Ribonucleic Acid Interference Knockdown of Interleukin 6 Attenuates Cold-Induced Hypertension

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Abstract—The purpose of this study was to determine the role of the proinflammation cytokine interleukin (IL) 6 in cold-induced hypertension. Four groups of male Sprague-Dawley rats were used (6 rats per group). After blood pressure was stabilized, 3 groups received intravenous delivery of adenoassociated virus carrying IL-6 small hairpin RNA (shRNA), adenoassociated virus carrying scrambled shRNA, and PBS, respectively, before exposure to a cold environment (5°C). The last group received PBS and was kept at room temperature (25°C, warm) as a control. Adenoassociated virus delivery of IL-6 shRNA significantly attenuated cold-induced elevation of systolic blood pressure and kept it at the control level for ≤7 weeks (length of the study). Chronic exposure to cold upregulated IL-6 expression in aorta, heart, and kidneys and increased macrophage and T-cell infiltration in kidneys, suggesting that cold exposure increases inflammation. IL-6 shRNA delivery abolished the cold-induced upregulation of IL-6, indicating effective silence of IL-6. Interestingly, RNA interference knockdown of IL-6 prevented cold-induced inflammation, as evidenced by a complete inhibition of tumor necrosis factor-α expression and leukocyte infiltration by IL-6 shRNA. RNA interference knockdown of IL-6 significantly decreased the cold-induced increase in vascular superoxide production. It is noted that IL-6 shRNA abolished the cold-induced increase in collagen deposition in the heart, suggesting that inflammation is involved in cold-induced cardiac remodeling. Cold exposure caused glomerular collapses, which could be prevented by knockdown of IL-6, suggesting an important role of inflammation in cold-induced renal damage. In conclusion, cold exposure increased IL-6 expression and inflammation, which play critical roles in the pathogenesis of cold-induced hypertension and cardiac and renal damage. (Hypertension. 2010;55:1484-1491.)

Key Words: cold exposure ■ inflammation ■ interleukin 6 ■ short-hairpin siRNA ■ RNAi ■ adenoassociated virus

It is well documented that cold temperatures have adverse effects on the human cardiovascular system. The prevalence of hypertension and related cardiovascular disease is higher in people who live in colder climates. The mortality and morbidity of cardiovascular diseases peak during the winter months in the United States. In addition, cold temperatures exacerbate hypertension in hypertensive patients. Therefore, it is important to fully understand the mechanism mediating cold-induced elevation of blood pressure (BP).

Cold-induced hypertension (CIH) represents an excellent model for studying environmentally induced hypertension. CIH is a “naturally occurring” form of experimental hypertension that requires no genetic manipulation, surgical intervention, or excessive drug or hormone administration. Previous studies have provided a central role for the sympathetic nervous system in initiating CIH via activation of the renin-angiotensin system. Activation of the renin-angiotensin system results in increased levels of angiotensin II and aldosterone. Other than its potant vasoactive effects, angiotensin II has been demonstrated to influence many inflammatory processes. Excessive aldosterone levels have also been shown to increase inflammation.

Located in the short arm of chromosome 7 (7p21) in humans, interleukin (IL) 6 is a pleiotropic cytokine with multiple biological roles in many different types of cell. The functions of IL-6 include induction of both local and systemic inflammatory responses, regulation of immune reaction, and hematopoiesis. In addition, IL-6 also induces proliferation and differentiation of T cells, as well as terminal differentiation of autoantibody-producing B cells. An overproduction of IL-6 thus exacerbates the immune reaction. Also, circulating IL-6 has been linked to central obesity, hypertension, and insulin resistance. In men, the proinflammatory cytokine IL-6 has been shown to be associated with elevated BP. Several reports have shown increased plasma levels of IL-6 in hypertensive patients. In addition, infusion of IL-6 into pregnant female rats induces preeclampsia (pregnancy-induced hypertension). Furthermore, angiotensin receptor blockers have been reported to reduce inflammatory mediator levels in hypertensive patients, specifically IL-6 and tumor necrosis factor (TNF)-α levels.

Although the association of IL-6 and hypertension has been reported, the cause-and-effect relationship is not clear. The purpose of this study was to examine the role of IL-6 in...
the development of hypertension using the CIH model. We hypothesized that in vivo knockdown of IL-6 by RNA interference (RNAi) silencing would decrease inflammatory infiltrates and attenuate cold-induced elevation of BP.

