Systemic Heme Oxygenase-1 Transgenic Overexpression Aggravates Pressure Overload-induced Cardiac Hypertrophy in Mice

Chang Chen^{1,*}, Rong Huo^{1,*}, Yan Tong¹, Yue Sheng¹, Hui-Bin Liu¹, Xu Gao², Osamu Nakajima³, Bao-Feng Yang¹ and De-Li Dong¹

¹Department of Pharmacology, the State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education, ²Department of Biochemistry, Harbin Medical University, Harbin, ³Research Laboratory for Molecular Genetics, Yamagata University, *Authors contributed equally to this work

Key Words

Heme oxygenase-1 • Cardiac hypertrophy • Pressure-overload • Transgenic overexpression

Abstract

Background/Aims: Heme oxygenase-1(HO-1) has been reported to protect against cardiac hypertrophy in cultured neonatal cardiomyocytes treated with HO-1 inducer, cardiac specific HO-1 transgenic mice, or animals treated with HO-1 inducer. The aim of the present study is to examine the effects of systemic HO-1 transgenic overexpression on pressure overload-induced cardiac hypertrophy in mice. **Methods:** Pressure-overload cardiac hypertrophy was induced by transverse aortic constriction (TAC) in WT (wild type) and systemic HO-1 transgenic overexpression (TG) mice. **Results:** We found that systemic HO-1 transgenic overexpression aggravated pressure overload-induced cardiac hypertrophy. Pressure-overload induced the more increases of heart weight/ body weight index, left ventricular weight/ body weight index, β -MHC protein expression, cardiac interstitial fibrosis in TG mice than in WT mice. Pressure-overload increased cardiac HO-1 protein expression in WT but not TG mice, but the cardiac

HO-1 protein level was still higher in TAC-treated TG mice than in TAC-treated WT mice. The basal cardiac calcineurin protein level in TG mice was lower than that in WT mice. Pressure-overload increased calcineurin protein expression in both WT and TG mice; however, pressure-overload induced more calcineurin protein expression in TG mice than in WT mice. **Conclusion:** This study shows for the first time that systemic HO-1 transgenic overexpression aggravates pressure overload-induced cardiac hypertrophy.

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Introduction

Heme oxygenase-1(HO-1), the inducible isoform of heme oxygenase, plays a protective role in cardiovascular diseases. HO-1 protects against cardiac ischemia and reperfusion injury [1], lowers blood pressure, and decreases lesion formation in atherosclerosis [2]. Recent several studies reveal that HO-1 protects against cardiac hypertrophy [3-6]. Hu et al reported HO-1 attenuated Ang II-induced cardiac hypertrophy both *in vitro* and *in vivo* [3]. Ndisang et al. reported that upregulating the heme

oxygenase system suppressed left ventricular hypertrophy in adult spontaneously hypertensive rats [4]. Wang et al observed the improved postinfarction survival, less cardiac hypertrophy and interstitial fibrosis in myocyte-restricted HO-1 transgenic mice which underwent coronary ligation to induce heart failure [6]. However, another group reported that HO-1 gene transfer to cardiomyocytes attenuated Ang II-mediated apoptosis but not hypertrophy [7].

Analyzing these studies about HO-1 in cardiac hypertrophy, we find that these applied models include the cultured neonatal cardiomyocytes treated with HO-1 inducer, HO-1 cardiac specific transgenic mice, or animals treated with HO-1 inducer. HO-1 is expressed in most tissues in the body and plays an important role in regulating the physiological functions; therefore, we wonder whether the same results of anti-hypertrophic effects of HO-1 could be obtained in the model of systemic HO-1 transgenic overexpression (TG) mice. In order to clarify the question, we set out to examine the effects of systemic HO-1 transgenic overexpression on pressure overload-induced cardiac hypertrophy in mice.

