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Pioglitazone, a specific ligand of peroxisome proliferator-activated receptor-gamma, accelerates gastric ulcer healing in rat

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Abstract

The peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a member of the nuclear hormone receptor superfamily that is involved in the control of inflammation and carcinogenesis. We determined the effect of the specific PPAR-γ ligand, pioglitazone (5–40 mg/kg intragastrically), on the healing of acetic-acid gastric ulcers in rats. At day 8 after ulcer induction, the ulcer area, the gastric blood flow and mucosal expression of proinflammatory cytokines such as interleukin-1β, tumour necrosis factor alpha (TNF-α) and cyclooxygenase-1, cyclooxygenase-2, constitutive nitric oxide synthase (cNOS), inducible nitric oxide synthase (iNOS) and heat shock protein 70 (HSP70) was determined. Pioglitazone reduced the area of gastric ulcers and raised significantly the gastric blood flow at the ulcer margin and downregulated the mRNA for interleukin-1β, TNF-α, cyclooxygenase-2 and iNOS while cyclooxygenase-1 mRNA was not affected. The expression of PPAR-γ mRNA was increased in the ulcerated gastric mucosa. We conclude that pioglitazone accelerates the healing of preexisting gastric ulcers due to the hyperemia at ulcer margin and the anti-inflammatory action including suppression of interleukin-1β, TNF-α, cyclooxygenase-2 and iNOS and by an overexpression of HSP70.

Keywords: PPAR-γ ligand; Ulcer healing; Interleukin-1β; TNF-α (tumor necrosis factor-α); Nitric oxide (NO) synthase; HSP70 (heat shock protein 70)

1. Introduction

Peroxisome proliferator-activated receptor γ (PPAR-γ) is a member of the nuclear hormone receptor superfamily whose activation has been linked to transcriptional control of numerous cellular processes with implications of this receptor in control of cell cycle, carcinogenesis, inflammation, atherosclerosis and immunomodulation (Auwerx, 1999). Several reports demonstrated an anti-inflammatory action of the specific ligands of PPAR-γ. It was shown that the activators of PPAR-γ inhibit the generation of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) by monocytes (Jiang et al., 1998). There is also evidence that the PPAR-γ ligands block the synthesis of the pro-inflammatory cytokines by inhibiting the activation of nuclear factor kappaB (NFκB) (Su et al., 1999). Another line of evidence for anti-inflammatory activities of PPAR-γ ligands originates from experiments with PPAR-γ-deficient mice exposed to acute mucosal injury (Nakajima et al., 2001). These experiments demonstrated that PPAR-γ-deficient mice are much more susceptible to mucosal injury than the wild animals (Nakajima et al., 2001). All these studies support the existence of an endogenous anti-inflammatory pathway in different organs that is mediated by PPAR-γ.

Recently, Kojima et al. (2002) demonstrated the expression of PPAR-γ in primary cultured rat gastric epithelial cells. Furthermore, Naito et al. (2001) found that pioglitazone, a specific PPAR-γ ligand counteracted the aspirin-induced gastric lesions but the precise role of PPAR-γ in the modulation of gastric mucosal integrity, especially gastroprotection and mucosal repair and ulcer healing has not been fully clarified to date.

Although PPAR-γ was detected in the rat gastric mucosa, there is no information about the effect of PPAR-γ ligands on the course of the ulcer healing. Therefore, the aim of the present study was to determine the effect of pioglitazone, the specific PPAR-γ ligand, on the healing of chronic gastric

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ulcers in rats and the mucosal expression of mRNA for proinflammatory cytokines (interleukin-1β, TNF-α), cyclooxygenase-1 and cyclooxygenase-2, heat shock protein 70 (HSP70) and constitutive (cNOS) and inducible nitric oxide synthase (iNOS).

2. Material and methods

2.1. Production of chronic gastric ulcers, determination of gastric blood flow and effect of pioglitazone on ulcer healing

Chronic gastric ulcers were induced in male Wistar rats (weight 180–220 g) by our modification of an acetic acid method (Brzozowski et al., 2001a,b) originally described by Okabe et al. (1987). In brief, the abdomen was opened under ether anesthesia and the stomach was exposed. A plastic tube (6 mm in diameter) was applied tightly to the serosal surface of the anterior wall, just proximal to the antral gland area. About 70 μl of 100% acetic acid was poured through the plastic mold onto the serosal surface of the stomach for 20 s. This produced an immediate necrosis of the entire thickness of the mucosa and submucosa within the area of acetic acid application (about 28 mm^2). Our previous studies (Brzozowski et al., 2001a,b) have shown that these ulcers become chronic within 2–3 days and then tend to heal within about 2 weeks without perforation or penetration to neighbouring organs. After ulcer induction, the abdomen was closed, and the rats were divided into four groups, each consisting of six to eight animals. The first group of rats (control) with gastric ulcers was treated intragastrically (i.g.) once daily with vehicle (saline), the next three groups of rats received i.g. pioglitazone (Takeda, Japan) at increasing doses from 5 up to 40 mg/kg. The rats were given pioglitazone or vehicle (saline) for 7 days and at day 8, were sacrificed.

