AAV-Based RNAi Silencing of NADPH Oxidase gp91^{phox} Attenuates Cold-Induced Cardiovascular Dysfunction

Xiuqing Wang,^{1,2} Lucille Skelley,² Bo Wang,² Ayesha Mejia,² Val Sapozhnikov,² and Zhongjie Sun^{1,2}

Abstract

Clinical observations and epidemiological surveys indicated that the prevalence of hypertension and heart diseases is increased in cold regions or during winter. Cold exposure increased NADPH oxidase gp91^{phox} protein expression in heart, kidneys, and aorta in rats. The aim of this study was to investigate if RNA interference (RNAi) silencing of gp91^{phox} would attenuate cold-induced hypertension and cardiovascular and renal damage. The recombinant adeno-associated virus serotype 2 (AAV-2) vector carrying gp91^{phox}-shRNA (gp91-shRNA) was constructed for inhibiting gp91^{phox} protein expression in cold-exposed rats. Blood pressure (BP) was monitored using a telemetry system. BP was increased in the Control-shRNA and PBS groups within 1 week of exposure to moderate cold (5°C) and reached a plateau after 7 weeks. The cold-induced increase in BP was attenuated significantly by intravenous delivery of gp91-shRNA (1.25 × 10¹⁰ particles/rat, 0.5 mL). One single dose of gp91-shRNA controlled hypertension for up to 10 weeks. In addition, gp91-shRNA reversed cold-induced vascular dysfunction. gp91-shRNA abolished the cold-induced up-regulation of gp91^{phox} protein expression in heart, kidneys, and aorta, confirming effective silencing of gp91^{phox}. The cold-induced increases in NADPH oxidase activity and superoxide production were eliminated by silencing of gp91^{phox}, suggesting that the cold-induced up-regulation of NADPH oxidase activity may be attributed to the increased gp91^{phox} protein expression. RNAi silencing of gp91^{phox} abolished cold-induced cardiac and renal hypertrophy and attenuated aortic, coronary, and renal remodeling. The up-regulation of gp91^{phox} may play a critical role in cold-induced cardiovascular dysfunction and organ damage. AAV delivery of gp91-shRNA may be a new and effective therapeutic approach for cold-related cardiovascular disorders.

Introduction

CLINICAL OBSERVATIONS and epidemiological surveys have established that people who live in cold (northern) regions have increased prevalence of hypertension and cardiovascular (CV) diseases (*e.g.*, stroke and myocardial infarction) (Baker-Blocker, 1982; Thorvaldsen *et al.*, 1995; Gorjanc *et al.*, 1999; Fu *et al.*, 2002; Velázquez Monroy *et al.*, 2002). There is seasonal variation in blood pressure (BP) in normal subjects and hypertensive patients, with higher BP in winter (Brennan *et al.*, 1982; Hata *et al.*, 1982; Verdon *et al.*, 1993; Minami *et al.*, 1996). Cold temperatures exacerbate hypertension in hypertensive patients (Hata *et al.*, 1982; Verdon *et al.*, 1993; Minami *et al.*, 1996; Donaldson *et al.*, 1997) and trigger myocardial infarction and stroke. Of the four seasons, the cold winter has the highest morbidity and mortality from CV disease (Baker-Blocker, 1982; Seretakakis *et al.*, 1997; Gorjanc *et al.*, 1999; Crawford *et al.*, 2003). Unfortunately, the adverse effects of cold temperatures on CV function have been ignored

despite much supportive clinical and epidemiological data. Therefore, it is important to fully understand the mechanism of cold-induced CV dysfunction, which is essential to the development of preventive and therapeutic approaches.

Our previous studies showed that the sympathetic nervous system activates the rennin-angiotensin system (RAS), which plays a role in cold-induced elevation of BP (Sun *et al.*, 1995, 1997, 2003, 2005; Sun, 2010). Inhibition of the sympathetic nervous system or the RAS by pharmacological blockades or by knockout of angiotensinogen or AT1 receptor genes reduced cold-induced elevation of BP, but failed to attenuate cold-induced cardiac hypertrophy (Sun *et al.*, 1995, 1997, 2003, 2005; Sun, 2010). Therefore, cold-induced CV dysfunction is not fully understood. Our preliminary studies showed that gp91^{phox} (Nox2) protein expression is up-regulated in heart, kidneys, and arteries. It is not clear if gp91^{phox} is involved in cold-induced CV dysfunction.

Recent studies suggest that the hypertrophic response is mediated by increased oxidative stress (Takemoto *et al.*, 2001;

¹Department of Physiology, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

²Department of Medicine, University of Florida College of Medicine, Gainesville, FL 32610.

Sawyer *et al.*, 2002). NADPH oxidase-derived reactive oxygen species is involved in endothelial dysfunction, organ hypertrophy, and fibrosis, which are seen in hypertension and diabetes (Levy *et al.*, 1990; Paravicini and Touyz, 2008). The NADPH oxidase is the primary source of superoxide in blood vessels and kidneys (Görlach *et al.*, 2000; Griendling *et al.*, 2000; Touyz and Schiffrin, 2004; Tain and Baylis, 2006). Although Nox1 and Nox4 are found in vasculature, the gp91^{phox} containing NADPH oxidase (Nox2) selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall (Görlach *et al.*, 2000). Our preliminary study indicated that cold exposure up-regulated gp91^{phox} protein expression, but did not alter Nox1 and Nox4 expression in arteries (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/hum).

