The Expression of Heme Oxygenase–1 and Inducible Nitric Oxide Synthase in Aorta During the Development of Hypertension in Spontaneously Hypertensive Rats

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Background: The aim of this study was to observe the time-course changes of heme oxygenase–1 (HO-1) and inducible nitric oxide synthase (iNOS) induction in aorta during the development of hypertension, as well as the relationship of HO-1/carbon monoxide (CO) system and iNOS/nitric oxide (NO) system in spontaneously hypertensive rats (SHR).

Methods: The systolic blood pressure (SBP) was determined in conscious rats by the tail-cuff method. The tissue HO-1 and iNOS mRNA and protein levels were estimated with reverse transcription polymerase chain reaction and Western blot method.

Results: The expression of HO-1 and iNOS in aorta increased with the SBP elevation during the development of SHR and was attenuated when the hypertension was lowered with the vasodilator hydralazine. At 8 weeks, only HO-1 was induced, whereas at 12 and 16 weeks, both HO-1 and iNOS were observed. The level of plasma nitrite/nitrate was associated with the change in iNOS expression in SHR. In addition, the SBP of 8-week-old SHR was significantly increased after pretreatment with zinc protoporphyrin IX for 7 consecutive days. Chronic blockade of iNOS activity by aminoguanidine resulted in significant up-regulation of HO-1, but the pressor effect was blunt.

Conclusions: These results suggest that the up-regulation of HO-1 and iNOS in aorta is a compensatory mechanism for the elevation of SBP during the development of hypertension in SHR. The expression of HO-1 is earlier than that of iNOS. Our data suggest that the HO-1/CO system takes over and acts as a major modulator for the regulation of SBP when the iNOS/NO system is suppressed. Am J Hypertens 2004;17:1127–1134 © 2004 American Journal of Hypertension, Ltd.

Key Words: Blood pressure, heme oxygenase, nitric oxide synthase, spontaneously hypertensive rats.

Nitric oxide (NO), generated from L-arginine by NO synthase (NOS), is reported to play many physiologic roles. Three NOS isoforms have been described—two that are constitutive (endothelial [eNOS] and neuronal [nNOS]) and one that is inducible (iNOS). These constitutive isoforms generate NO under basal conditions and in response to several physiologic stimuli, whereas iNOS is expressed by exposure to bacterial endotoxin (lipopolysaccharide) and some proinflammatory cytokines. Similar to NO, carbon monoxide (CO) increases cyclic GMP (cGMP), acts as a neurotransmitter in the brain, decreases vascular tone, inhibits platelet aggregation, and prevents production of proinflammatory cytokines and endothelial cell apoptosis. The major cellular source of CO is heme oxygenase (HO), a ubiquitously expressed protein that catalyzes the oxidative degradation of heme to biliverdine, CO, and iron. Among the three known HO isoforms (HO-1, HO-2, and HO-3), HO-1 is highly inducible by a vast array of stimuli, including oxidative stress, heat shock, ultraviolet radiation, ischemia-reperfusion, heavy metals, lipopolysaccharide, cytokines, and NO and its substrate, heme. The substance HO-2 is constitutively expressed and is present in high levels in the brain and testes. Although HO-3 closely resembles HO-2, it is characterized by much lower catalytic activity.

An impairment of endothelium-dependent relaxations has been observed in different vessels from spontaneously hypertensive rats.
hypertensive rats (SHR). This suggests that endothelial function might be impaired in hypertension. However, numerous studies have demonstrated that NO synthesis can be increased in SHR, probably as a counter-regulatory mechanism activated to compensate for the increase in blood pressure (BP). Therefore, the precise role of NO in hypertension is not clear, although a role of HO-1 in hypertension has been implied. Moreover, some investigators have reported up-regulation of HO-1 expression in hypertension and have suggested that the HO-1 system contributes to BP regulation in SHR. To date, no systematic study has been carried out to correlate the actual expression levels of HO-1 and iNOS and their relationship during the development process of hypertension in SHR. Thus, the aim of this study was to examine the changes over time in HO-1 and iNOS induction with increasing elevation of BP and to observe the relationship of these two systems in SHR.

