

Inhalation of Budesonide/Formoterol Increases Diaphragm Muscle Contractility

Chiyohiko Shindoh¹, Rie Shishido¹, Natsu Narumi¹, Naomi Murai¹ and Masahito Miura¹

ABSTRACT

Background: Although budesonide/formoterol (BUD/FORM) is used clinically as a steroid/ β_2 -agonist single inhaler, it has not yet been clarified whether BUD/FORM has inotropic effects on diaphragm muscles after inhalation.

Methods: We examined the effects of BUD/FORM inhalation, endotoxin injection, and BUD/FORM inhalation plus endotoxin injection on diaphragm contractile properties and nitric oxide (NO) production. After these three treatments, the diaphragm muscle was dissected, and its contractile properties were measured. Histochemistry for the reduced form of nicotinamide adenine dinucleotide phosphate diaphorase was performed for each muscle to assess NO production.

Results: The force-frequency curves showed an upward shift 1 h after inhalation ($p < 0.05$) in the BUD/FORM inhalation only group. The force-frequency curves showed a downward shift 4 h after injection ($p < 0.001$) in the endotoxin injection groups. In the BUD/FORM inhalation plus endotoxin injection groups, a downward shift in the force-frequency curves at 4 h after endotoxin injection was prevented. NO production was inhibited in the BUD/FORM inhalation plus endotoxin injection group compared with that of the endotoxin injection only groups.

Conclusions: BUD/FORM inhalation has an inotropic effect on diaphragm muscle, protects diaphragm muscle deterioration after endotoxin injection, and inhibits NO production. Increments in muscle contractility with BUD/FORM inhalation are induced through a synergistic effect of an anti-inflammatory agent and β_2 -agonist.

KEY WORDS

asthma, diaphragm muscle, endotoxin, inhaled corticosteroid, long-acting beta2-agonist

ABBREVIATIONS

arbitrary unit, a.u.; BUD/FORM, budesonide/formoterol; CSA, cross-sectional area; CT, contraction time; Esham, E1, E2, and E4, saline injection as a sham, and after 1, 2, and 4 h of endotoxin injection, respectively; 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; Ssham, S1, S2, and S4, sham inhalation with lactose, and after 1, 2, and 4 h of BUD/FORM inhalation, respectively; SEsham, SE1, SE2, and SE4, sham inhalation with lactose and saline injection, and after 1, 2, and 4 h of BUD/FORM inhalation and endotoxin injection, respectively; TNF- α , tumor necrosis factor- α ; TT, twitch tension; TT/CT, slope during contraction time; (TT/2)/HRT, slope during half relaxation time.

INTRODUCTION

Budesonide/formoterol (BUD/FORM) in a single inhaler is used to treat patients with bronchial asthma.

In a study by Pauwels *et al.*,¹ the greatest reduction in exacerbation occurred in patients receiving both a high dose of inhaled corticosteroid (ICS) and the addition of a long-acting β_2 -agonist (LABA). Single in-

¹Department of Clinical Physiology, Health Sciences, Tohoku University Graduate School of Medicine, Miyagi, Japan.

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Correspondence: Professor Chiyohiko Shindoh, MD, Department of Clinical Physiology, Health Sciences, Tohoku University Gradu-

ate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8575, Japan.

Email: cshindoh@med.tohoku.ac.jp

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haler therapy with budesonide and formoterol is a clinically effective and well-tolerated treatment for patients with asthma that is not fully controlled by inhaled glucocorticosteroids alone. A previous study found a greater increase in morning peak flow with a single-inhaler (35.7 L/min) and separate-inhaler (32.0 L/min) of budesonide and formoterol compared with budesonide alone.² Cellular studies have shown that both glucocorticoids and β_2 -agonists (10^{-8} mol/L) activate C/EBP- α and the glucocorticoid receptor with different kinetic profiles and inhibit proliferation, and the combination of lower drug doses (10^{-12} to 10^{-9} mol/L) results in a synchronized activation of transcription factors and enhances the antiproliferative effect.³ Therefore, in patients with persistent asthma symptoms despite treatment with inhaled glucocorticoids, the addition of formoterol to budesonide therapy has been shown to improve symptoms and lung function and may be beneficial.

Moreover, BUD/FORM in a single inhaler is as safe and effective in long-term (12 months) asthma treatment as budesonide plus formoterol via separate inhalers. A lower number of withdrawal symptoms in patients using BUD/FORM may reflect better adherence to treatment compared with budesonide inhalation and formoterol as a rescue inhalation.⁴ In addition, BUD/FORM (80 μ g/4.5 μ g, 2 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 μ g, 2 inhalations *b.i.d*) plus terbutaline (0.4 mg) as needed.⁵ These reports have shown the safety and compliancy of the BUD/FORM single inhaler in asthma treatment. However, it has not yet been clarified whether BUD/FORM has inotropic effects on diaphragm muscles after inhalation.

We have previously studied the effects of endotoxin injection on diaphragm muscle contractile properties and showed that endotoxin injection induces a decrement in diaphragm muscle contractility.⁶ This deterioration may be caused by a network of cytokines such as tumor necrosis factor- α (TNF- α)⁶ and free radicals such as nitric oxide (NO) and oxygen derived intermediates, including superoxide and the hydroxyl radicals.⁷ Therefore, BUD/FORM inhalation is expected to confer a protective effect against diaphragm muscle deterioration in contractile properties and NO production induced by endotoxin.

In the current study, we examined the effects of BUD/FORM inhalation and BUD/FORM inhalation plus endotoxin injection on diaphragm contractile properties and NO production. We first investigated the effects of BUD/FORM only inhalation in normal animals and measured diaphragm contractile properties for the following 4 h. We then assessed whether BUD/FORM inhalation and endotoxin injection have protective effects against endotoxin-induced deterioration of diaphragm muscle.

