

Original Article

Effects of *in vivo* treatment with K_v7.4 activator, URO-K10, on the impaired relaxation of pulmonary arteries in the monocrotaline-induced pulmonary hypertensive rats

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ABSTRACT Pulmonary arterial hypertension (PAH) is a fatal disease marked by increased pulmonary vascular resistance and right ventricular (RV) failure. Impaired vascular relaxation and vasoconstrictive signaling, including Rho-associated kinase (ROCK2) upregulation and myosin phosphatase target subunit 1 (MYPT1) downregulation, contribute to disease progression. We investigated the therapeutic effects of URO-K10, a novel K_v7.4 channel activator, in a monocrotaline-induced rat model of PAH (PAH-MCT). In PAH-MCT rats, chronic URO-K10 administration improved body weight gain, and significantly reduced RV hypertrophy. Functional studies revealed enhanced pulmonary artery relaxation, while relaxation after high K⁺-induced contraction showed only partial recovery. Immunoblot analysis demonstrated that ROCK2 upregulation was reversed by URO-K10, but MYPT1 remained downregulated and MLC2 diphosphorylation persisted. Interestingly, treatment with 8-Br-cGMP restored delayed relaxation and reduced MLC2 phosphorylation in URO-K10-treated PAH-MCT while not in the untreated PAH-MCT rats, suggesting that cGMP supplementation can compensate for the recovery from impaired endogenous signaling by the URO-K10 application. These findings suggest that URO-K10 improves pulmonary hemodynamics and RV remodeling via K_v7.4 activation and downregulation of ROCK2. However, incomplete recovery of MYPT1 and MLC2 phosphorylation highlights the complexity of contractile regulation in PAH. K_v7.4 activation represents a promising therapeutic approach but may require combination strategies to fully restore vascular function in PAH.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive and often fatal disease characterized by increased pulmonary vascular resistance, leading to elevated pulmonary arterial pressure and eventual right ventricular (RV) failure [1]. The estimated prevalence of PAH is 15–50 cases per million individuals, with idiopathic PAH accounting for a significant proportion of cases [2,3].

Clinical manifestations include dyspnea, fatigue, chest pain, and syncope, with disease progression leading to severe functional impairment and poor prognosis [4]. The pathophysiology of PAH involves endothelial dysfunction, excessive vasoconstriction, chronic inflammation, and abnormal vascular remodeling [5,6].



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Author contributions: All authors contributed to the conception and design of the study, and reviewed and approved the final manuscript. S.B.O. performed the isometric myography measurements. S.B.O. and Y.K.J. analyzed the histological changes. S.B.O. and N.C. conducted the immunoblotting assays. S.J.K. drafted the initial version of the manuscript. H.Y.Y. designed and supervised the vascular smooth muscle study. S.J.K. oversaw the entire study process.

Altered contractility: ROCK upregulation and MYPT1 downregulation in PAH

Pulmonary arterial contractility is different from the systemic arteries such as mesenteric arteries (MAs). Pulmonary arteries (PAs) exhibit slower relaxation than MAs after depolarization, which correlates with higher Rho-associated coiled-coil containing protein kinase 2 (ROCK2) and lower myosin phosphatase target subunit (MYPT1) expression, leading to increased diphosphorylated myosin regulatory light chain (MLC2) [7]. In addition, we have reported an impaired relaxation of PAs from the rat PAH model induced by monocrotaline injection (PAH-MCT). In the study, the recovery from tonic contraction induced by high K^+ (80 mM KCl) by washout with physiological salt solution (PSS) was significantly slower in PAH-MCT than the control group (CON) [8].

Phosphorylation of Ser19 (S19-p) on MLC2 is essential for arterial contraction, and additional Thr18/Ser19 diphosphorylation (T18/S19-pp) is associated with vasospastic diseases. In the PAH-MCT rats, we observed delayed relaxation of PAs, which linked to increased S19-p and T18/S19-pp levels. Proteomics and immunoblot analyses showed decreased soluble guanylate cyclase (sGC) and MYPT1 along with increased ROCK2. The downregulated sGC and MYPT1 and the upregulated ROCK indicate the decrease of pro-relaxing regulation and the increase of the pro-contractile signals, respectively [8].

Despite the availability of pharmacological treatments such as prostacyclin analogs, phosphodiesterase-5 inhibitors, and endothelin receptor antagonists, the current therapies remain palliative and do not fully reverse pulmonary vascular remodeling [9]. Therefore, it is strongly requested to investigate novel therapeutic targets to restore pulmonary arterial function and attenuate disease progression. Among the mechanisms regulating the vascular contractility, here we focused on the K^+ channel activity in pulmonary arterial smooth muscle cells (PASMCs).

K^+ channels and PAH

Previous studies have implicated ion channel dysfunction in PAH pathogenesis [10]. In general, Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels induces vasoconstriction, which is antagonized by activation of K^+ channels such as the voltage-gated K^+ (K_v) channels [11]. Among the members of K_v channel family, downregulation of $K_v1.5$ and $K_v2.1$ was reported in PAH, which could result in sustained membrane depolarization, increased Ca^{2+} influx, and excessive vasoconstriction [12,13]. While the previous studies have focused on the $K_v1.5$ and $K_v2.1$ channel subtype, K_v7 family K^+ channels, particularly $K_v7.4$ and $K_v7.5$, have recently gained attention due to their role in modulating vascular tone in systemic and pulmonary circulations [14].

The expression of $K_v7.4$ (KCNQ4) in PASMCs remains a subject of debate. Some studies have reported downregulation of

$K_v7.4$ in PAH, suggesting that its reduced activity contributes to membrane depolarization and increased cytosolic calcium concentration ($[Ca^{2+}]_i$) [15]. Conversely, recent studies have provided evidence showing no change in $K_v7.4$ expression in human patients with PAH [16]. Among the latter studies, we have demonstrated that the pharmacological activation of $K_v7.4$ using URO-K10 resulted in enhanced PA relaxation and suppression of PASMC proliferation [17]. URO-K10 is a relatively selective and potent activator of $K_v7.4$ [18]. These findings highlight $K_v7.4$ as a potential therapeutic target for modulating pulmonary vascular resistance in PAH.

