Introduction

Bronchial asthma is a chronic inflammatory disease characterized by airway obstruction in response to allergens, chronic eosinophilic airway inflammation, mucin hypersecretion, and non-specific airway hyperresponsiveness (AHR) (Galli, 2008). Evidence reveals that these inflammatory responses are mediated by T-helper type 2 (Th2) cells, mast cells, B cells, and eosinophils (Cohn, 2004; Medoff, 2008). Upon challenge with various allergens, these inflammatory cells infiltrate into the airway and produce Th2 cytokines, such as IL-5, IL-13, eotaxin, MCP-1, and TGF-β, in bronchoalveolar lavage fluid. The goblet cell metaplasia, thickness of airway smooth muscle, and airway fibrosis were markedly decreased in limonene-treated mice. Furthermore, AHR to acetylcholine was significantly abrogated in limonene-treated mice. These results indicate that limonene has a potential to reduce airway remodeling and AHR in asthma model.

Keywords: Limonene, asthma, Dermatophagoides farinae, airway inflammation, Th2 cytokine

Abstract

Limonene is one of the main flavonoids which is reported to inhibit the inflammatory response by suppressing the production of reactive oxygen species. The aim of this study was to evaluate whether limonene can inhibit Dermatophagoides farinae-induced airway hyperresponsiveness (AHR), eosinophil infiltration and other histological changes in the lung. T helper (Th) 2 cytokine production and airway remodeling in a mice model of asthma. Treatment with limonene significantly reduced the levels of IL-5, IL-13, eotaxin, MCP-1, and TGF-β, in bronchoalveolar lavage fluid. The goblet cell metaplasia, thickness of airway smooth muscle, and airway fibrosis were markedly decreased in limonene-treated mice. Furthermore, AHR to acetylcholine was significantly abrogated in limonene-treated mice. These results indicate that limonene has a potential to reduce airway remodeling and AHR in asthma model.

Keywords: Limonene, asthma, Dermatophagoides farinae, airway inflammation, Th2 cytokine
associated with the primary characteristic of asthma, airway hyperreactivity (Das, 2001; Eddleston, 2007).

Limonene is a major flavonoid isolated from yuzu (Citrus Junos Sieb. ex Tanaka) peel which has been used in Chinese medicine. Limonene reduces oxidative stress (Victor Antony Santiago, 2011), platelet aggregation (Jeon, 2006), and exerts anti-tubercular activity (Bueno-Sanchez, 2009). Our recent study has shown that limonene inhibits the inflammatory responses by suppressing the NF-κB pathway in eosinophil-stimulated eosinophil (Hirota, 2010). Furthermore, limonene from yuzu fruit attenuated the level of ROS in the eosinophil, then prevented the eosinophil chemotaxis. However, there is no report about the therapeutic efficacy of limonene in the treatment of allergic airway inflammation. We isolated limonene from C. junos Tanaka and examined the effectiveness of limonene in reducing airway inflammatory reactions and improving asthma symptoms in Dermatophagoides farinae (Der f)-induced airway inflammation model.

Materials and methods

Animals

Inbred male, specific pathogen-free BALB/c mice (3 weeks of age) (~20 g body weight) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Mice were housed under conventional conditions at the animal facility of Kochi Medical School in filter-topped macrolon cages with a bedding of wood chips, temperature of 23°C, 50–60% relative humidity, and a 12-h light/dark cycle. They received standard lab chow and acidified tap water ad libitum. The mice were maintained until they were 6 weeks old. All research adhered to the animal facility guidelines of Kochi Medical School (A000586).

Preparation of limonene

Limonene was extracted from yuzu fruit (Citrus Junos Sieb. ex Tanaka) grown in Kochi Fruit Tree Experimental Station. In order to extract limonene, yuzu cold-pressed peel was prepared according to the method described previously (Sawamura, 1988). Briefly, the flavedo was prepared according to the method described previously (Sawamura, 1988). The flavedo was centrifuged (4000 g, 15 min) after being saturated with sodium chloride. The supernatant, dehydrated with anhydrous sodium sulfate, was stored overnight at 5°C and then filtrated. Oil won by steam distillation under 5 mmHg was stored overnight at 5°C and then filtrated. Oil won by steam distillation under 5 mmHg with subsequent solvent extraction was prepared by the procedure reported previously (Kusunose, 1980). This yield was 2.39% of the flavedo by weight. Limonene was dissolved in dimethyl sulfoxide (DMSO), then diluted with pure water (final concentration; 0.01% w/w) for this experiment. Gas chromatography–mass spectrometry analyses showed that its purity was 78.13%.

