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Impaired gastric ulcer healing in diabetic rats: role of heat shock protein, growth factors, prostaglandins and proinflammatory cytokines

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Abstract

Gastric mucosa of diabetic rats is highly vulnerable to acute injury, but little is known about the influence of diabetic conditions on the healing of gastric ulcers. In this study, streptozotocin (70 mg/kg injected intraperitoneally) was used to induce diabetes mellitus in rats. Four weeks after streptozotocin injection, gastric ulcers were induced using the acetic acid method, and 10 days later, the healing rate and the gastric blood flow (GBF) were measured by planimetry and hydrogen (H_2)-gas clearance method, respectively. Six major groups of rats with gastric ulcers were used: (1) vehicle (saline); (2) streptozotocin alone; (3) insulin (4 IU/day intraperitoneally); (4) streptozotocin plus insulin; (5) pentoxifylline, an inhibitor of synthesis and release of tumor necrosis factor-α (TNFα); and (6) aspirin, a non-selective inhibitor of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), and rofecoxib, the highly selective COX-2. In the diabetic rats, a significant delay in ulcer healing (~ by 300%), accompanied by a decrease in the gastric mucosal blood flow was observed. The prolongation of the healing in diabetic animals was associated with an increase in gastric mucosal expression and release of TNFα, interleukin-1β (IL-1β), suppression of the vascular endothelial growth factor (VEGF), platelet endothelial cell adhesion molecule-1 (PECAM-1) and the mucosal overexpression of heat shock protein 70 (HSP 70). Administration of insulin reversed the delay in ulcer healing and significantly decreased the expression of IL-1β and TNF-α, while producing the rise in the expression of VEGF and PECAM. Pentoxifylline, an inhibitor of TNF-α, which by itself accelerated ulcer healing in non-diabetic rats, counteracted the increase in the area of gastric ulcer induced by streptozotocin, raised significantly gastric blood flow and suppressed the plasma TNF-α levels. Aspirin and rofecoxib, that significantly suppressed the mucosal prostaglandin E_2 generation in ulcer area, delayed significantly the rate of ulcer healing and decreased the GBF at ulcer margin in non-diabetic rats, and these changes were significantly augmented in diabetic animals. We conclude that: (1) Experimental diabetes dramatically impairs ulcer healing, depending upon the increased release of proinflammatory cytokines and the attenuation of angiogenesis that can limit the ulcer healing effects of locally produced HSP 70 and TNF-α. (2) Insulin reversed this impairment of ulcer healing in diabetic rats, mainly due to the enhancement of angiogenesis and reduction in expression of cytokines in the ulcer area. (3) Classic non-steroidal anti-inflammatory drugs such as aspirin prolonged ulcer healing under diabetic conditions due to suppression of endogenous prostaglandins and the fall in the microcirculation at the ulcer margin and these effects were mimicked by selective, so called ‘‘safe’’ COX-2 inhibitor, rofecoxib, suggesting that both COX isoforms are important sources of prostaglandins that are essential in the ulcer healing in diabetes.

Keywords: Streptozotocin; Diabetes mellitus; Cytokines; Heat shock protein; Growth factor; Ulcer

1. Introduction

Diabetes mellitus is a metabolic disease affecting a large number of people of all ages, races and socio-economic classes throughout the world. It is caused by either a relative or absolute insulin deficiency with possible impaired tissue responsiveness to insulin, which affects numerous organ systems in the body. Patients with long-standing diabetes

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mellitus, may develop autonomic neuropathy and demonstrate a variety of gastrointestinal symptoms such as functional dyspepsia, abdominal pain, vomiting, diarrhea, constipation and delayed gastric emptying (Bytzer et al., 2002; O'Reilly and Long, 1987). However, little attention has been paid to peptic ulcer in diabetics because the incidence of ulcers among diabetics is paradoxically low (Masuda et al., 1976) in view of increased prevalence of *Helicobacter pylori* infection in these patients (Quatrini et al., 2001). Recent studies indicate, however, that peptic ulcers related to the diabetic state are more severe and often associated with complications such as gastrointestinal bleeding (Pietzsch et al., 2002).

An accepted model of insulin-dependent diabetes mellitus has been previously studied by inducing diabetes in rats via injection of streptozotocin. This model went to demonstrate an increased vulnerability of the gastric mucosa against various ulcerogens such as ischemia–reperfusion injury, stress and non-steroidal anti-inflammatory drugs (Korolkiewicz et al., 1999; Tashima et al., 2000a,b). The mechanisms that underlie this increased susceptibility of multifactor damage under diabetic conditions included the impairment of the antioxidative system (Goldin et al., 1997) and suppression of basic fibroblast growth factor production in the gastric mucosa (Takeuchi et al., 1997), as well as impaired duodenal HCO_3^- secretion (Takehara et al., 1997), attenuation of angiogenic response and dysfunction of capsaicin-sensitive afferent neurons involved in the protection of the gastric mucosa (Tashima et al., 1998). Pentoxifylline, a methylxanthine derivative has been widely used for the treatment of peripheral and cerebro-vascular diseases (Samlaska and Winfield, 1994). This compound was recently implicated in the mechanism of gastric mucosal integrity and ulcer healing by the demonstration that it prevented the indomethacin-induced gastric mucosal damage (Santucci et al., 1994) and accelerated the healing of preexisting gastric ulcers (Shimizu et al., 2000). These gastroprotective and ulcer healing actions of pentoxifylline were attributed, in part, to the reduction of neutrophil infiltration and the attenuation of inflammatory response mainly due to the suppression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) by this agent (Santucci et al., 1994; Shimizu et al., 2000). Recently, an increased COX-2 expression was shown in the gastric mucosa exposed to ischemia–reperfusion (Brzozowski et al., 1999a,b) and at the ulcer edge during healing of experimental gastric ulcers (Shigeta et al., 1998, Takahashi et al., 1998). However, little attempts have been made to determine the relative involvement of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) products in gastric ulcerogenesis and the effects of various COX-2 inhibitors on the rate of ulcer healing under diabetic conditions, though Wallace et al. (2000) suggested that the inhibition of both COX-1 and COX-2 is required for the formation of gastric lesions by non-steroidal anti-inflammatory agents.

This study was designed to analyze the healing of gastric ulcers, the gastric blood flow (GBF) at ulcer margin and changes in the mucosal expression of heat shock protein 70 (HSP 70), proinflammatory cytokines (interleukin-1β [IL-1β], TNF-α), growth factors (TGF-α) and angiogenesis (platelet endothelial cell adhesion molecule-1 [PECAM-1]) in rats with experimental diabetes with and without treatment with insulin. An attempt was made to determine the effect of treatment with pentoxifylline and COX inhibitors on the ulcer healing, prostaglandin E_2 generation in the gastric mucosa, plasma of IL-1β and TNF-α levels and gastric blood flow at ulcer margin in rats with or without diabetes induced by streptozotocin.