Building on these previous findings, the present study aimed to investigate whether *in vivo* treatment with URO-K10 in PAH-MCT rats could improve the phenotypes of increased pulmonary vascular resistance, such as RV hypertrophy and body weight retardation. Also, we invested the restoration of PA contractility, with a particular focus on the possibility of recovery from the impaired relaxation. Additionally, we sought to determine whether the dysregulated signaling proteins involved in contractile regulation, previously identified in our prior studies [8], could be normalized following URO-K10 administration *in vivo*.

METHODS

Animal model preparation

All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee of Seoul National University (approval number: SNU-230424-3-3). Monocrotaline (Sigma) was dissolved in 2 ml of 1 M HCl and adjusted to pH 7.4 using 2 M NaOH solution. This aqueous solution was diluted to 17 ml with distilled water. 7 weeks-old male Sprague-Dawley rats were randomly assigned and treated with a single intraperitoneal injection of monocrotaline (60 mg/kg) to induce PAH-MCT model or an appropriate amount of saline as CON. After 21 days, both the PAH-MCT and the age-matched CON rats were sacrificed for further analysis, after measuring their body weight.

To establish the PAH-MCT/UK10 model for PAH, monocrotaline was administered via a single intraperitoneal injection. On day 3 post-injection, a small osmotic pump (RWD 2004W, 0.25 μ l/hr, RWD Life Science) was filled with either 0.9% saline (vehicle) or a solution of URO-K10 (100 μ M) diluted in dimethyl sulfoxide and 15% ethanol/saline. The pump was then incubated in a water bath set at 37°C with saline for 24 h before implantation, ensuring the continuous administration of URO-K10 (33 μ g/kg/hr) throughout the disease progression.

Isolation of PA and MA

After rapid excision of the rat lung tissue, it was preserved in

ice-cold normal Tyrode (NT) solution (140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose, 1.8 mM CaCl₂, and 1 mM MgCl₂, adjusted to pH 7.4 using NaOH). The tissue was then secured in a 100 mm silicone-coated Petri dish and observed under a microscope. The lung and bronchial tissues were carefully removed to expose and isolate the PA. The excised PA was further refined by removing surrounding adipose and other connective tissues.

Blood pressure measurement

Arterial blood pressure (systolic and diastolic) was measured at the conclusion of the study using a computerized tail-cuff method (CODA 4; Kent Scientific Corporation). Prior to measurement, the rats underwent a three-day habituation period, during which they were placed on a plastic rack mounted on a thermostatically controlled hot plate maintained at 37°C. Following habituation, a test session was conducted, comprising 20 consecutive measurements of blood pressure and heart rate. All measurements were performed between 18:00 and 20:00 h to minimize diurnal variations.

Right ventricle hypertrophy measurement

The hearts were rapidly excised from anesthetized rats and perfused with NT solution using the Langendorff system. After complete removal of blood from the heart, the left and right ventricles were visually identified and dissected. The septum (S) was considered part of the left ventricle (LV). The excised heart tissues were weighed, and RV hypertrophy was assessed as the ratio of RV weight to the sum of left ventricular and septal weight (RV/(LV + S)).

For histological analysis, the hearts were perfused using the Langendorff system to remove blood, and only the left lung was dissected and fixed in 4% paraformaldehyde. The heart and lung tissues were then transversely sectioned and embedded in paraffin. Tissue sections were cut at a thickness of 5 µm and stained with hematoxylin and eosin (H&E). Cardiac fibrosis was evaluated using Masson's trichrome staining. Stained tissue slides were scanned at 400× magnification using the Aperio AT2 scanner (Leica), and images were analyzed with Aperio ImageScope 12.3 software (Leica).

Isometric tension recordings

The second or third branches of the PA and MA of rats were carefully dissected into approximately 2 mm segments and mounted on a multi-wire myograph system (620M; Danish Myo Technology) using 25 µm tungsten wires. The mounted arterial segments were stabilized in PSS (118 mM NaCl, 4 mM KCl, 24 mM NaHCO₃, 1 mM MgSO₄, 0.44 mM NaH₂PO₄, 5.6 mM glucose, and 1.8 mM CaCl₂) and equilibrated at 37°C for at least 15

min under a gas mixture of 21% O₂, 5% CO₂, and N₂.

To assess the contractility and structural integrity of the arteries, the passive tension-internal circumference relationship was determined for each arterial segment. The initial tension was then set by calculating the internal circumference corresponding to an intravascular pressure of 30 mmHg and 100 mmHg in a relaxed state for PAs and MAs, respectively. To induce vascular contraction during the experimental procedure, PSS containing 80 mM KCl (replacing an equimolar amount of NaCl with KCl) was used. This 80K-induced contraction was employed to evaluate the functional integrity of the arterial segments.

Western blotting and detection of phosphorylated protein

Extracted proteins from the right ventricle and PA were mixed with Laemmli sample buffer and heated at either 95°C for 5 min, depending on the antibody used. The extracted proteins were separated using 8% SDS-PAGE and subsequently transferred onto a PVDF membrane (Bio-Rad) in a transfer buffer containing 25 mM Tris, 192 mM glycine, 0.01% SDS, and 20% methanol. This ensured efficient migration of proteins from the gel to the membrane.

Following transfer, the membranes were blocked in a solution containing 1× TBS, 1% Tween-20, and 5% non-fat milk at room temperature for 1 h with gentle shaking to prevent non-specific binding, ensuring that the antibodies specifically bound to the target proteins. The membranes were then incubated overnight at 4°C with primary antibodies. The list of primary and secondary antibodies used for contraction and relaxation assessment is provided in Table 1.

Protein signals were detected using the ECL Plus western blotting detection reagent (Merck), and images were acquired using the Amersham Imager 600 (Amersham Biosciences). The band intensity was analyzed using ImageJ v1.8.0 software (National Institutes of Health) to quantitatively assess the relative expression levels of each protein.

To show the phosphorylation states of MLC2 depending on the duration of membrane depolarization, the PAs were fixed with 10% trichloroacetic acid immediately after the incubation with high KCl bath solution. Arteries were fixed for 15 min. and washed three times with cold phosphate-buffered saline. The fixed arteries were homogenized for detecting phosphorylation through immunoblotting as described above.

