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Sex specific effects of heme oxygenase-2 deficiency on renovascular hypertension

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Abstract

Background—Heme oxygenase-2 (HO-2) is the main isoform responsible for the breakdown of heme and release of carbon monoxide (CO) in the vasculature. Vascular derived CO protects against excessive vasoconstriction due to agents such as angiotensin II (Ang II) and in states of deficiency of nitric oxide (NO). The current study was designed to determine the role of HO-2 in the development of renovascular hypertension using HO-2 knockout mice.

Methods—Polyurethane cuffs were placed around the left renal artery of male and female HO-2 wild-type (WT), heterozygous (HET), and knockout (KO) mice between 16–24 weeks of age to induce renovascular hypertension. After 3 weeks, blood pressure was measured for 5 days after which time both clipped and unclipped kidneys were harvested.

Results—No differences were observed in the blood pressure of sham mice between the different genotypes of both sexes. Cuffing of the left renal artery resulted in a significant increase in blood pressure in all genotypes of both sexes. In male mice, the increase in blood pressure was significantly greater in HET and KO mice as compared to WT mice ($P<0.05$). This effect was not observed in female mice. Renovascular hypertension resulted in a significant increase ($P<0.05$) in cardiac hypertrophy in male mice which was not different between the genotypes. In female mice, HET and KO mice exhibited significantly greater ($P<0.05$) cardiac hypertrophy as compared to WT mice.

Conclusion—These results demonstrate a sex specific effect of HO-2 deficiency on the development of renovascular hypertension and its effects on the heart in response to the increase in blood pressure.

Keywords

Angiotensin II; blood pressure; renin; bilirubin; carbon monoxide

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INTRODUCTION

Heme oxygenase-2 (HO-2) is the constitutively expressed isoform of heme oxygenase which is the major isoform responsible for the catabolism of heme to biliverdin, iron, and carbon monoxide (CO) in the brain and vasculature (8; 13; 19). Several studies have demonstrated the important role of HO-2 derived CO to protect against excessive vasoconstriction (13; 29; 30). HO-2 mediated CO production has also been demonstrated to play an important role in maintaining vasodilatation in conditions of nitric oxide (NO) deficiency (22; 23).

Renovascular hypertension is a form of hypertension which occurs when blood flow to one or both kidneys is reduced. Renal arterial stenosis leading to reductions in renal blood flow can be caused by fibromuscular dysplasia as well as atherosclerosis occurring in the renal artery (9). Occlusions resulting in the development of renal vascular hypertension are estimated to occur at a frequency between 16–50% depending on the specific patient population with the greatest percentage occurring in patients with occlusive disease of the aorta and legs (9).

The pathological cause of 2-kidney, 1-clip renovascular hypertension is believed to depend on increased renin release from the stenotic or clipped kidney. The increase in renin release results in increased plasma renin levels and increases in angiotensin II (Ang II) production (6). The Ang II then acts on the unclipped kidney to increase sodium and water reabsorption despite significant down-regulation of endogenous renin in this kidney. The actions of Ang II on the unclipped kidney in 2-kidney, 1-clip hypertension have been demonstrated to be mediated by the angiotensin type 1 receptor (5). Vascular CO generation had been previously demonstrated to play a critical role to limit vasoconstriction to pressor agents such as Ang II (13). Thus, HO-2 deficiency may be predicted to enhance the blood pressure response to renovascular hypertension; however, previous studies have demonstrated no effect of HO-2 deficiency on the development of Ang II-dependent or L-NAME hypertension (26). We wanted to determine the specific effect of HO-2 deficiency on the development of renovascular hypertension. In order to accomplish this, we performed 2-kidney, 1-clip renovascular hypertension in HO-2 deficient mice.

METHODS

Animals

Experiments were performed on 16- to 24-week-old male and female HO-2 wild-type (WT), heterozygous (HET), and knockout mice (KO) obtained from our breeding colony at the University of Mississippi Medical Center (26). HO-2 KO mice were derived as initially described by Poss et al and maintained on a mixed C57Bl/6 × 129/Sv background by breeding of HET × HET mice (20). The mice were fed a standard diet containing 0.29% NaCl and were provided water ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health.

Renovascular Hypertension

Renovascular hypertension or 2-kidney, 1-clip Goldblatt hypertension was produced via placement of a small segment of polyurethane tubing, sliced lengthwise around the left renal artery as previously described in detail (17). Mice were anesthetized with isoflurane and the left renal artery accessed by retroperitoneal approach. The renal cuff was placed on the left renal artery and then secured by tying with a 7-0 silk suture to close the gap in the cuff. Sham operated animals received an identical surgery without placement of the renal cuff.

