

Fasudil reduces airway smooth muscle contractility and lung resistance in ex vivo sheep lungs

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Abstract

Objectives

The present study aims to further our understanding of the mechanism of action of fasudil on airway smooth muscle (ASM) and lung function in a large animal (sheep) lung model.

Methods

Muscle organ bath and whole lung plethysmography were employed in the present study. With respect to lung function, we specifically examined the effectiveness of nebulized fasudil in reaching the resistance airways to reduce lung resistance. **Main results**

We found that fasudil significantly reduced ASM active force in a dose-dependent manner. The amount of active force reduction was accompanied by a comparable decrease in the level of activation-induced phosphorylation of the 20 kDa myosin light chain (MLC20), suggesting that fasudil deactivates ASM mainly through myosin regulation. Inhalation pretreatment of fasudil also effectively prevented the increase in spontaneous bronchoconstriction that occurs at low tidal volumes without deep inspirations in isolated sheep lungs.

Conclusion

Fasudil effectively reduced ASM contractility and airway resistance and elastance in the whole lung. The results provide support for the use of fasudil in the treatment of human asthma in clinical trials and enhance the feasibility for repurposing fasudil for asthma treatment.

Introduction

Rho-kinase (ROCK) inhibitors have been shown to be potent smooth muscle relaxants. However, there is currently no asthma medication based on the inhibition of ROCK, in spite of the evidence for the involvement of ROCK in airway inflammation, remodeling, and hyperresponsiveness. ROCK inhibition has been shown to reverse histamine- and PGF₂α-induced airway hyperresponsiveness (1), reduce airway resistance and suppress markers of inflammation and remodeling (2) in ovalbumin-sensitized guinea pigs. In a mouse model of asthma, ROCK inhibitors have been shown to reduce airway hyperresponsiveness and resistance (3–5), as well as to suppress pulmonary eosinophilia, cytokines release (6–8), and airway inflammation (9, 10). Results from the above cited studies are all based on small animal models. There is a need for large animal models, especially in the assessment of the effect of nebulized ROCK inhibitors on the mechanics of the lung that is comparable in size to the human lung.

Fasudil, a ROCK inhibitor, has been used clinically for the treatment of cerebral vasospasm in Japan and China for decades. Safety of the drug is well known. Many clinical trials, some of which are ongoing, were designed to assess the feasibility of using ROCK inhibitors for treating other diseases, especially those related to smooth muscle dysfunction. Double-blind, placebo controlled clinical trials have

demonstrated effectiveness of ROCK inhibitors in treating pulmonary hypertension (11, 12). Positive results have been obtained in clinical trials using ROCK inhibitors in the treatment of glaucoma and ocular hypertension (13–18). Other clinical trials have also demonstrated the effectiveness of ROCK inhibitors in the treatment of vascular smooth muscle related diseases such as coronary artery disease and heart failure, as well as acute ischemic stroke (19–22). Although smooth muscle dysfunction is likely involved in asthma pathophysiology, clinical trials using ROCK inhibitors for treating asthma are still to be carried out.

One goal of the present study is to examine the mechanism of fasudil in relaxing airway smooth muscle (ASM) through inhibition of the 20 kDa myosin light chain (MLC20) phosphorylation. It is known that ROCK enhances smooth muscle contractility through enhancement of MLC20 phosphorylation and other signaling pathways such as actin formation and stabilization (23). However, in this specific case of contractility down-regulation by fasudil, it is not known how much of the active-force reduction in ASM can be accounted for by MLC20 dephosphorylation. Another goal of the present study is to assess the feasibility of delivering nebulized fasudil into the lungs to achieve bronchoprotection in an ex vivo sheep lung. Results from the present study will facilitate the push for clinical trials of repurposing fasudil in the treatment of asthma.

Methods

Tissue source and ethics

Sheep tracheas and lungs were obtained from a local abattoir and transported to our laboratory in modified Krebs solution cooled to 4°C. Isolated whole lungs were obtained from approximately 8-month-old sheep; sex information was not available. Use of sheep lungs and tracheal tissues for this project was approved by the Ethics Committee for Animal Care and the Biosafety Committee of the University of British Columbia and conformed to the guidelines set out by the Canadian Council on Animal Care.

Solutions and reagents

Modified Krebs solution contained: 118 mM NaCl, 4 mM KCl, 1.2 mM NaH₂PO₄, 22.5 mM NaHCO₃, 2mM MgSO₄, 2mM CaCl₂, and 2 g/L dextrose. Acetylcholine chloride (MilliporeSigma Canada Ltd, Oakville, ON) was used to induce stable constriction. Fasudil (LC Laboratories, Woburn, MA) was dissolved in Krebs solution for muscle strip experiments, and nebulized for whole-lung experiments.

Sheep tracheal muscle strip preparation

Tracheal tissues were stored in Krebs solution at 4°C and used within 4 days. The in situ length of the trachealis muscle was measured in tracheal rings before cutting open the C-shaped cartilage. The opened trachea was pinned at both ends on a dissection plate while maintaining muscle length at its in

situ length. Smooth muscle strips (5 mm to 10 mm long, 1–2 mm wide, and 0.2 mm thick) were dissected by removing epithelium and connective tissue and attached to a force/length transducer (Duo Mode, Model 300C, Aurora Scientific Inc.) using aluminum foil clips affixed to both ends of each strip. Strips were mounted at their in situ length in organ baths at 37°C containing Krebs solution aerated with carbogen (95% O₂–5%CO₂) and equilibrated for at least 30 min. A simplified scheme for the procedure is illustrated in Fig. 1A.

