RNAi inhibition of mineralocorticoid receptors prevents the development of cold-induced hypertension

Zhongjie Sun,1,2 Mahajoub Bello-Roufai,2 and Xiuqing Wang1,2

1Department of Physiology, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; and 2Departments of Medicine and Physiology and Functional Genomics, College of Medicine, University of Florida, Gainesville, Florida

Submitted 11 November 2007; accepted in final form 21 February 2008

Sun Z, Bello-Roufai M, Wang X. RNAi inhibition of mineralocorticoid receptors prevents the development of cold-induced hypertension. Am J Physiol Heart Circ Physiol 294: H1880–H1887, 2008. First published February 22, 2008; doi:10.1152/ajpheart.01319.2007.—The objective was to determine whether the mineralocorticoid receptor (MR) plays a role in the initiation and development of cold-induced hypertension (CIH) by testing the hypothesis that the RNA interference (RNAi) inhibition of the MR attenuates CIH. The recombinant adeno-associated virus (AAV) carrying a short-hairpin small-interference RNA for MR (MRshRNA) or a scrambled sequence (ControlshRNA) was constructed. Six groups of albino mice were used (6 mice/group). Three groups were exposed to cold (6.7°C), whereas the remaining three groups were kept at room temperature (RT; warm) as controls. In each temperature condition, three groups received an intravenous injection of MRshRNA, ControlshRNA, or virus-free PBS, respectively, before exposure to cold. The viral complexes (0.35 × 1011 particles/mouse, 0.5 ml) or PBS (0.5 ml) was delivered into the circulation via the tail vein. The blood pressure (BP) of the mice treated with ControlshRNA or PBS increased significantly during exposure to cold, whereas the BP of the cold-exposed MRshRNA-treated mice did not increase and remained at the level of the control group kept at RT. Thus AAV delivery of MRshRNA prevented the initiation of CIH. MRshRNA significantly attenuated cardiac and renal hypertrophy. MRshRNA decreased the cold-induced increase in MR protein expression to the control level in the hypothalamus, kidneys, and heart, indicating an effective prevention of the cold-induced upregulation of MR. RNAi inhibition of MR resulted in significant decreases in the plasma level of norepinephrine, plasma renin activity, and plasma level of aldosterone in cold-exposed mice. MR played a critical role in the initiation and development of CIH. AAV delivery of MRshRNA may serve as a new approach for the prevention of cold-induced hypertension.

short-hairpin RNA; aldosterone; blood pressure; adeno-associated virus; cardiac hypertrophy; RNA interference

COLD TEMPERATURES HAVE adverse effects on the human cardiovascular system. People who live in cold regions have a high incidence of hypertension and related cardiovascular diseases (3, 4, 6, 11, 13, 25, 36). Of the four seasons, the cold winter has the highest mortality and morbidity from cardiovascular diseases (1, 24, 25, 36). Cold temperatures make hypertension more severe in hypertensive patients and trigger myocardial infarction and stroke (6, 7, 9, 13, 25, 36, 37). Chronic cold exposure causes hypertension and cardiac hypertrophy in rats within 1–3 wk (26, 27, 38, 39), namely cold-induced hypertension (CIH). CIH is a natural form of experimentally induced hypertension that does not require surgery, an administration of large doses of hormones or drugs, or genetic manipulation (26, 28). The characteristics of CIH are similar to those of human essential hypertension (26, 28, 29). Therefore, it is important to determine the mechanism of CIH. Previous studies from our laboratory suggested that CIH may be associated with the hyperactivity of the renin-angiotensin-aldosterone system (RAAS) (27, 30–33). A recent study from our laboratory indicated that inhibition of the mineralocorticoid receptor (MR) prevented the progression of CIH and renal damage (40). However, it is not known whether the MR is involved in the onset of CIH. The aim of this study was to determine the role of the MR in the initiation and development of CIH. Although pharmacological MR blockers such as spironolactone can inhibit the MR, they interact with androgen and progesterone receptors, causing an unwanted side effect due to the lack of specificity. These drugs are short lasting (<24 h). In addition, eplerenone was found to increase serum triglycerides, cholesterol, alanine aminotransferase, γ-glutamyl transpeptidase, creatinine, and uric acid (12). To overcome these limitations of pharmacological MR blockers, we used the RNA interference (RNAi) approach to achieve long-term and specific inhibition of MR expression. We hypothesized that RNAi inhibition of MRs attenuates or prevents the initiation and development of CIH.

