Heme Oxygenase-1 Gene Expression Modulates Angiotensin II–Induced Increase in Blood Pressure

Liming Yang, Shuo Quan, Alberto Nasjletti, Michal Laniado-Schwartzman, Nader G. Abraham

Abstract—The heme-heme oxygenase (HO) system has been implicated in the regulation of vascular reactivity and blood pressure. This study examines the notion that overexpression of HO decreases pressor responsiveness to angiotensin II (Ang II). Five-day-old Sprague-Dawley rats received an intraleft ventricular injection of \( \approx 5 \times 10^7 \text{cfu/mL} \) of retroviruses containing human HO-1 sense (LSN-HHO-1), rat HO-1 antisense (LSN-RHO-1-AS), or control retrovirus (LXSN). Three months later, rats were instrumented with femoral arterial and venous catheters for mean arterial pressure (MAP) determination and Ang II administration, respectively. Rats injected with LSN-HHO-1, but not with LXSN, expressed human HO-1 mRNA and protein in several tissues. BP increased with administration of Ang II in rats expressing and not expressing human HO-1. However, the Ang II-induced pressor response (mm Hg) in LSN-HHO-1 rats (16±3, 27±3, and 38±3 at 0.5, 2, and 10 ng) was surpassed \( (P<0.05) \) in LXSN rats (23±1, 37±2, and 52±2 at 0.5, 2, and 10 ng). Importantly, treating LSN-HHO-1 rats with the HO inhibitor tin mesoporphyrin (SnMP) enhanced \( (P<0.05) \) the Ang II-induced pressor response to a level not different from that observed in LXSN rats. Rats injected with LSN-RHO-1-AS showed a decrease in renal HO-1 protein expression and HO activity relative to control LXSN rats. Administration of Ang II (0.1 to 2 ng) caused small (4 to 5 mm Hg) but significant increases in MAP in rats injected with LSN-RHO-1-AS \( (P<0.05) \) compared with rats injected with LXSN. These data demonstrate that overexpression of HO-1 brings about a reduction in pressor responsiveness to Ang II, which is most likely due to increased generation of an HO-1 product, presumably CO, with the ability to inhibit vascular reactivity to constrictor stimuli. (*Hypertension.* 2004;43:1221-1226.)

Key Words: blood pressure | retrovirus | genes | angiotensin II | oxidative stress

Heme oxygenases (HO) are a family of enzymes involved in the enzymatic conversion of heme to CO and biliverdin, which is further metabolized by biliverdin reductase to the antioxidant bilirubin.\(^1\) HO activity arises primarily from two distinct genes, the inducible HO-1 and the constitutively expressed HO-2.\(^2,3\) Among the metabolic products of heme, CO has many functions, including vascular relaxation,\(^3,4\) inhibition of platelet aggregation,\(^5\) and modulation of the NO-cGMP signaling system.\(^6\) Exogenous administration of CO relaxes isolated blood vessels\(^7,8\) and treatment with heme decreases blood pressure in hypertensive rats,\(^9\) whereas the administration of HO inhibitors increases arterial blood pressure in normotensive rats.\(^7\) These observations suggest that one or more HO-derived products play a role in the regulation of vascular tone and arterial blood pressure.

Previous studies have documented induction of vascular, cardiac, and renal HO-1 in response to angiotensin II (Ang II) in vitro and in vivo.\(^10-12\) HO-1 expression is markedly increased in aortic adventitial and endothelial cells from rats with Ang II-induced hypertension; treatment with losartan, a selective Ang II type-1 (AT1) receptor antagonist, blocks the upregulation of HO-1 expression.\(^13\) Ang II induces renal oxidant stress and HO activity caused by upregulation of HO-1 in renal proximal tubules.\(^10\) In a rat model of radiation-induced nephropathy, elevated glomerular HO-1 expression can be prevented by treatment with AT1 receptor antagonists, suggesting that Ang II may be a mediator of HO-1 induction.\(^14\) In contrast, Ishizaka and Griendling have shown that treatment of rat vascular smooth muscle cells with Ang II decreased HO-1 mRNA levels and this decrease was blocked by the AT1 receptor antagonist losartan.\(^15\) It is conceivable then that Ang II-mediated upregulation of HO-1 subserves mechanisms that counteract the action of Ang II.

