Angiotensinogen Gene Knockout Delays and Attenuates Cold-Induced Hypertension

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Abstract—The aim of the present study was to assess our hypothesis that the renin-angiotensin system (RAS) is responsible for cold-induced hypertension and cardiac hypertrophy. Two groups of wild-type (WT) mice and 2 groups of angiotensinogen gene knockout (Agt-KO) mice (6 per group) were used. After blood pressures (BP) of the four groups were measured 3 times at room temperature (25°C), 1 WT and 1 Agt-KO group were exposed to cold (5°C). The remaining groups were kept at 25°C. BP of the cold-exposed WT group increased significantly in 1 week of cold exposure and rose gradually to 168±7 mm Hg by week 5, whereas the BP of the Agt-KO group did not increase until week 3. The cold-induced increase in BP (ΔBP) was decreased significantly in the Agt-KO mice (19±3 mm Hg) compared with that of the WT mice (61±5 mm Hg) by 5 weeks of exposure to cold. Both WT and Agt-KO groups had cardiac hypertrophy in cold to the same extent. Agt-KO caused a significant increase in nitric oxide (NO) production. Thus, the RAS may inhibit NO formation. Chronic cold exposure decreased NO production, which may be mediated partially by activation of the RAS. These results strongly support that the RAS plays a critical role in the development of cold-induced hypertension but not cardiac hypertrophy. Moreover, the role of the RAS in cold-induced hypertension may be mediated in part by its inhibition on NO production. The findings also reveal the possible relation between the RAS and NO in cardiovascular regulation. (Hypertension. 2003;41:322-327.)

Key Words: hypertension, experimental ■ hypertrophy ■ renin-angiotensin system ■ mice ■ nitric oxide

Epidemiologic surveys and clinical observations have established that people who live and work in cold areas have a high incidence of hypertension and related cardiovascular diseases.1–8 It has been reported3–5,7,8 frequently that people who live in cold areas have a high incidence of hypertension and related cardiovascular diseases. It was originally assumed that cold-induced elevation of blood pressure was a direct vasoconstrictive effect of sympathetic nervous system (SNS). Indeed, the SNS is activated by chronic cold exposure and the plasma and urine levels of catecholamines are increased significantly in cold-exposed rats.19,21,22,25 However, vascular contractive responsiveness to the α1-adrenoceptor agonist phenylephrine is decreased significantly in cold-exposed rats.26–28 The pressor response to genetic manipulation. Thus, it is important to understand fully the mechanism contributing to this unique model of hypertension because it is naturally occurring. It is notable that the elevated blood pressure of rats after 7 weeks of exposure to cold does not return to precold exposure level during 4 weeks after removal from cold.23 Thus, an elevation of blood pressure induced by a longer period of cold exposure might not be reversible after return to thermoneutral temperature. Intermittent exposure of rats to cold also induces hypertension,24 with a sigmoid relation between the hours per day exposed to cold and systolic blood pressure. Cold-induced hypertension (CHI) is a prototypic model of environmentally induced hypertension. Since the underlying cause of human hypertension remains unknown and its causes are multifarious, the use of various models, each of which induces the disease by a different mechanism yet with the same end result, is advantageous.
phenylephrine was also significantly decreased by chronic cold exposure. Several studies have shown that blockade of the renin-angiotensin system (RAS) at different sites could attenuate or prevent the cold-induced elevation of blood pressure. Antisense oligodeoxynucleotides to angiotensinogen mRNA reduces elevated blood pressure of cold-exposed rats. It was therefore suggested elsewhere that the hyperactivity of the SNS initiated CIH probably by activation of the RAS. Indeed, plasma renin activity (PRA) is increased during exposure to cold, and abolishment of the rise in PRA by renal denervation prevents the development of CIH. Thus, cold exposure activates the RAS. It has been reported that angiotensin II (AngII) acts as a growth factor, increases protein synthesis, and is involved in cardiac hypertrophy. Therefore, we hypothesized that the RAS is responsible for the development of CIH and cardiac hypertrophy. The aim of this study was to assess this hypothesis by using angiotensinogen gene knockout (Agt-KO) mice chronically exposed to cold.