The most commonly used Nox inhibitor is the iodonium derivative diphenylene iodonium (DPI). The other Nox inhibitor is apocynin. However, both DPI and apocynin are nonselective Nox inhibitors. There currently is no gp91^{phox}-specific inhibitor. RNA interference (RNAi) is a powerful tool for inhibiting a target protein expression. Therefore, we designed short hairpin RNA (shRNA) carried by adeno-associated virus serotype 2 (AAV-2) for *in vivo* specific inhibition of gp91^{phox} expression. Our recent studies showed that shRNA carried by the AAV-2 vector is an effective approach for long-term inhibition of protein expression in animal models (Wang *et al.*, 2006; Crosswhite and Sun, 2010).

The purpose of this study was to investigate if the up-regulation of gp91^{phox} plays a role in cold-induced CV dysfunction by RNAi silencing of gp91^{phox} expression.

Materials and Methods

Construction of recombinant AAV with gp91^{phox}-shRNA

Three shRNA sequences specifically targeting on gp91^{phox} (accession no. AF298656) were designed using Dharmacon software and synthesized by Integrated DNA Technologies (Coralville, IA). The inhibition efficiency of the three gp91^{phox}-shRNA sequences was tested in H9C2 cells.

The gp91^{phox}-shRNA sequence that achieved the greatest inhibition of gp91^{phox} expression was chosen for the *in vivo* study. This sequence was designed to target on rat gp91^{phox} at 5'-CCA TCG AGC TTC AGA TGA A-3' (nucleotides 842–860). This gp91^{phox}-shRNA was then constructed into AAV-2 vector (Stratagene, La Jolla, CA) under the control of RNA polymerase III promoter U6 (AAV.U6-gp91-shRNA), as described in our recent studies (Wang *et al.*, 2006; Crosswhite and Sun, 2010). AAV.U6-gp91-shRNA plasmid DNA was then packaged with pHelper and pAAV-RC to produce recombinant AAV-gp91-shRNA. The titer was determined by real-time PCR as described by Rohr *et al.* (2002). AAV-2 with a scrambled shRNA sequence was also constructed and served as a control (AAV.U6-Control-shRNA). The scrambled shRNA has been tested by BD Bioscience (Palo Alto, CA) and found not to match with any known gene sequences.

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. The project was approved by the In-

stitutional Animal Care and Use Committee. Four groups of Sprague-Dawley rats (220–250 g, 6 rats/group) were used in this experiment. All rats were housed individually and provided with Purina Laboratory Chow (no. 5001) and tap water.

All animals were implanted with BP devices (aortic cannulation) under anesthesia [sodium pentobarbital 65 mg/kg, intraperitoneally (IP)]. Following a 1-week recovery, three groups of animals were exposed to a moderate cold environment (5±0.2°C) continuously, as we described recently (Crosswhite and Sun, 2010), whereas the remaining group was kept at room temperature (25±2°C) and served as a control. The three cold-exposed groups of rats received intravenous (IV) delivery of AAV-gp91-shRNA (1.25×10¹⁰ particles/rat, 0.5 mL), AAV-Control-shRNA (1.25×10¹⁰ particles/rat, 0.5 mL), and phosphate-buffered solution (PBS; 0.5 mL/rat), respectively, 3 days before exposure to cold. The group kept at room temperature (warm) received PBS (0.5 mL/rat) and served as a control (PBS-Warm).

BP measurement. BP was monitored continuously using a telemetry system (DSI, St. Paul, MN) as described in our recent study (Wang and Sun, 2010). Body weight was measured weekly.

Hypotensive response to acetylcholine (Ach) and sodium nitroprusside (SNP). Hypotensive responses to Ach and SNP (Sigma-Aldrich, St. Louis, MO) were tested during week 10. The dose-response curve was generated for Ach (0, 1, 2.5, 10, 20, 150, 250, and 280 µg/kg, 0.25 mL, IV). The dose-response curve was also generated for SNP (0, 10, 20, 50, and 100 µg/kg, 0.25 mL, IV). A sufficient time (10 min) was allowed between doses to ensure a full recovery of BP.

Tissue preparations. At the end of week 10, all animals were euthanized (sodium pentobarbital, 100 mg/kg, IP) and perfused transcardially with heparinized saline. Following perfusion, heart, kidneys, and aorta were removed. Heart and kidney were weighed. One part of heart, kidney, and aorta was saved for quantification of gp91^{phox} protein expression by western blot analysis. Small parts of these organs were excised rapidly and embedded with O.C.T for assessing *in situ* superoxide production. Some animals were perfused with 4% paraformaldehyde, and tissues were fixed in 4% paraformaldehyde overnight at 4°C for histological examination and trichrome staining (collagen assay).

