Eukaryotic elongation factor 2 kinase mediates monocrotaline-induced pulmonary arterial hypertension via reactive oxygen species-dependent vascular remodeling

Satoshi Kameshima1), Kyosuke Kazama1), Muneyoshi Okada1), Hideyuki Yamawaki1)*

1)Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan

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*Correspondence to: Hideyuki Yamawaki, Ph.D.

Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Higashi 23 bancho 35-1, Towada, Aomori 034-8628, Japan.

Phone: +81-176-23-4371

FAX: +81-176-24-9456

E-mail: yamawaki@vmas.kitasato-u.ac.jp
Abstract

Pulmonary arterial (PA) hypertension (PAH) is a progressive and lethal disease that is caused by increased vascular contractile reactivity and structural remodeling. These changes contribute to increasing pulmonary peripheral vascular resistance, finally leading to right heart failure and death. Eukaryotic elongation factor 2 kinase (eEF2K) is a Ca\textsuperscript{2+}/calmodulin-dependent protein kinase. We previously revealed that eEF2K protein increases in mesenteric artery from spontaneously hypertensive rats and partly mediates the development of hypertension via a promotion of reactive oxygen species (ROS)-dependent vascular inflammatory responses, proliferation and migration of vascular smooth muscle cells. However, a role of eEF2K in pathogenesis of PAH is unknown. In the present study, we tested the hypothesis that eEF2K may be involved in the pathogenesis of PAH. PAH was induced by a single intraperitoneal injection of monocrotaline (MCT; 60 mg/kg) to rats. A specific eEF2K inhibitor, A-484954 (2.5 mg/kg/day) was intraperitoneally injected for 14 days. Long-term A-484954 treatment inhibited MCT-induced increased PA pressure. It was revealed that A-484954 inhibited MCT-induced PA hypertrophy and fibrosis but not impairment of endothelium-dependent and -independent relaxation. Furthermore, A-484954 inhibited MCT-induced NADPH oxidase (NOX)-1 expression and ROS generation as well as matrix metalloproteinase (MMP)-2 activation. In conclusion, the present results suggest that eEF2K at least partly mediates the MCT-induced PAH via stimulating vascular structural remodeling perhaps through NOX-1/ROS/MMP-2 pathway.
Keywords: eukaryotic elongation factor 2 kinase; vascular remodeling; reactive oxygen species; matrix metalloproteinase; pulmonary hypertension.
Introduction

Pulmonary hypertension is a mortal disease, which leads to pressure overload-induced right heart failure. Pulmonary hypertension is classified into five groups including pulmonary arterial (PA) hypertension (PAH), left heart disease-induced pulmonary hypertension, chronic lung disease and/or hypoxia-induced pulmonary hypertension, chronic thromboembolic pulmonary hypertension, and pulmonary hypertension due to unclear multifactorial mechanisms. PAH is caused by an abnormal narrowing of lung small intrapulmonary artery. It is demonstrated that this vascular abnormal narrowing is triggered by an increased vascular contractile reactivity and hyperplasia as well as medial hypertrophy. So far, drugs targeting various molecules such as endothelin receptor, phosphodiesterase 5, and prostacyclin have been clinically used. The combination therapy using these drugs dramatically improves the prognosis and quality of life in the PH patients. However, there are limitations in the present drug therapy, and the most progressive primary PH patients cannot be completely recovered unless they have a lung transplantation.

