Heart Failure

Heme Oxygenase-1 Inhibits Angiotensin II–Induced Cardiac Hypertrophy In Vitro and In Vivo

Chien-Ming Hu, PhD; Yen-Hui Chen, DVM, VMDr; Ming-Tsai Chiang, MS; Lee-Young Chau, PhD

Background—Heme oxygenase-1 (HO-1) is a stress-response enzyme implicated in cardioprotection. To explore whether HO-1 has a role in cardiac remodeling response, the effect of its overexpression on angiotensin II (Ang II)-induced cardiac hypertrophy was examined.

Methods and Results—HO-1 was induced in cultured rat neonatal cardiomyocytes by treatment with cobalt protoporphyrin IX (CoPPIX) or a recombinant adenovirus carrying the human HO-1 gene. Ang II–induced myocyte hypertrophy assessed by increments in cell size, [3H]leucine uptake, and protein content was suppressed by HO-1 overexpression. Cotreatment of cells with tin protoporphyrin IX, a HO inhibitor, significantly reversed the suppressive effect of HO-1. Bilirubin, one of the byproducts of heme degradation by HO-1, mediated the suppressive effect through the inhibition of Ang II–induced production of reactive oxygen species, as detected by a 2′,7′-dichlorofluorescein probe. The antihypertrophic effect of HO-1 was also demonstrated in rats receiving chronic Ang II infusions. Cotreatment of animals with CoPPIX significantly attenuated Ang II–induced left ventricular hypertrophy and hyperdynamic contractions, whereas concomitant treatment with tin protoporphyrin IX abolished CoPPIX-mediated cardioprotection in vivo.

Conclusions—HO-1 attenuates Ang II–induced cardiac hypertrophy both in vitro and in vivo, and bilirubin mediates, at least in part, the antihypertrophic effect of HO-1 via inhibition of reactive oxygen species production after Ang II stimulation. (Circulation. 2004;110:309-316.)

Key Words: heme oxygenase ■ angiotensin ■ hypertrophy

Cardiac hypertrophy represents an adaptive remodeling response to increased cardiac wall stress caused by blood pressure or volume overload. Although it is initially a favorable mechanism compensating for cardiac functions, the pathological hypertrophy frequently progresses to a state of congestive heart failure. During cardiac remodeling, cardiomyocytes respond to various extracellular stimuli, such as mechanical stress, cytokines, growth factors, and vasoactive peptides, by undergoing hypertrophic changes that are characterized by the enlargement of cell size, increases in protein synthesis, and the expression of fetal genes. Angiotensin II (Ang II) has been implicated in the pathogenesis and progression of cardiac hypertrophy and heart failure. Studies on cultured neonatal cardiomyocytes have demonstrated that Ang II directly induces cell enlargement and fetal gene expression. Continuous infusion of Ang II in rats also results in an increase in left ventricular (LV) mass and alterations of cardiac functions independent of its pressure-raising effects. It has been shown that mechanical stretches induce the release of Ang II from cardiomyocytes, supporting its role in an autocrine mechanism mediating the load-induced cardiac hypertrophy.

Heme oxygenase-1 (HO-1) is a stress-inducible enzyme catalyzing the degradation of heme to liberate free iron, carbon monoxide (CO), and biliverdin in mammalian cells. Numerous studies have demonstrated the importance of HO-1 as a cytoprotective defense mechanism against oxidative insults through the antioxidant activity of biliverdin and its metabolite, bilirubin, as well as the anti-inflammatory actions of CO. Over the past few years, compelling evidence has supported a vital role of HO-1 in regulating cardiac function. It has been shown that HO-1 null mice developed right ventricular infarcts with organized mural thrombi on exposure to chronic hypoxia. Isolated hearts from HO-1 heterozygous gene knockout mice were also more susceptible to ischemic/reperfusion (I/R) injury. Conversely, overexpression of HO-1 in heart significantly reduced postischemic myocardial injury, and direct gene transfer of HO-1 into myocardium before I/R also conferred long-term myocardial protection in rats. In view of the important function of HO-1 in cardioprotection, we were interested in additionally exploring its role in the cardiac remodeling process. Recently, some studies have supported the implication of reactive oxygen species (ROS) in the hypertrophic effect of Ang II. It is therefore intriguing to test whether HO-1 affects myocyte hypertrophy induced by Ang II both in vitro and in vivo. In this report, we provide evidence indicating that the induction
of HO-1 exerts a potent antihypertrophic effect by inhibiting the production of ROS after Ang II stimulation.

