Coincidental increase of leukotriene B_4 between cerebral cortex and lung tissue of sensitized rats[1]

DENG Yang-Mei[2], XIE Qiang-Min, CHEN Ji-Qiang, BIAN Ru-Lian

Zhejiang Respiratory Drugs Research Laboratory of State Drugs Administration of China, Medical School of Zhejiang University, Hangzhou 310031, China

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ABSTRACT

AIM: To explore the changes of leukotrienes (LT) in cerebral cortex and lung tissues in ovalbumin-induced rat asthma model and effects of different anti-asthma drugs on the changes. **METHODS:** Aerosol antigen-induced changes of inflammation in bronchoalveolar lavage fluids (BALF), pulmonary and brain histologic section in sensitized rats were investigated. Changes of LTB_4 and LTC_4 in lung and cerebral cortex homogenates were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC). **RESULTS:** The number of inflammatory cells in BALF and the score of lung and brain histological examination from antigen- challenged rats were significantly higher than that from control group (P<0.05). Dexamethason (DXM, 0.5 mg/kg, ip) and ketotifen fumarate (KF, 5 mg/kg, ig) markedly reduced total leukocyte number in BALF, and inhibited eosinophil accumulation, reduced the infiltration of eosinophils, and improved mucous edema and epithelial lesion of bronchi and bronchioles. In addition, RP-HPLC results shown LTB_4 in lung and cerebral cortex homogenates were increased in antigen-challenged rats [(4.1±2.4) ng/g and (1.5±0.9) ng/g, respectively] compared with control group [(1.55±0.21) ng/g and (0.7±0.3) ng/g, respectively, P<0.05], both DXM (0.5 mg/kg, ip) and KF (5 mg/kg, ig) reduced LTB_4 amount in lung[(1.4±0.6) ng/g and (1.8±0.7) ng/g] and cerebral cortex homogenates [(0.5±0.4) ng/g and (0.7±0.4) ng/g] in asthma rats. LTC_4 content in lung homogenates in asthma rats was increased compared with control group [(1.9± 0.9) ng/g and (0.5±0.3) ng/g, respectively] (P<0.05), but it has no change in cerebral cortex homogenates. DXM (0.5 mg/kg, ip) and KF (5 mg/kg, ig) reduced LTC_4 amount in lung homogenates in asthma rats [(0.8±0.6) ng/g and (1.0±0.3) ng/g, respectively] (P<0.05). **CONCLUSION:** The results indicate there is coincidental increase of LTB_4 between central nervous system and lung tissues in asthma rats. DXM and KF can inhibit the change.

INTRODUCTION

Leukotrienes (LT) are important inflammatory mediators in the pathophysiology development of asthma. Many inflammatory cells such as eosinophil, mast cell, neutrophil, and lymphocyte can produce LT when challenged by antigens or other stimulating factors[1]. LT can be divided into LTB_4 and cys-LT (LTC_4, LTD_4, and LTE_4). LTB_4 causes adhesion and chemotactic movement of leukocytes and stimulates aggregation, enzyme release, and generation of superoxide in neutrophils. The cys-LT are able to induce profound modification of vascular permeability through direct action on endothelial cells, and are among the

[1] Project supported by the National Natural Science Foundation of China, No 39970857.
[2] Correspondence to DENG Yang-Mei.
Phn/Fax 86-571-8721-7380. E-mail dym999@zju.edu.cn
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most potent bronchoconstricting agents tested in human, the potency of which is 1000 times higher than histamine[2]. Both cys-LT and LTB_4 have proinflammatory effects.

LT are also formed in the central nervous system (CNS) of human and animals. They take part in much pathophysiology of brain, such as aging process of brain[3,4], viral infection of CNS[5], cerebral ischemia and reperfusion lesion[6], malignant tumor of brain and neural development[7]. Although LT are important mediators in asthma, their effect in CNS of asthma animal is still unknown.

