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Combination Therapy With Fasudil and Sildenafil Ameliorates Monocrotaline-Induced Pulmonary Hypertension and Survival in Rats

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Background: Pulmonary hypertension (PH) is a fatal disease characterized by pulmonary artery (PA) remodeling, elevated PA pressure and right ventricular (RV) failure. It has been previously demonstrated that treatment with a Rho-kinase inhibitor, fasudil, ameliorates PH in animal models. Here, whether combination therapy with fasudil and sildenafil further ameliorates PH in rats was examined.

Methods and Results: PH was induced in Sprague-Dawley rats by the use of a single subcutaneous monocrotaline (MCT) injection, which caused PA remodeling, elevated RV systolic pressure (RVSP), and RV hypertrophy (RVH). While fasudil and sildenafil monotherapy inhibited the development of MCT-induced PH in the prevention and treatment protocols, their combination therapy further improved RVSP and RVH. Moreover, a histological examination demonstrated significant improvements of PA remodeling in the combination group compared with the monotherapy groups. An echocardiographic examination also revealed significant reduction in RV diameter in the combination group compared with the monotherapy groups. Mechanistic experiments revealed significant inhibition of Rho-kinase activity in PA trunk, lung and RV tissues in the combination group as well as in the monotherapy groups. Finally, the combination therapy markedly improved the long-term survival compared with the monotherapy groups.

Conclusions: These results indicate that the combination therapy with fasudil and sildenafil shows the synergistic effects through the inhibition of Rho-kinase activity for the treatment of PH. (*Circ J* 2014; **78:** 967–976)

Key Words: Combination therapy; Fasudil; Hypertrophy; Pulmonary hypertension; Sildenafil

Pulmonary arterial hypertension (PAH) is a fatal disease, characterized by pulmonary vascular remodeling and right ventricular (RV) failure.[1–3] The pathogenesis of PAH is complex, including endothelial dysfunction, vascular smooth muscle cells (VSMCs) proliferation, and inflammatory cell migration.[4–6] Currently, we obtained new therapeutic options such as prostanoid analogues, endothelin receptor blockers, and phosphodiesterase type 5 (PDE5) inhibitors.[7] However, no single agent has been shown to provide entirely satisfactory results. Recently, it has been proposed that combination therapy might provide synergistic benefits by inhibiting multiple signaling pathways;[8] however, there is no direct evidence as to the beneficial combination that will improve the long-term survival in PAH.[9]

Recent advances in molecular biology have elucidated the substantial involvement of intracellular signaling pathways mediated by small GTP-binding proteins (G proteins) such as Rho.[10–12] Rho-kinase, a downstream molecule of Rho, is involved in various cellular functions, including VSMC contraction, actin cytoskeleton organization, cell adhesion, and gene expressions.[13–18] Additionally, Rho-kinase causes VSMC proliferation, vascular contraction and remodeling by multiple mechanisms.[19,20] We have previously demonstrated that Rho-kinase is substantially involved in the pathogenesis of pulmonary hypertension (PH) in animal models.[21–23] Indeed, Rho-kinase is an important therapeutic target in cardiovascular diseases,[14] and Rho-kinase inhibition ameliorates PH in animals and humans.[23–26]

Pulmonary vascular remodeling is an important pathogenesis of PH, in which another signaling pathway through the nitric oxide (NO) and cGMP is involved.[27,28] Pulmonary vascular cGMP levels are increased by inhibiting the phosphodiesterases (PDEs) that are responsible for cGMP hydrolysis in the pulmonary vasculature.[27] Importantly, a recent study demon-

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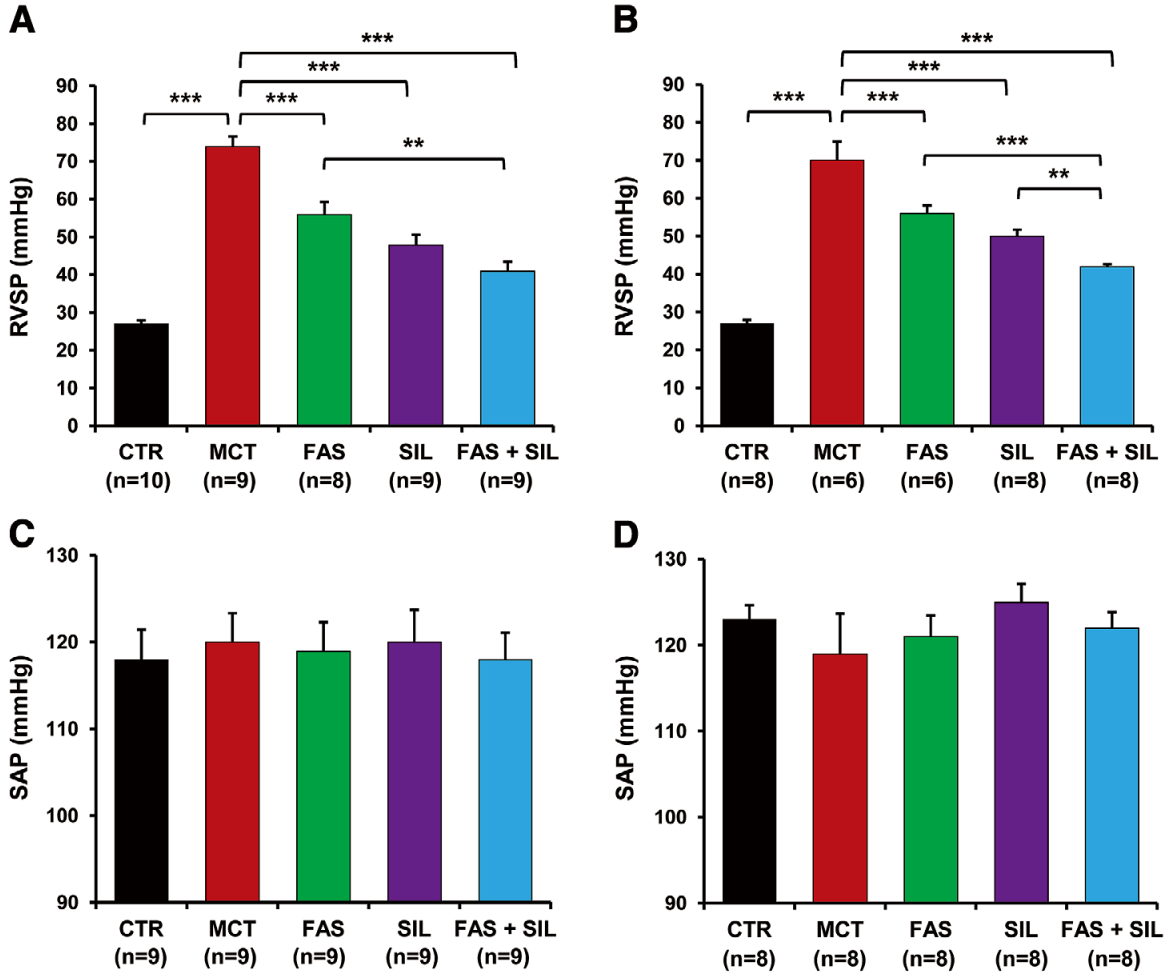

Figure 1. Combination therapy improves hemodynamic variables. (**A**,**B**) Compared with the control (CTR) group, right ventricular systolic pressure (RVSP) was elevated in the monocrotaline (MCT) group. The combination therapy significantly attenuated the MCT-induced increase in RVSP compared with each monotherapy in both the prevention and treatment protocols. (**C**,**D**) MCT did not affect systolic arterial pressure (SAP) in any of the groups in the prevention or treatment protocol. Results are expressed as mean±SEM. **P<0.01, ***P<0.001.

strated that sildenafil, a PDE5 inhibitor, inhibits Rho-kinase activity in an animal model of PH.[29] We hypothesized that combination therapy with fasudil and sildenafil could further improve the long-term survival in PH by synergistic effects between Rho-kinase and NO/cGMP/PDE5 signaling. In the present study, we therefore tested our hypothesis that combination therapy with fasudil and sildenafil improves RV function and long-term survival in monocrotaline (MCT)-induced PH in rats.