**Methods**

**Animals**

This study was carried out according to the Guidelines of the National Institute of Health on the care and use of laboratory animals. The protocol for which this study was a part, was approved by the Institutional Animal Care And Use Committee.

Two groups of wild-type (WT) and 2 groups of Agt-KO mice (6 mice per group) (Jackson Laboratories) were used. Agt-KO is produced by gene targeting leading to a null mutation of gene and back-crossed to establish a congenic line with C57BL/6J-Agt genetic background. Body weight and systolic blood pressure were measured 3 times during a 1-week control period at room temperature (warm, 25°C). Systolic blood pressure was measured from the tail of each unanesthetized mouse by the tail-cuff method. It has been confirmed that the noninvasive tail-cuff method is effective and reliable in determining of systolic blood pressure in rats and mice.

**Experimental Protocol**

After the control period, 1 group of WT and 1 group of Agt-KO mice were moved into a cold climate-controlled walk-in chamber (5°C). The remaining groups were kept in a warm chamber (room temperature, 25°C) and served as control animals. Relative humidity was controlled automatically at 45±5% in both thermal environments. Blood pressure and body weight were measured weekly in all mice during exposure to cold. During the 1st, 3rd, and 5th weeks of exposure to cold, a 48-hour urine sample was collected for measurement of urinary output of nitrite/nitrate. At the end of week 5 of exposure to cold, all mice were killed by decapitation, and blood was collected in EDTA for measurement of plasma concentrations of nitrite/nitrate and norepinephrine. The plasma level of norepinephrine was measured by high-pressure liquid chromatography (HPLC) as described earlier. The heart was removed and weighed. Brain hypothalamus, liver, heart, and kidneys were saved at −80°C for measurement of Agt mRNA by real-time reverse transcriptase–polymerase chain reaction (RT-PCR).

**Measurement of Nitrite/Nitrate**

Nitric oxide (NO) was measured by using an NO-analyzing system (Antek). Three injections (10 μL/injection) for a sample were required, and the concentration of nitrite/nitrate was determined by averaging the three readings. Urinary creatinine concentration was measured by using a Creatinine Analyzer (Beckman) so that urinary output of nitrite/nitrate could be expressed as micromoles per gram of creatinine.

**Quantitative Real-Time RT-PCR**

Quantitative real-time RT-PCR (QRT-RT-PCR) was performed with the use of the 5700 Sequence Detector (PE Biosystems). Specific quantitative assays for mice Agt mRNA and 36B4 were developed with the use of Primer Express software (PE Biosystems), following the recommended guidelines based on cDNA sequences from GenBank. Sense and antisense primers for angiotensinogen gene were 5’-GTACAGACAGCACCCTACTT-3’ and 5’-AAACAATTCTCCGTGACGTG-3’, respectively. The precise amount of total RNA added to each reaction (based on optical density) and its quality were checked by the quantification of the endogenous RNA control 36B4 (also known as RPLP0). Each sample was then normalized on the basis of its 36B4 content.

**Statistical Analysis**

Data for blood pressure were analyzed between the cold-exposed and warm-adapted groups of the same strain of mice by a repeated-measures, 1-way ANOVA. Data for body weight, heart weight, tissue Agt mRNA content, plasma concentrations of nitrate/nitrite, and norepinephrine and urinary nitrate/nitrate output were analyzed by a 3-way ANOVA (main factors: time, temperature, and strain), followed by 2- or 1-way ANOVA. The Newman-Keuls procedure was used to assess the significance of differences between means. Significance was set at the 95% confidence limit.

An expanded Methods section can be found in an online supplement available at http://www.hypertensionaha.org.