Statistical analysis

The total number of PA samples and the number of rats for the isometric tension assay are presented as *n* and *N*, respectively. Data analysis for all experiments was performed using Origin Pro 2016 (OriginLab) and GraphPad Prism 8.0 (GraphPad Software). All values are expressed as the mean ± standard deviation. One-

Table 1. List of the antibodies used for immunoblot assay

Antibody	Source	Cat No.	Dilution
Primary antibodies			
Rabbit anti-Phospho-Myosin Light Chain 2	Cell Signaling Technology, Danvers, MA, USA	3672S	1:1,000
Rabbit anti-Phospho-Myosin Light Chain 2 (Ser19)	Cell Signaling Technology, Danvers, MA, USA	3671S	1:1,000
Rabbit anti-Phospho-Myosin Light Chain 2 (Thr18/Ser19)	Cell Signaling Technology, Danvers, MA, USA	3674S	1:1,000
Rabbit anti-Guanylate Cyclase α subunit (soluble)	Cayman Chemicals, Ann Arbor, MI, USA	160895	1:1,000
Rabbit anti-Guanylate Cyclase β 1 subunit (soluble)	Cayman Chemicals, Ann Arbor, MI, USA	160897	1:1,000
Rabbit anti-PKG-1 (C8A4)	Cell Signaling Technology, Danvers, MA, USA	3248	1:1,000
Rabbit anti-ROCK1	Abcam, Cambridge, UK	ab45171	1:1,000
Rabbit anti-ROCK2	Cell Signaling Technology, Danvers, MA, USA	9029S	1:1,000
Rabbit anti-MYPT1	Cell Signaling Technology, Danvers, MA, USA	2634	1:1,000
Mouse anti- β -actin	Sigma-Aldrich, St. Louis, MO, USA	A1978	1:10,000
Secondary antibodies			
HRP-linked anti-Mouse IgG	Cell Signaling Technology, Danvers, MA, USA	7076S	1:5,000
HRP-linked anti-Rabbit IgG	Cell Signaling Technology, Danvers, MA, USA	7074S	1:5,000
Rabbit anti-Goat IgG, HRP conjugate	Millipore, Billerica, MA, USA	AP106P	1:5,000

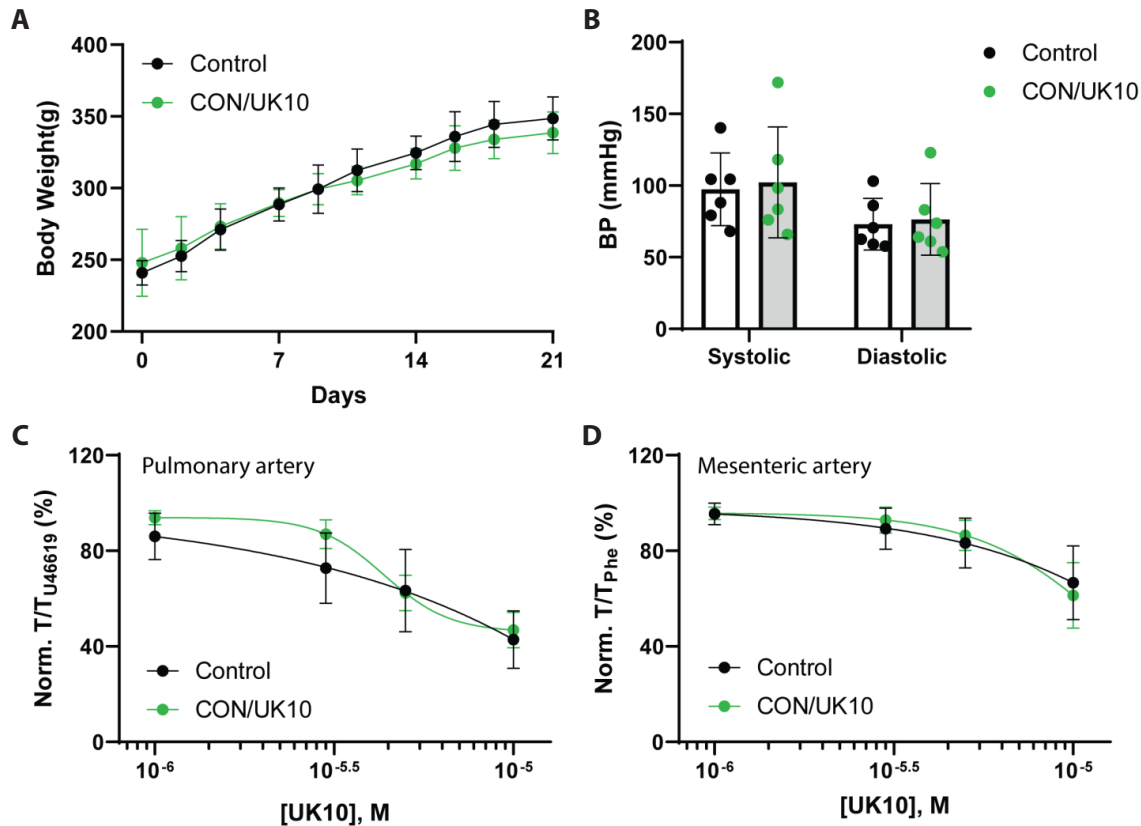


Fig. 1. Effects of the *in vivo* URO-K10 (UK10) application on body weight, systemic arterial blood pressure (BP) and relaxation responses of PA and MAs. (A) The body weights measured over three weeks in the control (CON, $n = 10$), the PAH-MCT ($n = 10$), and the UK10-osmotic pump implanted (CON/UK10, $n = 10$) rats. (B) Comparison of systolic and diastolic BP values between the CON and CON/UK10 rats (age, 11th week, $n = 6$). (C, D) Concentration-dependent relaxant effects of URO-K10 in pulmonary arteries pre-contracted with U46619 (20 nM) and mesenteric arteries pre-contracted with phenylephrine (5 μ M), respectively. Data are presented as mean $\pm$ SD. PA, pulmonary artery; MA, mesenteric artery; PAH-MCT, monocrotaline-induced pulmonary arterial hypertensive rats.

way analysis of variance (ANOVA) was used to compare three or more groups, followed by Tukey's *post-hoc* test for multiple comparisons. Also, a paired Student's *t*-test was used to com-

pare MLC2 phosphorylation between vehicle- and 8-Br-cGMP-incubated PAs. Statistical significance was set at $p < 0.05$ for all analyses. Asterisks in all figures indicate statistical significance (* p

< 0.05 , $**p < 0.01$, $***p < 0.001$). The application of large language models was not included except the literature search and the improvement of writing styles [19].

