GATA-3 is a Key Factor for Th1/Th2 Balance Regulation by Myristicin in a Murine Model of Asthma

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Myristicin, 1-allyl-3,4-methylenedioxy-5-methoxybenzene, was one of the major essential oils of nutmeg. However, its anti-allergic effect in the Th1/Th2 immune response was poorly understood. Recently, it was shown that T-bet and GATA-3 was master Th1 and Th2 regulatory transcription factors. In this study, we have attempted to determine whether myristicin regulates Th1/Th2 cytokine production, T-bet and GATA-3 gene expression in ovalbumin (OVA)-induced asthma model mice. Myristicin reduced levels of IL-4, Th2 cytokine production in OVA-sensitized and challenged mice. In the other side, it increased IFN-\(\gamma\), Th1 cytokine production in myristicin administrated mice. We also examined to ascertain whether myristicin could influence eosinophil peroxidase (EPO) activity. After being sensitized and challenged with ovalbumin (OVA) showed typical asthmatic reactions. These reactions included an increase in the number of eosinophils in bronchoalveolar lavage fluid, an increase in inflammatory cell infiltration into the lung tissue around blood vessels and airways, and the development of airway hyper-responsiveness (AHR). The administration of myristicin before the last airway OVA challenge resulted in a significant inhibition of all asthmatic reactions. Accordingly, these findings provide new insight into the immunopharmacological role of myristicin in terms of its effects in a murine model of asthma.

Key words – Asthma, ovalbumin, myristicin, MMP-9, GATA-3, T-bet, IL-4, IL-5, airway hyper-responsiveness

Introduction

Asthma is a chronic inflammatory lung disease that is characterized by airway hyper-responsiveness to allergens, airway edema, and increased mucus secretion. Bronchial asthma is an immune-mediated disorder characterized by airway hyperresponsiveness (AHR) and eosinophilic airway inflammation. T-helper type 2 (Th2) cells were dominant in the airways and Th2 cytokines such as IL-4, IL-5 and IL-13 play a pivotal role in the pathophysiology of asthma [6,10,11,15,28,37,41]. Asthma can also be induced by irritative hazards not only in atopy in mouse model. Ovalbumin(OVA)-induced asthma is characterized by AHR and inflammation of the airways [19]. This inflammation was associated with the infiltration of eosinophils, neutrophils, and lymphocytes into the bronchial lumen and lung tissues [4,19]. These cellular infiltrates released various chemical mediators that can cause AHR [5,7,12]. Recruitment of these inflammatory cells from the blood to the site of inflammation was regarded as a critical event in the development and persistence of airway inflammation. Inflammatory cells have to cross the basement membrane and move through connective tissue until they finally reach inflammatory sites, and require the involvement of

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adhesion molecules, cytokines, chemokines, and enzymes such as matrix metalloproteinases (MMPs) in this journey. The MMPs are a family of zinc- and calcium-dependent endopeptidases capable of proteolytically degrading many of the components of the extracellular matrix [26]. MMPs are produced not only by structural cells [25,39], but also by inflammatory cells [8,17,21,34]. They were secreted in their latent forms, followed by proteolytic processing to the active forms [26]. Of the MMP family, MMP-2 (gelatinase A, 72-kDa) and MMP-9 (gelatinase B, 92-kDa) share similar domain structures and in vitro matrix substrate specificities [20], and appear to induce the migration of eosinophils, lymphocytes, neutrophils, and dendritic cells across basement membranes during tissue injury and repair [29,33]. Recruitment of leukocytes from the circulating blood into tissues requires a series of cell adhesion molecules, including ICAM-1 and VCAM-1, which are shown to play important roles in the induction of airway inflammation.

