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Effects of Long-Acting β_2-Agonist and Corticosteroid Inhalation on Diaphragm Muscle in Mice

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Abstract

Background and Objective: Although a combination of inhaled corticosteroid (ICS) and inhaled long-acting β2-agonist (LABA) reduces exacerbation of asthma, whether these affect diaphragm muscle contraction is still unclear.

Methods: We investigated the effects of ICS and LABA inhalation separately, endotoxin injection-only, and ICS or LABA inhalation plus endotoxin injection on diaphragm contractile properties and nitric oxide (NO) production during 4 h, using BALB/c mice (n=84). In this study, budesonide was used as the ICS, and formoterol fumarate dehydrate was used as the LABA. After administrations of these drugs, the diaphragm muscles were dissected, and their contractile properties, including force/frequency (F/f) curves and twitch contraction, were measured by electrical pulse stimulation in an isometric condition. A reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry was performed to assess NO production, which induces cellular damages in diaphragm muscle fibers.

Results: The ICS inhalation did not significantly shift the F/f curves; but the LABA inhalation significantly shifted them upward compared with shams at 1 h ($p < 0.01$), 2 h ($p < 0.01$), and 4 h ($p < 0.05$) after inhalation, that is, it showed an inotropic effect. Although endotoxin injection resulted in a downward shift in F/f curves at 4 h ($p < 0.01$) and induced NO production, the endotoxin plus either ICS or LABA inhalation prevented downward shifts of the F/f curves and inhibited NO production.

Conclusions: These results indicate that the inhalation of LABA potentiates diaphragm muscle contractility more than ICS inhalation and that both ICS and LABA inhalation inhibit NO production induced by endotoxin injection.

Keywords: Bronchial asthma; Diaphragm muscle; Inhaled corticosteroids; Long-acting β_2-agonists; Nitric Oxide

Abbreviations: AU: Arbitrary Unit; ICS/LABA: Combination of ICS and LABA; CT: Contraction Time; F/F Curves: Force-Frequency Curves; HRT: Half-Relaxation Time; ICS: Inhaled Corticosteroid; INOS: Inducible NO Synthase; LABA: Long-Acting β_2-Agonist; NADP: Nicotinamide Adenine Dinucleotide Phosphate; NADPH: NADP Reduced Form; NO: Nitric Oxide; TT: Twitch Tension; TT/CT: Slope During Contraction Time; (TT/2)/HRT: Slope During Half-Relaxation Time

Introduction

Inhalation therapy with a combination of inhaled corticosteroid (ICS) and a long-acting β_2-agonist (LABA) is important in the treatment of patients with bronchial asthma. In patients who have persistent symptoms of asthma, despite treatment with inhaled glucocorticoids, the addition of LABA to ICS therapy alleviates symptoms and improves lung function [1]. In the study herein reported, the greatest reduction in exacerbation was found to occur when the subject received a higher than usual dose of ICS, that is, quadruplicate doses of ICS instead of duplicate doses of ICS with the addition of an LABA were found to be required for asthmatic crisis prevention.

It has been reported that single-inhaler therapy with a combination of ICS and LABA (ICS/LABA) is a clinically effective and well-tolerated treatment for patients with asthma that is not fully controlled by inhaled ICS alone [2]. In addition, ICS/LABA in a single inhaler is as safe and effective in long-term (12 months) treatment of asthma as ICS plus LABA via separate inhalers [3]. ICS/LABA (80 μg/4.5 μg, two inhalations b.i.d) for both maintenance and relief improves asthma control with a lower steroid load compared with a higher dose of budesonide (160 μg, two inhalations b.i.d) plus terbutaline (0.4 mg) as needed [4]. A combination of lower doses of drugs (10-12 to 10-9 mol/L) results in synchronized activation of transcription factors and an enhanced antiproliferative effect [5]. Thus, the safety and compliancy of the ICS/LABA single inhaler in the treatment of asthma has been sufficiently demonstrated, and combination therapy has recently been recommended for treatment of not only asthma, but also chronic obstructive pulmonary disease (COPD) [6,7].

As well as having synergistic effects and relieving symptoms of patients with bronchial asthma, we have recently reported that ICS/LABA inhalation increases diaphragm muscle contractility and prevents deterioration induced by endotoxin injection [8]. However, it remains to be clarified whether ICS or LABA inhalation has an inotropic effect on diaphragm muscles and whether ICS or LABA inhalation has a protective effect against diaphragm muscle deterioration of contractile properties and nitric oxide (NO) production induced by endotoxin. Given this background, we examined the effects of ICS and LABA inhalation alone on diaphragm contractile properties for 4 h after each

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inhalation, and then examined whether ICS or LABA inhalation plus endotoxin injection has protective effects against endotoxin-induced deterioration of diaphragm muscle.