Previous studies from our laboratory showed that induction of HO-1 by heavy metals or by its substrate, heme, increased HO activity and decreased blood pressure in spontaneously hypertensive rats (SHR).\(^9,16-18\) A recent study documented the feasibility of using a retroviral vector to deliver human HO-1 gene to newborn SHR, which continue to express it through adulthood.\(^19\) The development of hypertension in SHR expressing human HO-1 was significantly attenuated.\(^19\) To explore the effect of increasing and suppressing HO-1 expression on blood pressure regulation in normotensive rats, we injected Sprague Dawley (SD) newborns with a retrovirus

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From the Department of Pharmacology, New York Medical College, Valhalla.
Correspondence to Nader G. Abraham, PhD, professor of pharmacology, New York Medical College, Valhalla, NY 10595. E-mail nader_abraham@nymc.edu
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carrying either human HO-1 gene in its sense orientation (LSN-HHO-1) or rat HO-1 in its antisense orientation (LSN-RHO-AS) and examined changes in blood pressure following the administration of Ang II.

Materials and Methods
Extended method and details can be found in an online supplement available at http://www.hypertensionaha.org.

Animals
Newborn SD rats (Charles River, Wilmington, Mass) were injected at day 5 (20 μL) and day 12 (40 μL) with concentrated retroviruses (~5×10⁷ cfu/mL) into the left cardiac ventricle. Following injection, the rats were allowed to recover and returned to their cages with the appropriate mothers for continued weaning. The animals were weaned at 21 days and housed in their own cages. At different time points, rats were taken to measure the expression of human and rat HO-1 mRNA and protein in various tissues. When rats transduced with LSN-RHO-1-AS reached the age of 1 month, some were treated with heme (10 mg/kg IP, 24 hours), and the kidney and aorta were taken to measure HO-1 and HO-2 expression by Western blotting; kidney microsomal heme content and HO activity were also measured.

Experiments were conducted in 2 sets of 12 to 13 week old rats (350 to 375 g body weight). The first set included 6 rats transduced with retroviral vector LXSN and 6 rats transduced with LSN-HHO-1. The second set included 6 control LXSN- and 6 LSN-RHO-1-AS-transduced rats. Each rat was anesthetized with sodium pentobarbital (65 mg/kg IP), and arterial and venous catheters were implanted for blood pressure determination and Ang II or tin mesoporphyrin (SnMP) administration, respectively. The arterial catheter (PE-50), filled with heparinized normal saline, was introduced through the femoral artery and advanced into the lower abdominal aorta. The venous catheter was implanted into the femoral vein. Both catheters were tunneled subcutaneously to an exit point at the nape of the neck and sealed with a steel pin until use. All animals received ampicillin (30 mg/kg per 12 hours SC) for at least 3 days after surgery. A postsurgical recovery period of at least 4 days was allowed before the experiments began.

At the end of each experiment, rats were euthanized and renal expression of HHO-1 and HO-1 was measured. Rats that showed no detectable expression of HHO-1 were excluded from the study.

Statistical Analysis
The data are presented as mean±SE. Statistical significance (P<0.05) between the experimental groups was determined by unpaired Student t test or by ANOVA analysis.

Results
Expression of Human HO-1 in LSN-HHO-1-Transduced Rats
Rats were injected intravenicularly with concentrated LSN-HHO-1 and LXSN at days 5 and 12. At different time points (0.5 to 4 months) after injection, tissues from some rats were removed and analyzed for human HO-1 and neo' gene transcription by reverse-transcription polymerase chain reaction (RT-PCR). Figure 1A and 1B shows that expression of HHO-1 was evident only in rats injected with LSN-HHO-1. Expression of the transgenes human HO-1 and neo' genes lasted up to 5 months (duration of the experiments). Due to the difficulties in delivering the retroviral preparation into the left cardiac ventricle of a newborn, about 25% to 35% of the rats did not express HHO-1 or neo'. The rats not expressing HHO-1 were excluded from the study.

We also determined tissue distribution of human HO-1 mRNA expression in rats injected with LSN-HHO-1. RT-PCR analysis (Figure 1C) showed that human HO-1 mRNA was positive in all detected tissues (kidney, aorta, heart, femoral artery, lung, and liver). To assure the integrity of RNA from kidney tissues, amplification of β-actin mRNA was also performed using specific primers (Figure 1C). These data indicate that retrovirus-mediated gene transfer to newborn rats is a successful means for expressing the human HO-1 gene in adulthood.

