

Sitagliptin exerts anti-inflammatory and anti-allergic effects in ovalbumin-induced murine model of allergic airway disease

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Abstract Sitagliptin, a new oral glucose lowering medication, is used for treatment of type 2 diabetes mellitus. The anti-inflammatory property of sitagliptin is reported, yet no studies have been done on asthma. In the present study, the effect of sitagliptin on allergic asthma was investigated using ovalbumin (OVA)-induced asthma model in mice. Swiss male albino mice sensitized and challenged to ovalbumin were treated with sitagliptin (8 mg/kg administered orally twice a day). Drug treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. Sitagliptin treatment markedly decreased inflammatory cell accumulation in bronchoalveolar lavage (BAL) fluid and in the lungs, as revealed by histopathological examination. Furthermore, the levels of interleukin (IL)-13 in BAL fluid, total and OVA specific immunoglobulins (Ig)-E in serum, were significantly reduced as compared to the OVA group. In addition, sitagliptin significantly increased superoxidase dismutase (SOD) and reduced glutathione (GSH) activities with significant decrease in malondialdehyde (MDA) content in the lung. Importantly, sitagliptin decreased mRNA expression of the inflammatory cytokines tumor necrosis factor- α (TNF- α) and transforming growth factor- β_1 (TGF- β_1) in lung tissues as compared to the OVA group. Moreover, nitric oxide content as well as the mRNA expression of inducible nitric oxide synthase (iNOS) was

remarkably decreased by sitagliptin treatment. Sitagliptin attenuates the allergic airway inflammation suggesting that sitagliptin may have applications in the treatment of bronchial asthma.

Keywords Sitagliptin · Asthma · Allergic airway inflammation · Nitric oxide · Cytokines

Abbreviations

BAL	Bronchoalveolar lavage
DPP-4	Dipeptidylpeptidase-4
IgE	Immunoglobulin E
IL	Interleukin
OVA	Ovalbumin
MDA	Malondialdehyde
GSH	Reduced glutathione
SOD	Superoxide dismutase
TNF- α	Tumor necrosis factor- α
TGF- β_1	Transforming growth factor- β_1
iNOS	Inducible nitric oxide synthase

Introduction

Bronchial asthma is a chronic inflammatory disease of airways characterized by reversible bronchial obstruction, airway hyperresponsiveness (AHR), leukocyte infiltration, and plasma exudation. Inhaled pollutants including allergens, viruses, bacteria, fungi, tobacco smoke, and ozone potentiate the risk of developing bronchial asthma. These inhaled pollutants are reported to initiate allergic symptoms (Holgate 2008).

The inflammatory aspects of the disease are complex. The increase in eosinophils and T lymphocytes in the bronchial mucosa and bronchoalveolar lavage (BAL) fluid are distinctive features of the inflammatory response in patients

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with asthma and appear to correlate with the severity of the disease (Secor et al. 2005). Previous reports indicate that T helper (Th) cells play a pivotal role in the pathophysiology of the allergic airway response (Wills-Karp 1999). Th cells are important regulators of immune and inflammatory reactions via the release of cytokines. Th1 cytokines include tumor necrosis factor- α (TNF- α) and interferon- γ (INF- γ), while the Th2 cytokines include interleukin (IL)-4, IL-5, and IL-13 (Roh et al. 2008). The specific Th1 cytokines enhance Th1 cell-mediated allergic airway inflammation, while the specific Th2 cytokines enhance induction of allergic responses in asthma (Nakajima and Takatsu 2007). Furthermore, elevated Th2 cytokines have been reported to induce immunoglobulin-E (IgE) switching in B cells, replacement of mast cells and eosinophils, and inflammation in the lung tissue (Yuk et al. 2007).

Sitagliptin is a selective dipeptidylpeptidase-4 (DPP-4/CD26) inhibitor indicated for the treatment of type II diabetes mellitus. Gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP-1) are rapidly inactivated by DPP-4-mediated cleavage, thus limiting their duration of action (Mest and Mentlein 2005). DPP-4 inhibitors protect endogenous GLP-1 and GIP from N-terminal degradation, thereby prolonging their bioactivity. DPP-4/CD26 has been reported to be expressed in the serosal submucosal glands of the human bronchus (van der Velden et al. 1998) and in human BAL fluid (van der Velden et al. 1999). Also, DPP-4/CD26 is expressed on a fraction of resting T cells, and it is strongly up-regulated following T cell activation. It truncates several chemokines as well as neuropeptides and influences immune responses via modulation of cell adhesion and T cell activation, suggesting an involvement of DPP-4/CD26 in asthmatic and airway inflammation (Morimoto and Schlossman 1998). It was shown that the deficiency of DPP-4/CD26 expression remarkably reduces T-cell recruitment to the lung during experimental asthma (Kruschinski et al. 2005) and that especially DPP-4-positive F344 rats, activated T-cells are recruited to the lungs after induction of asthma (Skrupuletz et al. 2007).

Moreover, sitagliptin inhibited immediate-type hypersensitivity allergic reaction through inhibition of histamine release and proinflammatory cytokine production in rat peritoneal mast cells (Nader 2011). Hence for better understanding of the role of DPP-4 inhibitor in asthma, this study was designed to explore the effect of sitagliptin on ovalbumin (OVA)-sensitized murine model of asthma.

Materials and methods

Animals

Adult male Swiss albino mice (4 weeks old and 25–35 g in weight) were used in this study. They were purchased from

“Urology and Nephrology Center,” Mansoura University, Egypt. The animals were housed 5 per cage in a laminar air flow room maintained under a temperature of 22 ± 2 °C throughout the study. Animals received water and standard chow ad libitum. All procedures used in the *in vivo* assays were approved by the committee on Animal Experimentation of Mansoura University (Mansoura, Egypt) according to guidelines established by NIH Guidelines for the Laboratory Animal Care.

