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

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Kameshima S, Kazama K, Okada M, Yamawaki H. Eukaryotic elongation factor 2 kinase mediates monocrotaline-induced pulmonary arterial hypertension via reactive oxygen species-dependent vascular remodeling. Am J Physiol Heart Circ Physiol 308: H1298–H1305, 2015. First published March 13, 2015; doi:10.1152/ajpheart.00864.2014.—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 Ca²⁺/calmodulin-dependent protein kinase. We previously revealed that eEF2K protein increases in the mesenteric artery from spontaneously hypertensive rats and partly mediates the development of hypertension via a promotion of ROS-dependent vascular inflammatory responses and proliferation and migration of vascular smooth muscle cells. However, a role of eEF2K in the 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⁻¹·day⁻¹), 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 endothelial-dependent and -independent relaxation. Furthermore, A-484954 inhibited MCT-induced NADPH oxidase-1 activation and ROS generation as well as matrix metalloproteinase-2 activation. In conclusion, the present results suggest that eEF2K at least partly mediates MCT-induced PAH via stimulation of vascular structural remodeling perhaps through NADPH oxidase-1/ROS/matrix metalloproteinase-2 pathway.
eukaryotic elongation factor 2 kinase; vascular remodeling; reactive oxygen species; matrix metalloproteinase; pulmonary hypertension

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 (17, 18). PAH is caused by an abnormal narrowing of lung small intrapulmonary arteries (IPAs). It has been demonstrated that this vascular abnormal narrowing is triggered by an increased vascular contractile reactivity and hyperplasia as well as medial hypertrophy (18). So far, drugs targeting various molecules such as the endothelin receptor, phosphodiesterase 5, and prostacyclin have been clinically used. Combination therapy using these drugs dramatically improves the prognosis and quality of life in patients with pulmonary hypertension. However, there are limitations in the present drug therapy, and the most progressive primary pulmonary hypertension patients cannot completely recover 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 GTP-derived ribosomal movement from the A to P site in the process of protein translation. eEF2K-induced phosphorylation of eEF2 changes it into an inactive state, which, in turn, inhibits the protein translation. Expression and activation of eEF2K increase in tumor tissues from the pancreas and breasts, where eEF2K facilitates the proliferation and viability of tumor cells through positively modulating the cell cycle and autophagy (30). Thus, it has been proposed that eEF2K affects the pathogenesis of other cell proliferation-associated diseases than cancer. We previously reported that eEF2K partly mediates the development of hypertension in spontaneously hypertensive rats (SHRs) through the promotion of vascular inflammation, impaired contractile reactivity, and structural remodeling via ROS-dependent mechanisms (26). Furthermore, we demonstrated that eEF2K mediates platelet-derived growth factor-BB-induced vascular smooth muscle cell proliferation and migration (24). Accordingly, we hypothesized eEF2K may also mediate the development of PAH via structural and/or functional changes of IPAs. In the present study, we investigated the effects of A-484954, a selective eEF2K inhibitor, on the pathogenesis of monocrotaline (MCT)-induced PAH, especially focusing on the vasculature, and, for the first time, determined that eEF2K mediates PAH through ROS-dependent mechanisms.

MATERIALS AND METHODS

Animal experiments. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Kitasato University.Male Wistar rats (5 wk old, Clea Japan, Tokyo, Japan) were randomly divided into three groups: a control group (Cont group; n = 9), an MCT-injected group (MCT group, n = 9), and an A-484954-treated MCT-injected group (MCT + A-484954 group, n = 10). PAH was induced by a single intraperitoneal injection of MCT (60 mg/kg) as previously described (9, 14). Rats in the Cont group received a single injection of saline. A solvent of A-484954, 0.5% carboxymethyl cellulose, or A-484954 (2.5 mg·kg⁻¹·day⁻¹) 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...
Muscle contractility was recorded isometrically with a force-displacement transducer with a 95% O2-5% CO2 mixture at 37°C and pH 7.4. Smooth muscle contraction was pharmacologically evaluated by subcutaneous injection. No rats died 14 days after the MCT injection.