Methods

For a full description of the Materials and Methods, please see the online Data Supplement at http://hyper.ahajournals.org.

Adenoassociated Virus IL-6 Generation

The procedure for constructing the recombinant adenoassociated virus (AAV) 2 carrying the rat IL-6 small hairpin (sh)RNA sequence was described in detail in the online Data Supplement.

Additional AAV Generation

AAV carrying Scrambled shRNA (ScrshRNA) was constructed and used as a control construct. Scrambled shRNA has been confirmed by BD Biosciences not to match any known gene sequence.

Packaging of Recombinant Plasmids of AAV With IL-6-shRNA

AAV.IL-6 and AAV.ScrshRNA were packaged with pHelper and pAAV-Helper to produce recombinant viruses as described in our

Figure 1. IL-6 shRNA delivery attenuated cold-induced elevation of blood pressure. Intravenous injections of AAV.ScrshRNA, AAV.IL-6 shRNA, and PBS were administered before exposure to cold. Systolic BP was measured weekly. Data = mean ± SE; *P<0.05, **P<0.01, ***P<0.001 vs the ScrshRNA cold. n=6.

Figure 2. IL-6 shRNA delivery decreased IL-6 expression in the kidney. Western blot analysis of IL-6 protein expression in kidney homogenates (A and B). IHC analysis of IL-6 expression in the kidney (C and D). Arrows indicate IL-6 expression (brown staining). *P<0.05, **P<0.01, ***P<0.001 vs the PBS warm group; ++P<0.01, +++P<0.001 vs the IL-6 shRNA cold group. Data = mean ± SE. n=6.
For the virus packaging procedure, please see the online Data Supplement.

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 Institutional Animal Care and Use Committee. Four groups of male Sprague-Dawley rats (145 to 180 g, 6 rats per group) were allowed to acclimate for a week. After acclimation, resting systolic BP was measured twice weekly at room temperature from the tail of each unanesthetized rat by using a tail-cuff method with slight warming (28°C) but not heating of the tail using a CODA 6 BP Monitoring System (Kent Scientific). The volume-based tail-cuff measurements of BP have been validated by using a telemetry system.25 After 2 stable BP readings were obtained, the 4 groups of rats received a single injection via the tail vein of AAV.IL-6 shRNA, AAV.ScrshRNA, PBS, and PBS, respectively. The AAV complexes were delivered at $1.2 \times 10^8$ plaque-forming units per rat (0.5 mL). After injection, 3 groups (AAV.IL-6 shRNA, AAV.ScrshRNA, and 1 PBS) were moved into to a climate-controlled walk-in chamber (5 ± 2°C), whereas the remaining PBS group was kept in an identical chamber maintained at room temperature (25 ± 2°C, warm). BP and body weight were measured at least once per week until week 8 postinjection, when all of the animals were euthanized. For detailed procedures, please see the online Data Supplement.

Western Blot Analysis of IL-6 and TNF-α Protein Expressions in Tissue
IL-6 and TNF-α protein expressions in kidneys and arteries were measured using Western blot, as described in our previous studies.23,24 For details, please see the online Data Supplement.

Figure 3. IL-6 shRNA delivery attenuated macrophage and T-cell infiltration in the kidney. Semiquantitative analysis of macrophage infiltration in the kidney, as measured by the density of CD-68 expression (A) and the average count of CD-68+ cells (B). The IHC analysis of macrophage infiltration in the kidney as measured by CD-68 staining (C). Semiquantitative analysis of T-cell infiltration in the kidney as measured by the density of CD-3 expression (D) and the average count of CD-3+ cells (E). The IHC analysis of T-cell infiltration in the kidney was measured by CD-3 staining (F). Arrows indicate positive staining of CD-68 or CD-3. Photos were taken at ×200. Scale bars indicate 100 μm. *P<0.05, **P<0.01, ***P<0.001 vs the PBS Warm group; +P<0.05, ++P<0.01, +++P<0.001 vs the IL-6 shRNA cold group. Data=mean±SE. n=6.
Immunohistochemical Analysis of IL-6 Expression and Macrophage and T-Cell Infiltration

IL-6 expression and macrophage and T-cell infiltration were analyzed using IL-6 antibody and CD-3 and CD-68 markers, respectively. For details, please see the online Data Supplement.

