

PROTECTIVE ROLE OF NITRIC OXIDE IN INDOMETHACIN-INDUCED GASTRIC ULCERATION BY A MECHANISM INDEPENDENT OF GASTRIC ACID SECRETION

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Gastric ulceration was induced in rats by i.p. injection of the non-steroidal anti-inflammatory drug (NSAID), indomethacin (IND) (30 mg kg⁻¹). Pyloric ligation was carried out in each animal before injection to enable collection of the gastric juice. Three hours later, the animals were killed and their stomachs were removed. In the gastric juice, the amounts of mucin, pepsin and HCl were assessed. Gastric mucosa were scrapped for the determination of nitric oxide (NO) (as nitrite) after evaluation of the gastric ulcer index.

The influence of arginine (ARG) (300 mg kg⁻¹), a NO precursor, N^G-nitro-L-arginine methyl ester (L-NAME) (50 mg kg⁻¹), a non-selective constitutive nitric oxide synthase/inducible nitric oxide synthase (cNOS/iNOS) inhibitor, and the selective iNOS inhibitor aminoguanidine (AMG) (50 mg kg⁻¹) were studied. Each NO modulator was injected i.p. 30 min before IND administration. Results indicated that IND elevated gastric acidity by 80% of the normal group, decreased non-significantly mucosal nitrite by 22% and exhibited a remarkably high ulcer index ($\chi = 17$). Neither mucin nor pepsin levels were significantly altered. In comparison with the IND group, pretreatment with L-NAME caused a significant decrease in gastric HCl, further decrease in mucosal nitrite (50% of normal) and a two-fold increase in the ulcer index score ($\chi = 34$), despite the decrease in HCl. AMG did not alter gastric acidity, decreased mucosal nitrite by 38% of the normal value and failed to alter significantly the ulcer index of IND. On the other hand, pretreatment with ARG did not alter the gastric acidity and raised mucosal nitrite by 10% above normal. Surprisingly, ARG improved the gastric ulcer score ($\chi = 1$) almost similar to the normal score ($\chi = \text{zero}$). Therefore, this study creates a new pathway for the potential treatment of NSAID gastric ulceration through modulation of NO synthesis, regardless of the effect on gastric acidity. © 2001 Academic Press

KEY WORDS: nitric oxide, indomethacin, gastric ulcer, arginine, NOS inhibitors.

INTRODUCTION

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is limited by their ulcerogenic activity as well as their ability to interfere with ulcer healing. These effects are mainly mediated through inhibition of prostaglandin synthesis [1]. Recently, many strategies have been adopted to minimize the NSAID-induced gastropathy. These include the development of NSAIDs that only inhibit the inducible isoform of cyclooxygenase (COX-2) [2] and coupling of NSAIDs with a nitric oxide (NO)-releasing molecule [3]. NO donors have been repeatedly shown to protect gastric mucosa against damage

induced by various agents [4–6]. However, the role of NO in regulation and maintenance of the functions of gastric mucosa has not yet been fully understood.

The present investigation was therefore designed to explore the biochemical effects of the NO precursor, L-arginine (ARG), and the nitric oxide synthase (NOS) inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective constitutive NOS/inducible NOS (cNOS/iNOS) inhibitor, and the selective iNOS inhibitor aminoguanidine (AMG) on indomethacin (IND)-induced gastric ulceration, gastric acid secretion as well as mucin and pepsin secretions. The level of the mucosal NO was examined after each treatment. The ultimate goal was to understand the role of NO in the regulation of gastric acid secretion and to search for a simple protective mechanism against NSAID-induced gastropathy.

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MATERIALS AND METHODS

Animals

Male Wistar albino rats weighing 150–200 g were used in this study. The rats were treated in humane conditions and were allowed free access to water and diet *ad libitum* until the beginning of the experiment. All experimental protocols were approved by the Animal Care Committee of the University of Cairo.

Induction of ulcer

Rats were deprived of food, but not water, for about 18–24 h before the experiment. For each rat, the abdomen was then incised and the pylorus was ligated under ether anesthesia. Indomethacin (30 mg kg⁻¹ suspended in 1% Tween 80) was given intraperitoneally immediately after pyloric ligation. Three hours later, animals were killed with an ether overdose. A normal group that was treated similarly but without administration of indomethacin was included in the study.

