Vascular Responses in Male and Female Hypertensive Rats With Hyperhomocysteinemia

Chia-Hung Yen, Ying-Tung Lau

Abstract—We studied vascular responses in spontaneously hypertensive rats (SHR) of both genders after methionine (Met) loading to test whether or not there were gender differences. SHRs were divided into 5 groups: male control (MSHR), female control (FSHR), methionine-loaded (+Met) males (MSHR[+Met]) and females (FSHR[+Met]), and male SHR with both 17β-estradiol (E2) and Met administration (MSHR[E2,+Met]). Treated groups received Met (1g/kg body weight per day) in water for 6 weeks. Systolic blood pressure (SBP) was monitored weekly. Aortic contractile (phenylephrine-induced) and relaxant (acetylcholine-induced as endothelium-dependent relaxation, or EDR) responses as well as endothelial suppression (with nitric oxide synthase inhibitor) were evaluated at the end of experiments. Serum homocysteine (Hcy) level was also determined. Met overloading caused a nearly 3-fold increase in serum Hcy in each gender (moderate hyperhomocysteinemia, or HHcy). As age increased, SBP increased in all groups; FSHR(+Met) had the least elevation and significantly less increase of SBP than FSHR at the end of 6 weeks. There was also a significant increase of EDR in FSHR(+Met) compared with both FSHR and MSHR(+Met). FSHR(+Met) had the highest level of endothelium suppression. Furthermore, EDR in MSHR(+E2,+Met) was significantly higher than that in MSHR(+Met). Direct Hcy feeding appeared to reduce the development of hypertension in female SHR in 3 weeks. Hence, SBP development was partially alleviated, whereas EDR and endothelial suppression were enhanced in female SHR with HHcy. E2 could mimic the gender-dependent effect of HHcy on EDR enhancement in MSHR; moreover, reduction of SBP development occurred in Hcy-fed FSHR. (Hypertension. 2002;40:322-328.)

Key Words: gender ■ relaxation ■ blood pressure ■ endothelium ■ rats, spontaneously hypertensive ■ plasma

Moderate hyperhomocysteinemia (HHcy) is an independent risk factor of myocardial infarction, stroke, and atherosclerosis. Plasma Hcy level is affected by genetic disorders, kidney disease, smoking, high methionine (animal protein) diet, and vitamin deficiencies (for review, see Eikelenboom et al). Male sex and postmenopausal status are two demographic characteristics of HHcy as well. In general, male subjects have higher plasma Hcy levels than female subjects. After menopause, both the plasma Hcy concentration1 and the incidence of coronary artery disease2 in women increase. Administration of estrogen in men and postmenopausal women is associated with a reduction in both the plasma Hcy level3-7 and the incidence of coronary artery disease. Furthermore, men and women have different responses in the Hcy increment after oral methionine loading. Thus gender difference appears to play a role in Hcy metabolism and vascular responses in humans with HHcy. However, the effect of HHcy and gender dependence, if any, on blood pressure (BP) is unclear.

To test the effect of moderate HHcy under hypertensive conditions, spontaneously hypertensive rats (SHR) were examined. The SHR has a higher BP than its normotensive counterpart (the Wistar-Kyoto rat, WKY), and the BP of SHR is readily modifiable; for example, estrogen or SOD-fusion protein administration suppresses the development of BP in SHR but not in WKY. In addition, plasma nitric oxide (NO) level, aortic nitrate level, and the activity of endothelial NO synthase (eNOS) in the aorta are higher in SHR compared with WKY. The response of in vivo NOS inhibitor treatment on BP is also greater in SHR than in WKY. Since the interaction between NO and Hcy is important concerning the effect of moderate HHcy, the more sensitive NO/NOS level in SHR also makes it an ideal model. Furthermore, elevated Hcy can induce oxidative damage by the production of free radical species (H2O2, O2-) from autooxidation. In the same methionine loading protocol, SHR have more arteriosclerosis-like alterations compared with WKY. Therefore, the SHR is more sensitive to oxidative stress-induced vascular damage and is useful for investigating the effects of moderate HHcy on BP and concurrent vascular responses.

