Gastroesophageal reflux disease (GERD) is one of the most common diseases affecting about 40% of the world's population (1-3). The major mechanism by which GERD develops depends upon the tension of the lower esophageal sphincter (LES). When there is a transient decrease in the tension of the LES, gastric contents may leak into the esophagus (4-7). This disease is considered to be a failure of the physiological anti-reflux barrier to protect against frequent and abnormal amounts of gastroesophageal reflux (8, 9). The pathogenesis of GERD is complex, resulting from an imbalance between defensive factors protecting the esophagus such as; the anti-reflux barrier, esophageal acid clearance, esophageal tissue resistance, and the aggressive factors from the stomach and duodenum such as gastric acid, pepsin and duodenal contents.
which can damage the esophagus (10-13). The esophageal damage is developed in response to the prolonged exposure of the esophagus to acid and digestive enzymes found in the gastric fluid (pepsin), or duodenal contents containing for instance, bile salts regurgitated into the stomach. Gastric acid and alkaline contents may induce and promote irritation and result in symptoms and morphological changes to the esophageal mucosa (9, 10, 14).

The esophageal defense against acid damage consists of the anti-reflux mechanisms, including the mucosal barrier, which is created by special physiological properties of the gastroesophageal junction (4). The resistance to damage of the stratified squamous epithelium of the normal esophagus is governed by several factors. Besides this structural component, the functional component of mucosal resistance includes the ability of the esophageal epithelium to buffer and extrude hydrogen ions. The intraepithelial buffering capacity is accomplished by proteins, as well as bicarbonates (12, 14, 15).

Other functional defense mechanisms include the removal of the reflux material from the esophagus and the restoration of physiological pH of the esophageal mucosa after exposure to acid (6-9). Circulating blood delivers oxygen, nutrients and bicarbonate and removes hydrogen ion, CO₂ and other metabolites, thereby maintaining the proper mucosal acid-base balance. Previous studies documented that blood flow to the esophageal mucosa increases in response to an increase of mucosal secretory activity and the stress-induced increase of the luminal acid (11).

Among the endogenous aggressive factors of gastric origin, the reflux of gastric acid and pepsin play a major role. It has been demonstrated that acid alone induces minor injury with a pH of less than 3 (13, 16). However, when acid is combined with even small amounts of pepsin, the damage to the mucosal barrier occurs due to increased hydrogen ion permeability which causes local hemorrhage (16). In addition to gastric and pepsin, the alkaline duodenal content refluxed into esophagus could cause injury to the esophageal mucosa (17). Persistent reflux of gastric contents into the esophagus can induce breaks, disruptions and erosions of the mucosal cell layer (17, 18), as particular lines of the esophageal mucosal defense system becomes unable to withhold and repair local damage. Experimental studies demonstrated that bile acids induced esophageal damage is exaggerated in the presence of acid and pepsin (17-19).

Ingestion of various drugs could also contribute to the imbalance between offensive and defensive factors and the pathogenesis of reflux esophagitis. It is well established that the use of commonly used medications such as nonsteroidal anti-inflammatory drugs (NSAIDs), may increase the vulnerability of gastrointestinal mucosa to the development of peptic lesions, such as erosions and even ulceration as well as serious ulcer complications such as bleeding and perforation (20), however, the influence of NSAIDs on gastroesophageal reflux has not been fully explored. NSAIDs such as aspirin, naproxen, indomethacin and ibuprofen have been identified as risk factor for development of erosive esophagitis (21-24). It has also been demonstrated that aspirin makes the esophageal mucosa more sensitive to the injurious action of acid and pepsin (25) suggesting that this NSAID can act synergistically with gastroduodenal factors responsible for the damage of esophageal mucosa.

Since NSAIDs, aside from Helicobacter pylori (26), are considered as potential independent risk factor of gastrointestinal bleedings and peptic ulcer disease, the new strategy against adverse effect of classic NSAIDs includes the novel nitric oxide (NO)-releasing NSAIDs that were developed by the coupling of nitric oxide (NO)-releasing moiety to standard NSAIDs (27-32). In contrast to classic NSAIDs, the new NO-releasing derivatives of flurbiprofen, ketoprofen, diclofenac and NO-releasing aspirin have been shown to exhibit lower gastrointestinal toxicity than parent compounds, and they spare the gastrointestinal tract even when administered repeatedly for several weeks (33-35). The NO-NSAID were shown to inhibit non specifically both cyclooxygenases (COX)-1 and COX-2, with a comparable potency to parent NSAID but with slight modification has been described previously in detail (36, 37).

Affer carefully assessing previous research on the matter, it is our belief that the effects on esophageal mucosal damage in the model of gastric reflux taking into account the new derivatives of ASA, particularly the NO releasing ASA have not been fully explored. Therefore, using a modified method of gastric acid reflux as proposed by Omura et al. (38) and selecting the optimal time of reflux esophagitis to cause reproducible mucosal damage, we attempted to determine the effect of pretreatment with NO-ASA in comparison with conventional ASA on the esophageal mucosal injury and esophageal blood flow (EBF) in rodent model of acute acid reflux esophagitis in rats. The involvement of endogenously generated prostaglandins and the contribution of cyclooxygenase (COX)-1 and COX-2 to the development of esophageal damage by acid reflux with and without NO-ASA or ASA was determined by the employment of non-selective and highly selective COX-1 and COX-2 inhibitors administered to animals with reflux esophagitis in the doses that were previously reported to inhibit mucosal PG biosynthesis in vivo (39). Moreover, an attempt was made to determine not only the gross but also morphology of esophageal mucosa in rats with reflux esophagitis administered with COX inhibitors and NO releasing ASA. Furthermore, the effect of concurrent administration of NO donor combined with ASA was determined in order to check whether addition of exogenous NO could influence the esophageal damage and accompanying changes in the EBF comparable with those evoked by NO-ASA. In addition, we attempted to assess the effect of the treatment with NO-ASA, ASA and non-selective and selective COX-1 and COX-2 inhibitors on the esophageal expression of proinflammatory cytokines IL-1β and TNF-α and plasma levels of IL-1β and TNF-α and IL-6 in animals with gastric reflux.

