INVOLVEMENT OF PPAR- γ ACTIVATION IN NO-MEDIATED GASTRIC ULCER HEALING IN RATS

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ABSTRACT

The present study aimed to investigate the involvement of PPAR-γ activation in the protection against stress-induced ulcer and underlying mechanism(s). Rats were randomly assigned into five groups; vehicle, PPAR-γ agonist (rosiglitazone), either alone or in combination with PPAR-γ antagonist (BADGE) or a nitric oxide synthase inhibitor (L-NAME) pretreated groups (the pretreatment regimen was one week), in addition to control group. Gastric lesions were induced by cold restraint stress (CRS) and ulcer indices were determined. Serum TNF-α level, gastric juice parameters, mucosal content of (lipid peroxides "MDA", total nitrite/nitrates "NO$_x$", superoxide dismutase "SOD" and catalase) were determined in addition to histopathological examination. It was found that rosiglitazone proved to be protective against development of ulcerative lesions as evidenced by significant improvement of stomach histology and reduction in ulcer index, gastric juice acidity, MDA, SOD and serum TNF-α levels, with concomitant increases in mucin concentrations, gastric mucosal total NO$_x^-$ content and catalase activity. Concurrent administration of either BADGE or L-NAME with rosiglitazone abolished the protective effect of this PPAR-γ agonist on gastric ulceration.
This protective effect seems to be mediated via NO production that may contribute to the observed antisecretory, antioxidant and anti-inflammatory actions of PPAR- γ ligand.

Keywords: Gastric ulcer, PPARγ; NO; TNF-α, Antioxidants.

INTRODUCTION

Stress-related mucosal bleeding is a significant cause of morbidity and mortality in critically ill patients [1]. Stress induces acute gastric mucosal lesions by complex psychological factors lead to decrease blood flow to the mucosa, increase in muscular contractility, leukocyte activation and increased free radical generation [2].

A sudden reduction in gastric blood flow and increased free radical formation play fundamental roles in ulcer production [3]. Nitric oxide (NO) is the main regulator of gastric blood flow that participates in maintenance of mucosal integrity [4]. Reactive oxygen species (ROS) play a major role in oxidative damage of mucosa produced in all types of ulcer including stress-induced gastric ulcer [5]. ROS are involved in the formation of gastric mucosal damage was due to an enhancing effect on lipid peroxidation and attenuation of mucosal antioxidative mechanisms [6]. It was proposed that the ulcerogenesis possibly depend upon the interplay between ROS generation and NO action [7].

Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a member of the nuclear hormone receptor superfamily whose activation has been linked to transcriptional control of numerous cellular processes and various cytokies [8]. PPAR-gamma activation may modulate the production of inflammatory cytokines such as TNF-α and might control immune cell differentiation and function [9]. Moreover, several studies have shown that PPAR-γ activation have cytoprotective and antioxidant activities [10].

Although, PPAR-γ is shown to be expressed in several tissues and cells including gastrointestinal tract [11] and gastric epithelial cells [12], there are little information about the gastroprotective effect of PPAR-γ activation in stress-induced ulcer. Therefore this study was conducted in a trial to support the gastroprotective role of PPAR-γ activation in stress-induced ulcer and to investigate the underlying mechanism(s) through which this effect is mediated. Moreover, this study was designed to determine the relation between the proposed protective effect of PPAR- γ activation and NO pathway in the context of CRS-induced gastric ulcer.

MATERIALS AND METHODS

Animals

Forty wistar male albino rats weighing 150–200 g were used after acclimatization for a period of 2 weeks. They were kept under constant environmental conditions and were exposed to 12 hours dark/light cycle while food and water were available.

Experiments were conducted in accordance with the international ethical guidelines for animal care of the United States Naval Medical Research Centre, Unit No. 3, Abbaseya, Cairo, Egypt, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC international). The adopted guidelines are in accordance with "Principles of Laboratory Animals Care" (NIH publication No. 85-23, revised 1985). The study protocol was approved by members of "The Research Ethics Committee" and by the head of Pharmacology and Toxicology Department, Faculty of Pharmacy, Minia University, Egypt.

