Online Supplemental Methods and Data

Inhibition of Phosphodiesterase-1 Attenuates Cold-Induced Pulmonary Hypertension (CIPH)

Patrick Crosswhite and Zhongjie Sun

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

Running title: PDE1 and CIPH

Total characters: 22,400; **Total words:** 3268

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 S.L. Young Blvd.
Oklahoma City, OK 73126-0901
USA
Zhongjie-sun@ouhsc.edu
Tel. 405-271-2226 x 56237
Fax. 405-271-3181

Disclosures: Nothing to disclose. The authors confirm that there are no conflicts of interest.

Online Supplemental Methods

Animals. This study was carried out according to the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals. The project was approved by the Institutional Animal Care and Use Committee. Six groups of male Sprague–Dawley rats were used (150–180g, 6 rats/group). All rats were housed individually in wire-mesh cages and were provided with Purina laboratory chow (No. 5001) and tap water *ad libitum* throughout the experiment.

Animal study protocols. Following a one-week control period, 3 groups of rats were moved to a climate-controlled walk-in chamber maintained at moderately cold (5.0±1°C). The remaining groups were kept in an identical chamber maintained at room temperature (25±1°C, warm) and served as controls. The humidity of both environments was maintained at 45±5%. After eight weeks of cold exposure, animals were anesthetized (ketamine/xylazine, 85/10 mg/kg, IP) and osmotic mini-pumps (Model 2ML1, Alzet) were placed subcutaneously with a catheter inserted into the external jugular vein for continuous *i.v.* infusion of drug. Briefly, 3 groups in each temperature condition received 8-IBMX (PDE-1 inhibitor, 8.5 mg/kg/day[1]), apocynin (NADPH oxidase inhibitor, 25 mg/kg/day[2-3]) or the vehicle (dimethyl sulfoxide -DMSO, 50%). The doses of drugs have been validated for effective inhibition of PDE-1 and NADPH oxidase activity, respectively.[1-3] Body weight was measured weekly and 24-hr urine samples were collected before and during drug delivery using metabolic cages.

After one week of drug infusion, the animals' right ventricular systolic blood pressure (RVBP) was measured under anesthesia (ketamine/xylazine, 75/5 mg/kg, IP) by maneuvering a PA-C40 (Data Sciences International, Minneapolis, MN) transmitter into the RV *via* the left jugular vein. The RVBP was recorded continuously for 20 minutes (1 reading/min). The data presented in Figure 1A is the average value of RVBP over a 20-min period. The RVBP is a reliable indicator of pulmonary arterial blood pressure (PAP) and has been used by numerous investigators for evaluating PH[4-8]. After obtaining stable RVP, the animals were euthanized with overdose of ketamine/xylazine (200/20 mg/kg, IP) for tissue collections. Because RV hypertrophy (RVH) is a hallmark of pulmonary hypertension, we examined the RV weight and wall thickness.

Measurements of Systemic Blood Pressure

Systolic blood pressure (BP) was measured using a CODA 6 BP monitoring system (Kent Scientific) as described in our previous study[9]. The animals were handled twice a day to minimize handling stress. The animals were trained for the tail-cuff measurements. No signs of stress were seen during BP measurements. The volume-based tail-cuff measurements of BP have been validated by using a telemetry system[10]. The tail-cuff procedure is a common method used by our laboratory[9, 11-13] and others[14-15] to delineate cold-induced elevation of BP. It has been confirmed by the intra-arterial cannulation that the noninvasive tail-cuff method is effective and reliable in monitoring systolic BP in animals exposed to cold.[16-18]

Measurement of *In Situ* Superoxide Production

The *in situ* superoxide production was measured in pulmonary arteries using the oxidation sensitive dye dihydroethidium (DHE, Sigma-Aldrich, Atlanta, GA, USA). Dihydroethidium

enters the cells and is oxidized by O_2^- 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. Briefly, unfixed pulmonary artery rings were embedded in OCT, frozen at −80 °C, and cut at 10 μm using a cryostat. Sections were incubated in PBS (37°C) in a humidified chamber for 30 min followed by incubation with DHE (10^{-5} M in PBS) in the dark for 30 min. The preparations were counterstained with the nuclear stain 4, 6-diamidin-2-phenylindol dichlorohydrate (DAPI, 3×10^{-7} M, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 37°C for 5 min and mounted on slides. The images were captured with FITC filter using Leica TCS NT Confocal fluorescence microscopy, the average intensity was measured at 400 X magnification in three randomly chosen fields (15 cells each) from three independent experiments. EB and DAPI fluorescence were quantified using the Image J software as described in our recent studies.[19-22]