Preparation of DEPs suspensions

DEPs (4.6 g) were extracted using Soxhlet extraction for 24 h with dichloromethane. An extract (0.5 g) was suspended in 2.5 ml of DMSO and then diluted with 10 mM phosphate buffered saline, pH 7.4 (PBS) (final concentration; 1840 µg/ml) and filtered to remove particulate matter. Aliquots (100 µl) were stored at −20°C before use.

Preparation of Der f solution

The mite, Der f crude extract (Der f, LSL, Tokyo, Japan) was purchased and adjusted to 40 µg/ml protein concentrations (BSA conversion) diluted with PBS. Aliquots (100 µl) were stored at −20°C before use.

Immunization, challenge, and limonene delivery in lung tissue

Thirty animals were randomly divided into three groups of ten:

• DER group: mice received intratracheal injection of 4 µg of Der f plus 62.5 µg DEPs (suspended in saline solution, total volume 100 µl) on day 1, 2, 7, 8, 14, 15, 21, 22, 28 and 29.

• DER-LIM group: mice were exposed to 0.01% limonene (1 mg/kg, once a day) using an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan; output 0.8 ml/min) for 20 min in a Plexiglas exposure chamber (22.5 × 29.5 × 16.0 cm). After treatment with limonene, mice received intratracheal injection of 4 µg of Der f plus 62.5 µg DEPs suspended in saline solution on day 1, 2, 7, 8, 14, 15, 21, 22, 28 and 29.

• The control group (C): mice were sensitized neither with Der f nor DEPs; they received only saline solution.

At 48 h after the final challenge (day 31), all mice were subjects to AHR induction using acetylcholine (ACh), lung specimen sampling for histological examination, blood sampling for measurement of serum total IgE and serum Der f-specific IgG1 levels, and bronchoalveolar lavage (BAL) for the measurement of cytokines levels in BALF (Figure 1).

Collection of blood samples, measurement of total IgE, allergen-specific IgG1

On day 29, blood samples were drawn from mice in order to determine the levels of serum total IgE and allergen-specific IgG1. Samples were kept at −80°C in the freezer until analyses were performed with the use of specific ELISA kits for mice (mouse IgG kit from Morinaga Co. Ltd., Yokohama, Japan). Mouse IgG, kit was prepared as follows: 1 µg of Der f solution was added to each well of ELISA plate, then incubated at 4°C overnight. After washing with PBS, each of the wells was coated with 1% BSA solution. Thereafter, they were washed with PBS tween. Ten-fold diluted sera were applied onto the ELISA plate, and then Der f specific-IgG1 was detected with horseradish peroxidase conjugated antibody. Mice were sacrificed using a high dose of pentobarbital on day 31.

Measurement of airway function

Measurement of AHR to intravenous ACh was performed as previously described (Wakahara, 2008; Hirota, 2011).
Cytokine assays

The mouse IgG1 in serum was measured using hand made kit. Briefly, serum IgG1 was bound with coated Der f antigen, and then it was detected with horse radish peroxidase conjugated antibody. Total IgE in serum was measured using commercial kit (Morinaga Co. Ltd., Yokohama, Japan) according to manufacture’s protocol. Interferon γ (IFN-γ), IL-5, IL-13, eotaxin, TGF-β and MCP-1 levels in BALF were determined by ELISA (R&D Systems, Minneapolis, MN). The lower limits of detection for the cytokines were as follows: IFN-γ (>2 pg/ml), IL-5 (>5 pg/ml), IL-13 (>1.5 pg/ml), eotaxin (>3 pg/ml), TGF-β >1.7 pg/ml) and MCP-1 (>2 pg/ml).