Gastric blood flow was measured at day 8 after ulcer induction, using a hydrogen (H_2)-gas clearance technique as described previously (Brzozowski et al., 2001a,b). At day 8 after ulcer induction, the rats were lightly anesthetized with ether, the abdomen was opened and the stomach was exposed to measure gastric blood flow at the ulcer margin. Double needle electrodes were inserted through the serosa into the ulcer margin and into the 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 then used to calculate the blood flow rate in the tissue. 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.

After the rats had been anaesthetized and blood flow measured, the stomach was removed and the gastric wall was rinsed with phosphate-buffered saline and the area of gastric ulcers was measured by planimetry. Then the biopsy samples were taken from the ulcer margin and gastric mucosa, noninvolving gastric ulcer, the tissue being snap-frozen in liquid nitrogen, and then stored at −80 °C until the time of RNA extraction.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from mucosal samples using a guanidium isothiocyanate/phenol chloroform single step 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 StrataScript reverse transcriptase (Stratagene) and oligo-(dT)-primers (Stratagene) as described previously (Konturek et al., 2002). Briefly, 5 μg of total RNA was uncoiled by heating (65 °C for 5 min) and then reverse transcribed into complementary DNA (cDNA) in a 50-μl reaction mixture that contained 50 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 0.3 μg oligo-(dT)-primer, 1 μl RNase Block Ribonuclease Inhibitor (40 U/μl), 2 μl of a 100-mM mixture of deoxyadenosine triphosphate (dATP), deoxyribothymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 5 μl 10× RT buffer (10 mM Tris–HCl, pH = 8.3, 50 mM KCl, 5 mM $MgCl_2$). The resultant cDNA (2 μl) was amplified in a 50-μl reaction volume containing 2 U Taq polymerase, 200 μM (each) dNTP (Pharmacia, Germany), 1.5 mM $MgCl_2$, 5 μl 10× polymerase chain reaction buffer (50 μM KCl, 10 μM Tris–HCl, pH = 8.3) and specific primers used at final concentration of 0.5 μM. 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 interleukin-1 beta, TNF-α, PPAR-γ, cyclooxygenase-1, cyclooxygenase-2, iNOS, cNOS, HSP70 and β-actin were based on the basis of the published cDNA (Konturek et al., 2000, 2002; Brzozowski et al., 2001a,b). The primers were synthesized by GIBCO/Life Technologies (Eggenstein, Germany).

Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using 100-bp ladder (Takara Biomedicals) as a standard size marker. The gel was then photographed under UV transillumination. The intensity of polymerase chain reaction products was measured using video image analysis system (Kodak Digital Science). The signals for examinated mRNAs were standardized against that of the β-actin mRNA from each sample and the results were expressed as investigated PCR-product/β-actin mRNA ratio.

Table 1
Sequences of primers used in experiments and products size

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
PPAR-γ	5′ TGA TAT CGA CCA CTG GAA CC 3′ GTC CTC TCA GCT GTT CGC CA	60	793
COX-1	5′ AGC CCC TCA TTC ACC CAT TT 3′ CAC GGA CGC CTG TTC TAC GG	60	561
COX-2	5′ ACA ACA TTC CCT TCC TTC 3′ CCT TAT TTC CTT TCA CAC C	56	201
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
HSP70	5′ GTG AAG ATC TGC GTC TGC TTG 3′ TTT GAC AAC AGG CTG GTG AAC C	60	590
cNOS	5′ TAC TTG AGG ATG TGG CTG 3′ GTC TTC TTC CTG GTG ATG	60	840
iNOS	5′ CAG TGG CAA CAT CAG GTC 3′ GGT CTC GGA CTC CAA TCT	60	439

