Heme oxygenase-1 is upregulated in the rat heart in response to chronic administration of angiotensin II

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Ishizaka, Nobukazu, Toru Aizawa, Ichiro Mori, Jun-Ichi Taguchi, Yoshio Yazaki, Ryozo Nagai, and Minoru Ohno. Heme oxygenase-1 is upregulated in the heart in response to chronic administration of angiotensin II. Am J Physiol Heart Circ Physiol 279: H672–H678, 2000.—Heme oxygenase (HO) is a heme-catabolizing enzyme that converts heme into biliverdin, iron, and carbon monoxide. HO-1, an inducible form of HO, is thought to act as an endogenous antioxidant defense mechanism. To determine whether chronic administration of angiotensin II affects HO-1 expression in the heart, expression and localization of HO-1 were investigated in the heart of rats receiving angiotensin II infusion (0.7 mg · kg−1 · day−1) via osmotic minipump for up to 7 days. Angiotensin II induced formation of granulation tissue, characterized by myofibroblast proliferation, fibrous deposition, and inflammatory cell migration. Angiotensin II also upregulated cardiac HO-1 expression. Immunohistochemistry revealed that HO-1 was intensively expressed in the granulation tissue. The selective AT1-receptor antagonist, losartan, completely, but hydralazine only partially, suppressed angiotensin II-induced granulation tissue formation and HO-1 upregulation. Chronic norepinephrine infusion (2.8 mg · kg−1 · day−1) did not induce granulation tissue formation or HO-1 upregulation. Our data suggest that angiotensin II upregulates cardiac HO-1 expression in the newly formed inflammatory lesion, which may represent an adaptive response to angiotensin II-induced cardiac damage. norepinephrine; blood pressure; immunohistochemistry; oxidative stress

Heme oxygenase-1-deficient mice were hypersensitive to cytotoxicity caused by hydrogen peroxide (23) and that transformed lymphoblastoid cells derived from a human case of HO-1 deficiency was extremely sensitive to hemin-induced cell injury (35) provide further evidence that HO-1 acts favorably against oxidant-induced cellular injury.

The produced carbon monoxide has been hypothesized to serve a physiological role in regulating vascular tone (4), which is mediated by a cGMP-signaling pathway and by calcium-activated potassium channels (33). The HO system is also present and regulated in the heart (12, 20, 22, 25). The cardiac HO system may have a role in preventing arteriosclerosis (7), improving cardiac xenograft survival (28), regulating blood pressure (20), and modulating nitric oxide-mediated myocardial preservation (17). The recent finding that hypoxia induced severe right ventricular dilatation in HO-1 null mice also indicated the cardioprotective role of HO-1 in the stressed condition (36).

Using hypertensive rat model with chronic ANG II infusion, Ishizaka et al. (10) reported that pressure overload upregulated aortic HO-1 protein expression and activity. Through its antioxidant and anti-inflammatory properties, increased aortic HO-1 may act favorably against the tissue damage elicited by ANG II and pressure overload. It has been shown that the activated renin-angiotensin system and/or elevation of blood pressure participate in the initial signaling, which may mobilize inflammatory cells in the heart (21). We hypothesized that continuous infusion of ANG II may upregulate cardiac HO-1 expression, which may have a role in modulating the extent of cardiac injury mediated by increased circulating ANG II and/or hypertension. Therefore, the purpose of this study was to investigate whether or not HO-1 is regulated in the heart of hypertensive rats receiving ANG II infusion. To delineate the specific effects of ANG II and the elevation of blood pressure, we also made hypertensive rat model with chronic norepinephrine (NE) infusion. Here we report that HO-1 is upregulated in the heart of

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ANG II-induced hypertensive rats. Immunohistochemistry revealed that marked HO-1 expression was seen in the myofibroblasts and migrated inflammatory cells in the heart of rats with ANG II infusion.