Western blot analysis

The procedure for western blotting was described previously (Wang *et al.*, 2006; Wang and Sun, 2009). In brief, heart, kidney, and aorta were homogenized and lysed with cell lysis buffer (10 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.1% NP40, and 1% proteinase inhibitor cocktail) on ice for 30 min. An equal amount of protein was loaded in 10% Tris-HCl gel (Bio-Rad, Hercules, CA) followed by electronic transfer. After blocking with 5% milk (in Tris-buffered saline with Tween-20), the membranes were incubated with antibody against Nox1, gp91^{phox} (Nox2), and Nox4 (1:1,000; BD Transduction Laboratories Inc., Franklin Lakes, NJ) at 4°C overnight. The membrane was then incubated with horseradish peroxidase-conjugated secondary anti-mouse antibody (1:2,000) for 1 hr at room temperature. Proteins were

FIG. 1. RNAi silencing of $gp91^{phox}$ attenuated cold-induced elevation of BP and vascular dysfunction. BP was monitored using a telemetry system. (A) Mean aortic BP. (B) Hypotensive responses to ACh. (C) Hypotensive responses to SNP. Data are presented as percentage decreases in mean arterial pressure. Data are means $\pm$ SEM. $n=6$; $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. the PBS-Warm group. Data were analyzed using a two-way ANOVA (treatment and time) followed by a one-way ANOVA.

FIG. 2. RNAi silencing of $gp91^{phox}$ attenuated cold-induced cardiac and renal hypertrophy. (A) Heart weight normalized by 100 g of the body weight. (B) Kidney weight normalized by 100 g of the body weight. Data are means $\pm$ SEM. $n=5-6$; $*p<0.05$, $**p<0.01$, $***p<0.001$. Data were analyzed using a one-way ANOVA followed by an unpaired Student's t test.

visualized by ECL (Amersham, Little Chalfont, Buckinghamshire, UK), exposed to an X-ray film, and developed with an X-ray processor (Canon SRA-101A).

Evaluation of in situ superoxide level

Dihydroethidium (DHE) (Sigma-Aldrich) fluorescence dye can enter the cells and be oxidized by superoxide to yield ethidium, which binds to DNA to produce bright red fluorescence. Unfixed heart, kidney, and aorta were embedded in O.C.T., frozen at -80°C , and sectioned at $10\ \mu\text{m}$ using a cryostat (Microm International Model HM505 E; Microm International GmbH, Walldorf, Germany). Sections were incubated in a humidified chamber (37°C) for 30 min with HEPES-buffered solution (130 mM NaCl, 5 mM KCl, 1.2 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES, pH 7.3), and then incubated for an additional 30 min in HEPES-buffered solution containing DHE (10^{-5} M). The sections were

FIG. 3. RNAi silencing of gp91^{phox} attenuated cold-induced superoxide production. The frozen sections (10 μ m) of heart (A, D), kidney (B, E), and aorta (C, F) were incubated with DHE. The images were captured using confocal microscopy. Scale bars = 50 μ m (original magnification, 400 $\times$). (A–C) Representative fluorescence images of superoxide (DHE staining) in heart, kidney, and aorta. (D–F) Quantification of superoxide levels in heart, kidney, and aorta. *** $p < 0.001$. Data were analyzed using a one-way ANOVA followed by an unpaired Student's *t* test.

counterstained with the nuclear stain 4,6-diamidin-2-phenylindol dichlorohydrate (DAPI; 3×10^{-7} M) for 5 min at 37°C. Five sections of each group were examined, and the signal was captured with TRITC and UV filters using Leica TCS NT Confocal microscopy at 400 $\times$ magnification.

Analysis of the collagen level

The paraffin-embedded sections (5 μ m) of heart, kidneys, and aorta were used for quantifying connective tissue fibrosis and collagen deposition using trichrome stains (Sigma-Aldrich) as described in our recent study (Wang and Sun, 2009). The collagen level was semiquantified using NIH Imaging J software.

Measurement of NADPH oxidase activity by lucigenin-enhanced luminometer

NADPH oxidase activity was measured as we described recently (Wang and Sun, 2009). In brief, the tissue homoge-

nate was centrifuged at 11,000 *g*, and the supernatant was mixed (1:1, vol/vol) with PBS containing 20 μ M lucigenin (Sigma), before addition of NADPH (0.1 mM) (Sigma) as the substrate. The emitted luminescence was detected by luminometer (Gen5, BioTek, Winooski, VT). The data were expressed as fold changes versus the baseline level of NADPH oxidase activity (prior to the addition of NADPH).

Statistical analysis

Data were analyzed using a two-way ANOVA followed by a one-way ANOVA or an unpaired Student's *t* test. Significance was set at a 95% confidence limit.

Results

RNAi silencing of gp91^{phox} attenuated cold-induced elevation of BP and vascular dysfunction

Preliminary studies showed that cold exposure up-regulated vascular gp91^{phox} protein expression, but did not

FIG. 4. RNAi silencing of gp91^{phox} attenuated the cold-induced up-regulation of NADPH oxidase activity. NADPH activity in heart (A), kidneys (B), and aorta (C) is shown. Data are fold changes vs. the baseline level of NADPH oxidase activity (prior to the addition of NADPH). Data are means $\pm$ SEM. $n=6$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data were analyzed using a one-way ANOVA followed by an unpaired Student's t test.

affect Nox1 or Nox4 protein expression (Supplementary Fig. S1). Therefore, we investigated if the up-regulation of gp91^{phox} is involved in cold-induced hypertension (CIH) CV and renal damage by RNAi silencing of gp91^{phox} expression.