**Methods**

**Cardiomyocyte Culture**

Neonatal cardiomyocytes were isolated and cultured according to the method described by Simpson and Savion, with some modifications. Briefly, the ventricles of neonatal Wistar rats (1 to 2 days old) were digested with pancreatin (1.25 mg/mL) at 37°C. Cardiomyocytes were isolated and cultured in DMEM containing 10% fetal bovine serum and 0.1 mmol/L bromodeoxyuridine. After 3 days, cells were incubated in serum-free medium containing transferrin (5 μg/mL), insulin (5 μg/mL), and 0.1 mmol/L bromodeoxyuridine for 24 hours before treatment with indicated agents.

**Preparation of Recombinant Adenovirus**

A recombinant adenovirus containing human HO-1 (Adv-HO-1) was prepared as described previously.

**Measurement of Cardiomyocyte Surface Area**

Cells were viewed using a video camera (Nikon) attached to a microscope and projected onto a monitor. The surface area was determined with image analysis software (MetaMorph Imaging System, Meta Imaging Series 4.5) and calculated as the mean of 100 to 120 cells from randomly selected fields.

**[3H]Leucine Incorporation**

Cardiomyocytes were cultured in 6-well plates and incubated with [3H]leucine (1 μCi/mL) in the presence of indicated agents for 48 hours. After 2 washes with ice-cold PBS, cells were then treated with 5% trichloroacetic acid at 4°C for 1 hour. Protein precipitates were washed twice with ice-cold water and dissolved in 1 mL of 100 mmol/L NaOH, and radioactivities were determined with a liquid scintillation counter.

**Determination of ROS Production**

Cardiomyocytes were preloaded with 30 μmol/L 2',7'-dichlorofluorescein diacetate at 37°C for 75 minutes and treated with the indicated agents for an additional 2 hours in serum-free medium. After washing, cells were lysed with 10 mmol/L Tris-HCl, pH 7.4, containing 0.5% Tween-20, followed by centrifugation at 10 000g for 10 minutes. Fluorescence intensities of the supernatants were determined with a spectrophotometer (HITACHI, F-4010).

**Western Blot Analysis**

Western blot analysis was performed as described previously.

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**Figure 1.** CoPPIX inhibits Ang II–induced myocyte hypertrophic response. A, Myocytes were incubated with indicated concentrations of CoPPIX for 48 hours. B, Myocytes were incubated with 3 μmol/L CoPPIX for indicated times. C, Myocytes were treated with indicated concentrations of Ang II and CoPPIX for 48 hours. HO-1 expression was determined by Western blot analysis. HO-2 serves as an internal control. D through F, Effects on myocyte surface area, [3H]leucine incorporation, and cellular protein content were determined. Data are mean±SEM of 4 independent experiments performed in triplicate. *P<0.02 vs control cells; †P<0.05 vs Ang II–treated cells.
Confocal Immunofluorescence
Cardiomyocytes were rinsed with PBS, fixed with 1% paraformaldehyde for 30 minutes, and permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature. Cells were then incubated with a rabbit polyclonal antibody against desmin (dilution 1:100) for 30 minutes at 37°C. After washing, cells were treated with anti-rabbit FITC-conjugated IgG (dilution 1:100) for another 20 minutes and viewed with a confocal microscope (Bio-Rad MRC-1000).

Animal Experiments
Male Wistar rats (150 to 200 g) received infusions of either saline or Ang II (250 ng/kg body weight per min) through mini-osmotic pumps (Alzet 2002) implanted subcutaneously for 2 weeks. During this period, animals were or were not intraperitoneally administered cobalt protoporphyrin IX (CoPPIX) (1 mg/kg body weight), tin protoporphyrin IX (SnPPIX) (1 mg/kg body weight), or SnPPIX in combination with CoPPIX, as indicated, every 2 days. The concentrations of CoPPIX and SnPPIX used were determined by a pilot study showing insignificant effects on body weight and hemodynamic measures. At day 14, the systolic blood pressure (BP) was measured by tail-cuff method. Animals were then anesthetized with avertin (400 mg/kg body weight, IP), and heart function was assessed by echocardiography (ATL HDI 5000 SonoCT Ultrasound system with 15-MHz linear transducer). Animals were then euthanized, and tissues were collected, fixed in 4% paraformaldehyde, and paraffin embedded. All procedures were performed in accordance with institutional guidelines.