Chemicals

Rosiglitazone was a kind gift from GlaxoSmithKline Inc. (Mississauga, Canada). BADGE and L-NAME were obtained from Sigma Chemical (St Louis, MO, USA). All other chemicals were obtained of high purity from commercial sources.

Induction of gastric ulceration

At the end of the experiment, Rats were deprived of food for 24 h prior to the experiment in mesh-bottomed cages to minimize coprophagia but allowed free access to water except for the last hour before the experiment. Pyloric ligation was carried out under light ether anesthesia according to a previous method described by **[13]**. After pyloric ligation, the animals were restrained by fixing the four limbs to a wooden board and placed in a refrigerator at 4°C for 3 hours to induce CRS ulcer **[14]**. All experiments were performed at the same time of the day to avoid variations due to diurnal rhythms of putative regulators of gastric functions.

Experimental procedures

Rats were divided randomly into 5 groups (8 rats each) as the following:

I. Control (non-stressed) group; in which rats received 0.5% CMC orally (the vehicle of drugs) for one week and left freely wandering in their cages for 3 hours after being subjected to pyloric ligation.

II.   CRS group; in which rats received 0.5% CMC orally for one week then, subjected to 3hours of CRS after pyloric ligation.

III.   CRS + Rosi group; in which rats were pretreated with the PPARγ agonist, rosiglitazone (3 mg/kg/day, orally) for one week [15]. Then, subjected to 3hours of CRS after pyloric ligation.

IV.   CRS + BADGE+ Rosi group; in which rats were concurrently pretreated with the PPARγ antagonist, BADGE (30 mg/kg/day orally) [16] and rosiglitazone (3 mg/kg/day, orally) for one week then, subjected to 3hours of CRS after pyloric ligation .

V.   CRS + L-NAME group; in which rats were concurrently pretreated with the NO synthase inhibitor, L-NAME (50 mg/kg/day) [17] and rosiglitazone (3 mg/kg/day) orally for one week then subjected to 3hours of CRS after pyloric ligation.

After completion of 3 hours of CRS, rats were sacrificed under ether anesthesia, and blood samples were taken from the jugular vein. The stomachs were removed, opened along the greater curvature and the gastric content of each stomach was collected. The stomachs were washed with ice-cold saline and examined for gross gastric mucosal lesions using a magnified lens by an observer not aware of the experiment.

Assessment of gastric mucosal lesions

The gastric mucosal lesions were expressed in the form of ulcer index (U.I.) according a method previously described [18].

Histological examination

Stomachs tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological examination using light microscopy.

Analysis of the gastric juice

The gastric juice collected after opening the stomachs was centrifuged at 1000 g for 10 minutes to remove any solid debris and the volume of the supernatant was measured. The supernatant was then analyzed for the following:

Determination of free and total acid outputs (FAO & TAO) of the gastric juice

The free acidity was determined by titration of a given volume of the gastric juice against 0.1N sodium hydroxide up to 5.5 as guided by a pH meter. The total acidity was determined by completing the titration in the above procedure for determining free acidity to pH 7 as guided by the pH meter [19]. Free and total acid outputs were calculated by multiplying the respective acid concentration by the volume collected at the end of the experiment and was expressed as mEq/3h [20].

Determination of the proteolytic activity and mucins in gastric juice

The proteolytic activity and mucins can be determined colorimetrically according previously described methods [21, 22], respectively.

Biochemical analysis of gastric mucosa

Stomachs were scraped, homogenized in cold potassium phosphate buffer solution (PBS) (0.05 M, pH 7.4) and centrifuged at 2000 rpm for 10 minutes at 4°C. The supernatants were kept at -80°C for performing the following analysis.

Determination of gastric mucosal total nitrite/nitrate (total NO_x^-) level:

A commercially available kit (Biodiagnostic, Egypt) was used for the colorimetric determination of total NO_x^- level at 540 nm (as an indicator of NO) in gastric mucosal homogenate based on a previously described method [23].

Determination of gastric mucosal lipid peroxides

Malondialdehyde (MDA) levels in the gastric mucosa were determined as an indicator of lipid peroxidation by the thiobarbituric acid method according to a previously described method [24].

