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Nonsteroidal antiinflammatory drugs interact with horseradish peroxidase in an in vitro assay system for hydrogen peroxide scavenging

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Abstract

The established horseradish peroxidase/guaiacol in an in vitro assay system was used for investigation of the reactivity of nonsteroidal antiinflammatory drugs with hydrogen peroxide. Although the drugs rapidly seemed to react in the selected conditions, difficulties were encountered in attempts to quantify the reaction and an interaction with horseradish peroxidase was suspected. A more specific assay system based on the absolute specificity of the enzyme glutathione peroxidase for glutathione was subsequently used which demonstrated that none of the investigated nonsteroidal antiinflammatory drugs was able to scavenge hydrogen peroxide. An original procedure to further evidence the interaction was developed thereafter, based on the reaction of 5-aminosalicylic acid with similar hemoproteins. This led to the demonstration that nonsteroidal antiinflammatory drugs were substrates for horseradish peroxidase and explained their reactivity in the horseradish peroxidase/guaiacol assay system. The compound 5-aminosalicylic acid showed an unusual behaviour that was attributed to its ability to both scavenge hydrogen peroxide and interact with horseradish peroxidase. It was concluded that the lack of specificity of horseradish peroxidase for its donor substrate may lead to erroneous results in assays for hydrogen peroxide scavenging of some drugs. An alternative method is however available and a simple spectroscopic assay can evidence the interaction with horseradish peroxidase.

Keywords: Free radical; Hydrogen peroxide; Non-steroidal anti-inflammatory drug; Horseradish peroxidase; Glutathione peroxidase; Scavenging

1. Introduction

Free radicals play an important role in pathophysiological processes such as inflammation (Weissman et al., 1980; Black, 1989). The stimulation of phagocytic cells induces the production of derived oxygen species such as superoxide anion and hydrogen peroxide (Dougherty et al., 1980). In a number of conditions, damages to physiologically important molecules, cells and tissues, may result, therefore aggravating the inflammatory response (Blake et al., 1981; Weiss et al., 1984; Weiss, 1989; Mege et al., 1985; Burkhardt et al., 1986). Nonsteroidal antiinflammatory drugs were reported to posses free radical scavenging properties which could act in addition to the inhibition of cyclooxygenase (Bragt, 1984; Rommain et al., 1985; Vapaatalo, 1986; Aruoma and Halliwell, 1988; Breda and Maureen, 1992; Maffei et al., 1993). Such additional effects could be of interest in the treatment of chronic inflammatory diseases and lead to the development of more active compounds (Ahnfelt-Rønne, 1991). In order to get a general view of these properties, we examined the reactivity of a series of therapeutically used nonsteroidal antiinflammatory drugs in various in vitro systems. The scavenging activity of these drugs against hydroxyl radicals was recently assessed showing interferences with some components of the assay system that could be solved by modification of experimental conditions (Parij et al., 1995).

In the present paper, the hydrogen peroxide scavenging properties were first assessed using the classical horseradish peroxidase (EC 1.11.1.7) detection system. Some problems resulting from an interaction of the drugs with the enzyme of the assay system were again encountered. An alternative but more specific system using glutathione peroxidase (EC 1.11.1.9) was then used to further document the phenomenon and an experimental procedure was finally developed to confirm the interaction.

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2. Materials and methods

2.1. Chemicals and apparatus

The following reagents were obtained from Sigma Chemicals (Bornem, Belgium) and were of the highest purity available: horseradish peroxidase type VI-A (230–330 U/mg protein), guaiacol, glutathione, glutathione peroxidase from bovine erythrocytes (300–700 U/mg protein), trizma base, 5,5̂-dithiobis-2-nitrobenzoic acid, and 5-aminosalicylic acid. Hydrogen peroxide was purchased from Merck Belgolabo (Overijse, Belgium). The drugs were donated by their respective manufacturers: flurbiprofen and ibuprofen (Boots Pharmaceuticals, Nottingham, UK), ketoprofen and acemetacin (Rhône-Poulenc Rorer, Paris, France), naproxen (Sarva Syntex, Brussels, Belgium), diclofenac Na (Ciba Geigy, Groot-Bijgaarden, Belgium), flufenamic acid (Trenker, Brussels, Belgium), indomethacin and sulindac (Merck Sharp and Dohme, Rahway, NJ, USA), niflumic acid (Upsa Medica, Brussels, Belgium), tolmetin Na (Cilag, Schaffhausen, Switzerland), piroxicam (Pfizer, Brussels, Belgium), tenoxicam (Roche, Basle, Switzerland), and acetylsalicylic acid (Bayer, Leverkussen, Germany). The poorly water soluble drugs were dissolved by addition to the aqueous suspension of the minimum volume of a sodium carbonate solution (0.4 M) and the pH was thereafter rapidly adjusted to 7.4. The solutions were made in freshly deoxygenated water daily. UV-visible measurements were performed on a Shimadzu UV-160 apparatus.