Blood Pressure

Blood pressure was directly measured via microrenthane catheters implanted into the carotid artery using aseptic surgical technique 3 weeks after renal artery cuff surgery as previously described (28). This method is consistent with recommendations of the American Heart Association for measuring blood pressure in conscious animals (14). The mice were allowed two days to recover from surgery and then mean arterial blood pressure (MAP) was recorded from conscious, freely moving mice three hours per day for five consecutive days. Blood pressures are presented as the average daily pressure over the entire five day recording period. Mice were euthanized after blood pressure measurement at which time body and organ weights were measured. Tissues were frozen in liquid nitrogen and stored at -80°C until use.

Real-time PCR

RNA was isolated from the clipped and unclipped kidneys using a commercially available kit according to manufactures' guidelines (Tri-Reagent, Molecular Research Center, Inc, Cincinnati, OH). Following DNase treatment, RNA (1 μg) was used in a reverse transcription reaction using an oligo-dT primer with AMV reverse transcriptase in a 20 μL final volume. Real time PCR was performed on 1 μL of reverse transcription reaction prepared as above using a syber-green containing PCR mix (BioRad, Hercules, CA). The sequences for primers utilized for real-time PCR are given in Table 1. PCR was performed as follows: 94°C -30 sec, 60°C for 30 sec, 72°C for 30 sec for 45 cycles. Fluorescence data was acquired during the 72°C elongation step. All samples were normalized to levels of endogenous 18s rRNA. Data are presented as the negative difference in threshold cycles ($-C_T$) between the clipped and unclipped kidney. Any target gene in which the difference in threshold cycle was lower in the clipped kidney as compared to the unclipped kidney (indicating more RNA) would have a double negative value or be expressed as a positive value. Any target gene that was expressed less in the clipped kidney (greater $-C_T$) than in the unclipped kidney would have a negative value. Real time PCR was performed on RNA samples from 3 mice in each group.

Western Blots

Western blots were performed on lysates prepared from the unclipped kidney collected at the end of the experimental protocol. Samples of 30 μg of protein were boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) for 5 min and electrophoresed on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 2 hours at room temperature and then incubated with primary antibodies overnight at 4°C . Membranes were incubated with either Alexa 680 (Molecular Probes) or IRDye 800 (Rockland, Gilbertsville, PA) secondary antibodies for 1 hour at room temperature. Membranes were visualized using an Odyssey infrared imager (LI-COR, Lincoln, NE) which allows for simultaneous detection of two fluorophores. Densitometry analysis was performed using Odyssey software (LI-COR, Lincoln, NE). Antibodies for Western blots were as follows: mouse anti-interleukin 1 (IL-1, 1:1,000, Abcam, Cambridge, MA); mouse anti-transforming growth factor 1, (TGF-1, 1:1,000 Abcam) and rabbit anti- α -actin antibody (1:5,000, Abcam). Protein levels were normalized to α -actin and expressed as a ratio.

Statistics

Mean values $\pm$ SE are presented. Significant differences between mean values were determined by 2 way ANOVA followed by a post hoc test (Student-Newman-Keuls). A $P < 0.05$ was considered to be significant.

RESULTS

Renovascular hypertension is exacerbated in male HO-2 HET and KO mice but not in female mice

In male mice, there were no significant differences between the genotypes in mice undergoing sham surgery with blood pressures averaging 112 ± 2 vs. 118 ± 2 vs. 116 ± 2 mmHg in WT, HET, and KO mice, respectively (Figure 1A). Three weeks after undergoing clipping of the left renal artery, blood pressures were significantly ($P < 0.05$) increased in each genotype as compared to levels in corresponding sham controls (Figure 1A). Blood pressures after clipping were significantly increased ($P < 0.05$) in HET and KO mice as compared to WT mice and averaged: 150 ± 3 vs. 147 ± 3 vs. 138 ± 4 mmHg in each group, respectively (Figure 1A).

In female mice there were no significant differences between the genotypes in mice undergoing sham surgery with blood pressures averaging 115 ± 1 vs. 111 ± 3 vs. 109 ± 4 mmHg in WT, HET, and KO mice, respectively (Figure 1B). Three weeks after undergoing clipping of the left renal artery, blood pressure was significantly ($P < 0.05$) increased to the same extent in each genotype as compared to levels in corresponding sham controls (Figure 1B) with pressures averaging 140 ± 4 vs. 141 ± 6 vs. 137 ± 4 mmHg in each group, respectively (Figure 1B).