Determination of gastric mucosal antioxidant (Superoxide dismutase and catalase) activities:

Superoxide dismutase (SOD) and catalase (CAT) enzymes activities were determined using commercially available kits following the instructions of the manufacturer (Biodiagnostic, Egypt) and based on previously described colorimetric methods [25, 26] .

Determination of serum TNF-α level

Serum TNF-α concentration was measured by enzyme-linked immunosorbent assay (ELISA) using rat TNF-α assay kit (Biosource, USA) following the manufacturer's instructions and based on a previously described method [27].

Statistical Analysis

The data are expressed as means ± S.E.M Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramar post analysis test for multiple comparisons with P < 0.05 being considered as statistically significant.

RESULTS

Effect of CRS and various pretreatments on gastric mucosal lesions formation

Cold restraint stress induced a remarkably high ulcer index compared to the control group. Pretreatment of rats with the PPAR-γ agonist, rosiglitazone significantly reduced the intensity of CRS-induced ulcers and profoundly decreased the ulcer index score compared to CRS non-treated group. Concurrent administration of either the PPARγ antagonist, BADGE or the NOS inhibitor, L-NAME with rosiglitazone completely abolished the gastroprotective effect afforded by rosiglitazone alone and increased the ulcer index compared to CRS rats pretreated with rosiglitazone (Fig 1).
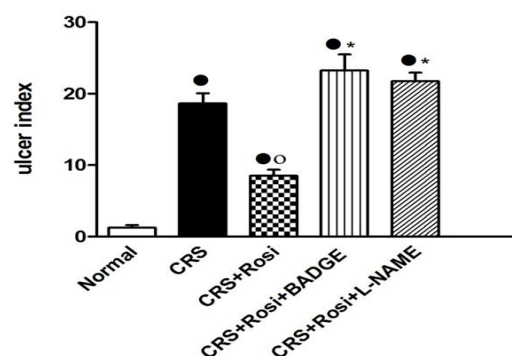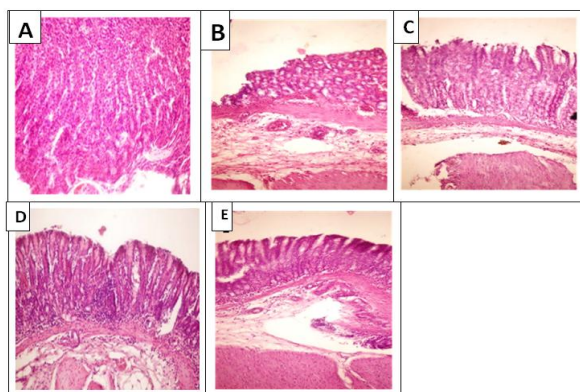

Figure1: Effect of CRS on gastric lesions development and its alteration by various pretreatments. •: Significantly different from control group; ○: Significantly different from CRS group; *: Significantly different from CRS+Rosi group, p≤0.05. Data represent the mean ± SEM of observations from 8 rats.

Effect of CRS and various pretreatment on stomachs histology.

Histological changes were screened to support the classical marker of gastric ulceration. Stomach of control (non-stressed) group showing normal histological structures without any abnormalities (Fig 2A). Stomach of CRS group showing focal necrosis, sloughing of gastric mucosa as well as submucosal oedema associated with leucocytic cells infiltration (Fig 2B). Stomach of CRS rats pretreated with rosiglitazone showing slight submucosal edema and apparent normal gastric mucosa (Fig 2C). Stomach of CRS rats pretreated with rosiglitazone and BADGE showing atrophy, intense leucocytic cells infiltration and submucosal oedema (Fig 2D). Stomach of CRS rats pretreated with L-NAME showing atrophy of gastric mucosa and sub mucosal edema (Fig 2E).

Figure 2: Effects of CRS and various pretreatments on stomach histology (A-E).

Cold restraint stress caused a significant increase in gastric juice FAO, TAO and proteolytic activity with concomitant reduction in the volume and mucin concentration. Pretreatment of CRS rats with rosiglitazone significantly decreased the FAO, TAO and proteolytic activity with concomitant increase in the volume and mucin concentration compared to CRS group.

