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Involvement of glucocorticoid receptor and peroxisome proliferator activated receptor-γ in pioglitazone mediated chronic gastric ulcer healing in rats

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ABSTRACT

Evidences suggest Peroxisome Proliferator Activated Receptor-gamma (PPAR-γ) ligand, pioglitazone results in the attenuation of gastric mucosal injury. But the molecular mechanism through which these agonists actually elicit gastroprotection through modulating inflammatory responses has not yet been established. Chronic gastric ulcer induced in rats by intraluminal application of acetic acid resulted in elevation of proinflammatory cytokines gene expression, such as, TNF-α (Tumor Necrosis Factor-α), IL-1β (Interleukin-1β) and the protein levels of nuclear p65 subunit of NF-κB (Nuclear Factor-κB) but decreased levels of PPAR-γ gene expression. Pioglitazone treatment reduced the severity of ulceration, repressed levels of TNF-α, IL-1β and nuclear p65 subunit as well as increased the abundance of PPAR-γ in gastric mucosa. Moreover, it significantly upregulated protein levels of glucocorticoid receptor demonstrating its possible involvement in pioglitazone mediated ulcer healing along with PPAR-γ. Administration of pioglitazone reverted back the decreased levels of both PPAR-γ and glucocorticoid receptor, resulting in their redistribution to the nucleus from the cytosol in course of ulcer healing. Moreover, pharmacological inhibition of glucocorticoid receptor function by its antagonist (RU486) inhibited pioglitazone mediated downregulation of TNF-α and IL-1β gene expression confirming involvement of glucocorticoid receptor in pioglitazone mediated ulcer healing. Co-immunoprecipitation studies further established association of PPAR-γ with glucocorticoid receptor during ulcer healing which was enhanced following pioglitazone administration. Thus, the present study is first of its kind bearing direct relevance to the participation of both PPAR-γ and glucocorticoid receptor and their physical association in influencing amelioration of inflammatory responses during pioglitazone mediated gastric ulcer healing.

1. Introduction

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a member of the nuclear receptor superfamily that regulates a number of metabolic processes including lipid metabolism and insulin sensitization, as well as inflammation and cell proliferation (Debril et al., 2001; Fajas et al., 2001). PPAR-γ is predominantly expressed in the adipose tissue and colon, whereas stomach, small intestine, liver and pancreas express lower but significant levels (Braissant et al., 1996). The broad pattern of its distribution indicates its possible involvement in multiple biological processes. Recently, growing evidence implicates PPAR-γ in the regulation of the immune response, particularly in inflammation control and has gained importance as a potential therapeutic target in the management of gastrointestinal inflammation.

Studies have demonstrated that PPAR-γ ligands reduced mucosal damage and prevented the inflammatory response of intestinal inflammation (Slomiany and Slomiany, 2002; Okada et al., 2002). The role of PPAR-γ has also been implicated in the control of gastric mucosal damage induced by ischemia reperfusion injury and chronic gastric ulcer (Konturek et al., 2003a,b). PPAR-γ activation by pharmacological agonists such as thiazolidinedione class of insulin sensitizers, have been shown to exert anti-inflammatory activities in various other cell types by inhibiting the expression of pro-inflammatory genes like nitric oxide (NO), IL-6 and TNF-α (Reilly et al., 2000; Ricote et al., 1998). Other studies have suggested that activation of PPAR-γ can also antagonize NF-κB action in macrophages resulting in the downregulation of proinflammatory cytokines (Wang et al., 2001). In these different animal models, PPAR-γ ligand therapy reduced a wide variety of inflammatory indices but the underlying mechanism by which PPAR-γ activation brings about these effects were not fully established. Thus the present study was designed to elucidate the molecular mechanism of pioglitazone (a thiazolidinedione class of PPAR-γ agonists), mediated amelioration of the inflammatory responses involved in chronic gastric ulcer.

Ligand-bound nuclear receptors, including the PPARs, repress the expression of inflammatory-response genes via a mechanism termed ligand-dependent transrepression (Pascual and Glass, 2006) which involve protein — protein interactions between ligand bound receptors

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and transcription factors or co-regulatory complexes located at the promoter element of the target genes. Glucocorticoid Receptor, another member of the nuclear receptor superfamily, also participates in the regulation of cell proliferation, inflammation and immune responses by modulating the activation of NF-κB (Barnes, 2006). Interestingly, the proinflammatory genes that are repressed by PPAR-γ overlap but are not identical to the genes that are down regulated by glucocorticoid receptor (Ogawa et al., 2005). Therefore, the possibility of involvement of glucocorticoid receptor in pioglitazone mediated chronic ulcer healing was also explored.

Thus, in the present study we investigated the functional relevance and regulation of PPAR-γ in the process of gastric ulcer healing. Since, the molecular mechanisms mediating the anti-inflammatory action of PPAR-γ in gastric ulcer healing are, at present, not fully understood, we tried to elucidate the molecular mechanism through which activation of PPAR-γ results in the modulation of inflammatory responses involved in chronic gastric ulcer. We also tried to delineate the co-operation of another member of the nuclear receptor superfamily, Glucocorticoid Receptor, along with PPAR-γ to repress the inflammatory events during chronic gastric ulcer healing.

2. Materials and methods

2.1. Materials

Chemicals were obtained from M/s. Sigma Chemicals, St Louis, MO, USA unless otherwise mentioned. Antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). Pioglitazone was gifted by Dr. Reddy's Laboratories (India) and Bisphenol A Diglycidyl Ether (BADGE) was obtained from Cayman Chemical (Ann Arbor, Michigan, USA).

2.2. Experimental animals

Adult Sprague Dawley rats of either sex, weighing 180–200 g were housed in environmentally controlled rooms (25 ± 2 °C, 12 h light and dark cycle) in raised bottom mesh cages to prevent coprophagy. Animals were fed with chow pellets and water *ad libitum*. All experimental protocols were approved by the Institutional Ethical Committee following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) which complies with International norms of Indian National Science Academy (INSA).