MATERIAL AND METHODS

Male Wistar rats weighting 190-250 g and fasted for 24 hours before the experiment were used in all studies on the development of acute esophagitis and esophago-protection. The experimental procedures of this study were approved by the Institutional Animal Care and use Committee of Jagiellonian University Medical College in Cracow. All experimentations were followed in accordance with statements of the Helsinki Declaration regarding handling of experimental animals.

Development of reflux esophagitis and experimental groups

The model of reflux esophagitis utilized in our present study with slight modification has been described previously in detail by Omura et al. (38). Briefly, under ether anesthesia in about 140 rats that underwent overnight fasting, a midline laparotomy was performed to expose the stomach, and then both the pylorus and the transitional junction between the fore-stomach and the corpus were first exposed and later ligated with 2-0 silk thread but without pyloric ring with a piece of 18 Fr Nelaton catheter as originally proposed by Omura et al. (38) (see Fig. 1). After the surgical preparation was completed, the EBF was measured to confirm that esophageal blood vessels were not ligated and then
the abdomen cavity was temporarily closed. In a separate series of experiments, the time-course relationship between changes in EBF and the degree of esophageal mucosal lesions and duration of development of acute esophagitis was investigated. In these series of experiments animals were treated with vehicle (saline) only. The rats were then anesthetized second time at 2 hrs, 4 hrs, 8 hrs, 12 hrs, and 24 hrs after the onset of the reflux esophagitis induction. Then, a laparotomy was performed and the EBF was measured. After EBF determination was completed the animals were killed with an overdose of pentobarbital (50 mg/kg i.p.) at 2 hrs, 4 hrs, 8 hrs, 12 hrs and 24 hrs, and the esophagus with the proximal portion of stomach was gently dissected, then removed and pinned open for the assessment of macroscopic lesions. The esophageal biopsy samples with and without mucosal lesions present were gently rinsed with saline, excised and the fixed in 10% buffered formalin. Samples were embedded in paraffin, and 3 μm sections were prepared and stained with H&E for microscopic assessment.

After these series of experiments were completed, we selected the duration model of acute esophagitis based on the time of reflux being 4 hrs from the time of the procedure of ligature. With this particular time of 4 hrs, the esophageal lesions were fully developed and the experiments were the most reproducible with a given satisfactory index of the lesions in control animals. Animals with reflux esophagitis were randomized into the six experimental groups (A-F), consisting of 6-10 rats per group and they received i.g. the following pretreatment with: A) vehicle (saline), B) non-selective COX-1 and COX-2 inhibitor, ASA (100 mg/kg) or indomethacin (5 mg/kg), C) COX-1 selective inhibitor, SC-560 (5 mg/kg), D) selective COX-2 inhibitor, celecoxib (10 mg/kg), E) NO-ASA (NicOx S.A., Sophia Antipolis, France; 100 mg/kg), and F) GTN (10 mg/kg i.g.), a NO donor combined with ASA (100 mg/kg, i.g.) (17). In separate group of animals with reflux esophagitis, the pretreatment with ASA applied i.g. in graded doses ranging from 12.5 mg/kg up to 100 mg/kg were determined.

Measurement of esophageal blood flow (EBF) and assessment of esophageal injury

Upon the termination of gastric acid reflux in most experiments at the standard time of 4 hrs after the ligation as described above, the animals were anesthetized with pentobarbital, their abdomens were opened by midline incision, and the abdominal part of the esophagus was exposed for measurement of EBF by means of the H2-gas clearance technique as described previously (19, 34). For this purpose double electrode of an electrolytic regional blood flow meter (Biotechnical Science, Model RBF-2, Osaka, Japan) was inserted into esophageal mucosa. The measurements were made in three areas of the mucosa and the mean values of the EBF was calculated and expressed as a percent changes of those compared in the control vehicle-treated animals. The animals were euthanized by pentobarbital overdose and the esophagus was removed. Immediately after its removal it was opened longitudinally, rinsed with saline and pinned open for macroscopic examination. The lesion index score was calculated (macroscopic degree of injury 0-6) after gross inspection of the esophagus under a dissecting microscope (19) by a researcher blind to the experimental grouping. The total area (mm2) of lesions that had developed in the esophagus was determined under a dissecting microscope (10x) with a square grid, and graded with the lesion index (LI), as follows: 0) no visible lesions; 1) a few erosions and bleedings; 2) total area of lesions <15 mm2; 3) total area of lesions <30 mm2; 4) total area of lesions <40 mm2; 5) total area of lesions 45 mm2; 6) perforation.