Transduction of the human HO-1 gene resulted in expression of human HO-1 protein as evidenced by Western blot analysis with specific human HO-1 antibody. As seen in Figure 2, human HO-1 protein expression was detected in all 5 kidneys and 3 out of 5 aorta from rats injected with LSN-HHO-1. In contrast, no human HO-1 protein was expressed in kidneys or aortas from rats injected with the control retrovirus LXSN. More importantly, basal expression of the rat HO-1 was not affected by the expression of human HO-1 as indicated by comparable expression levels in LSN-HHO-1– and LXSN-transduced rats. Furthermore, no change in HO-2 protein expression was found in kidneys or aortas from either LSN-HHO-1– or LXSN-transduced rats (Figure 2A).

HO activity was measured in kidneys from rats injected with LSN-HHO-1 or LXSN. Figure 2B shows that renal HO activity, measured as the production rate of bilirubin from heme, in rats injected with LSN-HHO-1 was significantly higher than that in rats injected with LXSN (P<0.05). As seen in Figure 2C, cellular heme levels were reduced by about 40% in kidneys from rats injected with LSN-HHO-1 as compared with that in kidneys from rats injected with LXSN (P<0.05). These results indicate that transduction of the human HO-1 gene by retrovirus-mediated gene transfer is functional; it brought about expression of human HO-1 protein and an increase in total HO activity, which led to a reduction in cellular heme levels.
HO-1 Protein Expression and HO Activity in RHO-1-AS-Transduced Rats

To evaluate whether transduction of RHO-1 in the antisense orientation following injection of the concentrated retrovirus LSN-RHO-1-AS into newborn rats results in suppression of HO-1 expression and HO activity, kidneys of 1-month-old rats were removed and analyzed. As shown in Figure 3A, renal HO-1 protein levels in LSN-RHO-1-AS-transduced rats were lower than those in LXSN-transduced rats (control). Heme treatment (10 mg/kg IP, 24 hours) increased renal HO-1 protein in both groups of rats (LSN-RHO-1-AS and LXSN-transduced rats) (Figure 3A). The protein levels of HO-2 were not affected by transduction of LSN-RHO-1-AS nor were they altered following heme treatment in either group (Figure 3A). Moreover, basal and heme-induced HO activity in LSN-RHO-1-AS transduced rats was significantly lower than basal and heme-induced HO activity in control LXSN-transduced rats (Figure 3B). Thus, transduction of rat HO-1 in the antisense orientation via injection of retrovirus into newborn rats attenuated the levels of expression of HO-1 protein and decreased HO activity in adult rats.

Effect of Ang II on Mean Arterial Pressure in LSN-HHO-1 and LSN-RHO-1-AS-Transduced Rats

Mean arterial pressure (MAP) was measured directly via cannulated femoral arteries in unrestrained, awake rats 3 months after injection of the concentrated viruses into newborn rats. There was no significant difference in basal MAP between rats injected with LSN-HHO-1 (92±4 mm Hg) and with LXSN (96±5 mm Hg). Following basal MAP measurement, Ang II (0.1 to 10 ng) was injected as a bolus into the femoral vein (each injection being made only when the blood pressure had returned to its basal value) to construct a dose-response curve. MAP was increased in a dose-dependent manner. Figure 4 displays the data on MAP increases after the administration of Ang II at various doses. Ang II induced greater increase of MAP in rats injected with LXSN than in rats injected with LSN-HHO-1 (P<0.05). In some rats that were injected with LSN-HHO-1, the pressor responsiveness to Ang II was not different from that of rats injected with LXSN.
injected with the LXSN. Further analysis revealed that these rats showed no detectable levels of expression of HHO-1, and thus, were excluded from the study.

To examine whether HO activity contributes to the attenuated pressor responsiveness to Ang II, the HO inhibitor SnMP (10 mg/kg body weight), was injected into rats 30 minutes before the administration of Ang II. As seen in Figure 4, pretreatment of LSN-HHO-1-transduced rats with SnMP caused a significant increase in Ang II–induced MAP elevation relative to that in LSN-HO-1 transduced rats not receiving the HO inhibitor ($P<0.05$). These data suggest that the increased HO activity displayed by rats transduced with LSN-HHO-1 contributed to the attenuated pressor responsiveness to Ang II.