In the present study, we observed that the inhalation of LABA potentiates diaphragm muscle contractility more than ICS inhalation and that both LABA and ICS inhibit NO production induced by endotoxin injection.

Methods

Animal preparation

A total of sixty BALB/c mice (Charles River Laboratories Japan, Inc., Yokohama, Japan) weighing 24.5 ± 0.3 (mean ± SE) g were used for contractile measurements. The mice were divided into the following eight groups: sham (i.e., lactose inhalation-only; n=5), ICS inhalation-only (n=15), LABA inhalation-only (n=15), E-sham (sham for endotoxin injection, i.e., saline injection in the same volume as that of endotoxin; n=5), endotoxin injection-only (n=5), EL-sham (sham for endotoxin injection and ICS or LABA inhalation, i.e., saline injection in the same volume as that of endotoxin and lactose inhalation; n=5), ICS inhalation plus endotoxin injection (n=5 animals), and LABA inhalation plus endotoxin injection (n=5).

We first examined the effects of ICS or LABA dry powder inhalation on diaphragm muscle contractility. In the ICS and LABA inhalation-only groups, animals inhaled budesonide (36 μg; total of 100 μg with lactose) as ICS or formoterol fumarate dehydrate (1 μg; total of 100 μg with lactose) as LABA using a dry powder insufflator (DP-4-M for mouse, Penn-Century Inc., Wyndmoor, PA, USA) with an air pump (200 μL of air, AP-1; Penn-Century Inc.). These drugs were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). We determined the inhalation dosages of ICS and LABA for the current study based on previous reports. The dose of budesonide (36 μg; total of 200 μg with lactose) was calculated as approximately 4% deposition (40 μg) in the lung of 1000 μg aerosol inhalation in mice [9]. Furthermore, the ICS/LABA ratio (35:1) was used based on human therapeutic formulation [10]. Therefore, the doses of 36 μg of budesonide and 1 μg of formoterol fumarate dehydrate were used in the present study.

Animals were lightly anesthetized in a glass jar, in which isoflurane for animals (Invet, Osaka, Japan) had been deposited. As a control, the sham inhalation group was inhaled with 100 μg of lactose only. The animals were then removed from the jar and fixed on a surgery board perpendicularly with a face mask with gauze containing isoflurane. Immediately after fixation, the epiglottis of the animal was extended using a laryngoscope (LS-2 for mouse; Penn-Century Inc.), and dry powder inhalation was achieved by two puffs in front of the opening of the vocal cords during spontaneous breathing. Diaphragm muscles were dissected and measured for contractility at 1 h (ICS1), 2 h (ICS2), and 4 h (ICS4) later (n=5 each) for the ICS inhalation-only group, or at 1 h (LABA1), 2 h (LABA2), and 4 h (LABA4) later (n=5 each) for the LABA inhalation-only group. We next examined the effects of endotoxin-only injection and ICS or LABA inhalation plus endotoxin injection on diaphragm muscle contractility. In the endotoxin injection-only group, animals were given an intraperitoneal injection of Escherichia coli endotoxin (20 mg/kg, 055:B5; Sigma Chemical Co., St. Louis, MO, USA) in 0.5 mL of saline, with measurement of muscle contractility at 4 h (E4) later (n=5 each). As a control for endotoxin injection, the E-sham group was injected with 0.5 mL of saline only. In the ICS and LABA inhalation plus endotoxin injection groups, animals initially inhaled ICS (36 μg) or LABA (1 μg) using a dry powder insufflator (DP-4-M for mouse; Penn-Century Inc.) with an air pump (200 μL of air, AP-1; Penn-Century Inc.), immediately followed by an intraperitoneal injection of E. coli endotoxin (20 mg/kg) in 0.5 mL of saline

The diaphragm muscles were then dissected and measured for muscle contractility 4 h (ICSE4) later (n=5 each) for the ICS inhalation plus endotoxin injection group, and at 4 h (LABAE4) later (n=5 each) for the LABA inhalation plus endotoxin injection groups. As a control for ICS or LABA inhalation and endotoxin injection, the EL-sham group was inhaled with 100 μg lactose and injected with 0.5 mL saline. Because we had previously shown that force-frequency curves (F/f) are maximally decreased at 3 to 4 h and then recover 6 h after endotoxin injection [11], we measured and analyzed diaphragm muscle contractility 4 h after endotoxin administration. All procedures of the present study were performed according to the protocol approved by the Institutional Committee for Use and Care of Laboratory Animals at Tohoku University (2011-Idou-28).

