Lansoprazole Protects and Heals Gastric Mucosa from Non-steroidal Anti-inflammatory Drug (NSAID)-induced Gastropathy by Inhibiting Mitochondrial as Well as Fas-mediated Death Pathways with Concurrent Induction of Mucosal Cell Renewal*

Received for publication, January 16, 2008, and in revised form, March 25, 2008. Published, JBC Papers in Press, March 28, 2008, DOI 10.1074/jbc.M800414200

Pallab Maity‡, Samik Bindu†, Vinay Choubey‡, Athar Alam‡, Kalyan Mitra‡, Manish Goyal‡, Sumanta Dey‡, Mithu Guha‡, Chinmay Pal‡, and Uday Bandyopadhyay‡†

From the ‡Department of Infectious Diseases and Immunology, Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Jadavpur, Kolkata 700032, West Bengal, India and the †Division of Drug Target Discovery and Development, ‡Electron Microscopy Unit, Central Drug Research Institute, Chatter Manzil Palace, Mahatma Gandhi Marg, Lucknow-226001, Uttar Pradesh, India

We have investigated the mechanism of antiapoptotic and cell renewal effects of lansoprazole, a proton pump inhibitor, to protect and heal gastric mucosal injury in vivo induced by indomethacin, a non-steroidal anti-inflammatory drug (NSAID). Lansoprazole prevents indomethacin-induced gastric damage by blocking activation of mitochondrial and Fas pathways of apoptosis. Lansoprazole prevents indomethacin-induced up-regulation of proapoptotic Bax and Bak and down-regulation of antiapoptotic Bcl-2 and BclxL to maintain the normal proapoptotic/antiapoptotic ratio and thereby arrests indomethacin-induced mitochondrial translocation of Bax and collapse of mitochondrial membrane potential followed by cytochrome c release and caspase-9 activation. Lansoprazole also inhibits indomethacin-induced Fas-mediated mucosal cell death by down-regulating Fas or FasL expression and inhibiting caspase-8 activation. Lansoprazole favors mucosal cell renewal simultaneously by stimulating gene expression of prosurvival proliferating cell nuclear antigen, survivin, epidermal growth factor, and basic fibroblast growth factor. The up-regulation of Flt-1 further indicates that lansoprazole activates vascular epidermal growth factor-mediated controlled angiogenesis to repair gastric mucosa. Lansoprazole also stimulates the healing of already formed ulcers induced by indomethacin. Time course study of healing indicates that it switches off the mitochondrial death pathway completely but not the Fas pathway. However, lansoprazole heals mucosal lesions almost completely after overcoming the persisting Fas pathway, probably by favoring the prosurvival genes expression. This study thus provides the detailed mechanism of antiapoptotic and prosurvival effects of lansoprazole for offering gastroprotection against indomethacin-induced gastropathy.

Non-steroidal anti-inflammatory drugs (NSAIDs), commonly used for the treatment of arthritis and other musculoskeletal disorders are considered to be one of the most important causative factors for gastric damage (1–3). Although various mechanisms have been suggested for NSAID-induced gastric ulcer (4–7), recent studies suggest that increased apoptotic cell death and simultaneous block of mucosal cell renewal play major roles in the development of mucosal lesion (8–10). Healthy gastric mucosa is always under equilibrium between cell death and cell renewal (11, 12) and mucosal injury is developed when this balance is disturbed due to an increase in apoptosis and/or inhibition of cell proliferation (11, 12). NSAIDs are shown to induce apoptosis in gastric mucosal cells through reactive oxygen species generation, cytochrome c release, activation of caspase-3, inhibition of survivin expression, and induction of Ca²⁺ signaling (8, 10, 13, 14). Caspase-3 activation is generally mediated through two main pathways, viz. the mitochondrial (internal) and death receptor (external) pathways (15, 16). In the mitochondrial pathway, up-regulation or activation of pro-apoptotic proteins and/or down-regulation or inactivation of antiapoptotic proteins lead to opening of mitochondrial permeability transition pores (MPTP) to release cytochrome c for the activation of caspase-9 (15, 17–20). Activated caspase-9 in turn activates caspase-3 for the execution of apoptosis (15, 17). The death pathway is initiated by binding the death ligand to its receptor leading to receptor oligomerization and finally activation of caspase-8 (16). Caspase-8 can directly activate caspase-3 or can converge on the mitochondrial pathway through cleaving Bid (16). In addition to induction of apoptosis, NSAIDs also inhibit cell proliferation in several systems (21, 22). Proton pump inhibitors such as, omeprazole, lansoprazole, etc. are the most popular drugs used for the therapeutic control.

* This work was supported by funds from Prof. Siddhartha Roy from the J. C. Bose National Award (to P. M.) and the Council of Scientific and Industrial Research (CSIR), New Delhi, through Suprainstitutional Project SIP 0007. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 91-33-24733491; Fax: 91-33-24730284; E-mail: ubandyo_1964@yahoo.com.