2. Materials and methods

2.1. Induction of chronic gastric ulcers

Male Wistar rats (180–250 g) were used. The Animal Care Local Ethical Committees at the Jagiellonian and Erlangen-Nuremberg Universities accepted all procedures performed in that study. Animals were given streptozotocin (Fluka-Sigma-Aldrich Poznan, Poland) in a single intraperitoneal injection at a dose of 70 mg/kg as described previously (Tashima et al., 1998). Two weeks after the injection of streptozotocin, when fasting blood glucose levels rose to 332 ± 25 mg/dl indicating diabetes, the gastric ulcers were produced using our modified acetic acid method originally proposed by Okabe et al. (1971). Briefly, the animals were anesthetized with ether, and their stomachs were exposed with a round plastic mold (6 mm in diameter), which was placed tightly on the anterior serosal surface of the stomach at the antro-oxyntic border. The amount of 75 μl of 100% acetic acid was poured in the mold and allowed to remain on the gastric wall for 25 s. This produced immediate necrosis of the entire mucosa and submucosa (but not serosa) within the area where the acetic acid was applied (approximately 28 mm^2). The excess of acetic acid was then removed via cotton swab and then the serosa was gently washed with saline. Our previous studies have documented that these ulcers became chronic within 2–3 days and healed completely within 2–3 weeks (Konturek et al., 1997). After the induction of acetic ulcers, the animals were allowed to recover from anesthesia and received only water the day of the operation. Following the treatment, groups were divided as follows: (1) vehicle (saline) control; (2) streptozotocin alone (3) insulin (4 IU/day) alone; (4) streptozotocin plus insulin (4 IU/day); (5) pentoxyffiline; and (6) apirin or rofecoxib. The involvement of TNFα in the mechanism of ulcer healing was determined in additional groups of non-diabetic and diabetic rats with gastric ulcers treated with pentoxifylline (20 mg/kg day i.g.), the inhibitor of synthesis and release of this cytokine (Shimizu et al., 2000). Ten days after ulcer induction, the rats were sacrificed and the area of gastric

ulcers was measured by planimetry as previously described (Konturek et al., 1997).

The involvement of endogenous prostaglandins in ulcer healing of diabetic and non-diabetic animals was determined in several groups of rats with gastric ulcers, consisting each of six to eight animals, which received daily intragastric treatment for 10 days either with: (1) vehicle (saline); (2) aspirin (30 mg/kg day); and (3) rofecoxib (5 mg/kg day), the highly selective COX-2 inhibitor (Brzozowski et al., 2000). At the dose used in the present study, aspirin has been shown previously to inhibit gastric PGE_2 generation capability by ~90% without causing by itself any mucosal damage (Brzozowski et al., 2001). The dose of rofecoxib was selected on the basis of previous studies showing that this agent remained without significant effect on prostaglandin E_2 generation in the non-ulcerated gastric mucosa but significantly attenuated this generation in ulcerated gastric mucosa (Brzozowski et al., 2000, 2001). Rofecoxib was first dissolved in methanol to obtain the stock solution 25 mg/ml and then diluted to the desired concentration with isotonic saline. Control rats received the corresponding vehicle. Our preliminary studies (data not included) showed that none of the cyclooxygenase inhibitors used in this study produced by itself any gastric lesions at the doses tested.

2.2. Measurement of gastric blood flow

Gastric blood flow was measured using a hydrogen (H_2)-gas clearance technique as described previously (Konturek et al., 1997) for measurement of gastric blood flow, rats were lightly anesthetized with ether, their abdomens were opened, and the stomach was exposed to measure the blood flow at the ulcer margin. Double-needle electrodes were inserted through the serosa into the ulcer margin and intact oxyntic mucosa. One electrode was used for the generation of H_2 gas and the other for the measurement of tissue H_2. With this method, the H_2 generated is carried away by the blood, and the polarographic current detector gives the decreasing tissue H_2 content as the clearance curve, which is used to calculate the blood flow rate in the tissue. The blood flow was expressed as the percentage of the basal flow rate in the gastric mucosa of the control rats with the saline applied to the serosa through the plastic mold.

2.3. Determination of plasma cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)

Immediately after GBF measurement, a venous blood sample was withdrawn from the vena cava into EDTA-containing vials and used for the determination of plasma tumor necrosis factor-α and interleukin-1β by solid phase sandwich ELISA (BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions. Briefly, each sample (50 μl) was incubated with biotinylated antibodies specific for rat TNF-α and IL-1β, 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 (Konturek et al., 2002).

2.4. Determination of PGE_2 generation in the gastric mucosa

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 PGE_2 by specific radioimmunoassay (RIA) as described previously (Brzozowski et al., 1999a,b). 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 s) with scissors, then washed and centrifuged for 10 s, 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 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 RIA. PGE_2 was measured in duplicate using RIA kits (New England Nuclear, Munich, Germany). The capability of the mucosa to generate PGE_2 was expressed in nanograms per gram of wet tissue weight.

2.5. Determination of mRNA for HSP 70, vascular endothelial growth factor (VEGF), TGF-α, TNF-α and IL-1β

The stomachs were removed, and mucosal specimen scrapings were done using a glass slide and immediately snap frozen in liquid nitrogen and stored at −80 °C until analysis. Total RNA was extracted from mucosal samples by the method of Chomczynski and Sacchi (1987) using the extraction kit from Stratagene (Heidelberg, Germany). Following precipitation, RNA was resuspended in RNase-free water and its concentration was estimated by absorbance at a 260 nm wavelength. RNA samples were stored at −80 °C until analysis. Single stranded cDNA was generated from 5 μg of total cellular RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Stratagene) in accordance with the procedure described by our group previously (Konturek et al., 2002). The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) at the specifications described in Table 1. The nucleotide sequence of the primers for β-actin, HSP 70, VEGF, TGF-α, TNF-α and IL-1β (Table 1) was based on the sequences of the published cDNAs (Hunt and Morimoto, 1985; Masahiro et al., 1999; Nudel et al., 1983; Shirai et al., 1988). These primers were synthesized by GIBCO BRL/Life Technologies (Eggenstein, Germany). Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide.

