Inhibitory Effects of Non-steroidal Anti-inflammatory Drugs on Human Myeloperoxidase

C. N. THERON, SUSAN LUBBE, A. VAN ZYL

SUMMARY

Myeloperoxidase with an A420/280 ratio of 0,48 was prepared from normal human leucocytes. This partially purified preparation catalysed guaiacol oxidation, iodination of bovine serum albumin and de-iodination of 125I-thyroxine.

Non-steroidal anti-inflammatory drugs (naproxen, indomethacin and flufenamic acid) showed a significant inhibitory effect on myeloperoxidase-catalysed iodination at concentrations of 10^{-4}M and higher. Guaiacol also inhibited myeloperoxidase-catalysed iodination, and its iodination inhibition curve was nearly identical to that obtained with the anti-inflammatory drugs.

At concentrations between 10^{-6}M and 10^{-4}M the anti-inflammatory drugs had very little or no effect on thyroxine de-iodination. Flufenamic acid and indomethacin, however, inhibited de-iodination significantly at a concentration of 10^{-4}M.

It is postulated that non-steroidal anti-inflammatory drugs may inhibit myeloperoxidase-catalysed protein iodination by acting as oxidizable cofactors which compete with other oxidizable substrates for oxidants formed by the peroxidase-hydrogen peroxide complex. In view of this and because the myeloperoxidase-hydrogen peroxide system may be involved in inflammatory tissue damage, the possibility should be considered that the action of non-steroidal anti-inflammatory drugs is at least partly attributable to a radical scavenging effect or to sequestration of oxidants.


Current ideas about the mode of action of non-steroidal anti-inflammatory drugs are based mainly on evidence that they inhibit prostaglandin synthesis. There is, however, evidence that highly reactive free radicals may also play an important role in inflammatory diseases. In the latter context it is important to take a closer look at the function of peroxidases, and more particularly the myeloperoxidase-hydrogen peroxide system in neutrophils.

Myeloperoxidase, hydrogen peroxide and an oxidizable cofactor such as halide combine to form a potent microbicidal system which kills micro-organisms after they have been ingested by neutrophils. The system probably exerts its microbicidal effect by halogenating essential macromolecules on the invading organism. But whatever the precise nature of its microbicidal action may be, it seems clear that the myeloperoxidase system fulfils an essential function in normal host defences against infections (for review see Klebanoff).

Myeloperoxidase may also be implicated in tissue damage associated with inflammatory processes. This notion is supported by evidence that the myeloperoxidase system is toxic to normal mammalian cells and to tumour cells. Furthermore, both myeloperoxidase and hydrogen peroxide are released into the extracellular fluid by neutrophils during phagocytosis or immune stimulation.

If the microbicidal functions of leucocytes are involved in inflammatory processes — and this seems very likely — it would obviously be important to find out whether anti-inflammatory drugs affect such functions. There is in fact some evidence that anti-inflammatory drugs, notably phenylbutazone, inhibit the bactericidal capacity of neutrophils. Olofsson and Olsson showed that phenylbutazone and sodium salicylate inhibit myeloperoxidase-catalysed iodination of yeast cells in vitro, and suggested that this may explain the reduced bacterial killing capacity of neutrophils exposed to phenylbutazone.

Van Zyl and Louw showed that non-steroidal anti-inflammatory drugs inhibit iodination of bovine serum albumin (BSA) catalysed by purified thyroid peroxidase, lactoperoxidase and chloroperoxidase. Van Zyl et al. also demonstrated an inhibitory effect of some anti-inflammatory drugs on BSA iodination catalysed by sonicated leucocytes. They ascribed this to an effect on the catalytic activity of myeloperoxidase.

To extend our previous investigations we prepared myeloperoxidase from normal human leucocytes. In this article we describe the effects of three non-steroidal anti-inflammatory drugs on myeloperoxidase-catalysed BSA iodination and thyroxine de-iodination.

MATERIALS AND METHODS

Human Leucocytes

Fresh, heparinized human blood from healthy donors was obtained from the Western Province Blood Transfusion Service. Leucocytes were isolated essentially as described by Olsson et al. The isolated leucocytes were suspended in a small volume of balanced salt solution and kept frozen in liquid nitrogen until further use. The balanced salt solution contained 6,8 g NaCl, 0,4 g KCl, 0,2 g CaCl2, 0,1 g MgSO4, 0,125 g NaH2PO4, 2,2 g NaHCO3 and 0,1 g glucose made up to 1 litre with distilled water.

