Stomach

Gastric damage and granulocyte infiltration induced by indomethacin in tumour necrosis factor receptor 1 (TNF-R1) or inducible nitric oxide synthase (iNOS) deficient mice

M H L P Souza, H Paula Lemos, R B Oliveira, F Q Cunha

Background: Tumour necrosis factor α (TNF-α) is involved in non-steroidal anti-inflammatory drug induced gastropathy. Nitric oxide (NO) is a mediator of gastrointestinal mucosal defence but, paradoxically, it also contributes to mucosal damage.

Aims: We optimised the C57BL/6 mouse model of indomethacin induced gastropathy to evaluate the role of TNF-α and inducible nitric oxide synthase (iNOS) generated NO in gastric damage and granulocyte infiltration using tumour necrosis factor receptor 1 (TNF-R1+/−) or iNOS (iNOS+/−) deficient mice.

Methods: Different doses of indomethacin (2.5, 5, 10, 20 mg/kg) were administered and animals were assessed 6, 12, or 24 hours later. Gastric damage was measured by the sum of all erosions in the gastric mucosa, and gastric granulocyte infiltration was determined by myeloperoxidase (MPO) activity. Other groups of wild-type mice received thalidomide, dexamethasone, fucoidin, L-NAME, or 1400W, and then indomethacin was administered. Additionally, indomethacin was administered to TNF-R1−/− or iNOS−/−. Gastric damage and MPO activity were evaluated 12 hours later.

Results: Indomethacin induced dose and time dependent gastric damage and increase in MPO activity in wild-type mice, with the greatest effect at a dose of 10 mg/kg and after 12 hours. Treatment with thalidomide, dexamethasone, or fucoidin reduced gastric damage and MPO activity induced by indomethacin. After indomethacin administration, TNF-R1−/− had less gastric damage and MPO activity than controls. Genetic (knockout mice) or pharmacological (1400W and L-NAME) inhibition of iNOS activity reduced indomethacin induced gastric damage, despite no reduction in MPO activity.

Conclusion: TNF-α, acting via TNF-R1, is involved in indomethacin induced gastric damage and granulocyte infiltration. Furthermore, iNOS generated NO is involved in gastric damage induced by indomethacin.

Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most widely used drugs in the world. NSAID induced gastric damage is the major side effect associated with usage of this type of drug. Although the mechanism of NSAID induced gastropathy is generally believed to be related to the ability of these agents to inhibit gastric prostaglandin generation, there is a great deal of evidence to suggest that neutrophils are important in the pathogenesis of the gastric damage induced by NSAIDs. It is becoming increasingly appreciated that tumour necrosis factor α (TNF-α) plays a critical role in NSAID induced gastric injury by modulating neutrophil infiltration. The activities of TNF-α are mediated by two distinct cell surface receptors: TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2). Several studies have shown that TNF-R1 is the dominant signalling receptor for the inflammatory effect of TNF-α. However, the role of TNF-R1 in NSAID induced gastric damage has not been studied.

Nitric oxide (NO) is a crucial mediator of gastrointestinal mucosal defence but, paradoxically, it also contributes to mucosal damage. This can be illustrated by the ability of different NO concentrations to produce completely opposite effects in the same tissue. In general, the neuronal and endothelial nitric oxide synthase (NOS) isoforms produce low amounts of NO. In contrast, the inducible form of NOS (iNOS) produces NO in higher quantities. Piotrowski et al showed that when animals received an ulcerogenic dose of indomethacin, there was a 12-fold increase in gastric epithelial expression of iNOS activity compared with controls, and this increase was positively correlated with damage to the epithelium. On the other hand, Wallace and Miller stated that NO plays a critical role in modulating several components of mucosal defence, including increased gastric blood flow, reduced neutrophil adhesion, and increased mucus secretion. However, the relationship between iNOS and NSAID induced gastric damage has not been studied extensively and requires further clarification.

The recent development of genetically engineered mice with gene deletions (knockout) makes it theoretically possible to identify the specific inflammatory mediators that may be involved in NSAID induced gastropathy. In the present study, we optimised a C57BL/6 mouse model of indomethacin induced gastropathy to evaluate the role of TNF-α and iNOS generated NO in gastric damage and granulocyte infiltration using TNF-R1 and iNOS deficient mice.

