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Binding of human myeloperoxidase to red blood cells: Molecular targets and biophysical consequences at the plasma membrane level

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ABSTRACT

Myeloperoxidase (MPO) is an oxidant-producing enzyme that can also bind to cellular surface proteins. We found that band 3 protein and glycophorins A and B were the key MPO-binding targets of human red blood cells (RBCs). The interaction of MPO with RBC proteins was mostly electrostatic in nature because it was inhibited by desialation, exogenic sialic acid, high ionic strength, and extreme pH. In addition, MPO failed to interfere with the lectin-induced agglutination of RBCs, suggesting a minor role of glycan-recognizing mechanisms in MPO binding. Multiple biophysical properties of RBCs were altered in the presence of native (i.e., not hypochlorous acid-damaged) MPO. These changes included transmembrane potential, availability of intracellular Ca^{2+}, and lipid organization in the plasma membrane. MPO-treated erythrocytes became larger in size, structurally more rigid, and hypersensitive to acidic and osmotic hemolysis. Furthermore, we found a significant correlation between the plasma MPO concentration and RBC rigidity index in type-2 diabetes patients with coronary heart disease. These findings suggest that MPO functions as a mediator of novel regulatory mechanism in microcirculation, indicating the influence of MPO-induced abnormalities on RBC deformability under pathological stress conditions.

1. Introduction

Myeloperoxidase (MPO) is a 145 kDa polycationic heme-containing glycoprotein (pI > 10), which is stored mainly in the azurophilic granules of neutrophils and secreted into the extracellular space at inflammatory sites as a result of neutrophil degranulation [1]. MPO displays peroxidase activity, which results in the production of highly reactive oxidants (HOCl, HOBr, and other free radicals) from H_2O_2 and halides (Cl^-, Br^-, I^-) and pseudohalide (SCN^-) [2,3]. As a polycationic protein, MPO binds to the negatively charged surface of pathogens [4] and, due to its aforementioned enzymatic activity, initiates the destruction of cell membranes and cell death. This ensures that the MPO-dependent antibacterial system of leukocytes is one of the most effective in the body [1]. However, MPO can associate not only with the surface of pathogens, but also with the surface of endothelial cells [5,6], fibroblasts [7], macrophages [8], epithelial cells [9], neutrophils [5,10], platelets [11,12], and low-density and very low-density lipoproteins [13]. When it binds to a cell's surface, MPO can alter the

Abbreviations used: MPO, myeloperoxidase; MPO-Cl, MPO, modified by HOCl; RBCs, red blood cells; 4-ABH, 4-aminobenzoic acid hydrazine; TEA, tetraethylammonium; DIDS, disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; SNA, *Sambucus nigra* agglutinin; STA, *Solanum tuberosum* agglutinin; WGA, *Triticum vulgaris* agglutinin; VAA, *Viscum album* agglutinin; PBS, phosphate buffered saline; RBCGs, red blood cell ghosts; EPR, electron paramagnetic resonance; AFM, atomic force microscopy; IR, rigidity index; IHD, ischemic heart disease; DM, diabetes mellitus; B3, band 3 protein; Gp, glycophorin.

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cell's functional properties regardless of its enzymatic activity. For example, it has been shown that the interaction of MPO with integrins on the neutrophil surface leads to the increased tyrosine phosphorylation of multiple cellular proteins. This finding implies that MPO activates protein tyrosine kinases in these cells, stimulating degranulation and respiratory burst [10]. Recently, we [11] and others [12] have shown that MPO binding to platelets triggers the reorganization of the platelet cytoskeleton and alters their aggregation activity.

It is assumed that any cell in the blood can be a target for MPO, provided the cell is in close proximity to the activated or lysed neutrophils. With regard to red blood cells (RBCs), recently it has been shown that MPO binds to plasma membranes *in vitro* and *in vivo* [14]. However, it is still unknown which RBC surface structures MPO interacts with and whether MPO modifies the cell's structural and functional properties. Given the relatively high level of MPO (>1 μg/ml) in the blood serum from patients with cardiovascular diseases [15,16] and such patients' problems with microcirculation and the biophysical properties of RBCs [17], a causal relationship between these parameters may be suggested. This study was designed to examine the hypothesis that MPO binding to the surface of RBCs is responsible for abnormalities in the cells associated with cardiovascular diseases. We show that native MPO binds specifically to the major integral proteins of RBCs (band 3 protein and glycophorins A and B), and this induces multiple changes in the biophysical properties of the cells (e.g., membrane fluidity, transmembrane potential, intracellular Ca^{2+}, cellular size and morphology, sensitivity to hemolysis, and cellular deformability). We also report that there is a strong correlation between the circulating level of MPO and the rigidity index of RBCs in patients with combined ischemic heart disease and diabetes. These findings provide new insights into the pathogenesis of syndromes associated with inflammatory stress.

