Inhibition of Phosphodiesterase-1 Attenuates Cold-Induced Pulmonary Hypertension

Patrick Crosswhite, Zhongjie Sun

Abstract—Chronic exposure to cold caused pulmonary arterial hypertension (cold-induced pulmonary hypertension [CIPH]) and increased phosphodiesterase-1C (PDE-1C) expression in pulmonary arteries (PAs) in rats. The purpose of this study is to investigate a hypothesis that inhibition of PDE-1 would decrease inflammatory infiltrates and superoxide production leading to attenuation of CIPH. Three groups of male rats were exposed to moderate cold (5±1°C) continuously, whereas 3 groups were maintained at room temperature (23.5±1°C, warm; 6 rats/group). After 8-week exposure to cold, 3 groups in each temperature condition received continuous intravenous infusion of 8-isobutyl-methylxanthine (8-IBMX) (PDE-1 inhibitor), apocynin (NADPH oxidase inhibitor) or vehicle, respectively, for 1 week. Cold exposure significantly increased right-ventricular systolic pressure compared with warm groups (33.8±3.2 versus 18.6±0.3 mmHg), indicating that animals developed CIPH. Notably, treatment with 8-IBMX significantly attenuated the cold-induced increase in right ventricular pressure (23.5±1.8 mmHg). Cold exposure also caused right-ventricular hypertrophy, whereas 8-IBMX reversed cold-induced right ventricular hypertrophy. Cold exposure increased PDE-1C protein expression, macrophage infiltration, NADPH oxidase activity, and superoxide production in PAs and resulted in PA remodeling. 8-IBMX abolished cold-induced upregulation of PDE-1C in PAs. Interestingly, inhibition of PDE-1 eliminated cold-induced macrophage infiltration, NADPH oxidase activation, and superoxide production in PAs and reversed PA remodeling. Inhibition of NADPH oxidase by apocynin abolished cold-induced superoxide production and attenuated CIPH and PA remodeling. In conclusion, inhibition of PDE-1 attenuated CIPH and reversed cold-induced PA remodeling by suppressing macrophage infiltration and superoxide production, suggesting that upregulation of PDE-1C expression may be involved in the pathogenesis of CIPH. (Hypertension. 2013;61:xxx-xxx.) • Online Data Supplement

Key Words: blood pressure • macrophage infiltration • NADPH oxidase • pulmonary artery • remodeling • right ventricle • smooth muscle cell proliferation • superoxide

The development of pulmonary hypertension (PH) is multifactorial with genetic background and environmental stress being 2 critical components. The adverse effects of cold temperatures on the human cardiovascular system are well documented. 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. Exposure to cold temperatures is an important public health concern, particularly for those dying from cardiorespiratory diseases. In the United States, cold weather is associated with increased mortality from pulmonary and cardiovascular diseases. Cold exposure causes pulmonary vasospasm in patients with Raynaud’s phenomenon. Cold exposure has been demonstrated to induce PH in several animal models including broilers, rats, and bovine. However, the underlying mechanism of cold-induced PH remains poorly understood.

Phosphodiesterases (PDEs) are a family of enzymes that catalyze the breakdown of the second messengers, cGMP and cAMP. Currently, 11 different families of PDEs have been described, each with several different subtypes. Three different PDE-1 variants have been described, PDE-1A, PDE-1B, and PDE-1C. Previously, these PDEs were given the term CaM-PDEs for their reliance on calcium-calmodulin for activity. PDE-1A and 1B hydrolyze cGMP more efficiently, whereas PDE-1C hydrolyzes both cGMP and cAMP equally. The PDE family has been a focus of drug development in recent years, especially for cardiovascular diseases, because of the favorable effects second messengers have in the vasculature that include increasing vasodilation and decreasing smooth muscle cells (SMC) proliferation. Early investigations revealed that PDE-1 inhibition increased aortic vasodilation. Recently, Schermuly et al showed that PDE-1C protein expression is increased in proliferating pulmonary vasculature in human idiopathic pulmonary hypertension. For these reasons, PDE-1 has generated significant interest in PH research.