Fasudil-induced relaxation and dose–response relationship

To verify muscle viability, preparations were periodically activated isometrically by electric field stimulation (EFS) (every 5 min for 10 s; 60 Hz; current density adjusted to elicit maximal muscle response) for five activations. If the final active stress was less than 100 kPa, the tissue was discarded. Strips were exposed to acetylcholine chloride (ACh, 30 µM) for 15 min to induce a stable contraction. Fasudil was then added cumulatively from 10 nM to 100 µM in half-log increments. At each concentration, fasudil was added after the isometric force had reached a plateau (defined as a change of less than 1 mN over 3 min) and force was recorded to construct a dose–response relationship. See Fig. 1A for an illustration of the methods.

Measurement of MLC20 phosphorylation

For each set of experiment, four muscle strips from the same trachea were frozen under different experimental conditions. The conditions under which tissue strips were frozen were as follows: 1) Rest, representing baseline phosphorylation; 2) ACh, corresponding to peak contraction induced by 30 µM acetylcholine; 3) ACh-Fasudil, in which strips were frozen at the plateau phase following addition of 100 µM fasudil to the ACh-pre-contracted strip, and 4) ACh-TC (time control), in which strips were frozen at the same time point as the fasudil-treated group but without fasudil exposure. The corresponding conditions are labeled (1 to 4) on the illustrated force trace in Fig. 1B. Force was continuously recorded under each condition. This set of experiment was repeated 8 times using different tracheas (n = 8).

Trachealis strips were snap-frozen in prechilled acetone at dry ice temperature (– 80°C), while still attached to the transducer. The muscle strips were then quickly removed from the apparatus and placed in prechilled acetone containing 5% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) and stored at -80°C. Total protein was extracted from these muscle strips within 2 weeks of storage. Prior to extraction, the strips were brought to room temperature. TCA was removed from the strips by two washes (30 minute each) of freshly prepared acetone at room temperature containing 10 mM DTT. The muscle strips were then submerged in freshly prepared extraction buffer (150 µl/mg wet tissue weight). The extraction buffer contained 6.4 M urea, 5 mM DTT, 10 mM EGTA, 1 mM EDTA, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 26.4 mM tris base, and 29.3 mM glycine. The muscle tissue in extraction buffer was sonicated and then placed on a rotator at 4°C. After continuously inverting for 2 hours, the tubes containing tissue extract were centrifuged at 20000 g for 30 minutes at 4°C. BioRad QuickStart

Bradford protein assay was performed on the supernatant to determine the concentration of total protein extracted from each muscle sample. A portion of the supernatant was further diluted to 0.1 mg total protein/ml with additional extraction buffer containing 0.02% Bromophenol Blue and stored at -80°C until native gel electrophoresis and Western analysis.

Phosphorylated and non-phosphorylated MLC20 were separated on 10% acrylamide and 40% glycerol mini-gel with 3% acrylamide-urea stacking gel. Muscle samples were thawed and loaded into lanes at 2 µg total protein per lane. Electrophoresis was performed at 200 V for 2.5 hours at room temperature in 100 mM glycine, 50 mM Tris, and β-mercaptoethanol (1 ml/L buffer). An additional 0.5 ml of β-mercaptoethanol was added to the inner chamber. At the end of electrophoresis, proteins were transferred to 0.2 µm nitrocellulose membrane at 25 V at 4°C overnight. Non-specific binding sites were blocked with tris-base saline (TBS)-5% bovine serum albumin (BSA) for 1 hour at room temperature. Western immunoblots were developed using a monoclonal mouse primary antibody to smooth muscle MLC20 (clone MY-21, MilliporeSigma Canada Ltd, Oakville ON) (1:5,000) followed by a goat anti-mouse IRDye 800 conjugated secondary antibody (LI-COR, Lincoln, NE, USA) (1:10,000) in TBST (Tween)-5% BSA. The membranes were washed 4 times with TBST for 5 minutes between primary and secondary antibodies and after secondary antibody. The membrane was then scanned on a LI-COR Odyssey CLx Infrared Imaging System (Lincoln, NE, USA). The integrated intensity of each band was obtained using the software LI-COR Image Studio Lite Ver 5.2 with subtraction of background intensity. The fraction of MLC20 phosphorylation in each sample was calculated by determining the ratio of the integrated intensity of the lower band (containing phosphorylated MLC20) to the sum of the lower and upper (containing unphosphorylated MLC20) bands. MLC20 phosphorylation due to activation was calculated by subtracting the value of fractional phosphorylation measured in an Acetylcholine-activated strip by the resting MLC20 phosphorylation measured in a resting strip from the same trachea.

Bronchoprotective experiments using trachealis strips

To examine the bronchoprotective effect of fasudil, trachealis strips were exposed to fasudil (100 µM) for 5 min, followed by EFS activations every 5 min for 10 s, this was repeated 12 times (1 h). Active forces were recorded throughout. A greater reduction in active force in the presence of fasudil relative to control would be taken as a bronchoprotective effect of fasudil. Figure 1C illustrates the protocol.

Ex vivo sheep lung preparation

Freshly excised lungs were placed in modified Krebs solution at 4°C and used for experiments on the same day. Before placement in the ventilation chamber of the plethysmograph, lungs were fully inflated by positive pressure (approximately 40 cmH₂O) to resolve visible surface atelectasis. Small leaks (typically due to knife cuts during tissue harvest) were repaired using tissue glue (sometimes with parafilm); lungs with large leaks were discarded.