RESULTS

Insignificant effect of chronic administration of URO-K10 alone in rats

To evaluate how chronic administration of URO-K10 affects physiological changes in the control rats, we implanted the URO-K10 containing osmotic pump at day 3 (CON/UK10) and monitored their body weight and blood pressure (2 or 3 days intervals, three times per week) to compare with those of the age-matched CON (body weight: $n = 10$, blood pressure: $n = 6$). The treatment with URO-K10 alone had no significant effect on the body weight and systemic blood pressure (Fig. 1A, B).

Then, we compared the relaxation effects of URO-K10 on the PAs isolated from CON and CON/UK10. The myography study

showed no difference between the two groups in the concentration-dependent relaxation responses to URO-K10 following a thromboxane A₂ analog U46619 (20 nM)- and phenylephrine (5 μ M)-induced contractions in PAs and MAs, respectively (Fig. 1C, D).

Mitigation of RV remodeling and growth retardation by URO-K10 treatment in PAH-MCT rats

A previous study reported that PAH-MCT rats exhibit weight loss compared to the growth rate of normal control [20]. We monitored the body weight changes at 2- or 3-day intervals in age-matched groups ($n = 10$, Fig. 2A). Consistent with the previous study, the PAH-MCT rats showed significantly retarded weight gain compared to CON. In contrast, rats receiving continuous URO-K10 administration via mini-osmotic pump at days 3 post-injection (PAH-MCT/UK10, $n = 6$) exhibited an increased rate of weight gain, suggesting a potential beneficial effect of URO-K10 on overall physiological homeostasis in the PAH model (Fig. 2A).

Additionally, the measurement of systemic blood pressure revealed that PAH-MCT rats exhibited significantly elevated levels

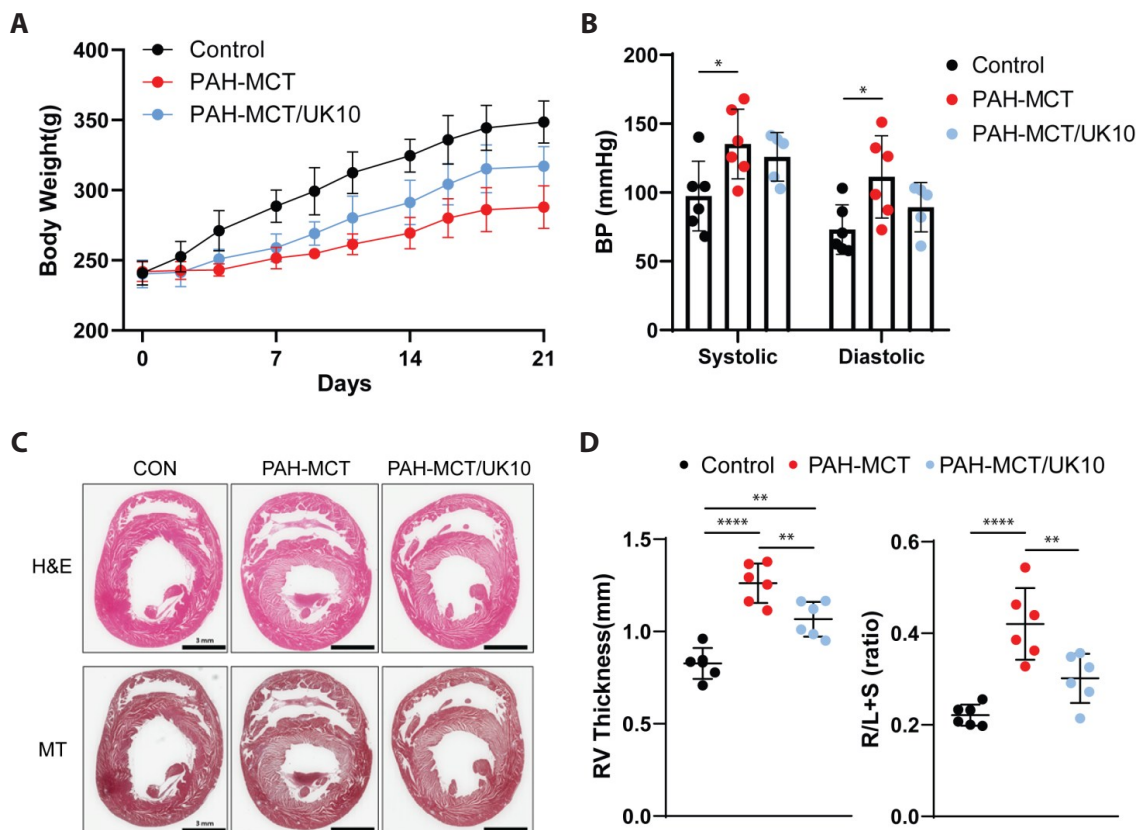


Fig. 2. Effects of the *in vivo* URO-K10 (UK10) application on body weight, systemic arterial blood pressure (BP) and right ventricular (RV) hypertrophy in PAH-MCT rats. (A) The body weights measured over three weeks in the CON, PAH-MCT, and the UK10 administered PAH (PAH-MCT/UK10) rats ($n = 10$). (B) Comparison of systolic and diastolic BP values among the CON, PAH-MCT and PAH-MCT/UK10 rats ($n = 6$). (C) Hematoxylin and eosin (H&E) and Masson's trichrome (MT) stained microscopic images of the RV from the CON, PAH-MCT, and PAH-MCT/UK10 rats. Magnification: 400 $\times$, Scale bar: 3 mm. (D) Comparison of RV wall thickness and RV hypertrophy weight (g) ratio among CON ($n = 6$), PAH-MCT ($n = 6$), and PAH-MCT/UK10 ($n = 6$). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test. Data are presented as mean $\pm$ SD. PAH-MCT, monocrotaline-induced pulmonary arterial hypertensive rats; CON, control. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

compared to CON, indicating another pathological condition apart from the PAH. The appearance of systemic hypertension was reversed in the PAH-MCT/UK10 group, suggesting the amelioration of PAH-induced hemodynamic disturbances by the chronic administration of URO-K10 ($n = 6$, Fig. 2B).