Drugs and chemicals

Ovalbumin (OVA; chicken egg, grade V), aluminum hydroxide hydrate, and urethane were purchased from Sigma, St. Louis, MO, USA. The highly water soluble sitagliptin phosphate monohydrate was obtained as tablets from the Medical Center hospital pharmacy of Mansoura University.

Immunization and challenge for induction of airway inflammation and asthma

Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20- μ g OVA emulsified in 1 mg of aluminum hydroxide in a total volume of 200 μ l. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3 % (w/v) OVA. Control mice were exposed to aerosolized saline. Aerosolization was performed for 30 min by placing the mice in custom-built cylindrical chamber (20 $\times$ 30 $\times$ 35 cm) connected to the ultrasonic air nebulizer (Omron, Vernon Hills). The outlet of the chamber was connected to a vacuum pump, and a constant flow rate of 15 ml/min was ensured by a flow meter (Hoffer Flow Controls, USA).

Animal grouping and drug treatment

Mice were divided into four groups each having ten animals. These groups were group (1): mice were OVA sensitized, saline challenged, and treated orally with drug vehicle (control group). Group (2): mice were OVA sensitized, saline challenged, and treated orally with sitagliptin which showed no relevant differences when compared to the control group under vehicle (Sita group). Group (3): mice were OVA sensitized, OVA challenged, and treated orally with drug vehicle (OVA group). Group (4): mice were OVA sensitized, OVA challenged, and treated orally with sitagliptin (8 mg/kg/twice a day) (OVA-Sita group). This treatment schedule and dose level were chosen based on the negative results obtained in pilot experiments using lower doses of sitagliptin (1, 2, and 4 mg/kg), which failed to decrease total and differential cell counts in BAL fluid (data not shown), taking in consideration previous study of Nader (2011).

Drug treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge.

Bronchoalveolar lavage (BAL) and lung preparation

The animals were anaesthetized using urethane (1.8 mg/kg) 24 h after the last challenge. Blood samples were collected from the mice via the retro-orbital venous plexus of the eye. Then, the collected blood samples were left to coagulate for 20 min; after which, the coagulated blood was centrifuged at 1,000 g for 10 min to separate the serum which is stored at -80°C for measurement of IgE.

After blood collection, the chest was opened, and the trachea with the heart–lung package was excised from the thorax; then, the left main bronchi were clamped. A cannula was inserted into the trachea in situ, the right lung was lavaged three times with portions of 1-ml PBS solution, and BAL fluid was collected for subsequent analysis. Thereafter, the left lung was collected for histopathological examination. The inferior lobe was dissected for NO and quantitative RT-PCR measurement.

Inflammatory cell counts in BAL fluid

The BAL samples were centrifuged for 5 min at 1,000 g and 4°C to collect whole cells in the pellet. The supernatant was carefully removed and stored at -80°C for subsequent analysis. The pellet of cells that remains after centrifugation of BAL samples was resuspended in an equal volume of fresh PBS (100 μl). Total inflammatory cell number was assessed by counting cells in at least five squares of a haemocytometer. Smears of BAL cells prepared with Cyto-spin II (Shandon, Runcorn, UK) were stained with Diff-Quik solution (B4132-1A; Dade Behring Inc., Deerfield, IL) for differential cell counting. Approximately 200 cells were counted in each of four different random locations at 40 magnifications. Differential cell count was performed through quantification of the slides for eosinophils, lymphocytes, neutrophils, and macrophages.

Lung tissue histopathology

The lung tissue was fixed in 10 % neutral buffered formalin for 24 h. The lung tissue was embedded in paraffin and then cut into 4-mm-thick sections, stained with H&E solution and with periodic acid-Schiff (PAS) stain for measuring mucus production. The tissue was subsequently mounted and cover-slipped with Dako-mounting medium. The degree of airway inflammatory cell infiltration was scored in a double-blind screen (Myou et al. 2003). The degree of peribronchiole and perivascular inflammation was evaluated by a subjective scale of 0–4. Briefly, the scoring system for cell infiltration was 0, no cells; 1, a few cells; 2, a ring of

cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; and 4, a ring of cells > 4 cells deep. Goblet cell hyperplasia in the airway epithelium was quantified based on a five-point system: 0, no goblet cells; 1, <25 % of the epithelium; 2, 25–50 % of the epithelium; 3, 50–75 % of the epithelium; and 4, >75 % of the epithelium. Quantitative analysis of mucus production was performed through the use of an image analyzer (Leica Microsystem Imaging solution Ltd., Cambridge, UK).

Measurement of IL-13 in BAL fluid

The amount of IL-13 present in BAL fluid was measured using enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, USA) according to the manufacturer's directions.

Measurement of total IgE and OVA-specific antibodies in serum

The total and OVA-specific serum IgE ELISA kits (R&D Systems, Minneapolis, USA) were performed according to the manufacturer's directions. Serum samples in duplicate were diluted to 1:100. IgE levels were measured from optical density readings at 450 nm, and IgE concentrations were calculated from a standard curve that was generated using recombinant IgE (52,000 pg/ml).

Measurement of oxidative stress in lung tissue

Measurement of malondialdehyde (MDA)

The MDA concentration was determined as an indicator of lipid peroxidation in the lung tissue. The tissue samples of lung were weighed and homogenized (1:10, w/v) in 0.1-M phosphate buffer (pH 7.4) in an ice bath. The homogenate was centrifuged at 3,000 g for 20 min at 4°C . Subsequently, MDA content in the supernatants was measured according to the method described by Ohkawa et al. (1979). In brief, the reaction mixture consisted of 40 μl of 8.1 % sodium dodecyl sulfate, 300 μl of 20 % acetic acid solution adjusted to pH 3.5 with NaOH, 300 μl of 0.8 % aqueous solution of thiobarbituric acid, and 40 μl of the tissue supernatant. The mixture was heated at 95°C for 60 min. After cooling on ice, 200 μl of distilled water was added, and the sample was centrifuged at 15,000 g for 20 min. The supernatant was taken out, and its absorbance was measured at 532 nm. The level of MDA was derived from a standard curve prepared from serial dilutions of a 3.2- μM stock solution of 1,1,3,3-tetraethoxypropane that were treated in identical manner as the lung homogenate samples. MDA was expressed as nmol/g tissue.