Solubilization of Myeloperoxidase

Frozen leucocytes obtained from 6 litres of blood were thawed and the suspension (150 ml) was dialysed overnight at 4°C against 0,5% Triton X-100 in 0,05M phosphate buffer.
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The olive green pellets were suspended in 40 ml 0.5M NaCl, 0.1M phosphate buffer (pH 5.8), and homogenized with an Ultra Turrax homogenizer (two 30-second bursts at maximum speed). The homogenate was then divided into 1 - 2 ml aliquots and the leucocytes were further disrupted by ultrasonication with an MSE ultrasonicator. Homogenates were cooled by keeping the tubes immersed in crushed ice.

After sonicating the leucocyte homogenate was centrifuged at 105 000 g for 1 hour at 5°C. This resulted in a clear green supernatant with high peroxidase activity and white pellets without appreciable peroxidase activity. The pellets were discarded and the soluble myeloperoxidase in the supernatant was further purified.

**Purification of Myeloperoxidase**

To purify myeloperoxidase we used a two-step method consisting of gel filtration followed by ion exchange chromatography.

**Gel filtration:** The solution of crude myeloperoxidase was concentrated by pressure dialysis to a volume of 25 ml and applied to a 80 x 3 cm Sepharose 4B column equilibrated with 0.5M NaCl and 0.1M phosphate buffer (pH 6.0). Protein fractions (6 ml) were eluted with the same buffer at a flow rate of 36 ml per hour. Absorbance at 260, 280 and 420 nm was measured spectrophotometrically for each fraction and peroxidase activity was estimated by means of a guaiacol assay.

**Ion exchange chromatography** was performed essentially as described by Zglicynski et al.\(^{15}\) The myeloperoxidase-containing fractions eluted from the Sepharose column were pooled and dialysed overnight at 4°C against 0.1M phosphate buffer (pH 6.0). It was then applied to a column containing 10 g Cellex-CM (Biorad) equilibrated with 0.1M phosphate buffer (pH 6.0). Protein was eluted with a 500 ml linear 0.1 - 0.4M NaCl gradient in a 0.1M phosphate buffer gradient (pH 6.0 - 7.8). Absorbance measurements and estimations of myeloperoxidase activity were done on each fraction.

**Guaiacol Assay for Peroxidase Activity**

The reagent mixture for the guaiacol assay consisted of 100 \(\mu\)l 0.089M guaiacol (Merck) and 200 \(\mu\)l 0.5% bovine serum albumin (Pentex) dissolved in 0.1M phosphate buffer (pH 7.0). To this was added a small volume of myeloperoxidase-containing fractions and 0.1M phosphate buffer to give a final volume of 2.0 ml. To start the reaction, 20 \(\mu\)l 0.01M hydrogen peroxide was added and the reagents rapidly mixed on a vortex stirrer. The mixture was then quickly transferred to a cuvette and the change in absorbance at 470 nm measured spectrophotometrically 0.5 minute after adding the hydrogen peroxide.

**Iodination Experiments**

Myeloperoxidase-catalysed protein iodination was studied at pH 7.0 in the presence of NaCl. The reaction mixture consisted of the following reagents (final concentrations given in brackets): sodium phosphate buffer (0.1M, pH 7.0); BSA (5 mg/ml); KI (10^{-3}M); carrier-free ^{131}I (0.5 \(\mu\)Ci); NaCl (0.1M); purified myeloperoxidase (0.8 \(\mu\)g/ml); and H\(_2\)O\(_2\) (10^{-3}M). All the reagents were dissolved in 0.1M phosphate buffer (pH 7.0). When a hydrogen peroxide-generating system was required, glucose and glucose oxidase were added, as described by Van Zyl and Louw.\(^{16}\)

Each anti-inflammatory drug was dissolved in 1.0M tris. From this stock solution a series of dilutions were made in 0.1M phosphate buffer (pH 7.0) to give final drug concentrations ranging from 10^{-7} to 10^{-5} in the iodination reaction mixture. For each tube containing a drug, an appropriate reagent control, consisting of 1.0M tris diluted as for the corresponding drug dilution, was included. The final volume of each reaction mixture (after adding the drug or reagent control) was 500 \(\mu\)l. Appropriate blanks, which contained all reagents except myeloperoxidase, were included for each experiment.

