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Effects of *HyangsosanGamibang* on the PPARγ in the bronchial asthma mouse model

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喘息 白鼠에서 香蘇散加味方의 PPARγ에 대한 효과

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목적 : OVA에 유도된 천식 쥐 모델에 향소산가미방을 투여한 후 PPARγ의 변화를 조사하여 향소산가미방의 천식 치료 기전을 알아보고자 하였다.
대상 및 방법 : 8주된 암컷 BALB/c 마우스에 첫 날과 14일 후에 20 μg의 OVA를 알루미니움 하이드록사이드 1mg과 혼합한 후 총 200 μl를 복강 내로 주입하여 감작시켰다. 처음 감작시킨 날로부터 21,22,23일 후에 천식 모델에 사용하는 초음파 분무기를 이용하여 세 번째 감작시켜서 천식 마우스 모델을 만들었다. 천식 마우스 모델을 만드는 기간 중 OVA를 복강 내로 주입한 후 19일째에 24시간의 간격으로 향소산가미방을 7일 동안 경구 투여하여 향소산가미방의 효과를 조사하였다.
기관지폐포세척술은 마지막 감작 후 72시간 후에 실행하고 기관지폐포 세척액의 총 세포수를 측정하였다. PPARγ의 발현은 천식 마우스 모델의 폐와 향소산가미방을 투여한 마우스 모델의 폐를 적출한 후 Western blotting 방법을 이용하여 측정하였다. 병리 조직학적 검사는 hematoxylin 2 and eosin-Y 염색을 이용하여 조사하였다.
결과 : 정상 군과 비교하여 OVA감작 천식 쥐 모델에서는 72시간 후에 총 세포 수가 증가하였다. 특히 OVA감작 천식 군에서 증가된 호산구의 수가 향소산가미방을 투여 한 쥐 군에서는 유의하게 감소하였다. OVA감작 천식 쥐 모델에서 72시간 후에 정상 군과 비교하여 핵 내에서의 PPARγ 단백질의 발현이 약간 증가하였다. 그러나 향소산가미방을 투여한 쥐 모델에서는 세포질과 핵 내에서 PPARγ 단백질의 발현이 유의하게 증가하였다. 조직학적 검사상 정상 군과 비교하여 OVA감작된 천식 쥐 모델에서는 폐포, 세기관지, 기도내강 주변에 많은 염증 세포들이 있었다. 그러나 가미향소산을 투여 한 후에는 염증 세포들이 유의하게 감소하였다.
결론 : 가미향소산은 PPARγ 작용제로서 역할을 하며, 천식에 대한 치료제 또는 예방제를 개발하는데 후보 물질이 될 것으로 사료된다.
Key words : peroxisome proliferator-activated receptors γ, *Hyangsosangamibang*, asthma

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I. INTRODUCTION

Asthma is well known a chronic inflammatory disorder of the airways in which many cell types play a role[1]. Recently, peroxisome proliferator-activated receptors (PPAR), in particular, PPARγ, have been shown to play a critical role as immunomodulators due to their anti-inflammatory actions[2,3]. Studies have demonstrated that activation of PPARγ reduces expression of various cytokines, airway hyperresponsiveness, and activation of eosinophils[4-6]. Besides PPARγ is involved in airway inflammation and remodeling in patients with asthma[5,7].

Many kinds of asthma treatments have been in the process of development with PPARγ agonist in the west[4,5,8,9]. There are various treatments of asthma and bronchitis in Korean Oriental medicine, some treatments have reported the relationship with the cytokines and IgE. Even now, there is no study about the mechanism with PPARγ for these medicines[10-12]. Among many asthma medicines, Hyangsosangamibang(HSSGMB) consisting of twenty-two herbs has been used for treatment of asthma and bronchitis . Six herbs of HSSGMB have been already reported on concerning their relationship with asthma[13-18].

In the present study, OVA-induced asthma model of asthma was used for studying changes of administration of HSSGMB in asthma treatments.

