

# Effect of therapeutic plasma concentrations of non-steroidal anti-inflammatory drugs on the production of reactive oxygen species by activated rat neutrophils

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## Abstract

The release of reactive oxygen specie (ROS) by activated neutrophil is involved in both the antimicrobial and deleterious effects in chronic inflammation. The objective of the present investigation was to determine the effect of therapeutic plasma concentrations of non-steroidal anti-inflammatory drugs (NSAIDs) on the production of ROS by stimulated rat neutrophils. Diclofenac (3.6  $\mu$ M), indomethacin (12  $\mu$ M), naproxen (160  $\mu$ M), piroxicam (13  $\mu$ M), and tenoxicam (30  $\mu$ M) were incubated at 37°C in PBS (10 mM), pH 7.4, for 30 min with rat neutrophils ( $1 \times 10^6$  cells/ml) stimulated by phorbol-12-myristate-13-acetate (100 nM). The ROS production was measured by luminol and lucigenin-dependent chemiluminescence. Except for naproxen, NSAIDs reduced ROS production: 58  $\pm$  2% diclofenac, 90  $\pm$  2% indomethacin, 33  $\pm$  3% piroxicam, and 45  $\pm$  6% tenoxicam (N = 6). For the lucigenin assay, naproxen, piroxicam and tenoxicam were ineffective. For indomethacin the inhibition was 52  $\pm$  5% and diclofenac showed amplification in the light emission of 181  $\pm$  60% (N = 6). Using the myeloperoxidase (MPO)/H<sub>2</sub>O<sub>2</sub>/luminol system, the effects of NSAIDs on MPO activity were also screened. We found that NSAIDs inhibited both the peroxidation and chlorinating activity of MPO as follows: diclofenac (36  $\pm$  10, 45  $\pm$  3%), indomethacin (97  $\pm$  2, 100  $\pm$  1%), naproxen (56  $\pm$  8, 76  $\pm$  3%), piroxicam (77  $\pm$  5, 99  $\pm$  1%), and tenoxicam (90  $\pm$  2, 100  $\pm$  1%), respectively (N = 3). These results show that therapeutic levels of NSAIDs are able to suppress the oxygen-dependent antimicrobial or oxidative functions of neutrophils by inhibiting the generation of hypochlorous acid.

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Research supported by Laboratório  
de Análises Clínicas, Faculdade  
de Ciências Farmacêuticas  
(NAC-FCF), UNESP, and CAPES.

Publication supported by FAPESP.

Received April 22, 2004  
Accepted January 14, 2005

## Key words

- Non-steroidal anti-inflammatory drugs
- Neutrophils
- Myeloperoxidase
- Hypochlorous acid
- Reactive oxygen species
- Chemiluminescence

## Introduction

The innate immune response comprises a series of events which prepare the organism to act against intruding agents. The liberation of vasoactive substances and acute phase protein and the migration of neutrophil polymorphonuclear leukocytes (PMN) to the inflammatory site are among the first events when pathogens, chemical agents and physical factors challenge the body. Indeed, neutrophils, which represent 50 to 70% of the total circulating leukocytes, constitute the first line of defense against infectious agents or non-self substances that penetrate the body's physical barrier (1). Upon activation, non-mitochondrial oxygen uptake is initiated by the PMN, resulting in the production of reactive oxygen species (ROS). This process, known as oxidative burst, is the result of the assembly of the multi-enzyme NADPH-oxidase system that promotes the one-electron reduction of oxygen to superoxide anion (2). Next, this species is reduced to hydrogen peroxide in a reaction catalyzed by superoxide dismutase and finally hydrogen peroxide is used by the enzyme myeloperoxidase (MPO) to oxidize chloride to hypochlorous acid (HOCl) (3-5). This highly oxidant chemical has been proposed to be the main agent responsible for the antimicrobial action of PMN. However, it is also an important component regarding its action on the tissue damage that characterizes chronic inflammation. HOCl reacts with unsaturated fatty acids and cholesterol generating chlorohydrins, promotes the oxidation of proteins through the chlorination of tyrosine and tryptophan residues and mediates the production of protein carbonyls via the breakdown of chloramines to aldehydes (6-10). Moreover, there is accumulating evidence demonstrating that some intracellular signaling pathways are redox sensitive and ROS are involved in regulating the production of some biochemical mediators of inflammation (11,12). In this respect, non-steroidal anti-inflammatory drugs (NSAIDs) have been studied in terms of their effect on ROS produc-

tion since, in addition to having a widely accepted mechanism of action by inhibition of the enzymes involved in the production of pro-inflammatory lipid-derived mediators, these compounds may also interact with the oxidants produced by phagocytic cells (13-16).