Determination of mucosal generation of PGE2, and plasma proinflammatory cytokines

In groups of rats exposed to acid reflux without or with pretreatment with non-selective COX-1 and COX-2 inhibitor ASA, the esophageal mucosal samples from were taken by biopsy (about 200 mg) from grossly unchanged esophageal mucosa without mucosal lesions immediately after the animals were sacrificed to determine the mucosal generation of prostaglandin E2 by radioimmunoassay (RIA) as described previously (39). The mucosal samples were placed in preweighed Eppendorf vial and 1 ml of Tris buffer (50 mm, pH 9.5) was added to each vial. The samples were finely minced (during 15 s) with scissors, washed and centrifuged for 10 min, the pellet being resuspended again in 1 ml of Tris. Then, each sample was incubated on a Vortex mixer for 1 min and centrifuged for 15 s. The pellet was weighed and the supernatant was transferred to a second Eppendorf vial containing indomethacin (10 mM) and kept at -20°C until the radioimmunoassay. The capability of the esophageal mucosa to generate prostaglandin E2 was expressed in nanograms of wet tissue weight (39, 40).

At the termination of the experiments, immediately after EBF measurements, a venous blood sample was withdrawn from the vena cava into EDTA containing vials and used for the determination of plasma TNF-α, IL-1β and IL-6 by a solid phase sandwich ELISA (Biosource International Inc. Camarillo, CA, USA) according to the manufacturer's instructions. Briefly, each sample (50 µl) was incubated with biotinylated antibodies specific for rat TNF-α, IL-1β and IL-6, was washed three times with an assay buffer and finally conjugated with streptavidin peroxidase to form a complex with stabilized chromogen as described in our previous study (20, 39, 40).

Expression of IL-1β and TNF-α mRNA transcripts in the esophageal mucosa determined by reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNAs for interleukin-1β and TNF-α were determined by reverse transcriptase-polymerase chain reaction in the esophageal mucosa of the intact rats or those exposed to reflux esophagitis with or without pretreatment with a non-selective and selective COX-1 and COX-2 inhibitors. Samples of the gastric oxyntic mucosa (about 200 mg) were scraped off on ice using a glass slide and then immediately snapped frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated from the esophageal mucosa according to Chomczynski and Sacchi (1987) (41) using a rapid guanidinium isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Stratagene GmbH, Heidelberg, Germany).

First strand cDNA was synthesized from total cellular RNA (5 µg) using 200 U Strata Script TM reverse transcriptase and oligo (dt) primers (Stratagene GmbH, Heidelberg, Germany). The primers for the rat β-actin and IL-1β were synthesized by Biometra (Gottingen, Germany). The IL-1β primer sequences were designed according to the published cDNA sequences for primer sequences were as follows: up-stream, 5’ GCT ACC TAT GTC TTG CCC GT; downstream, 3’ GAC CAT TGC TGT TTC CTA GG. The expected length product was 546 bp. The TNF-α primer sequences were as follows: up-stream, 5’TAC TGA ACT TCG GGC TGA TTG GTC C; downstream, 3’ CAG CCT TGT CCC TTG AAG AGA ACC. The expected length product was 296 bp. Concomitantly, amplification of control rat β-actin was performed on the same samples to verify the RNA integrity.

DNA amplification was carried out under the following conditions; denaturation at 94°C for 1 min, annealing at 60°C for 45 s and extension at 72°C for 45 s. Each PCR-product (8 µl)
was electrophoresed on 1.5% agarose gel stained with ethidium bromide, and then visualized under UV light. The location of the predicted PCR product was confirmed by using a 100-base pair ladder (Gibco BRL/Life Technologies, Eggenstein, Germany) as a standard marker. A comparisons between different treatment groups were made by determining the IL-1β and TNF-α/β-actin ratio of the immunoreactive area by densitometry.

**Statistical analysis**

Results are expressed as means ±S.E.M. Statistical analysis was done using analysis of variance and two way ANOVA test with Tukey post hoc test where appropriate. Differences of p<0.05 were considered significant.

**RESULTS**

**Time sequence of changes in esophageal injury and esophageal blood flow (EBF) during development of reflux esophagitis**

Fig. 2 shows the effects of duration of reflux esophagitis on the alterations in EBF and the degree of macroscopic esophageal mucosal injury as determined by the area of esophageal mucosa occupied by the damage and expressed by a lesion index. As shown in Fig. 2, the significant fall in the EBF and a significant increase in the mucosal injury index were observed already at 2 hrs after the onset of reflux of gastric juice. Mucosal injury at 2 hrs was characterized by the appearance of local mucosal edema with a small number of local hemorrhagic erosions, however, the average damage score was low. Four hours after induction of RE, further developments of esophageal mucosal lesions were observed which increased the lesion score to the mean value of 3.5±0.5 and further significant fall in EBF by about 15% when compared to that observed at 2 hrs after the end of the ligation procedure to induce reflux. With a prolongation of the time of acid exposure to the esophageal mucosa up to 8 hrs, 12 hrs and 24 hrs, the EBF was decreased by about 40% from control and the lesion score rose significantly to the values of 4.1±0.6, 4.2±0.8 and 4.2±0.4, respectively, comparing to those obtained in control animals at 2 hrs (Fig. 2). The increase in lesion index observed at 8 hrs, 12 hrs and 24 hrs of the mucosal lesion score was not significantly higher than this observed at 4 hrs of reflux. On the basis of this experiment, the decision was made to choose the time of 4 hrs as the standard time of observation for further experimentations. This time after initiation of reflux was also employed for the evaluation of ability of various NSAIDs including ASA, indomethacin, celecoxib, NO-ASA, and GTN combined with ASA to influence the esophageal mucosal injury induced by gastric reflux and the accompanied changes in EBF.