Although a significant amount of data has indicated that female subjects are less prone to cardiovascular diseases and such protection is partly due to estrogen (for review, see Kauser and Rubanyi), it is not known whether the effects of HHcy can be alleviated by estrogen. These questions are...
approached by comparing male and female SHRs under moderate HHcy conditions and testing the effect of estrogen on vascular response in male SHRs.

Methods

Animals and Treatments

SHRs were obtained from the National Science Council and maintained in the Animal Center of Chang Gung University. Eleven-week old SHRs with similar heart rate (HR) and BP were randomized into 5 groups: male SHR (MSHR), male SHR with methionine administration (MSHR [+Met]), female SHR (FSHR), female SHR with methionine administration (FSHR [+Met]), and male SHR with both 17β-estradiol (E2) and methionine (Met) administration (MSHR [+E2, +Met]). During the experimental period, body weight and water uptake were measured weekly. HR and systolic BP (SBP) were monitored weekly by the tail-cuff method (UR5000). MSHR (+Met) and FSHR (+Met) received L-methionine (1 g/kg body weight per day) and succinylsulfathiazole (SST; 0.5 g/kg body weight per day) in tap water for a period of 6 weeks, starting at age 13 weeks. SST was used to inhibit the proliferation of bacteria and thus reduce folate production in the rat intestine. In other experiments, plasma homocysteine level ([Hcy]) was raised by administration of Hcy in tap water (300 μmol/L) for 3 weeks in female SHR. MSHR (+E2, +Met) received E2 (once per week subcutaneously) suspended in cholesterol-free corn oil (2 mg/kg body weight per day) in addition to Met (1 g/kg body weight per day) and SST (0.5 g/kg body weight per day).

Determination of Serum Hcy Level

Rats were killed, and blood was collected and total serum Hcy concentrations were measured by high-performance liquid chromatography with fluorometric detection (excitation at 385 nm and emission at 515 nm) according to the Ubbink method.

Aortic Contractile and Relaxant Response

Thoracic aorta were isolated and cleaned immediately and cut into 2-mm lengths in Krebs solution with the composition (in mmol/L) of 118.4 NaCl, 25 NaHCO3, 11.66 glucose, 4.75 KCl, 1.18 MgSO4, 7H2O, 2.5 CaCl2, 2H2O, 1.19 KH2PO4, and 0.02 EDTA maintained at pH 7.4 and bubbled with 95% O2–5% CO2. Aortic rings were carefully mounted on the isometric force transducer in the organ chamber (95% O2–5% CO2, at 37°C) and were equilibrated for 90 minutes with resting tension of 1.8 g. Phenylephrine (PE, 10 μmol/L) was used to induce vasoconstriction, and acetylcholine (ACh, 30 μmol/L) was then used to assess the integrity of endothelium. After reequilibration for 30 minutes, PE (1 μmol/L) was added; under fully developed tension, cumulative ACh dose (10–8 to 3×10–5 mol/L) was used and vasodilator response obtained. After washout and reequilibration, cumulative PE dose (10–10 to 10–7 mol/L) was applied and vasoconstrictor response was obtained. Next, the rings were incubated with NOS inhibitor [L-NNA] (0.1 mmol/L) for 30 minutes followed by PE (1 μmol/L) treatment. Endothelium suppression, the increase of contractile tension in the presence of L-NNA, calculated as [(Tension (with L-NNA)−Tension (control))/Tension (control)]×100%, of each gender, was compared (MSHR versus FSHR [+Met]; FSHR versus FSHR [+Met]) to assess the effect of HHcy on basal NO release.

Drugs

All drugs were purchased from Sigma. 17β-Estradiol (E2) was dissolved in pure DMSO and then mixed with cholesterol-free corn oil (2:1000 vol/vol); SST was dissolved in solution of alkali hydroxides by addition of NaOH.

Statistical Analysis

Data are expressed as mean±SEM. Differences between mean values of multiple or two groups were analyzed either by ANOVA with a Tukey-Kramer multiple comparisons test or by Student’s t test. All comparisons were computed with the GraphPad Instat 2.0 software program. Significance was accepted at P<0.05.

