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Novel and superior treatment of pulmonary hypertension with netrin-1 derived, modified and improved small peptides

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ABSTRACT

Pulmonary hypertension (PH) is a severe and lethal cardiorespiratory disorder with limited therapeutic options to effectively stop or regress the development of the disease. We have previously demonstrated that netrin-1 protects against cardiac injuries via modest and stable production of nitric oxide (NO) and attenuation of oxidative stress. In view of the intermediate roles of NO deficiency and oxidative stress in the pathogenesis of PH, we have recently shown novel and potent attenuating effects on PH of netrin-1 and netrin-1 derived small peptides. Currently, we investigated therapeutic effects on PH of netrin-1 derived peptides with modifications to increase their stability, permeability and resistance to oxidative stress, which are anticipated to have improved efficacies in alleviating PH. Indeed, modified peptides of V1P, V2P, V3P, V1S, V1T, V1D, V1C turned out to be superior or more robust in alleviating all of the pathophysiological and molecular features of PH in hypoxia exposed mice either substantially or completely, with peptides V1S and V1C attenuating both mPAP and RVSP to below baseline levels. All modified peptides completely attenuated right heart hypertrophy more effectively than netrin-1 and the original peptides. They were also more effective in abrogating characteristic vascular remodeling (medial thickening, muscularization, increases in cell proliferation and fibrosis), and production of total ROS and mitochondrial superoxide. eNOS uncoupling activity was abolished by the modified peptides, which was accompanied by restoration in NO bioavailability. Taken together, these novel findings demonstrate that modified, netrin-1 derived small peptides are superior in treating PH, with improved or more robust effects in attenuating all of the mechanistic pathways and hallmark phenotypes of PH. Since these modified peptides pocess properties being more easily deliverable with enhanced stability and availability, they might be more readily translatable to clinical practice for the treatment of PH for which new therapeutics are urgently in need.

1. Introduction

Pulmonary hypertension (PH) is a devastating, lethal cardiorespiratory disease featured by remodeling of pulmonary arterioles, increased pulmonary arterial pressure, increased right ventricular systolic pressure, right heart hypertrophy, and eventually right heart failure [1,2]. Patients often die young due to lack of effective therapeutics to stop or regress the development of PH, or lack of donor lungs for end stage disease that requires lung transplantation; hence with the disease being referred to "cardiovascular cancer". At the 6th World Symposium on Pulmonary Hypertension in 2017, definitions of the disease were updated to include pulmonary vascular resistance $\geq$3 Wood Units in the definition of all forms of pre-capillary PH associated with mPAP >20 mmHg [3]. The Task Force also added a new category to group 1 for "pulmonary arterial hypertension (PAH) long-term responders to calcium channel blockers" [3]. Despite intensive efforts on original and translational research [4], therapeutic options for PH have remained limited to effectively manage the disease in clinical practice.

Produced by endothelial nitric oxide synthase (eNOS) in the endothelial cells, nitric oxide (NO) plays a central role in modulating vascular homeostasis, via multifaceted functions including mediation of vasodilatation, and inhibition of inflammatory responses and endothelial cell apoptosis [5–9]. It is also primarily responsible for regulation of vascular tone in the pulmonary vasculature [10]. Increase in pulmonary vasoconstriction has been linked to a decline in NO bioavailability [10]. Therefore, treatments targeting NO signaling has been developed for

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PH, including inhaled NO, PDE-5 inhibitors (sildenafil and tadalafil) and sGC simulator (riociguat) that have been shown to significantly alleviate symptoms of PH [1,11,12], although these regimes have limitations of not affecting later stage of the disease highlighted by extensive remodeling of pulmonary blood vessels. These limitations might be related to the fact that these medications do not necessarily attenuate oxidative stress, which is believed to play an important role in pulmonary vascular remodeling. We have recently demonstrated novel and robust attenuating effects on PH of netrin-1 and netrin-1 derived small peptides, which can restore NO bioavailability while diminishing oxidative stress, therefore resulting in substantial protective effects on PH at pathophysiological and molecular levels in the classical model of hypoxia induced PH. In the present study, we examined potentially augmented efficacies on treating PH of novel, modified netrin-1 peptides, which are designed to be more stable and active under oxidative stress to exert prolonged activation of netrin-1 receptor DCC for augmented protective effects against PH.

Of note, netrin-1, originally discovered as an axon-guiding protein during neuronal development, can trigger robust protective effects on cardiovascular system to result in enhanced angiogenesis and cardioprotection, via production of NO upon DCC/ERK1/2 mediated activation of coupled eNOS, and via inactivation of NOX4 to attenuate ROS production/oxidative stress, eNOS uncoupling, and mitochondrial dysfunction [13–19]. The advantages of netrin-1 and its derived peptides over traditional therapies for PH are their dual mechanisms of restoring NO bioavailability while diminishing oxidative stress. We have shown that the small peptides contain core sequences that can effectively bind to netrin-1 receptor DCC to activate downstream signaling events, as the full-length netrin-1 protein does, offering more clinically applicable therapeutic option in view of the pharmaceutical convenience of peptide drugs. Indeed, we have shown that netrin-1 and netrin-1 derived native peptides of V1, V2 or V3, are highly effective in treating PH in hypoxia exposed mice [20]. Nonetheless, some of the structures of the small peptides make them vulnerable to oxidation and degradation under oxidative stress. Therefore, we designed modified peptides with increased stability and resistance to oxidative stress (see Methods section), and examined their potential, augmented efficacies in treating PH that would be more relevant to chronic and long-term treatment of PH in patients. Indeed, using the hypoxia induced PH model in mice, our data from the current study indicate furtherly enhanced efficacies of the netrin-1 derived, modified peptides in the treatment of PH, completely alleviating all of the phenotypical and molecular features of PH. These data no doubt provide evidential support to clinical translation of these novel and improved peptides, derived and modified from netrin-1, in the treatment of the devastating cardiorespiratory disorder of PH.

2. Methods

2.1. Experimental design

The Institutional Animal Care and Usage Committee at the University of California, Los Angeles (UCLA), approved all animal experiments. Male C57BL/6 mice of 9–12 weeks old (Charles River Laboratories, Wilmington, MA) were used for experimentation and one group harvested as controls (n = 5–6). A group of animals were kept under hypoxic condition (n = 5–8) and then harvested after 21 days for comparison. The hypoxic environment was maintained by continuous mixed gas flow (normobaric 10% O_2, in 5% CO_2 balanced with 90% N_2). The animals were fed and watered ad libitum, and cages were changed twice weekly. After the chamber was closed, the mixed gas was flushed to recover the hypoxic environment as quickly as possible. Animals were maintained at 20°C with a 12:12-hr light-dark cycle. Age and body weight matched animals as for the control and hypoxia groups, were subcutaneously implanted osmotic minipumps to enable continue and stable release of netrin-1 derived, novel and modified small peptides at 15 ng/day (peptide V1P, peptide V2P, peptide V3P, peptide V1S, peptide V1T, peptide V1D and peptide V1C; vehicle: 3.8 mL H_2O, 120 μl of 5 M NaCl, 40 μl acetic acid) under hypoxia and then harvested after 21 days. The original netrin-1 derived small peptides are V1-9aa (304–312 aa of human netrin-1; CDCRHNTAG), V2-10aa (368–377 aa of human netrin-1, CLNCRHNTAG), and V3-11aa (423–433 aa of human netrin-1, CPCKDGVTGIT), synthesized by GenicBio Limited (Shanghai, China) [18]. To generate more stable and permeable peptides, the V1, V2 and V3 peptides were modified by conjugation to polyethylene glycol (PEG), into V1P, V2P or V3P [21,22], which enables cell permeability (Table 1). In addition, V1 peptide was mutated to generate modified peptides that are more resistant to oxidative stress, named V1S, V1T, V1D and V1C, based on the replacement amino acids modified into the peptides (Table 1). To be more specific, V1 peptide starts with a cysteine, which is known to be sensitive to oxidation. It was replaced by a serine (V1S) or threonine (V1T), which are known to be stable under oxidative environment. Another modification is to add D-amino acid (V1D) at the end, which also stabilizes the peptide (Table 1). The last one V1C is a modification of cyclization which stabilizes the peptide as well (Table 1).

2.2. Hemodynamic analyses

After hypoxia exposure for three weeks, the control group and the hypoxia exposed mice with or without infusion of modified netrin-1 peptides were anesthetized with intraperitoneal injection of pentobarbital at 60 mg/kg. Then, the animals were intubated and connected to a respirator (95% O_2, 5% CO_2). Hemodynamic analyses were carried out by an open chest method using the 1.4 F catheter (mikro-tip® catheter transducer; Model SPR-671, Millar Instruments, Houston, TX). The mean pulmonary arterial pressure (mPAP) and right ventricular systolic blood pressure (RVSP) were recorded using a Power Lab data acquisition system (ADInstruments Inc., CO).

