \text{OD (MPO-ANCA)}}{\text{OD (MPO + ceruloplasmin)}} \times 100\%
\]

**RESULTS**

**Influence of various glycans on the chlorination activity of MPO**

As shown in Figure 2, the enzyme activity of intact MPO increased gradually and reached steady state at one hour. Intact MPO exhibited highest activity of chlorination with an absorbance value of 0.50 ± 0.04. The enzyme activity of deglycosylated MPO decreased significantly throughout the incubation period. Removal of β-galactopyranoside (0.35 ± 0.02 vs. 0.50 ± 0.04, P < 0.001) and α-linked sialic acid (0.35 ± 0.02 vs. 0.50 ± 0.04, P < 0.001) exhibited the most remarkable decline in enzyme activity of MPO (Table 1).

Removal of β-mannopyranosyl, α1,6 linked mannopyranosyl, chitobiose core as well as innermost GlcNAc also decreased the enzyme activity of MPO significantly, but not as significant as that observed with β-galactopyranoside and α-linked sialic acid. The removal of α1,6 fucose (0.47 ± 0.05 vs. 0.50 ± 0.04, P = 0.282) and α1–2,3 mannopyranosyl (0.46 ± 0.04 vs. 0.50 ± 0.04, P = 0.199) had no significant effect on the enzyme activity of MPO (Table 1).

**Microbicidal functions of deglycosylated MPOs on ATCC6538**

Removal of innermost GlcNAc, β-galactopyranoside and α-linked sialic acid weakened the bactericidal effects of MPO were weakened (Fig. 3). Removal of α-linked sialic acid had the most remarkable effect on the bactericidal activity of MPO (P < 0.001). Removal of innermost GlcNAc (P = 0.002) and β-galactopyranoside (P = 0.001) also attenuated the bactericidal effect of MPO.

**Influence of various glycans on the binding between ceruloplasmin and MPO**

As shown in Figure 4, ceruloplasmin could bind to MPO in a dose dependent manner within the physiological reference range of ceruloplasmin (0 to 640 μg/mL). Intact MPO exhibited
the strongest binding capacity to ceruloplasmin, with the highest binding absorbance value of 1.06 ± 0.11. After the removal of glycans on MPO, the binding capacity of MPO to ceruloplasmin decreased significantly, except for α1,6 fucose (P > 0.05), irrespective of the concentrations of ceruloplasmin. Removal of different glycans led to variable decline in MPO binding with ceruloplasmin. The most significance effect was observed on removal of innermost GlcNAc at the asparagine residues of MPO (0.37 ± 0.04 vs. 1.06 ± 0.11, P < 0.001) (Table 2).

### Influence of various glycans on the inhibitory effect of MPO-ANCA on the binding between deglycosylated MPO and ceruloplasmin

The binding between ceruloplasmin and MPO was reversed by MPO-ANCA. The reversal effect of MPO-ANCA on the binding between intact MPO and ceruloplasmin was 88.3 ± 1.0% (Table 3). After treatment of MPO with different glycosidases, the reversal effect of MPO-ANCA changed accordingly. Removal of α-linked sialic acid (71.2 ± 5.1% vs. 88.3 ± 1.0%, P = 0.009), α1-2,3 linked mannoyparanosyl (62.2 ± 8.6% vs. 88.3 ± 1.0%, P = 0.048), chitobiose core (73.6 ± 1.9% vs. 88.3 ± 1.0%, P = 0.001), β-mannoyparanosyl (69.9 ± 5.6% vs. 88.3 ± 1.0%, P = 0.039) and innermost GlcNAc (77.9 ± 1.9% vs. 88.3 ± 1.0%, P = 0.002) from MPO significantly reduced the inhibitory effect of MPO-ANCA on the binding between MPO and ceruloplasmin (Table 3). However, the removal of the other three glycans, i.e., α1,6 mannoyparanosyl, β-galactopyranoside and α1,6 fucose had no significant effect on the reversal ability of MPO-ANCA (P > 0.05).

### DISCUSSION

As a peroxidase, the most vital physiological function of MPO is determined by its enzymatic activity, to catalyze the production of hypochlorous acid and reactive oxygen species, and to participate in the antimicrobial mechanism in circulation and at sites of inflammation. A recent study indicated glycosylation as being critical to the enzymatic activity of MPO; our results are consistent in this respect. We further investigated the effect of each glycan on the oxidation function of MPO.

