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ENDOTHELIN-1, INTERLEUKIN-4 AND NITRIC OXIDE SYNTHASE MODULATORS OF GASTRIC MUCOSAL INJURY BY INDOMETHACIN: EFFECT OF ANTIULCER AGENTS

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Endothelin-1 (ET-1), nitric oxide, and cytokines are recognized mediators of the inflammatory processes associated with gastric mucosal injury. In this study, we investigated mucosal expression of ET-1, interleukin-4 (IL-4), and the activity of constitutive nitric oxide synthase (cNOS) during indomethacin-induced gastric mucosal injury, and evaluated the effect of antiulcer agents on this process. The experiments were conducted with groups of rats pretreated intragastrically with ranitidine (100 mg/kg), ebrotidine (100 mg/kg), sulglycotide (200 mg/kg) or vehicle, followed 30 min later by an intragastric dose of indomethacin (60 mg/kg). The animals were killed 2 h later and their mucosal tissue subjected to macroscopic damage assessment and the measurements of epithelial cell apoptosis, ET-1, IL-4, and cNOS. In the absence of antiulcer agents, indomethacin caused multiple hemorrhagic lesions and extensive epithelial cell apoptosis, accompanied by a 20.7% reduction in IL-4, a 3.1-fold increase in mucosal expression of ET-1 and a 4.2-fold decline in cNOS. Pretreatment with H2-receptor antagonist, ranitidine produced a 15.7% reduction in the mucosal damage caused by indomethacin, 29.5% decrease in epithelial cell apoptosis and a 19.6% reduction in ET-1, while the expression of IL-4 increased by 10.8% and that of cNOS showed a 2-fold increase. The H2-blocker, ebrotidine, also known for its gastroprotective effects, reduced the indomethacin-induced lesions by 90.2%, epithelial cell apoptosis decreased by 61% and ET-1 showed a 58.2% decline, while IL-4 increased by 30.6% and that of cNOS showed a 3.1-fold increase. Pretreatment with gastroprotective agent, sulglycotide, led to a 51.2% reduction in the extent of mucosal damage caused by indomethacin, a 43.9% decrease in apoptosis, and a 63.5% decrease in ET-1, while the expression of cNOS increased by 3.4-fold and the level of IL-4 showed a 32.2% increase. The results suggest that an increase in vasoconstrictive ET-1 level combined with a decrease in regulatory cytokine, IL-4, and a loss of compensatory action by cNOS may be responsible for gastric mucosal injury caused by indomethacin. Our findings also point to a value of ebrotidine and sulglycotide in countering the untoward gastrointestinal side effects of NSAID therapy.

Key words: *Gastric mucosa, indomethacin, injury, ET-1, IL-4, cNOS, ranitidine, ebrotidine, sulglycotide.*

INTRODUCTION

Reduced mucosal blood flow and the injury to vascular endothelial cells, combined with oxyradical generation and the inhibition of prostaglandin synthesis, are well recognized early events associated with the use of NASIDs (1—4). The increased mucosal vascular tone and gastric mucosal microcirculatory disturbances have been intimately linked to the release from the endothelium of a potent vasoactive peptide, ET-1, known for its stimulatory effect on proinflammatory cytokine generation and the alterations in nitric oxide, a key mediator of signaling events linked to apoptotic cell death (5—10).

The endothelins (ET) produced by endothelial cells are family of 21-amino acid, cysteine-rich peptides containing two intramolecular disulfide bridges (11, 12). At the present, the existence of three active isoforms of endothelin, ET-1, ET-2, and ET-3, and two distinct endothelin receptors, ET_A and ET_B, is well documented (13, 14). The ET_A receptor mediates vasoconstriction and displays a high affinity for ET-1, while the ET_B receptor exhibits an equal affinity for ET-1 and ET-3 and its activation results in vasoconstriction (ET_{B2} subtype) as well as vasodilatation (ET_{B1} subtype) associated with the production of nitric oxide and prostacyclin (7, 12, 15). Studies indicate that ET receptors are also found in gastric and intestinal mucosa, and that ET-1 plays a major role in the pathogenesis of stress ulcer and gastric mucosal injury induced by local ischemia-reperfusion, indomethacin and ethanol (2, 15, 16—18). Moreover, new data suggest that the cytotoxic effects of indomethacin in gastric mucosa are manifested by an increased mucosal generation of proinflammatory TNF-α, disturbances in nitric oxide signaling pathway, and apoptotic caspase activation (19, 20).

Interestingly, enhanced ET-1 levels accompany local and systemic inflammations, and there are in vivo and in vitro studies indicating that ET-1 affects formation of several proinflammatory cytokines, including TNF-α, IL-2 and IL-6 (7—9). The expression of these mediators of inflammatory process is controlled at the translational level by IL-4, a pleiotropic cytokine that exerts a wide range of biological effects on target cells through the specific IL-4 receptor (21, 22). IL-4-induced receptor dimerization leads to a rapid onset of a chain of tyrosine protein transphosphorylation events that culminate in activation of JAK-STAT pathway (22, 23). The phosphorylated STAT factors then dimerize, translocate to the cell nucleus, and modulate transcription of target genes leading to such cellular events as suppression of apoptosis, proliferation, and differentiation (21—23).