**Effect of native ASA and non-selective and selective COX-1 and COX-2 inhibitors on esophageal lesion index, gross and microscopic injury and alterations in EBF**

Fig. 3 shows the data on the administration of ASA applied i.g, in graded doses ranging from 12.5 mg/kg up to 100 mg/kg on the mean lesion score and accompanying changes in the EBF and the content of PGE2 in the esophageal mucosa of rats with esophagitis. The mean value of EBF in the intact esophagus without reflux reached the value of 72.6±2.5 ml/min/100g of tissue, and this was considered to be the control. The lesion score at 4 hrs of duration of reflux was similar to that presented in Fig. 2. The pretreatment with ASA administered i.g. at the dose of 12.5 mg/kg failed to affect both the lesion index and the EBF as compared to vehicle. However, when ASA was administered in graded doses starting from 25 mg/kg up to 100 mg/kg, the dose-dependent increase in the lesion score was observed accompanied by the progressive fall in the EBF. The quantitative data on the effect of non-selective and selective COX-1 and COX-2 inhibitors on the lesion score index and the accompanying changes in EBF are presented in Fig. 4. The pretreatment with ASA (100 mg/kg i.g.) or indomethacin (5 mg/kg i.g), resulted in a significant rise in the lesion index followed by the significant fall in the EBF in rats with acute esophagitis induced by gastric reflux. The pretreatment with SC-560 (5 mg/kg i.g.), the selective COX-1 inhibitor, also significantly increased the lesion index and reduced the EBF as it was observed in the case of ASA and indomethacin (Fig. 4). In contrast, the pretreatment with

![Fig. 1. The scheme of methods employed in this study to induce esophagitis. Both, the duodenum and the transitional between the forestomach and the glandular portion was ligated with 2-0 silk thread to enhance reflux of gastric acid into the esophagus.](image-url)
celecoxib (10 mg/kg i.g.), which produced a smaller fall in the EBF, caused significantly less mucosal damage as compared with those observed in indomethacin- or SC-560-pretreated rats at 4 hrs of gastric reflux. At the same experimental conditions, the pretreatment with NO-ASA significantly attenuated the lesion index and significantly raised the EBF as compared to respective values in vehicle-control and those recorded in ASA-, indomethacin-, SC-560- and celecoxib-pretreated animals.

The macroscopic appearance of the intact esophagus and that exposed to vehicle (saline) control, ASA, indomethacin, SC-560, celecoxib and NO-releasing ASA is presented in Fig. 5A-G. As shown in Fig. 5A, the intact esophageal mucosa showed the normal appearance with no lesions present. In contrast, the exposure of esophagus for 4 hrs to the reflux of gastric content resulted in the formation of mucosal damage (Fig. 5B), and these lesions were significantly potentiated by the pretreatment with ASA (100 mg/kg i.g.), indomethacin (5 mg/kg i.g.) or SC-560 (5 mg/kg i.g.) (Figs. 5C, 5D and 5E). The pretreatment with celecoxib (10 mg/kg i.g.) also increased the area of esophageal lesions and significantly decreased the EBF but the lesion index was significantly smaller and the values of EBF were significantly higher in celecoxib group than those recorded in ASA- and indomethacin-pretreated rats exposed to gastric reflux (Fig. 5F). In contrast, the pretreatment with NO-ASA (100 mg/kg i.g.) markedly reduced the area of esophageal damage when compared with that treated with conventional ASA, indomethacin, SC-560 and celecoxib (Fig. 5G). The extent of injury was significantly smaller in NO-ASA pretreated animals than those pretreated with vehicle Figs. 5B and 5G).

The histological determination of esophageal mucosa stained with H&E documented that no microscopic mucosal changes were observed in intact animals (Fig. 6A). The normal esophagus exhibited a thin epithelial layer with squamous cells and only few inflammatory cells were found in submucosal layers. In contrast, at 4hrs after induction of reflux esophagitis, the mucosal damage and hyperemia of epithelial layers and edema in mucosa and submucosa was observed in vehicle (saline)-control animals (Fig. 6B). In ASA- and SC-560 -treated rats (Figs. 6C and 6D), a marked mucosal thickening accompanied by further exaggeration of
the damage, edema and heavy inflammatory cell infiltration were observed. In celecoxib group (Fig. 6E), the esophageal damage, edema and neutrophil infiltration were still observed, however these changes were significantly less pronounced as in case of group pretreated with ASA and SC-560. As shown in Fig. 6F, the mucosal damage, neutrophil infiltration and edema in the mucosa and submucosa were significantly reduced in rats pretreated with NO-ASA and then exposed to acid reflux.

Effect of pretreatment with ASA alone, NO-ASA and GTN combined with ASA on the lesion index and accompanying changes in EBF

Table 1 shows the macroscopic lesion index of esophageal injury and the alterations in EBF following reflux esophagitis in rats pretreated with vehicle, ASA alone and ASA combined with GTN, a donor of NO. Treatment with ASA (100 mg/kg i.g.) significantly increased the index of mucosal lesions, and
decreased the EBF when compared to those in the vehicle-treated animals. In contrast, pretreatment with NO-ASA given in similar dose as conventional ASA, significantly reduced the esophageal lesions index score and this effect was accompanied by a significant increase in EBF. Concurrent treatment with GTN (10 mg/kg i.g) in combination with ASA reversed the fall in the EBF and increase in mucosal lesion index caused by native ASA alone. This reduction in the lesion index and the rise in the EBF is almost identical to these evoked by NO-ASA.