Further evidence for the role of HO in regulating pressor responsiveness to Ang II is provided by data from experiments with rats injected with the LSN-RHO-1-AS. LSN-RHO-1–transduced rats were instrumented with arterial and venous catheters, and MAP was measured before and after Ang II administration. There was no significant difference ($P>0.05$, $n=6$ in each group) in basal MAP between rats injected with LSN-RHO-1-AS (99.2±5.6 mm Hg) and rats injected with LXSN (97.1±4.6 mm Hg). However, at low doses, Ang II caused significantly greater ($P<0.05$) increases of MAP in rats injected with LSN-RHO-1-AS (18.8±2.1, 33.5±1.4, 40.5±2.3 mm Hg) compared with rats injected with LXSN (12.8±2.6, 30.3±0.5, 36.3±2.1 mm Hg at 0.1, 1, and 2 ng, respectively). However, injection of 5 and 10 ng of Ang II elicited comparable elevation of MAP in rats transduced with LSN-RHO-1-AS and LXSN. Augmentation in pressor responsiveness to Ang II in rats transduced with RHO-1-AS compared with that in control LXSN-transduced rats, although small in magnitude, provides additional support for a role of HO-1 in regulating the pressor effect of Ang II.

Discussion

In the present study, we report the successful long-term expression of human HO-1 in the sense orientation and rat HO-1 in the antisense orientation in vivo via retrovirus-mediated gene transfer. Previously, we showed that these retroviral vectors are effective in altering HO expression and activity in vitro. Thus, overexpression of human HO-1 gene in rat endothelial cells infected with LSN-HHO-1 is associated with increased CO production and decreased cellular heme content.$^{20,24}$ In contrast, endothelial cells infected with LSN-HO-1-AS demonstrated decreased HO-1 protein expression associated with a decrease in HO activity and an increase in heme content.$^{23-26}$ We also showed that the human HO-1 gene can be expressed in adult SHR by injecting the retroviral vector LSN-HHO-1 into newborns,$^{19}$ a method used by others, for example, to overexpress or suppress angiotensin receptors.$^{27}$ In a previous study, long-term expression of human HO-1 resulted in attenuation of hypertension in SHR.$^{19}$ The present study builds on the previous findings and further attempts to elucidate the mechanisms that may contribute to the effect of HO-1 expression on blood pressure.

The ability of a retrovirus to integrate into chromosomal DNA makes it an ideal vehicle for long-term gene expression. However, low viral titers limit their application. Thus, we have modified the method of retrovirus concentration and obtained a high retroviral titer (up to $5\times10^7$ cfu/mL). The results showed that injection of concentrated retroviruses into newborn rats led to high transduction efficiency and long-term expression of the transgene. Successful transduction of the human HO-1 gene to newborn rats resulted in the expression of human HO-1 protein in several tissues throughout the duration of the experiments without altering the expression of the rat HO-1 or HO-2. It also resulted in a significant increase in total HO activity throughout the duration of the experiments. On the other hand, transduction of rat HO-1 in the antisense orientation to newborn rats led to decreased HO-1 protein expression as well as a significant decrease in total HO activity. These results indicate that retrovirus-mediated gene transfer is an effective approach for manipulating HO-1 expression levels and HO activity in vivo.

Transfer of the human HO-1 gene to newborn rats did not change, in adulthood, the basal MAP as compared with control (LXSN) rats, but it did affect the Ang II–induced increase in blood pressure. In rats expressing human HO-1 gene, the increase in MAP, induced by Ang II, was significantly lower than that in the control (LXSN) rats. Pretreatment of rats overexpressing HO-1 with the HO inhibitor SnMP offset the inhibitory effect of HHO-1 expression on pressor responsiveness to Ang II. These data suggest that increased HO activity is responsible for the attenuated pressor responsiveness to Ang II in rats transduced with the human HO-1.

The regulatory role of HO-1 on the Ang II pressor effect was further substantiated in rats injected at 5 and 12 days of age with a retroviral vector carrying the antisense cDNA of the rat HO-1. Kidneys from adult rats expressing HO-1 antisense exhibited a reduction in renal HO-1 protein levels and a 40% inhibition of HO activity. In these rats, the pressor responsiveness to Ang II at doses of 0.1 to 2 ng was slightly but significantly magnified compared with the control LXSN injected rats. It is not surprising that the response to Ang II in rats injected with LSN-HO-1-AS was modestly affected given the fact that HO-1 is an inducible enzyme. In most tissues, including the kidney, basal levels of expression of HO-1 are low.$^{28}$ Nevertheless, small but significant potentiation of the pressor response was apparent, further suggesting that reduction in HO-1 expression and HO activity results in removal of tonic inhibitory influence on the actions of Ang II.