In this study, we investigated the course of gastric mucosal apoptotic events during indomethacin-induced injury by analyzing the mucosal expression of ET-1 and IL-4, and the activity of constitutive nitric oxide synthase (cNOS), and evaluated the effect of antiulcer agents, ranitidine, ebrotidine, and sulglycotide, on this process.

MARERIALS AND METHODS

Animals

This study was conducted with Sprague-Dawley rats weighing 250—270 g and cared for by the professional personnel of the Research Animal Facility. The animals were deprived of food for 24 h before the experiment, and water was withheld for 2 h before the procedure. All studies were carried out with groups of 10 animals per treatment (19). By means of Teflon-fitted tubing attached to a 2-ml syringe, the animals received intragastric pretreatment with either ranitidine at 100 mg/kg, ebrotidine at 100 mg/kg, sulglycotide at 200 mg/kg, or the saline vehicle. This was followed 30 min later by an ulcerogenic dose of indomethacin at 60 mg/kg (19, 20). The animals in each group were killed 2 h after indomethacin, their stomachs dissected, and the mucosal tissue used for the assessment of macroscopic damage (19), quantification of ET-1 and IL-4 expression, and the assays of cNOS activity and epithelial cell apoptosis.

Apoptosis assay

Quantitative measurements of gastric epithelial cell apoptosis were carried out with a sandwich enzyme immunoassay directed against cytoplasmic histone-associated DNA fragments (Boehringer Mannheim). Gastric muucosal epithelial cells were prepared from gastric mucosal scraping (19). The cells were incubated in the lysis buffer in accordance with the manufacturer's instruction, centrifuged at 20,000 × g for 10 min, and the diluted supernatant containing the cytoplasmic histone-associated DNA fragments was reacted in the microtitrator wells with immobilized antihistone antibody. After being washed, the retained complex was reacted with anti-DNA peroxidase, and the immunocomplex-bound peroxidase probed with ABTS reagent for spectrophotometric quantitization. The values were expressed in apoptotic units per milligram of protein (absorbance at 405 nm/mg protein (19).

IL-4 assay

Quantitative measurement of IL-4 was conducted using a solid-phase enzyme-linked immunosorbent system (Bio-Source International). The individual specimens of gastric mucosal scrapings were homogenized with 5 volumes of the sample buffer and centrifuged, and the resulting supernatant diluted at 1 : 3 (24). Sample diluents were pipetted to the microtitrator wells precoated with antibody specific for rat IL-4, and following incubation the complex was probed with biotinylated second antibody. After washing, the retained complex was then reacted with streptividine-peroxidase and incubated with tetramethylbenzidine (TMB) reagent for spectrophotometric IL-4 quantitization (24).

cNOS assay

Constitutive nitric oxide synthase activity of gastric mucosa was measured using a NOS-detect kit (Stratagene). The individual specimens of gastric mucosa were homogenized in a sample buffer containing either 10 mM EDTA (inducible NOS) or 6 mM $CaCl_2$ (cNOS), and centrifuged at

800 × g for 10 min (10, 25). The aliquots of the resulting supernatants were incubated for 30 min at 25°C in the presence of L-[2,3,4,5-^{3}H] arginine (50 μCi/μl), 10 mM NADPH, 5 μM tetrahydrobiopterin, and 50 mM Tris-HCl buffer, pH 7.4, in a final volume of 250μl. The reaction was terminated by adding to each sample a 0.4 ml of stop buffer followed by 0.1 ml of equilibrated Dowex-50 W (Na^+) resin. The mixtures were transferred to spin cups, centrifuged and the formed L-[3H] citrulline contained in the flow through was quantitated by scintillation counting.

ET-1 assay

For quantitative measurement of ET-1, the individual specimens of gastric mucosal tissue following lyophilization were homogenized with 4 volumes of 1 M acetic acid containing 10 μg/ml of pepstatin (10, 18). The homogenate was heated for 5 min at 100°C to inactivate proteases, and centrifuged at 10,000 × g for 20 min at 4°C. The resulting supernatant was applied to a Sep-Pack C-18 reverse phase cartridges, washed with 10 ml of 0.1% trifluoroacetic, and the adsorbed ET-1 was eluted with 3 ml of methanol-water-trifluoroacetic acid (90:10:0.1, v/v/v). The eluates were dried under vacuum, reconstituted in the assay buffer, and subjected to immunometric ET-1 quantitization using double antibody sandwich technique according to the manufacturer's (Alexis Corporation) instructions. The sample aliquots were applied to the microtitrator wells coated with ET-1 capture antibody, an acetylcholinesterase: Fab' conjugate which binds selectively to a different epitope of ET-1 molecule added, and the complex was incubated at 4°C for 16 h. After washing, the wells were probed with Ellman's reagent, incubated at room temperature for 2 h, and ET-1 quantitated spectrophotometrically at 412 nm (10).

Antiulcer drugs

The antiulcer agents, ranitidine and ebrotidine, were kindly donated by Ferrer Internacional, S.A., Barcelona, Spain, while the sulglycotide was provided by Crinos Industria Farmacobiologica, Villa Guardia, Italy. The drugs were stored at 4°C in the dark and were suspended in saline shortly before experimentation. The drugs or vehicle were given in a volume of 1 ml.

Data analysis

All experiments were carried out in duplicate, and the results are expressed as the means ± SD. The significance level was set at $p < 0.05$. The Mann-Whitney 'U' test was used to compare the scores between the groups. The protein content of samples was measured with the BCA protein assay kit. The tests were performed using Soft Stat, STATISTICA, software.