Plethysmograph and ventilation

A custom-built plethysmograph was used (24). Temperature inside the plethysmograph was maintained at 36°C to 39°C, and humidity was maintained at 100% during experiment. The trachea was connected to a tube that went through the airtight ventilation chamber of the plethysmograph such that one end of the tracheal opening was exposed to atmospheric pressure while the rest of the lung sat inside the chamber exposed to experimentally controlled transpulmonary pressure. With the dorsal side up, the lung was inflated by negative pressure inside the plethysmograph. Airflow was measured by a pneumotachograph connected to the trachea; pneumotachograph calibration has been described previously (24).

Calculation of lung resistance and elastance

Lung resistance and elastance at each tidal oscillation frequency were calculated using a linear single compartment model as described by Bates (25). Transpulmonary pressure (P_L) was modeled as

$$P_L = E_L V + R_L \dot{V} + P_0$$

where E_L is the lung elastance, R_L is the lung resistance, V is the tidal volume (obtained by integrating airflow through the trachea), $\dot{V}$ is the rate of airflow through the trachea, and P_0 is an offset pressure that sets transpulmonary pressure to zero when the flow is zero. Additional details have been described previously (26).

Experimental procedures for ex vivo sheep lungs

To facilitate delivery of fasudil or vehicle (Krebs solution) into the airways, lungs were ventilated for 30 min at 7.5 cmH₂O P_L and 0.25 Hz before lung function measurements. During this preparatory phase, nebulized Krebs solution containing fasudil (10 mM) or Krebs solution alone (control) was inhaled into tidally ventilated lungs. To enhance deposition, deep inspirations (DIs) were administered once every 5 min during the preparatory phase.

After the preparatory phase, lungs were fully inflated to a transpulmonary pressure of approximately 40 cmH₂O to resolve surface atelectasis. Lungs were then ventilated at low tidal volume (120 cm³) at 7.5 cmH₂O P_L and at 0.25 Hz without intermittent DIs for 30 min. At 10 min intervals, lung resistance and elastance were measured at 0.25 Hz and 2 Hz of ventilation frequency.

Statistical analysis

Data were analyzed using one-way ANOVA, one-way repeated-measures ANOVA with Tukey's HSD test, and two-way repeated-measures ANOVA with Holm-Sidak multiple comparison tests (as appropriate). Data are presented as means $\pm$ SE unless otherwise indicated. IC_{50} values were determined by fitting concentration-response curves to a four-parameter log-logistic model using the 'drc' package in R (version 4.5.0). The model was fitted to the mean response at each concentration to estimate the half-

maximal inhibitory concentration (IC_{50}). Statistical significance was set at $p < 0.05$. Analyses were performed using SigmaPlot (version 14.0).

Results

Inhibition of ASM active stress by fasudil

Figure 2A shows that active stress (kPa) produced by 30 μ M acetylcholine measured immediately before drug administration was similar between groups (mean $\pm$ SEM): 172.5 ± 17.8 (control) vs. 159.8 ± 22.0 (fasudil). The difference is not statistically significant.

Figure 2B shows the inhibition effect on active stress of ASM by fasudil in a dose-dependent manner. The IC_{50} for fasudil was 6.5 ± 0.5 μ M. Trachealis strips for the control and test were dissected from the same tracheal segment. The time-control is necessary because the ACh-induced active force produced by the muscle strips gradually decreases over time.

Effect of fasudil on ACh-induced contraction and on MLC20 phosphorylation

Figure 3A shows resting stress (Rest), ACh-induced stress (ACh), time control reduction for ACh-induced stress (ACh-TC), and ACh-induced stress in the presence of fasudil (ACh-Fasudil). The incubation time required to reach the force plateau after fasudil administration (100 μ M) varied from one pair of tracheal strips to another, the average being 994.6 ± 100.4 seconds. However, for each pair (ACh-TC vs. ACh-Fasudil), the incubation time used was the same. That is, the time at which the active force was measured (and the strip flash frozen for MLC20 phosphorylation assessment) was the same for ACh-Fasudil and ACh-TC for any particular pair of strips. The active force (mean $\pm$ SEM) under each condition was (in kPa): Rest: 3.2 ± 0.8 ; ACh: 283.5 ± 23.3 ; ACh-TC: 241.0 ± 26.8 ; and ACh-Fasudil: 85.5 ± 16.2 . Therefore, the average active stress of time control at the end of the incubation period was $\sim 15\%$ less than the initial maximal stress. In the presence of fasudil, the decrease was $\sim 70\%$ from maximal stress, and $\sim 65\%$ from its time control (Fig. 3A).

Figure 3B shows a typical image obtained from Western blotting. There are two bands (top and bottom) for each condition. The top bands indicate un-phosphorylated and bottom the phosphorylated MLC 20.

Figure 3C shows the quantification of the bands shown in Fig. 3B. As expected, at plateau of response to ACh, the MLC20 phosphorylation was significantly increased compared to resting condition ($p < 0.001$). In the presence of fasudil, MLC20 phosphorylation was significantly reduced comparing to the time control ($p = 0.027$) and that at plateau of ACh ($p = 0.001$). In fact, fasudil reduced the MLC20 phosphorylation to the resting level ($p = 0.154$). Time control and maximal plateau force were not statistically different ($p = 0.274$).

Figure 3D shows the values of MLC20 phosphorylation measured in 3 activated groups after the resting MLC20 phosphorylation was subtracted. This allows visualization of MLC20 phosphorylation due to activation alone. We found all comparisons are statistically significant. The most meaningful comparison is between time control and fasudil treated after their resting values were subtracted. The mean value of MLC20 phosphorylation for time control was 17% and the mean value for fasudil treated was 7%. The reduction is 59%. At moment of snap freezing, the mean value of active stress in fasudil treated strips was 85.4 kPa, comparing to time control (232.1 kPa), a 63% reduction in active stress, which is comparable to the reduction in MLC20 phosphorylation (59%) from its time control (Fig. 3D).