Methods

Animals

Male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY), whose stock originated from the Charles River Breeding Laboratories (Tokyo, Japan), were purchased from the National Laboratory Animal Breeding and Research Center of the National Science Council, Taiwan. The animals were housed individually in clear plastic cages and kept in an environmentally controlled room maintained at room temperature of 23 ± 1°C, relative humidity 55 ± 5%, and on a light–dark cycle of 12 h light, 12 h dark. Hydralazine (20 mg/kg/day orally) was administered from age 8 weeks to 12 and 16 weeks. Aminoguanidine (30 mg/kg/day orally) was given from age 5 weeks to 8 weeks and 16 weeks. The drugs (hydralazine and aminoguanidine) were dissolved in drinking water, and the concentration was adjusted for daily water intake and body weight to obtain an average daily dose. Zinc protoporphyrin-IX (ZnPP, HO inhibitor, Aldrich Chemical Co., Milwaukee, WI) was dissolved in 50 mmol/L Na₂CO₃ solution immediately before use. The ZnPP was prepared in drinking water, and the concentration was adjusted for daily water intake and body weight to obtain an average daily dose. Zinc protoporphyrin-IX (ZnPP, HO inhibitor, Aldrich Chemical Co., Milwaukee, WI) was dissolved in 50 mmol/L Na₂CO₃ solution immediately before use.

Measurement of BP

Systolic BP (SBP) was determined in conscious rats by the tail-cuff method using an automatic BP monitoring system (UR-5000, UETA, Tokyo, Japan).

Tissue Preparation

The animals were killed with a lethal injection of sodium pentobarbital (50 mg/kg body weight) at 4, 8, 12, and 16 weeks. The thoracic aorta was flushed out with ice-cold phosphate-buffered saline (PBS), pH 7.4, immediately snap-frozen in liquid nitrogen, and stored at −70°C until processed. The composition of the PBS was as follows (in mmol/L): NaCl 140, KCl 3, Na₂HPO₄ 10, and KH₂PO₄ 2.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction Analysis

Thoracic aortas were ground in a mortar under liquid nitrogen, and total RNA was extracted using the Trizol reagent (GIBCO BRL, Life Technologies). Briefly, 200 μL of chloroform was added per 1 mL of Trizol reagent, and the samples were then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was taken and 500 μL of isopropanol added. The samples were left at −20°C overnight and then centrifuged for 10 min at 10,000 g at 4°C to obtain RNA pellets. The total RNA (2 μg) extracted from rat aorta was reverse-transcribed using the SuperScript II RT First Strand Synthesis kit (Invitrogen, Life Technologies), according to the manufacturer’s directions. A 2.5-μL quantity of product was amplified by polymerase chain reaction using Taq DNA polymerase (Invitrogen, Life Technologies) and a primer pair. The reaction was performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Boston, MA). The primers and the size of the HO-1 product were as follows: 5′ CACCCATATACCGCTACCT 3′ (forward) and 5′TCTGT-CACCTGTGCTTTGAC 3′ (reverse), 209 bp. For iNOS, the primers were 5′-GATCATAAACCTGAAGCCCG-3′ (forward) and 5′-GCCCCTTTTTGCTCCTCATAGG-3′ (reverse), and the size of the expected product was 578 bp. For GAPDH, the primers were 5′-CGGAGTCAAGGATTG-TCGTAT-3′ (forward) and 5′-AGCCTTCTCCATGGTG-GTGAAGAC-3′ (reverse), and the size of the expected product was 306 bp. The PCR conditions were as follows: denaturation at 94°C for 5 min, amplification for 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min (iNOS) or of 94°C for 1 min, 55°C for 30 sec, and 72°C for 1 min (HO-1), followed by a final extension at 72°C for 10 min. A Gene Ruler 100-bp DNA Ladder Plus (Invitrogen) was used to determine the size of the PCR products. The products were run on a 2% agarose gel, which was then stained with ethidium bromide (Sigma Chemical, St. Louis, MO) and viewed under ultraviolet light on a transilluminator. Densitometry of the bands was performed using a Bio-Rad Gel Doc 2000 (Bio-Rad), and the results were expressed as the optical density of the band of interest divided by that for the GAPDH band.