In the present investigation, we studied the effect of therapeutic plasma concentrations of NSAIDs on ROS generation by stimulated rat neutrophils. Using the purified MPO model we found that the NSAIDs are both able to inhibit the formation of HOCl and to scavenge it. Additionally, diclofenac has a pro-oxidant activity by promoting the generation of superoxide anion.

## Material and Methods

### Chemicals

Phorbol-12-myristate-13-acetate (PMA), luminol, lucigenin, sodium borohydride, 5,5'-dithio-bis(2-nitrobenzoic acid, DTNB), sodium hypochloride, and Ficoll-Hypaque 1077 were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide (30%) was purchased from Peróxidos do Brazil (São Paulo, SP, Brazil). MPO (E.C. 1.11.1.7) was purchased from Calbiochem (San Diego, CA, USA). Diclofenac and naproxen were purchased from Purifarma S.A. (São Paulo, SP, Brazil), piroxicam from Natural Farma S.A. (Belém, PA, Brazil), tenoxicam from S.P. Farma (São Paulo, SP, Brazil) and indomethacin from Sigma. All the reagents used for buffer preparation were of analytical grade.

### Isolation and purification of rat neutrophils

A 5% oyster glycogen solution (dissolved in 0.85% NaCl) was injected into the peritoneum of anesthetized rats. The animals were kept with food and liquid *ad libitum* and sacrificed 12 h later and 20 ml calcium-free Dulbecco's phosphate-buffered saline (PBS-D) (17) containing 10 IU heparin per ml was injected into the peritoneal cavity. The exu-

date was collected and centrifuged for 3 min at 200 g. The cell pellet was layered on Ficoll-Hypaque 1077 and centrifuged for 25 min at 1000 g. PMN cells were collected, washed and kept in ice-cold PBS-D until required.

#### Cytotoxic effect of NSAIDs

PMN ( $1 \times 10^6$  cells/ml) were incubated at 37°C in PBS-D with or without the NSAIDs for 30 min. Twenty-five microliters of the supernatant was used to measure the activity of lactate dehydrogenase (18).

#### Chemiluminescence

PMN ( $1 \times 10^6$  cells/ml) were pre-incubated at 37°C in PBS-D with 10  $\mu\text{mol/l}$  luminol or 10  $\mu\text{mol/l}$  lucigenin and with therapeutic or 10 times higher concentrations of the NSAIDs (tests) or without the NSAIDs (controls) for 30 min. The reaction was started by adding 100 nmol/l PMA and chemiluminescence was measured for 30 min (19).

#### Chemiluminescence assay using purified MPO

*Chlorinating activity.* MPO (50 mU/ml) was incubated at 37°C in PBS-D with 6  $\mu\text{mol/l}$  luminol and 67  $\mu\text{mol/l}$   $\text{H}_2\text{O}_2$  plus the NSAIDs (tests) or without the NSAIDs (controls). The reaction was started by adding  $\text{H}_2\text{O}_2$  and chemiluminescence measured for 5 min.

*Peroxidation activity.* The assay was carried out as described for chlorinating activity, but chloride was absent in the buffer (PBS/ $\text{MgSO}_4$ ). The reactions were carried out in a BIOOrbit model 1251 luminometer (Turku, Finland) (20).

#### Oxygen uptake by neutrophils

PMN ( $4 \times 10^6$  cells/ml) were incubated at 37°C in PBS-D with or without 36  $\mu\text{mol/l}$  diclofenac. A Yellow Spring 5300 oxygen

monitor (YSI, Cincinnati, OH, USA) was set at 100% and the reaction was started by adding 100 ng/ml PMA.

#### Hypochlorous acid scavenging (5-thio-2-nitrobenzoic acid assay)

*Control.* The absorbance of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm was measured before and after the addition of 15  $\mu\text{mol/l}$  HOCl to 48  $\mu\text{mol/l}$  TNB in PBS-D at 37°C. The decrease in absorbance at 412 nm is correlated with the concentration of HOCl.