2.3. Induction of acetic acid induced chronic gastric ulcer

Animals were deprived of food for 18 h before subjecting to ulcerogen but water was provided *ad libitum*. Under pentobarbital anaesthesia (30 mg/kg, i.p.) chronic gastric ulcer was induced by luminal application of acetic acid (40%, 0.2 ml) injected in between the anterior and posterior walls of the stomach as described previously with minor modifications (Okabe and Pfeiffer, 1971). Well defined gastric ulcers developed after 3 days of acetic acid application and the respective treatments were started from this day onwards and continued for a period of 10 days. Sham-operated rats were subjected to the same surgical procedure without application of acetic acid. Animals were sacrificed and stomachs were removed; the ulcerated area (sq mm) was observed under Trinocular zoom microscope and measured using Biovis image analyzer software.

2.4. Treatment schedule

Animals were divided into different groups, each comprising of 6 animals. The respective groups comprised of sham, 3 days and 13 days ulcer control group. Treatment of graded doses of PPAR-γ agonist, pioglitazone (20, 40 and 80 mg/kg, p.o.) and antagonist BADGE (20 mg/kg, p.o) were started from the 3rd day of ulceration and continued for 10 days. In another set of experiment, after induction of ulcer glucocorticoid receptor antagonist, RU 486 (25 mg/kg, p.o.) was administered and in a separate group it was administered 30 min prior to pioglitazone treatment (40 mg/kg, p.o.) for 10 days. All the drugs were administered once daily. Sham operated animals as well as 13 days ulcer control group were provided with vehicle daily.

2.5. Estimation of alteration in gene expression by RT-PCR

Total RNA was extracted from mucosal samples using TRIZOL Reagent (Invitrogen Life Technologies, Germany). cDNA was generated from 5 μg of total RNA using RETROscript kit (Ambion Inc, USA.) following manufacturer's instructions. Genes for PPAR-γ, TNF-α, IL-1β and β-Actin were amplified with specific primer sets as previously described (Konturek et al., 2003b). cDNA samples were annealed at 94 °C (5 min) and amplified for 35 cycles with the following cycling conditions: 94 °C for 1 min; respective annealing temperature for TNF-α — 70 °C, IL-1β — 65 °C, PPAR-γ — 60 °C and β-Actin — 55 °C for 1 min; 72 °C for 1 min followed by a final extension at 72 °C for 10 min and was run on Bioer XP Cycler. PCR products were electrophoresed on a 1.0% agarose gel using 100-bp ladder (Amersham Biosciences, UK) and intensity was measured using Biovis gel documentation software and expressed as relative intensity of PCR-product/β-Actin ratio.

2.6. Isolation of cytosolic and nuclear fraction from gastric tissue and estimation of alteration of NF-κB, PPAR-γ and glucocorticoid receptor protein expression by Western Blot analysis

Cytosolic and nuclear protein extracts were prepared according to the previously described method (Sanchez et al., 1994) separately for each independent experiment. Each independent experiment comprising of different experimental groups was repeated thrice. Briefly, gastric tissues pooled from three animals per experimental group were homogenized in buffer (10 mM HEPES, 1 mM EDTA, 0.5 mM DTT, 0.25 mM PMSF, 50 mM NaF, 2 mM sodium-orthovanadate, and 5 mg/ml of leupeptin and pepstatin). The homogenate was centrifuged (1000 *g* for 10 min) and the supernatant was further centrifuged (100,000 *g* for 1 h) for obtaining the cytosolic fraction. The low speed nuclear pellet was washed twice in homogenization buffer supplemented with 250 mM sucrose. After salt extraction for 1 h it was centrifuged at 8000 *g* for 30 min and the supernatant was considered as the nuclear fraction. Equal amount of protein was quantified (Lowry et al., 1951) and immunoblotted onto Hyperfilm ECL (Amersham Biosciences, UK). The membrane was incubated overnight with goat anti-PPAR-γ, rabbit anti-glucocorticoid receptor and rabbit anti-NF-κB polyclonal primary antibody at a dilution of 1:500, 1:1000 and 1:1000 respectively. After detection with the desired antibodies against the proteins of interest the membrane was stripped with stripping buffer (25 mM Glycine pH 2.0, 2% SDS for 30 min at room temperature) and reprobed overnight with rabbit anti β-actin polyclonal primary antibody at a dilution of 1:500 to confirm equal loading of protein. Further, membrane was probed with corresponding secondary antibodies. Thereafter, chemiluminescence was detected using ECL-Plus detection system (Amersham Biosciences, UK). The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software.

2.7. Interaction of PPAR-γ and glucocorticoid receptor by co-immunoprecipitation studies

For co-immunoprecipitation studies cytosolic and nuclear extracts of 13 days ulcer control and pioglitazone (40 mg/kg) treated gastric tissue (500 μg protein) were precleared with 20 μl of protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After centrifugation, the supernatants were incubated with 2 μg anti-glucocorticoid receptor

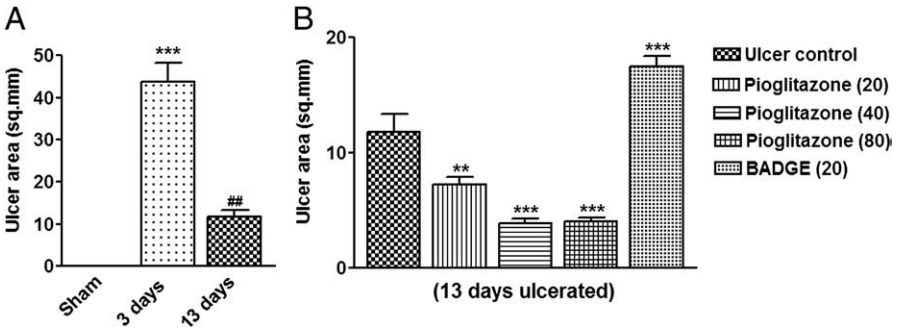

Fig. 1. A. Day dependent alteration in ulcer area induced by intraluminal application of acetic acid. Data expressed as mean ± SEM. *** Statistically significant at $P<0.001$ in comparison to sham on analysis by Kruskal–Wallis non-parametric test and ##$P<0.01$ in comparison to 3 days ulcer control on analysis by Mann–Whitney test. $n=6$ in each group. B. Dose dependent effect of PPAR-γ agonist, pioglitazone and antagonist, BADGE against chronic gastric ulcer. Treatment was started from the third day of ulcer formation and continued for 10 days. Data expressed as mean ± SEM. Statistical analysis was done by One Way ANOVA followed by Newman–Keul's Multiple Comparison Test. ** Statistically significant at $P<0.01$ and ***$P<0.001$ in comparison to 13 days ulcer control. $n=6$ in each group.