2.3. Western blot analysis

Shock frozen tissue from rat stomach was homogenized in lysis buffer (100 mmol Tris–HCl, pH 7.4, 15% glycerol, 2 mmol EDTA, 2% SDS, 100 mmol DL-dithiothreitol by the addition of 1:20 dilution of aprotinin and 1:50 dilution of 100 mmol 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) by electroblotting. Bovine serum albumin (3%; 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. Specific primary antibody against cyclooxygenase-1 (mouse monoclonal, 1:200 dilution; Santa Cruz, USA), cyclooxygenase-2 (goat polyclonal, 1:200 dilution; Santa Cruz), cNOS (mouse monoclonal; dilution 1:200), HSP70 (dilution 1:2000; mouse monoclonal, StressGen Biotechnologies, Canada) or β-actin (mouse monoclonal, dilution 1:5000; Sigma Aldrich) was added to the membrane, followed by an anti-mouse-immunoglobulin G, anti-rabbit-immunoglobulin G or anti-goat-immunoglobulin G horseradish peroxidase conjugated secondary antibody (dilution 1:20,000; Promega, WI, USA) dissolved in 1% non-fat milk in TBS–Tween-20 buffer. Incubation of 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 SuperSignal 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 determination of the examinated protein/β-actin protein ratio of the immunoreactive area by densitometry.

2.4. Statistical analysis

All values are expressed as mean ± S.E.M. Statistical analysis was determined by two-way analysis of variance (ANOVA) followed by non-parametric Mann–Whitney test. Significance was set at $P < 0.05$.

3. Results

3.1. Effect of pioglitazone on ulcer healing

Fig. 1 shows the effects of pioglitazone given in graded doses ranging from 5 to 40 mg/kg i.g. on the

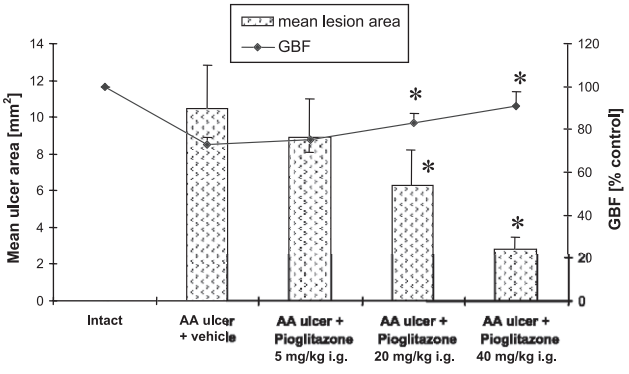

Fig. 1. Effect of vehicle (control group) and increasing doses of pioglitazone (5–40 mg/kg) given i.g. on the mean area of chronic acetic acid (AA) gastric ulcers and the mucosal gastric blood flow (GBF) at the ulcer edge. Means ± S.E.M. of six rats. Asterisk indicates a significant change compared with the value obtained with vehicle.

area of gastric ulcers and gastric mucosal blood flow as determined on day 8 after ulcer induction. Administration of pioglitazone was associated with a dose-dependent acceleration of ulcer healing and this was accompanied by a significant increase in the gastric blood flow at the ulcer margin. The most pronounced inhibition of the ulcer area was observed at a dose of 40 mg/kg of pioglitazone.

3.2. Expression of PPAR-γ mRNA in the normal and ulcerated gastric mucosa

In the intact gastric mucosa, the expression of PPAR-γ mRNA was not detectable. In contrast, in ulcerated gastric mucosa a significant upregulation of PPAR-γ mRNA was observed (Fig. 2).

3.3. Effect of pioglitazone on the mRNA expression of interleukin-1β, TNF-α, cyclooxygenase-1, cyclooxygenase-2, cNOS and iNOS in the gastric mucosa

In intact gastric mucosa, the mRNA expression of TNF-α and interleukin-1β was negligible. In ulcerated mucosa, increased mRNA levels of TNF-α and interleukin-1β were detected and these signals were significantly diminished in a dose-dependent manner by treatment with pioglitazone. Ratio of interleukin-Iβ and TNF-α mRNA to β-actin mRNA revealed that the strongest inhibition of the mRNA expression of TNF-α and interleukin-1β was observed with pioglitazone administered at a dose of 40 mg/kg i.g. At this dose, TNF-α levels were so low that were not detected by RT-PCR method anymore (Fig. 3).

As can be seen in Fig. 4, the expression of cyclooxygenase-1 was detected in the intact gastric mucosa and the induction of gastric ulcer combined with therapy with PPAR-γ ligand did not influence the levels of cyclooxygenase-1 in the gastric mucosa. In contrast, cyclooxygenase-2 was not detected or detected only as a weak signal in the intact gastric mucosa. Induction of experimental ulcers resulted in a significant upregulation of cyclooxygenase-2 expression. Treatment with pioglitazone significantly attenuated the mRNA expression of cyclooxygenase-2, as compared to that in vehicle-treated animals.