Quantification of NADPH Oxidase Activity

NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity in pulmonary arteries was assessed using the lucigenin-enhanced chemiluminescence method as we described recently.[19-21] To prevent autoxidation of lucigenin, a low concentration (5 μM) of lucigenin was used, as previously described[23] with the following modifications. Tissue sections (40 um) were rinsed in ice-cold PBS and kept in 96-well plate in cold saline on ice for 10 min. Sections were incubated with lucigenin in the dark for 15 min. Background counts were then obtained by measuring chemiluminescence using a luminometer (Biotek synergy 2 Luminometer) for 5 min (with a 2-min dark adjustment). To evaluate NAD(P)H oxidase activity, 100 μM NAD(P)H was then added to samples, and luminescence was measured for an additional 8 min. Background counts (with lucigenin) were subtracted from each value.

Morphometric measurements and IHC analysis of SM α-actin expression and macrophage infiltration. The histological and IHC analysis of macrophage infiltration and SM α-actin expression were performed as described in our recent studies.[9, 24] Lung and pulmonary artery (PA) tissue were post-fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm thickness. Basically, the morphometric measurements were taken approximately at the beginning of the 3rd order branch of pulmonary arteries (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.

Activation of inflammatory cascades could contribute to the cardiovascular disease in rodents and humans. Macrophage infiltration was assessed using a CD-68 marker (1:100, Abcam) as described in our recent studies.[9, 24] PA SMC proliferation was assessed by semi-quantifying protein expression of α-SM actin using α-SMA specific antibody (1:500, Abcam). α-SMA protein expression was determined by measuring the density and the total area and of α-SMA-positive staining using the image J software as described in our recent studies. [9, 24]

Western blot analysis of PDE-1 and PDE-5 protein expression in tissue. Pulmonary arteries and kidneys were collected for western blot analysis of protein expression of PDE-1A (1:500, Santa Cruz), PDE-1B (1:500, Santa Cruz), and PDE-1C (1:500, Santa Cruz) as we described previously.[9, 24]. Kidney cortex was used for western blot analysis. PDE-5 protein expression was measured in lungs (1:500, Cell Signaling)

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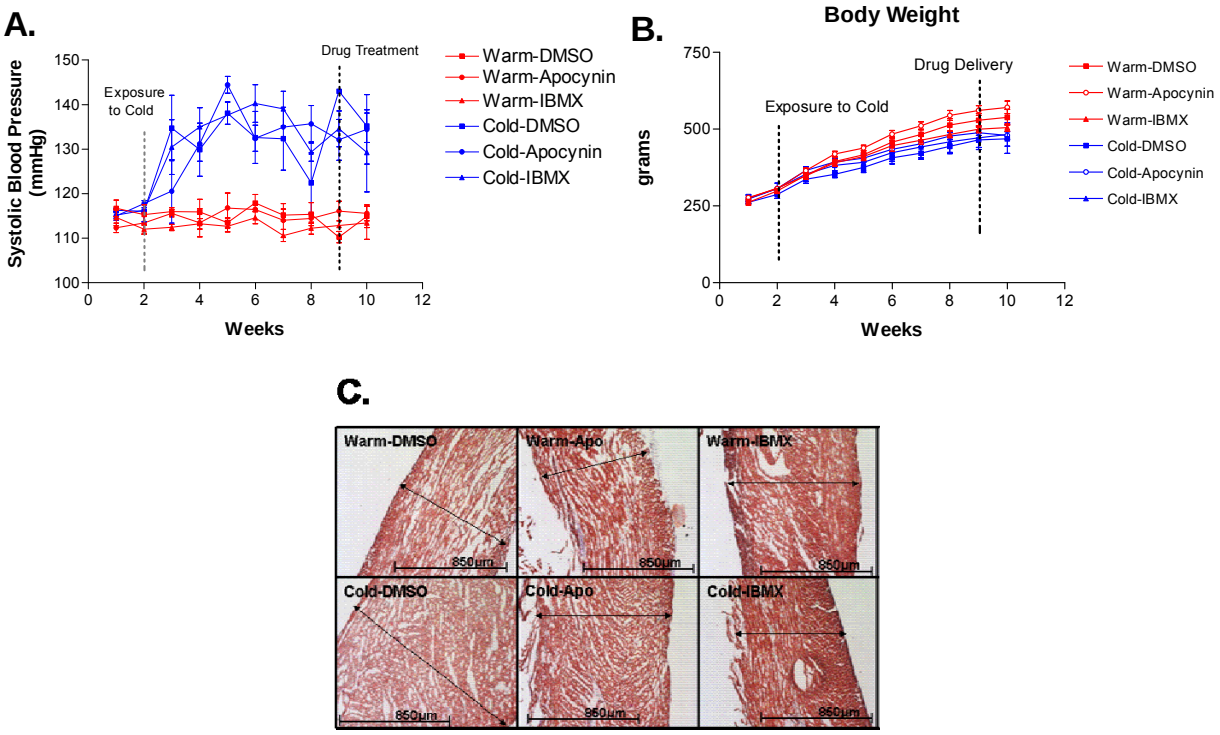
Online Supplemental Data