2.2. Horseradish peroxidase assay

The procedure was adapted from Aruoma et al. (1989) and performed at 25°C. Reaction mixtures contained in a final volume of 3.0 ml the following reagents at the final concentrations stated: a KH_2PO_4/KOH buffer pH 7.4 (10 mM), hydrogen peroxide (500 μM), 150 μl of a guaiacol solution at 100 μl/50 ml (89.4 mM), one of the drugs (generally 0–4 mM, sometimes 0–40 mM), and 100 μl of an horseradish peroxidase solution at 2×10^{-3} mg/ml ($\cong 1.5 \times 10^{-9}$ M). Drugs and hydrogen peroxide were allowed to stand during at least 30 min before addition of horseradish peroxidase. Absorbances at 436 nm were monitored during 30 min for each drug concentration.

2.3. Glutathione peroxidase assay

This assay was conducted essentially as described by Miles and Grisham (1994). Reaction mixtures contained in a volume of 1.0 ml the following reagents at the final concentrations stated: hydrogen peroxide (200 μM), a KH_2PO_4/KOH buffer at pH 7.4 (10 mM) and one of the drugs (0–4 mM). The mixture was allowed to stand during 1 h at 37°C. 100 μl of glutathione (1 mM) and 100 μl of a glutathione peroxidase solution at 1 U/100 μl ($\cong 1.2 \times 10^{-8}$ M) were then added and the mixture incubated for an additional 5 min at 37°C. The tubes were thereafter diluted 10-fold with a 0.2 M tris-HCl buffer at pH 8.5 for obtaining 12 ml of solution. After addition of 1 ml DTNB (1 mM), the absorbance at 412 nm was measured.

2.4. Interaction of the drug with horseradish peroxidase

This method was derived from Yamada et al. (1991) and performed at 25°C. The spectrum of the native horseradish peroxidase enzyme (HRP^{3+}, λmax: 500 and 645 nm) was first recorded with a reaction mixture containing: horseradish peroxidase 6.0 mg of the native enzyme ($\cong 4.65 \times 10^{-5}$M) in 2.8 ml of a 20 mM KH_2PO_4/KOH buffer at pH 7.0 containing 150 mM NaCl. 100 μl of an hydrogen peroxide solution (100 μM) were then added and the spectrum of the compound II formed was recorded after 1 min (λmax: 527 and 555 nm). Thereafter, 100 μl of a drug solution were added (drug concentration varying from 0.4 to 5 mM, ensuring complete restoration of the spectrum of the native enzyme) and the spectrum was again recorded.

3. Results

3.1. Hydrogen peroxide scavenging by the horseradish peroxidase / guaiacol assay

The horseradish peroxidase assay system is based on the oxidation by hydrogen peroxide (H_2O_2) of the native enzyme (HRP^{3+}) to compounds I (HRP^{5+}) and II (HRP^{4+}). The first is very unstable and rapidly passes into compound II. A substrate added in the system which can react with these last compounds restores the native enzyme. In the selected system, guaiacol acts as substrate