2.3. Tissue harvest, right heart assessment and histological examinations

Following hemodynamic measurements, the mice were euthanized for resection of the heart and lung tissues, which were weighed to record the weight of the RV free wall, and the LV plus septum (LV + S). The ratio of the RV free wall to the free LV wall and the ventricular septum (RV/LV + S) was calculated to evaluate right heart hypertrophy by hypoxia treatment and its reversal by netrin-1 derived, modified peptides. Middle region of the left lung tissues of all mice were submerged in ice-cold saline, perfused and subsequently fixed in 4% paraformaldehyde overnight followed by incubation for 24 h in 10% sucrose, and then embedded in paraffin for sectioning. In addition, the superior lobe from the right lung were immersed in Tissue Plus® OCT compound (Thermo Fisher Scientific, Cat# 23730571, Houston, TX, USA) medium. Cryostat transverse cuts (5 μm) of lung OCT blocks were freshly prepared under −20 °C for determination of ROS production and NO bioavailability using DHE and DAF fluorescent imaging respectively.

The lung paraffin sections of 5 μm thickness were stained with H&E. In each section, six randomly selected fields were examined using Nikon Eclipse Ti confocal microscope (Tokyo, Japan) for morphological analysis. Pulmonary arterioles ranging in 50–200 μm in diameter were observed. Percentage of medial wall thickness of pulmonary arterioles was measured using NIH Image J software. The medial thickness was categorically quantified for blood vessels with different diameters of 50–100 μm and 100–200 μm, and a separate figure was made for all blood vessels under the diameter of 200 μm. The pulmonary arterial medial wall thickness was calculated as % medial wall thickness = (wall thickness × 2/external diameter) × 100 [23,24]. For all histological analyses described here and below, at least 6–16 images were captured from each animal, and at least 6 individual fields of each image were analyzed.

Table 1
Structures, modifications and functions of netrin-1 derived peptides.

Peptide Name	Peptide Core Sequence	Modification	Function
V1-9aa	C–DCRHN–TAG––	Cx(1–2)Cx(3–4)Tx(0–1)G	Minimal cardioprotective sequence
V2-10aa	CLNCRHN–TAG––	Cx(1–2)Cx(3–4)Tx(0–1)G	Minimal cardioprotective sequence
V3-11aa	C–PCKDGVT–GIT	Cx(1–2)Cx(3–4)Tx(0–1)G	Minimal cardioprotective sequence
V1T	T–DCRHN–TAG––	Cys⟶ Thr	Resistant to oxidative stress
V1S	S–DCRHN–TAG––	Cys ⟶ Ser	Resistant to oxidative stress
V1P	C–DCRHN–TAG––PEG	Conjugation to PEG	Increased cell permeability
V2P	CLNCRHN–TAG––PEG	Conjugation to PEG	Increased cell permeability
V3P	C–PCKDGVT–GIT–PEG	Conjugation to PEG	Increased cell permeability
V1C	C–DCRHN–TAG–– (S—S)	Cys-Cys cyclization	Increased peptide stability
V1D	Mirror: C–DCRHN–TAG–– L-peptide / C–DCRHN–TAG–– D-peptide	L-peptide ↓ D-peptide	Increased peptide stability

2.4. Immunofluorescent assay and immunohistochemistry

Paraffin embedded lung sections of 5 μm thickness were dewaxed and rehydrated, followed by heat-mediated antigenic retrieval using a pH 6.0 citrate buffer for 20 min. Immunofluorescent assay and immunohistochemistry examining smooth muscle alpha actin (SMA) and proliferating cell nuclear antigen (PCNA) expression were performed by incubating the lung sections with a rabbit antibody for SMA (1:200, Cat# ab5694, Abcam, Cambridge, MA, USA) or a mouse antibody for PCNA (1:50, Cat# ab29, Abcam, Cambridge, MA, USA). The sections were then incubated for 1 h with FITC-labelled goat anti-mouse IgG secondary antibody or Alexa Fluor goat anti-rabbit IgG secondary antibody and cover slipped. Fluorescent images were taken using Nikon Eclipse Ti confocal microscope (Tokyo, Japan). Fluorescent intensity was quantified in six randomly selected fields in each section using the NIH Image J software. For DAB staining, sections were incubated with secondary antibodies (1:200, Cat# PK-6102-mouse, NC9293436-rabbit, Vecta stain ABC kit, Vector labs, Burlingame, CA, USA) and stained using a 3,3-diaminobenzidine (DAB) substrate according to the manufacturer's instructions (MilliporeSigma, Cat# D3939, St. Louis, MO, USA).

2.5. Masson's Trichrome staining

In parallel experiments, paraffin embedded lung tissue sections of 5 μm thickness were deparaffinized by sequential washes in xylene (2x), descending alcohol from 100% to 50%, and then distilled water. Then, the sections were subjected to Masson's Trichome staining according to the manufacturer's instructions (MilliporeSigma, Cat# HT-15-1KT, St. Louis, MO, USA). Images were captured using Nikon Eclipse Ti confocal microscope (Tokyo, Japan). The perivascular and peribronchial collagen deposition in the lung was assessed as the percentage of fibrous tissue area in the visual field in Masson's Trichrome stained lung tissue sections. Blue is specific for collagen. Muscle fibers and cytoplasm were dyed red, and nuclei were dyed black. The images of were analyzed using NIH Image J software in six randomly selected microscopic fields for each section.

2.6. Determination of NO bioavailability

Lungs freshly isolated from mice were perfused slowly via the pulmonary artery to flush blood with Krebs HEPES buffer (KHB; pH 7.35) as described above, and immersed in Tissue Plus® OCT compound (Thermo Fisher Scientific, Cat# 23730571, Houston, TX, USA). Cryostat transverse cuts (5 μm) of lung sections were freshly prepared under −20-°C. For determination of NO bioavailability using DAF fluorescent probe as a recognized method detecting intracellular levels of NO [25], lung tissue sections were incubated with 20 μM 4-Amino-5-Methylamino-2′, 7′-Difluorofluorescein Diacetate (DAF-FM DA) (Thermo Fisher Scientific, Cat# D23844, Houston, TX, USA) for 20 min in dark at 37-°C. After being washed for 3 times, the sections were coverslipped. The fluorescent images were captured using a Nikon Eclipse Ti confocal microscope (Tokyo, Japan) at excitation and emission wavelengths of 495 and 515 nm respectively, and six randomly selected fields in each section were quantified with the NIH Image J software.

2.7. Determination of reactive oxygen species (ROS) production

Briefly the lungs harvested from mice were perfused slowly from pulmonary artery to flush blood from the interlobular pulmonary arteries and basal segments with Krebs HEPES buffer (KHB; pH 7.35) and immersed in Tissue Plus® OCT compound (Thermo Fisher Scientific, Cat# 23730571, Houston, TX, USA) medium. Cryostat transverse cuts (5 μm) of lung sections were freshly prepared under −20 °C. Levels of superoxide in the lungs were determined using dihydroethidium (DHE) fluorescent imaging as we previously published [26–28]. DHE stock solution was prepared by dissolving DHE (MilliporeSigma) in dimethylsulfoxide (DMSO) at a concentration of 2 mM. The stock solution was stored in the dark and diluted in KHB to a final concentration of 2 μM immediately prior to use. The tissue sections were washed with KHB and incubated in 2 μM DHE solution in a light-protected humidified chamber at 37 °C for 30 min. Excessive DHE was rinsed off twice with KHB and the images were immediately captured with Nikon Eclipse Ti confocal microscope (Tokyo, Japan) at excitation and emission wavelengths of 520 and 610 nm, respectively. Fluorescent intensity of acquired digital images were quantified by NIH Image J software. Superoxide production and eNOS uncoupling activity were also determined using Electron Spin Resonance (ESR) as described below.

2.8. Determination of mitochondrial superoxide production

Mitochondrial superoxide production was examined by fluorescent

imaging using MitoSOX (Invitrogen, Cat# M36008, Carlsbad, CA), a red fluorescent dye that detects superoxide production within mitochondria. MitoSOX™ Red reagent permeates live tissues where it selectively targets mitochondria. It is rapidly oxidized by superoxide but not by other reactive oxygen species (ROS) and reactive nitrogen species (RNS). The oxidized product is highly fluorescent upon binding to nucleic acid. The stock solution was prepared by dissolving MitoSOX in DMSO at a concentration of 5 mM. The stock solution was diluted in KHB to make a 5 μM working MitoSOX solution prior to use. Freshly prepared lung sections were washed twice with KHB and then incubated with 5 μM MitoSOX in KHB for 10 min in dark at 37°C. After incubation, the slides were rinsed with KHB and imaged using a Nikon Eclipse Ti confocal microscope (Tokyo, Japan) at excitation and emission wavelengths of 510 and 580 nm, respectively. In each case, six randomly selected fields in each section were selected for examination. Fluorescent intensity of acquired digital images were quantified by NIH Image J software.

