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Effects of nonsteroidal anti-inflammatory drugs on glutathione
S-transferases of the rat digestive tract

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Nonsteroidal anti-inflammatory drugs (NSAIDs) have been demonstrated to reduce cancer rates in oesophagus, stomach and colon of humans and animals. Earlier, we showed that high human gastrointestinal tissue levels of glutathione S-transferase (GST), a family of detoxification enzymes consisting of class α, μ, π and θ isoforms, were inversely correlated with cancer risk. We investigated whether the NSAIDs indomethacin, ibuprofen, piroxicam, acetyl salicylic acid (ASA), and sulindac, supplemented in the diet for 2 weeks at 25, 400, 400, 400, and 320 ppm, respectively, influenced gastrointestinal GSTs in male Wistar rats. In cytosolic fractions of oesophagus, stomach, intestine and liver, GST activity towards 1-chloro-2,4-dinitrobenzene was measured, GST isozyme levels were determined by densitometrical analysis of Western blots after immunodetection with monoclonal antibodies, and glutathione levels were determined by HPLC. GST activity and GST μ levels were increased (1.2–1.8 X) in oesophagus and small intestine by indomethacin, ibuprofen, piroxicam and sulindac. GST α levels were induced (1.2–2.8 X) in stomach by piroxicam, in small intestine by indomethacin, ibuprofen, piroxicam and sulindac, and in liver by piroxicam. GST π levels were raised (1.9–3.6 X) in stomach by ibuprofen, ASA, and sulindac, and in small intestine by indomethacin, piroxicam, ASA, and sulindac. Glutathione levels were raised (1.2–2.3 X) by indomethacin and ASA in small intestine and by piroxicam in oesophagus. Enhancement of GSTs in the upper part of the digestive tract, resulting in a more efficient detoxification, may explain in part the anticarcinogenic properties of NSAIDs.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs*) are among the most prescribed drugs worldwide. They have anti-inflammatory, analgesic, and antipyretic activities. They are used clinically for the treatment of patients with (e.g.) acute and chronic rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, gouty arthritis, bursitis, tendositis, and inflammatory arthritis (1).

In addition to their therapeutic use, there is strong epidemiological evidence that NSAIDs may have anticarcinogenic effects in humans. Sulindac caused regression of adenomatous polyps in patients with familial adenomatous polyposis (FAP) (2–4), whereas no effect on sporadic colonic polyps was found (5). Epidemiological studies suggest that regular, prolonged use of aspirin-based NSAIDs may reduce the risk of development and mortality of oesophageal, gastric, colonic, or rectal cancer (6–9), although in one prospective study no support for such an association was found (10). Several NSAIDs are currently evaluated in clinical trials. Effects of NSAIDs on neoplastic growth in the colon of animals and humans, including possible mechanisms involved, were recently reviewed (11,12).

Many animal studies have revealed significant protection against development of chemically induced cancers by treatment with NSAIDs. Ibuprofen inhibited carcinogenesis in rat colon (13), mouse forestomach and lung (14). Indomethacin inhibited tumorigenesis in rat colon (15–19), stomach (16,20), mammary gland (21), urinary bladder (22,23), and liver (24,25), as well as in mouse oesophagus (26,27). Piroxicam reduced tumour incidence in the colon (13,15,17–19,28–31), small intestine (18), and liver (25) of the rat. Dietary acetyl salicylic acid inhibited carcinogenesis in rat colon (32–34) and bladder (35), whereas sulindac reduced tumour multiplicity in the rat colon (36) and mouse forestomach (14).

A generally accepted mechanism of action of NSAIDs is the inhibition of cyclooxygenases, the rate-limiting enzymes that catalyse the formation of prostaglandin precursors from arachidonic acid (12,37). Prostaglandins play a role in the control of cell proliferation and regulation of immune functions (38–41). However, doses of NSAIDs required to suppress inflammation may exceed substantially the doses necessary to inhibit prostaglandin synthesis, suggesting that the anticarcinogenic properties of these drugs may be achieved through additional unidentified mechanisms (42).