Induction of gastric ulcer also induced a significant increase of iNOS mRNA as compared to intact rats, where the signal for iNOS mRNA was very low and almost undetectable (Fig. 5). Treatment with pioglitazone at a dose of 5 mg/kg i.g. caused further significant increase in iNOS mRNA expression, but with administration of this PPAR-γ ligand at higher doses (20 and 40 mg/kg i.g.), a significant

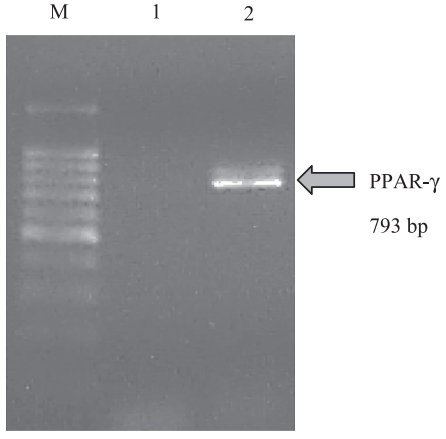

Fig. 2. Representative RT-PCR analysis showing expression of PPAR-γ mRNA in the intact gastric mucosa (lane 1) and at the ulcer edge at day 8 after ulcer induction (lane 2). M—DNA size marker.

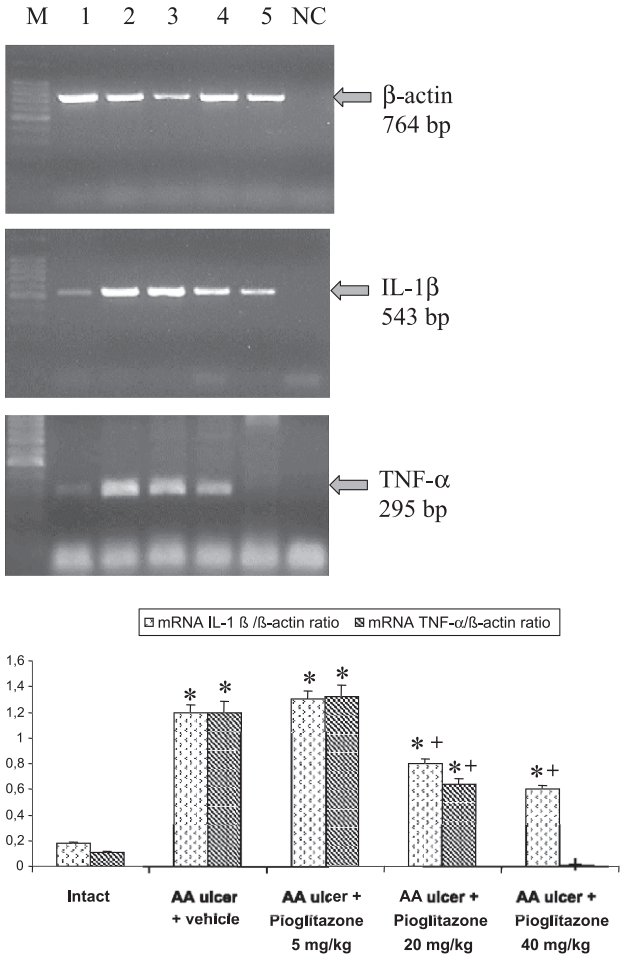

Fig. 3. Expression of mRNA for β-actin, interleukin-1β and TNF-α analyzed by RT-PCR. Lane 1—intact gastric mucosa; lane 2—ulcer + vehicle; lane 3—ulcer + pioglitazone (5 mg/kg i.g.); lane 4—ulcer + pioglitazone (20 mg/kg i.g.); lane 5—ulcer + pioglitazone (40 mg/kg i.g.). M—DNA marker size (upper panel). The ratio of interleukin-1β or TNF-α mRNA to β-actin mRNA in intact mucosa, gastric ulcer + vehicle, ulcer + pioglitazone at a dose of 5, 20 or 40 mg/kg given intragastrically (lower panel). Mean ± S.E.M. of six tests on six rats. Asterisk indicates significant increase above the value obtained in intact gastric mucosa. Cross indicates significant decrease below the value obtained in vehicle-treated rats.