Measurements of muscle contraction

Measurements of muscle contraction have been described in a previous paper [11]. In brief, muscle strips were dissected from the right and left hemidiaphragms of each animal under isoflurane (Invet) for animal anesthesia and mounted in separate organ baths containing Krebs-Henseleit solution oxygenated with a 95% O_2 to 5% CO_2 gas mixture. Both muscle strips were simultaneously stimulated with supramaximal currents by a constant current stimulus isolation unit (SS-302J; Nihon Kohden Co, Tokyo, Japan) driven by a stimulator (SEN-3201; Nihon Kohden Co.). The elicited tensions were measured by a force transducer (UL-100GR; Minebea Co., Fujisawa, Japan). The length of each muscle strip was changed by moving the position of the force transducer with a micrometer-controlled rack and pinion gear (Mitsutoyo Co, Kawasaki, Japan) and measured with a micrometer in close proximity to the muscle. The optimal length (Lo) of the muscle strip was defined as the muscle length at which

twitch tension development was maximal, and this Lo was maintained in the following measurements (isometric conditions). The cross-sectional area of the strip was calculated by dividing the muscle mass by the product of the strip muscle length (Lo) and muscle density (1.06 g/cm^3) [12], and tension (N/cm^2) was calculated as force (N) per cross-sectional area (cm^2).

The diaphragm F/f curves were assessed by sequentially stimulating muscles at 1, 10, 20, 30, 50, 70, 100, and 120 Hz. The tensions of both muscle strips were recorded by a hot-pen recorder (RECTI-HORIZ-8K; San-ei Co., Tokyo, Japan). When the muscle is stimulated by a 1 time/sec (1 Hz), it is called a twitch, and when it is stimulated more than 120 times/sec (120 Hz), it is called a tetanus. Thus, the F/f curve is a type of spectrum relationship between provoked tensions (force) and stimulating impulses (frequency).

Twitch contraction was elicited by single pulse stimulation (0.2-msec pulse duration). Twitch kinetics were assessed by twitch tension (TT; N/cm^2), contraction time (CT; msec), and half-relaxation time (HRT; msec) during a single muscle contraction. For analysis of contractile velocity of twitch contractions, TT/CT and (TT/2)/HRT were calculated from the curve of the twitch contraction trace.

Muscle fatigue was then assessed by examining the rate of the decrease in tension 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 the final tension as a percentage (%) of the initial tension. After completion of this protocol, the muscle strip was removed from the organ bath and weighed after removal of fatty tissue.

Reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry

Twenty-four other BALB/c mice weighing 23.7 ± 0.5 g (Charles River Japan) were used for NADPH diaphorase histochemistry. NADPH diaphorase histochemistry of diaphragm muscle was performed in sham animals (n=2 animals), at 1, 2, and 4 h in diaphragm muscle in the ICS or LABA inhalation-only groups (n=2 animals for each time point), in the E-sham group (n=2 animals), at 4 h in diaphragm muscle in the endotoxin injection-only group (n=2 animals), in the EL-sham group (n=2 animals), and at 4 h in the ICS or LABA inhalation plus endotoxin groups (n=2 animals for each time point). The diaphragm was quickly excised, and the tissue pieces were frozen in OCT compound (Tissue-Tek; Sakura Finetechnical Co, Ltd, Tokyo, Japan). Cryosections (10 μm in thickness) were cut from the diaphragm in the OCT compound. The histochemical procedure for NADPH diaphorase has been described elsewhere [13]. Briefly, the specimens were placed in the reaction solution consisting of 1.0 mM β-NADPH (Oriental Yeast Co., Ltd., Tokyo, Japan), 0.2 mM nitroblue tetrazolium (Wako Pharmaceutical Co.), 100 mM tris-HCL buffer (pH 8.0), and 0.2% Triton X-100 for 30 min at 37°C. The reactions were terminated by rinsing the sections in phosphate-buffered saline. The sections were then covered with a coverglass and photographed under a microscope (Axiolab A1; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with a charge-coupled device camera (AxioCam ERc 5s; Carl Zeiss MicroImaging GmbH). Because inducible NO synthase (iNOS) requires NADPH as a coenzyme, we evaluated the degree of staining in the histochemical reaction for NADPH diaphorase as an indicator of NO production [14]. 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.)