Methods

Animals

Adult male Sprague-Dawley rats (6 weeks of age, 150~170 g body weight [BW]) were purchased from SEIMI Corporation (Sendai, Japan). All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University. Animals were housed under climate-controlled conditions on a 12 : 12-h light–dark cycle with access to chow and water. They were allowed 1 week to adjust to the new environment prior to the experiment.

Monocrotaline

Monocrotaline (MCT; Sigma-Aldrich Co, St. Louis, MO, USA) was dissolved in 1N HCl, and the pH was adjusted to 7.4 with 1N NaOH.[23,30] The solution was administered as a single subcutaneous (SC) injection (60 mg/kg) in a volume of 3 ml/kg. Control, age-matched rats received an equal volume of isotonic saline.[23, 30]

Experimental Protocols

A total of 203 male rats (7 weeks of age) were used for the present study, for hemodynamic measurements, morphometric analysis and echocardiographic measurement in the prevention and treatment protocols. All rats were given a subcutaneous injection of either MCT (60 mg/kg) or 0.9% saline, where MCT induced severe PH in 3 weeks.[23] In the prevention protocol, animals were orally treated with a vehicle; fasudil ($30\,mg \cdot kg^{-1} \cdot day^{-1}$), sildenafil ($10\,mg \cdot kg^{-1} \cdot day^{-1}$), or both for

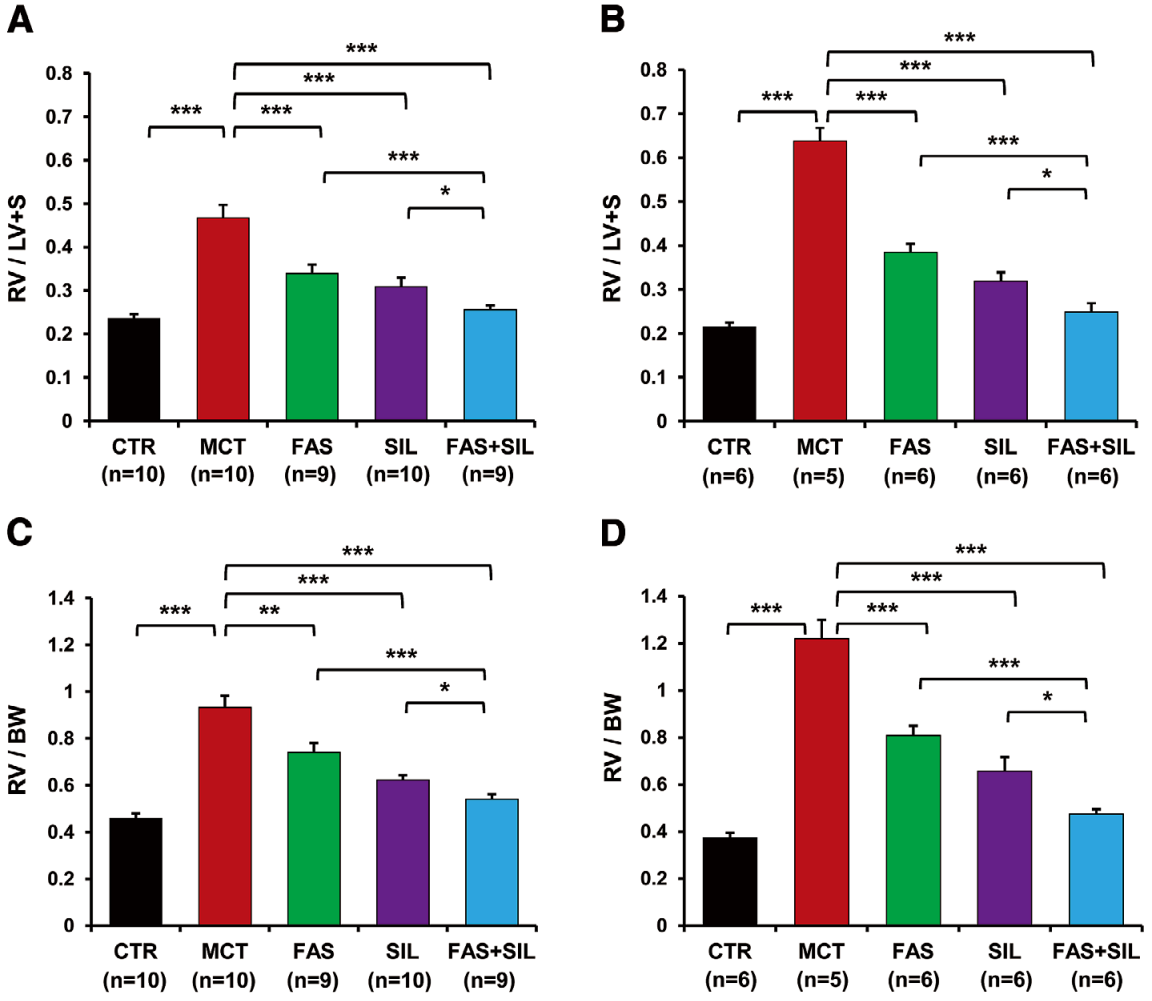

Figure 2. Effects of combination therapy on right ventricular hypertrophy. (**A**–**D**) The ratio of right ventricle to left ventricle plus septum weight [RV/(LV+Sep)] and the ratio of right ventricle to body weight (RV/BW) were increased in the monocrotaline (MCT) group compared with the control (CTR) group. The combination therapy significantly attenuated the development of right ventricular hypertrophy compared with the monotherapy. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001.

3 weeks concomitantly with a MCT injection.[23] In the treatment protocol, animals were orally treated with the same drugs or vehicle, starting at day 21 after MCT administration when severe PH had already been established.[23] Fasudil and sildenafil were provided by Asahi Kasei Pharma Co (Shizuoka, Japan) and Pfizer (Groton, CT, USA), respectively. This protocol resulted in the creation of 5 groups: control group (saline SC and vehicle orally), MCT group (MCT SC and vehicle orally), FAS group (MCT SC and fasudil orally), SIL group (MCT SC and sildenafil orally) and FAS+SIL group (MCT SC and combination with fasudil and sildenafil orally). Hemodynamic, echocardiographic, histologic and Western blot analyses were performed 3 weeks after the MCT injection in the prevention protocol and 9 weeks after the MCT injection in the treatment protocol.[23] Water and food intake were measured every week.

Hemodynamic Measurements

Hemodynamic measurements were performed by using a Millar Mikrotip® catheter (SPR 320, size 2F; Millar Instruments, Houston, TX, USA),[31] transducer and the PowerLab data acquisition system (AD Instruments, Sydney, Australia) connected to a polygraph system (AB 601G; Nihon Kohden, Tokyo, Japan). All animals were anesthetized with inhaled isoflurane by an anesthesia unit (Univentor 400; Univentor High Precision Instrument, Zejtun ZTN, Malta) and placed on a heating pad to maintain body temperature in the physiological range (37°C) throughout the study. The RV systolic pressure (RVSP) was measured by insertion of a Milllar catheter into the right jugular vein, then through the right atrium into the RV. Systolic arterial pressure (SAP) was also measured when the catheter was inserted into the left carotid artery.