Table 1
The nucleotide sequence of primers for RT-PCR employed in the study

Primer	Sequence	Annealing temperature [°C]	bp
β-actin	5′ TTG TAA CCA ACT GGG ACG ATA TGG 3′ GAT CTT GAT CTT CAT GGT GCT AGG	60	764
HSP 70	5′ -GTG AAG ATC TGC GTC TGC TTG 3′ -TTT GAC AAC AGG CTG GTG AAC C	60	590
TGF-α	5′ -ATGGTCCCCGCGGCCGGACA 3′ -GACCACTGTCTCAGAGTGGCAGCAGGCAGTCCTTCCTTT	66	476
VEGF	5′ TTGAACACCGAGCAGT 3′ GGTCCTCTGCCATTCT	56	271
IL-1β	5′ GCT ACC TAT GTC TTG CCC GT 3′ GAC CAT TGC TGT TTC CTA GG	62	543
TNF-α	5′ TAC TGA ACT TCG GGG TGA TTG GTC C 3′ CAG CCT TGT CCC TTG AAG AGA ACC	62	295

Location of the predicted products was confirmed by using a 100-bp ladder (Takara, Shiga, Japan) as a standard size marker (Konturek et al., 2001).

2.6. Western blot analysis

We investigated the protein expression of HSP 70 and PECAM-1. The expression of PECAM-1 was performed to assess the angiogenesis. Platelet endothelial cell adhesion molecule-1 is an important marker of endothelial cells and a modulator or angiogenesis in vitro and in vivo (Newman, 1997).

Shock frozen tissue from rat stomachs was homogenized in lysis buffer (100 mM Tris–HCl, pH 7.4, 15% glycerol, 2 mM EDTA, 2% sodium dodecyl sulfate [SDS], 100 mM D,L-dithiothreitol (DDT) by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at 12 000 × *g* for 15 min. Approximately 50 μg of total protein extracts was loaded on SDS–polyacrylamide gels and run 40 mA, followed by transfer on nitrocellulose membrane (Protran, Schleicher & Schuell, Germany) and by electroblotting. The concentration of 3% bovine serum albumin (Sigma Aldrich, Germany) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris–HCl, pH 7.4, 0.1% Tween-20) was used to block filters for at least 1 h at room temperature. The specific primary antibody against heat shock protein 70 (mouse monoclonal, 1:200 dilution; StressBen Biotechnologies Canada) or β-actin (mouse monoclonal, 1:5000; Sigma Aldrich, Germany) was added to the membrane. This was followed by an anti-mouse-immunoglobulin G horseradish peroxidase-conjugated secondary antibody (dilution 1: 20 000; Promega, USA) dissolved in 1% non-fat milk in TBS–Tween-20 buffer. Incubation of the primary antibody was followed by three washes with TBS–Tween-20 Buffer for 10 min. Incubation of the secondary antibody was followed by five washes for 10 min. Immunocomplexes were detected by the Super-Signal West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany). Comparison between different treatment groups was made by determining the heat shock protein/β-actin ratio of the immunoreactive area by densitometry as described elsewhere (Konturek et al., 2001).

2.7. Statistical analysis

Results are expressed as means ± S.E.M. Statistical comparison was performed with Student's *t* test. Comparison involving more than two groups was performed using ANOVA. Differences with P value < 0.05 were considered significant.

3. Results

3.1. Ulcer healing in rats with streptozotocin-induced diabetes

Fig. 1 shows the effects of the streptozotocin-induced diabetes on gastric ulcer area and gastric mucosal blood flow at ulcer margin recorded at day 10 after ulcer induction in these animals. Diabetic conditions with a fasting blood glucose level of 332 ± 25 mg/dl impaired the healing of gastric ulcers, which was associated with a significant reduction in gastric mucosal blood flow. Reducing the blood glucose level to 186 ± 14 mg/dl by daily injections of insulin (4 IU/rat/day) caused the reversal of ulcer healing in the diabetic rats to the extent observed in the vehicle-control non-diabetic rats. Insulin treatment in diabetic rats resulted in a significant increase of gastric mucosal blood flow, which reached similar values to those achieved in vehicle-treated animals without streptozotocin treatment. 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 the area of gastric ulcer and the associated alterations in the gastric mucosal blood flow.

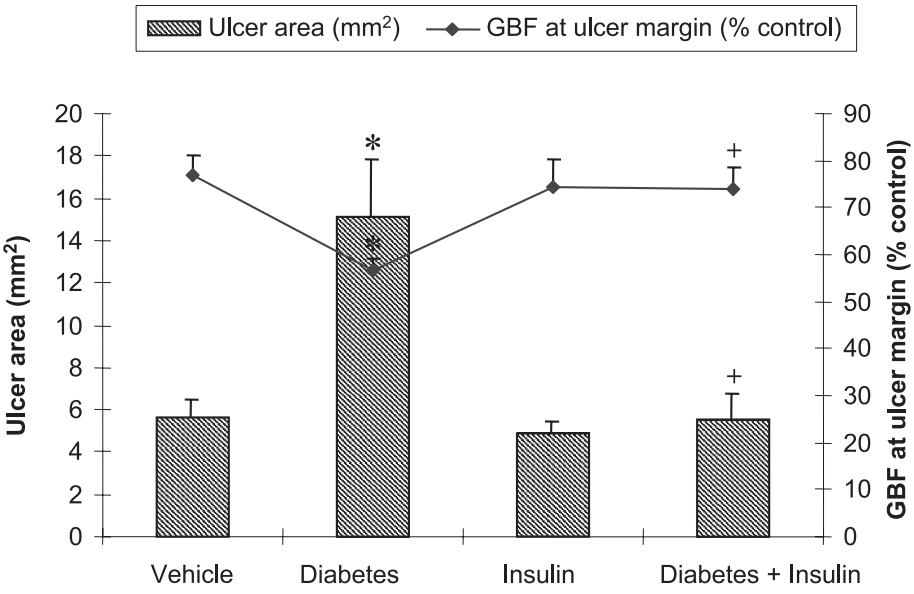

Fig. 1. Healing course and changes in the gastric mucosal blood flow in rats with and without streptozotocin-induced diabetes mellitus treated or not, with insulin (4 IU/day s.c.) or vehicle (saline). Mean ± S.E.M. from six to eight rats per group. Asterisk means a significant difference from the corresponding value in vehicle treated control rats with gastric ulcers. A cross denotes a statistically significant difference from the corresponding values in diabetic rats without insulin treatment.

