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N-phenylmaleimide derivatives as mimetic agents of the pro-inflammatory process: myeloperoxidase activation

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Abstract:
Myeloperoxidase (MPO) is an important enzyme that catalyzes the reaction between hydrogen peroxide and chloride to generate hypochlorous acid, which oxidizes a range of biomolecules and has been associated with inflammatory diseases. The synthetic compounds *N*-phenylmaleimide (NFM) and 4-methyl-*N*-phenylmaleimide (Me-NFM) increased the MPO activity *in vitro* (of isolated enzyme and in isolated cells after animal treatment) and *in vivo* assays. MPO-induction may represent a good model system to investigate the molecular and cellular mechanisms of oxidative cell injury induced by activated neutrophils, and the interactions between damaging species involved in the respiratory burst.

Key words:
N-phenylmaleimides, myeloperoxidase, inflammation, neutrophils, hypochlorous acid

Abbreviations: H_2O_2 – hydrogen peroxide, HBSS – Hank's buffered salt solution, HOCl – hypochlorous acid, HRP – horseradish peroxidase, Me-NFM – 4-methyl-*N*-phenylmaleimide, MPO – myeloperoxidase, NFM – *N*-phenylmaleimide, PBS – phosphate buffered saline, ROS – reactive oxygen species

Introduction

Phagocyte white blood cells (neutrophils, monocytes, macrophages and eosinophils) have a crucial role in host defense, since they are highly specialized to carry out phagocytosis and the destruction of foreign microorganisms. The activated neutrophils produce a series of reactive molecules prior to the superoxide anions during the respiratory burst [18]. The superoxide anions are then converted to other reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radicals (•OH) and singlet oxygen ($^1O_2$) [33, 34].

In the neutrophils there are a number of cytotoxic enzymes, in particular myeloperoxidase (MPO), which belongs to a mammalian family of peroxidases, located within azurophilic granules [16, 17]. It is a 140-kDA heme enzyme abundantly expressed in polymorphonuclear cells, accounting for up to 5% of total cellular protein, released during the phagocytosis [16].

The gene family of mammalian peroxidases includes myeloperoxidase, eosinophil peroxidase, lactoperoxidase, which are present in exocrine secretions and its relationships to host defense mechanisms and human disease, and thyroid peroxidase, which is involved in the thyroid hormone synthesis. These peroxidases are significantly homologous with the fungal chloroperoxidase and the horseradish peroxidase [14].

The peroxidases, mainly the MPO reacts with halide ions (Cl^-, Br^-, I^-) and pseudo-halide thiocyanate ions (SCN^-) to produce hypochlorous acid (HOCl) and their respective hypohalous acids [21].

Hipochlorous acid oxidizes a range of biomolecules, it reacts with nitrogen contained in biological macromolecules, principally amino groups, to form chloramine derivatives that can degrade to aldehydes; with tiols and methionyl residues, nucleotides, unsaturated lipids, amino acids, apolipoproteins and lipoproteins [17, 34].

MPO has been reported to be a relevant factor in tissue injury in many inflammatory processes such as rheumatoid arthritis, arteriosclerosis, cancer, renal and pulmonary disease, neurodegeneration, and vasculitis, as well as organ damage after renal ischemia reperfusion, among others [13, 19, 20, 24]. In contrast, this enzyme is essential to kill microorganisms, it plays a protective role in innate immunity against infectious diseases, it induces platelet aggregation and detoxification of microbial toxins and is involved in the modulation of cell signaling pathways [2, 17–19].

Cyclic imides such as *N*-phenylmaleimides, a type of stable cyclic α,β-unsaturated ketone, are solid yellow compounds, obtained easily through the reaction of maleamic acids and acetic anhydride in a medium containing sodium acetate as a catalytic agent. These maleimides contain an imide ring and a general structure –CO–N(R)–CO– that confers hydrophobic and neutral characteristics. The pharmacological activity of cyclic imides has been reported, including antibacterial, antifungal, antinociceptive, anti-spasmodic and antitumoral [6, 30].