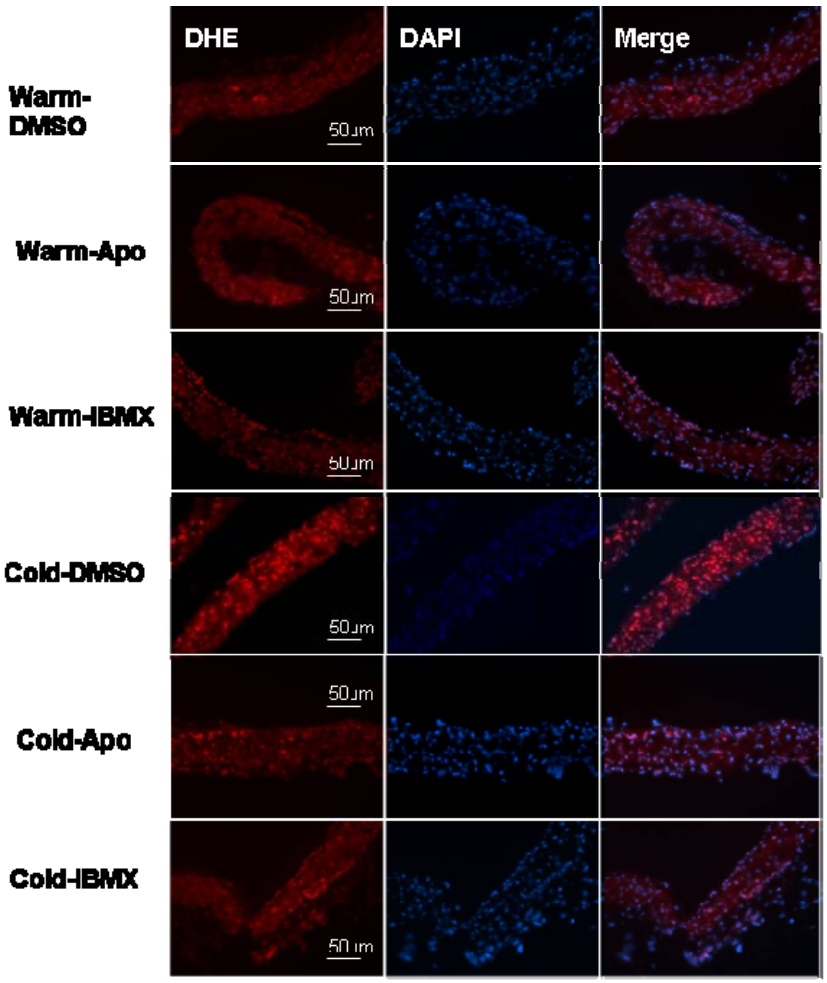


Supplemental Figure S1. 8-IBMX and apocynin did not significantly affect the systemic blood pressure (BP) and body weight. Systemic BP was increased within one week of exposure to cold and remained elevated thereafter **(A)**. Systemic BP was not significantly altered during the week of drug administration.