Inhibitors of carcinogenesis often have an enhancing effect on carcinogen detoxification systems such as glutathione S-transferases (GSTs; EC 2.5.1.18) (43–45). The soluble glutathione S-transferases are a gene family of dimeric enzymes comprised of four classes: α, μ, π and θ (43,44). They catalyse the binding of a large variety of electrophiles to the sulphydryl group of glutathione (GSH). Since most reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, GSTs take considerable importance as a mechanism for carcinogen detoxification (43,44). Enhancement of the activity of this system may result in a more efficient elimination of carcinogens and may ultimately lead to the prevention of cancer.

The present study was designed to investigate the effects of dietary administration of indomethacin, ibuprofen, piroxicam, acetyl salicylic acid and sulindac on glutathione and glutathione S-transferases in the rat oesophagus, intestine, stomach and liver.

Materials and methods

Animal treatment

Forty-eight male Wistar rats (183±2 g; Central Laboratory Animal Centre, University of Nijmegen, The Netherlands) were housed in pairs on wooden shavings in macrolon cages, maintained at 20–25°C and 30–60% relative humidity. A ventilation rate of seven air cycles/h and a 12 h light/dark cycle.
were used. The rats were randomly assigned to one of the dietary treatment groups. All groups were fed powdered RMH-TM lab chow (Hope Farms, Woerden, The Netherlands) from the same batch. After acclimatization for 7 days the animals were fed either the basal diet (control group) or one of the five experimental diets. Food and water were available ad libitum. Food cups were replenished every 2-3 days. Food consumption and gain in body weight were recorded daily.

Diet
Selection of NSAIDs as well as feeding period and dose levels were based on studies by others, showing reduction of tumour incidence in humans and inhibition of chemically induced carcinogenesis in animal models, where NSAIDs were adjusted 2 weeks prior to carcinogen treatment (13,18,21,32,36). The following six diet groups (eight animals per group) were studied: (a) RMH-TM lab chow only or supplemented with (b) 25 ppm indomethacin, (c) 400 ppm ibuprofen, (d) 400 ppm piroxicam, (e) 400 ppm acetyl salicylic acid, or (f) 320 ppm sulindac. The NSAIDs were purchased from Sigma Chemical Company, St. Louis, MO, USA. A food processor was used to obtain a homogenous mixture of test compound and powdered lab chow. After receiving the diets for 2 weeks the rats were killed by decapitation. The study protocol was approved by the local ethical committee for animal experiments of the University of Nijmegen.

Tissue preparation
All handling was performed on ice. After decapitation, oesophagus, stomach, intestine (proximal, middle, and distal small intestine and colon) and liver were dissected out and simultaneously frozen in liquid nitrogen and stored at -20°C until use. For preparation of the cytosolic fraction the tissue was thawed quickly using cold running water. The mucosal surface of stomach and intestine was collected by scraping with a scalpel and was homogenized in buffer A (4 ml/g tissue) in a glass/glass Potter-Elvehjem tube. The liver was homogenized in buffer A (4 ml/g tissue) with 10 strokes at 1000 rpm of a motor-driven glass/Teflon homogenizer (Braun, Germany). The homogenate was centrifuged at 9000 g (4°C) for 30 min. The resulting supernatant fraction was transferred to an ultracentrifuge tube and spun at 150000 g (4°C) for 60 min. The supernatant was homogenized in 5 ml buffer A per gram tissue in a glass/glass Potter-Elvehjem tube. These homogenates were centrifuged at 150 000 g for 60 min (4°C). Aliquots of the 150 000 g supernatant, representing the cytosolic fraction, were frozen in liquid nitrogen and stored at -20°C.

Assay
Protein concentration was assayed in quadruplicate by the method of Lowry et al. (46) using bovine serum albumin as the standard. GST activity was determined in triplicate according to Habig et al. (47), using 1-chloro-2,4-

Discussion
In the present study we have demonstrated that NSAIDs are able to induce glutathione S-transferases, especially in the upper part of the rat digestive tract.

