Human eNOS gene delivery attenuates cold-induced elevation of blood pressure in rats

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Wang, Xiuqing, Robert Cade, and Zhongjie Sun. Human eNOS gene delivery attenuates cold-induced elevation of blood pressure in rats. Am J Physiol Heart Circ Physiol 289: H1161–H1168, 2005.—We previously showed that chronic cold exposure inhibits endothelial nitric oxide synthase (eNOS) expression and decreases nitric oxide (NO) production. The aim of the present study was to evaluate the possible role of the NO system in the development of cold-induced hypertension (CIH) by testing the hypothesis that adenoviral delivery of human eNOS gene increases NO production and attenuates CIH in rats. The effect of in vivo delivery of adenovirus carrying human eNOS full-length cDNA (rAdv.heNOS) on CIH was tested using four groups of Sprague-Dawley rats (6 rats/group). Blood pressure (BP) did not differ among the four groups during the control period at room temperature (24°C). Two groups of rats received intravenous injection of rAdv.heNOS (1 × 109 plaque-forming units/rat), and the other two groups received the same dose of rAdv.LacZ to serve as controls. After gene delivery, one rAdv.heNOS-treated group and one rAdv.LacZ-treated group were exposed to cold (6°C) while the remaining groups were kept at 24°C. We found that the BP of the rAdv.heNOS group increased significantly within 1 wk of exposure to cold and reached a peak level at week 5 (152.2 ± 6.4 mmHg). In contrast, BP (118.7 ± 8.4 mmHg) of the cold-exposed rAdv.heNOS group did not increase until 5 wk after exposure to cold. The rAdv.heNOS increased plasma and urine levels of NO significantly in cold-exposed rats, which indicates that eNOS gene transfer increased NO production. Notably, rAdv.heNOS decreased plasma levels of norepinephrine and plasma renin activity in cold-exposed rats, which suggests that eNOS gene transfer may decrease the activities of the sympathetic nervous system and the renin-angiotensin system. Immunohistochemical analysis showed that the transferred human eNOS was expressed in both endothelium and adventitia of mesenteric arteries. We conclude that 1) eNOS gene transfer attenuates CIH by increasing NO production and inhibiting the sympathetic nervous system and the renin-angiotensin system; and 2) the NO system appears to mediate this nongenetic, nonpharmacological, nonsurgical model of hypertension.

endothelial nitric oxide synthase; hypertension; transfer; expression; norepinephrine; plasma renin activity

People who live in cold (northern) areas have a high incidence of hypertension and related cardiovascular diseases (7, 12, 21, 34, 35, 44, 54). Of the four seasons that occur in the US, the cold winter has the highest mortality and morbidity from cardiovascular diseases (3, 43, 44). Cold temperatures make hypertension more severe in hypertensive patients (7, 18, 21, 23, 35, 44, 56). Appropriate control of the cold-related increase in blood pressure (BP) will decrease the high mortality rates of hypertension and related cardiovascular diseases in cold regions or in winter. Thus it is important to fully understand the mechanism mediating the cold-induced elevation of BP.

Cold-induced hypertension (CIH) represents a prototypical model of environmentally induced hypertension. It is a “naturally occurring” form of experimental hypertension that is induced without genetic manipulation, administration of excessive doses of drugs or hormones, or surgical intervention (17, 46–48, 52). Previous studies have shown that cold exposure activates the sympathetic nervous system (SNS; Refs. 39, 49, 50, 52) but downregulates vascular α1-adrenergic receptors (14–16). Numerous studies suggest that the SNS initiates CIH via activation of the renin-angiotensin system (RAS; Refs. 39, 50–52). Carnio and Branco (8) reported that nitric oxide (NO) may be involved in the acute BP response to short-term (1–3 h) cold exposure. Our recent studies (51, 53) indicate that chronic cold exposure inhibits endothelial NO synthase (eNOS) expression and decreases NO production, which occurs simultaneously with cold-induced elevation of BP. We hypothesized that human eNOS gene transfer increases eNOS expression and NO production and thus attenuates CIH. The aim of this study was to evaluate the possible role of the NO system in the development of CIH.