Bilirubin Determination
Bilirubin accumulation in the culture medium was determined as described. To measure tissue content, bilirubin was extracted from heart tissue and analyzed by high-performance liquid chromatography with an Inertsil ODS-3 column (5 μm; 150x4.6 mm) (GL Sciences Inc). The mobile phase (CH3CN/H2O/CF3COOH, 900/100/1) was pumped at a constant flow rate of 1 mL/min. The retention time for bilirubin appeared at 29 minutes, as detected by the absorbance at 450 nm.

Cardiomyocyte Cross-Sectional Area Determination
Heart sections were incubated with 50 μg/mL of fluorescein-conjugated wheat germ agglutinin (FITC-WGA) (Molecular Probes) in PBS containing 1 mmol/L CaCl2 for 1 hour. After washing, LV cardiomyocyte membrane staining was examined by fluorescence microscope, and the cross-sectional area was determined with the image analysis software described above.

Statistical Analysis
Data were expressed as mean±SEM. The statistical significance of difference was analyzed by ANOVA followed by the Tukey-Kramer test.
Results

HO-1 Overexpression Suppressed Ang II–Induced Hypertrophic Response in Cardiomyocytes

CoPPIX, a HO-1 inducer, was found to induce HO-1 protein expression in cultured cardiomyocytes in a dose-dependent manner (Figure 1A). The induction reached a maximal level at 18 hours and was sustained up to 48 hours (Figure 1B). Ang II alone did not induce HO-1 expression (Figure 1C) but dose-dependently induced myocyte hypertrophy, as demonstrated by increased cell size, \[^{3}H\]leucine incorporation, and cellular protein content (Figures 2D through 2F). CoPPIX (3 \mu mol/L) cotreatment significantly inhibited the hypertrophic effects of Ang II, suggesting the possible involvement of HO-1 in this process. To confirm the role of HO-1, cardiomyocytes were infected with empty Adv or Adv-HO-1. As shown in Figures 2A and 2B, HO-1 was overexpressed in

Figure 3. Bilirubin mediates the anti hypertrophic effect of HO-1.
A, Myocytes treated without (control) or with CoPPIX (3 \mu mol/L), Adv (10 MOI), and Adv-HO-1 (10 MOI) for 48 hours. Levels of bilirubin accumulation in the culture medium were determined. Data are mean ± SEM of 4 independent experiments performed in triplicate. *P<0.01 vs control cells. B, Myocytes treated without or with Ang II (0.1 \mu mol/L) in the absence (vehicle) or presence of CoPPIX (3 \mu mol/L), Adv-HO-1 (10 MOI), bilirubin (10 \mu mol/L), and \([Ru(CO)_{3}Cl_{2}]_2\) (50 \mu mol/L) were incubated with \[^{3}H\]leucine (1 \mu Ci/mL). C, Myocytes treated without or with Ang II in the absence or presence of bilirubin (B) (1 to 10 \mu mol/L) were incubated with \[^{3}H\]leucine. Incorporated \[^{3}H\]leucine levels were determined after 48 hours. Data are mean ± SEM of 4 independent experiments performed in triplicate. *P<0.01 vs control cells; †P<0.01 vs cells treated with Ang II alone.

Figure 4. Effect of bilirubin on Ang II–induced ROS production.
A, Myocytes preloaded with 2′,7′-dichlorofluorescein diacetate were treated without (control) or with Ang II (0.1 to 1 \mu mol/L) in the absence or presence of 10 \mu mol/L bilirubin (B) for 2 hours. H\(_2\)O\(_2\) (50 \mu mol/L) was used as a positive control. After 2 hours, cells were lysed, and fluorescence intensities were determined.
B, Myocytes were treated with 0.1 \mu mol/L Ang II in the absence or presence of bilirubin (1 to 10 \mu mol/L). Data are mean ± SEM of 3 independent experiments performed in duplicate. *P<0.01 vs control cells; †P<0.01 vs cells treated with Ang II alone.
myocytes infected with Adv-HO-1 but not with Adv. In addition, Ang II–induced increases of [3H]leucine incorporation were abolished in cells infected with Adv-HO-1 (Figure 2C). However, the suppressive effect of Adv-HO-1 was significantly reversed by treatment with the HO inhibitor, SnPPIX. The antihypertrophic effect of HO-1 transduction on Ang II–treated myocytes was also demonstrated by desmin immunostaining shown in Figure 2D.