**Table 1.** Esophageal mucosal lesion index and esophageal blood flow (EBF) in rats with acute reflux esophagitis without or with intragastric pretreatment with aspirin (ASA) (100 mg/kg i.g) or NO-aspirin (NO-ASA) (100 mg/kg i.g) or glyceryl trinitrate (GTN) (10 mg/kg i.g) in combination with ASA (100 mg/kg i.g). Results are mean±S.E.M. of 6-8 rats. Asterisk indicates significant change (p<0.05) as compared to the value recorded in vehicle-saline pretreated rats. Cross indicates significant change (p<0.02) as compared to the value in ASA-pretreated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesion index (0-6)</th>
<th>EBF (%) control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>3.2±0.4</td>
<td>58±6</td>
</tr>
<tr>
<td>ASA</td>
<td>5.8±0.8*</td>
<td>48±3*</td>
</tr>
<tr>
<td>NO-ASA</td>
<td>1.4±0.3</td>
<td>78±5</td>
</tr>
<tr>
<td>GTN plus ASA</td>
<td>1.8±0.5*</td>
<td>76±4</td>
</tr>
</tbody>
</table>

**Fig. 6.** Microscopic appearance of histological findings for the esophagus. The esophageal mucosa in intact rats (A) and those pretreated i.g. 30 min before the initiation of reflux esophagitis with vehicle (B), ASA (100 mg/kg) (C), SC-560 (5 mg/kg) (D), celecoxib (10 mg/kg) (E) and NO-releasing ASA (100 mg/kg) (F). Note, the intact rat shows the thin epithelial layer with squamous cells and few inflammatory cells reflecting the normal appearance of the esophageal mucosa (A). Vehicle-pretreated rats exposed to reflux esophagitis showed the mucosal thickening, edema, mucosal damage, basal hyperplasia and leukocyte (neutrophil and eosinophil) infiltration (B). This damage to esophageal structure was exaggerated in rat with reflux esophagitis pretreated with ASA (B vs. C) and SC-560 (B vs. D). In celecoxib-pretreated group, the mucosal damage and neutrophil infiltration were less pronounced, however the mucosal thickening and edema were present. In contrast to ASA- and SC-560-pretreated rat, the pretreatment with NO-releasing ASA significantly attenuated the damage induced by reflux esophagitis as reflected by partial preservation of the mucosal architecture (C and D vs. F).

**Mucosal expression of IL-1β and TNF-α by RT-PCR and plasma levels of IL-1β, TNF-α and IL-6 in the rats with esophagitis treated with NO-ASA and COX-inhibitors**

As shown in Fig. 7, the plasma levels of IL-1β, TNF-α and IL-6 reached significantly higher values after 4 hrs of RE in vehicle-treated rats when compared to those recorded in the intact animals. The control concentration of IL-1β in the intact animals were 15±2 pg/ml and a significant increase in IL-1β level was observed averaging about 75 pg/ml in vehicle pretreated rats with gastric reflux. Pretreatment with ASA (100 mg/kg i.g.) prior to onset of reflux esophagitis significantly raised the plasma level of IL-1β when compared to that measured in the vehicle pretreated animals with reflux. The treatment with NO-ASA (100 mg/kg i.g) restored the plasma IL-1β to the level observed in vehicle pretreated rats, exposed to RE. Treatment with indomethacin (5 mg/kg i.g.) and celecoxib (10 mg/kg i.g) significantly raised the plasma level of IL-1β in comparison to the level recorded in vehicle-treated rats though the increase in this cytokine level in the animals pretreated with celecoxib were significantly less pronounced when compared to that in ASA, indomethacin and SC-560 treated rats. Similarly, the concentration of TNF-α and IL-6 remained at low levels (0.5±0.01 pg/ml and 1.2±0.2 pg/ml) in intact animals but following 4 hrs of reflux esophagitis, there was a remarkable rise in TNF-α and IL-6 concentration, reaching the value of about 4.0±0.7 pg/ml and 8.1±1.7 pg/ml, respectively (Fig. 7). Pretreatment with ASA or indomethacin resulted in a significant increase in plasma TNF-α and IL-6 level when compared with those measured in the vehicle-treated rats subjected to 4 hrs of reflux esophagitis. In celecoxib-pretreated rats, a significantly
smaller increase in the plasma TNF-α and IL-6 were observed as compared to the levels of these cytokines in ASA or indomethacin treated rats. The pretreatment of animals with NO-ASA significantly decreased the plasma concentrations of TNF-α and IL-6 below the value recorded in vehicle-treated animals exposed to gastric reflux (Fig. 7).

Fig. 8 (left panel A) shows the expression of IL-1β mRNA in the intact esophageal mucosa and in that exposed to acid reflux with or without the pretreatment with NO-ASA or native ASA. The weak signal for IL-1β mRNA was detected in intact esophageal mucosa and this was significantly increased with acid reflux, the effect that was further enhanced in the esophageal mucosa exposed to ASA. In NO-ASA pretreated rats, the signal for IL-1β mRNA expression was significantly inhibited. The ratio of IL-1β mRNA to β-actin confirmed that mRNA for IL-1β was significantly up-regulated in rats exposed to acid reflux and those pretreated with ASA when compared to intact gastric mucosa while NO-ASA inhibited the expression of mRNA for IL-1β (Fig. 8, right panel). As shown in Fig. 8 (left panel B), TNF-α mRNA was detected in the gastric mucosa of all rats including control animals and those exposed to acid reflux with and without ASA and NO-ASA administration. Signal intensity for TNF-α mRNA was weakly detectable in intact animals but it was increased in reflux esophagitis and further significantly augmented in rats pretreated with ASA. The pretreatment with NO-ASA inhibited the mRNA for TNF-α in the esophageal mucosa of rats with acid reflux. The ratio of TNF-α mRNA to β-actin mRNA confirmed that expression of mRNA for TNF-α was increased in rats with reflux esophagitis and further overexpressed in those pretreated with ASA as compared to intact rats. The ratio of TNF-α to β-actin mRNA confirmed that TNF-α mRNA was significantly inhibited in NO-ASA-pretreated animals and exposed 30 min later to acid reflux as compared to that in rats exposed to acid reflux with native ASA administration (Fig. 8, right panel).