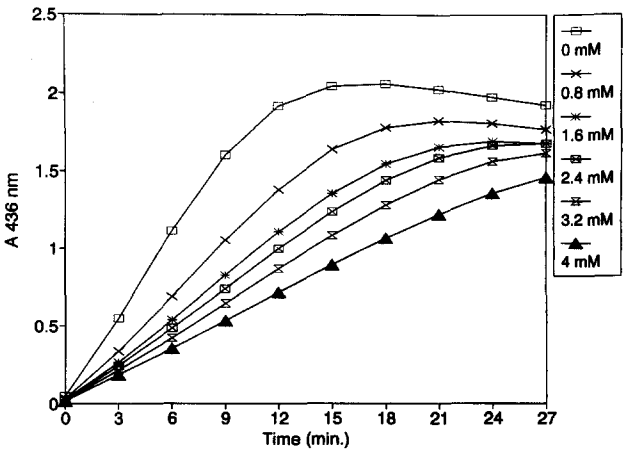

Fig. 1. Inhibition of guaiacol oxidation (A 436 nm) by indomethacin concentrations ranging from 0.8 to 4 mM. Reactions were followed during 30 min at 25°C.

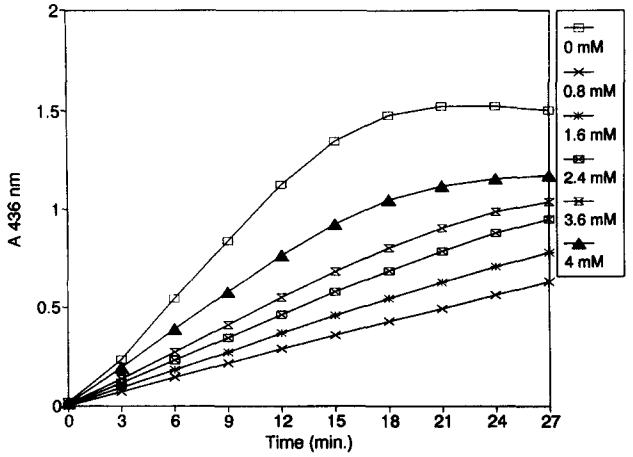

Fig. 2. Effect of increasing 5-aminosalicylic acid concentrations (0.8 to 4 mM) on guaiacol oxidation (A 436 nm). Reactions were followed during 30 min at 25°C.

giving an oxidation product possessing absorbing at 436 nm. The following reactions take place:

$$HRP^{3+} + H_2O_2 \rightarrow HRP^{5+} + H_2O \quad (1)$$

$$HRP^{5+}\ (\text{comp. I}) + \text{guaiacol} \rightarrow \text{Ox. guaiacol} + HRP^{4+}\ (\text{comp. II}) \quad (2)$$

$$HRP^{4+}\ (\text{comp. II}) + \text{guaiacol} \rightarrow \text{Ox. guaiacol} + HRP^{3+} \quad (3)$$

Any compound added to the system and able to react with hydrogen peroxide will decrease colour formation. As a matter of fact, first experiments showed that most drugs reacted in a concentration range of 0.5–4.0 mM. This was the case for: acetylsalicylic acid, acemetacin, diclofenac, flufenamic acid, flurbiprofen, indomethacin, naproxen, niflumic acid, piroxicam, sulindac, tenoxicam and tolmetin. Only ibuprofen and ketoprofen did not react at a significant rate in this concentration range, but an effect was noted for concentrations up to 40 mM.

In order to compare the reactivity of the different drugs, we examined the possibility of determining rate constants and checked the concentration dependency of the colour

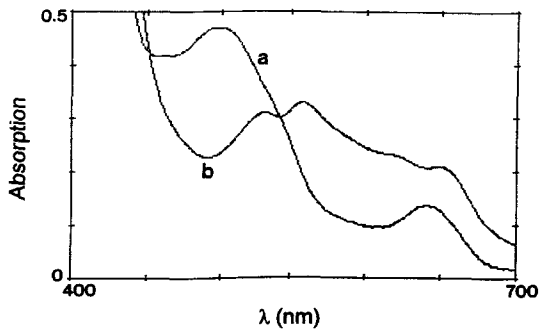

Fig. 3. Visible spectra of the horseradish peroxidase native enzyme (curve a) (λmax: 500 and 645 nm) and of compound II (curve b) (λmax: 527 and 555 nm).