Mean aortic BP was elevated significantly within 1 week of exposure to cold in the Control-shRNA and PBS groups (Fig. 1A). BP continued to increase at 3 weeks and reached a plateau after 5 weeks. In contrast, BP of the gp91-shRNA-treated group did not increase until week 2 after exposure to cold (Fig. 1A). The cold-induced increase in BP is significantly decreased in the gp-shRNA-treated group compared with the Control-shRNA and PBS groups (Fig. 1A). Thus, RNAi silencing of gp91^{phox} delayed and attenuated CIH, although it did not decrease BP to the level of the PBS-Warm group (Fig. 1A).

Cold exposure significantly decreased the hypotensive responses to Ach and SNP (Fig. 1B and C), indicating vascular endothelial dysfunction and vascular smooth muscle dysfunction, respectively. RNAi silencing of gp91^{phox} significantly increased the hypotensive responses to Ach and SNP (Fig. 1B and C), suggesting improvements in cold-induced vascular endothelial dysfunction and smooth muscle dysfunction.

RNAi silencing of gp91^{phox} attenuated cold-induced cardiac and renal hypertrophy

Heart and kidney weights were increased significantly in cold-exposed rats, indicating that cold exposure caused cardiac and renal hypertrophy (Fig. 2). It is interesting that gp91-shRNA abolished cold-induced cardiac and renal hypertrophy (Fig. 2). Viral delivery did not affect the body weight gain (data not shown).

RNAi silencing of gp91^{phox} attenuated cold-induced superoxide production

The *in situ* superoxide production was assessed using DHE staining (red fluorescence). The superoxide level was increased significantly in the heart (Fig. 3A and D), kidneys (Fig. 3B and E), and aorta (Fig. 3C and F) in cold-exposed groups versus that of the warm PBS group, indicating that cold exposure stimulated superoxide generation. gp91-shRNA prevented the cold-induced increase in superoxide levels (Fig. 3A–F).

RNAi silencing of gp91^{phox} attenuated the cold-induced up-regulation of NADPH oxidase activity

NADPH activity of the heart (Fig. 4A), kidneys (Fig. 4B), and aorta (Fig. 4C), as assessed by lucigenin-enhanced chemiluminescence, was markedly increased significantly in cold-exposed rats. gp91-shRNA abolished the cold-induced up-regulation of NADPH oxidase activity.

gp91-shRNA effectively silenced gp91^{phox} protein expression

Cold exposure increased gp91^{phox} protein expression in the left ventricle (Fig. 5A and B), kidneys (Fig. 5C and D), and abdominal aorta (Fig. 5E and F). gp91-shRNA abolished the cold-induced up-regulation in gp91^{phox} protein expression in these organs, confirming effective silencing of gp91^{phox} gene. It is noted that gp91-shRNA kept gp91^{phox} protein at, but not below, the control level (PBS-Warm) (Fig. 5A–F). Nox1 or Nox4 was not affected by cold exposure or gp91-shRNA (Supplementary Fig. S1). Nox5 was not measured, because it is not expressed in rodents (Schulz and Munzel, 2008).

FIG. 5. gp91-shRNA effectively silenced gp91^{phox} protein expression. (A, B) Western blot analysis of gp91^{phox} protein expression in hearts. (C, D) Western blot analysis of gp91^{phox} protein expression in kidneys. (E, F) Western blot analysis of gp91^{phox} protein expression in aorta. Data are means $\pm$ SEM. $n=6$; *** $p < 0.001$. Data were analyzed using a one-way ANOVA followed by an unpaired Student's t test.

FIG. 6. RNAi silencing of gp91^{phox} attenuated the cold-induced coronary artery hypertrophy and collagen deposition. (A) Representative images of trichrome staining (scale bar = 100 pixels or 64.2 μ m). Arrows indicate collagen (blue staining) built up around intramyocardial coronary artery. (B) Quantification of medial wall thickness of intramyocardial coronary artery. (C) Quantification of collagen around coronary arteries (percentage of collagen deposition area in a given heart section). Data are means $\pm$ SEM. $n=6$; ** $p < 0.01$, *** $p < 0.001$. Data were analyzed using a one-way ANOVA followed by an unpaired Student's t test.

FIG. 7. RNAi silencing of $gp91^{phox}$ attenuated the cold-induced aortic hypertrophy and collagen deposition. **(A)** Representative images of trichrome staining (scale bar=100 pixels or $64.2\ \mu\text{m}$). Arrows indicate collagen staining (blue) in adventitial layers. **(B)** Quantification of medial wall thickness of aorta. **(C)** Quantification of collagen built up in adventitial layers (percentage of collagen deposition area in vessel wall area). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data were analyzed using a one-way ANOVA followed by an unpaired Student's t test.

Manganese superoxide dismutase (Mn-SOD) protein expression was not affected by either cold exposure or $gp91$ -shRNA (Supplementary Fig. S2). The AAV transfection rate was about 60–76% (Supplementary Fig. S3). The reporter genes [green fluorescent protein (GFP) and LacZ] were still expressed at the time of animal sacrifice, indicating that AAV is a long-term vector (Supplementary Figs. S3 and S4).

RNAi silencing of $gp91^{phox}$ attenuated cold-induced coronary artery hypertrophy and collagen deposition

Trichrome staining revealed collagen deposition around the intramyocardial coronary arteries. Cold exposure increased the medial wall thickness of coronary arteries and the collagen deposition (fibrosis formation) (Fig. 6), indicating coronary remodeling. RNAi silencing of $gp91^{phox}$ prevented cold-induced coronary remodeling (Fig. 6).