MATERIALS AND METHODS

Animal models. To prepare the rat hypertension model, an osmotic minipump (Alzet model 2001; Alza Pharmaceutical, Palo Alto, CA) was implanted into each male Sprague-Dawley rat as described previously (11). ANG II (Sigma, St. Louis, MO) was infused at the rate of 0.7 mg · kg⁻¹ · day⁻¹. ANG II was infused beginning 2 days before pump implantation and during ANG II infusion. To examine the effect of the subpressor dose of ANG II, 0.25 mg · kg⁻¹ · day⁻¹ ANG II was infused. To examine another model of hypertension, NE (Sigma) was infused at the rate of 2.8 mg · kg⁻¹ · day⁻¹, or the nonspecific vasodilator, hydralazine (15 mg · kg⁻¹ · day⁻¹; Sigma), was given in the drinking water, beginning 2 days before pump implantation and during ANG II infusion. To examine the effect of the subpressor dose of ANG II, 0.25 mg · kg⁻¹ · day⁻¹ ANG II was infused. To examine another model of hypertension, NE (Sigma) was infused at the rate of 2.8 mg · kg⁻¹ · day⁻¹ using the same minipump system through a catheter that was placed in the superior vena cava via the left external jugular vein (10).

RNA isolation and Northern blot analysis. Total RNA was isolated from the homogenized heart by the acid guanidinium thiocyanate-phenol chloroform method using Isogen (WAKO, Osaka, Japan). Equal amounts of total RNA (15–20 μg) were subjected to electrophoresis in a 1.0% agarose gel containing 6.5% formaldehyde. RNA was covalently bonded to the membranes with ultraviolet crosslinking (UV Stratalinker 1800; Stratagene Cloning Systems, La Jolla, CA). Rat HO-1 cDNA (a kind gift from Dr. S. Shibahara, Tohoku University School of Medicine, Japan) was labeled with [α-³²P]dCTP (NEN of Medicine, Japan) was labeled with [α-³²P]dCTP (NEN) and was used at a 1:2,000 dilution. The ECL Western blotting system (Amersham, Arlington Heights, IL) was used for detection. Antibodies against rat macrophage/monocyte (ED1; Chemicon International, Temecula, CA), rat HO-1 (StressGen), and human α-smooth muscle actin (α-SM actin, Sigma) at 1/200, 1/200, and 1/1,000 dilutions, respectively. The slides were then washed and incubated with biotinylated secondary antibodies. After treatment of the slides with the avidine-biotinylated HRP complex (Vector Laboratories, Burlingame CA), the antigens were visualized with the 3,3-diaminobenzidine tetrahydrochloride (Dako, Carpenteria, CA) system. Counterstaining was performed with methyl green (Dako).

For semiquantification of the granulation tissue (areas of fibrosis, myocardial necrosis, and myofibroblast proliferation), the Masson’s trichrome-stained heart sections were scanned using a photoimaging system (Canon, Tokyo, Japan). The ratio of the inflammatory areas (granulation/fibrosis) to the total myocardium area was calculated using the image analysis software (NIH). Scattered areas of the inflammatory area were not included in this calculation, and thus,
Methodology:

Assay of HO activity.

Hearts were homogenized in 250 mmol/l sucrose containing 50 mmol/l Tris · HCl (pH 7.5), and homogenates were centrifuged at 18,800 g at 4°C for 10 min. The supernatant was removed and recentrifuged at 100,000 g at 4°C for 60 min, and the precipitated microsomal fraction was suspended in 100 mmol/l potassium phosphate buffer (pH 7.4). Biliverdin reductase was crudely purified by the method of Tenhunen et al. (31). Heme oxygenase activity was assayed according to the method of Yoshida et al. (37).

Statistical analysis.

Data are expressed as means ± SE. ANOVA followed by a multiple comparison test was used for comparisons of initial data before being expressed as a percentage of the control using the statistical analysis software Statistica ver. 5.1J for Windows (StatSoft, Tulsa, OK). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of ANG II, NE, and inhibitors on systolic blood pressure.

Both ANG II and NE caused a significant raise in the blood pressure (Fig. 1A) and in the heart rates (Fig. 1B) by day 1, and the increase continued through day 7. Blood pressure and heart rate at day 1 were slightly higher in the NE-infused rats than in the ANG II-infused rats; however, the differences were not statistically significant. Both the nonspecific vasodilator, hydralazine, and the specific AT1-receptor inhibitor, losartan, effectively normalized ANG II-induced hypertension at day 7 (Fig. 1C). Treatment with either hydralazine alone or losartan alone had no significant effect on blood pressure or heart rate (data not shown).

Effect of continuous ANG II infusion on heme oxygenase-1 (HO-1) mRNA levels in the heart.

A: representative Northern blot. B: data from 4 to 6 animals are summarized in the line graph. *$P < 0.05$ vs. sham-operated control.

Effect of continuous ANG II infusion on HO-1 protein levels in the heart.