§ The abbreviations used are: NSAID, non-steroidal anti-inflammatory drug; MPTP, mitochondrial permeability transition pores; pNA, p-nitroaniline; PCNA, proliferating cell nuclear antigen; MAPK, mitogen-activated protein kinase; BrdUrd, bromodeoxyuridine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PI, propidium iodide; JC-1, 5,5’,6,6’-tetrachloro-1’,3’,3’-tetrachlorobenzimidazolocarbocyanine iodide; RT, reverse transcriptase; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor.
Antiapoptotic Effect of Lansoprazole

of gastroduodenal ulcer (23, 24). Irreversible inactivation of H\(^+\),K\(^-\)-ATPase of the parietal cells to block acid secretion is considered to be the main event underlying antulcer effects of proton pump inhibitors (25, 26). Previously, our group has reported that in addition to antisecretory action, omeprazole also offers potent antioxidant and antiapoptotic effects to protect gastric mucosa from stress-induced gastric ulcer (27). Its antioxidant effect is mediated by scavenging of \(\cdot OH\), whereas its antiapoptotic function is indicated by blocking stress-induced DNA fragmentation during ulceration (27). Omeprazole is also therapeutically effective in healing peptic ulcers caused by NSAIDs (23). In rat models, omeprazole offers gastroprotection against indomethacin-induced gastric damage, where acid inhibition by omeprazole does not play a major function (27). This suggests that omeprazole has some independent pathways for its gastroprotective effect. The aim of the present study is to find out whether proton pump inhibitors protect and heal the gastric mucosa from NSAID-induced gastric damage by preventing apoptosis and stimulating the cell renewal process. Using a indomethacin-induced gastric injury model (4, 28), evidence has been presented to show that the proton pump inhibitor, lansoprazole, protects and heals the gastric mucosa from indomethacin-induced damage by preventing indomethacin-induced activation of mitochondrial as well as Fas-mediated cell death (apoptotic) pathways with concurrent induction of mucosal cell renewal process.

**EXPERIMENTAL PROCEDURES**

**Materials**—Indomethacin, mitochondria isolation kit, caspase-3, and caspase-8 assay kits were obtained from Sigma. Caspase-9 assay kit was procured from Biovision (Biovision, Mountain View, CA). Bax antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Gold (10 nm)-labeled anti-rabbit IgG and Ready-To-Go RT-PCR beads were obtained from GE Healthcare. JC-1 (5' , 6', 6'-tetrachloro-1', 3', 3'-tetraethylbenzimidazolcarbocyanine iodide) was purchased from Molecular Probes (Eugene, OR). The custom-made primers were purchased from Sigma Genosys. RNeasy kit was obtained from Qiagen (Hilden, Germany). 2 × PCR master mixture was from Fermentus. APO-BrdUTM TUNEL Assay Kit was purchased from Invitrogen. All other reagents were of analytical grade purity.

**Animals Used**—Sprague-Dawley rats (180–220 g) were used throughout the experiments. The animals were maintained at 24 ± 2 °C with 12-h light and dark cycles. The animals were fasted for 24 h before the start of the experiments with water *ad libitum* to avoid a food-induced increased acid secretion and its aggravating effect on gastric lesions. All the *in vivo* studies were done in accordance with guidelines of the institute animal ethics committee.

**Indomethacin-induced Acute Gastric Ulcer and Healing**—Indomethacin-induced gastric mucosal ulcers were developed as described earlier (27, 28). All the animals were divided into control, NSAID (indomethacin)-treated, lansoprazole-pre-treated, indomethacin-treated, and lansoprazole-pre-treated control groups (n = 8). Gastric ulcers were induced in the fasted animals with oral administration of indomethacin (dissolved in slightly alkaline distilled water) at a dose of 48 mg kg\(^{-1}\) body weight. The control group received the vehicle (water) only. In the lansoprazole-pretreated NSAID (indomethacin)-treated group, lansoprazole was administered at a dose of 20 mg kg\(^{-1}\) body weight intraperitoneally (this dose was selected from the dose-response curve, which causes more than 90% protection of indomethacin-induced mucosal injury), 30 min prior to indomethacin treatment. The lansoprazole-pretreated control group received lansoprazole (20 mg kg\(^{-1}\) body weight) intraperitoneally 30 min before oral administration of vehicle. After 4 h of indomethacin or vehicle administration, the animals were sacrificed under proper euthanasia and stomachs were collected. The mucosal injury was scored as ulcer index as follows: 0 = no pathology; 1 = one pinhead ulcer. The sum of the total scores divided by the number of animals gave the ulcer index. The stomachs were then used for subsequent experiments. For the healing studies, the same procedure for the induction of ulcer with indomethacin was followed. The 4-h indomethacin treatment, when the ulcer index is maximum, is considered the “0” h of healing. At this time point some of the animals were sacrificed, the stomachs were dissected out for assessment of ulcer index, and processed for subsequent experiments. Some of the animals at this time were intraperitoneally administered lansoprazole (20 mg kg\(^{-1}\) body weight). The lansoprazole-treated animals were considered as the lansoprazole-induced healing group, whereas, the animals that received no lansoprazole were considered as the indomethacin-treated autohealing group. At different times of healing, *i.e.* 0, 4, 8, 12, 20, and 24 h, the stomachs were dissected out from animals of both groups for assessing the ulcer index and performing subsequent experiments.

**Assay of Apoptosis in Gastric Mucosal Cells After Drug Treatment**—Gastric mucosal cells were isolated as described earlier (29). Mucosa from control, NSAID (indomethacin)-treated, lansoprazole-pretreated NSAID (indomethacin)-treated, and lansoprazole-pretreated control stomach was scrapped in Hanks’ balanced salt solution, pH 7.4, containing 100 units/ml penicillin and 100 μg/ml streptomycin. Mucosa was minced finely and suspended in Hanks’ balanced salt solution, pH 7.4, containing 0.1% collagenase and 0.05% hyaluronidase. The suspension was incubated for 30 min at 37 °C in a 5% CO\(_2\) environment with shaking. The suspension was then filtered through a sterile nylon mess. The filtrate was centrifuged at 600 × g for 5 min and the cell pellet was washed with Hank’s balanced salt solution, pH 7.4, for subsequent studies. Apoptosis was detected in the isolated gastric mucosal cells using a commercially available APO-BrdUTM TUNEL Assay kit. In brief, cell were first fixed with 1% paraformaldehyde in phosphate-buffered saline, pH 7.4, followed by treatment with 70% ethanol in ice. The cells were then loaded with DNA labeling solution containing terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay kit. In brief, cells were then stained with Alexa Fluor® 488 dye-labeled anti-BrdUrd antibody. The cells were finally stained with propidium iodide (PI) solution containing RNase A and visualized under a fluorescence microscope (Leica, DM-2500, Leica Microsystems GmbH, Wetzlar, Germany) using appropriate filters for Alexa Fluor 488 and PI.
Histological Study of Gastric Mucosa—Stomachs from the animals of different groups (control, indomethacin-treated, and lansoprazole-pretreated indomethacin treated) were washed in phosphate-buffered saline, pH 7.4, and fixed overnight in 10% formalin. The fixed tissue was dehydrated in a series of graded ethanol and finally embedded in paraffin. The paraffin-embedded tissue was used for semi-thin sections (5 μm). The semi-thin sections were collected on a glass slide and double stained with hematoxyline and eosin. The stained sections were examined under a microscope (Leica DM-2500, Leica Microsystems GmbH, Wetzlar, Germany) equipped with high-resolution digital camera.