Increased resistance in pulmonary circulation would place an excessive load on the right ventricle, which in turn must generate higher pressure to maintain pulmonary circulation. As a result, the RV hypertrophy and dysfunction would eventually progress to RV failure [21]. In this study, the H&E and Masson's trichrome staining of the PAH-MCT hearts exhibited an increase in RV wall thickness compared to controls; however, the PAH-MCT/UK10 showed significantly reduced RV thickening (Fig. 2C). Addition-

ally, RVH was assessed by comparing ventricular weights. In the PAH-MCT, RV weight was significantly increased compared to controls, whereas PAH-MCT/UK10 showed attenuated RVH ($n = 6$, Fig. 2D).

Partial recovery of PA contractility changes in PAH by URO-K10

Using the wire myography, we compared the relaxation effect of URO-K10 following the U46619-induced contraction in CON and PAH-MCT PAs. In the CON, 10 μ M URO-K10 treatment did not achieve complete relaxation. Notably, in the PAH-MCT/UK10 group, URO-K10 effectively restored the PA tension to the base-

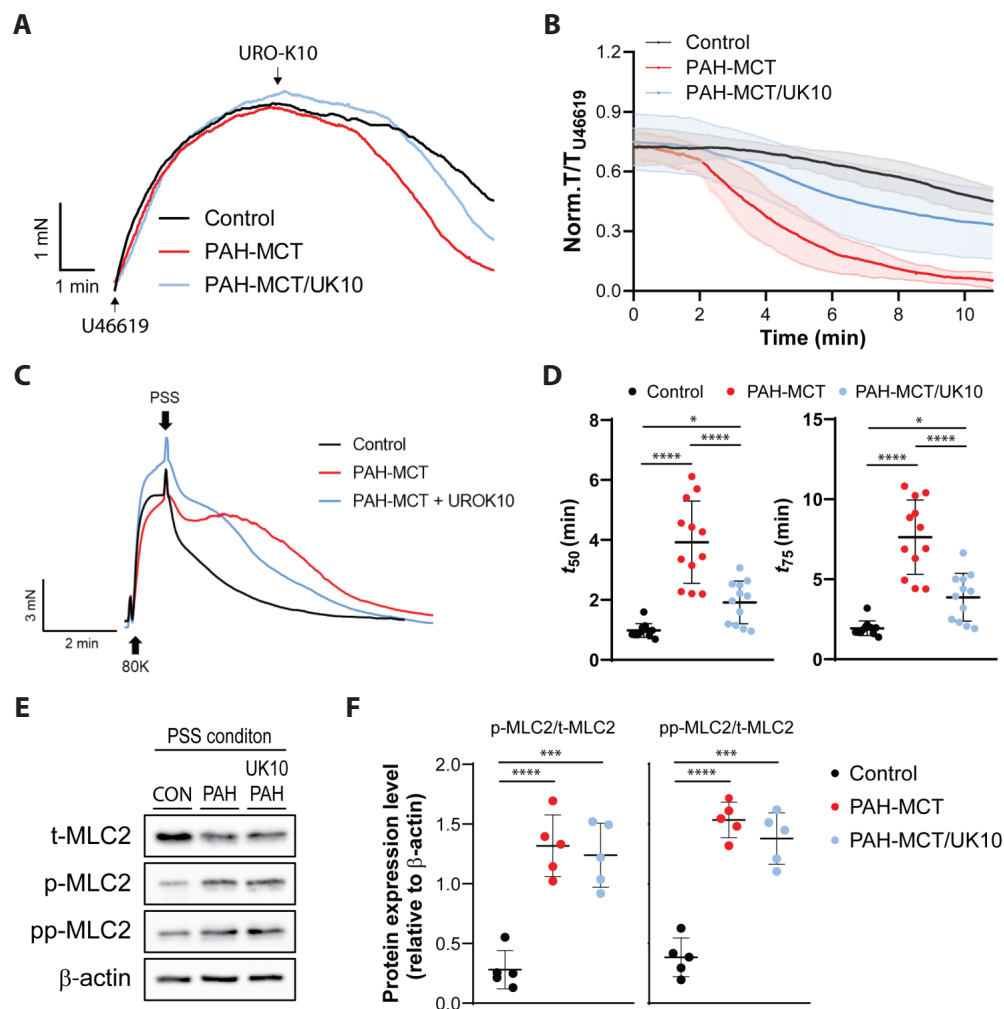


Fig. 3. Relaxing effects of URO-K10 (UK10) on PAs from CON, PAH-MCT and PAH-MCT/UK10 rats. (A) Representative traces of U46619-contraction and UK10-induced relaxation in PAs analyzed by wire-myography. (B) Summary of the traces of PA tone during the relaxation induced by URO-K10 ($n = 5$, $N = 3$). The arterial tone was normalized to the peak amplitude of the U46619-contraction (Norm.T/ T_{U46619}). (C) Representative traces of 80K-contraction and the relaxation during washout with PSS. (D) The 50% and 75% relaxation time to baseline (t_{50} and t_{75} , in min) analyzed in PAs from CON, PAH-MCT and PAH-MCT/UK10 rats ($n = 12$, $N = 4$). (E) Western blot analysis of mono- and di-phosphorylated states of MLC2 in PAs from the three groups. (F) Summary of total MLC2 expression normalized to β -actin and the levels of p-MLC2 (S19-p) and pp-MLC2 (T18/S19-pp) normalized to the expression of MLC2. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test. Data are presented as mean $\pm$ SD. PA, pulmonary artery; CON, control; PAH-MCT, monocrotaline-induced pulmonary arterial hypertensive rats; PSS, physiological salt solution; MLC2, myosin regulatory light chain. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

line levels ($n = 5$, $N = 3$, Fig. 3A, B). These findings are consistent with the previous finding that URO-K10 exerted greater vasorelaxant effect in the PAH-MCT, potentially due to the upregulated K_v7.4 as previously reported [17].