Several types of inflammatory models allow hypothesis testing, including *in vitro* tests with an enzyme, for example MPO, to characterize the role of a range of mediators and cells involved in the inflammatory process and to study new drugs and/or therapeutic strategies for inflammation treatment [22, 27]. In this context, the carrageenan-induced air pouch is a known model of acute inflammation which enables the evaluation of cell migration and pro-inflammatory mediators in cavity lavages [4, 11]. In addition, cell culture and other *in vitro* tests are widely used as models to study induced oxidative stress and the activities of pro-inflammatory enzymes.

In this study we evaluated N-phenylmaleimides as new selective biomarkers to MPO inducer using *in vitro* and *in vivo* experiments.

Materials and Methods

Chemicals

Sodium chloride (NaCl); potassium chloride (KCl); calcium chloride ($CaCl_2$); potassium phosphate buffer (KH_2PO_4), sodium phosphate buffer (NaH_2PO_4); sodium borohydride; hydrogen peroxide (H_2O_2); sodium hypochlorite (NaOCl); dimethyl sulfoxide (DMSO); ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Merck (Darmstad, Germany); carrageenan; May-Grünwald-Giemsa stain; Trypan blue, *o*-dianisidine, heparin, hexadecyltrimethylammonium bromide (HTMAB); sodium azide, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), horseradish peroxidase and myeloperoxidase were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. Ultrapure Milli-Q water was used throughout.

Animals

Swiss mice, weighing 18–22 g and male Wistar rats weighing 160–200 g, were housed under standardized conditions (room at constant temperature (25°C) with alternating 12 h periods of light and darkness and 50–60% humidity) and fed a standard mouse diet with water *ad libitum* before use. All experiments adhered to the guidelines on ethical standards of investigation of experimental procedures in animals [35]. This study was approved by the Committee for Ethics in Animal Research (CEUA) of our university and performed in accordance with the norms of the Brazilian College of Animal Experimentation (COBEA), protocol number 23080.007040/2006-39. Efforts were made to reduce the number of animals used.

Drugs

The synthesis of N-maleimide and *N*-substituted maleimide was carried out in a previously reported two-step procedure [15]. Reactions of maleic anhydride with the appropriate amine in the presence of glacial acetic acid at room temperature generated the corresponding *N*-substituted maleamic acid. The maleamic acid intermediates were subsequently cyclized to the corresponding *N*-phenylmaleimides (Fig. 1) by heating in the presence of acetic anhydride containing catalytic amounts of sodium acetate [7].

The cyclic imides were synthesized at Itajaí Valley University, and their structures were confirmed by spectroscopic methods. The compounds were solubilized in DMSO and used at different concentrations, depending on the test. In each case the effect of the solvent alone was monitored. The solvent did not affect the experimental systems used.

Assay for MPO activity – *in vitro* test

Obtaining of MPO enriched lung homogenate

The lungs were obtained from male Wistar rats. The tissue was homogenized in ice-cold phosphate buffer (50 mM, pH 6.0), containing 0.5% HTMAB and freeze-thawed three times. The samples were centrifuged at 12,000 × g at 4°C for 20 min. The major peroxidase activity in the supernatant is mainly of the MPO, although other peroxidases such as eosinophil peroxidase may be present [22, 23, 31].

Enzyme assay

The reaction medium containing 50 mM NaH_2PO_4 at 25°C, 0.167 mg/ml *o*-dianisidine × 2HCl, and 0.005% H_2O_2 with or without increasing concentrations of maleimides. The reaction was started by the addition of the supernatant containing MPO (4 mg protein/ml).

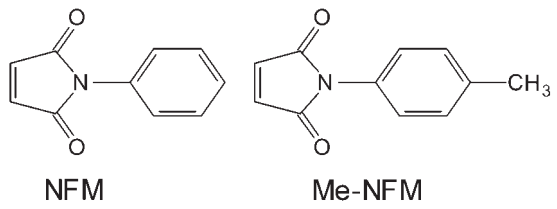

Fig. 1. Structure of maleimides

Sodium azide (250 μM) was used as a control that run in parallel with the experiments, inhibiting completely the activity. The activity was determined by the slope of the absorption curve set at 450 nm. The same procedure was performed to evaluate the effect of both maleimides on commercial purified MPO activity as well as to build an analytical curve of MPO activity [25, 31].