RESULTS

Intragastric administration of indomethacin at an ulcerogenic dose of 60 mg/kg caused within 2 h extensive multiple hemorrhagic lesions, affecting mostly the glandular portion of the mucosa, and involving 29.3 ± 1.5 mm^2 of

the corpus area (*Fig. 1*). Pretreatment with gastroprotective agent, sulglycotide (26), produced a 51.2% reduction in the extent of damage caused by indomethacin, the H2-receptor antagonist, ranitidine reduced lesion formation by a 15.7%, while ebrotidine, recognized for its acid inhibitory and gastroprotective effects (19, 27), elicited a 90.2% reduction in lesions (*Fig. 1*).

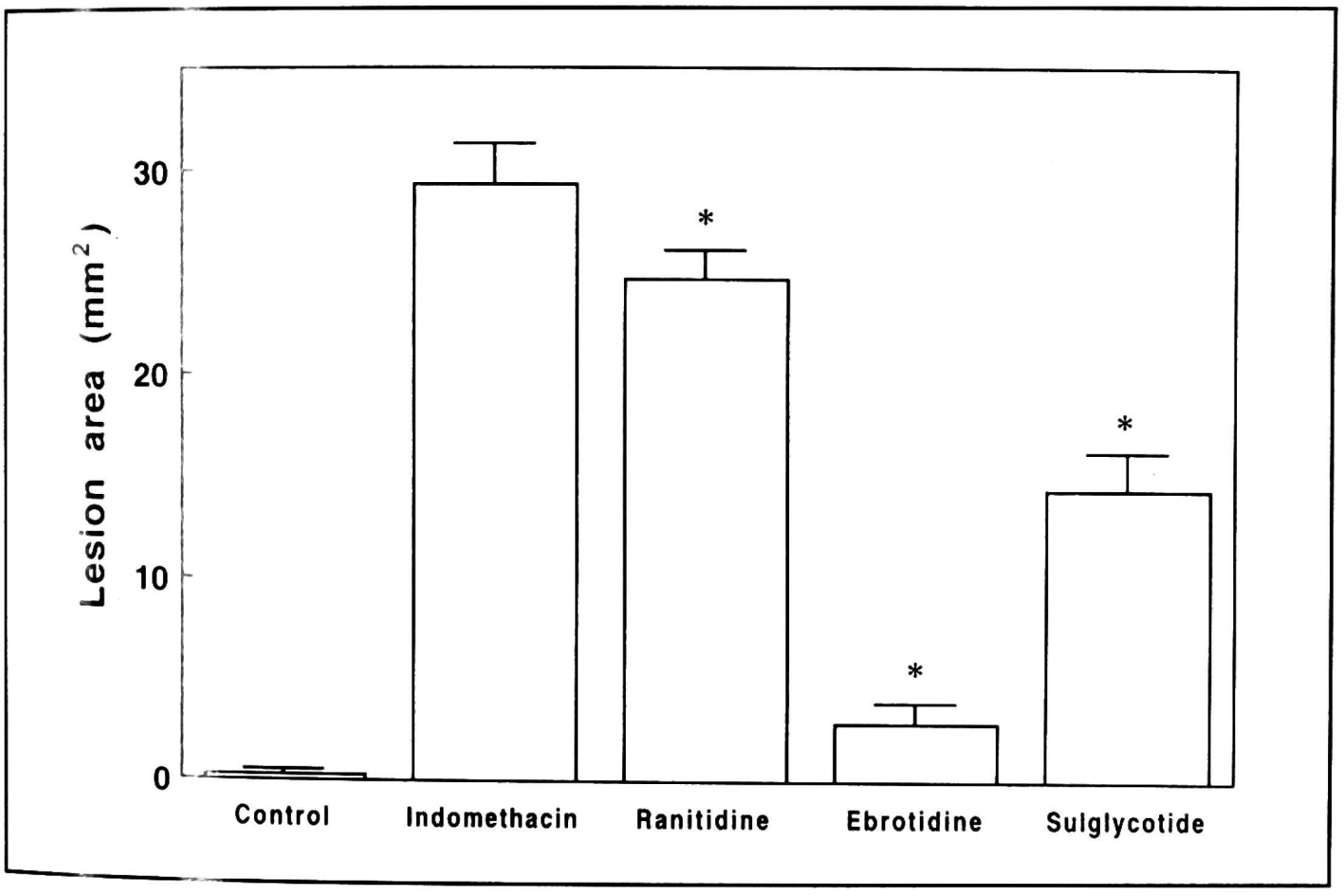

Fig. 1. Effect of antiulcer agents on gastric mucosal lesions caused by intragastric administration of an ulcerogenic dose (60 mg/kg) of indomethacin. Ranitidine (100 mg/kg), ebrotidine (100 mg/kg) or sulglycotide (200 mg/kg) were administerd 30 min before indomethacin. Values represent the means ± SD of analyses carried out with 10 animals in each group. * $P < 0.05$ compared with that of indomethacin.