Next, the rate of relaxation after a transient contraction was analyzed. As previously reported, relaxation during the washout period after 80 mM KCl (80K) was significantly slower in PAH-MCT PAs compared to CON. Interestingly, the rate of post-80K relaxation showed a recovery tendency in the PAH-MCT/UK10 ($n = 12$, $N = 4$, Fig. 3D). However, when compared with CON PAs, the time to relaxation (t_{50} and t_{75}) remained delayed although less than the PAH-MCT PAs ($n = 5$, $N = 3$, Fig. 3B).

The levels of MLC2 phosphorylation state, especially the di-phosphorylated (T18/S19-pp) form has been suggested to be responsible for the delayed relaxation in pathological vessels [8]. To investigate the changes in MLC2 phosphorylation, we conducted immunoblot analysis using specific antibodies targeting the mono-phosphorylated (S19-p) and di-phosphorylated (T18/S19-pp) forms of MLC2. After 15 min of incubation with PSS, arterial tissues were fixed and homogenized for a comparative analysis of phosphorylation states. Perplexingly, the elevated levels of S19-p and T18/S19-pp were not significantly reversed in the PAs of PAH-MCT/UK10 while slightly less than those of the PAH-MCT

PAs ($n = 5$, Fig. 3E, F).

Reversed upregulation of ROCK2 in PAH-MCT/UK10

According to our previous study, the increased phosphorylation of MLC2 in PAH-MCT PAs was associated with the upregulated ROCK2 as well as the downregulated MYPT1, soluble guanylate cyclase (sGC β), and PKG [8]. Immunoblot analysis showed that in the PAH-MCT/UK10, the expression levels of sGC β , PKG and MYPT1 were not significantly recovered. Interestingly, however, the upregulated ROCK2 in PAH-MCT was normalized in the PAH-MCT/UK10 ($n = 6$, Fig. 4).

Effects of 8-Br-cGMP on Delayed relaxation in PAH-MCT/UK10

The incomplete recovery of post-80K relaxation and the persistent phosphorylation levels of MLC2 in PAH-MCT/UK10 might be due to the downregulated sGC/PKG activity despite the reversed level of ROCK2. Therefore, we examined whether the pharmacological complementation using a membrane-permeable 8-Br-cGMP could affect the relaxation and the MLC2 phosphorylation in PAH-MCT/UK10 PAs.

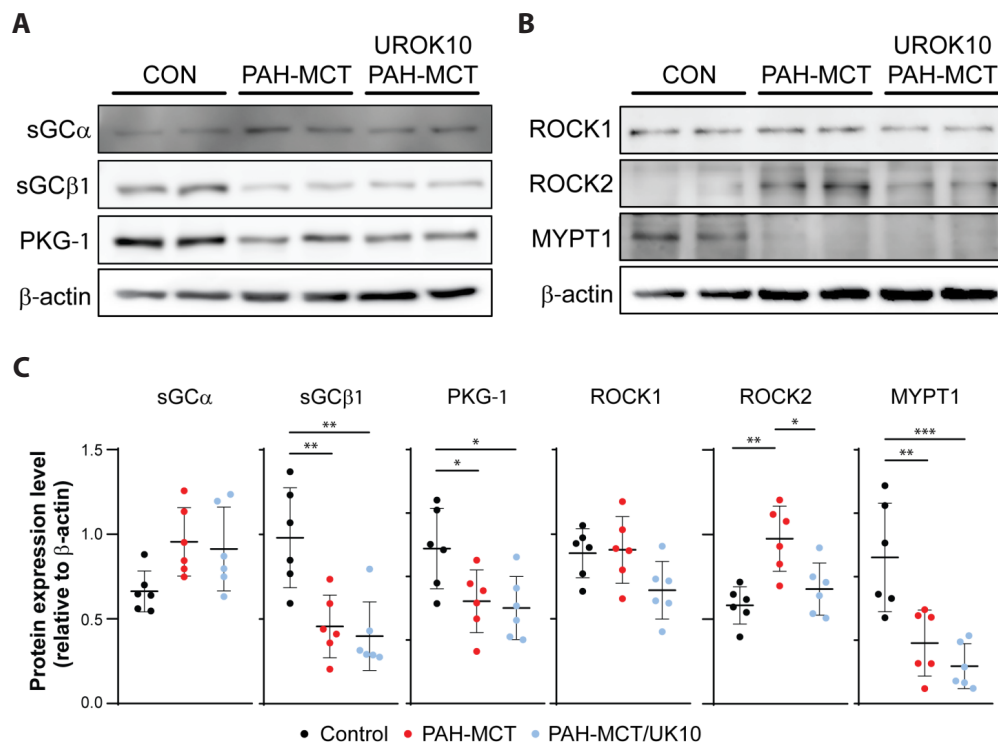


Fig. 4. Expressions of sGC, PKG, MYPT1 and ROCK in the PAs of CON, PAH-MCT, and PAH-MCT/UK10 rats. (A) Representative immunoblot analysis of key proteins involved in cGMP-dependent signaling molecules (sGC α , sGC β 1, PKG). (B) Representative immunoblot analysis of ROCK (ROCK1, ROCK2) and MYPT1. (C) Summary of the protein levels normalized to β -actin. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test. Data are presented as mean $\pm$ SD. sGC, soluble guanylate cyclase; MYPT1, myosin phosphatase target subunit 1; ROCK, Rho-associated kinase; PA, pulmonary artery; CON, control; PAH-MCT, monocrotaline-induced pulmonary arterial hypertensive rats; UK10, URO-K10. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To allow the equilibrium with the exogenous agent, 30 μ M 8-Br-cGMP was applied 15 min before the 80K-contraction in the myography experiment. In the CON group, the pretreatment with 8-Br-cGMP did not significantly alter the post-80K relaxation speed of PAs ($n = 8$, $N = 3$, Fig. 5A, B). However, in the PAH-MCT/UK10 group, the delayed post-80K relaxation was effectively reversed ($n = 9$, $N = 3$, Fig. 5C, D). It should be noted that the slower post-80K relaxation in PAH-MCT PAs could not be reversed by the pretreatment with 8-Br-cGMP ($n = 5$, $N = 3$, Fig.

5E).

To further investigate potential changes in MLC2 phosphorylation, we conducted immunoblot analysis of the S19-p and T18/S19-pp forms of MLC2. After 15 min of incubation with 8-Br-cGMP in PAH-MCT/UK10, the previously elevated levels of S19-p and T18/S19-pp were restored to baseline ($n = 5$, Fig. 5F, G).

