Previous studies have demonstrated that the gastric mucosa of diabetic rats is highly vulnerable to acute injury but the influence of nonsteroidal anti-inflammatory drugs (NSAID) and their new nitric oxide (NO) releasing derivatives of aspirin (NO-ASA) on the ulcer healing under diabetic conditions has been little studied. In this study streptozocin (STZ, 70 mg/kg injected intraperitoneally) was used to induce diabetes mellitus in rats. Four weeks after STZ injection, gastric ulcers were induced using the acetic acid method and rats with gastric ulcers received the treatment with 1) aspirin (ASA, 30 mg/kg-d i.g.), 2) NO-ASA applied in equimolar dose of 50 mg/kg-d i.g., 3) rofecoxib (5 mg/kg-d i.g.), the selective cyclooxygenase-(COX)-2 inhibitor and 4) SNAP (5 mg/kg-d i.g.), a donor of NO, combined with ASA (30 mg/kg-d i.g.). Ten days after the induction of the ulcers, the healing rate and the gastric blood flow (GBF) were measured by planimetry and hydrogen (H₂)-gas clearance method, respectively and the plasma cytokine such as IL-1β, TNF-α and IL-10 were determined. In addition, the effect of insulin (4 IU/day/rat i.p.) with or without the blockade of NO-synthase by L-NNA (20 mg/kg-d i.p.) on the ulcer healing and the GBF in non-diabetic and diabetic rats was determined. In the diabetic rats, a significant delay in ulcer healing (~ by 300%) was observed with an accompanied decrease in the GBF at ulcer margin. The prolongation of the healing in diabetic animals was associated with an increase in the plasma cytokine (IL-1β, TNF-α and IL-10) levels. ASA and rofecoxib, that significantly suppressed the mucosal prostaglandin (PG) E₂ generation in ulcer area, delayed significantly the rate of ulcer healing and decreased the GBF at ulcer margin, while elevating plasma IL-1β, TNF-α and IL-10 concentrations in non-diabetic rats and these alterations were significantly augmented in diabetic animals. In contrast to ASA, the treatment with NO-ASA failed to influence both, the ulcer healing and GBF at ulcer margin and significantly attenuated the plasma levels of IL-1β, TNF-α and IL-10 as compared to those recorded in ASA- or rofecoxib-treated animals. Co-treatment of SNAP with native ASA abolished the deleterious effect of ASA on ulcer healing, GBF at ulcer.
margin and luminal NO release in diabetic rats. Administration of insulin in rats with diabetes, opposed the delay in ulcer healing, and the fall in the GBF at ulcer margin and these effects were counteracted by the concurrent treatment with L-NNA. We conclude that: 1) ulcer healing is dramatically impaired in experimental diabetes and this effect involves the fall in the gastric microcirculation at the ulcer margin and increased release of proinflammatory cytokines; 2) classic NSAID such as ASA and selective COX-2 inhibitors such as rofecoxib, prolong ulcer healing under diabetic conditions probably due to suppression of endogenous PG and the fall in the GBF at the ulcer margin suggesting that both COX isoforms, namely, COX-1 and COX-2, are important sources of PG during ulcer healing in diabetes; and 3) NO-ASA counteracts the impairment of ulcer healing in diabetic rats induced by ASA, mainly due to the release of NO that compensates for PG deficiency resulting in enhancement in the GBF at ulcer margin and suppression of cytokine release in the ulcer area.

Key words: ulcer healing, diabetes mellitus, nitric oxide, aspirin, rofecoxib, interleukin-
Ibeta, tumor necrosis factor alpha

INTRODUCTION

Previous studies documented that streptozotocin (STZ)-induced diabetes in rats is an accepted model of insulin-dependent diabetes, in which an increased vulnerability of the gastric mucosa against various ulcerogens such as ischemia/reperfusion injury, stress and non-steroidal antiinflammatory drugs (NSAID) can be observed (1 - 4). The mechanism underlying the increased susceptibility of gastric mucosa in diabetic animals to damage is multifactorial and includes the impairment of the antioxidative system in the gastric mucosa (1), impaired duodenal HCO$_3^-$ secretion (5), the suppression of bFGF production in the gastric mucosa (6), the attenuation of angiogenesis and the dysfunction of capsaicin-sensitive afferent neurons involved in the protection of gastric mucosa (7).