The coadministration of BADGE with rosiglitazone to CRS rat group completely abolished the antisecretory effects of rosiglitazone and produced a significant rise in gastric acidity and proteolytic activity with concomitant reduction in the volume and mucin concentration. While, concurrent administration of L-NAME with rosiglitazone produced a significant increase in TAO and proteolytic activity without any significant change on the other gastric juice parameters namely, volume, FAO and mucin levels compared to rosiglitazone-treated CRS group (table 1).

Effect of CRS and various pretreatments on gastric juice parameters

Table 1: Effect of CRS and various pretreatments on gastric juice parameters, Data represent the mean ± SEM of observations from 8 rats. ●: significantly different from control group; ○: significantly different from CRS; *: significantly different from CRS+Rosi group, P≤0.05.

Groups	Volume(ml/3h)	FAO(mEq/3h)	TAO(mEq/3h)	Proteolytic activity(mg/ml)	Mucin content (mg % hexose)
Control	2.2±0.18	47.8± 2.09	61.5± 2.57	145.9± 3.98	73.8± 6.57
CRS	0.7±0.07●	69.9± 3.31●	85.3± 3.88●	247.1± 4.95●	45.1± 3.07●
CRS+Rosi	1.3±0.11●○	55.3± 3.99○	67.8± 2.58○	195.8± 6.39●○	62.5± 2.59○
CRS+Rosi+BADGE	0.7±0.10●*	81.3± 3.76●*	98.1± 2.79●*	259.8± 3.97●*	42.5± 3.60●*
CRS+Rosi+L-NAME	0.8±0.07●	67.3± 3.12●	87.5± 2.77●*	241.5± 4.27●*	48.5± 4.24●

Effect of CRS and various pretreatments on the gastric mucosal lipid peroxides

Cold restraint ulcer significantly elevated the gastric mucosal MDA concentration to about three folds the value observed for the control group. while, rosiglitazone pretreatment significantly reduced the gastric mucosal MDA concentration compared CRS group. Coadministration of either BADGE or L-NAME with rosiglitazone, completely abolished the decrease in gastric mucosal MDA concentration mediated by rosiglitazone in CRS rats (Fig 3).
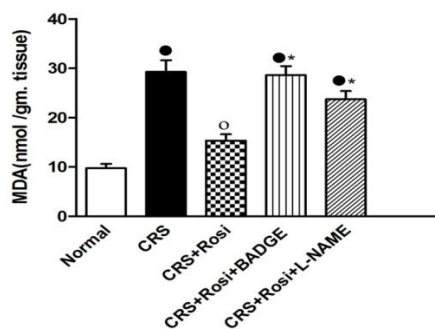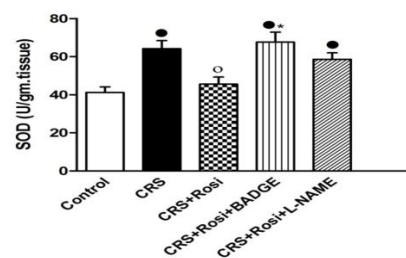

Figure 3: Effect of CRS and the various pretreatments on gastric mucosal MDA level.

●: Significantly different from control group; ○: Significantly different from CRS group; *: Significantly different from CRS+Rosi group, p≤0.05.

Data represent the mean ± SEM of observations from 8 rats.

Effect of CRS and various pretreatments on the gastric mucosal CAT and SOD levels

In comparison to the control group, CRS significantly increased the gastric mucosal SOD level. Pretreatment of CRS rats with rosiglitazone prevented the CRS-induced increase in SOD activity and kept the value near to the control level. Concurrent administration of BADGE but not L-NAME with rosiglitazone to CRS rats significantly increased the gastric mucosal SOD activity compared to rosiglitazone alone (Fig 4).

Figure 4: Effect of CRS and the various pretreatments on gastric mucosal SOD activity. ●: Significantly different from control group; ○: Significantly different from CRS group; *: Significantly different from CRS+Rosi group, p≤0.05.

Data represent the mean ± SEM of observations from 8 rats.