With the development of the research of the mechanism of asthma, and based on the long time of work on the respiratory system, there raised the hypothesis that immune and inflammatory response of CNS might occur in animal asthma model, and play an important role in the pathological status of asthma. Our Lab has found that in CNS Th1/Th2 cytokines ratio is imbalance in asthma rats, that is, IL-4/γ-INF ratio is changed to Th2 direction[8]. We want to explore if LT also have correlative change between CNS and lung tissue in asthma rat. reverse phase high-performance liquid chromatography (RP-HPLC) method was used to analysis LT content in cerebral cortex and lung tissue homogenates. Meanwhile, glucocorticoid and H_1 receptor antagonist were used to observe the effects of different anti-asthma drugs.

MATERIALS AND METHODS

Animal and reagents Sprague-Dawley (SD) rats of either sex weighing 180 g±20 g were purchased from Laboratory Animal Center of Medical School of Zhejiang University (Grade II, Certificate No 20020610). Dexamethasone sodium phosphate (DXM, Suzhou Sixth Pharmaceutical Factory); ketotifen fumarate (KF, Shanghai Sixteenth Pharmaceutical Factory); egg albumin grade V, LTB_4, LTC_4, and prostaglandin B_2 (PGB_2) were purchased from Sigma, USA; methanol and acetonitrile (Tianjing Siyou Pharmaceutical Co Ltd, China) were of HPLC grade.

Sensitization, treatment, and challenge regimens As described previously[8], all of SD rats except control group were sensitized by sc injection on experiment d 0 with 1 mg antigen ovalbumin mixed with aluminium hydroxide adjuvant 100 mg in saline 1 mL at footpad, neck, back, and groin.

From d 14 after sensitization, one time every day for 6 d and 1 h before antigen challenge, rats in treatment group were ip injected with DXM 0.5 mg/kg, or ig with KF 5 mg/kg. Rats in control and model group were ip injected with saline.

After treatment, rats were placed in a plastic box (45 cm×45 cm×15 cm) and challenged by exposure to an aerosol of ovalbumin (10 g/L in saline) which was generated in a jet nebulizer (partical size 1-5 mm; BARI, MASTER, Germany) for 20 min, and one time every day for 6 d. Control rats were similarly exposed to an aerosol of saline.

Bronchoalveolar lavage (BAL) and cell counts Twenty-four hours after the last antigen challenge, rats were decapitated and the right lung was ligated, BAL was performed via a tracheal cannula with 5 mL of sterile Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (*D*-Hanks'). The fluid was recovered through a plastic syring and immediately centrifuged (Eppendorf Centrifuge 5804R, Germany) at 400×*g* for 10 min at 4 ºC. The pellet was resuspended with 5 mL of saline, and the total cell number was counted in a hemocytometer. Differential cell counts were made from centrifuged preparations stained with Giemsa stain. A minimal of 200 cells was counted at a magnification (×400) and classified based on morphologic criteria as mononuclear cells, eosinophils and neutrophils. Data are summarized as the percentage of inflammatory cells out of the total cell number counted.

Lung and brain tissue histology The right lung which had been ligated and right hemisphere were removed and immersed in 10 % buffered formalin. All the tissues were embedded in paraffin wax and cut into 5-μm-thick sections followed by hematoxylin and eosin (HE) stain and observed under a magnification of ×400. As previous described[8], score of eosinophil infiltration, mucous edema, and epithelial lesion was as absent (scale 0), rare (scale 1), mild (scale 2), moderate (scale 3), and severe (scale 4).