Eukaryotic elongation factor 2 (eEF2) kinase (eEF2K), a member of calmodulin kinase family proteins, phosphorylates a downstream substrate, eEF2. eEF2 facilitates a GTP-derived ribosomal movement from A to P site in the process of protein translation. eEF2K-induced phosphorylation of eEF2 makes it inactive state, which in turn inhibits the protein translation. Expression and activation of eEF2K increase in the tumor tissues from pancreas and breasts, where eEF2K facilitates proliferation and viability of tumor cells through positively modulating the cell
cycle and autophagy (30). It is thus proposed that eEF2K affects the pathogenesis of other cell proliferation-associated diseases than cancer. We previously reported that eEF2K partly mediates the hypertension development in spontaneously hypertensive rats (SHR) through promotion of vascular inflammation, impaired contractile reactivity and structural remodeling via reactive oxygen species (ROS)-dependent mechanisms (26). Furthermore, we demonstrated that eEF2K mediates platelet-derived growth factor-BB-induced vascular smooth muscle cells proliferation and migration (24). Accordingly, we hypothesized eEF2K may also mediate the development of PAH via structural and/or functional changes of intrapulmonary arteries. In this study, we investigated the effects of A-484954, a selective eEF2K inhibitor, on pathogenesis of monocrotaline (MCT)-induced PAH especially focusing on vasculature, and for the first time determined that eEF2K mediates PAH through ROS-dependent mechanisms.
Materials and methods

Animal experiments

Animal care and treatment were performed in accordance with the institutional guidelines of The Kitasato University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar rats (5-week-old, Clea Japan, Tokyo, Japan) were randomly divided into three groups; control group (Cont, n = 9), MCT-injected group (MCT, n = 9) and A-484954-treated MCT-injected group (MCT + A-484954, n = 10). PAH was induced by a single intraperitoneal injection of MCT (60 mg/kg) as described previously (9, 14). Control rats received a single injection of saline. A solvent of A-484954, 0.5% carboxymethyl cellulose (CMC) or A-484954 (2.5 mg/kg/day) was intraperitoneally injected once daily from the day of MCT injection. We chose the intraperitoneal administration by referring to our previous studies (9, 14). Intraperitoneal administration makes it easier to get a stable PAH model in a shorter period of time (14 days) compared to subcutaneous injection. Any rats did not die 14 days after the MCT injection. There is no report using A-484954 in vivo. Accordingly, we chose the dose (2.5 mg/kg/day) by referring to the results of our previous study using NH125, another eEF2K inhibitor, in vivo (hypotensive effect was observed in SHR at 300 μg/kg) and in vitro (inhibitory effect on eEF2K activation was observed in cultured vascular smooth muscle cells at 1 μM) (26). Since we confirmed that 10 μM A-484954 significantly inhibited eEF2K activation in cultured vascular smooth muscle cells (24), we used 2.5 mg/kg A-484954 that is approximately ten times of in vivo NH125 dose. After a measurement of PA pressure at day 14, rats were euthanized by exsanguination under a deep urethane (1.5 g/kg, i.p.)
anesthesia, and heart and lung were isolated. After right ventricle and left ventricle + septum were separated from the heart, wet weight of each tissue was measured. Intrapulmonary arteries (IPAs) isolated from left lung were used for measurement of isometric contraction. Right middle lobes of lung were used for histological and biochemical examinations.

Measurement of mean PA pressure

Mean PA pressure was measured under urethane (1.5 g/kg, i.p.) anesthesia as described previously (9, 14). The catheter filled with a heparin-saline solution was inserted from right external jugular vein into the root of pulmonary artery through right atrial, tricuspid valve, and right ventricle as described previously (9, 14). Catheter was connected to MLT0670 BP transducer (ADInstruments, Colorado Springs, CO). Mean PA pressure was measured and digitally recorded using ML117 BP Amp (ADInstruments) and ML825 PowerLab 2/25 system (ADInstruments).

Measurement of isometric contraction

After two intrapulmonary arterial rings (diameter; 500-1000 μm) were isolated from each rat, they were placed in normal physiological salt solution (PSS), which contained (mM): NaCl 136.9, KCl 5.4, CaCl₂ 1.5, MgCl₂ 1.0, NaHCO₃ 23.8, glucose 5.5 and EDTA 0.001. The PSS was saturated with a 95% O₂-5% CO₂ mixture at 37 °C and pH 7.4. Smooth muscle contractility was recorded isometrically with a force-displacement transducer (Nihon Kohden, Tokyo, Japan) as described
previously (13, 29). Each arterial ring was attached to a holder under a resting tension of 0.5 g as

described previously (9). After equilibration for 30 min in a 3 ml organ bath, each ring was

repeatedly exposed to 72 mM KCl solution until the responses became stable.

