Emerging role of a peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonist in bronchial asthma in vivo: antioxidant activity and the down-regulation of inflammatory factors

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ABSTRACT

Objectives: To investigate the effects of a peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonist, rosiglitazone, on induced bronchial asthma in a murine model. Material and methods: Adult male guinea pigs were administered ovalbumin 100 mg/kg S.C. and 100 mg/kg I.P. Treatment with rosiglitazone (5 mg/kg/day, PO) was assessed for 21 days. On day 21, the animals were challenged with the same dose of ovalbumin and the ratio of FEV1 (forced expiratory volume in one second) to FVC (forced vital capacity), FEV1/ FVC, was measured using a spirometer. In addition, serum levels of interleukin-5 (IL-5) and immunoglobulin E (IgE) were assessed. The activities of superoxide dismutase (SOD) and catalase and the level of reduced glutathione (GSH) were determined in lung tissue homogenates. Furthermore, histopathological examination of the lung was performed. Results: treatment with rosiglitazone resulted in a profound improvement in lung function and histopathological features. These improvements were accompanied by a significant decrease in the serum levels of IL-5 and IgE. Moreover, the activities of antioxidant enzymes, including SOD and catalase, and the GSH level were significantly increased in the lung tissues of treated animals compared to those of untreated asthmatic animals. Conclusion: PPAR-γ agonist rosiglitazone may have potential in the development of therapies for bronchial asthma.

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INTRODUCTION

Bronchial asthma is a disease characterized by reversible airway obstruction in response to allergens, chronic eosinophilic airway inflammation, and nonspecific airway hyper responsiveness (AHR) [1]. Typically, airflow obstruction diseases such as bronchial asthma are diagnosed using spirometer by demonstrating a lower than normal ratio of FEV1 (forced expiratory volume in one second) to FVC (forced vital capacity), FEV1/ FVC [2]. The pathological symptoms and clinical hallmarks of allergic asthma are the result of a Th2-type dominated cytokine profile with increased levels of IL-4, IL-5, and IL-13[3]. These cytokines may induce airway inflammation and AHR directly, through effects on airway smooth muscle and bronchial mucosa [4], or indirectly, through effector cells such as B-cells, mast cells, and eosinophils [5-6], and further sustain the Th2 response [7]. Among the Th2 cytokines, IL-5 is a major factor that promotes eosinophilia by priming the cells for heightened responsiveness, enhancing their adhesion to the endothelium, increasing eosinophilic...
cytotoxicity, and prolonging the viability of mature eosinophils.\(^8\)

Moreover, the importance of IgE in airway inflammation and the development of bronchial asthma has been explored in different animal models and in patients suffering from bronchial asthma. Furthermore, in patients suffering from bronchial asthma, IgE levels in serum or bronchoalveolar lavage fluid are often increased and may correlate with the incidence or severity of the disease.\(^1\)

Pulmonary cells have several ways of alleviating the effects of oxidative stress and diminishing the expression of the receptor superfamily containing transcription factors that regulate gene expression.\(^9\) Early investigation of PPAR-\(\gamma\) focused on its role in regulating adipocyte differentiation and lipid and glucose metabolism.\(^9\) Recent studies have demonstrated that this receptor has a pivotal role in regulating the immune response.\(^10\) PPAR-\(\gamma\) is now being investigated as a potential target in a variety of lung-related diseases.\(^11\) Moreover, PPAR-\(\gamma\) ligands have been shown to exert anti-inflammatory effects on immune cells,\(^12-13\) as well as on specific cells in the lung, such as alveolar macrophages,\(^14\) airway epithelial cells, and airway smooth muscle cells.\(^15\)

Furthermore, several investigators have demonstrated that rosiglitazone, a member of the thiazolidinedione (TZD) drug class, reduces glucose, fatty acid and insulin blood concentrations. At the same time, rosiglitazone inhibits the growth of many cells, particularly cancerous airway epithelial cells, through binding to PPAR receptors and affecting DNA expression.\(^15\) Based on this evidence, we hypothesized that the potent PPAR-\(\gamma\) agonist rosiglitazone, which has anti-inflammatory and antioxidant activities, would be beneficial in treating respiratory diseases such as bronchial asthma.

Therefore, we used a murine asthma model to evaluate the role of the PPAR-\(\gamma\) agonist rosiglitazone in the regulation of the allergic immune response, more specifically in the involvement of IL-5, and to investigate the molecular mechanisms by which PPAR-\(\gamma\) reduces oxidative stress in bronchial asthma.