Assay of Caspase-3, Caspase-8, and Caspase-9 Activity—Caspase-3 and caspase-8 activities were measured in the cytosolic fraction of the gastric mucosal homogenate using commercially available kits and according to the manufacturers’ protocol (Sigma) as described earlier (30). In brief, gastric mucosa from control and drug-treated rats was homogenized in caspase lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM dithiothreitol) followed by centrifugation at 16,000 × g for 15 min. The supernatant was mixed with 85 μl of assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol) in the presence of 200 μM substrate (Ac-DEVD-pNA, for caspase-3 or Ac-IETD-pNA for caspase-8). It was then incubated at 37 °C for 4 h and absorbance was taken at 405 nm. Caspase-9 activity was also measured in the cytosolic fraction of the mucosal lysate, using a commercially available kit and according to the manufacturer’s protocol (Biovision, CA). In brief, gastric mucosa was homogenized in caspase lysis buffer provided with the kit. The homogenate was centrifuged at 16,000 × g for 15 min to get clear supernatant. The supernatant was mixed with 50 μl of 2× reaction buffer provided with the kit in the presence of substrate (LEHD-pNA, 200 μM final concentration). The mixture was incubated at 37 °C for 4 h and absorbance was taken at 405 nm. Caspase activity was expressed as nanomole of pNA release/mg of cytosolic protein/h.

Semiquantitative RT-PCR Analysis for the Expression of Various Apoptotic, Antiapoptotic, and Growth Factors—Equal amounts of mucosal tissue (30 mg) from control or NSAID-induced ulcerated or lansoprazole-pretreated, NSAID-treated, or lansoprazole-pretreated control stomach was used for total RNA isolation using a commercially available kit (RNaseasy kit, Qiagen, Germany). RNA (2 μg) was used to prepare cDNA using oligo(dT)18. Equal amounts of cDNA was used for PCR amplification using specific forward and reverse primers of Bcl-2, BclXL, Bax, Bak, Bcl, Fas, FasL, proliferating cell nuclear antigen (PCNA), survivin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), Flt-1, and actin. The PCR-amplified products were resolved in 2% agarose gel and documented in a gel-doc system (Alpha Infotech). The intensity of each band was quantified with densitometric software (Lab Image Beta version, Kapelan GmbH, Germany). The intensity of each band was normalized with that of actin.

Immunogold Electron Microscopy for Bax Translocation—Gastric mucosal section from control or NSAID-induced ulcerated or lansoprazole-pretreated NSAID-treated animals was washed with 0.1 M sodium phosphate buffer, pH 7.4, and quickly transferred for fixation in a solution of 4% (w/v) paraformaldehyde and 0.01% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 4 h at room temperature. Samples were washed with phosphate buffer and then dehydrated in an ascending series of ethanol followed by impregnation in LR-White resin and polymerized at 60 °C for 24 h. After proper selection of the area of interest from stained semi-thin sections (1 μm), ultra thin sections (50–70 nm) were made using an Ultra Cut ultra-microtome (Leica Microsystems GmbH, Wetzlar, Germany) and collected onto nickel grids. Sections in the grids were first washed with phosphate-buffered saline, pH 7.4, and then blocked with 0.1% bovine serum albumin and 0.1% teleost fish gelatin in phosphate-buffered saline. The sections were then incubated with 1:10 diluted rabbit polyclonal Bax antibody for 2 h at room temperature in blocking buffer. After washing 5 times with the same buffer, sections were incubated with 10-nm gold-coupled goat anti-rabbit IgG (1:20) at room temperature for 1 h. The sections were then washed 5 times with blocking buffer, contrasted with uranyl acetate, and examined under a FEI Tecnai-12 Twin Transmission Electron Microscope equipped with a SIS Mega View II CCD camera at 80 kV (FEI Company, Hillsboro, OR). The primary antibody incubation step was omitted in negative control samples.