Data Analysis

Data were obtained from both halves of the diaphragm; therefore, the number of muscle samples used was n=10 per treatment/time point for F/f curves, twitch kinetics, and fatigability. The mean values of tension for each frequency of F/f curves, twitch kinetics, fatigability, and mean density of muscle fibers were compared by Student's t-test. To compare the entire configuration of each F/f curve at each time point or concentration, analysis of variance with Fisher's protected least significant difference post hoc test was performed. Data are presented as means ± SE. A p value less than 0.05 was considered significant [15,16].

Results

Changes in contractile properties in the ICS inhalation-only and the LABA inhalation-only groups. In the ICS inhalation-only group, there were no overall significant changes in the F/f curves compared within the group (Figure 1a). Regarding tensions, those at 10, 20 and 30 Hz of ICS1 and ICS2 were equal or lower than those of sham, and those of ICS4 were higher than those of sham at frequencies of 1-30 Hz. Tensions at higher frequencies at 70-120 Hz for ICS2 were higher than those of sham; however, these standard errors of ICS2 and sham overlapped, and therefore, were not significant. We speculate that the tendencies at ICS2 were related to the fact that NO production decreased from ICS1 to ICS2, as described below. These observations indicated that ICS does not have an inotropic effect on diaphragm muscle during 4 h of inhalation.

On the other hand, in the LABA inhalation-only group, the F/f curves significantly shifted upward at LABA1 ($p < 0.01$) and then significantly shifted downward at LABA2 ($p < 0.01$) and at LABA4 ($p < 0.05$) compared with those of the sham group (Figure 1b). Tensions at 1, 20, 30, 70, 100, and 120 Hz (each $p < 0.05$) at LABA1, at 1 and 20 Hz (each $p < 0.05$) at LABA2, and at 1 Hz ($p < 0.05$) at LABA4 were significantly increased compared with those in the sham animals. These observations indicated that LABA has an inotropic effect on diaphragm muscle during 4 h of inhalation. Table 1 shows data of twitch contraction and fatigability in both groups. In the ICS inhalation-only group, there was a significant decrease in CT at ICS1 ($p < 0.05$) and ICS2 ($p < 0.01$) compared with that in the sham animals, and a significant decrease in HRT at ICS1 ($p < 0.001$) and ICS2 ($p < 0.01$) compared with that in shams. There was also a significant decrease in TT/CT at ICS4 ($p < 0.05$) and in (TT/2)/HRT at ICS4 ($p < 0.01$) compared with those at ICS1. However, these changes might not have greatly affected the F/f curves. In the LABA inhalation-only group, there was a significant increase in TT at LABA1 ($p < 0.05$), LABA2 ($p < 0.05$), and LABA4 ($p < 0.05$) compared with those in the sham animals, and these increases in TT might have contributed to an increase in F/f curves, as shown in Figure 1b.

Changes in NADPH histochemistry in the ICS inhalation-only and the LABA inhalation-only groups

Figure 2 shows NADPH histochemistry staining of both groups. In the ICS inhalation-only group, NADPH diaphorase histochemistry showed a slight increase in the mean density of staining at ICS1 (78.8 ± 1.6 a.u., $p < 0.05$) compared with those in the sham animals (50.9 ± 1.9 a.u.), but the increases in density for ICS2 (67.9 ± 2.6 a.u.) and ICS4 (60.3 ± 4.1 a.u.) were not significant. Production of NO was observed early during ICS inhalation, but as mentioned below, the increased density at ICS1 was less than that of endotoxin-induced NO production at E4.

In the LABA inhalation-only group, NADPH diaphorase histochemistry showed that there was no significant increase in the mean density of staining at LABA1 (53.5 ± 2.2 a.u.), LABA2 (52.5 ± 2.4 a.u.), and LABA4 (48.2 ± 2.4 a.u.) compared with that in the sham animals. This indicated that LABA inhalation alone did not induce NO

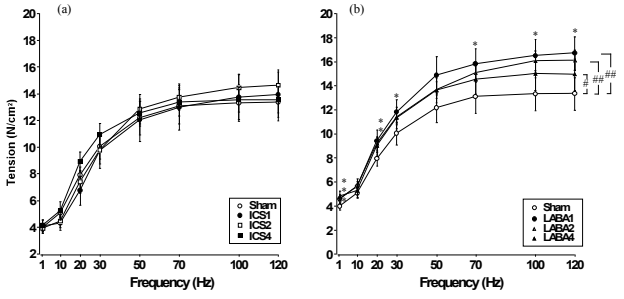

Figure 1: Changes in F/f curves in the ICS inhalation-only (a) and in the LABA inhalation-only groups (b). There were no significant shifts in F/f curves from ICS1 to ICS4 compared with shams. However, F/f curves at LABA1 (##, $p<0.01$), LABA2 (##, $p<0.01$), and LABA4 (#, $p<0.05$) significantly shifted upward compared with sham animals. *$p<0.05$ compared with tensions of each frequency in shams.