Measurement of superoxide dismutase (SOD)

The SOD activity was estimated according to the method described by Misra and Fridovich (1972). In brief, epinephrine undergoes autooxidation rapidly at pH 10.0 to produce adrenochrome, a pink colored product that was detected at 480 nm in kinetic mode using UV–vis spectrophotometer. The amount of enzyme required to produce 50 % inhibition was defined as one unit of enzyme activity. The SOD activity was expressed as units/mg protein.

Measurement of reduced glutathione (GSH)

The concentration of GSH in the lung homogenate was measured according to the method described by Ellman 1959. Briefly, a 100- μ l aliquot of lung homogenate was mixed with 100 μ l of 5 % meta-phosphoric acid, and the mixture was centrifuged at $2,000\times g$ for 5 min. A 100- μ l portion of the clear supernatant was mixed with 1.9 ml of 0.1-M phosphate buffer pH 8.0 and 20 μ l of 0.02-M 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in 0.1-M phosphate buffer pH 8.0, and the absorbance of the resulting product was read at 412 nm on a spectrophotometer. The concentration of GSH in the sample was derived by reference to a calibration curve of GSH prepared from serial dilutions of a 240-nM GSH stock solution that were treated in identical manner as the lung homogenate samples. The result was expressed as μ mol/g tissue.

Quantitative RT-PCR analysis

Lung tissues, approximately 20 mg of lungs, were flash frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted using a single-step isolation method (Chomczynski and Sacchi 1987), using a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Then, RNA concentration and purity were determined by measuring the change in optical density at 260 and 280 nm using UV-visible spectrophotometer (Labomed, USA). The pure RNA samples were reverse transcribed using Qiagen Quantitect Reverse Transcription Kit according to manufacturer's instructions. RT-PCR was performed with a thermocycler Rotor Gene Q (Qiagen, Hilden, Germany), and the DNA-binding dye SYBR Green I was used for the detection of PCR products. The PCR was performed using 23- μ l PCR master mix composed of 12.5 μ l of SYBR Green PCR Master Mix + 1 μ l of the forward primer + 1 μ l of the reverse primer + 8.5 μ l of RNase-free water. The PCR procedure involved initial activation step at 95°C for 5 min. This is followed by denaturation for 5 s at 95°C and annealing for 10 s at 60°C . These cycles were repeated for 40 cycles. PCR efficiency of both the target genes [tumor necrosis factor- α (TNF- α), transforming growth factor- β_1 (TGF- β_1), inducible

nitric oxide synthase (iNOS)] and reference or the housekeeping gene (β -actin) was calculated from the derived slopes of the standard curves. Levels of TNF- α , TGF- β_1 , and iNOS mRNA were normalized relative to β -actin in the same sample.

Primers for the TNF- α , TGF- β_1 , iNOS, and β -actin are listed in the following:

TNF- α (sense: CCACCACGCTCTTCTGTC; anti-sense: ATCTGAGTGTGAGGGTCTGG), TGF- β_1 (sense: GCTAATGGTGGACCGCAAC; anti-sense: CACTGCTTCCCGAATGTCTG), iNOS (sense: GGAGCCTTTAGACCTCAACAGA; anti-sense: GGCTGGACTTTTCACTCTGC), β -actin (sense: TGGGTATGGAATCCTGTGG; anti-sense: GCCTGTGTTGGCATAGAGG).

Measurement of nitric oxide in lung tissue

The concentration of total nitrate/nitrite (NO_x) was measured in lung homogenates as an indicator of NO production using NO assay kit according to the supplier's specifications (R&D Systems, Minneapolis, USA). The amount of NO_x in the test samples was determined by interpolation of the result into the standard curve of nitrite prepared from serial dilutions of a 2,000- μ M stock solution. All assays were performed in duplicate and expressed as μ M/g protein.

Statistical analysis

The results were presented as mean $\pm$ standard error of mean (S.E.M), where n equals the number of mice. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test.

Results

Effect of sitagliptin on inflammatory cell count in BAL fluid

As shown in Table 1, OVA challenge induced a significant increase in total and differential cell counts as compared to the control group. Sitagliptin treatment markedly decreased total cell ($P<0.001$), eosinophils ($P<0.01$), lymphocytes ($P<0.001$), neutrophils ($P<0.01$), and macrophages ($P<0.001$) count in the BAL fluid in comparison to the OVA group.

Effects of sitagliptin on the lung tissue histopathology

Eosinophil infiltration into lung tissue

OVA challenge induced marked infiltration of inflammatory cells into the peribronchiole and perivascular connective

Table 1 Effects of sitagliptin treatment on total and differential cell counts in BAL fluid of OVA-sensitized and OVA-challenged mice

	Cell count in BAL fluid ($\times 10^4$ /lung)				
	Total cell	Lymphocytes	Eosinophils	Neutrophils	Macrophages
Control	10.8 $\pm$ 2.40	8.07 $\pm$ 1.87	0	2.12 $\pm$ 0.80	0
Sita	12.5 $\pm$ 1.60	7.09 $\pm$ 3.01	0	2.56 $\pm$ 0.40	0
OVA	89.2 $\pm$ 7.40***	73.64 $\pm$ 6.37***	2.58 $\pm$ 0.60**	10.67 $\pm$ 1.13***	1.24 $\pm$ 0.15***
OVA+Sita	21.52 $\pm$ 3.25 ^{SSS}	15.66 $\pm$ 2.13 ^{SSS}	0.33 $\pm$ 0.07 ^{SS}	5.7 $\pm$ 0.74* ^{SS}	0.11 $\pm$ 0.05 ^{SSS}