Right Ventricular Hypertrophy

The RV was dissected from the left ventricle (LV) and septum (S). These samples were weighted to determine the extent of RV hypertrophy (RVH), expressed as the RV/(LV+S) ratio, and also in terms of BW, expressed as RV/BW.[23,32]

Morphometric Analysis

After the hemodynamic measurement, the lungs were perfused

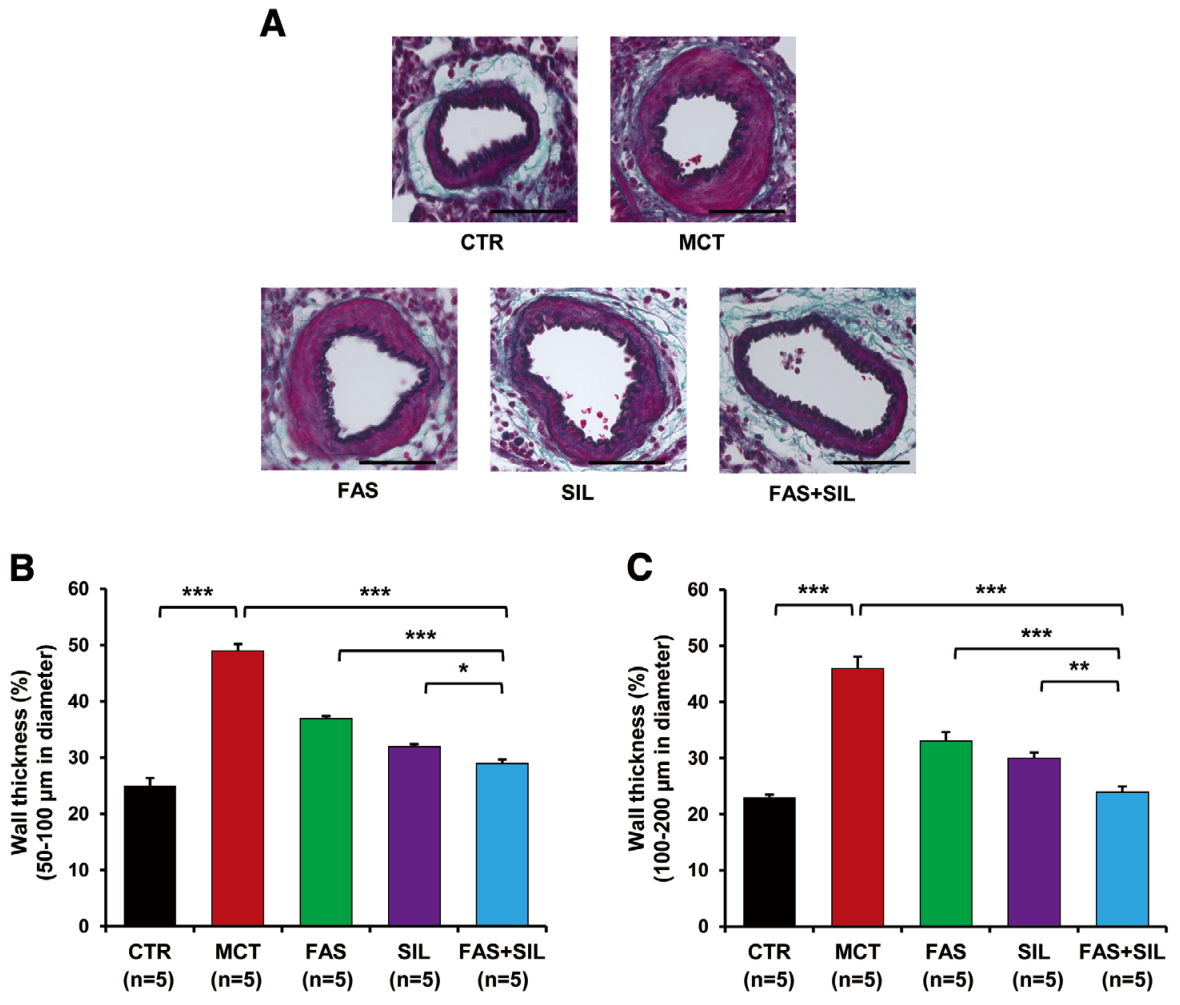

Figure 3. Inhibitory effects of the combination therapy on pulmonary microvascular remodeling. (**A**) Representative photomicrographs showing macroscopic features of pulmonary arteries by Elastica-Masson staining. Scale bars, 50 μm. (**B**,**C**) Quantitative analysis of medial wall thickness of pulmonary arteries. Compared with the control (CTR) group, medial thickness of pulmonary arteries was markedly increased in the monocrotaline (MCT) group. The combination therapy significantly attenuated the development of medial wall thickening compared with each monotherapy. Results are expressed as mean±SEM. *P < 0.05, **P<0.01, ***P<0.001.

with physiological salt solution and were fixed with 10% formalin for morphometric analysis. Paraffin sections (5 μm) were obtained from the left lung and stained with Elastica Masson for examinations by light microscopy (BZ-9000; KEYENCE, Elmwood Park, NJ, USA). The measurement of medial thickness was assessed in pulmonary arteries (PA) with an external diameter of 50~200 μm. The PA were divided into 2 groups (50~100 and 100~200 μm in diameter) for analysis. Medial thickness was expressed as follows: percent wall thickness = [(medial thickness×2)/external diameter]×100.[23] More than 20 PA per animal were measured.

Echocardiographic Measurements

Echocardiographic measurements were performed after 3 weeks of MCT injections. Rats were anesthetized using inhaled isoflurane and placed in a supine position. Two-dimensional (2D) and M-mode (motion mode) images were recorded by the Toshiba echocardiographic system (SSA-770A; Toshiba Corporation, Tokyo, Japan) through the right parasternal long axis view of the heart.[33] M-mode images were used to determine the RV end-systolic diameter (RVESD) and the RV end-diastolic diameter (RVEDD) as measures for RV dilatation. RV fractional shortening (RVFS) was also determined as a measure for RV contractility.[34]

Western Blot Analysis

PA, lungs and RV tissues (100 mg each) were homogenized individually with 1 ml of 10% TCA (trichloroacetic acid) buffer with autohomogenizer (Precellys 24; Bertin Technologies, France) and centrifuged. Then, the cell lysate was washed with PBS (phosphate buffered saline) and re-suspended in Urea/SDS buffer. Protein concentration of the supernatant was determined by a Pierce BCA protein assay kit (Thermo Scientific, IL, USA). Equal amounts of protein were loaded in each lane of polyacrylamide/SDS gels (Mini Protean TGX Gels; BIO-RAD Laboratories Inc, CA, USA), which were then electrophoresed