RNAi silencing of $gp91^{phox}$ attenuated cold-induced aortic hypertrophy and collagen deposition

Cold exposure increased medial wall thickness of aorta and perivascular collagen deposition (Fig. 7). RNAi inhibition of $gp91^{phox}$ abolished cold-induced aortic remodeling.

RNAi silencing of $gp91^{phox}$ attenuated cold-induced kidney damage

Representative histological images of kidneys are presented in Fig. 8A and B. Glomerular sclerosis (blue fibrotic

tissue) and collapsed capillary structure were found in kidneys of cold-exposed rats, indicating severe glomerular injury (Fig. 8A and C). Renal tubular damage, including blocked tubules in the outer medulla (red protein deposition casts), was also found in cold-exposed rats (Fig. 8B and D), indicating impairment of tubular function. RNAi silencing of $gp91^{phox}$ abolished cold-induced kidney damage and remodeling (Fig. 8A–D). Cold exposure significantly increased plasma creatinine, indicating functional damage of the kidneys (Fig. 8E). RNAi silencing of $gp91^{phox}$ improved kidney function (Fig. 8E).

Discussion

It is well established that the prevalence of hypertension and heart diseases is increased in cold regions or during winter (Baker-Blocker, 1982; Thorvaldsen *et al.*, 1995; Gorjanc *et al.*, 1999; Fu *et al.*, 2002; Velázquez Monroy *et al.*, 2002). The mortality and morbidity of CV disease is peaked in the winter (Baker-Blocker, 1982; Seretakakis *et al.*, 1997; Gorjanc *et al.*, 1999; Crawford *et al.*, 2003). Cold weather exacerbates hypertension (Hata *et al.*, 1982; Verdon *et al.*, 1993; Minami *et al.*, 1996; Donaldson *et al.*, 1997) and triggers myocardial infarction and stroke in hypertensive patients. Thus, cold temperatures have adverse effects on the human CV system. However, the mechanism of cold-induced CV dysfunction is not fully understood. We noticed that cold exposure up-regulated NADPH oxidase $gp91^{phox}$ protein expression in aorta, heart, and kidneys (Fig. 5). RNAi silencing of $gp91^{phox}$

FIG. 8. RNAi silencing of gp91^{phox} attenuated the cold-induced kidney damage. Light microscopy images of renal cortex (20× original magnification; **A**) and renal outer medullary regions (10× original magnification; **B**). (**A**) Glomerular sclerosis (blue fibrotic tissue and collapsed capillary structure, single arrow). (**B**) Protein deposition casts in enlarged renal tubules and/or blocked tubules (double arrows). (**C**) Quantification of glomerulosclerosis (percentage of sclerotic glomeruli of total glomeruli in a section). (**D**) Quantification of protein casts in renal tubules (percentage of protein cast area of the total area in a section). (**E**) Serum level of creatinine (mg/dL). **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Data were analyzed using a one-way ANOVA followed by an unpaired Student's *t* test.

gene effectively prevented the cold-induced up-regulation of gp91^{phox} protein expression, delayed and attenuated cold-induced elevation of BP, and abolished cold-induced vascular dysfunction and cardiac, vascular, and renal hypertrophy and remodeling (Figs. 1–8). These findings demonstrate, for the first time, that up-regulation of NADPH oxidase gp91^{phox} plays a critical role in the pathogenesis of CIH, vascular dysfunction, and organ damage. Renal function was impaired in cold-exposed rats (Fig. 8E). RNAi silencing of gp91^{phox} significantly improved the renal function in cold-exposed animals (Fig. 8E). Because RNAi silencing of gp91^{phox} attenuated cold-induced cardiac remodeling, one would expect improved cardiac function in cold-exposed rats treated with gp91-shRNA. The limitation of this study is that the *in vivo* cardiac function was not assessed due to technical challenges.

It is noted that silencing of gp91^{phox} delayed and attenuated, but did not prevent, cold-induced elevation of BP (Fig. 1), suggesting that a mechanism other than the up-regulation of gp91^{phox} also contributes to CIH. In contrast, the up-regulation of gp91^{phox} may be responsible for cold-induced

cardiac and renal hypertrophy, because they can be abolished by RNAi silencing of gp91^{phox} expression (Fig. 2). These data suggest that cold-induced cardiac and renal hypertrophy may be independent of CIH. On the other hand, prevention of the up-regulation of gp91^{phox} protein expression by gp91-shRNA abolished cold-induced cardiac, vascular, and renal remodeling although BP remains elevated. Therefore, up-regulation of gp91^{phox} is the key player in cold-induced organ damage and remodeling. It was reported that overproduction of superoxide (O₂^{•−}) leads to oxidative damage, fibrosis formation, and tissue remodeling (Levy *et al.*, 1990; Sawyer *et al.*, 2002; Paravicini and Touyz, 2008). Indeed, cold-induced up-regulation of gp91^{phox} is associated with increases in NADPH oxidase activity and O₂^{•−} generation (Figs. 3 and 4). It seems that the up-regulation of gp91^{phox} protein expression may account for the cold-induced increases in NADPH oxidase activity and O₂^{•−} generation, which can be abolished by silencing of gp91^{phox}. The alteration in O₂^{•−} levels may not be due to Mn-SOD, because it was not affected by either cold exposure or silencing of gp91^{phox} (Supplementary Fig. S2).

