ROLE OF CASPASE-3 AND NITRIC OXIDE SYNTHASE-2 IN GASTRIC MUCOSAL INJURY INDUCED BY INDOMETHACIN: EFFECT OF SUCRALFATE

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Background: Apoptosis is the process of programmed cell death characterized by a series of distinct biochemical and morphological changes which involve activation of caspase proteases cascade that remains under the regulatory control of nitric oxide. Here, we investigated the activity of a key apoptotic protease, caspase-3, and the expression of inducible nitric oxide synthase (NOS-2) and tumor necrosis factor-α (TNF-α) associated with gastric epithelial cells apoptosis during indomethacin-induced gastric mucosal injury, and evaluated the effect of antiulcer agent sucralfate on this process. Methods: The experiments were conducted with groups of rats pretreated intragastrically with 200 mg/kg sucralfate or the vehicle, followed 30 min later by an intragastric dose of indomethacin at 60 mg/kg. The animals were killed 2 h later and their gastric mucosal tissue used for macroscopic assessment, assays of epithelial cells apoptosis and TNF-α, and the measurements of caspase-3 and NOS-2 activities. Results: In the absence of sucralfate, indomethacin caused multiple hemorrhagic lesions occupying 29.3 mm² of the corpus area, and accompanied by a 20-fold enhancement in gastric epithelial cells apoptosis and a 47% increase in mucosal expression of TNF-α, while NOS-2 showed an 11.9-fold induction and the activity of caspase-3 increased 3.9-fold. Pretreatment with sucralfate produced a 59.7% reduction in the extent of mucosal damage caused by indomethacin, a 41.2% decrease in the epithelial cells apoptosis and a 33.4% reduction in TNF-α, while the activity of caspase-3 decreased by 45% and that of NOS-2 showed a 44.7% decline. Conclusions: The results implicate caspase-3 in the process of indomethacin-induced gastric epithelial cells apoptosis, and point towards participation of NOS-2 in the amplification of the cell death signaling cascade. Our findings also show that sucralfate protection against gastric mucosal injury caused by indomethacin involves the suppression of NOS-2 and the apoptotic events propagated by caspase-3.

Key words: indomethacin, mucosal injury, apoptosis, TNF-α, caspase-3, NOS-2, sucralfate.

INTRODUCTION

Sucralfate, a basic aluminum salt of sucrose octasulfate, is a potent gastroprotective agent, widely used in peptic ulcer therapy (1, 2). Over the
years, numerous studies demonstrated that the beneficial effects of the drug in ulcer healing occur through the stimulation of endogenous prostaglandin generation due to rapid activation of mucosal phospholipase A\textsubscript{2} for arachidonic acid release, stimulation of mucus glycoprotein synthesis and secretion, and the inhibition of peptic erosion of mucus gel (3—6). Moreover, the evidence indicates that sucralfate causes enhancement in the efficacy of growth factors during mucosal repair process, and affects the regulatory cytokines associated with cell cycle events which stimulate cellular proliferation (7—9). These effects of sucralfate can be achieved even in the presence of inhibitors of endogenous prostaglandin generation such as indomethacin (6, 10, 11).

Although the damaging effects of indomethacin are most often ascribed to the impairment in prostaglandin synthesis and the disturbances in mucosal blood flow and superoxide radicals generation (5, 12—14), more recent data point towards the detrimental action of indomethacin the processes associated with cellular proliferation, cell cycle progression and apoptosis (15, 16). Other recently recognized cytotoxic effects of indomethacin are triggered by an increase in mucosal generation of proinflammatory cytokines and the disturbances in nitric oxide signaling pathway (16—19).

Of the three calmodulin-dependent nitric oxide synthase (NOS) isozymes, the two constitutively expressed isoforms of NOS (cNOS) are Ca\textsuperscript{2+}-dependent and provide precise pulses of NO for a fine modulation of the cellular processes, including the inhibition of apoptotic signals (21—24). The inducible isoform of NOS, known as iNOS or NOS-2, is Ca\textsuperscript{2+}-independent and, once induced, provides a high output of NO generation for host defense. However, its massive and sustained activation may have also cytotoxic consequences causing transcriptional disturbances and the induction of aspartate-specific proapoptotic casteine proteases, termed caspases (23—27).