**MATERIALS AND METHODS**

**Animals**

Adult male guinea pigs weighing 400-450 g were obtained from the animal house of the faculty of medicine, Cairo University (Cairo, Egypt). The animals were housed in a pathogen-free environment in the animal house of October University for Modern Sciences and Arts, Egypt, in a 12/12 h light/dark cycle with food and water available ad libitum. Procedures involving animals and their care conformed to the institutional guidelines and complied with national and international laws on the care and use of laboratory animals.

**Chemicals and drugs**

Rosiglitazone was purchased from Cayman Chemical, Ann Arbor, MI, USA. IL-5 and IgE detection kits were obtained from Biosource, Belgium. All other chemicals were purchased from Sigma Co., USA.

**Experimental design**

All animal experiments in this study were carried out in accordance with the ethics guidelines established by the Experimental Animal Care. Male guinea pigs 400-450 g were used and divided into three groups each containing eight animals. The induction of bronchial asthma was performed according to the method of Piper and Vane\(^16\), 1969 with modification. On day 1, the first and second groups were immunized by injection of Grade V chicken ovalbumin (100 mg/kg IP and 100 mg/kg SC), while the third group was negative control group (control) and injected with saline only throughout the experiment.

Starting on the second day of immunization to avoid dampening sensitization, the first immunized group was treated with rosiglitazone (5 mg/kg/day, PO) for 21 days (treated...
group), while the second immunized group received from the second day oral saline for the same period (asthma group). On day 21, the two immunized groups (asthma and treated groups) were challenged with another dose of ovalbumin, injected by the IP and SC routes. The control group underwent the same schedule for challenge, but received saline rather than ovalbumin.

**Assessment of respiratory functions**

One hour after ovalbumin injection, guinea pigs were anesthetized by I.P. injection of urethane. After full anesthesia, the animal trachea was exposed and cannulated using a Y-shaped cannula. One arm of the Y-shaped cannula was connected to a spirometer (AD instruments pty ltd, power lab, 4/30, Australia). The animal was allowed to breathe freely through the other arm of the cannula while monitoring the following ventilatory parameters: FVC (forced vital capacity), FEV1 (forced expiratory volume in one second) and FEV1/FVC.

**Quantitative determination of interleukin-5 (IL-5)**: The IL-5 level was determined based on the method of Tavernier et al. [17]. Briefly, diluted samples or IL-5 standards (10 µl) were mixed with 100 µl of 50 µg/l anti-IL-5 immunoglobulin G labeled with horseradish peroxidase in 10 mmol/l ethylene diamine tetra acetic acid. A 100-µl aliquot of the mixture was transferred to each of the wells, which had been previously coated with anti-IL-5 immunoglobulin G. The plate was then incubated for 60 min at room temperature without shaking and then washed three times with 10 mmol/l PBS (pH 7.0) containing 0.1 mol/l NaCl.

The micro-plate-bound horseradish peroxidase activity was determined by adding 100 µl of 0.15 mol/l citric acid sodium phosphate buffer (pH 4.9) containing 2.0 g/l of o-phenylenediamine and 0.02% (v/v) hydrogen peroxide and incubating for 20 min at room temperature. The reaction was stopped by adding 100 µl of 1 mol/l sulfuric acid, and the absorbance was measured with a micro-plate reader at a wavelength of 450 ± 2 nm. The amount of guinea pig IL-5 in the serum was calculated from the standard curve [24]. The results were expressed as pg/ml.

**Enzyme immunoassay for the quantitative determination of immunoglobulin E**

The technique used was a "sandwich" type solid-phase enzyme immunoassay based on two monoclonal antibodies that are specific for different epitopes of the IgE molecule, according to the method of Crowther [18]. In this method, one of the antibodies was conjugated to horseradish peroxidase and the other was immobilized on the inner surface of micro-plate wells. IgE molecules from the serum sample were allowed to bind to both immobilized antibodies and the anti-IgE peroxidase conjugate. Then, the wells were washed with 10 mmol/l PBS wash buffer to remove any material not bound to the inner surface of the wells. The quantity of the bound conjugate is directly proportional to the IgE concentration of the tested sample. Color appears during incubation with the substrate solution, and the intensity is directly proportional to the IgE concentration in the sample. The enzymatic reaction was stopped by dispensing acidic solution (1 N HCl) into the wells.

The optical density of the solution in the wells is directly proportional to the IgE concentration in the samples. The standard curve was plotted using the IgE concentrations as the calibrators (x-axis) and their corresponding OD values (y-axis) [18]. The results were expressed as ng/ml.