Histological studies and mucin, collagen analysis

Left lungs were fixed with 10% buffered formalin, and the tissues were embedded in paraffin. Fixed tissues were cut at 4 µm, placed on glass slides, and deparaffinized. For light microscopy and morphometry, the lung sections were stained with hematoxylin and eosin (HE) to assess the eosinophils infiltrate, Periodic Acid-Schiff (PAS) staining to detection of mucin, Masson’s trichrome (MT) to determine collagen deposition in the lungs. Airway smooth muscle thickness was determined in MT-stained lung sections by measuring the thickness of the smooth muscle cell layer beneath the airway epithelial cell basement membrane at three sites tangential to each airway cross section examined. Subepithelial fiber thickness was determined in MT-stained lung sections by measuring the thickness of the bronchiolar wall from the base of the columnar epithelium to the outer limit of the adventitia (Kuhn, 2000). Morphometry was performed by individuals blinded to the protocol design. Cell counts were performed with the computerized image analyzer program (BX50, Olympus, Tokyo, Japan). A minimum of 10 fields throughout the upper and lower right lung tissue were randomly examined for the morphometric analyses.

Statistical analysis

Data are expressed as mean ± SD. Statistical comparisons were performed using one-way ANOVA, followed by Tukey’s test using SPSS version 11 software (SPSS Inc, Chicago, IL). A value of p < 0.05 was accepted as an indication of statistical significance.

Results

Effects of limonene on the production of allergen-specific IgG1 and total IgE in Der f and DEPs challenged mice

It is well-known that airways exposure to allergen such as Der f in sensitive mice species induces an increase in the serum level of allergen specific IgG1. In this experiment, significantly lower levels of Der f specific-IgG1 were observed in the DER-LIM group (p < 0.01) groups as compared with the DER group (Figure 2a). Similarly, serum total IgE level in the DER-LIM group was lower...
as compared with the DER group, but not significantly ($p > 0.05$) (Figure 2b).

**Limonene suppresses the recruitment of inflammatory cells into the airway**

To examine the effect of limonene on cells chemotaxis, i.e. recruitment of inflammatory cells into the airway, total and differential cell counts were performed in BALF. The DER group showed a significantly increase of number of total cells, macrophages, eosinophils, neutrophils and lymphocytes as compared with the control group ($p < 0.001$) (Figure 3). In addition, treatment with limonene significantly reduced the total number of inflammatory cells ($p < 0.001$), macrophages ($p < 0.05$), eosinophils ($p < 0.001$) in BALF as compared with the DER group. The observed reduction in eosinophils chemotaxis into the airway was well-correlated with the histological changes in the lung parenchyma.

**Limonene reduces the levels of Th2 cytokines involved in the pathophysiology of airway inflammation in BALF**

To examine the effect of limonene on cytokine production, the level of cytokines were measured in BALF. IL-5, IL-13, eotaxin, TGF-$\beta$, and MCP-1 expressions were significantly reduced in DER-LIM as compared with the DER group (69, 41, 30, 52 and 66%, respectively) (Figure 4a–4e), whereas IFN-$\gamma$ was significantly increased in DER-LIM as compared with the DER group (59%) (Figure 4f).

**Limonene attenuates the development of AHR**

To evaluate the AHR in mice, ACh was administered intravenously and bronchoconstriction was measured using a bronchospasm transducer. An increase in the intensity of bronchoconstriction was observed in dose-dependent way, particularly in DER mice, while this process was markedly attenuated by limonene treatment in DER-LIM mice ($p < 0.01$). In addition, no significant difference was observed between DER and control mice ($p > 0.05$) (Figure 5).