Concentration-responses curve was obtained by a cumulative application of acetylcholine (ACh; 1

nM to 30 μM) or sodium nitroprusside (SNP; 100 pM to 3 μM) to the artery precontracted with 100

nM noradrenaline (NA). 100 nM NA is an almost maximally effective concentration in each group.

The normalized precontraction by 72 mM KCl-induced contraction was as followings (%): before

ACh: Cont 74.7 ± 4.6 (n = 14 rings); MCT 103.8 ± 5.4 (n = 9 rings); MCT + A-484954 105.1 ± 8.1 (n = 10 rings), before SNP: Cont 70.9 ± 3.9 (n = 15 rings); MCT 82.7 ± 2.2 (n = 8 rings); MCT +

A-484954 82.7 ± 9.6 (n = 14 rings). E_{max} (maximal relaxation) and pD2 (-log EC_{50}) values were

calculated using Sigma Plot software (Jandel Scientific, Richmond, CA) (Table 2).

Histology

Lung tissues were fixed in 10% neutral buffered formalin. Thin paraffin sections (4 μm)

were made and stained with hematoxylin and eosin as described previously (9, 27). The images were

obtained using a light microscope (BX-51, Olympus, Tokyo, Japan). For evaluating vascular

hypertrophy, the luminal to vessel area ratio (%) of three IPAs (diameter; 50-100 μm) from each lung

section was calculated using Image J software (NIH, Bethesda, MD).
Azan staining

Azan staining was performed as described previously (15). Lung tissues were fixed in 10% neutral buffered formalin and thin paraffin sections (4 μm) were made. The deparaffinized sections were immersed in 5% potassium dichromate solution for 1 h and stained with azocarmine G (Waldeck GmbH & Co KG Division Chroma, Münster, Germany) at room temperature overnight. The sections were immersed in 12-tungsto-(VI)-phosphoric acid n-hydrate solution for 1 h and stained with aniline blue-orange G (Waldeck GmbH & Co KG Division Chroma) for 15 min. The images were obtained using a light microscope (BX-51). For evaluating intrapulmonary arterial fibrosis, the fibrotic area to vessel area ratio (%) of three IPAs from each lung section was calculated using Image J software (NIH).

Western blotting

Western blotting was done as described previously (25). Protein lysates were obtained by homogenizing tissue samples with Triton-based lysis buffer. Protein concentration was measured using a bicinchoninic acid method (Pierce, Rockford, IL). Equal amount of proteins (10-15 μg) were separated by SDS-PAGE (7.5%) and transferred to a nitrocellulose membrane (Pall, Ann Arbor, MI). After blocked with 3% bovine serum albumin (for phosphorylation-specific antibodies) or 0.5% skim milk (for others), membranes were incubated with the following primary antibodies (1:500 dilution): phoso-eEF2K (Ser500), total eEF2K, NADPH oxidase (NOX)-1, and 4-hydroxy-2-nonenal.
(4-HNE) at 4 ℃ overnight and visualized using horseradish peroxidase-conjugated 2nd antibodies (1:10,000 dilution, 45 min at room temperature) and the EZ-ECL system (Biological industries, Kibbutz Beit Haemek, Israel). Equal loading of protein was ensured by measuring total actin expression. The resulting bands were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

Gelatin zymography

The activity of matrix metalloproteinase (MMP)-2 in lung tissues was measured by a gelatin zymography as described previously (15). Protein samples were separated by SDS-PAGE (7.5%) containing 1.8 mg/ml gelatin under a non-reducing condition. After washed for 1 h in the buffer containing 50 mM Tris-HCl (pH 7.4), 2.5% Triton X-100, 5 mM CaCl₂, and 1 μM ZnCl₂, the gels were incubated in the buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM CaCl₂, and 1 μM ZnCl₂ at 37 ℃ for 6 h. Then the gels were stained with 0.1% Coomassie Blue G-250 (Merck KGaA, Darmstadt, Germany) for 20 min and washed with ion-exchanged water until the bands were visible.