**Results**

**Blood Pressure and Body and Heart Weights**

Resting systolic blood pressure (95±3 mm Hg) of the Agt-KO mice was significantly (P<0.05) lower than that (112±5 mm Hg) of the WT mice during the control period in warm (25°C) (Figure 1A). When a WT and an Agt-KO group were exposed to cold (5°C), the blood pressure of the WT group increased significantly (P<0.05) above its precold exposure measurement within week 1 of cold exposure. The blood pressure of this group continued to increase thereafter and rose to 168±7 mm Hg by week 5. However, blood pressure of the Agt-KO group did not increase until week 3 of exposure to cold. The cold-induced increase in blood pressure (ΔBP=19±3 mm Hg, compared with precold exposure level) of the Agt-KO mice was significantly (P<0.01) less than that (61±5 mm Hg) of the WT mice by week 5 (Figure 1A). Thus, the cold-induced elevation of blood pressure was delayed and significantly attenuated in the Agt-KO mice. Blood pressures of both groups kept in warm (25°C) remained unchanged during the period of observation. Body weights of the four groups did not differ significantly from one another throughout the experiment (Figure 1B). Both Agt-KO and WT mice, in cold or in warm, grew at approximately the same rate. Heart weights (mg/g body wt) of both cold-exposed groups were increased significantly above that of the WT group maintained at 25°C (Figure 2). There was no significant difference of heart weight between the Agt-KO and WT groups, either in cold or in warm.

**RT-PCR Analysis of Agt mRNA**

Agt mRNA expression was increased significantly in brain hypothalamus, heart, kidneys, and liver of the cold-exposed WT mice compared with that of the WT mice kept in warm (25°C) (Table). Agt mRNA was not detectable in both Agt-KO groups.
Plasma Level of Norepinephrine
Plasma concentration of norepinephrine was increased significantly in both Agt-KO and WT groups exposed to cold compared with that of the warm-adapted WT group (Figure 3). No significant difference of plasma concentration of norepinephrine was found between Agt-KO and WT group in either temperature condition.

Plasma Level of Nitrite/Nitrate
Plasma level of nitrite/nitrate was significantly greater in the Agt-KO group than in the WT group kept in warm (25 °C) (Figure 4). Chronic cold exposure decreased concentration of nitrite/nitrate in plasma of both Agt-KO and WT mice compared with their counterparts kept at 25°C. However, plasma concentration of nitrite/nitrate was significantly greater in the cold-exposed Agt-KO group than in the cold-exposed WT group. Thus, cold exposure failed to decrease plasma level of nitrite/nitrate of the Agt-KO mice to that of the cold-exposed WT mice.

Urinary Output of Nitrite/Nitrate
Urinary nitrite/nitrate output was significantly greater in the Agt-KO group than in the WT group kept at 25 °C at all of the 3 time points of observation (Figure 5). Chronic cold exposure significantly decreased urinary nitrite/nitrate output in the WT group. Urinary nitrite/nitrate output was decreased significantly in the cold-exposed Agt-KO group compared with its counterpart in warm during the 3rd and 5th weeks but not the 1st week of cold exposure. Urinary nitrite/nitrate output was significantly greater in the Agt-KO group than in the WT group in cold. Thus, cold exposure failed to decrease the urinary nitrite/nitrate output of the Agt-KO mice to the level of the cold-exposed WT mice.

Discussion
Chronic exposure to cold induced a significant elevation of blood pressure (Figure 1) and cardiac hypertrophy (Figure 2) in the WT mice. Thus, the WT mice had hypertension in cold (5°C). Agt gene knockout delayed and attenuated the increase in blood pressure seen with exposure to cold (Figure 1). This supports our hypothesis that the RAS plays a critical role in the development of CIH. Agt mRNA was not detectable in

![Quantitative Real Time RT-PCR Analysis of Agt mRNA Expression in Brain Hypothalamus, Heart, Liver, and Kidneys of 4 Groups of Mice](quantitative_real_time_rtpcr_analysis_of_agt_mrna_expression_in_brain_hypothalamus,heart,liver,and_kidneys_of_4_groups_of_mice.png)

This was measured when the animals were sacrificed at 5 weeks after exposure to cold. Data are mean±SEM; n=6. WT indicates wild-type mice; AGT-KO, angiotensinogen gene knockout mice; —, not detectable. *P<0.05 compared with the wild-type group in warm; **P<0.01 compared with the wild-type group in warm.

![Plasma concentration of norepinephrine (mean±SEM) of the 4 groups of mice designated.](plasma_concentration_of_norepinephrine_meansem_of_the_4_groups_of_mice_designated.png)

Figure 1. Systolic blood pressure (A) and body weight (B) of Agt-KO and WT groups exposed to cold (5°C) and counterparts maintained at warm (25°C). Values are mean±SEM; n=6.