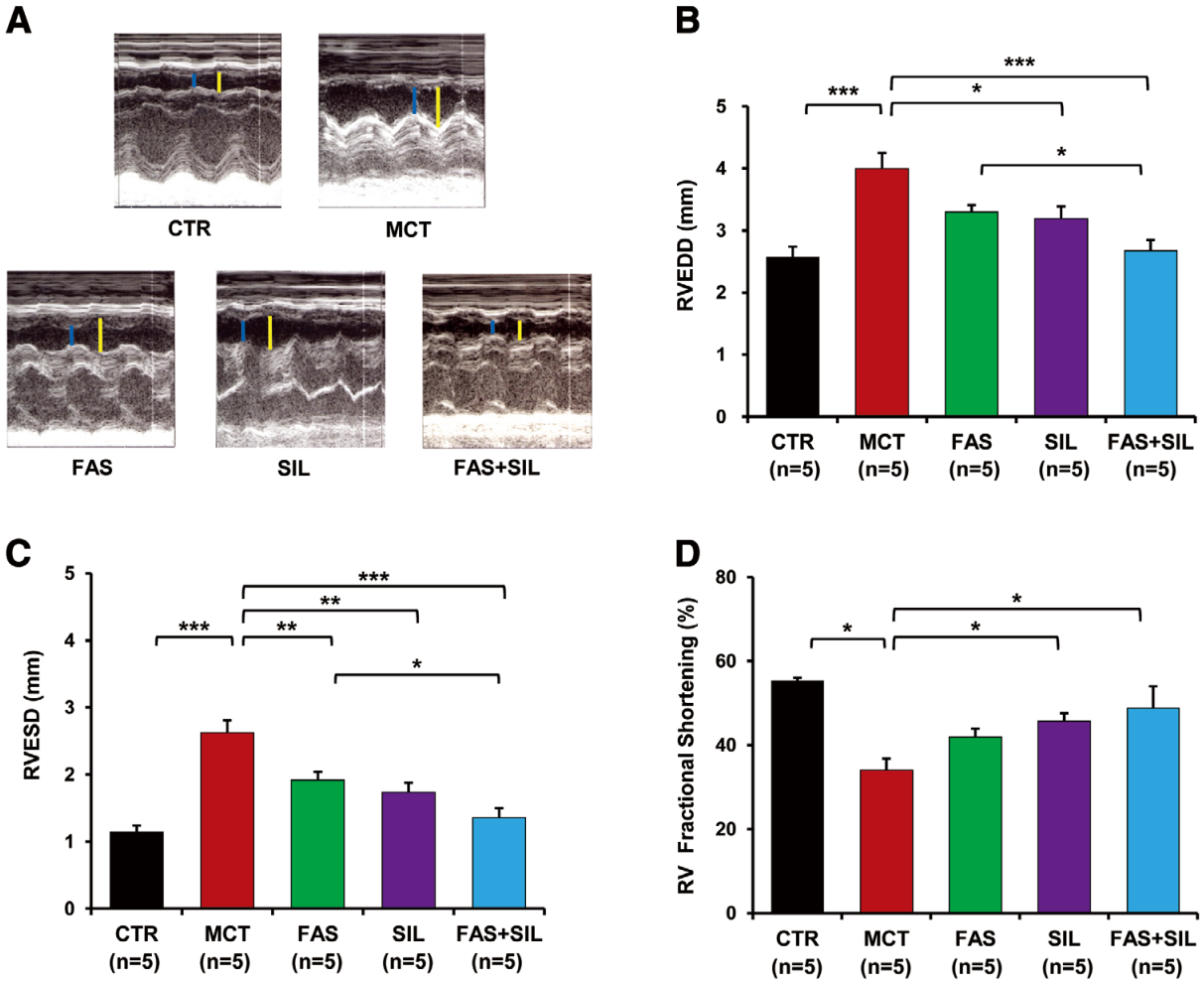

Figure 4. Effects of the combination therapy on RV function. (**A**) Representative echocardiographic images of the right ventricle (RV). Vertical yellow bar shows the RV end-diastolic diameter (RVEDD) and the vertical blue bar shows the RV end-systolic diameter (RVESD). (**B**,**C**) The RVEDD and RVESD were markedly increased in the monocrotaline (MCT) group compared with the control (CTR) group. The combination therapy significantly reduced RVEDD and RVESD compared with each monotherapy. (**D**) RV contractility assessed by RV fractional shortening was significantly reduced in the MCT group compared with the CTR group, and was significantly improved by the combination therapy compared with each monotherapy. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001.

and transferred to a PVDF membrane (Amersham Hybond-P; GE Healthcare, UK). Lysates were analyzed by using a Western blot. Equal loading was confirmed by re-probing the membrane with a monoclonal anti-α-tubulin antibody (Sigma-Aldrich Co, St. Louis, MO, USA) and an anti-β actin antibody (Abcam). Rho-kinase activity was analyzed by Western blot analysis for MYPT (myosin phosphatase targeting subunit) using a purified mouse anti-MYPT1 antibody (BD transduction Laboratories, USA) and for phosphorylated MYPT1 using a rabbit polyclonal anti-phospho-MYPT1 (Thr696) antibody (Millipore, MA, USA).[23] Extracellular-signal regulated kinases (ERK) activity was also analyzed by Western blot analysis for ERK1/2 protein using a p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology, Danvers, MA 01923) and for phosphorylated ERK1/2 using a rabbit polyclonal phosphor-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) antibody (Cell Signaling Technology, Danvers, MA 01923). The protein expression of endothelial NO synthase (eNOS) using purified mouse anti-eNOS/NOS type III antibody (BD transduction Laboratories, USA) and β-actin as an internal control in lungs was also analyzed by Western blot analysis. Western blots were visualized using a horseradish peroxidase-conjugated secondary antibody and subsequent ECL detection (GE Healthcare, UK) with a LAS-4000 mini instrument (Fujifilm, Tokyo, Japan), and then quantified by densitometric analysis using ImageJ software (NIH).

Statistical Analysis

All results are expressed as mean±SEM. The log-rank test was used to determine the P value for Kaplan-Meier survival curves. Analyses were performed using SPSS, version 19.0 (Chicago, IL, USA) and the R version 3.0.1. Differences in all other parameters were evaluated by ANOVA followed by both Dunnett's and Fisher's post-hoc test. A value of P<0.05 was considered to be statistically significant.

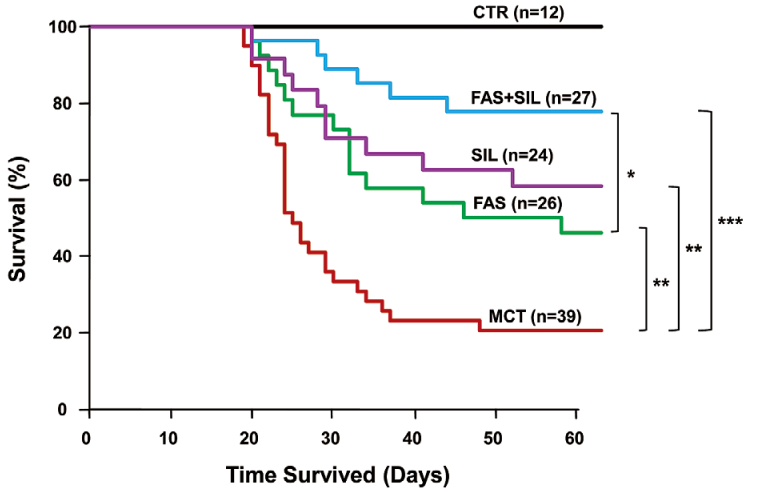

Figure 5. Combination therapy improves the survival. The Kaplan-Meier curve shows that the combination therapy improved long-term survival compared with the monotherapy. Control group (CTR); monocrotaline group (MCT); fasudil group (FAS); sildenafil group (SIL); and combination group (FAS+SIL). *Log-rank P<0.05, **Log-rank P<0.01, ***Log-rank P<0.001.

Results

Combination Therapy Improves Hemodynamic Variables

We measured hemodynamics at 3 weeks after the MCT injection in the prevention protocol and at 9 weeks after the injection in the treatment protocol (**Figure S1**). A catheter examination revealed that RVSP was significantly elevated in the MCT group compared with the control group both in the prevention and treatment protocols (**Figures 1A**,**B**). In the prevention protocol, the monotherapy with fasudil or sildenafil significantly reduced RVSP, and their combination therapy further reduced RVSP compared with each monotherapy (**Figure 1A**). In the treatment protocol, the monotherapy with fasudil or sildenafil significantly reduced RVSP, which was further reduced by their combination therapy (**Figure 1B**). In contrast, there was no significant difference in systemic arterial pressure among the groups in the prevention or treatment protocols (**Figures 1C**,**D**).

Combination Therapy Ameliorates Right Ventricular Hypertrophy

RVH is a characteristic of PH. We confirmed a significant development of RVH in the MCT group compared with the control group both in the prevention and treatment protocols (**Figure 2**). In the prevention protocol, the monotherapy with fasudil or sildenafil significantly inhibited the development of RVH, which was further inhibited by their combination therapy (**Figures 2A**,**C**). Also, in the treatment protocol, the monotherapy with fasudil or sildenafil ameliorated the developed RVH, which was further inhibited by their combination therapy (**Figures 2B**,**D**).

Combination Therapy Inhibits Pulmonary Vascular Remodeling

A histological examination showed that medial wall thickening of the PA was reduced in the combination group compared with other groups (**Figure 3A**). Quantitative analysis of the PA demonstrated that MCT caused a significant increase in the medial wall thickness of the PA (**Figures 3B**,**C**). In contrast, the monotherapy with fasudil or sildenafil significantly inhibited the development of medial wall thickening, which was further inhibited by their combination therapy (**Figures 3B**,**C**).