Measurement of In Situ Vascular Superoxide Production

The in situ superoxide production was measured in aortas using the oxidation-sensitive dye dihydroethidium (DHE). For details, please see the online Data Supplement.

Statistical Analysis

The data for BP and body weight were analyzed by a repeated-measures 1-way ANOVA. The protein expression and reactive oxygen species levels 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

IL-6 shRNA Delivery Attenuated the Cold-Induced BP Increase

IL-6 shRNA significantly decreased cold-induced elevation of systolic BP compared with the PBS cold and ScrshRNA cold groups (Figure 1). RNAi knockdown of IL-6 maintained BPs at the level of the PBS warm group (control). It is noted that one single dose of AAV IL-6 shRNA controlled hypertension for ≥7 weeks (length of the study; Figure 1). Body weight was not significantly different between groups both before and after the single injection of viral complexes (Figure S2, available in the online Data Supplement), suggesting that AAV-2 had no adverse effects on body weight gains.

IL-6 shRNA Delivery Decreased Cold-Induced Increases in IL-6 Expression in the Kidney

The PBS cold group showed a significant increase in IL-6 protein expression compared with the PBS warm group (Figure 2A and 2B), suggesting that cold exposure may increase inflammation. IL-6 shRNA significantly decreased the cold-induced increase in IL-6 expression versus the ScrshRNA cold group and the PBS cold group (Figure 2A and 2B). The immunohistochemical (IHC) analysis revealed that cold exposure increased IL-6 expression (brown staining) around the glomeruli and tubules in the ScrshRNA Cold group and the PBS Cold group versus the PBS Warm group (Figure 2C and 2D). IL-6 expression was decreased significantly in the IL-6shRNA Cold group (Figure 2C and 2D). These results indicate that IL-6 gene was effectively silenced by IL-6shRNA.

IL-6shRNA Delivery Attenuated Cold-Induced Macrophage and T-Cell Infiltration in the Kidney

The IHC analysis showed that CD-68 staining and the number of CD-68+ cells were increased significantly in kidneys in PBS cold and ScrshRNA cold groups (Figure 3A through 3C), indicating that cold exposure increased macrophage infiltration. Macrophages infiltrated in the renal tubules and glomeruli (Figure 3C). IL-6 shRNA abolished the cold-induced increase in macrophage infiltration (Figure 3A through 3C). Cold exposure also increased T-cell (CD-3+ cell) infiltration in kidneys, which could be abolished by IL-6 shRNA (Figure 3D through 3F). T cells infiltrated in the renal tubules (Figure 3F). These results suggest that that silence of IL-6 prevented cold-induced inflammation.

IL-6shRNA Delivery Attenuated Cold-Induced Kidney Damage

Figure 4 showed glomerular collapses in the ScrshRNA cold and PBS cold groups, indicating that chronic exposure to cold caused structural damage in kidneys. RNAi knockdown of IL-6 abolished cold-induced glomerular collapses (Figure 4A and 4B).

IL-6 shRNA Delivery Abolished the Cold-Induced Increase in IL-6 and TNF-α Expression in Aorta

Western blot analysis showed significant increases in IL-6 and TNF-α protein expression in aortas in the ScshRNA cold and PBS cold groups compared with the PBS warm group (Figure 5), indicating that cold exposure may increase inflammation in the aorta. IL-6 shRNA delivery abolished the cold-induced increases in IL-6 and TNF-α in aortas (Figure...
This result suggests that IL-6 may mediate the cold-induced increase in vascular TNF-α.

IL-6 shRNA Delivery Abolished the Cold-Induced Increase in Vascular Superoxide Production

In situ vascular superoxide production was evaluated using DHE staining. Cold exposure significantly increased superoxide production in aortas (Figure 6). RNAi silencing of IL-6 abolished the cold-induced increase in vascular superoxide production.