II. MATERIALS AND METHODS

1. Asthma animal model

Female BALB/c mice, 8 weeks of age and free of murine specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejon, Korea). This study was approved by the Institutional Animal Care and Use Committee of Chonbuk National University. Mice were sensitized by intraperitoneal injection of 20 μg ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) in a total volume of 200 μl on days one and 14. On days 21, 22, and 23 after the initial intraperitoneal injection of ovalbumin, 5 mice in each group, the mice were challenged with saline as a control and an aerosol of 3% (w/v) OVA in saline as a asthma model using an ultrasonic nebulizer (NE-U12, Omron, Japan) for 30 minutes[8].

2. HSSGMB preparation

HSSGMB which is a mixture of twenty-two traditional drugs as shown in Table 1 was obtained from Won-Kwang Oriental Medical Hospital (Iksan, Chonbuk, Korea) and classified and identified by local experts. Amounts of the twenty-two traditional drugs studied in this work are shown in Table 1.

Table 1. The Ratio of the Component in HSSGM.

Component	Ratio (g)
Rhizoma Cyperi(Cyperus rotundus)	4
Rhizoma Pinelliae (Pinellia ternata)	4
Radix Peucedani (Angelica decursiva(MIQ.) or Peucedanum praeruptorum DUNN)	4
Radix Platycodi (Platycodon grandiflorum(JACQ)	4
Radix Bupleuri (Bupleurum Chinense)	4
Bulbus Fritillariae (Fritillaria verticillata WILD)	4
Folium Perillae (Perilla frutescens(L.))	4
Semen Armeniacae Amarum (Prunus armeniaca var. ansu MAXIM)	4
Pericarpium Citri Reticulatae (Citrus unshiu MARCOR)	4
Radix Glycyrrhizae (Glycyrrhiza uralensis FISCH.)	4
Fructus Schisandrae (Schisandra chinensis BAILL.)	4
Herba Ephedrae (Ephrdra sinica STAPF)	4
Radix Ophiopogonis (Ophiopogon japonicus KER-GAWL.)	4
Fructus Ponciri Seu Aurantii Immaturus (Poncirus trifoliata (L.) RAF.)	3
Herba Asari (Asarum Sieboldii MIQ.)	3
Radix Scutellariae (Scutellaria baicalensis GEORGI)	2
Cortex Mori Radicis (Mrus alba L.)	6
Cortex Lycii Radicis (Lycium chinense MILL.)	6
Radix Asteris (Aster tataricus L. f.)	5
Flos Farfarae (Petasites japonicus (SIEB. et ZUCC.) MAX.)	5
Herba Raraxaci (Taraxacum Mongolicum H. AND NAZZ.)	8
Herba Houttuyniae (Houttuynia cordata THUNB.)	8
total	204.0

Two hundreds and four grams of HSSGMB were added to 1800 ml of water and boiled for 2 hours, filtered and then concentrated to 400 ml. The sterile extract (30.0 g) was stored at -70℃.

3. HSSGMB administration

HSSGMB(200 mg/kg body weight/day) dissolved in distilled water and was administered 7 times by oral gavage at 24 hour intervals from day 19 after intraperitoneal injection of OVA, that is beginning 2 days before the first challenge (5 mice in each group) (Fig. 1).

Mice were sensitized on days 1 and 14 by intraperitoneal injection of OVA -aluminum hydroxide. On days 21, 22 and 23 after the initial peritoneal injection, the mice were challenged for 30 minutes with an aerosol of OVA in saline using an ultrasonic nebulizer. In the case of treatment with HSSGMB, oral gavage was given 7 times at 24-h intervals

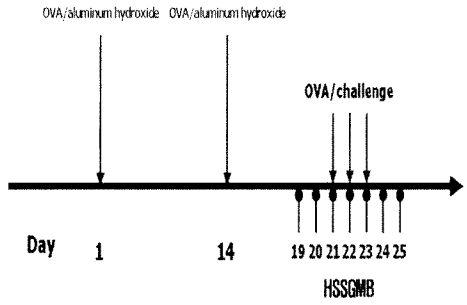

Fig. 1. Schematic diagram of the experimental protocol.

on days 19-25, beginning 2 days before the first challenge.