The mammalian caspase family of proteases consists of three subfamilies of proteins homologous to interleukin-1β converting enzyme, and synthesized as single chain inactive zymogens that require cleavage adjacent to aspartates for activation (25—28). The activation of caspases and subsequent proteolysis of specific set of cellular proteins is recognized as an irreversible commitment to the execution phase of apoptosis characterized by cytoplasmic shrinkage, breakdown of the nuclear envelope, condensation of chromatin structure, and DNA fragmentation (26, 27).

In this study, we investigated gastric mucosal activity of a key apoptotic protease, caspase-3, and the expression of NOS-2 associated with the epithelial cells apoptosis triggered by the enhancement in TNF-α during indomethacin-induced gastric mucosal injury, and evaluated the effect of sucralfate on this process.
MATERIALS AND METHODS

Animals

The study was conducted with Sprague-Dawley rats weighing 250 to 275 g and cared for by the professional personnel of Research Animal Facility. The animals were deprived of food for 24 h before the experiment, and water was withheld 2 h before the procedure. All studies were carried out with groups consisting 10 animals per treatment. By means of Teflon-fitted tubing attached to a 2-ml syringe, the animals received intragastric pretreatment with sucralfate at 200 mg/kg or the saline vehicle. This was followed 30 min later by an ulcerogenic dose of indomethacin at 60 mg/kg (16). 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 (16), assays of NOS-2 and caspase-3 expression, and quantization of TNF-α and epithelial cells apoptosis.

Caspase-3 activity assay

Caspase-3 activity assays were carried out with gastric epithelial cells using Quanti Zyme assay system (Biomat Res. Lab., Inc.). The assay is based on the ability of the active enzyme to cleave DEVD-pNA (Asp-Glu-Val-Asp-p-nitroanilide) substrate. Gastric mucosal cells were collected by scraping the mucosa with a blunt spatula. The scrapings were minced by passage through a 50-mesh grid, and the epithelial cells were dispersed by homogenization and settled by centrifugation (16). The cell aliquots were incubated at 4°C with the lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA) according to the manufacturer's instruction and the lysates were centrifuged at 10,000 × g for 10 min. The aliquots of the resulting cytosolic fraction, diluted with the reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM EDTA, and 10% glycerol) to contain 30 μg of protein, were incubated in the microtiter wells with 50 μM of DEVD-pNA for 1 h at 37°C, and the caspase-3 activity measured spectrophotometrically at 405 nm.

NOS-2 activity assay

NOS-2 activity of gastric mucosa was measured by monitoring the conversion of L-[3H]arginine to L-[3H]citrulline using NOS-detect Assay Kit (Stratagene). The mucosal tissue was homogenized in a sample buffer containing 10 mM EDTA, and centrifuged at 800 × g for 10 min (29). The aliquots of the resulting supernatants were incubated for 30 min at 25°C in the presence of L-[2,3,4,5-3H]arginine (50 μCi/μl), 10 mM NADPH, 5 μM tetrahydrobiopterin, and 50 mM Tris-HCl buffer, pH 7.4, in a final volume of 250 μl. The reaction was terminated by adding to each sample a 0.4 ml of stop buffer followed by 0.1 ml of equilibrated Dowex-50W resin (30). The mixtures were transferred to spin cups, centrifuged and the formed L-[3H]citrulline contained in the flow through was quantitated by scintillation counting (29).

Apoptosis assay

For quantitative measurements of epithelial cells apoptosis, the gastric mucosal scrapings were minced by passage through a 50-mesh grid, the cells were dispersed by homogenization and collected by centrifugation (16). The assays were carried out with a sandwich enzyme immunoassay directed against cytoplasmic histone-associated DNA fragments according to the manufacturer's (Boehringer Mannheim) instruction. The cells were lysed in the lysis buffer, centrifuged, and the diluted supernatant containing the cytoplasmic histone-associated DNA fragments reacted in the
microtiter wells with immobilized anti-histone antibody, and the complex processed for spectrophotometric detection (16).

**TNF-α expression assay**

TNF-α was quantitated with a solid-phase enzyme-linked immunosorbent assay according to the manufacturer's (Genzyme) instruction. The wells were precoated with monoclonal anti-TNF-α to capture TNF-α from the mucosal homogenates, and after washing away the excess of reagent the wells were probed with horseradish peroxidase-conjugated polyclonal anti-TNF-α, the complex was then incubated with TMB substrate solution and TNF-α quantitated spectrophotometrically (16).