IL-6 shRNA Delivery Abolished the Cold-Induced Increases in IL-6 Expression in the Heart and Prevented Collagen Deposition Around the Coronary Arteries

IHC analysis showed a significant increase in cardiac IL-6 expression in the PBS cold and ScrshRNA cold groups (Figure 7A and 7B), suggesting that cold exposure increased inflammation in the heart. IL-6 shRNA significantly decreased IL-6 expression compared with the PBS cold and ScrshRNA cold groups (Figure 7A), confirming effective silencing of the IL-6 gene. A significant decrease in collagen deposition (blue staining) around the coronary arteries of the IL-6 shRNA cold group was observed compared with the PBS cold and ScrshRNA cold groups (Figure 7C and 7D), suggesting that RNAi silencing of IL-6 prevented heart remodeling. IL-6 shRNA decreased the cold-induced increase in heart weight but did not reduce it to the control level (Figure S1), suggesting partial attenuation of cold-induced cardiac hypertrophy.

Discussion

The present data demonstrated, for the first time, that chronic exposure to cold caused inflammation in kidneys, hearts, and blood vessels, as evidenced by increased levels of IL-6 and TNF-α expression and leukocyte infiltration. Interestingly, RNAi knockdown of IL-6 attenuated cold-induced inflammation and elevation of BP, suggesting that that inflammation plays a vital role in the pathogenesis of CIH and that IL-6 is a key mediator in this process. The finding is significant because of its potential to provide novel preventive and therapeutic strategies for cold-related cardiovascular and renal dysfunctions, which are important for people who live in cold regions and during the winter. The prolonged attenuation of CIH is likely to be because of the long-term expression vector, AAV. AAV is an effective and nonpathogenic vector that has been used to deliver therapeutic genes to the cardiovascular system and kidneys for long-term control of hypertension. AAV can express for months after gene delivery.

Our previous studies have established that overactivation of the renin-angiotensin system is responsible for the development of CIH. In addition to its vasoconstrictor properties, angiotensin II has been demonstrated to exert proinflammatory effects in the vasculature by inducing production of integrins, adhesion molecules, cytokines, and growth factors through activation of the transcription factor nuclear factor-κB. Nuclear factor-κB, in turn, regulates a variety of proinflammatory genes at the transcriptional level. Angiotensin II has also been shown to directly contribute to the upregulation of IL-6 and has the ability to influence various stages of the inflammatory process. It will be interesting to test whether inflammation mediates the role of the renin-angiotensin system in CIH.

The molecular mechanisms involved in the pathogenesis of hypertension are not fully understood. Inflammation has been shown to increase the generation of reactive oxygen species.
IL-6 and Cold-Induced Hypertension

Patients with cardiovascular disease have increased protein expression and plasma concentration of many inflammatory markers, including selectins (P, E, and L selectins), intracellular adhesion molecule 1, and vascular cell adhesion molecule. Human hypertension is associated with increased levels of TNF-α and IL-6, as well as intracellular adhesion molecule 1, vascular cell adhesion molecule, and selectins. However, the role of IL-6 in inflammation in the context of hypertension is largely unknown. The present study revealed that IL-6 is likely a key mediator of inflammation, because knockdown of IL-6 prevented the cold-induced increases in TNF-α and leukocyte infiltration. The molecular mechanism leading to increased levels of macrophages and T cells in cold-exposed animals needs to be further investigated, but increased levels of IL-6 could upregulate intracellular adhesion molecule 1, vascular cell adhesion molecule, and the selectins through nuclear factor-κB activation leading to the recruitment of inflammatory infiltrates. These inflammatory factors are directly responsible for the recruitment of leukocytes, including macrophages and lymphocytes, to the vasculature and have been demonstrated to be upregulated in several animal models of hypertension.

In the present study, systolic BP was monitored using the tail-cuff method. The tail-cuff procedure is a common method used by our laboratory and others to delineate CIH. It has been confirmed by the intra-arterial cannulation that the noninvasive tail-cuff method is effective and reliable in monitoring BP responses to cold.