Figure 2 presents the results of apoptotic DNA fragmentation assays conducted with epithelial cells isolated from gastric mucosa of the indomethacin-treated animals in the absence and the presence of pretreatment with antiulcer agents. The quantitative measurements of cytosolic histone-associated DNA fragments revealed that, compared with controls (1.3 unit/mg protein), intragastric administration of indomethacin evoked a marked increase in epithelial cell apoptosis (26.5 unit/mg protein). Pretreatment with sulglycotide caused a 43.9% decrease in the extent of DNA fragmentation elicited by indomethacin, ebrotidine produced a 61% reduction,

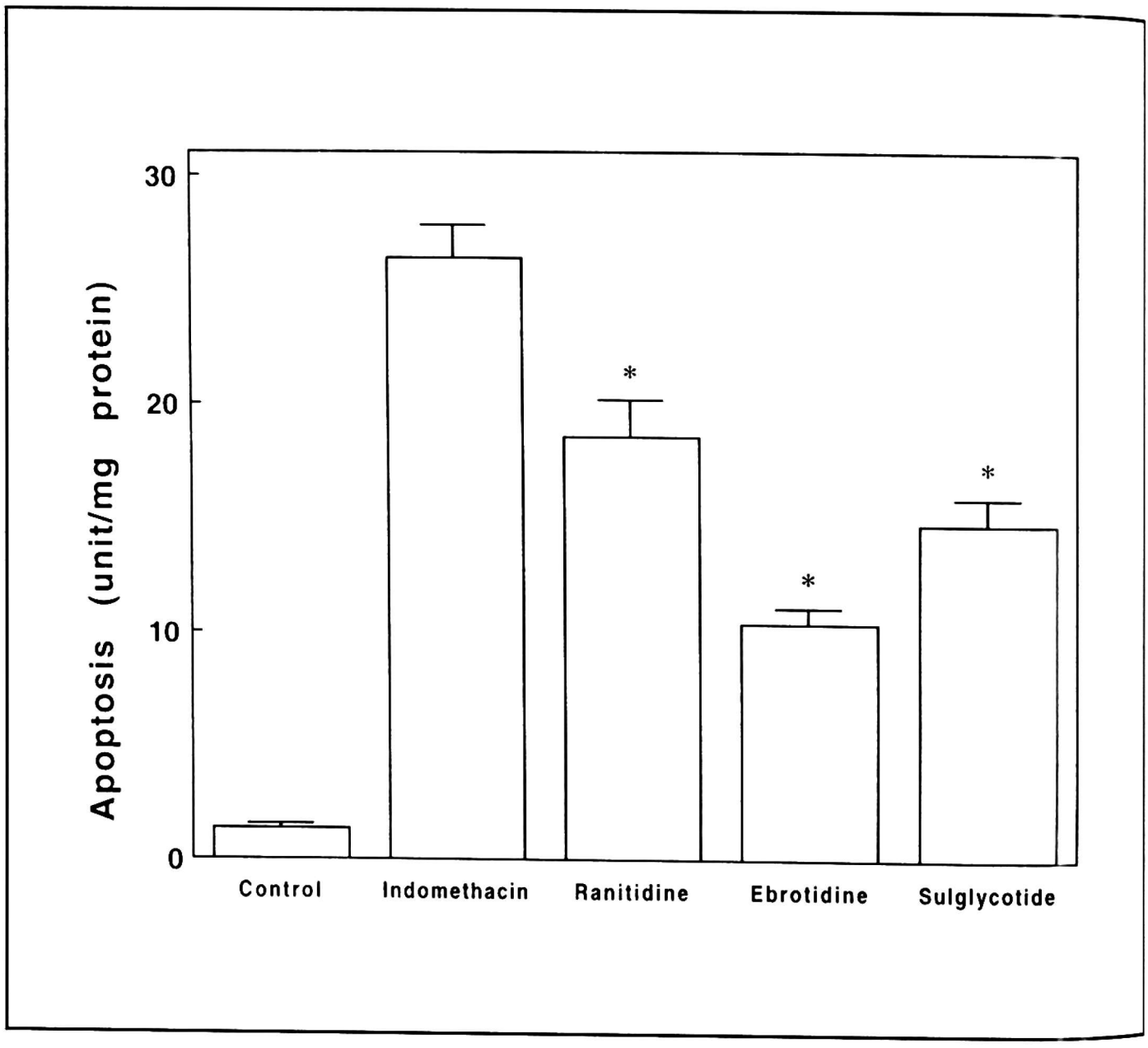

Fig. 2. Effect of ranitidine, ebrotidine and sulglycotide on gastric epithelial cell apoptosis during indomethacin-induced mucosal injury. Pretreatment with the antiulcer agents was carried out 30 min before indomethacin. Values represent the means ± SD of duplicate analyses performed with 10 animals in each group. * $P < 0.05$ compared with that of indomethacin.

whereas pretreatment with ranitidine elicited a 29.5% reduction in the extent of indomethacin-induced apoptosis.

The expression of gastric mucosal cNOS activity during indomethacin--induced mucosal injury in the absence and the presence of pretreatment with antiulcer agents is shown in *Fig. 3*. The assays of Ca^{2+} – dependent NOS activity, monitored by conversion of [^{3}H]arginine to [^{3}H]citrulline, revealed that, compared with controls, the animals subjected to an ulcerogenic dose of indomethacin showed a 4.2-fold decline in the gastric mucosal expression of cNOS activity. This effect of indomethacin was countered by the pretreatment with antiulcer agents, with ranitidine causing a 2-fold increase in cNOS activity over that of indomethacin, sulglycotide producing a 3.4-fold increase, and ebrotidine eliciting a 3.1-fold increase.