NO deficiency has been suggested to be involved in hypertension (9, 25, 26, 30). NO therapy for hypertension may be beneficial and highly desirable. However, the short half-life (several seconds) and high reactivity of NO and the tolerance and other unwanted effects (e.g., hypotension) caused by NO donors often limit their effectiveness in clinical applications (9). Thus eNOS is an attractive target for cardiovascular gene therapy (10), and eNOS gene transfer may provide the possibility for long-term control of hypertension by continuous NO production. Intracerebroventricular injection of adenoviral vectors with eNOS gene into cerebrospinal fluid of dogs suffering from subarachnoid hemorrhage resulted in eNOS gene expression in the peri-ischemic area (38). Several studies using local gene delivery with adenoviral vectors have demonstrated that eNOS gene transfer restored NO-dependent vasomotor dysfunction in arteries of eNOS gene-knockout mice as well as in spontaneously hypertensive rats and ANG II-hypertensive models (2, 29, 36). For ex vivo gene transfer, adenoviral vectors have several advantages over other vectors such as the ability to infect quiescent cells, a broad host range, ease of preparation of high-titer viral stock, and high-level transgenic expression (6). Adenoviruses are easy to produce and are widely used for experimental gene transfer, although there are
presently many limitations to their successful use in human gene therapy (42).

In the present study, we successfully constructed the recombinant adenovirus with human eNOS full-length cDNA to test the effects of in vivo eNOS transfer on CHF in rats.

**METHODS**

Construction of recombinant adenovirus with human eNOS full-length cDNA. Recombinant adenovirus containing human eNOS full-length cDNA (rAdv.heNOS) or LacZ reporter gene (rAdv.LacZ) driven by cytomegalovirus promoter was constructed using an Adeno-X DNA System (BD Biosciences Clontech) as described recently (57). The prAdv.heNOS was identified by PCR using the Adeno-X PCR primer and the human eNOS-specific primer.

Western blot analysis of human eNOS expression. Human embryonic kidney (HEK-293) cells were used to determine the transfection efficiency of rAdv.heNOS and the dose-dependent expression of the transgene, human eNOS. HEK-293 cells were plated in four wells (1 x 10^5 cells/well). The first three wells were treated with rAdv.heNOS at 1 x 10^8 multiplicity of infection (MOI) of 100, 5 x 10^7 (MOI of 50), and 1 x 10^7 (MOI of 10) plaque-forming units (PFU), respectively. The last well was treated with rAdv.LacZ at 1 x 10^8 PFU (MOI of 100) as a control. Human abdominal aortic endothelial (HAAE1) cells were plated in two wells (1 x 10^5 cells/well). The first well was treated with rAdv.heNOS at 2 x 10^8 PFU (MOI of 200), and the second well was treated with rAdv.LacZ at 2 x 10^8 PFU (MOI of 200) as a control. The cells were incubated at 37°C in a humidified atmosphere maintained with 5% CO_2 for 2 days. The lysate was collected and boiled for 5 min and then centrifuged at 12,000 g for 5 min at room temperature. The supernatant was processed for Western blot analysis of human eNOS protein expression as described recently (53, 57). Mouse anti-human eNOS primary antibody (1:1,000 dilution) and goat anti-mouse IgG-horseradish peroxidase (1:2,000 dilution; BD Transduction Laboratories) were used to reveal human eNOS.

**Immunocytochemical analysis for rAdv.heNOS expression in cells.** Rat heart (H9c2) cells and HAAE1 cells (1 x 10^5 cells/well) were transfected with rAdv.heNOS or rAdv.LacZ (MOI of 200) and incubated at 37°C in a humidified atmosphere maintained with 5% CO_2 for 2 days. Human eNOS was revealed using a monoclonal antibody specific for human eNOS (BD Transduction Laboratories). Immunocytochemical analysis was performed as described in the instruction manual for the BD Adv-X Rapid Titer Kit (BD Biosciences Clontech).