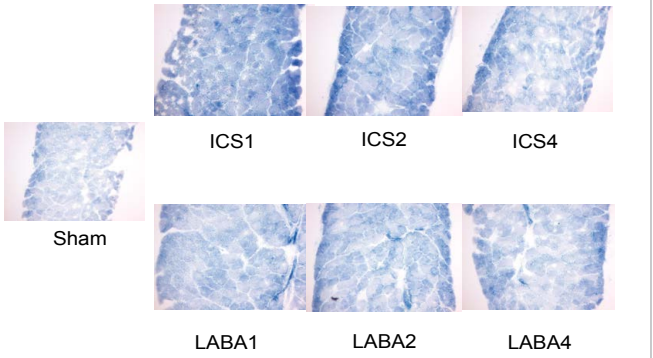

Figure 2: Photographs of NADPH diaphorase histochemistry in the ICS inhalation-only (ICS1, ICS2, and ICS4) and LABA inhalation-only (LABA1, LABA2, and LABA4) groups and shams. Although there were slight increases in the mean density of staining at ICS1 compared with shams, there were no significant increases in the mean density of staining from LABA1 to LABA4. All scale bars indicate 50 μm.

production in the cross-sectional view of diaphragm muscle fibers.

Changes in contraction properties in the endotoxin-only and ICS or LABA inhalation plus endotoxin groups

In the endotoxin injection-only group, there was a significant downward shift at E4 ($p < 0.001$) in the F/f curves compared with those in the E-sham group (Figure 3a). Regarding values of tension, those at 10 ($p < 0.05$), 20 ($p < 0.01$), 30 ($p < 0.01$), 50, 70, 100, and 120 Hz (each $p < 0.01$) at E4 were significantly decreased compared with those in the E-sham group. These changes indicated that endotoxin deteriorated diaphragm muscle contraction in the 4-h follow-up period.

In the ICS or LABA inhalation plus endotoxin group, there were significant upward shifts ($p < 0.05$) in the F/f curves at ICSE4, and also further significant upward shifts in the F/f curves at FE4 compared with those in the EL-sham group ($p < 0.01$, Figure 3b). Values of tension at LABAE4 showed significant increases at 1 ($p < 0.05$), 30 ($p < 0.01$), 50, 70, 100 (each $p < 0.05$), and 120 Hz ($p < 0.01$) of F/f curves compared with those in the EL-sham group. These observations indicate that both inhalations prevented the decrease in F/f curves induced by endotoxin.

Table 2 shows data of twitch kinetics of the endotoxin-only and ICS or LABA inhalation plus endotoxin groups. In the endotoxin injection-only group, there were significant decreases in HRT at E4 ($p < 0.01$) and in fatigability at E4 ($p < 0.05$) compared with those in the E-sham group. In the ICS inhalation plus endotoxin group, there were significant increases in TT/CT ($p < 0.05$) and in (TT/2)/HRT at ICSE4 ($p < 0.001$) compared with those in the EL-sham group. In the LABA inhalation plus endotoxin group, there was a significant increase in TT ($p < 0.05$), a decrease in fatigability ($p < 0.05$), an increase in TT/CT at LABAE4 ($p < 0.01$), and an increase in (TT/2)/HRT at LABAE4 ($p < 0.05$) compared with those in the EL-sham group. These changes of twitch kinetics might have contributed to the changes of each F/f curve.

NADPH diaphorase histochemistry showed that cross-sectional views of diaphragm muscle at E4 (148.7 ± 4.9 a.u.) had significantly stronger staining than in the E-sham group (45.4 ± 2.2 a.u, $p < 0.001$; Figure 4). This increased staining density at E4 in the endotoxin injection-only group indicated that NO production was strongly induced by endotoxin injection. However, there was no significant staining at 4 h in the ICS or LABA inhalation plus endotoxin groups at either ICSE4 (55.4 ± 2.7 a.u.) or LABAE4 (48.2 ± 2.4 a.u.), and they were not significantly different compared with the EL-sham group (39.7 ± 1.9 a.u, Figure 4). These changes in staining corresponded well with the changes in diaphragm muscle F/f curves, and it is concluded that ICS and LABA inhalation prevented endotoxin-induced NO production.