Table 1. Changes in body weight, mean pulmonary arterial pressure, right ventricular weight, left ventricular + septal weight, and the right ventricle-to-(left ventricle + septum) ratio

<table>
<thead>
<tr>
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<th>Cont group</th>
<th>MCT group</th>
<th>MCT + A-484954 group</th>
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<tbody>
<tr>
<td>Body Weight, g</td>
<td>226.3 ± 4.3</td>
<td>199.9 ± 3.8</td>
<td>191.8 ± 5.2†</td>
</tr>
<tr>
<td>Pulmonary Artery Pressure, mmHg</td>
<td>15.0 ± 0.8</td>
<td>25.6 ± 0.9†</td>
<td>17.8 ± 2.1§</td>
</tr>
<tr>
<td>Right Ventricular Weight, g</td>
<td>0.17 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.18 ± 0.01‡</td>
</tr>
<tr>
<td>Left Ventricular + Septal Weight, g</td>
<td>0.59 ± 0.02</td>
<td>0.54 ± 0.01*</td>
<td>0.53 ± 0.02†</td>
</tr>
<tr>
<td>Right Ventricle-to-(Left Ventricle + Septum) Ratio, %</td>
<td>28.4 ± 1.3</td>
<td>39.2 ± 1.8†</td>
<td>34.8 ± 1.4†</td>
</tr>
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n, Number of animals. Cont, control (saline); MCT, monocrotaline. *P < 0.05 vs. the Cont group; †P < 0.01 vs. the Cont group; ‡P < 0.05 vs. the MCT group; §P < 0.01 vs. the MCT group.

with subcutaneous injection. No rats died 14 days after the MCT injection. There have been no reports using A-484954 in vivo. Accordingly, we chose the dose of A-484954 (2.5 mg·kg⁻¹·day⁻¹) by referring to the results of our previous study (26) using NH-125, another eEF2K inhibitor, in vivo (a hypotensive effect was observed in SHR at 300 μg/kg) and in vitro (an inhibitory effect on eEF2K activation was observed in cultured vascular smooth muscle cells at 1 μM). Since we confirmed that at 10 μM A-484954 significantly inhibited eEF2K activation in cultured vascular smooth muscle cells (24), we used 2.5 mg/kg A-484954, which is ~10 times the in vivo NH-125 dose. After PA pressure was measured on day 14, rats were euthanized by exsanguination under deep urethane (1.5 g/kg ip) anesthesia, and the heart and lungs were isolated. After the right ventricle and left ventricle + septum were separated from the heart, the wet weight of each tissue was measured. IPAs isolated from the left lung were used for measurement of isometric contraction. Right middle lobes of the lung were used for histological and biochemical examinations.

Measurement of mean PA pressure. Mean PA pressure was measured under urethane (1.5 g/kg ip) anesthesia as previously described (9, 14). A catheter filled with heparin-saline solution was inserted from the right external jugular vein into the root of the pulmonary artery through the right atrium, tricuspid valve, and right ventricle as previously described (9, 14). A catheter filled with heparin-saline solution was inserted from the right external jugular vein into the root of the pulmonary artery precontracted with 100 nM norepinephrine (NE). NE at 100 nM was an almost maximally effective concentration in each group. The normalized precontraction by 72 mM KCl-induced contraction was as described (15). Lung tissues were fixed in 10% neutral buffered formalin and stained with azocarmine G (Waldeck, Division Chroma, Münster, Germany) at room temperature overnight. Sections were immersed in 5% potassium dichromate solution for 1 h and stained with aniline blue-orange G (Waldeck, Division Chroma) for histological examination. Sections were immersed in 5% potassium dichromate solution for 1 h and stained with azocarmine G (Waldeck, Division Chroma) for histological examination.

Histology. Lung tissues were fixed in 10% neutral buffered formalin. Thin paraffin sections (4 μm) were made and stained with hematoxylin and eosin as previously described (9, 27). Images were obtained using a light microscope (BX-51, Olympus, Tokyo, Japan). To evaluate vascular hypertrophy, the luminal-to-vessel area ratio (in %) of three IPAs (diameter: 50–100 μm) was measured. IPAs isolated from the left lung were used for measurement of isometric contraction. Right middle lobes of the lung were used for histological and biochemical examinations.

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

Measurement of isometric contraction. After two intra-PA rings (diameter: 500–1,000 μm) were isolated from each rat, they were placed in normal physiological salt solution, which contained (in mM) 136.9 NaCl, 5.4 KCl, 1.5 CaCl₂, 1.0 MgCl₂, 23.8 NaHCO₃, 5.5 glucose, and 0.001 EDTA. The physiological salt solution 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 previously described (13, 29). Each arterial ring was attached to a holder under a resting tension of 0.5 g as previously described (9). After equilibration for 30 min in a 3-ml organ bath, each ring was repeatedly exposed to 72 mM KCl solution until responses became stable. A concentration-response curve was obtained by the cumulative application of ACh (1 nM–30 μM) or sodium nitroprusside (SNP; 100 pM–3 μM) to the artery precontracted with 100 nM norepinephrine (NE). NE at 100 nM was an almost maximally effective concentration in each group. The normalized precontraction by 72 mM KCl-induced contraction was as follows: before ACh, 47.7 ± 4.6% in the Cont group (n = 14 rings), 103.8 ± 5.4% in the MCT group (n = 9 rings), and 105.1 ± 8.1% in the MCT + A-484954 group (n = 10 rings); and before SNP, 70.9 ± 3.9% in the Cont group (n = 15 rings), 82.7 ± 2.2% in the MCT group (n = 8 rings), and 82.7 ± 9.6% in the MCT + A-484954 group (n = 14 rings). Maximal relaxation (Eₘ₉₅) and −log EC₅₀ (pD₂) values were calculated using Sigma Plot software (Jandel Scientific, Richmond, CA).