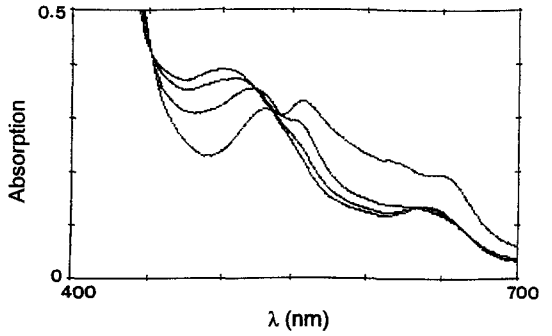

Fig. 4. Transformation of compound II (λmax: 527 and 555 nm) into the native enzyme (λmax: 500 and 645 nm) in presence of 4 mM of naproxen after 0, 20, 40 and 60 min.

decrease. The reaction was therefore performed with increasing drug concentrations and followed during 30 min. Fig. 1 illustrates with indomethacin the typical kind of graph obtained showing a clear influence of drug concentration on guaiacol oxidation. In spite of this, no satisfactory quantitative relationship could be established. The best we could obtain was a linear relationship during a very short period of time in the course of the reaction, but it occurred not always at the same moment for the different drugs examined and even for the same drug. We also did not observe any difference in the extent of phenomenon when the peroxidase was added directly to the reaction mixture (without pre-incubation) and when drugs were first incubated with hydrogen peroxide before addition of the peroxidase (such as recommended in the original procedure). These facts encouraged us to examine the possibility of interactions of the drugs with some other components of the assay system.

Although all examined drugs gave graphs similar to the one of Fig. 1, 5-aminosalicylic acid (which was examined as a reference compound in the next assay) had not the same kind of concentration dependency. Such as indicated in Fig. 2, a bi-phasic phenomenon was evidenced: indeed, a first important decrease in colour formation with increas-

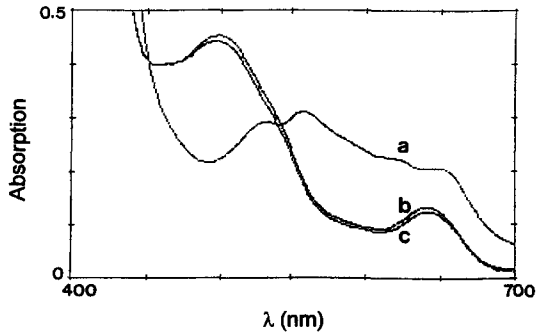

Fig. 5. Transformation of native horseradish peroxidase (curve b, λmax: 500 and 645 nm) into compound II (curve a, λmax: 527 and 555 nm) by addition of 100 μM hydrogen peroxide and return to the native enzyme in the presence of 0.4 mM of flufenamic acid (curve c) after 2 min at 25°C.

ing drug concentration was directly followed by a more progressive increase in absorbance. This suggested the occurrence of a more complex mechanism than for other drugs.

3.2. Hydrogen peroxide scavenging assessed by the glutathione / glutathione peroxidase assay

As the enzyme glutathione peroxidase (GPx) has an absolute specificity for glutathione (GSH) as electron-donating substrate (Eq. 4), other compounds would theoretically not be able to react with this peroxidase.

$$H_2O_2 + 2GSH \xrightarrow{Gpx} H_2O + GSSG \quad (4)$$

Miles and Grisham (1994) experimented such a system to assess the scavenging activity of 5-aminosalicylic acid. The decrease in hydrogen peroxide concentration after incubation with the scavenger was evaluated by determination of the quantity of glutathione consumed by reaction with 5,5′-dithiobis-2-nitrobenzoic acid, giving rise to 5-thio-2 nitrobenzoic acid absorbing at 412 nm. Any compound able to decompose hydrogen peroxide will therefore attenuate the oxidation of glutathione and the increase in absorbance.

Application of this assay to nonsteroidal antiinflammatory drugs showed that none of the derivatives tested up to a concentration of 4 mM reacted. We also observed that the test compound 5-aminosalicylic acid had only a very slight scavenging effect in the system at concentrations less than 4 mM, but better reacted at higher concentrations (up to 40 mM). However, nonsteroidal antiinflammatory drugs did not better react in this rather high concentration range.