METHODS

ANIMAL PREPARATION

Experiments were performed on 60 mice divided into three groups ($n = 20$ of animals for each group) using BALB/c mice weighing 24.2 ± 0.4 g (Charles River Japan, Tokyo, Japan). In the BUD/FORM inhalation only groups, animals inhaled BUD/FORM (36 μ g/1 μ g, a total of 200 μ g with lactose) using a dry powder insufflator (DP-4-M, Penn-Century, Wyndmoor, PA, USA) with an air pump (200 μ L of air, AP-1, Penn-Century). The dose of BUD (36 μ g) was calculated as approximately 4% deposition (40 μ g) in the lung of 1000 μ g aerosol inhalation in mice.⁸ The concentration combination of BUD/FORM (36 μ g/1 μ g) was approximately one-fourth that of a commercially available dry powder inhaler (BUD/FORM [160 μ g/4.5 μ g for one puff]; Symbicort[®]), which was provided by AstraZeneca (Lund, Sweden). Animals were lightly anesthetized in a glass jar into which diethyl ether drops were added. The animals were then removed from the jar and perpendicularly fixed on a surgery board with a face mask treated with diethyl ether. Immediately after fixation, the epiglottis of the animal was extended using a laryngoscope (LS-2 for mouse, LMS, Tokyo, Japan), and dry powder was administered through two puffs in front of the opening of the vocal cords during spontaneous breathing. Diaphragm muscles were dissected and contractility was measured immediately after sham inhalation with lactose (200 μ g) (Ssham), and after 1 (S1), 2 (S2) and 4 h (S4) of BUD/FORM inhalation ($n = 5$ animals each). In the endotoxin injection only groups, animals were intraperitoneally injected with *Escherichia coli* endotoxin (20 mg/kg, 055: B5, Sigma Chemical, St. Louis, MO, USA) in 0.5 mL of saline, and muscle contractility was measured after saline (0.5 mL only) injection as a sham experiment (Esham), and after 1 (E1), 2 (E2) and 4 h (E4) of endotoxin injection ($n = 5$ animals each). In the BUD/FORM inhalation plus endotoxin groups, animals initially inhaled BUD/FORM (36/1 μ g) using a dry powder insufflator (DP-4-M, Penn-Century) with an air pump (200 μ L of air, AP-1, Penn-Century), followed immediately by intraperitoneal injection of *E. coli* endotoxin (20 mg/kg) in 0.5 mL of saline. Diaphragm muscles were then dissected, and muscle contractility was measured immediately after sham inhalation with lactose (200 μ g) and saline injection (0.5 mL only) (SEsham), and after 1 (SE1), 2 (SE2) and 4 h (SE4) BUD/FORM inhalation plus endotoxin injection ($n = 5$ animals each). We have previously shown that the force-frequency curves of diaphragm muscle maximally decreased at 3 to 4 h and then recovered at 6 h after endotoxin injection.⁶ Therefore, we measured and analyzed diaphragm muscle contractility 4 h after endotoxin administration. Written approval was obtained from the Tohoku University Animal Facility.

MEASUREMENTS OF MUSCLE CONTRACTION

Muscle strips (width, 3 to 4 mm; length, 8 to 11 mm; weight, 0.008 to 0.010 g) were dissected from the right and left hemidiaphragm of each animal under diethyl ether anesthesia 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). The composition of the aerated Krebs-Henseleit solution in mEq/L was as follows: Na⁺, 153.8; K⁺, 5.0; Ca²⁺, 5.0; Mg²⁺, 2.0; CL⁻, 145.0; HCO₃⁻, 15.0; HPO₄²⁻, 1.9; SO₄²⁻, 2.0; glucose, 110 mg%; d-tubocurarine, 10 µM; and regular crystalline zinc insulin, 50 U/L. Both muscle strips were simultaneously stimulated with supramaximal currents of 200 to 250 mA (*i.e.*, 1.2 to 1.5 times the current required to elicit maximal twitch tension and pulse duration of 0.2 ms) by a constant current stimulus isolation unit (SS-302J; Nihon Kohden, Tokyo, Japan) driven by a stimulator (SEN-3201; Nihon Kohden). The elicited tensions were measured using a force transducer (UL-100GR; Minebea, 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, Aichi, 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 (CSA) of the strip was calculated by dividing the muscle mass by the product of the muscle strip length (Lo) and muscle density (1.06 g/cm³),⁹ or CSA (cm²) = (muscle mass (g)/1.06)/Lo (cm). Tension (N/cm²) was calculated as force (N) per CSA (cm²).

The diaphragm force-frequency relationship was assessed by sequentially stimulating muscles at 1, 10, 20, 30, 50, 70, 100 and 120 Hz. Each stimulus train was applied for approximately 1 s, and adjacent trains were applied at approximately 10-s intervals. The tensions of the muscle strips were recorded by a hot-pen recorder (RECTI-HORIZ-8K; San-ei, Osaka, Japan). The force-frequency curves of the groups are displayed as elicited tensions (N/cm²) versus stimulation frequencies (Hz).

Twitch contraction was elicited by a single pulse stimulation (0.2-ms pulse duration), and the twitch contraction trace was recorded at high speed (10 cm/s). Twitch kinetics were assessed by twitch tension (TT, N/cm²), contraction time (CT, the time required to develop peak tension, ms) and half relaxation time (HRT, the time required for peak tension to decrease by 50%, ms) during a single muscle contraction. For the analysis of contractile velocity of twitch contractions, TT/CT (slope during contraction time) and (TT/2)/HRT (slope during half relaxation time) were

calculated from the curve of the twitch contraction trace.

Muscle fatigue was assessed by examining the rate of tension decrease over a 5-min period of rhythmic contraction, which was induced by applying trains of 20-Hz stimuli (train duration, 0.3 s; rest duration, 0.7 s) at a rate of 60 trains/min. Muscle fatigue was expressed as a percentage of the final tension (%) compared with initial tension. If this value was lower than that at 0 h, we concluded that the muscle was easily fatigued; however, if this value was higher than that at 0 h, we concluded that the muscle was fatigue resistant. Following this procedure, the muscle strip was removed from the organ bath, the tendons and attached fat tissues were removed, and the muscle was weighed.

NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADPH) DIAPHORASE HISTOCHEMISTRY

Twenty-seven BALB/c mice weighing 23.7 ± 0.5 g (Charles River Japan, Tokyo, Japan) were divided into three groups for NADPH diaphorase histochemical analysis. NADPH diaphorase histochemistry of diaphragm muscle was performed at 0, 1, 2, and 4 h in the BUD/FORM inhalation only group (*n* = 3 animals each), and at 0, 2, and 4 h of diaphragm muscle in both the endotoxin injection only group (*n* = 3 animals each) and in the BUD/FORM inhalation plus endotoxin group (*n* = 3 animals each). The diaphragm was quickly excised, and the tissue pieces were frozen in optimal cutting temperature compound (OCT, Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) in a dry ice and acetone bath. Cryosections (10 µm in thickness) were cut from the diaphragm, and immersed in 0.3% Triton X-100 containing phosphate buffer for histochemistry. To test for the presence of NADPH diaphorase, the sections were dipped in freshly prepared 1.0 mM β-NADPH (Oriental Yeast, Tokyo, Japan) and 0.2 mM nitroblue tetrazolium (Wako Pharmaceutical, Osaka, Japan) in 100 mM Tris-HCL buffer pH 8.0, containing 0.2% Triton X-100 for 30 min at 37°C.¹⁰ Reactions were stopped by rinsing the sections with PBS. The sections were covered with a mixture of glycerol and PBS (2 : 1), and photographed using an Olympus microscope with positive color film (Velvia 100, Fujichrome, Tokyo, Japan). Inducible NO synthase (iNOS) requires NADPH as a coenzyme, which is stained in a histochemical reaction for NADPH diaphorase.¹¹

We measured the mean density of the CSA of each muscle fiber using software of image analyzer (NIH Image, National Institute of Health, Bethesda, USA). We counted more than 30 muscle fibers in each photograph. The densities were averaged and expressed in arbitrary units (a.u.).

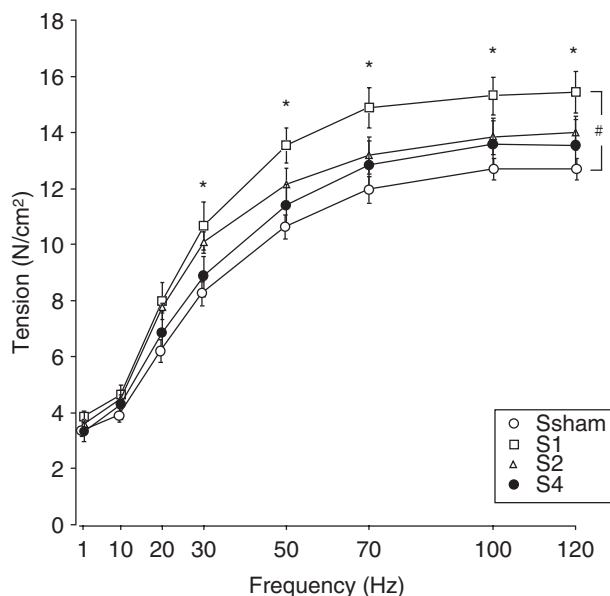


Fig. 1 Changes in force-frequency curves in the BUD/FORM inhalation only groups at Ssham (open circles), S1 (open squares), S2 (open triangles) and S4 (closed circles). * $p < 0.05$ compared with each frequency (Hz) at Ssham; # $p < 0.05$ compared with the force-frequency curve at Ssham.

DATA ANALYSIS

Data were obtained from both halves of the diaphragm in each animal ($n = 5$ animals). Therefore, the number of muscle samples used was $n = 10$ per treatment/time point for force-frequency curves, twitch kinetics and fatigability. Mean values of tensions for each frequency of force-frequency curves, twitch kinetics, fatigability, and mean density of muscle fibers were compared using the Student's *t*-test. To compare the entire configuration of each force-frequency curve at each hour or concentration, analysis of variance (ANOVA) with Fisher's protected least significant difference *post hoc* test were performed. Data are presented as means $\pm$ SEM (standard error of the mean). *P* values < 0.05 were considered to be significant.

RESULTS

CHANGES IN CONTRACTION PROPERTIES IN THE BUD/FORM INHALATION ONLY GROUP

In the BUD/FORM inhalation only group, the force-frequency curve at S1 was significantly shifted upward (15.5 ± 1.5 N/cm² peak) compared with that at Ssham (12.7 ± 0.4 N/cm² peak, $p < 0.05$) (Fig. 1). The force-frequency curve at S2 was shifted downward (14.0 ± 0.6 N/cm² peak) compared with that at S1, and the force-frequency curve at S4 (13.5 ± 0.9 N/cm² peak) was shifted between Ssham and S2. According the changes of the force-frequency curve at S1, significant changes in tension were also observed at 30, 50, 70, 100 and 120 Hz for S1 (each $p < 0.05$) com-

pared with those at Ssham (Fig. 1).

In the BUD/FORM inhalation only groups, there were no significant differences in TT, CT, and HRT of twitch contraction and fatigability at each time point compared with values at Ssham (Fig. 2). No significant differences were observed in TT/CT and (TT/2)/HRT of twitch contraction (Fig. 3).

NADPH diaphorase histochemistry showed that in the BUD/FORM inhalation only group at S2 (92.8 ± 6.3 a.u.), there was a slight but non-significant increase in mean density compared with the level at Ssham (82.7 ± 5.1 a.u.), and the staining disappeared at S4 (83.72 ± 6.6 a.u., Fig. 4). This slight increase in staining at S2 in the BUD/FORM inhalation only group suggested that NO production may have been induced by BUD/FORM. However, this staining did not correspond with the time course changes of diaphragm muscle contractility.

CHANGES IN CONTRACTION PROPERTIES IN THE ENDOTOXIN ONLY AND BUD/FORM INHALATION PLUS ENDOTOXIN GROUPS

For the endotoxin injection only groups, the force-frequency curves were significantly shifted downward at E2 (9.6 ± 0.6 N/cm² peak, $p < 0.001$) and at E4 (6.9 ± 0.7 N/cm² peak, $p < 0.001$) compared with those at Esham (12.3 ± 0.6 N/cm² peak) (Fig. 5A). Significant decreases in tensions were observed at 10, 20, and 30 Hz (each $p < 0.05$) at E1, at 10, 20, 30, 50, 70, 100, and 120 Hz (each $p < 0.05$) at E2, and at 10 ($p < 0.01$), 20 ($p < 0.001$), 30 ($p < 0.01$), 50, 70, 100, and 120 Hz (each $p < 0.001$) at E4 compared with Esham (Fig. 5A). Therefore, low frequency fatigue (at 10, 20, and 30 Hz) occurred at E1, and tensions of high frequency (70 to 120 Hz) at E1 did not exhibit significant changes. These changes indicated that the intraperitoneal administration of endotoxin deteriorated diaphragm muscle contraction in the 4 h follow-up period. However, in the BUD/FORM inhalation plus endotoxin groups, the force-frequency curve was significantly shifted upward at SE4 (14.9 ± 0.6 N/cm² peak, $p < 0.05$) compared with that at SEsham (12.7 ± 0.4 N/cm² peak), and the force-frequency curve at SE4 (14.9 ± 0.6 N/cm² peak, $p < 0.01$) was also significantly shifted upward compared with that at SE1 (11.6 ± 0.7 N/cm² peak) (Fig. 5B). Tensions of force-frequency curves at SE4 showed significant increases at 1 and 10 Hz (both $p < 0.05$) compared with those at SEsham, and they were increased at 20, 30, 50, 70, 100, and 120 Hz ($p < 0.05$) compared with those at SE1.