**Animal study protocols.** This study was carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The project, of which this study was a part, was approved by our Institutional Animal Care and Use Committee.

Twenty-four Sprague-Dawley rats (280–300 g body wt) were divided into four groups (6 rats/group). BP and body weight were measured one time during the control period at room temperature (24°C). Systolic BP was measured from the tail of each unanesthetized rat via the tail-cuff method with slight warming (28°C) but not heating of the tail. All rats were handled frequently (4 times/day) to minimize handling stress. Animals did not appear stressed during BP measurement. The tail-cuff method is a common method used by us (47, 49, 50–52) and by others (41, 58) to delineate cold-induced elevation of BP. For consistency, the tail-cuff method was used in the present study. It has been confirmed by intraarterial cannulation that the noninvasive tail-cuff method is effective and reliable in monitoring systolic BP in rats exposed to cold (17, 48).

After the control period, two groups received administration (via jugular vein) of rAdv.heNOS (1 x 10^8 PFU/rat iv; 0.5 ml) while under anesthesia (65 mg/kg ip pentobarbital sodium). The other two groups received rAdv.LacZ (1 x 10^8 PFU/rat iv; 0.5 ml) to serve as controls. After the rats recovered from anesthesia, one rAdv.heNOS-treated group and one rAdv.LacZ-treated group were moved into a cold climate-controlled walk-in chamber (6 ± 2°C). The remaining groups were kept in an identical warm chamber (room temperature, 24 ± 2°C) and served as controls. Relative humidity was controlled automatically and maintained at 45 ± 5% in both thermal environments. BP was measured weekly during exposure to cold.

During the first, third, and fifth weeks of exposure to cold, a 24-h urine sample was collected for measurement of urinary output of nitrate and nitrite (NO_3^-; an index of NO production). Urinary creatinine concentration was measured using a creatinine analyzer (Beckman) so that urinary NO_3^- outputs could be normalized to micromoles per gram of creatinine. At the end of week 5 of exposure to cold, all animals were killed by decapitation, and blood was collected in chilled tubes that contained EDTA. Plasma was separated by centrifugation and saved for determination of plasma NO_3^- levels, norepinephrine (NE) levels, and plasma renin activity (PRA). The brain stem was removed, and the rostral ventrolateral medulla (rVLM) was carefully dissected, homogenized in nitrogen-free water, and centrifuged. The supernatant was used for measuring NO_3^- levels. Total protein was assayed by the Lowry method.

Plasma and urine NO_3^- values were measured using a NO analyzing system (Antek Instruments) as described previously (51). PRA was measured by radioimmunoassay (49, 50, 52). Plasma NE levels were measured by high-pressure liquid chromatography (HPLC) as described previously (50, 52).

**Immunohistochemical analysis for in vivo expression of human eNOS.** An additional 16 Sprague-Dawley rats (4 rats/group) were used and treated as described (see Animal study protocols). At 1 and 5 wk after gene delivery, two rats from each group were deeply anesthetized (100 mg/kg ip pentobarbital sodium) and perfused transcardially with heparinized saline followed by 4% paraformaldehyde (in PBS). Brains, hearts, and superior mesenteric arteries were removed and fixed in 4% paraformaldehyde (in PBS) for 1–4 h at 4°C according to the size of the samples. Sections (6 μm) were cut using a cryostat machine and mounted onto poly-L-lysine-coated slides. Human eNOS was revealed using human eNOS-specific antibody (BD Clontech). The immunohistochemical procedure was performed as described in the DAKO Envision System manual (model K1390; DAKO). Human eNOS expression (density) was measured and photographed using an M4 computerized imaging system (Imaging Research; St. Catharines, Ontario, Canada).

**X-gal staining of LacZ.** LacZ gene expression in mesenteric arteries was visualized by using X-gal staining as described by van Tuyl et al. (55). Pictures were taken using a Nikon digital camera.