Bilirubin Mediates the Antihypertrophic Effect of HO-1

Treatment of cardiomyocytes with either CoPPIX or Adv-HO-1 resulted in a significant accumulation of bilirubin in the culture medium (Figure 3A). To determine which heme degradation byproduct mediates the antihypertrophic effect of HO-1, cardiomyocytes were treated with Ang II in the presence of bilirubin or tricarbonyldichlororuthenium (II) dimmer, [Ru(CO)3Cl2], a CO releasing compound.19 It was found that bilirubin, but not [Ru(CO)3Cl2], inhibited Ang II–induced [3H]leucine incorporation to a similar extent as treatment with either CoPPIX or Adv-HO-1 (Figure 3B). The inhibitory effect of bilirubin was dose-dependent, as shown in Figure 3C. Experiments were then performed to test whether bilirubin affects Ang II–induced ROS production in cardiomyocytes. As shown in Figures 4A and 4B, Ang II significantly enhanced the generation of intracellular ROS in a dose-dependent manner and bilirubin dose-dependently reduced the increase of ROS induced by Ang II. Additional experiments demonstrated that neither CoPPIX nor bilirubin had an effect on the hypertrophic response induced by leukemia inhibitory factor (LIF), which stimulates cell growth independent of ROS production20 (Figure 5), indicating that the antihypertrophic effect of HO-1/bilirubin is primarily mediated through its antioxidant activity.

HO-1 Suppresses Ang II–Induced Cardiac Hypertrophy In Vivo

To examine the antihypertrophic effect of HO-1 in vivo, the effect of CoPPIX on cardiac hypertrophy induced by chronic Ang II infusion in rats was assessed. As shown in Figures 6A and 6B, animals receiving Ang II treatment for 2 weeks did not significantly affect HO-1 expression in heart. Animals subjected to CoPPIX administration every 2 days during the
same period, however, expressed higher levels of cardiac HO-1. In parallel with HO-1 expression levels, cardiac bilirubin levels detected in the CoPPIX-treated group were substantially higher than in the control group or in the Ang II–treated group (Figure 6C). The elevated bilirubin production induced by CoPPIX, however, was reversed by the cotreatment with SnPPIX. Subsequent physiological assessment of the animals revealed that the body weight gains after the 2-week treatment period were not significantly different between the various groups (Table). Nevertheless, systolic BP was substantially increased in animals treated with Ang II. Neither CoPPIX nor CoPPIX/SnPPIX cotreatment significantly affected Ang II–induced BP elevation. On the other hand, the LV mass/body weight and heart weight/body weight ratios were significantly greater in Ang II–treated animals compared with control rats (P<0.01). Likewise, morphological examination of LV myocytes using FITC-WGA staining revealed that the cross-sectional areas of the cardiomyocytes were significantly increased in Ang II–treated animals (Figure 7). Echocardiograms also clearly showed that Ang II infusions led to a 24% increase in percentage of fractional shortening (P<0.01) (Table), indicating a LV hyperdynamic contraction, which normally presents as an early adaptive response to cardiac hypertrophy. Nevertheless, CoPPIX cotreatment significantly reduced Ang II–induced LV hypertrophy and hyperdynamic response in animals, and this protective effect was again counteracted by cotreatment with SnPPIX.

### Physiological and Echocardiographic Measurements of Wistar Rats After Various Treatments

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<th>Control (n=6)</th>
<th>Ang II (n=8)</th>
<th>CoPPIX (n=6)</th>
<th>Ang II/CoPPIX (n=8)</th>
<th>SnPPIX (n=4)</th>
<th>SnPPIX/CoPPIX (n=4)</th>
<th>SnPPIX/CoPPIX/Ang II (n=7)</th>
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<tr>
<td>Body weight gain, g</td>
<td>98.0±6.3</td>
<td>99.6±6.2</td>
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<td>90.0±2.3</td>
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<td>Systolic BP, mm Hg</td>
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<td>166.5±8.1*</td>
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<td>178.9±7.1*</td>
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<td>108.0±2.8</td>
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<td>0.36±0.01*</td>
<td>0.28±0.01</td>
<td>0.30±0.01</td>
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<td>0.31±0.01</td>
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<tr>
<td>Heart weight/body weight, %</td>
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<td>0.39±0.01*</td>
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<td>0.30±0.01</td>
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<td>Fractional shortening, %</td>
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<td>47.25±1.05</td>
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<td>42.96±1.22</td>
<td>51.97±1.85*</td>
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Male Wistar rats received subcutaneous infusions of either saline or Ang II for 2 weeks. During the same period, some animals were coadministered CoPPIX, SnPPIX, or SnPPIX/CoPPIX every 2 days as indicated. On day 14, the systolic BP was measured by tail-cuff method, and LV mass and heart function were assessed by echocardiography. Animals were then killed, and the hearts were dissected out and weighted. No. of animals in each group is indicated in parentheses. Data are mean±SEM. Statistical analysis was performed with ANOVA followed by the Tukey-Kramer test.