Materials

Reagent sources were as followings: MCT (Wako Pure Chemical, Osaka, Japan); ACh (Daiichi-Sankyo, Tokyo, Japan); NA and SNP (Sigma-Aldrich, St. Louis, MO); eEF2K inhibitor (A-484954), 7-amino-1-cyclopropyl-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]
pyrimidine-6-carboxamide (Merck KGaA). A-484954 is a small molecule inhibitor identified from a
chemical library (3). It is a cell permeable pyrido-pyrimidinedione derivative that inhibits the activity
of eEF2K through competitively binding to the ATP-binding legion of eEF2K (5). In an in vitro
enzymatic assay using a mixture of eEF2K and radio-labeled ATP, IC50 value of A-484954 against
eEF2K is approximately 0.28 μM (3), which indicates that A-484954 has high substrate specificity
for eEF2K compared with other inhibitors. MCT was dissolved in 1 N HCl. The pH was neutralized
with 1 N NaOH, and the volume was adjusted with saline to achieve a concentration of 24 mg/ml.
ACh, NA, and SNP were dissolved in distilled water. A-484954 was suspended in 0.5% CMC.

Antibody sources were as followings: phospho-eEF2K (Ser500) (No. EP44511) (ECM
Biosciences, Versailles, KY); total eEF2K (No. GTX107879) (Gene Tex, Irvine, CA); NOX-1 (No.
s-25545) (Santa Cruz Biotechnology, Dallas, TX); total actin (No. MAB1501) (Sigma-Aldrich);
4-HNE (No. MHN-020P) (Japan Institute for the Control of Aging, Shizuoka, Japan).

Statistical analysis

Data were shown as mean ± SEM. Statistical evaluations were performed by one-way
ANOVA followed by Bonferroni’s test. Values of p<0.05 were considered statistically significant.
Results

Effects of long-term A-484954 treatment on MCT-induced increases in mean PA pressure

We first examined the effects of long-term A-484954 (2.5 mg/kg/day, 14 days) treatment on MCT-induced increases in mean PA pressure of rats. Mean PA pressure was significantly elevated in the MCT group compared with Control (from 15.0 ± 0.8 mmHg, n = 8 to 25.6 ± 0.9 mmHg, n = 8, p<0.01). A-484954 significantly inhibited it (17.8 ± 2.1 mmHg, n = 9, p<0.01, Table 1).

Effects of A-484954 on MCT-induced impairment of vasorelaxing function in IPAs

Impaired relaxation in IPAs is important for PAH development. Therefore, we examined the effects of A-484954 on MCT-induced impairment of endothelium-dependent and -independent relaxation. In IPAs from Control, ACh (1 nM to 30 μM, closed circle, n = 14 rings, Fig. 1A) and SNP (100 pM to 3 μM, closed circle, n = 15 rings, Fig. 1B) induced relaxation of the NA-induced precontraction in a concentration-dependent manner (ACh: pD2 = 6.13 ± 0.04, Emax = 73.0 ± 3.3%; SNP: pD2 = 7.61 ± 0.02, Emax = 95.5 ± 1.8%, Table 2). In IPAs from MCT, the relaxation induced by ACh (open circle, n = 9 rings, p<0.05: 1 μM, p<0.01: 3-30 μM, Fig. 1A) and SNP (open circle, n = 8 rings, p<0.01, Fig. 1B) was significantly impaired from Control (ACh: pD2 = 5.99 ± 0.20, Emax = 45.8 ± 6.2%; SNP: pD2 = 6.40 ± 0.30, Emax = 70.4 ± 8.0%, Table 2). Long-term A-484954 treatment did not inhibit the MCT-induced impairment of relaxation either by ACh (open square, n = 10 rings, Fig. 1A) or SNP (open square, n = 14 rings, Fig. 1B) (ACh: pD2 = 6.19 ± 0.10, Emax = 48.0 ± 6.6%;
SNP: pD₂ = 6.74 ± 0.28, E_max = 68.6 ± 6.5%, Table 2).