We reported in a previous work that a variety of phytochemicals exhibit profound immunoregulatory activities both in vitro and in vivo, particularly in DCs (Dendritic cells) [13,14]. Myristicin, 1-allyl-3,4-methylenedioxy-5-methoxy benzene, is an active constituent of nutmeg, parsley, carrot, and black pepper [38]. Myristicin was known to induce glutathione S-transferase and to inhibit the tumorigenesis induced by benzo(a)pyrene in the mouse lung, suggesting that myristicin acts as a chemopreventive [2,40]. Recently, it was reported that liver injury caused by lipopolysaccharide is potentially prevented by myristicin treatment [23]. These findings offered useful information concerning the spics that contain myristicin [16]. Myristicin has also been shown to be effective in the treatment of a wide range of liver diseases including hepatitis and cirrhosis, as well as gall bladder diseases and certain dermatological conditions. Non-toxicity was one of the most important properties of this compound, and this property has been verified in a variety of animal models, using different modes of administration [23]. In this study, we have attempted to characterize the effects of a non-cytotoxic concentration of myristicin in a murine model of asthma. Our findings demonstrated, for the first time, that myristicin treatment inhibit asthmatic syndrome, and suppress the OVA-induced gelatinolytic activity of MMP-9, and the translocation of GATA-3 in the cytosol. In a recent study, it was suggested that T-bet might protect against asthma through increased expression of GATA-3 mRNA in asthmatic airways [3,22,27,32,35,36]. This study investigated the role of myristicin on T-bet and GATA-3 in a murine model of asthma.

Materials and Methods

Animals and experimental protocol
Female BALB/c mice, 6-8 weeks of age and free of murine-specific pathogens, were obtained from the Charles River Laboratories (Yokohama, Japan). All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the Pusan National University Medical School.

Administration of myristicin
Mice were immunized intraperitoneally (i.p.) with 20 µg of OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) on day 1 and 15. Mice were challenged for 30 min via the airway with OVA (50 mg/ml of saline) each day from days 21-23 on consecutive days. Bronchoalveolar lavage (BAL) was obtained at 24 hr after the last challenge. At the time of lavage, the mice (6 mice in each group) were killed with an overdose of ether. The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed saline solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4°C. A part of each pool was then centrifuged, and the supernatants were kept at -70°C until use. Mice were injected i.p with 1 or 10 mg/kg/day in 200 µl of myristicin (Mry 1 or Mry 10) (Sigma, St Louis, Mo) each day from days 18-20 on consecutive days.

Total cell counting
The total cell numbers were counted with a hemacytometer. Smears of BAL cells prepared with Cytospin II (Shandon, Runcorn, UK) were stained with Diff-Quik solution (Dade Diagnostics of P.R. Inc, Aguada, PR) for differential cell counting. Two independent, blinded investigators counted the cells, using a microscope. Approximately 200 cells were counted in each of four different random locations.

Measurement of eosinophil peroxidase
The suspension of BAL cells and the pulmonary homo-
genates were frozen/thawed three times using liquid nitrogen and a water bath at 37°C to obtain the EPO. The BAL fluid was centrifuged to 4°C for 10 min and serially diluted in a 96-well plate (75 µl/well) followed by the addition of 150 µl of substrate (1.5 mM o-phenylenediamine and 6.6 mM H₂O₂ in 0.05 M Tris-HCl, pH 8.0). After 30 min at room temperature, the reaction was stopped by the addition of 75 µl of 30% H₂SO₄ and the absorbance of the samples was determined at 492 nm on an ELISA reader.

**Histology**

At 48 h after the last challenge, lungs were removed from the mice after they had been sacrificed. Prior to the removal of the lungs, the lungs and trachea were filled intratracheally with a fixative (4% paraformaldehyde) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) paraformaldehyde. 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 sequentially stained with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI).

**T cell counting by flow cytometric analysis**

Equal amount of BAL fluids in each group, 5 x 10⁵ cells were incubated in staining buffer (PBS with 2% FBS and 0.1% sodium azide) containing FITC-conjugated anti-CD3e Ab (BD Pharmingen) for 15 min on ice to block the non-specific binding. Cells stained with the appropriate isotype-matched immunoglobulin were used as negative controls. After staining, the cells were fixed with 4% w/v paraformaldehyde and analyzed by FACScalibur, using CellQuest software (BD Biosciences).

- T cell number = BAL Fluids Total cell x T cell (%)
- T cell (%) = T cell number/FACS reading cells(10⁶)x100

**RNA preparation and quantitative RT-PCR**

The total RNA from lung tissues was isolated with the use of a rapid extraction method (TRI-Reagent) (Invitrogen Life Technologies, CA, U.S.A), as previously described.[14] Real-time PCR was performed on cDNA samples using the SYBR Green system (Bio Rad, Richmond, CA). Primers used were T-bet sense 5'-CAA CAA CCC CTG TGC CAA AG-3', T-bet antisense 5'-TCC CCC AAG CAG TTG ACA GT-3; GATA-3 sense 5'-GAG GTG GAC GTA CTG TTT AAC ATC G-3, GATA-3 antisense 5'-GGC ATA CCT GGC TCC CGT-3. Cycling conditions were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles each corresponding to 15 s at 95°C and 1 min at 60°C. Analysis used the sequence detection software supplied with the instrument. The relative quantitation value is expressed as 2^ΔΔCt, where ΔCt is the difference between the mean CT value of duplicates of the sample and of the GAPDH control.

