Pulmonary Hypertension

AAV Delivery of Tumor Necrosis Factor-α Short Hairpin RNA Attenuates Cold-Induced Pulmonary Hypertension and Pulmonary Arterial Remodeling

Patrick Crosswhite, Kai Chen, Zhongjie Sun

Abstract—Cold temperatures are associated with increased mortality and morbidity of cardiovascular and pulmonary disease. Cold exposure causes lung inflammation, pulmonary hypertension (PH), and right ventricle hypertrophy, but there is no effective therapy because of unknown mechanism. Here, we investigated whether RNA interference silencing of tumor necrosis factor (TNF)-α decreases cold-induced macrophage infiltration, PH, and pulmonary arterial (PA) remodeling. We found for the first time that continuous cold exposure (5.0°C) increased TNF-α expression and macrophage infiltration in the lungs and PAs right before elevation of right ventricle systolic pressure. The in vivo RNA interference silencing of TNF-α was achieved by intravenous delivery of recombinant AAV-2 carrying TNF-α short hairpin small–interfering RNA 24 hours before cold exposure. Cold exposure for 8 weeks significantly increased right ventricle pressure compared with the warm controls (40.19±4.9 versus 22.9±1.1 mm Hg), indicating that cold exposure caused PH. Cold exposure increased TNF-α, interleukin-6, and phosphodiesterase-1C protein expression in the lungs and PAs and increased lung macrophage infiltration. Notably, TNF-α short hairpin small–interfering RNA prevented the cold-induced increases in TNF-α, interleukin-6, and phosphodiesterase-1C protein expression, abolished lung macrophage infiltration, and attenuated PH (26.28±1.6 mm Hg), PA remodeling, and right ventricle hypertrophy. PA smooth muscle cells isolated from cold-exposed animals showed increased intracellular superoxide levels and cell proliferation along with decreased intracellular cGMP. These cold-induced changes were prevented by TNF-α short hairpin small–interfering RNA. In conclusions, upregulation of TNF-α played a critical role in the pathogenesis of cold-induced PH by promoting pulmonary macrophage infiltration and inflammation. AAV delivery of TNF-α short hairpin small–interfering RNA may be an effective therapeutic approach for cold-induced PH and PA remodeling. (Hypertension. 2014;64:1141-1150.)

Key Words: hypertension, pulmonary □ inflammation □ interleukin-6 □ macrophage □ phosphodiesterase-1C □ proliferation □ smooth muscle cells

The adverse effects of cold temperatures on the systemic circulation are well documented.1-4 However, whether cold exposure affects the pulmonary circulation is largely unexplored. Lungs are open to the environment and are susceptible to cold air stimulation. Clinical studies suggest that inhaled cold air causes pathophysiological responses such as vasoconstriction in the respiratory tract mucosa, which may contribute to cold-related respiratory diseases.5 Exposure to cold temperatures is an important public-health concern, particularly for those dying from cardiorespiratory diseases.6 In the United States, cold weather is associated with increased mortality from pulmonary and cardiovascular diseases.7,8 Cold exposure causes pulmonary vasoconstriction in patients with Raynaud phenomenon.9 Cold weather provokes respiratory systems.10-12 Cold air causes pulmonary vascular responses.13 We recently showed that exposure to cold increased pulmonary arterial (PA) pressure in rats, namely cold-induced pulmonary hypertension.14 Prolonged elevation of PA pressure is detrimental to the pulmonary vascular system and the right heart. However, the negative effect of cold temperature on the pulmonary circulation is poorly studied and the underlying mechanism is largely unknown. The study of cold-induced pulmonary hypertension has important implications for people who live in cold regions or during winter.

Tumor necrosis factor (TNF)-α is a proinflammatory cytokine that plays diverse roles in many pathological processes. TNF-α is produced largely by monocytes and macrophages in response to inflammatory stimuli.15,16 On release, TNF-α binds to its receptors, which are present on nearly all cells including macrophages and can regulate immune function, apoptosis, autoimmunity, and fever induction.17 The receptors and ligands in the TNF-α family couple directly to signaling pathways that involve cell...
proliferation, survival, and differentiation, where their rapid response is critical to coordinating the proliferation and protective functions of various cells. As such, TNF-α and its receptors have been the focus of intense biological research to understand their role in a variety of conditions including atherosclerosis, rheumatoid arthritis, and autoimmune disease.

