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Luteolin alleviates bronchoconstriction and airway hyperreactivity in ovalbumin sensitized mice

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Abstract. *Objective and Design:* Asthma is an inflammatory disease of the airways and the current focus in managing asthma is the control of inflammation. In this study, we attempted to investigate the anti-asthmatic potential of a plant derived natural compound, luteolin.
Material: We used a murine model of airway hyperreactivity, which mimicked some of the characteristic features of asthma. Male BALB/c mice (8–9 weeks) were used for this study.
Treatment: Mice (n = 6) were sensitized by intraperitoneal (i.p.) injection of 10 mg of ovalbumin (OVA) on days 0, 7 and 14 followed by aerosol inhalation (5% OVA) treatments daily beginning from day 19 to day 23. To study its preventive effect, luteolin (0.1, 1.0, and 10 mg/kg body weight; daily) was administered orally during the entire period (0 to 23 day) of sensitization. To study its curative effect, mice were first sensitized and then luteolin (1.0 mg/kg body weight daily) was given orally from day 26 to 32. The airway hyperreactivity, immunoglobulin E (IgE) in the sera, and cytokines (IFN-γ, IL-4 and IL-5) in the bronchoalveolar lavage fluid (BALF) were measured.
Results: Both during sensitization and after sensitization, luteolin, at a dose of 0.1 mg/kg body weight, significantly modulated OVA-induced airway bronchoconstriction and bronchial hyperreactivity ($p < 0.05$). Luteolin also reduced OVA-specific IgE levels in the sera, increased interferon gamma (IFN-γ) levels and decreased the interleukin-4 (IL-4) and interleukin-5 (IL-5) levels in the BALF.
Conclusion: Our study showed that luteolin treatment during and after sensitization significantly attenuated the asthmatic features in experimental mice. Therefore, luteolin could be used either as a lead molecule to identify an effective anti-asthma therapy or as a means to identify novel anti-asthma targets.

Key words: Asthma – Luteolin – Bronchial hyperreactivity – Immunoglobulin E – Cytokines

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Introduction

Asthma is an inflammatory disease of the airways, which affects millions of people worldwide. The disease is reaching epidemic proportions and young lives are increasingly rendered unproductive. Asthma is characterized by difficulty in breathing due to constriction of smooth muscles of the bronchi as a result of inflammation. It is characterized by elevated levels of immunoglobulin E in the blood and infiltration of eosinophils into the airways. The development of the disease is mediated by cytokines- IL-4 and IL-5 [1], IgE [2–4], eosinophils and various other mediators e.g. leukotrienes, cyclooxygenase products, phospholipases [5, 6] all of which lead to the symptoms of asthma. In contrast, IFN-γ inhibits this process [7].

The current focus in managing asthma is to control inflammation using anti-asthmatic drugs with low side effects [8]. There is a need for novel drugs for the treatment of asthma which may have fewer side-effects.

Luteolin is a flavonoid, which has been attributed with anti-inflammatory [9, 10] and anti-allergic properties [9]. In vitro studies have elucidated some of the molecular mechanisms by which luteolin modulates the inflammatory response. Luteolin has been reported to inhibit: a) the process of signal transduction leading to the inhibition of nuclear transcription factor-kappa B (NF-κB) mediated gene expression [10], b) the production of the proinflammatory cytokine, IL-5 [11] and c) the release of mediators such as leukotrienes and prostaglandins [10]. In addition, luteolin suppresses the expression of TNF-α induced ICAM molecules on endothelial cells [12]. Because of this profile of action, we postulated that luteolin might have a preventive and/or therapeutic role in attenuating asthma. We used a sensitized murine model that shares some of the features of asthma following antigen challenge including low airway conductance, bronchial hyperreactivity to methacholine, high serum IgE levels, and increased IL-4 and IL-5 levels in bronchoalveolar fluid.

Materials and methods

Animals

BALB/c male mice (eight-nine weeks old, 18–22 g body weight) were obtained from National Institute of Nutrition, Hyderabad, India. Approval for these experimental protocols was obtained from the Institutional Animals Ethical Committee. The mice were acclimatized for one week under the laboratory conditions (25 ± 2 °C, 60% humidity and at usual day/night cycle) before starting the experiment.

