Human Heme Oxygenase-1 Gene Transfer Lowers Blood Pressure and Promotes Growth in Spontaneously Hypertensive Rats

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Abstract—Heme oxygenase (HO) catalyzes the conversion of heme to biliverdin, with release of free iron and carbon monoxide. Both heme and carbon monoxide have been implicated in the regulation of vascular tone. A retroviral vector containing human HO-1 cDNA (LSN-HHO-1) was constructed and subjected to purification and concentration of the viral particles to achieve $5 \times 10^9$ to $1 \times 10^{10}$ colony-forming units per milliliter. The ability of concentrated infectious viral particles to express human HO-1 (HHO-1) in vivo was tested. A single intracardiac injection of the concentrated infectious viral particles (expressing HHO-1) to 5-day-old spontaneously hypertensive rats resulted in functional expression of the HHO-1 gene and attenuation of the development of hypertension. Rats expressing HHO-1 showed a significant decrease in urinary excretion of a vasoconstrictor arachidonic acid metabolite and a reduction in myogenic responses to increased intraluminal pressure in isolated arterioles. Unexpectedly, HHO-1 chimeric rats showed a simultaneous significant proportionate increase in somatic growth. Thus, delivery of HHO-1 gene by retroviral vector attenuates the development of hypertension and promotes body growth in spontaneously hypertensive rats. (Hypertension. 2001;38:210-215.)

Key Words: hypertension ■ heme oxygenase-1 ■ retrovirus ■ gene transfer ■ growth

As the key enzyme in heme degradation, heme oxygenase (HO) activity governs cellular heme concentration. To date, 3 HO isoforms (HO-1, HO-2, and HO-3), the products of 3 distinct genes, have been identified in mammals.1,2 HO and its metabolic products have been implicated in the regulation of numerous biological processes.1–4 CO derived from HO activity has been shown to function as a neurotransmitter,5 a vasodilator,6 and an endogenous modulator of the NO-cGMP signaling system in brain.7 Recent studies indicate that administration of HO inhibitors increases arterial pressure in normotensive rats.8 It has also been documented that CO arising from heme via metabolism by HO exerts a vasodilatory effect,8,9 that increased expression of HO attenuates reactivity to constrictor agonists,10 and that HO inhibitors magnify myogenic tone in gracilis muscle arterioles.11 These observations suggest that endogenous HO-derived CO plays a role in the regulation of basal tone and contributes to setting the level of arterial blood pressure.

Induction of HO-1 by heavy metals or by its substrate heme was shown to increase HO activity and to decrease blood pressure (BP) in spontaneously hypertensive rats (SHR),12,13 thus establishing a reciprocal relationship between HO gene expression and hypertension in SHR. However, the specificity of the effects of these inducers has not been unequivocally documented. The development of gene transfer techniques has provided the opportunity to deliver a functional HO-1 gene and to evaluate the direct effect(s) of this gene on BP. Other investigators have used viruses as efficient vehicles for transfer of genes that modulate vascular functions.14–16 The present study was undertaken to establish chimeric rats expressing the human HO-1 (HHO-1) gene, with the use of a retroviral vector, and to investigate the impact of augmentation of HO activity on the development of hypertension in SHR.

Methods

Construction of the Retroviral Recombinant LSN-HHO-1

The HHO-1–expressing replication-deficient retrovirus vector LSN-HHO-1 was constructed with the use of the backbone of LXSN17 vector, as previously described.18 Exponentially growing PA317 packaging cells in 60-mm-diameter tissue culture dishes were used for transfection and preparation of viral particles. Individual G418-resistant clones were selected, and initial viral titer assays were measured by infecting NIH-3T3 cells as described previously.18 A...
clone of packaging cell line PA317/LSN-HHO-1 (PA317/HHO-1) producing the highest viral titer of 1.4 × 10^6 colony-forming units (CFU)/mL was used in the experiments described below. PA317/LSN-HHO-1, and the viral control; PA317/LXSN cells were grown in T150 flasks (Fisher Scientific) until subconfluence, and the supernatants were harvested and subjected to low-grade centrifugation at 6000g for 16 hours at 4°C in 250-mL bottles (Nalgene) as previously described. After centrifugation, the pellet was suspended at 1% of the original volume in Hanks' balanced salt solution (HBSS), and the suspension was filtered through a 0.45-μm filter. To achieve higher titer, the same centrifugation was repeated in 1.5-mL Eppendorf tubes, the viral pellet was resuspended in 10 μL HBSS, and the concentrated viral particles (10 μL of 1 × 10^6 CFU/mL) were used for intracardiac delivery into SHR pups. Aliquots of the control vector or HHO-1 vector suspension were used for viral titration assay with the use of NIH-3T3 or stored at −80°C. This procedure resulted in >40% recovery of infectious viral particles, and the final viral titer after concentration ranged from 5 × 10^6 to 1.2 × 10^7 CFU/mL of control or HHO-1 vectors.