Preliminary studies in our laboratory indicate that cold exposure increases pulmonary TNF-α expression, lung macrophage infiltration, and PA blood pressure and causes PA remodeling. We hypothesized that inhibition of TNF-α would decrease cold-induced pulmonary macrophage infiltration and inflammation, PA remodeling, and PH.

Methods
For details, see the expanded methods section in the online-only Data Supplement.

Results
Exposure to Cold Increased Right Ventricle Pressure and Upregulated TNF-α Protein Expression in PAs
Exposure to moderate cold (5±0.1°C) significantly increased right ventricle (RV) systolic pressure, a reliable indicator of PA pressure, in a time-dependent manner (Figure 1A). RV pressure started to elevate slightly at 5 days and reached a significant high level by 7 days after exposure to cold (Figure 1A), indicating that cold exposure caused PH. In contrast, TNF-α protein expression in PAs was increased as early as 3 days after exposure to cold (Figure 1B and 1C). Macrophage infiltration was also increased in the lungs and PAs from 3 days of exposure to cold (Figure S1 in the online-only Data Supplement). Interleukin (IL)-6 protein expression in PAs was not increased until 14 days after exposure to cold (Figure S2). Thus, cold exposure caused pulmonary inflammatory responses. Notably, TNF-α expression was upregulated right before elevation of PA pressure.

TNF-α Short Hairpin Small–Interfering RNA Attenuated the Cold-Induced Increase in RV Pressure and RV Hypertrophy
The recombinant AAV-2 carrying the rat TNF-α short hairpin small–interfering RNA (TNFshRNA; AAV.TNFshRNA)
and scrambled (AAV.ScrshRNA) were constructed as we described previously.23,24 RV systolic pressures in the cold PBS (40.14±4.9 mm Hg) and cold ScrshRNA (37.91±3.1 mm Hg) groups were elevated significantly versus the control groups (Figure 2; Figure S3). Cold-exposed animals treated with TNFshRNA, however, had a significantly decreased RV systolic pressure (26.28±1.6 mm Hg) that was similar to the 3 warm groups. It is noted that 1 single injection of TNFshRNA prevented the cold-induced increase in RV pressure for ≤8 weeks (length of the study; Figure 2).

It seems the AAV constructs were mainly trapped in the pulmonary tissues after delivery via jugular veins because AAV.GFP was expressed in lungs but not in systemic tissues (aortas and kidneys; Figure S4). Gene delivery did not affect body weight gain in rats kept in either temperature environment (Figure S5A), indicating that the AAV construct did not affect animals’ growth.

RV hypertrophy is a hallmark of PH. Cold-exposed rats developed RV hypertrophy as evidenced by a significant increase in the RV weight compared with the warm controls, whereas TNFshRNA prevented RV hypertrophy (Figure S5B). Trichrome staining indicated that collagen was not detectable in the RV (Figure S6). No obvious fibrosis was found.

**TNFshRNA Attenuated Cold-Induced PA Remodeling**

PA remodeling is common in almost all forms of PH. We examined small PAs in the lungs with a diameter of 50 to 80 μm, which is in line with the third order of PA branching (resistance PAs). The lumen diameter was significantly decreased in the cold PBS (40.3±4.9 μm) and cold ScrshRNA (44.7±4.7 μm) groups compared with warm PBS (63.2±4.2 μm), warm ScrshRNA (72.4±6.1 μm), and warm TNFshRNA (70.4±4.6 μm; Figure 3A and 3B). TNFshRNA