rabbit polyclonal antibody or normal rabbit IgG for 4 h at 4 °C followed by overnight incubation with protein A/G-agarose beads. The immune complexes were washed with PBS containing 0.02% Tween 20 and pelleted by centrifugation at 2000 g for 2 min (Kardassis et al., 2002). Expression of PPAR-γ was detected in the resulting immunoprecipitates by Western blot analysis as mentioned previously.

2.8. Statistical analysis

Data are expressed as mean ± SEM. Analysis was performed with Prism version 3.0 software using one-way analysis of variance (ANOVA) followed by Newman–Keul's multiple comparison test, Kruskal–Wallis non-parametric test and Mann Whitney test as mentioned where applicable. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Pioglitazone promotes gastric ulcer healing through PPAR-γ activation

To investigate the role of PPAR-γ in gastric ulcer healing, the effect of specific agonist and antagonist of PPAR-γ (pioglitazone and BADGE respectively) was studied against acetic acid-induced ulceration. As evident from Fig. 1A, significant decrease in ulcer area (11.80 sq mm) was observed in 13 days ulcerated animals ($P<0.01$) when compared to 3 days ulcer control group (43.81 sq mm). Graded doses of pioglitazone (20, 40 and 80 mg/kg, p.o) further potentiated ulcer healing by decreasing ulcer area significantly (7.22, 3.92 and 4.02 sq mm respectively) showing 38.81%, 66.77% and 65.89% protection ($P<0.01$, $P<0.01$ and $P<0.001$), respectively. Therefore, on the basis of the above observation 40 mg/kg was found to be the median effective dose and thus selected for further studies. Moreover, a dose dependent effect (10, 20 and 40 mg/kg, p.o.) of BADGE was also observed (data not shown) from which 20 mg/kg, p.o was identified as the effective dose as mortality was observed at a dose of 40 mg/kg, p.o. There was an aggravation in ulceration (17.52 sq mm) with blockade of PPAR-γ activity by the antagonist, BADGE (20 mg/kg, p.o) [$F(4,25=43.87$] indicating the involvement of PPAR-γ activation in gastric ulcer healing. The results are graphically represented in Fig. 1B.

3.2. Pioglitazone regulates alteration of gene expression of TNF-α, IL-1β and PPAR-γ in gastric mucosa

We next investigated the effect of PPAR-γ activation on proinflammatory gene expression involved in gastric ulceration. As evident from Fig. 2, induction of ulcer caused a significant increase in the expression levels of TNF-α [$F(3,8=78.51$)] and IL-1β [$F(3,8=187.3$)] in 3 day and 13 days ulcer control groups whereas, pioglitazone at a dose of 40 mg/kg, p.o. significantly downregulated the expression of TNF-α ($P<0.01$) and IL-1β ($P<0.01$). Moreover, a significant decrease in the levels of PPAR-γ [$F(3,8=556.2$)]was observed with induction of ulcer in 3 days and also in 13 days ulcer control group. Administration of pioglitazone however, upregulated the expression of PPAR-γ ($P<0.001$) significantly in comparison to 13 days ulcer control group as shown in Fig. 2 suggesting that the induction of this nuclear receptor expression might have mediated amelioration of inflammation during healing of chronic gastric ulcer.

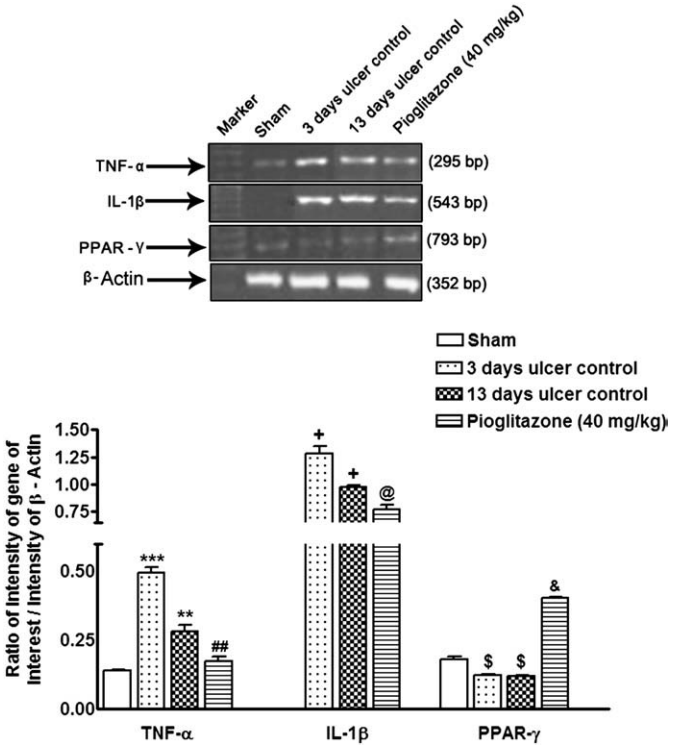

Fig. 2. Alterations in the levels of gene expression of TNF-α, IL-1β and PPAR-γ in gastric mucosa in response to pioglitazone treatment. Effect of pioglitazone (40 mg/kg, p.o.) on the levels of expression of TNF-α, IL-1β and PPAR-γ was analyzed by RT-PCR. Data expressed as mean ± SEM of three separate sets of independent experiments. The results in the histogram are expressed as ratio of relative intensity of gene of interest to β-Actin. Statistical analysis was done by One Way ANOVA followed by Newman–Keul's multiple comparison test. ** Statistically significant at $P<0.01$and ***$P<0.001$ in comparison to sham and ##$P<0.01$ in comparison to 13 days ulcer control for TNF-α; + Statistically significant at $P<0.001$ in comparison to sham and @$P<0.001$ in comparison to 13 days ulcer control for IL-1β; $ Statistically significant at $P<0.001$ in comparison to sham and &$P<0.001$ in comparison to 13 days ulcer control for PPAR-γ.