FIGURE 1. Lansoprazole protects NSAID (indomethacin)-induced gastric mucosal cell apoptosis as measured by APO-BrdUrd TUNEL assay. Red fluorescence represents the PI-stained nuclei, and green fluorescence represents the Alexa Fluor 488-stained nick end label, an indicator of apoptotic DNA fragmentation. The merged picture represents the merging of PI-stained nuclei with the Alexa Fluor 488-stained nick end label. In control cells, no such green fluorescence was observed, suggesting no apoptotic DNA fragmentation. In NSAID-induced ulcerated mucosal cells, profuse green fluorescence suggests extensive typical apoptotic DNA fragmentation. The lansoprazole-pretreated NSAID-treated cells also show very low amounts of green fluorescence suggesting less occurrence of DNA fragmentation. Lansoprazole-pretreated control gastric mucosal cells show no green fluorescence, suggesting no incidence of DNA fragmentation.
Measurement of Mitochondrial Transmembrane Potential (ΔΨm)—Mitochondrial transmembrane potential was measured as described (30). Mitochondria were isolated from gastric mucosa of control and drug-treated rats using the mitoisolation kit (Sigma) by following the protocol mentioned therein and isolated mitochondria were incubated in the dark with JC-1 for 10 min at 25 °C. The fluorescence of each sample was measured in a PerkinElmer LS50B spectrofluorometer (excitation 490 nm, slit, 5 nm; emission 590 nm for J-aggregate, 530 nm for J-monomer, slit, 7.2 nm) (31). To relate the fluorescence measurements at 590 nm to the mitochondrial potential in millivolts (mV), fluorescence values were calibrated using antimycin A assuming −200 mV in control cells (31, 32).

Measurement of Cytochrome c Release by Enzyme-linked Immunosorbent Assay—Cytochrome c released into the cytosol was measured using an enzyme-linked immunosorbent assay-based rat cytochrome c assay kit (R&D Systems, Minneapolis, MN). In brief, 5% gastric mucosal homogenate was prepared in ice-cold phosphate-buffered saline, pH 7.4, supplemented with protease inhibitor mixture in a ratio of 100 μl/ml of phosphate-buffered saline. The homogenate was spun at a low speed to discard cell debris. The supernatant was then centrifuged at 105,000 × g for 1 h to get the clear cytosolic supernatant (33) and this was used for the measurement of cytochrome c release according to the protocol described in the product insert.

Transmission Electron Microscopy for Subcellular Morphology—Control or NSAID-treated or lansoprazole-pretreated NSAID-treated mucosa was cut into small pieces (1 mm³) and fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.4, for 4 h at room temperature (24 °C). The tissues were then washed in 0.1M sodium phosphate buffer and for post-fixation placed in 2% osmium tetroxide in 0.1M sodium phosphate buffer, pH7.4, for 2h at room temperature. The tissues were then dehydrated in ascending grades of ethanol, followed by embedding in Epon 812, and polymerized at 60 °C for 24 h. Ultra-thin sections (50–70 nm) were obtained using an Ultra.

<table>
<thead>
<tr>
<th>Time after NSAID exposure (Hr)</th>
<th>Gastric mucosal injury</th>
<th>Ulcer index</th>
<th>Caspase-3 activation (pNA release nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Lansoprazole</td>
<td>+ Lansoprazole</td>
<td>-Lansoprazole</td>
</tr>
<tr>
<td>0</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td>8.8 ± 0.87</td>
</tr>
<tr>
<td>2</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td>15.2 ± 1.43</td>
</tr>
<tr>
<td>3</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td>30.3 ± 3.5</td>
</tr>
<tr>
<td>4</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td>50.1 ± 5.7</td>
</tr>
</tbody>
</table>

Black arrow indicates mucosal erosion.

*, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control; ***, p < 0.01; ****, p < 0.001 versus indomethacin.

**TABLE 1**

Lansoprazole prevents NSAID-induced gastric mucosal injury and caspase-3 activation

<table>
<thead>
<tr>
<th>Time after NSAID exposure (Hr)</th>
<th>Gastric mucosal injury</th>
<th>Ulcer index</th>
<th>Caspase-3 activation (pNA release nmol/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Lansoprazole</td>
<td>+ Lansoprazole</td>
<td>-Lansoprazole</td>
</tr>
<tr>
<td>0</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td>8.8 ± 0.87</td>
</tr>
<tr>
<td>2</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td>15.2 ± 1.43</td>
</tr>
<tr>
<td>3</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td>30.3 ± 3.5</td>
</tr>
<tr>
<td>4</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td>50.1 ± 5.7</td>
</tr>
</tbody>
</table>

Black arrow indicates mucosal erosion.

*, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control; ***, p < 0.01; ****, p < 0.001 versus indomethacin.
cut Ultra-microtome (Leica Microsystems GmbH, Wetzlar, Germany) and picked up onto 200 mesh copper grids. The sections were double stained with uranyl acetate and lead citrate and visualized under a FEI Tecnai-12 Twin Transmission Electron Microscope equipped with a SIS Mega View II CCD camera at 80 kV (FEI Company, Hillsboro, OR).

Statistical Analysis—All data were presented as mean ± S.E. Data were analyzed by unpaired Student’s t test with a two-tailed distribution or by one-way analysis of variance followed by multiple comparison t tests for evaluation of differences between groups. The p value ≤ 0.05 was considered as significant.

RESULTS
Lansoprazole Prevents Indomethacin-induced Caspase-dependent Gastric Mucosal Cell Apoptosis in Vivo to Protect Mucosal Injury—The result of the TUNEL assay (Fig. 1) clearly indicates that indomethacin (NSAID) induces apoptosis of gastric mucosal cells in vivo, whereas pretreatment with lansoprazole effectively inhibits apoptosis induced by indomethacin. Red fluorescence represents PI-stained nuclei and green fluorescence (Alexa Fluor 488) represents nick end labeling for apoptotic DNA fragmentation. Merging of PI-stained nuclei with the corresponding Alexa Fluor 488-stained end labeling suggests that end labeling is localized within the nucleus, indicating extensive apoptotic DNA fragmentation within the nuclei of mucosal cells after treatment with indomethacin. Lansoprazole pretreatment blocks indomethacin-induced apoptosis in gastric mucosal cells as evident from low levels of Alexa Fluor 488 (green) fluorescence (Fig. 1). However, lansoprazole alone has no effect on gastric mucosal apoptosis in control cells. It is further evident that indomethacin-induced gastric mucosal apoptosis is mediated through a caspase-dependent pathway because indomethacin induces the activation of caspase-3 (Table 1). The time course studies of the images of histological injury with the corresponding ulcer index and caspase-3 activation induced by indomethacin and their protection by lansoprazole are shown in Table 1. Histological studies indicate that indomethacin causes a time-dependent increase in mucosal damage as shown by loss of integrity of the surface epithelium with increased cell shading (Table 1). Lansoprazole pretreatment can almost completely block this injury as evidenced by the intact surface epithelium similar to the 0 h control tissue. The increased mucosal injury by indomethacin is reflected by a similar time-dependent increase in ulcer index and its protection by lansoprazole. Moreover, this damage is associated with increased apoptosis as evidenced by the gradual activation of caspase-3, an important marker for apoptosis. Lansoprazole pretreatment can block apoptosis as indicated by the significant prevention of caspase-3 activation (Table 1).