**Figure 3.** Tumor necrosis factor-α short hairpin small-interfering RNA (TNFshRNA) attenuated cold-induced pulmonary artery remodeling. A, Hematoxylin and eosin staining was performed in paraffin-embedded lung sections (5 μm). Morphometric analysis of third order pulmonary arteries (50–80 μm) was performed to evaluate the (B) lumen diameter, (C) lumen area, and (D) medial layer thickness. Data represent mean±SEM. *P<0.05, **P<0.01, and ***P<0.001 vs warm PBS; ^P<0.05, ^^^P<0.001 vs cold PBS. Photos are shown at ×400. Arrows indicate pulmonary artery and scale bars are 50 μm (n=6).
prevented the cold-induced decreases in lumen diameter (57.4±3.6 μm). Cold exposure also decreased lumen area, which can be partially rescued by TNFshRNA (Figure 3A and 3C). We also measured the medial layer thickness, which is primarily determined by proliferation of the smooth muscle cells. Cold PBS and cold ScrshRNA groups had significant increases in medial layer thickness (23.3±0.8 and 21.3±1.2 μm, respectively) versus the warm PBS (16.1±0.48 μm), warm ScrshRNA (14.8±0.44 μm), and warm TNFshRNA (15.8±0.7 μm) groups (Figure 3A and 3D). TNFshRNA prevented the cold-induced increase in medial layer thickness (15.93±0.7 μm).

**TNFshRNA Prevented the Cold-Induced Increase in Lung TNF-α Expression**

To evaluate the effectiveness of the AAV.TNFshRNA construct, we measured TNF-α protein expression in the lung. Cold PBS and cold ScrshRNA showed significantly increased lung TNF-α protein expression compared with the 3 warm control groups (Figure 4A and 4B) demonstrating that cold exposure upregulates TNF-α protein expression. TNFshRNA prevented the cold-induced increase in TNF-α mRNA and protein expression in the lung (Figure 4A and 4B; Figure S7), indicating effective silencing of lung TNF-α. In addition, TNFshRNA also prevented the cold-induced upregulation of plasma TNF-α (Figure S8).

Unexpectedly, cold exposure did not increase protein expression of lung IL-6 (a proinflammatory cytokine) significantly, and TNFshRNA did not have obvious effect on lung IL-6 expression (Figure 4A and 4C).

**TNFshRNA Prevented the Cold-Induced Increases in TNF-α, IL-6, and Phosphodiesterase-1C Expression in PAs**

TNF-α protein expression was increased in PAs of the cold PBS and cold ScrshRNA groups compared with the warm control groups (Figure 5A and 5B). TNFshRNA prevented the cold-induced increase in TNF-α in PAs, indicating effective silencing of TNF-α. In contrary to IL-6 expression in the lung (Figure 4A and 4C), IL-6 expression in the PAs was increased by cold exposure, which can be prevented by TNFshRNA (Figure 5A and 5C).

It was reported that phosphodiesterase-1 (PDE-1) may be involved in the development of PH.25 We examined PDE-1 expression in PAs. Cold exposure increased PDE-1C protein expression (Figure 5A and 5D) but did not affect PDE-1A or PDE-1B expression (not shown), in PAs compared with the warm controls. Interestingly, TNFshRNA prevented the cold-induced increase in PDE-1C expression in PAs.

PDE-1C hydrolyzes the second messenger cGMP, a vasodilator in the pulmonary circulation. We measured the intracellular level of cGMP in isolated PA smooth muscle cells (PASMCs).
PASMCs isolated from cold PBS and cold ScrshRNA groups had a lower cGMP level compared with cells from the warm controls (Figure 5E). TNFshRNA almost abolished the cold-induced decrease in intracellular cGMP in PASMCs (Figure 5E).

TNFshRNA Prevented Cold-Induced Infiltration of Macrophages in the Lungs
To further evaluate the relationship between cold exposure and inflammation, we examined the macrophage infiltration in the lungs using a CD-68 antibody. Cold exposure clearly increased both the density of CD-68 staining and the actual count of CD-68+ cells in the cold PBS and cold ScrshRNA groups compared with the warm controls (Figure 6A–6C), indicating that cold exposure increased macrophage infiltration in the lungs. TNFshRNA prevented the cold-induced increases in pulmonary macrophage infiltration (Figure 6A–6C).

TNFshRNA Prevented the Cold-Induced Increase in Superoxide in Isolated PASMCs
The relationship among TNF-α, superoxide, and cold exposure is unclear. We measured superoxide levels in freshly isolated PASMCs as described recently.26,27 Cold exposure significantly increased superoxide levels in PASMCs compared with the 3 warm control groups (Figure 7 and 7B). TNFshRNA prevented the cold-induced increase in superoxide in PASMCs (Figure 7A and 7B).