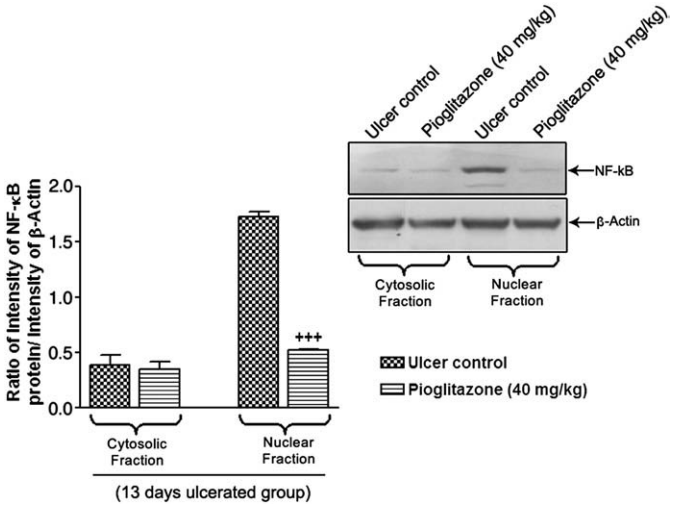

Fig. 3. Effect of pioglitazone on levels of expression of p65 subunit of NF-κB. Western Blot analysis of expression of p65 subunit of NF-κB in cytosolic and nuclear protein extract from 13 days ulcerated and pioglitazone treated gastric tissues. Data expressed as mean ± SEM of three separate sets of independent experiments. The blots are representative and the results in the histogram are expressed as ratio of relative intensity of levels of protein expression of p65 subunit of NF-κB to β-Actin. Statistical analysis was done by One Way ANOVA followed by Newman–Keul's multiple comparison test. +++Statistically significant at $P<0.001$ in comparison to 13 days ulcer control in nuclear fraction.

3.3. Pioglitazone downregulates nuclear levels of p65 subunit of NF-κB during gastric ulcer healing

In order to delineate whether the anti-inflammatory effect of pioglitazone is mediated through inhibition of NF-κB activation, the expression levels of the p65 subunit of this transcription factor was observed in both the cytosolic and nuclear fractions of gastric mucosa isolated from 13 days ulcer control and pioglitazone treated group. A significant decrease in the levels of expression of p65 subunit [$F(3,8 = 118.5)$] by pioglitazone treatment in the nuclear fraction was observed in comparison to 13 days ulcer control group whereas, no significant change in the levels of expression was observed in the cytosolic fraction of both the control and pioglitazone treated groups as demonstrated in Fig. 3. This observation led us to speculate that pioglitazone might not inhibit translocation of p65 subunit from the cytosolic to the nuclear fraction, rather it might be facilitating nuclear degradation of p65 subunit resulting in the inhibition of NF-κB mediated response.

3.4. Pioglitazone leads to the redistribution of PPAR-γ from cytosolic to the nuclear fractions

We hypothesized that PPAR-γ, like other nuclear receptors, existed in both the cytosolic and nuclear compartments and that ligand activation by pioglitazone might enrich the nuclear content of PPAR-γ. So in order to gain insight into the activation status of the receptor, distribution of PPAR-γ in the cytosolic and nuclear fractions in course of ulcer healing was observed. Western Blot analysis of cytosolic and nuclear protein extract from normal gastric mucosa indicated that PPAR-γ protein was distributed evenly between the cytosol and nucleus. There was a significant decrease in the expression of PPAR-γ [$F(3,8 = 7.0)$] in 3 days ($P<0.05$) and 13 days ($P<0.05$) ulcer control groups when compared to sham in the cytosolic fraction. However, treatment with pioglitazone (40 mg/kg, p.o.) significantly ($P<0.05$) up regulated PPAR-γ receptor expression in comparison to the 13 days ulcer control group as evident from Fig. 4.

In the nuclear fraction, during ulceration the expression of PPAR-γ [$F(3,8 = 37.56)$] was significantly decreased in 3days ($P<0.001$) and 13 days ulcer control group ($P<0.001$). Pioglitazone administration for