Renovascular hypertension results in augmented cardiac hypertrophy in female but not in male HO-2 deficient mice

In male mice, no differences in cardiac hypertrophy determined by the ratio of heart weight to body weight (HW:BW) were detected between the genotypes under basal conditions (Figure 2A). However, 3 weeks after induction of renovascular hypertension cardiac hypertrophy was significantly increased ($P < 0.05$) in each genotype as compared to those observed in control sham mice (Figure 2A).

In female mice, sham HET and KO mice exhibited significantly greater ($P < 0.05$) cardiac hypertrophy as compared with WT mice (Figure 2B). Induction of renovascular hypertension significantly increased ($P < 0.05$) cardiac hypertrophy as compared to sham in each genotype (Figure 2B). Cardiac hypertrophy in response to 3 weeks of renovascular hypertension was significantly ($P < 0.05$) enhanced in HET and KO mice as compared to WT mice (Figure 2B).

No differences in the renal response to clipping of the left renal artery as determined by the ratio of the clipped to unclipped kidney weight were observed between the genotypes in either male (Figure 2C) or female (Figure 2D) mice.

Alterations in the response of components of the renin-angiotensin system in response to renovascular hypertension

The effect of clipping of the left renal artery on the expression level of renin, angiotensin converting enzyme (ACE), angiotensin type 1A receptor (AT1A), and the endothelin B receptor (ETB) in the clipped and unclipped kidneys of male WT, HET and KO mice was determined by measurement of gene expression with real-time PCR. Renin levels were significantly elevated in the clipped kidney as compared to the unclipped kidney and the response was similar between all genotypes (Figure 3A). The levels of ACE were increased in the unclipped kidney as compared to the clipped kidney in both WT and KO mice (Figure 3B). However, the levels of ACE were increased to a greater degree in the clipped kidney as compared to the unclipped kidney in HET mice (Figure 3B). The levels of the AT1A receptor were increased in the clipped kidney as compared to the unclipped kidney and this

difference was significantly ($P<0.05$) attenuated in the clipped kidney of KO mice as compared to the other two genotypes (Figure 3C). The levels of the ETB receptor were expressed at a higher level in the unclipped kidney of all mice but the level was significantly ($P<0.05$) enhanced in the unclipped kidney of KO mice (Figure 3D).

Inflammatory and endoplasmic reticulum (ER) stress response of the unclipped kidney in response to renovascular hypertension

The inflammatory response of the unclipped kidney in response to renovascular hypertension was determined in male mice via measurement of interleukin-1 (IL1) and transforming growth factor ($\text{TGF-}\beta$) levels by Western blot and tumor necrosis factor (TNF- α) levels by real-time PCR. No differences in the levels of IL1 were detected in the unclipped kidneys between the different genotypes (Figure 4A&B). The levels of pro-TGF- β were significantly ($P<0.05$) increased in the unclipped kidney of WT as compared to KO mice (Figure 4A&C). However, the levels of active TGF- β were significantly ($P<0.05$) increased in the unclipped kidney of KO as compared to WT mice (Figure 4A&D). TNF- α levels were greater in the unclipped versus the clipped kidney for all genotypes and this difference was significantly ($P<0.05$) enhanced in HET mice as compared to both WT and KO mice (Figure 5A). The ER stress response of the unclipped kidney was evaluated by measuring the levels of ER stress markers glucose-regulated protein-78 (GRP78) and X-box binding protein-1 (XBP1). GRP78 levels were greater in the unclipped versus the clipped kidney for all genotypes and this difference was significantly ($P<0.05$) enhanced in HET mice as compared to both WT and KO mice (Figure 5B). XBP1 levels were also greater in the unclipped versus the clipped kidney for all genotypes and this difference was significantly ($P<0.05$) enhanced in HET mice as compared to both WT and KO mice (Figure 5B).