Lansoprazole Accelerates Healing of Indomethacin-induced Gastric Mucosal Damage through the Inhibition of Apoptosis—To investigate whether inhibition of caspase-3-mediated apoptosis leads to acceleration of healing of indomethacin-induced gastric damage, we further studied the effect of lansoprazole on the healing of gastric lesions with respect to caspase-3 activation (Fig. 2). The results indicate that in the absence of lansoprazole, the mucosa shows a time-dependent autohealing of ulcer wounds, which is associated with decreased apoptotic activity as shown by decreased caspase-3 activity. However, lansoprazole pretreatment significantly stimulates the percent of ulcer healing as well as prevents caspase-3 activation. At 24 h, the mucosa shows 60% autohealing with a 40% decrease in caspase-3 activity, whereas after

Antiapoptotic Effect of Lansoprazole MUCOS C-21, 2008—VOLUME 283 • NUMBER 21
JOURNAL OF BIOLOGICAL CHEMISTRY 14395

FIGURE 2. Lansoprazole accelerates healing of NSAID-induced mucosal damage along with the inhibition of caspase-3 activity. Detailed methodology for healing studies has been described under “Experimental Procedures.” *#, p < 0.02; ##, p < 0.01; ###, p < 0.001 versus NSAID.

FIGURE 3. Lansoprazole blocks NSAID-induced inhibition of cell survival/proliferation mediators. A, RT-PCR of PCNA, survivin, EGF, bFGF, Flt-1, and actin (internal control) gene expression in the gastric mucosal cells. B, densitometric analysis of the RT-PCR data. ***, p < 0.001 versus control; ###, p < 0.001 versus NSAID.
treatment with lansoprazole, the healing was increased to 90% with a further (60%) decrease in caspase-3 activity (Fig. 2). The data further indicate that although lansoprazole stimulates ulcer healing to nearly 90% at 24 h it is not completely blocking the activation of caspase-3 suggesting that the arrest of gastric mucosal apoptosis is not the sole factor for mucosal repair by lansoprazole.

Effect of Lansoprazole on Cell Survival/Proliferation during Indomethacin-induced Gastric Damage—Because cell survival/proliferation plays an important role in maintaining gastric mucosal integrity (11, 34), it is therefore interesting to study whether lansoprazole has any role on this process. The expression of several cell survival/proliferation mediators was studied by RT-PCR in indomethacin-induced gastric damage and after pretreatment with lansoprazole (Fig. 3, A and B). The expression of PCNA, the most reliable marker for studying in vivo cell proliferation, is significantly decreased during indomethacin-induced mucosal injury (p < 0.001), whereas pretreatment with lansoprazole can almost completely protect it. This suggests that inhibition of cell survival/proliferation occurs during development of mucosal damage, which is effectively blocked by pretreatment with lansoprazole. This result further directed us to identify the mechanism behind the suppression of cell survival during ulceration. It is interesting to note that indomethacin down-regulates the expression of survivin (p < 0.001), an important pro-survival factor in the family of inhibitors of apoptosis. In addition, indomethacin also down-regulates the expression of EGF, bFGF, and Flt-1 (receptor for vascular epidermal growth factor) in gastric mucosal cells (p < 0.001 in all cases). Interestingly, lansoprazole pretreatment prevents indomethacin-induced suppression of cell proliferation, as evidenced by the restoration of PCNA expression (p < 0.001) (Fig. 3, A and B). Lansoprazole also significantly blocks indomethacin-induced changes of survivin (p < 0.001), EGF (p < 0.001), bFGF (p < 0.01), and Flt-1 (p < 0.001) (Fig. 3B). But lansoprazole alone has no effect on the expression of PCNA, survivin, EGE, bFGF, and Flt-1 in control stomachs (Fig. 3, A and B). Thus, lansoprazole appears to reverse or prevent the NSAID induced changes of the expression of pro-survival genes to protect gastric mucosa.