4. Measurement of cell count using bronchoalveolar lavage

At 72 hours after the last challenge in each group, bronchoalveolar lavage (BAL) was performed. The catheter was inserted into the trachea and secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. Total cell numbers were counted with a hemocytometer. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. Interinvestigator variation was <5%. The mean number from the two investigators was used to estimate the cell differentials[8].

5. Preparation of mouse lung nuclei for analysis of PPARγ and Western blot analysis

Lungs were homogenized in eight volumes of a lyses buffer (1.3 M sucrose, 1.0 mM $MgCl_2$, 10 mM potassium phosphate buffer, pH 7.2). The homogenate lung was centrifuged at 1000 × *g* for 15 min after being filtered through four layers of gauze. The resulting pellets were re-suspended in 2.4 M sucrose (1.0 mM $MgCl_2$, 10 mM potassium phosphate buffer, pH 7.2) and centrifuged at 100,000 × *g* for 1 hour to maintain a final 2.2 M sucrose concentration. The resulting nuclear pellets were washed once with a solution containing 0.25 M sucrose (0.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.2) and centrifuged at 1000 × *g* for 10 min. The pellets were solubilized with a solution (50 mM Tris-HCl (pH 7.2), 0.3 M sucrose, 150 mM NaCl, 2 mM EDTA, 20% glycerol, 2% Triton X-100, 2 mM PMSF, protein inhibitor cocktails). The mixture was kept on ice for 2 hours with gentle stirring and centrifuged at 12,000 × *g* for 30 min. For Western analysis, the resulting supernatant (30 μg of protein per lane) which was used as solubilized nuclear proteins for detection of PPARγ (Santa Cruz Biotechnology) were loaded on a 10% SDS-PAGE gel[8].

Separated proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) by the wet transfer method after electrophoresis at 120 V for 90 min. Nonspecific sites were blocked with 5% non-fat dry milk in TBST buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 hours, and the blots were then in-

cubated with antibody against PPARγ, overnight at 4°C. Anti-rabbit horseradish peroxidase conjugated IgG was used to detect binding of antibodies. The membranes were stripped and reblotted with anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane[8].

The binding of the specific antibodies was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (Amersham Pharmacia Biotech).

6. Histology

At 72 h after the last challenge, lungs were removed from the mice after sacrifice. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI).

7. Densitometric and statistic analyses

All immunoreactive and phosphorylation signals were analyzed by densitometric scanning (LAS-3000; Fuji Film, Tokyo, Japan). Data were expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by the Fisher's test.

Ⅲ. RESULTS

1. Effect of HSSGMB on cellular changes in BAL fluids

Numbers of total cells (macrophages, lymphocytes, neutrophils, and eosinophils) were increased significantly at 72 hours after OVA inhalation compared with numbers of total cells after saline inhalation (Fig. 2). Numbers of total cells were decreased significantly after the administration of HSSGMB compared with OVA inhalation group. Especially, the increased numbers of eosinophils in BAL fluids after OVA inhalation were reduced by the administration of HSSGMB.

2. Effect of PPARγ on HSSGMB

Western blot analysis revealed that PPARγ levels in nuclear level were slightly increased 72 h after OVA inhalation compared with the levels after saline inhalation (Fig. 3). On the other hand, PPARγ levels in cytosolic level were decreased significantly. PPARγ levels in both cytosolic and nuclear cell were remarkably increased after the administration of HSSGMB over 72hrs OVA inhalation.