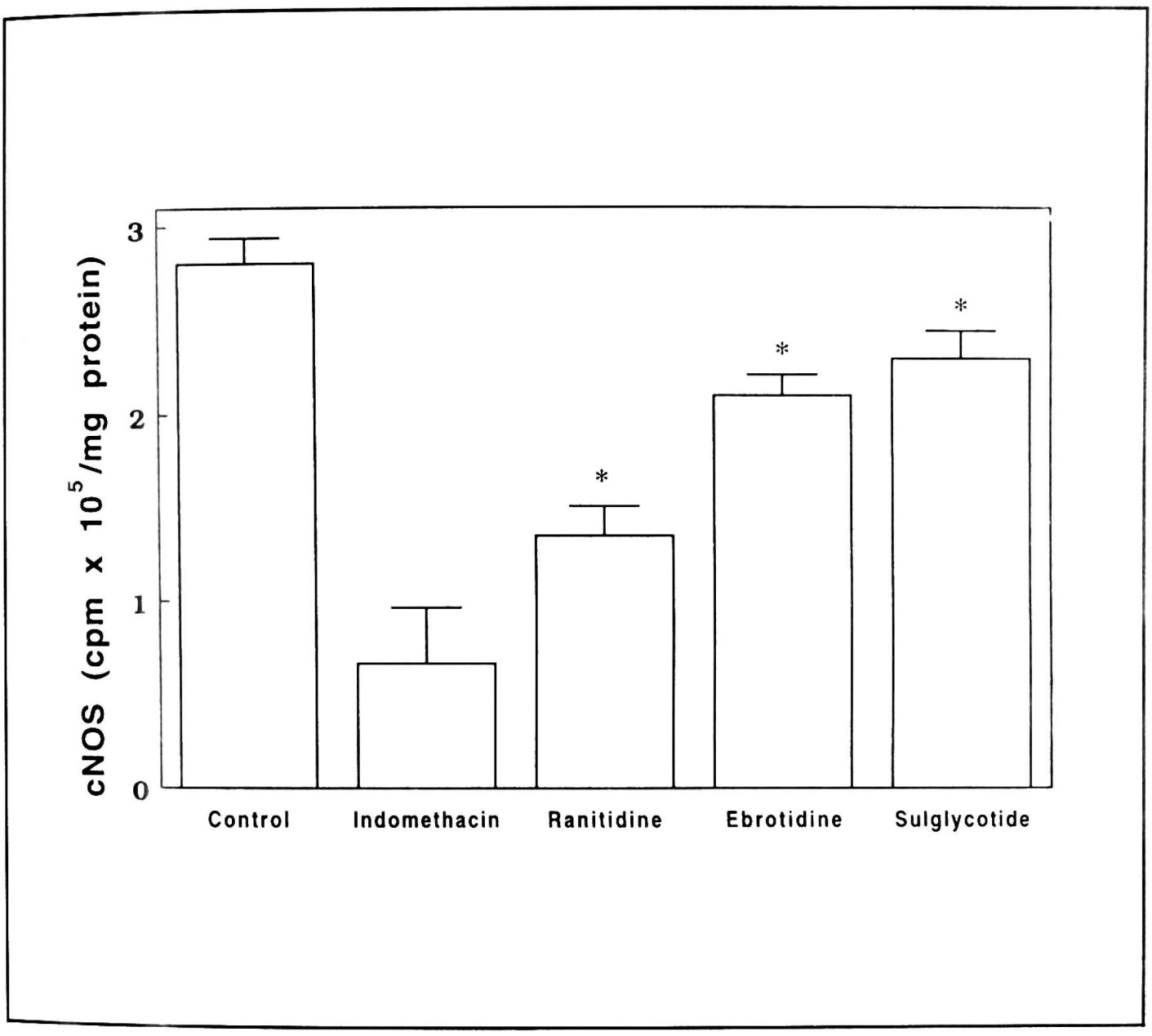

Fig. 3. Effect of ranitidine, ebrotidine and sulglycotide on the expression of cNOS activity in gastric mucosa during indomethacin-induced mucosal injury. Pretreatment with the antiulcer agent was carried out 30 min before indomethacin. Values represent the means ± SD of duplicate analyzes performed on 10 animals in each group. * $P < 0.05$ compared with that of indomethacin.

Figure 4 shows the data on gastric mucosal expression of Et-1 during indomethacin-induced injury in the absence and the presence of pretreatment with antiulcer agents. The results of immunometric assays established a mean value for ET-1 in the controls at 0.61 pg/mg protein, while that in gastric mucosa of the animals subjected to indomethacin treatment reached the mean value of 1.66 pg/mg protein. This represents a 3.1-fold increase over that of the controls. Pretreatment with sulglycotide produced a 63.5% reduction in gastric mucosal ET-1 level induced by indomethacin; ebrotidine evoked a 58.2% reduction, whereas ranitidine elicited only a 19.6% reduction in ET-1 expression evoked by indomethacin.