Lung and brain homogenates preparation After BAL, the lung artery were perfused with *D*-Hanks' liquid to remove blood cells. Then the left lung and hemisphere were scissored into 1 mm×1 mm×1 mm, homogenated (DY89-I Homogenater. Linbo Xinzhi SCITETH research institute) in ice-cold Hanks' buffer (pH 7.5). Samples were added with methanol (1:1, v/v) to precipitate proteins and centrifuged at 3500×*g* for 10 min at 4 ºC, supernatant was diluted with ultra pure water (Water Pro Ps, LABCONCO) to obtain a methanol concentration of 25 % and extracted on Sep-Pak

C^{18} column (Waters) prewashed with 20 μL of ethanol and 20 μL of water. After 200 ng PGB_2 was added as internal standard, then diluted through 0.1 % edetic acid, ultra pure water, 15 % ethanol, petroleum ether and methanol in sequence. The methanolic fraction was concentrated under argon and stored at -80 ºC, and the residual mixture was dissolved in methanol before RP-HPLC assay. To minimize absorption of LT, only tubes, vials and pipette tips made of polypropylene were used. All steps of the procedure were performed under 4 ºC.

RP-HPLC system RP-HPLC was performed used HP1100 separation module consisting of multiple solvent delivery system, and equipped with UV detector, analytical pump, on-line degasser, and column thermostat. Samples were separated by a Waters symmetry C_{18} reversed-phase column (4.6 mm×250 mm, 5 μm particles) which was protected by a Waters sentry C_{18} guard column (3.9 mm×20 mm, 5 μm particles). The absorbance of the column affluent was monitored using a dual wave-length absorbance detector adjusting to 270 nm and 280 nm for LTB_4 and LTC_4, respectively. The peak areas were calculated with a chromatography manager program. The mobile phase for LTC_4 was acetonitrile/water (70:30, v/v) and trifluoroacetic acid (TFA) was added in it to terminal concentration of 0.5 mmol/L[9]. The mobile phase for LTB_4 was methanol/water/acetic acid (70:30:0.01, v:v:v) adjusted to pH5.6 with NH_4OH. A flow rate of 1 mL/min and 2 mL/min at 35 ºC for LTB_4 and LTC_4 were used respectively.

Based on the peak areas, the LTB_4 concentration of biological samples tested was estimated by internal standard PGB_2. And the LTC_4 concentration in same sample of LTB_4 was estimated by LTC_4 standard curves, and was transformed based on the recovery rate of PGB_2 in the LTB_4 detection performance. All these results were expressed as ng of LT per g wet weight of lung or brain.

Statistical analyses Data were presented as mean±SD. Statistical analyses were performed with Student's *t*-test. A probability value (*P*) of less than 0.05 was considered statistical significance. All statistical calculations were performed using a Sigmastate statistical package.

RESULTS

Antigen-induced changes of airway inflammatory cells in BALF In sensitized rats, 6 d after ovalbumin-aerosol challenge, antigen induced a significant increase of eosinophils, and neutrophils. The number of inflammatory cells in BALF in antigen challenged group was significantly higher than that in control group ($P<0.05$). DXM (0.5 mg/kg, ip) markedly reduced total leukocyte numbers in BALF, and almost completely inhibited eosinophil accumulation. KF (5 mg/kg, ig) also inhibited eosinophil accumulation, but it had no effect on lymphocyte number (Tab 1).

Histological changes of pulmonary and brain tissues in ovalbumin-induced asthma rats Pulmonary histological examination found characteristic of inflammatory cell infiltration around the airways and blood vessels. Eosinophil numbers in the epithelium and subepithelial connective tissue of bronchi, bronchioles, and peripheral small arteries were increased. The mucous edema and epithelial lesion of bronchi and bronchioles were also observed. The score of histological examination in antigen-challenged group was higher than that in control group, and DXM and KF reduced the numbers of eosinophils, improved mucous edema and epithelial lesion of bronchi and bronchioles (Fig 1, Tab 2). The slices of the brain tissues used did not reveal any sighs of leukocyte and eosinophils infiltration or malignant transformation.

Tab 1. Antigen-induced change of airway inflammatory cells in bronchoalveolar lavage fluids and inhibitory effects of different anti-asthma drugs. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control group; $^eP<0.05$, $^fP<0.01$ *vs* sensitized+ovalbumin.