It is noted that chronic exposure to cold for 8 weeks is sufficient to cause glomerular collapse, a sign of renal structural damage. RNAi knockdown of IL-6 effectively prevented cold-induced glomerular damage, suggesting that IL-6 may mediate inflammatory responses that lead to renal vascular damage and, ultimately, glomerular collapse. Previous studies from our laboratory have demonstrated that chronic cold exposure also leads to cardic hypertrophy. The mechanism of cold-induced cardiac hypertrophy is unknown but is independent of high BP, because prevention of CIH does not attenuate the development of cold-induced cardiac hypertrophy. The present data revealed that inflammation may play a role in the pathogenesis of cold-induced cardiac hypertrophy, because knockdown of IL-6 attenuated the cold-induced increase in heart weight. Interestingly, cold exposure also resulted in formation of fibrosis around the coronary artery, which may ultimately impair the coronary circulation. It is notable that cold-induced formation of fibrosis was abolished by inhibition of IL-6, suggesting an important role for inflammation in this pathological process.

TNF-α is a primary proinflammatory cytokine that has been shown to increase the production of cytokines, including IL-6, and to initiate inflammatory cascades. Both TNF-α and IL-6 blockade therapies can reduce inflammation and improve prognosis in patients with arthritis, a chronic inflammatory disease. Although TNF-α blockers have been shown to decrease IL-6 levels, this is the first study demonstrating that knockdown of IL-6 prevented cold-induced upregulation of TNF-α. Additional investigation into the molecular mechanism mediating the regulation of TNF-α by IL-6 would be of particular interest.

**Perspectives**

The current study yields 2 major findings in the pathogenesis of CIH. First, chronic exposure to cold caused inflammation in the cardiovascular system and kidneys, as evidenced by upregulation of proinflammatory cytokines (IL-6 and TNF-α)
and an increase in leukocyte infiltration. Second, RNAi knockdown of IL-6 abolished cold-induced inflammation and attenuated cold-induced elevation of BP and organ damage. These results suggest that inflammation plays a critical role in the development of CIH and that IL-6 is a critical mediator in this pathological process. This study also reveals that AAV.RNAi knockdown of IL-6 may serve as a new approach for the effective control of hypertension.

**Sources of Funding**
This work was supported by National Institutes of Health grant R01 NHLBI-077490.

**Disclosures**
None.

**References**
31. Crosswhite G, Sun IL-6 and Cold-Induced Hypertension.
Ribonucleic Acid Interference Knockdown of Interleukin 6 Attenuates Cold-Induced Hypertension
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*Hypertension*. 2010;55:1484-1491; originally published online April 12, 2010; doi: 10.1161/HYPERTENSIONAHA.109.146902

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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RNAi Inhibition of Interleukin-6 Attenuates Cold-Induced Hypertension

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Supplemental Methods

**AAV.IL-6 generation.** Three sets of complimentary rat IL-6 siRNA oligos flanked by BamHI and EcoRI restriction sites were designed using Gene Link RNAi Explorer software (Accesion No: NM_012589) and synthesized by IDT DNA (Coralville, IA, USA). The complimentary oligos were then annealed and ligated into pAAV-U6shRNA (Adeno-associated virus-2 vector, previously published) vector forming pAAV.IL-6shRNA. The fidelity was measured by sequencing of the vector/insert sequence using the upstream primer U6-p39 5'-GGT CCT AAG GTA GCG AAA GC-'3 and compared the sequence using Blast. The pAAV.IL-6shRNA constructs were then transfected into 3T3-L1 adipocytes (ATCC) and inhibition efficiency was determined via Western blot analysis using a rabbit polyclonal anti-IL-6 primary antibody (Abcam Inc.). One construct was selected and then transfected into and amplified by *E.coli*. The *E.coli* was then lysed and the plasmid DNA was removed and purified via CsCl gradient and ultracentrifugation.

**Packaging of recombinant plasmids of adeno-associated virus with IL-6-shRNA.** One IL-6shRNA that achieved the greatest inhibition was chosen for the *in vivo* study. Adeno-associated virus-2 vector (Stratagene, La Jolla, CA USA) with RNA polymerase III promoter U6 and IL-6-shRNA were constructed as previously described by our lab1. AAV.U6-IL-6shRNA was then packaged with pHelper and pAAV-RC to produce recombinant AAV.IL-6shRNA. The titer was determined by real-time PCR1,2.

**Additional AAV generation.** AAV.ControlshRNA was purchased from and tested by BD Biosciences (Palo Alto, CA. USA) not to match any known gene sequence.