ACh stimulation. ACh stimulation was performed as previously described (15). Lung tissues were fixed in 10% neutral buffered formalin, and thin paraffin sections (4 μm) were made. Deparaffinized sections were immersed in 5% potassium dichromate solution for 1 h and stained with azocarmine G (Waldeck, Division Chroma, Münster, Germany) at room temperature overnight. Sections were immersed in 12-tungstos-(V)-phosphoric acid n-hydrate solution for 1 h and stained with aniline blue-orange G (Waldeck, Division Chroma) for 15 min. Images were obtained using a light microscope (BX-51). To evaluate intra-PA fibrosis, the fibrotic area-to-vessel area ratio (in %) was calculated as relative to the precontraction induced by ACh (n = 9–14 rings, A) and sodium nitroprusside (SNP; n = 8–15 rings, B), respectively, in intrapulmonary arteries (IPAs) of rats. After saline [control (Cont) group] or MCT (60 mg/kg) was intraperipherically injected to rats, 0.5% carboxymethyl cellulose (OMC; MCT group) or A-484954 (MCT + A-484954 group; 2.5 mg·kg⁻¹·day⁻¹) was intraperipherically injected daily. After 14 days, IPAs were isolated and divided into two rings. Contraction was expressed as a percentage of the precontraction induced by 100 nM norepinephrine (NE). ACh (1 nM–30 μM) or SNP (100 pM–3 μM) was cumulatively applied. *P < 0.05 vs. the Cont group; **P < 0.01 vs. the Cont group.
of three IPAs from each lung section was calculated using ImageJ software (NIH).

**Western blot analysis.** Western blot analysis was performed as previously described (25). Protein lysates were obtained by homogenizing tissue samples with Triton X-100-based lysis buffer. Protein concentration was measured using the bicinchoninic acid method (Pierce, Rockford, IL). Equal amount of proteins (10–15 g) was separated by SDS-PAGE (7.5%) and transferred to nitrocellulose membranes (Pall, Ann Arbor, MI). After being blocked with 3% BSA (for phosphorylation-specific antibodies) or 0.5% skim milk (for others), membranes were incubated with the following primary antibodies (1:500 dilution): phospho-eEF2K (Ser500), total eEF2K, NADPH oxidase (NOX)-1, and 4-hydroxy-2-nonenal (4-HNE) at 4°C overnight and visualized using horseradish peroxidase-conjugated secondary 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 previously described (15). Protein samples were separated by SDS-PAGE (7.5%) containing 1.8 mg/ml gelatin under a nonreducing condition. After being washed for 1 h in buffer containing 50 mM Tris·HCl (pH 7.4), 2.5% Triton X-100, 5 mM CaCl₂, and 1 mM ZnCl₂, gels were incubated in buffer containing 50 mM Tris·HCl (pH 7.4), 5 mM CaCl₂, and 1 mM ZnCl₂ at 37°C for 6 h. Gels were then stained with 0.1% Coomassie blue G-250 (Merck, Darmstadt, Germany) for 20 min and washed with ion-exchanged water until bands were visible.

**Materials.** Reagent sources were as follows: MCT (Wako Pure Chemical, Osaka, Japan), ACh (Daiichi-Sankyo, Tokyo, Japan), NE and SNP (Sigma-Aldrich, St. Louis, MO), and eEF2K inhibitor A-484954 (7-amino-1-cyclopropyl-3-ethil-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carboxamide; Merck). A-484954 is a small-molecule inhibitor identified from a chemical library (3). It is a