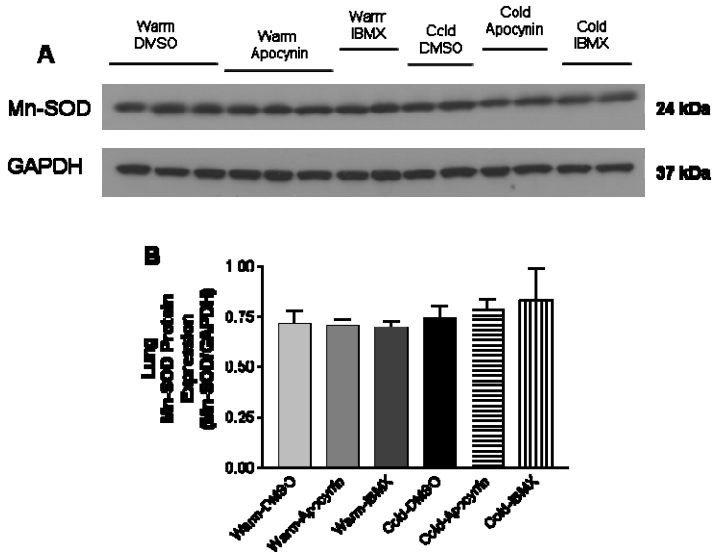
All warm groups showed an increase in body weight compared to the cold groups but there was no significance amongst the 3 warm groups and amongst the 3 cold groups **(B)**. In our experiments, an average lower body weight due to cold exposure is regularly observed.[9, 13]

Exposure to cold significantly increased the RV wall thickness, which can be attenuated by treatment with IBMX **(C)**. For quantitative analysis, please refer to Figure 1C.

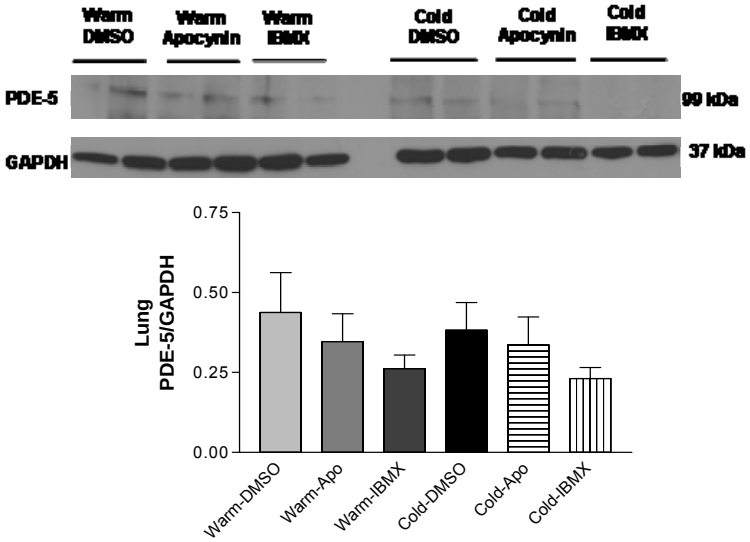
The data for blood pressure and body weight were analyzed by a repeated measures one-way analysis of variance (ANOVA). Tukey's multiple comparison tests were used to assess the significance of differences between means. Significance was set at a 95% confidence limit. N=6.