2.9. ESR determination of eNOS uncoupling activity

Electron spin resonance (ESR) is the gold standard of specific and quantitative detection of superoxide in biological systems. In brief, fresh isolated lung tissues were homogenized on ice in lysis buffer containing 1:100 protease inhibitor cocktail, and centrifuged at 12,000 g for 10 min. Protein content of the supernatant was determined using a DC™ protein assay kit (Bio-Rad, Cat# 500–0114 for reagent B, Cat# 500–0113 for reagent A, Cat# 400–0115 for reagent S, Hercules, CA, USA). eNOS uncoupling activity was determined from lung homogenates using ESR as we previously published [9,15–18,26,28–36]. Total superoxide production was calculated from the SOD-inhibitable fraction. For the determination of eNOS uncoupling activity, superoxide production was measured in the presence or absence of NOS inhibitor L-NAME (a NOS inhibitor, 10 μM, Cayman Chemical, Cat# 80587, Ann Arbor, MI, USA). When eNOS is healthy or coupled, L-NAME reduces NO production to result in less buffering effect of superoxide, hence an increase in superoxide detected. When NOS is "uncoupled", L-NAME treatment leads to reduction in superoxide production, which is derived from eNOS.

2.10. Statistical analysis

Statistical analyses of data were completed using Prism software. One-way ANOVA was used to compare means among different groups with a Newman-Keuls or Dunnett's Post-Hoc test. A statistical probability (p) value of < 0.05 was considered significant.

3. Results

3.1. Augmented therapeutic effects on PH of modified small peptides derived from netrin-1: attenuation of mPAP, RVSP and right ventricular hypertrophy

At first, we examined potential augmented protective effects on hypoxia induced elevations in mPAP and RVSP of the novel, modified small peptides derived from netrin-1 (modification designs see Methods section). Indeed, these modified peptides had augmented efficacies comparing to the original netrin-1 derived small peptides in attenuating hypoxia induced increases in mPAP in the PH mice, most notably for peptide V1S and peptide V1C (Fig. 1A). Likewise, these modified peptides also had improved efficacies in alleviating hypoxia induced elevation in RVSP, especially for peptide V1S and peptide V1C as well (Fig. 1B). All of the modified peptides substantially alleviated increases in mPAP and RVSP in hypoxia induced PH mice, with peptides V1S and V1C inhibiting both mPAP and RVSP to below control levels (Fig. 1A–B). The peptides V1T and V1D also attenuated RVSP to control levels (Fig. 1B) (see below).

As shown in Fig. 1A, mean pulmonary arterial pressure (mPAP) in the hypoxia group was 40.68 ± 3.56 mmHg, which was significantly higher than the normoxia group at 20.14 ± 1.49 mmHg. Compared to hypoxia, the mPAP was substantially or completely reduced by treatment with novel, modified peptides derived from netrin-1: V1P (21.19 ± 1.96 mmHg), V2P (25.38 ± 2.28 mmHg), V3P (20.22 ± 1.30 mmHg), V1S (16.58 ± 1.61 mmHg), V1T (24.52 ± 2.69 mmHg), V1D (22.89 ± 3.24 mmHg), or V1C (18.75 ± 1.16 mmHg) (Fig. 1A). Of note, V1S and V1C turned out to be superiorly effective in attenuating mPAP to below control levels.

Similarly in Fig. 1B, the right ventricular systolic pressure (RVSP) was significantly elevated in the hypoxia group at 42.38 ± 1.43 mmHg, comparing to normoxia group at 31.23 ± 2.29 mmHg. Intriguingly, the increased RVSP was completely attenuated after the treatment with novel, modified small peptides derived from netrin-1: V1P (30.78 ± 2.71 mmHg), V2P (30.49 ± 1.39 mmHg), V3P (29.27 ± 1.97 mmHg), V1S (23.62 ± 0.52 mmHg), V1T (29.03 ± 2.96 mmHg), V1D (29.41 ± 3.88 mmHg), or V1C (26.88 ± 2.12 mmHg) (Fig. 1B), also with V1S and V1T reducing RVSP to below control levels.

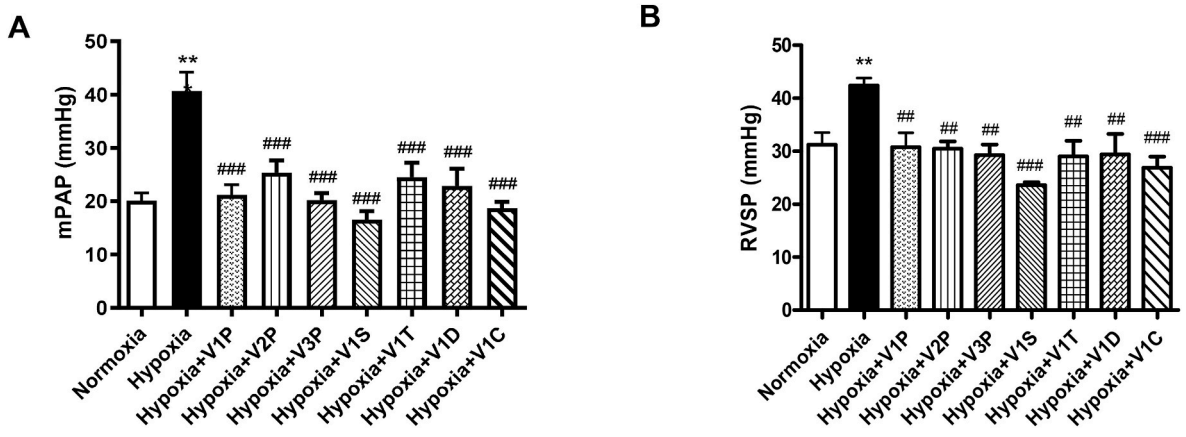

Fig. 1. Novel, modified peptides derived from netrin-1 exerted augmented effects in attenuating mPAP and RVSP in pulmonary hypertensive mice. Pulmonary hypertension was induced in mice by exposure to normobaric hypoxia (10% O_2) for three weeks. Osmotic pump was used to deliver modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, V1C continuously. At the end of 3 weeks, mPAP and RVSP measurements were performed by an open chest catheterization. (A) mean pulmonary arterial pressure (mPAP) (B) Right ventricular systolic pressure (RVSP). Hemodynamic analyses indicate that increased mPAP and RVSP in hypoxia induced pulmonary hypertensive mice were substantially or completely attenuated by treatment with modified peptides derived from netrin-1. Data are shown as Mean ± SEM. ***$p < 0.001$ vs. Control; ##$p < 0.01$, ###$p < 0.001$ vs. Normoxia (n = 4), Hypoxia (n = 6), Hypoxia + V1P (n = 6), Hypoxia + V2P (n = 6), Hypoxia + V3P (n = 4), Hypoxia + V1S (n = 4), Hypoxia + V1T (n = 4), Hypoxia + V1D (n = 4), Hypoxia + V1C (n = 7).

At the end of hypoxia treatment, mouse lungs were freshly isolated and measured for weights of RV and LV plus septum. As shown in Fig. 2, the RV/LV + S ratio was markedly increased in mice exposed to hypoxia (0.415 ± 0.009) compared to normoxia group (0.275 ± 0.017). Compared to the hypoxia group, RV/LV + S ratio was completely reduced by treatment with novel, modified small peptides derived from netrin-1: V1P (0.265 ± 0.014), V2P (0.256 ± 0.014), V3P (0.259 ± 0.010), V1S (0.267 ± 0.013), V1T (0.256 ± 0.013), V1D (0.252 ± 0.017), or V1C (0.273 ± 0.011) (Fig. 2).

3.2. Augmented therapeutic effects on PH of modified small peptides derived from netrin-1: attenuation of pulmonary vascular remodeling

Pulmonary vascular remodeling was assessed by examination of medial thickness. The percentage medial thickness in blood vessels that have an external diameter less than 200 μm was significantly increased in the hypoxia group (64.05 ± 1.66) compared to the control group (26.56 ± 3.07) (Fig. 3A & B). Compared to hypoxia, percentage medial thickness in blood vessels that have an external diameter less than 200 μm was near completely attenuated in animals treated with novel, modified small peptides derived from netrin-1: V1P (32.25 ± 2.40), V2P (33.49 ± 0.69), V3P (33.63 ± 0.69), V1S (32.82 ± 0.92), V1T (31.25 ± 1.81), V1D (31.96 ± 1.88), or V1C (32.92 ± 1.39) (Fig. 3A & B).

