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Diaphragm Muscle Contraction Decrease in a Mouse Model of Ovalbumin-Induced Allergic Airway Inflammation

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Abstract

Objective: We investigated diaphragm contractile and inflammatory properties of mice with OVA sensitization and challenge.

Methods: BALB/c mice were sensitized to OVA by intraperitoneal (i.p.) injection at 0 and 7 days, and challenged with aerosolized OVA on 21, 22, and 23 days (O/O group). Budesonide/Formoterol combination was inhaled on days 21, 22, and 23 before OVA challenge on those same days (O/OC group). Control mice were sensitized and challenged with an aerosolized saline (O-group). The diaphragm contractile and inflammatory properties were measured on day 24. NOS activity in the diaphragm muscle was evaluated by NADPH diaphorase staining. IL-4 and IL-13 levels of BALF, as well as lung tissue and diaphragm muscle homogenates were measured by ELISA.

Results: Force-frequency (F/f) curves of O/O and O/OC shifted downward in comparison with O- ($p<0.05$). NADPH diaphorase staining results of O/O and O/OC showed a significantly higher density compared with O-. The IL-4 level of diaphragm muscle homogenates increased significantly in the O/O compared with the O- and O/OC.

Conclusions: OVA sensitization and challenge decreased diaphragm muscle contraction, increased NOS activity, IL-4 levels of diaphragm in a mouse model. Budesonide/Formoterol combination could protect diaphragm muscle weakness and inflammation. According to the traditional concept of the contemporary Immunology, neither autoimmune diseases nor allergic diseases can be cured completely. Nevertheless, a fortunate coincidence led me to discovery of a novel concept that eliminations of the causes of these diseases are possible. In other words, combinations of pathogenic antibodies with responsible cells, namely, cytolytic T lymphocytes in cases of autoimmune diseases and mast cells in cases of allergic diseases, can be decomposed by replacing the pathogenic antibodies with non-specific antibodies. In more detail, intradermal injections with a non-specific antigen preparation induce production of non-specific antibodies in the body of the patient. Repetitions of the injections bring about an accumulation of them. Accumulated non-specific antibodies will occupy most of the receptors on the surface of responsible cells. When the accumulation reaches the sufficient level, virtually no pathogenic antibodies would remain on the receptors. That is, no causes of the diseases remain. Naturally, where there is no cause, there is no disease. Details are demonstrated elsewhere.

Keywords: OVA; Diaphragm muscle; NOS; ICS/LABA; Inflammation

Abbreviations: TNF-: Tumor Necrosis Factor-alpha; Th2: T helper-2; IL: Interleukin; NO: Nitric oxide; BAL: Broncho Alveolar Lavage; OVA: Ovalbumin; IP: Intraperitoneal; PBS: Phosphate Buffered Saline; F/f: Force-frequency; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; NOS: Nitric Oxide Synthase; AU: Arbitrary Unit; Inos: Inducible NO Synthase; TLR: Toll-like Receptor.

Introduction

Asthma is a chronic airway disease characterized by airway inflammation, narrowing, hyper responsiveness, and remodeling [1]. It is well known that inspiratory resistive breathing reduces diaphragm muscle contractility, and bronchoconstriction leads to diaphragm muscle fatigue in an asthma patient [**2**,**3**]. And it is well known that airway inflammation, bronchoconstriction and airway hyper responsiveness increase in an ovalbumin (OVA) sensitized and challenged mice [4-6]. However, research has not yet shown clearly whether OVA sensitization and challenge reduces diaphragm muscle contractility, ICS/LABA combination protects diaphragm muscle weakness or not. In this study we investigated diaphragm muscle contractility and inflammatory profile of OVA sensitized and challenged mice and the effect of ICS/LABA.

Methods

Animal preparation

61 male BALB/c mice weighing 26.0 0.6 g at 6-8 weeks of age (CLEA Japan, Inc., Tokyo, Japan) were randomly divided into three groups-control group treated with saline (O- group), allergic group sensitized and challenged with OVA (O/O group), treatment group sensitized

and challenged with OVA plus Budesonide/Formoterol inhalation (O/OC group). The experimental protocol was approved by the Ethics Committee for Animal Experimentation at Tohoku University (2013-Idou-441).