Patients with long-standing diabetes mellitus develop a variety of gastrointestinal symptoms such as abdominal pain, vomiting, diarrhea, constipation and delayed gastric emptying (8, 9). However, little attention has been paid to the incidence and healing rate of peptic ulcer in diabetics because peptic ulcers among diabetics are considered infrequent (10). Recent studies indicate, however, that peptic ulcers occurring in the course of diabetic state are more severe and often associated with complications such as gastrointestinal bleeding (11). Moreover, in the diabetic patients a significantly higher prevalence of H. pylori infection, a leading cause of peptic ulcer, was observed (12).

Non-steroidal anti-inflammatory drugs (NSAID) such as aspirin (ASA) are widely used but the major limitation of their clinical application are serious side-effects such as induction of acute hemorrhagic erosions, aggravating effect on stress ulceration and interfering with healing of preexisting ulceration (13 - 16). Recently, a new class of NSAID has been developed by adding of nitric oxide.
(NO) moiety to the native NSAID (17 - 20). The rationale behind this strategy is that NO released from these derivatives exerts beneficial influence on gastric mucosa by enhancing the mucosal defense ability and prevention of pathogenic events resulting from the suppression of prostanoid synthesis such as the reduction in mucosal microcirculation and the leukocyte-endothelial adherence (21 - 23). Recent studies in human volunteers proved the concept that the addition of NO-donating moiety to aspirin results in new chemical entity that maintains cyclooxygenase-1 and platelet inhibitory activity while nearly avoiding gastrointestinal damage (24). However, the effects of NO-releasing NSAID and selective COX-2 inhibitors such as rofecoxib (25), on the process of ulcer healing under diabetic conditions have not been extensively studied.

This study was designed to determine 1) the effect of classic NSAID such as aspirin (ASA) and the nitric oxide releasing ASA (NO-ASA) or specific COX-2 inhibitor such as rofecoxib on the course of healing of gastric ulcers in rats with the experimental diabetes; 2) to compare the effect of ASA and NO-ASA with that of S-nitroso-N-acetyl-D-L-penicillamine (SNAP), an NO donor added to native ASA and to determine the effect of suppression of NO-synthase with L-NNA on the ulcer healing in diabetic rats and 3) to evaluate the functional changes such as gastric blood flow (GBF) at the ulcer margin as well as alterations in the release of proinflammatory and anti-inflammatory cytokines (interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-10) under diabetic conditions without or with treatment with ASA, NO-ASA or rofecoxib.

MATERIAL AND METHODS

Induction of chronic gastric ulcers

Male Wistar rats weighing 180-250 g were employed in this study. The Animal Care Local Ethical Committee at the Jagiellonian University Medical College accepted all procedures performed in that study. Animals were given streptozocin (Fluka - Sigma-Aldrich Co., Poznan, Poland) in a single intraperitoneal injection at a dose of 70 mg/kg as described previously (7). Two weeks after the injection of streptozocin, when fasting blood glucose levels rose to 338 ± 22 mg/dl indicating diabetes, the gastric ulcers were produced using our modified acetic acid method originally proposed by Okabe et al. (26). Briefly, animals were anesthetized with ether, the stomach was exposed and a round plastic mold (6 mm in diameter) was placed tightly on the anterior serosal surface of the stomach at the antro-oxyntic border and 75 µl of 100% acetic acid was poured into the mold and allowed to remain on the gastric wall for 25 sec. Our previous studies documented that these ulcers became chronic within 2-3 days and healed completely within 2-3 weeks (27). After the application of acetic ulcers, the animals were allowed to recover from anesthesia and received only water at the day of operation.

Following treatment groups were used: 1) vehicle-treated (control) rats with gastric ulcer without or with ASA (30 mg/kg-d i.g.) or rofecoxib (5 mg/kg-d i.g.), 2) diabetic rats with gastric ulcers without or with ASA (30 mg/kg-d i.g.) or rofecoxib (5 mg/kg-d i.g.), 3) vehicle-treated (control) rats with gastric ulcer without or with NO-ASA applied in the equimolar dose to ASA (50 mg/kg-d i.g.), 4) diabetic rats with gastric ulcers without or with ASA (30 mg/kg-d i.g.) combined
with NO donor, SNAP (5 mg/kg i.g.), 5) diabetic rats with gastric ulcers without or with NO-ASA (50 mg/kg-d i.g.), 6) non-diabetic rats with gastric ulcer treated daily with insulin (4 IU/day i.p.); 7) diabetic rats with gastric ulcers treated daily with insulin (4 IU/day i.p.). At day 10 after ulcer induction, the rats were sacrificed and the area of gastric ulcers was measured by planimetry as described previously (27). The NO-ASA (NCX 4016; 2-(Acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester) was kindly provided by Mrs. Nathalie Baudry from NicOx (S.A., Sophia Antipolis, France).

