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 and IL-13 levels of BALF, as well as lung tissue and 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 and IL-13 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).

![Image](image.png)

**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% O2 and 5% CO2 gas mixture (37.0 ± 0.5°C, pH 7.40 ± 0.05). Both muscle strips were simultaneously homogenized, and total protein from lung tissue and diaphragm muscle were extracted by a kit (Minute™, Invent Biotechnologies, Inc., Eden Prairie, MN, USA). Mean values of tensions for each frequency of force-frequency curves were compared by 1-way ANOVA with Fisher’s LSD post-hoc analysis.

**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/cm² peak) was significantly shifted downward compared with control group O- (18.5 ± 1.9 N/cm² peak) and OVA plus Budesonide/Formoterol inhalation group O/OC (18.8 ± 1.3 N/cm² 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.](image)

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).

![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.](image)

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 indicated 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.
Budesonide/Formoterol reduce the resistive breathing through bronchodilatation 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 bronchodilatation of Formoterol and anti-inflammatory effect of Budesonide.

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Disclosure

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

References


