Action of the 4-Nitro-2-Phenoximethanesulphophonanilide (Nimesulide) on Neutrophil Chemotaxis and Superoxide Production

Study Carried out at the Laboratório de Investigação em Reumatologia (LIM-17) e Serviço de Reumatologia do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo. This research has been partially funded by Fundação para o Desenvolvimento da Reumatologia and by the Conselho dos Fundos Remanescentes da Sociedade Brasileira de Reumatologia.

4-nitro-2-phenoximethanesulphophonanilide (nimesulide) is a nonsteroidal anti-inflammatory agent that has been employed in the treatment of inflammatory diseases because of its specific actions on the inflammatory response mechanisms caused by injury. The objectives of this paper were to determine the action of this agent on two notable neutrophil functions, chemotaxis and production of the superoxide anion. These two functions were studied after the neutrophils were pre-incubated with three different concentrations of 4-nitro-2-phenoximethanesulphphonanilide (0.1; 0.3 and 0.5 mM). The results obtained herein demonstrated that 4-nitro-2-phenoximethansulphphonanilide-exposed peripheral blood neutrophils from healthy subjects produced significantly less superoxide when challenged by phorbolmyristate acetate (PMA at 50 ng/ml) or formyl-methionyl-leucyl-phenilalanine (FMLP 10⁻⁷ M) and opsonized zymosan (1mg/ml). Additionally, the agent was equally effective in reducing the PMN chemotaxis when challenged by C5a factor (2% zymosan activated solution), FMLP 10⁻⁹ M and leukotrien (3. 10⁻⁷ M). The results obtained suggest that in addition to its interference in the metabolism of the arachidonic acid, the 4-nitro-2-phenoximethanesulphophonanilide may interfere in a more direct fashion with the neutrophil function. This specific action may contribute to its anti-inflammatory activity.

Key words: neutrophil, superoxide, chemotaxis, 4-nitro-2-phenoximethanesulphophonanilide.

INTRODUCTION

Polimorphonuclear leukocytes (PMN) are essential elements for the complete inflammatory response. Accumulation of PMN leukocytes at the inflammatory site of injury is characteristic of the acute inflammatory response. During phagocytosis or upon chemical stimulation, PMN leukocytes not only releases several inflammatory substances as lisosomal enzymes and arachidonic acid metabolites, but it also exhibits an increase in oxygen consumption. The latter is recognized as “respiratory burst”(3,4). This increase in oxygen uptake generates the production of superoxide anion (O₂⁻), a free radical formed by a stepwise reduction of the molecular oxygen and its reaction products.

The oxidase enzyme system (nicotinamide, adenine dinucleotide phosphate, NADPH-oxidase), that is responsible by the PNM superoxide generation, is a multicomponent enzyme complex with cytosolic and membrane components. Following cellular activation, this complex becomes coupled to the plasma membrane, which also forms the lining membrane of the phagosomes (7,21,35).
The superoxide generated by the activated cells is released into the extracellular space and inside the phagocytic vesicles (17). This anion is not believed to be directly involved in bactericidal protection and tissue damage. However, its reactive by-products, namely, hydrogen peroxide, hydroxyl-radicals, hypochlorous acid, N-chloramine and perhaps singlet oxygen, all have an important role in microbicidal activity, inflammation, and in reperfusion injury (5,16,22,33). Whereas superoxide anion is able to inactivate endothelial-derived vascular relaxing factor, its by-products have the ability to reversibly or irreversibly damage compounds of all biochemical classes (22,23,25, 32). These are, nucleic acids, proteins, free amino acids, lipids, lipoproteins, carbohydrates, a large variety of cellular and extracellular macromolecules such as hyluronic acid and collagen (8, 20, 22, 23, 24, 25, 31, 32).

PMN leukocytes can be activated by chemotactic factors, aggregated immueglobulins and also by non-immune stimuli such as lecithin, ionophores and detergents (35,38). Once attracted to the inflammatory site, PMN leukocytes can cause irreversible tissue damage by means of releasing proteolytic enzymes and by means of the production of the so-called oxygen-derived active species (OAS). It has been proposed that some non-steroid anti-inflammatory drugs (NSAID), especially 4-nitro-2-phenoximethanesulphonanilide (nimesulide) may exert its therapeutic effects independently of suppression of prostaglandin synthesis (28).