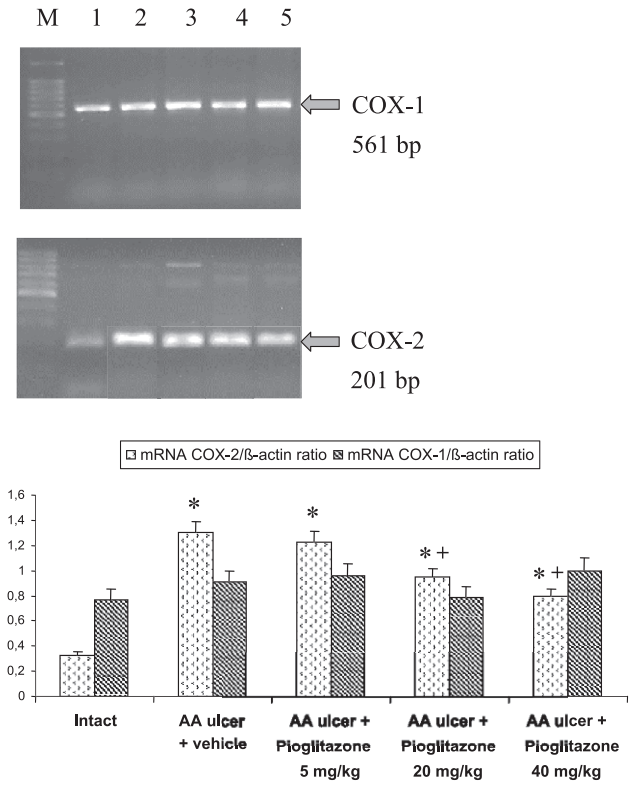

Fig. 4. RT-PCR expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2. Lane 1—intact gastric mucosa; lane 2—ulcer + vehicle; lane 3—ulcer + pioglitazone (5 mg/kg i.g.); lane 4—ulcer + pioglitazone (20 mg/kg i.g.); lane 5—ulcer + pioglitazone (40 mg/kg i.g.). M—DNA marker size (upper panel). The ratio of cyclooxygenase-2 or cyclooxygenase-1 mRNA to β-actin mRNA in the intact gastric mucosa, gastric ulcer + vehicle, ulcer + pioglitazone at a dose of 5, 20 or 40 mg/kg given intragastrically (lower panel). Asterisk indicates significant increase above the value in the intact mucosa. Cross indicates significant decrease below the vehicle control.

inhibition of iNOS mRNA was observed. The signal for cNOS mRNA was significantly decreased in ulcerated mucosa as compared to that detected in intact gastric mucosa. Treatment with a lowest dose of pioglitazone failed to influence significantly the expression of cNOS mRNA, which remained at the decreased level. Semiquantitative ratio of cNOS mRNA over β-actin mRNA revealed that the treatment with higher doses of pioglitazone (20 and 40 mg/kg i.g.) resulted in significant upregulation of cNOS mRNA levels toward the level observed in the intact gastric mucosa (Fig. 5).

3.4. Effect of pioglitazone on the protein expression of cyclooxygenase-2, cNOS and HSP70

As shown in Fig. 6, cyclooxygenase-2 protein expression was not detected in the normal gastric mucosa. Induction of gastric ulcer raised markedly the protein expression of cyclooxygenase-2. Treatment with pioglitazone caused a dramatic decrease in cyclooxygenase-2 protein expression as compared to that observed in ulcerated mucosa without this PPAR-γ treatment. This decrease was observed already at the lowest dose of pioglitazone (5 mg/kg i.g.).

Fig. 7 shows that the high level of cNOS protein expression was detected in intact gastric mucosa, but the induction of gastric ulcer resulted in a dramatic decrease in cNOS protein expression. There was no significant change in cNOS protein expression in rats with gastric ulcers treated with pioglitazone at a lowest dose of 5 mg/kg i.g. as compared with that obtained in vehicle-control animals. Interestingly, a significant increase in gastric mucosal protein expression of cNOS was observed in rats with gastric ulcers and treated with higher doses of pioglitazone (20 and 40 mg/kg) as compared to that detected in vehicle-treated animals without or with pioglitazone applied in lowest dose of 5 mg/kg i.g.

As shown in Fig. 8, there was a low level of HSP70 mRNA and protein expression detected in the intact gastric mucosa. A significant increase in HSP70 mRNA and