Control (OVA-sensitized, saline-challenged mice), Sita (OVA-sensitized, saline-challenged mice received sitagliptin 8 mg/kg/twice daily), OVA (OVA-sensitized/-challenged mice), OVA + Sita (OVA-sensitized/-challenged mice received sitagliptin 8 mg/kg/twice daily). Sitagliptin treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. BAL fluid was collected from mice 24 h after the last OVA challenge. Values represent mean $\pm$ S.E.M, $n=10$

BAL bronchoalveolar lavage, Sal saline, OVA ovalbumin, Sita sitagliptin

* $P<0.05$; ** $P<0.01$; *** $P<0.001$ as compared to control group; ^{SS} $P<0.01$; ^{SSS} $P<0.001$ as compared to OVA group (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)

tissue (Fig. 1a–h). The majority of the infiltrated inflammatory cells were eosinophils. Sitagliptin significantly attenuated eosinophil-rich leukocyte infiltration as compared to the OVA group. In addition, sitagliptin similarly blocked changes in the structural cells of the airways, including increases in airway smooth muscle mass, and epithelial cell disruption. There was a significant decrease in the inflammatory score in the sitagliptin group as compared to the OVA group ($P<0.001$) (Fig. 2a)

Airway goblet cell hyperplasia and mucus production

Overproduction of mucus and goblet cell hyperplasia were observed in the bronchial airways of OVA group, but not in the control mice (Fig. 1e–h). Mice of the OVA group had a staining score of 2.33 ± 0.21 , while control mice had a staining score of 0.75 ± 0.16 . In particular, there was a greater reduction in the number of PAS-stained goblet cells in the sitagliptin-treated asthmatic mice, leading to a significant decrease in the production of mucus as compared to the OVA group mice ($P<0.001$) (Fig. 2b).

Effect of sitagliptin on IL-13 production in BAL fluid

Exposure to OVA resulted in a significant elevation in the level of IL-13 in BAL fluid as compared to the control mice ($P<0.001$) (Fig. 3). Sitagliptin significantly decreased the level of IL-13 as compared to that in the OVA group. These results suggest that sitagliptin inhibited the OVA-induced increase in Th2 cytokines levels in murine model of asthma.

Effect of sitagliptin on serum IgE

The level of serum total and OVA-specific IgE in mice was increased significantly by airway challenge with OVA as compared to those of the control group. The administration

of sitagliptin significantly reduced the concentration of serum total and OVA-specific IgE as compared to the OVA group (Fig. 4).

Effect of sitagliptin on oxidative stress in lung homogenate

Airway challenge with OVA resulted in a significant increase ($P<0.001$) in MDA level in lung, while GSH and SOD activities were markedly decreased ($P<0.001$; $P<0.01$) (Fig. 5). These changes were all reduced significantly by sitagliptin treatment.

Effect of sitagliptin on TNF- α and TGF- β_1 mRNA expression level in lung homogenate

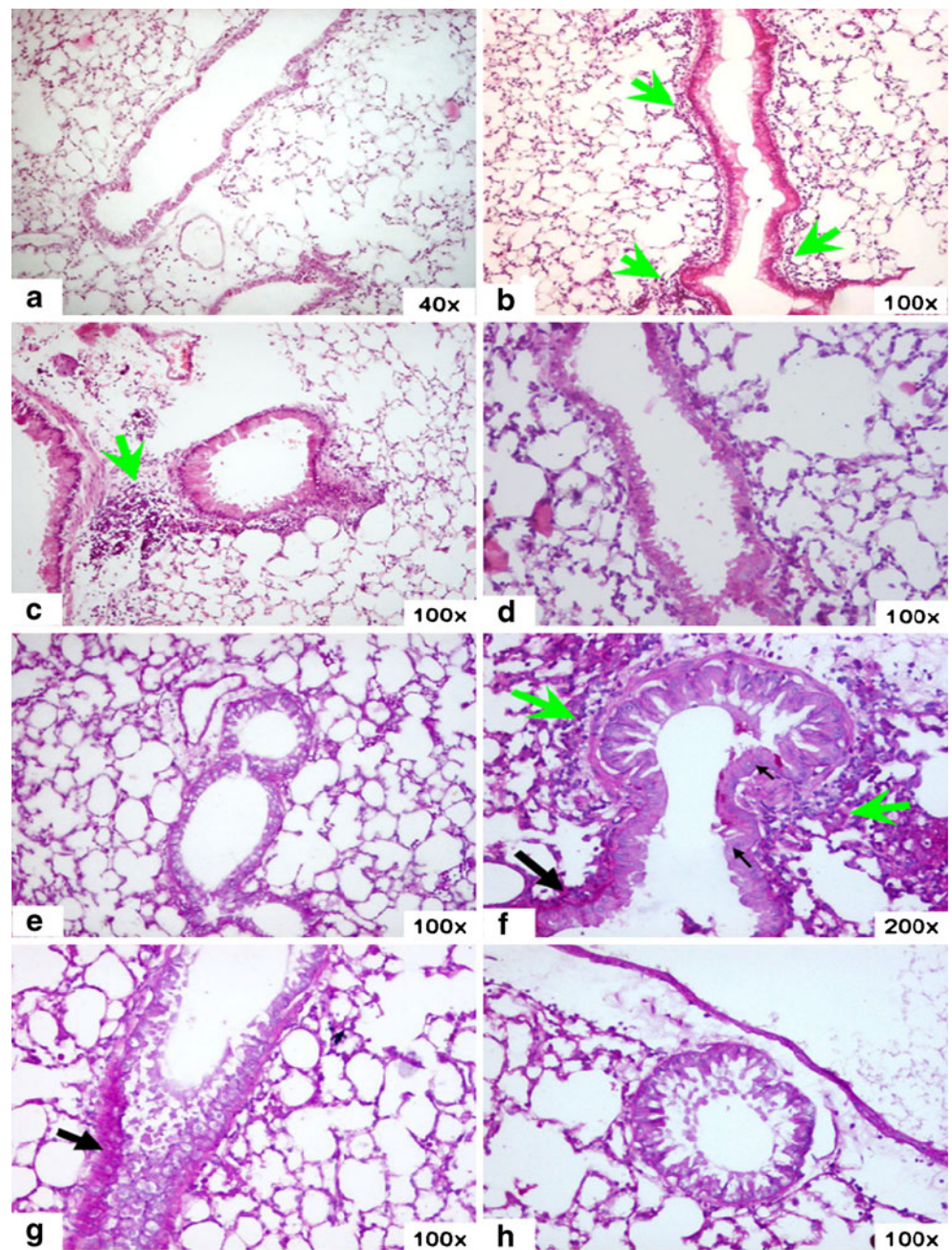
OVA challenge resulted in a significant increase in the mRNA expression levels of TNF- α and TGF- β_1 as compared to the control group ($P<0.001$ and $P<0.05$, respectively). Sitagliptin significantly decreased the expression of TNF- α ($P<0.001$) as well as TGF- β_1 ($P<0.05$) as compared to the OVA group (Fig. 6).