These NSAID may act directly on the neutrophils inhibiting aggregation, chemotaxis to the inflammation site, lysosomal enzyme release, OAS production or by ameliorating the effects of OAS on tissue components (1,26,30). In keeping with this hypothesis, 4-nitro-2-phenoximethanesulphonanilide (nimesulide) administered in a pharmacologically active dose in rats displays an intermediate potency inhibiting prostaglandin synthetase (13,34). Additionally, it does not affect the level of cytoprotective prostaglandins or thromboxane B2 despite its potent anti-inflammatory effect (14,34).

Therefore, the present work was undertaken to determine the influences of 4-nitro-2-phenoximethanesulphonanilide on two different neutrophilic functions, namely, chemotaxis and superoxide production.

METHODS

Chemical reagents

The reagents were obtained from Sigma Chemical Company.

PMA (phorbol-mirystate-acetate), FMLP (formyl-methionil-leucyl-phenylalanine), zymosan, ferrocyanochrome C (type VI), leukotriene B4, SOD (superoxide dismutase).

Zymosan was employed in the final concentration of 1mg/ml after an incubation period for 30 minutes with fresh human serum (2:1w/v) at 37°C, washed twice and resuspended in HBSS.

PMA and FMLP were dissolved in DMSO (dimethyl sulfoxide) and subsequently diluted in medium with a resulting concentrations of DMSO (0.1% or less). These concentrations did not produce any detectable effects on PNM viability or any effects of cytochrome C reduction. The final concentrations in the superoxide assay were PMA=50 ng/ml; FMLP = 10^-7 M, ferrocyanochrome C=100μM; superoxide dismutase final concentration =40μM/ml.

In the migratory assay, the stimulant substances were: C5a = 2% zymosan activated plasma; FMLP = 1 10^-5 M; LTB4 = 3 , 10^-7 M.

The drug 4-nitro-2-phenoximethanesulphonanilide (nimesulide) was employed in different concentrations near to therapeutic doses.

Isolation of PNM leukocytes

Peripheral blood with heparin was obtained from healthy subjects and PNM leukocytes were isolated by Ficoll-Hypaque density gradient as described by Boyum (11). Mononuclear cells were removed and red cells were lysed twice in the PMN-red cell pellet with a cold isotonic NH4Cl solution. The cells were washed twice with a Hanks balanced salt solution (HBSS) and resuspended with the same buffer solution supplemented with a 10% heat-inactivated fetal calf serum. Subsequently, the cells were counted and cell concentration was adjusted to 10^6 cells/ml. Cellular viability was confirmed by the Trypan blue dye exclusion method.

4-nitro-2-phenoximethanesulphonanilide (nimesulide)

PNM from healthy subjects were incubated with different concentrations of 4-nitro-2-phenoximethanesulphonanilide (0.1; 0.3 and 0.5 mM) for 30 minutes at 37°C before the superoxide production and chemotaxis assays.

Superoxide production assay

After the PMN were incubated with 4-nitro-2-phenoximethanesulphonanilide (nimesulide) superox-
ide ($O_2^-$) production was measured as superoxide dismutase inhibitable reduction of cytochrome C (37). The reaction mixture contained 10⁶ non-stimulated and stimulated neutrophils in HBSS and 100μmol of cytochrome C. In order to control 90U samples, SOD was added to determine the level of nonspecific cytochrome C reduction. The mixtures were incubated at 37°C for 20 minutes and the reaction was arrested by placing the tubes in a frozen solution. The absorbance of the supernatant fluids was determined spectrophotometrically at 550nm.

PMA 50 ng/ml, FMLP (10⁻⁷ M) or opsonized zymosan (1mg/ml) were used for cell stimulation diluted in HBSS. The amount of produced superoxide was calculated using an extinction coefficient of 21.1 mM·cm⁻¹ (36). The percentage decrease in the amount of $O_2^-$ produced in the presence of the drug was calculated relative to control tubes. These tubes demonstrated no spontaneous reduction of ferricytochrome C by a stimulus substance or drugs in the absence of PMN and no significant PMN activity in the absence of stimuli.

