Effect of Kaempferol on Cyclooxygenase 2 (Cox2) and Cytosolic Phospholipase A2 (cPLA2) Protein Expression in BALB/c Mice

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ABSTRACT

Kaempferol, a phytochemical found in many edible plants, is known to alleviate diseases such as cancer, allergy, and inflammation. The objective of this study was to investigate whether kaempferol could reduce omega-6 and ovalbumin-mediated allergic reactions at lung and trachea in BALB/c mice.

Mice were allocated into five groups: 1) control group (CON); 2) positive control group with orally administration of omega-6 (POS); 3) bovine serum albumin (BSA) sensitization group (with BSA injection and ovalbumin inhalation); 4) BSA+K10 group: BSA injection, 10 μg/g of kaempferol administration and ovalbumin inhalation; and 5) BSA+K20 group: BSA injection, 20 μg/g of kaempferol administration and ovalbumin inhalation.

Results revealed that serum histamine level was the highest (p<0.01) in BSA group. In lung tissue and trachea, cyclooxygenase 2 (Cox2) expression was significantly (p<0.05) higher in the BSA group compared to that in other groups. However, phosphorylated cytosolic phospholipase A2 (p-cPLA2) expression in the trachea was not significantly different among groups.

Taken together, results of this study suggest that kaempferol might be useful for alleviating inflammation reaction associated with Cox2 expression. However, the exact mechanism of action involved in the effect of kaempferol on inflammatory response remains unclear.

Keywords: Allergy; Cyclooxygenase; Cytosolic phospholipase A2; Inflammation; Kaempferol

INTRODUCTION

Diseases play a very important role in livestock mortality and production rate.1 In mono-gastric animal farming, disease is one of the important factors that affect productivity. Respiratory illness can occur when...
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animals are exposed to air contaminants. Indoor air qualities in animal houses are very poor compared to the general outdoor environment due to high concentration of ammonia and hydrogen sulfide. This can cause great damage to domestic and foreign livestock industry. Feed additives have been used to enhance the growth and immunity of pigs.

Mast cells are major causes of allergic diseases. Mast cells are degranulated by immunoglobulin E (IgE). During degranulation of mast cells, histamine, leukotrienes, prostaglandins, and other allergen-induced cytokines are secreted. They can activate the immune system and cause allergic symptoms. In addition, the number of CD19+CD23+B2 lymphocytes can affect antibody response in allergic asthma. Hyaluronan may help alleviate fungal sensitized asthma in a mice model. Cyclooxygenase (COX) in mast cells produces leukotrienes and prostaglandins that cause inflammation by modifying arachidonic acid. There are two types of COX: Cox1 and Cox2. Cox2 is induced by inflammatory stimulation in a short time. When these substances enter the airways, they activate inflammatory cells and promote mucous secretion of the airway mucosa, causing an allergic reaction. Identifying substances that can inhibit allergen-inducing substances will be an important means to alleviate allergies.

Kaempferol, a phytochemical belonging to flavonoids of, is known to be able to alleviate diseases such as cancer. Kaempferol also has many beneficial roles, including inhibiting matrix metalloproteinases and transcription factors, modulating the expression of adhesion molecules and cellular mechanisms, affecting pro-inflammatory enzyme activities, and activating anti-oxidation and anti-inflammation. It has been reported that kaempferol has inhibitory effect on inflammation and hypersensitivity in murine airway of an asthma model by inhibiting Th2 cytokines (IL-5 and IL-13). In a study on the effect of flavonols on the release of inflammatory mediators, kaempferol inhibited the release of IL-6, IL-8, TNF-a, tryptase, and histamine. It also suppressed the increase of intracellular calcium ion and phosphorylation of protein kinase C theta.

For mono-gastric animals such as pigs and chicken, the ratio of fatty acid composition in the feed is reflected by intramuscular fatty acid composition. Corn is known to be rich in omega-6 fatty acid. In animal feed, corn has a high proportion of 50-70%. For pigs that ingest a lot of corns in the feed, the ratio of omega-6 fatty acid will increase and the balance of omega-3/omega-6 can collapse. The collapse of omega-3/omega-6 balance can have negative impacts on animals.