Material and Methods

Animals
C57BL/6 mice (weight 18–20 g) were fasted for 18–24 hours before the experiments. Breeding pairs of mice with a targeted disruption of the TNF-α receptor R1 (p55) and iNOS genes were obtained from Jackson Laboratories (Bar Harbor, ME). The experimental protocol was approved by the Ethics Committee on Animal Care of the Federal University of Ceará, Brazil.

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; NO, nitric oxide; TNF-α, tumour necrosis factor α; TNF-R1, tumour necrosis factor receptor 1; TNF-R2, tumour necrosis factor receptor 2; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; TNF-R1−/−, TNF-R1 deficient mice; iNOS−/−, iNOS deficient mice.
Harbor, Maine, USA). Breeding stock backcrossed to C57BL/6 was obtained and the genotype of p55 and iNOS mice was determined by polymerase chain reaction of DNA, as previously described. Animals were housed in a sterile laminar flow cabinet until the start of the experiments, in temperature controlled rooms, and received water and food ad libitum. All animal treatments and surgical procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals, National Institutes of Health (Bethesda, Maryland, USA).

Drugs
Drugs used in this study were indomethacin (Prodome Química e Farmacêutica, São Paulo, São Paulo, Brazil), Tris (Merck, São Paulo, São Paulo, Brazil), 1400W (Cayman Chemical Co, Ann Arbor, Michigan, USA), L-NAME, thalidomide, dexamethasone, fucoidin, 3,3′, 5,5′-tetramethylbenzidine and hexadecyltrimethyl-ammonium bromide (all from Sigma Chemicals, St Louis, Missouri, USA).

Gastric damage induced by indomethacin in mice
Gastric damage was induced in wild-type C57BL/6 mice by intragastric installation of indomethacin (2.5, 5, 10, 20 mg/kg) dissolved in Tris buffer. The control group received only vehicle (Tris buffer). Animals were killed 6, 12 or 24 hours later by decapitation after light anaesthesia. Other groups of wild-type mice were treated with fucoidin, a sulphated fucosylated polysaccharide that binds to and blocks the function of L- and P-selectins12 13 (two doses of 25 mg/kg, five minutes before and six hours after, intravenously) or saline, and then indomethacin was administered (10 mg/kg). Twelve hours later, animals were sacrificed and their stomachs rapidly removed, opened by an incision along the greater curvature, and pinned onto a wax platform. Haemorrhagic or ulcerative lesions were counted and their length measured with analogue callipers. A gastric damage score (lesion index) was then calculated as the sum of the lengths of all linear erosions, which was measured by an observer (HPL) who was unaware of the treatment given to the animals. After scoring the damage, a sample of the corpus region of each stomach was excised for measurement of myeloperoxidase (MPO) activity. MPO is an enzyme found primarily in the azurophilic granules of neutrophils and therefore has been used extensively as a biochemical marker of granulocyte infiltration into various tissues, including the gastrointestinal tract.

Role of TNF-α in indomethacin induced gastric damage in mice
Gastric damage was induced in TNF-R1 deficient mice (TNF-R1−/−) or wild-type C57BL/6 mice by intragastric installation of indomethacin (10 mg/kg). Other groups of wild-type mice were treated with the TNF-α synthesis inhibitor thalidomide16 (50 mg/kg, one hour before, and 25 mg/kg, six h after, intraperitoneally), or with the glucocorticoid dexamethasone (two doses of 1 mg/kg, one hour before and six hours after, intraperitoneally). One hour later, indomethacin was administered (10 mg/kg). The control group received only vehicle. After a further 12 hours, gastric damage was determined as described above. Thereafter, full...
the saline group (ANOVA and Newman-Keuls test). Results are expressed as means (SEM) of at least five animals per group. *p<0.05 compared with the saline group (ANOVA and Newman-Keuls test).

### Role of iNOS derived NO in indomethacin induced gastric damage in mice

Gastric damage was induced in iNOS deficient (iNOS−/−) or wild-type C57BL/6 mice by intragastric instillation of indomethacin (10 mg/kg). Other groups of wild-type or iNOS−/− mice were treated with L-NAME (100 mg/kg, one hour before, and 50 mg/kg, six hours after indomethacin administration, intraperitoneally), and then indomethacin was administered (10 mg/kg). Another group of wild type mice was treated with a selective inhibitor of inducible NOS, 1400W (1.5 mg/kg, one hour before, intravenously), and then mice received indomethacin (10 mg/kg). The control group received only Tris buffer. Results are expressed as means (SEM) of at least five animals per group. *p<0.05 compared with the saline group (ANOVA and Newman-Keuls test).