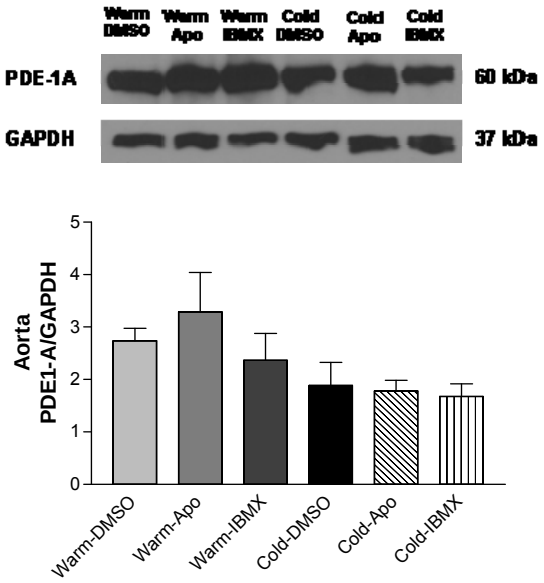
Supplemental Figure S2. Superoxide production was viewed using the dye DHE. DAPI was used to view nuclear staining. **A,** Photomicrograph of PA segments showing DHE staining, DAPI staining, and a merge of DHE and DAPI, respectively. Red fluorescence is indicative of superoxide production. Blue staining indicates nuclei. For quantitative analysis, see Figure 4A.



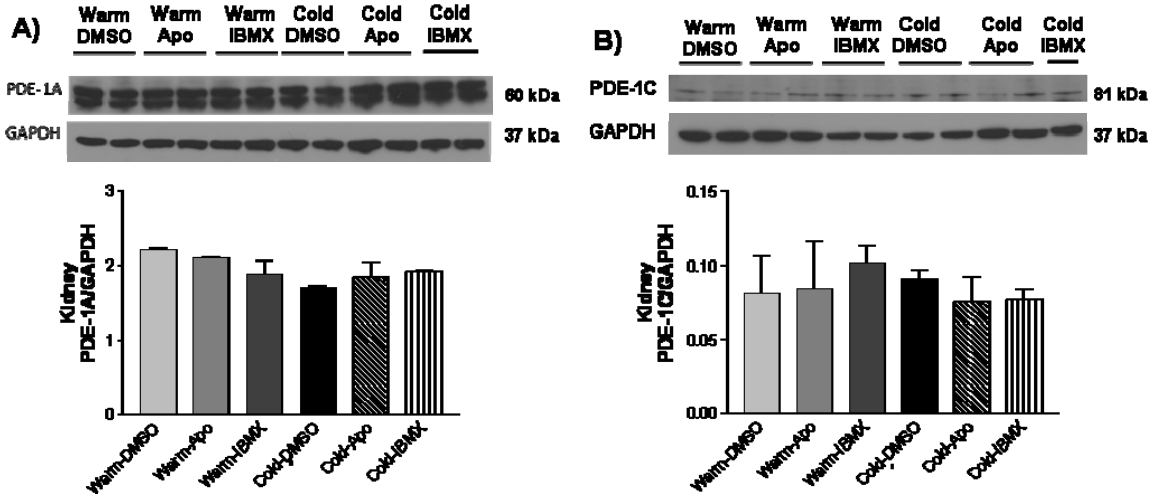
Supplemental Figure S3. Mn-SOD protein expression in lungs was not altered by cold exposure or treatment with IBMX or apocynin. Western blot analysis of Mn-SOD protein expression **(A)** and quantification of Mn-SOD protein expression **(B)**. N=3.