Effects of the NOS modulators

Three groups of rats were given intraperitoneally 30 min before pyloric ligation either the NO precursor, L-arginine (300 mg kg⁻¹), or the NOS inhibitors L-NAME (50 mg kg⁻¹) or AMG (50 mg kg⁻¹). Doses of drugs were in homology with previous reports [7, 8].

Collection of the gastric juice

After the animals were killed, their stomachs were removed following ligation of the oesophocardiac junction, washed with distilled water and dried between filter paper and opened along the greater curvature. The gastric juice was drained and centrifuged at 3500 rpm [9] and used for the following determinations:

Measurement of gastric acidity

Titrate acidity in the supernatant of the gastric juice was determined by titration with 0.01 M NaOH using phenol red as an indicator. Units are expressed as mEq l⁻¹ [10].

Determination of pepsin activity

Peptic activity is a major factor involved in the proteolytic activity of gastric secretion. Measured pepsin activity represents the amount of liberated tyrosine (μmol) per 1 ml of gastric juice per minute, using 1:100 diluted gastric juice and 2% bovine albumin in N/100 HCl as a substrate. In detail, each sample of the gastric juice was first diluted 1:100 with N/100 HCl. One ml of the diluted juice was added to 5 ml of 2% bovine serum albumin solution. The mixture was incubated at 37 °C for exactly 10 min in a water bath. At the end of the incubation period, 10 ml of 0.3 M trichloroacetic acid was then added and the mixture was boiled for 5 min. The solution was then centrifuged for 5 min at 3000 rpm and filtered. To 1 ml of the filtrate 2 ml of 0.5 N NaOH and 0.2 ml of Folin reagent were added. The color that developed after

20 min was measured colorimetrically at 680 nm. A blank experiment was similarly carried out using 1 ml of N/100 HCl. In addition, a standard solution containing 0.2 ml working tyrosine standard made up to 1 ml with N/100 HCl was also included [11].

Determination of mucin content

The method adopted is based on the determination of the hexose component of the mucin. It depends on the reaction of carbohydrate in concentrated sulfuric acid with Orcinol (5-methyl resorcinol) to give a colored product that can be measured colorimetrically. In detail, 0.25 ml of 1:20 diluted juice was added to an equal volume of 1.6% Orcinol and 2 ml of 60% sulfuric acid. The mixture was boiled in a water bath for 10 min and then cooled on ice. The optical density was measured at 425 nm. Results are expressed as mg hexose dl⁻¹ [12].

Determination of gastric ulcer index

After collection of the gastric juice, the stomach of each animal was then rinsed with saline and inflated on cork. The ulcer index was expressed as the sum of the length (mm) of all lesions in the fundic region [13].

Determination of mucosal nitric oxide

Finally, the gastric mucosa of each rat was scrubbed off, weighed and its nitric oxide content was determined as nitrite by diazotization with sulfanilic acid at acidic pH and subsequent coupling with N-1-naphthyl-ethylene diamine to give a colored product that was measured colorimetrically at 548 nm [14].

Statistical analysis

Data are presented as mean \pm SEM for 6–10 animals. Statistical significance between groups was evaluated using analysis of variance (ANOVA). In all data analysis, *P* values of 0.05 or less were considered significant

RESULTS

As shown in Table I, IND elevated significantly gastric acidity by 80% above that of the normal group. This elevation was normalized by pretreatment with L-NAME. Pretreatment with AMG or ARG failed to significantly modify the elevated gastric acid secretion. Neither IND nor any pretreatment had a significant effect on mucin content or peptic activity of the gastric juice.

With regard to the effects on gastric ulcer formation, IND treatment induced a remarkably high ulcer index ($x = 17$) (Fig. 1). Pretreatment with L-NAME further aggravated the ulcer formation as reflected by a two-fold increase in the ulcer index score ($x = 34$). AMG pretreatment failed to alter significantly the ulcer index score as compared to the IND index. On the other hand, ARG profoundly improved the gastric ulcer score ($x = 1$) to almost that of the normal score ($x = 0$).