**Statistical analysis.** The data for BP and urinary NO_3^- outputs were analyzed by a two-way ANOVA (temperature and treatment) for repeated measurements (in time). The data for plasma levels of NE and NO_3^- and PRA were analyzed by two-way ANOVA (treatment and temperature). Tukey’s tests were used to assess the significance of differences between means. Significance was set at the 95% confidence limit.

**RESULTS**

rAdv.heNOS. The prAdv.heNOS contained human eNOS full-length cDNA and Adeno-X backbone sequences (Fig. 1).

Expression and subcellular localization of human eNOS protein in cultured cells. Western blot analysis demonstrated that human eNOS expression in HEK-293 cells was increased with the increase of the MOI (from 10 to 100) of rAdv.heNOS (Fig. 2A). Thus human eNOS was expressed in HEK-293 cells in a dose-dependent manner at 48 h after transfection with rAdv.heNOS. Human eNOS protein expression was not found in HEK-293 cells transfected with rAdv.LacZ. However, HAAE1 cells transfected with rAdv.LacZ showed minimal (basal) human eNOS expression (Fig. 2B). Transfection with...
rAdv.heNOS increased human eNOS protein expression by threefold in HAAE1 cells.

Immunostaining with human eNOS-specific antibody showed that human eNOS was mainly expressed in the cytoplasm and membrane in rat H9c2 cells (Fig. 2C) and HAAE1 cells (data not shown). These results indicate the correct subcellular localization of human eNOS. H9c2 cells transfected with rAdv.LacZ showed negative staining with human eNOS (Fig. 2D). No cytopathic efficiency was found in HAAE1 and H9c2 cells transfected with rAdv.heNOS even at a higher titer (MOI of 200).

**Blood pressure.** Resting systolic BP did not differ significantly among the four groups during the control period at room temperature (Fig. 3). BP of the rAdv.LacZ-treated group increased significantly (P < 0.05 or 0.001) within 1 wk of exposure to cold. BP of this group continued to increase thereafter and reached a peak level at week 5 (152.2 ± 6.4 mmHg). In contrast, BP of the cold-exposed rAdv.heNOS group did not increase until 5 wk after exposure to cold (118.7 ± 8.4 mmHg). The repeated-measures ANOVA revealed a significant (P < 0.001) treatment-temperature interaction (F = 3.72). BP of the eNOS-cold group was significantly (P < 0.05 or 0.001) lower than that of the LacZ-cold group. Thus eNOS gene transfer significantly attenuated the cold-induced elevation of BP. BP of the eNOS-cold group was slightly but significantly (P < 0.05) greater than that of the LacZ-warm group at week 5 (Fig. 3). The eNOS gene delivery also slightly decreased BP in rats kept at room temperature (warm). BP of the eNOS-warm group was significantly (P < 0.05) decreased compared with the LacZ-warm group during all time points of observation except week 2.

**Urinary NOx output.** Urine NOx levels were significantly (P < 0.001) decreased in the LacZ groups during weeks 1, 3, and 5 of exposure to cold (Fig. 4). The eNOS gene transfer significantly (P < 0.001) increased urine NOx levels in the cold-exposed group compared with the LacZ-cold group. Urinary NOx output in the eNOS-cold group reached a maximal level (610 ± 19 μmol/g of creatinine) at week 3 and then decreased to a level (563 ± 21 μmol/g of creatinine) that was significantly (P < 0.05) lower than the LacZ-warm (control) group at week 5. Urinary NOx output in the eNOS-cold group was significantly (P < 0.05) decreased at week 5 compared with weeks 3 and 1. The eNOS gene delivery also significantly (P < 0.05) increased urinary NOx output in rats kept at room temperature compared with the LacZ-warm group. However, the increase in urine NOx levels induced by eNOS gene transfer (compared with the LacZ groups in their respective environments) was significantly (P < 0.05) greater in the cold-exposed rats than in rats kept at room temperature (Fig. 4).