Measurement of gastric blood flow and determination of luminal NO content

Gastric blood flow (GBF) was measured using a hydrogen (H₂) gas clearance technique as described previously (28). For measurement of GBF, rats were lightly anesthetized with ether, the abdomen was opened and the stomach was exposed to measure the blood flow at the ulcer margin. The blood flow was expressed as the percentage of the basal flow recorded in the gastric mucosa of control rats with saline applied to the serosa through the plastic mold.

The luminal concentration of NO was quantified indirectly as nitrate (NO₃⁻) and nitrite (NO₂⁻) levels in the gastric contents using the nitrate/nitrite kit purchased from Cayman Lab, Michigan, USA (29, 30). This method is based on the Griess reaction and generation of chromophore absorbing at 595 nm, according to the original procedure described previously (29, 30). For this purpose, the gastric content was aspirated just before the removal of the stomach following the i.g. injection of 1 ml of saline to wash out the luminal content. After centrifugation for 10 min at 3000 rpm, the samples were mixed with Griess reagent from the commercially available kit.

Determination of plasma IL-1β, TNF-α and IL-10 levels and the generation of PGE₂ in the gastric mucosa

Immediately after GBF measurement, a venous blood sample was withdrawn from vena cava into EDTA-containing vials and used for determination of plasma TNF-α, IL-1β and IL-10 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-10, washed three times with assay buffer and finally conjugated with streptavidin peroxidase to form a complex with a stabilized chromogen as described in our previous study (25).

The samples of the oxyntic gland area were taken by biopsy (about 100 mg) immediately after the animals had been killed to determine the mucosal generation of PGE₂ by specific radioimmunoassay (RIA) as described previously (31). Briefly, the mucosal sample was placed in preweighed Eppendorf vials, and 1 ml of Tris buffer (50 mM, pH 9.6) was added to each vial. The samples were finally minced (about 15 sec) with scissors, then washed and centrifuged for 10 sec, the pellet being resuspended again in 1 ml of Tris. Then each sample was incubated on a Vortex mixer (Unipan, Warsaw, Poland) for 1 min and centrifuged for 15 sec. The pellet was weighed, and the supernatant was transferred to a second Eppendorf vial containing indomethacin (10 mM) and kept at -20°C until RIA. PGE₂ was measured in duplicate using RIA kits (New England Nuclear, Munich, Germany). The capability of the mucosa to generate PGE₂ was expressed in nanograms per gram of wet tissue weight.

Statistical analysis

Results are expressed as means ± SEM. Statistical comparison was performed with Student's t-test. Comparison involving more than two groups was performed by ANOVA. Differences with p-value <0.05 were considered significant.
RESULTS

Effect of daily treatment of ASA and NO-ASA on the healing rate of gastric ulcers and the accompanying changes in the GBF at ulcer margin and luminal release of NO

Fig. 1 shows the effects of the STZ-induced diabetes with respect to gastric ulcer area and gastric mucosal blood flow at ulcer margin recorded without or with the treatment with ASA or NO-ASA at day 10 after inducing ulcers in these animals. Diabetic conditions with a fasting blood glucose level of 338 ± 22 mg/dl produced a significant increase in the area of acetic acid ulcers and this effect was accompanied by a significant decrease in the GBF at ulcer margin. Treatment with ASA (30 mg/kg-d i.g.) further significantly increased the area of gastric ulcers in rats with or without diabetes and decreased the GBF at ulcer margin as compared to those in vehicle-treated non-diabetic and diabetic rats. In contrast,

Fig 1. Effect of the treatment with vehicle (saline), aspirin (ASA, 30 mg/kg-d i.g.) and NO-ASA (50 mg/kg-d i.g.) on the area of gastric ulcer induced by acetic acid at day 10 upon ulcer induction and the accompanying changes in the gastric mucosal blood flow (GBF) in rats with and without streptozocin (STZ, 70 mg/kg i.p.)-induced diabetes mellitus. Mean ± SEM from 6-8 rats per group. Asterisk means a significant change from the corresponding value in vehicle-treated non-diabetic rats. Cross denotes a statistically significant difference from the corresponding values in diabetic rats without ASA administration. Double cross indicates a significant change as compared with the corresponding values in diabetic rats treated with ASA.
daily treatment with NO-ASA (50 mg/kg-d i.g.) failed to enhance the area of gastric ulcers and did not significantly influence the GBF at ulcer margin as compared to the values recorded in vehicle-treated non-diabetic rats.