Reactions were started by adding hydrogen peroxide and rapidly mixing the components on a vortex stirrer. Tubes were then incubated at 37°C for 20 minutes. At the end of incubation a 25 \(\mu\)l sample of each reaction mixture was chromatographed on paper in butanol-acetic acid-water to separate unbound iodide from protein-bound iodide. The radioactivity in each fraction was then measured with an Autogamma spectrometer and the percentage BSA iodination calculated from the total counts of bound and unbound ^{131}I.\(^{17}\)

**Thyroxine De-iodination**

Protocols for myeloperoxidase-catalysed de-iodination were essentially similar to those for iodination. The only differences were that the final reaction volume was 250 \(\mu\)l and that 2 \(\mu\)Ci ^{131}I-thyroxine replaced BSA, KI and ^{131}I. Paper chromatography was again used to separate radiolabelled components.

**Anti-inflammatory Drugs**

Flufenamic acid (Arlef) was obtained from Parke Davis, Johannesburg, naproxen (Naprosyn) from Syntex Laboratories, Palo Alto, California, and indomethacin (Indocid) from MSD, Johannesburg.

**RESULTS**

**Properties of Myeloperoxidase**

Gel filtration and ion exchange chromatography (Figs 1 and 2) yielded a clear, pale green enzyme preparation. The catalysed guaiacol oxidation, protein iodination and thyroxine de-iodination in the presence of hydrogen peroxide. Enzyme preparations obtained during successive stages of purification showed a progressive increase in specific activity for classic peroxidase-catalysed reaction (guaiacol oxidation) and a concomitant increase in the 420/
280 nm absorbance ratio (Table I). The two fractions with the highest specific peroxidase activity had 420/280 nm absorbance ratios of 0.48 and 0.37. A value of 0.80 for this ratio in the case of myeloperoxidase is generally accepted as an indication of the absence of contaminating proteins.

However, on the basis of its A420/280 ratio and specific activity, we considered the preparation to be satisfactory for preliminary studies of myeloperoxidase-catalysed iodination and de-iodination.

**Effect of Myeloperoxidase Concentration on Iodination**

To determine the smallest amount of enzyme needed for effective iodination of BSA, we studied the effect of myeloperoxidase concentration on iodination. The results are shown in Fig. 3.

At pH 6.0 the maximum efficiency of iodination was 66%. This was achieved at a myeloperoxidase concentration of 5 µg/ml reaction mixture. However, near maximum efficiency was already obtained with an enzyme concentration of 1 µg/ml. This curve agrees very closely with the one obtained by Dubin and Silberring under nearly similar conditions.

At pH 7.0 the maximum efficiency of iodination was only 50% but near maximum efficiency was obtained with an enzyme concentration of 0.8 µg/ml. Since a linear relationship between enzyme concentration and iodination was observed up to a concentration of 0.8 µg/ml, we used this concentration for all further iodination and de-iodination experiments described here.

**TABLE I. ABSORBANCE RATIO (A420/280) AND SPECIFIC ACTIVITY OF MYELOPEROXIDASE AT DIFFERENT STAGES OF PURIFICATION**

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>A420/280 ratio</th>
<th>Specific activity for guaiacol oxidation (A470/0.5 min/mg protein)</th>
<th>Increase in specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucocyte homogenate</td>
<td></td>
<td>1.57</td>
<td></td>
</tr>
<tr>
<td>Crude soluble myeloperoxidase</td>
<td>0.026</td>
<td>15,75</td>
<td>10-fold</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>0.21</td>
<td>29.6</td>
<td>20-fold</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>0.48</td>
<td>262.5</td>
<td>168-fold</td>
</tr>
</tbody>
</table>

Fig. 1. Elution profile of protein fractions after separation of crude soluble myeloperoxidase on Sepharose 4B.

Fig. 2. Elution profile after ion exchange chromatography on Cellex-CM of pooled peroxidase-containing fractions from Sepharose 4B.