In the endotoxin injection only groups, TT of twitch contraction was significantly decreased at E4 compared with that at Esham ($p < 0.01$) and at E1 ($p < 0.05$) (Fig. 6A). However, there were no significant changes in CT and HRT of twitch contraction and fatigability (Fig. 6B, C, D). In the BUD/FORM inhalation plus endotoxin groups, TT was significantly in-

BUD/FORM Inhalation and Diaphragm Muscle

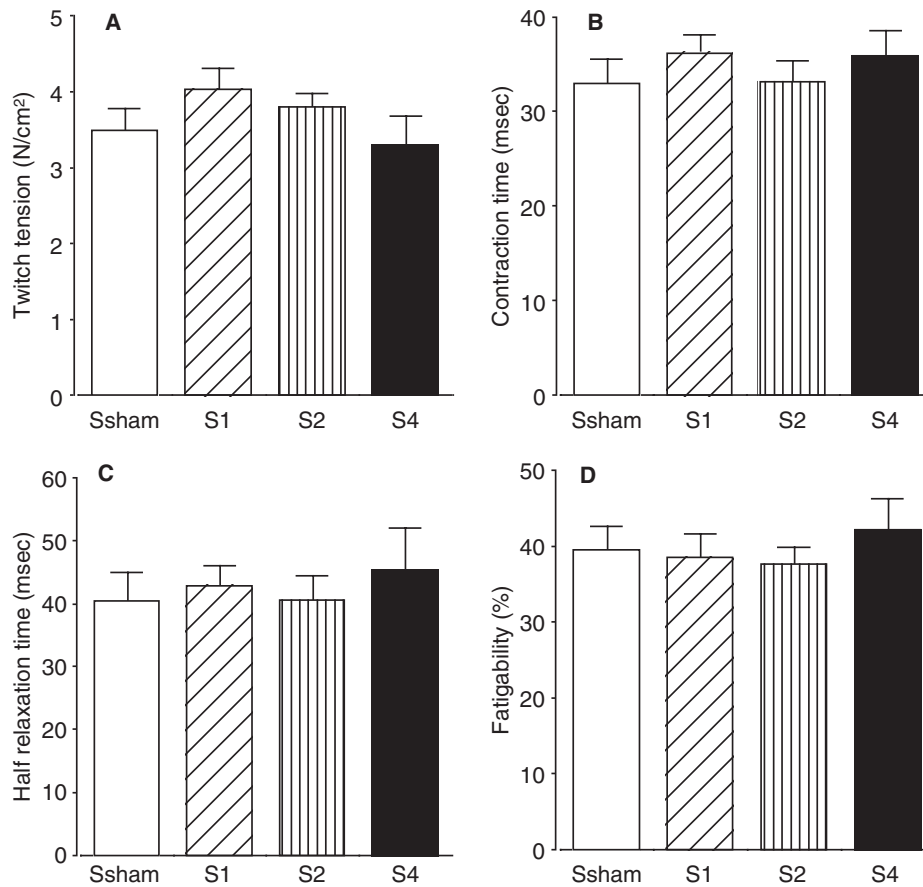


Fig. 2 Changes in twitch kinetics in the BUD/FORM inhalation only groups at Ssham, S1, S2, and S4. There were no significant changes in TT (A), CT (B), HRT (C) or fatigability (D). TT, twitch tension; CT, contraction time; HRT, half relaxation time.

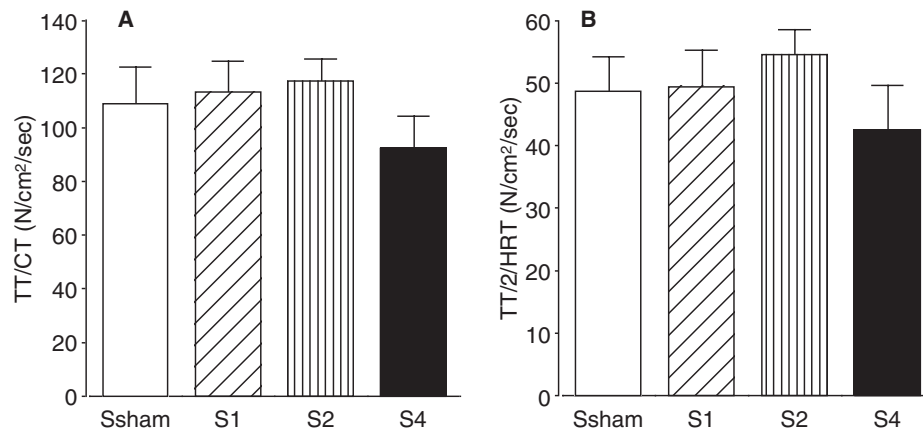


Fig. 3 Changes in twitch kinetics in the BUD/FORM inhalation only groups at Ssham, S1, S2, and S4. There were no significant changes in TT/CT (A) and (TT/2)/HRT (B). TT, twitch tension; CT, contraction time; HRT, half relaxation time.

creased at SE4 ($p < 0.05$) compared with that at SE-sham (Fig. 6A); however, there were no significant changes in CT, HRT and fatigability (Fig. 6B, C, D).

In the endotoxin injection only groups, TT/CT of twitch contraction was significantly decreased at E4 compared with that at Esham ($p < 0.01$) and at E1 ($p <$

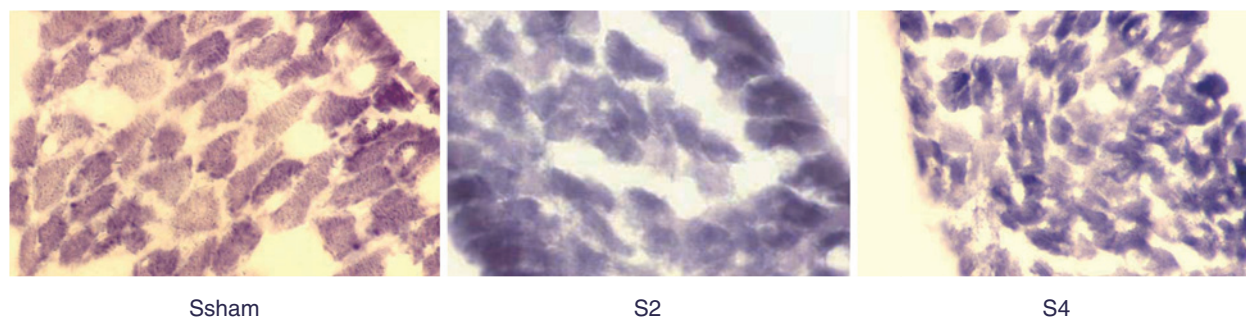


Fig. 4 NADPH diaphorase histochemistry in the BUD/FORM only inhalation groups. Histochemistry shows that those at S2 samples are slightly stained compared with staining at Ssham and then the staining becomes lighter at S4.