As shown in Fig. 2, the luminal release of NO was significantly reduced in vehicle-treated rats with the diabetes along with the marked delay in ulcer healing and a significant fall in the GBF at ulcer margin as compared to those not subjected to experimental diabetes. Treatment with ASA in diabetic rats, which markedly delayed ulcer healing and significantly decreased the GBF at ulcer margin, produced a further significant decrease in the luminal NO content as compared to that attained in diabetic rats treated with vehicle. In contrast, treatment with NO-ASA, which failed to delay the ulcer healing and also reversed the fall in the GBF at ulcer margin caused by native ASA, raised the luminal NO

![Figure 2](https://example.com/fig2.png)

Fig. 2. Effect of the treatment with vehicle (saline), aspirin (ASA, 30 mg/kg-d i.g.), NO-ASA (50 mg/kg-d i.g.) and ASA combined with S-nitroso-N-acetyl-D-L-penicillamine (SNAP; 5 mg/kg-d i.g.), a NO donor, on the area of gastric ulcer induced by acetic acid at day 10 upon ulcer induction and the accompanying changes in the gastric mucosal blood flow (GBF) and luminal NO concentration in rats with diabetes induced by single injection of streptozocin (STZ, 70 mg/kg i.p.). Mean ± SEM from 6-8 rats per group. Asterisk means a significant change from the corresponding value in vehicle-treated non-diabetic rats. Cross denotes a statistically significant difference from the corresponding values in diabetic rats without ASA administration. Double cross indicates a significant change as compared with the corresponding values in diabetic rats treated with vehicle or ASA. Double asterisk indicates a significant change as compared with the corresponding values in diabetic rats treated with ASA.
content by about 5 folds as compared to that measured in ASA-treated diabetic rats. Concurrent treatment with SNAP (5 mg/kg-d i.g.) added to ASA abolished almost completely the prolongation of ulcer healing and the fall in the GBF at ulcer margin produced by native ASA in rats with experimental diabetes.

**Effect of daily treatment with rofecoxib, the selective COX-2 inhibitor, on the ulcer healing and the accompanying changes in the GBF at ulcer margin and the generation of PGE\(_2\) in the gastric mucosa**

*Fig. 3* shows the effect of daily treatment with rofecoxib (5 mg/kg-d i.g.) on the mean area of gastric ulcer and the accompanying changes in the GBF at ulcer margin and the mucosal generation of PGE\(_2\) in the gastric mucosa. Diabetes resulted in a similar delay in ulcer healing and the fall in the GBF at ulcer margin as presented in *Fig. 2*. The PGE\(_2\) generation in intact non-ulcerated gastric mucosa of the vehicle-treated rats at day 10 upon ulcer induction averaged 65±7 ng/g wet tissue weight and this was significantly increased in the ulcerated gastric mucosa excised from the margin of gastric ulcer (98 ± 6 ng/g wet tissue weight).

![Fig. 3](image_url)

*Fig. 3. Effect of the treatment with vehicle (saline) and rofecoxib (5 mg/kg-d i.g.) on the area of gastric ulcer induced by acetic acid at day 10 upon ulcer induction and the accompanying changes in the gastric mucosal blood flow (GBF) and the generation of PGE\(_2\) in the gastric mucosa in rats with diabetes induced by single injection of streptozocin (STZ, 70 mg/kg i.p.). Mean ± SEM from 6-8 rats per group. Asterisk means a significant change from the corresponding value in vehicle-treated non-diabetic rats. Cross denotes a statistically significant difference from the corresponding values in diabetic rats without rofecoxib administration.*
ASA or NO-ASA inhibited mucosal PGE\(_2\) generation by about 85% in the intact non-ulcerated gastric mucosa with the extent similar to that measured in ulcerated gastric mucosa (data not shown).