**Antiulcer drug**

The antiulcer drug, sucral fate, was kindly provided by Chugai Pharmaceutical Co. Ltd., Tokyo, Japan. The agent was stored at 4°C in the dark and was suspended in saline shortly before experimentation. The drug or vehicle were given orally 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 (29). The tests were performed using Soft Stat, STATISTICA, software for Macintosh PC.

**RESULTS**

The apoptotic processes induced by NSAID and leading to the loss of gastric mucosal integrity were assessed using gastric epithelial cells of the rats subjected to intragastric administration of ulcerogenic dose (60 mg/kg) of indomethacin in the absence and the presence of pretreatment with antiulcer agent, sucral fate. At this dose indomethacin caused extensive multiple hemor-
rhagic lesions of the glandular mucosa, concentrated mainly along the mucosal folds, and occupying $29.3 \pm 1.5 \text{ mm}^2$ of the corpus area, while the animals pretreated with gastroprotective dose (200 mg/kg) of sucralfate (7) showed a 59.7% reduction in the extent of mucosal damage caused by indomethacin (Fig. 1).

![Fig. 2. Effect of sucralfate on gastric epithelial cells apoptosis during indomethacin-induced mucosal injury. Sucralfate was administered 30 min before indomethacin. Values represent the means $\pm$ SD of duplicate analyses performed on 10 animals in each group. *P < 0.05 compared with that of indomethacin.](image)

The results of apoptotic DNA fragmentation assays conducted with the epithelial cells isolated from gastric mucosa of the control and indomethacin-treated animals in the absence and the presence of pretreatment with sucralfate are presented in Fig. 2. The apoptotic DNA fragmentation assays for the control group gave a mean value of 1.4 unit/mg protein, while the apoptotic index in the epithelial cells of the animals subjected to indomethacin reached the mean value of 26.5 units/mg protein. However, pretreatment with sucralfate caused a 41.2% decrease in DNA fragmentation caused by indomethacin.

The effect of sucralfate pretreatment on the expression of gastric mucosal TNF-$\alpha$ during indomethacin-induced mucosal injury is summarized in Fig. 3.

![Fig. 3. Effect of sucralfate on the expression of gastric mucosal TNF-$\alpha$ during indomethacin-induced mucosal injury. Pretreatment with sucralfate at 200 mg/kg was carried out 30 min before indomethacin. Values represent the means $\pm$ SD of duplicate analyses performed on 10 animals in each group. *P < 0.05 compared with that of indomethacin.](image)
In the absence of sucralfate, indomethacin-induced mucosal injury was accompanied by a 47% increase in TNF-α expression over that of the controls, while the animals subjected to the pretreatment with sucralfate showed a 33.4% decline in gastric mucosal TNF-α level induced by indomethacin.

The data on the activity of apoptotic protease, caspase-3, in gastric epithelial cells of the control and indomethacin-treated animals in the absence and the presence of sucralfate pretreatment are presented in Fig. 4. The assays established a mean value for the activity of caspase-3 in the controls at 6.5 pmol/min/mg protein, while that in the epithelial cells of the animals subjected to indomethacin treatment gave a mean value of 25.5 pmol/min/mg protein. On the other hand, pretreatment with sucralfate caused a 45% reduction in the extent of caspase-3 activity induced by indomethacin.

The expression of gastric mucosal NOS-2 activity during indomethacin-induced mucosal injury is depicted in Fig. 5. The assays, conducted in the presence of EDTA, revealed that comparing to the controls the
animals subjected to an ulcerogenic dose of indomethacin showed an 11.9-fold increase in the gastric epithelial expression of NOS-2 activity, while the pretreatment with sucralfate reduced the indomethacin-induced increase in NOS-2 expression by 44.7%.

The analysis of correlation between the expression of caspase-3 activity and gastric epithelial cells apoptosis in response to indomethacin with sucralfate pretreatment is presented in Fig. 6, while the data on correlation between the expression of NOS-2 and the caspase-3 activity with indomethacin in the

![Graph showing correlation between Caspase-3 and Apoptosis](image)

**Fig. 6.** Correlation between the expression of caspase-3 activity and gastric epithelial cells apoptosis induced by intragastric application of indomethacin in the presence of pretreatment with sucralfate. Values for caspase-3 activity are expressed in pmol/min/mg protein, and the extent of epithelial cells apoptosis in apoptotic units/mg protein.
presence of sucralfate pretreatment are shown in Fig. 7. The statistical evaluation of the data of caspase-3 vs. apoptosis with sucralfate pretreatment demonstrated a positive correlation with $r = 0.74$ and $p < 0.01$, whereas evaluation of the data on NOS-2 vs. caspase-3 gave a positive correlation values of $r = 0.72$ and $p < 0.01$.