**Limonene reduces allergen-induced lung histopathological changes**

To evaluate whether limonene affects the allergen induced histopathological changes, right lung specimens were stained with HE (Figure 6e–6g). Sections from the control mice displayed normal structure and no pathological changes under a light microscope. Der $f$ and DEPs challenges induced marked marked perivascular and peribronchial infiltration of eosinophils into the lungs of DER mice (Figure 6a), a trait of allergic airway inflammation. However this process was reduced by limonene.
Limonene inhibits AHR in Der f-treated mice 377

To evaluate whether limonene affects mucin production in bronchial goblet cells, lung specimens were stained with PAS (Figure 6h–6j). A marked goblet cells hyperplasia was observed in lung specimens from DER mice, while the number of those cells was reduced in DER-LIM mice (Figure 6b).

To evaluate whether limonene affects airway remodeling from fibroblast proliferation, lung specimens were stained with MT (Figure 6k–6m); the airway fibrosis was significantly decreased in limonene treated DER-LIM mice (p < 0.05) (Figure 6c). In addition, the smooth muscle thickness was also significantly decreased in the DER-LIM mice (p < 0.05) (Figure 6d). These results indicate that treatment with limonene efficiently inhibited the infiltration of inflammatory cells, attenuated allergic airway inflammation and collagen deposition.
mice; portions in asthmatic patients (Walker, 1992; Wegmann, kines such as IL-5 and IL-13, are activated in greater pro-

nene treatment inhibited Der f-induced allergic airway 
tion, limonene-treatment significantly inhibited AHR 
as compared with asthmatic mice (Figure 2). In addi-

IgE was significantly lower in limonene-treated mice 
2001). This study showed that the serum levels of total 
gic inflammation, is thought to play a central role in 

Asthma is an escalating public health problem in children 
and adults; patients have an exaggerated immune 
response to allergens leading to lung inflammation 
and AHR (Hartert, 2008). Previous in vitro studies have 
demonstrated the anti-inflammatory activity of limonene 
(Souza, 2003; Hirota, 2010; Yoon, 2010).

Limonene is a monoterpene present in citrus fruit 
and is used as flavouring agent of foods; it is listed as 
a safe agent (Sun, 2007), thus, limonene is considered 
to be a chemical with fairly low toxicity. The American 
National Toxicology Program investigated its toxicity; 
no adverse effect was noted at doses <1650 mg/kg 
administered daily to rats and mice for 3 weeks 
(Jameszon, 1990). We hypothesized that limonene 
could possibly attenuate airway inflammation in Der 
f-exposed mice.

Allergen-specific IgG1 and IgE, a marker of aller-
gic inflammation, is thought to play a central role in 
the allergic response and the spreading epidemic of 
esthma has heightened interest in this immunoglobu-
lin (Hanania, 2008). In asthmatic patients, allergen-
specific IgG1 and IgE level correlates with the disease 
severity and bronchial hyperresponsiveness (Oettgen, 
2001). This study showed that the serum levels of total 
IgE was significantly lower in limonene-treated mice 
as compared with asthmatic mice (Figure 2). In addi-

Antioxidants are thought to play a significant role in 
mediating the pathogenesis of asthma. The intake 
of antioxidant foods is thought to be beneficial to pre-
vent some episodes of asthma and a number of epide-
miologic studies have reported a positive association 
between dietary antioxidant status and lung function, 
and the protective effect of dietary antioxidant supple-
mentation on asthma (Britton, 1995; Hatch, 1995; 
Soutar, 1997; Allen, 2009). The antioxidant activity of 
limonene (Fraternale, 2007; Roberto, 2010) could have 
contributed to the reduction of Der f and DEP-induced 
allergic inflammation and AHR that we observed in this 
study.

In conclusion, results from this study showed that 
limonene treatment inhibited the production of IgE, 
representative Th-2 cytokines, chemokines MCP-1 and 
TGF-β1, allergen-specific IgG1, IgE; which resulted in 
the inhibition of AHR, reduction of collagen deposition 
and the influx of inflammatory cells into the lungs of Der f 
and DEPs challenged mice.

These findings suggest that limonene may potentially 
be beneficial as a prophylactic and therapeutic agent for 
esthma. However, further research is to be performed in 
order to evaluate the beneficial effects of limonene in 
asthmatic human subjects.

Discussion

Inhalation Toxicology Downloaded from informahealthcare.com by Mahidol University, Faculty of Medicine on 01/07/13 For personal use only.