Groups	Dose	*n*	10^{-8}×Amount of total leukocyte $/L^{-1}$	10^{-8}×Amount of eosinophil $/L^{-1}$	10^{-8}×Amount of neutrophil $/L^{-1}$	10^{-8}×Amount of lymphocyte $/L^{-1}$
Control	Saline 4 mL/kg	8	0.8 ± 0.3^f	0.03 ± 0.04^f	0.10 ± 0.04^f	0.70 ± 0.26^f
Sensitized+ovalbumin	Saline 4 mL/kg	10	2.2 ± 0.5^c	0.46 ± 0.19^c	0.38 ± 0.06^c	1.4 ± 0.4^c
Sensitized+ovalbumin +dexamethasone	0.5 mg/kg	8	1.4 ± 0.5^{bf}	0.08 ± 0.06^{bf}	0.22 ± 0.08^{cf}	1.2 ± 0.4^b
Sensitized+ovalbumin +ketotifen fumarate	10 mg/kg	6	1.7 ± 0.4^{be}	0.14 ± 0.07^{ce}	0.28 ± 0.08^{ce}	1.24 ± 0.22^c

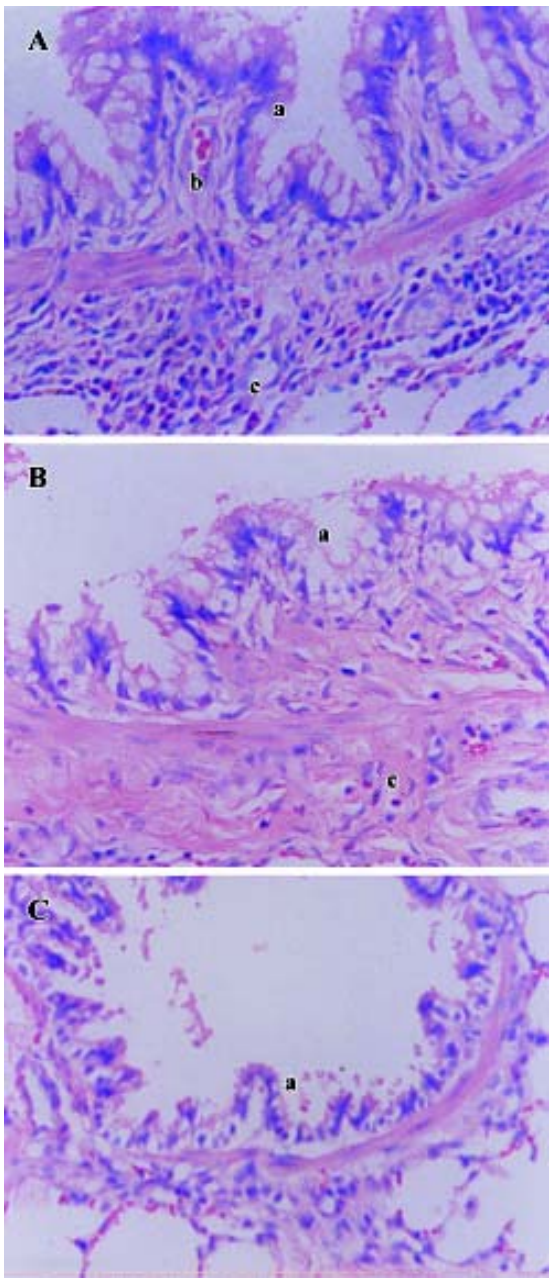

Fig 1. The histopathological changes of pulmonary sections in antigen-induced asthmatic rats, and different anti-asthma drugs on it. A: Sensitized+ovalbumin; B: Sensitized+ovalbumin+ketotifen fumarate; C: Sensitized+ovalbumin+dexamethasone (a: mucosa; b: artery; c: eosinophil. HE stain, ×400).