Lansoprazole Prevents Indomethacin-induced Mitochondrial Pathway of Gastric Mucosal Apoptosis—As caspase-3 activation is generally mediated through mitochondrial (internal) as well as death receptor (external) pathways (15, 16), studies were carried out to see whether the blocking effect of lansoprazole on indomethacin-induced caspase-3 activation is mediated through these pathways. Semiquantitative RT-PCR analysis (Fig. 4, A and B) indicates that indomethacin significantly
down-regulates the expression of antiapoptotic Bcl-2 ($p < 0.001$) and Bcl$_{xL}$ ($p < 0.001$) proteins and also up-regulates the expression of proapoptotic Bax ($p < 0.001$) and Bak ($p < 0.001$) proteins. Lansoprazole pretreatment, on the other hand, significantly attenuates indomethacin-induced changes of Bax ($p < 0.001$) and Bak ($p < 0.001$) as well as Bcl-2 ($p < 0.001$) and Bcl$_{xL}$ ($p < 0.001$). Indomethacin treatment significantly increases the pro- to antiapoptotic protein ratio (Fig. 4C), which leads to the activation of proapoptotic counterparts, such as Bax and Bak. Lansoprazole, by correcting the expression of these apoptosis-associated proteins decreases the ratio of pro- to antiapoptotic proteins (Fig. 4C), as a result of which the activation and mitochondrial translocation of proapoptotic proteins are blocked. However, lansoprazole alone has no effect on the expression of Bax and Bak or Bcl-2 and Bcl$_{xL}$ in gastric mucosal cells from control rat. The mitochondrial translocation of Bax has been evident by immunogold electron microscopy as shown in Fig. 4D. Mucosal cells from the control rat show very scant amounts of Bax molecules (black spot, arrowhead) present within the cytosol, whereas cells from indomethacin-treated rats show significant up-regulation of Bax and most of the Bax molecules are associated with the mitochondria, suggesting mitochondrial translocation during indomethacin-induced gastric mucosal apoptosis. Lansoprazole pretreatment significantly reverses indomethacin-induced changes of Bax expression and subsequent mitochondrial translocation (Fig. 4D). Mitochondrial translocation of Bax opens the MPTP (18–20), as a result of which mitochondrial membrane potential ($\Delta\Psi$) is decreased (18, 35). Results (Fig. 5) clearly indicate that indomethacin treatment decreases $\Delta\Psi$ in the mucosal cells and lansoprazole pretreatment significantly prevents it ($p < 0.001$). In intact healthy mitochondria with higher $\Delta\Psi$ (more than $-140$ mV), JC-1 accumulates in mitochondrial matrix to form J-aggregates (36), showing intense fluorescence emission at 590 nm. Mitochondria with open transition pores would be at low $\Delta\Psi$ and the accumulation of JC-1 would be less in the matrix, leading to less availability of JC-1 to form aggregates, showing weak fluorescence emission at 590 nm. Fluorescence emission at 530 nm was recorded for the measurement of the JC-1 monomer (31). Calibration of fluorescence values at 590 nm to millivolts (mV) assuming $-200$ mV in healthy control cells (31) indicates that mitochondria from indomethacin-treated rat possess $\Delta\Psi$ of $-41$ mV, whereas mitochondria from lansoprazole-pretreated rat show $\Delta\Psi$ of $-146$ mV. Moreover, the ratio of red fluorescence (590 nm, JC-1, aggregate) to green fluorescence (530 nm, JC-1, monomer) (Fig. 5, inset) was found to be decreased by indomethacin treatment ($p < 0.001$), whereas lansoprazole pretreatment significantly restores this ratio close to normal ($p < 0.001$) (Fig. 5, inset). Thus, lansoprazole significantly prevents indomethacin-induced reduction of $\Delta\Psi$.

Again, indirect enzyme-linked immunosorbent assay for the cytochrome c level in the cytosol indicates that the cytochrome c level is significantly higher after indomethacin treatment ($p < 0.001$) compared with the control value (Fig. 6A), indicating the release of cytochrome c from mitochondria to the cytosol. Interestingly, lansoprazole pretreatment causes a significant block of indomethacin-induced release of cytochrome c to the cytosol ($p < 0.001$). Cytochrome c once released into the cytosol activates caspase-9 through the formation of the apoptosome complex (15, 17, 37). Results shown in Fig. 6B indicate that indomethacin treatment significantly activates caspase-9 ($p < 0.001$), whereas lansoprazole pretreatment can significantly prevent the activation ($p < 0.01$). Again, lansoprazole alone did not show any effect on $\Delta\Psi$, cytochrome c release, and caspase-9 activation in control mucosa (Figs. 5 and 6).

Lansoprazole Prevents the Indomethacin-induced Fas-mediated Death Receptor (CD95) Pathway of Apoptosis—Studies were carried out to check whether the death receptor pathway

![FIGURE 5. Lansoprazole prevents NSAID-induced disruption of mitochondrial membrane potential ($\Delta\Psi$). $\Delta\Psi$ was measured by JC-1 fluorescence study as described under “Experimental Procedures.” Inset shows the ratio of red (590 nm, J-aggregate) to green (530 nm, J-monomer) fluorescence of JC-1. ***,$p < 0.001$ versus control; ###,$p < 0.001$ versus NSAID.](http://www.jbc.org/)

![FIGURE 6. A, lansoprazole prevents NSAID-induced release of cytochrome c. Cytochrome c in the cytosol was measured by indirect enzyme-linked immunosorbent assay. B, lansoprazole prevents NSAID-induced activation of caspase-9. The assay of caspase-9 activity has been described under “Experimental Procedures.” ***,$p < 0.001$ versus control; ###,$p < 0.001$ versus NSAID.](http://www.jbc.org/)
is also involved in gastric mucosal apoptosis induced by indomethacin and whether lansoprazole has any effect on this pathway. Results shown in Fig. 7, A and B, indicate that indomethacin significantly ($p < 0.001$) up-regulates the expression of Fas (CD95) as well as its ligand, FasL (CD95L), in mucosal cells during the development of gastric lesions. Lansoprazole pretreatment, on the other hand, prevents indomethacin-induced changes of both Fas and FasL ($p < 0.001$ in both cases) (Fig. 7, A and B). Interestingly, indomethacin treatment causes significant activation of caspase-8 ($p < 0.001$) in the gastric mucosa (Fig. 7C) and lansoprazole pretreatment significantly prevents this activation ($p < 0.001$). Here also lansoprazole alone did not show any change in the expression of Fas and FasL as well as caspase-8 activation in control stomach.