The generation of PGE\(_2\) in the gastric mucosa of diabetic rats was significantly decreased as compared to those without diabetes (Fig. 3). Treatment with rofecoxib in non-diabetic rats, which by itself suppressed the PGE\(_2\) generation in the ulcerated gastric mucosa, significantly increased the area of gastric ulcers while producing a significant fall in the GBF at ulcer margin as compared to the respective values measured in vehicle-control gastric mucosa. Such treatment with rofecoxib in rats with diabetes, which also suppressed significantly the mucosal generation of PGE\(_2\), resulted in a marked delay in ulcer healing and a further significant fall in the GBF at ulcer margin as compared to the respective values recorded in diabetic rats without rofecoxib administration. In animals with diabetes, rofecoxib tended to decrease the luminal NO concentration as compared to the value obtained in vehicle control animals but this decrease failed to reach statistical significance (2.9±0.5 µM/L in vehicle-treated vs 2.1±0.4 µM/L in rofecoxib-treated animals).

Effect of daily treatment with insulin with or without the combination with L-NNA on the healing rate of gastric ulcers and the accompanying changes in the GBF at ulcer margin

Daily administration of insulin in the non-diabetic rats, reduced the blood glucose concentration from 78 ± 9 mg/dl in vehicle-control without insulin to the value of 66 ± 7 mg/dl, but it failed to significantly influence, either the area of gastric ulcer and the associated alterations in the GBF at ulcer margin. Daily injections of insulin (4 IU/rat i.p.) to the diabetic rats, while reducing blood glucose from 338 ± 22 mg/dl to 186 ± 14 mg/dl, counteracted in part, the delay in ulcer healing under diabetic conditions and increased significantly the GBF as compared to those without the concomitant treatment with insulin (Fig. 4). Insulin treatment in diabetic rats, produced a significant rise in the GBF at ulcer margin, which reached similar values to that achieved in vehicle-treated animals without diabetes. L-NNA, which by itself prolonged ulcer healing and significantly decreased the GBF at ulcer margin, reversed almost completely the insulin-induced acceleration of ulcer healing and the accompanying rise in the GBF at ulcer margin in diabetic animals (Fig. 4).

Effect of daily treatment with native ASA, NO-ASA and rofecoxib on the plasma IL-1β, TNF-α and IL-10 levels in rats with or without diabetes

As shown in Fig. 5, the plasma IL-1β, TNF-α and IL-10 levels reached significantly higher values at day 10 upon ulcer induction in non-diabetic rats treated with ASA and rofecoxib as compared to those recorded in vehicle-treated control rats. The plasma levels of IL-1β, TNF-α and IL-10 were significantly
decreased in non-diabetic rats treated with NO-ASA. Diabetes resulted in a significant increase in the plasma IL-1β, TNF-α and IL-10 levels as compared to non-diabetic animals treated with vehicle. Treatment with ASA and rofecoxib in diabetic animals produced a significant enhancement in the plasma IL-1β, TNF-α and IL-10 levels over those recorded in ASA- and rofecoxib-treated animals without diabetes. In contrast, NO-ASA attenuated significantly the rise in the plasma IL-1β, TNF-α and IL-10 levels as compared to the respective values recorded in ASA- and rofecoxib-treated rats subjected to diabetes.

**DISCUSSION**

This study shows that diabetes delays ulcer healing due to the significant reduction in the gastric microcirculation around the ulcer possibly involving the
impairment in the mucosal PGE$_2$ generation and NO-release into the gastric lumen as well as the increased release of proinflammatory cytokines such as TNF-α and IL-1β. Classic NSAID such as ASA and the selective COX-2 inhibitor, rofecoxib, that suppressed the PGE$_2$ generation in the ulcerated gastric mucosa, and enhanced the plasma levels of IL-1β and TNF-α, caused further impairment of ulcer healing and accompanying fall in the GBF in diabetic rats. In contrast to the effects of classic NSAID and COX-2 inhibitor, the treatment with NO-ASA raised luminal NO content and enhanced significantly the GBF at ulcer margin, failing to augment the ulcer healing. These effects could be attributed to the suppressive effect of this NO-derivative of ASA on the proinflammatory cytokines, especially IL-1β and TNF-α activity in the ulcer area. The importance of NO in the mechanism of ulcer healing activity of NO-ASA under the diabetic conditions is supported by the observation that the co-treatment with NO-donor, SNAP, added to classic NSAID such as ASA counteracted the deleterious effect of native ASA on ulcer healing and the accompanying fall in the gastric