The bronchoprotective effect of fasudil simulated in trachealis strips

Figure 4A shows the effect of pre-incubation of fasudil (100 μ M) on active force generation by electrical field stimulations performed every five minutes in tracheal strips over a one-hour period (solid circles). The untreated control (open circles) showed no significant change over the same time period. The asterisks indicate statistically significant difference. Figure 4B shows the resting tension in the control and test groups. The resting tension was steadily low and no significant difference was found in the two groups (2.4 ± 0.8 vs. 2.2 ± 0.6 in mN). Figure 4C is a re-plot of Fig. 4A, except that the active force generated by a particular tracheal strip in the fasudil-treated group was expressed as a percentage of its paired control. The paired strips were adjacent strips dissected from the same tracheal segment. The large standard errors seen in Fig. 4A compared with those in Fig. 4C reflect variation in individual tracheas. Once the difference in individual animals is eliminated by paired normalization, the bronchoprotective effect of fasudil becomes more apparent.

Bronchoprotective effect of fasudil in ex vivo lungs

Figure 5A illustrates the protocol for testing the bronchoprotective effect of fasudil. During the 30-min preparatory phase of the protocol, an ex vivo lung was tidally ventilated at FRC at 0.25 Hz with nebulized Krebs solution (control) or fasudil (10 mM). Every 5 min a deep inspiration was applied to facilitate uptake of the drug deep into the lung. It should be pointed out that although 10 mM of fasudil was inhaled by the lung, the actual concentration of the drug “seen” by the bronchial smooth muscle is not known. Therefore, direct comparison of the ASM response in muscle bath to fasudil and the response of ex vivo lungs to nebulized fasudil should take this unknown into account. During the following 30-min recording period, the lung was not ventilated and the lung resistance and elastance were measured at 4 time-points (indicated by the vertical down arrows). This protocol took advantage of the fact that, in an unventilated ex vivo sheep lung, bronchoconstriction occurs spontaneously and gradually over time.

Figure 5B shows that in the absence of fasudil (control, open circles) lung resistance and elastance increased significantly over time. However, in the presence of fasudil (solid circles), no significant increase was detected. At a ventilation frequency of 0.25 Hz, lung resistance and elastance stem from

both tissue and airway sources. At 2 Hz, lung resistance and elastance reflect mostly the airway properties.

Discussion

Fasudil, a ROCK inhibitor, has a relaxant effect on airway smooth muscle. This makes it a potential drug for the treatment of asthma. We have shown that, in airways and lung sections from human asthmatics and age-matched controls, the protein expression of both isoforms of ROCK, ROCK1 and ROCK2, are elevated in the bronchial smooth muscle and in the pulmonary arterial smooth muscle from human asthmatic lungs (27). Over-active ROCK signaling, therefore, may be a consistent feature of asthma pathophysiology, and the use of ROCK inhibitor may be the correct strategy in our search for new drugs in the treatment of bronchial hyperresponsiveness associated with asthma.

Results from the present study show that fasudil is able to relax pre-contracted ASM in a dose-dependent fashion (Fig. 2). The reduction in active force has also been shown (Fig. 3) to be mainly due to inhibition of MLC20 phosphorylation by the ROCK inhibitor. Potentiation of MLC20 phosphorylation is only part of the downstream effects of ROCK signaling. Other effects of ROCK include actin polymerization and stabilization of cytoskeletal filaments (23). One would, therefore, expect inhibitors of ROCK to reduce active force in ASM by at least two ways: reduction in MLC20 phosphorylation and disruption of the cytoskeletal filaments. Because MLC20 phosphorylation is directly proportional to active force in ASM (28), our results (Fig. 3A and 3D) suggest that the primary mechanism by which fasudil reduces ASM force is through dephosphorylation of MLC20, at least under the condition that exposure of ASM to fasudil is limited to ~ 60 min in our present experimental protocol. Long term or repeated exposure of ASM to fasudil may produce different results, as it may result in actin depolymerization and cytoskeletal disruption that may lead to further decrease in ASM active force independent of MLC20 phosphorylation.

Pre-exposure of fasudil results in a decrease in the ability of ASM to produce force (Fig. 4A and C). This suggests that fasudil has a bronchoprotective property. This bronchoprotective effect can be seen even with a brief period (5 min) of fasudil pre-exposure (Fig. 4C). A better demonstration of fasudil's bronchoprotective effect is shown in Fig. 5. In sheep lungs with spontaneous ASM tone, lung resistance will increase over time in the absence of deep inspiration (29). The present result corroborates our previous observation (Fig. 5B, open circles, control observation). In the presence of fasudil (closed circles), the spontaneous increase in lung resistance and elastance are suppressed, demonstrating fasudil's bronchoprotective effect, even in the absence of deep inspiration. Lung resistance measured at a ventilation frequency of 0.25 Hz mostly reflects the sum of tissue resistance and airway resistance, whereas lung resistance measured at 2 Hz mostly reflects airway resistance (30). Figure 5B shows that fasudil is able to reduce airway resistance (at 2 Hz) if the lung is pre-exposed to the drug.

It is known that lung elastance increases as ventilation frequency increases (30). Our present data (Fig. 5B) follows this common observation. Figure 5B also shows that, in the absence of fasudil, the lung

elastance increases over time. Bronchoconstriction is likely a reason for this increase. Our recent finding from the same ex vivo sheep lung model (29) has demonstrated that air trapping occurs in lungs with spontaneous airway tone in the absence of deep inspirations. The present finding that fasudil prevents the increase of elastance over time suggests that fasudil improves lung function partially through reducing air trapping in the lung.