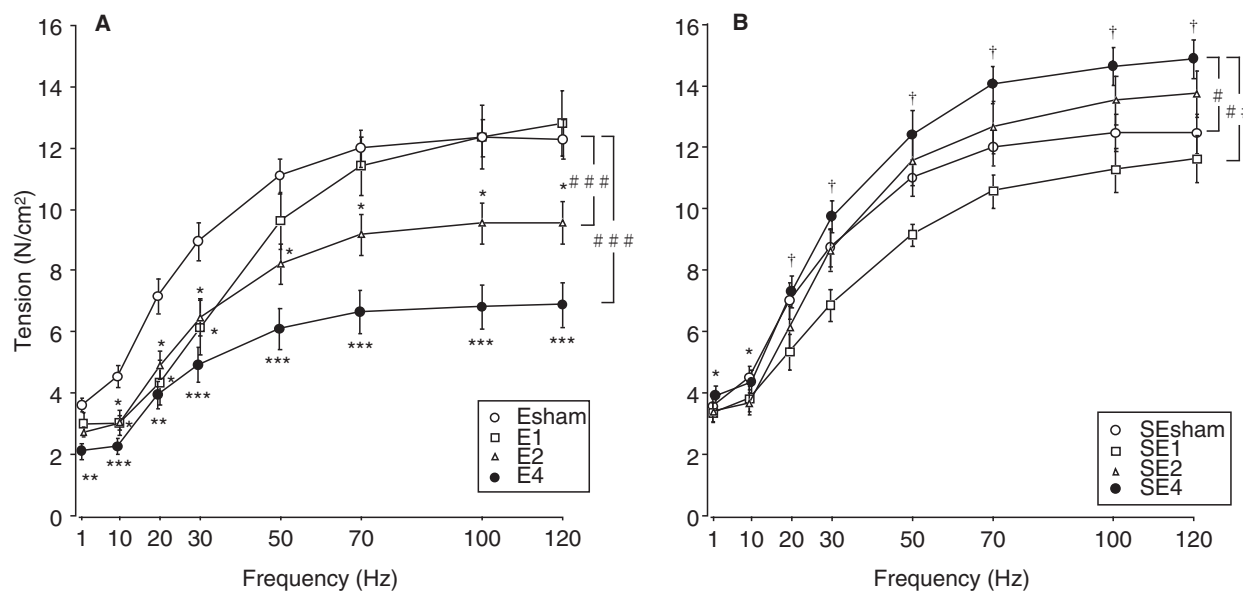


Fig. 5 (A) Changes in force-frequency curves in the endotoxin only injection groups at Esham (open circles), E1 (open squares), E2 (open triangles) and E4 (closed circles). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with each frequency at Esham; ### $p < 0.001$ compared with the force-frequency curve at E4. (B) Changes in force-frequency curves in the BUD/FORM inhalation plus endotoxin injection groups at SEsham (open circles), SE1 (open squares), SE2 (open triangles), and SE4 (closed circles). * $p < 0.05$ compared with each frequency at SEsham; † $p < 0.05$ compared with each frequency at SE1; # $p < 0.05$, ## $p < 0.01$ compared with the force-frequency curve at SEsham.

0.05) (Fig. 7A). $(TT/2)/HRT$ was significantly decreased at E4 compared with that at Esham ($p < 0.01$) and at E1 ($p < 0.001$), and $(TT/2)/HRT$ at E2 was also significantly decreased compared with that at E1 ($p < 0.05$) (Fig. 7B). In the BUD/FORM inhalation plus endotoxin groups, $(TT/2)/HRT$ at SE4 was significantly increased ($p < 0.05$) compared with that at SEsham (Fig. 7B).

NADPH diaphorase histochemistry showed that cross-sectional views of diaphragm muscle gradually exhibited stronger staining from Esham (80.8 ± 4.3 a.u.) and E2 (126.9 ± 5.9 a.u., $p < 0.01$ compared with Esham) to E4 (197.6 ± 2.4 a.u., $p < 0.001$ compared with Esham) in the endotoxin injection only groups.

However, diaphragm muscle was not strongly stained at SEsham (90.5 ± 5.7 a.u.), SE2 (85.6 ± 6.7 a.u.) and SE4 (91.8 ± 5.1 a.u.) in the BUD/FORM inhalation plus endotoxin groups (Fig. 8). This increased staining at E4 indicated that NO production was strongly induced by endotoxin injection. However, NO production was depressed in the BUD/FORM inhalation and endotoxin groups for 4 h (from SEsham to SE4) in muscle fibers of the diaphragm. These changes corresponded well with the time course changes in diaphragm muscle contractility both in the endotoxin injection only and the BUD/FORM inhalation plus endotoxin groups. These data indicate that BUD/FORM protects against endotoxin-induced effects on