The percentage medial thickness in differently sized pulmonary arteries in the lung is shown in Fig. 3C & D. The percentage medial thickness in blood vessels that have an external diameter of 50–100 μm was significantly increased in the hypoxia group (74.16 ± 0.94) compared with the control group (31.94 ± 1.91) (Fig. 3C). Compared to hypoxia, percentage medial thickness in blood vessels that have an external diameter less than 50–100 μm was near completely (much more than original peptides) attenuated in animals treated with novel, modified small peptides derived from netrin-1: V1P (38.77 ± 4.563), V2P (37.39 ± 0.9110), V3P (32.52 ± 3.027), V1S (37.29 ± 6.211), V1T (36.32 ± 3.272), V1D (37.01 ± 3.167) or V1C (37.58 ± 2.410) (Fig. 3C). The percentage medial thickness in blood vessels that have an external diameter of 100–200 μm was significantly increased in the hypoxia group (58.33 ± 1.12) compared with the control group (29.02 ± 1.48), and this response was completely attenuated by treatment with novel, modified small peptides derived from netrin-1: V1P (25.99 ± 1.65), V2P (30.50 ± 1.26), V3P (34.55 ± 1.53), V1S (30.77 ± 2.31), V1T (28.28 ± 2.02), V1D (25.91 ± 2.42) or V1C (30.50 ± 2.84) (Fig. 3D).

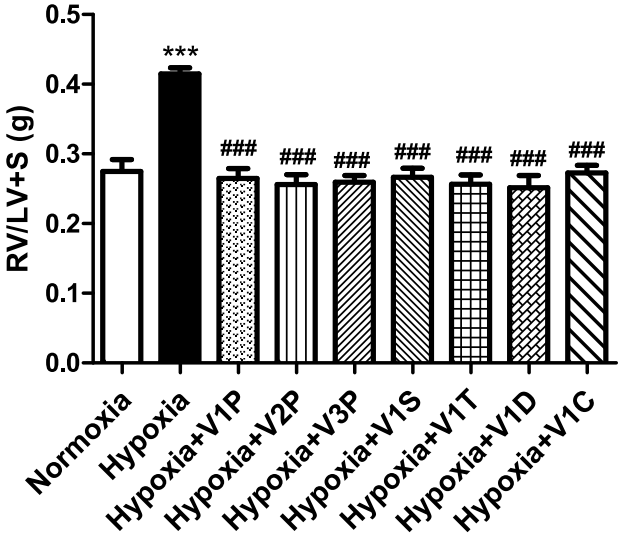

Fig. 2. Novel, modified peptides derived from netrin-1 exerted augmented effects in attenuating right heart hypertrophy in pulmonary hypertensive mice. Pulmonary hypertension was induced in mice by exposure to normobaric hypoxia (10% O_2) for three weeks. Osmotic pump was used to deliver modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, V1C continuously. Right ventricle hypertrophy as indicated by increased RV/LV + S ratio in hypoxia induced pulmonary hypertensive mice was completely attenuated by treatment with the modified netrin-1 peptides. Data are shown as Mean ± SEM. RV = right ventricle; LV = left ventricle; S = septum.***$p < 0.001$ vs. Control; ###$p < 0.001$ vs. Hypoxia. Normoxia (n = 5), Hypoxia (n = 6), Hypoxia + V1P (n = 5), Hypoxia + V2P (n = 5), Hypoxia + V3P (n = 5), Hypoxia + V1S (n = 5), Hypoxia + V1T (n = 5), Hypoxia + V1D (n = 5), Hypoxia + V1C (n = 6).

In order to examine potential lung vascular muscularization and cell proliferation, lung sections were stained with antibodies specific for smooth muscle actin (SMA) and proliferative cell nuclear antigen (PCNA). Both immunofluorescent and immunohistological analyses of lung tissues revealed vascular muscularization as evidenced by increased SMA-positive staining in hypoxia-treated mouse pulmonary arterioles. The expression of SMA by DAB staining in the lung sections was significantly increased in the hypoxia group (10.18 ± 0.56) compared to the control group (2.66 ± 0.02) (Fig. 4A & C). Compared to hypoxia, the expression of SMA by DAB staining was near completely reduced by treatment with novel, modified small peptides derived from netrin-1: V1P (3.00 ± 0.12), V2P (3.33 ± 0.20), V3P (3.42 ± 0.20), V1S (2.60 ± 0.12), V1T (2.80 ± 0.17), V1D (2.60 ± 0.15), or V1C (3.24 ± 0.46) (Fig. 4A & C).

Likewise, the expression of PCNA by DAB staining was markedly elevated in the hypoxia group (5.64 ± 0.38) compared with the control group (0.75 ± 0.02) (Fig. 4B & D), indicating increased cell proliferation. Compared to hypoxia, the expression of PCNA by DAB staining was substantially reduced by treatment with novel, modified small peptides derived from netrin-1: V1P (1.88 ± 0.24), V2P (1.74 ± 0.27), V3P (2.01 ± 0.33), V1S (1.56 ± 0.21), V1T (1.88 ± 0.17), V1D (1.68 ± 0.37), or V1C (1.82 ± 0.30) (Fig. 4B & D).

In addition, collagen deposition as assessed by Mason's Trichrome staining was found to be markedly increased in the peribronchial and perivascular areas in the hypoxia treated mouse lung (10.93 ± 0.23) compared to control group (3.99 ± 0.72) (Fig. 5A & B). Compared to hypoxia, percentage fibrosis was near completely reduced by treatment with novel, modified small peptides derived from netrin-1: V1P (4.25 ± 0.33), V2P (4.85 ± 0.29), V3P (4.57 ± 0.22), V1S (5.18 ± 0.33), V1T (4.72 ± 0.37), V1D (4.61 ± 0.37), or V1C (4.69 ± 0.25) (Fig. 5A & B).

3.3. Augmented therapeutic effects on PH of modified small peptides derived from netrin-1: restoration of NO bioavailability and attenuation of total and mitochondrial superoxide production

Endothelial nitric oxide (NO) bioavailability was assessed in mouse lungs *in situ* by fluorescent imaging using the NO-specific fluorescent probe DAF-FM DA. The image analysis of lung sections stained with DAF-FM DA showed decreased NO bioavailability in the hypoxia group (32.85 ± 2.82) compared to control group (55.76 ± 3.05) (Fig. 6A & B). Whereas, NO bioavailability was substantially or completely restored by treatment with novel, modified small peptides derived from netrin-1: V1P (53.82 ± 5.21), V2P (47.19 ± 2.02), V3P (58.62 ± 3.25), V1S (50.79 ± 7.22), V1T (47.31 ± 4.66), V1D (50.24 ± 7.07), or V1C (51.28 ± 6.80) (Fig. 6A & B).

We next determined the effects of hypoxia on total and mitochondrial superoxide production in lung tissue sections. Total superoxide in lung tissue sections was determined using DHE fluorescent imaging. The image analysis of lung sections stained with DHE showed increased superoxide levels in the hypoxia group (51.69 ± 3.70) compared to control group (18.85 ± 1.83) (Fig. 7A & C). Whereas, superoxide production was substantially attenuated by treatment with novel, modified small peptides derived from netrin-1: V1P (31.05 ± 2.68), V2P (28.61 ± 3.30), V3P (23.62 ± 4.35), V1S (30.86 ± 4.49), V1T (26.64 ± 5.57),