Effect of sitagliptin on NO production and iNOS mRNA expression in lung homogenate

As shown in Fig. 7a, OVA-challenged mice induced a significant increase in iNOS mRNA expression ($P<0.01$) as compared to the control group (Fig. 7b). Sitagliptin significantly downregulated ($P<0.01$) the OVA-induced increase in iNOS mRNA expression in the lung tissues (Fig. 7a).

Similarly, NO production in lung tissues was significantly higher in OVA-sensitized–OVA-challenged mice compared to the control group ($P<0.001$), but this OVA-triggered NO production was significantly decreased by

Fig. 1 Effects of sitagliptin on histological examination of lung tissues of OVA-sensitized and OVA-challenged mice. In particular, OVA-induced inflammatory cell recruitment and mucus hypersecretion. **a** Control, stained with H&E; **b, c** inflammatory cell infiltrate (green arrows); **d** lung tissue from Sitagliptin-treated mice with no inflammatory infiltrate; **e** PAS-stained section of control with no goblet cell hyperplasia; **f, g** PAS-stained sections of (OVA)-induced reaction showing goblet cell hyperplasia (black arrow); **h** PAS-stained section showing effect of sitagliptin treatment with no goblet cell hyperplasia. Control (OVA-sensitized, saline-challenged mice), Sita (OVA-sensitized, saline-challenged mice received sitagliptin 8 mg/kg/twice daily), OVA (OVA-sensitized/-challenged mice), OVA + Sita (OVA-sensitized/-challenged mice received sitagliptin 8 mg/kg/twice daily). Sitagliptin treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. Lung tissues were collected from mice 24 h after the last OVA challenge



treatment with sitagliptin ($P < 0.01$) as compared to the OVA group (Fig. 7b).

Discussion and conclusions

The present study used an OVA-induced murine model of allergic airway inflammation to test the potential anti-asthmatic effect of sitagliptin. Results have shown that sitagliptin significantly inhibits increases in total inflammatory cell and eosinophil numbers in BAL fluid induced by OVA, and reduces the levels of total and OVA-specific IgE in serum, and IL-13 in BAL fluid. This was supported by

histopathological examination results which showed that sitagliptin substantially decreased both OVA-challenged eosinophilia in lung tissue and mucus hypersecretion by airway goblet cells.

To explore the possible mechanism(s) of the protective action of sitagliptin against OVA-induced asthma, oxidative stress and inflammatory factors were evaluated in the lung tissue. Sitagliptin effectively reduces oxidative burden during the inflammatory response to OVA challenge. Also, sitagliptin attenuated OVA-induced production of inflammatory cytokines which induce, enlarge, and facilitate the entire or focal inflammatory reaction.

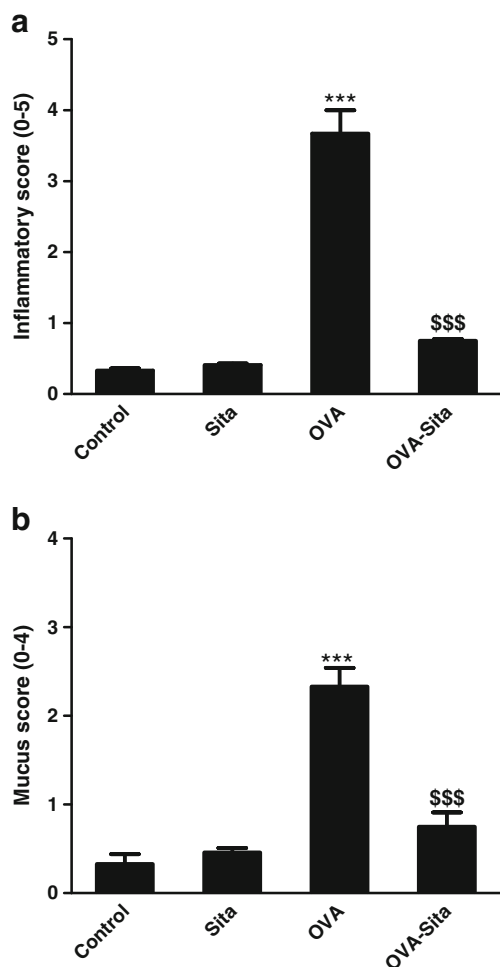


Fig. 2 Effects of sitagliptin on **a** scoring the extent of inflammation by quantitative analysis of inflammatory-cell infiltration in lung sections and **b** scoring of mucus production in lung sections. Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section. Control (OVA-sensitized, saline-challenged mice), Sita (OVA-sensitized, saline-challenged mice received sitagliptin 8 mg/kg/twice daily), OVA (OVA-sensitized/-challenged mice), OVA + Sita (OVA-sensitized/-challenged mice received sitagliptin 8 mg/kg/twice daily). Sitagliptin treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. Lung tissues were collected from mice 24 h after the last OVA challenge. *** $P < 0.001$ as compared to control group; \$\$\$ $P < 0.001$ as compared to OVA group (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)