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brain hypothalamus, liver, heart, and kidneys of \textit{Agt-KO} mice, confirming complete deletion of the \textit{Agt} gene (Table). Blood pressure is lower in the \textit{Agt-KO} mice than in the WT mice at room temperature (25°C), supporting that the RAS is involved in normal blood pressure regulation.

Chronic cold exposure increased \textit{Agt} mRNA expression in both central and peripheral organs (Table). It has been shown that PRA is increased during the first 3 weeks of exposure to cold.\textsuperscript{33} Thus, the RAS is activated by chronic cold exposure.\textsuperscript{19,33} Several pharmacological studies\textsuperscript{20,22,29} have suggested that the hyperactivity of the RAS may be involved in the initiation or development of CIH, since blockade of the RAS at different sites can attenuate or prevent the elevation of blood pressure during cold exposure. Likewise in this model, development of hypertension is prevented by bilateral renal denervation, which abolishes the cold-induced rise in PRA.\textsuperscript{19} It is noted that renal \textit{Agt} mRNA level is increased in the cold-exposed WT mice (Table). Sigmund\textsuperscript{41} has recently clarified that kidney RAS is an important determinant of blood pressure. Thus, the kidney RAS may be crucial in the development of CIH.

Interestingly, \textit{Agt-KO} failed to prevent cardiac hypertrophy, although it delayed and attenuated the cold-induced elevation of blood pressure (Figure 1A, Figure 2). The blood pressure (116±4 mm Hg) of the cold-exposed \textit{Agt-KO} mice was at the level of the WT mice kept in warm (25°C, room temperature) at 5 weeks after exposure to cold. Consistently, our previous studies showed that the antihypertensive agents, such as propranolol,\textsuperscript{18} captopril,\textsuperscript{29} losartan potassium,\textsuperscript{30} and l-arginine,\textsuperscript{42} effectively reduced or prevented the cold-induced elevation of blood pressure but failed to prevent or attenuate cardiac hypertrophy. Thus, the occurrence of cold-induced cardiac hypertrophy is independent of either pressure overload or tachycardia.\textsuperscript{18,42} It was reported\textsuperscript{34,35} that AngII increased cardiac protein synthesis in the adult rat heart, and that cardiac AngII may play an important role in isoproterenol-induced left ventricular hypertrophy. However, the present study supports that the RAS does not contribute to cold-induced cardiac hypertrophy because the \textit{Agt-KO} mice, in absence of the cardiac \textit{Agt} gene (Table), had cardiac hypertrophy in cold approximately to the same extent as did the cold-exposed WT mice (Figure 2). This is in agreement with pharmacological studies that neither captopril (ACE inhibitor)\textsuperscript{29} nor losartan potassium (AT\textsubscript{1} receptor blocker)\textsuperscript{30} prevented the cold-induced increase in heart weight in rats. It has been reported that pressure-overload induces cardiac hypertrophy in AT\textsubscript{1a} receptor--deficient mice.\textsuperscript{43} These mice still contain AT\textsubscript{1b} and AT\textsubscript{2} receptors, which may be involved in cardiac hypertrophy. Thus, the present study shows, for the first time, that the hypertrophy occurs in mice with RAS completely deleted. Plasma level of norepinephrine was elevated in both \textit{Agt-KO} and WT groups in cold. However, our previous work suggests that cold-induced cardiac hypertrophy is not due to the increased activity of the SNS, since neither α-receptor nor β-receptor blockers affect heart weight of cold-exposed rats. It should be mentioned that although chronic cold exposure similarly increased heart weight of WT and \textit{Agt-KO} mice, the quality of the change in the hearts of these two genotypes was not known. Thus, additional study is required to examine the histology of the heart.

Chronic cold exposure significantly increased plasma concentration of norepinephrine in both \textit{Agt-KO} and WT groups (Figure 3). Plasma level of norepinephrine did not differ between \textit{Agt-KO} and WT mice in either temperature condition, suggesting that \textit{Agt-KO} did not affect the sympathetic outflow. It is notable that \textit{Agt-KO} decreased cold-induced increase in blood pressure against the background of an increased concentration of norepinephrine in plasma. This is particularly interesting from the point of view of the interrelation of the SNS and RAS in the development of CIH. Previous studies\textsuperscript{17–19,21,22} from this laboratory have suggested that the SNS initiates CIH through activation of the RAS.