Effects of A-484954 on MCT-induced PA hypertrophy

PA hypertrophy also plays an important role in the development of PAH. We next examined the effects of A-484954 on MCT-induced hypertrophy in the IPAs. MCT caused decreases in luminal to vessel area ratio of IPAs (from 64.0 ± 5.0 %, n = 9 to 31.4 ± 3.0 %, n = 9, p<0.01, Fig. 2AB).

A-484954 significantly inhibited it (58.8 ± 8.0 %, n = 10, p<0.01, Fig. 2AB).

Effects of A-484954 on MCT-induced expression and activation of eEF2K in lung tissues

Firstly, we confirmed that A-484954 treatment significantly inhibited increased phosphorylation (Ser500) (n = 9-10, p<0.05, Fig. 3) but not expression of eEF2K in MCT (n = 9-10, Fig. 3). eEF2K activity is regulated by multiple phosphorylation sites. It is known that the phosphorylation of Ser500 site leads to activation of eEF2K, which results in the phosphorylation of signaling molecules including ERK, Akt, and p38 (20, 24).

Effects of A-484954 on MCT-induced NOX-1 expression and ROS generation in lung tissues

ROS contribute to the development of MCT-induced PAH (28). We examined the effects of A-484954 on MCT-induced expression of NOX-1, a major ROS producing enzyme as well as ROS generation by Western blotting using antibody against 4-HNE, which is the end product of lipid
peroxidation by ROS (22). The expression of NOX-1 protein (n = 9, p<0.01, Fig. 4A) and 4-HNE
production (n = 4, p<0.05, Fig. 4B) were significantly increased in MCT compared with Control.
A-484954 significantly inhibited the increased expression of NOX-1 protein (n = 10, p<0.05, Fig. 4A) and 4-HNE production (n = 4, p<0.05, Fig. 4B). It should be noted that mRNA expression of
NOX-2 and -4 was unchanged (n = 3-4, data not shown). In addition, we investigated the expression
of antioxidant enzymes including extracellular (EC)-superoxide dismutase (SOD), manganese
(Mn)-SOD, and copper and zinc (Cu/Zn)-SOD. EC-SOD expression decreased in MCT compared
with Control, whereas A-484954 did not prevent it. For Mn-SOD and Cu/Zn-SOD, there are no
differences between the groups (n = 6-7, data not shown).

**Effects of A-484954 on MCT-induced MMP-2 activation and fibrosis in lung tissues**

Finally, we examined activity of MMP-2, a down-stream enzyme of ROS related to vascular
remodeling in lung tissues, and intrapulmonary arterial fibrosis. MMP-2 activity (n = 7-9, p<0.01,
Fig. 5A) and intrapulmonary arterial fibrosis (n = 6, p<0.01, Fig. 5BC) were significantly increased
in MCT compared with Control. A-484954 significantly inhibited the increased MMP-2 activation (n
= 8, p<0.05, Fig. 5A) and intrapulmonary arterial fibrosis (n = 6, p<0.01, Fig. 5BC). It should be
noted that MMP-9 activity was not changed in MCT (n = 2-3, data not shown).
Discussion

PAH is characterized by a persistent increase in PA pressure, that is mainly caused by increased vascular contractile reactivity and hyperplasia as well as hypertrophy (18). In this study, A-484954 inhibited MCT-induced increased PA pressure (Table 1) and intrapulmonary arterial hypertrophy (Fig. 2), but not MCT-induced impairment of endothelium-dependent and -independent relaxation (Fig. 1). Moreover, A-484954 inhibited MCT-induced expression of NOX-1 and ROS generation in lung tissues (Fig. 4). These results suggest that eEF2K mediates vascular structural changes through ROS-dependent mechanisms, but not through functional disorders in MCT-induced PAH. We have previously demonstrated that eEF2K partly mediates elevation of systemic blood pressure in SHR via promotion of ROS in mesenteric arterial smooth muscle cells (26), which is in accordance with the present results.