Fig. 3. Percentage BSA iodination obtained with different concentrations of purified myeloperoxidase at pH 6.0 and pH 7.0. The reaction mixture (total volume 500 µl) contained BSA (5 mg/ml); KI (10⁻³M); carrier-free I² (0.5 µCi); NaCl (0.1M); H₂O₂ (10⁻³M) and different amounts of purified myeloperoxidase. The reagents were dissolved in 0.1M phosphate buffer pH 6.0 or pH 7.0.
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<table>
<thead>
<tr>
<th>Condition</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>2.5 mg – 5 mg once daily</td>
</tr>
<tr>
<td>Oedema of cardiac failure</td>
<td>5 mg – 10 mg once daily</td>
</tr>
<tr>
<td>Oedema of renal disease</td>
<td>5 mg – 20 mg once daily</td>
</tr>
<tr>
<td>Oedema of late pregnancy or pre-eclamptic toxaemia</td>
<td>2.5 mg – 5 mg once daily</td>
</tr>
</tbody>
</table>
Effect of Anti-inflammatory Drugs and Guaiacol on Iodination and De-iodination

The effects of anti-inflammatory drugs on myeloperoxidase-catalysed BSA iodination and $^{131}$I-thyroxine de-iodination are shown in Figs 4 - 6. Briefly stated, these effects were as follows:

All three drugs inhibited BSA iodination at pH 7.0 in the presence of added hydrogen peroxide (10<sup>-7</sup>M) or a hydrogen peroxide-generating system (Figs 4 - 5). The inhibitory effects of the three drugs on iodination did not differ greatly, although naproxen was slightly less effective than indomethacin and flufenamic acid in the presence of a hydrogen peroxide-generating system (Fig. 5).

In contrast to their inhibitory effect on BSA iodination the anti-inflammatory drugs had very little or no effect on the de-iodination of $^{131}$I-thyroxine at low drug concentrations. At 10<sup>-7</sup>M flufenamic acid and indomethacin inhibited de-iodination significantly.

Guaiacol, a classic oxidizable cofactor for peroxidases, showed an inhibitory effect on BSA iodination which was nearly identical to that observed for the anti-inflammatory drugs (Fig. 7).

DISCUSSION

We have shown that indomethacin, flufenamic acid and naproxen inhibit myeloperoxidase-catalysed protein iodination. These findings extend and confirm certain earlier observations. Olofsson and Olsson<sup>13</sup> for example, demonstrated that phenylbutazone and sodium salicylate inhibit <i>in vitro</i> iodination of yeast cells in a system containing purified myeloperoxidase. As early as 1973 Saeed and Warren<sup>14</sup> provided evidence that several non-steroidal anti-inflammatory drugs, including indomethacin, inhibit horseradish peroxidase-catalysed oxidation of <i>O</i>-dianisidine. More recently, Van Zyl and Louw<sup>15</sup> showed that non-steroidal anti-inflammatory drugs had an inhibitory effect on several purified peroxidases. Other very recent experiments in our unit indicate that anti-inflammatory drugs...
inhibit in vitro iodination catalysed by sonicated human leucocytes. This was ascribed to an inhibitory effect on myeloperoxidase, an assumption which our present observations support.

Because the myeloperoxidase system plays an important role in the antimicrobial functions of neutrophils, the question arises whether in vitro inhibition of this system by anti-inflammatory agents has any clinical significance. Would long-term treatment with these drugs, for example, weaken normal host defences against infection?

On the weight of existing evidence the answer to this question would most probably have to be no. As Klebanoff pointed out, inhibition of myeloperoxidase-catalysed halogenation does not necessarily imply a discernible decrease in the bactericidal capacity of leucocytes in vivo. In the intact neutrophil other microbialic substances (e.g. hydrogen peroxide or superoxide anion) may compensate for decreased myeloperoxidase activity. Furthermore, we are not aware of clinical evidence that therapy with non-steroidal anti-inflammatory agents may cause an increased susceptibility to infection. Finally, it should be borne in mind that we observed near 100% inhibition only at non-therapeutic drug concentrations.