Fig. 5. Effect of the treatment with vehicle (saline), aspirin (ASA, 30 mg/kg-d i.g.), rofecoxib (5 mg/kg-d i.g.) and NO-ASA (50 mg/kg-d i.g.) on the plasma IL-1β, TNF-α and IL-10 levels in diabetic rats with gastric ulcer induced by acetic acid at day 10 upon ulcer induction. Mean ± SEM from 6-8 rats per group. Asterisk means a significant change from the corresponding value in vehicle-treated non-diabetic rats. Cross denotes a statistically significant difference from the corresponding values in non-diabetic rats.
microcirculation at ulcer margin. Finally, the treatment with insulin reversed the impaired ulcer healing in diabetic animals, mainly due to the normalization of hyperglycemia and an enhancement in the GBF at ulcer margin. This beneficial effect of insulin could be mediated by NO because the insulin-induced acceleration of ulcer healing under diabetic conditions was significantly attenuated by the concurrent treatment with L-NNA, the potent NO-synthase inhibitor.

An accepted model of insulin-dependent diabetes mellitus has been previously studied by inducing diabetes in rats via injection of streptozocin. This model demonstrates an increased vulnerability of the gastric mucosa against various ulcerogens such as ischemia-reperfusion injury, stress, and non-steroidal anti-inflammatory drugs (2 - 4, 32). As shown in previous studies, the NSAID exhibit potent anti-inflammatory and analgesic actions but the use of these drugs in humans is limited due to untoward effects such as formation of gastric mucosal bleeding erosions and ulcerations, potentiation of ulcerogenic response to various noxious stimuli and impairment of healing of preexisting ulcers (13 - 16, 32). This deleterious action of conventional NSAID especially of ASA, was attributed to their topical irritating effect, activation of neutrophils, fall in the microcirculation, enhancement in gastric motility and the reduction in mucosal generation of PGE$_2$ (13 - 16). In contrast, newly developed NO-releasing NSAID, distributed by NicOx Company (Sophia Antipolis, France), that is produced by adding an nitroxy-butyl moiety to ASA, exhibit lower gastrointestinal toxicity despite inhibiting both COX-1 and COX-2 activity in the gastric mucosa and exerting anti-thrombotic effects comparable to their parent NSAID (33 - 35).

The relationship between diabetes mellitus, the NSAID therapy including the new NO-releasing derivatives of NSAID and the healing of preexisting gastric ulcerations has not been established. Our present study confirmed previous reports that STZ-induced diabetes impairs healing process of gastric ulcer (6, 36) and shows for the first time, that this delay in ulcer healing in diabetic rats can be markedly attenuated by NO-ASA which also is capable of counteracting the significant reduction in the GBF at ulcer margin in these animals. This latter observation seems to be of importance, since the gastric microcirculation, especially at the area of the ulcer margin, plays an important role in the healing process by supplying oxygen and nutrients and by removing toxic substances for the ulcer area most probably reactive oxygen species (37).