Discussion

In the present study, it was revealed that LABA inhalation has a greater inotropic effect on diaphragm muscle contraction than ICS inhalation, as shown by the fact that the F/f curves of the ICS inhalation-only group did not significantly change after inhalation; however, the F/f curves of the LABA inhalation-only group significantly shifted upward. Although NADPH diaphorase histochemistry in the LABA inhalation-only group was unchanged, that in the ICS inhalation-only group showed a significant increase at ICS1. However, this increase in NADPH diaphorase histochemistry at ICS1 was less than that at E4. Because the observations in a previous study indicated that ICS/LABA combined inhalation increased F/f curves and decreased NO production [8], we attempted to clarify whether these activities were caused by ICS or LABA individually or in an additive fashion in the case of the diaphragm.

Although we did not measure blood concentrations in the present study, there is a possibility that the observed changes of muscle contractile properties might have been elicited by blood circulation of both drugs referred to in the previous reports [16,17].

We found that LABA inhalation had a greater inotropic effect on muscle contraction than ICS and that both ICS and LABA each had a preventive effect on endotoxin-induced inflammation of diaphragm muscle. We have previously reported that other β2-agonists, such as procaterol [18] and tulobuterol [19], shift F/f curves upward, and in the present study, we similarly showed that LABA has an inotropic effect. Generally, skeletal muscles, including the diaphragm and other respiratory muscles, are known to have β2 receptors [20,21]. The inotropic effect of β2-agonists is induced via β2 receptors in cell membranes and this increases cyclic adenosine monophosphate, which is a pathway to facilitate diaphragm muscle contraction [22].

However, we found that ICS did not show an inotropic effect. Before the advent of ICS in the treatment of asthma, severe patients were treated by oral steroid administration. Several reports have shown steroidal and myofibrillar changes induced by systemic administration [23]. Oral steroid administration causes generalized muscle atrophy

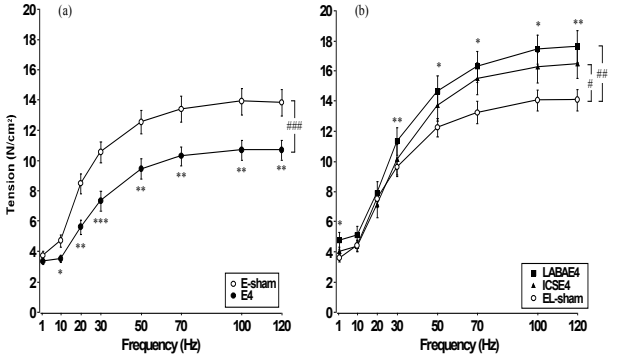

Figure 3: Changes in F/f curves in the endotoxin-only injection (a) and in the ICS or LABA inhalation plus endotoxin injection groups (b). The F/f curves significantly shifted downward at E4 (###, $p<0.001$) compared with those of the E-sham. *$p<0.05$, **$p<0.01$, ***$p<0.01$ compared with each frequency in the E-sham. The F/f curves at ICSE4 (#, $p<0.05$) and LABAE4 significantly shifted upward (##, $p<0.01$) compared with those of the EL-sham. *$p<0.05$, **$p<0.01$ compared with each frequency in the EL-sham.

	Sham	ICS1	ICS2	ICS4	LABA1	LABA2	LABA4
TT (N/cm^2)	4.2 ± 0.4	4.3 ± 0.5	4.0 ± 0.3	4.0 ± 0.6	4.4 ± 0.3*	4.3 ± 0.3*	4.6 ± 0.4*
CT (msec)	39.4 ± 1.9	34.3 ± 1.7*	32.4 ± 1.5**	42.1 ± 2.0	34.8 ± 3.3	41.1 ± 1.8	36.1 ± 1.8
HRT (msec)	45.0 ± 4.6	34.2 ± 2.7***	36.9 ± 2.9**	54.0 ± 5.3	44.9 ± 6.8	50.5 ± 6.2	40.1 ± 2.9
Fatigue (%)	39.9 ± 3.6	33.4 ± 2.7	32.5 ± 2.2	36.8 ± 2.4	33.4 ± 1.9	37.9 ± 2.4	36.6 ± 2.4
TT/CT (N/cm^2/sec)	95.3 ± 13.3	127.4 ± 13.9	125.2 ± 8.0	93.5 ± 10.9†	129.0 ± 9.9	106.8 ± 9.0	131.0 ± 15.6
(TT/2)/HRT (N/cm^2/sec)	43.0 ± 7.2	64.9 ± 7.8	55.6 ± 3.0	39.0 ± 5.6††	55.8 ± 7.6	45.9 ± 4.6	58.9 ± 5.6