**Gastric MPO activity**

The extent of granulocyte accumulation in the gastric mucosa was measured by assaying MPO activity, as previously described. Briefly, 50–100 mg of gastric tissue were homogenised in two volumes of ice cold buffer (0.1 M NaCl, 20 mM NaPO₄, 15 mM Na EDTA), pH 4.7, and centrifuged at 3000 rpm for 15 minutes. The pellet was then subjected to hypotonic lysis (900 µl of 0.2% NaCl solution followed 30 seconds later by addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose). After further centrifugation, the pellet was resuspended in 50 mM NaPO₄ buffer, pH 5.4, containing 0.5% H-TAB, and re-homogenised. The homogenate was then frozen and thawed three times and centrifuged again at 10 000 rpm for 15 minutes at 4°C. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using tetramethylbenzidine (1.6 mM) and H₂O₂ (0.5 mM). Firstly, the results were reported as total number of neutrophils by comparing absorbance of the tissue supernatant with that of rat peritoneal neutrophils processed in the same way. To this end, neutrophil migration was induced in the peritoneum of rats by injecting carrageenin (300 µg/animal). A standard curve relating neutrophil numbers (>90% purity, 12 500 to 195.3 neutrophils/50 µl) and absorbance was obtained by processing purified neutrophils, as described above, and assaying for MPO activity. The correlation between the number of neutrophils and units of MPO was determined using the technique described by Bradley and colleagues. The neutrophil standard curve was processed using 0.0005% hydrogen peroxide as substrate for MPO. A unit of MPO activity was defined as that converting 1 µmol of hydrogen peroxide to water in one minute at 22°C.

### Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by the Newman-Keuls test, when appropriate. Statistical significance was set at p<0.05.

### RESULTS

Intragastric administration of indomethacin induced linear haemorrhagic erosions in the corpus of the animals’ stomachs. As shown in fig 1, indomethacin induced gastric lesions and increased gastric MPO activity dose dependently, with the greatest effect at a dose of 10 mg/kg (fig 1A, B). The time course of both gastric damage and the increase in gastric MPO activity induced by indomethacin, at a dose of 10 mg/kg, peaked at 12 hours (fig 1C, D).

Gastric damage (fig 2A) and gastric MPO activity (fig 2B) induced by indomethacin (10 mg/kg) were significantly reduced by treatment with fucoidin. Figure 3 shows that thalidomide or dexamethasone treatment significantly inhibited both the gastric damage (fig 3A) and gastric MPO activity (fig 3B) induced by indomethacin. Furthermore, TNF-R1 deficient mice presented less gastric damage (fig 3C) and gastric MPO activity (fig 3D) than wild-type mice.

Treatment with L-NAME or 1400W reduced the gastric damage (fig 4A) induced by indomethacin. Gastric MPO activity induced by indomethacin was increased with L-NAME treatment and unchanged with 1400W treatment (fig 4B). In addition, after indomethacin administration, iNOS deficient mice had less severe gastric damage and similar indomethacin induced gastric MPO activity compared with wild-type mice (fig 4C, D).

Table 1 shows that L-NAME treatment did not change indomethacin induced gastric damage in iNOS deficient mice but increased gastric MPO activity compared with iNOS deficient mice.

### DISCUSSION

The recent development of genetically engineered mice makes this species particularly attractive as an animal model to define the pathogenic mechanism responsible for NSAID induced gastric damage. In the present study, we optimised a mouse model of indomethacin induced gastropathy and used TNF-R1 or iNOS deficient mice to evaluate the role of TNF-α and iNOS derived NO in the gastric damage and granulocyte infiltration induced by indomethacin. This last event was analysed because indomethacin induces gastric

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**Figure 2** Gastric damage and gastric myeloperoxidase (MPO) activity induced by indomethacin were significantly reduced by treatment with fucoidin. Wild-type mice were treated with saline or fucoidin. Five minutes later, indomethacin (10 mg/kg) was administered. Gastric lesions (A) and gastric MPO activity (B) were determined 12 hours later. The control (C) group received only Tris buffer. Results are expressed as means (SEM) of at least five animals per group. *p<0.05 compared with the saline group (ANOVA and Newman-Keuls test).