DISCUSSION

Gastric esophageal reflux disease (GERD) is one of the most important gastrointestinal disorders of our population nowadays and the mechanisms responsible for this disease should be extensively examined (42, 43). In this study we attempted to develop the experimental model of esophagitis that serves as a
accompanying by the inhibition of COX-1 and the suppression of protection and the subsequent increase in the EBF was demonstrated by NO-ASA in the stomach of experimental gastroesophageal reflux. Similar protective activity is protective against mucosal injury induced by animals and humans (27, 29-32). Mechanism of this protection involves several mechanisms, mainly undisturbed epithelial barrier of the esophageal mucosa against aggressive factors of reflux esophagitis. The maintenance of integrity of the epithelial barrier of the esophageal mucosa against aggressive factors involves several mechanisms, mainly undisturbed mucosal microcirculation which is potentially responsible for maintaining normal mucosal acid-base balance. An increase in the esophageal blood flow takes place in response to an activation of the esophageal secretory activity and the presence of luminal refluxate from the stomach and duodenum (8, 13). An imbalance between defensive and offensive factors is proposed as a major mechanism in the development of reflux esophagitis (4-8). Recently, we reported that the inhibition of COX/PG and NOS/NO systems and the deactivation of sensory nerves by NO-ASA against the damage induced reflux esophagitis may determine the involvement of NO in the mediation of the expression and release of proinflammatory cytokines such as IL-1β and TNF-α. Furthermore, the plasma level of proinflammatory cytokine IL-6 was also markedly diminished in animals pretreated with NO-ASA. On contrary, the expression of mRNA for IL-1β and TNF-α and their plasma levels were markedly increased in rats with acute gastric acid reflux and this effect was further potentiated in those treated with non-selective and selective COX-1 inhibitors. This supports the notion that inhibition of COX-1 stimulates pro-inflammatory factors resulting in the aggravation of mucosal injury and the impairment of mucosal recovery from the damage (44, 46).

The esophagoprotective activity of NO-ASA shown in this paper is consistent with previous data from our and others laboratories that NO accelerated experimental ulcer healing, whereas inhibition of endogenous NO generation by NO-synthase inhibitor impaired healing of chronic gastric ulcers (34, 35, 37). The mechanism through which NO released from ASA induces inhibition of esophageal mucosal lesion is complex and not entirely clear but could be attributed to its well established potent vasodilatory properties and an ability to increase the mucosal blood flow (31, 34, 35). NO was shown to suppress the release of pro-inflammatory cytokines and contribute to the enhanced generation of mucosal esophageal PGE₂ (18, 19).

Similar protective activity of NO-ASA has been recently demonstrated in rodent stomach (27-29, 31). Our results emphasize the protective role of NO released from NO-ASA in the action of new ASA derivative on esophageal mucosa against acute reflux esophagitis. The present investigations attempted to determine the involvement of NO in the mediation of the esophageal protective action of NO-ASA and for this purpose the exogenous donor of NO, GTN was employed to mimic the effect of NO releasing ASA. The combination of native ASA with GTN induced the same degree of esophageal protection as NO-ASA and enhanced significantly the EBF to the level observed after application of NO-ASA. These protective and microcirculatory effects of GTN combined with ASA could be attributed to a very well recognized circulatory and the anti-inflammatory activity of NO, which was apparently released from GTN (31). Further studies are needed to assess which isofrom of COX, is involved in esophageal protection and

![Fig. 8. Determination of IL-1β (A) and TNF-α (B) and β-actin (C) mRNA expression by RT-PCR (left panel) and the ratio of IL-1β and TNF-α mRNA over β-actin mRNA (right panel) in the intact esophageal mucosa (lane 1) and in those pretreated i.g. with vehicle (saline) (lane 2), and NO-ASA (100 mg/kg) (lane 3) or ASA (100 mg/kg) (lane 4) and exposed to reflux esophagitis for 4 hrs; M - DNA size marker. Mean±S.E.M. of 4-6 rats. Asterisk indicates a significant change (p<0.05) as compared with vehicle control gastric mucosa. Cross indicates a significant change (p<0.05) as compared with vehicle and ASA treatment. Double crosses indicate a significant change (p<0.05) as compared to the value obtained in vehicle-control and NO-ASA groups.

complementary tool for a clinical investigation offering insight into the mechanism of damage to the mucosa of the esophagus by aggressive factors and COX inhibitors. The model of gastroesophageal reflux described in this paper seems to be especially suitable for examination of the early mechanisms of acute esophageal injury caused by gastric reflux and does not entirely reflect the changes associated with chronic esophagitis or long term GERD. In this experimental model, the gastric acid secreted by parietal cells and pepsin activity in the stomach but not intestinal alkaline content, are the most important pathogenic factors of reflux esophagitis. The maintenance of integrity of the epithelial barrier of the esophageal mucosa against aggressive factors involves several mechanisms, mainly undisturbed mucosal microcirculation which is potentially responsible for maintaining normal mucosal acid-base balance. An increase in the esophageal blood flow takes place in response to an activation of the esophageal secretory activity and the presence of luminal refluxate from the stomach and duodenum (8, 13). An imbalance between defensive and offensive factors is proposed as a major mechanism in the development of reflux esophagitis (4-8). Recently, we reported that the inhibition of COX/PG and NOS/NO systems and the deactivation of sensory nerves rendering the esophageal mucosa highly susceptible to damage by the exposure to exogenously administered acid-pepsin or to the combination of acid, pepsin and bile solution (19).