HOCl acid scavenging activity

Preparation of HOCl acid

HOCl (70 μM) was prepared immediately before use by adjusting a solution of NaOCl to pH 6.6 with KH_2PO_4 (50 mM). The concentration of HOCl was further determined spectrophotometrically at 290 nm using the molar absorption coefficient of 350 M^{-1} cm^{-1} [8].

Preparation of 5-thio-2-nitrobenzoic acid (TNB)

TNB was prepared according to a previously described procedure [8]. Briefly, to a 1 mM solution of DTNB in 50 mM potassium phosphate buffer (pH 6.6), containing 5 mM EDTA, 20 mM sodium borohydride was added. The resulting solution was incubated at 37°C for 30 min. The concentration of TNB was determined by measuring the absorbance at 412 nm using the molar absorption coefficient of 13,600 M^{-1} cm^{-1}.

HOCl acid scavenging assay

The assay was performed at room temperature in a cuvette containing a 70 μM TNB solution, with or without *N*-phenylmaleimides (0–100 μM). The absorbance was measured at 412 nm before and 5 min after the addition of HOCl (25 μM).

MPO activity induction in isolated neutrophils

Isolation of neutrophils

Neutrophils were isolated at room temperature, after washing the exudate dorsal skin (induced air pouch cavity) of six non treated animals with 1.0 ml of sterile PBS. The isolated neutrophils were resuspended in HBSS, pH 7.35, containing 1.6 mM $CaCl_2$. The viability of neutrophils in the cell preparations was considered 95%, determined by the Trypan blue method.

Induction of MPO

Neutrophils (1×10^6) were incubated with HBSS in 24-well microplates without compounds for 1.3 h, and were maintained at 37°C in a 5% CO_2 humidified atmosphere. Briefly, increasing concentrations of the compounds (25–100 μM) dissolved in DMSO (0.01% final concentration) were added and diluted with HBSS followed by incubation for 1 h. The detergent hexadecyltrimethylammonium bromide (HTMAB – 0.5 %) was used to lyse the cells to release MPO. The enzyme activity was determined by the 3,3-dimethoxybenzidine (*o*-dianisidine) oxidation method monitored at 450 nm. The method consists of the addition of *o*-dianisidine (0.125 mg/ml) and H_2O_2 (0.001%) dissolved in phosphate buffer (pH 6.2) and incubation for 15 min at 37°C. The reactions were stopped by adding 1% sodium azide solution and the plates were centrifuged at 258 × g for 10 min at room temperature. The myeloperoxidase activity was expressed as percent of oxidation of *o*-dianisidine/1×10^6 cells [12, 32].

Assay for leukocyte migration, exudation, and MPO activity – *in vivo* test

Experimental protocol

In this experimental protocol, different groups of animals received air injection on three alternate days to induce the air pouch. On the sixth day, the animals received carrageenan (Cg, 1%) by subcutaneous route (*sc*) and 24 h later the animals were sacrificed by ether overdose [4]. The animals were fixed into a surgery table and an incision in the dorsal skin was made perforating the air pouch. The cavity was washed with sterile PBS (pH 7.6, composition mmol: NaCl 137, KCl 2.7 and phosphate buffer salts 10) containing heparin (20 IU/ml) [4]. In this protocol a dose-response assay was carried out.

The animals were divided in groups and were treated (0.5 h before Cg) with different doses of Me-NFM (100–400 μM) administered by intraperitoneal route (*ip*). The same procedure with NFM (100–400 μM, *ip*) was performed, also administered 0.5 h before Cg. The leukocyte migration, as well as MPO activity, was analyzed 24 h after carrageenan injection. In summary, the animals were equally divided in four experimental groups as following: Group 1: positive control group: animals treated only with carrageenan (Cg, 1%), Group 2: negative control group: animals treated only with sterile saline (NaCl, 0.95%), Group 3: animals treated with Me-NFM plus Cg, Group 4: animals treated with NFM plus Cg.

Quantification of leukocyte migration

After killing the animals, samples of the air pouch cavity fluid were collected to determine the total and differential leukocyte contents. Total leukocyte counts were determined in a Newbauer chamber, and cytospin preparations of exudates were stained with May-Grünwald-Giemsa for the differential count, which was performed with an optical microscope under an oil immersion objective and 100× magnifications [26].