As mentioned in the Introduction, many studies of the effect of ROCK inhibitor on airway and lung function have been based on small animal models. The use of sheep lung model in the present study demonstrates that fasudil can be administered locally (via inhalation of nebulized fasudil) to achieve bronchodilation and bronchoprotection in lungs that are comparable in size of that of humans. By avoiding systemic application, any side effects of the drug can be minimized.

The present study only explored short-term effects of fasudil. Exposure of the drug to ASM was largely limited to 1 hour. Longer exposure could lead to other effects. The anti-inflammatory effects of ROCK inhibitors (2, 9, 10) was not investigated in the present study.

In conclusion, fasudil has bronchodilatory and bronchoprotective effects due to its relaxant effect on ASM through dephosphorylation of the MLC20. The present results support further studies that could lead to the repurpose of fasudil, or any suitable ROCK inhibitors, in the treatment of asthma.

Declarations

Competing Interest

None of the authors declared any conflict of interest.

Ethics, Consent to Participate, and Consent to Publish declarations

Not applicable.

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Author Contribution

CS and YY designed the experiments, YY, LW and PC conducted the experiments, YY and CS wrote the main text, YY prepared all figures 1, 2, 4, and 5; LW prepared figure 3. All authors reviewed and edited the manuscript.

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Data Availability

Raw data available upon reasonable request.

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Figures

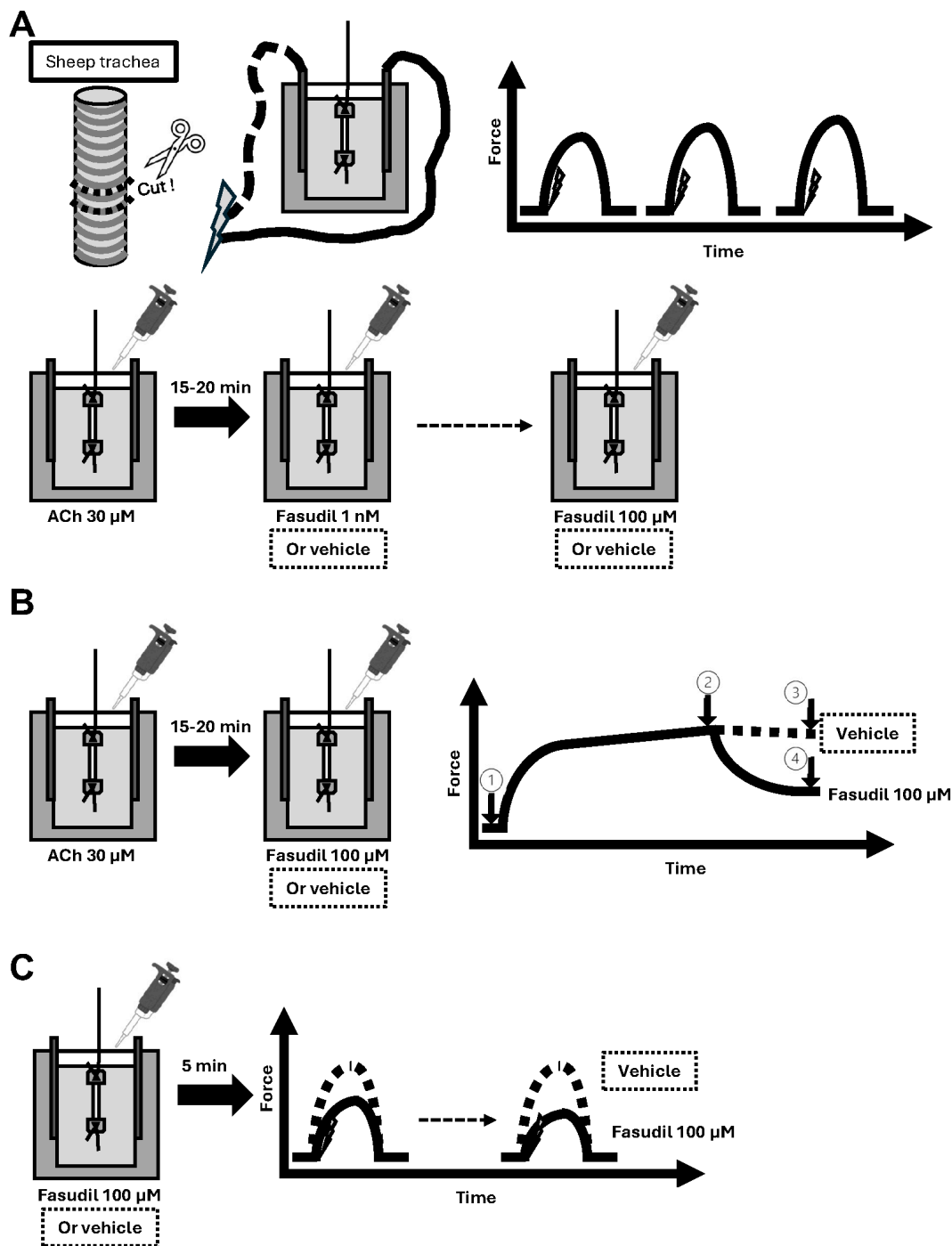


Figure 1

Schematic illustration of experimental protocol. A) Dose-response relationship of fasudil. B) Measurement of MLC20 phosphorylation during the bronchodilatory effect of fasudil. Numbers ①–④ indicate the specific time points for tissue freezing: “Rest” (baseline), “ACh” (peak contraction induced by 30 μ M ACh), “ACh-TC” (time control; frozen at the same time point as ACh-Fasudil without fasudil exposure), and “ACh-Fasudil” (frozen at the plateau reached approximately 20 min after adding 100 μ M

fasudil to the ACh-contracted strip). C) Bronchoprotective effect of fasudil evaluated using electrical field stimulation (EFS) in tracheal strips. Dotted lines in B) and C) indicate time control.