**Measurement of cytokines**

Levels of IFN-γ, IL-4 and IL-5 were quantified in the supernatants of BAL fluids by enzyme immunoassays performed according to the manufacturer’s protocol of the manufacturer (R&D Systems, Inc., Minneapolis, MN).

**Determination of airway responsiveness to methacholine**

Airway responsiveness was measured in mice 24 hrs after the last challenge in an unrestrained conscious state, as described previously [34]. Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (2.5 to 50 mg/ml) was nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization. Enhanced pause (Penh), calculated as (expiratory time/relaxation time-1) x (peak expiratory flow/peak inspiratory flow), according to protocol of the manufacturers, is a dimension-less value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure of airway responsiveness to methacholine. Results were expressed as the percent increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) was expressed as 100%. Penh values were averaged for 3 min after each nebulization and evaluated.

**Zymography**

Gelatin zymography was used to assess the activity of MMP-9. The supernatant was diluted three times with nonreducing loading buffer (400 mmol/L Tris-HCL, 5% sodium dodecyl sulfate, 20% glycerol, 0.006% bromophenol blue). The sample were mixed with 15 µL of equal amounts of loading buffer, and proteins were separated by polyacrylamide gel electrophoresis (0.75 mm; constant current
20 mA) consisting of an 8% sodium dodecyl sulfate solution with 1% gelatin (Bio-Rad). The gels were incubated in a renaturing buffer (2.5% Triton X-100 buffer) for 30 min to remove the sodium dodecyl sulfate. After rinsing, the gels were incubated (37°C for 20 hr) in an enzyme activation buffer (50 mmol/L Tris-HCl [pH 7.3], 200 mmol/L NaCl, and 0.02% Tween 20). The gels were then stained with Coomassie brilliant blue R250 stain and destained (5% methanol, 7% acetic acid in PBS solution), and the gelatinolytic activity was detected as clear bands. The molecular weight of the gelatinolytic bands was estimated relative to the prestained molecular-weight markers (see BluePlus2 Prestained Standard; Invitrogen, Carlsbad, CA).

Densitometric analysis and statistics

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean ± S.E.M. ANOVA was used to compare experimental groups to control values while comparisons between multiple groups were performed using Tukey’s Multiple Comparison test. Statistical significance was indicated by a P value less than 0.05, P<0.01, P<0.001.

Results

Myristicin decreases the increased numbers of inflammatory cells in BAL fluids of OVA-induced asthma

The total cell numbers in BAL fluids were increased about 10-fold, compared to those of the control group at 2 days after the last OVA challenge. The number of neutrophils, eosinophils, lymphocytes, and macrophages in BAL fluids was increased to compare to those of the control group at 2 days after the OVA challenge. Interestingly, the number of neutrophils, eosinophils, lymphocytes, and macrophages observed in BAL fluids in the myristicin treated of mice group decreased to below the level of OVA-challenge group. The administration of the myristicin with OVA resulted in a significantly reduction of neutrophils, eosinophils, lymphocytes, and macrophages and total cells elicited in the airway lumen 2 days after OVA inhalation (Fig. 1).

Myristicin decreased the level of eosinophils in BAL fluids

Eosinophil peroxidase (EPO) is a major parameter of indirectly eosinophil number. Eosinophil peroxidase (EPO) are heme-containing oxidoreductases that function in vivo primarily to catalyze the reaction: H2O2 + X + H+→HOX + H2O. The levels of eosinophils in BAL fluids were increased at 24 hr after OVA inhalation compared with the levels after saline inhalation (Fig. 2). The increased levels of these cells were significantly reduced by about 68.7% (Myr 1 mg) and 73.3% (Myr 10 mg) upon administration of myristicin.

Myristicin inhibits CD3ε+ T cell number in BAL Fluids

Numbers of CD3ε+ T cell in BAL fluids were increased significantly at 24 hr after OVA inhalation (78.0%) compared with the numbers after saline inhalation (31.1%) (Fig. 3). The increased numbers of CD3ε+ T cell were significantly reduced by the administration of Myristicin (52.4%).