Materials and Methods

Generation of systemic HO-1 transgenic overexpression mice

The systemic HO-1 transgenic overexpression mice (TG) were generated by pronuclear microinjection of fertilized eggs from a C57BL/6 F1 parents with a transgenic construct expressing the mouse HO-1 cDNA (mHO-1 cDNA) under the control of chicken α -actin promoter [8]. Transgenic mice were identified by analysis of tail DNA by PCR using an upper primer from the α -actin promoter (5'-GCC TTC TTC TTT TTC CTA CAG CTC-3') and a lower primer from the mHO-1 cDNA (5'-GGC ATG CTG TCG GGC TGT GGA C-3'). The schematic of TG mice generation has been described in our previous study [9]. Meanwhile, we compared the basal physiological parameters, such as the body weight, heart rate, systolic blood pressure, diastolic blood pressure, mean blood pressure, heart weight/body weight index, left ventricular weight/body weight index, ventricular wall thickness between wild type (WT) and TG mice and found no differences. The data were shown in Table 1. Twelve-week old TG and WT mice were used. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Harbin Medical University, PR China.

Pressure-overload cardiac hypertrophy

The pressure-overload heart hypertrophy models were obtained by subjecting the animals to transverse aortic constriction (TAC) as described in our previous work [10]. TG or wild type (WT) mice (C57/BL6), weighing 22-26g, were anesthetized with pentobarbital (65mg/kg, intraperitoneal in-

Parameters	N	WT	TG
BW(g)	10	25.7±1.3	25.0±1.4
HR(beats/min)	6	638±24.2	690±18.2
SBP(mmHg)	6	117±6.0	110±5.7
DBP(mmHg)	6	64.7±3.5	67.3±4.5
MBP(mmHg)	6	82.4±3.9	81.2±4.6
HW/BW (mg/g)	10	5.83±0.17	5.79±0.33
LVW/BW(mg/g)	10	4.14±0.17	4.21±0.31
Left ventricle wall thickness (mm)	6	1.35±0.125	1.37±0.120

Table 1. Comparison of basal physiological parameters between 12-week old wild type (WT) and transgenic mice (TG). BW, body weight; HR, heart rate; SBP: systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure. HW, heart weight; BW, body weight; LVW, left ventricular weight. HR, SBP, DBP, and MBP of WT and TG mice were measured in conscious state.

jection). Animals were placed in the supine position and a mid-line cervical incision was made to expose the trachea. After successful endotracheal intubation, the cannula was connected to a volume cycled rodent ventilator (UGO BASILE S.R.L. Italy). The chest was opened and the thoracic aorta was identified. A 7-0 silk suture was placed around the transverse aorta and tied around a 26-gauge blunt needle which was subsequently removed. This intervention increased systolic pressure about 1.5 folds and induced cardiac hypertrophy [10]. The chest was closed and the animals were kept ventilated until recovery of autonomic breath. After 4 weeks, surviving animals were sacrificed and the heart was quickly excised and weighted in cold (4°C) buffer. The left and right ventricles were separated and weighted, and left ventricle tissue was then rapidly frozen in liquid nitrogen and stored at -80°C for subsequent western blot analysis. All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of Harbin Medical University, PR China.

Western Blot for HO-1, HO-2, calcineurin and β -MHC

The concentration of proteins from left ventricle was determined with BCA Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada). The samples were subjected to electrophoresed in 10% SDS-PAGE and the separated proteins were transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked in 5% non-fat milk overnight at 4°C, then incubated with the primary antibodies against HO-1(Santa Cruz biotechnology, Inc, USA), HO-2 (Santa Cruz Biotechnology, Inc, USA), calcineurin-A (Santa Cruz Biotechnology, Inc, USA), β -MHC (β -myosin heavy chain) (Sigma, USA). After washed, the membrane was incubated with secondary antibody (invitrogen, USA). Western blot bands were quantified by using Odyssey infrared imaging system (LI-COR, Lincoln, NE).