**Determination of antioxidant activities in lung homogenate**

The left lung was removed immediately after blood collection, rinsed with saline and gently dried using filter paper. The tissues were finely sliced and homogenized in 10% (w/v) phosphate-
buffered saline, pH 7.8. The homogenates were centrifuged at 4000 rpm at 4° C for 20 minutes using a Remi C-24 high-speed, cooling centrifuge. The clear supernatant was used for assaying the levels of antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) and reduced glutathione (GSH) using a spectrophotometer (Shimadzu UV-PC 1601, Japan).

**Determination of SOD activity:** SOD activity was measured using the method of Niskikimi et al. [19]. Briefly, freshly prepared 0.93 µM phenazinemethosulfate was added to a cuvette containing 0.1 M sodium pyrophosphate buffer (pH 8.3), 0.3 mM nitroblue tetrazolium, and 0.47 mM NADH and the sample. The increase in absorbance at a wavelength of 560 nm was measured. The SOD activity was expressed as U/g wet tissue.

**Determination of catalase activity:** Catalase activity was measured as described by Clairborne [20]. In brief, the supernatant of the previously prepared lung homogenate was added to a cuvette containing 50 mM phosphate buffer (pH 7.8), and H2O2 was added to a final concentration of 10 mM. The disappearance of H2O2 was determined by measuring the absorbance at 240 nM. The difference in the absorbance per unit of time is a measure of catalase activity. Activity was expressed as μmol/sec/g wet tissue.

**Determination of lung GSH content**
The reduced glutathione (GSH) level was determined using the method of Prins and Loose, [21]. Briefly, 0.5 ml of previously prepared homogenate was added to 0.5 ml of tungstate solution after centrifugation at 2000 rpm for 5 minutes. The supernatant (200 µl) was added to a tube containing Tris buffer followed by the addition of 0.2 ml of DTNB reagent. After 30-60 seconds, the color had developed and the optical density was measured at wavelength of 412 nm. GSH content was expressed as mg/g wet tissue.

**Histopathological examination of the lung**
The right lung was removed for histopathological evaluation, fixed overnight in 10% formalin and embedded in paraffin. The tissues were sliced into 4-μm sections and stained with hematoxylin and eosin (H&E). The degree, extent and distribution of lung inflammation were evaluated as follows: mild (1), moderate (2) and severe (3) for degree of inflammation; focal, patchy and diffuse for the extent of inflammation; and interstitial, perivascular and peribronchial for the distribution of inflammation.

**Statistical analysis**
Data are expressed as the mean ± standard error of the mean (SEM). Comparisons between different groups were carried out by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. The level of significance was set at p < 0.05. Graphpad software InStat (version 2) was used to carry out statistical analysis.

**RESULTS**
**Effect of rosiglitazone on respiratory functions**
The FEV1/FVC ratio ranged from 87.7 % to 97.8%, with an average of 95.06% ± 3.6, in the control group and from 21.2% to 29.9%, with an average of 24.7% ± 3.029, in the asthma group. Rosiglitazone treatment significantly improved the deteriorated respiratory function tests compared to untreated asthmatic animals. The FEV1/FVC ratio ranged from 41% to 56.6%, with an average of 49.8% ± 5.799, in the rosiglitazone treatment group (Table 1).

**Effect of rosiglitazone on serum levels of IL-5 and IgE**
Quantitative determination revealed that the serum levels of IL-5 and IgE were significantly elevated in asthmatic animals compared to control animals. The inhibitory effect of rosiglitazone treatment on IL-5 and IgE were indicated when rosiglitazone administered resulted in significantly decreased serum levels of IL-
5 and IgE compared to the asthma group (Figures 1a and 1b, respectively; p < 0.05).

**Effect of rosiglitazone on the tissue SOD and catalase activities**
The mean activities of SOD and catalase in the lung tissue homogenates of the asthma group were significantly lower than in the control group. Our results showed that the rosiglitazone treatment significantly elevate the SOD and catalase enzymes activities compared to the asthma group (Figures 2a and 2b, respectively; p < 0.05).

**Effect of rosiglitazone on the tissue GSH level**
The mean level of reduced glutathione in lung tissue homogenates of the asthmatic animal group was significantly lower compared to the control group. However, in the animal group treated with rosiglitazone elicited significant increase in the level of reduced glutathione compared with untreated asthmatic animal group (Figure 3; p < 0.05).