Combination Therapy Ameliorates RV Function and Long-Term Survival

An echocardiographic examination showed that RV dilatation, as evaluated by RV end-diastolic (EDD) and end-systolic dimension (ESD), was developed together with reduced RVFS in the MCT group compared with the control group (**Figures 4A–D**). Those MCT-induced changes were improved by the monotherapy with fasudil or sildenafil, and further improved by their combination therapy (**Figures 4A–D**).

In the prevention protocol, no animals died during the 3 weeks of the follow-up period in the MCT group. In contrast, in the treatment protocol, 79% of the animals died by 9 weeks in the MCT group (**Figure 5**). We noted pleural effusion in the dead animals, indicating that the potential cause of death was RV failure. Importantly, the monotherapy with fasudil or sildenafil significantly improved the survival rate, which was further improved by the combination therapy in the treatment protocol (**Figure 5**). We also measured BW, food and water intake that might be related to their longevity. BW was significantly reduced in the MCT group compared with the control group in both the prevention (**Figure S2A**) and treatment protocols (**Figure S3A**). Additionally, the amount of water and food intake was significantly reduced in the MCT group compared with the control group in both the prevention (**Figures S2B**,**C**) and treatment protocols (**Figures S3B**,**C**).

Combination Therapy Inhibits Rho-Kinase Activity

To elucidate the mechanism involved in the significant improvement of PH, RV function and survival in the combination group, we performed Western blot analysis to quantify Rho-kinase activity in tissues from PA trunk, lung, and RV. The MCT group showed a significant increase in Rho-kinase activity in the PA trunk compared with the control group (**Figures 6A**,**B**). In contrast, the monotherapy with fasudil or sildenafil significantly inhibited the Rho-kinase activity in the PA trunk, which was further inhibited by their combination therapy (**Figure 6B**). Additionally, the monotherapy with fasudil or sildenafil showed marked inhibition of Rho-kinase activity in the lung and RV tissue, with no further inhibition by their combination therapy (**Figures 6C**,**D**).

ERK1/2 signaling plays an important role in the regulation of VSMC proliferation and pulmonary vascular remodeling.[35] Thus, we performed Western blot analysis to measure ERK1/2

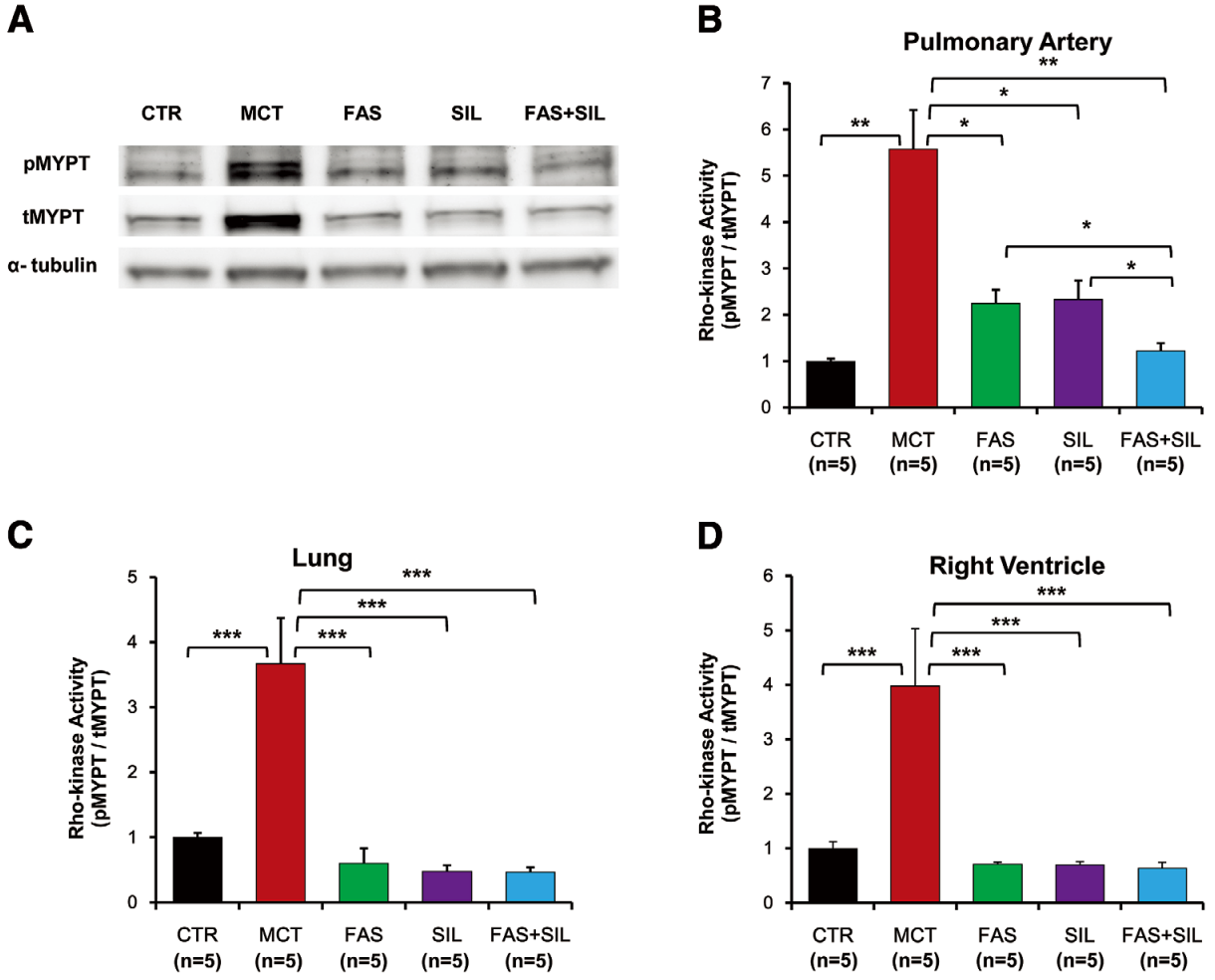

Figure 6. Inhibitory effects of the combination therapy on Rho-kinase activity. The combination therapy significantly inhibits monocrotaline (MCT)-induced Rho-kinase activation. (**A**) Rho-kinase activity was assessed by the ratio of phosphorylated MYPT (pMYPT) per total MYPT (tMYPT). (**B**–**D**) Rho-kinase activity (pMYPT/tMYPT) is shown by bar graphs and expressed as the ratio relative to the control group (CTR). Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001.

activity in the tissues from PA trunk and lung. The MCT group showed a significant increase in the ERK1/2 activity compared with the control group (Figures S4A,B). In contrast, the monotherapy with fasudil or sildenafil significantly attenuated the ERK activity, which was further inhibited by the combination therapy in the lung but not in the PA (Figures S4A,B).

We also measured eNOS expression in the lung tissue by using Western blot analysis. It was observed that eNOS expression was significantly reduced in the MCT group compared with the control group (Figure S5). In contrast, the monotherapy or the combination therapy with fasudil and sildenafil significantly increased the eNOS expression (Figure S5).

Discussion

The major finding of the present study is that the combination therapy with fasudil and sildenafil has a significant efficacy in the treatment of MCT-induced PH in rats. In the present study, we performed 2 protocols to examine the efficacy of the combination therapy, which showed significant effects over the monotherapy by improving RVSP, RVH and pulmonary vascular remodeling. Importantly, the combination therapy significantly improved RV functions and long-term survival through Rho-kinase inhibition.