Protein Purification and Western Blot Analysis

The thoracic aortas were ground in a mortar containing liquid nitrogen. The powdered tissue was suspended in 1 mL of lysis buffer (50 mmol/L HEPES, 5 mmol/L EDTA, and 50 mmol/L NaCl, pH 7.5) containing protease inhibitors (10 μg/mL of aprotinin, 1 mmol/L PMSF, and 10 μg/mL of leupeptin) and agitated at 4°C for 1 h. After centrifugation at 10,000 g at 4°C for 30 min, the protein concentration in the supernatant was determined using BCA protein assay kit (Pierce, Rockford, IL).
Samples containing equal amounts of protein (40 μg) were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels and subsequently blotted onto nitrocellulose membrane (Millipore). Membranes were blocked with Tris-buffered saline buffer (TBS), pH 7.4, containing 0.1% Tween-20 and 5% skimmed milk, then incubated overnight at 4°C with mouse monoclonal anti–HO-1 (1:1000 dilution, StressGen Biotechnologies Corp.) or anti-iNOS antibody (1:1000 dilution, Transduction Laboratory) in TBS containing 0.1% Tween-20. The membranes were subsequently incubated with horseradish peroxidase–conjugated anti-mouse IgG antibody (1:1000 dilution, Cell Signaling). The blots were detected with the enhanced chemiluminescence method (Pierce, Rockford, IL), and the membrane exposed to x-ray film (Kodak, Rochester, NY) for 5 min. The density of the respective bands was quantified by densitometric scanning of the blots using Image-Pro Plus software (Media Cybemetrics, Inc.).

Measurement of Plasma Nitrite/Nitrate Concentration

After the rats were anaesthetized, 1 mL blood was withdrawn from the abdominal artery and immediately centrifuged (3000 g for 10 min). Plasma was stored at −70°C until use. A sample of thawed plasma was deproteinized with 2 volumes of 4°C 99% ethanol and centrifuged (3000 g for 10 min). These plasma samples (100 μL) were injected into a collection chamber containing 5% VCl₃. This strong reducing environment converts both nitrate and nitrite to NO. A constant stream of helium gas carried NO into a NO analyzer (Seivers 270B NOA; Seivers Instruments Inc., Boulder, CO), where the NO reacted with ozone, resulting in the emission of light. Light emission is proportional to the NO formed; standard amounts of nitrate were used for calibration.

Statistical Analysis

All data were expressed as mean ± SEM. Statistical significance was assessed using the Mann-Whitney U test. Differences were considered statistically significant at values of \( P < .05 \).

Results

Relationship Between Age and SBP

As shown in Figure 1A, from ages 4 through 16 weeks, SBP (mean ± SEM) increased progressively from 122.5...
± 5.2 to 205.6 ± 6.3 mm Hg in SHR and from 115.3 ± 6.7 to 152 ± 0.8 mm Hg in WKY rats. The SBP was significantly higher than that of age-matched WKY except at 4 weeks of age.

**HO-1 Expression in WKY Rats and SHR During Development**

As shown in Figures 1B and 1C, HO-1 mRNA was essentially undetectable at age 4 weeks in either strain but was seen in both strains at 8, 12, and 16 weeks. Levels of HO-1 mRNA in SHR were significantly higher than in WKY rats at ages 8, 12, and 16 weeks. Changes in HO-1 protein pattern were similar to those of HO-1 mRNA (Fig. 1D and 1E).

**Expression of iNOS in SHR and WKY Rats During Development of Hypertension**

The expression of iNOS in SHR and WKY rats is shown in Figures 2A and 2B. We found that iNOS mRNA was not detectable in WKY rats of any age. However, in SHR, iNOS mRNA was first seen clearly at age 12 weeks and its levels were maximal at 16 weeks. In addition, changes in iNOS protein expression were similar to those of iNOS mRNA (Fig. 2C and 2D).