During the last decade, many studies have shown significant protection against the development of cancer by NSAIDs. Compelling evidence is presented in several epidemiological studies, suggesting that NSAIDs have significant protective activity against human oesophageal, gastric, and colonic cancer (11,12). Regression of colon adenomas during treatment with NSAIDs, particularly sulindac, occurred in patients with familial adenomatous polyposis coli who are at high risk for
Effects of NAIDs on glutathione S-transferases

Table I. Daily food consumption, NSAID-intake and gain in body weight of male Wistar rats receiving diets supplemented with indomethacin, ibuprofen, piroxicam, ASA or sulindac

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (ppm)</th>
<th>Food consumption (g/day)</th>
<th>Total NSAID-intake (mg/day/kg b.)</th>
<th>Gain in body weight (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>16.1±0.3</td>
<td>—</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>25</td>
<td>15.9±0.5</td>
<td>2.0±0.1</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>400</td>
<td>17.0±0.2*</td>
<td>34.0±0.5</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>400</td>
<td>14.7±0.3*</td>
<td>29.4±0.5</td>
<td>1.2±0.2b</td>
</tr>
<tr>
<td>ASA</td>
<td>400</td>
<td>16.3±0.5</td>
<td>32.7±0.4</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>Sulindac</td>
<td>320</td>
<td>14.8±0.3*</td>
<td>29.6±0.7</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

Values given are means ±SEM. The one-tailed Wilcoxon rank sum test was used to assess statistical significance of differences between control and treated groups. aP<0.05, and bP<0.01.

Table II. Effects of indomethacin, ibuprofen, piroxicam, ASA or sulindac on rat alimentary tract glutathione S-transferase activity

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Glutathione S-transferase activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oesophagus</td>
</tr>
<tr>
<td>Control</td>
<td>49±5</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>66±5b</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>63±6a</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>88±7c</td>
</tr>
<tr>
<td>ASA</td>
<td>45±5</td>
</tr>
<tr>
<td>Sulindac</td>
<td>63±4a</td>
</tr>
</tbody>
</table>

PSI, proximal small intestine; MSI, middle small intestine; DSI, distal small intestine; aP<0.05, bP<0.01, and cP<0.005.

Table III. Effects of indomethacin, ibuprofen, piroxicam, ASA or sulindac on rat alimentary tract glutathione S-transferase α levels

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Glutathione S-transferase α level (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oesophagus</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>ND</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>ND</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>ND</td>
</tr>
<tr>
<td>ASA</td>
<td>ND</td>
</tr>
<tr>
<td>Sulindac</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not detectable; aP<0.05, bP<0.01.

Table IV. Effects of indomethacin, ibuprofen, piroxicam, ASA or sulindac on rat alimentary tract glutathione S-transferase μ levels

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Glutathione S-transferase μ level (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oesophagus</td>
</tr>
<tr>
<td>Control</td>
<td>3469±326</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>4738±343b</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>4792±268a</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>5432±356c</td>
</tr>
<tr>
<td>ASA</td>
<td>4008±399</td>
</tr>
<tr>
<td>Sulindac</td>
<td>5223±461f</td>
</tr>
</tbody>
</table>

aP<0.05, bP<0.01 and cP<0.005.

development of colonic cancer (2-4). NSAIDs such as aspirin, indomethacin, piroxicam, and sulindac were repeatedly shown to inhibit chemically induced tumours of the colon (15,17-19,29-31,51-53), oesophagus (26,27), bladder (54), breast (21), and liver (24) in laboratory animals.