The objective of this study was to investigate whether kaempferol could reduce omega-6 and ovalbumin-mediated allergic reactions at lung and trachea in BALB/c mice with omega-3/omega-6 balance broken. Kaempferol and omega-6 rich oil were orally administered to mice to investigate the effect of kaempferol on respiratory immunity.

MATERIALS AND METHODS

Measuring Fatty Acid Compositions in Soybean Oil, Corn Oil, and Grape Seed Oil

Fatty acid compositions of soybean oil (Incheon, Koea), corn oil (Gyeoggi-do, Korea), and grape seed oil (Gyeonggi-do, Korea) were analyzed to select omega-6 source according to published method with some modifications. For methylation, 0.5 g of oil was mixed with 2 mL of 14% boron-trifluoride (sigma, St Louis, MO, USA) and 2 mL of methanol. The mixture was vortexed every 5 min and incubated at 80°C for 2 h. After cooling down at room temperature, 3 mL of DW and hexane were added to the mixture followed by vortexing and centrifugation at 2000xg for 5 min at 4°C. The supernatant was collected with a Pasteur pipette to a brown screw cap vial and stored at -20°C until analysis.

Methylated samples were passed through a GC (Gas chromatography; Shimadzu GC 2010, Shimadzu). GC analysis conditions were as follows: column—Famewax column (30 mx0.32 mm ID×0.25 μm), Restek; injection temperature—250°C; detector temperature—250°C; detector— Flame ionization detector (FID); oven temperature—150°C; carrier gas—N2, H2. Air; split ratio—30.0: 1; gas flow rate—1.65 mL/min; injection volume—1 μL. Results were obtained based on the percentage of total peak area.

Experimental Animals

This study was approved by the Animal Ethics Committee of Chonbuk National University (CBNU-2017-00101), Republic of Korea.

Three-week old male BALB/c mice (10-16 g) were purchased. All mice were provided feed and water ad libitum. They were adapted for one-week in the...
conventional environment. These animals were then grouped (4 mice per group) with two repetitions. The animal experiment was carried out according to a previous study. As shown in Figure 1, the experiment was carried out for 4 weeks. Before beginning the experiment, grape-seed oil was administered to break the omega-3/omega-6 balance in mice. Mice were sensitized by injecting 100 µL bovine serum albumin (BSA) first. They were then administered oil on the 21st day. BSA was mixed with Imject alum adjuvant (5% BSA: Imject alum adjuvant, 1:1, v:v) (Thermo Fisher Scientific, Schwerte, Germany). Both orally administration and injection were performed at the same time of each day. At three days before dissection, kaempferol and oil were orally administered. Inhalation of 5% ovalbumin was performed every day using a nebulizer. Experiments were carried out in the following order. First, oil and kaempferol were orally administered to mice. After 1-1.5 h, ovalbumin was inhaled for 20 min. It was then cleaned with ventilation after 10 min of standing.

**Measurement of Histamine Release**

To measure histamine levels, blood samples were collected into heparin tubes from celiac arteries followed by centrifugation at 2000 x g. Supernatants were stored at -20°C. Histamine levels in plasma samples (diluted 8-fold) were measured using histamine ELISA kit (Histamine ELISA kit, IBL, Hamburg, Germany) following the manufacturer’s instructions. All samples and standards were measured in duplicates.

**Measurement of Cox2 and p-cPLA2 Protein Expression Levels**

Mouse lung and trachea were homogenized in 1 mL RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxy cholic acid, 1% triton X-100, pH 7.5) and centrifuged at 12,000 rpm for 20 min. Supernatant were collected and stored at -20°C until analysis. Before western blotting, proteins were quantified using DC Protein assay kit (BioRad, USA) followed the manufacturer’s instructions. Equal amounts of protein samples were subjected to 8% SDS-PAGE for protein separation. They were then transferred to PVDF membranes (Immuno-Blot PVDF membrane Roll, BioRad, USA). Cox-2 and p-cPLA2 proteins were detected using primary Cox-2 (M-19) goat polyclonal IgG and p-cPLA2(Ser 505) rabbit polyclonal IgG antibody with secondary donkey anti-goat IgG-HRP and goat anti-mouse IgG-HRP antibody followed by incubation with chemiluminescent substrate reagent (Super signal West Pico Chemiluminescent Substrate, Thermo Scientific, USA). X-ray films were developed, fixed, and dried in a darkroom. These films were scanned using a Perfection V700 Photo (EPSON, Japan). Images were analyzed using myImage Analysis Software (Thermo scientific, version 1.1, USA).