DISCUSSION

The goal of this study was to determine the effect of HO-2 genotype on the development of renovascular hypertension in male and female mice. In male mice, loss of either 1 or both copies of the HO-2 gene enhanced the development of renovascular hypertension 3 weeks after clipping of the left renal artery. In females, HO-2 genotype did not have a significant effect on the development of renovascular hypertension. Sex or gender differences in blood pressure have been extensively examined in rodents (21). As in humans, male rats and mice usually exhibit higher levels of blood pressure as compared to females until females reach menopause (21). Consistent with this finding is the recent report that infusion of Ang II results in a greater pressor response in male as compared to female mice (27). While there was no significant difference in the degree of hypertension developed by male and female mice following renal artery clipping, there was a significant effect of HO-2 genotype on the blood pressure response in male mice. The mechanism responsible for the effect of HO-2 genotype on the development of renovascular hypertension is not known. We have previously demonstrated that HO-2 HET and KO mice exhibit an attenuated renal blood flow response to a bolus infusion of Ang II as compared to WT mice (26). These results would suggest that alterations in renal hemodynamics of the unclipped kidney in response to the increased renin release and subsequent increase in Ang II are likely not the source of the increase in blood pressure observed in these genotypes. It is possible that HO-2 deficiency could contribute to enhanced tubular sodium and water reabsorption in response to the elevated Ang II levels in the unclipped kidney.

The influence of sex on the development of renovascular hypertension has not been extensively examined. The effect of sex on the outcome of renal artery stent placement for the treatment of renovascular hypertension has been examined in a few small clinical studies. Harjai et al failed to observe any effect of sex on the blood pressure response and

incidence of restenosis in a small patient population examined 6 months after stent placement (12). However, a more recent retrospective analysis did identify female sex as an independent predictor of failure to achieve blood pressure improvement following renal artery stent placement (2). We did not detect any sex differences in the degree of stenosis of the clipped kidney as determined by kidney weight in the current study. Furthermore, we did not detect any statistically significant effect of HO-2 genotype on the degree of renal stenosis after clipping of the left renal artery.

One of the harmful consequences of hypertension is the effect of high blood pressure on organs of the body which results in end organ damage. The heart is a target of this damage as it is required to pump against an increased afterload resulting in compensatory cardiac hypertrophy. In the present study, renovascular hypertension resulted in a significant increase in cardiac hypertrophy in mice of both sexes. In male mice, HO-2 genotype did not have any significant effect on the development of cardiac hypertrophy in response to renovascular hypertension. In female mice, HO-2 deficiency resulted in significantly greater hypertrophy in both sham and clipped mice. This effect of HO-2 gene deficiency was similar to that observed in female mice in response to chronic inhibition of NO production with L-NAME (26). The important modulatory role of HO-2 in the heart was recently demonstrated in a model of beta-adrenergic receptor mediated cardiotoxicity (7). These results suggest a potential role for HO-2 in protecting females from hypertension induced cardiac hypertrophy.

Like the heart, the kidney is also an organ which is targeted for injury by hypertension. The effect of renovascular hypertension on the unclipped kidney of male mice was examined using known markers of renal injury. HO-2 deficiency resulted in higher levels of active TGF- β in the unclipped kidney of KO as compared to WT mice. TGF- β is an important growth factor which promotes fibrosis in the kidney (4; 15). TGF- β is anchored to the extracellular matrix in its pro form and is then activated via through several mechanisms including proteolytic cleavage, reactive oxygen species and heat. Once activated it can bind to a membrane receptor and activate several intracellular signaling pathways including Smads and protein kinases to promote fibrosis (10). The levels of another renal marker of inflammation, TNF- α , were also increased in the unclipped kidney of HET mice. TNF- α has both pro-inflammatory and immunosuppressive roles in the kidney depending upon which TNF receptor subtype is activated. Blockade of TNF with etanercept, a TNF-receptor 2 blocker reduced albuminuria and leukocyte infiltration without effects on blood pressure in Ang II-dependent hypertension (18). The increase in TGF- β and TNF- α levels with HO-2 deficiency observed in the present study is consistent with previous observations that HO-2 deficiency increased renal oxidative stress and promoted renal injury in a model of diabetic nephropathy (11).

Endoplasmic reticulum (ER) stress has been linked to fibrosis in several organs including the kidney (16). ER stress has also been demonstrated to play an important role in the development of renal damage in both obesity and diabetes (1; 25). In the present study, markers for ER stress were increased in the unclipped as compared to the clipped kidney with the greatest increase observed in the unclipped kidney of HET mice. The importance of the ER stress response in promoting injury in the unclipped kidney in renovascular hypertension has yet to be fully examined. However, recent studies have suggested a link between ER stress and increased levels of reactive oxygen species (ROS) generation (24). HO-2 deficiency has been associated with increased levels of ROS generation which may play an important role in the promotion of ER stress response in the unclipped kidney of HET mice (11).