**Histopathological examination of the lung tissue**
Histological examination of the degree, extent and anatomical distribution of allergic inflammation revealed moderate and diffuse interstitial inflammation in the lungs of the untreated asthmatic guinea pig group (Figure 4b). This inflammation was also observed in the perivascular and peribronchial zones and was composed mainly of lymphocytes, neutrophils and eosinophils (Figures 4c and 4d). Although the resolution of inflammation in the rosiglitazone-treated group was only partial, the overall histopathological lesion was lower in this group compared to the untreated asthmatic guinea pig group (Figures 4e and 4f). Rosiglitazone-treated animals exhibited a mild degree of interstitial inflammation, which was only observed focally in the lung (Figures 4b).

**DISCUSSION**
Bronchial asthma is characterized by a wide variety of pathophysiologic features that can be attributed to the release of potent inflammatory mediators, such as lipid-derived products and proinflammatory cytokines [22]. Studies in guinea pigs, mice and primates have provided increasing evidence for the important role of IL-5 in the induction and maintenance of eosinophilic airway infiltration that is associated with bronchial asthma. Down-regulation of IL-5 production may be one of the reasons for the beneficial effects of the treatment of allergic patients with corticosteroids [23-24]. As a result, IL-5 may be an ideal target for novel therapeutic approaches in the treatment of asthma. In the present study, treatment of ovalbumin-sensitized guinea pigs with rosiglitazone resulted in a significant decrease in the elevated level of IL-5 compared to the untreated asthmatic animal group (Figure 1a). In accordance with our observations, several studies have reported a significant decrease in the level of IL-5 in different asthma models upon activation of PPAR-γ [25-26]. Furthermore, PPAR-γ agonists reduce the ability of IL-5 to induce eosinophil survival and chemotaxis [27]. Recently, a direct inhibitory effect of PPAR-γ ligands on the expression of IgE heavy chain germline transcripts in the human B cell line DND39 was described [28]. Similarly, Ruhl et al. [29] reported a significant inhibition of IgE production by the PPAR-γ ligand ciglitazone in vitro using purified peripheral blood mononuclear cells and in vivo using ovalbumin-sensitized mice that were treated with ciglitazone. Similarly, the increased level of IgE in ovalbumin-sensitized mice was significantly reduced by administration of rosiglitazone or pioglitazone [30]. Interestingly, several reports have also demonstrated that local administration of PPAR-γ agonists had similar beneficial effects on pathological conditions, including serum levels of IgE, airway hyperresponsiveness and lung eosinophilia [31-32].
Considering the results of these studies and our in vivo findings in ovalbumin-sensitized guinea pigs, which show a significant decrease in the serum level of IgE after treatment with rosiglitazone (Figure 1b), we propose that PPAR-γ plays an important role in decreasing IgE production, which significantly contributes to the pathogenesis of bronchial asthma.

In the last decade, increases in oxidative stress and oxidant–antioxidant imbalance have emerged as major contributors to the pathogenesis of bronchial asthma [33-34]. Lung epithelia are being increasingly damaged by free radicals. To treat this damage, free-radical scavengers must be present at the site of free radical production and in adequately high concentrations over sufficiently long periods of time [33]. Consequently, localized increases in the levels of enzymatic and non-enzymatic antioxidants in pulmonary tissue could be beneficial in the treatment of bronchial asthma.

In our study, treatment of ovalbumin-sensitized guinea pigs with rosiglitazone significantly improved the deteriorated activities of both SOD and catalase enzymes (Figure 2a and 2b; respectively). In accordance with our findings, PPAR-γ ligands have been shown to reduce oxidative stress by stimulating the activity and expression of both SOD and catalase [28-35]. Similar to the improvement of SOD and catalase activities, the level of GSH in lung tissue homogenates was significantly increased in rosiglitazone-treated, ovalbumin-sensitized guinea pigs (Figure 3). The improvement in the GSH level was previously reported with various PPAR-γ agonists [36-37].

In addition to our present findings, it appears that there is a negative correlation between antioxidant (SOD, catalase, and GSH) levels and the FEV1\FVC ratio in the asthma group, suggesting that increased antioxidant activity in the pulmonary tissue leads to improved respiratory function. These data agree with other studies in which the correlation between oxidant or antioxidant levels and disease severity was investigated. In this regard, Wood et al., [36] reported that SOD activity was negatively associated with asthma severity in patients with mild disease. In addition, Kanazawa et al., [37] reported that enhanced production of superoxide anion by neutrophils in asthmatics was inversely correlated with FEV1. Ovalbumin-sensitized guinea pigs treated with rosiglitazone showed significant improvement in the FEV1\FVC ratio, which appeared to be positively correlated with the improvement of antioxidant (catalase, SOD and GSH) levels. Based on these observations, we hypothesize that rosiglitazone has a beneficial effect on bronchial asthma induced in ovalbumin-sensitized guinea pigs, in part through the improvement of antioxidant activities in pulmonary tissue.