Quantification of MPO activity

In-house assays of both MPO were employed according to the methods developed by Rao et al. [22]. Using conventional reagents, the concentration of each enzyme was estimated by means of colorimetric measurements (absorbance of 450 nm) in an ELISA plate reader (Organon Teknika, Roseland, NJ, USA). One unit of MPO is defined as the activity of the enzyme that oxidizes 1 mol of H_2O_2/min. The results were expressed as mU/ml (MPO). Detailed descriptions of these assays have been published previously [12].

Statistical analysis

All results are the mean ± SEM. All statistical tests were performed using Instat® version V2.05a, and the statistical significance was assessed by using ANOVA followed by the Dunnett's test. When necessary we also used the Student's *t* test for the *in vivo* assay; p values < 0.05 were considered to be significant.

Results

The compound Me-NFM significantly induced an increase of MPO activity, whose results of the kinetic curve are presented in Figure 2A. This assay was carried out with the supernatant obtained from lung homogenate. The activation was significant only above 75 μM. The NFM maleimide also promoted the enzyme activation, although this result was not statis-

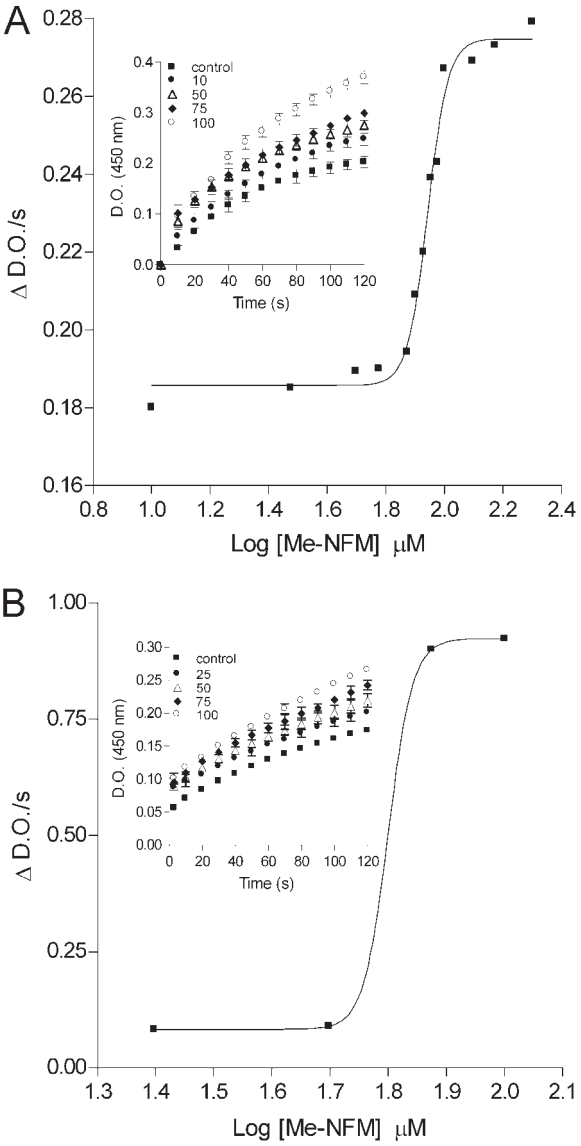

Fig. 2. Effect of Me-NFM maleimide on MPO activity. (**A**) Enzyme obtained from the lung supernatant, (**B**) Enzyme obtained commercially. The enzyme activities were measured spectrophotometrically following the oxidation of *o*-dianisidine by hypohalous acids produced by the enzymes. The values of the enzyme activities were calculated from the slope of the curves shown in the inset. Optical density of control groups was taken as 100% of oxidation of *o*-dianisidine. Data are presented as the mean ± SEM of three identical experiments performed in triplicate

tically significant (data not shown). In this assay, the Me-NFM increased effectively the MPO activity in a concentration-dependent manner ($K_{0.5}$ 84 ± 0.3 μM), showing a maximum induction (85%) at 100 μM.