Nox2 (gp91^{phox}) was first described in neutrophils and macrophages and is often referred to as the phagocyte NADPH oxidase. Recent studies indicated that gp91^{phox} is also expressed in nonphagocytic cells, including neurons (Serrano *et al.*, 2003), cardiomyocytes (Heymes *et al.*, 2003), skeletal muscle myocytes (Javesghani *et al.*, 2002), hepatocytes (Reinehr *et al.*, 2005), endothelial cells (Jones *et al.*, 1996; Görlach *et al.*, 2000; Li and Shah, 2002), and hematopoietic stem cells (Piccoli *et al.*, 2005). The NADPH oxidase is the major source of superoxide in blood vessels and kidneys (Görlach *et al.*, 2000; Griendling *et al.*, 2000; Touyz and Schiffrin, 2004; Tain and Baylis, 2006). Increased superoxide levels inactivate the vasodilator nitric oxide leading to endothelial dysfunction and vasoconstriction, which are associated with several CV diseases, including hypertension (Touyz, 2004; Paravicini and Touyz, 2008). Cold exposure caused vascular endothelial and smooth muscle cell dysfunction as evidenced by disrupted hypotensive responses to Ach and SNP, respectively, in cold-exposed rats (Fig. 1). It appears that cold-induced vascular dysfunction is mainly mediated by the up-regulation of gp91^{phox} protein expression, because it can be restored by silencing of gp91^{phox}. Indeed, the cold-induced increases in vascular NADPH oxidase activity and superoxide generation were abolished by silencing of gp91^{phox} (Fig. 4).

It is interesting that up-regulation of a single NADPH oxidase gp91^{phox} plays a critical role in cold-induced CV dysfunction. The gp91^{phox} gene expression is inducible (Newburger *et al.*, 1988; Nicotera *et al.*, 1993; Krijnen *et al.*, 2003). The gp91^{phox} expression was increased in phagocytes in response to interferon- γ (Newburger *et al.*, 1988), in myofibroblasts after carotid artery injury (Nicotera *et al.*, 1993), and in cardiomyocytes after acute myocardial infarction (Krijnen *et al.*, 2003). The gp91^{phox} expression is also increased in response to angiotensin II in adipose tissue, aorta, resistance artery smooth muscle cells, and heart (López-Barneo *et al.*, 1999; Touyz *et al.*, 2002). The present study showed that cold exposure resulted in up-regulation of gp91^{phox} protein expression (Fig. 5). Our previous studies showed that cold exposure activates the RAS (Sun *et al.*, 2003, 2005; Sun, 2010). However, the RAS may play a role in CIH, but not cold-induced cardiac and renal hypertrophy, because inhibition of the RAS reduces cold-induced elevation of BP, but does not attenuate organ hypertrophy (Sun *et al.*, 1995, 1997, 2003, 2005; Sun, 2010). In contrast, the up-regulation of gp91^{phox} played a critical role in both CIH and cardiac and renal hypertrophy and remodeling (Figs. 1–8). Thus, the activation of the RAS cannot fully explain the up-regulation of gp91^{phox}. Further studies are required to determine the mechanism of cold-induced up-regulation of gp91^{phox}.

The present study convincingly demonstrated that one single dose of AAV-gp91-shRNA attenuated hypertension and CV and renal damage for at least 10 weeks (length of the study). The reporter genes were still expressed at the endpoint of the study (Supplementary Figs. S3 and S4), indicating that AAV is indeed a long-term vector. AAV also had a decent transfection rate (Supplementary Figs. S3 and S4). AAV-2 is a clinical-grade vector and has been approved by the U.S. Food and Drug Administration for clinical trial in humans (Flotte *et al.*, 2011; Mingozzi and High, 2011). We and others have shown that AAV is a safe vector for animal studies (Wang *et al.*, 2006; Wang and Sun, 2009; Crosswhite and Sun, 2010; Flotte

et al., 2011; Mingozzi and High, 2011). shRNAs delivered by AAV-2 have been used for regulating gene expression in cultured cells (Tenenbaum *et al.*, 2004; Michel *et al.*, 2005) and in animal models (Hasuwa *et al.*, 2002; Wang *et al.*, 2006; Sun *et al.*, 2008). It was reported that high doses of shRNAs, particularly the 25-mer shRNAs, caused toxicity and morbidity in animals within 1 month (Grimm *et al.*, 2006). In our study, AAV-2 delivery of 19-mer gp91^{phox}-shRNA at the dose of 1.25×10^{10} particles/rat did not cause obvious adverse effects in rats. The viral complexes did not affect the body weight gain.

Clinical Perspective

The present findings are of clinical significance and entail translational potential. The results will arouse clinicians' awareness of the adverse effects of cold temperatures on the CV function. There currently is no specific inhibitor for gp91^{phox}. To the best of our knowledge, this is the first study showing that gp91-shRNA, a gp91^{phox}-specific inhibitor, effectively prevented the cold-induced up-regulation of gp91^{phox} expression and superoxide production. The inhibition efficiency was over 50% (Figs. 4 and 5). It is noted that one single dose of AAV-gp91^{phox}-shRNA attenuated cold-induced CV dysfunction for at least 10 weeks (length of the study). The present findings suggest that AAV-2 delivery of gp91-shRNA may serve as a novel approach for ameliorating hypertension, vascular dysfunction, and organ damage.