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**Figure 3** Gastric MPO activity induced by indomethacin was increased in iNOS deficient mice. The recent development of genetically engineered mice makes this species particularly attractive for the study of indomethacin induced gastropathy. Figure 3 shows that thalidomide or dexamethasone treatment significantly inhibited both the gastric damage (fig 3A) and gastric MPO activity (fig 3B) induced by indomethacin. Furthermore, TNF-R1 deficient mice presented less gastric damage (fig 3C) and gastric MPO activity (fig 3D) than wild-type mice.

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**Table 1** Treatment with L-NAME or 1400W reduced the gastric damage (fig 4A) induced by indomethacin. Gastric MPO activity induced by indomethacin was increased with L-NAME treatment and unchanged with 1400W treatment (fig 4B). In addition, after indomethacin administration, iNOS deficient mice had less severe gastric damage and similar indomethacin induced gastric MPO activity compared with wild-type mice (fig 4C, D).
damage is a neutrophil dependent process.\textsuperscript{2,6} Our data showed that TNF-\(\alpha\), acting via TNF-R1, mediates gastric damage and granulocyte infiltration, measured as MPO activity, in indomethacin induced gastropathy. Furthermore, iNOS generated NO is involved in gastric damage induced by indomethacin.

Indomethacin induced dose and time dependent gastric erosions and an increase in gastric MPO activity in mice, with the greatest effect 12 hours after administration. Gastric damage observed was associated with increased MPO activity because the severity and time course of the gastric damage were coincident with such infiltration. Moreover, treatment with fucoidin, a sulphated fucosylated polysaccharide that binds to and blocks the function of L- and P-selectins,\textsuperscript{12,13} reduced both gastric MPO activity and gastric damage induced by indomethacin. Similarly, other studies have shown that NSAID induced gastric damage in rats depends on neutrophil mucosal infiltration.\textsuperscript{2,5} Furthermore, treatment with anti-ICAM-1, P-selectin, and E-selectin attenuated the severity of indomethacin induced gastric in rats,\textsuperscript{19} and mice deficient in L- or P-selectins developed less indomethacin induced gastric damage.\textsuperscript{4}

We observed that TNF-\(\alpha\), acting via TNF-R1, is an important mediator in NSAID induced gastric damage and granulocyte infiltration as both events were reduced in TNF-R1\(-/-\) mice. The involvement of TNF-\(\alpha\) in this event was further supported by the observation that thalidomide, a drug that inhibits TNF-\(\alpha\) production,\textsuperscript{16} and also dexamethasone, reduced both gastric MPO activity and gastric damage induced by indomethacin. These results are in agreement with previous studies that showed a reduction in gastric susceptibility to indomethacin in rats treated with pentoxifylline, thalidomide, or dexamethasone.\textsuperscript{2,5} With regard to the TNF-R1 receptor, there is much evidence that it is the main receptor that mediates the inflammatory effect of TNF-\(\alpha\), being responsible for neutrophil migration and activation, including NO production.\textsuperscript{7} Furthermore, nuclear factor \(\kappa\)B, an important transcription factor for inflammatory events, including gastrointestinal inflammatory diseases,\textsuperscript{20} is activated by TNF-\(\alpha\) via the TNF-R1 receptor. The glucocorticoid dexamethasone, apart from inhibiting TNF-\(\alpha\) production, also inhibits release of the majority of other inflammatory cytokines, such as interleukin\(\beta\) and chemokines,\textsuperscript{21} and lipoxygenase products,\textsuperscript{22,23} which are mediators thought to be involved in the gastrointestinal inflammatory process.\textsuperscript{22} Our results do not eliminate the possibility that these molecules may also mediate indomethacin induced gastropathy in mice. In spite of the many studies showing involvement of NO in the gastric damage induced by chemical substances, such as serotonin or compound 48/80,\textsuperscript{24} by stress,\textsuperscript{25} or by Helicobacter pylori infection,\textsuperscript{26} the role of iNOS derived NO in NSAID induced gastric damage has not been completely elucidated. There is evidence that low doses of NO releasing substances protect against NSAID induced gastropathy\textsuperscript{27} and increase the healing rate of gastric ulcers.\textsuperscript{28} However, high doses of these substances could induce extensive haemorrhagic mucosal damage.\textsuperscript{29,30} Furthermore, there are studies showing that L-NAME, a non-specific NOS inhibitor, at doses of 15–50 mg/kg,
increases indomethacin induced gastric damage,\textsuperscript{31} reduces gastric blood flow,\textsuperscript{32} and delays healing of chronic gastric ulcers.\textsuperscript{33} Our results showed that iNOS generated NO is implicated in indomethacin induced gastric damage as iNOS deficient mice exhibited less gastric damage when indomethacin was administered, and wild-type mice treated with l-NAME at a dose of 100 mg/kg, which inhibits both cNOS and iNOS activity,\textsuperscript{34} or with 1400W, a selective inhibitor of iNOS,\textsuperscript{17} decreased indomethacin induced gastric damage. The fact that both genetic (knockout mice) and pharmacological (1400W) specific inhibition of iNOS reduced gastric damage indicates that the effect of l-NAME on indomethacin induced gastric damage was also determined by its effect on iNOS activity, and not by causing a decrease in gastric blood flow, which—due its inhibitory effect on cNOS—could, in turn, render less conspicuous the haemorrhagic expression of the gastric damage. Confirmation that the l-NAME effect was due to inhibition of iNOS was demonstration that l-NAME treatment did not enhance reduction of gastric damage caused by indomethacin in iNOS deficient mice (table 1). These results are in apparent contradiction with others described in the literature which showed an increase in indomethacin induced gastric damage by a non-specific NOS inhibitor, l-NAME, at a dose of 50 mg/kg.\textsuperscript{31} One possible explanation for this discrepancy is that at this dose, l-NAME is unable to efficiently inhibit iNOS activity.