TNFshRNA Prevented the Cold-Induced Increase in Proliferating Cell Nuclear Antigen Expression
Proliferating cell nuclear antigen (PCNA) is expressed in the cell nuclei during DNA synthesis and replication and is thus considered as a marker of cell proliferation. We evaluated the expression of PCNA in isolated PASMCs using both immunocytochemical staining (Figure 8A and 8B) and Western blot (Figure 8C and 8D) analysis. PCNA expression was increased in the cold PBS and cold ScrshRNA groups compared with warm controls (Figure 8A–8D), suggesting increased PASMC proliferation. TNFshRNA prevented the cold-induced increase in PASMC proliferation (Figure 8A–8D).

Discussion
This study demonstrated, for the first time, that chronic exposure to cold upregulated TNF-α expression and increased...
macrophage infiltration in PAs and lungs (Figures 4–6; Figure S1), indicating that cold exposure caused pulmonary inflammation. Cold-induced pulmonary inflammation was not the consequence of PH because the upregulation of TNF-α and macrophage infiltration preceded the elevation of PA pressure (Figure 1; Figure S1). Interestingly, RNA interference (RNAi) inhibition of TNF-α protein expression abolished cold-induced inflammation, PH, PA remodeling, and RV hypertrophy (Figures 2–6; Figure S5). Thus, these findings revealed a previously unidentified role of TNF-α in the pathogenesis of cold-induced pulmonary hypertension.

One single injection of TNFshRNA not only prevented the PA remodeling but also abolished the increase in PA blood pressure because of exposure to cold. The AAV-2 vector we used to deliver TNFshRNA is an effective and non-pathogenic vector that has been repeatedly to deliver therapeutic genes to the cardiovascular system for long-term control of cardiovascular disease.24,27,28 Other investigators have shown that AAV-2 is an efficient vector for delivering genes to pulmonary vessels (SMCs) and lungs for the treatment of PH.29–31 AAV delivery of TNFshRNA prevented the cold-induced upregulation of TNF-α mRNA and protein expression in lungs and PAs, indicating effective silencing TNF-α (Figure 4 and 5; Figure S7). Indeed, TNFshRNA attenuated the cold-induced increase in TNF-α levels in plasma (Figure S8). Therefore, the results suggest that AAV-based RNAi silencing of TNF-α may be new preventive and therapeutic strategy for cold-related impairment of the pulmonary circulation.

The current experiment clearly showed that cold exposure caused PA remodeling. The major PA remodeling seemed to be the increase in medial layer thickness that is primarily composed of SMCs. PASMCs isolated from cold-exposed animals had elevated protein expression of PCNA (Figure 8), suggesting that cold exposure increased PASMC proliferation. The cold-induced PASMC proliferation may be because of upregulation of TNF-α and inflammation because RNAi silencing of TNF-α prevented the cold-induced increase in PCNA (Figure 8). Proliferation of PASMCs results in PA hypertrophy and is a feature common to almost all forms of PH.32–34 PA remodeling can lead to narrowing or obliteration of the PAs and increase the PA resistance and the PA pressure.

Figure 6. Tumor necrosis factor-α short hairpin small–interfering RNA (TNFshRNA) prevented cold-induced infiltration of macrophages in the lungs. A, Immunohistochemical analysis of infiltration of macrophages in paraffin-embedded lung sections (5 µm) using anti–CD-68 primary antibody. B, Densitometry analysis of the CD-68 staining and (C) the actual count of CD-68–positive cells. Photos are shown at x400. Arrows indicate positive staining (brown). Scale bars indicate 50 µm. Data represent mean±SEM. *P<0.05, **P<0.01, and ***P<0.001 vs warm PBS; ^P<0.05, ^^^P<0.001 vs cold PBS (n=3).
The unique finding of this study is that cold exposure results in a robust inflammatory response characterized by increased levels of the proinflammatory cytokines TNF-α (lungs and PA), IL-6 (PA only), and lung macrophage infiltration. RNAi silencing of TNF-α effectively prevented the cold-induced increases in IL-6 expression and macrophage infiltration, suggesting that the upregulation of TNF-α plays a critical role in cold-induced inflammation. Tuder et al. first identified inflammatory infiltrates in the plexiform lesions of patients with PH in 1994 and other studies since have shown an increase in additional inflammatory mediators including intracellular adhesion molecules, vascular adhesion molecules, selectins, and integrins. The monocrotaline-induced PH model also shows massive mononuclear infiltration. Therefore, inflammation is an important pathological mechanism of PH.