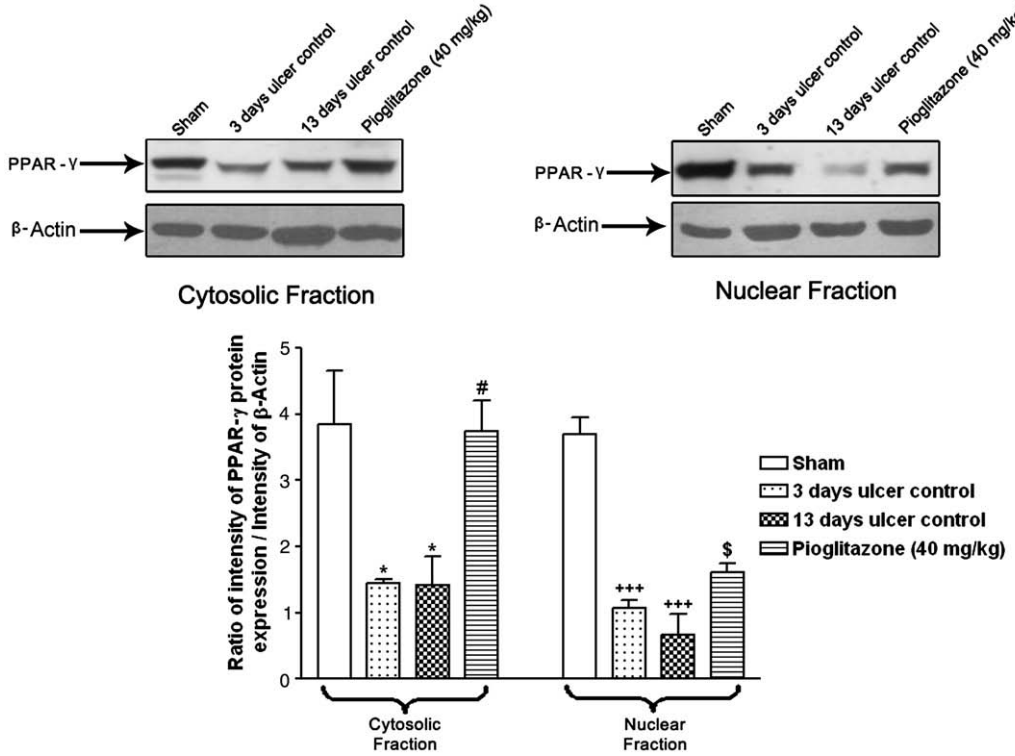

Fig. 4. Effect of pioglitazone on expression of PPAR-γ in cytosolic and nuclear fraction. Pioglitazone mediated alteration in expression of PPAR-γ was observed by Western Blot analysis in cytosolic and nuclear fraction. Data expressed as mean ± SEM of three separate sets of independent experiments. The blots are representative and the results in the histogram are expressed as ratio of relative intensity of levels of protein expression of PPAR-γ to β-Actin. Statistical analysis was done by One Way ANOVA followed by Newman–Keul's multiple comparison test. * Statistically significant at $P<0.05$ in comparison to sham and #$P<0.05$ in comparison to 13 days ulcer control in cytosolic fraction. In nuclear fraction, +++ statistically significant at $P<0.001$ in comparison to sham and \$$P<0.05$ in comparison to 13 days ulcer control.

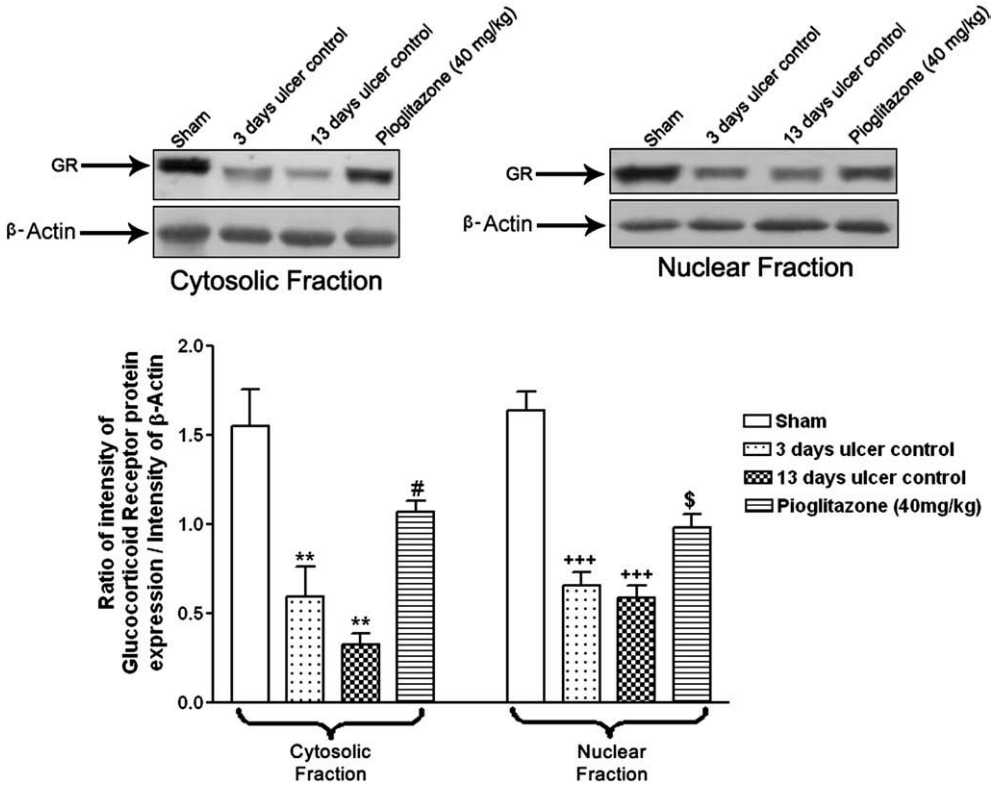

Fig. 5. Effect of pioglitazone on expression of Glucocorticoid Receptor in cytosolic and nuclear fraction. Cellular fractionation studies were performed to observe the changes in levels of protein expression of Glucocorticoid Receptor on administration of pioglitzone. Data expressed as mean ± SEM of three separate sets of independent experiments. The blots are representative and the results in the histogram are expressed as ratio of relative intensity of levels of protein expression of Glucocorticoid Receptor to β-Actin. Statistical analysis was done by One Way ANOVA followed by Newman–Keul's multiple comparison tests. In cytosolic fraction, ** statistically significant at $P<0.01$ in comparison to sham and #$P<0.05$ in comparison to 13 days ulcer control. +++ Statistically significant at $P<0.001$ in comparison to sham and \$$P<0.05$ in comparison to 13 days ulcer control in nuclear fraction.

10 days significantly upregulated ($P<0.05$) receptor expression in comparison to the 13 day ulcerated group as shown in Fig. 4 indicating the involvement of activation of the receptor in progression of ulcer healing.

3.5. Pioglitazone leads to the translocation of glucocorticoid receptor from the cytosolic to the nuclear fraction

We then considered the possibility of glucocorticoid receptor involvement in pioglitazone mediated gastric ulcer healing. A significant decrease in the expression level of glucocorticoid receptor [$F(3,8=14.68)$] in the cytosolic fraction was also observed in 3 days ($P<0.01$) and 13 days ulcer control group ($P<0.01$). However, expression of the receptor ($P<0.05$) increased significantly with pioglitazone treatment as clearly evident from Fig. 5.