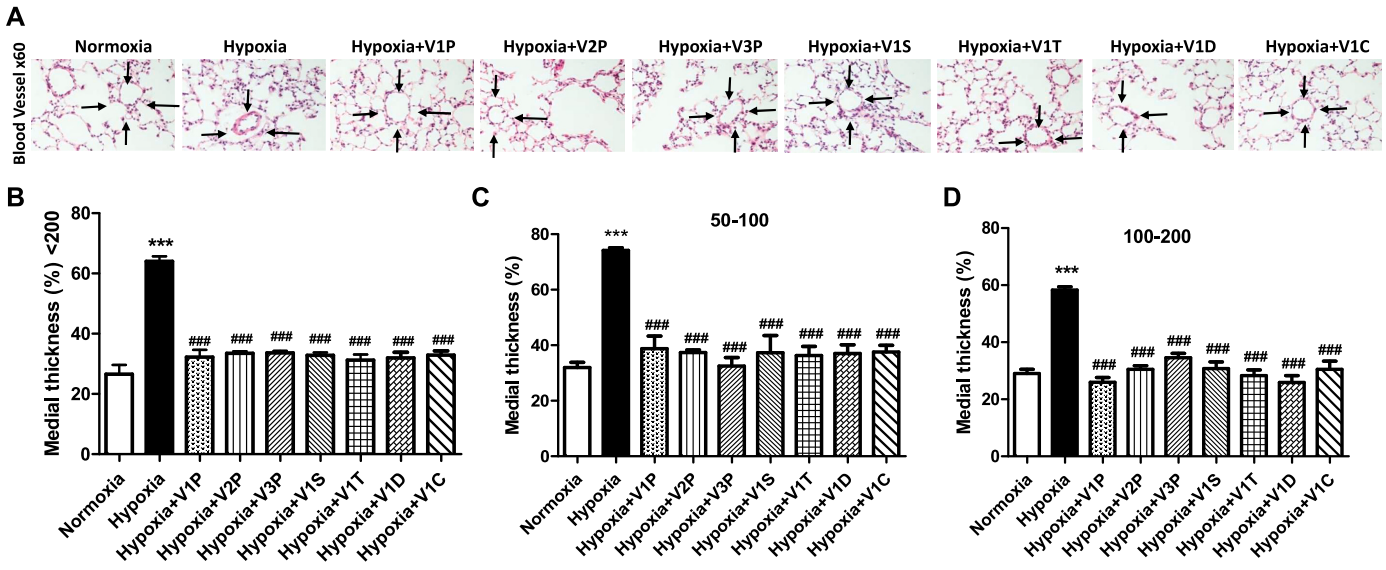

Fig. 3. Novel, modified peptides derived from netrin-1 exerted augmented effects in attenuating vascular remodeling of medial thickening in pulmonary hypertensive mice. Pulmonary hypertension was induced in mice by exposure to normobaric hypoxia (10% O_2) for three weeks. Osmotic pump was used to deliver modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, V1C continuously. (A) Representative images of H&E staining (60× magnification) of lung tissue sections. (B) Medial thickness (<200 vessel diameter) of the pulmonary vasculature. (C–D) Medial thickness in 50–100 and 100–200 diameter sized blood vessels. Data indicate that vascular remodeling of medial thickening in hypoxia induced pulmonary hypertensive mice was completely attenuated by treatment with the modified netrin-1 peptides. Data are shown as Mean ± SEM. ***p < 0.001 vs. Normoxia; ###p < 0.001 vs. Hypoxia. Normoxia (n = 5), Hypoxia (n = 5), Hypoxia + V1P (n = 5), Hypoxia + V2P (n = 5), Hypoxia + V3P (n = 5), Hypoxia + V1S (n = 5), Hypoxia + V1T (n = 5), Hypoxia + V1D (n = 5), Hypoxia + V1C (n = 5).

V1D (31.40 ± 2.42), or V1C (25.45 ± 2.88) (Fig. 7A & C).

To further evaluate mitochondrial damage and its potential contribution to the enhanced intracellular superoxide generation in the lung tissues of PH mice, mitochondrial superoxide production was assessed using the MitoSOX Red fluorescent probe. As shown in Fig. 7B & D, hypoxia exposure significantly increased mitochondrial superoxide production in the lung sections, compared to the normoxia group (67.88 ± 5.25 vs. 30.78 ± 1.764). Whereas, mitochondrial superoxide production was substantially or completely abrogated by treatment with novel, modified small peptides derived from netrin-1: V1P (31.90 ± 5.11), V2P (27.59 ± 7.15), V3P (35.98 ± 6.39), V1S (37.88 ± 5.34), V1T (34.06 ± 8.99), V1D (45.27 ± 1.75), or V1C (35.96 ± 4.58) (Fig. 7B & D).

3.4. Augmented therapeutic effects on PH of modified small peptides derived from netrin-1: recoupling of eNOS

Electron spin resonance (ESR) was used to examine eNOS uncoupling activity as we previously published [9,15–18,26,28–36]. Under normal conditions when eNOS is coupled, the addition of the eNOS inhibitor L-NAME will increase the measured superoxide production, as eNOS is producing NO to scavenge superoxide; however, when eNOS is uncoupled and producing superoxide, its inhibition will lead to a decrease in measured superoxide. We examined whether eNOS was uncoupled in hypoxia-induced PH in mice and whether novel, modified peptides derived from netrin-1 can recouple eNOS, therefore contributing to improvement in NO bioavailability and attenuation in oxidative stress. While total superoxide production by ESR determination was markedly increased by hypoxia to 30.02 ± 2.32 μM/min/mg protein comparing to the normoxia group (12.87 ± 1.63 μM/min/mg protein), it was completely abrogated by treatment with novel, modified small peptides derived from netrin-1 to control or below control levels: V1P (13.38 ± 2.52), V2P (9.03 ± 3.20), V3P (6.49 ± 3.32), V1S (7.53 ± 2.86), V1T (11.15 ± 2.84), V1D (5.32 ± 1.77), or V1C (9.10 ± 3.28) (Fig. 8A). Of note, eNOS was markedly uncoupled as reflected by L-NAME inhibitable production of superoxide in Fig. 8B, which was completely reversed by treatment with novel, modified small peptides derived from netrin-1 (Fig. 8B). Taken together, these data indicate that via augmented restoration of eNOS coupling activity to improve NO bioavailability and further reduce oxidative stress comparing to the original netrin-1 peptides, the novel, modified netrin-1 peptides are superior and more effective in alleviating development of PH, which is characterized by attenuated changes in hemodynamic parameters of elevated mPAP and RVSP, right heart hypertrophy, as well as features of vascular remodeling of medial thickness with increases in SMA and PCNA expression.

4. Discussion

In the present study, we demonstrate for the first time that modified, netrin-1 derived peptides have robust and superior treatment efficacies on pulmonary hypertension (PH), which are even more potent than netrin-1 and the original unmodified peptides derived from netrin-1. These modified peptides are more stable and resistant to oxidative stress mediated degradation, making them even more suitable for pharmaceutical applications. Indeed, these netrin-1 derived, modified peptides are superior in further alleviating all of the pathophysiological and molecular features of PH in a classical model of hypoxia exposed mice, including substantial or complete attenuation of hemodynamic changes of elevated mPAP and RVSP, right heart hypertrophy, remodeling of small vessels in the lung characterized by medial thickening, muscularization and proliferation, elevated production in total ROS and mitochondrial superoxide, as well as eNOS uncoupling activity. Comparing to the responses induced by netrin-1 and its original peptides, these modified peptides exerted more robust inhibitory effects on phenotypes and molecular changes of PH [20]. Combined with their improved stability, permeability, and resistance to oxidative stress, these findings demonstrate that these modified peptides can be used as novel and more effective therapeutic strategies for the treatment of PH. The peptide modifications and related designing principles, and the underlying molecular mechanisms for the protection against PH, are