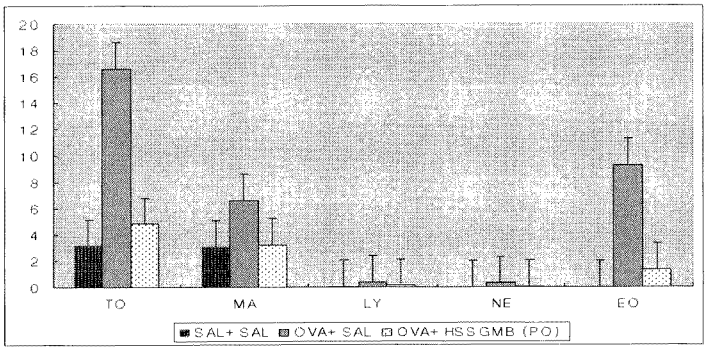

Fig. 2. Effect of HSSGMBon cellular changes in BAL fluids. Effect of HSSGMB on total cells (TO), macrophage (MA), lymphocyte (LY), neutrophil (NE) and eosinophils (EO) in bronchioloaveolar lavage fluids (BAL) was shown. Sampling was performed at 72h after treatment with HSSMMB. The numbers of each cellular component were counted. Bars represent the mean ± SD from two independent experiments.

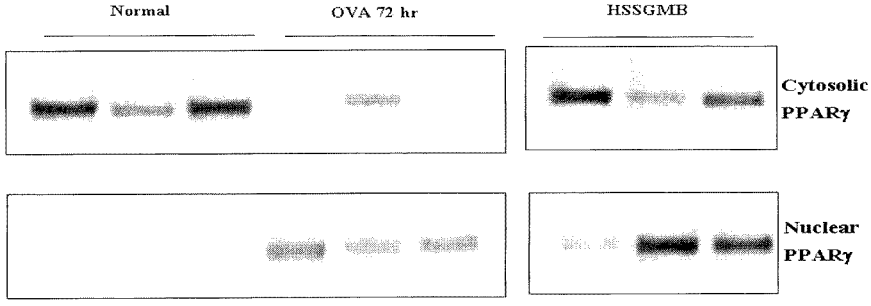

Fig. 3. Western blot analysis. Effect of HSSGMB on PPARγ protein expression in cytosolic and nuclear protein extracts of lung tissues of OVA -sensitized and -challenged mice. PPAR protein levels were determined by Western blotting as described in "Materials and Methods".

3. Histology

Through the histological examinations, mice that were OVA-induced asthma had more inflammatory cells around alveoli, bronchioles, and airway lumen than those after saline instillation. However, inflammatory cell around alveoli, bronchioles, and airway lumen decreased markedly after administration of HSSGMB (Fig 4).

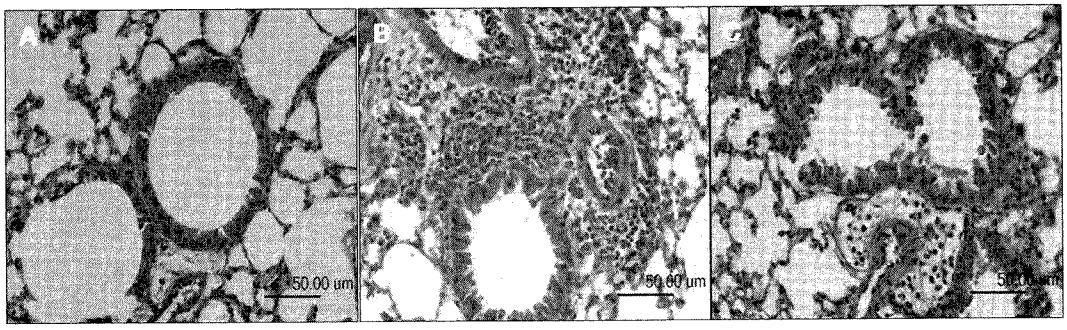

Fig. 4. Histologic examination of many inflammatory cells around the alveoli, bronchioles, and airway lumen of mice with OVA-induced asthma after administration of HSSGMB. On histologic examination, there were many inflammatory cells around the alveoli, bronchioles, and airway lumen of mice with OVA-induced asthma (B) compared with inflammatory cells after saline instillation (A). However, inflammatory cell around alveoli, bronchioles, and airway lumen markedly decreased after administration of HSSGMB (C).