Combination Therapy Inhibits Pulmonary Vascular Remodeling

MCT is known to cause endothelial injury of PA with subsequent proliferation of pulmonary VSMC, infiltration of inflammatory cells and pulmonary vascular remodeling.[30] Rho-kinase plays an important role in mediating various cellular functions, including VSMC contraction, actin cytoskeleton organization,[10] and cytokinesis,[14] thereby developing cardiovascular diseases.[12] Importantly, Rho-kinase is substantially involved in the vascular effects of various vasoactive factors.[12] Rho-kinase inhibitors have an inhibitory effect on the proliferation and migration of VSMC and infiltration of inflammatory cells.[14] It has also been shown that statins enhance eNOS mRNA stability by cholesterol-independent mechanisms involving the inhibition of Rho geranylgeranylation.[36] Importantly, Rho-kinase inhibitors

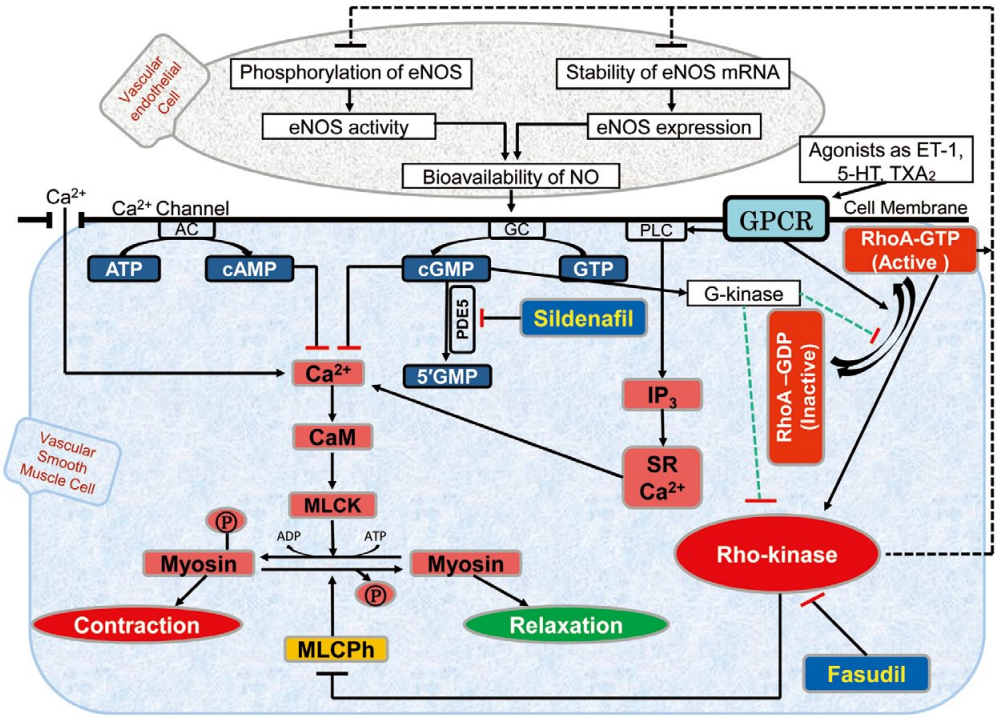

Figure 7. Additive and synergistic effects of the combination therapy. Additive and synergistic Rho-kinase inhibition by the combination therapy with fasudil and sildenafil. 5-HT, serotonin; AC, adenylate cyclase; CaM, calmodulin; ET-1, endothelin 1; eNOS, endothelial nitric oxide synthase; GC, guanylate cyclase; GPCR, G-protein coupled receptor; IP_3, inositol 1,4,5-triphosphate; MLCK, myosin light chain kinase; MLCPh, myosin light chain phosphatase; NO, nitric oxide; PDE-5, phosphodiesterase-5; VSMC, vascular smooth muscle cell; PLC, phospholipase C; SR, sarcoplasmic reticulum; TXA_2, thromboxane A_2.

induce vasodilation especially where vasoconstrictor tone is increased by a variety of mechanisms including hypoxia, NOS inhibition and other mechanisms.[37] In contrast, sildenafil inhibits pulmonary vascular remodeling by a different mechanism targeting the eNOS-NO-cGMP pathway.[38] Thus, Rho-kinase inhibition and PDE5 inhibition might have additive and synergistic effects in terms of vascular dilatation and remodeling (Figure 7). Indeed, in the present study, the combination therapy with fasudil and sildenafil significantly inhibited MCT-induced pulmonary vascular remodeling.

Combination Therapy Improves RV Function and Survival

PH causes RV failure, characterized by an increased RV pressure and reduced RV fractional shortening, and ultimately premature death.[39] Fasudil suppresses Rho-kinase-mediated vasoconstriction by dephosphorylation of myosin phosphatase in PA of rats.[40] In addition, sildenafil increases the cGMP level through PDE5 inhibition and causes vasodilation of PA in rats.[41] In severe PH, increased pulmonary vascular resistance causes RV pressure overload and develops an imbalance between RV oxygen demand and supply, resulting in RV failure. RV dysfunction is also augmented by LV dysfunction due to myocardial ischemia and by an interventricular interaction through the ventricular septal wall.[42] These changes in RV dysfunction are associated with multiple cellular changes, such as oxidative stress, apoptosis, inflammation, fibrosis and metabolic remodeling.[43,44] Importantly, Rho-kinase activity is increased in most of the cardiovascular diseases, including myocardial ischemia and remodeling.[45] Rho-kinase inhibition by fasudil protects cardiovascular tissues by activating the phosphatidylinositol 3-kinase/protein kinase Akt/eNOS pathway that has anti-apoptotic and anti-inflammatory effects and by decreasing the ventricular tachycardia.[46–48] In contrast, PDE5 is strongly expressed in vascular beds and cardiac myocytes, which blocks NO-mediated cGMP generation.[49] Importantly, sildenafil exerts cardioprotective effects by increasing NO generated by eNOS or iNOS, thereby opening mitochondrial ATP-sensitive potassium channels or by decreasing the ventricular arrhythmia.[50–52] In the present study, we were able to demonstrate that the combination therapy with fasudil and sildenafil significantly improved RV function and long-term survival compared with each monotherapy (Figure 7).

Additive and Synergistic Effects of the Combination Therapy

Rho-kinase plays an important role in the pathogenesis of PH directly by activating its substrates and indirectly by mediating the signal transduction of various inflammatory mediators.[12,23] In contrast, the NO/cGMP pathway plays crucial roles in vasoconstriction and pulmonary vascular remodeling.[19,27,53] Importantly, this pathway is closely associated with the Rho/Rho-kinase system as it regulates the cGMP-dependent phosphorylation of RhoA in VSMC.[54] Indeed, it has been shown that Rho-kinase inhibitors increase vascular eNOS expression and exert cGMP-mediated vasodilatation.[46,55,56] In the present study, we also confirmed that eNOS expression was significantly increased by the monotherapy or the combination therapy with fasudil and sildenafil compared with the MCT group. Thus, fasudil blocks vasoconstriction through the dephosporylation of myosin phos-

phatase,[40] and sildenafil causes relaxation by a cGMP-dependent mechanism.[29] Consistently, we demonstrated a significant inhibition of Rho-kinase-mediated MYPT phosphorylation by combination therapy with fasudil and sildenafil.

It was previously demonstrated that the NO/cGMP pathway regulates RhoA by phosphorylating them by cGMP-dependent protein kinase (PKG).[29,54] This phosphorylation prevents the translocation of active GTP-bound RhoA to the membrane, resulting in the inhibition of Rho-kinase activity.[29,54] Thus, sildenafil activates the NO/cGMP pathway and thereby indirectly inhibits Rho-kinase through the PKG or G-kinase.[29,54] In contrast, fasudil directly inhibits Rho-kinase through dephosphorylation of myosin phosphatase.[40] In the present study, we were able to demonstrate a significant inhibition of Rho-kinase activity in the PA by the combination therapy compared with each monotherapy. Thus, we propose additive and synergistic Rho-kinase inhibition by the combination therapy with fasudil and sildenafil (**Figure 7**).