Sensitization

Mice (n = 6 in each group) were immunized/sensitized with or without (sham-sensitized) 0.2 ml of 10 µg ovalbumin (Sigma Chemicals Co. St Louis, MO, USA) and 2 mg aluminum hydroxide intraperitoneally on days 0,7 and 14 using a modified protocol previously described by Sakai et al. [13] (Fig. 1). Five days after the final i.p. injection, the mice were subjected to aerosolized OVA (5%) or Phosphate Buffered Saline (PBS) (for sham-sensitized group) inhalation for 20 min daily beginning from day 19 to day 23 (Fig. 1). Mice were placed in a plexiglas chamber (20 × 20 × 20 cm dimensions) and exposed to an aerosol generated from a nebulizer (de Vilbiss, USA) with an airflow of 4 l/min.

Treatment with Luteolin

To study the preventive effect, luteolin (dissolved in 50% hydro-alcohol) (Sigma Chemicals Co. St Louis, MO, USA) (0.1, 1.0, and 10.0 mg/kg body weight, 20 µl volume) or vehicle (i.e., 50% hydro-alcohol) was administered orally to each group of mice (n = 6) daily, starting from the first day of sensitization (Fig. 1A). To study the curative effect, mice were first sensitized as described before followed by oral luteolin (1.0 mg/kg body weight) treatment daily from day 26 to 32 (Fig. 1B).

Measurement of specific airway conductance

Specific airway conductance of the mice was measured by a modified technique developed in our laboratory (A R and B G; manuscript under preparation), based on dual-chamber constant-volume body plethysmograph [14]. Briefly, each mouse was placed in a two-chambered acrylic box. The posterior chamber housed the body and the anterior chamber held the head of the animal. A pneumotachograph (Hans Rudolph Series 8421B, Hans Rudolph Inc. Missouri, USA) with a transducer (Validyne MP 45 + 2 cm H_2O, USA) and an amplifier (Validyne CD15, USA) was attached to the anterior chamber for sensing the airflow from the mouth of the mouse. This two-chambered box was kept inside an outer box with a capacity of approximately 2 litres. Changes in the outer box pressure were determined using a similar type of transducer and amplifier (Validyne, USA). These two amplified signals of the outer box pressure and airflow from the mouth were fed to an oscilloscope (Tektronix 6116, USA) and computed for a (X–Y) plot. The signals were displayed as loops and the X and Y values of the slope of the loops provided the data for computing SGaw. On an average, 20 loops were examined and readings were taken from three similar overlapping loops. The values of SGaw were calculated by the formula [14]:

$$\text{SGaw} = \tan\theta \cdot \text{calibration factor}$$

where, $\tan\theta = Y \div X$

calibration factor =

$$\frac{\text{ml air/sec/division on ordinate}}{\text{ml air/division on abscissa}} \div \frac{1}{\text{Pressure (barometric)-Pressure (vapor)}}$$

Measurement of ovalbumin-induced bronchoconstriction

Bronchoconstriction was measured in terms of percent decrease in SGaw after OVA challenge as compared to pre-challenge (basal) values. Each individual mouse was housed in the body box and challenged for 60 s with a dose of aerosolized vehicle (0.9% saline) and the initial basal values of the airway conductance were taken. The aerosol was generated with a nebulizer (de Vilbiss, USA) at 4 l/min air current. The mouse was then challenged for 60 s with aerosolized OVA (5 mg/ml in saline) and the final value of the airway conductance were taken. The difference in the initial and final values gave the percent airway conductance from which percent fall of the airway conductance (SGaw) can be derived. The data are presented in terms of percentage of basal specific airway conductance.