The presence of increased numbers of eosinophils in BAL fluid is a characteristic of asthma. Sitagliptin-treated mice had significantly reduced lung eosinophilia in BAL fluid and lung tissue as compared to OVA-challenged mice. This effect of sitagliptin treatment may result from a decrease in the levels of Th2 cytokines (IL-13) responsible for eosinophil recruitment (Pope et al. 2001) into the lung in asthma.

Previous investigations have shown that Th2 cytokines IL-4, IL-5, and IL-13 play important roles in the lungs of

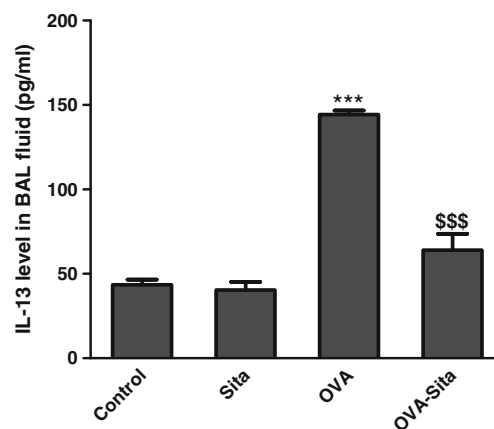


Fig. 3 Effects of sitagliptin on the level of IL-13 in BAL fluid of OVA-sensitized and OVA-challenged mice. Control (OVA-sensitized, saline-challenged mice), Sita (OVA-sensitized, saline-challenged mice received sitagliptin 8 mg/kg/twice daily), OVA (OVA-sensitized/-challenged mice), OVA + Sita (OVA-sensitized/-challenged mice received sitagliptin 8 mg/kg/twice daily). Sitagliptin treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. Lung tissues were collected from mice 24 h after the last OVA challenge. Values represent mean $\pm$ S.E.M, $n = 10$. *** $P < 0.001$ as compared to control group; \$\$\$ $P < 0.001$ as compared to OVA group (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)

asthmatic subjects because all enhance IgE production, IL-5 increases eosinophil accumulation, and IL-13 directly stimulates mucus hypersecretion and airway hyperreactivity (Brightling et al. 2002; Lee et al. 2010). IL-4, IL-13, and TGF- β are secreted additionally by eosinophils as well as IgE-activated mast cells and exert direct effects on fibroblasts, epithelial cells, and airway smooth muscle. Elevated serum IgE level has been reported to be important in the development of asthmatic responses (Hamelmann et al. 1999; Ishii et al. 2004). Activation of T-cells with allergen-specific IgE plus airway challenge enhances its function, leading to increased Th2-cytokine (IL-4, IL-5, and IL-13) production and eosinophilic airway inflammation (Hamelmann et al. 1999). Our present results showed that sitagliptin inhibited both total and allergen specific IgE production levels in serum as well as BAL fluid level of IL-13. So, sitagliptin has an effect on allergic asthma that developed in an IgE-dependent manner. Previously, Kruschinski et al. (2005) reported that in two CD26-deficient F344 rat sub-strains, the number of CD4⁺ T lymphocytes was markedly reduced compared to wild-type F344. The decrease in T cell recruitment observed in the CD26-deficient rats was associated with significantly reduced OVA-specific IgE-titres and blunted inflammatory status. This novel and remarkable finding indicates that CD26 is a necessary prerequisite for constituting a full-blown T cell component in response to the challenge with antigens such as OVA. This report suggested a role for CD26 in the pathogenesis of asthma via T cell-dependent processes such as antibody production.