In the nuclear fraction [$F(3,8=33.53)$], there was a significant decrease in the expression of the receptor in 3days ($P<0.001$) and 13 days ulcer control group ($P<0.001$). Significant upregulation of glucocorticoid receptor expression ($P<0.05$) was observed in animals treated with pioglitazone when compared with 13 days ulcer control group (Fig. 5) indicating the possible activation of this receptor in promoting gastric ulcer healing by pioglitazone.

3.6. Glucocorticoid receptor antagonist (RU 486) failed to inhibit the expression of TNF-α and IL-1β during chronic ulcer healing

Apart from PPAR-γ, involvement of glucocorticoid receptor activation in pioglitazone mediated ulcer healing was further confirmed by studies with glucocorticoid receptor specific antagonist RU486. Treatment with RU 486 (25 mg/kg, p.o) for 10 days showed no significant effect on TNF-α [$F(3,8=13.65)$] and IL-1β [$F(3,8=7.44)$] gene expression in ulcerated condition whereas, pioglitazone (40 mg/kg, p.o) significantly suppressed the levels of expression of these proinflammatory cytokines. Moreover administration of RU 486 (25 mg/kg, p.o) prior to pioglitazone treatment abolished the effect of pioglitazone as it failed to revert back the increased expression of TNF-α and IL-1β in ulcerated condition as evident from Fig. 6.

3.7. Association of PPAR-γ and glucocorticoid receptor during ulcer healing

Further, to confirm whether physical association of PPAR-γ and glucocorticoid receptor is involved in promoting ulcer healing mediated by pioglitazone, co-immunoprecipitation studies were performed. Association between PPAR-γ and glucocorticoid receptor in both the cytosolic and nuclear fraction of 13 days ulcer control and pioglitazone treated groups were observed but a strong association was detected in the nuclear fraction rather than the cytosolic counterpart as evident from Fig. 7. Further, administration of pioglitazone enhanced the interaction between nuclear PPAR-γ and glucocorticoid receptor in comparison to the ulcer control group. However, their association in the cytosolic fraction remained unaltered despite ligand activation signifying possible interaction between the two receptors in activated condition.

4. Discussion

Regulation of inflammatory response is an essential element in the pathogenesis of a variety of inflammatory disorders including chronic gastric ulcer. Amelioration of these inflammatory events might accelerate healing of chronic gastric ulcer. Thus, we hypothesized that modulation of PPAR-γ activation might promote resolution of inflammation during chronic gastric ulcer healing. In this regard the functional relevance of PPAR-γ in the process of gastric ulcer healing was investigated by pharmacological manipulation of receptor

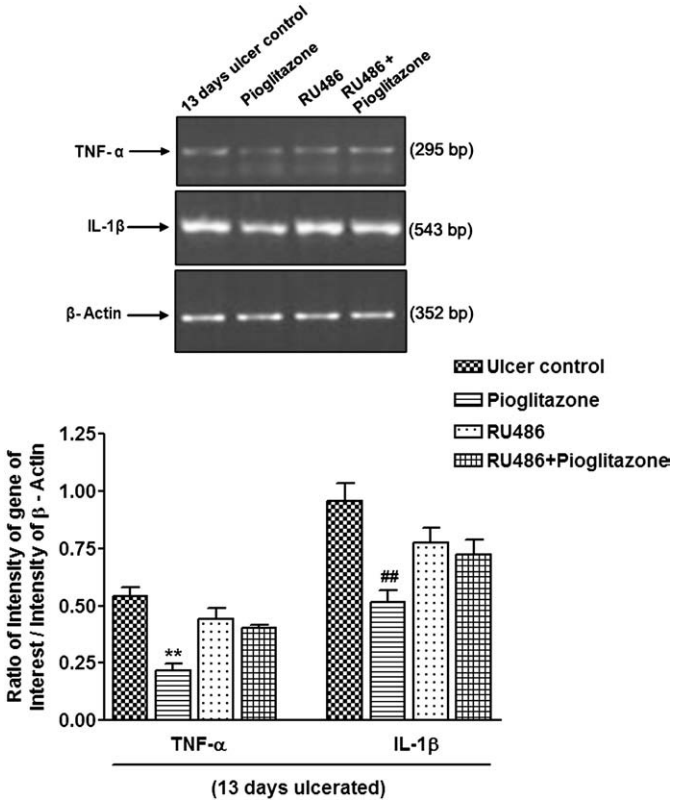

Fig. 6. Effect of Glucocorticoid Receptor antagonist (RU486) on expression of TNF-α and IL-1β. RU 486 (25 mg/kg, p.o.) was administered alone and in a separate group it was administered 30 min prior to pioglitazone treatment (40 mg/kg, p.o.) for 10 days after ulcer induction. Data expressed as mean ± SEM of three separate sets of independent experiments. The results in the histogram are expressed as ratio of relative intensity of gene of interest to β-Actin. Statistical analysis was done by One Way ANOVA followed by Newman–Keul's multiple comparison test. **Statistically significant at $P<0.01$ in comparison to 13 days ulcer control for TNF-α and ## for IL-1β.

activation by pioglitazone, a selective agonist of PPAR-γ. Pioglitazone is a member of the thiazolidinedione class of insulin-sensitizing drugs which are used in the management of type II diabetes but recent evidences suggest that these PPAR-γ agonists may function as potent anti-inflammatory agents. However, the underlying molecular mechanisms by which PPAR-γ agonists bring about gastroprotective effects are not fully understood. So, in this study we also tried to delineate the molecular mechanism through which pioglitazone mediates chronic gastric ulcer healing. The present study is the first to demonstrate the participation of glucocorticoid receptor in pioglitazone mediated resolution of inflammatory responses during chronic gastric ulcer healing in which glucocorticoid receptor functionally interacts with PPAR-γ in a ligand dependent fashion to enhance gastric ulcer healing.