Acknowledgments

This work was supported by NIH grants R01 HL077490, HL105302, and HL102074.

Author Disclosure Statement

The authors confirm that there are no conflicts of interest.

References

- Baker-Blocker, A. (1982). Winter weather and cardiovascular mortality in Minneapolis-St. Paul. *Am. J. Public Health* 72, 261–265.
- Brennan, P.J., Greenberg, G., Miall, W.E., and Thompson, S.G. (1982). Seasonal variation in arterial blood pressure. *Br. Med. J. (Clin. Res. Ed.)* 285, 919–923.
- Crawford, V.L., McCann, M., and Stout, R.W. (2003). Changes in seasonal deaths from myocardial infarction. *QJM* 96, 45–52.
- Crosswhite, P., and Sun, Z. (2010). Ribonucleic acid interference knockdown of interleukin 6 attenuates cold-induced hypertension. *Hypertension* 55, 1484–1491.
- Donaldson, G.C., Robinson, D., and Allaway, S.L. (1997). An analysis of arterial disease mortality and BUPA health screening data in men, in relation to outdoor temperature. *Clin. Sci. (Lond.)* 92, 261–268.
- Flotte, T.R., Trapnell, B.C., Humphries, M., *et al.* (2011). Phase 2 clinical trial of a recombinant adeno-associated virus vector expressing $\alpha 1$ antitrypsin: interim results. *Hum. Gene Ther.* 22, 1239–1247.
- Fu, S., Cao, Y., Li, Y., *et al.* (2002). Hypertensive epidemiology in Heilongjiang Province in China. *Chin. Med. J. (Engl.)* 115, 498–501.
- Gorjanc, M.L., Flanders, W.D., Vanderslice, J., *et al.* (1999). Effects of temperature and snowfall on mortality in Pennsylvania. *Am. J. Epidemiol.* 149, 1152–1160.