Ⅳ. DISCUSSION

Through this study, HSSGMB resulted as PPARγ agonist which can be a treatment of asthma. Several studies have clearly demonstrated that PPARγ plays an important role in the control of inflammatory responses, acting on T cells, macrophages, dendritic cells, and mast cells[5,19-25]. In addition to that, research has disclosed the involvement of PPARγ in many facets of the disease such as decreasing antigen induced airway hyperresponsiveness, lung inflammation, eosinophilia, cytokine production and serum levels of antigen-specific IgE. Airway remodeling is characterized by the increase in subepithelial membrane (SBM) and collagen deposition. A positive correlation between PPARγ expression, membrane thickening and collagen deposition in the epithelium has been reported[6]. Therefore, PPARγ is an indicator of airway inflammation, remodeling in asthma and administration of HSSGMB, which can improve airway remodeling in asthma patients through the PPARγ mechanism[23].

HSSGMB has been clinically used for treatment of asthma and bronchitis . It has been reported that two herbs of HSSGMB can relax histamine-induced contraction of tracheal smooth muscle[13,18] and four herbs of HSSGMB can decrease eosinophil, IgE, cytokines in association with asthma [11-17].

In this study, PPARγ levels of OVA inhalation in both cytosolic and nuclear were remarkably increased after dose of HSSGMB. Induction of asthma through OVA-challenge increased expression of PPARγ for a control of anti-inflammation and administration of the agonists which was increased after receptor

expression of PPARγ. Up-regulation of PPARγ expression is already observed in human asthmatic airways[7] and PPARγ agonist resulted to reduce of all asthmatic features. These findings indicated that PPARγ was associated with anti-inflammatory responses in asthma and HSSGMB coud play a role as a PPARγ agonist.

A number of synthetic compounds had been developed that act as agonists of PPARγ including the thiazolidinediones, rosiglitizone, ciglitizone, and triglitazone, and GW1929[27]. In vitro, PPARγ agonists inhibit macrophage production of the inflammatory cytokine tumor necrosis factor-α and the induction of inducible nitric oxide synthethas[28]. In addition, PPARγ agonists have been shown to down regulate the production of type-1 helper T cell (TH1) cytokines IFN-γ and IL-2, and to inhibit T cell proliferation. type-2 helper T cell (TH2) cytokines IL-4 and IL-5 in vitro[8,28]. Moreover PPARγ agonists attenuated airway hyperresponsiveness[29]. Signal transduction through the PPARγ had a direct and immediate effect on effectors cell functions in the inflamed lung. Many studies provide strong support for a role for PPARγ in regulating airway inflammation and AHR. Therefore, compounds represents treatments for human asthma[4]. The role of PPARγ agonist is well known and more experiments are performed about which HSSGMB plays various roles as PPARγ agonist. In addition, it is considered that additional experiments are needed about what kinds of ingredients mainly perform functions as PPARγ agonists, through making an analysis of the constituents of HSSGMB.

In conclusion, HSSGMB could play a role as a PPARγ agonist and could be candidate in effective treatment and preventive medicine in asthma through the PPARγ mechanism for bronchial asthma.

V. SUMMARY

This experiment was intended to find out the mechanism of HSSGMB by medicating HSSGMB on to the mouse which was induced OVA.

1. Numbers of total cells were significantly increased after 72 hours, after OVA inhalation comparing with those of the control group. Especially, the increased numbers of eosinophils in BAL fluids 3 days after OVA inhalation were significantly reduced by the administration of HSSGMB.
2. Western blot analysis revealed that PPARγ levels in nuclear level were remarkably increased after dose of OVA inhalation, comparing with those of the control group. 72 hours after inhalation OVA, HSSGMB were given to the mouse. As a result, PPARγ levels in cytosolic and nucleus were markedly increased.
3. Through the histological examination, in-

flammatory cell around alveoli, bronchioles, and airway lumen markedly decreased after administration of HSSGMB.

4. In conclusion, HSSGMB could play a role as a PPARγ agonist and also this could be an effective treatment in asthma patients.

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