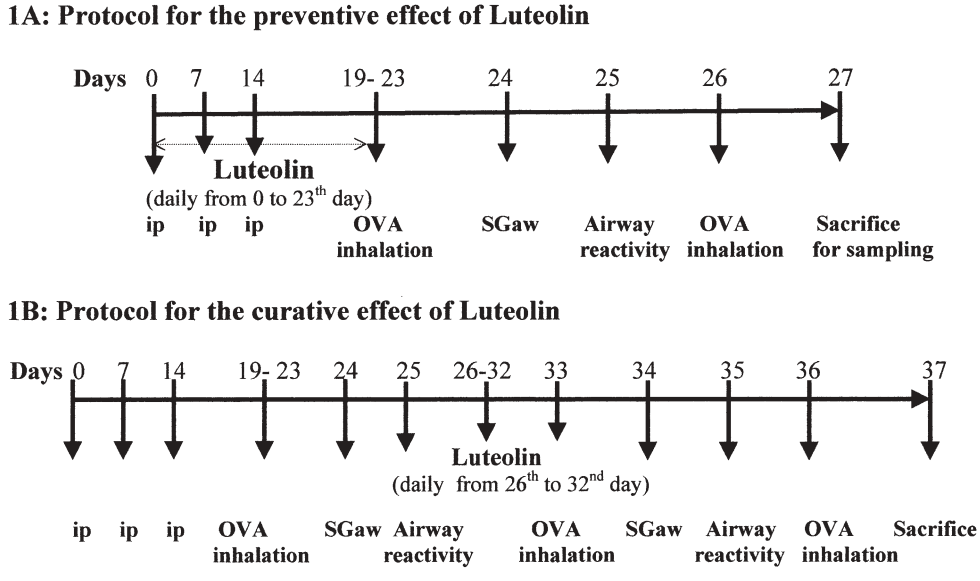

Fig. 1. Protocol for sensitization of mice and treatment with luteolin as described in the Materials and methods.

Measurement of airway reactivity to methacholine

Airway reactivity to methacholine was determined by measuring the concentration of inhaled methacholine that produced 35% fall in SGaw in intact mouse sitting in the body plethysmograph [14]. In this experiment, the animals housed in the body plethysmograph were exposed for 60 s to increasing concentrations of inhaled aerosolized methacholine (3.1, 6.25, 12.5, 50 and 100 mg/ml in PBS). Intervals between the doses were adjusted to allow full recovery of airway conductance. The aerosol was generated with a nebulizer (deVilbiss, USA) at 4 l/min air current. A dose-response curve for decrease in SGaw with increasing concentrations of methacholine was plotted for each individual animal. The methacholine concentration that produced a 35% decrease in SGaw (PD_{35}MCh) was determined and was used as a measure of airway reactivity to methacholine. PD_{35}MCh values were measured in all the groups of mice before and after the sensitization period.

Sampling of sera and BAL fluid

Serum and BALF were collected after final measurements of specific airway conductance and airway reactivity. The mice were challenged with OVA aerosol as before and were sacrificed 24 h later using an overdose of sodium pentobarbitone (100 mg/kg body weight, i.p.). Blood was collected and sera was separated by centrifugation at 3000 rpm for 10 min at 4 °C. The trachea was canulated and the lungs were lavaged with 0.5 ml of saline. Lungs of each animal were lavaged 4 times. About 1.8 ml of BALF was recovered per mouse. The samples were stored at −20 °C until analysis was carried out.

Measurement of ovalbumin-specific Immunoglobulin E

OVA-specific serum IgE levels were measured by Enzyme Linked Immuno Sorbent Assay (ELISA) [15] immediately after sampling. Briefly, a 96 well microtitre plate (Maxisorp Nunc, Denmark) was coated with 10 µg of OVA in 50 µl coating buffer and kept overnight at 4 °C. The wells were washed three times with PBS and blocked with 50 µl blocking buffer (1% Bovine Serum Albumin (BSA) in PBS) and kept at 37 °C for two hours. After washing three times with PBST (0.05% Tween 20 in PBS), 100 µl serum (diluted 1 : 3 in blocking buffer) was added to the wells and kept overnight at 4 °C. The wells were washed three times with PBST and 50 µl of horseradish peroxidase labelled anti IgE antibody (1 : 1000 dilution, Pharmingen, USA) was added and kept at 37 °C for two hours. After washing four times with PBST, 50 µl of substrate (3, 3′, 5, 5′ tetramethylbenzedine and H_2O_2) was added. The reaction was stopped by adding 2.4 M H_2SO_4 to each well. Readings were taken using an ELISA plate reader (Spectramax 190, Molecular Devices, USA) at 450 nm. The results were given in Optical Density (OD_{450}) values.