**Lansoprazole Accelerates Healing through the Complete Inhibition of the Mitochondrial Pathway but Not the Death Receptor Pathway with Concurrent Stimulation of Cell Renewal**—Time course studies of caspase-9 and caspase-8 activity during healing of gastric lesions indicate that autohealing of indomethacin-induced mucosal lesions is associated with a 40% decrease of caspase-9 activity (Fig. 8A) and only a 25% decrease of caspase-8 activity at 24 h of healing (Fig. 8B). Lansoprazole pretreatment on the other hand further inhibits caspase-9 activity and bring it back to almost normal at 20 h of healing (Fig. 8A), whereas at the same time, caspase-8 activity is still higher than the control level (Fig. 8B). The persisting activation of caspase-3, which was noted even after 24 h of healing (Fig. 2), probably resulted from the sustained activation of caspase-8. However, RT-PCR analysis of PCNA expression during healing (Fig. 8, C and D) indicates that lansoprazole stimulates the rate of cell proliferation by favoring cell renewal to accelerate the healing process, compared with autohealing. Thus, during the healing phase, despite the operation of the death receptor pathway as evident from persistent caspase-8 activation, the healing of gastric mucosa results from the action of cell renewal/proliferation factors.

**Lansoprazole Restores Normal Cellular Architecture**—The protection of indomethacin-induced gastric mucosal damage by lansoprazole was monitored by transmission electron microscopy. Transmission electron microscopy studies indicate that inhibition of indomethacin-induced apoptosis as well as stimulation of cell survival by lansoprazole lead to the restoration of normal mucosal cell architecture (Fig. 9). The control mucosa (first row, first column) shows well defined cytoarchitecture with cell organelles finely distributed within the cells along with cell-cell contact. The nuclei (first row, second column) remain intact with visible and well defined nucleolus. The mitochondria (first row, third column) appear well organized with clear appearance of cristae and double membrane. The indomethacin-induced injured mucosa (second row, first column) shows typical characteristics of apoptosis such as disintegration of cellular organelles, dissociation of cell-cell contact, appearance of cytoplasmic vacuoles with cytoplasmic conden-
Antiapoptotic Effect of Lansoprazole

The salient points of the present study are: 1) lansoprazole blocks indomethacin-induced activation of mitochondrial and death receptor pathways to protect gastric mucosa from indomethacin-induced gastric damage and 2) lansoprazole also prevents indomethacin-induced suppression of mucosal cells survival to stimulate cell proliferation and healing of the ulcer wound. This is perhaps the first detailed report to establish a novel antiapoptotic and cell proliferating role of a proton pump inhibitor to protect and heal the gastric damage induced by a non-steroidal anti-inflammatory drug.

Results presented in this study indicate that indomethacin activates the caspase-3-dependent pathway for apoptosis in gastric mucosal cells and lansoprazole prevents this activation to block indomethacin-induced gastric damage. Time course study also suggests that the activation of caspase-3 correlates well with the severity of mucosal injury, as revealed by the macroscopic (ulcer index) and microscopic (histological) analysis of gastric mucosa (Table 1). However, in caspase-dependent apoptosis, caspase-3 is generally activated by two main pathways, such as, the mitochondrial (internal) pathway through the activation of caspase-9 and death receptor (external) pathway through the activation of caspase-8 (15, 16). The mitochondrial pathway involves up-regulation of proapoptotic proteins and/or down-regulation of antiapoptotic proteins of the Bcl-2 family (19, 20, 38). The present study shows that indomethacin significantly up-regulates Bax and Bak and down-regulates Bcl-2 and Bcl_{\text{xi}} to stimulate apoptosis in the gastric mucosal cells. Indomethacin-induced up-regulation of Bax and Bak has also been evident in the gastric cancer cell line (39) and down-regulation of Bcl-2 has also been demonstrated earlier in chronic myeloid leukemia cells (40). However, the molecular mechanism behind the up-regulation of Bax and Bak and down-regulation of Bcl-2 and Bcl_{\text{xi}} is still not clear. Reactive oxygen species that are augmented during indomethacin-induced gastric ulceration (4, 7) may have a signaling role in the expression of pro- and antiapoptotic proteins. However, up-regulation of proapoptotic proteins and down-regulation of antiapoptotic proteins leads to activation of proapoptotic counterparts, which are generally inactivated by the association of antiapoptotic members. This activation causes the translocation of proapoptotic proteins toward mitochondria where they play a contributory role in the opening of MPTP to release some apoptotic members. This activation causes the translocation of proapoptotic proteins toward mitochondria where they play a contributory role in the opening of MPTP to release some apoptotic proteins (18, 20). In this present study, immunogold electron microscopy at the single cell level shows mitochondrial translocation of Bax during the development of indomethacin-induced mucosal injury. Opening of MPTP by Bax translocation leads to a decrease of $\Delta \Psi \text{m}$ (18). Indomethacin treatment in this study was found to decrease $\Delta \Psi \text{m}$ of the gastric mucosal cells following Bax translocation. However, opening of MPTP leads to cytochrome $c$ release into the cytosol to induce formation of the apoptosome complex to activate caspase-9 (15, 17, 18, 20), which in turn activates caspase-3 to execute the final stage of apoptosis (15, 17, 20). Activation of caspase-3 by indomethacin as shown in this study was also reported earlier (14, 41). Lansoprazole blocks the mitochondrial pathway of apoptosis through the inhibition of Bax and Bak expression as well as stimulation of Bcl-2 and Bcl_{\text{xi}} expression, thereby inhibiting Bax transloca-
Antiapoptotic Effect of Lansoprazole

FIGURE 9. Ultrastructural analysis of gastric mucosal cells by transmission electron microscopy showing that lansoprazole protects NSAID-induced gastric mucosal cell damage. Control stomach section (first row) shows well formed cellular architecture, with intact membranes and well defined cell-cell interaction. Chromatin is finely distributed with the well defined nucleoli. Mitochondrion appear healthy with intact cristae and visible double membrane. NSAID-treated ulcerated stomach section (second row) shows typical characteristics of apoptosis such as, disruption of cytoarchitecture with dissociation of cell-cell contact, chromatin condensation, and appearance of cytoplasmic vacuoles. Mitochondria are disintegrated with the disappearance of cristae and appearance of electron-dense granules. The lansoprazole-pretreated NSAID-treated stomach section (third row) shows the restoration of normal cytoarchitecture, along with the restoration of nuclear and mitochondrial morphology.