Several hypotheses have been proposed to explain the mechanism of chemoprevention by NSAIDs: (a) NSAIDs reduce the gastrointestinal permeability of carcinogens and their metabolites (55), (b) NSAIDs are scavengers of reactive oxygen species involved in initiation and promotion of cancer (56), (c) NSAIDs can bind to cytochrome P450-monoxygenases, thereby inhibiting P450-mediated activation of procarcinogens to reactive electrophilic intermediates (57,58). On the other hand, ibuprofen and indomethacin are able to induce prokaryotic cytochrome P450BM_3 (CYP102) (59). (d) In parallel with the inhibition of tumour growth, aspirin, indomethacin,
and piroxicam, reduce the prostaglandin levels in the colon of rodents treated with carcinogens (15,17,60), by inhibition of cyclooxygenases, the rate-limiting enzymes in the synthesis of prostaglandins (61,62). On the other hand, GSTs are involved in the synthesis of prostaglandin D₂, E₂ and F₂α (63). (e) NSAIDs can inhibit the induction of ornithine decarboxylase activity and tissue levels of putrescine, two markers of tumour promotion (64,65). (f) In addition, NSAIDs may inhibit the activity of enzymes such as phosphodiesterases or cyclic GMP-AMP protein kinases (66), which may be central to cancer initiation and promotion.

Much of the research on NSAIDs and cancer prevention at this moment is focused on the hypothesis that prostaglandins may play a key role in the regulation of neoplasia. However, there is no direct evidence that NSAIDs prevent tumour development solely through inhibition of cyclooxygenases (67), and prevention of cancer could be due to multiple mechanisms. Another way of action of NSAIDs, in addition to the possibilities cited above, may be the enhancement of carcinogen detoxification by GSTs, as shown in this study. A more efficient detoxification could lead to a reduction of biologically active compounds and thus prevent carcinogenesis. No information about the possible effects of NSAIDs on oesophageal, gastric, intestinal, and hepatic GST enzyme activity has been reported before. In our study, GST activity in the oesophagus and proximal small intestine was increased by indomethacin, ibuprofen, piroxicam, and sulindac. This may be of direct significance in the protection against cancer in these organs. However, organs such as the colon could also benefit from a more efficient detoxification in the upper part of the digestive tract, since lower levels of carcinogens may now reach the colon.

In human organs at high risk for cancer development low GST levels were measured, and vice versa (68). Recent data, mostly obtained from animal studies, have indicated that many naturally occurring dietary anticarcinogens are able to elevate the levels of GSTs (44,45, and references therein). Enhancement of GSTs in humans was found after consumption of cruciferous vegetables with cancer preventing properties such as broccoli and Brussels sprouts (69,70) and a reduction of oxidative DNA damage in humans was measured after consumption of brussels sprouts (71). In this respect the chemopreventive properties of NSAIDs could very well be mediated in a similar way by the enhancement of GSTs.

In addition to the measurement of GST activity, it may be important to register changes in the levels of GST isoenzymes, which have different though partly overlapping substrate specificities. The isoenzymes showed a tissue specific distribution. Class α GSTs are abundant in liver and small intestine, whereas class Pi GSTs are present in stomach, small- and large intestine (43,45). In contrast, class μ enzymes seem to be less organ specific since they were detected at high levels in all tissues examined. GST α was not detectable in the oesophagus and colon, and GST π was undetectable in both oesophagus and liver and none of the NSAIDs had any effect on this. The increase in GST activity in oesophagus and PSI by indomethacin, ibuprofen, piroxicam and sulindac was paralleled by a rise in GST μ level in the same order of magnitude. This was expected, since GST μ is the most prominent of all GSTs in the rat (Tables III—V). In two of the sites in which both GST activity and GST μ levels were induced, GST α level was increased as well. The molecular basis for these inductions is not clear yet.

Indomethacin, ibuprofen, piroxicam, and sulindac each induced the GST enzyme activity as well as GST α, GST μ or GST π levels in at least one organ. Indomethacin, ibuprofen and sulindac were equally efficient in inducing glutathione S-transferases, in seven out of 28 possibilities (25%). Piroxicam appeared to be the most active, with inductions seen in 32% of all possibilities. Acetyl salicylic acid showed an increase of glutathione S-transferase enzyme activity or isoenzyme levels in only 11% of all possibilities, which makes it the least
This work was supported by grant KUN 94-715 (EMMvL) from the Dutch Cancer Society. The authors would like to thank H.M.J.Roelofs for her excellent technical assistance.

Acknowledgements

The authors would like to thank H.M.J.Roelofs for her excellent technical assistance.

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