LTB_4 and LTC_4 content change in lung and cerebral cortical homogenates of antigen-induced asthmatic rats Content of LTB_4 and LTC_4 in lung homogenates from antigen-challenged rats were markedly higher compared with samples from control and drug treatment rats ($P<0.05$). LTB_4 in cerebral cortical homogenates in antigen-challenged rats were also increased significantly ($P<0.05$), but LTC_4 had no difference from that of control rats in cerebral cortical homogenates ($P>0.05$). DXM (0.5 mg/kg) and KF (5 mg/kg) decreased LTB_4 content not only in lung homogenates, but also in cerebral cortex homogenates in antigen-challenged rats (Tab 3). DXM and KF reduced LTC_4 content in lung homogenates of antigen-challenged rats (Tab 4).

DISCUSSION

The brain was once considered as an "immunologically privileged" organ because the cerebrospinal fluid, the blood-brain barrier, and the meninges effectively shield the CNS from other tissues[10]. But recent evidence shows that both microglia and astrocytes secrete numerous cytokines, it is widely accepted that these cells participate actively in an integrative communication between resident immune cells of the CNS and those of the periphery. Leukocytes can also enter CNS under physiological conditions. Chemokines play specific roles in directing the recruitment of leukocytes into inflammatory foci within the CNS.

There has a close relation between CNS and periphery in asthma animal, and there are also inflammatory changes in CNS. Extended exposure to allergen exacerbates asthma symptoms, in part via complex interactions between inflammatory cells and mediators. One consequence of the interactions is the triggering of local and CNS neuronal activity that might further exacerbate the asthma-like symptoms by causing bronchoconstriction, mucous secretion, increased microvascular leak, and cough. In a rhesus monkey model of allergic asthma, caudomedial nucleus tractus solitarius neurons undergo intrinsic increases in excitability, indicating that CNS might contribute to the exaggerated symptoms[11]. There is a significant interplay change of Th1 and Th2 cytokine between CNS and pulmonary airway in asthma rats[8]. Fan *et al*[12] found that c-*fos* mRNA expression and c-fos protein production are enhanced in CNS of asthma rat. All these results indicated that inflammatory reaction in CNS took part in the pathophysiologic change in asthma.

As important inflammatory mediators in asthma, LT have important pathophysiological effect in periphery. In this study, the eosinophils amount was increased in the BALF, and lung pathological examination indicated there were many changes characteristic in asthma, which means that the model of asthma is

Tab 2. Score of histological examination in pulmonary tissue of antigen-induced asthmatic rats and inhibitory effects of different anti-asthma drugs. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group. $^{f}P<0.01$ *vs* sensitized+ovalbumin.

Groups	Dose	*n*	Eosinophil infiltration	Mucous edema	Epithelial lesion
Control	Saline 4 mL/kg	8	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}
Sensitized+ovalbumin	Saline 4 mL/kg	10	3.6 ± 0.5^{c}	2.7 ± 0.5^{c}	2.0 ± 0.9^{c}
Sensitized+ovalbumin+dexamethasone	0.5 mg/kg	8	0.5 ± 0.5^{bf}	0.25 ± 0.46^{cf}	0.12 ± 0.35^{f}
Sensitized+ovalbumin+ketotifen fumarate	5 mg/kg	6	1.5 ± 0.5^{cf}	0.7 ± 0.5^{cf}	0.3 ± 0.5^{f}

Tab 3. LTB_4 in lung and cerebral cortical homogenates in antigen-induced asthmatic rats and effects of different anti-asthma drugs. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group. $^{f}P<0.01$*vs* sensitized+ovalbumin.

Groups	Dose	*n*	LTB_4 of Lung homogenates/ $ng\cdot g^{-1}$ wet weight	LTB_4 of cerebral cortical homogenates/ $ng\cdot g^{-1}$ wet weight
Control	Saline 4 mL/kg	8	1.55 ± 0.21^{e}	0.7 ± 0.3^{e}
Sensitized+ovalbumin	Saline 4 mL/kg	10	4.1 ± 2.4^{b}	1.5 ± 0.9^{b}
Sensitized+ovalbumin+dexamethasone	0.5 mg/kg	8	1.4 ± 0.6^{f}	0.5 ± 0.4^{e}
Sensitized+ovalbumin+ketotifen fumarate	5 mg/kg	6	1.8 ± 0.7^{e}	0.7 ± 0.4^{e}

Tab 4. LTC_4 in lung and cerebral cortical homogenates in antigen-induced asthmatic rats and effects of different anti-asthma drugs on it. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group. $^{e}P<0.05$, $^{f}P<0.01$*vs* sensitized+ovalbumin.