**Chemotaxis assay**

The migratory assay was performed employing a multiwell Boyden chamber as reported elsewhere (29). Aliquots of cell suspension containing 1.5 millions neutrophils were placed in the upper chamber that was separated from the chemotactic agent in the lower chamber by a 8μm average pore size nitrate filter. The chemotactic agent was substituted by HBSS to measure random migration. Stimuli substances used were FMLP (10⁻⁴ M), LTB4 (3 · 10⁻⁷ M) and C5a 25 of zymosan activated plasma. The cells were allowed to migrate in humidified air for 60 minutes at 37°C. Removal of the filters for fixation and staining of the cells followed. Neutrophil migration within the filter was determined under a light microscope employing the “leading front method” (39). The distance from the top of the filter to the farthest point containing two cells was measured under a 40 X magnification light microscope objective.

Duplicate wells were always run for each individual variable. Five fields were counted and averaged for each filter.

**Statistical analysis.**

Means and standard errors of means (S.E.M.) of all data are presented and compared using the Student’s t test or analysis of variance with the significant probability levels of less than 0.05.

**RESULTS**

**SUPEROXIDE PRODUCTION BY NEUTROPHILS**

The production of superoxide by PMN leukocytes from healthy subjects was efficiently reduced after the pre-incubation with 4-nitro-2-phenoximethanesulphonanilide (nimesulide) at 0.3 and 0.5 mM. This reduction was more remarkable when induced by PMA (50ng/ml) and it was also observed when FMLP (10⁻⁷ M) and opsonized zymosan were employed as stimuli (figure 1). The inhibition of superoxide production by 4-nitro-2-phenoximethanesulphonanilide (nimesulide) at 0.1mM was statistically significant only when induced by FMLP.

**INFLUENCE OF 4-NITRO-2-PHENOXIMETHANESULPHONANILIDE ON CHEMOTAXIS**

In order to investigate the influence of 4-nitro-2-phenoximethanesulphonanilide on chemotaxis, neutrophils were incubated with the drug for 30 minutes at 37°C and immediately resuspended in HBSS with 1% bovine serum albumin for testing. The results depicted in figure 2 indi-

![Figure 1 - Superoxide production anion (O2-) by PMN from healthy subjects pre-incubated for 30 minutes at 37°C, with medium; and with phenoximethanesulphonanilide at 0.1; 0.3; 0.5 mM; PMA at 50 ng/ml; FMLP at 10⁻⁷ M and opsonized zymosan at 1mg/ml. These compounds were employed as stimuli. Values are shown as mean + S.E.M. of the seven individual experiments performed in duplicate. * < 0.05.](image-url)
cate that the migratory assay was performed with C5a (25 zymosan activated plasma, FMLP 10-9 M), LTB4 3.10-7 M). The drug 4-nitro-2-phenoximethanesulphonanalide was inhibitory in all concentrations tested.

DISCUSSION

The results obtained herein demonstrate that 4-nitro-2-phenoximethanesulphonanalide (nimesulide) has a broader but less specific inhibitory action on two PMN functions, namely, chemotaxis and superoxide production. This action proved to occur independently of the type of stimulus employed to activate the neutrophil.

PMN cells were stimulated by different substances with different mechanisms of action.

PMA, opsonized zymosan, FMLP, LTB4, C5a (2% zymosan activated plasma). PMA is believed to directly activate the intracellular protein kinase C bypassing a receptor-mediated signal transduction (19,27). Zymosan stimulates phagocytosis, the complement activation product C5a, LTB4, and the synthetic polypeptide FMLP derived from bacterial endotoxin, all react with distinct and specific sites on the PMN cell membrane (2,6,15,39).

4-nitro-2-phenoximethanesulphonanalide was tested in near therapeutic doses and as in other reports, the authors herein verified no superoxide production activity with 4-nitro-2-phenoximethanesulphonanalide concentrations below 10^{-4} M. The inhibitory drug effect observed on superoxide production and chemotaxis was dose dependent.