Our studies further indicate significant up-regulation of Fas and FasL in gastric mucosal cells during indomethacin-induced mucosal damage (Fig. 7). This may lead to Fas-FasL-mediated activation of caspase-8, which in turn can activate caspase-3 or converge to the mitochondrial pathway through cleaving Bid. tBid at the outer mitochondrial membrane binds with Bax to induce cytochrome c release through a functional translocase of the outer membrane complex (42). Thus, death signaling can merge with the mitochondrial pathway in several instances (16, 42). Lansoprazole, by inhibiting indomethacin-induced changes of Fas and FasL expression, blocks the Fas-mediated pathway for the activation of caspase-8 as evident in this present study. Thus, lansoprazole prevents indomethacin-induced gastric mucosal apoptosis through inhibition of both mitochondrial and Fas-mediated death receptor pathways.

The integrity of gastric mucosa is under dynamic equilibrium between cell death through apoptosis and cell renewal through proliferation (11, 12). Now the question arises about the status of the cell survival mechanism during indomethacin-induced gastric mucosal damage. Indomethacin was found to inhibit the cell survival mechanism as revealed by decreased expression of PCNA. This inhibition of cell survival is mediated through suppression of cell survival/proliferation mediators, such as survivin, EGF, bFGF, and Flt-1, as evident from semiquantitative RT-PCR analysis. Inhibition of survivin expression by indomethacin has also been reported earlier (14). The importance of survivin in healthy gastric mucosal cells may be explained by its regulatory role in both proliferation as well as apoptosis (14). The gastric mucosal cells are continuously undergoing apoptosis and rapidly replaced by newly proliferating cells (9). This rapid death and renewal process are regulated by some factors to maintain mucosal integrity and tissue homeostasis. Survivin may be one of such regulators, the increased expression of which may be the target to control NSAID-induced gastric damage (14, 43). EGF and bFGF are members of peptide growth factor family, which are essential for maintaining normal mucosal health and functional status as well as regeneration following injury (11, 34, 44). EGF stimulates cell proliferation and migration, increases mucus secretion, and inhibits gastric acid secretion (11, 44), whereas bFGF possesses angiogenic and mitogenic properties, which are involved in cell migration and differentiation (11, 44). Flt-1 (FMS-related tyrosine kinase 1, receptor for vascular endothelial growth factor) is responsible for angiogenesis, endothelial cell proliferation, and survival (45) and essential for gastroprotection and mucosal healing. Inhibition of these important cell survival/proliferation mediators by indomethacin as revealed by RT-PCR studies (Fig. 3) significantly suppresses cell survival during the development of indomethacin-induced mucosal damage. Lansoprazole, by preventing changes of the expression of these mediators (Fig. 3), significantly restores cell survival in the gastric mucosa to prevent indomethacin-induced damage. Stimulation of gastric mucosal cell proliferation by lansoprazole is found to be mediated through activation of p44/42 mitogen-activated protein kinase (MAPK) (46). When growth factors bind with their specific receptors on the cell surface lead to receptor dimerization, resulting in subsequent autophosphorylation and activation of receptor tyrosine kinase and cause activation of the MAPK system (47). It is probable that up-regulation of growth factors or their receptors, such as EGF, bFGF, and Flt-1 in the gastric mucosal cells leads to activation of growth factor signaling, causing activation of MAPK to enhance the transcription of the gene essential for cell survival or proliferation. Subcellular structural analysis suggests that during protection of indomethacin-induced gastric damage by
lansoprazole, the restoration of the near normal cytoarchitecture is evident. However, the activation of cell survival/proliferation machineries in the gastric mucosal cells by lansoprazole is finely controlled and never leads to tumorigenic development as evident from morphology and expression of proliferation mediators in the healed gastric mucosal cells even after 48–72 h of healing.

In conclusion, this study for the first time reveals that lansoprazole protects and heals gastric mucosa from indomethacin-induced gastric damage by its novel antiapoptotic action mediated through regulation of various factors involved in mitochondrial and Fas death pathways of apoptosis. Moreover, indomethacin suppresses the cell survival mechanism in the gastric mucosa and lansoprazole blocks this inhibition and restores the cell survival system for effective mucosal protection and healing. Thus, in addition to inhibition of acid secretion, lansoprazole also offers gastroprotective action through inhibition of apoptosis as well as stimulation of cell survival/proliferation.

Acknowledgments—We thank Dr. Ranajit K. Banerjee and Dr. Alok K. Dutta for editing this manuscript.

REFERENCES

Lansoprazole Protects and Heals Gastric Mucosa from Non-steroidal Anti-inflammatory Drug (NSAID)-induced Gastropathy by Inhibiting Mitochondrial as Well as Fas-mediated Death Pathways with Concurrent Induction of Mucosal Cell Renewal

Pallab Maity, Samik Bindu, Vinay Choubey, Athar Alam, Kalyan Mitra, Manish Goyal, Sumanta Dey, Mithu Guha, Chinmay Pal and Uday Bandyopadhyay

doi: 10.1074/jbc.M800414200 originally published online March 28, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M800414200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 9 of which can be accessed free at http://www.jbc.org/content/283/21/14391.full.html#ref-list-1