Concerning the effect of treatments on mucosal nitrite

Table I
HCl level, mucin content and pepsin activity in the gastric juice of normal, indomethacin, L-NAME, aminoguanidine, and L-arginine treated rats. Results are mean \pm SEM of 6–10 animals

	Normal	IND	L-NAME	AMG	ARG
HCL	48.5 \pm 15.2	87.3 \pm 7.7 ^b	44.3 \pm 6.3 ^a	64.0 \pm 13.7	62.4 \pm 5.1
Pepsin	9.3 \pm 0.9	7.6 \pm 1.1	7.5 \pm 1.2	7.4 \pm 0.8	8.0 \pm 1.0
Mucin	444.3 \pm 39.5	439.4 \pm 49.7	481.4 \pm 51.2	434.8 \pm 44.1	473.6 \pm 46.3

^aSignificantly different from IND at $P < 0.01$. ^bSignificantly different from normal at $P < 0.05$. Units are mEq l⁻¹ for HCl, mg hexose dl⁻¹ for mucin, μ mol ml⁻¹ min⁻¹ for pepsin.

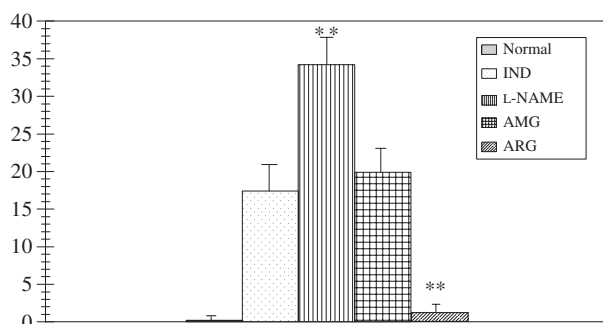


Fig. 1. Ulcer index (mm) of normal, indomethacin, L-NAME, aminoguanidine, and L-arginine treated rats. Results are mean \pm SEM of 6–10 animals. (**) Significantly different from indomethacin at $P < 0.01$.

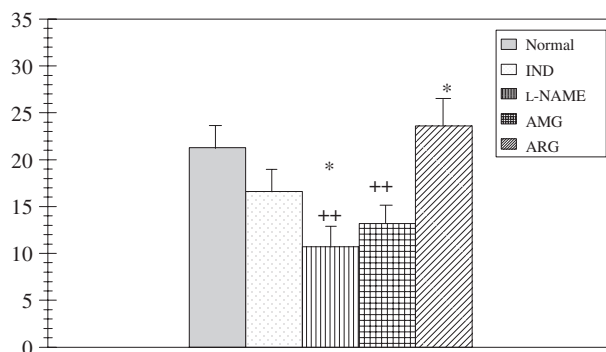


Fig. 2. Level of mucosal nitrite (μ g g⁻¹ tissue) in normal, indomethacin, L-NAME, aminoguanidine, and L-arginine treated rats. Results are mean \pm SEM of 6–10 animals. (*) Significantly different from indomethacin at $P < 0.05$. (++) Significantly different from normal at $P < 0.01$.

(Fig. 2), IND decreased non-significantly mucosal nitrite by 22%. In contrast, all pretreatments, as anticipated, affected mucosal nitrite content either by reduction (50% with L-NAME and 38% with AMG) or elevation (10% with ARG) as compared with the values of the normal group.

DISCUSSION

The present study refers to the importance of mucosal NO in the protection against NSAID-induced gastric ulceration. Evidence is clearly demonstrated by the prevention of IND-induced gastric ulcer formation by pretreatment

with the NO precursor, L-arginine, and the aggravation of ulcer formation by pretreatment with the NOS inhibitor, L-NAME. These effects were, interestingly, independent of gastric acid secretion or other measured factors such as pepsin activity or mucin content. Rather, they are well correlated with mucosal nitrite level.