Measurement of IFN-γ, IL-4 and IL-5

The cytokines IFN-γ, IL-4 and IL-5 were measured in the BALF using ELISA kits (BD Pharmingen, USA) as per the manufacturer's protocol immediately after sampling. Briefly, the plates were coated with the respective capture antibodies of the three cytokines IFN-γ or IL-4 or IL-5 and kept overnight at 4 °C. The wells were washed four times with PBS and blocked with 100 µl of blocking buffer (3% BSA/PBS) and kept at room temperature (27 °C) for three hours. After washing three times with PBST, 100 µl BALF was added to the wells and kept for 2 h at 27 °C. The wells were washed thrice with PBST and 100 µl of detection antibody (biotinylated anti-IFN-γ or IL-4 or IL-5 along with streptavidin conjugated horseradish peroxidase) (different dilutions for the three cytokines as per instructions) was added and incubated for 1 h at 27 °C. After washing three times with PBST, 100 µl of substrate (3, 3′, 5, 5′ tetramethylbenzedine containing H_2O_2) and was added to the wells and the plate was left for 15 min at 27 °C. The reaction was stopped by adding 2.4 M H_2SO_4 to each well. Readings were taken using ELISA plate reader (Spectramax 190, Molecular Devices, USA) at 450 nm. The results were presented as OD_{450} values.

Statistics

All the data were presented as mean ± SEM. The normality of the samples was confirmed by Kolmogorov-Smirnov test. Student's T test was performed to find the level of significance. Statistical analyses were done as described previously [16, 17]. The non parametric Mann-Whitney U-test was applied for the difference between two groups and a p-value less than or equal to 0.05 was considered to be significant.

Results

Luteolin reduces the OVA-induced acute constriction in sensitized mice

To determine the preventive effect of luteolin, mice were dosed with luteolin (0.1, 1.0 and 10.0 mg/kg body weight) or vehicle during sensitization, as described in Materials and Methods. Following challenge, OVA-sensitized mice treated with vehicle showed a 43% fall in specific airway conductance (SGaw) as compared to their basal values, whereas sham sensitized mice showed no change (Fig. 2). Interestingly, treatment with luteolin markedly prevented OVA-induced decreases in SGaw. The dose of 0.1 mg/kg luteolin markedly reduced the fall in SGaw induced by OVA and only a 7% decrease was recorded ($p < 0.01$). Further increase of the doses did not show any greater reduction in OVA-induced decreases in SGaw ($p < 0.01$).

To examine the curative effect of luteolin, mice were first sensitized and then treated with luteolin (1.0 mg/kg) for one week. Luteolin was found to reverse OVA-induced decreases in SGaw (Fig. 2).

Luteolin reduces airway reactivity to methacholine

To examine the effect of luteolin on OVA-induced bronchial hyperreactivity we determined PD35 MCh following methacholine challenge as described in materials and methods. As shown in Figure 3, there was a significant fall in MCh PD_{35} values (1.3 ± 0.5 mg/ml) in following OVA challenge of sensitized mice compared to normal mice (97 ± 1.0 mg/ml, $p < 0.01$). Treatment with luteolin (0.1, 1.0 and 10.0 mg/kg during sensitization) markedly attenuated the development of airway hyperreactivity to methacholine. PD_{35} MCh values increased following luteolin treatment to 72.1 ± 1.9, 42.4 ± 4.2 and 10.9 ± 4.8 mg/ml, respectively ($p < 0.01$). In addition, when the mice were first sensitized and then treated with luteolin (1.0 mg/kg) for one week, PD_{35} MCh values increased significantly ($p < 0.01$) suggesting that luteolin inhibited the development of OVA-induced airway hyperreactivity.

Luteolin reduces ovalbumin specific IgE

To investigate the effect of luteolin on serum IgE levels, we compared the levels of the OVA-specific IgE in serum of mice