O/O and O/OC mice were sensitized with an intraperitoneal (i.p.) injection of 100 μg Chicken OVA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1 mg aluminum hydroxide in 20 μl phosphate-buffered saline (PBS) on days 0 and 7. O/O and O/OC mice were challenged for 30 minutes with aerosolized 1% OVA using a nebulizer PARI-emotion (PARI Japan, Osaka, Japan) on days 21, 22 and 23. O/OC mice were inhaled Budesonide (LKT Laboratories, Inc., St Paul, USA) 36 μg and Formoterol (LKT Laboratories, Inc., St Paul, USA) 1 μg combination with Lactose 163 μg by Dry powder insufflator model DP-4 (Penn-Century, Inc., Glenside, USA) and air pump model AP-1 (Penn-Century, Inc., Glenside, USA) on days 21, 22, and 23 before OVA challenge on those same days. O-mice were administrated 20 μl PBS by an i.p. injection on day 0, 7 and administrated for 30 minutes with PBS on days 21, 22 and 23 (Figure 1).

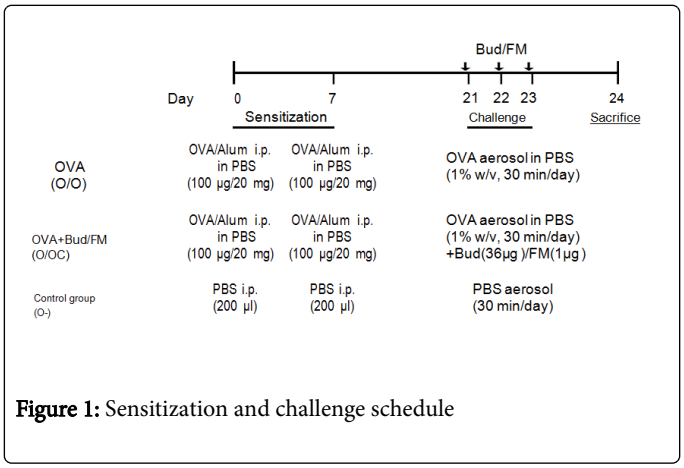

Figure 1: Sensitization and challenge schedule

Measurements of muscle contraction

Muscle contraction properties were measured on Day 24. Mice of each group were sacrificed by decapitation under deep anesthesia and diaphragm muscle was resected to evaluate the muscle contraction. Muscle strips were dissected from the right and left hemi diaphragm of each animal and mounted in separate organ baths containing Krebs-Henseleit solution oxygenated with a 95% O_2 and 5% CO_2 gas mixture (37.0 ± 0.5°C, pH 7.40 ± 0.05). Both muscle strips were simultaneously stimulated with supramaximal currents of 200 to 250 mA through an isolation unit (SS-302J; Nihon Kohden Co., Tokyo, Japan) and driven by a stimulator (SEN-3201; Nihon Kohden Co., Tokyo, Japan). These electrical stimulations were controlled by computer. The elicited tensions were measured by a force transducer (UL-100GR; Minebea Co., Nagano, Japan). The length of each muscle strip was altered by moving the position of the force transducer with a micrometer-controlled rack and pinion gear (accuracy of displacement, 0.05 mm; Mitsutoyo Co., Aichi, Japan) and measured with a micrometer in close proximity to the muscle.

The diaphragm force-frequency curves were assessed by sequentially stimulating muscles at 1, 10, 20, 30, 50, 70, 100 and 120 Hz. Each stimulus train was applied for 1 s, and adjacent trains were applied at 10-s intervals by computer. The tensions of the muscle strips were recorded by a thermal pen recorder (RECTI-HORIZ-8K; NEC San-ei, Tokyo, Japan). The force-frequency curves of the groups were displayed as elicited tensions (N/cm^2) versus stimulation frequencies (Hz). Twitch contraction was elicited by single pulse stimulation (0.2-m sec pulse duration) and twitch kinetics were assessed by twitch tension (TT; N/cm^2), contraction time (CT; the time required to develop peak tension, m sec), and half-relaxation time (HRT; the time required for peak tension to decrease by 50%, m sec) during a single muscle contraction. Muscle fatigue was assessed through the rate of tension decrease over a 5-min period of rhythmic contraction, which was induced by applying trains of 20-Hz stimuli at a rate of 60 trains/min. Muscle fatigue was expressed as a percentage of the final tension (%) compared with initial tension. We determined according to a previously described method [7,8].