In support of this conclusion, several reports from different laboratories have demonstrated the importance of endogenous NO in the protection of gastric mucosa. Two studies from Pique's laboratory [15, 16] have shown that NO plays a vasodilatory role in the gastric microcirculation during acid secretion. Other studies have accredited the role of NO as an endogenous modulator of leukocyte adhesion [17]. In support, Calatayud *et al.* [18] have recently shown that transdermal nitroglycerin protected against indomethacin-induced gastric ulceration through maintenance of mucosal blood flow and reduction of leukocyte–endothelial cell rolling and adherence. In addition, Wallace [19] stated that reduction of gastric blood flow is the main predisposing factor in the induction of NSAID gastropathy.

Reports have not restricted the role of NO to gastric protection, but also include the acceleration of ulcer healing. Konturek *et al.* [20] have shown that glyceryl trinitrate was capable of ulcer healing and suppression of NO synthesis resulted in impaired ulcer healing. It is possible that NO directly accelerates ulcer repair by promoting the growth of smooth muscles as suggested by Hogaboam *et al.* [21].

Our data show a close agreement between the prevention of ulcer formation produced by L-arginine against indomethacin-induced ulceration and the maintenance of mucosal NO. This agreement was also found for the effect of the NOS inhibitor, L-NAME, which aggravated ulcer formation. It is noticeable that, although AMG, a selective iNOS inhibitor, lowered significantly gastric mucosal nitrite, it failed to significantly alter indomethacin-induced damage. This suggests that cNOS may be the major enzyme involved.

Other than the role of NO in maintenance of blood flow, NO may protect against NSAID damage by promotion of prostaglandin synthesis. A mutual interaction was shown to exist between NOS and cyclooxygenase (COX) enzymes. NO donors were shown to enhance COX activity whereas NOS inhibitors blocked PGE₂ production [22]. In addition, indomethacin, a COX inhibitor, reduced the level of cyclic GMP that was

increased by NO donors [23]. This may explain the 22% decrease in mucosal NO by indomethacin administration observed in our study.

Results of the studies investigating the effect of NOS inhibitors on gastric acid secretion are quite controversial. While Pique *et al.* [16] found that inhibition of NOS by L-NMMA did not affect either basal or pentagastrin-stimulated acid secretion, Kato *et al.* [24] showed that inhibition of NO synthesis by L-NAME stimulated the increase of acid secretion induced by pentagastrin by using an *ex vivo* stomach chamber. In accordance, Martinez-Cuesta *et al.* [25] demonstrated the role of NO in the endotoxin-induced inhibition of pentagastrin-stimulated acid secretion and that L-NAME antagonized this inhibition. In contrast, Bilski *et al.* [26] observed that NO inhibition failed to affect basal acid secretion but reduced acid secretion stimulated by pentagastrin or meat feeding. Thus, in the scope of these controversial reports, it seems that control of gastric acid secretion is not of prime importance as a mechanism for the prevention of gastric ulceration induced by NO donors or aggravation of gastric ulceration by NO inhibitors. This conclusion complies well with the data presented in this study. While L-NAME antagonized the indomethacin-stimulated acid secretion, L-arginine administration failed to modify significantly acid secretion. We assume that not only pharmacological factors, but also hemodynamic factors determine the influence of NO precursors and inhibitors on gastric acid secretion. We postulate that the inhibitory effect of L-NAME on indomethacin-induced gastric acid secretion is possibly related to local gastric ischemia and that the prevention of gastric ulceration by L-arginine profoundly observed in this study is not directly related to an effect on gastric acid secretion. On the same line, other NO donors, such as nitrofenac and glyceryl trinitrate, were shown to accelerate gastric ulcer healing by a mechanism independent of modification of gastric acid secretion [27]. Although other studies have reported that indomethacin also increases pepsinogen secretion, we did not find in this study significant changes in pepsin activity after indomethacin administration. This may be related to differences in the other studies in the timing of measurement of pepsin activity and/or duration of treatment with indomethacin, as indomethacin was given to rats just in a single dose.

In conclusion, results of this study show that L-arginine almost completely protects against indomethacin-induced gastric ulceration by a mechanism independent of modulation of acid secretion, mucin content or pepsin activity, but via maintenance of mucosal NO. Thus, this study focuses the attention for an alternative pathway for treating the universal problem of NSAID-induced gastropathy.

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