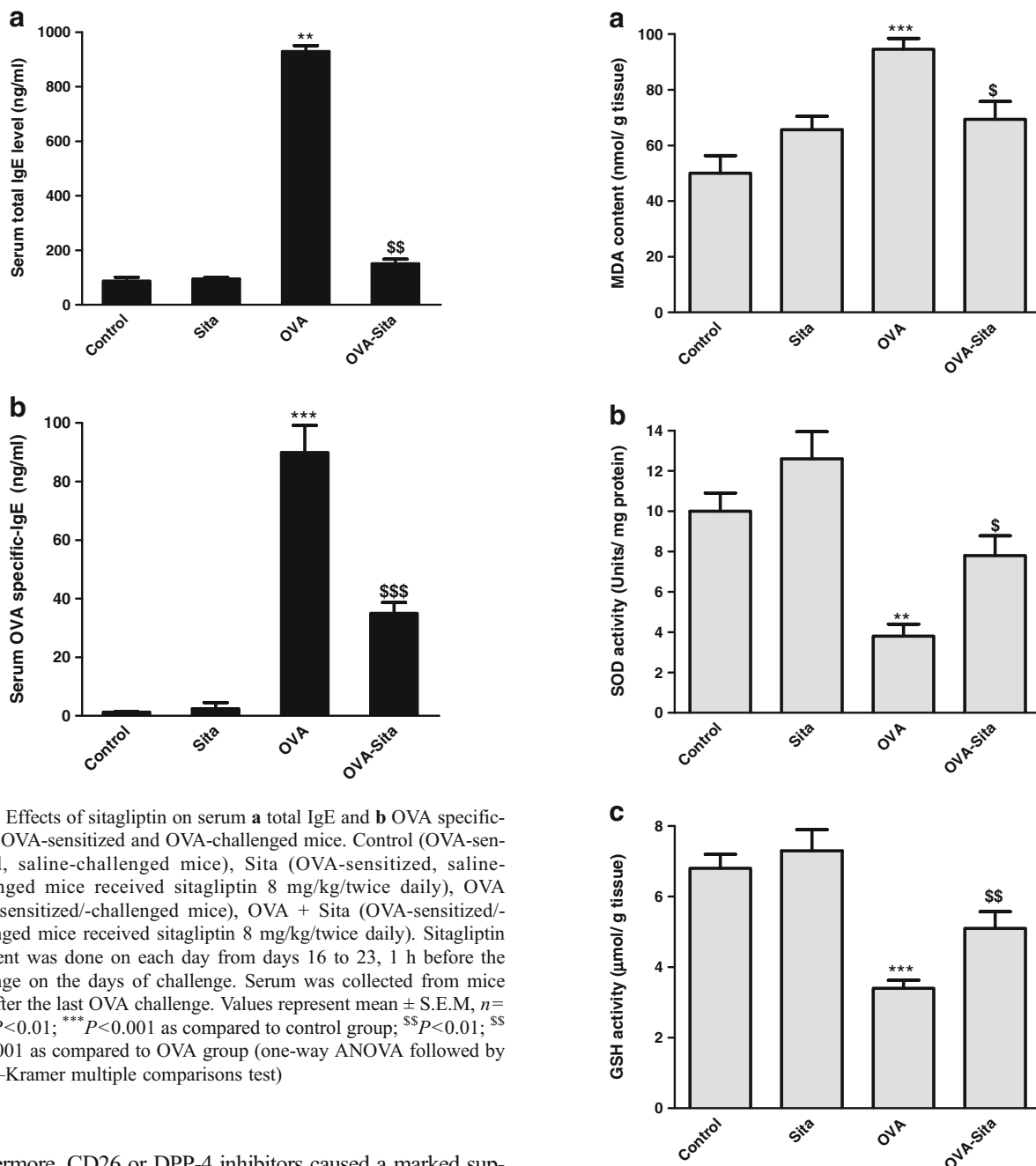


Fig. 4 Effects of sitagliptin on serum **a** total IgE and **b** OVA specific-IgE in OVA-sensitized and OVA-challenged mice. Control (OVA-sensitized, saline-challenged mice), Sita (OVA-sensitized, saline-challenged mice received sitagliptin 8 mg/kg/twice daily), OVA (OVA-sensitized/-challenged mice), OVA + Sita (OVA-sensitized/-challenged mice received sitagliptin 8 mg/kg/twice daily). Sitagliptin treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. Serum was collected from mice 24 h after the last OVA challenge. Values represent mean $\pm$ S.E.M, $n=10$. ** $P<0.01$; *** $P<0.001$ as compared to control group; \$\$ $P<0.01$; \$\$\$ $P<0.001$ as compared to OVA group (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)

Furthermore, CD26 or DPP-4 inhibitors caused a marked suppression of paw swelling in rats with adjuvant arthritis due to anti-proliferative effects on T cells (Williams et al. 2003).

In addition, histopathological results of the present study revealed that infiltrated inflammatory cells or mucus generation of goblet cells induced by airway challenge with OVA is ameliorated by sitagliptin administration. These results indicate that mucus hypersecretion is attenuated by the suppressive effects of sitagliptin on cytokine production and eosinophilia.

Eosinophils, alveolar macrophages, and neutrophils from asthmatic patients produce more ROS than those from normal subjects (Teramoto et al. 1996). The overproduction of ROS or depression of protective mechanisms results in bronchial hyperreactivity, which is characteristic of asthma

Fig. 5 Effect of sitagliptin on MDA content, SOD, and GSH activities in lungs of OVA-sensitized and OVA-challenged mice. Control (OVA-sensitized, saline-challenged mice), Sita (OVA-sensitized, saline-challenged mice received sitagliptin 8 mg/kg/twice daily), OVA (OVA-sensitized/-challenged mice), OVA + Sita (OVA-sensitized/-challenged mice received sitagliptin 8 mg/kg/twice daily). Sitagliptin treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. Lung tissues were collected from mice 24 h after the last OVA challenge. Values represent mean $\pm$ S.E.M, $n=10$. ** $P<0.01$; *** $P<0.001$ as compared to control group; \$ $P<0.01$; \$\$\$ $P<0.001$ as compared to OVA group (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)

(Sadeghi-Hashjin et al. 1996). Airway epithelial cells are known to produce ROS in response to many stimuli,

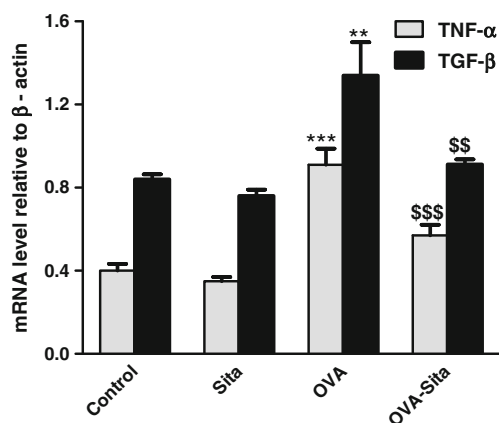


Fig. 6 Effects of sitagliptin on mRNA expression levels of TNF- α and TGF- β_1 in the lung tissues of OVA-sensitized and OVA-challenged mice. Control (OVA-sensitized, saline-challenged mice), Sita (OVA-sensitized, saline-challenged mice received sitagliptin 8 mg/kg/twice daily), OVA (OVA-sensitized/-challenged mice), OVA + Sita (OVA-sensitized/-challenged mice received sitagliptin 8 mg/kg/twice daily). Sitagliptin treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. Lung tissues were collected from mice 24 h after the last OVA challenge. Values represent mean $\pm$ S.E.M, $n=10$. * $P<0.05$; *** $P<0.001$ as compared to control group; $^{\$}P<0.05$; \$\$\$ $P<0.001$ as compared to OVA group (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)