NADPH diaphorase staining

Mice of each group were sacrificed by decapitation under deep anesthesia and diaphragm muscle was resected to evaluate a NOS activity by NADPH diaphorase staining [9,10]. The diaphragm was quickly excised, and the tissue pieces were frozen in O.C.T. Compound (Tissue-Tek; Sakura Fine technical Co., Ltd., Tokyo, Japan) in a thermos containing dry ice and acetone. Cryosections (10 μm in thickness) were cut from the diaphragm in the O.C.T. Compound. The histochemical procedure for NADPH diaphorase consisted of dipping the sections in freshly prepared 1.0 mM -NADPH (Oriental Yeast Co., Ltd., Tokyo, Japan) and 0.2 mM nitroblue tetrazolium (Wako Pharmaceutical Co., Osaka, Japan) in 100 mM tris-HCL buffer, pH 8.0, containing 0.2% Triton X-100 for 30 min at 37°C. The reactions were stopped by rinsing the sections in phosphate buffered saline (PBS). The sections were covered with a mixture of glycerol and PBS (2:1) and photographed by a microscope (Axiolab A1; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with a charge-coupled device camera (Axio Cam ERc 5S; Carl Zeiss MicroImaging GmbH, Göttingen, Germany). The mean density of the cross-sectional views of each muscle fiber was measured using image analyzer software (NIH Image, National Institutes of Health, Bethesda, MD, USA). More than 30 muscle fibers were counted in each photograph, and the densities were averaged and expressed in arbitrary units (a.u.). NADPH diaphorase staining was performed as described previously [7].

Cytokines in BALF by ELISA

BALF, lung tissue and diaphragm muscle were collected to evaluate levels of IL-4 and IL-13 on day 24. Study mice were sacrificed under deep anesthesia, BALF, lung tissue and diaphragm muscle was collected. Lung tissue and diaphragm muscle were homogenized, and total protein from lung tissue and diaphragm muscle were extracted by a kit (Minute™, Invent Biotechnologies, Inc., Eden Prairie, MN, USA). Levels of IL-4 and IL-13 in BAL fluid, and total protein of lung tissue homogenates and diaphragm muscle homogenates were analyzed by ELISA using a Diaclone IL-4 mouse ELISA kit (Gen-Probe Diaclone SAS, Besancon, France) and a Diaclone IL-13 mouse ELISA kit (Gen-Probe Diaclone SAS, Besancon, France).

Data analysis

Data were analyzed using JMP ver11.0 (SAS Institute, North Carolina, USA). Mean values of tensions for each frequency of force-frequency curves, twitch kinetics, fatigability, Levels of IL-4 and IL-13 were compared by 1-way ANOVA with Fisher's LSD post-hoc analysis.

Results

Muscle contraction

The force-frequency curve of OVA group O/O (14.6 ± 1.5 N/cm^2 peak) was significantly shifted downward compared with control group O- (18.5 ± 1.9 N/cm^2 peak) and OVA plus Budesonide/Formoterol inhalation group O/OC (18.8 ± 1.3 N/cm^2 peak) (p<0.05, each) (Figure 2).

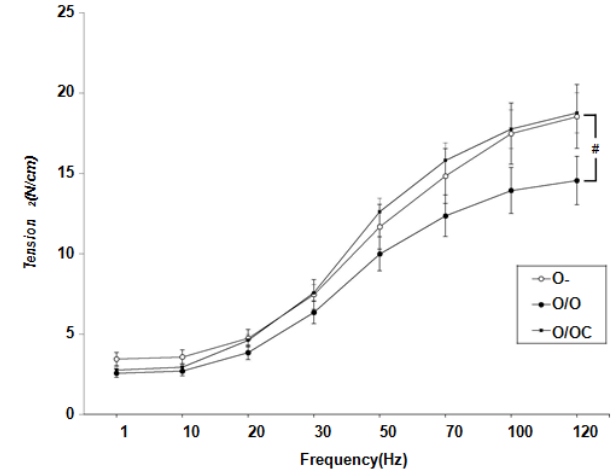

Figure 2: Changes in force-frequency curves of OVA group (O/O: closed circles), Budesonide/Formoterol inhalation group (O/OC: closed squares), control group (O-: open circles). #p<0.05.