Our previous studies showed that the development of cold-induced cardiac hypertrophy is independent of high blood pressure (BP) because the prevention or attenuation of CIH does not attenuate cold-induced increases in heart weight (27, 32, 33). Thus cold-induced cardiac hypertrophy may be due to endocrine changes associated with cold exposure. The cardiac MR has been reported to be involved in the pathogenesis of hypertrophy. Thus an additional aim of this study was to determine whether the MR plays a role in the development of cold-induced cardiac hypertrophy. We hypothesized that RNAi inhibition of MRs attenuates cold-induced cardiac hypertrophy.

METHODS

Recombinant adeno-associated virus with short-hairpin small-interference RNA for MR and a scrambled sequence. The recombinant adeno-associated virus (AAV) carrying short-hairpin small-interference RNA for MR (AAV.MRshRNA) or a scrambled sequence (AAV.ControlshRNA) was constructed as described previously (40). Briefly, the MRshRNA was designed using Dharmacon’s software,
synthesized by Integrated DNA Technologies (Coralville, IA), and cloned into an AAV serotype-2 (AAV-2; Startagen, La Jolla, CA) under the control of a human RNA polymerase II U6 promoter (AAV.MRshRNA) (40). Scrambled shRNA was cloned into AAV-2 vector and used as a control viral construct (AAV.ControlsRNA). Scrambled shRNA has been proved, by BD Clontech (Palo Alto, CA), not to match with any other known gene sequences. The constructs were packaged with PHelper and pAAV-RC to produce recombinant vectors. Titers were determined by real-time PCR as described by Rohr et al. (23).

**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. Male albino mice of 8 wk of age were used in the experiment. All mice 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 protocol.** Six groups of albino mice (6 mice/group) were used. The animals were handled daily to minimize handling stress. The animals did not appear stressed during BP measurement. Resting systolic BP was measured at room temperature (RT) from the tail of each anesthetized mouse using the tail-cuff method. The tail-cuff procedure is a common method used by our laboratory (27, 30–33) and others (17, 43) to delineate CIH. 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 (5, 29).

During a 2-wk control period, BP, body weight (BW), food intake, water intake, and urine output were measured. After the control period, three groups were moved into a climate-controlled walk-in chamber (6.7 ± 2°C), whereas the other three groups were kept in an identical chamber maintained at RT (25 ± 2°C, warm). In each temperature condition, one group received a bolus injection of AAV.MRshRNA (0.35 × 10^11 particles/mouse, 0.5 ml iv via tail vein), one group received AAV.ControlsRNA (0.35 × 10^11 particles/mouse, 0.5 ml iv), and the last group received phosphate-buffered saline (PBS; 0.5 ml iv) 48 h before exposure to cold. BP was measured 24 h after the injection to ensure that the gene delivery did not affect animal-resting BP at RT. BP and BW were measured at least once a week throughout the experiment. Food intake, water intake, and urine output were measured during weeks 3 and 5 of exposure to cold. Urinary Na⁺ was measured by flame photometry as described in previous studies from our laboratory (32, 40).

At day 32 after exposure to cold, all animals were deeply anesthetized with pentobarbital sodium (120 mg/kg ip) for blood collection, followed by transcardiac perfusion with PBS. The heart and kidneys were removed and weighed. The left kidney, the apical region of the heart, and half of the brain were fixed in 4% paraformaldehyde in PBS for immunohistochemical (IHC) and histological analyses. The rest of the tissues were snap frozen and saved for Western blot analysis of MR and AAV protein expression.

**Measurements of plasma norepinephrine, plasma renin activity, and plasma aldosterone.** The plasma norepinephrine (NE) level was measured using HPLC with an electrochemical detector as described in previous studies from our laboratory (27, 32). Plasma renin activity (PRA) and plasma aldosterone levels were measured using radioimmunoassay as described previously (32, 41).

**Western blot analysis of MR and AAV capsid protein expression.** Tissues were homogenized in lysis buffer (50 mM Tris, 150 mM NaCl, 1% sodium dodecyl sulfate, 1% sodium deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylene diamine tetracetic acid, and 1% Triton X-100) containing a protease inhibitor complex and centrifuged 5 min at 10,000 g. Supernatants were collected and immediately mixed with an equal volume of electrophoresis loading buffer for Western blot analysis of MR and AAV capsid protein expression.