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Preliminary studies in our laboratory indicated that cold exposure causes cold-induced pulmonary hypertension (CIPH) and pulmonary artery (PA) remodeling and upregulates expression of PDE-1C. Increased PDE-1 activity decreases intracellular cGMP levels, which may increase PA resistance and SMC proliferation. Cold exposure also increased inflammation in the cardiovascular system.2 The aim of this study is to investigate a hypothesis that inhibition of PDE-1 would decrease inflammatory infiltrates and superoxide production and attenuate CIPH.

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

Animal Study Protocols
Six groups of male Sprague–Dawley rats were used (150–180 g, 6 rats/group). Three groups of rats were exposed to a climate-controlled walk-in chamber maintained at moderate cold (5.0±1°C). The remaining groups were kept in an identical chamber maintained at room temperature (23.5±1°C, warm) and served as controls. After 8 weeks of exposure to cold, 3 groups in each temperature condition received continuous intravenous infusion of 8-isobutyl-methylxanthine (8-IBMX) (PDE-1 inhibitor, 8.5 mg/kg per day),18 apocynin (NADPH oxidase inhibitor, 25 mg/kg per day),19,20 and vehicle (dimethyl sulfoxide [DMSO], 50%), respectively. The doses of drugs have been validated for effective inhibition of PDE-1 and NADPH oxidase activity, respectively.18–20 Body weight was measured weekly. After 1 week of drug infusion, the animals’ right-ventricular (RV) systolic blood pressure was measured under anesthesia. The RV blood pressure is a reliable indicator of pulmonary arterial blood pressure and has been used by numerous investigators for evaluating PH.*** For details, refer to the Methods and Data in the online-only Data Supplement.

Western Blot Analysis of PDE-1 and PDE-5 Protein Expression in Tissue
Protein expression of PDE-1A, PDE-1B, PDE-1C, and PDE-5 was measured as we described previously.2,26

Morphometric Measurements and Immunohistochemical Analysis of CD-68 and SM α-Actin Expression
The histological and immunohistochemical analysis of macrophage infiltration and SM α-actin expression were performed as described in our recent studies.2,26 For details, refer to the Methods and Data in the online-only Data Supplement.

Measurement of In Situ Superoxide Production
The in situ PA superoxide production was assessed using dihydroethidium staining as we described recently.2,26 For details, refer to the Methods and Data in the online-only Data Supplement.

Measurement of NADPH Oxidase Activity
NADPH oxidase activity was assessed using the lucigenin assay as we described recently.2,26 For details, refer to the Methods and Data in the online-only Data Supplement.

Measurement of Pulmonary cGMP Levels
The pulmonary cGMP levels were measured using a KGE 003 cGMP Parameter Assay Kit (R&D Systems) according to manufacturer instruction. The data were normalized with protein for each animal.

Statistical Analysis
Data were analyzed by 1-way ANOVA. Tukey multiple comparison tests were used to assess the significance of differences between means. Significance was set at a 95% confidence limit.

Results
8-IBMX and Apocynin Reduced CIPH
The RV systolic blood pressure measured at 9 weeks after exposure to cold was elevated significantly in the Cold-DMSO group (33.8±3.2 mmHg) compared with the Warm-DMSO, Warm-Apocynin, and Warm-IBMX groups (18.6±0.6, 20.7±0.5, and 19.6±0.5 mmHg, respectively). Thus, cold exposure caused CIPH. Treatments with IBMX and apocynin significantly decreased cold-induced elevation of RV pressure (23.5±1.8 and 24.2±0.6 mmHg, respectively), although they did not decrease RV pressure to the warm control levels (Figure 1A).