The mechanism(s) by which the expression of human HO-1 gene regulates the pressor responsiveness to Ang II remains to be clarified. CO, a metabolite of heme by HO, was reported to inhibit constrictor responsiveness to myogenic stimuli and attenuate the sensitivity of renal arterial vessels to vasoconstrictors.$^{4,29}$ On the other hand, biliverdin and bilirubin are antioxidants which may downregulate the activity of redox mechanisms involved in vascular actions of Ang II.$^{30}$ HO-1 overexpression may also minimize Ang II–induced oxidative stress as a result of lowering cellular heme. Finally, overexpression of HO-1
has been shown to affect the expression of numerous heme proteins, including cyclooxygenases (COX) and cytochrome P450 monoxygenases, possibly by altering the cellular-free heme level. Studies in our laboratory have demonstrated that increased HO-1 expression and HO activity downregulated COX-2 expression and activity in cultured endothelial cells in vitro and in kidney tissues in vivo. Decreased expression of COX-2 may lead to decreased production of endothelin-derived contracting factors, including endoperoxides and thromboxanes, which are known to increase constrictor mechanisms through activation of TP receptors in hypertension. Other studies showed that administration of the HO-1 inducer stannous chloride decreased the renal production of the vasoconstrictor cytochrome P450-derived eicosanoid 20-HETE as well as thromboxane synthase activity and urinary levels of the thromboxane B₂; such treatment was associated with a decrease in blood pressure in the SHR. It should be noted that changes in heme content and the levels of HO-1 protein with genetic interventions are modest and less than those obtained after bolus administration of heme or chemical inducer. Thus, genetic interventions result in a steady change of HO activity and heme content, which is regulated by an increase in the rate of heme synthesis. In a recent study, we demonstrated that a moderate increase in HO-1 expression obtained by gene transfer as opposed to chemical inducers does not affect COX-1 or nitric oxide synthase and significantly increases cGMP levels in microvessel endothelial cells. Such effects may also contribute to increasing vasodilator mechanisms that counteract Ang II actions. All of these direct and/or indirect changes, caused by overexpression of HO-1, might influence the responsiveness of MAP to Ang II. The fact that Ang II administration rapidly increases HO-1 expression and HO activity in several tissues further suggests that the heme-HO-1 system serves as a control mechanism to the pressor activity of Ang II.

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HEME OXYGENASE-1 GENE EXPRESSION MODULATES ANGIOTENSIN II-INDUCED INCREASE IN BLOOD PRESSURE

Liming Yang, Shuo Quan, Alberto Nasjletti, Michal Laniado-Schwartzman
and
Nader G. Abraham
Department of Pharmacology, New York Medical College
Valhalla, New York 10595

Corresponding author:
Nader G. Abraham, PhD
Professor of Pharmacology
New York Medical College
Valhalla, NY 10595
E-mail: nader_abraham@nymc.edu
Tel: (914) 594-4132
FAX: (914) 594-4119

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Methods

Cell culture. The amphotropic retroviral packaging cell line PA317 (ATCC) was used for generation of replication-deficient recombinant retroviruses. PA317 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin (P/S) (GIBCO-BRL, Grand Island, NY). NIH3T3 fibroblasts were cultured in DMEM with 10% FBS and P/S. All cells were incubated at 37°C in a 5% CO₂ humidified atmosphere, and maintained at subconfluency by passaging with trypsin-EDTA.