*Tests.* The NSAIDs were incubated at 37°C in PBS-D containing 15  $\mu\text{mol/l}$  HOCl for 10 min. Then 48  $\mu\text{mol/l}$  TNB was added and absorbance was measured. The scavenging of HOCl by NSAIDs is measured by the reduction of absorbance at 412 nm using the control as reference. TNB solutions were prepared daily by reduction of DTNB as described (21).

#### Statistical analysis

Data are reported as means  $\pm$  SD and the Student *t*-test was used to determine the difference between test and control preparations, with the level of significance set at  $P < 0.05$ .

#### Results

The NSAID concentrations reported here are the mean plasma level detected after administration of these drugs at their therapeutic oral dosage in different inflammatory diseases (22-24).

The toxicity of the drugs was assessed since their inhibitory effects on ROS production may be linked to non-stimulated neutrophil dysfunction. The assays were performed by measuring the liberation of the enzyme lactate dehydrogenase when the cells were incubated with the drugs (18). The concentrations of NSAIDs tested here were not toxic to the cells (Table 1).

Table 1. Cytotoxic effect of NSAIDs on rat neutrophils.

NSAID ( $\mu\text{M}$ )	Lactate dehydrogenase activity (U/l)
Diclofenac	
3.6 <sup>a</sup>	17 $\pm$ 3
36 <sup>b</sup>	22 $\pm$ 3
Indomethacin	
12 <sup>a</sup>	22 $\pm$ 1
120 <sup>b</sup>	27 $\pm$ 5
Naproxen	
160 <sup>a</sup>	20 $\pm$ 4
1600 <sup>b</sup>	25 $\pm$ 5
Piroxicam	
13 <sup>a</sup>	28 $\pm$ 2
130 <sup>b</sup>	32 $\pm$ 5
Tenoxicam	
30 <sup>a</sup>	24 $\pm$ 4
300 <sup>b</sup>	26 $\pm$ 3

Data are reported as the means  $\pm$  SD of 6 experiments. Lactate dehydrogenase activity was measured in the extracellular medium when neutrophils were incubated with NSAIDs. <sup>a</sup>Therapeutic plasma concentration; <sup>b</sup>ten times higher than the therapeutic plasma concentration. The control in the absence of NSAIDs was 19  $\pm$  1 U/l and neutrophil disrupted by sonication was 95  $\pm$  6 U/l. NSAIDs = nonsteroidal anti-inflammatory drugs.

Table 2. The effect of NSAIDs on luminol- and lucigenin-dependent neutrophil chemiluminescence.

NSAID ( $\mu\text{M}$ )	Luminol (% inhibition)	Lucigenin (% inhibition)
Diclofenac		
3.6 <sup>a</sup>	58 $\pm$ 2 *	Amplified <sup>c</sup>
36 <sup>b</sup>	65 $\pm$ 10 *	Amplified <sup>d</sup>
Indomethacin		
12 <sup>a</sup>	90 $\pm$ 2 *	52 $\pm$ 5 *
120 <sup>b</sup>	99 $\pm$ 2 *	72 $\pm$ 6 *
Naproxen		
160 <sup>a</sup>	NS	NS
1600 <sup>b</sup>	42 $\pm$ 7 *	NS
Piroxicam		
13 <sup>a</sup>	33 $\pm$ 3 *	NS
130 <sup>b</sup>	48 $\pm$ 2 *	NS
Tenoxicam		
30 <sup>a</sup>	45 $\pm$ 6 *	NS
300 <sup>b</sup>	61 $\pm$ 2 *	NS

Data are reported as the means  $\pm$  SD of 6 experiments. <sup>a</sup>Therapeutic plasma concentration; <sup>b</sup>ten times higher than the therapeutic plasma concentration; <sup>c</sup>amplification of 181  $\pm$  60%; <sup>d</sup>amplification of 280  $\pm$  90%. NSAIDs = nonsteroidal anti-inflammatory drugs.

\*P < 0.05 compared to controls: 3.0  $\pm$  0.7  $\times 10^5$  (mV·s) for luminol assay or 5.6  $\pm$  1.6  $\times 10^4$  (mV·s) for lucigenin assay (Student *t*-test). NS = nonsignificant.