Inflammation of the mucosal layer is a feature almost always associated with ulceration of gastric tissues. Administration of pioglitazone significantly augmented mucosal healing by decreasing ulcer lesions and its ulcer healing effect was reversed by the blockade of PPAR-γ activation by its antagonist BADGE. Ulceration led to induction of gene expression levels of proinflammatory cytokines, like, TNF-α and IL-1β and along with endogenous healing their levels were decreased after 10 days. Pioglitazone further reduced the levels of these proinflammatory cytokines in the ulcerated tissue thereby indicating to the involvement of PPAR-γ in modulating inflammatory mediators associated with chronic ulceration. This is in accordance with a previous study (Brzozowski et al., 2005) which also showed downregulation of the inflammatory responses mediated by pioglitazone through attenuation of the expression and release of pro-inflammatory cytokines and thus exhibiting gastroprotective effects. Furthermore, PPAR-γ expression might be altered during inflammatory or disease states (Katayama et al., 2003). Decreased expression of PPAR-γ was also observed in ulcerated condition. This suppression of receptor expression might in turn be related to high tissue levels of TNF-α as elevated TNF-α levels has been shown to significantly down regulate PPAR-γ gene expression in mature adipocytes (Tanaka et al., 1999).We observed that pioglitazone induced the expression of PPAR-γ in ulcerated gastric tissues where the levels of this nuclear receptor are not sufficient. It seems that the inductive effect of these ligands occur in tissues or under conditions where the existing levels of this nuclear receptor is low. Similar inductive action of thiazolidinediones on PPAR-γ expression has been shown in hepatic tissue by troglitazone (Davies et al., 1999) but the precise mechanism of how these ligands induce expression of its own receptor is not clear. We suggest that the decrease in PPAR-γ expression might be the cause of delayed ulcer healing in which inflammatory cytokines have an important role. In this study we have shown that pioglitazone strongly induced PPAR-γ gene expression that had been suppressed due to ulceration. The increase in PPAR-γ expression might allow for sufficient levels of this receptor to influence the regulation of the entire array of its target genes thus explaining one of the possible mechanisms for resolution of inflammatory responses involved in healing of gastric ulcer by pioglitazone.

One of the most important signaling pathways regulating expression of pro-inflammatory genes involves NF-κB and so in order to confirm whether the anti-inflammatory activity of PPAR-γ agonists is mediated by antagonizing the activation of NF-κB, we observed the translocation pattern of this transcription factor from the cytoplasmic to the nuclear fraction in course of ulcer healing. In the present study, on 10 days of ulceration nuclear translocation of p65 subunit of NF-κB was observed, whereas, pioglitazone administration significantly reduced the nuclear levels of expression of p65 subunit. But, the cytosolic fraction did not show any change in the abundance of p65 expression levels after treatment with pioglitazone in comparison to ulcer control. This suggests that pioglitazone might not inhibit nuclear translocation of NF-κB otherwise, pioglitazone mediated inhibition of nuclear translocation of p65 subunit would have resulted in a substantial increase in its levels in the cytosolic fraction. This strongly indicates a possibility of degradation of the nuclear p65 levels by pioglitazone during chronic ulcer healing. Recent studies have also shown post-induction repression of NF-κB activity through degradation of p65/RelA by proteasome in the nucleus in a DNA-binding dependent manner which actively promotes transcriptional termination (Tanaka et al., 2007; Saccani et al., 2004). However, other additional mechanisms might also be involved in pioglitazone mediated termination of NF-κB response. The anti-inflammatory activity of PPAR-γ agonists have previously been shown to be mediated by antagonizing the activation of this transcription factor through direct interaction of PPAR-γ with subunits p65, p50 or both of NF-κB (Chung et al., 2000) or inhibition of IκB protein degradation (Feinstein et al., 2002). Thus, our observations suggest that pioglitazone results in the termination of NF-κB activation which in turn prevents the induction of inflammatory response genes involved in gastric ulceration.

PPAR-γ is a member of the nuclear-receptor superfamily. In the inactivated state, these nuclear receptors are considered to be complexes, bound with cytosolic co-repressor proteins. Upon ligand mediated activation, PPAR-γ dissociate from the co-repressors followed by recruitment of co-activators leading to its nuclear translocation (Zhu et al., 1996). In the present context, alteration in the pattern of PPAR-γ activation was characterized by observing the translocation of PPAR-γ from the cytosolic to the nuclear fraction in both ulcerated and pioglitazone treated groups. PPAR-γ existed in both the cytosolic and nuclear compartments in normal gastric mucosa but during ulceration a decrease in the receptor expression was observed in both the compartments. However with progression of ulcer healing levels of