MR expression was normalized with the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as an internal control. Briefly, equal volumes of mixtures (30 μl and 3 μl/sample for MR and GAPDH, respectively) were loaded for all groups. Membranes were blocked overnight at 4°C before incubation with primary antibodies. For MR, the membranes were incubated with rabbit polyclonal anti-MR primary antibody (dilution, 1:500; Santa Cruz Biotechnology) overnight and then with goat anti-rabbit secondary antibody (dilution 1:2,000; Santa Cruz Biotechnology) for 2 h. For GAPDH, the membranes were incubated with mouse monoclonal anti-GAPDH primary antibody (dilution 1:30,000; Santa Cruz Biotechnology) for 1 h and then with goat anti-mouse secondary antibody for 2 h. Enhanced chemiluminescence was added to the membranes and exposed to photosensitive films. The films were imaged by using a Bio-Rad transilluminator. Protein band intensities were quantified using Quantity One version 4.1.1.1 c system (Bio-Rad, Hercules, CA).

AAV capsid protein expression was similarly analyzed using anti-AAV antibodies (VP1, VP2, and VP3; Strategen).

**IHC analysis of MR expression.** After a 24-h fixation with 4% paraformaldehyde, the heart and kidneys were incubated overnight in 70% ethanol at 4°C and processed for paraffin embedding. Sections (5 μm) were used for the immunostaining of MR. Brains were dehydrated in 20% sucrose for 48 h before embedding with optimal cutting temperature. For immunostaining, the sections were treated for 30 min with 0.25% Triton X-100 for antigen retrieval, 5 min with peroxidase-blocking solution (Dako), and 15 min with protein blocker (Biocare). The sections were then incubated with polyclonal goat anti-MR primary antibody (dilution 1:100; Santa Cruz Biotechnology) for 1 h and then with a donkey anti-goat secondary antibody (dilution 1:200; Santa Cruz Biotechnology) for 30 min. The sections were examined and photographed using a Zeiss Axioplan 2 photomicroscope coupled with a digital color camera. In the hypothalamus, the MR expression level was evaluated by calculating the percentage of MR-positive neurons in 150 neurons in each of three different areas.

**Statistical analysis.** The data for BP and BW were analyzed by a repeated-measures two-way ANOVA followed by one-way ANOVA. The remaining data were analyzed by a two-way ANOVA (main factors, temperature and treatment). The Newman-Keuls procedure was used to assess the significance of difference between means. Significance was set at a 95% confidence limit.

**RESULTS**

**Effect of AAV delivery of MRshRNA on BP and BW.** BP did not differ significantly among the six groups during the control period at RT (Fig. 1A). No significant difference in BP was found between any two groups at 24 h after gene delivery. The BP of the ControlshRNA- and PBS-treated groups increased significantly within 4 days of exposure to cold. The BP of these two groups continued to increase thereafter and reached a plateau (∼140 mmHg) by 3 wk of exposure to cold. No significant difference in BP was found between these two groups throughout the experiment. In contrast, the BP of the MRshRNA-treated group did not increase significantly and remained at the level of the PBS-warm group during exposure to cold. One dose of MRshRNA kept the BP at the control level for at least 32 days (length of the study; Fig. 1A). MRshRNA or ControlsRNA did not significantly change BP in mice maintained at RT (warm).

Despite the observed differences in BP, all animals grew at approximately the same rate (Fig. 1B). No significant difference in BW was found between any two groups at any time points (P > 0.05), indicating that cold exposure or treatments with viral complexes did not affect the animals’ growth.
Food intake, water intake, urine output, and urinary sodium output. Food intake, water intake, and urine output did not differ among the six groups during the control period and among all the warm-kept groups throughout the experiment (P > 0.05). Although cold exposure did not affect BW, it significantly increased food intake, water intake, and urine output in all three groups compared with their corresponding warm-kept groups (P < 0.01) during weeks 3 and 5 (Fig. 2, A–C). No significant difference was found in food intake, water intake, and urine output among the three groups in either temperature condition. Cold exposure increased urinary sodium output significantly (data not shown). However, no significant difference was found in the urinary sodium output between the MRshRNA- and ControlshRNA- or PBS-treated groups in either temperature environment, indicating that MRshRNA did not affect urinary sodium output.