Animal Treatment

Pregnant SHR mothers were purchased from Taconic Laboratories (Germantown, NY). Five-day-old SHR from the same litter were divided into 3 treatment groups: vehicle (HBSS), LXSN (viral control), and LSN-HHO-1 (experimental). Treatments were administered by bolus injection of 10 μL HBSS with or without LXSN or LSN-HHO-1 viral particles (1 × 10^6 CFU/mL) directly into the left ventricle under methoxyflurane anesthesia with 96% survival, as previously described. Animals were weaned at 21 days of age; males were separated and used for all experiments. Systolic BP was measured by tail-cuff sphygmomanometry twice weekly, starting at 4 weeks of age. Total body weight gain and average daily food intake were measured for all treatment groups. Daily food intake was estimated by measuring the weight of food used by each cage divided by the number of animals in that cage. At 12 weeks of age, animals were subjected to radiography (n = 6) and measurements of nose-to-tail length and fibula length. At different times (4, 8, 12, 16, and 20 weeks), rats were killed, and tissues, including kidney, liver, lung, brain, and aorta, were isolated for determination of HHO-1 expression and HO activity.

Analysis of HHO-1 mRNA and Protein Expression

Detection of HHO-1 mRNA in virally infected tissues was done by RT/PCR with the use of the following primers: forward: 5'-CAGGCAGAATGCTGAGTTC-3' and reverse: 5'-GATGTGGACGCAACGCAGT-3', with oligo(dT)18 used as reverse transcription primers. Cycling parameters for amplifying RT products were as follows: 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, for 30 cycles, and then extension at 72°C for another 5 minutes. PCR products were mixed with 5 μL of 2× gel loading buffer and applied to a 5% polyacrylamide gel.

The number of HHO-1 mRNA molecules in the kidney samples was estimated by competitive RT-PCR with the use of an internal standard, as previously described. Briefly, chimerical primers composed of 2 specific sequences of the HHO-1 DNA separated by 30 bp allowed the introduction of a 30-bp deletion at the 5′ end of the competitor sequence with the use of exonuclease activity of T4 DNA polymerase and T4 DNA ligase activity to facilitate cloning. The product of this deletional mutagenesis was purified, amplified, and used as an internal standard for quantification of HHO-1 mRNA. A constant amount of internal standard (mHHO-1) at 10 fg concentration was mixed with total RNA from SHR tissues at 500, 200, 100, 50, 25, 5, and 1 ng. The PCR products were applied to a 5% polyacrylamide gel, the gel was exposed to x-ray film, and bands representing PCR products were quantified after normalization with GAPDH mRNA levels, as previously described.

Primers used for detection of endogenous rat HO-1 and GAPDH mRNA and cycling parameters were essentially as described previously. For detection of human and rat HO-1 and HO-2–immunoreactive proteins, 20 μg of cell lysates or tissue homogenates was electrophoresed on a 12% polyacrylamide gel. Specific bands corresponding to HHO-1, rat HO-1, and rat HO-2 proteins were identified with the use of specific monoclonal antibodies (Stressgen Biotechnology) as described. HO activity was measured in cell sonicates or tissue homogenates as the amount of bilirubin generated from heme per milligram of protein per 30 minutes.

Immunocytochemical Analysis of HHO-1 Protein Expression

The rats were anesthetized with 0.2 mL pentobarbital per 100 g of rat weight. The femoral artery was catheterized, and direct BP was measured. Then the animals were perfused through the same line at a rate of 7.23 mL/min with 3 infusions: (1) with 50 mL of 0.9% saline, (2) with 20 mL of vascular rinse for cryoprotection, and (3) with 150 mL of 4% paraformaldehyde. Liver, kidney, lung, aorta, and brain were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin sections, and stained with hematoxylin and eosin. To detect HHO-1–specific protein adducts, sections were immunostained with polyclonal anti–H HO-1 antibody, incubated for 3 hours at 37°C with FITC-labeled rat anti-mouse monoclonal antibodies (Biosource International), and viewed with a confocal fluorescence laser scanner (Molecular Dynamics Inc) as described.

Measurement of Pressure-Diameter Relationship in Isolated Gracilis Muscle Arterioles

The gracilis antibiotic muscle was removed, and segments of the first-order arterioles (1 to 2 mm in length) were isolated and transferred to a water-jacketed vessel chamber (1 mL volume) containing Krebs’ buffer and prepared for evaluation of the pressure-diameter relationship as previously described. The intraluminal pressure was allowed to increase in a stepwise fashion, and the internal diameter of the arterioles was recorded at each pressure step. Before an experiment was concluded, the superfusion buffer was changed to calcium-free Krebs-Ringer bicarbonate buffer containing 1 mMol/L EGTA. Vascular diameters at each pressure level were expressed as the percentage of passive diameter.