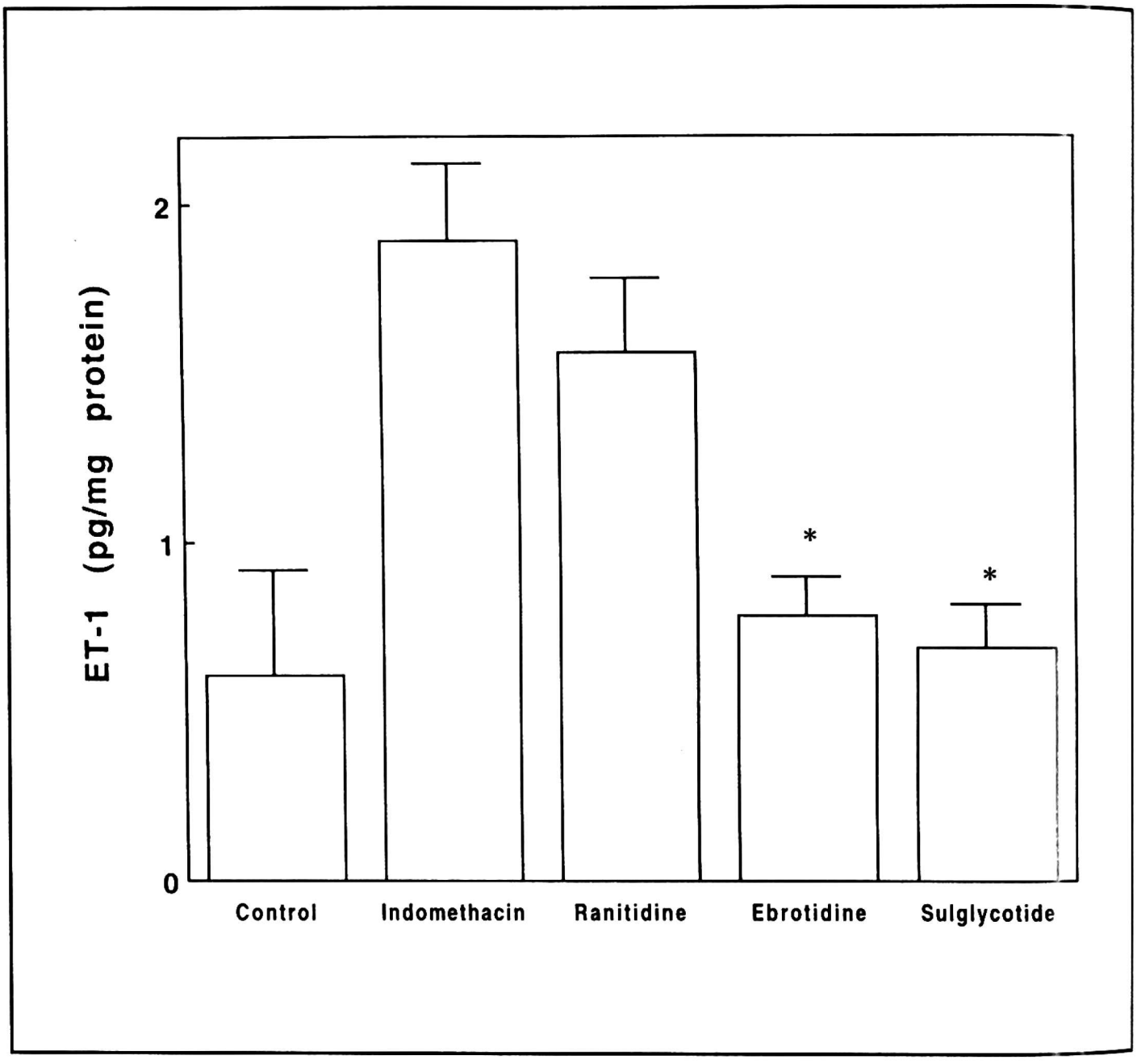

Fig. 4. Effect of ranitidine, ebrotidine and sulglycotide on the expression of gastric mucosal ET-1 during indomethacin-induced mucosal injury. Pretreatment with the antiulcer agents was carried out 30 min before indomethacin. Values represent the means ± SD of duplicate analyses performed on 10 animals in each group. * $P < 0.05$ compared with that of indomethacin.

The expression of gastric mucosal IL-4 in response to intragastric administration of ulcerogenic dose of indomethacin in the absence and the presence of pretreatment with antiulcer agents is depicted in *Fig. 5*. Compared with controls, the animals subjected to ulcerogenic dose of indomethacin showed a 20.7% reduction in gastric mucosal expression of IL-4, and the level increased by only a 10.8% following pretreatment with ranitidine. On the other hand, pretreatment with sulglycotide evoked a 32.2% increases in IL-4 over that shown in the mucosa of the animals in the indomethacin group, and ebrotidine produced a 30.6% increase.