Compared with the 3 warm control groups, the Cold-DMSO group showed a significant increase in the ratio of RV/(left ventricle+septum) after normalizing to body weight (Figure 1B), suggesting that cold-exposed rats developed RV

Figure 1. 8-IBMX and apocynin reduced right-ventricular blood pressure (RVP). A, In vivo RVP. After 1 week of drug infusion, animals were anesthetized and RV systolic blood pressure was monitored for 20 minutes (1 reading/min) using a telemetry system (DSI). B, RV weight. The free RV was separated from the left ventricle (LV) for calculating the ratio of RV/(LV+septum). C, Morphometric measurements of the RV wall thickness (for histological micrographs, refer to Figure S1C). *P<0.05, **P<0.01, ***P<0.001 vs Warm-DMSO; ΔP<0.05, ΔΔP<0.01, ΔΔΔP<0.001 vs Cold-DMSO; #P<0.05, ##P<0.01, ###P<0.001 vs Warm-IBMX.)
hypertrophy (RVH). IBMX attenuated cold-induced RVH, whereas treatment with apocynin slightly, but not significantly, decreased RVH (Figure 1B). The RV wall thickness was increased by cold exposure (Figure 1C; Figure S1C in the online-only Data Supplement). IBMX prevented the cold-induced increase in RV wall thickness. Trichrome staining showed that there is no significant difference in collagen levels between groups (not shown). IBMX and apocynin did not affect systemic blood pressure or body weight gain (Figure S1A and S1B).

**Figure 2.** 8-IBMX and apocynin reversed cold-induced remodeling of pulmonary arteries (PAs). A, Photomicrograph of lung and PAs (H&E staining). B, PA medial layer thickness. C, PA lumen diameters. D, Ratio of the medial layer to lumen diameter. PAs with a diameter of 60 to 80 µm were selected for morphological analysis. Sections were viewed at ×200. **P<0.01, ***P<0.001 vs Warm-dimethyl sulfoxide (DMSO); +P<0.05, +++P<0.001 vs Warm-Apocynin; ^P<0.05, ^^P<0.01, ^^^P<0.001 vs Cold-DMSO; #P<0.05, ##P<0.01 versus Warm-IBMX.

**Figure 3.** 8-IBMX and apocynin attenuated cold-induced pulmonary artery (PA) smooth muscle cell (SMC) proliferation. A, The immunohistochemical analysis of α-SM actin (α-SMA) staining in the smooth muscle layer of small PAs of the lungs. The negative control (without primary antibody) showed no α-SMA staining indicating antibody specificity. Arrows indicate α-SMA–positive staining (brown). Sections were viewed at ×400. B, Semiquantitative analysis of the α-SMA staining in small PAs. **P<0.01 vs Warm-dimethyl sulfoxide (DMSO); +P<0.05, +++P<0.001 vs Warm-Apocynin; ^P<0.05, ^^P<0.01 vs Cold-DMSO.
8-IBMX and Apocynin Reversed Cold-Induced Remodeling of Pulmonary Arteries (PAs)

We examined small PAs with diameters of 60 to 80 μm. Cold exposure increased medial layer thickness of small PAs in the Cold-DMSO group (22.7±1.3 μm) compared with Warm-DMSO, Warm-Apocynin, and Warm-IBMX groups (17.4±0.8, 15.6±0.6, and 17.7±0.3 μm, respectively; Figure 2A and 2B). Cold exposure also caused narrowing of the PA lumen in the Cold-DMSO group (32.2±3.0 μm) compared with Warm-DMSO, Warm-Apocynin, and Warm-IBMX groups (56.8±4.6, 55.9±3.5, and 62.1±3.1 μm, respectively; Figure 2A and 2C). IBMX or apocynin significantly reduced medial layer thickness (19.0±0.9, and 16.9±0.8 μm, respectively) and increased lumen diameter (62.7±4.2, and 59.5±4.3 μm, respectively) of small PAs in cold-exposed rats. Cold exposure increased the ratio of the medial layer thickness to the lumen diameter which can be abolished by 8-IBMX or apocynin (Figure 2D).

8-IBMX and Apocynin Attenuated Cold-Induced PA SMC Proliferation

Alpha smooth muscle actin (α-SMA) is a marker of proliferating SMCs. We used immunohistochemical with an α-SMA specific antibody to semiquantify the expression of α-SMA in small resistance PAs (40–80 μm). The α-SMA expression in PA medial layer of the Cold-DMSO group was increased significantly compared with the 3 warm groups (Figure 3A and 3B). IBMX and apocynin significantly reduced α-SMA expression in PAs of cold-exposed rats (Figure 3A and 3B), suggesting decreased PA SMC proliferation.