A and C: effect of Hyd. B and D: effect of Los. A and B: representative immunoblots. C and D: data from 4 to 7 animals are summarized in the bar graphs. *

Fig. 4. Effects of Los and Hyd on ANG II-induced HO-1 protein upregulation. In addition to continuous ANG II (0.7 mg · kg$^{-1}$ · day$^{-1}$) infusion, some rats received either Los or Hyd in drinking water beginning 2 days before ANG II infusion. After ANG II infusion for 7 consecutive days, rats were euthanized and protein samples were prepared (see MATERIALS AND METHODS). A and C: effect of Hyd. B and D: effect of Los. A and B: representative immunoblots. C and D: data from 4 to 7 animals are summarized in the bar graphs. *

* $P < 0.01$ vs. sham-operated control; † $P < 0.05$ and ‡ $P < 0.01$ vs. ANG II-infused rat.
Effect of ANG II infusion on HO-1 expression in the heart. HO-1 mRNA was significantly increased as early as 3 days after ANG II infusion and was increased further for up to 7 days (Fig. 2). This increase in HO-1 mRNA expression was accompanied by an increase in HO-1 protein (Fig. 3, A and C), whereas HO-2, the constitutive form of HO, was unchanged at 7 days after ANG II infusion (Fig. 3B). HO activity in the microsomal fractions was elevated in the heart of rats that received ANG II infusion for 7 days compared with the sham-operated control (control vs. ANG II: 3.2 ± 0.6 vs. 5.2 ± 0.4 bilirubin generation · mg protein⁻¹ · h⁻¹, n = 5, respectively, P < 0.01). Effect of antihypertensive drugs, subpressor dose of ANG II, and NE. Losartan completely blocked the ANG II-induced HO-1 protein upregulation (Fig. 4, B and D), which indicated that ANG II-induced HO-1 upregulation was an AT₁ receptor-specific event. In contrast, hydralazine only partially suppressed the ANG II-induced HO-1 protein upregulation (Fig. 4, A and C). These data suggested that ANG II could upregulate cardiac HO-1 via its pressor-independent effect. The finding that subpressor dose of ANG II infusion resulted in a small but significant upregulation of HO-1 protein (Fig. 5, A and C) further supported this notion. In contrast, NE did not upregulate HO-1 protein expression (Fig. 5, B and D), which suggested that increase of circulating ANG II played a pivotal role in HO-1 upregulation.

Histology and immunohistochemistry of the heart. Masson’s trichrome staining revealed that ANG II infusion induced a right ventricle-dominant granulation tissue formation, which was characterized by the proliferation of myofibroblasts, migration of inflammatory cells, and fibrotic scar (Fig. 6A). Losartan completely inhibited ANG II-induced inflammatory changes; however, hydralazine only partially inhibited these changes. Subpressor dose of ANG II induced similar inflammatory changes, though to a lesser extent. In contrast, irrespective of marked elevation of the blood pressure,
these inflammatory changes were not seen in the heart of NE-infused rats (Fig. 6B). Immunohistochemistry showed that spindle-shaped, fibroblast-like cells in the granulation tissue were α-SM actin positive, and therefore, were identified to be myofibroblasts (Fig. 7B). These spindle-shaped myofibroblasts were strongly positive for HO-1 staining, and some cardiomyocytes surrounding the granulation tissue were also positive for HO-1 staining (arrowheads in Fig. 7C). HO-1 was also induced in scattered monocytes/macrophages in these areas (Fig. 7D).

**DISCUSSION**

This study demonstrated that chronic infusion of ANG II, but not NE, upregulated HO-1 expression in the heart. The finding that hydralazine only partially blocked ANG II-induced HO-1 upregulation suggested that ANG II increased cardiac HO-1 expression in a pressor-independent manner, and that this response was augmented by pressor-effect of ANG II. Histological analysis showed the formation of granulation tissue, presenting as myofibroblast proliferation, fibrous deposit, and migration of monocytes/macrophages, in the heart of rats with ANG II infusion. Intense expression of HO-1 in and around the granulation tissue may explain, at least partially, why HO-1 upregulation and granulation tissue formation was seen in a parallel manner.