Groups	Dose	*n*	LTC_4 of Lung homogenates/ $ng\cdot g^{-1}$ wet weight	LTC_4 of cerebral cortical homogenates/ $ng\cdot g^{-1}$ wet weight
Control	Saline 4 mL/kg	8	0.5 ± 0.3^{f}	0.7 ± 0.5
Sensitized+ovalbumin	Saline 4 mL/kg	10	1.9 ± 0.9^{c}	0.9 ± 0.6
Sensitized+ovalbumin+dexamethasone	0.5 mg/kg	8	0.8 ± 0.6^{e}	0.8 ± 0.4
Sensitized+ovalbumin+ketotifen fumarate	5 mg/kg	6	1.0 ± 0.3^{be}	0.9 ± 0.5

successful. At the same time, we also found that LTB_4 in cerebral cortex and lung tissue homogenates increased in asthma rat, showing that inflammatory mediators took part in the reaction in CNS of asthma.

Theoretically, several types of cells could sustain LT synthesis in brain, including neuron, glia (astroglia and oligodendroglia), vessal wall constituents, ependymal cells and, under certain condition, infiltrating leucocytes. Resident macrophages are additional potential sites if synthesis, though their spare occurrence would make any such activity of little consequence[13]. The synthesis of LT in CNS is most closely correlative to many cytokines. Platelet-activating factor induced cysteinyl-LT synthesis *in vivo* and also enhanced the synthesis of astrocytes in culture state[14]. So, the increased LTB_4 may interact with other cytokines or transmitters in CNS, so as to regulate the inflammatory reaction of CNS in asthma animal.

Confusedly, as the production of 5-lipoxygenase pathway, the change of LTB_4 and LTC_4 in cerebral cortex is not coincident, although both of their content were increased in lung tissue in asthma model, LTC_4 in CNS

has no difference compared to normal group. What's more, we did not find any leukocyte infiltration or malignant tumor cells in CNS, then there derived a problem, that is, what make this difference in LTB_4 and LTC_4 production in CNS? As we know that the profile of LT synthese is dictated by a cell's complement of distal LT synthases (LTA_4-hydrolase and LTC_4-synthase), and varies with the cell type[15]. We guess the distal LT synthases distributed differently in CNS, and the LTC_4 synthase requires reduced glutathione as a co-substrate[16]. So, the next step is to do some immunohistochemical examination and RT-PCR to examine the cell derivating of LT in CNS, which can help to interprete of the mechanism of asthma.

Glucocorticoids reduced cytokines that are involved in cell recruitment and the survival of inflammatory cells. In most but not all studies, inhaled glucocorticoids and oral glucocorticoids have been shown to reverse many of these inflammatory indices[17]. In our experiment, it is also found that DXM decreased not only the LTB_4 and LTC_4 amount in lung homogenates, but also the content of LTB_4 in brain homogenates. Whether the reduction in LT production induced by DXM is primarily due to a reduction in inflammatory cell numbers, or whether this substance also directly inhibits the production of LT, for example, by down-regulating cytosolic, calcium-dependent phospholipase A_2 was not addressed in this study. KF, a H_1 receptor antagonist and mast cells stabilizer, could also reduce the amount of LTB_4 and LTC_4 in lung homogenates and decrease the amount of LTB_4 in brain in asthma rats. Its effect may be due to its trans-blood-brain barrier capability and inhibiting synthesis of LT in CNS.

In a word, this experiment indicates that there is coincidental increase of LTB_4 between CNS and lung tissues in asthma rats.

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