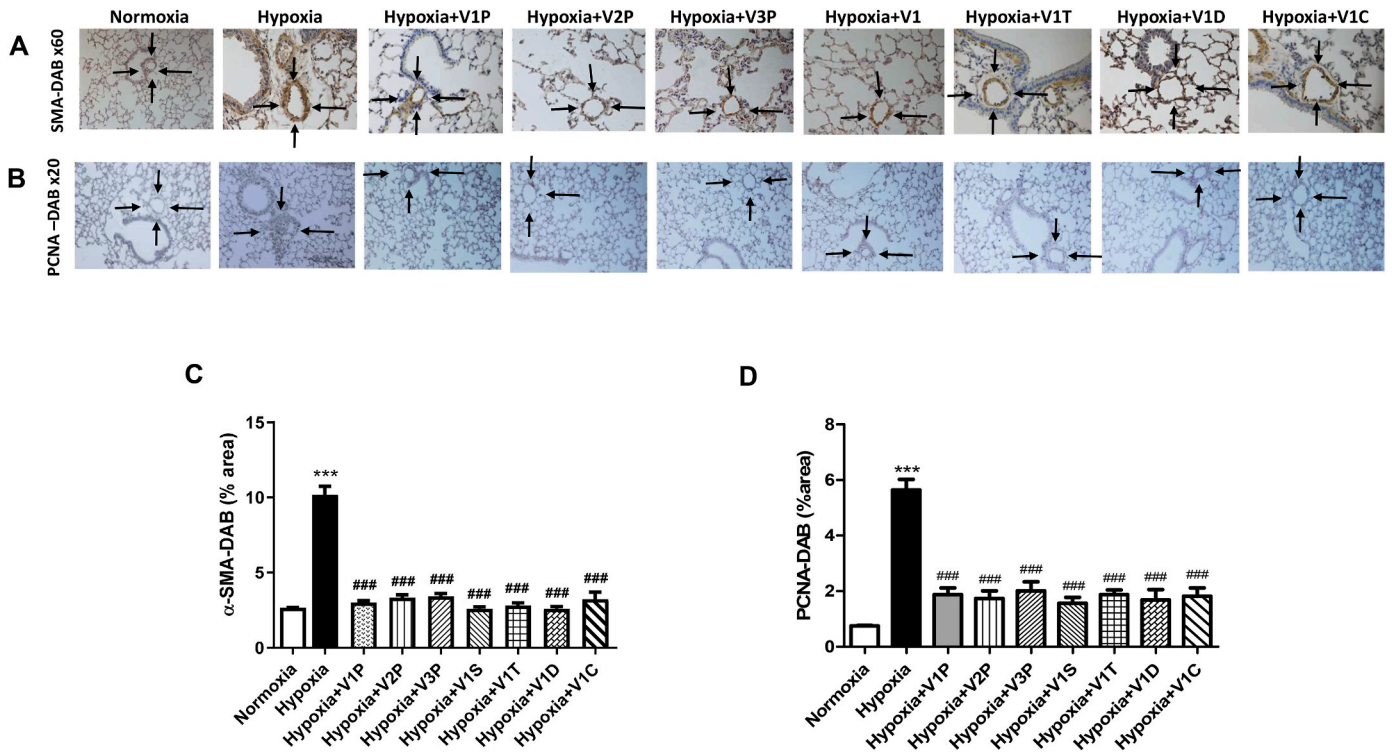

Fig. 4. Novel, modified peptides derived from netrin-1 exerted augmented effects in attenuating pulmonary vascular muscularization and cell proliferation in pulmonary hypertensive mice. Pulmonary hypertension was induced in mice by exposure to normobaric hypoxia (10% O_2) for three weeks. Osmotic pump was used to deliver modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, V1C continuously. (A,D) Representative images and quantitative data of smooth muscle actin (SMA) expression by immunohistochemical staining using DAB substrate. (B,E) Representative images and quantitative data of proliferating cell nuclear antigen (PCNA) expression by immunohistochemical staining using DAB substrate. (C,F) Representative images and quantitative data of collagen deposition by Masson's Trichrome staining. Data indicate that pulmonary vascular muscularization, cell proliferation and fibrosis in hypoxia induced pulmonary hypertensive mice were completely attenuated by treatment with the modified netrin-1 peptides. Data are shown as Mean ± SEM. ***p < 0.001 vs. Normoxia; ###p < 0.001 vs. Hypoxia. Normoxia (n = 5), Hypoxia (n = 5), Hypoxia + V1P (n = 5), Hypoxia + V2P (n = 5), Hypoxia + V3P (n = 5), Hypoxia + V1S (n = 5), Hypoxia + V1T (n = 5), Hypoxia + V1D (n = 5), Hypoxia + V1C (n = 5).

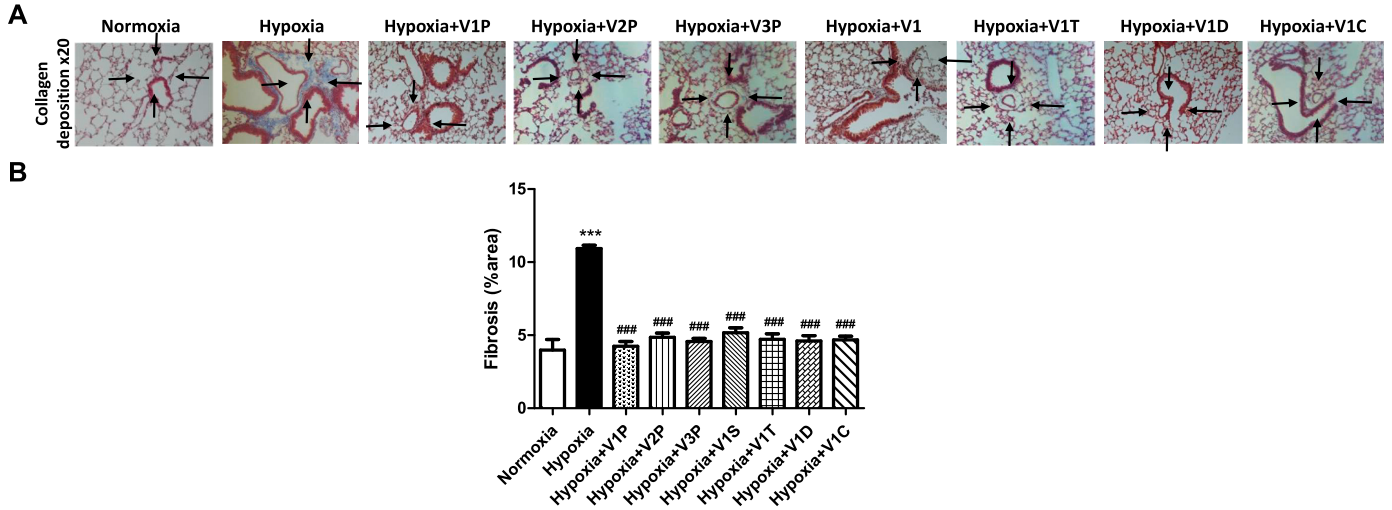

Fig. 5. Novel, modified peptides derived from netrin-1 exerted augmented effects in restoring NO bioavailability in pulmonary hypertensive mice. Pulmonary hypertension was induced in mice by exposure to normobaric hypoxia (10% O_2) for three weeks. Osmotic pump was used to deliver modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, V1C continuously. (A,B) Representative images and quantitative data of collagen deposition by Masson's Trichrome staining. Data indicate that pulmonary perivascular and peribronchial fibrosis in hypoxia induced pulmonary hypertensive mice was completely attenuated by treatment with the modified netrin-1 peptides. Data are shown as Mean ± SEM. ***p < 0.001 vs. Normoxia; ###p < 0.001 vs. Hypoxia. Normoxia (n = 5), Hypoxia (n = 6), Hypoxia + V1P (n = 4), Hypoxia + V2P (n = 4), Hypoxia + V3P (n = 4), Hypoxia + V1S (n = 4), Hypoxia + V1T (n = 4), Hypoxia + V1D (n = 4), Hypoxia + V1C (n = 5).

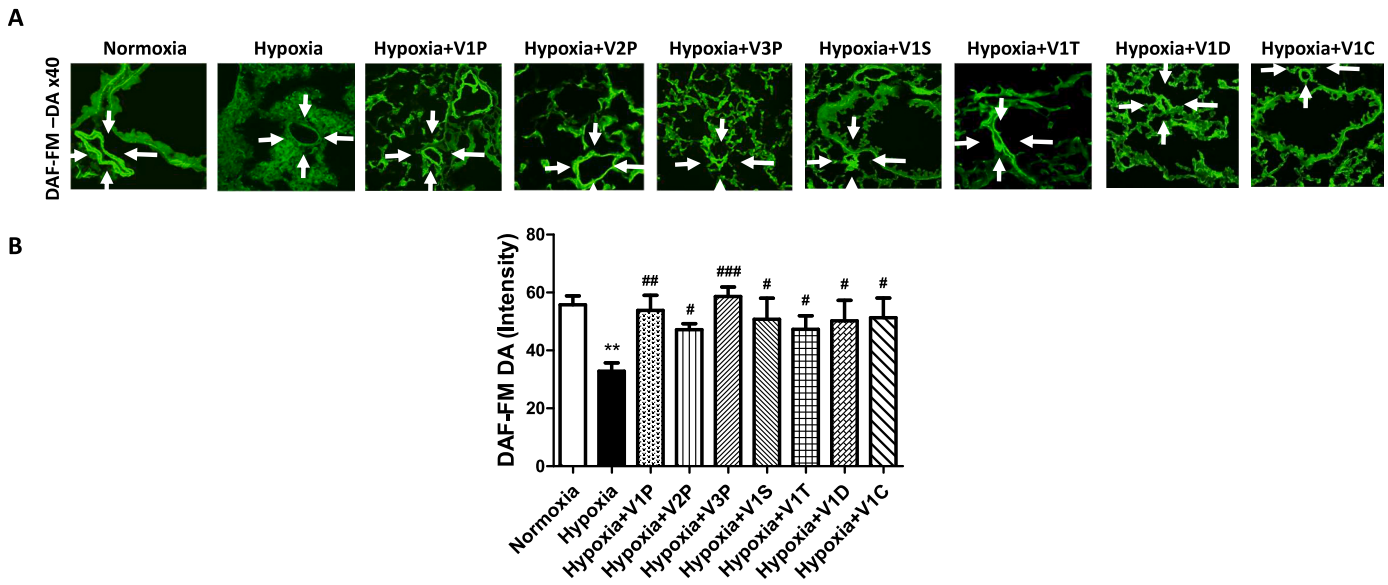

Fig. 6. Novel, modified peptides derived from netrin-1 exerted augmented effects in attenuating pulmonary perivascular and peribronchial fibrosis in pulmonary hypertensive mice. Pulmonary hypertension was induced in mice by exposure to normobaric hypoxia (10% O_2) for three weeks. Osmotic pump was used to deliver modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, V1C continuously. (A,B) Representative images and quantitative data of NO bioavailability by DAF-FM fluorescent staining. Data are shown as Mean ± SEM. **p < 0.01 vs. Control; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. Hypoxia. Normoxia (n = 5), Hypoxia (n = 6), Hypoxia + V1P (n = 4), Hypoxia + V2P (n = 4), Hypoxia + V3P (n = 4), Hypoxia + V1S (n = 4), Hypoxia + V1T (n = 4), Hypoxia + V1D (n = 4), Hypoxia + V1C (n = 5).