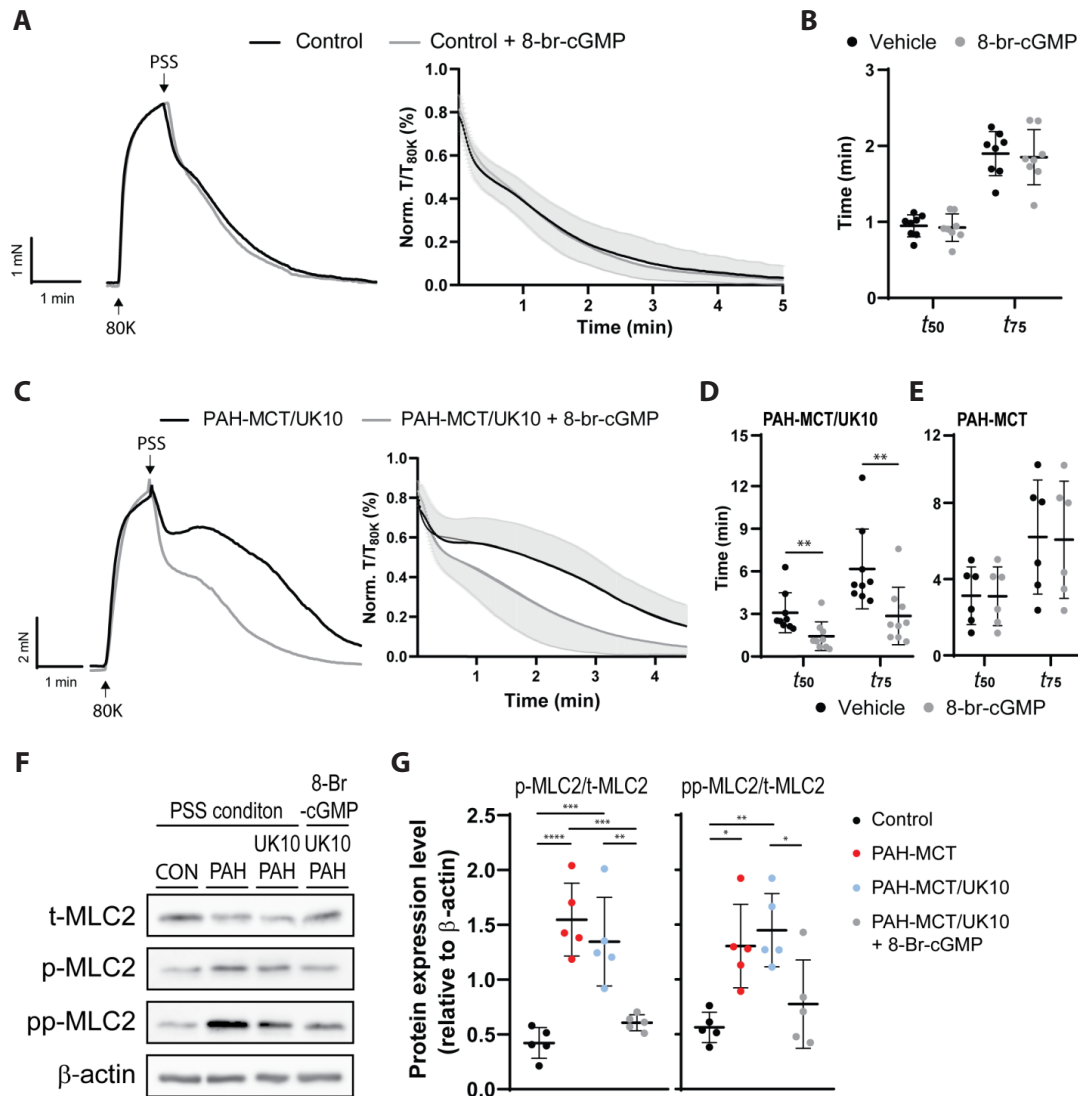


Fig. 5. Effects of 8-Br-cGMP on the recovery of impaired vasorelaxation and MLC2 phosphorylation in PAs of PAH-MCT/UK10 rats. (A) Representative traces of 80K-contraction and washout relaxation (left panel) and their normalized summary (right panel) of the CON PAs pretreated with 8-Br-cGMP (30 μ M, gray traces) or non-treated (black traces). (B) The 50% and 75% relaxation time to baseline (t_{50} and t_{75} in min) measured in CON PAs with or without 8-Br-cGMP ($n = 8$, $N = 3$). (C) Representative traces of 80K-contraction and washout relaxation (left panel) and their normalized summary (right panel) of the PAH-MCT/UK10 PAs pretreated with 8-Br-cGMP (gray traces) or not (black traces). (D, E) The $t_{1/2}$ and $t_{3/4}$ summary in PAs pretreated with 8-Br-cGMP (gray) or not (black), obtained from PAH-MCT/UK10 ($n = 8$, $N = 3$, D) or PAH-MCT ($n = 6$, $N = 3$, E) rats. (F) Immunoblot analysis of mono- and di-phosphorylated states of MLC2 in PAH-MCT/UK10 PAs pretreated with 8-Br-cGMP or not. (G) Summary of the immunoblot study for MLC2 and its phosphorylation. The expression levels were normalized to β -actin and the relative levels of p-MLC2 (S19-p) and pp-MLC2 (T18/S19-pp) again normalized to the MLC2. Statistical analysis was performed using paired Student's t-test or one-way ANOVA followed by Tukey's *post-hoc* test. Data are presented as mean $\pm$ SD. MLC2, myosin regulatory light chain; PA, pulmonary artery; CON, control; PAH-MCT, monocrotaline-induced pulmonary arterial hypertensive rats; UK10, URO-K10; CON, control; PSS, physiological salt solution. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DISCUSSION

In PAH, the dysfunction of key vasodilatory pathways, including the NO/cGMP and PGI₂ signaling cascades, is a hallmark of the disease [22]. The increase in RhoA/Rho kinase (ROCK2) signaling and the downregulation of MYPT1 further exacerbate excessive smooth muscle contraction and heightened vascular resistance, leading to increased pulmonary vascular stiffness [8]. Previous studies have reported that pulmonary vascular relaxation via the NO/cGMP pathway is impaired in PAH, whereas the response to K_v7 activators is enhanced [16].