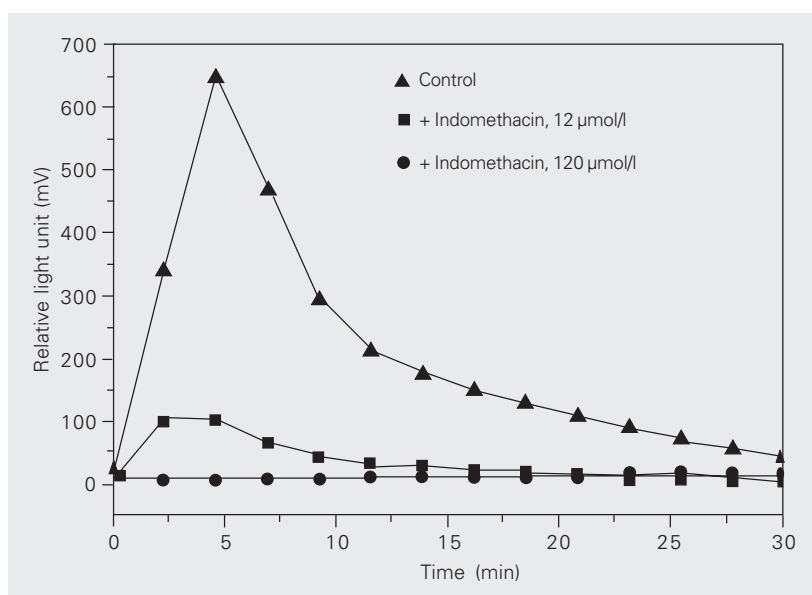


Figure 1. Typical light emission of luminol-dependent neutrophil chemiluminescence and inhibition by indomethacin. The neutrophils were stimulated with phorbol-12-myristate-13-acetate. The traces are representative of 6 experiments.

The effects of the NSAIDs at their therapeutic or 10 times higher plasma concentrations on total ROS production were studied in luminol- or lucigenin-dependent chemiluminescence assays of rat neutrophils stimulated with PMA. The reactions were triggered by the stimulus and monitored for 30 min. The integrated light emission was taken as the analytical parameter. A typical light-emission profile and the suppressive effect of indomethacin at the therapeutic or 10 times higher plasma concentration are shown in Figure 1 for illustration.

Table 2 presents the inhibitory effects on luminol and lucigenin chemiluminescence observed when the NSAIDs were added to the reaction medium. In agreement with results reported for human neutrophils, NSAIDs at therapeutic concentrations are effective inhibitors of luminol chemiluminescence.

cence (19,25,26). Except for indomethacin and diclofenac, lucigenin chemiluminescence was not affected by NSAIDs. Moreover, diclofenac had the opposite effect, increasing lucigenin chemiluminescence. Since lucigenin is supposed to be a specific probe for superoxide anion (27-29), which is the primary reactive species formed during the oxidative burst, we also studied the effect of these drugs on oxygen consumption by activated neutrophils. In agreement with the chemiluminescence assay, diclofenac had a small but statistically significant ( $P < 0.05$ ) promoting effect on superoxide anion generation, since the oxygen uptake increased (Figure 2).

The luminol-dependent chemiluminescent assay lacks specificity regarding the ROS generated upon neutrophil activation (30); therefore, experiments were performed to determine the effect of the drugs on the activity of the MPO/H<sub>2</sub>O<sub>2</sub> enzymatic system. Two parameters were studied, i.e., peroxidation activity and chlorinating activity, in the absence and in the presence of chloride anion in the buffer, respectively. The reactions were monitored for 5 min and triggered by adding H<sub>2</sub>O<sub>2</sub>. The integrated light emission was taken as the analytical parameter. Both the peroxidation and chlorinating activities were strongly inhibited by the NSAIDs at the concentrations tested (Table 3).

The scavenging of pure HOCl by the NSAIDs was studied in the TNB assay. In this experiment, the suppression of TNB oxidation to DTNB is an indication of the capacity of NSAIDs to scavenge HOCl. For illustration, a typical absorption spectrum showing the oxidation of TNB to DTNB by HOCl and the effect of diclofenac is shown in Figure 3. The HOCl scavenging property of the NSAIDs studied here is shown in the Table 3. Piroxicam, tenoxicam and the higher dose of diclofenac were able to react with HOCl. On the other hand, indomethacin and naproxen did not scavenge HOCl in a statistically significant manner (Table 3).