Nitrite and nitrate are stable NO metabolites, which have been used as indices of NO formation.\textsuperscript{44–46} It is interesting to note that \textit{Agt-KO} resulted in a significant increase in NO production at room temperature (25°C, warm) (Figures 4 and 5). This suggests that the RAS may suppress NO formation in normal physiological conditions. The present study showed that nitrite/nitrate level was decreased in both urine and blood of the WT mice during exposure to cold (Figures 4 and 5). Thus, chronic cold exposure decreases NO production. These findings are both interesting and significant because NO
dysfunction may contribute to blood pressure response to cold exposure. Cold exposure failed to decrease the NO production of the Agt-KO group to that of the cold-exposed WT group. Thus, cold-induced decrease in NO production of WT mice may be mediated partially by the RAS. It has been reported that AngII could inhibit endothelial nitric oxide synthase (eNOS) and decrease NO formation.\textsuperscript{37,48} This may be mediated by AT\textsubscript{1} receptors, since activation of AT\textsubscript{1} receptors stimulates the production of NO.\textsuperscript{49,50} It is known that chronic cold exposure activates the RAS.\textsuperscript{17,19,33} upregulates AT\textsubscript{1} receptors,\textsuperscript{22,51,52} and downregulates AT\textsubscript{2} receptors,\textsuperscript{52} which may be involved in cold-induced decrease in NO production. Thus, the role of RAS in CIH may be mediated in part by its inhibition on NO production. It should be mentioned that CIH and subsequent vascular hypertrophy may impair endothelial system and cause NO dysfunction, which can further amplify CIH and related cardiovascular disorders. It has been shown that aortic eNOS protein content is decreased significantly after 8 weeks of exposure to cold.\textsuperscript{53}

Chronic cold exposure inhibited NO production of Agt-KO mice during and after the 3rd week of exposure to cold (Figures 4 and 5). The mechanism by which this occurs is not known but is apparently unrelated to the RAS. Consistently, blood pressure of the cold-exposed Agt-KO group began to elevate at 3 weeks of exposure to cold (Figure 1A). These results suggest that the cold-induced reduction of NO production may contribute to the slight increase in blood pressure of the cold-exposed Agt-KO mice. It has been reported that the NO system may participate in cold-induced elevation of blood pressure.\textsuperscript{54}

Agt-KO and WT mice grew at approximately the same rate throughout the experiment (Figure 1B). Body weights of the two cold-exposed groups did not decrease, suggesting that both Agt-KO and WT mice adapted well to chronic cold exposure. Previous studies\textsuperscript{20,26} from this laboratory have shown that rats could maintain their core temperature during chronic exposure to cold (5\textdegree C).

**Perspectives**

As mentioned in the introduction, CIH in animals may have implications for humans. A report\textsuperscript{1} from the World Health Organization (WHO) indicates that the incidence of hypertension and related cardiovascular diseases is significantly higher in the north (ie, cold) than in the south (ie, warm) areas in China. Cold winter weather makes hypertension more severe and is associated with an increase in cardiovascular mortality rates.\textsuperscript{3–5,7–8} Thus, the present results are significant because they provide a potential target for preventive and therapeutic interventions that are particularly important for people who live and work in cold areas, especially those who work daily in cold environments (meat packer, butchers, custodians of freezer lockers, ice cream manufacturers, and so forth). The findings may help to control cold-induced elevation of blood pressure and related cardiovascular diseases (stroke, myocardial infarction, and so forth) more appropriately and more effectively in hypertensive patients in winter. Although CIH has been studied extensively, little is known about the pathogenesis of cold-induced cardiac hypertrophy, which is independent of either pressure overload or tachycardia. Cardiac hypertrophy is an identified risk factor for myocardial infarction. Thus, it will be interesting and important to investigate the mechanisms of cold-induced hypertrophy by using the nonpharmacological, nonsurgical, nongenetic model of hypertension, CIH.

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