**Tissue NOx content in rVLM.** The NOx level in the rVLM was significantly (P < 0.01) decreased in the LacZ-cold group compared with the LacZ-warm group (see Fig. 8). The eNOS gene transfer significantly (P < 0.05) increased the NOx level in the rVLM in the cold-exposed group compared with the LacZ-cold group. The eNOS gene transfer slightly but significantly (P < 0.05) increased NOx levels in the rVLM in the
group kept at room temperature (warm) compared with the LacZ-warm group.

Plasma NOx and NE levels and PRA. These parameters were measured when animals were killed at 5 wk after gene delivery. Chronic cold exposure decreased plasma NOx levels significantly in the LacZ-treated group compared with the LacZ-warm group (Fig. 5A). Plasma NOx levels were significantly (P < 0.001) greater in the eNOS-cold group than in the LacZ-cold group, which indicates that eNOS gene delivery markedly increased NO production in the cold-exposed rats. Plasma NOx levels were slightly but significantly (P < 0.05) greater in the eNOS-warm group than in the LacZ-warm group. The increase in plasma NOx induced by eNOS gene transfer (compared with the LacZ group in their respective environment) was significantly (P < 0.05) greater in the cold-exposed rats than in rats kept at room temperature.

Cold exposure increased plasma NE concentration significantly (P < 0.001) in the LacZ-treated group (Fig. 5B). The eNOS gene delivery significantly (P < 0.001) decreased plasma NE levels in cold-exposed rats. However, plasma NE levels were significantly higher in the eNOS-cold group than in the LacZ-cold group. Plasma NE levels were slightly but significantly (P < 0.05) greater in the eNOS-warm group than in the LacZ-warm group. The increase in plasma NE induced by eNOS gene transfer (compared with the LacZ group in their respective environment) was significantly (P < 0.05) greater in the cold-exposed rats than in rats kept at room temperature.

Cold exposure increased PRA significantly (P < 0.001; Fig. 5C) in the LacZ groups. The eNOS gene delivery significantly decreased PRA in the cold-exposed rats. However, PRA was significantly (P < 0.05) higher in the eNOS-cold group than in the LacZ-cold group. PRA was slightly but significantly (P < 0.05) decreased in the eNOS-warm group compared with the LacZ-warm group.

In vivo human eNOS expression in cardiac and vascular tissues. Immunohistochemical analysis showed that human eNOS was expressed in hearts and mesenteric arteries of rAdv.heNOS-treated rats in both temperature conditions. Human eNOS staining (for density) was stronger in hearts and mesenteric arteries in cold-exposed rats than in rats kept at room temperature. Figure 6 shows human eNOS staining in mesenteric arteries of cold-exposed rats. The brown staining appeared in both the endothelial layer and adventitia of mesenteric arteries in rAdv.heNOS-treated but not rAdv.LacZ-treated rats. Human eNOS density was significantly (P < 0.05) greater at week 1 (Fig. 6A) than at week 5 after gene delivery (Fig. 6C). No human eNOS staining was detected in mesenteric arteries of rats treated with rAdv.LacZ (Fig. 6, B and D). Human eNOS staining also was found in cerebral blood vessels but not in nervous system tissues in rats treated with rAdv.heNOS (figures not shown).
X-gal staining of in vivo LacZ expression in transgenic animals. X-gal staining showed that the LacZ reporter gene was still expressed in mesenteric arteries (Fig. 7A) of rats treated with rAdv.LacZ at 5 wk after gene delivery. No X-gal staining was found in mesenteric arteries (Fig. 7B) of rats treated with rAdv.heNOS, which indicates absence of the LacZ gene.

DISCUSSION

We previously showed that chronic cold exposure inhibits eNOS expression and decreases NO production (51, 53). The present study demonstrated for the first time that human eNOS gene delivery increases NO production and attenuates cold-induced elevation of BP (see Fig. 3). These results suggest that the NO system may mediate the initiation and development of CIH. Human eNOS gene transfer was successful as evidenced by strong expression of human eNOS gene in hearts and blood vessels (see Fig. 6). The transferred human eNOS gene was also functional as evidenced by the increased production of NO in rats treated with rAdv.heNOS (see Figs. 4 and Fig. 5A). NO generated from eNOS has a relatively short half-life and is rapidly converted to NOx. Therefore, measurement of NOx is a good indicator of NO production. The increased NO production in the rAdv.heNOS-treated groups was not due to adenoviral inflammation, because both groups used adenoviral vectors. In fact, no visible inflammation was found in heart, blood vessels, lungs, kidneys, or liver during autopsy.