Statistical Analysis

All in vitro experiments were performed with triplicate culture dishes, and each experiment was repeated at least 3 times unless stated otherwise. Thus, each point represents a minimum of 9 dishes from 3 separate experiments. Data are presented as mean ± SE. Statistical significance between different groups was determined at P < 0.05 by repeated-measures ANOVA and Student’s t test.

Results and Discussion

A retroviral vector containing HHO-1 cDNA (LSN-HHO-1) was constructed with the use of the LXSN vector as previously described. This vector was functional as evidenced by its ability to express HHO-1 mRNA and to increase HO activity in cultured rat lung microvessel endothelial (RLMV) cells (Figure 1A and 1B). The expression was robust and was maintained for several passages. Importantly, neither the parent RLMV cells nor RLMV cells infected with the control vector LXSN expressed HHO-1.

HHO-1 mRNA was detected by RT/PCR in the kidney, liver, lung, and brain of 12-week-old SHR treated with LSN-HHO-1 but not with LXSN viral particles (Figure 1C). Rat HO-1 mRNA expression was comparable in SHR treated with LXSN and LSN-HHO-1 viruses. The average level of expression of HHO-1 mRNA in kidneys, estimated by competitive RT/PCR with the use of an internal standard, was 1.81 × 10^6 molecules of HHO-1 mRNA per nanogram total RNA.

Immunoblot analysis using specific antibodies that discriminate between human and rat HO-1 revealed expression of HHO-1 protein only in tissues of SHR treated with

Sabaawy et al Human HO-1 Gene Transfer Lowers Blood Pressure in SHR
LSN-HHO-1 but not in those treated with LXSN viral particles (Figure 1D). Rat HO-1 and HO-2 protein expression was comparable in tissues of SHR treated with LSN-HHO-1 or LXSN viral particles. HO-1 protein expression was shown to increase in SHR tissues by immunohistochemical analysis. We confirmed the immunoblot data (Figure 1D) showing that HHO-1 was detected in the kidney, liver, heart, lung, and brain of SHR after single intracardiac delivery of LSN-HHO-1 by the advent of increased expression of HO-1 in the kidney and aorta of LSN-HHO-1–treated SHR detected by immunohistochemistry (Figure 2). Expression of HHO-1 in rat tissues was associated with increased HO activity (eg, renal HO activity was 0.78±0.2 and 0.44±0.1 nmol bilirubin per milligram protein per 30 minutes in rats treated with LSN-HHO-1 and LXSN, respectively). Similarly, we observed an increase in HO activity in both liver and lung microsomes from SHR treated with LSN-HHO-1 compared with SHR treated with LXSN viral particles; liver HO activity was 0.69±0.2 and 0.48±0.1, and lung HO activity was 0.86±0.08 and 0.68±0.1 nmol bilirubin per milligram protein per 30 minutes in rats treated with LSN-HHO-1 and LXSN, respectively. The levels of HO activity in LXSN-treated SHR were comparable to those of vehicle-treated SHR.

BP increased as a function of time in all treatment groups; however, up to 20 weeks of age, the BP of LSN-HHO-1–treated SHR was significantly lower than that of vehicle-treated or LXSN-treated SHR (Figure 3). The fact that hypertension is attenuated in SHR expressing the HHO-1 gene implies that a mechanism dependent on the function of this human gene lowers BP in SHR. Thus, the attenuating influence of LSN-HHO-1 treatment on the development of hypertension in SHR is most likely the functional manifestation of increased HO activity consequent to HHO-1 expression. This is in agreement with reports that other interventions that increase HO activity in SHR as a result of inducing HO-1 expression also attenuate the development of hypertension.12

The association of increased HO activity and attenuation of hypertension in SHR expressing the HHO-1 gene suggests that, in these animals, the heme-HO system either interferes with the expression of a prohypertensive mechanism(s) or promotes the expression of an antihypertensive mechanism(s). The development of hypertension in SHR has been
linked to increased expression of a prohypertensive mechanism mediated by a metabolite of arachidonic acid derived from the cytochrome P450 system, presumably, 20-hydroxyeicosatetraenoic acid (20-HETE).22,23 HO has been implicated as a major regulator of cytochrome P450 hemoproteins, including those responsible for the formation of 20-HETE, by limiting the amounts of heme and/or by producing CO, which strongly binds to the heme moiety of cytochrome P450, causing enzyme inhibition.