Previous studies have demonstrated that the Ras/ERK signaling pathway regulates a variety of processes including VSMC proliferation[35] and pulmonary vascular remodeling.[57] In the present study, we observed ERK1/2 activation in the MCT-treated rats. The phosphorylated ERK1/2 needs to be translocated to the nucleus for many of its actions. Recently, it has been demonstrated that the nuclear translocation of phosphorylated ERK1/2 depends on the Rho-kinase activity.[58] Fasudil inhibits VSMC proliferation by blocking the nuclear translocation of ERK1/2.[59] All of these reports support our present study finding that treatment with fasudil inhibited the phosphorylation of Rho-kinase and ERK1/2. In contrast, sildenafil inhibits VSMC proliferation through activating the cGMP/PKG pathway, where cGMP activates PKG to induce the mitogen-activated protein kinase phosphatase-1 (MKP-1) enzyme that deactivates ERK1/2.[60,61] In the present study, we were able to demonstrate that sildenafil reduced ERK1/2 activity in both the PA trunk and lung. Taken together, the combination therapy with fasudil and sildenafil significantly inhibits ERK1/2 activity and Rho-kinase activity compared with each monotherapy (**Figure 7**).

Study Limitations

Several limitations should be mentioned for the present study. First, the MCT-induced PH model might not fully represent PH in humans and thus the effectiveness of the combination therapy should be evaluated in other PH models with different etiologies. Second, relatively high doses of fasudil and sildenafil were used in the present study MCT-induced PH model in rats. Thus, it remains to be examined whether the combination therapy of clinical doses of fasudil and sildenafil also exerts further beneficial effects on PH.

Conclusions

In conclusion, the combination therapy with fasudil and sildenafil significantly improves pulmonary vascular remodeling, RV dysfunction, and survival in MCT-induced PH in rats.

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Disclosures

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References

1. Humbert M, Sitbon O, Simonneau G. Treatment of pulmonary arterial hypertension. *N Engl J Med* 2004; **351:** 1425–1436.
2. Rubin LJ. Primary pulmonary hypertension. *N Engl J Med* 1997; **336:** 111–117.
3. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest* 2012; **122:** 4306–4313.
4. McLaughlin VV, McGoon MD. Pulmonary arterial hypertension. *Circulation* 2006; **114:** 1417–1431.
5. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, et al. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* 2004; **43:** 13S–24S.
6. Satoh K, Kagaya Y, Nakano M, Ito Y, Ohta J, Tada H, et al. Important role of endogenous erythropoietin system in recruitment of endothelial progenitor cells in hypoxia-induced pulmonary hypertension in mice. *Circulation* 2006; **113:** 1442–1450.
7. Chin KM, Rubin LJ. Pulmonary arterial hypertension. *J Am Coll Cardiol* 2008; **51:** 1527–1538.
8. Badesch DB, Abman SH, Simonneau G, Rubin LJ, McLaughlin VV. Medical therapy for pulmonary arterial hypertension: Updated ACCP evidence-based clinical practice guidelines. *Chest* 2007; **131:** 1917–1928.
9. Hoeper MM, Markevych I, Spiekerkoetter E, Welte T, Niedermeyer J. Goal-oriented treatment and combination therapy for pulmonary arterial hypertension. *Eur Respir J* 2005; **26:** 858–863.
10. Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, et al. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science* 1997; **275:** 1308–1311.
11. Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, et al. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J* 1996; **15:** 1885–1893.
12. Satoh K, Fukumoto Y, Shimokawa H. Rho-kinase: Important new therapeutic target in cardiovascular diseases. *Am J Physiol Heart Circ Physiol* 2011; **301:** H287–H296.
13. Loirand G, Guerin P, Pacaud P. Rho kinases in cardiovascular physiology and pathophysiology. *Circ Res* 2006; **98:** 322–334.
14. Shimokawa H, Takeshita A. Rho-kinase is an important therapeutic target in cardiovascular medicine. *Arterioscler Thromb Vasc Biol* 2005; **25:** 1767–1775.
15. Hall A. Rho GTPases and the Actin Cytoskeleton. *Science* 1998; **279:** 509–514.
16. Chihara K, Amano M, Nakamura N, Yano T, Shibata M, Tokui T, et al. Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. *J Biol Chem* 1997; **272:** 25121–25127.
17. Narumiya S. The small GTPase Rho: Cellular functions and signal transduction. *J Biochem* 1996; **120:** 215–228.
18. Fukumoto Y, Shimokawa H. Recent progress in the management of pulmonary hypertension. *Circ J* 2011; **75:** 1801–1810.
19. Fukumoto Y, Tawara S, Shimokawa H. Recent progress in the treatment of pulmonary arterial hypertension: Expectation for Rho-kinase inhibitors. *Tohoku J Exp Med* 2007; **211:** 309–320.
20. Satoh K, Shimokawa H, Berk BC. Cyclophilin A: Promising new target in cardiovascular therapy. *Circ J* 2010; **74:** 2249–2256.
21. Abe K, Tawara S, Oi K, Hizume T, Uwatoku T, Fukumoto Y, et al. Long-term inhibition of Rho-kinase ameliorates hypoxia-induced pulmonary hypertension in mice. *J Cardiovasc Pharmacol* 2006; **48:** 280–285.
22. Nagaoka T, Morio Y, Casanova N, Bauer N, Gebb S, McMurtry I, et al. Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats. *Am J Physiol* 2004; **287:** L665–L672.
23. Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, et al. Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ*