including weed pollens and viruses (Boldogh et al. 2005). These ROS oxidize cell membrane phospholipids and generate lipid hydroperoxides which impair membrane functions such as transport, which result in dysfunction of channel proteins and the loss of fluidity leading to tissue injury (Cienciewicki et al. 2008). Therefore, attenuation of intracellular ROS during inflammation may have potential advantages with regard to the treatment of lung inflammatory diseases. The results of the present study have shown that sitagliptin decreased oxidative stress as it controlled the levels of SOD and GSH to the values near control with resultant decrease in MDA content. Therefore, sitagliptin can be considered to reduce oxidative burden during the inflammatory response to OVA challenge which is likely related to its general effect on inhibition of PMN infiltration and hence ROS generation.

TNF- α and TGF- β_1 have been implicated in the inflammatory response seen in asthma. These are proinflammatory and powerful chemotactic agent for neutrophils, eosinophils, macrophages, and mast cells (Berger et al. 2005).

Furthermore, there is increased expression of TNF- α and TGF- β_1 in the airways of asthmatic patients compared to normal subjects (Thomas 2001; Xie et al. 2007). TNF- α can also increase microvascular permeability and activate T cells, eosinophils, and mast cells (Thomas 2001).

In the present study, there was overexpression of the mRNA level of TNF- α and TGF- β_1 in lung tissue induced by OVA sensitization and challenge, and the inhibition of overexpressed TNF- α - and TGF- β_1 -mRNA by sitagliptin

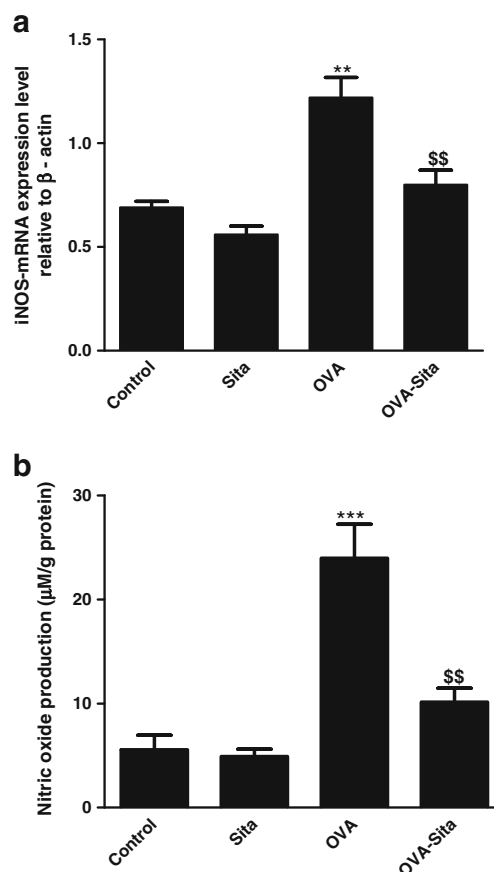


Fig. 7 Effects of sitagliptin on **a** mRNA expression levels of iNOS and **b** nitric oxide (NO) production in lung tissues of OVA-sensitized and OVA-challenged mice. Control (OVA-sensitized, saline-challenged mice), Sita (OVA-sensitized, saline-challenged mice received sitagliptin 8 mg/kg/twice daily), OVA (OVA-sensitized/-challenged mice), OVA + Sita (OVA-sensitized/-challenged mice received sitagliptin 8 mg/kg/twice daily). Sitagliptin treatment was done on each day from days 16 to 23, 1 h before the challenge on the days of challenge. Lung tissues were collected from mice 24 h after the last OVA challenge. Values represent mean $\pm$ S.E.M, $n=10$. ** $P<0.01$; *** $P<0.001$ as compared to control group; \$\$ $P<0.01$ as compared to OVA group (one-way ANOVA followed by Tukey–Kramer multiple comparisons test)

treatment seems to be a mechanism by which it can decrease the concentration of inflammatory mediators.

NO is another mediator involved in the pathophysiology of asthma; excessive production of NO in asthma may be cytotoxic and may also contribute to the pathologic changes (e.g., airway edema, mucus production, and inflammatory cellular infiltration) seen in asthma, particularly during asthma exacerbations (Ricciardolo 2003). The non-invasive measurement of NO in exhaled air seems accurately to reflect inflammation in the airways and may be of value in monitoring airway diseases such as asthma, adult respiratory distress syndrome, and lung fibrosis (Beckman and Koppenol 1996). Although high NO output is poorly correlated with the degree of bronchoconstriction, this parameter correlates well with other inflammatory markers, including airway

eosinophilia; excessive NO may recruit eosinophils in the airway and shift the balance toward Th2 cells, thus exacerbating in inflammation of the airway (Kharitonov et al. 1994; Moon et al. 2008). In the present study, a significant increase in iNOS gene expression was observed in OVA-challenged mice compared to the control ones. This result was confirmed by analysis of NO content analysis. Sitagliptin pretreatment significantly reduced the OVA-triggered up-regulation of iNOS and NO levels.

In conclusion, these results provide further evidence that oral administration of sitagliptin may play a role in treatment of allergic diseases such as asthma. This effect may be attributed to its ability to modulate nitric oxide expression and production. Also, sitagliptin can enhance antioxidant status and downregulate some inflammatory cytokines expression through exerting anti-inflammatory/immunomodulatory activities. Further clinical studies are needed to prove this effect.

Conflict of interest statement The authors declare no conflict of interest.

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