We showed that MMP-2 activity was elevated in the MCT-injected rat lung tissues, and that A-484954 treatment significantly inhibited it (Fig. 5A). MMP-2 plays a pivotal role in the development of PA hypertrophy and fibrosis. MMP-2 promotes migration of PA endothelial cells and smooth muscle cells, PA smooth muscle cells proliferation, trans-differentiation of adventitial fibroblast into myofibroblast, collagen synthesis, and lymphocyte infiltration into PA media (2). In PA smooth muscle cells from the patients with idiopathic PAH, it was demonstrated that production and activity of MMP-2 increased (11). Another study revealed that high concentration of proMMP-2 was detected in the urine from PAH patients (1). Moreover, expression and activity of MMP-2 were
elevated in the experimental models of pulmonary hypertension (4, 6, 10). On the other hand, MMP-9, an inducible gelatinase, also plays an important role in the pathogenesis of PAH similar to MMP-2 (7). However, MMP-9 activity was not changed by MCT in this study (n = 2-3, data not shown). A report that MMP-9 activity was not increased in PAH (6) supports our results. We suppose that MMP-9 activity may vary dependent on the condition and stage of PAH. It was previously reported that a specific MMPs inhibitor, batimastat prevented the development of hypoxic pulmonary hypertension (8). ROS, the crucial molecule in the pathogenesis of PAH, are important for activation of MMPs (19). In the present study, we showed that A-484954 significantly attenuated MCT-induced pulmonary arterial fibrosis (Fig. 5BC). Poiani et al. revealed that collagen synthesis increased in pulmonary artery from hypoxia-induced pulmonary hypertensive rat (16). Accordingly, it is suggested that eEF2K mediates PA hypertrophy via NOX-1/ROS/MMP-2 pathway leading to fibrosis.

We showed that not only phosphorylation (Ser500) but also expression of eEF2K increased by an MCT injection (Fig. 3). We suppose that the increased eEF2K expression may lead to increased phosphorylation of eEF2K. We previously showed that eEF2K mediates the activation of ERK, Akt, and p38 in rat mesenteric arterial smooth muscle cells (24). It was also reported that these signaling molecules mediate the gene expression of NOX (21). Thus, it is supposed that eEF2K may mediate NOX-1 expression via the activation of ERK, Akt, and p38. Moreover, it was reported that NF-xB and JNK, a downstream signaling molecule for ROS, facilitated MMPs activity (23). We
thus propose that eEF2K may mediate MMP-2 activation via the activation of these signaling molecules. ERK, Akt, and p38 are signaling molecules associated with cell proliferation and migration. We previously demonstrated that eEF2K mediates proliferation and migration of rat mesenteric arterial smooth muscle cells via activation of these molecules (24). Thus, eEF2K may mediate pulmonary arterial hypertrophy via the similar mechanisms in pulmonary arterial smooth muscle cells. A report by Florian et al. that NOX-1 mRNA expression was elevated which mediated the proliferation and migration of PA smooth muscle cells from MCT-injected rats (28) supports our results.

MCT-induced PAH model used in this study is not a complete mimetic for human PAH. For example, the plexiform legion, a characteristic feature in pulmonary artery of human PAH, is not observed in this model. A plexiform legion in addition to medial hypertrophy leads to vascular stenosis, that is a new therapeutic target for PAH. It was reported that MCT injection plus pneumonectomy or hypoxia exposure plus SU-5416, a selective vascular endothelial growth factor receptor blocker caused a plexiform legion (12). Therefore, these models should be examined in the future. In addition, we did not examine the right heart function in this study. Right heart failure is the major predictor for mortality in the PAH patients. If the effects of A-484954 persist for a long period of time, it may improve the right heart failure. That is also our important future target.

In summary, the present results suggest that eEF2K may at least partly mediate PA hypertrophy via NOX-1/ROS/MMP-2 pathway leading to fibrosis, which contributes to the
development of PAH (Fig. 6). Our results suggest eEF2K as a novel pharmaco-therapeutic target for PAH.
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None.


regulates the development of hypertension through oxidative stress-dependent vascular inflammation.


