Hydrogen peroxide scavenging activity by non-steroidal anti-inflammatory drugs

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Abstract

Hydrogen peroxide (H$_2$O$_2$) has been shown to be formed during inflammatory processes and is implicated in its pathophysiology. Thus, a putative scavenging activity against this reactive oxygen specie (ROS) by anti-inflammatory drugs may be of great therapeutical value. The present study was undertaken to evaluate the scavenging activity for H$_2$O$_2$ by several non-steroidal anti-inflammatory drugs (NSAIDs), namely indomethacin, acemetacin, etodolac, tolmetin, ketorolac, oxaprozin, sulindac and its metabolites sulindac sulfide and sulindac sulfone. The H$_2$O$_2$ scavenging assay was performed by measuring H$_2$O$_2$-elicited lucigenin chemiluminescence using a microplate reader. The specificity of the method was confirmed by the use of catalase, which completely prevented the H$_2$O$_2$-induced lucigenin chemiluminescence. The endogenous antioxidants melatonin and reduced glutathione (GSH) were used as positive controls. The obtained results demonstrated that all the studied NSAIDs display H$_2$O$_2$ scavenging activity, although in different extents. The ranking order of potency found was sulindac sulfone > sulindac sulfide > GSH > sulindac > indomethacin > acemetacin > etodolac > oxaprozin > ketorolac ≈ melatonin ≈ tolmetin.

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Keywords: NSAIDs; Hydrogen peroxide scavenging activity; Microplate chemiluminescence assay

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Introduction

During the course of inflammatory processes, hydrogen peroxide (H$_2$O$_2$) may be generated in large amounts. This occurrence may be due to the activation of mast cells, macrophages, eosinophils, and neutrophils, which generate superoxide radical (O$_2^-$), predominantly via NADPH oxidase (Barnes, 1990; Hampton et al., 1998). The O$_2^-$ is then rapidly converted into H$_2$O$_2$ by superoxide dismutase (SOD). H$_2$O$_2$ can easily penetrate the membranes of surrounding cells, whereas O$_2^-$ usually cannot. H$_2$O$_2$ is not an inherently reactive compound. However, H$_2$O$_2$ can be converted into highly reactive and deleterious products: (i) H$_2$O$_2$ may undergo transition-metal-dependent conversion into the extremely deleterious hydroxyl radical (HO•) (Halliwell and Gutteridge, 1985); (ii) myeloperoxidase, a polymorphonuclear neutrophil (PMN) characteristic enzyme generates hypochlorous acid (HOCl) from H$_2$O$_2$ and chloride ions (Winterbourn, 1985). Membrane lipids are particularly susceptible to oxidation by HO•, not only due to their high polyunsaturated fatty acid content (De Groot and Noll, 1987; Schinella et al., 2002), but also due to their association in the cell membrane with enzymatic and non-enzymatic systems able to generate ROS (Schinella et al., 2002). A single HO• can result in the formation of many molecules of lipid hydroperoxides in the cell membrane, which may severely disrupt its function, and lead to cell death. ROS, via lipid peroxidation, causes the release of arachidonic acid from membrane phospholipids and may thus increase the formation of prostaglandins and leucotrienes (Hemler et al., 1979; Taylor et al., 1983). Lipid peroxidation may also alter protein structure, thus altering antigenicity, which may provoke immune responses (Barnes, 1990). The HOCl plays an important role in the bactericidal function (Hampton et al., 1998). However, when produced in excess, HOCl is also highly reactive toward a range of biological substrates and may cause deleterious effects in the surrounding tissue (Hampton et al., 1998; Pullar et al., 1999).

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most important therapeutical agents used for the treatment of inflammatory processes. Among them, indomethacin, acemetacin, etodolac, tolmetin, ketorolac, oxaprozin, and sulindac have been widely used, due their inhibitory activity against cyclooxygenase (COX) enzymes that catalyse the formation of prostaglandin precursors from arachidonic acid (Fernandes et al., 2004). However, taking into account the above-mentioned rationale, it may be postulated that a putative scavenging activity against H$_2$O$_2$ by anti-inflammatory drugs may also be of great therapeutical value. Thus, the aim of the present study was to evaluate the scavenging activity for H$_2$O$_2$ by the NSAIDs indomethacin, acemetacin, etodolac, tolmetin, ketorolac, oxaprozin, sulindac and its metabolites sulindac sulfide and sulindac sulfone. The endogenous antioxidants catalase, melatonin and reduced glutathione (GSH) were used as positive controls of the assay system.

Methods

Chemicals

Indomethacin, acemetacin, etodolac, tolmetin, ketorolac, sulindac, 30% hydrogen peroxide, reduced glutathione (GSH), melatonin, and lucigenin, were obtained from Sigma Chemical Co. (St. Louis, USA). Oxaprozin was a gift from Helsinn Healthcare SA, Switzerland. Sulindac sulfide and sulindac sulfone were a gift from Merck Research Laboratories, USA.