The Me-NFM also induced an increase in activity of the purified MPO obtained commercially. The effect was significant from 50 μM (Fig. 2B). The activity increase was concentration-dependent and a $K_{0.5}$ of 62.85 ± 3.52 μM was obtained and showing a maximum induction (45%) at 100 μM. The results obtained with NFM were significant only with concentrations above 75 μM (data not shown).

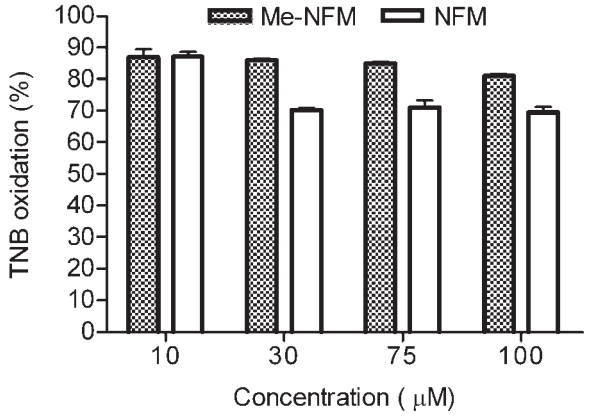

Fig. 3. Effect of Me-NFM and NFM maleimide on the oxidation of TNB into DTNB by HOCl. The assay was monitored spectrophotometrically at 412 nm. The amount of TNB unchanged after incubation was calculated and expressed as percentage of the initial value. Data are presented as the mean ± SEM of three identical experiments performed in triplicate

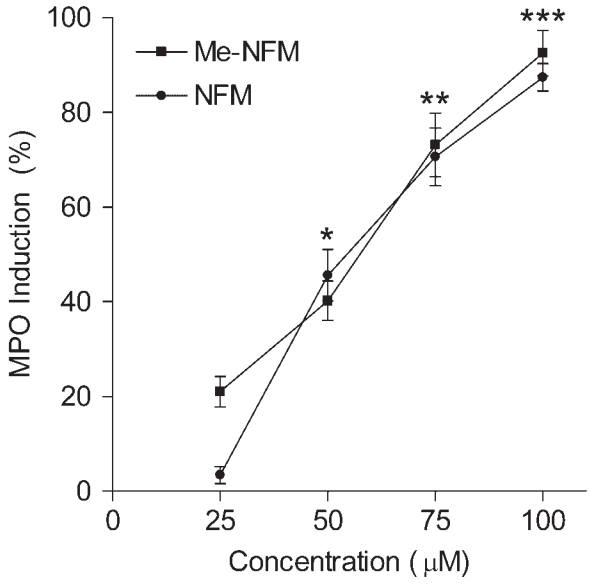

Fig. 4. Effect of Me-NFM and NFM maleimide on MPO activity after neutrophil lysing obtained from mouse air pouch model. The enzyme activity was measured spectrophotometrically following the oxidation of *o*-dianisidine by HOCl produced by the enzyme. Optical density of control groups was taken as 100% of oxidation of *o*-dianisidine. Data are presented as the mean ± SEM of three identical experiments. * $p < 0.05$; ** $p < 0.01$, and *** $p < 0.001$, compared to control groups, using ANOVA followed by the Dunnett's test

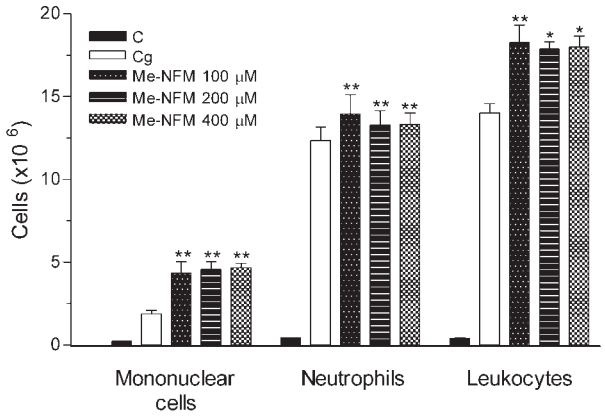

Fig. 5. Effect of Me-NFM maleimide (100 to 400 μM) on leukocyte migration in the mouse air pouch model. The compound was administered to different groups of animals 0.5 h before the inflammation induction by carrageenan (1%). C – response in animals treated only with sterile saline (NaCl 0.9 %). Cg – response in animals treated only with carrageenan. N = 5 animals. Data are presented as the mean ± SEM of three identical experiments. * $p < 0.05$; ** $p < 0.01$, compared to control groups, using ANOVA followed by the Dunnett's or Student's *t* tests