Effects of AAV delivery of MRshRNA on cold-induced cardiac and renal hypertrophy. Cold exposure increased heart weight to approximately the same extent in the ControlshRNA- and PBS-treated groups (Fig. 3A). MRshRNA significantly decreased the cold-induced increase in heart weight compared with the ControlshRNA- or the PBS-cold groups. Nevertheless, the heart weight was significantly greater in the MRshRNA-cold group than in the PBS-warm group, indicating that MRshRNA failed to decrease heart weight to the warm control level. MRshRNA did not affect heart weight significantly in the mice maintained at RT (warm).

Cold exposure significantly increased kidney weight in the ControlshRNA- and PBS-treated groups (Fig. 3B). MRshRNA significantly decreased the cold-induced increase in kidney weight but failed to decrease it to the warm control level. MRshRNA did not significantly affect kidney weight in the mice maintained at RT (warm).
Effects of AAV delivery of MRshRNA on plasma NE, PRA, and plasma aldosterone. The plasma NE concentration, PRA, and plasma aldosterone concentration were significantly increased in the ControlshRNA- and PBS-cold groups (Fig. 4, A–C), indicating that cold exposure activated the sympathetic nervous system (SNS) and the RAAS. AAV delivery of MRshRNA significantly decreased the plasma NE concentration, PRA, and plasma aldosterone concentration of cold-exposed rats to the control levels. MRshRNA did not affect these parameters in rats kept at RT (warm).

Detection of AAV proteins and the effects of AAV.MRshRNA on MR expression. The expression of AAV structural (capsid) proteins VP1, VP2, and VP3 was used to assess gene delivery and tissue transfection efficiency. VP1, VP2, and VP3 were strongly expressed in hearts (Fig. 5A). A strong expression of VP2 and VP3 was also found in the kidneys. VP2 was expressed in the hypothalamus. Thus the expression of at least one of the AAV structural proteins was detected in the heart, kidneys, and brain hypothalamus, indicating that AAV was expressed in these tissues or cells.

Cold exposure significantly increased MR expression in the heart, kidneys, and hypothalamus in ControlshRNA- and PBS-cold groups compared with their corresponding warm-kept groups (Fig. 5, B–E). Thus MR expression was upregulated by cold exposure. MRshRNA significantly decreased MR protein expression in these tissues in cold-exposed rats. No significant difference in MR expression was found between the MRshRNA-cold and the PBS-warm groups (Fig. 5, C–E), indicating that MRshRNA abolished the cold-induced increase in MR expression.

IHC analysis of MR expression. The inhibitory effect of MRshRNA on MR expression in the heart, kidneys, and hypothalamus of cold-exposed mice was further examined by IHC. Cold exposure increased MR immunostaining in the hearts and kidneys in ControlshRNA- and PBS-treated groups. In the renal epithelial cells, MR staining was also found in the cytoplasm. MRshRNA greatly decreased MR staining in the hearts and kidneys of cold-exposed mice (Fig. 6). Cold exposure increased interstitial fibrotic tissue in the hearts of ControlshRNA- and PBS-treated
mice (Fig. 6). MRshRNA greatly decreased cardiac fibrosis in cold-exposed mice. For simplicity, the IHC photomicrographs for MRshRNA- and ControlshRNA-warm mice were not shown because their MR staining was not altered compared with PBS-warm mice.

Cold exposure significantly increased the number of MR-positive neurons in the hypothalamus as determined by the increased percentage of MR-positive neurons in ControlshRNA- and PBS-cold groups (Fig. 7, A and B). MRshRNA significantly decreased the number of MR-positive neurons in the hypothalamus in the cold-exposed group compared with the ControlshRNA-cold group. MRshRNA decreased the number of MR-positive neurons in the cold-exposed group to that of the PBS-warm group (MRshRNA cold vs. PBS warm, P > 0.05; Fig. 7B), indicating that AAV delivery of MRshRNA abolished the cold-induced upregulation of hypothalamic MR.

DISCUSSION

The most exciting finding of this study is that the RNAi inhibition of MR prevented the cold-induced elevation of BP. It is noted that one single dose of AAV.MRshRNA controlled CIH for up to 32 days (length of the study). The RNAi inhibition of MR also attenuated cardiac and renal hypertrophy. The MR was effectively inhibited by MRshRNA, as evidenced by the fact that the cold-induced increase in the MR was prevented in rats treated with AAV.MRshRNA. The prolonged antihypertensive effect of AAV.MRshRNA was probably due to the long-lasting AAV vector, which is safe, nonpathogenic, noninflammatory, and extremely stable (19, 20). No signs of inflammation were seen during an autopsy, and no viral effect was observed in the data analysis. Indeed, AAV.shRNA did not have a toxic effect on animal growth and BP regulation because AAV.ControlshRNA did not affect BW and BP in either temperature condition. A recent study from our laboratory indicated that RNAi inhibition of MR stopped the progression of hypertension in rats with established CIH although the BP was not lowered to normotensive level (40). The present study clearly shows that AAV delivery of MRshRNA may be a promising approach for the prevention of hypertension due to the overactive MR although there are many challenges before it can be used clinically.