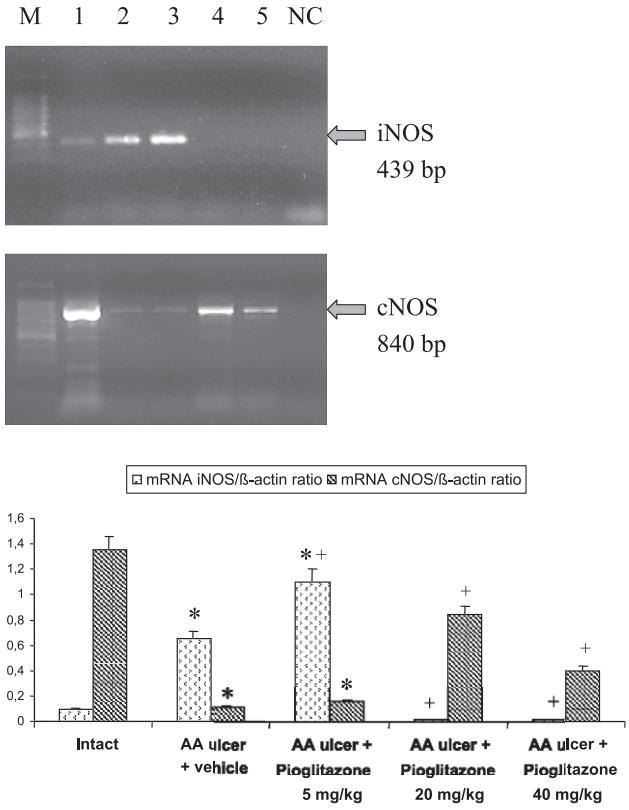

Fig. 5. Expression of mRNA for cNOS and iNOS and analyzed by RT-PCR. Lane 1—intact gastric mucosa; lane 2—ulcer + vehicle; lane 3—ulcer + pioglitazone (5 mg/kg i.g.); lane 4—ulcer + pioglitazone (20 mg/kg i.g.); lane 5—ulcer + pioglitazone (40 mg/kg i.g.). M—DNA marker size (upper panel). The ratio of iNOS or cNOS to β-actin mRNA in the intact gastric mucosa, gastric ulcer + vehicle or gastric ulcer + pioglitazone at a dose of 5, 20 or 40 mg/kg given intragastrically (lower panel). Mean ± S.E.M. of six tests on six rats. Asterisk indicates significant change as compared to intact rats. Cross indicates significant change as compared to vehicle- and pioglitazone (5 mg/kg intragastrically)-treated animals.

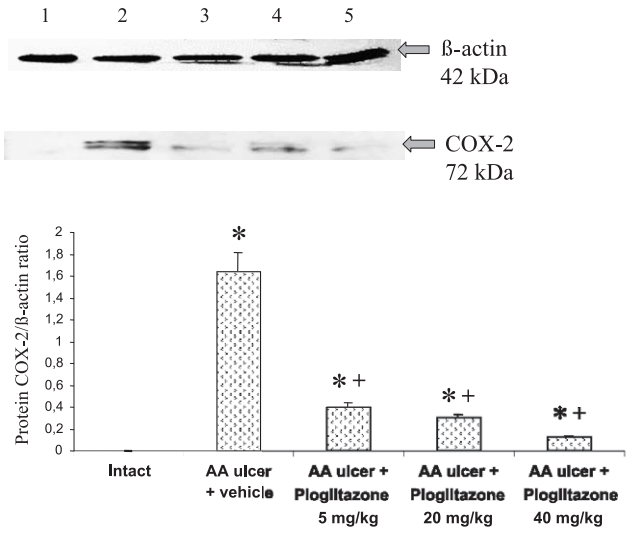

Fig. 6. Expression of protein for cyclooxygenase-2 and β-actin as analyzed by Western blot in the intact gastric mucosa and in rats with chronic gastric ulcers and treated daily with vehicle (control) or pioglitazone (5, 20 or 40 mg/kg intragastrically) (upper panel). Densitometric analysis of the expression of protein for cyclooxygenase-2 and β-actin (ratio) in the intact gastric mucosa and in ulcerated mucosa of rats treated with vehicle (control) or pioglitazone (5, 20 or 40 mg/kg intragastrically) (lower panel). Asterisk indicates significant change of the ratio compared with the value obtained in the intact gastric mucosa. Cross means significant change of the ratio compared with the value obtained in rats with gastric ulcers and treated with vehicle.

protein expression was found in vehicle-treated ulcerated gastric mucosa. Pioglitazone treatment, especially at doses 20 and 40 mg/kg, but not at 5 mg/kg, led to further