Cold exposure significantly increased the lung macrophage infiltration (Figure 6; Figure S1). Macrophages play a vital role in regulation of the immune response through their ability to release inflammatory mediators, including growth factors and complement proteins, and are also vital for antigen presentation to lymphocytes. Macrophages are also a major source for the production of TNF-α in the lungs. The increase in lung macrophage infiltration because of cold exposure and the subsequent production of inflammatory mediators including TNF-α may further activate downstream inflammatory pathways in the lung contributing to the pathogenesis of PH. Although this mechanism is not fully understood, inhibition of TNF-α may be preventing downstream activation of nuclear factor-κB, which is well accepted to induce both the innate and adaptive immune responses. Interestingly, macrophages are also the targets of TNF-α because TNFshRNA successfully prevented the cold-induced infiltration of macrophages in the lungs (Figure 6).

Cold exposure increased superoxide levels in isolated PASMCs (Figure 7). RNAi silencing of TNF-α abolished the cold-induced increase in superoxide production in PASMCs. Therefore, cold-induced increases in TNF-α in the pulmonary vasculature are likely contributing to the overproduction of superoxide in PASMCs. It is well established that increased superoxide production causes SMC proliferation and migration, 2 components that are vital in vascular remodeling pathologies. Furthermore, TNF-α is known to activate NADPH oxidase, a major source of vascular superoxide. Although there are several ways to block superoxide production in cells (including superoxide dismutase, diphenyl iodonium), this is the first report of viral-mediated TNF-α inhibition to suppress superoxide production in PASMCs.

The current study clearly demonstrates that PDE-1C expression increases in the PAs because of cold exposure...
and that TNF-α inhibition, an anti-inflammatory therapy, prevents the cold-induced PA PDE-1C expression (Figure 5). Although this study did not thoroughly investigate the relationship between TNF-α and PDE-1C, these findings suggest that inflammatory mediators, including TNF-α, may upregulate PDE-1C expression in response to cold exposure. PDE-1C belongs to a family of enzymes that hydrolyze second messengers, including cGMP, a vital vasodilator and inhibitor of vascular smooth muscle cell proliferation in the pulmonary circulation. The cold-induced decrease in cGMP may be contributing to PH in 2 ways: (1) decreased cGMP would decrease the vasodilatory response in PASMCs and (2) decreased cGMP would relieve its inhibitory effect on PASMC proliferation. The intracellular cGMP was decreased in PASMCs isolated from cold-exposed animals, which can be partially rescued by TNFshRNA. Therefore, the beneficial effects of TNF-α inhibition on cold-induced pulmonary hypertension, PASMC proliferation, and PA remodeling may be mediated, at least in part, by attenuation of cold-induced upregulation of PDE-1C and downregulation of intracellular cGMP in pulmonary vascular cells.

**Perspectives**

The prevalence of pulmonary and cardiovascular diseases is increased in cold regions or in winter. Cold weather is associated with increased mortality and morbidity from pulmonary and cardiovascular diseases. Cold air causes pulmonary vasoconstriction and provokes cardiovascular complications. The current study yields 2 significant findings in the pathogenesis of cold-induced PH. First, cold exposure significantly increased the expression of the proinflammatory cytokine TNF-α in the lungs and PAs. Second, inhibition of TNF-α attenuated the cold-induced increase in PA blood pressure, RV hypertrophy, PA remodeling, and pulmonary inflammation. These findings suggest that TNF-α may play a critical role in the pathogenesis of cold-induced PH and that inhibition of TNF-α may be a potential therapeutic strategy for cold-induced pulmonary vascular disorders.

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**Disclosures**

None.
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References


Novelty and Significance

What Is New?
- It is new and interesting that prolonged exposure to cold temperatures increased tumor necrosis factor (TNF)-α protein expression in the lungs and pulmonary arteries causing inflammation and leading to pulmonary remodeling and hypertension.
- This study demonstrates, for the first time, that in vivo AAV-based RNA interference inhibition of TNF-α attenuated cold-induced right ventricle hypertrophy as well as cold-induced increases in lung macrophage infiltration. These findings reveal a previously unidentified role of TNF-α in cold-induced lung inflammation and pulmonary hypertension (PH).