Among the eight different glycans, β-galactopyranoside and α-linked sialic acid appear to be essential, since the microbicidal effect of MPO-ANCA showed a significant attenuation after the removal of β-galactopyranoside and α-linked sialic acid. These findings underline the important role of glycans in the physiological functions of MPO. The rigid heme architecture is essential to the enzyme activity of peroxidase, and the glycans are closely configured around the heme pocket. The change of glycans may modify the overall protein structure, alter the
In the pathogenesis of autoimmune diseases, MPO contributes to inflammatory tissue damage. Ceruloplasmin levels within the physiological range inhibit the peroxidase activity of MPO by binding to it and forming binary complexes with cationic MPO. The modelled loop between ceruloplasmin domains (aa885–892) contacts MPO in the vicinity of heme pocket and affect the interaction of MPO with its substrates. Sialic acid and β-galactopyranoside are always located on the terminus of oligosaccharide chains. Their loss may have a considerable influence on the conformational structure, thereby attenuating the enzymatic activity of MPO.

Deglycosylated MPO is very likely to exist in human circulation via the mechanism of major-and minor-microheterogeneity of N-glycans on mature dimeric MPO. In a study on α1-acid glycoprotein (AGP), Ceci et al. suggested that the change in branching degree, namely major-microheterogeneity, as well as the change of fucosylation and sialic acid numbers, namely minor-microheterogeneity, both influence AGP’s biological activity, including its binding properties to pathogenic microorganisms and its immunomodulatory function. These findings imply that the different glycosylation patterns of MPO, mediated by microheterogeneity, may also influence the enzymatic activity of MPO in circulation. More infections in older people, together with high prevalence of MPO-ANCA associated vasculitis in older people support our hypothesis that deglycosylated MPO may be more common in older people, and attenuate the bactericidal function, and increase susceptibility to infections and vasculitis. This mechanism may enhance the pathogenesis of ANCA associated vasculitis.

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In summary, the loss of glycan terminus decreased the enzymatic activity and microbicidal effect of MPO, which may potentially lead to severe infections. The loss of innermost glycans decreased the binding of ceruloplasmin to MPO, which may be a key mechanism for the sustained inflammatory tissue damage in ANCA associated vasculitis. Further studies on MPO

| Table 3 Binding capacities between ceruloplasmin and myeloperoxidase (MPO) with and without MPO-anti-neutrophil cytoplasmic antibodies (ANCA) |
|-------------|----------------|----------------|---------------|----------------|
| Group       | Binding between MPO and ceruloplasmin OD (Mean ± SD) | Binding between MPO and ceruloplasmin with MPO-ANCA OD (Mean ± SD) | Reversal effect of MPO-ANCA (%) P-value | OD (Mean ± SD) |
| Intact MPO  | 0.54±0.018     | 0.063±0.004    | 88.3±1.0      | As control |
| De-Sialic Acid MPO | 0.43±0.086 | 0.121±0.008 | 71.2±5.1 | 0.009 |
| De-β-Galactopyranosyl MPO | 0.35±0.054 | 0.103±0.002 | 69.9±5.6 | 0.039 |
| De-β-Galactopyranoside MPO | 0.42±0.110 | 0.100±0.004 | 74.6±6.4 | 0.092 |
| De-β-Mannopyranosyl MPO | 0.31±0.011 | 0.068±0.005 | 77.9±1.9 | 0.002 |
| De-α1,2-Mannopyranosyl MPO | 0.407±0.086 | 0.150±0.029 | 62.2±8.6 | 0.048 |
| De-α1,6-Mannopyranosyl MPO | 0.406±0.085 | 0.060±0.001 | 84.6±3.5 | 0.231 |
| De-Chitobiose core MPO | 0.367±0.043 | 0.096±0.005 | 73.6±1.9 | 0.001 |
| De-α1,6Fucose MPO | 0.519±0.009 | 0.060±0.002 | 88.4±0.7 | 0.884 |

OD, optical density; SD, standard deviation.
glycans and their antigenicity are required to enhance our understanding of the mechanism of vasculitis.

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JW performed the deglycosylation of MPO, purified human MPO, total IgG and MPO-IgG, performed all tests of MPO, analyzed the data and wrote the manuscript. ZC designed and directed the experiments, analyzed the data and helped write the manuscript. JNL contributed reagents/materials/analysis tools. MHZ designed and directed the study. All authors read and approved the final manuscript. This work was supported by grants from the National Natural Science Foundation of China to the Innovation Research Group (81321064), the National Natural Science Fund of China (81330020, 81370801, 81400703), and the Capital of Clinical Characteristics and Applied Research Fund (Z16110000516039).

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