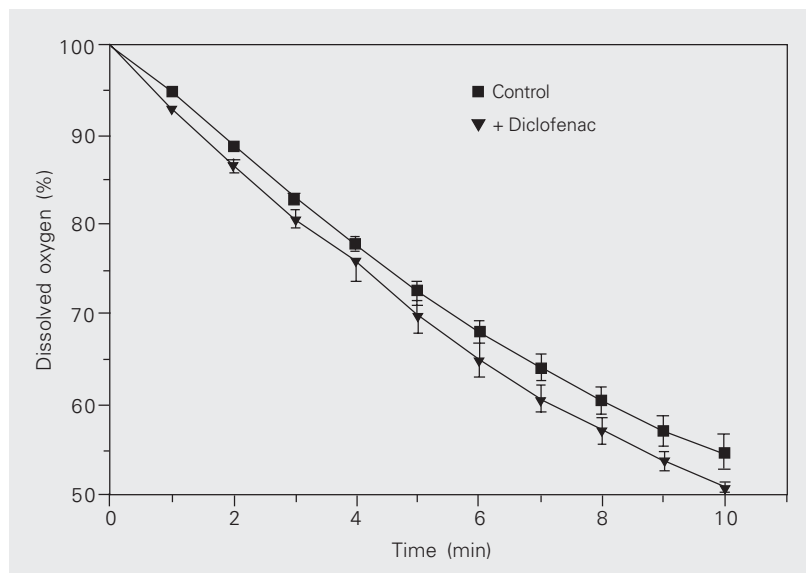


Figure 2. Effect of diclofenac on oxygen uptake by stimulated rat neutrophils. The traces are representative of 3 experiments.

Table 3. Inhibition of the peroxidation and chlorinating activities of myeloperoxidase by NSAIDs and reactivity with hypochlorous acid.

NSAID ( $\mu\text{M}$ )	Chlorination activity (% inhibition)	Peroxidation activity (% inhibition)	HOCl <sup>c</sup> ( $\mu\text{M}$ ) (scavenging)
Diclofenac			
3.6 <sup>a</sup>	45 $\pm$ 3 *	36 $\pm$ 10 *	NS
36 <sup>b</sup>	86 $\pm$ 1 *	71 $\pm$ 2 *	10.8 $\pm$ 1.5 *
Indomethacin			
12 <sup>a</sup>	100 $\pm$ 1 *	97 $\pm$ 2 *	NS
120 <sup>b</sup>	100 $\pm$ 1 *	99 $\pm$ 1 *	NS
Naproxen			
160 <sup>a</sup>	76 $\pm$ 3 *	56 $\pm$ 8 *	NS
1600 <sup>b</sup>	85 $\pm$ 3 *	62 $\pm$ 2 *	NS
Piroxicam			
13 <sup>a</sup>	99 $\pm$ 1 *	77 $\pm$ 5 *	11.7 $\pm$ 1.4 *
130 <sup>b</sup>	100 $\pm$ 2 *	89 $\pm$ 2 *	12.4 $\pm$ 1 *
Tenoxicam			
30 <sup>a</sup>	100 $\pm$ 1 *	90 $\pm$ 2 *	11.7 $\pm$ 1 *
300 <sup>b</sup>	100 $\pm$ 2 *	97 $\pm$ 1 *	12.7 $\pm$ 1.3 *

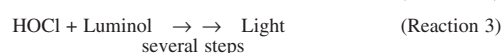
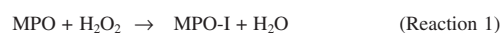
Data are reported as the means  $\pm$  SD of 6 experiments. <sup>a</sup>Therapeutic plasma concentration; <sup>b</sup>ten times higher than the therapeutic plasma concentration; <sup>c</sup>consumption of HOCl by NSAIDs after incubation for 10 min detected by the 5-thio-2-nitrobenzoic acid assay (see Experimental section). HOCl = hypochlorous acid; NSAIDs = nonsteroidal anti-inflammatory drugs.

\* $P < 0.05$  compared to controls:  $1.3 \pm 0.2 \times 10^5$  (mV·s) for chlorination activity or  $1270 \pm 160$  (mV·s) for peroxidation activity (Student *t*-test). NS = nonsignificant.