**Effects of Hydralazine on HO-1 and iNOS Expression**

To examine whether HO-1 and iNOS up-regulation were dependent on the elevation of BP, SHR were treated daily from age 8 weeks with hydralazine to ages 12 and 16 weeks. As shown in Figure 3A, in hydralazine-treated SHR, BP significantly decreased and reached approximately that in age-matched untreated WKY rats. In addition, the expression of HO-1 and iNOS mRNA (Figs. 3B and 3C) and protein (Fig. 3D and 3E) were also significantly reduced compared with those in age-matched untreated SHR.

**Effects of ZnPP on HO-1 and iNOS Expression**

As shown in Figures 1B and 1C, only HO-1 expression was observed in SHR at 8 weeks of age. To confirm the role of HO-1 at this stage, SHR were treated starting from 7 weeks of age with ZnPP for 7 consecutive days. In ZnPP-treated SHR, both BP and expression of HO-1 were significantly increased, but there was no appearance of iNOS expression at 8 weeks of age (Fig. 4).

**Effects of Aminoguanidine on HO-1 and iNOS Expression**

To clarify the role of iNOS during the hypertensive development in SHR, the rats were treated daily with aminoguanidine from age 5 weeks to ages 8 and 16 weeks. We observed that BP was significantly increased at 8 weeks of age, but there was no significant difference at 16 weeks when values were compared with those of age-matched untreated SHR (Fig. 5A). Meanwhile, the expression of HO-1 and iNOS in treated SHR at 8 and 16 weeks were significantly greater than in age-matched untreated SHR (Fig. 5B to 5E).

**Plasma Nitrite/Nitrate Levels**

The basal plasma nitrite/nitrate level was significantly greater in SHR than in WKY in the rats at 12 and 16
weeks. After administration of hydralazine from 8 weeks to 12 and 16 weeks in SHR, the plasma nitrite/nitrate level was markedly decreased. Similarly, the plasma nitrite/nitrate level was markedly decreased in SHR at 16 weeks when treated with aminoguanidine from 5 weeks of age but was unchanged at 8 weeks. After treatment of ZnPP...
The present study is the first in vivo experiment to demonstrate the expression of HO-1 and iNOS in aorta during the development of SHR. Up-regulation of HO-1 and iNOS expression were attenuated when BP was lowered with the vasodilator hydralazine. Our results imply that BP per se may play an important role in the modulation of HO-1 and iNOS expression in aorta during the development of SHR. The results also suggest that the increase of HO-1 and iNOS expression may exert an important compensatory effect on the elevation of BP. In addition, only HO-1 was induced at the early stage around 8 weeks and progressively maintained during the whole experimental period, whereas, iNOS was also expressed simultaneously at a later stage (12 to 16 weeks). For the other, the BP was significantly increased after administration of ZnPP for 7 consecutive days from age 7 weeks. Chronic blockade of iNOS activity by aminoguanidine from age 5 weeks to 16 weeks resulted in significant up-regulation of HO-1 expression, but a pressor effect was not found. These results suggest that the HO-1/CO system may compensate for the dysfunction of iNOS/NO system and play a major role in the regulation of BP during the development of hypertension in SHR.

As shown in Figure 1, our study results demonstrated that the development of hypertension is age-dependent in SHR. The BP of SHR at 8 weeks of age was higher than that at 4 weeks; it then progressively increased until reaching a steady state at 16 weeks. We also found that HO-1 and iNOS expression in aorta correlated with the change in BP (Fig. 1 and 2). On the contrary, when SHR were treated with hydralazine from age 8 weeks to ages 12 and 16 weeks, not only was the development of hypertension prevented but expression of HO-1 and iNOS was suppressed as compared with levels in untreated SHR (Fig. 1 and 2). Moreover, the level of plasma nitrite/nitrate was associated with the change in iNOS expression in aorta (Table 1). These data confirm the report by Chou et al that quinapril, an angiotensin converting enzyme inhibitor, markedly decreased BP and expression of iNOS in aorta.9 These studies suggest that hypertension is an important factor in the modulation of HO-1 and iNOS expression in aorta and that up-regulation of HO-1 and iNOS is activated to compensate for the elevation of BP in SHR. However, despite the increase in HO-1 and iNOS expression, arterial BP still increased in SHR. One possible explanation is that the vascular superoxide anion level in SHR increased in an age-dependent manner in concordance with the development of elevated BP.18 The superoxide anion can scavenge NO to form peroxynitrite, which leads to a decrease in the availability of NO or to injury of vascular bed, or both. The other possible explanation is...
that elements of the NOS/NO-sGC/cGMP system or the HO/CO-sGC/cGMP system were impaired. Therefore, despite the observed compensatory increase in HO-1 and iNOS expression, BP still increased during the development of hypertension in SHR.