DISCUSSION

Sucralfate is a potent antiulcer agent recognized for its mucosal strengthening action through the enhancement of prostaglandin synthesis and the events associated with efficacy of growth factor and stimulation of cellular prolifer-
ation and mucosal repair (1—9). Although the transient increase in gastric mucosal prostaglandin level has been associated for long with the gastroprotective effect of sucralfate, the literature data indicate that the protective action of the drug can be achieved even in the presence of indomethacin, a known inhibitor of prostaglandin synthesis (10, 11). Moreover, while the mucosal injury associated with the use of indomethacin and other NASIDs are most often ascribed to their inhibitory effect on prostaglandin synthesis, and the impairment in mucosal blood flow and superoxide radicals generation (5, 12—14), recent data point towards the detrimental effects of these compounds on the processes linked to cell cycle progression, induction of proinflammatory cytokine TNF-α expression, disturbances in nitric oxide, and apoptosis (15—19).

As the amplification and propagation of the cell death signaling cascade induced by TNF-α involve the activation of a family of specific cysteine proteases, known as caspases, which remains under the regulatory control of nitric oxide (21, 22, 31—33), in this study we assessed the effect of sucralfate pretreatment on the events associated with gastric mucosal injury induced by indomethacin by analyzing the interplay between the extent of epithelial cells apoptosis and the mucosal expression of TNF-α and NOS-2, and the activation of a key apoptotic protease, caspase-3. The results revealed that gastric mucosal injury by indomethacin are characterized by a massive induction in TNF-α and the enhanced apoptosis of gastric epithelial cells, accompanied by a 3.9-fold increase in caspase-3 activity and an 11.9-fold increase in NOS-2. On the other hand, the pretreatment with sucralfate produced a 41.2% decrease in epithelial cells apoptosis and a 33.4% decrease in TNF-α, while the activity of caspase-3 decreased by 45% and NOS-2 showed a 44.7% decline. Moreover, this effect of sucralfate was reflected in a 59.7% reduction in the mucosal damage elicited by indomethacin. In our interpretation, these findings indicate that the indomethacin-induced disturbances in mucosal homeostasis, in conjunction with the low intragastric pH, appear to induce the potentiation of the mucosal TNF-α expression which leads to the induction in NOS-2 and the enhanced apoptotic caspase-3 activation, resulting in the programmed cell death. This course of events is supported by the literature data demonstrating that acidic environment potentiates proinflammatory cytokine production that cause NK-κB activation and translocation to the nucleus, increasing its DNA binding activity, and ultimately resulting in an increased induction of NOS-2 gene expression (33). Apparently, sucralfate has the ability to suppress the apoptotic events resulting from the induction of NOS-2 and propagated by caspases-3 activation.

Our data on the expression of NOS activity point towards a distinct role for NOS-2 in the apoptotic process occurring during indomethacin-induced gastric mucosal injury. Our findings strongly suggest that NOS-2 participates
in the amplification of the cell death signaling cascade induced by TNF-α, as its enhanced expression with indomethacin ingestion, like that of TNF-α, shows a significant positive correlation with the extent of gastric epithelial cells apoptosis. It is relevant to note that the activation of NOS-2 and subsequent generation of the high and sustained level of NO is also recognized as a major factor of degenerative changes observed in the pathogenesis of liver disease, inflammatory processes, and endotoxic shock (20, 31, 34—36). Furthermore, the enhanced expression of NOS-2 leads not only to NO production, but also results in the formation of NO-related species such as nitrosothiols, peroxynitrite, and dinitrosyl iron complexes which exert a direct inhibitory effect on NK-κB (23), and hence cause transcriptional disturbances that lead to apoptosis (21, 31). Moreover, the apoptotic death pathway linked to overexpression of NOS-2 is characterized by a such typical apoptotic events as chromatin condensation, blebbing of cytoplasmic membranes, and DNA laddering (20, 37).