All the other reagents were of analytical grade.
H$_2$O$_2$ scavenging assay

The H$_2$O$_2$ scavenging activity was measured using a chemiluminescence (CL) methodology, by monitoring the H$_2$O$_2$-induced oxidation of lucigenin, accordingly to a previously described procedure (Chang et al., 2002; Jeng et al., 2002), with modifications. Reaction mixtures contained, in a final volume of 250 μL, the following reagents at the indicated final concentrations: 50 mM Tris buffer, pH 7.4, 3 mM lucigenin, and tested compounds, at various concentrations (0–5000 μM) and 2% H$_2$O$_2$. Ketorolac, oxaprozin, sulindac, sulindac sulfone, and tolmetin were dissolved in the Tris buffer. Acemetacin, indomethacin, etodolac and sulindac sulfide were dissolved in DMSO. Under the present experimental conditions, DMSO did not interfere with the assay. The assays were performed at ambient temperature. Blank values were measured in the absence of H$_2$O$_2$. The endogenous antioxidants melatonin and GSH were also assayed under the same conditions, for comparison. Catalase was used as a positive control to confirm H$_2$O$_2$ specificity. H$_2$O$_2$-elicited lucigenin oxidation results in a CL signal (Fig. 1) that was detected using a microplate reader (BIO-TEK, Synergy HT). The resulting CL was monitored for 5 minutes. Each study corresponds to four experiments, performed in triplicate.

Fig. 1. Schematic illustration of the reaction pathway leading to H$_2$O$_2$-induced lucigenin-chemiluminescence (adapted from Rakicioglu et al., 2001).
The effects are expressed as the percentual inhibition of the H$_2$O$_2$-induced lucigenin oxidation:

$$\text{Percentual Inhibition of}\ \text{The H}_2\text{O}_2\text{-induced Lucigenin Oxidation} = 100 - \left( \frac{\text{CL}_{\text{sample}} - \text{CL}_{\text{blank}}}{\text{CL}_{\text{control}} - \text{CL}_{\text{blank}}} \times 100 \right)$$

Results

Fig. 2 and Fig. 3 show the results obtained in the H$_2$O$_2$ scavenging assay. The obtained results demonstrated that all the studied NSAIDs and positive controls prevent H$_2$O$_2$-elicited lucigenin oxidation in a concentration dependent manner, although in different extents. The ranking order of potency found was sulindac sulfone $>$ sulindac sulfide $>$ GSH $>$ sulindac $>$ indomethacin $>$ acemetacin $>$ etodolac $>$ oxaprozin $>$ ketorolac $\approx$ melatonin $>$ tolmetin (IC$_{50s}$ of 941 $\pm$ 60, 1177 $\pm$ 13, 1384 $\pm$ 75, 1476 $\pm$ 50, 1781 $\pm$ 129, 2028 $\pm$ 93, 2224 $\pm$ 28, 2680 $\pm$ 195, $>$5000, $>$5000, $>$5000 $\mu$M (Mean $\pm$ SEM) respectively) (Table 1). The specificity of the method was confirmed by the use of catalase, which completely prevented the H$_2$O$_2$-induced lucigenin chemiluminescence (IC$_{50}$ = 0.050 $\pm$ 0.005 U/ml).

Discussion

The present results showed that the studied NSAIDs exhibit scavenging activity for H$_2$O$_2$, although with a variable potency. This effect is reported for the first time in the present study for these NSAIDs. Importantly, some reports from the literature indicating H$_2$O$_2$ scavenging activity when the horseradish
peroxidase assay is applied may give misleading results due to the inhibitory effect on this enzyme displayed for some of the tested drugs (Parij and Nève, 1996). Thus, when enzymatic systems are involved in such studies due attention should be always given to the possible enzyme inhibitory effects of the compounds under study. The present assay system circumvented such methodological shortcoming.

When compared to the endogenous compounds GSH and melatonin, the potency of the studied NSAIDs was similar or even higher, as it was demonstrated for sulindac and its metabolites. Of note, sulindac is not considered as a therapeutical drug per se, but rather as a pro-drug, which is converted in vivo, into to the metabolites sulindac sulfide (responsible for the anti-inflammatory effect) and sulindac sulfone (Fernandes et al., 2003). Acemetacin was another NSAID found to have high H2O2 scavenging