What Is Relevant?
- It is significant that inhibition of TNF-α attenuated cold-induced pulmonary hypertension, right ventricle hypertrophy, and pulmonary arterial remodeling, which provides a new therapeutic approach for the management of cold-induced PH.
- This study addresses an important role of TNF-α in cold-induced PH, which is a public-health concern but remains poorly understood.

Summary
Inhibition of TNF-α attenuated cold-induced PH and reversed cold-induced pulmonary arterial remodeling by suppression of inflammation in the pulmonary system and by decreasing superoxide production in the pulmonary artery smooth muscle cells, which suggests that upregulation of TNF-α protein expression may be involved in the pathogenesis of cold-induced PH.
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AAV Delivery of Tumor Necrosis Factor-α Short Hairpin RNA Attenuates Cold-induced Pulmonary Hypertension and Pulmonary Arterial Remodeling

Patrick Crosswhite, Kai Chen and Zhongjie Sun

Department of Physiology, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

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Address Correspondence to:
Zhongjie Sun, MD, PhD, FAHA
Professor of Physiology
Director, The Robert & Mary Cade Laboratory
BMSB 662A, Box 26901
Department of Physiology, BMSB 662A
College of Medicine
University of Oklahoma Health Sciences Center (OUHSC)
940 Stanton L. Young Blvd.
Oklahoma City, OK 73126-0901
USA
Zhongjie-Sun@ouhsc.edu
Tel. 405-271-2226 x56237
Fax. 405-271-3181
Online Supplemental Methods

Effects of cold exposure on pulmonary TNF-α expression, macrophage infiltration and PA blood pressure. Twenty-four rats were exposed to moderate cold (5±0.5°C) while 8 rats were kept at room temperature (warm, 23.5 ± 0.5°C). At 3, 5, 7 and 14 days after exposure to cold, 6 cold-exposed rats and 2 warm-adapted rats were anesthetized with ketamine/xylazine (85/15 mg/kg IP) for measuring RV pressure (see below). Following RV pressure measurement, rats were euthanized and perfused with saline. Lungs and PAs were collected for western blot analysis. A part of these tissues was processed for paraffin embedding for IHC analysis.

Generation of recombinant AAV.TNF. Adeno-associated virus-2 vector (AAV2, Stratagene, La Jolla, CA USA) with TNFshRNA was constructed similar to previously described. Briefly, the pAAV.TNFshRNA constructs were transfected into isolated pulmonary artery smooth muscle cells and inhibition efficiency was determined via Western blot analysis using an anti-TNF-α primary antibody (1μg/μl, Abcam Inc. Cambridge, MA, USA) as we previously described. AAV-carrying Scrambled shRNA (AAV.ScrshRNA) was and used as a control construct and was purchased from and confirmed by BD Biosciences (Palo Alto, CA. USA) not to match any known gene sequence. AAV carrying GFP (AAV.GFP) was also amplified and injected into two animals for additional controls (Fig. S4). AAV.TNFshRNA, AAV.ScrshRNA, and AAV.GFP were then packaged with pHHelper and pAAV-RC in modified AAV 293 cells to produce recombinant virus particles. The titer was determined by real-time PCR.

Animal Study protocols. This study was carried out according to the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals. This project was approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (IACUC). Six groups of male Sprague–Dawley rats (145–180g, 8 rats/group) were allowed to acclimate for one week and then anesthetized with ketamine/xylazine (85/15 mg/kg, IP) and the left jugular vein was exposed for injection. Three groups of rats were injected with either AAV.TNFshRNA, AAV.ScrshRNA or PBS (300 µl), allowed to recover for 24 hours, and moved to a climate controlled walk-in cold chamber (5.0±0.5°C). The viral particles were delivered IV at 1.2x10^8 PFU/300 µl/rat. The remaining three groups received identical injections but were kept in a climate chamber maintained 23.5±0.5°C (room temperature). After eight weeks of exposure to cold, the animals’ right ventricular blood pressure was determined prior to euthanasia via overdose of ketamine/xylazine (200/20 mg/kg, IP). Two additional animals were injected with AAV.GFP and kept in warm conditions for determining AAV localization.