In this study 24-hour urinary excretion of 20-HETE in 7-week-old SHR treated with LSN-HHO-1 was lower (P<0.01) than that of vehicle- or LXSN-treated SHR (1.29±0.06, 2.34±0.09, and 2.2±0.06 ng 20-HETE per milliliter in rats treated with LSN-HHO-1, LXSN, and vehicle, respectively; n=6). Hence, the renal excretion of 20-HETE, a vasoconstrictor eicosanoid, is reciprocally related to the expression of HO-1. Reduction in 20-HETE production may favor, at least in part, the lowering of BP in SHR since 20-HETE promotes vasoconstriction at renal and extrarenal sites24 and consequently may be a mediator of prohypertensive mechanisms in SHR, an experimental model in which 20-HETE production was reported to increase.22,23

Endogenous CO was proposed to inhibit myogenic vascular tone,11 which may explain, in some way, the lower BP of SHR treated with LSN-HHO-1. We studied pressure-diameter relationships in isolated gracilis muscle arterioles of 12-week-old SHR treated with LXSN or LSN-HHO-1 viral particles. Stepwise elevation of intraluminal pressure over the range of 40 to 100 mm Hg elicited pressure-dependent reductions in arteriolar diameter expressed as a percentage of the passive diameter in the absence of calcium. The pressure-induced constrictor response at both 80 and 100 mm Hg was less intense (P<0.01) in arterioles of SHR treated with LSN-HHO-1 than in arterioles of SHR treated with LXSN viral particles (Figure 4). Importantly, after treatment of the vessels with chromium mesoporphyrin (15 μmol/L), an inhibitor of HO, the intensity of the pressure-induced reduction in arteriolar diameter significantly increased (Figure 4). Previous studies have documented that CO of vascular origin reduces myogenic tone along with vascular reactivity to constrictor agonists.10 Consequently, increased production of CO in tissues of SHR expressing the HHO-1 gene is expected to promote vasodilation and thus foster the activity of antihypertensive mechanisms.

An additional antihypertensive process possibly linked to the expression of HHO-1 in SHR involves enhanced degradation of heme to biliverdin, which, along with bilirubin, is endowed with antioxidant activity. Inasmuch as recent studies suggest participation of reactive oxygen species in the pathogenesis of hypertension,25 increased biliverdin and bilirubin formation in SHR expressing HHO-1 may minimize the
hypertension by interfering with prohypertensive mechanisms linked to oxidative stress.

Beginning at 4 weeks of age, the body weight of SHR treated with LSN-HHO-1 viral particles surpassed that of SHR treated with vehicle alone or with LXSN viral control (Figure 5A). The nose-to-tail length and fibula length of SHR treated with LSN-HHO-1 also exceeded the corresponding values in SHR treated with vehicle or LXSN (Figure 5B); however, food intake was similar in all treatment groups (Figure 5A). SHR expressing the HHO-1 gene grew faster than SHR lacking the HHO-1 gene, particularly during the first 12 weeks. Importantly, the increase in somatic growth associated with HHO-1 expression in SHR was both proportionate and not associated with an increase in food intake. The latter observation, striking and most unexpected, implies that SHR expressing the HHO-1 gene are, in metabolic terms, more efficient than their counterparts lacking the HHO-1 gene and thus can develop somatically at a faster pace without consuming greater amounts of food.

Recent reports indicate that both humans and mice lacking the HO-1 gene display severe growth retardation. HO-1 gene expression has been shown to play a role in cell proliferation and cell death; indeed, previous studies demonstrated that elevation of HO-1 activity by gene transfer to rabbit coronary microvessel endothelial cells enhances cell proliferation and increases angiogenesis. In contrast, Lee et al. demonstrated that overexpression of HO-1 in pulmonary epithelial human cell line results in cell growth arrest, highlighting the cell-specific effects of HO-1 on cellular proliferation. A priori, a consequence of HO activity may have a direct impact on somatic growth by influencing the production and/or cellular actions of hormones and factors that stimulate or inhibit growth. Cherithandum et al. have found a significant correlation between hepatic levels of HO-1 and growth hormone in transgenic mice. Others showed that, in the rat, hormones such as thyroid hormone and insulin increase hepatic HO. Moreover, consensus binding sites for nuclear factor-kB, activator protein-1, activator protein-2, and interleukin-6 responsive elements, as well as other transcription factors, have been reported in the promoter region of the HO-1 gene.

Whether these transcription factors activate certain elements involved in promoting SHR growth remains to be investigated. Our study offers no information on the mechanism(s) responsible for the observed growth-promoting effect of HO-1 expression in SHR. This does not detract from the importance of our findings, which for the first time link the heme-HO system to the regulation of somatic growth in SHR.

In summary, this study demonstrates that delivery of the HHO-1 gene to SHR by means of a recombinant retrovirus vector attenuates the development of hypertension and accelerates somatic growth. These findings support the notion that one or more consequences of HO activity subserve vasodepressor and body growth-promoting functions. The study also highlights the usefulness of gene transfer approaches to the investigation of the functional tasks of the heme-HO system.

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