Regarding their function, IL-5 activates eosino-
phils while IL-13 is linked to AHR. On the other hand, 
chemoattractant cytokines such as MCP-1 and eotaxin 
are important for the influx of inflammatory cells (eosin-
ophils, neutrophils) into the lung during asthma. These 
processes contribute to the installation of airway inflam-
mation and AHR (Therien, 2008). Our study showed that 
treatment with limonene markedly reduced the levels 
of IL-5, IL-13 (Figure 4a and 4b) and also that of MCP-1 
and eotaxin (Figure 4c and 4d) in BALF. The reduced lev-
els of chemoattractant cytokines observed in this study 
(Figure 3) may explain the lower number of eosinophils in 
the lung specimens of limonene-treated mice (Figure 6).

IFN-γ is also linked to inflammation and the increase 
of its production has been reported in several mouse model 
of asthma. Results from this study showed a significant 
reduction of this Th-1 cytokine in limonene-treated mice 
as compared with the controls (Figure 3).

Taken together, these results may explain the inhibi-
tion of the development of Der f-induced allergic inflam-
mation observed in limonene-treated mice.

As mentioned above, IL-13 is linked to mucus hyper-
secretion by hyperplasic goblet cells that creates airway 
mucus plugs, especially in peripheral airways of asth-
matics (Liu, 2009). On the other side, TGF-β signaling 
activity is associated with the development of airway 
remodeling in asthma and correlates with thickening of 
the basement membrane (Matsukura, 2010; Tian, 2011). 
In our study, the histological analysis of lung specimens 
from mice showed a marked reduction of number of PAS 
positive goblet cells in DER-LIM mice and a reduced 
smooth muscle thickness in those mice (Figure 6).

In our study, the histological analysis of lung specimens 
from mice showed a marked reduction of number of PAS 
positive goblet cells in DER-LIM mice and a reduced 
smooth muscle thickness in those mice (Figure 6).

Figure 5. Limonene reduces airway responsiveness to 
intravenous acetylcholine. Bronchoconstriction with ACh (%) 
were measured among three groups. Figure 5 shows that AHR 
was significantly inhibited in the DER-LIM mice as compared 
with the DER mice ($p < 0.01$). Values represent the mean ± SD 
of five mice in each group. Area under the curve (AUC) for the 
dose–response to ACh (range: 62.5–2000 mg/kg); # $p < 0.05$ vs. DER 
mouse; **$p < 0.01$ vs. DER mouse.
Limonene inhibits AHR in Der f-treated mice

Figure 6. Limonene reduces eosinophilia (a,e,f,g), the number of PAS positive cells (b,h,i,j), airway collagen fibers (c,k,l,m), and smooth muscle thickness (d,k,l,m) in BALB/c mice induced airway inflammation. Figure 6 shows significantly lower numbers of PAS positive cells and eosinophils in the DER-LIM mice (vs. DER mice, \( p < 0.05 \), \( p < 0.05 \), respectively). In addition, limonene reduced collagen deposition and prevented the increase in smooth muscle thickness as compared with saline in DER mice (\( p < 0.05 \), \( p < 0.05 \), respectively). Representative photomicrographs in a 20 and 40 times magnification (inset) of HE (histology) (e,f,g), PAS (mucin) (h,i,j), MT (fibrosis) (k,l,m) staining of lungs from mice 31 days after intratracheal instillation with Der f. Mice were divided into three groups: saline-challenged mice treated with saline (C); Der f-challenged mice treated with saline (DER); Der f-challenged mice treated with limonene (1 mg/kg, once a day) (DER-LIM). The black arrows indicate representative eosinophils in the infiltrate (e,f,g), green arrows indicate subepithelial fibrosis (l,m), yellow arrows indicate smooth muscle cells (l,m), bars = 100 \( \mu \)m. Abbreviations: AL, alveolus; BR, bronchiole; V, blood vessel; G, goblet cell hyperplasia.
Declaration of interest

This study was supported by a grant from the Japanese Ministry of Economy. The authors declared no conflict of interest.

References


Limonene inhibits AHR in Der f-treated mice 381