Res 2004; **94:** 385–393.
24. Do e Z, Fukumoto Y, Takaki A, Tawara S, Ohashi J, Nakano M, et al. Evidence for Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ J* 2009; **73:** 1731–1739.
25. Satoh K, Fukumoto Y, Sugimura K, Miura Y, Aoki T, Nochioka K, et al. Plasma cyclophilin A is a novel biomarker for coronary artery disease. *Circ J* 2013; **77:** 447–455.
26. Fukumoto Y, Yamada N, Matsubara H, Mizoguchi M, Uchino K, Yao A, et al. Double-blind, placebo-controlled clinical trial with a Rho-kinase inhibitor in pulmonary arterial hypertension : A pilot efficacy trial. *Circ J* 2013; **77:** 2619–2625.
27. Zeng Z, Li Y, Jiang Z, Wang C, Li B, Jiang W. The extracellular signal-regulated kinase is involved in the effects of sildenafil on pulmonary vascular remodeling. *Cardiovasc Ther* 2010; **28:** 23–29.
28. Kuang T, Wang J, Pang B, Huang X, Burg ED, Yuan JX, et al. Combination of sildenafil and simvastatin ameliorates monocrotaline-induced pulmonary hypertension in rats. *Pulm Pharmacol Ther* 2010; **23:** 456–464.
29. Guilluy C, Sauzeau V, Rolli-Derkinderen M, Guerin P, Sagan C, Pacaud P, et al. Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol* 2005; **146:** 1010–1018.
30. Tawara S, Fukumoto Y, Shimokawa H. Effects of combined therapy with a Rho-kinase inhibitor and prostacyclin on monocrotaline-induced pulmonary hypertension in rats. *J Cardiovasc Pharmacol* 2007; **50:** 195–200.
31. Pacher P, Nagayama T, Mukhopadhyay P, Batkai S, Kass DA. Measurement of cardiac function using pressure-volume conductance catheter technique in mice and rats. *Nat Protoc* 2008; **3:** 1422–1434.
32. Itoh T, Nagaya N, Fujii T, Iwase T, Nakanishi N, Hamada K, et al. A combination of oral sildenafil and beraprost ameliorates pulmonary hypertension in rats. *Am J Respir Crit Care Med* 2004; **169:** 34–38.
33. Urboniene D, Haber I, Fang YH, Thenappan T, Archer SL. Validation of high-resolution echocardiography and magnetic resonance imaging vs. high-fidelity catheterization in experimental pulmonary hypertension. *Am J Physiol* 2010; **299:** L401–L412.
34. Mouchaers KT, Schalij I, de Boer MA, Postmus PE, van Hinsbergh VW, van Nieuw Amerongen GP, et al. Fasudil reduces monocrotaline-induced pulmonary arterial hypertension: Comparison with bosentan and sildenafil. *Eur Respir J* 2010; **36:** 800–807.
35. Jung SM, Park SS, Kim WJ, Moon SK. Ras/ERK1 pathway regulation of p27KIP1-mediated G1-phase cell-cycle arrest in cordycepin-induced inhibition of the proliferation of vascular smooth muscle cells. *Eur J Pharmacol* 2012; **681:** 15–22.
36. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol* 2001; **21:** 1712–1719.
37. Wang J, Weigand L, Foxson J, Shimoda LA, Sylvester JT. Ca^{2+} signaling in hypoxic pulmonary vasoconstriction: Effects of myosin light chain and Rho kinase antagonists. *Am J Physiol* 2007; **293:** L674–L685.
38. Zhao L, Mason NA, Morrell NW, Kojonazarov B, Sadykov A, Maripov A, et al. Sildenafil inhibits hypoxia-induced pulmonary hypertension. *Circulation* 2001; **104:** 424–428.
39. Dohi K, Onishi K, Gorcsan J 3rd, Lopez-Candales A, Takamura T, Ota S, et al. Role of radial strain and displacement imaging to quantify wall motion dyssynchrony in patients with left ventricular mechanical dyssynchrony and chronic right ventricular pressure overload. *Am J Cardiol* 2008; **101:** 1206–1212.
40. Jiang BH, Tawara S, Abe K, Takaki A, Fukumoto Y, Shimokawa H. Acute vasodilator effect of fasudil, a Rho-kinase inhibitor, in monocrotaline-induced pulmonary hypertension in rats. *J Cardiovasc Pharmacol* 2007; **49:** 85–89.
41. Clozel M, Hess P, Rey M, Iglarz M, Binkert C, Qiu C. Bosentan, sildenafil, and their combination in the monocrotaline model of pulmonary hypertension in rats. *Exp Biol Med* 2006; **231:** 967–973.
42. Voelkel NF, Quaife RA, Leinwand LA, Barst RJ, McGoon MD, Meldrum DR, et al. Right ventricular function and failure: Report of a National Heart, Lung, and Blood Institute working group on cellular and molecular mechanisms of right heart failure. *Circulation* 2006; **114:** 1883–1891.
43. Schafer S, Ellinghaus P, Janssen W, Kramer F, Lustig K, Milting H, et al. Chronic inhibition of phosphodiesterase 5 does not prevent pressure-overload-induced right-ventricular remodelling. *Cardiovasc Res* 2009; **82:** 30–39.
44. Voelkel NF, Gomez-Arroyo J, Abbate A, Bogaard HJ, Nicolls MR. Pathobiology of pulmonary arterial hypertension and right ventricular failure. *Eur Respir J* 2012; **40:** 1555–1565.
45. Sanada S, Asanuma H, Tsukamoto O, Minamino T, Node K, Takashima S, et al. Protein kinase A as another mediator of ischemic preconditioning independent of protein kinase C. *Circulation* 2004; **110:** 51–57.
46. Wolfrum S, Dendorfer A, Rikitake Y, Stalker TJ, Gong Y, Scalia R, et al. Inhibition of Rho-kinase leads to rapid activation of phosphatidylinositol 3-kinase/protein kinase Akt and cardiovascular protection. *Arterioscler Thromb Vasc Biol* 2004; **24:** 1842–1847.
47. Demiryurek S, Kara AF, Celik A, Babul A, Tarakcioglu M, Demiryurek AT. Effects of fasudil, a Rho-kinase inhibitor, on myocardial preconditioning in anesthetized rats. *Eur J Pharmacol* 2005; **527:** 129–140.
48. Bao W, Hu E, Tao L, Boyce R, Mirabile R, Thudium DT, et al. Inhibition of Rho-kinase protects the heart against ischemia/reperfusion injury. *Cardiovasc Res* 2004; **61:** 548–558.
49. Kukreja RC. Cardiovascular protection with sildenafil following chronic inhibition of nitric oxide synthase. *Br J Pharmacol* 2007; **150:** 538–540.
50. Rossoni G, Manfredi B, De Gennaro Colonna V, Berti M, Guazzi M, Berti F. Sildenafil reduces L-NAME-induced severe hypertension and worsening of myocardial ischaemia-reperfusion damage in the rat. *Br J Pharmacol* 2007; **150:** 567–576.
51. Nagy O, Hajnal A, Parratt JR, Vegh A. Sildenafil (Viagra) reduces arrhythmia severity during ischaemia 24h after oral administration in dogs. *Br J Pharmacol* 2004; **141:** 549–551.
52. Kukreja RC, Ockaili R, Salloum F, Yin C, Hawkins J, Das A, et al. Cardioprotection with phosphodiesterase-5 inhibition--a novel preconditioning strategy. *J Mol Cell Cardiol* 2004; **36:** 165–173.
53. Nakamura K, Shimizu J, Kataoka N, Hashimoto K, Ikeda T, Fujio H, et al. Altered nano/micro-order elasticity of pulmonary artery smooth muscle cells of patients with idiopathic pulmonary arterial hypertension. *Int J Cardiol* 2010; **140:** 102–107.
54. Gudi T, Chen JC, Casteel DE, Seasholtz TM, Boss GR, Pilz RB. cGMP-dependent protein kinase inhibits serum-response element-dependent transcription by inhibiting Rho activation and functions. *J Biol Chem* 2002; **277:** 37382–37393.
55. Rikitake Y, Liao JK. Rho GTPases, statins, and nitric oxide. *Circ Res* 2005; **97:** 1232–1235.
56. Takemoto M, Sun J, Hiroki J, Shimokawa H, Liao JK. Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. *Circulation* 2002; **106:** 57–62.
57. Liu AJ, Ling F, Wang D, Wang Q, and Lu XD, Liu YL. Fasudil inhibits platelet-derived growth factor-induced human pulmonary artery smooth muscle cell proliferation by up-regulation of p27kip1 via the ERK signal pathway. *Chin Med J* 2011; **124:** 3098–3104.
58. Wang N, Guan P, Zhang JP, Li YQ, Chang YZ, Shi ZH, et al. Fasudil hydrochloride hydrate, a Rho-kinase inhibitor, suppresses isoproterenol-induced heart failure in rats via JNK and ERK1/2 pathways. *J Cell Biochem* 2011; **112:** 1920–1929.
59. Chen XY, Dun JN, Miao QF, Zhang YJ. Fasudil hydrochloride hydrate, a Rho-kinase inhibitor, suppresses 5-hydroxytryptamine-induced pulmonary artery smooth muscle cell proliferation via JNK and ERK1/2 pathway. *Pharmacology* 2009; **83:** 67–79.
60. Li B, Yang L, Shen J, Wang C, Jiang Z. The antiproliferative effect of sildenafil on pulmonary artery smooth muscle cells is mediated via upregulation of mitogen-activated protein kinase phosphatase-1 and degradation of extracellular signal-regulated kinase 1/2 phosphorylation. *Anesth Analg* 2007; **105:** 1034–1041.
61. Tantini B, Manes A, Fiumana E, Pignatti C, Guarnieri C, Zannoli R, et al. Antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells. *Basic Res Cardiol* 2005; **100:** 131–138.

Supplementary Files

Supplementary File 1

Figure S1. Experimental protocols.

Figure S2. Time-course of body weight (BW) and intake of food and water in the prevention protocol.

Figure S3. Time-courses of body weight (BW) and intake of food and water in the treatment protocol.

Figure S4. Inhibitory effects of the combination therapy on extracellular-signal regulated kinases (ERK) activity.

Figure S5. Effects of the combination therapy on endothelial nitric oxide synthase (eNOS) expression.

Please find supplementary file(s);
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