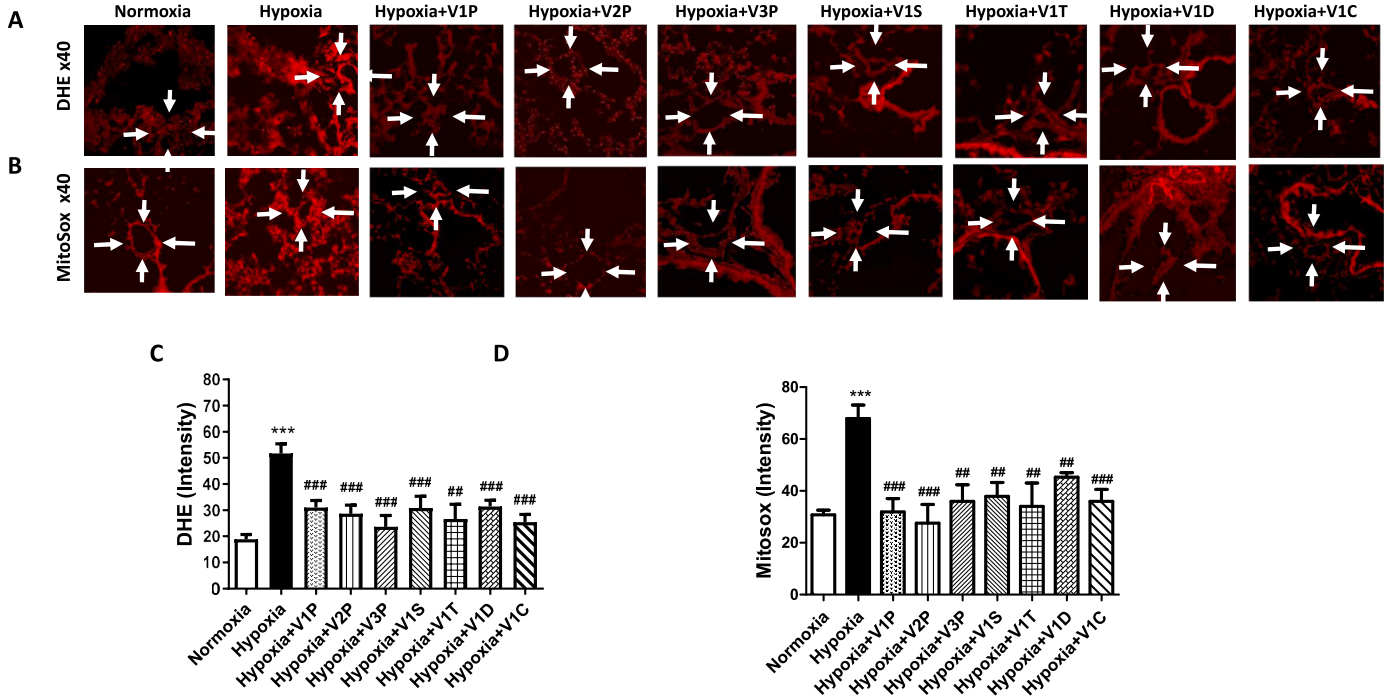

Fig. 7. Novel, modified peptides derived from netrin-1 exerted augmented effects in attenuating pulmonary total and mitochondrial superoxide production in pulmonary hypertensive mice. Pulmonary hypertension was induced in mice by exposure to normobaric hypoxia (10% O_2) for three weeks. Osmotic pump was used to deliver modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, V1C continuously. (A, C) Representative images and quantitative data of ROS production by DHE fluorescent staining. (B, D) Representative images and quantitative data of mitochondrial ROS production by MitoSox fluorescent staining. Data are shown as Mean ± SEM. ***p < 0.001 vs. Control; ##p < 0.01, ###p < 0.001 vs. Hypoxia. Normoxia (n = 5), Hypoxia (n = 6), Hypoxia + V1P (n = 4), Hypoxia + V2P (n = 4), Hypoxia + V3P (n = 4), Hypoxia + V1S (n = 4), Hypoxia + V1T (n = 4), Hypoxia + V1D (n = 4), Hypoxia + V1C (n = 5).

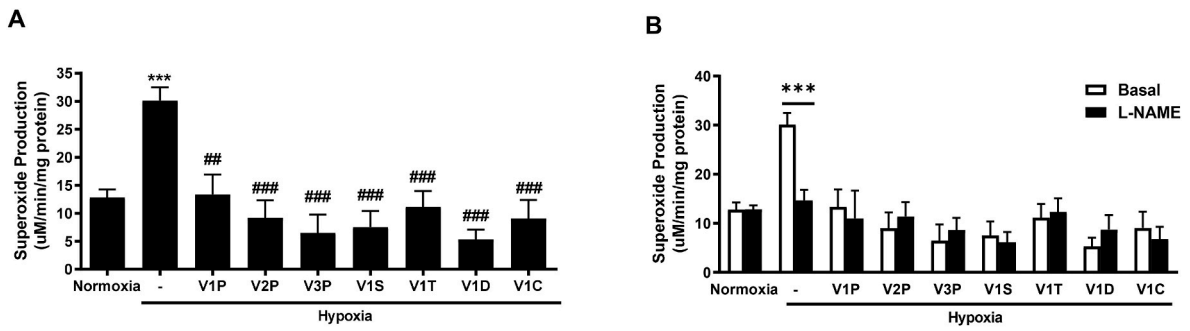

Fig. 8. Novel, modified peptides derived from netrin-1 abrogated superoxide production and eNOS uncoupling activity in pulmonary hypertensive mice. Pulmonary hypertension was induced in mice by exposure to normobaric hypoxia (10% O_2) for three weeks. Osmotic pump was used to deliver modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, V1C continuously. Lung tissues were used to measure total superoxide production and eNOS uncoupling activity using electron spin resonance (ESR) as we previously published. (A) Grouped data of total superoxide production determined by ESR, indicating increased superoxide production in hypoxia exposed mice, which was completely attenuated by novel, modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, or V1C. ***p < 0.001 vs normoxia, ##p < 0.01, ###p < 0.001 vs hypoxia. (B) Grouped data of superoxide production in the presence or absence of LNAME for assessment of eNOS uncoupling activity, indicating that uncoupling of eNOS (reflected by L-NAME sensitive superoxide production) in hypoxic pulmonary hypertensive mice was completely reversed by novel, modified peptides derived from netrin-1, V1P, V2P, V3P, V1S, V1T, V1D, or V1C. ***p < 0.001. Normoxia (n = 7), Hypoxia (n = 8), Hypoxia + V1P (n = 5), Hypoxia + V2P (n = 7), Hypoxia + V3P (n = 4), Hypoxia + V1S (n = 5), Hypoxia + V1T (n = 6), Hypoxia + V1D (n = 5), Hypoxia + V1C (n = 6).

presented in comprehensive details in Table 1 and Fig. 9. Induction of NO by the more stable and cell permeable peptides triggers augmented inhibitory responses on vascular remodeling in the lung, leading to preserved eNOS and endothelial function and ultimate alleviating effects on PH.

Treatment options for the devastating cardiorespiratory disorder of PH have long been limited, with most of the existing medications effective in reliving symptoms only. Most of the therapies target the early phase of the disease to improve vasodilation, while the remodeling phase of the disease, driven by oxidative stress, is not affected [37]. PH is a devastating cardiorespiratory disorder, with lack of effective therapies to stop or regress the disease to prevent lethal right heart failure and mortality especially in young patients. We have recently shown that netrin-1 and netrin-1 derived small peptides are able to induce significant protection against development and progression of PH [20]. We have previously demonstrated that netrin-1 and its derived small peptides, exert robust angiogenic and cardioprotective effects via activation of netrin-1 receptor DCC to lead to production of NO from coupled eNOS, as well as attenuation of oxidative stress through inhibition of NOX4/eNOS uncoupling axis during cardiac ischemia reperfusion injury [13–19]. Of note, activation of NOX4 has been implicated in the development of PH in hypoxia exposed mice [38,39], and that scavenging of ROS is effective in attenuating PH in hypoxia induced PH model [40,41]. The enhanced dual mechanisms of elevating NO while inhibiting oxidative stress, is considered to underlie augmented therapeutic efficacies on PH of the modified, netrin-1 derived peptides. More importantly, these mechanisms would enable netrin-1 and its peptides to stop or regress development of PH, which is more advantageous comparing to existing therapies that primarily function through alleviation of symptoms only.