## Discussion

It has been reported that some NSAIDs act as scavengers of ROS produced when neutrophils are stimulated with opsonized zymosan or via the soluble PMA stimulus (19,25,26). However, there is no report about whether their therapeutic plasma concentrations are able to affect neutrophil oxidative function. In the present study, we advanced in this area by studying the effects of therapeutic plasma concentrations of NSAIDs on ROS production by stimulated neutrophils. Besides the cell system assays, we also assessed the properties of NSAIDs as MPO inhibitors by the luminol-dependent chemiluminescent technique. We found that, except for naproxen, the therapeutic plasma concentrations of the NSAIDs tested here are sufficient to inhibit the luminol-dependent chemiluminescence generated by activated neutrophils. Similar results were obtained using cell-free systems, in which the pres-

ence or absence of chloride in the buffer dictates the mechanism of luminol chemiluminescence. In fact, it has been demonstrated that HOCl oxidizes luminol when the MPO/H<sub>2</sub>O<sub>2</sub> system is operating in chloride-containing buffers (Reactions 1-3). In contrast, in the absence of chloride, luminol oxidation occurs through a classical peroxidation mechanism via the intermediate compound-I and compound-II of MPO (Reactions 1 and 4-6) (31).



This property of MPO-catalyzed luminol oxidation is useful to assess whether a molecule is able to compete for the active site of the enzyme or whether it is only able to react with the generated HOCl. Here we obtained a similar inhibition pattern for each NSAID studied in both assays, which indicates that these compounds at their therapeutic plasma concentration are able to compete with chloride for the catalytic site of MPO, leading to the inhibition of HOCl production. Besides inhibiting HOCl production, therapeutic plasma levels of piroxicam and tenoxicam and the 10 times higher concentration of diclofenac were also able to react with pure HOCl. This additional chemical property of some NSAIDs could contribute to the suppression of this oxidant species at the inflammatory site.

As demonstrated by lucigenin-dependent chemiluminescence, the drugs tested here were less effective inhibitors of superoxide anion. Only indomethacin was able to inhibit its generation in a statistically significant manner. Interestingly, diclofenac was able to promote the generation of superoxide anion

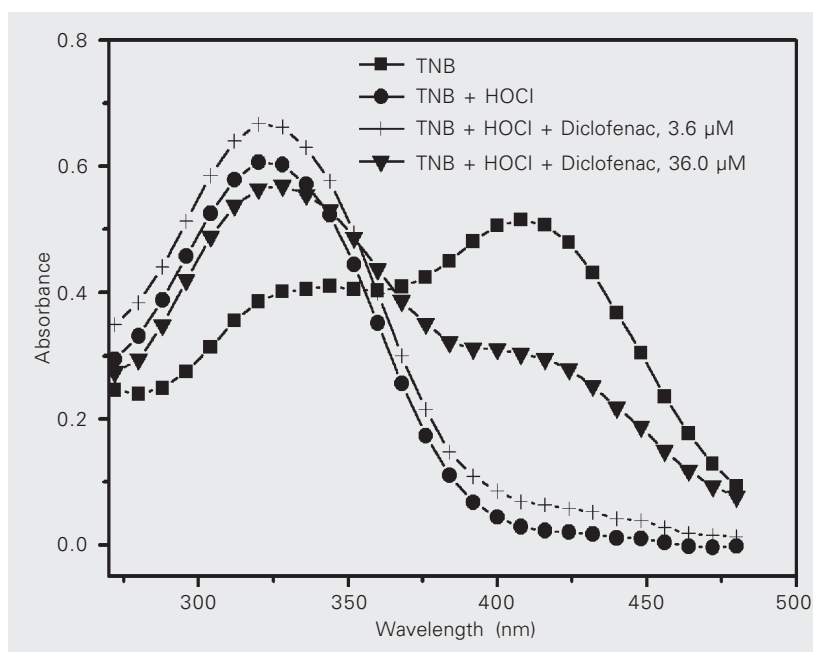


Figure 3. Scavenger of HOCl by diclofenac (TNB assay). The concentrations of diclofenac represent the therapeutic dose and a dose 10 times higher. The reduction of the absorbance decreases at 412 nm compared to control (TNB + HOCl trace) denotes the scavenging property of the diclofenac. The traces are representative of 6 experiments. TNB = 5-thio-2-nitrobenzoic acid; HOCl = hypochlorous acid.