The major finding of our present study is the observation that NO-ASA, the novel ASA derivative, which by itself failed to produce macroscopic mucosal damage despite suppressing of PGE$_2$ generation to the same extent as their native ASA, exhibits the beneficial effect on ulcer healing under diabetic conditions. In contrast to NO-ASA, topical application ASA at the same molar dose as NO-ASA, markedly prolonged ulcer healing in diabetes and this effect was accompanied by the significant fall in the GBF at ulcer margin. Moreover, the luminal NO content which was significantly lower in diabetes, significantly
declined in ASA-treated diabetic animals suggesting that ASA-induced fall in NO released into gastric lumen may be important event in NSAID gastropathy. The fall in NOx content observed in the gastric lumen of diabetic rats is in keeping with the recent studies by Pinheiro and Calixto (38) and by De La Cruz et al (39) who demonstrated that serum concentration of NOx is markedly decreased under the diabetic conditions induced by STZ. The mechanism of NO inhibition in diabetes remains unclear but it was proposed that sustained high blood glucose levels in diabetes stimulates oxidative stress mechanisms while decreasing NO production, thus contributing to the vascular dysfunction observed in diabetes mellitus (39). In another study, STZ-induced diabetes was accompanied by the depletion of nNOS content from nitrergic nerve fibers and this effect was reversible by insulin treatment (40). The importance of NO that was decreased in diabetic animals is further emphasized by the fact that the acceleration of ulcer healing and hyperemia at ulcer margin in diabetic animals treated with NO-ASA were mimicked in our present study, by the addition of NO-donor, SNAP (41) to native ASA. One possibility to explain the apparent fall in the luminal NO concentration induced by ASA in these animals could be that ASA suppressed the PGE\textsubscript{2} generation and this resulted in a concomitant decrease in NO released into gastric lumen. It was proposed that the protection of pancreas involves the activation of NO system which may exert stimulatory effect on PGE\textsubscript{2} generation indicating that the NO/PG system participates to the protective response in the pancreas (42). In another study, the suppression of COX activity in diabetic animals with indomethacin, another potent NSAID, significantly decreased NO concentration in carrageenin-treated rats (38). It is of interest, however, that in contrast to aspirin, rofecoxib failed to influence significantly the luminal NOx content in our diabetic rats suggesting that classic NSAID such as ASA can differ from selective COX-2 inhibitors (rofecoxib) with respect to their influence of NO release following diabetic conditions. An attempt has been made in this study to determine the role of COX-2-PG system in the mechanism of ulcer healing in diabetic animals by 2 ways; 1) by direct measurement of PGE\textsubscript{2} in the gastric mucosa and 2) by using pharmacological intervention such as administration of highly selective COX-2 inhibitor, rofecoxib to diabetic animals in order to compare its effect on ulcer healing with that exhibited by ASA, a non-selective COX-1 and COX-2 inhibitor. Previous studies revealed that the selective COX-2 inhibitors such as NS-398 and L-745,337 by themselves failed to cause gastric ulcerations and to inhibit PG synthesis (43). In agreement with recent observations (25, 44), we have shown that COX-2 inhibitor, rofecoxib, similarly to aspirin, suppressed PGE\textsubscript{2} at the site of the pre-existing ulcers. Furthermore, we found that diabetic animals by themselves exhibited a decrease in mucosal generation of PGE\textsubscript{2} at the ulcer margin and that rofecoxib further suppressed this generation in diabetic animals leading to a marked delay in healing observed predominantly in diabetic animals treated with non-selective and selective COX-2 inhibitor. Our finding that PGE\textsubscript{2}
generation is decreased in diabetes remains in agreement with previous reports that vascular production of prostacyclin is impaired in diabetic animals and that the production of PGE$_2$ and 6-keto PGF$_{1\alpha}$ is markedly decreased (by about 50%) at 2 and 4 weeks after the injection of STZ (39). The mechanism of STZ-induced inhibition of prostanoids requires further studies but it could be attributed to events associated with diabetes such as hyperglycemia (39,40), enhanced generation of reactive oxygen metabolites and/or impairment of the gastric microcirculation (45, 46). Paradoxically, COX-2 specific inhibitors that are widely proposed as an attractive therapeutic development in the treatment of rheumatoid arthritis, osteoarthritis or cardiovascular diseases, partly because they were shown to spare the COX-1 isoform responsible for gastrointestinal protection and failed to cause peptic ulcers or bleeding, appear in our hands to exert much less favorable effects on healing of pre-existing ulcers than simple NO-ASA, blocking both COX-1 and COX-2. Thus, our study demonstrates that PG derived from activity of both, COX-1 and COX-2 enzymes may contribute to the healing of chronic gastric ulcers, and the use of any inhibitor of COX activity, besides NO-NSAID, should be avoided when ulcer healing in diabetes is considered.

Since both ASA and rofecoxib inhibited the mucosal generation of endogenous PGE$_2$ while increasing the release of IL-1$\beta$ and TNF-$\alpha$, our study implies that endogenous PG could act via negative feedback mechanism to regulate the production and/or release of these proinflammatory cytokines. A failure in ulcer healing in rats with STZ-induced diabetes could be attributed to the hyperglycemia and increased production of proinflammatory cytokines such as IL-1$\beta$ and TNF-$\alpha$ being a result of sustained inflammatory reaction and the delaying healing process at the ulcer area. Indeed, the treatment with insulin reversed the impaired ulcer healing in diabetic animals, mainly due to the normalization of hyperglycemia, but not to the direct effect of insulin on the ulcer healing because insulin administered to non-diabetic rats failed to influence this healing. Moreover, the improvement of ulcer healing in diabetic rats by insulin appears to involve the NO pathway because co-administration of insulin with L-NNA, an NO-synthase inhibitor, reversed the effect of this hormone on ulcer healing and hyperemia at ulcer margin. This notion is supported by the earlier observations that hyperglycemia impaired the regulation by NO of the gastric hyperemia in diabetic animals (47, 48) and that insulin may restore the NOS expression and the activity that is lost in diabetic gastroparesis (49).