*P<0.01 vs control group.

**Figure 7.** HO-1 suppresses the increments of cardiomyocyte cross-sectional area in Ang II–treated rats. LV cardiomyocyte cross-sectional areas in rats treated with indicated agents were examined by FITC-WGA staining. A, Representative staining images. Bar=100 μm. B, Quantitative analysis of the cardiomyocyte sizes in different treated groups. The number of animals in each group is indicated in parentheses. *P<0.01 vs control group; †P<0.01 vs Ang II–treated group; #P<0.01 vs CoPPIX/Ang II–treated group.
Discussion
Our data clearly show that Ang II–induced cardiomyocyte hypertrophy can be abrogated by HO-1 overexpression. Although a previous study reported that Ang II–induced hypertension increases HO-1 expression in the heart,21 we found that Ang II per se cannot induce HO-1 expression in cardiomyocytes in vitro or in vivo. This discrepancy, however, does not diminish the finding that HO-1 induction in the heart can modulate the remodeling process. Although the upregulation of HO/CO system has been shown to lower BP in some studies of hypertensive rats,22–25 we noted that the treatment of Wistar rats with CoPPIX did not significantly affect Ang II–induced hypertension in our experiments. The lack of a BP-lowering effect after HO-1 induction was also previously reported in studies on normotensive rats and SHR rats with established hypertension.23–26 The cause of the discrepancies is not yet fully understood. Nevertheless, it has been shown that NO-mediated vasorelaxation is significantly suppressed by overproduction of CO, which has a lower affinity for soluble guanylate cyclase and competes with NO for this enzyme in the vasculature.27 Whether this is a possible mechanism underlying the sustained hypertensive state in CoPPIX/Ang II–treated animals remains to be determined. Although we did not find that CoPPIX significantly affected Ang II–induced hypertension, it did markedly attenuate cardiac phenotypic changes induced by Ang II. These observations indicate that cardiac hypertrophy is not a secondary event resulting from hypertension in our present experimental setting. We envision that the direct local effect of HO-1 on cardiac tissue is responsible for its antihypertrophic function in vivo. In accordance with this, a previous study has also demonstrated that HO-1 induction attenuates cardiac hypertrophy in stroke-prone SHR rats via a pressure-independent mechanism.28

Circumstantial evidence has supported the roles of bilirubin and CO, the byproducts of heme degradation, in the cytoprotective functions of HO-1 in vitro and in vivo.3 The data show that bilirubin exhibits suppressive effect on Ang II–induced hypertrophic response through the reduction of ROS production, which is implicated in the growth response signaling in cardiomyocytes. It is obvious that the antihypertrophic effect of HO-1 is attributable, at least in part, to the antioxidant activity of bilirubin. This observation is consistent with earlier reports showing that antioxidants are effective to prevent cardiomyocyte hypertrophy induced by Ang II.12,14 The cardioprotective function of bilirubin has also been demonstrated in a recent study showing that HO-1–derived bilirubin ameliorates myocardic dysfunction and reduces infarct size after I/R insult.8 Moreover, studies on human patients have revealed that higher serum bilirubin is associated with a lower risk of coronary heart disease.29–31 Although CO is not involved in the anti hypertrophic response, the suppressive effect of CO on endothelial cell apoptosis, vascular thrombosis, and leukocyte infiltration, as previously demonstrated in a cardiac transplant model,32 is likely to contribute to HO-1–mediated prolonged protection in a diseased heart.

We conclude that our findings have added a new dimension to the understanding of the cardioprotective function of HO-1. Nevertheless, the possibility that the high levels of free iron, CO, and bilirubin liberated from heme degradation may cause tissue injury via distinct pathways in certain circumstances with HO-1 overexpression cannot be completely excluded.33 Whether these possible adverse effects will occur when the HO-1 expression levels in the heart reach a certain threshold in vivo remains to be clarified. In any event, our data support the contention that HO-1 protects cardiomyocytes from Ang II–induced hypertrophic response via suppressing ROS production. Because the generation of ROS is widely regarded as one of the signals involved in growth stimulation effects by various agonists and under stress conditions in cardiomyocytes,34 the present findings support the possibility of using HO-1 as a therapeutic gene target for the long-term protection against hypertrophic changes after cardiac injury.

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References
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