Development of recombinant retroviral vectors. The construction of the retroviral vector LSN-HO-1, in which the human HO-1 cDNA is under the control of the retroviral LTR promoter, has been described previously ¹. The control retroviral vector, LXSN, was obtained from Clontech (Palo Alto, CA). The retroviral vector LSN-RHO-1-AS was constructed as follows: The 1030 bp (-59 to +971) rat HO-1 cDNA was released from the plasmid pRHO-1 ² by the digestion with XhoI and HindIII, and was inserted at the XhoI and HindIII sites of pGEM7zf(+) (Promega). The resulting plasmid pGEM-RHO-1 was linearized with BamHI, end-blunted with dNTP and Klenow enzyme, and digested with XhoI. The released RHO-1 fragment (XhoI-blunt-end) was ligated at the XhoI-Hpal sites of the retroviral vector pLXSN (Clontech), and was designated as LSN-RHO-1-AS in which the opposite-transcription oriented RHO-1 cDNA fragment (RHO-1-AS) was controlled by long terminal repeat (LTR) promoter.
Production and concentration of retroviruses. PA317 retroviral packaging cells were transfected with retroviral vectors (LXSN, LSN-HHO-1 or LSN-RHO-1-AS) using Lipofectamine (GIBCO-BRL). After treatment with G418 (600 µg/ml, GIBCO-BRL), individual G418-resistant clones were selected. For each isolated clone, the viral titer was determined by infection of NIH/3T3 fibroblasts. The retrovirus producing cell clones PA317/LXSN, PA317/LSN-HHO-1 and PA317/LSN-RHO-1-AS with viral titers of $1.4 \times 10^6$ to $1.1 \times 10^7$ cfu/ml were further concentrated by repeated centrifugation at 9,000×g for 12-14 hr at 4°C. Viral titers in concentrated retroviruses were $3-11 \times 10^9$ cfu/ml.

RT-PCR analysis. Reverse transcription (RT) was carried out using the Advantage™ RT-for-PCR Kit (Clontech). Poly-d(T)n was used as reverse transcription primer. Specific primers for amplifying different DNA fragments were described as follows: human HO1-UP (upstream), 5'-CAGGCAGAGAATGCTGAGTTC-3', human HO1-DN (downstream), 5'-GATGTTGAGCAGGAACGCAGT-3'; Neo-UP, 5'-AAGATGGATTGCACGCAGG-3', Neo-DN, 5'-GCAAGGTGAGATGACAGGAG-3' (313bp); PCR was performed using an AmpliTaq PCR kit (Perkin-Elmer, Norwalk, CT). For each RT-PCR, a sample without reverse transcriptase was processed in parallel and served as a negative control. Cycling parameters for amplifying RT products were as follows: 95°C, 1'; 60°C, 1'; 72°C, 1-3', for 30 cycles, and then extended at 72°C for another 5'. After amplification, PCR products were electrophoresed on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light.
**Western Blotting.** Protein levels were visualized by immunoblotting with 1:2000 dilution of monoclonal mouse antiserum directed against human HO-1 or polyclonal rabbit antiserum directed against rat HO-1 or HO-2 (Stressgen Biotechnologies, Victoria, BC). Polyclonal rabbit antiserum directed against the rat HO-1 has weak cross reaction with HHO-1. However, mouse anti-human HO-1 monoclonal antibody has no cross reaction with rat HO-1. Microsomal protein was prepared and immunoblotting was performed as described previously ¹.

**Measurement of HO activity.** Microsomal HO activity was assayed by the method of Abraham et al ³ in which bilirubin, the product of HO degradation, was extracted with chloroform and its concentration determined spectrophotometrically (model DW-2C, Aminco, Urbana, IL) using the difference in absorbance at wavelength from λ 460 to λ 530 nm with an absorption coefficient of 40 mM⁻¹cm⁻¹ ⁴.

**Heme content.** Microsomal heme was determined as the pyridine hemochromogen by using the reduced minus oxidized difference in absorbance at λ460 and λ600 nm with an absorption coefficient of 32.4 mM⁻¹cm⁻¹ ⁵.

**Blood pressure recording in conscious unrestrained rats:** After 4-5 days of recovery, experiments were performed in conscious unrestrained rats. Blood pressure was continuously measured via the arterial catheter connected to a pressure transducer (model P23ID, Statham Division, Gould Inc., Iowa) and recorded on a polygraph (model 7, Grass Instruments, Quincy, MA). After connecting the catheters, blood pressure was
recorded at rest for 1 h. The experiment started when the blood pressure was stable. Following basal MAP measurement, Ang II (0.1, 0.5, 1, 2, 5 and 10 ng in 0.1 ml saline) was injected as a bolus into the femoral vein (each injection being made only when the blood pressure had returned to its basal value) to construct a dose-response curve. SnMP (10 mg/kg, i.v.) was administered 30 min before a bolus of Ang II was injected.

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