The mechanism responsible for up-regulation of HO-1 and iNOS expression during the progression of hypertension in SHR is still not clear. In animal studies, arterial superoxide anion levels were increased in several hypertensive models including SHR. Although the source of superoxide anion is uncertain, several observations suggest that the NADH/NADPH oxidase system accounts for the majority of superoxide anion generation in the vessel wall. It has been reported that hydralazine decreases both BP and NADPH activity in the aorta of rats with experimentally induced hypertension. In the present study, it has been shown that BP and both HO-1 and iNOS expression were significantly reduced by treatment with hydralazine. Altogether, these data suggest that the superoxide anion may play an initial and critical factor in the induction of HO-1 and iNOS expression. Thus, the possibility that an increase in superoxide anions in SHR may be secondary to an increase in BP cannot be excluded and requires further study.

Another important finding of this study is that during development in SHR, HO-1 was expressed before iNOS (8 weeks v 12 weeks, respectively). This was shown by the fact that BP and HO-1 expression were significantly increased after treatment with HO-1 inhibitor ZnPP for 7 consecutive days at age 7 weeks. Moreover, Da Silva et al. and Levere et al. also found that administration of HO-1 inducer (SnCl$_2$, heme arginate) resulted in a marked decrease in BP in young SHR. Furthermore, the level of plasma nitrite/nitrate in ZnPP-treated SHR was not different from that in untreated SHR (Table 1). These findings together suggest that expression of HO-1 at an early stage in SHR was induced by the change in BP. In addition, after chronic blockade of iNOS by aminoguanidine from age 5 weeks to 16 weeks, there was a significant decrease in the level of plasma nitrite/nitrate (Table 1) and an increase in HO-1 expression, but there was no change in BP (Fig. 5). These results are in agreement with a report by Motterlini et al, which demonstrated that the HO-1/CO system can compensate for the dysfunction of the iNOS/NO system.

However, one question that is raised by the current study is why iNOS expression was not found in untreated SHR at 8 weeks of age but an elevation of BP was found after treatment with aminoguanidine from age 5 weeks to 8 weeks. This contradictory result may be caused by the inhibition of eNOS activity after the treatment of aminoguanidine at this dosage (30 mg/kg/day orally) or by the non-specific effects of aminoguanidine, or both.

In conclusion, the present study demonstrated that the expression of HO-1 and iNOS in SHR remains to be clarified and requires further study.

### Table 1. Plasma nitrite/nitrate levels in Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) under basal or hydralazine-, aminoguanidine-, or ZnPP-Treated conditions

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Basal</th>
<th>Hydralazine-Treated SHR</th>
<th>Aminoguanidine-Treated SHR</th>
<th>ZnPP-Treated SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>12.28 ± 0.21</td>
<td>11.46 ± 0.66</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>11.97 ± 0.95</td>
<td>12.00 ± 0.98</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>10.86 ± 1.1</td>
<td>17.51 ± 0.50*</td>
<td>12.44 ± 0.72†</td>
<td>12.82 ± 0.27†</td>
</tr>
<tr>
<td>16</td>
<td>13.02 ± 0.63</td>
<td>21.23 ± 0.92*</td>
<td>9.59 ± 0.92†</td>
<td>—</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM for six rats.
* P < 0.05, SHR v WKY; † P < 0.05 treated SHR v untreated SHR.

References