As the execution phase of apoptosis is associated with the activation of caspases, a family of aspartic acid-directed proteases (26, 27), we have sought further to explore the effect of pretreatment with sucralfate on the indomethacin-induced increase in caspase-3, a well recognized key protease of the apoptotic process (24, 26, 27). The data obtained demonstrated that enhanced gastric epithelial cells apoptosis evoked by indomethacin ingestion is associated with a marked (3.9-fold) increase in caspase-3 activity, and that pretreatment with sucralfate caused a significant (45%) decrease in this caspase activation. Thus, indomethacin displays its influence on apoptotic signal propagation consistent with that induced by TNF-α (26, 27), and the effect sucralfate occurs through the inhibition of this proinflammatory cytokine expression.

The essential role of TNF-α in the process of apoptotic caspases activation and subsequent DNA fragmentation is an area of intense investigations (26—28). The soluble form of TNF-α, produced by the proteolysis of membrane-associated TNF by a specific metalloproteinase, mediates its biological activity through type I TNF receptor (38, 39). The ligand binding induces TNF receptor trimerization triggering several downstream signaling events which culminate in the caspase recruitment. All known caspases, which to date number 12, preexist in the cytoplasm as zymogens that require cleavage adjacent to aspartates for their activation (27, 28). The implementation of the apoptotic program requires the participation of two classes of caspases, the initiator caspases, with long N-terminal prodomains (caspases-8, -9, and -10), which activate the executioner caspases with short N-terminal prodomains (caspases-3, -6, and -7), thut in turn cleave the targeted intracellular substrates (26, 46). The activation of caspases with long N-terminal prodomain (such as caspase-8 and -10) involves their recruitment by specialized adaptor molecules.
to form the death-inducing signaling complex which facilitates the autocatalytic process of procaspase zymogen activation (26, 46).

The activation of another long N-terminal prodomain initiator caspase, caspase-9, involves the regulatory proteins of the Bcl-2/Bax family and takes place upstream of DNA fragmentation, but downstream of the mitochondrial permeability transition (MPT), as the caspase inhibitor does not prevent induction of MPT (41). The Bcl-2/Bax proteins associate mainly with the outer mitochondrial membrane and nuclear envelope, and appear to posses ion channel activity responsible for maintaining electrical and osmotic mitochondrial homeostasis (26, 41, 42). Alteration of mitochondrial membrane permeability leads to mitochondrial swelling and the escape of cytochrome c and a 50 kDa protease termal AIF (apoptosis inducing factor) or Apaf-1, an acronym for apoptotic protease activating factor, into the cytosol, and triggering activation of death caspase cascade through formation of Apaf-1-caspase-9-cytochrome c complex. Binding of cytochrome c triggers activation of caspase 9, which then accelerates apoptosis by activating the executioner caspas, including caspase-3, which lack the long N-terminal prodomain required for the recruitment to Apaf-1 complex (26, 42, 43).

The process of caspase activation, associated with the mitochondrial swelling and redistribution of cytochrome c from the intermembrane space to the cytosol, apparently involves the efflux of intracellular K⁺ (44). Indeed, the swelling of mitochondria is indicative of dysregulation of osmotic homeostasis due to inner membrane permeability alterations (43). This is supported by studies on the interdependence between apoptotic DNA fragmentation and caspase-3 activation demonstrating that normal intracellular K⁺ concentrations inhibit both DNA fragmentation and caspase-3 protease activation, while a decrease of intracellular K⁺ of the magnitude observed in apoptotic cells leads to caspase-3 activation (44). It is of interest to note that K⁺ is the major determinant of mitochondrial matrix volume, and its transport across the inner membrane is controlled by a several well tuned channel systems (43). At least one of these, K⁺/H⁺ exchanger, is controlled by apoptosis inhibitor Bcl-Xl (45). This mitochondrial membrane protein, using the H⁺ gradient stimulates the exchanger to extrude K⁺ from the matrix and thus prevents the mitochondrial swelling (43, 45).

Our findings indicate that disturbances in this complex system may well be responsible for the TNF-α mediated activation of proapoptotic caspase-3, induction of NOS-2, and the enhanced gastric epithelial cells apoptosis with indomethacin ingestion. Consequently, gastroprotective agents, such as sucralfate, that inhibit caspase activation and cause perturbation of proapoptotic signaling by modulation of the NOS-2 expression may be of value in therapeutic intervention with the rampant and undesired increase in gastric epithelial cells apoptosis associated with the use of NSAIDs.
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