- Görlach, A., Brandes, R.P., Nguyen, K., *et al.* (2000). A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circ. Res.* 87, 26–32.
- Griendling, K.K., Sorescu, D., and Ushio-Fukai, M. (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ. Res.* 86, 494–501.
- Grimm, D., Streetz, K.L., Jopling, C.L., *et al.* (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441, 537–541.
- Hasuwa, H., Kaseda, K., Einarsdottir, T., and Okabe, M. (2002). Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Lett.* 532, 227–230.
- Hata, T., Ogihara, T., Maruyama, A., *et al.* (1982). The seasonal variation of blood pressure in patients with essential hypertension. *Clin. Exp. Hypertens. A* 4, 341–354.
- Heymes, C., Bendall, J.K., Ratajczak, P., *et al.* (2003). Increased myocardial NADPH oxidase activity in human heart failure. *J. Am. Coll. Cardiol.* 41, 2164–2171.
- Javesghani, D., Magder, S.A., Barreiro, E., *et al.* (2002). Molecular characterization of a superoxide-generating NAD(P)H oxidase in the ventilatory muscles. *Am. J. Respir. Crit. Care Med.* 165, 412–418.
- Jones, S.A., O'Donnell, V.B., Wood, J.D., *et al.* (1996). Expression of phagocyte NADPH oxidase components in human endothelial cells. *Am. J. Physiol.* 271, H1626–H1634.
- Krijnen, P.A., Meischl, C., Hack, C.E., *et al.* (2003). Increased Nox2 expression in human cardiomyocytes after acute myocardial infarction. *J. Clin. Pathol.* 56, 194–199.
- Levy, D., Garrison, R.J., Savage, D.D., *et al.* (1990). Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N. Engl. J. Med.* 322, 1561–1566.
- Li, J.M., and Shah, A.M. (2002). Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J. Biol. Chem.* 277, 19952–19960.
- López-Barneo, J., Pardal, R., Montoro, R.J., *et al.* (1999). K⁺ and Ca²⁺ channel activity and cytosolic [Ca²⁺] in oxygen-sensing tissues. *Respir. Physiol.* 115, 215–227.
- Michel, U., Malik, I., Ebert, S., *et al.* (2005). Long-term in vivo and in vitro AAV-2-mediated RNA interference in rat retinal ganglion cells and cultured primary neurons. *Biochem. Biophys. Res. Commun.* 326, 307–312.
- Minami, J., Kawano, Y., Ishimitsu, T., *et al.* (1996). Seasonal variations in office, home and 24 h ambulatory blood pressure in patients with essential hypertension. *J. Hypertens.* 14, 1421–1425.
- Mingozzi, F., and High, K.A. (2011). Therapeutic *in vivo* gene transfer for genetic disease using AAV: progress and challenges. *Nat. Rev. Genet.* 12, 341–355.
- Newburger, P.E., Ezekowitz, R.A., Whitney, C., *et al.* (1988). Induction of phagocyte cytochrome *b* heavy chain gene expression by interferon γ . *Proc. Natl. Acad. Sci. U.S.A.* 85, 5215–5219.
- Nicotera, T., Thusu, K., and Dandona, P. (1993). Elevated production of active oxygen in Bloom's syndrome cell lines. *Cancer Res.* 53, 5104–5107.
- Paravicini, T.M., and Touyz, R.M. (2008). NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* 31 Suppl 2, S170–S180.
- Piccoli, C., Ria, R., Scrima, R., *et al.* (2005). Characterization of mitochondrial and extra-mitochondrial oxygen consuming reactions in human hematopoietic stem cells. Novel evidence of the occurrence of NAD(P)H oxidase activity. *J. Biol. Chem.* 280, 26467–26476.
- Reinehr, R., Becker, S., Eberle, A., *et al.* (2005). Involvement of NADPH oxidase isoforms and Src family kinases in CD95-dependent hepatocyte apoptosis. *J. Biol. Chem.* 280, 27179–27194.
- Rohr, U.P., Wulf, M.A., Stahn, S., *et al.* (2002). Fast and reliable titration of recombinant adeno-associated virus type-2 using quantitative real-time PCR. *J. Virol Methods* 106, 81–88.
- Sawyer, D.B., Siwik, D.A., Xiao, L., *et al.* (2002). Role of oxidative stress in myocardial hypertrophy and failure. *J. Mol. Cell. Cardiol.* 34, 379–388.
- Schulz, E., and Munzel, T. (2008). NOX5, a new “radical”-player in human atherosclerosis? *J. Am. Coll. Cardiol.* 52, 1810–1812.
- Seretakakis, D., Lagiou, P., Lipworth, L., *et al.* (1997). Changing seasonality of mortality from coronary heart disease. *JAMA* 278, 1012–1014.
- Serrano, F., Kolluri, N.S., Wientjes, F.B., *et al.* (2003). NADPH oxidase immunoreactivity in the mouse brain. *Brain Res.* 988, 193–198.
- Sun, Z. (2010). Cardiovascular responses to cold exposure. *Front. Biosci. (Elite Ed.)* 2, 495–503.
- Sun, Z., Fregly, M.J., and Cade, J.R. (1995). Effect of renal denervation on elevation of blood pressure in cold-exposed rats. *Can. J. Physiol. Pharmacol.* 73, 72–78.
- Sun, Z., Cade, J.R., Fregly, M.J., and Rowland, N.E. (1997). Effect of chronic treatment with propranolol on the cardiovascular responses to chronic cold exposure. *Physiol. Behav.* 62, 379–384.
- Sun, Z., Cade, R., Zhang, Z., *et al.* (2003). Angiotensinogen gene knockout delays and attenuates cold-induced hypertension. *Hypertension* 41, 322–327.
- Sun, Z., Wang, X., Wood, C.E., and Cade, J.R. (2005). Genetic AT1A receptor deficiency attenuates cold-induced hypertension. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 288, R433–R439.
- Sun, Z., Bello-Roufai, M., and Wang, X. (2008). RNAi inhibition of mineralocorticoid receptors prevents the development of cold-induced hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 294, H1880–H1887.
- Tain, Y.L., and Baylis, C. (2006). Dissecting the causes of oxidative stress in an in vivo model of hypertension. *Hypertension* 48, 828–829.
- Takemoto, M., Node, K., Nakagami, H., *et al.* (2001). Statins as antioxidant therapy for preventing cardiac myocyte hypertrophy. *J. Clin. Invest.* 108, 1429–1437.
- Tenenbaum, L., Chtarto, A., Lehtonen, E., *et al.* (2004). Recombinant AAV-mediated gene delivery to the central nervous system. *J. Gene. Med.* 6 Suppl 1, S212–S222.
- Thorvaldsen, P., Asplund, K., Kuulasmaa, K., *et al.* (1995). Stroke incidence, case fatality, and mortality in the WHO MONICA project. *Stroke* 26, 361–367.
- Touyz, R.M. (2004). Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension* 44, 248–252.
- Touyz, R.M., and Schiffrin, E.L. (2004). Reactive oxygen species in vascular biology: implications in hypertension. *Histochem. Cell Biol.* 122, 339–352.
- Touyz, R.M., Chen, X., Tabet, F., *et al.* (2002). Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ. Res.* 90, 1205–1213.

- Velázquez Monroy, O., Rosas Peralta, M., Lara Esqueda, A., *et al.* (2002). [Arterial hypertension in Mexico: results of the National Health Survey 2000]. *Arch. Cardiol. Mex.* 72, 71–84.
- Verdon, F., Boudry, J.F., Chuat, M., *et al.* (1993). [Seasonal variations in arterial pressure in hypertensive patients]. *Schweiz. Med. Wochenschr.* 123, 2363–2369.
- Wang, X., and Sun, Z. (2010). RNAi silencing of brain klotho potentiates cold-induced elevation of blood pressure via the endothelin pathway. *Physiol. Genomics* 41, 120–126.
- Wang, X., Skelley, L., Cade, R., and Sun, Z. (2006). AAV delivery of mineralocorticoid receptor shRNA prevents progression of cold-induced hypertension and attenuates renal damage. *Gene Ther.* 13, 1097–1103.
- Wang, Y., and Sun, Z. (2009). Klotho gene delivery prevents the progression of spontaneous hypertension and renal damage. *Hypertension* 54, 810–817.

Address correspondence to:

Dr. Zhongjie Sun

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

E-mail: Zhongjie-sun@ouhsc.edu

Received for publication April 7, 2012;

accepted after revision June 14, 2012.

Published online: June 26, 2012.