**Animals.** 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 Institutional Animal Care and Use Committee. We used 4 groups of male Sprague–Dawley rats (145–180g, 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.** The rats were allowed to acclimate for a week. After the acclimation period, resting systolic BP and body wight were measured twice weekly at room temperature from the tail of each unanesthetized rat using the tail-cuff method with slight warming (28°C), but not heating of the tail. All rats were handled frequently (twice a day) to minimize handling stress. Animals did not appear stressed during the BP measurement. The tail-cuff measurements were performed at room temperature (25°C) between 8:00-12:00 am using a CODA 6 blood pressure monitor. The volume-based tail-cuff measurements of BP have been validated by using a telemetry system3. An average of 6 measurements was obtained for each session. After two steady state blood pressure readings were obtained, the rats were treated as described below:

Each group consisted of 6 rats.
- Group 1. 500μl AAV.ScshRNA (1.2 x 10^8 PFU/rat via tail vein) (Sc=scrambled sequence)
- Group 2. 500μl AAV.IL-6shRNA (1.2 x 10^8 PFU/rat via tail vein)
- Group 3. 500μl PBS via tail vein
Group 4. 500μl PBS via tail vein

Following injections, three groups (AAV.IL-6shRNA, AAV.ScrambledshRNA and one PBS) were moved into a climate-controlled walk-in chamber (5 ± 0.1°C), whereas the remaining PBS group was kept in an identical chamber maintained at RT (25 ± 0.1°C, warm). BP was measured 24 h after the injection to ensure that the gene delivery did not affect animal-resting BP at room temperature. BP and BW were measured at least once a week throughout the experiment. During week eight after viral injection, 3 animals from each group were deeply anesthetized with pentobarbital sodium (120 mg/kg i.p.) for blood collection, followed by transcardiac perfusion with heparinized phosphate buffered saline (PBS). The heart and kidneys were removed and weighed. The kidneys, heart and aorta were divided then OCT embedded for dihydroethidium (DHE) staining and for Western blot analysis of IL-6 and TNF-α protein expression. The remaining 3 animals from each group were then deeply anesthetized for blood collection, followed by transcardiac perfusion with PBS then perfused a second time with 4% paraformaldehyde (PFA) in PBS. The heart and kidneys were removed and weighed. The heart, kidneys, and aorta were fixed in 4% PFA in PBS overnight and paraffin embedded. 5μm sections were cut and slides were used for immunohistochemical (IHC) and hematoxylin and eosin stain (H&E) histological analyses.

Four groups were exposed to different temperature conditions as follows:
ScshRNA-Cold, rats treated with scrambled RNA and exposed to cold (5°C)
AAV.IL-6shRNA-Cold, rats treated with IL-6shRNA and exposed to cold (5°C)
PBS-Cold, rats treated with PBS and exposed to cold (5°C)
PBS-Warm, rats treated with PBS and kept at room temperature (warm, 25°C)

Western blot analysis of IL-6 and TNF-α protein expression in tissue. Tissues were frozen in liquid nitrogen, shattered, and powderized using a mortar and pestle. The frozen powder was then homogenized in lysis buffer (50 mM Tris, 150 mM NaCl, 1% nonidet p40, .5% sodium deoxycholate, .1% sodium dodecyl sulfate) containing a protease inhibitor complex, incubated on ice for 30 minutes and centrifuged for 10 min at 10,000 g. Supernatants were collected and immediately mixed with an equal volume of electrophoresis loading buffer for Western blot analysis of IL-6 and TNF-α proteins. TNF-α and IL-6 expression was normalized with the expression of β-actin, which served as an internal control. Briefly, tissue proteins were equalized after subjection to bicinchoninic acid (BCA) quantification. Equal protein concentrations were loaded for all groups. For IL-6, membranes were blocked for 2 hours at room temperature in 5% milk/TBS-T. Membranes were incubated with rabbit polyclonal anti-IL-6 primary antibody (dilution 1:1,000; Abcam Inc.) at 4°C overnight and then goat anti-rabbit secondary antibody (dilution 1:2,000 Santa Cruz Biotechnology) for 1 hour at room temperature. For TNF-α, membranes were blocked in 2% bovine serum albumin (BSA) for 2 hours at room temperature followed by incubation with rabbit polyclonal anti-TNF-α primary antibody (dilution 1:1,000 Cell Signaling) overnight in 4°C. Membranes were then incubated in goat anti-rabbit secondary antibody for 1.5 hours room temperature. For β-actin, the membranes were blocked in 5% milk/TBS-T for 2 hours at room temperature followed by incubation with mouse monoclonal anti-β-actin primary antibody (dilution 1:10,000; Abcam Inc) for 1 hour room temperature and then with goat anti-mouse secondary antibody (dilution 1:2,000; Santa Cruz Biotechnology) for 1 hour room temperature. Enhanced chemiluminescence (Amersham) was added to the
membranes and exposed to photosensitive films. The films were imaged by using an X-ray processor (Konica Minolta, SRX-101A). Protein band intensities were quantified using Image J software.