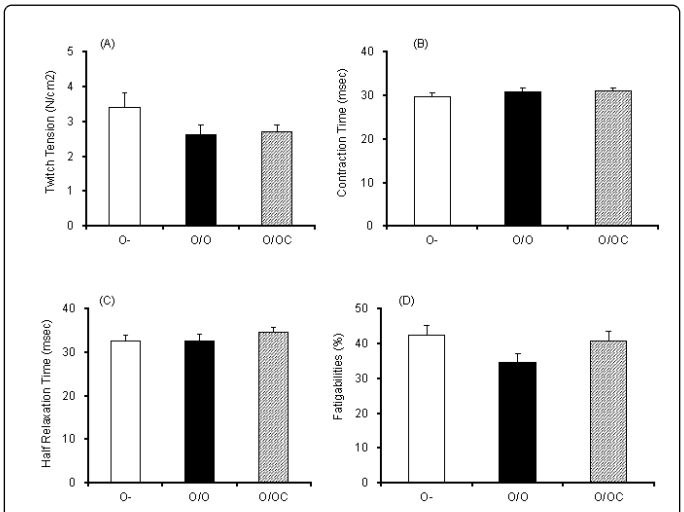

Figure 3: Changes in (A) Twitch Tension, (B) Contraction Time, (C) Half Relaxation Time and (D) Fatigabilities of the O/O, O/OC and O- groups.

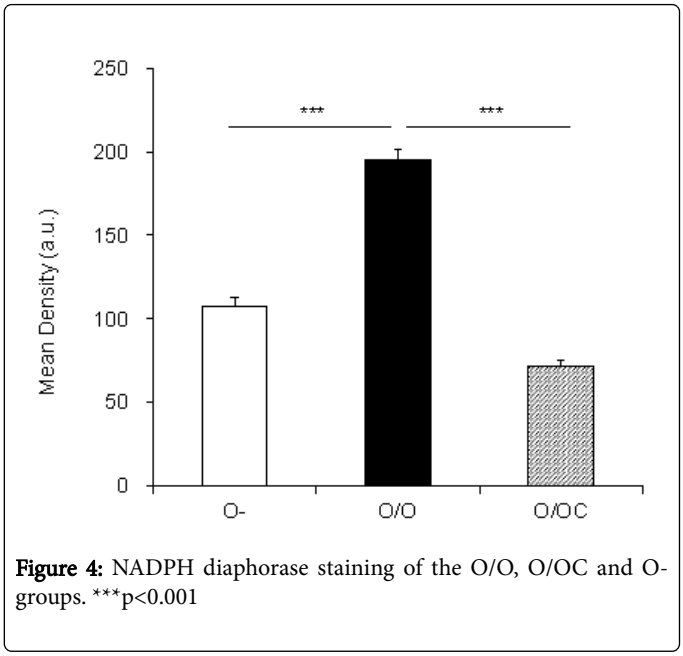

Figure 4: NADPH diaphorase staining of the O/O, O/OC and O- groups. ***p<0.001

This result suggested that OVA sensitization and challenge decreased diaphragm muscle contraction and Budesonide/Formoterol inhalation protected diaphragm muscle weakness. There were no significant changes in TT, CT and HRT of twitch contraction and fatigability (Figure 3).

NADPH diaphorase staining

NADPH diaphorase staining was used to detect NOS activity. The mean density of OVA group O/O (195.0 ± 6.1 a.u.) was significantly strengthened compared with control group O- (107.8 ± 5.1 a.u.) and OVA plus Budesonide/Formoterol inhalation group O/OC (71.3 ± 4.4 a.u.) (p<0.001, each) (Figure 4).

This result indicate that OVA sensitization and challenge increased NOS activity compared with O- and Budesonide/Formoterol inhalation protected OVA induced NOS activity.

Cytokines in BALF, lung tissue and diaphragm muscle homogenates by ELISA

IL-4 and IL-13 *levels* in BALF, in lung tissue homogenates and in diaphragm muscle homogenates were measured using *ELISA* for assessing inflammatory cytokines of Th2. Overall there was no significant difference between them in BALF and in lung tissue homogenates (Figures 5A and 5B). *IL-4 levels* of O/O *in* diaphragm muscle homogenates was significantly increased compared with control group O- and OVA plus Budesonide/Formoterol inhalation group O/OC (p<0.05, each) (Figure 5C).

This result indicates that OVA sensitization and challenge have inflammatory cytokine inductions of IL-4 in diaphragm muscle of mice. These results suggested that NOS activity affected induction of Th2 cytokines in the diaphragm muscle.