Measurement of the total cardiac tissue Fe content

Cardiac tissue Fe content was measured by using flame atomic absorption spectrometry (FAAS). The standard iron solution (GSB (G) 62020-90, (National Center for Standard Ma-

terials, Beijing, China)) was used to make the standard curve ($R=0.9991$). The animal was anesthetized with pentobarbital (65mg/kg, intraperitoneal injection) and the chest was opened. Left ventricle of heart was punctured with a 25-gauge cannula for the infusion of saline until the heart, liver and kidney were perfused clearly and became white. Samples of the heart, liver and kidney (500 ug) were digested with acid mixture containing $\text{HClO}_4\text{-HNO}_3$ (1:4, v/v), and placed on muffle furnace at 80°C to evaporate the reagents with simultaneously rinse for three to four times. Then the remainder was dissolved in 1% HCl in calibrated flask and diluted to 10mL volume, which was tested by flame atomic absorption spectrometry (Perkin Elmer Model 3110, USA) under the following conditions: wavelength, 248.3 nm; slit width, 0.2 nm; lamp current, 5mA. The results were obtained by using standard curve method.

Measurement of heart rate, systolic pressure, diastolic pressure of mice in conscious state

Tail-cuff method was used to measure the basal physiological parameters in conscious mice. Briefly, the mouse was put into the mouse cage to limit its activity. Then the mouse cage was put on the thermophore to maintain the mouse body temperature and the mouse tail was exposed to pressure transducer (BP-98A, Softron Beijing Biotechnology, China). When the mouse was fully sedate, the heart rate, systolic and diastolic pressure were measured.

Hemodynamic measurements

The hemodynamics of mice was measured by using the Scisense pressure-volume measurement system (Scisense Inc. Ontario, Canada). The pressure-volume sensor was inserted into left heart ventricle through the right carotid artery.

Histological analysis

The tissue samples were embedded in paraffin and subjected to standard hematoxylin and eosin (HE) staining or masson's trichrome staining.

Data analysis

For the westernblot analysis, the normalization was performed within the individual group. Data are presented as mean $\pm$ SEM. Significance was determined by using Student t-test for comparison of two groups and one-way ANOVA for comparison of two more groups in Sigma Stat Analysis Software. $P<0.05$ was considered significant.

Results

HO-1 protein level in systemic HO-1 transgenic overexpression mice

We first examined the HO-1 and HO-2 protein expression in the hearts of TG and WT mice. As shown in Fig. 1A, compared with WT, HO-1 but not HO-2 protein level in TG mice hearts significantly increased. Next, we examined the HO-1 protein expression in kidney,

Fig. 1. The HO-1 protein expression in the heart, kidney, liver, brain, aortic artery from systemic HO-1 transgenic overexpression (TG) mice increased. There was no difference of HO-2 protein expression in the heart from TG and WT (wild type) mice. ** $P<0.01$ vs WT.

brain, liver and artery of TG and WT mice. As shown in Fig. 1B, C, D and E, HO-1 protein expression in kidney, brain, liver and artery of TG mice was significantly higher than that of WT mice, suggesting that HO-1 transgenic overexpression was systemic.

Pressure-overload induces aggravated cardiac hypertrophy in systemic HO-1 transgenic overexpression mice

WT and TG mice were subjected to sham operation and pressure-overload stimulation by 4 week of TAC. As shown in Fig. 2A, both WT and TG mice had significant increases in heart weight/body weight (HW/BW), left ventricular weight/body weight (LVW/BW) and left ventricular wall thickness (LVWT) after 4 weeks TAC stimulation. However, the HW/BW index, LVW/BW index and LVWT in TAC-treated TG mice were

Fig. 2. Pressure-overload induced the aggravated cardiac hypertrophy in TG mice. (A) The upper panel showed the H&E-stained sections of the representative whole hearts from WT+sham, WT+TAC, TG+sham and TG+TAC mice. The panel below showed the summarized data of HW/BW index, LVW/BW index and left ventricle wall thickness (LVWT). HW/BW, heart weight/ body weight; LVW/BW, left ventricular weight/ body weight. ** $P < 0.01$ vs sham. (B) The left panel showed the representative H&E-stained sections of hearts and the right panel showed the summarized data of cross-sectional area of ventricular cardiomyocytes. Fifty cell cross-sections in each group were calculated. ** $P < 0.01$ vs sham.