2. Materials and methods

2.1. Reagents

4-Aminobenzoic acid hydrazine (4-ABH), NaOCl, sodium citrate, *o*-dianisidine, H_2O_2, Fluo-3/AM, tetraethylammonium (TEA), disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS), 4-chloro-1-naphthol, sialic acid, ionomycin, and neuraminidase from *Clostridium perfringens* were purchased from Sigma–Aldrich (St. Louis, USA). Sodium L-aspartate was from Alfa Aesar (Ward Hill, USA). High-affinity rat and rabbit polyclonal antibodies against human MPO were prepared as described previously [18]. Plant lectins from *Sambucus nigra* (SNA), *Solanum tuberosum* (STA), *Triticum vulgaris* (WGA), and *Viscum album* (VAA) were purchased from Lektinotest (Lviv, Ukraine).

2.2. Isolation of MPO and preparation of MPO, modified by HOCl (MPO-Cl)

Native MPO was isolated from the frozen leukocytic mass of healthy donors as described elsewhere [19]. The purity of the MPO was characterized by its Reinheit Zahl (RZ) value, a 430 nm/280 nm absorbance ratio, which was ~0.85.

The modification of the MPO using HOCl was performed in phosphate buffered saline (PBS) (10 mM Na_2HPO_4/KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) as previously described for human serum albumin [20]. Briefly, a solution of NaOCl was used as a source of highly reactive HOCl, which was formed by dissociation reactions in a neutral PBS considering pKa~7.5 for hypochlorous acid [21]. MPO (13.8 mM) was exposed to the NaOCl solution (1.38 mM) for 1 h at room temperature by mixing these solutions in equal volumes. At these conditions degradation of amino acid residues Met, Cys, His, Trp, Lys [22], and MPO heme destruction is expected [23]. In this regard the conversion of native MPO to its oxidized form (MPO-Cl) was monitored by the decrease of the intrinsic fluorescence (excitation at 285 nm and emission at 340 nm) due to the destruction of tryptophan residues and also by the decrease of the MPO Soret peak.

2.3. Isolation of RBCs and RBC ghosts (RBCGs)

Washed RBCs were obtained after two centrifugation cycles of venous blood collected in tubes containing 3.8% (w/v) trisodium citrate as anticoagulant at a ratio of 9:1 or capillary blood (100 μl) at 600 g in PBS (10 ml) for 5 min and stored in PBS with 10 mM D-glucose at 4 °C. These cells were used to prepare RBCGs by hypoosmotic hemolysis. One volume of washed RBCs was mixed with 20 volumes of cold hemolysis buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6) and incubated for 5 min at 4 °C. This sample was centrifuged twice at 30,000 g (30 min, 4 °C), resuspending the first RBCG pellet in 10 volumes of the hemolysis buffer before the second centrifugation. The final pellet of RBCGs was suspended in 3 volumes of the hemolysis buffer and used for downstream procedures.

2.4. Detection of MPO-binding proteins using Western ligand blot assay

RBCGs were lysed in 1/5 volume of a SDS-Tris sample buffer (125 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% 2-mercaptoethanol, 0.001% bromphenol blue, and 50% glycerol), and 100 μg of total protein was loaded per well of polyacrylamide gel [24]. The separated proteins were transferred on nitrocellulose membranes using a semi-dry method [25], and the membranes were blocked for 15 min with a blocking solution containing 1% BSA and 0.05% Tween 20 in PBS (BSA-T). To detect MPO-binding proteins, the membranes were exposed to 10–400 nM MPO or MPO-Cl in BSA-T for 30 min followed by HRP-labeled rabbit anti-human MPO antibody (1:1000–1:40000) for 1 h. Each step was accompanied by an intensive washing of the membranes with BSA-T for 5 min, three times. The peroxidase activity was visualized in a reaction with 4-chloro-1-naphthol and H_2O_2. It should be noted that in the absence of HRP-labeled antibody, basal MPO peroxidase activity was hardly visible. Control dot-blotting experiments showed no difference between MPO and MPO-Cl in binding to the HRP-labeled antibody against MPO. The identity of MPO-binding protein bands on SDS-PAGE gels was confirmed by mass spectrometry after *in situ* tryptic digestion [26].

2.5. ELISA assay for MPO binding with RBCG proteins

The surfaces of polystyrene 96-well plates were activated through 12 h of incubation with 100 μl 0.5% glutaraldehyde in PBS. To immobilize RBCG proteins, 100 μl of the RBCG lysate (protein concentration 20 μg/ml) in 0.05% Tween 20 was added to each well for 1 h at room temperature. Next, the wells were blocked with 0.2 ml BSA-T for 60 min, and 100 μl MPO (50 nM) was added in combination with tested treatments. These treatments included tonicity variation by NaCl (100–500 mM, 10 mM Na-phosphate buffer, pH 7.4), pH challenge in the range of 5.0–9.0 (100 mM NaCl, 10 mM Na-phosphate buffer), presence and absence of 0.1 mM sialic acid, and 0.1 ml HRP-labeled rabbit anti-human MPO antibody (1:2000). Afterwards, each incubation plate was washed 3-times with PBS. The peroxidase activity was determined to be the rate of *o*-phenylenediamine oxidation with the optical density decrease measured at 492 nm.