3.3. Study of the interaction of the drugs with horseradish peroxidase

We hypothesised that drugs could be able to react with compound II of the enzyme (HRP^{4+}) causing its reduction to the native state HRP^{3+} and making the assay unsuitable. A closely related type of interference during a peroxidase-catalysed reaction was described by Yamada et al. (1991) who studied the interaction between 5-aminosalicylic acid and hemoglobin (Hb), another well known member of the hemoprotein family. By recording the UV-visible spectra of the different forms of hemoglobin, they showed that the drug was able to reduce Hb^{4+} (product of the reaction between Hb^{3+} and hydrogen peroxide) to Hb^{3+}.

The experiment could be successfully reproduced with horseradish peroxidase that also has different redox states with well defined spectral characteristics in the visible region. Fig. 3 shows the spectrum of the native enzyme and of compound II, obtained after reaction of HRP^{3+} with hydrogen peroxide. By allowing nonsteroidal antiinflammatory drugs to react with compound II, we similarly observed that they were able to reduce compound II into the native enzyme. The process occurred at variable drug concentrations in the reaction mixture and sometimes in different experimental conditions. Indeed, an incubation period was necessary to observe the conversion of compound II into the native enzyme for several drugs including acetylsalicylic acid, flurbiprofen, ibuprofen, ketoprofen, naproxen and tolmetin (e.g., for naproxen in Fig. 4). However, for other tested drugs, namely acemetacin, 5-aminosalicylic acid, indomethacin, flufenamic acid, niflumic acid, piroxicam, sulindac and tenoxicam, the reaction was very rapid (Fig. 5).

4. Discussion

Several methods are available for the determination of small amounts of hydrogen peroxide in different systems (Heath and Tappel, 1976; Boveris et al., 1977; Free et al., 1983; Aruoma et al., 1989; Kettle et al., 1994; Miles and Grisham, 1994). The most satisfactory procedures are based on the catalysis by a hemoprotein (usually horseradish peroxidase) of the peroxide-dependent oxidation of electron-donating detector molecules yielding to compounds that can be detected by spectrophotometry (phenol red or NADP) (Pick and Keisari, 1980; Negri et al., 1991) or fluorimetry (scopoletin or homovanillic acid) (Boveris et al., 1977; Kettle et al., 1994). The large majority of published studies are ex vivo experiments measuring the influence of drugs on the quantity of hydrogen peroxide released by phagocytic cells like polymorphonuclear leukocytes and macrophages. On the other hand, in vitro assays are rather few (Heath and Tappel, 1976; Aruoma et al., 1989; Miles and Grisham, 1994), and data on the reactivity of drugs with hydrogen peroxide could only be found for a limited number of compounds (Aruoma et al., 1988, 1992; Das and Mira, 1992). Among existing procedures, the horseradish peroxidase/guaiacol method was selected due to its relative simplicity (Keilin and Hartree, 1951; Halliwell and De Rycker, 1978).

The first results obtained with the horseradish peroxidase/guaiacol system were encouraging as practically all drugs reacted in the system. However, difficulties later appeared with the standardisation and quantification of the results. When looking at literature, it was astonishing that among the authors that reported hydrogen peroxide scavenging by a drug (Aruoma et al., 1988, 1992; Das and Mira, 1992), none of them tried to derive quantitative data (e.g. rate constants), that would allow to compare the reactivity of different molecules. Another troublesome problem was the high velocity of the reaction evidenced by the fact that no difference could be observed in the extent of the reaction either the drug was pre-incubated with hydrogen peroxide or not. Results from previous experiments by other authors indeed indicated that either drugs

do not react with hydrogen peroxide (e.g., antiarrhythmic drugs and carnosol) (Das and Mira, 1992; Aruoma et al., 1992) or that they react very slowly with this species, at a physiologically nonsignificant rate (e.g., taurine, hypotaurine and their metabolic precursors) (Aruoma et al., 1988). Only carnosic acid and trolox were found to significantly react in the horseradish peroxidase/guaiacol system (Aruoma et al., 1992).