Fig. 3. (A) Effects of pressure-overload on β -MHC protein expression in WT and TG mice. The upper panel showed the representative western blot of β -MHC protein expression. The panel below showed the summarized data of β -MHC protein expression. * $P < 0.05$; ** $P < 0.01$. β -MHC, β -myosin heavy chain. (B) Analysis and quantification of cardiac fibrosis by using masson's trichrome method. The interstitial fibrosis was in blue staining. The degree of fibrosis was evaluated by the ratio of the area of blue staining to the area of picture. The upper panel showed the representative masson's trichrome staining and the panel below showed the summarized data of cardiac fibrosis. ** $P < 0.01$ vs sham, n=10 from three hearts in each group.

significantly higher than those in TAC-treated WT mice, indicating that cardiac hypertrophy induced by TAC in TG mice was aggravated. Moreover, we also measured the cross-sectional area of ventricular cardiomyocytes and the results were similar to those of HW/BW index, LVW/BW index and LVWT measurement (Fig. 2B). Consistent with these results, TAC induced increase in protein expression of β -MHC, the marker of cardiac hypertrophy, in WT and TG mice, and β -MHC protein expression in TAC-treated TG mice heart was significantly higher than that of TAC-treated WT mice (Fig. 3A). Pressure-overload induced hypertrophy is often accompanied by interstitial fibrosis. Using Masson's

trichrome staining, ventricular fibrosis was more intensified in TAC-treated TG mice than in TAC-treated WT mice (Fig. 3B).

Pressure-overload alters of HO-1 but not HO-2 protein expression

Because HO-1 is a stress responsive gene, stretch stress has been shown to increase HO-1 transcription and translation [11]. We examined HO-1 protein expression in WT and TG mice treated with TAC. As shown in Fig. 4A, HO-1 protein expression increased in TAC-induced cardiac hypertrophy in WT mice, however, there was no difference of HO-1 protein level in hearts

Fig. 4. Effects of pressure-overload on HO-1 and HO-2 protein expression. (A, B). Pressure overload increased cardiac HO-1 protein expression in WT but not TG mice. (C) In comparison with TAC-treated WT mice, the HO-1 protein level was higher in hearts of TAC-treated TG mice. $**P<0.01$. (D, E, F) Pressure-overload had no effects on HO-2 protein expression.

Fig. 5. Summarized data of total cardiac tissue Fe content in heart, liver and kidney in mice from all groups (n=5).

from TG mice and TAC-treated TG mice (Fig. 4B). We speculated that the pressure overload-induced stimulation of HO-1 expression was blunted in TG mice. In comparison with TAC-treated WT mice, the HO-1 protein level was still higher in hearts of TAC-treated TG mice (Fig. 4C). Meanwhile, we examined HO-2 protein expression in WT and TG mice treated with TAC. As shown in Fig. 4D, E, F, there was no difference of HO-2 protein expression in all groups.

HO degrades heme to generate carbon monoxide (CO), bilirubin, and Fe. Among these products, CO and bilirubin have been shown to be the beneficial factors in protection against cardiac hypertrophy [3, 6]. But excessive Fe has been reported to induce cardiac hypertrophy [12]. We measured the total cardiac tissue Fe content by using Flame Atomic Absorption Spectrometry. As shown in Fig. 5, there was no difference of total tissue Fe content in the heart, liver and kidney in all groups, indicating that either TAC treatment or HO-1 transgenic overexpression did not increase tissue Fe deposition, and the cardiac hypertrophy in the present study was not associated with Fe excessive deposition.