Despite these considerations we feel that this question should be approached with an open mind. Strauss et al. found that phenylbutazone inhibited both engulfment and killing of Escherichia coli by peritoneal leucocytes in guinea-pigs. One should also bear in mind that certain clinical syndromes, characterized by recurrent infections, are associated with defective bactericidal functions of neutrophils. Examples are chronic granulomatous disease in children, Chediak-Higashi syndrome and congenital or acquired myeloperoxidase deficiency. (For a review of this topic see Quie.) It is conceivable that in these instances long-term treatment with drugs which inhibit the myeloperoxidase system may further weaken the already depressed antibacterial capacity of leucocytes.

Our observation that anti-inflammatory drugs had a much greater inhibitory effect on protein iodination than on thyroxine de-iodination, also applies to the peroxidases investigated by Van Zyl and Louw. The differences between the inhibitory effects on the two reactions have an important bearing on the mechanism of inhibition.

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into two groups. The first group includes substances such as azide and cyanide which form complexes with the haem iron of peroxidase and inactivate the enzyme directly. The second group inhibits peroxidase-catalysed reactions indirectly by competing with other oxidizable cofactors for the available hydrogen peroxide. Members of the second group may inhibit some peroxidase-catalysed reactions and not others, depending on their relative oxidation-reduction potentials.

The differences which we observed between inhibition of iodination and of de-iodination can be explained by the assumption that the non-steroidal anti-inflammatory drugs belong to the second group of peroxidase inhibitors, defined above, i.e. they act as competitive cofactors for peroxidases. The fact that guaiacol, a classic peroxidase cofactor, produced an iodination inhibition curve which virtually matched that obtained with the anti-inflammatory drugs, strongly supports this assumption.

To explain how anti-inflammatory agents might inhibit iodination, it is necessary to consider the possible mechanisms by which the myeloperoxidase-hydrogen peroxide system iodinates proteins. One probable mechanism which has been postulated involves the release of hypochlorite and hydroxide when the myeloperoxidase-hydrogen peroxide complex reacts with chloride. Hypochlorite is very reactive and a potent oxidant. It can react with amino acids to produce chloramines, which by rearrangement will result in oxidative de-amination of the amino acids. Hypochlorite can also chlorinate phenolic compounds or oxidize iodide to yield molecular iodine (I₂). I₂ is of course also a strong oxidant and bactericidal substance which easily iodinates tyrosine and histidine in proteins. When the iodide concentration is low conditions would be appropriate for iodination of phenols (for review see Morrison and Schonbaum\(^2\)). Non-steroidal anti-inflammatory drugs may act as inhibitors of iodination by scavenging the oxidants which convert \(I^-\) to \(I_2\), or they may compete with tyrosine for \(I_2\), i.e. they may be iodinated.

The possibility that non-steroidal anti-inflammatory drugs may act as competitive cofactors which sequester (scavenge) oxidants released during peroxidatic reactions, open an interesting field for speculation.

As early as 1970 Otomo and Fuhira (quoted by Saeed and Warren\(^3\)) studied lipid peroxidation induced by radiation and hydrogen peroxide. They suggested that anti-inflammatory drugs may act by scavenging free radicals or peroxides. More recently, Fridovich\(^5\) has accumulated evidence that highly toxic oxygen radicals such as superoxide (\(O_2^-\)) and the hydroxyl radical (\(OH^+\)) may be important mediators of inflammatory processes.

Predominant ideas about the mode of action of non-steroidal anti-inflammatory drugs are still focused on inhibition of prostaglandin synthesis. One of the steps of prostaglandin synthesis involves the peroxidatic conversion of PGG₂ to PGH₂. Very recent work has shown that this reaction releases an extremely potent oxidant which may play a key role in the pathogenesis of inflammatory diseases.\(^6,7\) Furthermore, the anti-inflammatory action of a new compound, MK-447, correlates well with the ability of this compound and other radical scavengers to sequester this destructive oxygen moiety.\(^8\) While our observations do not provide direct evidence that non-steroidal anti-inflammatory agents act as radical scavengers, they do fit such a concept. A more rigorous and direct attempt to test this hypothesis may well be worth while.

We are indebted to Parke Davis for supplying us with flufenamic acid, to Syntex Laboratories for naproxen and to MSD for indomethacin. The help of the Director and staff of the Western Province Blood Transfusion Service in obtaining fresh blood is gratefully acknowledged. We also thank the South African Medical Research Council for their financial support and interest in our work.

REFERENCES