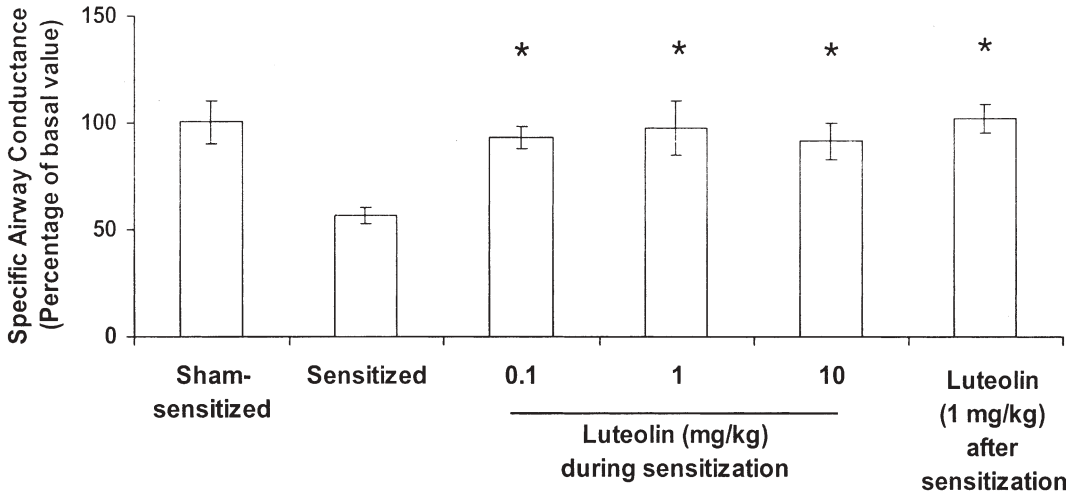

Fig. 2. Luteolin reduces bronchoconstriction in ovalbumin-sensitized mice. Experiments were performed with six groups of mice (n = 6) as described in Figure 1. Briefly, mice were treated with three different doses of luteolin (0.1, 1.0 and 10.0 mg/kg body weight) during sensitization. Another group was first sensitized and then orally treated with luteolin (1.0 mg/kg). A vehicle treated sham-sensitized group and a vehicle treated sensitized group were kept as controls. The percent airway conductance was measured using dual chamber whole body plethysmography as described in the Materials and Methods. Results are shown as the mean ± SEM. The significance of differences between means was determined by the Mann-Whitney U test. Comparisons were made between sensitized and luteolin treated groups. * $p < 0.01$.

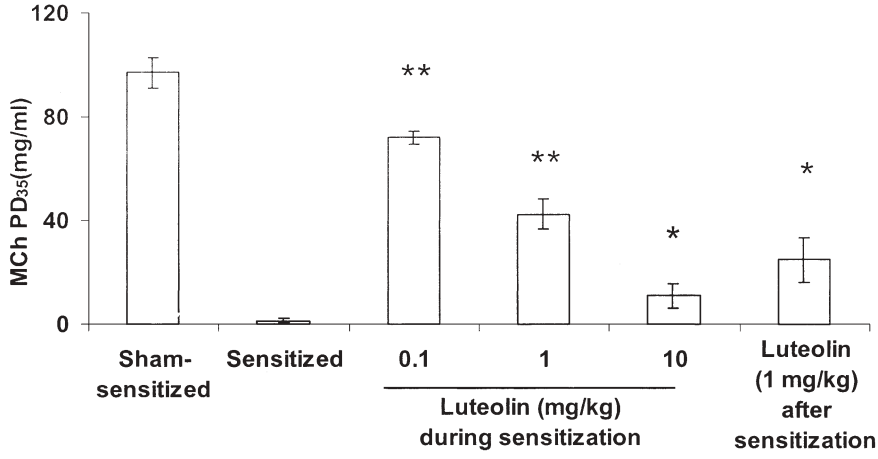

Fig. 3. Luteolin decreases airway hyperrectivity to methacholine. Experiments were performed with six groups of mice (n = 6) as described in Figure 2. – Mice were subjected to inhalation with increasing doses of methacholine. Methacholine dose-response curves were plotted and the MCh PD_{35} values were determined. Results are shown as the mean ± SEM. The significance of differences between values was determined by Student's t test. Comparisons were made between sensitized and the sham-sensitized, and sensitized and luteolin treated groups. * $p < 0.05$, ** $p < 0.01$.

treated with vehicle or with luteolin (0.1, 1.0 and 10.0 mg/kg) during sensitization and in sham-sensitized mice. As shown in Figure 4, the IgE values increased in sensitized mice when compared to sham-sensitized mice (3.3 ± 0.4 versus 0.24 ± 0.07; $p < 0.05$). Luteolin was found to decrease the serum IgE levels in sensitized mice when compared with vehicle treated sensitized group (0.74 ± 0.4, 0.93 ± 0.03, 0.97 ± 0.07 following doses of 0.1, 1.0 and 10.0 mg/kg respectively versus 3.3 ± 0.4 in sensitized, vehicle treated mice; $p < 0.05$). When serum IgE levels in mice, first sensitized and then treated with luteolin (1.0 mg/kg) for a week, were measured, the IgE level was found to be decreased (2.07 ± 1) as compared to sensitized mice ($p < 0.05$).