instead of inhibiting it and this effect seems to be related to the activation of NADPH-oxidase, as detected by the increased oxygen uptake by the stimulated neutrophils. These results show that further experiments are needed to identify the effects of NSAIDs on the neutrophil oxidative burst. Indeed, the reports are contradictory about the effect of diclofenac and indomethacin on ROS generation. For instance, Al-Arfaj et al. (32) reported an increase in both isolated neutrophil and whole blood chemiluminescence when subjects were treated with diclofenac. Similar results were reported for indomethacin when superoxide anion generated by neutrophils was measured by the reduction of ferricytochrome C (33). However, Parij et al. (19) have reported chemiluminescence suppression by both the luminol and lucigenin techniques.

Taken together, the results obtained in the luminol and lucigenin assays lead us to conclude that the major effect of NSAIDs on oxygen-dependent antimicrobial or oxidative neutrophil functions was to impair the generation of HOCl by competing with chloride for the active site of MPO without inhibiting the NADPH-oxidase enzymatic complex, except for indomethacin.

It was not our aim in this investigation to determine the reasons why some NSAIDs are more potent MPO inhibitors than others and additional experiments are needed to address this question. Nevertheless, the results showed that phenol derivatives such as piroxicam and tenoxicam were more effective than non-phenol structured compounds as diclofenac and naproxen, despite the higher concentration used for the last one. This is not unexpected since phenol compounds are typical MPO substrates (34). Moreover, indomethacin, which is also an MPO substrate, was as efficient as piroxicam and tenoxicam (15).

The results obtained for the cell system were more complex. Again, naproxen was an ineffective MPO inhibitor, and piroxicam

and tenoxicam were less efficient compared to the enzymatic system. Certainly, other factors are involved and an important difference between the enzymatic and cell systems is the presence of superoxide anion in the latter. In this respect, it has been postulated that superoxide anion is able to react with MPO-II, leading to the native form of MPO (35). Since MPO-II is an inactive chlorinating species, the recycling of this intermediate by superoxide anion may augment the production of HOCl in the cell system, which may explain the reduced HOCl suppression effect observed compared to the enzymatic system. It is also important to consider the inhibition of cyclooxygenases by NSAIDs, which could promote the accumulation of arachidonic acid. In fact, it has been proposed that arachidonic acid is able to directly activate NADPH oxidase, inducing intact neutrophils to generate superoxide anion through a protein kinase-independent pathway (36). Moreover, the ROS could be generated by the 5-lipoxygenase pathway through the B<sub>4</sub> leukotriene (37).

Endogenous oxidizing species such as HOCl play an important role in the pathophysiology of inflammatory disease, not only as terminal effectors, but also as secondary messengers in signal transduction. For instance, HOCl induced tumor necrosis factor- $\alpha$  production in peripheral blood mononuclear cells in a tyrosine kinase-dependent manner (38). Thus, the finding that therapeutic plasma levels of NSAIDs are able to suppress the generation of HOCl implies that the mechanism of the anti-inflammatory action of these drugs may be also linked to this reaction.

The MPO-catalyzed oxidation of NSAIDs may also have a direct influence on their toxic side effect. Recent research has focused on the pro-oxidant property of some NSAIDs. For example, the horseradish peroxidase-mediated oxidation of biomolecules like ascorbic acid, NADH and glutathione is promoted when these NSAIDs are added to the reac-

tion medium (39). This pro-oxidant activity is based on the accelerated oxidation of these biomolecules by NSAID radicals and on the generation of reactive metabolites by the peroxidase-catalyzed reaction. In this respect, the iminoquinone formed by the reaction between diclofenac and HOCl or activated neutrophils has been proposed as another possible cause of idiosyncratic reactions associated with the use of diclofenac (40). Accordingly, we observed that diclofenac reacted with HOCl and also to stimulate the production of superoxide anion.

We have demonstrated that therapeutic

plasma levels of classical NSAIDs are able to suppress the oxygen-dependent antimicrobial or oxidative functions of neutrophils by inhibiting the MPO-chlorinating activity and also by scavenging HOCl. The efficiency of inhibition is variable, but at least 50% inhibition was obtained with the drugs tested here. From a different point of view, our results support the idea that oxidized intermediates of NSAIDs may be formed when the drugs are administered therapeutically and these metabolites may be partially responsible for the helpful and adverse effects of NSAIDs.

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