Most peroxidases and particularly horseradish peroxidase are known to be enzymes with a low specificity (Flohé et al., 1976; De Mello et al., 1980; Smith et al., 1982; Porter and Bright, 1983; Halliwell and Gutteridge, 1989). This suggested to us that nonsteroidal antiinflammatory drugs could also act as substrates. A limited number of authors already mentioned such a possibility for other drugs. For example, Aruoma et al. (1992) suggested that carnosic acid and trolox could be acting as substrates for peroxidases. Miles and Grisham (1994) seem to be the first authors to apply an alternative system to assess the reactivity of certain aminosalicylates, including 5-aminosalicylic acid. They indeed proposed to use the absolute specificity of the selenoenzyme glutathione peroxidase for glutathione as an electron-donating substrate (Flohé et al., 1976). In such a system, the majority of molecules are supposed not to interfere with the detection of hydrogen peroxide. The fact that none of the nonsteroidal antiinflammatory drugs reacted in these conditions strongly suggested the presence of interaction in the first system, such as recently demonstrated with tenoxicam and leukocyte myeloperoxidase (Ichihara et al., 1985). The 5-aminosalicylic acid molecule, which was used as a test compound for demonstrating the interaction, presently showed some reactivity in the glutathione peroxidase system, but at rather high concentrations (4–40 mM). This observation was not in accordance with Miles and Grisham (1994) who reported that it does not react with hydrogen peroxide, but these authors used a concentration of 4 mM.

Two kinds of mechanisms could be evoked to explain the observed interaction. After the demonstration by Yamada et al. (1991) that aminosalicylates such as 5-aminosalicylic acid are able to reduce some forms of hemoglobin, namely Hb^{4+} to the resting enzyme Hb^{3+}, Miles and Grisham (1994) showed that these compounds are excellent substrates for peroxidase-catalysed reactions. The similarity between hemoglobin and horseradish peroxidase let us to assume that 5-aminosalicylic acid and maybe the other drugs tested could also be able to reduce the oxidised horseradish peroxidase to its resting form HRP^{3+}. This reaction is actually easy to follow as changes are associated with modifications in UV-visible spectra. By adapting the test described by Yamada et al. (1991), we actually showed that nonsteroidal antiinflammatory drugs behaved as horseradish peroxidase substrates. This most probably explains why the drugs although not reacting with hydrogen peroxide gave positive results in the horseradish peroxidase system.

Another explanation that could only apply to some of the investigated drugs is based on the observed reaction between horseradish peroxidase and auxin or indole-3-acetic acid (De Mello et al., 1980; Smith et al., 1982). Indeed, two examined nonsteroidal antiinflammatory drugs (indomethacin and acemetacin), classified in the 'indole acetic acid family', are structurally related with this natural horseradish peroxidase substrate. According to Smith et al. (1982), there seem to be two possible pathways for oxidation of indole acetic acids by the peroxidase: the first one is similar to the one previously described and the second is liable to occur in the absence of hydrogen peroxide and involves a direct reaction between peroxidase and the drugs. The two pathways occur at different enzyme/substrate ratios. We also tried to reproduce the experimental conditions for the second pathway with the two drugs but their very poor solubility in water prevented us to test this hypothesis.

The unique behavior of 5-aminosalicylic acid in the horseradish peroxidase test may be due to the fact that it is the sole compound which both reacts with hydrogen peroxide and interacts with the peroxidase and that these phenomenons simultaneously occur in the reaction mixture. The quantity of 5-aminosalicylic acid which reacts with hydrogen peroxide will not be available for reacting with HRP^{4+} and more guaiacol will consequently be oxidised. On the other hand, the quantity of 5-aminosalicylic acid which reacts with HRP^{4+} will not be available to react with hydrogen peroxide and more hydrogen peroxide will be present to oxidise HRP^{3+} into HRP^{5+}. This event leads to the production of increased amounts of oxidised guaiacol. Both mechanisms may contribute to increase guaiacol oxidation in place of decreasing it as the drug concentration increases.

In conclusion, the horseradish peroxidase/guaiacol assay for determination of the hydrogen peroxide scavenging activity of drugs may lead to erroneous results because of the lack of specificity of the peroxidase. Another assay method based on the better selectivity of glutathione peroxidase for its substrate can be applied to check the results obtained with this system. Moreover, the interaction of a drug with a horseradish peroxidase can easily be evidenced by a simple spectroscopic assay. Investigators who want to assess hydrogen peroxide scavenging are therefore encouraged to apply these last two procedures in case of a significant effect in the horseradish peroxidase based system.

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