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A new easy method for specific measurement of active myeloperoxidase in human biological fluids and tissue extracts

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ABSTRACT

The SIEFED ("Specific Immunological Extraction Followed by Enzymatic Detection") method already developed for the specific detection of the activity of equine myeloperoxidase (MPO) was adapted for the specific measurement of active human MPO in biological fluids or tissue extracts. The method consists of the extraction of MPO from aqueous solutions by immobilized anti-MPO antibodies followed by a washing (to eliminate the extraction medium and the biological fluid with their possible interfering molecules) and the measurement of the activity of MPO with a detection system containing a fluorogenic substrate, H_2O_2 and nitrite ions as reaction enhancer. The SIEFED was applied to study active MPO in human biological fluids (plasma, bronchoalveolar lavage fluid and supernatant from carotids extracts). The SIEFED for human MPO has a sensitivity limit of 0.080 mU/mL and showed good precision with intra- and inter-assay coefficients of variation below 10 and 20% respectively within a broad range of MPO activities establish from 0.156 to 473 mU/mL. The SIEFED for human MPO will be useful for the specific detection of active MPO in complex fluids and can be complementary to an ELISA to determine an active/total MPO ratio in healthy volunteers and patients especially in case of chronic or acute inflammatory diseases.

1. Introduction

Neutrophil myeloperoxidase (MPO) is involved in the intracellular bacterial destruction by producing potent oxidant molecules, mainly hypochlorous acid (HOCl) [1,2]. MPO is released in the extracellular milieu by dying or highly stimulated neutrophils in pathological conditions of acute and chronic inflammations [2–6]. In these conditions, MPO is able to exert a deleterious oxidant activity on neighbouring cells and tissues, and a therapeutic target should be to modulate its activity. Increased blood levels of MPO are now considered as markers of neutrophil activation and degranulation [7,8] and are measured by immunological methods (ELISA or RIA), which quantify the total concentration of the enzyme [9,10] without reflecting its true enzymatic activity. The activity of MPO is currently measured by the direct addition in the medium of H_2O_2 and a chromogenic or fluorogenic substrate as electron donors [11–13]. The oxidation of these electron donors is monitored by spectrophotometry or fluorimetry as a probe of the MPO activity [14,15]. Although these methods are suitable for measuring the activity of MPO *in vitro* in simple mixtures [13], they lack sensitivity, are cumbersome for cohort measurements and are not easily applicable to complex biological samples such as blood, plasma, or tissues, because of interferences with components endowed with peroxidasic activity (e.g. haemoglobin) [16,17], proteins (e.g. albumin, lipoproteins, ceruloplasmin) [18,19], reducing agents (e.g. ascorbic acid, glutathione) [16]. The physicochemical characteristics (pH, viscosity, etc.), the redox status or the colour of the biological fluids also interfere with the reading of the colorimetric or fluorescence response.

We designed a new technique, the SIEFED (for "Specific Immunological Extraction Followed by Enzymatic Detection") to measure the activity of an enzyme in biological fluids, and firstly developed it for equine MPO [20]. The major advantage of the SIEFED technique is its rapidity and an easy extraction of MPO out of the sample or reaction mixture by specific immobilized antibodies. The MPO binding to antibodies is followed by a washing

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step that eliminates the solution and its potential interfering capacity, before starting the detection of MPO enzymatic activity. In human, increasing evidence indicated that MPO plays important roles in the initiation and progression of arthritis, carcinogenesis, pulmonary, kidney, neurodegenerative, inflammatory bowel and especially cardiovascular diseases in which the enzyme is taken as an inflammatory marker and a prognostic agent [6]. In these diseases, an important clinical aspect is to know the active part of MPO, which is the real witness of the oxidant potential of the enzyme. We describe here the development and optimization of the SIEFED technique for the specific detection of the enzymatically active human MPO in complex media [plasma, bronchoalveolar fluids (BALs)] and tissue extracts (carotids). The SIEFED technique is based on the immunological capture of the antigen by antibodies. An interesting point of this study was to compare the active MPO content measured by SIEFED with the total MPO content measured by ELISA to determine an active/total MPO ratio for the tested samples.

2. Experimental

2.1. Reagents

Analytical grade phosphate salts, sodium and potassium chlorides, sodium hydroxide, sodium acetate, H_2O_2 (30%, w/v), ethanol and Tween 20 and human myeloperoxidase (Calbiochem) were purchased from Merck (VWR International, Leuven, Belgium). Bovine serum albumin (BSA) and sodium nitrite were purchased from Sigma–Aldrich (Bornem, Belgium). 96-Wells microtiter plates (Combiplate 8 EB) were from Thermo Scientific (Breda, The Netherlands). The fluorogenic substrate, Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine), was purchased from Molecular Probes (Invitrogen) (Merelbeke, Belgium).