3.2. Expression of heat shock protein 70 and changes in plasma level of IL-1β and TNF-α

Gene and protein expressions of heat shock protein 70 in the intact gastric mucosa were weakly detectable (Figs. 2 and 3). In contrast, a significant increase in HSP 70 mRNA and protein expressions in the ulcerated mucosa was observed in all ulcerated mucosa of vehicle-treated animals. In diabetic rats, both the mucosal mRNA and protein expressions of HSP 70 were dramatically increased. Treatment of those diabetic rats with insulin led to a significant decrease in HSP 70 expression as compared to the control level.

In rats treated with insulin, a significant increase in plasma levels of both cytokines was observed (Fig. 4). In diabetic rats, the administration of insulin resulted in a

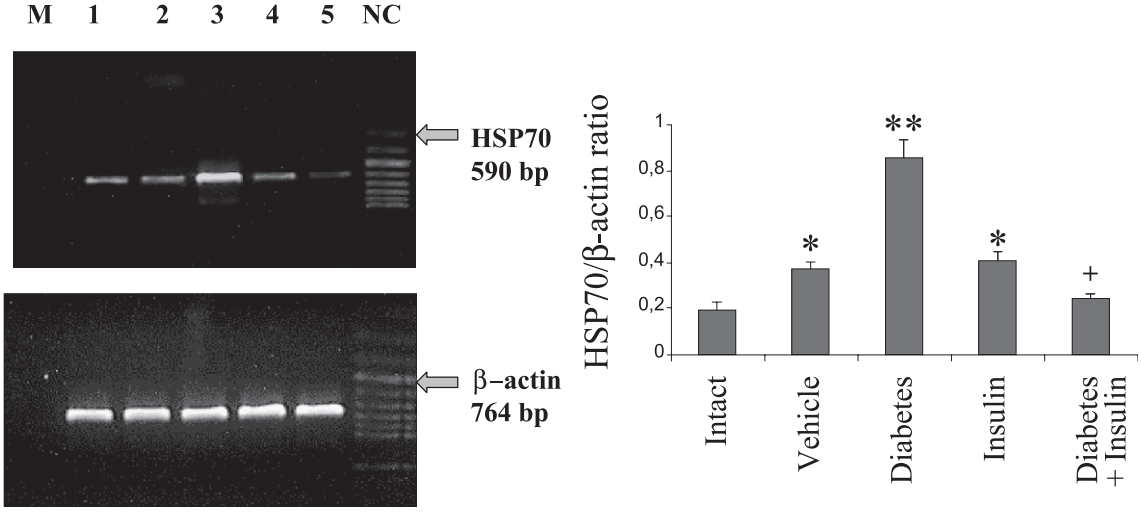

Fig. 2. Representation of RT-PCR for HSP 70 and densitometric analysis for HSP 70 in intact rats without ulcer (lane 1), non-diabetic rats with gastric ulcers (lane 2), diabetic rats with gastric ulcers without treatment with insulin (lane 3), non-diabetic rats with gastric ulcer treated with insulin (lane 4) and diabetic rats with gastric ulcers treated with insulin (lane 5). Assessment of the ratio of mRNA HSP 70/β-actin mRNA in studies is presented on the right panel. A single asterisk indicates significant change as compared to the value in intact rats. Double asterisks indicate significant rise above the value obtained in vehicle-treated control rats with gastric ulcer. A cross indicates a significant decrease as compared to the value recorded in diabetic rats without insulin treatment.

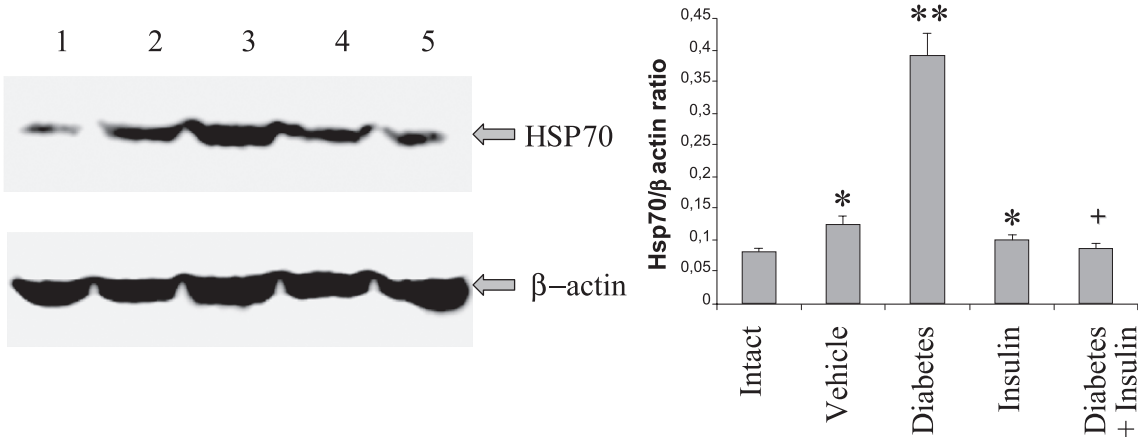

Fig. 3. Representation of Western blot for HSP 70 and densitometric analysis for HSP 70 expression in intact rats (lane 1), non-diabetic rats with gastric ulcers (lane 2), diabetic rats with gastric ulcers not treated with insulin (lane 3), non-diabetic rats with gastric ulcers treated with insulin (lane 4) and diabetic rats with gastric ulcers treated with insulin. Assessment of the ratio of mRNA HSP 70/β-actin mRNA in studies is presented on the right panel. A single asterisk indicates significant changes as compared to the value in intact rats. Double asterisks indicate a significant rise above the value obtained in vehicle-treated control rats with gastric ulcers. A cross indicates a significant decrease as compared to the value recorded in diabetic rats without insulin treatment.

significant decrease in plasma levels of both cytokines toward the values recorded in vehicle-treated rats with gastric ulcer. Insulin administered to control rats with gastric ulcer failed to affect plasma levels of either cytokines, and these results have not been included for the sake of clarity.

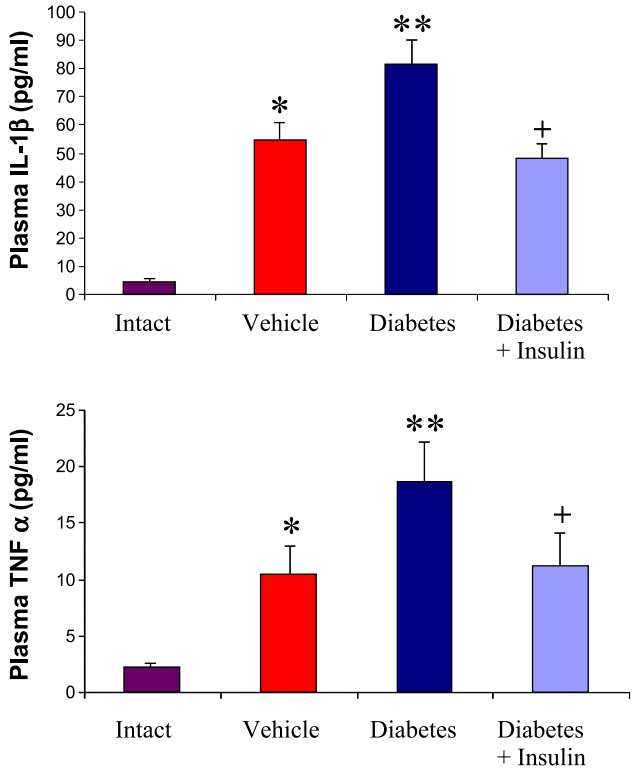

Fig. 4. Changes in plasma level of IL-1β and TNF-α in intact rats, in vehicle-treated control or diabetic rats with gastric ulcers without or with treatment with insulin. A single asterisk indicates a significant increase above the value obtained in intact rats. Double asterisks indicate a significant increase above the value recorded in vehicle-treated control rats. A cross indicates a significant decrease as compared to that obtained in diabetic rats without insulin treatment.

At the mRNA level, the expression of TNF-α and IL-1β was significantly upregulated in the ulcerated mucosa of the vehicle-treated control rats as compared to that in the intact mucosa (Fig. 5). In ulcerated diabetic rats, a further increase in IL-1β was traced, whereas the expression of TNF-α remained at an increased level that was similar to that observed in the vehicle-control animals. In non-diabetic rats treated with insulin, the mRNAs for TNF-α and IL-1β showed similar values to that recorded in vehicle-treated control animals. In diabetic rats treated with insulin, both TNF-α and IL-1β mRNA expression showed a small but significant decrease when compared to that in diabetic animals with ulcer but without insulin treatment (Fig. 5).

3.3. Expression of mRNA for TGF-α and VEGF and protein expression of PECAM-1

Expression of TGF-α and VEGF was detected as a weak signal in the intact gastric mucosa (Fig. 5). In vehicle-control rats with gastric ulcers, a significant increase in mRNA expression of both growth factors was observed. In ulcerated gastric mucosa of diabetic rats, the expression of VEGF was dramatically reduced in the gastric mucosa as compared to that traced in vehicle-treated rats with ulcer. In contrast, the expression of TGF-α remained at the increased level as in the vehicle-control group. The expression of these two growth factors remained elevated in the gastric mucosa of non-diabetic rats, while in diabetic rats treated with insulin, a significant increase in mucosal expression of VEGF mRNA was recorded. Likewise, the expression of TGF-α was not influenced by insulin and remained at the increased level comparable to that in the vehicle-treated animals (Fig. 5).

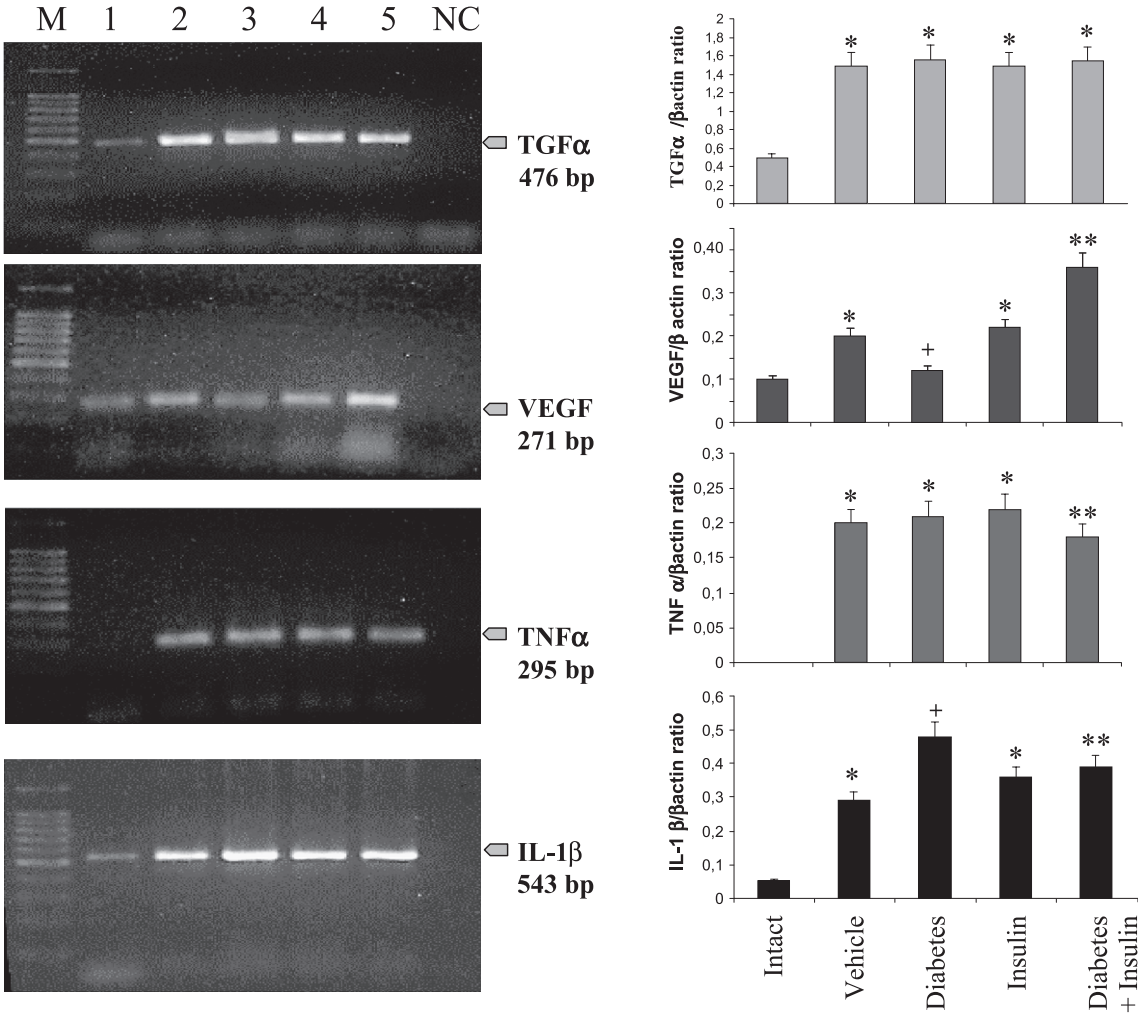

Fig. 5. Representation of RT-PCR for TGF-α, VEGF, TNF-α, IL-1β and densitometric analysis in intact rats (lane 1), vehicle treated control rats with gastric ulcers (lane 2), diabetic rats with gastric ulcers not treated with insulin (lane 3), non-diabetic rats with gastric ulcers treated with insulin (lane 4) and diabetic rats with gastric ulcers treated with insulin (lane 5). Assessment of the ratio of mRNA for TGF-α, VEGF, TNF-α and IL-1β mRNA to β-actin mRNA in studies is presented on the right panel. A single asterisk indicates a significant change as compared to the intact mucosa. Double asterisk indicates a significant change compared to the value obtained in diabetic rats without treatment with insulin. A cross indicates a significant change as compared to vehicle-control value.

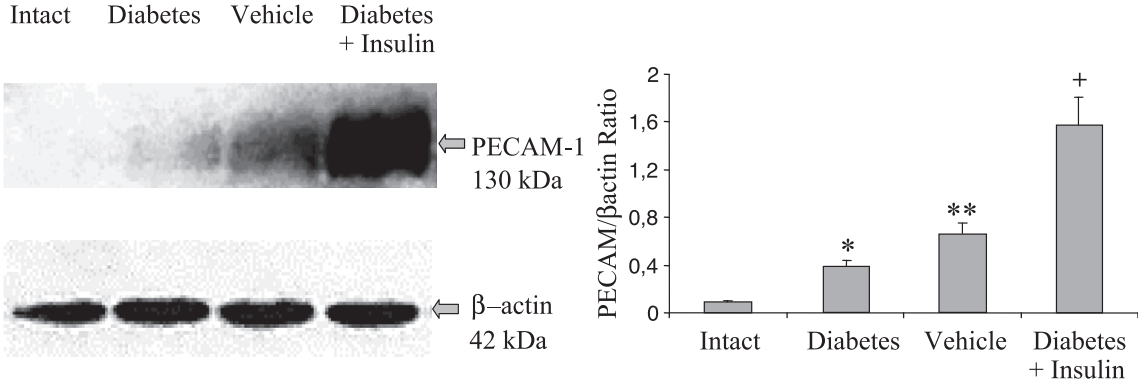

Fig. 6. Representation of changes in protein expression of PECAM-1 in intact rat gastric mucosa, in diabetic rats with gastric ulcer, in vehicle-treated control rats with gastric ulcer and in diabetic rats with gastric ulcer but treated with insulin. A single asterisk indicates a significant increase above the value in intact gastric mucosa. Double asterisks indicate a significant increase about the value recorded in diabetic rats with gastric ulcer not treated with insulin and a cross indicates a significant increase above the value in diabetic rats with gastric ulcer and not treated with insulin. Assessment of the ratio of mRNA for PECAM-1 to mRNA β-actin in studies is presented on the right panel.

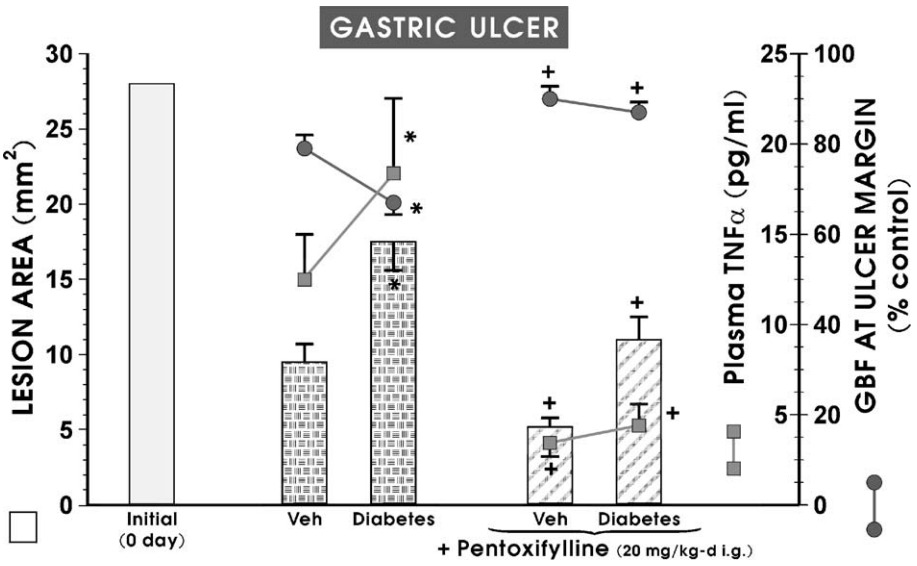

Fig. 7. Effect of daily intraperitoneal (i.p.) treatment with vehicle (saline) and pentoxifylline (20 mg/kg day) on the area of gastric ulcers at day 10 and the accompanying changes in the GBF at ulcer margin and plasma TNF-α levels in rats with or without diabetes induced by streptozotocin. Results are means ± S.E.M. of six to eight rats. Asterisk indicates a significant change as compared with the value obtained in vehicle-control gastric mucosa. Cross indicates a significant change as compared with the value obtained in rats without pentoxifylline treatment.

As shown in Fig. 6, the protein expression of PECAM-1 was negligible in the intact gastric mucosa. In ulcerated non-diabetic rats, the protein level of PECAM-1 was significantly higher than that in the intact mucosa. The expression of this protein was significantly smaller in diabetic rats as compared to that in non-diabetic animals. The ratio of PECAM-1 protein over β-actin protein reached significantly higher value in diabetic rats treated with insulin as compared to that in diabetic rats without insulin treatment (Fig. 6).

3.4. Effect of pentoxifylline on the ulcer healing, gastric blood flow at ulcer margin and plasma TNF-α levels

As shown in Fig. 7, the area of gastric ulcer was significantly increased while the gastric blood flow at the

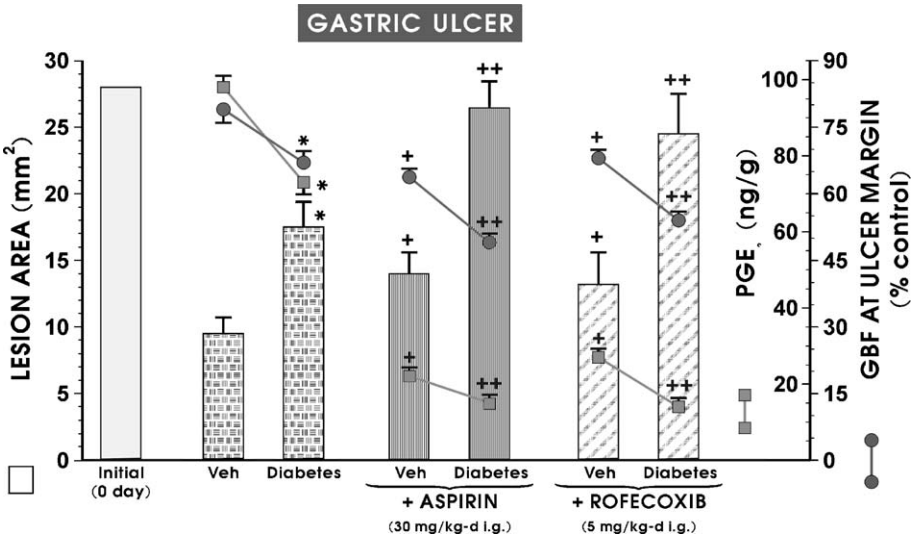

Fig. 8. Area of gastric ulcers at day 10 upon ulcer induction and accompanying changes in the gastric mucosal prostaglandin E2 generation and gastric blood flow (GBF) at ulcer margin in rats treated intragastrically (i.g.) with vehicle, aspirin (30 mg/kg day) or rofecoxib (5 mg/kg day). Results are means ± S.E.M. of six to eight rats. Asterisk indicates a significant change as compared with the value obtained in vehicle-treated animals. Cross indicates a significant change as compared with the values obtained in rats without treatment with COX inhibitor. Double cross indicates a significant change as compared with the respective values obtained in non-diabetic animals.

ulcer margin was significantly decreased in diabetic rats as compared to the respective values in vehicle-treated non-diabetic animals. Ten days of intragastric treatment with pentoxifylline (20 mg/kg day) significantly decreased the area of gastric ulcer in both non-diabetic and diabetic rats, as compared to those measured in animals without pentoxifylline treatment. Treatment with pentoxifylline produced a significant rise in the gastric blood flow at ulcer margin of non-diabetic and diabetic rats as compared to the respective values in rats not treated with pentoxifylline.

The results of the measurement of plasma TNF-α levels in non-diabetic and diabetic rats with gastric ulcer with and without concomitant treatment with pentoxifylline are presented in Fig. 7. Plasma TNF-α was negligible in the intact animals, but it rose significantly in vehicle-treated non-diabetic rats with gastric ulcer. Diabetes resulted in a further significant enhancement of the plasma tumor necrosis factor-α levels. Treatment with pentoxifylline suppressed significantly the plasma levels of this cytokine in diabetic and non-diabetic rats.

3.5. Effect of aspirin, the non-selective COX-1 and-2 inhibitor, and rofecoxib, the selective COX-2 inhibitor on the ulcer healing, gastric blood flow at ulcer margin and mucosal generation of prostaglandin E_2

Treatment with rofecoxib, the highly selective COX-2 inhibitor, resulted in similar prolongation of the ulcer healing in non-diabetic rats to that observed with aspirin administration (Fig. 8). At day 10 upon ulcer induction, all animals treated with aspirin exhibited the presence of ulcer, whose area remained significantly larger than that measured in vehicle-treated controls, and this effect was accompanied by a significant fall in the GBF at ulcer margin as compared to the respective values recorded in vehicle-treated gastric mucosa. Treatment with rofecoxib significantly delayed ulcer healing similarly as aspirin, and this effect was also accompanied by the decrease in the GBF at ulcer margin with the magnitude not significantly different than that measured at the ulcer margin of rats treated with aspirin. The PGE_2 generation in ulcerated gastric mucosa of the vehicle-treated rats without diabetes reached the value of 95 ± 6 ng/g of wet tissue weight, and this value was significantly reduced in diabetic animals (Fig. 8). Treatment with aspirin and rofecoxib, which significantly delayed ulcer healing in both diabetic and non-diabetic rats resulted in suppression of PGE_2 generation in the ulcerated mucosa of these animals.

4. Discussion

The relationship between diabetes mellitus and peptic ulcer has not been fully established. Our study confirms, at least in part, previous report that streptozotocin-induced diabetes impairs gastric ulcer healing (Takeuchi et al., 1997) and shows, for the first time, that the VEGF expression is strongly downregulated in the ulcerated mucosa of diabetic rats. This observation seems to be of importance since this growth factor is one of the most potent known angiogenic cytokines promoting all steps in the cascade process of angiogenesis during ulcer healing (Leung et al., 1989; Szabo et al., 1998). Moreover, there is also evidence supporting the gastroprotective role of VEGF in the gastric mucosal injury (Matsui et al., 2002). Based on these observations, the downregulation of mRNA expression for VEGF in gastric mucosa of diabetic rats could contribute to the impairment of ulcer healing in these animals due to an attenuation of angiogenesis. This is in keeping with our finding that the concomitant treatment with insulin led to a significant upregulation of VEGF mRNA in the ulcer area and accelerated ulcer healing in diabetic animals. The fact that insulin in non-diabetic rats failed to stimulate the expression of VEGF mRNA in contrast to that in diabetic animals indicates that the upregulation of mRNA for this growth factor in diabetic rats treated with insulin is probably attributed to the counteraction of hyperglycemia by this hormone rather than due to direct effect of insulin on expression of this growth factor. This is supported by our preliminary observation, not presented in this study, that administration of glucose to the levels to mimic the increase in plasma glucose levels in diabetic animals (about 300 mg/dl) for 7 days resulted in the downregulation of mRNA for VEGF in the gastric mucosa.

Our study confirms that the expression of TGF-α was significantly increased in the ulcer area, and this observation is consistent with previous observations showing the importance of this growth factor in the process of ulcer healing (Coffey et al., 1995; Konturek et al., 1997). However, the fact that a delay of healing of the gastric ulcers in the diabetic state, despite increased expression of TGF-α, indicates that the healing effects of this growth factor may be attenuated by some unknown mechanism. This militates against the major role of this factor in the observed delay in healing of gastric ulcers under diabetic conditions. One possibility is that the hyperglycemia in diabetic animals could induce some structural modifications of TGF-α or its receptors in the ulcer area, leading to the dysfunction of this growth factor during diabetes mellitus (Facchiano et al., 2002). Further studies are needed to answer the question why increased expression of TGF-α does not contribute to the release of a functionally active growth factor.

The delay in ulcer healing in diabetic rats was also associated with a significant reduction in the gastric blood flow, which plays an important role in the healing process by supplying oxygen and nutrients and by removing toxic substances from the ulcer area (Abdel-Salam et al., 2001). The attenuated hyperemic response in the ulcer margin

could be also due to the dysfunction of capsaicin-sensitive afferent neurons important for the protection of the gastric mucosa and the healing process (Tashima et al., 1998).

A failure in ulcer healing in rats with streptozotocin-induced diabetes could also be attributed to the increased production of proinflammatory cytokines such as IL-1β and TNF-α leading to sustained inflammatory reaction and the delayed healing at the ulcer area. Besides many biological activities, both IL-1β and TNF-α are involved in the induction of inflammation, injury and carcinogenesis in a variety of tissues including the gastric mucosa (Le and Vilcek, 1987; Diamond and Pesek, 1991; Troost et al., 2003). Moreover, these cytokines were recently implicated in the mechanism ischemia–reperfusion injury progressing into gastric ulcer (Brzozowski et al., 2000) and to mediate the delay in ulcer healing induced by *H. pylori* and its water extract (Brzozowski et al., 1999a,b). In addition, IL-1β is considered as a major factor responsible for the induction of ulcer recurrence (Watanbe et al., 2001). Our results are in keeping with the original finding of Takahashi et al. (1998) and Shigeta et al. (1998), who proposed that endogenous IL-1β can influence ulcer healing by triggering effect on the expression of COX-2 and certain growth factors including basic fibroblast growth factor and hepatocyte growth factor at the ulcer margin. Since IL-1β mRNA is upregulated in the ulcerated mucosa, it was postulated that enhanced expression of IL-1β could contribute to the ulcer healing by induction of COX-2 expression and the elevation of prostaglandin production (Shigeta et al., 1998). In our present study, we confirmed our (Brzozowski et al., 2000, 2001) and other (Takahashi et al., 1998) previous observations that both expression and release of IL-1β and TNF-α are increased during the process of ulcer healing. Furthermore, we found that the expression and release of these cytokines were remarkably enhanced in diabetic animals along with the marked prolongation of the ulcer healing and a potent fall in the gastric mucosal blood flow at the ulcer margin recorded in these animals. It is not excluded that in our study in diabetic animals, proinflammatory and anti-secretory cytokines could contribute to the activation of the genes for COX-2 as described in our previous report (Brzozowski et al., 2001). On the other hand, Nakamura et al. (1998) reported that the increase in IL-1β inhibited growth factor-stimulated healing of wounded epithelium suggesting that major mechanism by which cytokines are implicated in the mucosal repair and ulcer healing includes inhibition of growth factors responsible for mucosal regeneration and recovery from the damage. Thus, we conclude that IL-1β and TNF-α exert deleterious influence on the ulcer healing in diabetes. This notion is supported by our present observation that suppression of TNF-α by pentoxifylline (Shimizu et al., 2000) resulted in acceleration of ulcer healing and hyperemia at ulcer margin in both non-diabetic and diabetic animals.

An attempt has been made in this study to determine the role of COX-2/prostaglandin system in the mechanism of ulcer healing in diabetic animals by two ways: (1) direct measurement of prostaglandin E_2 in the gastric mucosa; and (2) by using pharmacological intervention such as administration of highly selective COX-2 inhibitor, rofecoxib, in diabetic animals in order to compare its effect on ulcer healing with that exhibited by aspirin, 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 (Schmassmann et al., 1997). In agreement with recent observations (Lesch et al., 1998; Brzozowski et al., 2001), we have shown that COX-2 inhibitor, rofecoxib, similarly to aspirin, suppressed prostaglandin E_2 at the site of the pre-existing ulcers. Furthermore, we found that diabetic animals exhibited a decrease in mucosal generation of prostaglandin E2 at the ulcer margin and that both aspirin and rofecoxib further suppressed this generation in non-diabetic and diabetic animals leading to a marked delay in healing observed predominantly in diabetic animals treated with these COX inhibitors. Thus, our study demonstrates that prostaglandins 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 should be avoided when ulcer healing in diabetes is considered. This is in keeping with the notion that the suppression of both COX-1 and COX-2 may be required to induce gastric mucosa lesions in rats (Wallace et al., 2000). Further studies on gene expression of COX-1 and COX-2 in this diabetic animal model are needed to clarify the relative contribution of COX isoforms in healing of these ulcers.

The present study demonstrates that diabetic conditions alter the mucosal expression of HSP 70, an important member of stress proteins (heat shock proteins), which play an essential role in the folding, assembly, translocation and maintenance of the integrity of polypeptides as molecular chaperones (Liang and MacRae, 1997). In comparison to the control group, we observed more pronounced HSP 70 expression in the ulcerated gastric mucosa in diabetic rats than that in non-diabetic animals, and this expression was reversed by the insulin treatment. These findings suggest that diabetes increases the cellular level of HSPs, which are dependent upon the deficiency of insulin. However, changes in the expression of HSP 70 differ in various organs of diabetic rats. Yamagishi et al. (2001) showed that HSP 70 content is markedly reduced in the liver but not in the brain, adrenals and pancreas. We believe that the mucosal upregulation of HSP 70 observed in the ulcer in diabetic rats as compared to that of the intact or non-diabetic rats could be a consequence of a stronger inflammatory reaction in the ulcer area of diabetic rats when compared to that in non-diabetic animals. Previous studies demonstrated that some proinflammatory cytokines such as IL-1β can enhance HSP 70 expression (Hao et al., 1999). Our results are at variance to those by Bitar et al. (1999) who demonstrated significant decrease in HSP 70 level in cutaneous wound in diabetic

conditions. Based on this finding, the authors postulated a possible link between impaired healing and decreased HSP 70 expression. Further studies are needed to clarify why the expression of this protein in gastric ulcers differs from that in skin wounds in diabetic conditions.

In conclusion, (1) diabetic rats exhibited an impaired ulcer healing, which was associated with attenuation of angiogenesis, mucosal overexpression and increased expression and release of proinflammatory cytokines IL-1β and TNF-α, (2) the delay in ulcer healing and the fall in gastric blood flow at ulcer margin in diabetic animals can be counteracted by the TNF-α antagonist, pentoxifylline, (3) hyperglycemia in diabetic rats appears to attenuate the protective and healing effects of HSP 70, as well as angiogenic growth factor expression, such as VEGF in the margin of gastric ulcer, (4) the treatment with insulin reverses 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 and (5) the decrease in the biosynthesis of endogenous prostaglandins in diabetic animals treated with or without non-specific or selective COX inhibitors could play an important role in the prolongation of ulcer healing under diabetic conditions.

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