<table>
<thead>
<tr>
<th>Tested compound</th>
<th>IC50 (µM)</th>
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<tbody>
<tr>
<td>Sulindac sulfide</td>
<td>1177±13</td>
</tr>
<tr>
<td>Sulindac sulfone</td>
<td>941±60</td>
</tr>
<tr>
<td>Sulindac</td>
<td>1476±50</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1781±129</td>
</tr>
<tr>
<td>Acemetacin</td>
<td>2028±93</td>
</tr>
<tr>
<td>Etodolac</td>
<td>2224±28</td>
</tr>
<tr>
<td>Oxaprozin</td>
<td>2680±195</td>
</tr>
<tr>
<td>Ketorolac</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Tolmetin</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Melatonin</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>GSH</td>
<td>1384±75</td>
</tr>
</tbody>
</table>
activity. However, one should bear in mind that in vivo, acemetacin is rapidly converted into indomethacin (Jones et al., 1991), which under the present assay conditions has a lower but still effective activity. The obtained results also indicate that the H$_2$O$_2$-scavenging effect will hardly contribute for ketorolac and tolmetin therapeutic outcome, due to their low activities.

In accordance with the present results, Wang and Jiao, 2000, have also reported a weak direct H$_2$O$_2$-scavenging effect of GSH. Nevertheless, in vivo, as it is well known, this endogenous antioxidant participates in the rapid metabolism of cellular H$_2$O$_2$ as the co-factor of the enzyme glutathione peroxidase. On the other hand, Gulcin et al., 2003 reported a strong direct H$_2$O$_2$-scavenging effect of melatonin (83% at 260 $\mu$M). In this case, the low amount of H$_2$O$_2$ used (40 mM) comparatively to the concentration used in the present study (about 600 mM), may explain the reported high effectiveness of melatonin.

The H$_2$O$_2$-scavenging activity observed in the present study should not be assumed as the total capacity of these drugs to counteract oxidative stress. In fact, many other reactive species are known to be produced during inflammatory processes. Among the various reactive species that may be produced in excess during the inflammatory processes, the ROS peroxyl radical (ROO$^\cdot$), HO$^\cdot$, O$_2$^--, H$_2$O$_2$, and HOCl as well as the reactive nitrogen species (RNS) nitric oxide (NO) and peroxynitrite anion (ONOO$^-$) also play important roles in their pathophysiology, which makes them potential targets for the chemotherapy of inflammation (Dedon and Tannenbaum, 2004). It was previously shown that sulindac and sulindac sulfone exhibit a strong scavenging activity against O$_2$^--, HO$^\cdot$, NO, and ONOO$^-$, while sulindac sulfide exhibits a potent scavenging activity over HOCl, O$_2$^--, HO$^\cdot$, NO, and ONOO$^-$ (Fernandes et al., 2003 and references therein). Concerning the other NSAIDs assayed in the present study, it was also previously demonstrated that tolmetin, ketorolac, and oxaprozin are not active against O$_2$^-- while acemetacin, indomethacin and etodolac are highly effective against this radical, exhibiting a concentration dependent effect (Fernandes et al., 2004 and references therein). Oxaprozin was the less active scavenger for HO$^\cdot$ being indomethacin, acemetacin, etodolac, tolmetin, and ketorolac shown to be highly active against this ROS. The scavenging effect against HOCl was not observed for indomethacin, acemetacin, etodolac, tolmetin, ketorolac and oxaprozin, while ROO$^\cdot$ was effectively scavenged by etodolac, with indomethacin, acemetacin, tolmetin, ketorolac and oxaprozin much less active. NO, and ONOO$^-$ were also effectively scavenged by etodolac, indomethacin, acemetacin, etodolac, tolmetin, ketorolac and oxaprozin (Fernandes et al., 2004).

Although the effects here described occur at relatively high concentrations as compared to those in plasma and synovial fluid of healthy humans receiving the drugs in pre-clinical tests (Netter et al., 1989; Urquhart, 1991), some NSAIDs were reported to accumulate in inflamed tissues, leading for example, to concentrations in the synovial fluid about 3 to 8 times higher than in control joints (Graf et al., 1975). It must also be taken into account that the scavenging effect of antioxidants is highly dependent on the amount of reactive species being produced (Valentão et al., 2002). Thus, it is possible that much lower concentrations of NSAIDs are active in vivo. Indeed, indomethacin was already shown to prevent H$_2$O$_2$-induced erythrocytic membrane lipid peroxidation at therapeutic concentrations (Orhan and Sahin, 2001), in spite of the high concentrations needed in vitro for this NSAID.

In conclusion, the present study, performed for the NSAIDs sulindac, sulindac sulfide, sulindac sulfone, indomethacin, acemetacin, tolmetin, etodolac, ketorolac and oxaprozin showed, for the first time, that these NSAIDs exhibit scavenging activity for H$_2$O$_2$. It may be postulated that the present results concerning H$_2$O$_2$ scavenging activity plus the ROS and RNS scavenging effects previously reported, indicate that these effects may contribute for the anti-inflammatory activity of the studied
NSAIDs, sulindac and its metabolites as well as acemetacin and indomethacin being the most promising compounds in this respect.

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References