Fig. 5. Western blot analysis of AAV capsid protein expression and mineralocorticoid receptor (MR) protein expression. A: representative Western blot of AAV capsid proteins VP1, VP2, and VP3 in heart, kidneys, and hypothalamus in the MRshRNA-cold group. B: representative Western blot of MR expression in heart, kidneys, and hypothalamus. Relative MR expression was normalized with GAPDH in heart (C), kidneys (D), and hypothalamus (E). These were measured at 32 days after exposure to cold. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the ControlshRNA-warm group; +++P < 0.01 and +++++P < 0.01 compared with the ControlshRNA-cold group; n = 6 animals.
The present finding also supports the hypothesis that the MR plays a critical role in the initiation and development of CIH because cold exposure upregulated the MR and the normalization of MR expression prevented the onset of CIH. Previous studies from our laboratory have shown that the hyperactivity of the SNS and the RAAS is essential for the development of CIH (27, 30–33). It has been confirmed that the SNS initiates CIH via the activation of the RAAS (27, 32, 33). It is interesting to note that the AAV delivery of MRshRNA prevented the cold-induced increase in the plasma NE level, PRA, and plasma aldosterone level. This finding is unexpected because it was originally assumed that RNAi inhibition of the MR would increase plasma aldosterone levels due to compensatory feedback. The decreased plasma aldosterone levels by gene delivery of MRshRNA were probably due to the inhibition of the SNS in cold-exposed mice, which decreased the activity of the

![Immunohistochemical analysis of MR protein expression in heart and kidneys.](image)

![Immunohistochemical analysis of MR protein expression in the hypothalamus.](image)

The present finding also supports the hypothesis that the MR plays a critical role in the initiation and development of CIH because cold exposure upregulated the MR and the normalization of MR expression prevented the onset of CIH. Previous studies from our laboratory have shown that the hyperactivity of the SNS and the RAAS is essential for the development of CIH (27, 30–33). It has been confirmed that the SNS initiates CIH via the activation of the RAAS (27, 32, 33). It is interesting to note that the AAV delivery of MRshRNA prevented the cold-induced increase in the plasma NE level, PRA, and plasma aldosterone level. This finding is unexpected because it was originally assumed that RNAi inhibition of the MR would increase plasma aldosterone levels due to compensatory feedback. The decreased plasma aldosterone levels by gene delivery of MRshRNA were probably due to the inhibition of the SNS in cold-exposed mice, which decreased the activity of the
It is noted that RNAi inhibition of MR prevented the cold-induced elevation of BP but only partially attenuated cardiac and renal hypertrophy. Previous studies from our laboratory indicate that cold-induced cardiac and renal hypertrophy are independent of pressure overload because the attenuation or prevention of CIH does not attenuate cold-induced increases in heart and kidney weights (27, 32, 33). Therefore, the partial attenuation of hypertrophy may be attributed to the direct inhibition of the MR rather than its preventive effect on BP. Indeed, the MR has been reported to be related to cardiac hypertrophy (14, 16, 25a). The MR may also be involved in some forms of renal hypertrophy (2, 10, 21). Nevertheless, the present study suggests that MR partially contributes to the pathogenesis of cold-induced cardiac and renal hypertrophy. Thus the mechanisms in addition to the MR may be involved in cold-induced cardiac and renal hypertrophy. This hypothesis that cold-induced cardiac and renal hypertrophy is largely mediated by thyroid hormones needs to be tested because cold exposure increases the secretion of thyroid hormones that are required for nonshivering thermogenesis.

In conclusion, cold exposure upregulated MR expression, which played a critical role in the initiation and development of CIH. AAV delivery of MRshRNA may serve as a new approach for the prevention of CIH.

GRANTS
This study was supported by National Heart, Lung, and Blood Institute Grant R01-HL-077490 (to Z. Sun).

REFERENCES