The beneficial effects of rosiglitazone on ovalbumin-sensitized guinea pigs were confirmed by histopathological findings because lung sections from ovalbumin-sensitized guinea pigs treated with rosiglitazone showed a profound improvement of the degree, extent and distribution of inflammatory cells in the lung. PPAR-γ expression has been reported to be dramatically up-regulated in several inflammatory cell types during inflammatory responses and can be induced by immunoregulatory molecules [38-39]. Overexpression of PPAR-γ potentiates the ability of diverse PPAR-γ agonists to inhibit the expression of inflammatory response genes, which is consistent with the mediating anti-inflammatory effects [40-41]. As bronchial asthma is characterized by infiltration of pulmonary tissue with activated inflammatory cells [6], we propose that rosiglitazone, through activation of overexpressed PPAR-γ receptors on activated inflammatory cells; can inhibit the expression of several inflammatory
mediators, chemoattractants and survival factors \cite{42}. These inhibitory effects could subsequently decrease the infiltration of inflammatory cells into the lung and improve the pathophysiological symptoms of asthma.

In accordance with our hypothesis, the potential anti-inflammatory effects and clinical benefits of PPAR-\(\gamma\) agonists in various pathological conditions are based on their abilities to inhibit the transcriptional activation of inflammatory response genes through activation of overexpressed PPAR-\(\gamma\) in activated inflammatory cells \cite{41}.

In conclusion, our results together with the results of previous studies have demonstrated that administration of PPAR-\(\gamma\) agonists improves the asthmatic features, through downregulation of the inflammatory reaction, associated with bronchial asthma. Moreover, PPAR-\(\gamma\) agonists enhance the antioxidant potential of pulmonary tissue. Therefore, it is anticipated that PPAR-\(\gamma\) ligands, particularly TZDs, will be able to serve as effective treatments for chronic respiratory diseases such as bronchial asthma. Clinical trials and further studies are needed to evaluate the definite clinical effects of PPAR-\(\gamma\) ligands based on abundant experimental studies.

References


**Table (1):- Average effect of rosiglitazone on respiratory functions**

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<th>Treated asthmatic animals by ROSI</th>
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<td>FEV1/FVC ratio</td>
<td>95.06 ± 3.9</td>
<td>24.7 ± 3.029</td>
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**Figure 1**: The levels of IL-5 (a) and IgE (b) in serum of OVA-induced guinea pigs of bronchial asthma (BA). The level of IL-5 and IgE in guinea pigs with BA was significantly higher than that of the control group (a and b, respectively). The level of IL-5 and IgE in guinea pigs with BA was decreased significantly in guinea pigs with rosiglitazone (ROSI) (a and b, respectively; \( P < 0.05 \)). Ovalbumin-sensitized guinea pigs were treated with a synthetic ligand for PPAR-\( \gamma \) (ROSI) (5mg/kg/day) for 21 days.
**Figure 2:** Lung SOD activities (a) and Catalase activities in lung tissue homogenates (b). The mean activities of SOD and catalase in BA group were significantly lower than control group (a and b, respectively). Ovalbumin-sensitized guinea pigs were treated with a synthetic ligand for PPAR-γ (rosiglitazone) (5mg/kg/day) for 21 days; ROSI significantly improves the activities of both enzymes as compared with BA group (a and b, respectively; P < 0.05).
Figure 3: The lung homogenate GSH contents in BA group were significantly lower than control group; ROSI significantly increased in the level of reduced glutathione compared with untreated asthmatic animal group ($P<0.05$).
Figure 4: H&E stained lung sections from the control group (a), asthma group (b-d) and treated group (e-f). In the diseased group, there was moderate-severe diffuse interstitial (b,X200) and perivascular (c,X400) and peribronchial (d,X200) chronic inflammatory infiltrate with occasional formation of lymphoid follicles(b). The infiltrate was mainly lympho-histiocytic, eosinophils and neutrophils especially in the perivascular (c) and peribronchial zones. After treatment, there was significant resolution of the inflammation (f,X10) that was mild and detectable only focally in the lung parenchymal tissue in the perivascular area (e,X400).