Figure Legends

**Fig. 1.** Effects of A-484954 on monocrotaline (MCT)-induced impairment of endothelium-dependent and -independent relaxation mediated by acetylcholine (ACh, n = 9-14 rings, A) and sodium nitroprusside (SNP, n = 8-15 rings, B), respectively, in intrapulmonary arteries (IPAs) of rats. After saline (Cont) or MCT (60 mg/kg) was intraperitoneally injected to rats, 0.5% CMC (MCT) or A-484954 (MCT + A-484954; 2.5 mg/kg/day) was intraperitoneally treated daily. After 14 days, IPAs were isolated and divided into two rings. Contraction was expressed as relative to the precontraction induced by 100 nM noradrenaline. ACh (1 nM to 30 μM) or SNP (100 pM to 3 μM) was cumulatively applied. *p<0.05 vs. Cont; **p<0.01 vs. Cont.

**Fig. 2.** Effects of A-484954 on MCT-induced pulmonary arterial (PA) hypertrophy. After saline (Cont) or MCT (60 mg/kg) was intraperitoneally injected to rats, 0.5% CMC (MCT) or A-484954 (MCT + A-484954; 2.5 mg/kg/day) was intraperitoneally treated daily. After 14 days, lungs were harvested. (A) Representative hematoxylin and eosin stained lung sections were shown (a: Cont, n = 9; b: MCT, n = 9; c: MCT + A-484954, n = 10). (B) Luminal to vessel area ratio (%) of IPAs (diameter: 50-100 μm) was calculated and shown in the bar graph (n = 9-10). **p<0.01 vs. Cont; ##p<0.01 vs. MCT. Scale bar: 50 μm.

**Fig. 3.** Effects of A-484954 on MCT-induced expression and phosphorylation of eukaryotic
elongation factor 2 kinase (eEF2K) in the lung tissues. After saline (Cont) or MCT (60 mg/kg) was intraperitoneally injected to rats, 0.5% CMC (MCT) or A-484954 (MCT + A-484954; 2.5 mg/kg/day) was intraperitoneally treated daily. After 14 days, lungs were harvested and immediately frozen in -80 °C. After extraction of protein, expression and phosphorylation of eEF2K were determined by Western blotting. The results were shown as fold increase relative to control (expression) or MCT (phosphorylation) (n = 9-10). Equal protein loading was confirmed using total actin antibody. **p<0.01 vs. Cont; #p<0.05 vs. MCT.

**Fig. 4.** Effects of A-484954 on MCT-induced expression of NADPH oxidase (NOX)-1 protein and reactive oxygen species (ROS) generation. After saline (Cont) or MCT (60 mg/kg) was intraperitoneally injected to rats, 0.5% CMC (MCT) or A-484954 (MCT + A-484954; 2.5 mg/kg/day) was intraperitoneally treated daily for 14 days. (A) Expression of NOX-1 protein (n = 9-10) in lung tissues was determined by Western blotting. (B) ROS generation (n = 4) in lung tissues was determined by Western blotting using 4-hydroxy-2-nonenal (4-HNE) antibody. Equal protein loading was confirmed using total actin antibody, and results were shown as fold increase relative to Control. *p<0.05 vs. Cont; **p<0.01 vs. Cont; #p<0.05 vs. MCT.

**Fig. 5.** Effects of A-484954 on MCT-induced activation of matrix metalloproteinase (MMP)-2 in the lung tissues and intrapulmonary arterial fibrosis. After saline (Cont) or MCT (60 mg/kg) was
intraperitoneally injected to rats, 0.5% CMC (MCT) or A-484954 (MCT + A-484954; 2.5 mg/kg/day) was intraperitoneally treated daily for 14 days. (A) MMP-2 activity (n = 7-9) in the lung tissues was determined by a gelatin zymography, and results were shown as fold increase relative to Control. (B) Representative azan stained lung sections were shown (a: Cont, n = 6; b: MCT, n = 6; c: MCT + A-484954, n = 6). (C) The fibrotic area to vessel area ratio (%) of IPAs was calculated and shown in the bar graph (n = 9-10). **p<0.01 vs. Cont; #p<0.05 vs. MCT; ##p<0.01 vs. MCT. Scale bar: 50 μm.