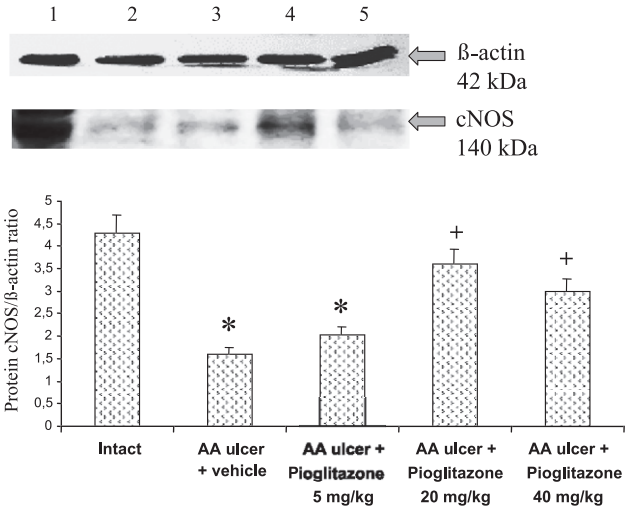

Fig. 7. Expression of protein for cNOS and β-actin as analyzed by Western blot in the intact gastric mucosa and in rats with chronic gastric ulcers and treated daily with vehicle (control) or pioglitazone (5, 20 or 40 mg/kg i.g.) (upper panel). Densitometric analysis of the expression of protein for cNOS and β-actin (ratio) in the intact gastric mucosa and in ulcerated mucosa of rats treated with vehicle (control) or pioglitazone (5, 20 or 40 mg/kg) (lower panel). Asterisk indicates significant change of the ratio compared with the value obtained in the intact gastric mucosa. Cross means significant change of the ratio compared with the value obtained in rats with gastric ulcers and treated with vehicle.

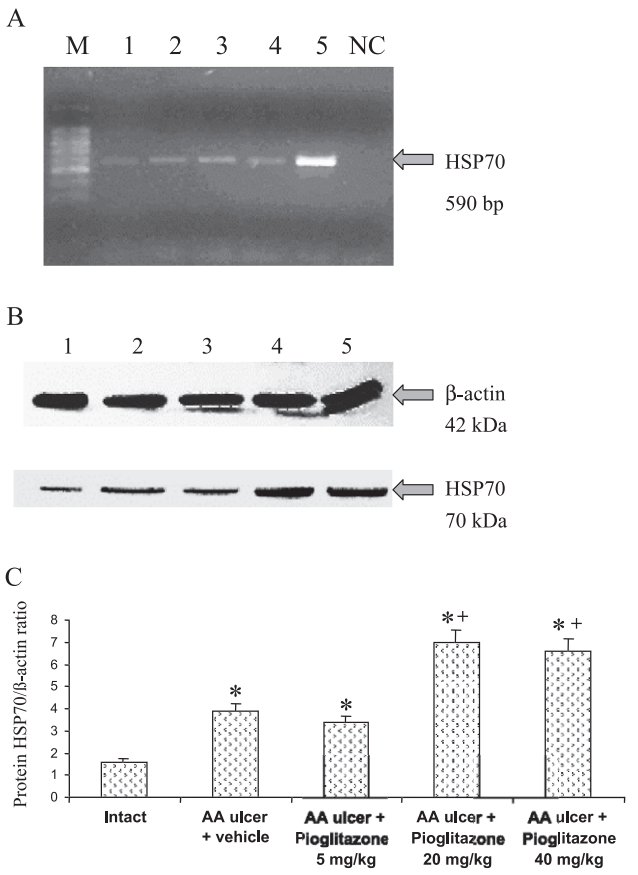

Fig. 8. (A, B, C) Expression of mRNA for HSP70 (A) and protein for HSP70 and β-actin (B) as analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot, respectively, in the intact gastric mucosa and in rats with chronic gastric ulcers and treated daily with vehicle (control) or pioglitazone (5, 20 or 40 mg/kg intragastrically). Densitometric analysis of the protein expression of HSP70 and β-actin (ratio) in the intact gastric mucosa and in ulcerated mucosa of rats treated with vehicle (control) or pioglitazone (5, 20 or 40 mg/kg) (C). Asterisk indicates significant change of the ratio of HSP70 and β-active protein compared with the value obtained in the intact gastric mucosa. Asterisk and cross mean significant change of the ratio HSP70/β-actin protein as compared with the value obtained in vehicle–control rats with gastric ulcers.

increase in expression of HSP70 protein in the gastric mucosa.

4. Discussion

We demonstrated that PPAR-γ is expressed at the margin of gastric ulcer and that pioglitazone, a PPAR-γ ligand, dose-dependently accelerates ulcer healing, the effect being accompanied by increased gastric mucosal blood flow at ulcer margin. These results suggest that PPAR-γ is involved in ulcer healing. This observation is supported by the fact that the mRNA expression for PPAR-γ was significantly upregulated at the ulcer edge compared to the intact gastric mucosa. To our knowledge, this is the first report showing the beneficial effect of PPAR-γ ligand on healing of chronic gastric ulcer.

The exact mechanism of action by which PPAR-γ ligand accelerates ulcer healing remains unknown. One possible mechanism could be the attenuation of gene expression of important proinflammatory cytokines, such as TNF-α and interleukin-1β. These cytokines TNF-α and interleukin-1β are expressed during inflammatory states and may contribute to the delay in ulcer healing via the inhibitory effect on the cell proliferation, angiogenesis and gastric microcirculation at the ulcer margin (Brzozowski et al., 1999; Nakamura et al., 1999; Shimuzu et al., 2000; Jenkinson et al., 2002). In the present study, we observed a dose-dependent decrease in mRNA expression for TNF-α and interleukin-1β in pioglitazone-treated animals and these results are keeping with previous reports showing a pronounced downregulation of inflammatory pathways by PPAR-γ ligands (Jiang et al., 1998; Nakajima et al., 2001). This supports the notion that pioglitazone accelerates ulcer healing via the mechanism involving the decrease in expression and release of interleukin-1β and TNF-α.