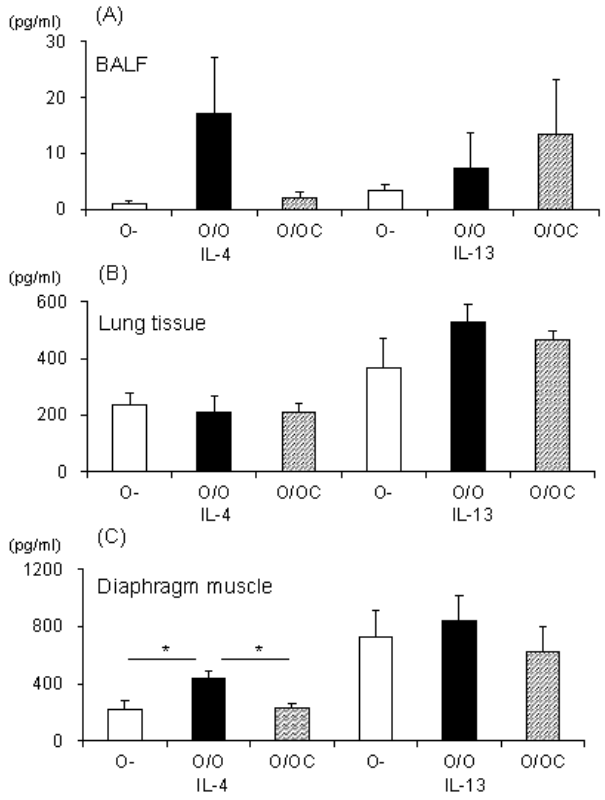

Figure 5: (A) IL-4 levels and IL-13 levels in BALF; (B) IL-4 levels and IL-13 levels in lung tissue, (C) IL-4 levels and IL-13 levels of the O/O, O/OC and O- groups in diaphragm muscle. *p<0.05.

Discussion

The main findings of this study are:1)OVA sensitized and challenged diaphragm muscle contraction was reduced: 2) OVA sensitization and challenge increased NOS activity in the diaphragm of mice: 3) OVA sensitization and challenge increased IL-4 in the diaphragm of mice: 4) Budesonide/Formoterol inhalation protected diaphragm muscle contractile dysfunction, OVA induced NOS activity and IL-4 in diaphragm muscle. F/f curve of OVA sensitized and challenged mice shift downward compared with control group. The implication is that OVA contribute to diaphragm muscle weakness. Although there has been no study that OVA affect the diaphragm muscle, it seems that mechanisms of diaphragm muscle contraction reduction are due to: 1) OVA increase resistive breathing of mice: 2) IL-4 is induced inside the diaphragm in response to resistive breathing: 3) iNOS is produced by IL-4: 4) muscle contraction is reduced by iNOS.

There were previously reported that OVA increased the breathing resistance of the mice [11], resistive breathing induced IL-4 expression in diaphragm muscle [12], Th2 cytokines such as IL-4 and IL-13 induce iNOS expression through the STAT-6 pathway [13-17], iNOS reduced diaphragm muscle contraction, inhibition of iNOS induction and inhibition of NOS activity prevented diaphragm muscle contractile dysfunction [18]. Study results of ours are in agreement with study that previously reported. Budesonide/Formoterol inhalation protected diaphragm muscle contractile dysfunction. We assume that Budesonide/Formoterol reduce the resistive breathing through bronchodilation of Formoterol and anti-inflammatory effect in airway of Budesonide. In particular, airway edema caused by the continuation of inflammation is improved with Budesonide as an ICS, bronchoconstriction caused by airway remodeling and airway hypersensitivity are improved with Formoterol as a LABA. Hence Budesonide/Formoterol protect the weakness of diaphragm muscle contraction through prevent the induction of IL-4 and iNOS. Our results support previous findings indicating that OVA sensitization and challenge exacerbate the airway inflammation [4,19,20]. ICS and ICS/LABA are used as a first choice for asthma treatment around the world. The effectiveness of ICS and ICS/LABA for asthma treatment is supported in plural large clinical trials [21-29].

In conclusion, OVA sensitization and challenge led to diaphragm muscle weakness and Budesonide/Formoterol inhalation protected diaphragm muscle weakness. OVA sensitization and challenge increased IL-4 levels and NOS activity in the diaphragm muscle, Budesonide/Formoterol inhalation decreased IL-4 levels and NOS activity through bronchodilation of Formoterol and anti-inflammatory effect of Budesonide.

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Kazunobu Yamaguchi: Contributed to the design and coordination of the study, prepared the manuscript, and read and approved the final manuscript.

Masahito Miura: Contributed to preparing the manuscript, and read and approved the final manuscript.

Chiyohiko Shindoh: Contributed to preparing the manuscript, and read and approved the final manuscript.

Disclosure

Kazunobu Yamaguchi is employee of AstraZeneca K.K. The authors report no other conflicts of interest in this work.

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