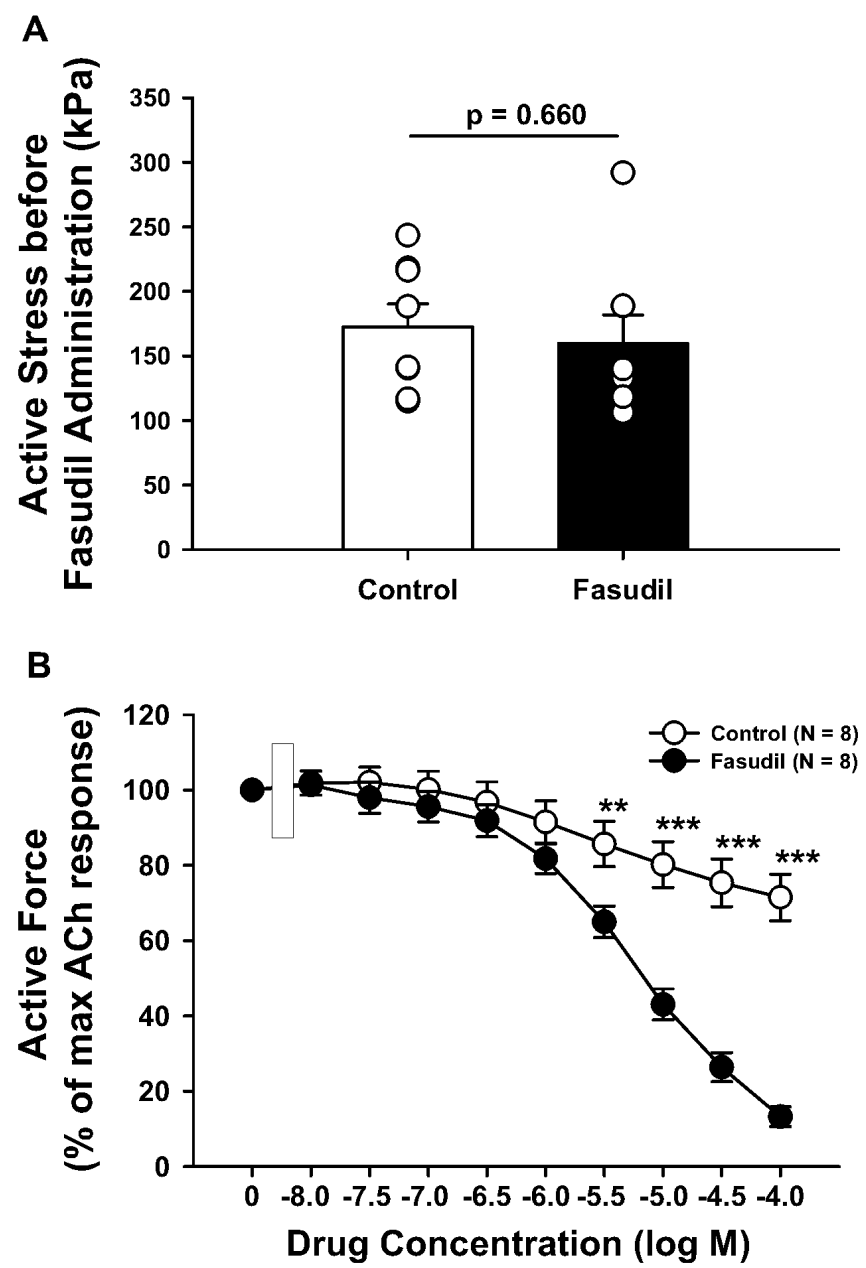


Figure 2

A) Active stress (kPa) produced by 30 μ M acetylcholine in control and fasudil-treated groups measured immediately prior to drug administration. No significant difference was observed between the two

groups (One-way RM ANOVA). B) Dose-response curves of fasudil and time control. Data were fitted using a four-parameter log-logistic model. The IC_{50} (drug concentration required to inhibit force by 50%) for fasudil was $6.5 \pm 0.5 \mu\text{M}$. Statistical significance was determined by two-way RM ANOVA. Difference between the control and fasudil-treated groups: ** $p < 0.01$; *** $p < 0.001$. All data are presented as mean $\pm$ SEM (n = 8 per group).