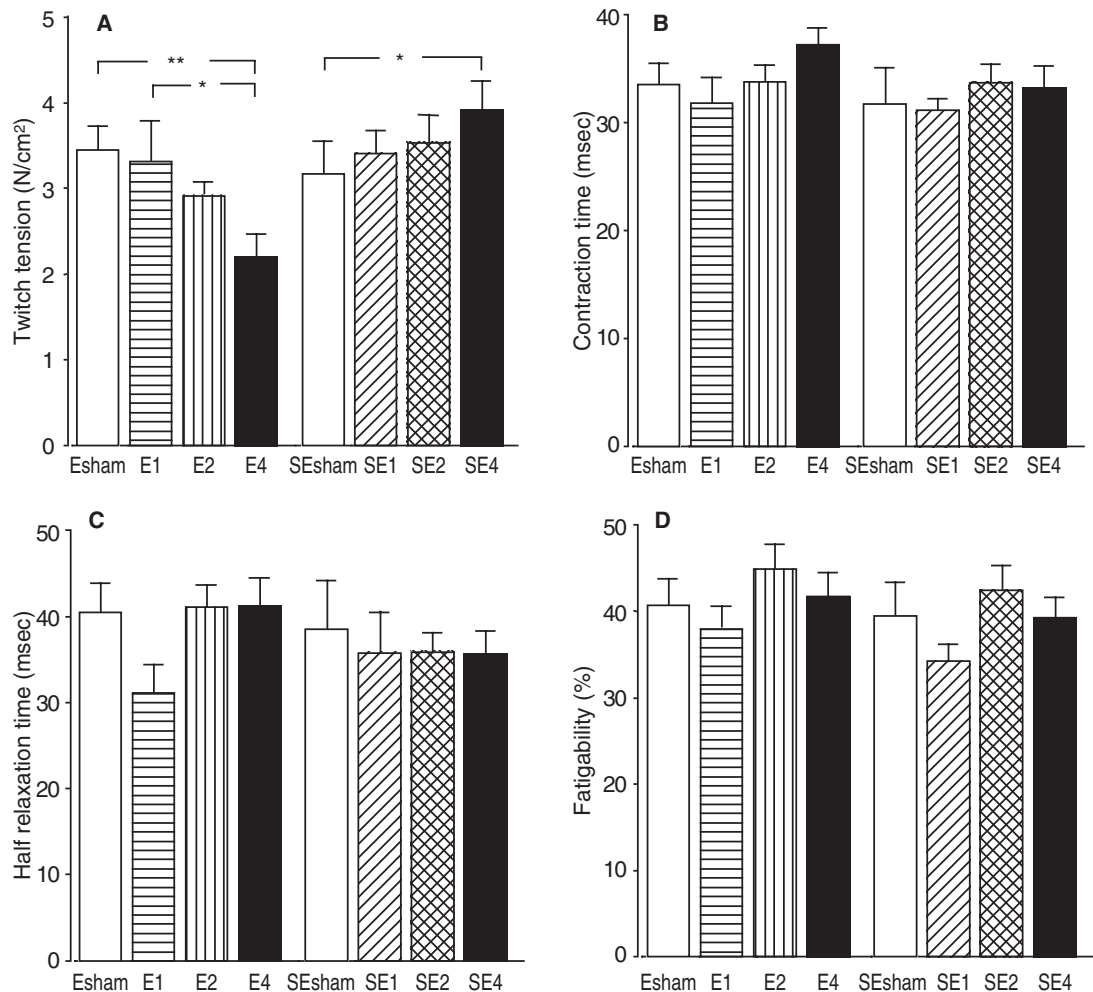


Fig. 6 Changes in twitch kinetics after endotoxin injection at Esham, E1, E2, and E4, and in the BUD/FORM inhalation plus endotoxin groups at SEsham, SE1, SE2, and SE4 as measured by TT (A), CT (B), HRT (C), and fatigability (D). * $p < 0.05$, ** $p < 0.01$ compared between two time points. TT, twitch tension; CT, contraction time; HRT, half relaxation time.

diaphragm muscle contractility.

DISCUSSION

In the present study, the force-frequency curves of the BUD/FORM inhalation only groups were significantly shifted upward at 1 h after inhalation, and then returned to a value similar to Ssham in normal diaphragm muscle. Second, in the endotoxin injection only groups, the force-frequency curves were significantly shifted downward at E4 compared with that at Esham; however, in the BUD/FORM inhalation plus endotoxin injection groups, the force-frequency curves were significantly shifted upward at SE4, indicating that BUD/FORM inhalation can inhibit the decrease in force-frequency curves induced by endotoxin. Third, NO production induced by endotoxin at E4 was inhibited by BUD/FORM inhalation plus endotoxin at SE4 according to NADPH diaphorase histochemistry. Therefore, BUD/FORM inhalation has

an inotropic effect on normal diaphragm muscle at 1 h and protects against both a decrease in force-frequency curves and NO production of diaphragm muscle induced by endotoxin.

There are some concerns regarding the dose of BUD/FORM and anesthesia in this study. As mentioned above in the Materials and Methods section, the dose of BUD/FORM (36 μg /1 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,⁸ and BUD/FORM was approximately one-fourth that of a dry powder inhaler (BUD/FORM [160 μg /4.5 μg for one puff]). We consider that this inhalation study should be similar to clinical use.

It has been reported that when 1000 μg of BUD was inhaled in humans, blood concentrations of BUD reached 4.8 nmol/L (2.1 ng/ml) at 12.6 minutes after inhalation.¹² Therefore, the observed changes in

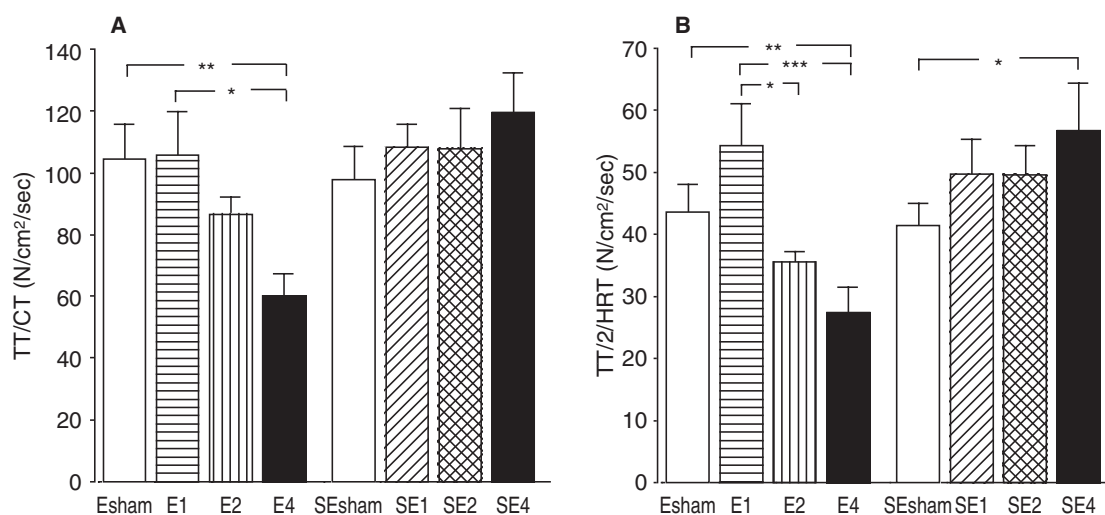


Fig. 7 Changes in twitch kinetics in the endotoxin injection groups at Esham, E1, E2, and E4, and in the BUD/FORM inhalation plus endotoxin groups at SEsham, SE1, SE2, and SE4 as measured by TT/CT (A), and (TT/2)/HRT (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared between two time points. TT, twitch tension; CT, contraction time; HRT, half relaxation time.