The neutrophil responses, chemotaxis and superoxide production both inhibited by 4-nitro-2-phenoximethanesulphonanalide are controlled at different cellular levels of the intracellular signal transduction process. The initial steps of the sequence are shared by both responses (binding of an agonist to its receptor and the interaction of the ligand-receptor complex with a GTP-binding protein). However, the shape change is unaffected by depletion of cytosolic calcium levels and by inhibitors of the protein kinase C while the superoxide production is inhibited under this condition (6,7). It is believed that 4-nitro-2-phenoximethanesulphonanalide interferes with superoxide generation through the decreased protein kinase C translocation from cytosol to neutrophil membranes which has no superoxide scavenger activity (9,10,12). The reduced neutrophil chemotactic activity produced by 4-nitro-2-phenoximethanesulphonanalide may be explained on the same basis. More clear studies are necessary.

The data reported herein support the conclusion of other studies which evaluated the 4-nitro-2-phenoximethanesulphonanalide's inhibitory activity on proteinase release from human leukocytes as well as of chemoluminescence of human leukocytes, rats peritoneal and broncho-alveolar leukocytes. The drug provoked a broad but less specific suppression of these responses, independently of the cell type, its stage of activation and the stimuli employed for leukocyte activation. Previous studies have also demonstrated the 4-nitro-2-phenoximethanesulphonanalide inhibitory action on superoxide production by human neutrophil induced by PMA, FMLP and by zymosan (9,10,12,17).

Our results showed that 4-nitro-2-phenoximethanesulphonanalide produced neutrophil chemotaxis inhibition. Capsoni et al observed no 4-nitro-2-phenoximethanesulphonanalide activity on PMN chemotaxis after cell stimulation performed as in the study herein (zymosan-activated serum and FMLP). However, the different micropore filter size (3μm by 8μm in diameter) and the higher concentration of the chemical compound employed in Capsoni's assays (FMLP at 10^{-8} M and 10% zymosan activated serum against FMLP at 10^{-4} and 2% zymosan activated serum) may account for these conflicting results. Previous assays in our laboratory also failed to demonstrate a 4-nitro-2-phenoximethanesulphonanalide inhibitory activity employing stimulant compounds in high concentrations.

Figure 2 – Chemotactic response of the neutrophils obtained from healthy subjects and pre-incubated with medium; and with phenoximethanesulphonanalide at 0.1; 0.3; 0.5 mM. Chemoattractants used were C5a (2% zymosan activated serum), FMLP at 10^{-9} M and LTB4 at 3·10^{-7} M. Values are shown as mean ± S.E.M. of the seven individual experiments performed in duplicate. * p < 0.05.
Our data suggest that inhibition of chemotaxis and superoxide production by stimulated neutrophils at the inflammatory site could be an additional anti-inflammatory mechanism by which the 4-nitro-2-phenoximethanesulphonanilide (nimesulide) works.

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REFERENCES


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**RESUMO**

O 4-nitro-2-fenoximetanosulfonanilide (nimesulide), antiinflamatório não esteróide, vem sendo utilizado no tratamento de quadros inflamatórios devido à sua atividade farmacológica nestes mecanismos de resposta as injúrias flogísticas. O objetivo do presente trabalho é estudar a ação deste fármaco sobre duas importantes funções neutrofílicas: quimiotaxia e produção de ânion superóxido após pré-incubação com doses crescentes da droga (0,1; 0,3 e 0,5mM). Os resultados obtidos demonstram que a pré-incubação de PMNs coletados do sangue periférico de indivíduos normais com o 4-nitro-2-fenoximetanosulfonanilide reduz significativamente a produção de superóxido por estimulação com acetato de forbol miristato (PMA 50ng/ml), formil metionil leucil fenilalanina (FMLP 10^{-7}M e Zimozan oxiposazonado (1mg/ml). Adicionalmente o fármaco se mostrou igualmente efetivo em reduzir a habilidade quimiotática de PMNs, frente ao fator CSA (2% de soro ativado por zimozan). FMLP (10^{-9}M) e leucotrieno B4 (3x10^{-7}M). Os resultados obtidos sugerem que além da sua atividade a nível do metabolismo do ácido araquidônico, o 4-nitro-2-fenoximetanosulfonanilide possa interferir diretamente sobre a função neutrofílica, o que provavelmente contribui para sua ação antiinflamatória.