The major observation of our present study is the evidence accumulated for the first time, that NO-ASA, in contrast to native NSAIDs such as conventional ASA or indomethacin, protects esophageal mucosa against mucosal injury induced by gastroesophageal reflux. Similar protective activity is demonstrated by NO-ASA in the stomach of experimental animals and humans (27, 29-32). Mechanism of this protection by NO-ASA against the damage induced reflux esophagitis may involve excessive release of NO from this NO derivative of ASA as it was shown to result in gastroprotection induced by those agents (27-35). Moreover, the NO-ASA-induced esophageal protection and the subsequent increase in the EBF was accompanied by the inhibition of COX-1 and the suppression of the PG generation and did not differ from the inhibitory effect of this agent and classic NSAIDs on gastric PG as described earlier (31). It is of interest that these esophago-protective actions of the NO-ASA was accompanied by a profound attenuation of the expression and release of proinflammatory cytokines such as IL-1β and TNF-α. Furthermore, the plasma level of proinflammatory cytokine IL-6 was also markedly diminished in animals pretreated with NO-ASA. On contrary, the expression of mRNA for IL-1β and TNF-α and their plasma levels were markedly increased in rats with acute gastric acid reflux and this effect was further potentiated in those treated with non-selective and selective COX-1 inhibitors. This supports the notion that inhibition of COX-1 stimulates pro-inflammatory factors resulting in the aggravation of mucosal injury and the impairment of mucosal recovery from the damage (44, 46).

The esophagoprotective activity of NO-ASA shown in this paper is consistent with previous data from our and others laboratories that NO accelerated experimental ulcer healing, whereas inhibition of endogenous NO generation by NO-synthase inhibitor impaired healing of chronic gastric ulcers (34, 35, 37). The mechanism through which NO released from ASA induces inhibition of esophageal mucosal lesion is complex and not entirely clear but could be attributed to its well established potent vasodilatory properties and an ability to increase the mucosal blood flow (31, 34, 35). NO was shown to suppress the release of pro-inflammatory cytokines and contribute to the enhanced generation of mucosal esophageal PGE₂ (18, 19).

Similar protective activity of NO-ASA has been recently demonstrated in rodent stomach (27-29, 31). Our results emphasize the protective role of NO released from NO-ASA in the action of new ASA derivative on esophageal mucosa against acute reflux esophagitis. The present investigations attempted to determine the involvement of NO in the mediation of the esophageal protective action of NO-ASA and for this purpose the exogenous donor of NO, GTN was employed to mimic the effect of NO releasing ASA. The combination of native ASA with GTN induced the same degree of esophageal protection as NO-ASA and enhanced significantly the EBF to the level observed after application of NO-ASA. These protective and microcirculatory effects of GTN combined with ASA could be attributed to a very well recognized circulatory and the anti-inflammatory activity of NO, which was apparently released from GTN (31). Further studies are needed to assess which isofrom of COX, is involved in esophageal protection and
whether selective blockade of COX-1, or COX-2 or both (47), might be responsible for the enhancement of esophageal damage induced by gastric reflux.

Our study possesses evident limitation related to the duration of inflammatory response since we have shown the ability of NO-releasing derivative of commonly used native ASA to protect the esophageal mucosa during early but not prolonged chronic stage of esophagitis. However, experimental surgical method of induction of reflux esophagitis via gastro-esophageal reflux of acid caused visible mucosal damage to the esophageal structure detectable as early as 2 hrs following surgical ligation. The method of lesion induction employed in our present study after original technique by Omura et al. (38), does not require the animal to be under anesthesia for a long time during experimentation as is the case of the method using esophageal perfusion of acid and bile (19) and therefore we postulate that this procedure most likely mimics the human scenario of acute esophagitis. Moreover, in our present study, the damage was achieved by endogenously secreted gastric acid but not exogenous artificial acid as in our previous report (19).

The ingestion of NSAIDs has been associated with serious complications and adverse effects such as an increase in the vulnerability of the gastrointestinal and esophageal mucosa in the development of peptic lesions including inflammation, erosions and bleeding ulcerations (20, 25, 47). These destructive effects of NSAIDs are related to inhibition of constitutive enzyme COX-1, mainly responsible for PG generation and esophageal protection. The COX-2 is induced through the mechanism involving cytokines, growth factors and endotoxins (40). Selective COX-2 inhibitors such as rofecoxib and celecoxib, were specifically designed to preferentially inhibit the enzyme COX-1, mainly responsible for PG generation and in addition, the increased expression of COX-2 and mPGES-1 was markedly increased in rat acid reflux esophagitis compared with normal esophagus, while only modest changes in expression of COX-1, cPGES, and mPGES-2 were observed. Similar as in our present study, the PGE2 levels were significantly elevated in esophagitis comparing to baseline and in addition, the increased expression of COX-2 and mPGES-1 on both days 3 and 21 were observed by these authors (48). Because celecoxib significantly reduced the reflux-induced increase in PGE2 synthetic activity on both days 3 and 21, they concluded (48) that PGE2 derived from COX-2 and mPGES-1 play a significant role in the development of chronic esophagitis but to lesser degree in acute esophageal lesions. The discrepancy between our present results and those reported by Hayakawa et al. (48) might be attributed to different method of esophagitis employed and short time of acute esophagitis selected (up to 4 and 24 hours) in our experiments comparing with longer time (3 and 21 days) of observation in their study.