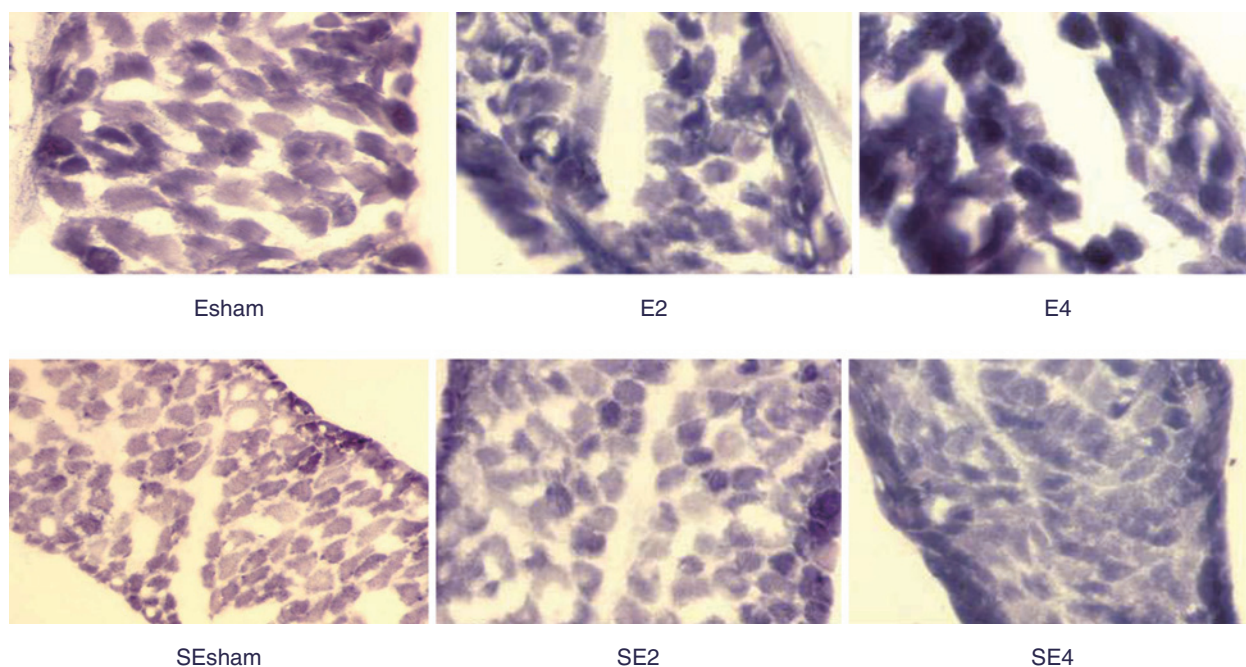


Fig. 8 NADPH diaphorase histochemistry of the endotoxin injection only groups at Esham, E2, and E4, and of the BUD/FORM inhalation plus endotoxin group at SEsham, SE2, and SE4. Histochemistry shows that cross-sectional views of diaphragm muscle are gradually more strongly stained from Esham and E2 to E4 in the endotoxin injection only groups. However, samples were not strongly stained from SEsham to SE4 in the BUD/FORM inhalation plus endotoxin groups.

muscle contractile properties in our study are thought to have been elicited by blood circulation of BUD/FORM after inhalation, which then reached diaphragm muscle.

Anesthesia of diethyl ether is thought to have irritative, secretory and contractive effects on the airways, and it might induce hypoxemia. In the current study,

we anesthetized mice in a jar in a short time of approximately 30 to 40 seconds while maintaining spontaneous breathing, and anesthesia was given in the same manner in all animals. Therefore, we suggested that the data obtained in our study could reflect the effects of BUD/FORM inhalation.

Pauwels *et al.* have published a pivotal report show-

ing that a combination of budesonide and formoterol single inhalation in patients with persistent symptoms of asthma despite treatment with inhaled glucocorticoids improves symptoms and lung function without lowering asthma control.¹ It has also been shown that the addition of an inhaled LABA to an ICS may potentiate the molecular mechanism of corticosteroid action, with increased nuclear localization of glucocorticoid receptors and additive or sometimes synergistic suppression of inflammatory mediator release.¹³ Furthermore, clinical trials have recently shown that using a BUD/FORM combination inhaler for regular maintenance treatment twice daily and also for rescue therapy for breakthrough symptoms can provide more effective asthma control.¹⁴ As a rescue therapy, formoterol is effective in relieving symptoms by relaxing airway smooth muscle, but it is also likely to have important inhibitory effects on mast cells, plasma exudation and neutrophilic inflammation. Therefore, it is likely that molecular interactions between β_2 -agonists and corticosteroids also enhance the effect of the combination therapy as rescue therapy.

Our results suggest that BUD/FORM inhalation has an additive or a synergistic effect, such as an inotropic effect on muscle contraction and an anti-inflammatory effect on diaphragm muscle. We have reported that β_2 -agonists, such as procaterol¹⁵ and tulobuterol,¹⁶ shift force-frequency curves upward; this inotropic effect may be caused by formoterol, which is a β_2 -agonist. This inotropic effect by β_2 -agonists is induced by β_2 receptors in cell membranes and increases cAMP.¹⁷ Additionally, an inhibitory effect on NO production appears to be induced by budesonide, which is transported into the cytoplasm, connects with a steroid receptor, and is then transported to the nucleus where it binds to DNA, which inhibits transcription of iNOS and inflammatory cytokines. Therefore, the combination of BUD/FORM inhalation protects diaphragm muscle tissue and maintains contractile properties.