IHC analysis of IL-6, CD-3 and CD-68 expression. After a 24-h fixation with 4% PFA, the heart, kidneys, and aorta were incubated overnight in 70% ethanol at 4°C and paraffin embedded. Sections (5 µm) were used for the immunostaining of IL-6, CD-3 and CD-68. For immunostaining, the sections were heated for 10 min with sodium citrate buffer (pH 6.0) for antigen retrieval, incubated for 15 min with 3% hydrogen peroxide solution (VWR International), and 10 min with protein blocker (Background Sniper; Biocare Medical). For IL-6, the sections were incubated with rabbit polyclonal anti-IL-6 primary antibody (dilution 1:1,000; Abcam Inc) overnight and then with a goat anti-rabbit secondary antibody (dilution 1:500; Santa Cruz Biotechnology) for 1 hour. For CD-3, the sections were incubated with rabbit polyclonal anti-CD-3 primary antibody (dilution 1:1000; Abcam Inc) overnight and then with a goat anti-rabbit secondary antibody (dilution 1:2000; Santa Cruz Biotechnology) for 1 hour. For CD-68, the sections were incubated with mouse monoclonal anti-CD68 primary antibody (dilution 1:100; Abcam Inc) overnight and then with a goat anti-mouse secondary antibody (dilution 1:500; Santa Cruz Biotechnology) for 1 hour. The sections were then examined and photographed using a Nikon Eclipse Ti-U photomicroscope coupled with a digital color camera. The expression of IL-6, CD-3 and CD-68 expression level was evaluated by calculating the percentage of staining for a given area. In addition, counts of actual positively stained cells were performed for CD-3 and CD-68. To determine the antibody specificity, sections from each tissue were subjected to primary antibody only, secondary antibody only, and PBS treatment. These slides were considered negative controls and were compared to non-control slides.

Measurement of In Situ Superoxide Production.
We measured superoxide production by fluorescence microscopy using the oxidation sensitive dye dihydroethidium (DHE, Sigma-Aldrich, Atlanta, GA, USA). Using OCT embedded aorta tissue, sections were cut at 10 µm thickness. Excess OCT was removed from the slides using PBS. DHE (10µg/ml) was then allowed to incubate for 20 minutes at 37°C in the dark. Excess DHE was then rinsed out using PBS. A nuclear stain was then applied using DAPI (Santa Cruz Biotechnology Inc, Santa Cruz, CA USA) and the slides were immediately viewed using a Nikon Eclipse Ti-U photomicroscope coupled with a digital color camera.

Statistical analysis. The data for BP and BW were analyzed by a repeated measures one-way analysis of variance (ANOVA). The protein expression and ROS levels were analyzed by one-way ANOVA. Turkey’s multiple comparison test was used to assess the significance of differences between means. Significance was set at a 95% confidence limit.

References


**Supplemental Results**

![Graph showing heart weight](image)

**S1. Heart weight.** Animals were euthanized at the end of week 8 of exposure to cold. Heart weight was adjusted for body weight (g/kg body weight) for each animal. Chronic exposure to cold increased the heart weight significantly. IL-6shRNA significantly attenuated the cold-induced increase in heart weight compared to the ScrshRNA Cold group. However, heart weight of the IL-6shRNA Cold group was significantly greater than that of the PBS Warm group, suggesting partial attenuation of cold-induced cardiac hypertrophy. *p<0.05, ***p<0.001 vs the PBS Warm group; +p<0.05 vs the IL-6shRNA Cold group. Data=means±SE. n=6.

![Graph showing body weight](image)

**S2. Body weight.**