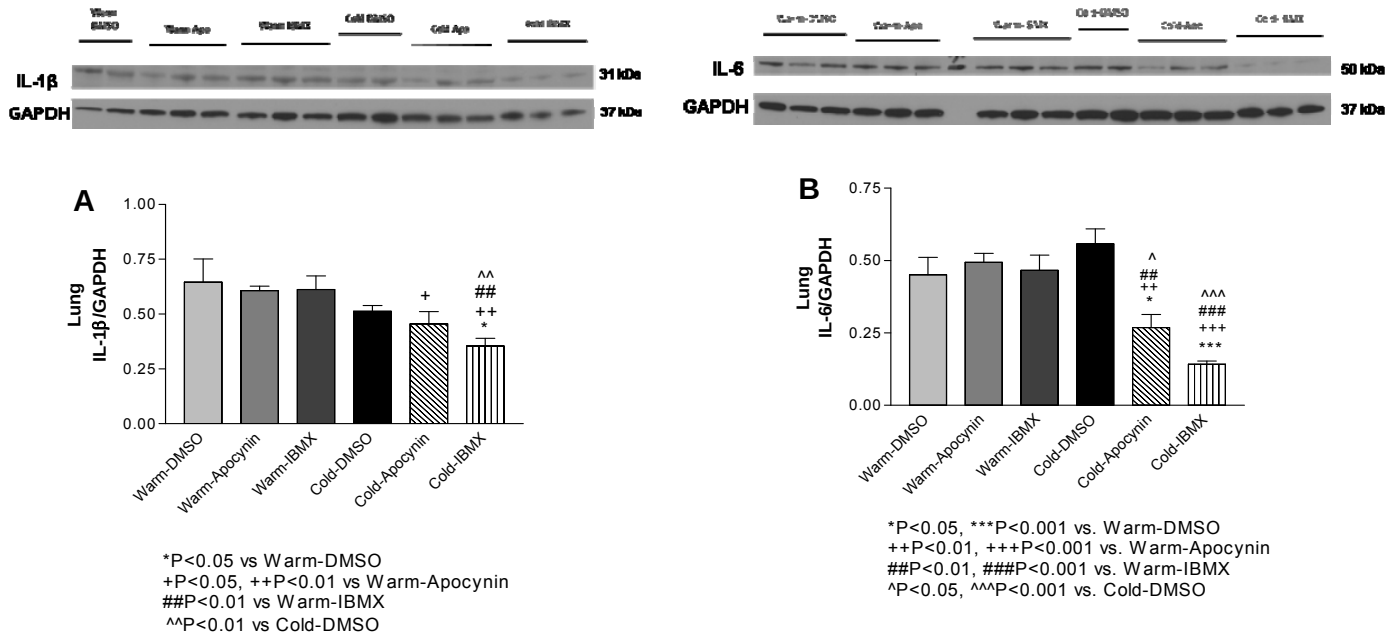
Supplemental Figure S4. PDE5 protein expression in lungs was not altered significantly by cold exposure or by treatment with Apocynin or IBMX although IBMX tends to decrease lung PDE5 expression. N=6.



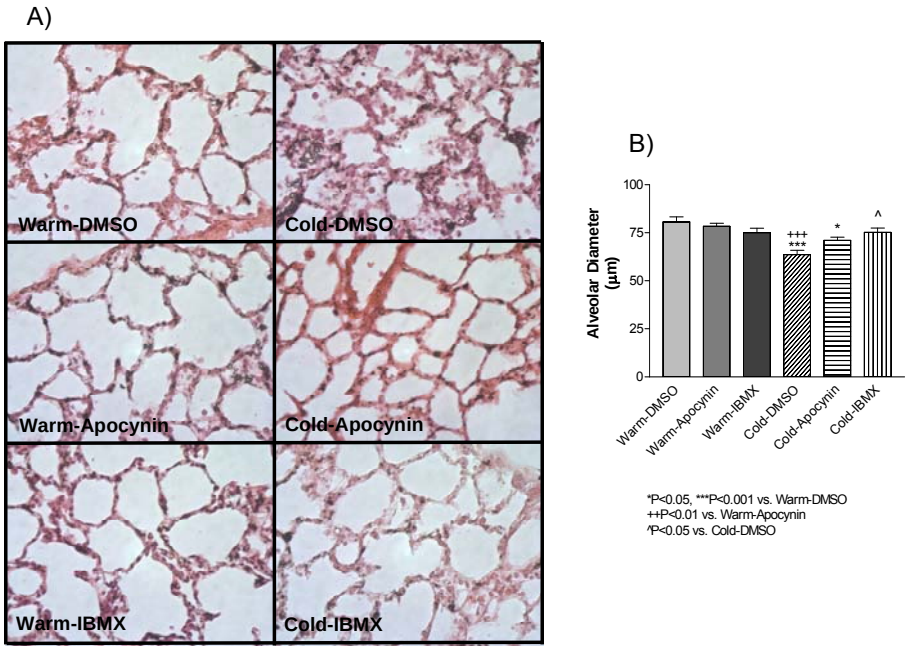
Supplemental Figure S5. PDE1A and PDE1C expression in aortas. Cold exposure tended to decrease PDE1A protein expression in aortas although no significant difference was found among groups. IBMX and apocynin did not affect PDE1A expression. PDE1C protein expression was not detectable in aortas. N=3.