Measurement of right ventricular blood pressure. Eight weeks after injection, animals were anesthetized with ketamine/xylazine (85/15 mg/kg IP) and the right jugular vein was exposed. A blood pressure recording device (PA-C40, DSI Inc. St. Paul, MN, USA) was inserted into the jugular vein and maneuvered into the right ventricle for monitoring blood pressure. After a stable signal was established, the right ventricular (RV) blood pressure was recorded once per minute for a minimum of 15 minutes using DSI Dataquest A.R.T software (DSI Inc). This RV pressure is a reliable indicator of pulmonary arterial blood pressure and has been used by numerous investigators for evaluation of pulmonary pressure. After recording the pressure, animals were euthanized immediately via overdose of ketamine/xylazine (200/20 mg/kg, IP) followed by exsanguination and perfused with heparin saline.
**Morphometric analysis of small pulmonary arteries.** Lung tissue was post-fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 µm thickness. Small and resistance PAs (50-80 µm in diameter) were examined. Basically, the morphometric measurements were taken approximately at the beginning of the 3rd order branch of PAs. At this position, the diameters small PAs were very close in the same group of animals. A total of 2-5 small PAs were examined randomly for each section in a series of 5 sections. The value was the average of 5-10 readings for each animal.

**Right ventricular hypertrophy index.** The right ventricle from each animal was dissected from the left ventricle (and septum), weighed and the hypertrophy index was calculated by normalizing the right ventricle weight to the left ventricle and septum weight.

**Western blot analysis of TNF-α, IL-6, and PDE-1C protein expression in tissue.** Pulmonary arteries and lungs were collected for Western blot analysis of protein expression of TNF-α (1µg/µl, Abcam, Cambridge, MA, USA), IL-6 (1:1000, Abcam) and PDE-1C (1:500, Santa Cruz Inc., Santa Cruz, CA, USA) as we described previously.1, 10

**Immunohistochemical (IHC) analysis of macrophage infiltration.** The IHC analysis was performed as described previously.1, 10 Briefly, macrophage infiltration was assessed using an anti-CD-68 marker (1:100, Abcam). Both density of the CD-68 staining and the number of CD-68 positive cells were measured.

**Isolation of pulmonary artery smooth muscle cells (PASMCs).** PASMCs were isolated using the method adapted from Ray et al, 2002.11 Briefly, PAs were placed in Fungizone solution (10 µl of 0.25 mg/ml Fungizone (Life Technologies, Carlsbad, CA, USA) in 10 ml Dulbecco’s Modified Eagle Medium (DMEM) (catalog #12430-054, Life Technologies). The adventitia was removed and the artery was placed in fresh DMEM and cut into small segments. The artery segments were then subjected to enzyme digestion (5.5 ml DMEM with 13.9 mg collagenase type II, 10.9 mg elastase, and 5 mg soybean trypsin inhibitor, Worthington Biochem, Lakewood, NJ, USA) for 1-1.5 hrs at 37°C. After digestion, the solution was filtered using a 70 µm filter, centrifuged in fresh medium, and transferred to 12-well plates. The media was changed after 24 hrs (and every 48 hrs thereafter) and the cells were allowed to grow unmolested for 5-7 days, depending on confluence. After the cells grew to confluence, they were trypsinized, counted and seeded into 6 well plates (3x10⁴ per well) for the PCNA and cGMP.

**Measurement of vascular superoxide production in PASMCs.** We measured superoxide production using the oxidation sensitive dye dihydroethidium (DHE, Sigma-Aldrich, Atlanta, GA, USA) in isolated PASMCs in 6 well plates. This method was provided in our previous studies.12-14,15 DHE enters the cells and is oxidized by O₂⁻ to yield ethidium bromide (EB) which binds to DNA to produce bright red fluorescence. EB emits red fluorescence (610 nm) when excited at 488 nm.