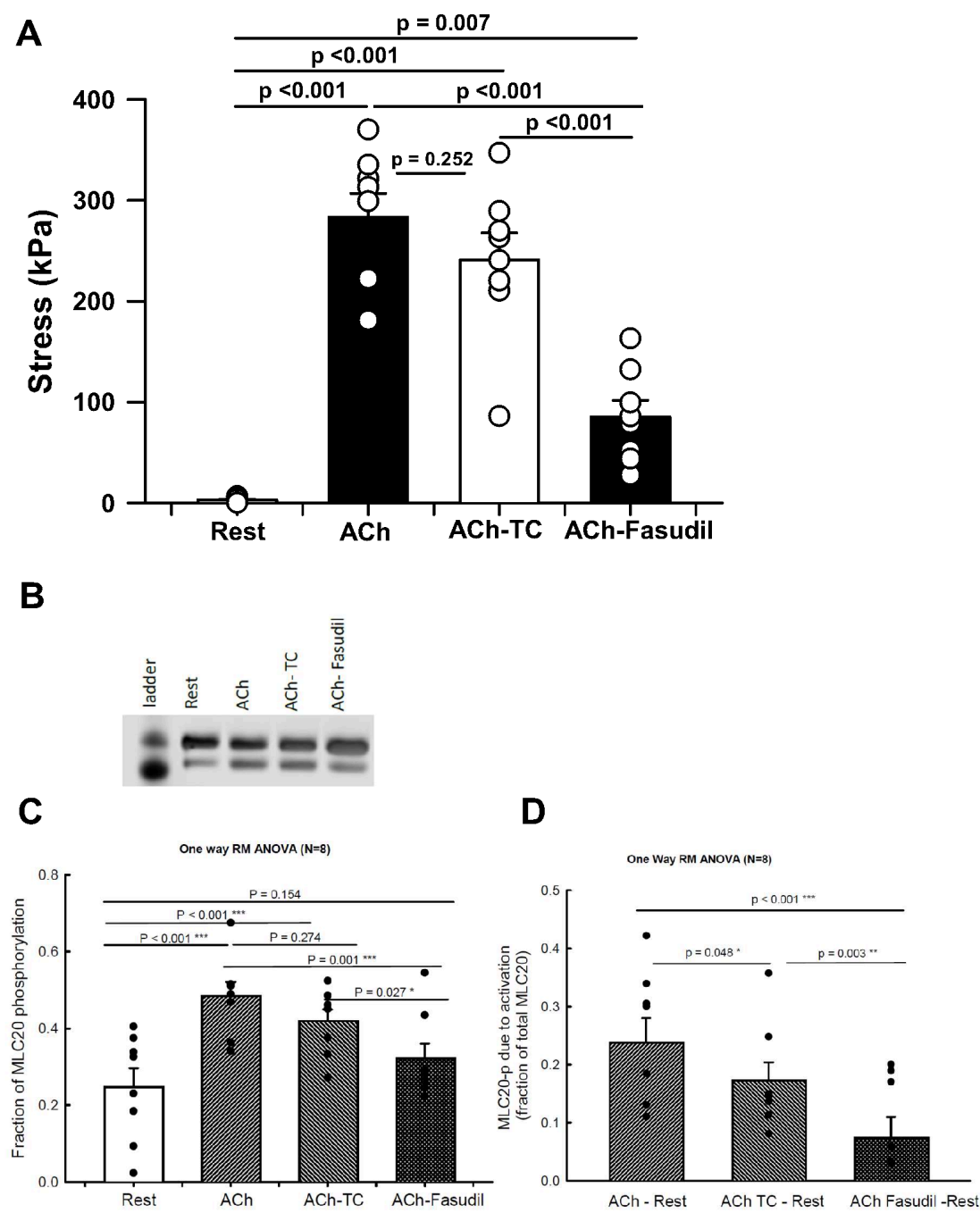


Figure 3

A) Active stress (kPa) at specific experimental stages: “Rest” (baseline), “ACh” (peak contraction induced by 30 μ M ACh), “ACh-TC” (time control; frozen at the same time point as ACh-fasudil without fasudil exposure), and “ACh-Fasudil” (frozen at the plateau reached approximately 20 min after adding 100 μ M fasudil to the ACh-contracted strip). B) Example of Western blot. (Uncropped blot can be found in the supplemental figure). C) Fraction of MLC20 phosphorylation under all 4 conditions. D) MLC20 phosphorylation due to activation (the resting values were subtracted. Data are presented as mean $\pm$ SEM (n = 8 per group). *, P<0.05; **, P<0.01; ***, P<0.001.