Intriguingly, all of the pathophysiological and molecular features of PH in hypoxia exposed mice were further alleviated by modified, netrin-1 derived small peptides comparing to netrin-1 and the original peptides. These include hemodynamic parameters determined by the gold standard of right heart catheterization technology. Hypoxia induced increases in mPAP and RVSP were all substantially or completely attenuated by osmotic minipump mediated continuous infusion into mice of modified, netrin-1 derived small peptides, with peptides V1S and V1C inhibiting mPAP and RVSP to below control levels and peptides V1T and V1D inhibiting RVSP to control levels. Right heart hypertrophy in hypoxia induced PH mice was also abolished to baseline by all of the modified, netrin-1 derived small peptides, which is more potent than netrin-1 and its original peptides [20]. The characteristic vascular remodeling responses of medial thickening, muscularization of vessels, and increases in cell proliferation and perivascular and peribronchial collagen deposition, were also substantially abrogated to near baseline by modified netrin-1 derived small peptides, which represent augmented inhibitory responses comparing to netrin-1 and the original peptides [20]. In addition, total ROS and mitochondrial superoxide production defined by both fluorescent imaging and ESR analysis, markedly increased in hypoxia exposed PH mice, was also substantially or completely alleviated by modified, netrin-1 derived small peptides to a greater extent. eNOS uncoupling activity was completely reversed, which was accompanied by marked restoration in NO bioavailability. Therefore, the superior protective effects on PH of modified, netrin-1 derived small peptides are attributed to recoupling of eNOS to result in restoration of eNOS function/improved NO bioavailability and more effective abrogation of oxidative stress, resulting in further alleviated pathophysiological features and phenotypes of attenuated vascular remodeling, hemodynamic changes and right heart hypertrophy. Indeed, the mechanistic insights identified share similarity with our previous findings regarding cardioprotective effects of netrin-1 and netrin-1 derived small peptides [13–19].

Importantly, the peptide drugs are of better translational implications since they are unlikely to trigger severe side effects especially for being derived from native protein of netrin-1. These modified peptides may also be used in lower dosing comparing to the native peptides due to its improved therapeutic efficacies. The peptides can be delivered intravenously or potentially orally based on progress on oral peptide medications in the field. All of these make these newer and more effective peptides ideal therapeutic options for the treatment of PH.

In conclusion, our data innovatively demonstrate significant and translational observations that netrin-1-derived, novel and modified small peptides have augmented and superior treatment efficacies on PH in hypoxia exposed mice. The improved netrin-1 peptides, with modifications to improve their stability, permeability and activity, exerted enhanced effects in attenuating all of the pathophysiological and molecular phenotypes of PH, with some of the peptides inhibiting parameters to below control levels such as for the mPAP, RVSP and right heart hypertrophy. Given the improved efficacies and pharmaceutical applicability, these novel, modified peptides derived from netrin-1 might prove to be superior candidates for immediate drug development for the treatment of the devastating cardiorespiratory disorder of PH.

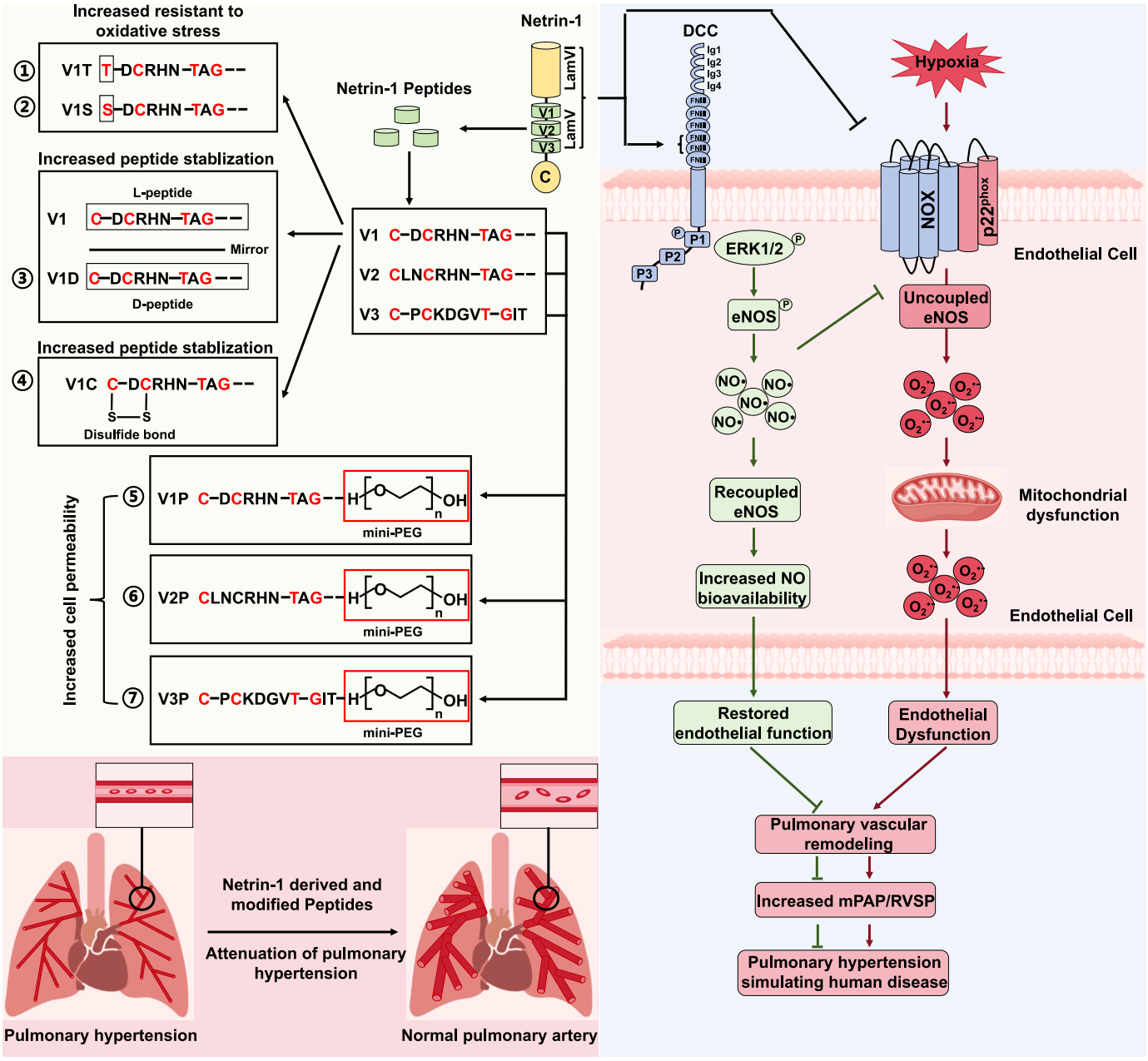

Fig. 9. Schematic summary of mechanistic insights into robust protective effects on pulmonary hypertension of netrin-1 derived, modified, and improved small peptides. The improved netrin-1 peptides, modified to enhance their stability (V1C, V1D), permeability (V1P, V2P, and V3P), and resistance to oxidative stress (V1T, V1S), exert augmented protective effects on pulmonary hypertension (PH) in attenuating all of the pathophysiological and molecular features of PH. In the murine model of PH, hypoxia induces activation of NOX isoforms, which leads to eNOS (endothelial nitric oxide synthase) uncoupling, mitochondrial dysfunction, persistent oxidative stress, and endothelial dysfunction, resulting in development of PH characterized by pulmonary vascular remodeling/increased pulmonary vascular resistance, and increased mean pulmonary arterial pressure (mPAP) and right ventricular systolic pressure (RVSP). The modified and improved netrin-1 peptides can attenuate these pathological changes via parallel mechanisms of DCC-dependent activation of nitric oxide (NO) production from coupled eNOS, and preservation of eNOS function from being uncoupled via inhibition of NOX activation (NO-dependent upon peptide application). Therefore, the modified and improved netrin-1 peptides is robustly effective in alleviating all of pathophysiological and molecular features of PH, including abrogation of hemodynamic changes in mPAP/RVSP, pulmonary vascular resistance and vascular remodeling. Taken together, these novel, modified peptides derived from netrin-1 represent promising drug candidates for immediate development of new therapeutics for PH.

CRediT authorship contribution statement

Priya Murugesan: Data curation, Formal analysis, Investigation, Writing – original draft. **Yuhan Zhang:** Data curation, Formal analysis, Writing – original draft. **Yixuan Zhang:** Data curation, Formal analysis. **Ji Youn Youn:** Data curation, Formal analysis. **Hua Cai:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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