Luteolin increases the ratio of IFN-γ to IL-4 and decreases IL-5 in the BAL fluid

To investigate the levels of IL-4 and IFN-γ in the BALF, we measured the cytokine levels by ELISA and compared between the different groups. As shown in Table 1, serum levels of IFN-γ were significantly elevated in OVA-sensitized mice treated with luteolin, whereas in the untreated OVA-sensitized mice, IgE levels were reduced. In the luteolin-treated group, there was an increase in the ratio of IFN-γ/IL-4 (25.8 ± 2.7, 15.5 ± 1.3, 13.2 ± 2.5 following doses of 0.1, 1.0 and 10.0 mg/kg luteolin, respectively as compared to 0.48 ± 0.1 in sensitized, vehicle treated mice) ($p < 0.01$) (Table 1). In the mice first sensitized and then treated with

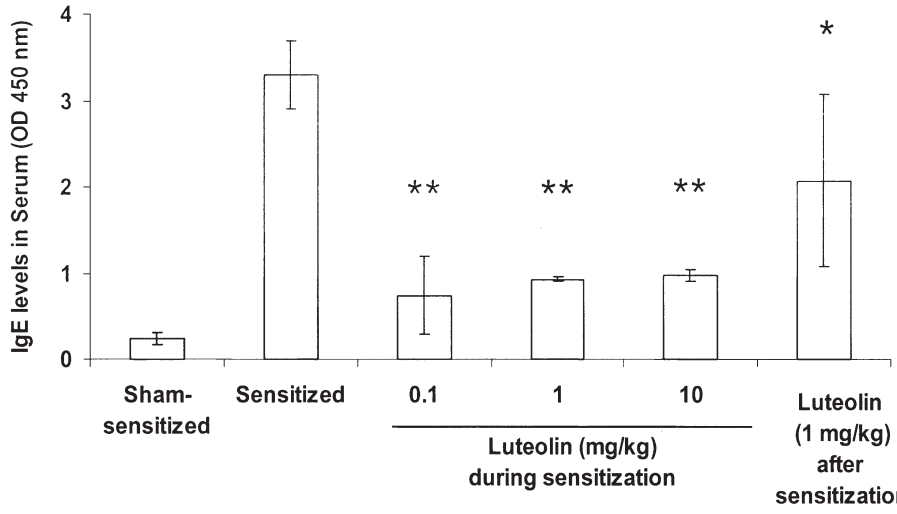

Fig. 4. Luteolin reduces ovalbumin specific IgE. Experiments were performed with six groups of mice (n = 6) as described in Figure 2. The mice were sacrificed and OVA-specific IgE levels in the serum were determined using ELISA. The results are given in OD values as mean ± SEM of six mice in each group. The significance of differences between values was determined by Student's t test. Comparisons were made between sensitized and luteolin treated groups * $p < 0.05$, ** $p < 0.01$.

Table 1. Luteolin upregulates IFN-γ levels and decreases IL-4 and IL-5 levels in the BALF.

Treatment	IFN-γ (pg/ml)	IL-4 (pg/ml)	IFN-γ: IL-4	IL-5 (pg/ml)
Sham-sensitized	2650 ± 50	130 ± 10	9.2 ± 5.1	118 ± 19
Sensitized	81 ± 3.8	171 ± 23.8	0.5 ± 1.0	149 ± 3.5
Luteolin (0.1 mg/kg)[a]	3533 ± 427**	136 ± 4.0	26 ± 2.7**	73 ± 13.4 *
Luteolin (1.0 mg/kg)[a]	2652 ± 114.3**	169 ± 13.9	16 ± 1.3**	43 ± 3.5*
Luteolin (10.0 mg/kg)[a]	1247 ± 412.5**	128 ± 8.8	13 ± 2.5**	235 ± 66.4
Luteolin (1.0 mg/kg)[b]	2067 ± 233.3**	130 ± 5.0	16 ± 2.2**	44 ± 2.3*

Experiments were performed with six groups of mice (n = 6) as described in Figure 2 legend and the levels of IFN-γ, IL-4 and IL-5 were measured in the BALF as described in the Materials and methods. The values are a mean ± SEM of six mice in each group. Mann Whitney U test was used to determine the significant differences in the ratios between the groups. Comparisons were made between sensitized and luteolin treated groups for each cytokine studied. * $p < 0.05$, ** $p < 0.01$. 'a' denotes treatment during sensitization; 'b' denotes treatment after sensitization.

luteolin (1.0 mg/kg) for one week, the ratio of IFN-γ/IL-4 was also increased (16 ± 2.2) ($p < 0.01$) as compared to sensitized, vehicle treated mice. This increase in the IFN-γ/IL-4 ratio was due to the increase in the IFN-γ levels. We also measured the concentration of IL-5 in the BALF by ELISA in the different groups (Table 1). In the luteolin-treated group, a decrease in the levels of IL-5 was seen (73.3 ± 13.4 and 43.3 ± 3.5 following doses of 0.1 and 1.0 mg/kg luteolin respectively as compared to 148.5 ± 3.5 in sensitized, vehicle treated mice) (Table 1). In the sensitized mice treated with luteolin (1.0 mg/kg) for one week, the IL-5 level was decreased (43.7 ± 2.3) when compared to sensitized, vehicle treated mice.