Pressure-overload alters calcineurin protein expression

Calcineurin (PP2B) is a calcium/calmodulin-activated, serine-threonine phosphatase that dephosphorylates the transcription factor, nuclear factor of activated T-cells (NFAT), which translocates into the nucleus to bind DNA and activate hypertrophic response genes. We and others have previously demonstrated that calcineurin expression level is correlated with cardiac hypertrophy [10, 13-15]. We examined calcineurin protein expression in WT, TG, WT and TG mice treated with TAC. As shown in Fig. 6A, relative to WT mice, the hearts of TG mice expressed lower calcineurin protein. Pressure-overload increased calcineurin protein expression in both WT and TG mice (Fig. 6B, C). Pressure-overload induced more calcineurin protein expression in TG mice than in WT mice (Fig. 6D).

Hemodynamic measurements

We also measured the hemodynamics by using the Scisense pressure-volume measurement system (Scisense Inc. Ontario, Canada). The hemodynamic results were

Fig. 6. Pressure-overload alters cardiac calcineurin protein expression in WT and TG mice. (A) TG mice expressed lower cardiac calcineurin protein than WT mice. $**P<0.01$ vs WT. (B, C) Pressure-overload increased calcineurin protein expression in both WT and TG mice. $**P<0.01$ vs sham. (D). Pressure-overload induced more calcineurin protein expression in TG mice than in WT mice. $**P<0.01$ vs WT+TAC.

shown in Table 2. TAC induced significant increases of left ventricular end-systolic pressure (ESP), left ventricular end-systolic volume (ESV) and end-diastolic volume (EDV) in WT and TG mice. However, there was no significant difference of hemodynamics between TAC treated WT and TG mice. We surmised that both TG and WT mice treated with TAC in the present experimental conditions were in the compensated state.

Discussion

The manifestation of cardiac hypertrophy, including the increases in the HW/BW index, LVW/BW index, β -MHC expression, interstitial fibrosis, and calcineurin expression, has shown that the aggravated cardiac hypertrophy occurred in systemic HO-1 transgenic overexpression (TG) mice under the pressure-overload conditions. In view of the deleterious role of oxidative stress in pressure-overloaded hearts and the protective effects of HO-1 against oxidation and cardiac hypertrophy, we had expected that the pressure overload-induced cardiac hypertrophy would be attenuated in the TG mice; however, on the contrary, the results showed that it was aggravated. To our knowledge, it is the first report that systemic HO-1 transgenic overexpression aggravates pressure overload-induced cardiac hypertrophy.

HO-1 is rapidly inducible during acute stress. The effect of HO-1 on cardiovascular system has always been attractive. Our precious work has shown that HO-1 is involved in the endothelial BK channel (big conductance Ca^{2+} -activated K channel) activation [16]. In the field of cardiac hypertrophy, several studies report that HO-1 protects against cardiac hypertrophy. In the myocyte-restricted HO-1 transgenic mice, Wang et al [6] found that HO-1 upregulation alleviated pathological remodeling, including improved postinfarction survival, less left ventricular dilatation and dysfunction, cardiac hypertrophy, fibrosis, oxidative stress, and apoptosis. In cultured rat neonatal cardiomyocytes, induction of HO-1 by treatment with cobalt protoporphyrin IX (CoPP) or a recombinant adenovirus carrying the human HO-1 gene attenuated Ang II-induced cardiac hypertrophy [3]. The antihypertrophic effect of HO-1 was also demonstrated in rats receiving chronic Ang II infusions. Cotreatment of animals with CoPP attenuated Ang II-induced left ventricular hypertrophy *in vivo* [3]. A recent work reports that Bach1, a transcriptional repressor of HO-1, protects against cardiac hypertrophy through increasing HO-1 expression [17]. On the other hand, Foo et al reported that HO-1 gene transfer inhibited Ang II-mediated cardiomyocyte apoptosis but not hypertrophy in cultured rat cardiac myocytes [7]. Presently, we find that systemic HO-1 transgenic overexpression aggravates pressure