### Table 2. $E_{\text{max}}$ and $pD_2$ values

<table>
<thead>
<tr>
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<th>Cont Group</th>
<th>MCT Group</th>
<th>MCT + A-484954 Group</th>
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<tbody>
<tr>
<td></td>
<td>Means ± SE</td>
<td>n</td>
<td>Means ± SE</td>
</tr>
<tr>
<td>ACh $E_{\text{max}}$, %</td>
<td>73.0 ± 3.3</td>
<td>14</td>
<td>45.8 ± 6.2*</td>
</tr>
<tr>
<td>pD₂</td>
<td>6.13 ± 0.04</td>
<td>14</td>
<td>5.99 ± 0.20</td>
</tr>
<tr>
<td>Sodium nitroprusside $E_{\text{max}}$, %</td>
<td>95.5 ± 1.8</td>
<td>15</td>
<td>70.4 ± 8.0*</td>
</tr>
<tr>
<td>pD₂</td>
<td>7.61 ± 0.02</td>
<td>15</td>
<td>6.40 ± 0.30</td>
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$\bar{n}$, Number of arterial rings. $E_{\text{max}}$, maximal relaxation; pD₂, $-\log EC_{50}$. *P < 0.01 vs. the Cont group.

Fig. 2. Effects of A-484954 on MCT-induced pulmonary arterial hypertrophy. After saline (Cont group) or MCT (60 mg/kg) was intraperitoneally injected to rats, 0.5% CMC (MCT group) or A-484954 (MCT + A-484954 group; 2.5 mg·kg$^{-1}$·day$^{-1}$) was intraperitoneally injected daily. After 14 days, lungs were harvested. A: representative hematoxylin and eosin-stained lung sections from Cont ($n = 9$; a), MCT ($n = 9$; b), and MCT + A-484954 ($n = 10$; c) groups. Scale bars: 50 μm. B: luminal-to-vessel area ratio (in %) of IPAs (diameter: 50–100 μm) was calculated ($n = 9–10$). **P < 0.01 vs. the Cont group; $$$P < 0.01$ vs. the MCT group.
cell-permeable pyrido-pyrimidinedione derivative that inhibits the activity of eEF2K through competitively binding to the ATP-binding region of eEF2K (5). In an in vitro enzymatic assay using a mixture of eEF2K and radiolabeled ATP, the IC_{50} value of A-484954 against eEF2K was ~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, NE, and SNP were dissolved in distilled water. A-484954 was suspended in 0.5% carboxymethyl cellulose.

Antibody sources were as follows: phospho-eEF2K (Ser^{500}, EP4451, ECM Biosciences, Versailles, KY), total eEF2K (GTX107879, Gene Tex, Irvine, CA), NOX-1 (sc-25545, Santa Cruz Biotechnology, Dallas, TX), total actin (MAB1501, Sigma-Aldrich), and 4-HNE (MHN-020P, Japan Institute for the Control of Aging, Shizuoka, Japan).

Statistical analysis. Data are presented as means ± SE. Statistical evaluations were performed by one-way ANOVA followed by Bonferroni’s test. P values of <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^{-1}·day^{-1}, 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 the Cont group (from 15.0 ± 0.8 mmHg, n = 8, to 25.6 ± 0.9 mmHg, n = 8, P < 0.01). A-484954 significantly inhibited mean PA pressure (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 the development of PAH. Therefore, we examined the effects of A-484954 on MCT-induced impairment of endothelium-dependent and -independent relaxation. In IPAs from the Cont group, ACh (1 nM—30 μM, n = 14 rings; Fig. 1A, solid circles) and SNP (100 μM–3 μM, n = 15 rings; Fig. 1B, solid circles) induced relaxation of NE-induced precontractions in a concentration-dependent manner (ACh: pD_{2} = 6.13 ± 0.04 and E_{max} = 73.0 ± 3.3%; and SNP: pD_{2} = 7.61 ± 0.02 and E_{max} = 95.5 ± 1.8%; Table 2). In IPAs from the MCT group, the relaxation induced by ACh (n = 9 rings, P < 0.05: 1 μM and P < 0.01: 3–30 μM; Fig. 1A, open circles) and SNP (n = 8 rings, P < 0.01; Fig. 1B, open circles) was significantly impaired compared with the Cont group (ACh: pD_{2} = 5.99 ± 0.20 and E_{max} = 45.8 ± 6.2%; and SNP: pD_{2} = 6.40 ± 0.30 and E_{max} = 70.4 ± 8.0%; Table 2). Long-term A-484954 treatment did not inhibit the MCT-induced impairment of relaxation either by ACh (n = 10 rings; Fig. 1A, open squares) or SNP (n = 14 rings, Fig. 1B, open squares) (ACh: pD_{2} = 6.19 ± 0.10 and E_{max} = 48.0 ± 6.6%; and SNP: pD_{2} = 6.74 ± 0.28 and 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 IPAs. MCT caused decreases in the relaxation induced by ACh (n = 9 rings, P < 0.05: 1 μM and P < 0.01: 3–30 μM; Fig. 1A, open circles) (ACh: pD_{2} = 6.13 ± 0.04 and E_{max} = 73.0 ± 3.3%; and SNP: pD_{2} = 7.61 ± 0.02 and E_{max} = 95.5 ± 1.8%; Table 2).