**Determination of cGMP in PASMCs.** The cGMP level was determined using a cGMP-specific ELISA assay kit (KGE003, R&D Systems, Minneapolis, MN, USA) and a microplate reader (BioTek Inc.). Lysates of PASMCs were used for measuring cGMP according to the manufacturer’s directions.
Semi-quantitative analysis of lung TNFα mRNA. TNFα mRNA was measured using RT-PCR as we described previously.10

Measurement of plasma TNFα. Plasma TNFα was measured using an ELISA kit according to the manufacturer’s instruction (R&D).

References


Online Supplemental Data

Supplemental Figure S1. Cold exposure increased macrophage infiltration in the lungs and around PAs. Macrophages were revealed using an anti-CD-68 marker. Macrophage infiltration was increased as early as 3 days after exposure to cold. (A) Photomicrographs of macrophage infiltration in the lungs. Arrows point to macrophages (dark brown staining). (B) Quantification of macrophage staining density. (C) Number of macrophage in the lungs. The data for this figure was obtained from rats exposed to cold for various lengths of time (3, 5, 7 or 14 days) while one group was kept in warm conditions (room temperature) as control. N=6.
Supplemental Figure S2. Cold exposure did not increase PA IL-6 expression until 14 days after exposure to cold. Western blot analysis of IL-6 expression in PAs. Data=means±SEM. *p<0.05 vs the Warm group. N=6.

Supplemental Figure S3. TNFshRNA attenuated the cold-induced increase in right ventricle (RV) systolic blood pressure. RV blood pressure was recorded 8 weeks after treatment with TNFshRNA using a telemetry system. Refer to Figure 2 for quantitative analysis of RV systolic blood pressure. N=6.
Supplemental Figure S4. **AAV.GFP was expressed in the lung.** Two animals were injected, via jugular veins, with the AAV.GFP construct in a similar manner to the AAV.TNFshRNA- and AAV.ScrshRNA-treated animals. After eight weeks, these animals were euthanized and perfused with paraformaldehyde. Lungs, kidneys, and aortas were psot-fixed, paraffin embedded, and sectioned at 5 μm. The tissue sections were stained with an anti-GFP specific antibody (Abcam, 1:500) followed by a secondary antibody conjugated to horseradish peroxidase (HRP, Abcam, 1:2000) to evaluate GFP expression. The AAV.ScrshRNA and PBS Control lung sections showed no positive GFP expression. Lung sections from AAV.GFP animals, however, showed strong GFP staining (brown color, indicated by arrows) throughout the lung samples. Therefore, the GFP viral construct successfully integrated into the lung tissue and produced GFP protein, indicating the successful construction of AVV constructs. In contrast, kidney and aorta sections did not show any GFP expression in the AAV.GFP-treated animals. Therefore, GFP was expressed in the lung but not systemically (kidneys, aorta), suggesting that the viral particles may be primarily trapped in the pulmonary circulation and lungs following delivery via jugular veins. The AAV-mediated gene expression still exists at 8 weeks after gene delivery (length of the study).
**Supplemental Figure S5.** **Body weight and right ventricle (RV) weight.** A) TNFshRNA did not affect body weight gain. Body weight was decreased in all cold groups, a phenomenon seen in cold-exposed animals. However, no significant difference was found between groups in either cold or warm environments, indicating that viral delivery did not affect the body weight gain. B) Cold exposure increased the RV weight significantly (RV hypertrophy). TNFshRNA prevented cold-induced RV hypertrophy (RVH). The right ventricle was dissected from the left ventricle (and septum) to determine the hypertrophic index. Data=means±SEM. **p<0.01 vs. Warm PBS; ^p<0.05 vs. Cold PBS. N=6.

**Supplemental Figure S6.** Trichrome staining indicated that collagen deposition was not detectable in the RV of rats kept at either temperature environment.
Supplemental Figure S7. TNFshRNA abolished the cold-induced increase in TNFα mRNA expression in lungs. TNFα mRNA was measured using RT-PCR. The bar graph showed semi-quantitative analysis of TNFα mRNA. Data=means±SEM. **p<0.01 vs. Warm PBS; ^^p<0.001 vs. Cold PBS. N=6.
Supplemental Figure S8. TNFshRNA abolished the cold-induced increase in plasma TNFα.
Plasma TNFα was measured using an ELISA kit (R&D). Data=means±SEM. **p<0.01 vs. Warm PBS; ^^p<0.001 vs. Cold PBS. N=6.