Besides many biological activities, both IL-1$\beta$ and TNF-$\alpha$ are involved in the induction of inflammation, injury and carcinogenesis in a variety of tissues including the gastric mucosa (50 -52). Moreover, these cytokines were recently implicated in the mechanism ischemia-reperfusion injury progressing into gastric ulcer (53), stress-induced gastric lesions (54) and to mediate the delay in ulcer healing induced by Helicobacter pylori and its water extract (55). We found that the release of these cytokines was remarkably enhanced in diabetic animals
treated with ASA and rofecoxib along with the marked prolongation of the ulcer healing and a potent fall in the GBF at the ulcer margin recorded in these animals. This is in keeping with the original observation by Takahashi et al (56) and Shigeta et al (57), who postulated that endogenous IL-1β may trigger expression of COX-2 and growth factors such as fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF). We confirmed others (56) and our own (25) findings that plasma IL-1β and TNF-α levels is increased in rats with gastric ulcer and we found that this increase is even more pronounced in those with diabetes. Furthermore, we demonstrated that release of these cytokines was remarkably enhanced in ASA-treated diabetic animals along with the marked prolongation of the ulcer healing and a potent fall in the GBF at ulcer margin recorded in these animals. Therefore, the possibility can not be excluded that proinflammatory cytokines could contribute to the upregulation of genes for COX-2 as documented in our previous study using non-diabetic rats (25). On the other hand, the major mechanism by which cytokines exert their deleterious influence on ulcer healing may involve the inhibition of growth factors responsible for the mucosal recovery from the damage repair and mucosal regeneration (58). In contrast to proinflammatory cytokines IL-1β and TNF-α, the IL-10 is considered to act as anti-inflammatory cytokine (60). It is of interest that the plasma IL-10 levels was also significantly increased in diabetic animals and even further elevated in those treated with ASA and rofecoxib indicating that this cytokine was trigger in similar fashion as proinflammatory cytokines IL-1β and TNF-α in non-diabetic and diabetic rats treated with ASA or COX-2 inhibitor. Moreover, the plasma IL-10 was significantly diminished in NO-ASA treated animals, possible reflecting attenuation of inflammation at ulcer margin by this NO-derivative of ASA. Since the GBF was elevated and luminal NO production was enhanced while plasma levels of IL-1β, TNF-α and IL-10 were suppressed in animals treated with NO-ASA as compared to those treated with native ASA or rofecoxib, it is likely that beneficial effect of NO-ASA in ulcer healing is not only due to excessive release of NO that may compensate for PG deficiency caused by native NSAID but also depends upon the suppression of the release of proinflammatory cytokines such as IL-1β and TNF-α, caused by ASA (45,46). Recently we have documented that suppression of TNF-α by pentoxyfilline exerted gastroprotection against stress-induced gastric lesions (60). The involvement of TNF-α in the NSAID-induced gastropathy is in keeping with recent observation by Fiorucci and coworkers (61) that gastric mucosal injury induced by oral NSAID administration is linked to TNF-α-dependent activation of ICE-like cysteine proteases and that NO-ASA is capable of protecting the gastric mucosa via inhibiting these key endopeptidases in the caspase cascade. These authors (61) have suggested that activation of ICE/caspase-1 pathway by native ASA is a limiting step in the process of maturation and secretion of proinflammatory cytokines such as IL-1β and that modulation by NO-NSAID of ICE-1 like cysteine endopeptidase could be explanatory for the beneficial action of NO-derivatives of NSAID. In contrast to
ASA, NO-ASA caused the S-nitrosilation/inhibition of caspase-1, resulting in an inhibition of the proinflammatory cytokine such as TNF-α, IL-1β, IL-8, IL-12 and IFN-γ release from endotoxin (LPS)-challenged monocytes due to the formation of NO (61).

In summary, we demonstrated that diabetic rats exhibited an impaired ulcer healing, process which was associated with the attenuation of GBF at ulcer margin and an increased release of proinflammatory (IL-1β and TNF-α), and anti-inflammatory cytokine (IL-10). This delay in ulcer healing in diabetic rats was markedly augmented by native ASA and rofecoxib supporting the notion that both COX-1 and COX-2 products are involved in the healing of preexisting ulcers under diabetic conditions. Furthermore the prolongation of ulcer healing and the fall in GBF at ulcer margin in diabetic animals were counteracted by insulin, possibly activating the NO-dependent pathway and by treatment with NO-releasing ASA that enhanced the GBF at ulcer margin and suppressed the activity of proinflammatory cytokines.

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