Table 1. Changes in twitch kinetics in the sham, ICS inhalation-only, and LABA inhalation-only groups
ICS1, ICS2, and ICS4 express 1 h, 2 h, and 4 h after ICS inhalation. LABA1, LABA2, and LABA4 express 1 h, 2 h, and 4 h after LABA inhalation.
TT, twitch tension; CT, contraction time; HRT, half-relaxation time.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with sham.
†$p < 0.05$, ††$p < 0.01$ compared with each value at ICS1.

	E-sham	E4	EL-sham	ICSE4	LABAE4
TT (N/cm^2)	3.8 ± 0.3	3.4 ± 0.3	3.7 ± 0.3	4.2 ± 0.4	4.7 ± 0.5§
CT (msec)	35.9 ± 1.6	34.7 ± 1.7	34.2 ± 2.2	31.0 ± 2.2	31.6 ± 1.7
HRT (msec)	43.7 ± 2.6	32.3 ± 3.2**	35.6 ± 2.8	28.8 ± 2.0	36.4 ± 4.4
Fatigue (%)	37.6 ± 1.9	45.9 ± 2.8*	36.0 ± 2.1	35.0 ± 2.1	27.8 ± 3.2†
TT/CT (N/cm^2/sec)	107.9 ± 9.6	97.3 ± 7.9	107.3 ± 7.8	139.4 ± 11.8†	156.8 ± 25.2††
(TT/2)/HRT (N/cm^2/sec)	44.7 ± 3.8	53.7 ± 3.2	51.3 ± 3.8	74.4 ± 5.2†††	73.5 ± 12.4†

Table 2: Changes in twitch kinetics in the endotoxin injection groups (E-sham and E4), and in the endotoxin injection plus ICS or LABA inhalation groups (EL-sham, ICSE4, and LABAE4)
E-sham and E4: sham and 4 h after saline + endotoxin injection
EL-sham, ICSE4 and LABAE4: sham and 4 h after ICS + endotoxin injection, and 4 h after LABA + endotoxin injection
TT, twitch tension; CT, contraction time; HRT, half-relaxation time.
*$p < 0.05$, **$p < 0.01$ compared with E-sham.
†$p < 0.05$, †$p < 0.01$, †††$p < 0.001$ compared with EL-sham.

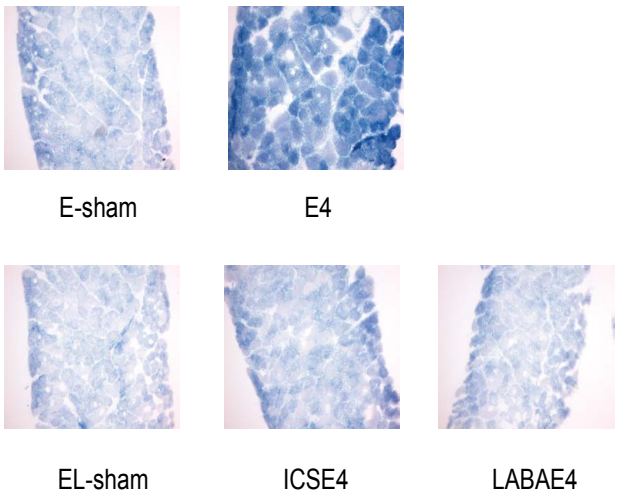

Figure 4: Photographs of NADPH diaphorase histochemistry in the endotoxin-only injection (E-sham, and E4) and in the ICS or LABA inhalation plus endotoxin injection groups (EL-sham, ICSE4, and LABAE4). In the cross-sectional views, diaphragm muscle fibers were stained more strongly at E4 than in the E-sham. However, there was no strong staining in EL-sham, ICSE4, and LABAE4. All scale bars indicate 50 μm. -25- -26-

and rhabdomyolysis, including that of respiratory muscles [24], and diffuse fiber atrophy predominantly affecting fast fibers [25,26]. However, as compared with the conventional administration of corticosteroid, the use of prodrugs/softdrugs and the mechanism (such as esterification) by which retention at the target site is achieved, minimize systemic exposure [27]. Furthermore, the concentration of inhaled glucocorticoids can be reduced when combined with β2 agonists, minimizing the side effects of the drugs [5]. Therefore, based on our results, the combination of ICS and LABA can be expected to alleviate diaphragm muscle contractile weakness caused by steroids. In addition, the regular use of low-dose ICSs is associated with a decreased risk of death from asthma [28].

contractility. Anti-infammatory effects such as inhibition of free radical species production were also investigated. With regard to contractile properties, it might be concluded that the increment in F/f curves by ICS/LABA combined inhalation is mainly caused by the effects of LABA inhalation.