Another important mechanism by which pioglitazone accelerates ulcer healing could be its inhibitory effect on the inducible nitric oxide synthase (iNOS). Previous studies revealed that PPAR-γ ligands inhibit the expression of iNOS and gelatinase B, in part, by antagonizing the activities of the transcription factors such as NFκB (Ricote et al., 1988). Moreover, it was shown that 15d-PGI_2, a PPAR ligand, inhibited directly NFκB gene expression by covalent modification of critical residues in IκB kinase and by modification of the DNA-binding domains of NFκB (Straus et al., 2000). Our present data support the finding that pioglitazone dose-dependently inhibited the gastric mRNA expression of iNOS, which was significantly upregulated in the ulcerated mucosa. Decrease in iNOS expression was accompanied by a compensatory increase in cNOS expression. This, again, remains in agreement with the previous reports showing the inhibitory effect of PPAR-γ ligands on the iNOS expression (Ricote et al., 1988; Simonin et al., 2002).

We found that pioglitazone lowered also significantly the protein expression for cyclooxygenase-2 and this could be due to the inhibition of NFκB pathway, which subsequently led to the decrease in cyclooxygenase-2 protein expression. Although we have previously shown that cyclooxygenase-2 is an important for ulcer healing (Brzozowski et al., 2001a), the suppressive effect on cyclooxygenase-2 expression induced by pioglitazone is probably overcome by anti-inflammatory properties of this agent. This is supported by the fact that PPAR-γ ligand suppressed the expression of proinflammatory cytokines such as TNF-α and interleukin-1β that are known to act as a potent cyclooxygenase-2 inducers (Brzozowski et al., 2001b). There is still a lot of controversy considering the role of NFκB in ulcer healing. Our results are contradictory to those obtained by Takahashi et al. (2001) who showed that prevention of NFκB activation in ulcerated mucosa caused impairment of ulcer healing in rats. As a possible mechanism, the authors postulated a downregulation of healing-promoting factors such as cyclooxygenase-2 and iNOS. In our study, the treatment with PPAR-γ agonist decreased strongly cyclooxygenase-2 protein expression, probably as a result of NFκB inhibition, but instead of the impairment of ulcer healing, the acceleration of the healing was observed. It seems very likely that pioglitazone can also function in a receptor-independent manner, thus contributing to the acceleration of ulcer healing. One of the mechanisms could be the stimulation of the expression of inducible heat shock protein (HSP70). Previous reports demonstrated that HSP70 is involved in mucosal regeneration in the course of ulcer healing (Tsukimi et al., 2001) by the observation that HSP70 expression in the ulcer margin was generally increased along with the time course of ulcer healing.

HSP70 has been suggested to exert its cytoprotective and ulcer healing promoting actions by protecting mitochondria and by interfering with the stress-induced apoptotic program (Rokutan, 2000).

Furthermore, the decrease in the expression of inducible HSP70 was observed at ulcer base immediately upon ulcer induction, but this expression returned to the normal level by the end of the healing stage, that is 8–12 days after ulcer induction (Guo et al., 2002). It is likely that anti-inflammatory action of HSP70 that showed enhanced expression in pioglitazone-treated stomach could be mediated via NFκB pathway. This notion is supported by the finding of Yoo et al. (2000), that anti-inflammatory effect of heat shock protein induction in respiratory epithelial cells depends upon the stabilization of IκBα through prevention of IκB kinase activation, both crucial proteins involved in activation of NFκB cascade.

Another important aspect is an angiogenesis, playing a crucial role in ulcer healing (Akimoto et al., 2002). PPAR-γ agonists increase the expression of proangiogenic factors such as vascular endothelial growth factor (VEGF) (Yamakawa et al., 2000). Although we did not determine the effect of pioglitazone on VEGF expression, the previous reports strongly suggest that pioglitazone may accelerate the ulcer healing process via stimulation of angiogenesis. However, this point requires further clarification in future studies.

We conclude that pioglitazone accelerates the healing of preexisting ulcers due to the hyperemia at ulcer margin and the anti-inflammatory action including suppression of proinflammatory cytokines, downregulation of cyclooxygenase-2 and iNOS at the level of mRNA and protein and an overexpression of HSP70, which plays an essential role in the mechanism of gastric defense and ulcer healing.

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