Seefeld *et al.* [23] reported that the motif compound of URO-K10 exhibited exceptional potency over established KCNQ activators like ML-213 and ICA-27243. Notably, its EC₅₀ value was reduced by a factor of 80 compared to ML-213 and by 500-fold relative to ICA-27243. Furthermore, unlike the classical K_v7 activators such as retigabine and flupirtine, URO-K10 induced a marked vasodilatory response in rat PAs and significant anti-proliferative effects in human pulmonary arterial smooth muscle cells (hPASMCs). This enhanced efficacy is attributed to URO-K10's ability to activate K_v7 channels independently of KCNE4, which typically modulates K_v7 activity and restricts vascular smooth muscle relaxation [17]. By bypassing this regulatory mechanism, URO-K10 achieves superior vasodilatory and anti-proliferative effects, suggesting its potential as a more effective therapy for pulmonary vascular diseases.

The present study showed beneficial effects of URO-K10 application *in vivo* on the pathophysiological progress of PAH-MCT rats. Although not perfect, URO-K10 administration restored the delayed body weight gain in PAH-MCT rats compared to the CON (Fig. 2A). Furthermore, it significantly alleviated RV hypertrophy, suggesting that pulmonary arterial resistance was reduced compared to the disease group (Fig. 2C, D). This therapeutic effect is likely due to the ability of URO-K10 to induce PA relaxation, which alleviated the afterload to the RV in PAH-MCT rats (Fig. 2). The pronounced relaxing effect of URO-K10 on PAH-MCT than CON PAs could be explained by our previous study showing the upregulated expression of K_v7.4 channels in PAH models [17].

Another focus of this study was to determine whether the *in vivo* administration of URO-K10 could restore the previously observed impairment in PA smooth muscle relaxation in the PAH-MCT model. Since the impaired post-80K relaxation was associated with the altered signaling pathways of contractility regulation and MLC phosphorylation states, we also investigated the expressions of sGC, PKG, MYPT1 and ROCK molecules, along with the levels of mono- and di-phosphorylation of MLC2.

Although somewhat disappointing, post-80K relaxation showed only a partial recovery in the PAH-MCT/UK10 rats (Fig. 3C). Furthermore, the downregulated sGC, PKG and MYPT1 were not recovered in the PAs from PAH-MCT/UK10 rats. Although the upregulated ROCK2 was reversed to the control level,

the increased phosphorylation states of MLC2 were not reversed in the PAH-MCT/UK10 PAs (Fig. 3E).

In our previous study, we observed that the impaired post-80K relaxation in PAH-MCT rats was restored by the ROCK inhibitor Y27632. Compared to these previous findings, it is difficult to explain why post-80K relaxation was not recovered in PAH-MCT/UK10 rats, despite the normalization of ROCK2 levels. It could be argued that the expression level of ROCK2 detected by the immunoblot assay might not exactly reflect the actual enzymatic activity of ROCK2. In addition, since Y27632 is a general inhibitor of both ROCK1 and ROCK2, the pharmacological inhibition of all ROCK isoforms could have exerted more prominent effect than the recovery of ROCK2 expression alone.

Nevertheless, one interesting finding was the restorative effect of 8-Br-cGMP administration on post-80K relaxation and the MLC2 phosphorylation. In our previous report, the impaired post-80K relaxation in PAH-MCT could not be recovered by the acute treatment with 8-Br-cGMP, but could be recovered by Y27632, a ROCK inhibitor [8]. Compared to PAH-MCT rats, the ROCK2 level was normalized in PAH-MCT/UK10 rats. Therefore, it can be interpreted that the supplementation of cGMP, the product of sGC, was sufficient to induce a relaxation-promoting effect in the PAH-MCT/UK10 rats showing the reversed ROCK2 level. Moreover, MLC2 phosphorylation, particularly diphosphorylation, which is known to contribute to slow relaxation, was also decreased upon 8-Br-cGMP treatment in the PA of PAH-MCT/UK10 (Fig. 5), while not in PAH-MCT PAs [8].

It should be noted that the MYPT1 expression remained decreased in the PAH-MCT/UK10 PAs (Fig. 4). As MYPT1 is a critical regulatory subunit of myosin phosphatase (MLCP), which plays a key role in smooth muscle relaxation [24], its persistent downregulation suggests that MLCP activity could remain decreased, potentially limiting the recovery of post-80K relaxation.

Mechanisms underlying URO-K10's effects on vasorelaxation

The direct effect of URO-K10 on vascular smooth muscle is considered to be relaxation promotion via membrane hyperpolarization induced by the activation of K⁺ channels such as K_v7.4 and the inhibition of L-type Ca²⁺ channels. However, another observed effect is that long-term administration of URO-K10 may induce the downregulation of contraction-relaxation regulatory signaling proteins, particularly ROCK2. These results suggest that URO-K10 may also suppress RhoA/ROCK signaling, thereby attenuating excessive contraction in PAH.

K⁺ channels in smooth muscle and immune cells could be a target to overcome the pathological changes in arteries [25,26]. However, it is not easy to directly explain how membrane hyperpolarization and the subsequent reduction in [Ca²⁺]_i in smooth muscle cells lead to the decreased expression of ROCK2. Elevated

$[Ca^{2+}]_c$ can activate various calcium-dependent pathways, which may influence ROCK expression indirectly. However, more direct signaling mechanisms such as calmodulin-dependent regulation of ROCK expression is not known yet. Further research is necessary to elucidate the precise mechanisms by which the activation of $K_v7.4$ and membrane hyperpolarization influences ROCK2.

Dysfunction or altered expression of $K_v7.4$ or 7.5 channels has been implicated in several cardiovascular diseases, such as hypertension and PAH. Consequently, K_v7 channels have emerged as potential therapeutic targets; pharmacological activation of these channels has been shown to induce vasodilation and lower vascular resistance, offering a promising strategy for treating hypertension and related cardiovascular disorders [27-30]. In the PAH-MCT, URO-K10 partly improved the relaxation responses, suggesting that $K_v7.4$ activation could counteract increased vascular tone in the disease model. Despite continuous *in vivo* administration of URO-K10, its ability to fully restore vascular tone regulation remains limited, indicating that additional factors may contribute to the relief of pathophysiology in PAH.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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