Furthermore, the F/f curves of the endotoxin injection-only group showed a significant downward shift at E4 compared with those of E-sham, and those of the ICS inhalation plus endotoxin injection and LABA inhalation plus endotoxin injection groups showed significant upward shifts. NO production shown by NADPH diaphorase histochemistry induced by endotoxin at 4 h was inhibited by ICS and LABA inhalation.

With regard to twitch kinetics, tensions from 10 Hz to 120 Hz in F/f curves are generally produced as convolutions of a twitch contraction. In the present study, although CT and HRT were decreased at ICS1 and ICS2 in the ICS inhalation-only group, this did not change the configurations of the F/f curves at ICS1 and ICS2. An increase in TT was observed in the LABA inhalation-only group, which was related to the upward shift of the F/f curves at LABA1, LABA2, and LABA4. In the endotoxin injection-only group, a decrease in HRT at E4 and a slight decrease in TT and CT were observed, which were related to the F/f curves at E4. At ICSE4 and LABAE4, an increase in TT and decrease in CT were observed, which appeared to be related to increases in F/f curves.

In contrast to oral drug delivery, which is inactivated by the liver (first-pass effect), drugs in inhalation are absorbed through the mucosa of the throat and lung, and pass through the heart, aorta, and diaphragmatic artery [15]. In fact, concerning blood concentrations after ICS and LABA inhalation, it has been reported that when 1000 μg of ICS was inhaled in humans, blood concentrations of ICS reached 4.8

nmol/L (2.1 ng/ml) 12.6 min after inhalation [16]. Additionally, when 852 μg of LABA was inhaled in rats, blood concentrations of LABA reached 28.0 nmol/L (33.3 ng/ml) 15 min after inhalation [17].

Steroids downregulate iNOS expression, suggesting a potential to downregulate NO-mediated inflammation in neonates with meconium aspiration syndrome [29]. Observation was limited to 4 h in this experiment, perhaps too short to show genomic effects of ICS. However, endotoxin administration induces many substances, such as superoxide and peroxynitrite anions [30] and tumor necrosis factor α mRNA [11], contributing to the deterioration of diaphragm muscle contractility. NO has an unpaired electron in 2p antibonding orbital. Therefore, NO is a free radical [31], and many oxidants interfere with Ca^{2+} uptake and extrusion mechanisms at the level of the plasma membrane [32]. This cellular damage is considered to be related to contractile deterioration of diaphragm muscle. Therefore, ICS and LABA are able to prevent diaphragm muscle deterioration by inhibiting NO production. The results of the present experiment support the possibility that ICS and LABA inhalation alone inhibit oxidative stress and induction of inflammatory cytokines. A recent national survey of endotoxin in United States housing found that household endotoxin exposure is a significant risk factor for increased asthma prevalence [33]. It is considered that daily inhalation of house dust containing endotoxin is involved in increased asthma exacerbation. However, we performed endotoxin injection instead of inhalation of endotoxin in our animal experiments.

Therefore, it may be important that ICS/LABA inhalation prevents airway inflammation and diaphragm deterioration when patients with bronchial asthma have increased pulmonary and systemic oxidative stress at rest and during exacerbation. ICS/LABA is thought to be able to mitigate diaphragm muscle deterioration by excessive breathing due to asthma exacerbation [34,35]. In patients with asthma using ICS, LABAs do not increase the risk of asthma-related hospitalization. There are few asthma-related deaths and intubations, and events are too infrequent to establish the relative effect of LABAs on these outcomes [36].

In conclusion, the inhalation of LABA rather than ICS inhalation potentiates diaphragm muscle contractility, and both ICS and LABA inhalations inhibit NO production induced by endotoxin injection. The increment in muscle contractility with ICS or LABA plus endotoxin appears to be induced by an inhibiting effect against NO production. Based on these results, we speculate that the combination of ICS and LABA inhalation may improve diaphragm muscle contractile properties as well as contributing to the reduction of exacerbation in patients with bronchial asthma.

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