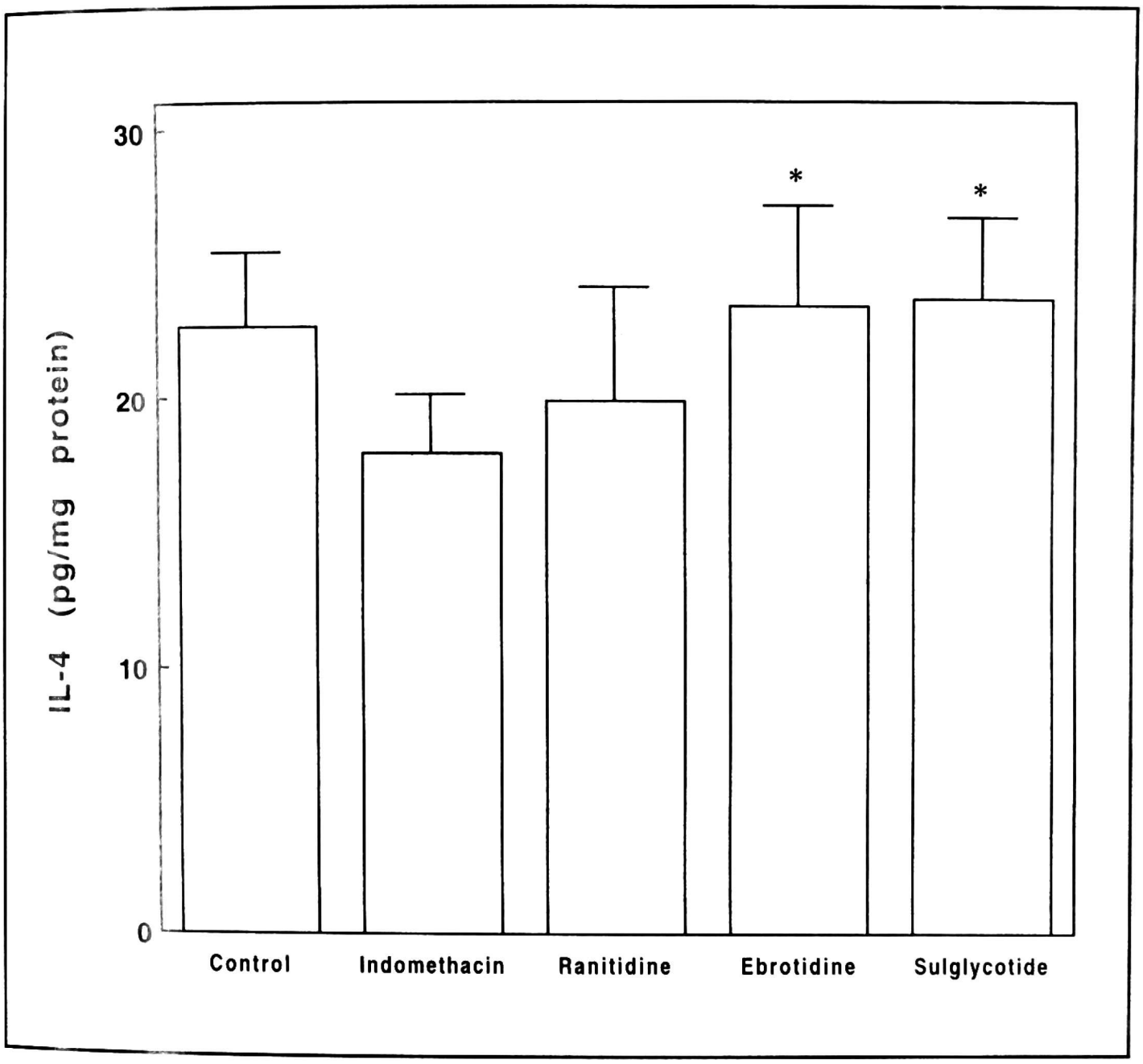

Fig. 5. Effect of ranitidine, ebrotidine and sulglycotide on the expression of gastric mucosal IL-4 during indomethacin-induced mucosal injury. Pretreatment with the antiulcer agents was carried out 30 min before indomethacin. Values represent the means ± SD of duplicate analyses performed on 10 animals in each group. * $P < 0.05$ compared with that of indomethacin.

DISCUSSION

Previous studies from our laboratory showed that gastric mucosal responses to indomethacin, manifested by a marked enhancement in proinflammatory TNF-α expression, lead to the activation of caspase death signaling cascade, induction in NOS-2, and result in massive epithelial cell apoptosis (19, 20). As the formation of TNF-α is controlled by ET-1 and IL-4 that remain under the influence of nitric oxide signaling pathway (21—23), we investigated further the course of events during gastric mucosal injury induced by indomethacin by analyzing the interplay between the extent of epithelial cell apoptosis, the mucosal expression of ET-1 and IL-4, and the activity of cNOS.

The results obtained revealed that gastric mucosal responses to indomethacin, characterized by a massive epithelial cell apoptosis, were accompanied by a 3.1-fold increase in mucosal expression of ET-1, 4.2-fold decrease in cNOS, and a 20.7% reduction in IL-4.

Pretreatment with gastroprotective agent, sulglycotide, elicited a 51.2% reduction in the extent of mucosal damage caused by indomethacin, and this effect of sulglycotide was reflected in a 43.9% decrease in epithelial cell apoptosis and a 63.5% reduction in ET-1, while the expression of IL-4 increased by 32.2% and cNOS showed a 3.4-fold increase. Ebrotidine, recognized for its acid suppressant and gastroprotective effects, produced a 90.2% reduction in the extent of mucosal lesion caused by indomethacin, and this was associated with a 61% reduction in apoptosis and a 58.2% reduction in ET-1, while the IL-4 expression increased by 30.6% and cNOS by a 3.1-fold. On the other hand, the pretreatment with H2-blocker, ranitidine, which produced only 15.7% reduction in the extent of mucosal damage caused by indomethacin, was reflected in its lesser value in countering indomethacin effects on epithelial cell apoptosis (29.5%), and the expression of ET-1 (19.6%) and IL-4 (10.8%). However, the activity of cNOS showed a 2-fold increase. The fact that the indomethacin-induced mucosal damage was associated with a marked decrease in cNOS activity, and the protective effect of antiulcer agents was reflected in its increased expression provides a strong indication as to the importance of cNOS activity in the maintenance of gastric mucosal homeostasis.