**Statistical Analysis**

All data analyses were performed using SAS software, Version 9.0 (SAS Institute, Cary, NC, USA). Analysis of Variance (ANOVA) followed by a Duncan’s Multiple Range Test was performed for statistical separation at pre-determined significance level of p<0.05 and p<0.01.

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**Figure 1. Treatment schedule for BSA and ovalbumin to induce inflammatory respiratory reaction. BSA: Bovine serum albumin**
RESULTS

Fatty Acid Compositions of Soybean Oil, Corn Oil, and Grape Seed Oil
Fatty acid compositions of all three oils were analysed through GC. They were administered to mice to break their omega-3/omega-6 balance.

A total of 20 fatty acids were detected in the standard. However, only 13 fatty acids were analyzed. In fatty acid compositions, linoleate (C18:2) is omega-6 fatty acid while α-linolenate (C18:3) and docosahexaenoate (C22:6) are omega-3 fatty acids. Percentage of omega-6 (omega-3) fatty acid in soybean oil, corn oil, and grape seed oil were 56.37 (6.72), 54.30 (1.58), and 64.55 (0.28), respectively. Grape seed oil had the highest ratio of omega-6 but the lowest ratio of omega-3. This result showed that grape seed oil would be the most suitable oil of the three to break the balance of omega-3/omega-6 fatty acids in mice.

Histamine Release
In this study, plasma was collected for histamine assay at 24 h after the last oral administration of kaempferol and BSA challenge. Histamine levels were determined using ELISA kit. As shown in Figure 2, BSA-treatment induced significantly higher level of histamine release in the plasma compared to the control. However, histamine level was decreased significantly after administration of kaempferol (p<0.01). The control and kaempferol groups showed similar levels of histamine. This indicated that kaempferol could sufficiently suppress BSA-sensitized and ovalbumin-induced histamine release.

Cox2 and p-cPLA2 Protein Expression
Protein was isolated from lung and trachea at the same time as blood collection. Cox2 and p-cPLA2 protein expression levels in mouse tissues were detected by western blotting using primary Cox2 and p-cPLA2 antibody, respectively. Cox2 was expressed both in the lung and trachea. However, p-cPLA2 was not detected in the lung tissue (not presented in figure). It was detected only in the trachea.

As shown in Figure 3, Cox2 expression in the lung was significantly higher in BSA group (p<0.05). Cox2 expression in the lung was not significantly different among CON, POS, K10, and K20 groups. In the trachea, higher expression in BSA group was also found (Figure 4). Kaempferol treated groups showed significantly lower Cox2 expression compared to BSA group (p<0.05). p-cPLA2 expression was not significantly different among groups.

Figure 2. Histamine release amount in the plasma of mouse. Histamine levels in POS, BSA+K10, and BSA+K20 groups were normal. Histamine level was significantly increased by injection of BSA with inhalation of ovalbumin. Significant improvements in histamine release were observed in the serum following administration of kaempferol. Data are presented as mean ± SD. ‘a-b’ indicates statistically significant difference (p<0.01).
CON: control treatment; POS: positive control treatment; BSA: bovine serum albumin treatment; BSA+K10: bovine serum albumin with 10 μg/g kaempferol treatment; BSA+K20: bovine serum albumin with 20 μg/g kaempferol treatment.
DISCUSSION

Several conclusions were deduced from this study. No significant increase in histamine was observed in the blood of mice after omega-3/omega-6 balance was broken by administering omega-6 rich oil. Histamine levels were elevated when BSA was injected with ovalbumin inhalation. Administration of kaempferol at 10 mg/kg and 20 mg/kg inhibited the increase in histamine level. When allergic reactions were induced after injection of BSA, expression level of COX2 was significantly increased in the lung and trachea while...
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treatment with kaempferol alleviated such increase. These results suggest that feeding kaempferol might have a positive effect in mitigating allergic inflammatory response of the respiratory tract.