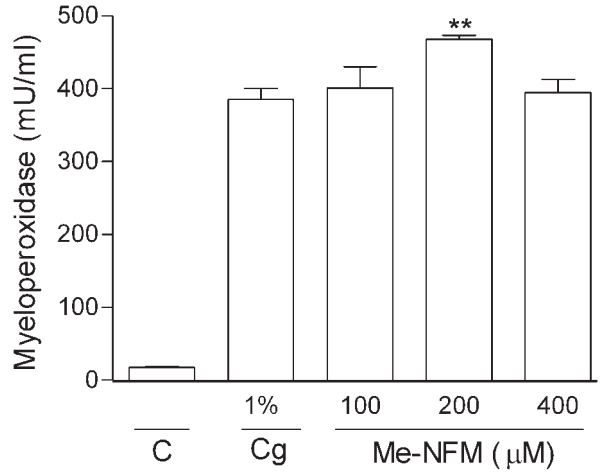

Fig. 6. Effect of different concentrations of Me-NFM on myeloperoxidase activity of neutrophils in a mouse air pouch model compared with carrageenan-only control (Cg). The enzyme activity was measured spectrophotometrically following the oxidation of *o*-dianisidine by HOCl produced by the enzyme. Optical density of control groups was taken as 100% of oxidation of *o*-dianisidine. Data are presented as the mean ± SEM of six identical experiments. ** $p < 0.01$, compared to control groups, using ANOVA followed by the Dunnett's test

Both malemides also promoted a significant increase (~30%) in horseradish peroxidase (HRP) activity tested up to 100 μM (data not shown), although with concentrations of maleimides higher than 50 μM, the HRP activity was drastically inhibited. This assay was accomplished to analyze the selectivity of the maleimides.

In Figure 3 are presented the poor HOCl scavenge activity of the compounds. They were tested up to 100 μM and in this condition the compounds were not able to scavenge significantly the reactive species.

The Me-NFM and also the NFM, caused a significant increase in myeloperoxidase activity at the concentrations studied (50 to 100 μM) when neutrophils were incubated with the compounds for 1 hour at 37°C in a 5% CO_2 humidified atmosphere (Fig. 4).

Furthermore, Me-NFM caused a significant increase of both total and differential leukocytes migration and MPO activity (*in vitro* and in *vivo* assays).

In the animals inflamed with carrageenan and pretreated with 4-methyl-N-phenylmaleimide (Me-NFM) (100–400 μM) an effective increase of leukocyte migration into the air pouch cavity was observed. The changes varied from 44.2 ± 3.6 to 47.5 ± 8.6% ($p < 0.01$), neutrophils from 26.6 ± 8.1 to 32.7 ± 11.0% ($p < 0.05$), and mononuclear cells from 130.1 ± 37.1 to 147.7 ± 14.8% ($p < 0.01$) in comparison with positive control group (animals treated only with carrageenan) (Fig. 5).

On the other hand, the inflamed animals pretreated with NFM at the same doses did not suffer changes in leukocyte migration in the case of neutrophils or mononuclear cells when compared with positive control group ($p > 0.05$) (data not shown).

Additionally, inflamed animals pretreated with Me-NFM at a dose of 200 μM also suffer a significant enhancement in the myeloperoxidase activity (~22%) ($p < 0.01$) (Fig. 6). On the other hand, inflamed animals pretreated with NFM at the same doses did not show changes in this inflammatory parameter ($p > 0.05$) (results not shown).

Discussion

Many *in vivo* and *in vitro* experiments are used to investigate the role of neutrophils in the inflammatory process, especially in relation to MPO activity. The ability to isolate murine neutrophils permits the search for new drugs with anti-inflammatory activity or new pharmacological tools to study the inflammatory process [9]. In this study, the activation of MPO enzyme was observed in the assay with isolated enzyme and that one obtained from lung supernatant

(Figs. 2A and 2B). The values of $K_{0.5}$ were 62.85 ± 3.52 μM and 84 ± 0.3 μM, respectively, for Me-NFM. The values of $K_{0.5}$ probably differ because in the supernatant there are other proteins.