8-IBMX and Apocynin Attenuated Cold-Induced Superoxide Production in PAs

Cold exposure increased PA superoxide production (Figure 4A; photomicrograph in Figure S2) and NADPH oxidase activity (Figure 4B) compared with warm controls. Treatment with apocynin reduced superoxide production and NADPH oxidase activity to the warm control levels (Figure 4A and 4B), indicating a role of NADPH oxidase in the cold-induced increase in superoxide in PAs. Interestingly, IBMX abolished the cold-induced increases in NADPH oxidase activity and superoxide production (Figure 4A and 4B), suggesting that upregulation of PDE-1 may increase NADPH oxidase activity and superoxide production in PAs of cold-exposed rats.

The mitochondrial superoxide dismutase protein expression was not altered significantly by cold exposure or treatments with IBMX or apocynin (Figure S3A and S3B), suggesting that the increases in PA superoxide levels may not be attributable to alteration of mitochondrial superoxide dismutase protein expression.

8-IBMX Abolished the Cold-Induced Increase in PDE-1C Protein Expression in PAs

Cold exposure increased protein expression of PDE-1C in PAs compared with the 3 warm control groups (Figure 5A and 5B). PDE-1A levels were unaffected by either cold exposure or drug treatments (Figure 5A and 5C), whereas PDE-1B levels were not detectable by Western Blot (not shown). PDE-5 protein expression in the lungs was not altered significantly by cold exposure or drug treatments (Figure S4). Thus, cold exposure selectively upregulates PDE-1C expression in PAs. Treatment with IBMX or apocynin abolished cold-induced increases in PDE-1C expression on PAs (Figure 5A and 5C). PDE-1A and PDE-1C protein expression in aortas and kidneys were not altered by cold exposure or by IBMX (Figures S5 and S6).

Cold exposure decreased the pulmonary cGMP levels, which can be abolished by IBMX (Figure 5D). These data indicate that the cold-induced decrease in pulmonary cGMP bioavailability can be rescued by PDE-1 inhibition.

8-IBMX Decreased Cold-Induced Macrophage Infiltration

Chronic cold exposure remarkably increased infiltration of CD-68-–positive macrophages around the small PAs of the lungs compared with the 3 warm groups (Figure 6A–6C). IBMX significantly attenuated cold-induced macrophage infiltration. Apocynin decreased macrophage infiltration, although the difference was not significant when compared with the DMSO-Cold group (Figure 6A and 6B). These findings indicate that cold exposure increased inflammation, which can be abolished by PDE-1 inhibition.

Cold exposure did not increase interleukin-1β or interleukin-6 levels in the lungs (Figure S7), suggesting that cold-induced macrophage infiltration may not be mediated by these cytokines. IBMX significantly decreased these cytokines (Figure S7).

Discussion

Although the adverse effect of cold exposure on the systemic circulation is well documented, its effects on the pulmonary circulation is poorly studied. The present study demonstrates that cold exposure caused CIPH and PA remodeling...
in rats, which is supported by several previous reports. PDE-1C was upregulated in PAs in response to cold exposure (Figure 5). Interestingly, inhibition of PDE-1 effectively attenuated CIPH and RVH and reversed PA remodeling in cold-exposed animals (Figures 1 and 2), indicating that upregulation of PDE-1 may be involved in the pathogenesis of CIPH. These findings suggest that inhibition of PDE-1 is an effective therapeutic strategy for cold-related impairment in the pulmonary circulation, which is important for people who live in cold regions or work outside during the winter months.