With regard to the role of NO in granulocyte infiltration induced by indomethacin, our results suggest that small amounts of NO released by cNOS are sufficient to downregulate indomethacin induced gastric granulocyte infiltration induced by indomethacin, as shown by the decrease in gastric MPO activity in iNOS deficient mice (table 1).

**Table 1** Effect of l-NAME treatment in indomethacin induced gastropathy in iNOS deficient mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Lesion index* (mm)</th>
<th>MPO activity* (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (Tris buffer)</td>
<td>0.0 (0.0)</td>
<td>0.64 (0.06)</td>
</tr>
<tr>
<td>Indomethacin (wild-type mice)</td>
<td>23.1 (3.7)</td>
<td>5.36 (0.26)</td>
</tr>
<tr>
<td>Indomethacin+saline (iNOS\textsuperscript{-/-})</td>
<td>12.5 (2.6)\textsuperscript{††}</td>
<td>6.46 (1.02)</td>
</tr>
<tr>
<td>Indomethacin+l-NAME (iNOS\textsuperscript{-/-})</td>
<td>9.3 (0.7)\textsuperscript{†††}</td>
<td>8.52 (0.74)\textsuperscript{†††}</td>
</tr>
</tbody>
</table>

*Data are mean (SEM) of 4–6 mice per group.
iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase.
\textsuperscript{††p}<0.01, indomethacin+saline (iNOS\textsuperscript{-/-}) versus indomethacin (wild-type mice).
\textsuperscript{†††p}<0.001, indomethacin+l-NAME (iNOS\textsuperscript{-/-}) versus indomethacin (wild-type mice).
infiltration. This interpretation is supported by the fact that we observed a reduction in gastric MPO activity only when both NOS isoforms (cNOS and iNOS) were inhibited by l-NAME in both wild-type and iNOS−/− mice. In line with our results, there is evidence in the literature that NO inhibits expression of cell adhesion molecules on endothelial cells, which is an important step in neutrophil migration.41–43

The fact that the reduction in indomethacin induced gastric lesions observed in iNOS−/−, in l-NAME or 1400W treated mice, was not accompanied by a similar reduction in granulocyte infiltration appears to contradict the hypothesis that NSAID induced gastric damage is a neutrophil dependent process. However, one possible explanation is that although neutrophils, which could be the source of the NO involved in gastric damage, are present in the gastric mucosa, iNOS in these cells is not able to produce NO and/or other reactive nitrogen species.

In summary, our results indicate that TNF-α, acting via TNF-R1, is involved in indomethacin induced gastric damage and neutrophil infiltration in mice. Furthermore, iNOS generated NO is involved in gastric damage induced by indomethacin.

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