These findings, together with our recent data on the enhanced expression of NOS-2 during gastric mucosal injury by indomethacin (20), underscore also the importance of cNOS and NOS-2 in regulation of the events involved in apoptotic cell death. Indeed, of the three NOS isoenzymes responsible for nitric oxide generation, the inducible isoform, NOS-2, provides a high NO output for host defense, but its sustained activation leads to the induction of apoptotic caspase cascade (28—31). Furthermore, the enhanced expression of NOS-2 results in the formation of NO-related species such as nitrosothiols, peroxynitrate, and dinitrosyl iron complexes which exert a direct inhibitory effect on NF-κB, and hence cause transcriptional disturbances that lead to apoptosis (32). On the other hand, the two constitutively expressed isoforms of NOS (cNOS) provide NO pulses for a fine modulation of the cellular processes, and play an active role in the inhibition of apoptogenic signals generated by caspase activation (28, 29). Apparently, this inhibitory effect of cNOS occurs through S-nitrosylation of the apoptotic caspase-3 activity which leads to the suppression of Bcl-2 cleavage, thus preventing the mitochondrial release of cytochrome c, and resulting in the inhibition of apoptosis (28, 29, 31).

There are also indications that cNOS is involved in the inhibition of the catalytic activity of caspase enzymes through a cGMP-dependent mechanism,

associated with phosphorylation-dephosphorylation event, that functions at the level of caspase zymogen activation that requires cleavage adjacent to aspartates (29). Hence, the potential of sulglycotide and ebrotidine in preventing the injurious effect of indomethacin may well depend on their ability to maintain mucosal cNOS activity at the level required for the suppression of apoptotic caspase activities. This interpretation of our results is supported by the data indicating that during ischemia-reperfusion injury the endothelial activity of cNOS is compromised (2, 33, 34).

The role of ET-1 in pathogenesis of gastric mucosal injury remains an active area of investigations, and over the years several mechanisms of its damaging action have been revealed, including generation of free radicals, up-regulation of PAF formation, and perturbations of calcium channel and phospholipase C activation (2, 12, 17, 34—36). Moreover, it has been demonstrated recently that ET-1 stimulates the biosynthesis of proinflammatory cytokine, TNF-α (7). This process, apparently, involves the activation by ET-1 of ET_A receptor which, in turn, leads to the activation of tyrosine kinase intracellular protein cascade and ultimately culminates in translation of the TNF-α gene (7, 37). Incidentally, there are reports suggesting that nitric oxide, a product of NOS, regulates both the interaction of ET-1 with its receptor and by directly displacing bound ET-1 from the ET_A receptor and by interfering with postreceptor pathways related to calcium mobilization (34, 38). The later event appears to be related to calcium channel activation by protein S-nitrosylation (39).

Since, as shown by us recently, the mucosal damaging effect of indomethacin are intimately linked to epithelial cell apoptosis and the agent displays its influence on apoptogenic signal propagation consistent with that induced by TNF-α (19, 20), our current findings on the enhanced expression of ET-1 strongly imply a key involvement for this vasoactive peptide in triggering gastric epithelial apoptosis. Indeed, gastric mucosal level of ET-1 increased dramatically (3.1-fold) in response to indomethacin, while the reduction in mucosal damage following pretreatment with sulglycotide and ebrotidine was also reflected in a marked (2.7- and 2.4-fold, respectively) decrease in the mucosal expression of ET-1, and ranitidine which produced limited (15.7%) reduction in the extent of mucosal damage caused by indomethacin elicited also less pronounced (19.6%) decrease in ET-1.

The processing pathway in ET-1 synthesis involves the proteolytic cleavage of the initial gene product, an inactive big ET-1 consisting of 39 amino acids, by a specific protease that removes 18 amino acids from its carboxyl terminal (12, 40). This protease, referred to as endothelin-converting enzyme (ECE), is a typical metallopeptidase with an exclusive cell surface location, and characterized by its sensitivity to phosphoramidon (12, 40). Hence, the activity of ECE as well as that of a specific metalloproteinase responsible for the production of

the soluble form of TNF-α (41) is important for apoptotic signal generation. In this connection, it is noteworthy that the inflammatory responses involving up-regulation of metalloproteinase gene expression are controlled at the translational level by the regulatory cytokine, IL-4 (21—23). Indeed, Il-4 not only is known to suppress the secretion of proinflammatory IL-1, IL-2, and IL-6, but also to block the synthesis and processing of metalloproteinases (7, 21, 22, 42).

From the results obtained in this study, it is apparent that indomethacin exerts detrimental effect on the gastric mucosal IL-4 expression, causing dysregulation of ET-1 production, induction of TNF-α, and triggering the apoptotic events that exacerbate the inflammatory process. This course of events, combined with the loss of compensatory action by cNOS, may be responsible for gastric mucosal injury caused by indomethacin. Hence, sulglycotide and ebrotidine may have a value in countering the untoward gastrointestinal side-effects of NSAID therapy.

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