The PDE-1C enzyme regulates intracellular signaling mechanisms by degrading both cGMP and cAMP. Reduced cGMP levels promote vasoconstriction, proliferation of vascular SMC and endothelial dysfunction, contributing to PH. The present study demonstrated that cold exposure decreased pulmonary cGMP levels and that treatment with IBMX for 1 week restored cGMP to warm control levels and most importantly attenuated CIPH and PA remodeling (Figures 2 and 6). It seems that IBMX and apocynin mainly affected the pulmonary circulating system and lungs because PDE-1A and PDE-1C expression in aortas and kidneys were not altered by the treatments (Figures S5 and S6). Therefore, after intravenous infusion via the jugular vein, the drugs were effectively taken up by the pulmonary circulation.

In addition to the regulation of cGMP levels, PDE-1C also has the ability to decompose cAMP. Decreased cAMP levels promote inflammation, including cytokine production that is known to activate NADPH oxidases (Figure S9). It was recently shown that decreased cAMP levels promoted superoxide production via activating the Rac1-NADPH oxidase pathway. Superoxide derived from the NADPH oxidase is a major oxidant source in the vasculature and can promote the development of oxidative stress. Although superoxide production is necessary for a variety of cellular processes (e.g., cell signaling, host defense), overproduction of superoxide can lead to oxidative damage in the vasculature contributing to endothelial dysfunction, vascular remodeling, and hypertension. The present study demonstrated that cold exposure increased NADPH oxidase activity and superoxide production in PAs (Figure 4). Treatment with the NADPH oxidase inhibitor apocynin and the PDE-1 inhibitor 8-IBMX significantly reduced NADPH oxidase activity and superoxide production in PAs and improved CIPH and reversed PA remodeling (Figures 2 and 6). Although apocynin acts to directly inhibit NADPH oxidase, the mechanism by which 8-IBMX decreased cold-induced superoxide production is unclear at this time. Based on literatures, inhibition of PDE-1C could decrease NADPH activity via 2 pathways: (1) increasing cAMP levels and preventing low cAMP-related activation of NADPH oxidase; and (2) eliminating low cAMP-associated inflammation and thus attenuating inflammation-related activation of NADPH oxidase (Figure S9).

Inhibition of NADPH oxidase activity by apocynin abolished the cold-induced increase in PDE-1C protein expression (Figure 5), suggesting that NADPH oxidase and superoxide may mediate cold-induced PDE-1C expression. On the other hand, inhibition of PDE-1 activity decreased NADPH oxidase activity and superoxide production (Figure 4B and 4C), which, in turn, decreased PDE-1C expression (Figure 5). As depicted in Figure S9, the upregulation of PDE-1C may promote NADPH oxidase activity and superoxide production, which then induce PDE-1C expression. Thus, this study revealed an important role of the NADPH oxidase and superoxide in the regulation of PDE-1C expression. Heumüller et al suggested that in vascular cells, apocynin functions more as an antioxidant than as a specific inhibitor of NADPH oxidase. As the changes in NADPH oxidase activity are minor...
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(≈15%; Figure 4C), the effects of apocynin may be partially
mediated by its antioxidant action.

One of the major pathological changes in PH patients is the
proliferation of SMCs in PAs, especially the small distal PAs.
Proliferation of PA SMCs leads to extensive vascular remodel-
ing and is a feature common to almost all forms of PH.44,46,47
Proliferating SMCs lead to narrowing of blood vessels, thus
resulting in increased PA resistance and PA remodeling. In
the present study, we showed that cold exposure increased PA
SMC proliferation and medial thickness and decreased small
PA lumen (Figures 2 and 3). The improved PA remodeling
by PDE-1 inhibition is likely a cumulative effect of several
results, including the following: (1) decreases in cold-induced
macrophage infiltration and inflammation; (2) reduction of
cold-induced increases in NADPH oxidase activity and super-
oxide production; (3) elimination of the cold-induced decrease
in cGMP bioavailability; and (4) reduction of PA pressure.
Although significant improvements in managing PA pressure
and endothelial function have been made with recent therapeu-
tics, treating PA remodeling has remained elusive. If PH is to
be managed effectively, the PA remodeling must be addressed.
To our knowledge, this is the first study demonstrating that
8-IBMX treatment reversed cold-induced PA remodeling.