HO system has been known to act cytoprotectively against various types of oxidative stress (32). HO system is thought to exert an antioxidant property through bilirubin generation and/or ferritin induction (32) and exert an antihypertensive property through carbon monoxide generation (20). Recent papers have shown that cardiac HO-1 may have roles in the prevention of arteriosclerosis (7) and improvement of cardiac xenograft survival (28). Several papers (18, 20, 22) have shown that HO-1 expression was regulated in the heart. However, there are only a few previous investigations that showed the effect of hemodynamic stress on cardiac HO-1 expression (12, 26). HO-1 was upregulated in both ventricles in response to pressure overload induced by pulmonary artery banding (12) and by the renal artery occlusion (25). In addition, HO-1 was upregulated in the heart of genetically hypertensive rats compared with their normotensive counterpart (26). All these experiments suggested the concept that hemodynamic and mechanical forces upregulate cardiac HO-1 expression. In contrast, however, NE failed to affect cardiac HO-1 expression in the present study. Hemodynamic stress-induced HO-1 regulation in the heart may be variable according to the type, duration, and intensity of applied physical forces. By what mechanism ANG II, but not NE, exerted a proinflammatory effect has not been investigated in the present study. Because ANG II, but not NE, increases superoxide...
production in the arterial wall (24), different levels of oxidative stress induced by either ANG II or NE may explain the different effects of these hormones.

It has been reported that ANG II plays a pivotal role in an adverse structural remodeling of the heart such as myocardial necrosis (30), myofibroblast proliferation, interstitial/perivascular fibrosis (2), and mobilization of inflammatory cells (21). The finding that hydralazine only partially suppressed ANG II-induced granulation tissue formation supported the notion that ANG II induces cardiac inflammation by its direct action (5, 13). In addition, our results also suggested that pressure overload may exacerbate ANG II-induced myocardial damage. Campbell et al. (3) reported that ANG II-stimulated expression of transforming growth factor-β1 (TGF-β1) in cardiac myofibroblasts. Because TGF-β1 stimulation increased HO-1 expression in fibroblasts in other tissue (15), the possibility that ANG II-induced TGF-β1 gene expression acts as an autocrine/paracrine stimulus to upregulate cardiac HO-1 should be addressed in future experiments.

The possible physiological role of ANG II-induced cardiac HO-1 upregulation may be threefold. First, because produced and released carbon monoxide may exert a vasodilatory effect by activating soluble guanylate cyclase (20), upregulated cardiac HO-1, together with aortic HO-1, may attenuate the pressor effect of ANG II. Second, because HO-1 has an anti-inflammatory property (28, 34), upregulated HO-1 may ameliorate ANG II-induced cardiac inflammatory response. It has been reported that the myocardial preservation afforded by nitric oxide was partially mediated by HO-dependent carbon monoxide generation in the setting of myocardial ischemia (17). Therefore, upregulated HO-1 may enhance protective cardiovascular homeostatic role of nitric oxide synthase in hypertension (8). The infiltrated inflammatory cells could modulate the synthetic and mitotic activity of the cardiac cells, which may result in cardiac hypertrophy (14, 27). Upregulated HO-1 or its product, carbon monoxide, may act against cell growth in epithelial cells (16) and in vascular smooth muscle cells (19). Therefore, third, upregulated cardiac HO-1 may counteract the hypertrophic effect of ANG II infusion and/or pressure overload.

We (1) recently found that administration of hemin, an HO inducer, resulted in a marked increase of HO-1 expression in various tissues (heart, carotid artery, aorta, liver, and kidney), which led to the complete normalization of blood pressure in the ANG II-infused rat (Ishizaka N, unpublished observation). To assess the cardioprotective role of upregulated HO-1 without affecting blood pressure, the adenoviral HO-1 gene transfer system is now under investigation in our laboratory. Yet et al. (36) recently reported an interesting observation that hypoxia induced myocardial dilatation and infarction of right ventricles in HO-1 null (HO-1−/−) mice but not in wild-type (HO-1+/+) mice. In their paper, ventricular section of HO-1 null mice showed inflammatory cell infiltration, myocardial degeneration, and collagen deposition. The fact that histological features in the heart of ANG II-infused rat resembled those in the heart of hypoxia-exposed HO-1 null mice may suggest the notion that ANG II-induced HO-1 upregulation in the granulation tissue might act protectively in the development of ANG II-induced cardiac inflammation and remodeling.

In conclusion, chronic ANG II infusion induced formation of granulation tissue (myofibroblast proliferation, inflammatory cell migration, fibrous tissue deposition), where HO-1 was intensively expressed. These ANG II-induced histological changes and HO-1 up-regulation were provoked in a pressor-independent manner and was augmented by pressure overload. Upreregulated HO-1 may exert anti-inflammatory and antioxidant effects in the ANG II-induced granulation tissue.

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REFERENCES