The adverse effects of traditional NSAID are related to topical mucosal injury which usually occurs after the ingestion of aspirin and other NSAIDs, especially under acidic conditions. The apparent reduction in gastrointestinal blood flow induced by NSAIDs such as ASA occurs also as a result of enhanced adherence of neutrophils to vascular endothelium in microvasculature via increased expression of adhesion molecules on endothelial cells (43, 44). In our present study native ASA dose-dependently augmented the damage induced by acid reflux and this effect was accompanied by potent suppression of PGE2 generation and a significant fall in the EBF. It has been documented that PG such as PGI2 are very potent vasodilators responsible for control of blood flow through resistance vessels and microcirculation and these eicosanoids are involved in the mediation of the vascular auto-regulatory responses including functional hyperemia in the gastrointestinal tract including the esophagus. Thus, it is likely that the mechanism of the esophageal lesions induced by ASA could be attributed to PG inhibition and vasoconstriction, ischemia and a final consequence tissue hypoxia leading to the impairment of the mucosal barrier. These damaging effects of ischemia on esophageal mucosa are potentiated by the presence of hydrogen ions and pepsin which alone can induce mucosal damage.

We have shown that at this acute stage of both COX-1 and COX-2 activity could be involved in the mechanism of acute esophagitis. This is supported by the fact that both non-selective and selective COX-1 inhibitors aggravated the esophageal damage and decreased the esophageal blood flow. An attempt has been made to determine the involvement of COX-2 in the mechanism of acute esophagitis by the use of celecoxib, the selective COX-2 inhibitor. While COX-1 inhibition is achieved by pretreatment with ASA, indomethacin and the selective inhibitor SC-560 potentiated the damage induced by gastric reflux, celecoxib was found to be superior to both non-selective and selective inhibitors as documented by the weaker aggravatory effect on esophagitis of celecoxib comparing to COX-1 inhibitors. Similarly, the degree of reduction of EBF observed after pretreatment with celecoxib in rats with gastric reflux was significantly smaller than that observed after pretreatment with selective COX-1 inhibitor, SC-560. Although there are many differences between COX-1 and COX-2, one notable distinction is that COX-2 is believed to be the principal COX isofrom, which is involved in inflammation. It is also debated whether COX-2 activity could also be detected in the non-inflamed stomach (47). Our data with celecoxib is only in partial agreement with study by Hayakawa et al. (48) who found that COX-2 inhibitors such as celecoxib and nimesulide significantly reduced the severity of chronic esophagitis (on day 21) but failed to affect acute esophageal lesions (on day 3). They demonstrated (48) that mRNA and protein expression of COX-2 and mPGES-1 was markedly increased in rat acid reflux esophagitis compared with normal esophagus, while only modest changes in expression of COX-1, cPGES, and mPGES-2 were observed. Similar as in our present study, the PGE2 levels were significantly elevated in esophagitis comparing to baseline and in addition, the increased expression of COX-2 and mPGES-1 on both days 3 and 21 were observed by these authors (48). Because celecoxib significantly reduced the reflux-induced increase in PGE2 synthetic activity on both days 3 and 21, they concluded (48) that PGE2 derived from COX-2 and mPGES-1 play a significant role in the development of chronic esophagitis but to lesser degree in acute esophageal lesions. The discrepancy between our present results and those reported by Hayakawa et al. (48) might be attributed to different method of esophagitis employed and short time of acute esophagitis selected (up to 4 and 24 hours) in our experiments comparing with longer time (3 and 21 days) of observation in their study.

The esophagus is the first anatomical part of GI-tract which undergoes exposure to NSAIDs. Esophageal ulcers are thought to be initiated by disruption of mucosal barrier by refluxate thus allowing the diffusion of hydrogen ion. Previous studies revealed that under experimental conditions and in humans, the ulceration of the esophageal mucosa can be induced after a few oral doses of a NSAID or by low dose of ASA (20). Indeed, in our study, the native ASA when applied i.g. induced a dose-dependent potentiation of damage induced by gastric reflux and this effect was accompanied by a parallel fall in EBF. These effects could be explained by the ability of ASA to inhibit COX-1 and the suppression of the generation of esophageal PG. In this light, the most important finding of this study is the discovery that NO-derivative of ASA protects esophageal mucosa against mucosal injury induced by acid refluxed from the stomach to the esophagus. Both, the NO-ASA-induced esophageal protection and accompanying increase in the EBF take place in the face of inhibition of COX-1 and suppressing of endogenous PG generation. Further studies are needed to determine whether NO-ASA could exert inhibitory action in progression of chronic esophagitis into Barrett's adenocarcinoma under experimental conditions described by our group recently (49).

In summary, we have shown that pretreatment of animals with NO-releasing ASA derivative or concomitant pretreatment by native ASA with an NO donor (glyceryl trinitrate) has beneficial effect on esophageal mucosa in reflux esophagitis. The mechanisms through which NO induces inhibition of
esophageal mucosa lesion may involve an increase in the esophageal blood flow. NO can also reduce the generation of proinflammatory factors, e.g. reactive oxygen metabolites (50) and contribute to an increased generation of esophageal mucosal PGE 
. Our present study implies that the beneficial effect of NO-releasing derivative of commonly used ASA to protect the esophageal mucosa during early stage of gastro-esophageal reflux depends upon the enhancement of the esophageal microcirculation, the inhibition of proinflammatory cytokines IL-1β, TNF-α and IL-6 induced by acidic refluxate and the replacement by vasodilatory NO as a compensatory mechanism to the decrease in endogenous PG induced by conventional ASA.

Conflict of interests: None declared.

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