An increase in NO production may contribute to attenuation of cold-induced elevation of BP by its direct vasodilatory effect. Interestingly, systemic eNOS gene delivery reduced plasma levels of NE in cold-exposed rats (see Fig. 5B), which suggests that increased NO production decreases the activity of the SNS. Notably, the cold-induced reduction in NO production (see Figs. 4, 5A, and 8) was accompanied by an increase in SNS activity (see Fig. 5B). Thus it is highly likely that NO is the mediator of the increased SNS activity in cold-exposed rats. It has been reported that a decrease in central NO in-
that the activated SNS initiates CIH via activation of the RAS. Numerous studies from this laboratory suggest that circulating rAdv.heNOS may not penetrate the blood-brain barrier (27). In addition, rAdv.heNOS expressed in the endothelial cells of cerebral vessels and NO can diffuse to adjacent brain areas freely. Indeed, eNOS gene delivery increased NO levels in the rVLM (Fig. 8) and inhibited sympathetic activity (see Fig. 5B). Another interesting finding is that eNOS gene transfer decreased PRA in cold-exposed rats (see Fig. 5C), which may be partially attributed to the inhibition of SNS activity. Numerous studies from this laboratory suggest that the activated SNS initiates CIH via activation of the RAS (39, 50–52), because vascular α1-adrenergic receptors are downregulated by cold exposure (14–16). Therefore, the decreased PRA may contribute to the antihypertensive effect of eNOS gene transfer.

The slight increase in BP of the eNOS-cold group at week 5 (see Fig. 3) is probably due to the diminished human eNOS expression (see Fig. 6C) that resulted in decreased NO production (see Fig. 4C). It is interesting to note that human eNOS gene delivery resulted in stronger human eNOS expression and a greater increase in NO production in cold-exposed rats than in rats kept at room temperature (warm; see Figs. 4 and 5A). The reason for this phenomenon is still under investigation. One possibility is that prolonged cold exposure may upregulate cofactors (i.e., protein kinase Akt) that enhance eNOS expression and activity (18) to compensate depressed eNOS (4).

Urine NOx levels were slightly increased in the eNOS-warm group at 1, 3, and 5 wk after eNOS gene delivery (see Fig. 4) and were accompanied by corresponding decreases in BP in this group (see Fig. 3). The urine NOx-to-creatinine ratio has been used as an indicator of in vivo NO formation (5, 28, 51). In this study, NO formation was also evaluated by measurement of plasma NOx levels, which validates the urine data. Although depressed NO bioavailability in human and animal models of hypertension has been reported (11, 33, 37), the role of the NO system in the pathogenesis of hypertension is still controversial. The present finding in CIH may not necessarily be applicable to other forms of hypertension. In some cases, endothelial NO dysfunction may be secondary to hypertension. In the CIH model, a cold-induced decrease in NO production occurs simultaneously with cold-induced elevation of BP (51, 53). Thus the cold-induced inhibition in NO formation does not seem to be caused by hypertension-associated vascular damage, because CIH is not established until 5 wk after exposure to cold (46, 52). In fact, cold-induced inhibition in the NO system may be due to endocrine changes (ANG II, endothelin, etc.) associated with cold exposure. The RAS may be involved in the cold-induced inhibition in the NO system (51, 53). For longer periods of cold exposure (e.g., 8 wk), CIH and subsequent vascular hypertrophy may further impair eNOS and perpetuate NO dysfunction (58).