Group	WT-sham	TG-sham	WT+TAC	TG+TAC
n	7	7	8	6
HR (bpm)	513±58	505±48	529±27	519±30
ESP (mmHg)	81±5.3	85±6.2	105±6.1*	113±8.2*
EDP (mmHg)	0.6±1.1	1.2±0.8	2.7±0.6	2.8±0.7
dPmax (mmHg/s)	11275±1706	10875±1820	12975±1552	11536±1865
dPmin (mmHg/s)	-7937±681	-8010±628	-9818±612	-8893±703
ESV (μL)	21.3±1.3	20.4±1.5	27±1.3*	28±1.7*
EDV (μL)	30.1±1.4	29.5±1.7	36.6±1.9*	37.3±1.8*
SV (μL)	9.4±0.79	9.6±0.69	9.7±2.08	10.1±2.12
CO	4937±867	4880±877	6061±1147	5976±1006
EF	31.3±2.4	30.3±2.5	26.6±5.1	25.4±4.6

Table. 2. Cardiac hemodynamics at 4 weeks after TAC and sham operation in anaesthetized mice. HR, heart rate; ESP, left ventricular end-systolic pressure; EDP, left ventricular end-diastolic pressure; dPmax, peak rate of pressure rise; dPmin, peak rate of pressure rise decline; ESV, left ventricular end-systolic volume; EDV, left ventricular end-diastolic volume; SV, stroke volume; CO, cardiac output; EF, ejection fraction. * $P<0.05$ vs sham.

overload-induced cardiac hypertrophy. Cardiac hypertrophy can be caused by multiple factors; these results also suggest that the mechanism of angiotensin II infusion-induced cardiac hypertrophy may be different from those by mechanical overload-induced hypertrophy.

HO-1 is widely expressed in the body and involved in a multitude of cellular processes, such as in neurons, cardiomyocytes, smooth muscle cells, kidneys et al. Relative to TG mice, the treatment with HO-1 inducer or a recombinant adenovirus carrying the HO-1 gene, or treatment of animals with HO-1 inducer belongs to the acute stress. The results of acute stress might be different from those of the long-term transgenic overexpression. Even for the transgenic overexpression, there are two types, systemic and specific overexpression. The systemically and specifically transgenic overexpression shows distinct consequences. For example, HO-1 induction by transfection *in vivo* with vectors encoding HO-1 or by HO-1 inducer increased vascular relaxation [18, 19] and HO-1 induction lowered blood pressure [20, 21]. However, Imai et al revealed that vascular smooth muscle cell-directed overexpression of HO-1 elevated blood pressure [22]. The different results of local and systemic HO-1 overexpression were also observed in the immunology field, Araujo et al found that, although local and systemic HO-1 overexpression improved cardiac allograft outcomes, systemic HO-1 led to a more robust protection and resulted in a significant blunting of host immune activation [23]. All above information implicates

that the effects of HO-1 on cardiac hypertrophy in the cultured neonatal cardiomyocytes treated with HO-1 inducer, HO-1 specific transgenic mice or animals treated with HO-1 inducer can not necessarily be extrapolated to systemic HO-1 transgenic overexpression model.

We found that TG mice expressed significantly lower calcineurin protein level than WT mice (Fig. 6A). But under the pressure-overload conditions, the more calcineurin protein expression was induced in TG mice (Fig. 6D). Activation of calcineurin/NFAT pathway plays a critical role in cardiac hypertrophy. Tongers et al reported that HO-1 inhibited the calcineurin/NFAT signaling cascade upstream of calcineurin, which may partly explain the lower calcineurin expression in TG mice [5]. However, the more calcineurin expression induced by pressure-overload in TG mice than WT mice is unexpected. It is a very interesting phenomenon; unfortunately, we do not elucidate the mechanism, which is the limitation of the present study.

Although HO-1 has been reported to protect against cardiac hypertrophy in cultured neonatal cardiomyocytes treated with HO-1 inducer, cardiac specific HO-1 transgenic mice, or animals treated with HO-1 inducer, the increases in the HW/BW index, LVW/BW index, β -MHC expression, the cross-sectional area of cardiomyocytes, interstitial fibrosis, and calcineurin expression have confirmed that systemic HO-1 transgenic overexpression aggravates pressure overload-induced cardiac hypertrophy in mice.

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