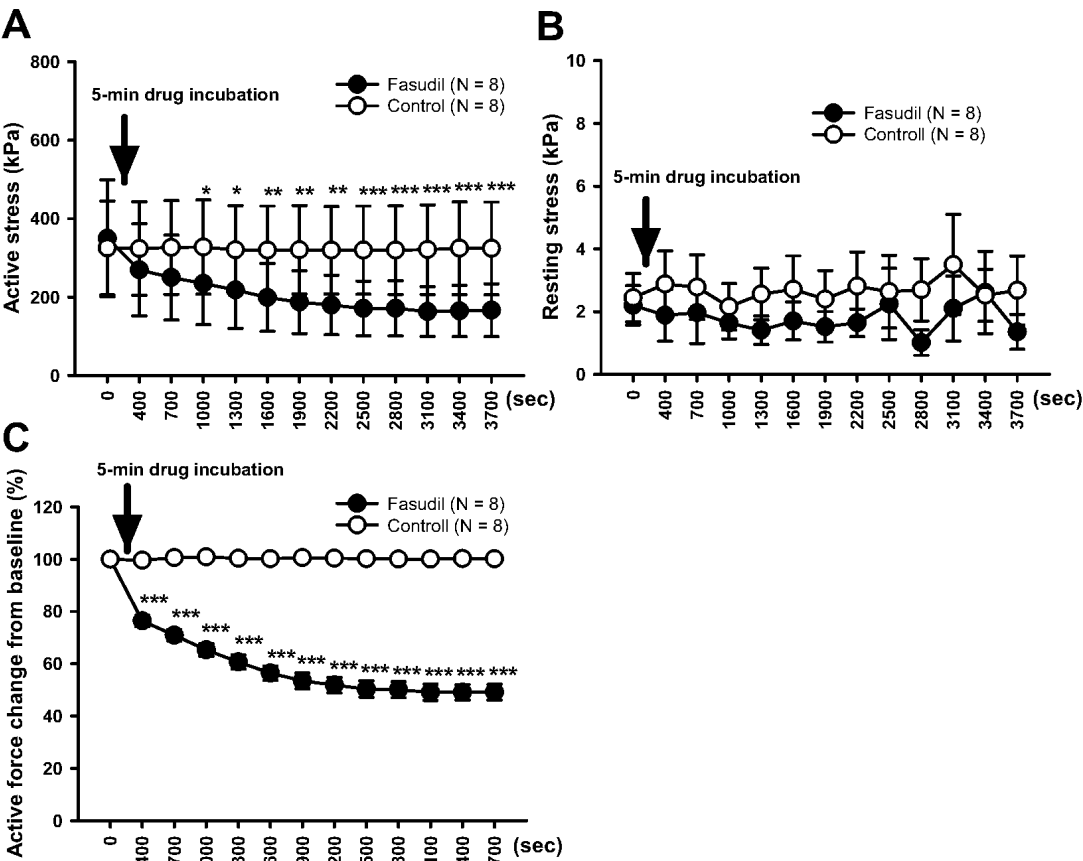


Figure 4

A) Active stress (kPa) produced by electrical field stimulation in tracheal strips pretreated with 100 μ M fasudil and in untreated control strips. B) Resting stress (kPa) for both groups. C) Percentage change in active force, normalized to the pre-treatment baseline of each individual strip, for control and 100 μ M fasudil-treated groups. Statistical analysis was performed using two-way RM ANOVA. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$. Data are presented as mean $\pm$ SEM ($n = 8$ per group).

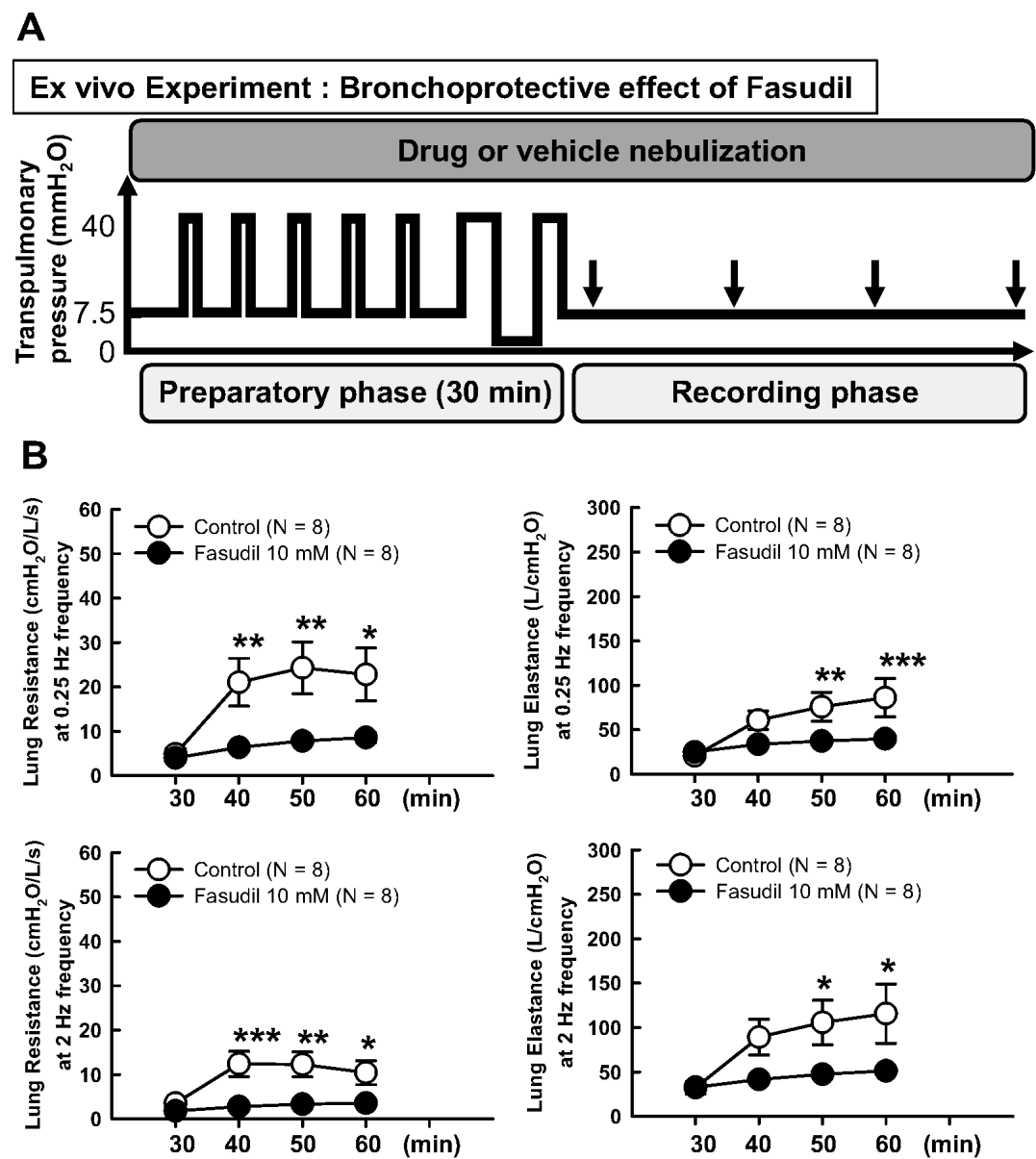


Figure 5

A) Schematic illustration of the experimental protocol for ex vivo lung ventilation protocol. Vertical downward arrows indicate measurement points for lung resistance and elastance at 10-min intervals. Lungs were tidally ventilated throughout the experiment, except during the application of six consecutive deep inspirations (DIs) during the preparatory phase. B) Time-course of lung resistance and elastance measured at ventilation frequencies of 0.25 and 2 Hz with nebulized Krebs (vehicle control, open circles) or fasudil (closed circles). Two-way RM ANOVA was performed followed by Holm-Sidak post hoc test. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$. Data are presented as mean $\pm$ SEM (n = 8 per group).

Supplementary Files

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