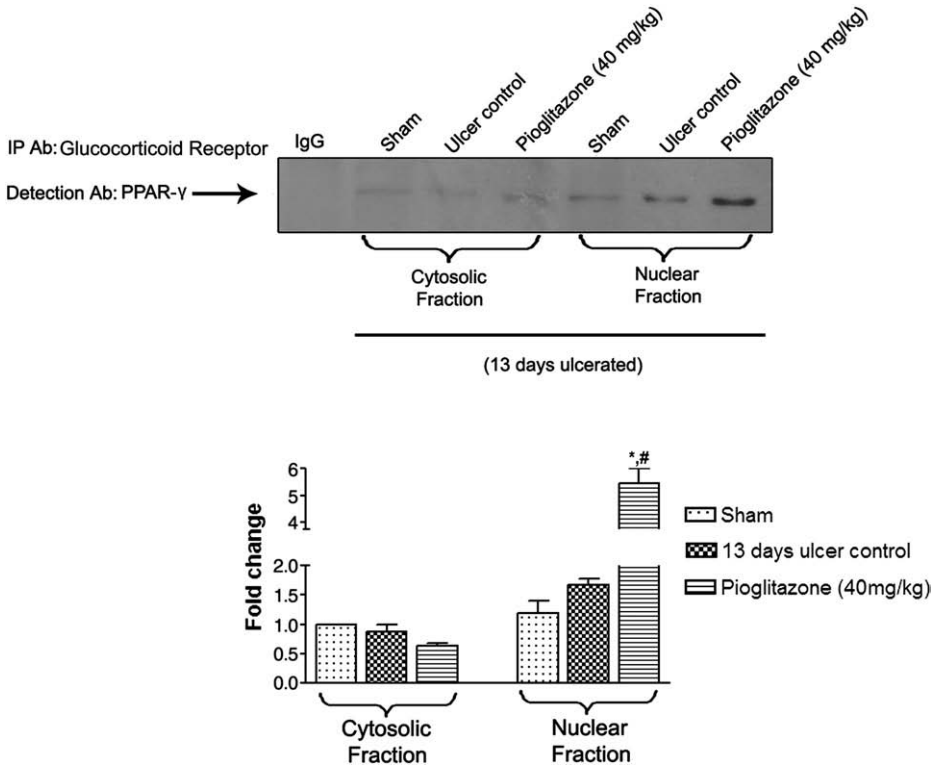

Fig. 7. Physical association of PPAR-γ and Glucocorticoid Receptor in the cytosolic and nuclear fraction of gastric tissues during ulcer healing by pioglitazone. Immunoprecipitation (IP) of glucocorticoid receptor was performed in the cytosolic and nuclear fractions with antibody against the receptor. Level of expression of PPAR-γ was detected in the immunoprecipitated samples. Data expressed as mean ± SEM of three separate sets of independent experiments. The blot is representative and the results in the histogram are expressed as fold change in the relative intensity of expression of PPAR-γ in comparison to sham in samples immunoprecipitated with Glucocorticoid receptor. Statistical analysis was done by One Way ANOVA followed by Newman–Keul's multiple comparison test. In nuclear fraction, *Statistically significant at $P<0.001$ in comparison to 13 days ulcer control and # statistically significant at $P<0.001$ in comparison to 13 days pioglitazone treated group in cytosolic fraction.

PPAR-γ expression was upregulated in the cytosolic fraction by pioglitazone treatment which increased the protein levels of PPAR-γ in a significant proportion in the nuclear fraction and in the cytosolic fraction as well. This indicates that pioglitazone treatment might activate PPAR-γ during chronic ulcer healing as evident from its distribution pattern to the nuclear compartment.

PPAR-γ can also modulate gene expression through positive or negative interference with the activity of other transcription factors, a mechanism generally referred to as "transcriptional crosstalk". Our study reports for the first time that apart from activation of PPAR-γ, gastroprotective effects mediated by pioglitazone also involves activation of glucocorticoid receptor during chronic gastric ulcer healing. Following ligand-receptor coupling, the ligand-glucocorticoid receptor complex translocates to the nucleus in a manner similar to PPAR-γ, leading to the induction or repression of target genes (Adcock, 2001). The cellular fractionation studies indicated that a significant level of glucocorticoid receptor was located in both the cytosolic and nuclear fraction of normal gastric mucosa. But with induction of ulcer a significant decrease in the levels of glucocorticoid receptor protein was observed in both of these compartments. When treated with pioglitazone, the levels of expression of glucocorticoid receptor increased in the cytosolic fraction. The increase did not restrict to the cytosolic counterpart but a significant level of glucocorticoid receptor appeared to increase in the nucleus too thereby indicating a possible role of activation of glucocorticoid receptor in pioglitazone mediated ulcer healing. Since, PPAR-γ and glucocorticoid receptor possess highly conserved ligand-binding domain essential for their transcriptional regulatory effects (Glass et al., 1997), a study was performed which confirmed that pioglitazone did not alter glucocorticoid receptor expression *per se* (data not included).

The involvement of glucocorticoid receptor in the regulation of inflammatory responses by pioglitazone in course of ulcer healing was further established by pharmacological inhibition of glucocorticoid receptor dependent actions by its antagonist RU 486 which resulted in sustained expression of TNF-α and IL-1β as observed with ulcerated condition. Furthermore, pioglitazone treatment coupled with glucocorticoid receptor antagonist failed to repress the elevated levels of expression of these proinflammatory mediators during ulcer healing. This indicates that pioglitazone facilitates resolution of chronic gastric ulcer through activation of endogenous glucocorticoid receptor along with PPAR-γ. Moreover, studies have also demonstrated the gastroprotective effects of endogenous glucocorticoids against the formation of chronic gastric ulcer (Filaretova et al., 2002) and the transcriptional activity of glucocorticoid receptor is known to be modulated by these glucocorticoids.

It is known that members of the nuclear-hormone-receptor super family, including PPAR-γ and glucocorticoid receptor, once activated by an agonist, can interact physically and modulate target gene transcription (Nie et al., 2005; Ialenti et al., 2005). In the present study we have seen strong association of PPAR-γ with glucocorticoid receptor in the nuclear fraction during ulcer healing which was further potentiated by pioglitazone. This signifies that the interaction between the two signaling pathways might be in an activated condition as their association is not pronounced in the cytosolic compartments even after administration of pioglitazone. It might be possible that this interaction between activated PPAR-γ and glucocorticoid receptor can result in an enhancement of the transcriptional activities of the individual interacting proteins leading to the repression of the inflammatory mediators involved in gastric ulcer. Moreover, it is also pertinent to mention that PPAR-γ and glucocorticoid receptor might interact with each other by possible sharing of the same co activators like the steroid receptor coactivator-1 (SRC-1) and cAMP-response element-binding protein-binding protein (CREB) (Needham et al., 2000; Kodera et al., 2000). The interaction of these

two signaling pathways demonstrated in our studies might have direct relevance to the mechanism through which pioglitazone is imparting ulcer healing effects.

Therefore, we conclude that pioglitazone suppressed the expression of inflammatory mediators like, TNF-α and IL-1β during chronic gastric ulcer healing ultimately leading to the repression of NF-κB transcriptional activity. This might be mediated through the combined upregulation and interaction of PPAR-γ and glucocorticoid receptor resulting in amelioration of gastric ulcer. Thus, PPAR-γ along with the co-operation of glucocorticoid receptor might repress overlapping distinct subsets of inflammatory response genes during chronic gastric ulcer healing. The physical association of PPAR-γ and glucocorticoid receptor further offers an insight to the molecular mechanism of action of PPAR-γ agonists in promoting gastric ulcer healing. Thus, understanding of the biological roles of these nuclear receptors in inflammatory responses will help us to exploit their potential for therapeutic intervention as gastric anti-inflammatory targets.

Conflict of interest statement

The authors declare no conflict of interest.

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