The present study identified HO-2 deficiency in male mice as a significant contributor to the exacerbation of renovascular hypertension. Male mice, which lost 50% of their HO-2 genes, exhibited a similar blood pressure increase as those mice which completely lacked the HO-2 gene. Inhibition of HO activity has been previously reported to increase blood pressure in a rat model of 2K, 1C renovascular hypertension (3). HO activity was also found to be increased in the clipped kidney and further chemical induction of HO-1 was able to attenuate stenosis and apoptosis in the clipped kidney which resulted in a lowering of blood pressure (3). These results when taken with those from our study highlight the importance of the renal HO system in the adaptation to renovascular hypertension. Our findings suggest that complete loss of HO-2 activity, which may not be clinically relevant, is not required in order to exacerbate renovascular hypertension and that significant augmentation of hypertension in response to renal artery stenosis can occur in conditions of partial HO-2 deficiency in males.

In summary, the results of our study indicate an important role for HO-2 in the attenuation of renovascular hypertension in males but not in females. While HO-2 genotype did not have any significant effect on the development of renovascular hypertension in females, it did play a significant role in the attenuation of cardiac hypertrophy in response to the hypertension. Male mice deficient in HO-2 exhibited an increase in markers of inflammation in the unclipped kidney which was exposed to the higher blood pressure. The expression level of ER stress related genes was increased in the unclipped kidney and this increase was enhanced in mice with partial deficiency of HO-2. The results of the present study suggest that HO-2 may have a sex specific role in the attenuation of blood pressure and target organ injury in renovascular hypertension.

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Figure 1.

Blood pressure response in A) male and B) female mice 3 weeks after sham surgery or clipping of the left renal artery, n=6/group. * = significant ($P < 0.05$) difference as compared to corresponding value in sham operated. † = significant ($P < 0.05$) difference as compared to corresponding value in WT mice.

Figure 2.

Cardiac hypertrophy in A) male and B) female mice 3 weeks after sham surgery or clipping of the left renal artery, n=6/group. Degree of renal stenosis as determined by the ratio of the clipped kidney to the unclipped kidney weight in C) male and D) female mice 3 weeks after sham surgery or clipping of the left renal artery, n=6/group. *= significant ($P<0.05$) difference as compared to corresponding value in sham operated. † = significant ($P<0.05$) difference as compared to corresponding value in WT mice.

Figure 3.

Quantitation of gene expression between the clipped and unclipped kidney in male mice via real time PCR. Expression of A) Renin, B) angiotensin converting enzyme (ACE), C) angiotensin type 1 receptor (AT1A), and D) endothelin B receptor (ETB). Each targeted gene was normalized to 18s rRNA in the clipped and unclipped kidney and this difference expressed as the $-\Delta C_t$ difference. Thus, genes expressed at a higher level in the clipped kidney have a positive value and those expressed higher in the unclipped kidney have a negative value. *= significant ($P < 0.05$) difference as compared to corresponding value in WT and HET mice. † = significant ($P < 0.05$) difference as compared to corresponding value in WT and KO mice, $n=4/\text{group}$.

Figure 4. Renal inflammation in the unclipped kidney of male mice as determined by Western blot. A) Representative Western blots for interleukin 1 (IL-1), transforming growth factor 1, (TGF-1) active and pro and -actin. B) Levels of IL-1. C) Levels of pro- TGF-1. D) levels of active- TGF-1. * = significant (P<0.05) difference as compared to corresponding value in KO mice. n=6 in WT and KO, n=4 in HET.

Figure 5.

Renal inflammation and endoplasmic reticulum (ER) stress response gene expression in clipped and unclipped kidneys in male mice via real time PCR. A) Expression of tumor necrosis factor- α (TNF- α). B) Expression of glucose-regulated protein-78 (GRP78). C) Expression of X-box binding protein-1 (XBP1). † = significant (P<0.05) difference as compared to corresponding value in WT and KO mice, n=4/group.

Table 1

List of PCR primers utilized for real-time PCR.

Primer	Forward (5'-3')	Reverse (5'-3')
ACE	CCCTAGAGAAAATCGCCTTCTTG	CGAAGATACCACCAGTCGAAGTT
AT1A	GCCAGCGTGTTCCTGCTC	GAGACTTCATCGGGTGGACAA
ETB	TTCACTCCCCAGTTGGTCTC	GTCTTAGTGGGTGGCGTCAT
GRP78	TGGAGTTCCCCAGATTGAAG	CCTGACCCACCTTTTCTCA
XBP-1	TCCGCAGCACTCAGACTATG	ACAGGGTCCAACCTTGCCAG
Renin	TCCACTACGGATCAGGGAGAGT	ACAGTGATTCCACCCACAGTCA
TNF-	GACAAGGCTGCCCGACTAC	GACGGCAGAGAGGAGGTTGA
18s	TAAGTCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC