GASTRIC ULCER HEALING AND STRESS-LESION PREVENTIVE PROPERTIES OF PIOGLITAZONE ARE ATTENUATED IN DIABETIC RATS

Diabetes mellitus increases susceptibility to acute gastric injury and impairs ulcer healing. Pioglitazone as an agonist of peroxisome proliferator-activated receptor gamma (PPARγ) is used as anti-diabetic drug and has additionally gastroprotective activities. However, the effect of pioglitazone on the protection and healing of gastric mucosa under diabetic conditions is poorly understood. The aim of the present study was: 1) to compare the effects of treatment with PPARγ ligand (pioglitazone) on healing of acetic acid-induced gastric ulcers and prevention of acute water immersion and restraint stress (WRS)-induced gastric lesions in normal rats and those with streptozotocin (STZ)-induced diabetes mellitus; 2) to assess the effects of pioglitazone on the mRNA expression of cyclooxygenase-2 (COX-2), c-NOS, interleukin-1β and hypoxia inducible factor-1 alpha (HIF-1α) in the gastric mucosa of rats with or without STZ-induced diabetes mellitus; 3) to investigate the involvement of endogenous NO and proinflammatory cytokines (IL-1β, TNF-α) in healing of chronic gastric ulcers and in prevention of acute stress lesions by pioglitazone in rats with or without STZ-induced diabetes mellitus. Diabetes was induced in rats by single injection of STZ (70 mg/kg i.p.) four weeks prior to production of gastric ulcers by acetic acid method or induction of stress lesions by 3.5 hours of WRS. Non-diabetic rats were used as controls. Two major animal groups (A and B) were tested; A) diabetic and non-diabetic rats with chronic gastric ulcers treated with 1) pioglitazone (40 mg/kg-d i.g.), 2) pioglitazone in combination of blocker of NO synthase (L-NNA 20 mg/kg-d i.p.), and 3) saline (vehicle-control); and B) diabetic and non-diabetic rats exposed to 3.5 hours of WRS and pretreated with 1) pioglitazone (40 mg/kg i.g.), 2) pioglitazone in combination of blocker of NO synthase (L-NNA 20 mg/kg i.p.), and 3) saline (vehicle-control). The gastric mucosal blood flow was assessed by H2-gas clearance method. The area of chronic acetic acid ulcers and number of acute WRS-induced gastric lesions were assessed by planimetry or by counting of number of lesions, respectively. In rats with chronic ulcers, the mRNA expression of HIF-1α, IL-1β and COX-2 was assessed by RT-PCR and protein expression of platelet endothelial cell adhesion molecule-1 (PECAM-1), COX-2 and cNOS was examined by Western blot. In rats with stress lesions, the protein expression of COX-2, cNOS, catalase, PPAR and heat shock protein 70 (HSP70) was examined by Western blot. In diabetic rats, a marked delay in ulcer healing and increased susceptibility to WRS lesions were observed and these effects were accompanied by a significant decrease in GBF. Pioglitazone significantly increased healing of chronic gastric ulcers and exerted a strong protective effect against WRS-induced lesions, but these effects were attenuated by NO-inhibition with L-NNA. Interestingly, the ulcer healing and gastroprotective effects of pioglitazone were weak under diabetic conditions, and this effect on ulcer healing was accompanied by impaired angiogenesis due to decreased PECAM-1 expression, attenuated expression of COX-2 and the increased expression of proinflammatory cytokines compared to those in diabetic rats treated with vehicle. We conclude that: 1) experimental diabetes in rats impairs healing of chronic ulcers and enhances acute stress ulcers due to an increase in the expression and release of proinflammatory cytokines such as TNF-α and IL-1β; 2) the ulcer healing effect of pioglitazone, which is, at least in part, mediated by endogenous NO, is significantly attenuated by L-NNA in diabetic rats despite increased COX-2 expression at the ulcer edge; 3) the formation of acute gastric lesions induced by WRS is also attenuated by pretreatment with pioglitazone due to increased GBF probably mediated by NO, as the administration of L-NNA reversed, in part, the preventive action induced by this PPARγ ligand; and 4) pioglitazone is effective both in healing of chronic ulcers and protection against WRS lesions though its action under diabetic conditions seems to be attenuated, possibly due to reduction in NOS-NO system, angiogenesis and increased expression and release of proinflammatory cytokines.

Key words: stress, gastric ulcer, diabetes mellitus, peroxisome proliferator-activated receptor gamma, hypoxia inducible factor-1alpha, cyclooxygenase-2, nitric oxide, cytokines

INTRODUCTION

Diabetes mellitus is a metabolic disease affecting a large number of people of all ages, races and socio-economic classes throughout the world. Patients with long-standing diabetes mellitus, may develop autonomic neuropathy and demonstrate a variety of gastrointestinal symptoms such as functional dyspepsia, abdominal pain, vomiting, diarrhoea, constipation
and delayed gastric emptying (1). Diabetes mellitus increases the susceptibility of gastric mucosa to ulcerogens and impairs gastric ulcer healing (2, 3).

An accepted experimental model of insulin-dependent diabetes mellitus has been previously studied by inducing diabetes in rats via injection of streptozotocin (STZ). It was demonstrated that gastric mucosa exhibits an increased vulnerability to the damage induced by various ulcerogens such as ischemia-reperfusion injury, stress and non-steroidal anti-inflammatory drugs (4, 5). The mechanisms that underlie this increased susceptibility to multifactor damage under diabetic conditions included the impairment of the mucosal antioxidative system and suppression of the production of a potent angiogenic factors such as basic fibroblast growth factor in the gastric mucosa, resulting in attenuation of angiogenic response as well as the impaired mucosal HCO₃⁻ secretion, and dysfunction of capsinic-sensitive afferent neurons involved in the protection of this mucosa (2, 5, 6).

Pioglitazone as a member of glitazone agents, has been widely considered as an anti-diabetic drug, because it decreases insulin resistance in muscle and adipose tissue by activating peroxisome proliferator-activated receptor gamma (PPARγ) which increases production of proteins involved in glucose uptake (7). Pioglitazone decreases also hepatic glucose production by improving hepatic insulin sensitivity (8). When added to insulin regimens, pioglitazone confers a small advantage in terms of HbA1c in type 2 diabetes patients with hypoglycaemia and weight gain. Other actions of this PPARγ agonist may however, involve side effects such as the risk of heart failure and fractures in women (9, 10).

It became recently apparent that the pleiotropic effects of glitazones include also its protective action against gastric mucosal damage. Previous studies showed that pioglitazone exerted a potent gastroprotective and hyperaemic actions on the gastric mucosa involving endogenous prostaglandins (PG), nitric oxide (NO) and attenuation of the expression and release of proinflammatory cytokines such as IL-1β and TNF-α (11-13).

However, to our best knowledge, none of the studies published before examined the effect of pioglitazone on the mucosal protection and ulcer healing in gastric mucosa under diabetic conditions. The aims of the present study were; 1) to compare the effects of treatment with PPARγ agonist (pioglitazone) on healing of acetic acid-induced gastric ulcers and prevention of acute water immersion and restraint stress (WRS)-induced gastric lesions in normal rats and those with STZ-induced diabetes mellitus; 2) to assess the effect of pioglitazone on the mRNA expression of COX-2, cNOS, IL-1β and hypoxia inducible factor alpha-1a (HIF-1α) in the gastric mucosa of rats with or without STZ-induced diabetes mellitus; and 3) to investigate the involvement of endogenous NO and proinflammatory cytokines (IL-1β, TNF-α) in healing of chronic gastric ulcers and in prevention of acute stress lesions by pioglitazone in rats with or without STZ-induced diabetes mellitus.

MATERIAL AND METHODS

Induction of diabetes

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 that were run according to the principles of Helsinki Declaration. Animals were given streptozotocin (STZ, Fluka-Sigma-Aldrich Poznan, Poland) in a single intraperitoneal injection at a dose of 70 mg/kg as described previously (2). Four weeks after the injection of STZ, when fasting blood glucose levels rose to 325 mg/dl indicating diabetes, the rats without and with diabetes were randomly assigned to 2 major groups (A and B) and subjected to procedure of chronic ulcer induction (A) and acute WRS lesions formation (B), respectively.

Induction of chronic gastric ulcers

The gastric ulcers were produced using our modified acetic acid method originally proposed by Okabe et al. (1971) (14). 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-oxynctic border. The amount of 75 μl of 100% acetic acid was poured in the mould 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 mm²). The excess of acetic acid was then removed using a 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. After the induction of acetic ulcers, the animals were allowed to recover from anaesthesia and received only water the day of the operation.

Following treatment groups were investigated: 1) diabetic and non-diabetic rats with chronic gastric ulcers treated with saline (vehicle-control), 2) diabetic and non-diabetic rats with chronic gastric ulcers treated with pioglitazone (40 mg/kg-d i.g.), 3) diabetic and non-diabetic rats with chronic gastric ulcers treated pioglitazone in combination with non-selective inhibitor of NO synthase (L-NNA 20 mg/kg-d i.p.).

Induction of gastric water immersion and restraint (WRS) lesions

The gastric WRS lesions were induced by placing rats with or without STZ-induced diabetes in the Bollman cages causing the immobilization and immersing them in water at 23°C to the rats xyphoid process as described previously (15). The rats were then sacrificed immediately after withdrawal from 3.5 hours of WRS. The stomachs were removed and the number of ulcerations was counted by a person blinded to the origin of coded specimens, and the average number of lesions per rat was calculated. The stress lesion was defined as a round or linear mucosal defect of at least 0.1 mm of diameter.

Following treatment series were used: 1) rats with or without STZ-induced diabetes pretreated 30 min prior the exposure to WRS with saline (vehicle-control); 2) rats with or without STZ-induced diabetes pretreated 30 min prior the exposure to WRS with pioglitazone (40 mg/kg i.g.), and 3) rats with or without STZ-induced diabetes pretreated 30 min prior the exposure to WRS with pioglitazone given in combination with the NO synthase inhibitor, L-NNA (20 mg/kg i.p.).

Measurement of the gastric blood flow (GBF)

After the termination of 3.5 hours of WRS, the animals were anesthetized, the abdomen was opened, the stomachs were exposed, and GBF was measured by the H₂-gas clearance technique as described previously (2). Briefly, double needle electrodes were inserted into the mucosa through the serosa with the tips located in the mucosa, one electrode being used for local generation of H₂-gas and the other for the measurement of tissue H₂. With this method the H₂ generated by water hydrolysis is carried away by the blood and the polarographic current detector shows the decreasing tissue H₂ as a clearance curve which is used to calculate absolute flow rate (ml/min 100g). The GBF was measured in three areas of the oxyntic
portion of the stomach not involving macroscopically visible mucosal lesions, and the mean value of three recordings was calculated and expressed as a percentage of the flow recorded in the intact mucosa.

Determination of mRNA for IL-1β, HIF-1α and COX-2

The stomachs were removed, and mucosal specimens were scraped off using a slide glass 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 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 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, Heidelberg, Germany) and oligo-(dT)-primers (Stratagene, Heidelberg, Germany) in accordance with the procedure described by our group previously (2, 11). The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) at the specifications shown in the Table 1. The nucleotide sequence of the primers for β-actin, IL-1β, HIF-1α and COX-2 were based on the sequences of the published cDNAs. The 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. The location of a reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The location of a reaction product was confirmed by using 100-bp ladder (Takara, Shiga, Japan) as a standard size marker. The gel was then photographed under UV transillumination. The intensity of PCR products was measured using video image analysis system (Kodak Digital Science) as described earlier. The signal for investigated mRNA was standardized against that of the β-actin mRNA from each sample and the results were expressed as analyzed mRNA/β-actin mRNA ratio as described earlier (15).

Western blot analysis

For Western blot analysis, proteins were extracted from the same gastric mucosa samples as mentioned above. Approximately 10 µg of total protein extracts was loaded on SDS-polyacrylamide gels and run at 40 mA, followed by transfer onto nitrocellulose membrane (Protran, Schleicher and Schuell, Germany) by electroblotting. Solution of 3% BSA (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 hour at room temperature. Specific primary antibody against cNOS (rabbit polyclonal, dilution 1:200; Santa Cruz, USA), PECAM-1 (rabbit polyclonal, dilution 1:200; Santa Cruz, USA), COX-2 (rabbit polyclonal, dilution 1:400; Santa Cruz, USA) PPARγ (rabbit polyclonal, dilution 1:500; Santa Cruz, USA), HSP70 (mouse monoclonal, dilution 1:7000; StressGen, USA), catalase (rabbit polyclonal, dilution 1:10000 Rockland, Germany) or β-actin (mouse monoclonal, dilution 1:3000; Sigma Aldrich, Germany) was added to the membrane, followed by an anti-rabbit-IgG or anti-mouse-IgG HRP-horseradish peroxidase conjugated secondary antibody (dilution 1:4000 or 1:20000) dissolved in 1% non-fat milk in TBS-Tween-20 buffer. Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 5 min. incubation of the secondary antibody was followed by 6 washes for 5 min according to manufacturer instructions. Immunocomplexes were detected by the SuperSignal West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

Determination of plasma cytokines TNF-α and IL-1β

Immediately after GBF measurement, a venous blood sample was withdrawn from vena cava into EDTA-containing vials and used for determination of plasma IL-1β and TNF-α by a solid phase sandwich ELISA (BioSource International Inc.,

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

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>ANNIAL. TEMP. °C</th>
<th>Dp</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5’TGT TAA CCA ACT GGG AGG ATA TGG 3’</td>
<td>60</td>
<td>64</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>5’TCT TGA CTC TCG CCT CTG 3’</td>
<td>58</td>
<td>330</td>
</tr>
<tr>
<td>COX-2</td>
<td>5’ACA ACA TTC CCT TCC TTC 3’</td>
<td>56</td>
<td>201</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’GAT CAT TGC TGT TTC CTA GGG 3’</td>
<td>62</td>
<td>343</td>
</tr>
</tbody>
</table>

Fig. 1. Healing course and changes in the gastric mucosal blood flow (GBF) at ulcer margin in rats with and without streptozotocin-induced diabetes mellitus without or without treatment with pioglitazone alone or in combination with L-NNA or vehicle (saline). Mean±S.E.M. from six 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 with pioglitazone treatment. Double crosses indicate a significant change from the respective values in non-diabetic rats.
Camarillo, CA, USA) according to the manufacturer's instructions. Briefly, each sample (50 µl) was incubated with biotinylated antibodies specific for rat IL-1β and TNF-α 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 (2).

**Statistical analysis**

Results are expressed as means±S.E.M. Statistical comparisons were performed with Student's t test. Comparisons involving more than two groups were performed by ANOVA. Differences with p-value <0.05 were considered significant.

**RESULTS**

As shown in Fig. 1, nine day treatment with pioglitazone significantly accelerated the ulcer healing and significantly raised the GBF at ulcer margin in non-diabetic rats as compared with the respective values recorded in vehicle-control animals. These effects were significantly attenuated by co-treating of these animals with the blocker of NO synthase, L-NNA. In rats with STZ-induced diabetes mellitus, a significant delay in ulcer healing was observed as compared to non-diabetic rats and this effect was accompanied by the fall in the GBF at ulcer margin. In rats with experimentally induced diabetes mellitus, pioglitazone treatment caused an acceleration of ulcer healing. However, this acceleration of ulcer healing significantly weaker than that observed in non-diabetic rats with gastric ulcers. The addition of L-NNA to pioglitazone completely abolished the ulcer healing and the GBF at ulcer margin effects of pioglitazone resulting in the marked prolongation of ulcer healing comparing to those observed in pioglitazone-treated animals (Fig. 1).

At mRNA level, in gastric ulcer edge of non-diabetic rats treated with vehicle, a significant upregulation of expression for IL-1β was observed and this effect was significantly attenuated in rats treated with pioglitazone (Fig. 2). In diabetic rats, the expression of IL-1β at the ulcer edge was significantly upregulated as determined by the semi-quantitative ratio of IL-1β over β-actin mRNA. This IL-1β expression remained elevated despite the treatment with pioglitazone in diabetic rats (Fig. 2). Similarly to IL-1β, the expression of HIF-1α mRNA was significantly upregulated at the ulcer edge in non-diabetic rats as compared to that in rats with intact gastric mucosa. However, treatment with pioglitazone caused a significant downregulation of transcript expression for HIF-1α. In rats with STZ-induced diabetes, the expression of HIF-1α mRNA was increased at the ulcer edge and remained at increased level despite pioglitazone treatment (Fig. 2). The expression of COX-2 mRNA was also significantly upregulated at the margin of gastric ulcer in non-diabetic and diabetic rats as assessed by the ratio of mRNA for COX-2 over that of β-actin. In non-diabetic rats, the treatment with pioglitazone caused a significant downregulation of transcripts for COX-2 (Fig. 2). In contrast, diabetic rats exhibited still increased expression of COX-2 despite pioglitazone treatment (Fig. 2).

Western blot analysis showed that pioglitazone causes upregulation of eNOS and PECAM-1 protein in vehicle-control rats with gastric ulcers (Fig. 3). The expression of COX-2 protein was detected as the strong signal in vehicle-control mucosal at ulcer margin and it was significantly decreased by the treatment with pioglitazone (Fig. 3). In diabetic rats with STZ-induced diabetes mellitus, pioglitazone also increased the expression of eNOS and PECAM-1 as confirmed by the ratio of mRNA and COX-2 protein expression with the respective values recorded in vehicle-control animals. The expression of COX-2 protein tended to increase in diabetic animals as compared with non-diabetic controls, however, this increase failed to reach statistical significance. The pioglitazone-induced inhibition of COX-2 protein expression was significantly smaller as compared with that achieved in vehicle-control animals without diabetes (Fig. 3).

As shown in Fig. 4, rats exposed to 3.5 h WRS demonstrated multiple gastric lesions and decreased GBF in gastric mucosa non-involving gastric lesions as compared to those obtained in control animals rats not exposed to WRS. The pretreatment with pioglitazone caused significant decrease in the number of acute WRS-induced gastric lesions and a significant rise in GBF (Fig. 2).
In rats with STZ-induced diabetes exposed to 3.5 hours of WRS, the number of gastric lesions was significantly increased as compared to that in rats without diabetes and exposed to WRS. Pretreatment with pioglitazone in rats with STZ-induced diabetes significantly reduced the number of gastric lesions and caused a significant reduction in GBF, but these effects were less pronounced as compared to the respective values obtained in rats without diabetes. The combined treatment with pioglitazone and L-NNA completely abolished the attenuation of the number and an increase in the GBF in diabetic rats exposed to WRS (Fig. 4).

As shown in Fig. 5, the WRS significantly increased the release of proinflammatory cytokines IL-1β and TNF-α. Pretreatment with pioglitazone caused significant decrease of circulating cytokines IL-1β and TNF-α. Addition of L-NNA to rats exposed to 3.5 h WRS and pretreated with pioglitazone caused an significant increase of circulating IL-1β and TNF-α as compared to the levels observed to pioglitazone pretreated rats without L-NNA administration (Fig. 4). Rats with STZ-induced diabetes exposed to WRS showed a marked increase in plasma IL-1β and TNF-α levels which was higher than in non-diabetic rats exposed to WRS (Fig. 4). Pioglitazone treatment caused a significant decrease in the level of circulating proinflammatory cytokines IL-1β and TNF-α, but this decrease in plasma cytokine levels were significantly smaller as compared to that observed in non-diabetic rats exposed to WRS and pretreated with pioglitazone. Addition of blocker of NO synthase, L-NNA, completely reversed the inhibitory effect of pioglitazone on the release of these proinflammatory cytokines in rats with diabetes (Fig. 5).

At the protein level, the exposure of non-diabetic or diabetic rats to WRS was associated with an increase in expression of HSP70 and a decrease of cNOS protein (Fig. 6). Pretreatment with pioglitazone significantly increased the expression of cNOS and HSP70 proteins toward their levels observed in intact gastric mucosa.

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**Fig. 3.** Effect of pioglitazone on gastric mucosal protein expression of cNOS, PECAM-1 and COX-2 in the ulcer area of non-diabetic and diabetic rats with gastric ulcer. Asterisk means a significant difference from the corresponding value in vehicle-control gastric mucosa. Cross indicates a significant change from the respective values in non-diabetic or diabetic rats treated with vehicle. The double crosses denote a statistically significant difference from the corresponding values in vehicle control gastric mucosa treated with vehicle.

**Fig 4.** Effect of pioglitazone on gastric mucosal lesions and accompanying changes in the GBF induced by the exposure of animals to 3.5 hours of water immersion and restraint stress (WRS) in non-diabetic and diabetic animals. Asterisk means a significant difference from the corresponding value in vehicle-control gastric mucosa. Cross and asterisk indicate a significant change from the respective values in non-diabetic rats treated with pioglitazone (40 mg/kg i.g.) Cross denotes a statistically significant difference from the corresponding values in animals without diabetes.
In clear contrast, the expression of HSP70 protein was significantly decreased in gastric mucosa of diabetic rats as compared to that in non-diabetic ones (Fig. 6). Pioglitazone increased the expression of HSP70 protein but this increase was significantly smaller than that observed in pioglitazone-pretreated non-diabetic rats. In non-diabetic and diabetic rats, the downregulation of cNOS protein was observed in rats exposed to WRS as compared to that recorded in intact gastric mucosa (Fig. 6). The pretreatment with pioglitazone reversed the downregulation of cNOS in non-diabetic and diabetic rats as determined by ratio of cNOS protein over β-actin protein (Fig. 6). The expression of catalase was significantly upregulated in non-diabetic rats exposed to WRS and this effect was further increased in pioglitazone-pretreated animals. In diabetic rats, an increase in the catalase protein was observed, though this increase was not that much pronounced as in gastric mucosa of non-diabetic rats. As shown by the ratio of catalase over β-actin protein, the pretreatment with pioglitazone reversed the fall in catalase protein observed in gastric mucosa of diabetic rats exposed to WRS (Fig. 6).

**DISCUSSION**

This study confirms that the healing of chronic gastric ulcers is impaired in rats with STZ-induced diabetes mellitus (2, 5, 11). The mechanisms responsible for the delay in ulcer healing under diabetic conditions is not fully understood. Our previous study showed that the expression of VEGF, a potent pro-angiogenic growth factor, is strongly downregulated in the ulcerated mucosa of diabetic rats (2). In the present study we demonstrated that the activation of PPARγ by a ligand pioglitazone significantly accelerates the ulcer healing by the mechanism involving NO because the blockade of NO with L-NNA completely reversed...
the beneficial effect of pioglitazone on ulcer healing. This observation is of importance, because diabetic patients who are treated with pioglitazone as insulin sensitizer could receive benefit from both, the improvement of diabetic conditions and the acceleration of ulcer healing. Pioglitazone is known to have multiple important antiinflammatory effects. Pioglitazone may influence cardiovascular pathophysiology at multiple stages of the disease process, including atherogenesis, plaque formation and development of vascular inflammation, plaque rupture and haemostatic disturbances (*i.e.* thrombus/embolism formation), as well as microangiopathy (16).

We have demonstrated that pioglitazone accelerates impaired ulcer healing in diabetic rats *via* decrease in generation of proinflammatory cytokines such as IL-1β and TNF-α, which may impair ulcer healing due to interaction with mucosal restitution and angiogenesis (2). Interestingly, the COX-2 expression which is a source of PG in ulcerated mucosa (17) was downregulated by pioglitazone treatment. Despite this phenomenon the ulcer healing was accelerated after pioglitazone treatment suggesting that COX-2 downregulation seems to be a consequence of the downregulated expression and subsequent release of proinflammatory cytokines, which are responsible for the activation of COX-2 expression. Angiogenesis plays a central role in the mechanism of ulcer healing (18). Our study shows that protein PECAM1 expression, as a marker of angiogenesis, was significantly increased in normal non-diabetic controls treated with pioglitazone suggesting that pioglitazone may exert angiogenic activity. On contrary, this glitazone treatment failed to affect this angiogenic response during of ulcer healing in diabetic rats. This probably would be an explanation why pioglitazone was less effective in treating diabetic gastropathy reflecting in our study an impairment of ulcer healing in diabetic rats. It is of interest that an increase in the expression of mRNA for HIF-1α was significantly decreased in rats treated with pioglitazone suggesting that this PPARγ agonist can counteract the formation of factor associated with hypoxia thus preserving the oxygen and nutrients delivery to the ulcerated tissue in order to accelerate ulcer healing.

The present study demonstrates that pioglitazone not only accelerates ulcer healing but also prevents the development of acute WRS-induced gastric lesions. The number of stress lesions in diabetes rats was significantly higher than in non-diabetic rats indicating an increased susceptibility of gastric mucosa to stress lesions under diabetic conditions. The pretreatment with pioglitazone significantly prevented the development of acute stress-induced gastric lesions and this effect was accompanied by increased gastric mucosal blood flow. Our study suggest that this protective effect of pioglitazone is mediated by NO because the combined treatment of pioglitazone with NO synthase inhibitor, L-NNA, completely abolished the preventive effect of pioglitazone on acute stress-induced gastric lesions. This observation strongly supports the notion that NO plays an important role in gastric protection against stress induced by pioglitazone.

Several previous studies indicated a central role of NO in protection of gastric mucosa, mainly due to an improvement of gastric mucosal microcirculation (19). The molecular analysis showed a significant downregulation of eNOS in gastric mucosa of diabetic and non-diabetic rats exposed to WRS. This observation indicates that the production of NO by constitutive NO is downregulated in the gastric mucosa exposed to stress. Another explanation for suppression of eNOS could be increased generation of NO by alternative pathway *i.e.* upregulation of iNOS, which was, however, not analysed in this study.

It is of interest that the treatment with pioglitazone caused a significant upregulation of eNOS in gastric mucosa of diabetic and non-diabetic rats exposed to WRS. This suggests that pioglitazone may have direct stimulatory effect on eNOS expression. This observation is corroborative with the recent study of Yasuda et al. (20), who demonstrated that anti-diabetic drug pioglitazone protects heart *via* the activation of NO/eNOS pathway. This indicates that pioglitazone may represent a novel strategy for the treatment of gastric ulceration induced by stress as well as satisfactory prevention of coronary artery disease in patients with diabetes mellitus. Catalase is responsible for subcellular breakdown of H₂O₂ (21, 22) and was markedly inhibited in our present study in gastric mucosa of diabetic rats and this effect was further reversed by treatment with pioglitazone. Moreover, our findings in the present study that pioglitazone counteracts the diabetes-induced inhibition of catalase enzyme activity further support the antioxidant capability of pioglitazone. Another important information of our present investigation was that pioglitazone increased the protein expression of HSP70. It has been shown that most HSPs exhibit a potent cytoprotective effects, being involved in many regulatory pathways, and behaving as molecular chaperones for preserving important cellular proteins and affording protection against major gastric pathogen *Helicobacter pylori* (23, 24). Moreover, HSPs are crucial for the maintenance of gastric mucosal cell integrity during normal gastric and endothelial cell development (25, 26). Here we present the evidence that HSP70 was overexpressed in gastric mucosa exposed to WRS and this effect was further augmented in pioglitazone-pretreated rats. These effects were greatly attenuated in diabetic animals who showed greater susceptibility to damage induced by WRS. Our observation is in keeping with that presented by Filipovic et al. (26) who documented the overexpression of HSP70 in different organs of the body exposed to various onsets of cold stress. This clearly indicates that PPARγ agonist, pioglitazone affects the HSP system and this may serve as explanatory for its gastroprotective activity against the formation of stress-induced gastric damage.

In summary, we have found that experimental diabetes impairs healing of chronic ulcers and aggravates acute stress-induced gastric lesions *via* mechanism involving an increase in expression and release of proinflammatory cytokines such as TNF-α and IL-1β. The increased generation of endogenous NO could serve as explanatory for the ulcer healing effect of anti-diabetic drug, pioglitazone, because both, the enhanced ulcer healing and attenuation of stress lesions as well as accompanying increase in the GBF were significantly attenuated by L-NNA. The COX-2 expression which was upregulated at the ulcer edge was diminished by the treatment with pioglitazone suggesting that this PPARγ agonist exhibits anti-inflammatory properties *via* the reduction of proinflammatory markers. The formation of acute gastric lesions induced by WRS is also attenuated by pretreatment with pioglitazone due to the increase in gastric blood flow probably mediated by NO as the administration of L-NNA reversed, in part, the preventive action induced by this PPARγ ligand. Therefore, we conclude that pioglitazone is an effective agent both, in healing of chronic ulcers and protection against stress lesions though its action under diabetic conditions seem to be attenuated, possibly due to reduction in NOS-NO system and angiogenesis, an overexpression of HIF-1α and the increased expression and release of proinflammatory cytokines.

Conflict of interests: None declared.

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