2.2. Purification of human MPO and rabbit anti-human MPO polyclonal antibodies

MPO was purified from human neutrophils by detergent enhanced extraction followed by two steps of chromatography, and antisera were obtained by rabbit immunization against the pure human MPO emulsified with complete Freud adjuvant as previously described [9,21]. Polyclonal antibody (IgG), purified from rabbit antisera by affinity chromatography on Protein A sepharose (GE Healthcare, The Netherlands), was characterized for its specificity and stability and firstly used to develop a radioimmunoassay for total MPO assay in human plasma [9].

2.3. Methodology of the SIEFED technique

Three buffers were used. The coating buffer was 20 mM K and Na phosphates (pH 7.4), 137 mM NaCl and 2.7 mM KCl. The blocking buffer was obtained by addition of 5 g/L BSA to the coating buffer. The diluting buffer was the blocking buffer added with 0.1% Tween 20. The washing solution was 154 mM NaCl with 0.1% Tween 20. Microtitration wells were coated overnight, at 4 °C with 100 μL/well of serial dilutions of a rabbit IgG solution (62.5–500 ng/well) diluted with the coating buffer to determine the optimal IgG concentration to be used in the SIEFED assay. After removing the coating solution, the blocking buffer was added, the plates were incubated for 150 min at room temperature and washed three times. Serial dilutions of a standard MPO solution (pure human MPO) or unknown samples were added (100 μL/well) and incubated for 1 or 2 h either at 22 °C or at 37 °C or for 20 h at 4 °C. After three washings, the peroxidasic activity of MPO was detected by adding 100 μL of a 40 μM Amplex Red solution freshly prepared in phosphate buffer (50 mM) at pH 7.5 and added with 10 μM H_2O_2 and 10 mM nitrite. After incubation in the darkness (30 min, 37 °C), the fluorescence was read with a Fluoroscan Ascent (Thermo Scientific) with excitation and emission wavelengths set at 544 and 590 nm, respectively. Controls (blank) were performed with the dilution buffer. The fluorescence value was directly proportional to the quantity of active MPO present in the sample.

2.4. Validation of the SIEFED technique

The stability of the reference human MPO (from Calbiochem) was tested after its dilution in phosphate buffer saline (PBS) or PBS added with 5 g/L BSA and 0.1% Tween 20 (dilution buffer) and either incubated for 2 h at 37 °C or submitted to several freezing–thawing cycles.

The cross-reactivity of the anti-MPO antibodies with albumin, haemoglobin, elastase or eosinophil peroxidase was measured as previously described [9]. The detection limit was defined as the lowest active MPO concentration (mean value ± SD) that was statistically different from the value obtained with buffer alone. The standard curve was calculated and plotted point to point by using the Ascent Software for Multiscan Ascent (Thermo Labsystems). The precision and reproducibility were estimated by the coefficients of variation (CV) within or between assays calculated for several determinations of the same sample in the same assay or in different assays performed on different days. Accuracy was estimated by the recovery of known amounts of purified MPO added to the samples (plasma, BALs and carotid extracts).

To assure that the SIEFED technique could be reproduced with other anti-MPO antibodies, the immunoreactivity of our antibody was compared with that of the primary MPO-antibody (polyclonal antibody from rabbit) used in the MPO-ELISA kit (ELIZEN MPO) purchased from the Zentech company (Belgium).

2.5. Sampling technique

To test the stability of the neutrophils in blood after sampling, blood was drawn from five healthy donors without anticoagulant and on three anticoagulants with centrifugation (10 min, 22 °C, 1000 × *g*) 1 or 3 h after sampling. The anticoagulants were EDTA (1.8 mg/mL blood, in 3.5 mL vacutainer tubes; Venosafe, Terumo, Belgium), lithium heparin (15 IU/mL blood), or citrate (3.2% Na-citrate solution). Blood in plain tubes was allowed to clot (3 h) at room temperature (22 °C) before serum collection. For all the other studies with healthy and pathological subjects, venous peripheral blood samples were obtained in vacutainer tubes on EDTA (1.8 mg/mL blood), and centrifuged within 30 min after drawing.

Bronchoalveolar lavages (BALs) were performed in intensive care patients for therapeutic purpose (bacterial agent determination) via the endotracheal tube, with four aliquots of 50 mL warm sterile 0.9% NaCl solution as previously described [22]. The first aliquot was discarded and the second used for bacterial studies. The aspirated liquids of the third and the fourth aliquots were strained through sterile gauze and centrifuged (10 min, 4 °C, 300 × *g*); the supernatant was used for SIEFED assay.

Carotid atherosclerotic plaques were collected during surgery for carotid thrombo-endatherectomy and rinsed with 0.9% NaCl solution before their freezing at −20 °C. After thawing, the carotid plaques were homogenised on ice in 20–25 mL of cold 10 mM Tris-buffer, pH 7.4. The extract was centrifuged (20 min, 4 °C, 17,000 × *g*) and the supernatant was used for SIEFED assay.

Plasma samples, supernatants from BALs and carotid extracts were freshly used or kept frozen in small aliquots at −20 °C. Immediately before the SIEFED assay, samples were thawed and diluted with the diluting buffer before loading into the wells.