An upregulation of PDEs has been suggested to play a role
in human PH.48 However, the pulmonary PDE-5 expression
was not altered significantly by cold exposure or by IBMX
(Figure S4). PDE-5 is primarily expressed in the arterial walls
of the lungs and penis and acts to decompose the second mess-
cenger cGMP. The first PDE-5 inhibitor, sildenafil citrate,
was originally used for the treatment of erectile dysfunction
but was approved by Food and Drug Administration in 2005
for the treatment of PH.36 It is marketed under the name of
Revatio.36 Although PDE-1 is expressed in a relatively low
level, a significant increase in expression is observed in prolif-
erating pulmonary vasculature.48

It has been shown that lung inflammation precedes the
development of PH in rodent models.49 Furthermore, the
recruitment of monocytes/macrophages was demonstrated
to be vital to vascular remodeling in hypoxia-induced PH.50
In this study, we found that cold exposure increased macro-
phage infiltration around PAs, indicating increased inflam-
mation. Interestingly, treatment with 8-IBMX attenuated
the infiltration of macrophages around the small PAs from the
lungs of cold-exposed animals (Figure 6). While examin-
ing macrophages in the lung tissue, we also found that the
alveolar diameter was decreased but cell infiltrates were
increased in cold-exposed rats, which can be abolished by
IBMX (Figure S8). Infiltration of macrophages in the lungs
of cold-exposed animals may promote a switch to a proin-
flammatory state that allows for leukocytes, cytokines, and
superoxide to initiate the proliferation and remodeling pro-
cess of the pulmonary vasculature (Figure S9). Significant
remodeling of the lung vasculature, especially the small PAs,
can lead to an increase in pulmonary vascular resistance
and, eventually, to the development of PH. Unexpectedly,
the levels of interleukin-1β and interleukin-6 in the lungs
were not altered significantly by cold exposure (Figure S7).
Additional studies are needed to explore the underlying
mechanism of cold-induced inflammatory responses and its
involvement in CIPH.
It is noted that both IBMX and apocynin decreased CIPH to a similar extent but only IBMX attenuated RVF weight (Figure 1), suggesting that cold-induced RVH may be independent of elevation of pulmonary arterial blood pressure. The antihypertrophic effect of IBMX is likely attributable to inhibition of PDE-1 in the heart. The NADPH oxidase may not contribute significantly to cold-induced RVH.

**Perspectives**

Chronic exposure to cold upregulated PDE-1C in small PAs and caused PH and PA remodeling. Inhibition of PDE-1 with 8-IBMX decreased cold-induced macropage infiltration and superoxide production in PAs and attenuated CIPH and PA remodeling. Therefore, inhibition of PDE-1 represents an effective therapeutic strategy for CIPH and related cardiovascular dysfunctions. The findings have major implications for people who live in cold regions or in winter. Further studies are warranted to determine the mechanistic link of PDE-1C and NADPH oxidase in the context of inflammation in the pathogenesis of CIPH.

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None.

**References**


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**Novelty and Significance**

**What Is New?**

- It is new and interesting that continuous exposure to cold temperatures increased phosphodiesterase (PDE)-1C protein expression and macrophage infiltration in pulmonary arteries, which leads to pulmonary arterial hypertension and remodeling.
- This study demonstrates, for the first time, that inhibition of PDE-1 attenuated cold-induced increases in macrophage infiltration and NADPH oxidase activity in pulmonary arteries, which reveals a previously unidentified role of PDE-1 in the regulation of inflammation and superoxide production.

**What Is Relevant?**

- It is significant that inhibition of PDE-1 attenuated cold-induced pulmonary arterial hypertension (CIPH) and pulmonary artery remodeling, which provides a new therapeutic approach for the management of CIPH and related cardiovascular disorders.
- This study addresses an important role of PDE-1 in CIPH, which is a public health concern but remains poorly explored.

**Summary**

Inhibition of PDE-1 attenuated CIPH and reversed cold-induced pulmonary artery remodeling by suppressing macrophage infiltration and superoxide production, which suggest that upregulation of PDE-1C expression may be involved in the pathogenesis of CIPH.
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