Myristicin induced Pathological change in a murine asthma model

Histological analyses revealed typical pathologic features of asthma in the OVA-exposed mice, as compared with the control, with the OVA-exposed mice showing numerous inflammatory cells, including eosinophils infiltrated
Fig. 2. Eosinophil peroxidase (EPO) activity in BAL fluids of OVA-sensitized and -challenged mice. EPO was an indicator of the numbers of Eosinophil levels. The increased levels of EPO were significantly reduced about 68.79 % (OVA + Myr 1 mg/kg/day), 73.35 % (OVA + Myr 10 mg/kg/day) compared with OVA-treated mice. The results were from one representative experiment out of 5 performed. This experiment used 5 mice (n = 5). ***P<0.001 vs. OVA.

Fig. 4. Myristicin inhibits lung inflammation and inflammatory cells infiltration. Mice were sensitized and challenged as described in Materials and Methods. Sections were obtained from the lungs of mice receiving the control (CON), OVA plus myristicin (OVA + Myr 1 mg/kg/day, 10 mg/kg/day) and OVA (OVA). Lungs were removed 2 days after the last airway challenge. Sections were stained by haematoxylin and eosin staining (x 200).

inhibits antigen-induced inflammation in the lungs, including the influx of eosinophils.

Myristicin decreased MMP-9 mRNA, T-bet and GATA-3 mRNA levels in lung tissues of OVA-sensitized and -challenged mice

RT-PCR analysis revealed that expression of MMP-9 and GATA3 mRNA in lung tissues was significantly increased at 24 hr after OVA inhalation compared with the levels after saline inhalation (Fig. 5). The increased mRNA expression of MMP-9, GATA-3 was decreased by the administration of myristicin. Conversely, myristicin treatment resulted in an increase in Th1 transcription factor T-bet.

Myristicin regulates on levels of IL-4, IL-5 and IFN-γ in lung tissues of OVA-sensitized and -challenged mice

BAL fluids were obtained 4 hr after the last airway challenge. The levels of IL-4 and IL-5 in the BAL fluids were significantly increased by airway challenge with OVA when compared with that of the control. The administration of myristicin reduced the concentration of IL-4, IL-5 secretion (Table 1). As shown in Table 1, the levels of Th2 cytokines, IL-4 and IL-5, were found to be increased in OVA-sensitized and -challenged mice, but that of the...
Th1 cytokine, IFN-γ (Table 1) was not changed as compared to saline-sensitized and -challenged mice. These results indicate that myristicin treatment inhibits Th2 cytokine levels in the BAL fluids.

Myristicin decreased airway hyper-responsiveness in OVA-challenged mouse

Airway responsiveness was assessed as the percent increase of Penh in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of percent Penh was shifted to the left compared with that of control mice (Fig. 6). In addition, the percent Penh produced by methacholine administration (at doses from 2.5 mg/ml to 50 mg/ml) increased significantly in the OVA-sensitized and -challenged mice compared with the controls. OVA-sensitized and -challenged mice treated with myristicin showed a dose-response curve of percent Penh that shifted to the right compared with that of untreated mice. The shift was dose-dependent. These results indicate that myristicin treatment reduces OVA-induced airway hyper-responsiveness.

Myristicin inhibits MMP-9 gelatinolytic activity

OVA-challenge induced a marked induction of matrix metalloproteinase-9 activity in BAL fluids in comparison to control mice (Fig. 7). When the administration of myristicin 1 mg/kg/day or 10 mg/kg/day, increased MMP-9 gelatinolytic activity was significantly inhibited (Fig. 7). These results indicate that myristicin regulates MMP-9 gelatinolytic activity in lung tissues.

Discussion

Airway remodelling was a potentially important consequence of asthma. This study was the first to provide experimental evidence demonstrating that myristicin inhibited OVA-induced airway inflammation in a murine model of asthma. Myristicin profoundly inhibited asthmatic reactions such as leukocytic recruitment into the airway and lung inflammation. We also demonstrated that myristicin regulates the Th1/Th2 balance, which can be mediated by the level of T-bet and GATA-3 levels.

OVA-induced asthma has been recognized as a disease
that results from chronic airway inflammation characteristically associated with the infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen. In our experiment, we demonstrated that OVA-induced asthma increased levels of eosinophil infiltration, eosinophils peroxidase activity, and the thicknesses of bronchial wall and area of smooth muscle. But they were significantly decreased by administration of myristicin.