Asthma is a chronic and multifactorial disease caused by a combination of genetic and environmental factors (air pollution or allergens). Various antigens inhaled into the airway can also induce inflammatory cells and mediators such as T cell, B cell, eosinophil, neutrophil, mast cell, cytokine, and chemokine, leading to hypersensitivity (increased airway resistance), hypersecretion of mucus, chronic inflammation mainly caused by eosinophil, and high serum IgE levels. The production of IgE has long been associated with immunological production of allergic asthma. B cells capture and process antigens from the microenvironment and regulate T cell function. In addition to IgE production, B cells are also involved in immune regulation during allergic reactions in animal studies.

Histamine is the most important mediator in the anaphylactic reaction which is called immediate type allergy. Histamine in the serum has a shrinkage effect by acting directly on the smooth muscle. It is the major mediator in allergic inflammation. In this study, ovalbumin and BSA acting as an allergen increased histamine level whereas kaempferol mitigated histamine release. This result is consistent with a previous study showing that serum histamine level is significantly lower in a murine allergic Rhinitis model after kaempferol administration.

High concentration of omega-6 administration can cause imbalance of omega-6 and omega-3 ratio. This can have negative effects on the health of mammalians. Linoleic acid (LA, C18:2, w-6) is conversed to arachidonic acid in human and other animals. Conversed and originally presented arachidonic acid is transferred to endoperoxide by COX2. They will eventually become prostaglandin, thromboxane, and prostacyclin. In epithelial cells, expression of COX2 can lead to inflammation. Expression of COX2 by inflammatory stimulation is synthesized by prostaglandins. It is known that prostaglandin can modulate vascular tone and mediate the inflammatory process, causing damage to the tissue. LPS-induced histamine can increase COX2 expression and generate prostaglandin I2 and E2. Prostaglandins can accumulate, causing inflammatory responses. It has been demonstrated that cPLA2 can induce eosinophil migration and airway hyper-responsiveness. That situation result in the production of arachidonic acid by cPLA2. Our results showed high expression of COX2 in BSA group while kaempferol treatment alleviated COX2 expression. These results may explain the effect of kaempferol on airway inflammation. However, further research is needed to understand the mechanisms involved in the effect of kaempferol.

Kaempferol belongs to the family of flavonoids. It is found in many kinds of edible foods. Recently, it has been found that kaempferol can significantly inhibit antioxidant enzyme activation and cancer cell growth affected by signal transduction pathways, leading to apoptosis. Although the mechanism of cell cycle arrest and cell death induced by kaempferol has not yet been elucidated, its potential use as chemotherapeutic agent for cancer and inflammation has been studied. In mice infected with OVA, kaempferol inhibited the production of IL-8 and eotaxin1, Tyk2 activity, SOCS3 expression, and STAT3 transactivation. In addition, the secretion of proinflammatory cytokines (Il-6, IL-8) and prostaglandins (PGE2 and PGF2a) from fetal membranes can be suppressed by kaempferol. As a result, kaempferol might be useful for relieving inflammation that causes early labor.

In conclusion, this study investigated the effect of kaempferol on inflammatory respiratory reaction in OVA-sensitized BALB/c mice. Kaempferol decreased histamine release in the plasma. COX2 and p-cPLA2 expression levels were also suppressed by kaempferol in lung tissues. In the trachea, COX2 and p-cPLA2 expression levels were not significantly different between BSA and kaempferol groups. However, Cox2 and p-cPLA2 expression patterns were higher in BSA group. These results suggest that kaempferol might be able to alleviate inflammatory respiratory reactions in lung and trachea. Therefore, kaempferol might be useful as an anti-inflammatory agent for BSA/OVA allergens. Further studies are needed to determine factors modulated by kaempferol and its effect on allergen mediators.

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