Howerver, CRS significantly decreased the gastric mucosal CAT activity compared to control group. Pretreatment of CRS rats with rosiglitazone significantly increased the CRS-induced reduction in CAT activity. Concurrent administration of either BADGE or L-NAME with rosiglitazone to CRS rats significantly decreased the gastric mucosal CAT activity compared with rosiglitazone-treated CRS rats (Fig. 5).
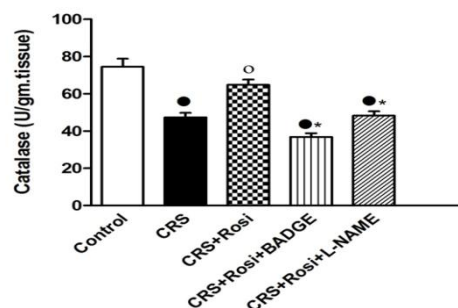

Figure 5: Effect of CRS and the various pretreatments on gastric mucosal catalase activity. ●: Significantly different from control

group; ○: Significantly different from CRS group; *: Significantly different from CRS+Rosi group, p≤0.05.

Data represent the mean ± SEM of observations from 8 rats.

Effect of CRS and various pretreatments on the gastric mucosal nitrite content

Cold restraint ulcer significantly reduced gastric mucosal total NO_x^- level compared to control group. Administration of rosiglitazone significantly increased gastric mucosal total NO_x^- content compared to CRS group. Concurrent administration of either BADGE or L-NAME with rosiglitazone, to CRS rats, prevented the increase in gastric mucosal total NO_x^- level mediated by rosiglitazone alone
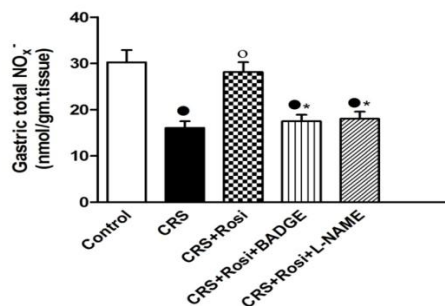
(Fig 6).

Figure 6: Effect of CRS and the various pretreatments on gastric mucosal total NO_x^- level. •: Significantly different from control group; ○: Significantly different from CRS group; *: Significantly different from CRS+Rosi group, p≤0.05.

Data represent the mean ± SEM of observations from 8 rats.

Effect of CRS and various pretreatments on serum TNF-α level

Cold restraint ulcer significantly increased serum TNF-α level compared to control group. Pretreatment of CRS rats with rosiglitazone significantly reduced serum TNF-α level compared to CRS group. Co-administration of either BADGE or L-NAME with rosiglitazone, to CRS rats, significantly increased the serum TNF-α level compared to rosiglitazone-treated CRS rats (Fig 7).
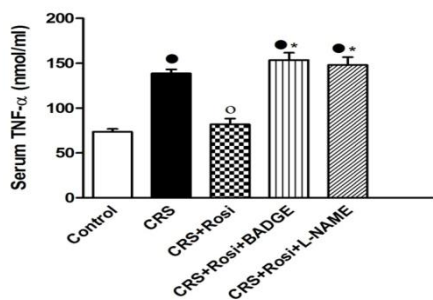

Figure 7: Effect of CRS and the various pretreatments on serum TNF-α level. •: Significantly different from control group; ○: Significantly different from CRS group; *: Significantly different from CRS+Rosi group, p≤0.05.

Data represent the mean ± SEM of observations from 8 rats.

DISCUSSION

Stress ulceration represents a serious complication in patients. Experimental studies have demonstrated that exposure of rat gastric mucosa to stress results in gastric mucosal lesion [28].CRS is a commonly used and clinically relevant experimental model for acute gastric damage in rats [29].

In the present study, non-treated rats exposed to CRS developed evident gastric lesions and deterioration in stomach histology which was presented as necrosis and sloughing of gastric mucosa

associated with leucocytic cells infiltration. In addition to a significant increases in gastric acidity, proteolytic activity with concomitant reduction in the volume of gastric juice and mucin concentration. The same finding was observed in previous studies [30, 31] which reported that CRS-induced ulceration may attributed to vagal stimulation which may lead to disturb the intact gastric mucosal barrier and enhance the gastric lesions [32, 33].