Discussion

In this report, we demonstrate for the first time that treatment with luteolin, a plant-derived natural compound, significantly reduced the antigen-induced bronchoconstriction and airways hyperreactivity in mice following oral administration during sensitization as well as after sensitization (Figs. 2, 3). This activity of luteolin may be correlated to some of the immunomodulatory and anti-inflammatory properties of the compound reported earlier [9, 10]. Asthma is a multifactorial complex genetic disease where multiple pathways are involved in the pathogenesis of bronchial hyperreactivity. In this context, it is possible that luteolin may be acting by any one, or a combination, of the following pathways: i) increased IFN-γ production, ii) decreased IgE production, iii) decreased IL-5 production, iv) affected cellular infiltration, and v) altered IgE receptor induced mediator release. Our results showed that although IL-4 levels in the BALF were not significantly decreased, the ratio of IFN-γ/IL-4 was increased in luteolin-treated mice (Table 1). It is, therefore, likely that luteolin, through higher IFN-γ production, is able to modulate the immune response towards the Th1 type. Accordingly, our results also showed a marked reduction in the IgE levels in the luteolin-treated mice (Fig. 4). In the future it would be important to study the mechanism (s) by which luteolin activates IFN-γ and subsequently affecting IgE levels.

Our finding that luteolin is effective in reducing the overall symptoms of antigen-induced bronchoconstriction (Fig.

2) and airways hyperreactivity to methacholine (Fig. 3) is important in the context of this study and earlier studies. It has been noted that with increasing doses of luteolin, the effect on OVA-induced hyperreactivity to methacholine was reduced (Fig. 3). Although the underlying mechanism for this observation is unclear at this time, however, it is interesting to note that the reduction in inhibition of OVA-induced hyperreactivity seen at higher doses of luteolin is associated with an attenuated increase in IFN-γ production and smaller increase in the serum IgE levels (Table 1). It is possible that at higher doses of luteolin some secondary pathways including the production of IL-13, IL-10, IL-9 and IL-12 may be activated. This remains to be investigated in the future.

In our study, luteolin was found to decrease the IL-5 levels in the BALF (Table 1). It has been shown that IL-5 is one of the major cytokines responsible for maturation, growth and infiltration of effector cells, such as eosinophils, into the lungs [18]. Earlier studies showed that luteolin was effective in reducing the release of histamine, leukotrienes, prostaglandin D_2 and other mediators from various effector cells including mast cells in vitro [10, 19, 20]. It also inhibited protein tyrosine phosphorylation to block nuclear transcription factor-NF-κB-mediated gene expression, such as ICAM-1 on endothelial cells [10, 12]. It is, therefore, likely that luteolin affects a number of pathways to be effective in reducing the bronchoconstriction and airways hyperreactivity as observed in our study.

In conclusion, this study shows that luteolin attenuates the characteristic features of asthma in a mouse model. The molecular mechanism of luteolin action, at least partly, can be attributed to its ability to increase IFN-γ level in BALF and decreasing OVA specific IgE antibody in the sera. Luteolin, therefore, can be used either as a lead molecule to identify an effective anti-asthma therapy or as a means to identify novel anti-asthma targets. in future.

Acknowledgement. This study was supported by Centre for Biochemical Technology, Council of Scientific and Industrial Research (CSIR), India.

Abbreviations

BALF: Broncho Alveolar Lavage Fluid
BSA: Bovine Serum Albumin
ELISA: Enzyme Linked Immuno Sorbent Assay
IFN-γ: Interferon gamma
IgE: Immunoglobulin E
IL-4: Interleukin-4
IL-5: Interleukin-5
i.p: Intraperitonial
OD: Optical Density
OVA: Ovalbumin
PBS: Phosphate Buffered Saline
SGaw: Specific Airways Conductance
TMB: 3, 3′, 5, 5′ tetramethylbenzedine

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