The rAdv.heNOS contained human eNOS DNA sequences (see Fig. 1B). The immunocytochemical data indicated that rAdv.heNOS can be expressed in HAAE1 and H9c2 cells as evidenced by expression of human eNOS protein (see Fig. 2C). Interestingly and importantly, the subcellular localization of human eNOS was mainly in the cytoplasm and the membrane (see Fig. 2C). This indicates that the recombinant human eNOS delivered by adenovirus may have efficiency functions, because the subcellular localization of natural (endogenous) eNOS is in the plasma membrane. This localization might render the enzyme more susceptible to activation by physical stimuli such as shear stress (24). Transfection with rAdv.heNOS also increased human eNOS expression in vascular endothelial cells (see Fig. 2B). The dose-dependent increase in human eNOS protein expression in HEK-293 cells (see Fig. 2A) suggests high transfection efficiency of rAdv.heNOS. The extensive portions of the early regions 1 and 3 (E1 and E3, respectively) of wild-type adenovirus had been deleted from the adenovirus type 5 genome (1, 20). No cytopathic efficiency occurred in HAAE1 and H9c2 cells transfected with rAdv. heNOS even at a high titer (MOI of 200), because these cells lack the necessary transcription factors (E1 genes). Thus the adenoviral vector we used provides a valuable safety feature.

It was previously reported (32) that intravenous injection of the naked eNOS plasmid DNA caused a reduction in BP of 21

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**Fig. 7.** X-gal staining of LacZ in superior mesenteric arteries of cold-exposed rats. X-gal staining was carried out when animals were killed at 5 wk after gene delivery. A: X-gal staining of LacZ in a mesenteric artery from a rat treated with rAdv.LacZ. LacZ expression (blue staining) in a mesenteric artery is indicated (arrow). B: X-gal staining in a mesenteric artery from a rat treated with rAdv.heNOS (negative LacZ staining). Original magnification, ×10.

**Fig. 8.** Tissue NOx content in the rostral ventrolateral medulla (rVLM; values are means ± SE; n = 6 rats). Tissue NOx content was measured when animals were killed at 5 wk after gene delivery. *P < 0.05; **P < 0.01 compared with LacZ-warm group; +P < 0.05; ++P < 0.01 compared with LacZ-cold group; #P < 0.05; ##P < 0.01 compared with eNOS-warm group.
mmHg in spontaneously hypertensive rats. However, eNOS plasmid failed to prevent the development of spontaneous hypertension (32). The present study showed that adenoviral delivery of human eNOS full-length cDNA almost abolished the development of CIH for at least 5 wk (length of the study) after gene delivery. Thus adenoviral-mediated eNOS gene transfer may be more efficient than plasmid delivery of eNOS.

To determine human eNOS expression in cardiac and vascular tissues, we used an eNOS antibody that specifically recognizes the human form in the immunohistochemical analysis. As shown in Fig. 6, human eNOS was strongly expressed in both the endothelium and adventitia of the mesenteric artery at 1 wk after gene delivery in the cold-exposed rats (see Fig. 6A). It is interesting and perhaps important that the adenovirus-delivered human eNOS gene can be expressed in the adventitial tissue where NO can easily diffuse to the adjacent smooth muscle. The results suggest that the recombinant adenovirus is an efficient vascular gene-transfer vector for in vivo studies. Vascular human eNOS expression was significantly decreased at 5 wk after gene delivery (see Fig. 6C). Adenovirus-mediated gene transfer usually reaches a peak level by 2 wk and decreases to a minimal level by 4 wk (45). However, the present study clearly demonstrated that human eNOS expression still existed with a reduced level at 5 wk after gene delivery (see Fig. 6C). X-gal staining indicates that the LacZ reporter gene delivered by the adenovirus also existed in the mesenteric artery at 5 wk after gene delivery (see Fig. 7A). These results suggest that the in vivo adenovirus-mediated gene delivery is successful.

In summary, chronic cold exposure inhibited the NO production that was accompanied by increased activities of the SNS and the RAS. The eNOS gene delivery increased NO production, inhibited the SNS and the RAS, and attenuated cold-induced elevation of BP. Thus the NO system may mediate the development of CIH.

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