Fig. 6. Summary of the present results. eEF2K mediates ROS production via upregulation of NOX-1 protein, which may lead to increased MMP-2 activity. ROS/MMP-2 may mediate increased PA pressure through PA hypertrophy and fibrosis. A-484954 can prevent these processes. This mechanism may be at least in part responsible for the pathogenesis of pulmonary arterial hypertension.
<table>
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<th>Group</th>
<th>BW (g) ('n = 9')</th>
<th>PAP (mmHg) ('n = 8')</th>
<th>RV (g) ('n = 9')</th>
<th>LV + S (g) ('n = 9')</th>
<th>RV / (LV+S) (%) ('n = 9')</th>
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<td>Cont</td>
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<td>MCT</td>
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<td>17.8±2.1##</td>
<td>0.18±0.01#</td>
<td>0.53±0.02**</td>
<td>34.8±1.4**</td>
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Table 1. Changes in body weight, mean pulmonary arterial pressure, right ventricular weight, left ventricle + septum weight, and right ventricle / (left ventricle + septum) ratio.

MCT: monocrotaline, BW: body weight, PAP: pulmonary arterial pressure, RV: right ventricle, LV + S: left ventricle + septum. *p<0.05 vs. Cont, **p<0.01 vs. Cont, #p<0.05 vs. MCT, ##p<0.01 vs. MCT.
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<td>$E_{\text{max}}$ (%)</td>
<td>95.5±1.8 (n = 15)</td>
<td>70.4±8.0** (n = 8)</td>
<td>68.6±6.5** (n = 14)</td>
</tr>
<tr>
<td>$pD_2$</td>
<td>7.61±0.02 (n = 15)</td>
<td>6.40±0.30 (n = 8)</td>
<td>6.74±0.28 (n = 14)</td>
</tr>
</tbody>
</table>

Table 2. $E_{\text{max}}$ and $pD_2$ values. ACh: acetylcholine, SNP: sodium nitroprusside. **p<0.01 vs. Cont. N represents the number of arterial ring.
Fig. 1

A

Contraction (%)

ACh (log M)

B

Contraction (%)

SNP (log M)
Fig. 2

Luminal area/vessel area (%)

(a) Cont
(b) MCT
(c) MCT + A-484954

Fig. 2
Fig. 3

- p-eEF2K (Ser500) / total eEF2K
- p-eEF2K (Ser500) / total actin
- eEF2K / total actin

MCT
A-484954
- - - + + + + + +

<table>
<thead>
<tr>
<th>Condition</th>
<th>eEF2K / total actin (relative to Cont)</th>
<th>p-eEF2K (Ser500) / total eEF2K (relative to MCT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>MCT</td>
<td>2.0</td>
<td>1.2</td>
</tr>
<tr>
<td>MCT + A-484954</td>
<td>2.0</td>
<td>0.8</td>
</tr>
<tr>
<td>MCT + A-484954</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**p < 0.01**

# p < 0.05
Figure 4

(A) NOX-1 / total actin

- Cont
- MCT
- MCT + A-484954

![Graph showing NOX-1 / total actin](image)

(B) 4-HNE adducts

- Cont
- MCT
- MCT + A-484954

![Graph showing 4-HNE / total actin](image)

Fig. 4
**Fig. 5**

**A**

Pro-MMP-2 and active-MMP-2 levels in response to MCT and A-484954 treatment.

**B**

Images showing fibrosis and vessel area percentage in different conditions.

(a) Control

(b) MCT treated

(c) MCT + A-484954 treated

**C**

Bar graph showing fibrosis/vessel area percentage.

(a) Control

(b) MCT treated

(c) MCT + A-484954 treated
MCT → p-eEF2K (Ser500) → A-484954

- NOX-1
- ROS → MMP-2 activity

Pulmonary arterial remodeling (fibrosis)
Pulmonary arterial hypertension

Fig. 6