Effects of A-484954 on MCT-induced expression and activation of eEF2K in lung tissues. First, we confirmed that A-484954 treatment significantly inhibited the increased phosphorylation (Ser^{500}, n = 9–10, P < 0.05; Fig. 3) but not expression of eEF2K in the MCT group (n = 9–10; Fig. 3).
eEF2K activity is regulated by multiple phosphorylation sites. It is known that phosphorylation of the Ser^500 site leads to the 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 blot analysis using an antibody against 4-HNE, which is the end product of lipid peroxidation by ROS (22). 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 the MCT group compared with the Cont group. 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 SOD, Mn-SOD, and Cu/Zn-SOD. Extracellular SOD expression decreased in the MCT group compared with the Cont group, whereas A-484954 did not prevent it. For Mn-SOD and Cu/Zn-SOD, there were no differences among groups (n = 6–7; data not shown).

**DISCUSSION**

PAH is characterized by a persistent increase in PA pressure, which is mainly caused by increased vascular contractile reactivity and hyperplasia as well as hypertrophy (18). In the present study, A-484954 inhibited MCT-induced increased PA pressure (Table 1) and intra-PA 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 the elevation of systemic blood pressure in SHRs via the 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 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 the migration of PA endothelial cells and smooth muscle cells, PA smooth muscle cells proliferation, transdifferentiation of adventitial fibroblasts into myofibroblasts, collagen synthesis, and lymphocyte infiltration into PA media (2). In PA smooth muscle cells from patients with idiopathic PAH, it has been demonstrated that the production and activity of MMP-2 are increased (11). Another study (1) revealed that a high concentration of proMMP-2 was detected in urine from PAH patients. Moreover, expression and activity of MMP-2 were elevated in 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 (4, 6, 10). A report (6) showing that MMP-9 activity was not increased in PAH supports our results. We suppose that MMP-9 activity may vary dependent on the condition and stage of PAH. It has been previously reported that a specific MMP inhibitor, batimastat, prevented the development of hypoxic pulmonary hypertension (8). ROS, which are crucial molecules in the pathogenesis of PAH, are important for the activation of MMPs (19). In the present study, we showed that A-484954 significantly attenuated MCT-induced PA fibrosis (Fig. 5, B and C). Poiani et al. (16) revealed that collagen synthesis increased in PAs from
hypoaxia-induced pulmonary hypertensive rats. Accordingly, it has been suggested that eEF2K mediates PA hypertension via a NOX-1/ROS/MMP-2 pathway leading to fibrosis.

We showed that not only the phosphorylation (Ser^500) but also expression of eEF2K increased by 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 has also been 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 has been reported that NF-kB and JNK, a downstream signaling molecule for ROS, facilitate MMP 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 have previously demonstrated that eEF2K mediates the proliferation and migration of rat mesenteric arterial smooth muscle cells via activation of these molecules (24). Thus, eEF2K may mediate PA hypertension via similar mechanisms in PA smooth muscle cells. A report by Veit et al. (28) showing that NOX-1 mRNA expression was elevated, which mediated the proliferation and migration of PA smooth muscle cells from MCT-injected rats, supports our results.

The MCT-induced PAH model used in this study is not a complete mimic for human PAH. For example, plexiform lesions, characteristic features in PAs of human PAH, were not observed in this model. Plexiform lesions in addition to medial hypertrophy lead to vascular stenosis, which is a new therapeutic target for PAH. It has been reported that MCT injection plus pneumonectomy or hypoxia exposure plus SU-5416, a selective vascular endothelial growth factor receptor blocker, caused plexiform lesions (12). Therefore, these models should be examined in the future. In addition, we did not examine right heart function in this study. Right heart failure is the major predictor for mortality in PAH patients. If the effects of A-484954 persist for a long period of time, it may improve right heart failure. This is also an important future target for us.

In summary, the present results suggest that eEF2K may at least partly mediate PA hypertension via a 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 pharmacotherapeutic target for PAH.

**DISCLOSURES**
No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**
Author contributions: S.K., K.K., M.O., and H.Y. conception and design of research; S.K., K.K., and M.O. performed experiments; S.K. analyzed data; S.K., M.O., and H.Y. interpreted results of experiments; S.K. prepared figures; S.K. drafted manuscript; S.K., K.K., M.O., and H.Y. approved final version of manuscript; H.Y. edited and revised manuscript.

**REFERENCES**