The current study demonstrated that the activation of PPAR-γ receptor by pretreatment with rosiglitazone markedly attenuated the ulcerative lesions induced by CRS which evident by improvement in stomach histology and suppression in ulcer index compared to CRS non-treated rats. This protective treatment was associated with significant decreases in FAO, TAO, pepsin activity in gastric juice together with concomitant increases in gastric juice volume and mucin. These results suggest that the protective effect of PPAR-γ receptor activation against CRS-induced ulcer seems to be multifactorial.

PPAR-γ stimulation by rosiglitazone reserved the oxidative changes induced by CRS. Rosiglitazone protective treatment significantly reduced gastric mucosal MDA and SOD activity and increase CAT activity, restoring their normal balance. These results are supported by the finding of several previous studies [34, 35] that indicated the antioxidant effect of PPAR-γ agonists.

The ROS lowering effect of the PPAR-γ agonist, rosiglitazone could be attributed to its inhibitory effect on TNF-α [36], an inflammatory mediator that increases ROS production during CRS [37]. This hypothesis was confirmed in the present study that showed a significant decrease in serum TNF-α by rosiglitazone pretreatment to CRS rats compared to CRS non treated rats. So, it could be suggested that the gastroprotective effect of PPAR-γ stimulation may be attributed to antioxidant and/or anti-inflammatory effects.

The pretreatment with rosiglitazone significantly prevented the development of acute stress-induced gastric lesions and this effect was accompanied by increased gastric mucosal total NO_x^- content. It could be possible to suggest that this protective effect of RRAR-γ stimulation is mediated by NO because the combined treatment of rosiglitazone with NOS inhibitor, L-NAME, completely abolished the protective effect afforded by rosiglitazone and aggravated the ulcerative lesions which was accompanied with significant deterioration of stomach histology and increases in aggressive factors (TAO, proteolytic activity, gastric mucosal MDA and serum TNF-α levels) with concomitant reductions in protective factors (NO and CAT levels). Similar results were reported by Konturek et al., [38] who suggested that the beneficial effect of pioglitazone, another PPAR-γ agonist, on the gastric mucosa may involve up-regulation of the constitutive NOS (cNOS) pathway and excessive NO release, hence suppressed NOS activity attenuates this protective effect [8]. This observation strongly supports the notion that NO plays an important role in gastric protection against stress induced ulcer through PPAR-γ receptor stimulation.

Nitric oxide is a well-established mediator in gastric mucosal defense and repair [39]. The significant reduction of gastric mucosal total NO_x^- content in CRS rats can contribute to reduced mucosal blood flow by the vasoconstriction response which leads to increase the production of free radicals, enhanced lipid peroxidation, impairment of antioxidizing enzyme activity and the increased generation of proinflammatory cytokines, such as TNFα [40]. This finding supports the hypothesis that the anti-oxidant and anti-inflammatory effects of PPAR-γ ligand are NO-dependant. Moreovere, The increased NO production observed in the present study could also explain the antisecretory effects of rosiglitazone on CRS rats. NO is well recognized for its ability to enhance gastric mucus/alkaline secretion and inhibiting gastric acid secretion [41].

The role of PPAR-γ activation in the protection against CRS-induced ulcer was clarified by concurrent administration of the PPAR-γ antagonist, BADGE with rosiglitazone to CRS group. It was found that concurrent administration of BADGE and rosiglitazone to CRS rats completely abolished the protective effect afforded by rosiglitazone when given alone as evidenced by the significant deterioration of stomachs histology and increase in ulcer index, gastric acidity,

pepsin activity, gastric mucosal MDA, SOD and serum TNF-α levels together concomitant decreases in mucin, total NO_x^- content and CAT activity. These results support the evidence presented by previous studies **[42, 35]** which demonstrated that the gastroprotective action of PPAR-γ ligand was dependent on the activation of the PPAR-γreceptor.

In conclusion, the present study highlighted the role of PPAR-γreceptor activation in protection against CRS-induced ulcer. This protective effect may be mediated via NO production through distinct signaling pathways that are PPAR-γreceptor dependent. These results provide further evidence that PPAR-γ ligands have the potential to modify oxidative stress and inflammation associated with CRS-induced ulcer and to modulate the production of NO, a crucial mediator in maintenance of gastric mucosal integrity.

ACKNOWLEDGMENT

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