Inhibition of NO production by BUD/FORM inhalation may indicate anti-inflammatory effects induced by endotoxin injection in the diaphragm muscle tissues. *In vitro* steroids down-regulate iNOS expression, thus suggesting a potential to down-regulate NO-mediated inflammation in neonates with meconium aspiration syndrome.¹⁸ In addition, allergen-induced sputum eosinophilia is significantly reduced by combination treatment to a greater extent than by budesonide alone. The effects on allergen-induced changes in sputum eosinophils, airway myofibroblast numbers, and smooth muscle produced by combination therapy suggest that the beneficial effects on airway inflammation responses and airway hyperresponsiveness by combination treatment are likely due to the known functional antagonistic effect of formoterol.¹⁹ Furthermore, budesonide and formoterol combination therapy synergistically controls serum-

induced proteoglycan production, primarily at the post-transcriptional level, and proteoglycan upregulation characteristic of asthmatic airways may be limited by combination therapy with budesonide and formoterol.²⁰

Furthermore, our results suggested that BUD/FORM inhalation plays a role in reducing asthma exacerbation due to excessive breathing by preventing diaphragm muscle deterioration. Rabe *et al.*²¹ found that both monocomponents of budesonide-formoterol given as needed contribute to enhanced protection from severe exacerbations in patients receiving combination therapy for maintenance. Although symptomatic control and rescue bronchodilator use may be improved by the addition of a LABA to ICS, there may be a lower risk of severe exacerbation and hospitalization from an ICS dose increase.²² However, exacerbation and loss of asthma control in patients using combination therapy strongly suggest the need to increase the ICS dose rather than relying on a long-acting bronchodilator.²³ Additionally, a previous study found that there were very few asthma-related deaths and intubations, and events were too infrequent to establish the relative effect of LABA on these outcomes.²⁴ Therefore, for many patients, the addition of a LABA to ICS has been remarkably effective in improving symptom control, lung function, and quality of life.

It has been reported that endotoxin administration induces systemic effects. TNF- α plasma concentrations are increased at 2 h after intravenous bolus injection of *E. coli* endotoxin (0.3 ng/kg) in humans, and endotoxemia upregulates iNOS in the vasculature.²⁵ Our results showed that BUD/FORM inhalation improved diaphragm muscle contractility and depressed NO production 4 h after endotoxin administration in the BUD/FORM inhalation plus endotoxin groups. Superoxide (O_2^-) and NO are known to rapidly react to form a stable peroxynitrite anion, and superoxide dismutase may protect vascular tissue stimulated to produce superoxide and NO under pathological conditions by preventing the formation of peroxynitrite.²⁶ We have previously reported that N-acetylcysteine²⁷ and PEG-superoxide dismutase²⁸ have an anti-oxidant effect, preventing diaphragm muscle deterioration induced by endotoxin. Furthermore, because TNF- α mRNA is expressed in diaphragm muscle cells after endotoxin administration,⁶ we also speculate that oxidative stress factors such as TNF- α and NO may contribute to endotoxin-induced diaphragm muscle contractile deterioration. Therefore, the results of our experiments support the possibility that BUD/FORM inhalation inhibits oxidative stress and induction of inflammatory cytokines such as NO production and TNF- α . Recently, a national survey of endotoxins in United States housing reported that household endotoxin exposure is a significant risk factor for increased asthma prevalence.²⁹

The most common risk factor in the development of asthma is induction of IgE against indoor allergens from pets containing endotoxin, which causes an imbalance in T-helper type 1 and T-helper type 2 (Th2) with skewing towards a Th2 response.³⁰ Therefore, endotoxin not only protects against the development of asthma but also enhances an already established inflammation. It may be important that BUD/FORM inhalation prevents airway inflammation and diaphragm deterioration when patients with bronchial asthma have increased pulmonary and systemic oxidative stress both at rest and under conditions of exacerbation.

Before the availability of ICSs, patients with severe asthma attacks were frequently treated with oral steroids, and several studies have shown adverse effects on muscular tissues. Afifi *et al.* reported a case of a 45-year-old housewife with a chronic asthmatic condition that had been treated with bronchodilators and a steroid (methylprednisolone 12 mg/day), causing her to experience muscular weakness in her legs, in which massive aggregates of glycogen in subsarcolemmal and intermyofibrillar sites and disarray and loss of myofibrils were observed.³¹ In a review of short-term treatment with high doses of steroids, generalized muscle, including respiratory muscles, was found to be accompanied by reduced respiratory muscle force.³² An ATPase stain for muscle fiber typing showed that diffuse fiber atrophy predominantly affecting fast fibers was present, although there was no indication that atrophy was confined to type IIb (fast-twitch) fibers.³³ Steroid administration (triamcinolone acetonide 1.2 mg/kg per day) of 8 days produced diaphragmatic atrophy as severe as that of the fast-twitch hindlimb muscle in an animal experiment.³⁴ In a small animal such as the rat, the percentages of type I (slow-twitch) and type II are approximately 29% and 71%, respectively,³⁵ and in humans, these percentages are approximately 36% and 64%, respectively.³⁶ Our study was exerted only in the initial 4 hours after BUD/FORM inhalation, and therefore, the pathological changes mentioned above could be avoided. Although there are some differences in the composition of muscle fiber typing within mice and humans, our results might be applicable to human diaphragm contractile function as suggested by our findings of BUD/FORM inhalation.

Furthermore, it has been reported that steroids, at doses typically administered in chronic severe asthma (from 9 to 24 mg/day), do not cause muscular weakness, and that malnutrition rather than corticosteroids is the most important contributory factor to type II muscle fiber atrophy in steroid-dependent asthma patients.³⁷ Knowledge of the pharmacokinetic properties of ICSs is important for the achievement of high levels of airway selectivity. There should also be an additional focus on the use of prodrugs/softdrugs relative to those of conventional corticosteroid mole-

cules, and determining mechanisms (such as esterification) by which retention at the target site is achieved while minimizing systemic exposure and the role of plasma protein binding.³⁸ If the above-mentioned effects on diaphragm muscle cannot be avoided, they might be decreased by a small dosage of inhalation (budesonide) and combination with a β_2 -agonist (formoterol). β_2 -agonists have a direct inotropic activity on diaphragm muscle contractility by inhalation.^{15,16} Roth *et al.* have reported that the concentration of inhaled glucocorticoids can be reduced when combined with β_2 agonists, minimizing the adverse effects of these drugs.³ Therefore, on the basis of our results, we conclude that the combination of budesonide and formoterol is expected to improve diaphragm muscle contractile weakness caused by steroids.

In conclusion, BUD/FORM inhalation has an inotropic effect on diaphragm muscle, protects diaphragm muscle deterioration after endotoxin injection, and inhibits NO production. Although our study performed endotoxin injection in an animal model, it has been reported that inhaled endotoxin enhances the response to allergen challenge in patients with asthma, i.e., it enhances inflammatory responses.³⁹ Therefore, BUD/FORM inhalation may improve diaphragm muscle contractile properties in patients with bronchial asthma by its inhibition of NO production, and contribute to reduction of exacerbation in patients with bronchial asthma.

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