In primary culture, when neutrophils were treated for 1 hour with Me-NFM, the total cellular MPO activity was determined in 1×10^6 neutrophils lysed with HTMAB plus *o*-dianisidine and H_2O_2 as the colorimetric agent for spectrophotometrical detection (Fig. 4). In this case the compounds showed similar performance in terms of MPO induction, since the $K_{0.5}$ obtained were similar: Me-NFM $K_{0.5}$ = 54.47 μM and NFM $K_{0.5}$ = 58.9 μM.

The compounds were also tested for their capability to scavenge HOCl showing very low reactivity with the reactive species. If the compounds were HOCl scavengers they could somehow change the reaction equilibrium of the enzyme.

The actions involving MPO activity were studied, in particular the inhibition of the enzyme as a potential target for the discovery of new drugs for anti-inflammatory therapies, since MPO-mediated damage is not limited to intraphagosomal microbes, but can trigger a variety of inflammatory diseases [2, 17, 28, 34].

The MPO released has been shown to be important in the leukocyte activation prior to neutrophils and adhesion molecules [10, 28]. MPO has also been described as an activator of the complement system cascade [20]. Many authors suggest that complete and partial myeloperoxidase deficiency is strongly associated with a high number of infections and cancer risk. MPO deficiency is a rare event, but humans in this condition have an increased susceptibility to systemic vasculitis [1] and infectious diseases.

HOCl is known for its ability to modify various molecules *via* chlorination. It is responsible for the sulfhydryl oxidation of plasma membrane proteins, inactivates α_1-antiprotease, activates collagenase, and depletes antioxidant substances [17].

MPO catalyzes the reaction of HOCl formation, a powerful oxidant agent, and it is the major antimicrobial component contained in neutrophils. The induction of MPO activity could be applied to support therapies, to enhance the efficacy of the microbicidal drugs. Therefore, is the induction or inhibition of MPO-activity good or bad? Although the MPO enzyme plays an important role in the body's defense mechanism, the main result of its activity is harmful to the human organism. In this case, MPO-induction may represent a good model system to investigate the molecular and cellular mechanisms of oxidative cell injury, induced by activated neutrophils, and the interactions between damaging species involved in the respiratory burst. The phorbol 12-myristate 13-acetate (PMA) is used to stimulate neutrophils and degranulation of specific granules in *in vivo* and *in vitro* tests, and the *N*-phenylmaleimide substances are a good alternative to enhance the activity of MPO, to simulate conditions of inflammation and sepsis. Also, it has been demonstrated that others substances such as cyclophosphamide metabolites that exert strong influence on the immune system by modulation of cytokines network [5] and carnosine that avoids age induced oxidative stress in erythrocytes [3].

N-phenylmaleimide appears to be a promising pharmacological tool to study new strategies for therapeutic interventions modulating the activity of MPO at different points: MPO active site blocking, irreversible inhibition through the use of oxidized inhibitors, and application of HOCl scavengers to prevent initiation and propagation of diseases triggered by this activation.

In vivo, the air pouch model of acute inflammation has been widely used to test the efficacy of anti-inflammatory drugs and the factors involved in inflammation due to a significant enhancement of neutrophils 24 h after carrageenan administration in the air pouch in mice. This inflammation model mimics the inflammatory response that occurs in patients with rheumatoid arthritis, an important inflammatory and chronic disease [29].

The enhancement of MPO levels is observed in the first phase of the inflammatory reaction in the animal inflammation models, such as pleurisy and air pouch, and the MPO levels are well correlated with neutrophil influx [4, 12].

In conclusion, the mechanism of MPO activation by *N*-phenylmaleimides is still a matter to be studied, however, they are good candidates as a pharmacological tool to study inflammatory reactions in animal inflammation models and *in vitro* tests, since the quantization of MPO mass and its activity, as well as analysis of its derivates (MPO-biomarkers), enables the discovery of new strategies for the treatment of inflammatory processes. In addition, to gaining a better understanding of the action of MPO and its effect on the body, its monitoring may serve as a prognosis of disease progression.

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