It was recently demonstrated that the administration of an MMP inhibitor reduces the migration of inflammatory cells through the endothelial and epithelial basement membranes [33]. Additionally, an MMP inhibitor regulated inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma [13]. In our murine model of asthma, intraperitoneally injected mice with 1 mg/kg/day or 10 mg/kg/day myristicin were evaluated the effect on the expression of MMP-9 and GATA3 mRNA. In this study, myristicin reduced levels of MMP-9, GATA3 in lung tissues of myristicin treated mice. Conversely, myristicin treatment resulted in increase in Th1 transcription factor T-bet.

T-bet, a member of the T-box family of transcription factors, was a master determinant of Th1 lineage [9, 24]. Indeed, T-bet deficient mice exhibited a profound lack of Th1 immune responses [18] and ectopic expression of T-bet in murine Th2 cells directs activation of IFN-\(\gamma\), as well as the upregulation of IL-12R\(^\beta1\) [1, 9, 18]. Th1 cytokines are known to inhibit allergic responses [30, 31]. GATA-3 belong to the GATA family of transcription factors. Six members (GATA-1 to GATA-6) of this family have been identified in avians, with homologues in mammals. Based on their expression profile and structure, the GATA proteins might be classified as haematopoietic (GATA-1 to GATA-3) [31] or nonhaematopoietic (GATA-4 to GATA-6). Naïve CD4+ T cells expressed low levels of GATA-3 mRNA. The expression of GATA-3 is, however, markedly upregulated in cells differentiating along the Th2 lineage, and is down-regulated in cells differentiating along the Th1 pathway [9].

Also, we examined Th1/Th2 cytokine production in BAL cells. Myristicin reduced the increased levels of IL-4, Th2 cytokine production in OVA-sensitized and -challenged mice. In the other side, it increased IFN-\(\gamma\), Th1 cytokine production in Myristicin administrated mice. Our data demonstrate that myristicin reduced the increased levels of GATA3 mRNA in OVA-sensitized and -challenged mice.
(Fig. 4). Also, it suggest that myristicin treatment is a novel, selective way to simultaneously suppress GATA-3 and increase T-bet expression in asthmatic reactions in vivo [18]. Taken together, these suggest that T-bet, GATA-3 might be a regulator gene for asthma via Th1/Th2 balance.

In conclusion, our results strongly indicated that myristicin reduces allergic airway inflammation and hyperresponsiveness due to the alteration of Th1/Th2 polarization via the suppression of GATA-3 and increase of T-bet expression. Therefore our data suggested that myristicin might offer a new therapeutic approach to allergic airway diseases.

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Reference


초록: Myristicin이 Ovalbumin으로 유도한 천식 생쥐모델에서 Th1/Th2 Balance를 조절하는 GATA-3에 미치는 효과

이규1,2, 이창민1,2, 정인덕1,2, 정영일2,3, 전성학2, 박희주4, 최병환5.

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Myristicin은 육두구에서 발견되는 고농축 질량 중 하나인 물질이다. 하지만 Th1/Th2 면역반응에서 육두구의 항갈레르기 효과는 아직 밝혀지지 않았다. 최근에 Th1/Th2 전사인자로서 T-bet, GATA-3가 밝혀졌는데 이전 실험에서 myristicin이 ovalbumin(OVA)으로 유도한 천식(астhma) 생쥐모델에서 Th1, Th2 싸이토카인과 유전자가 발현을 조절할 수 있는가에 대하여 알아보고었다. 또한 기판기 폐포 세포세척액을 취하여 백혈구의 수적 변화, 세포를 황조T세포(T2 cell)가 생성하는 IL-4, IL-5의 생산에 미치는 영향과 폐조직에서 matrix metalloproteinase (MMP)-9 활성을 측정하였다. 그 결과 기판기 폐포 세포세척액에서 OVA로 감작하여 천식을 유도한 실험군에서는 호산구의 현저한 증가, Th2 형 싸이토카인(IL-4, IL-5)의 증가가 관찰되었다. 그러나 myristicin을 투여한 그룹에서는 OVA의 감작에 의하여 증가한 각종 염증성 지표들이 감소하거나 정상화 되었다. 또한 OVA에 의하여 증가된 기도저항성이 myristicin 투여에 의하여 감소하였으며 폐조직의 염증성 소견도 덜었게 감소되었다. 이와 같은 연구 결과는 myristicin가 천식의 치료에 유용하게 쓰일 수 있을음을 시사해준다.