Supplemental Figure S6. PDE1A and PDE1C protein expression in kidney cortex. PED1A and PDE1C protein expression was not altered by either cold exposure or treatments with IBMX or apocynin. N=6.



Supplemental Figure S7. IL-1β and IL-6 in the lungs. The lung IL-1β and IL-6 protein levels were not altered significantly by cold exposure. IBMX significantly decreased IL-1β and IL-6 expression. Apocynin also significantly decreased IL-6 protein expression in the lungs. IL-10 was not detectable in lungs. **A,** Lung IL-1β protein expression. **B,** Lung IL-6 protein expression. N=3.



Supplemental Figure S8. The alveolar diameter was decreased due to chronic cold exposure which can be reversed by 8-IBMX treatment. Chronic cold exposure decreased the average diameter of lung alveoli compared with the warm controls **(A)**. Treatment with 8-IBMX restored the alveolar diameter almost to the control levels **(B)**. N=3.

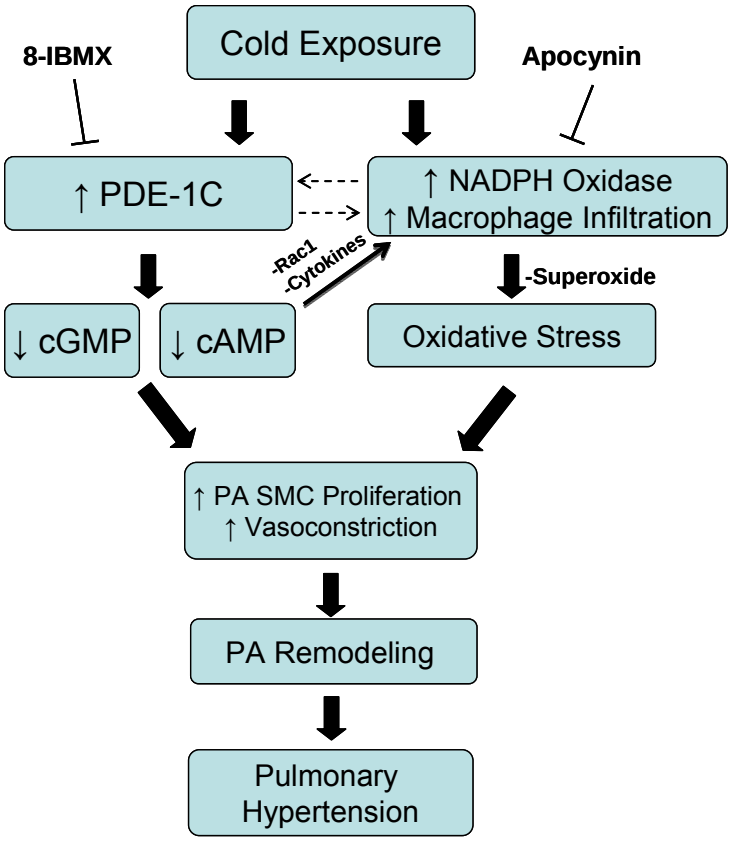


Figure S9. ***The postulated relationship PDE-1C and NADPH oxidases in CIPH.*** Cold exposure increases PDE-1C expression in the pulmonary vasculature leading to degradation of second messengers, cGMP and cAMP. Decreased cGMP levels can increase PA SMC proliferation and decrease vasodilation resulting in occlusion of small PAs. Occlusion of the small PAs increases pulmonary vascular resistance (PVR) and pulmonary arterial blood pressure (PAP) resulting in pulmonary hypertension. Increased PDE-1C also decreases cAMP levels that result in activation of NADPH oxidases (*via* Rac1 and inflammatory cytokines) and increased production of superoxide, leading to oxidative stress in the pulmonary vasculature. Increased oxidative stress can further contribute to CIPH pathogenesis. Inhibition of PDE-1 with 8-IBMX or inhibition of NADPH oxidase with apocynin ultimately attenuates PA remodeling and CIPH. Although PDE-1 inhibition decreased NADPH oxidase activity and superoxide production in PAs, the underlying mechanism remains to be determined (represented by dashed arrows).