Results

Physiological Variables After Methionine Loading

Body weight showed a clear difference between genders in control SHRs (MSHR versus FSHR) as they aged between week 11 and 18 (Figure 1A). Male rats were significantly heavier at week 11 and gained more weight. After Met loading, the difference narrowed from 108±15 g (n=6) in control to 69±5 g (n=6) in Met-loaded SHRs (Figure 1B) as the result of the suppression of weight gain in male rats. There was also a significant difference between genders in the HR of control SHRs (Figure 1C). FSHR had higher HR than MSHR between week 13 and 18. This difference was significantly reduced in Met-loaded SHRs (Figure 1D).

Blood pressure was determined weekly after Met loading for male and female SHRs (results are illustrated in Figure 2). In control rats (Figure 2A), SBP increased from 160 mm Hg to ≈200 mm Hg for each gender in a parallel manner as reported previously. In treated SHRs (Figure 2B), each gender began with SBP (157 mm Hg) identical to that in the control animals. After Met loading, MSHR (+Met) had a rapid initial increase in SBP, significantly higher than control at week 13 (P<0.01) and reaching a plateau of 210 mm Hg. FSHR (+Met) had a gradual and diminished increase in SBP, reaching a plateau of 180 mm Hg, significantly lower than FSHR at weeks 14 (P<0.05), 15 (P<0.005), and 18 (P<0.005). Thus, Met loading appears to aggravate the development of hypertension in male SHRs while suppressing it in female SHRs.

Serum Homocysteine Levels

The relation between Met loading and accumulation of Hcy in SHRs was examined. We found that homocysteine concentration ([Hcy]) in the serum of control SHRs was 4.0±0.2 μmol/L (n=4), whereas [Hcy] in the serum of Met-loaded SHRs was 11.5±2.3 μmol/L (n=12), significantly higher (P<0.05) than that of control. There was no difference of [Hcy] between genders. Thus, Met loading of SHRs produced a significant (~3-fold) increase in serum [Hcy] of each gender.

Endothelium-Dependent Relaxation and Endothelium Suppression Under Hyperhomocysteinemia

We next examined vascular contractile and relaxant properties in Met-loaded male and female SHRs. Figure 3 demonstrates the results of ACh-induced endothelium-dependent relaxation (EDR) of control (Figure 3A) and treated (Figure 3B) SHRs in aortic rings precontracted with PE (1 μmol/L). The stimulated EDR for rings of FSHR (+Met) was significantly higher than that of MSHR (+Met) over a wide range of ACh concentrations (Figure 3B). Table 1 summarizes the dose-dependent pattern (Rmax and ED50) of ACh-induced EDR. There was no significant gender difference of Rmax (degree of relaxation) in control rats; however, Met loading in female SHRs produced a significant increase in Rmax when compared with both FSHRs and MSHR (+Met). Furthermore,
the sensitivity (reduction of ED₅₀) to ACh-induced relaxation also increased in FSHR(+/H11001 Met).

We next examined vasoconstriction induced by PE (1 μmol/L) in the absence and presence of L-NNA (0.1 mmol/L, a selective inhibitor of endothelial NOS). The increase of contractile tension in the presence of L-NNA, termed endothelium suppression, was determined and is shown in Figure 3C. Female SHR had higher endothelium suppression in both control SHR (open columns) and treated SHR (hatched columns) compared with MSHR and MSHR(+/H11001 Met), respectively. The results that control SHR showed gender differences were consistent with earlier reports. However, neither the magnitude of endothelium suppression nor the gender difference of endothelium suppression showed significant difference between the control and treated groups.

Contractile Response Under Hyperhomocysteinemia
Contractile response of aortic rings was also altered. While the maximal tension induced by PE was similar in each gender with or without Met loading, the sensitivity was significantly increased in MSHR(+/H11001 Met) such that it became significantly higher than that of MSHR as well as FSHR(+/H11001 Met) (Table 2). There was no significant change in female rats.

Estrogen Injection in Male SHR Under Hyperhomocysteinemia
The effect of gender on Met loading could involve an E₂-dependent mechanism; therefore, we studied male SHR coadministered E₂ and Met loading. After 6 weeks of treatment, EDR of aortic rings of MSHR(+E₂+Met) was significantly enhanced compared with MSHR(+/H11001 Met) (Figure 4). The relaxation response to ACh of MSHR(+E₂+Met) was not significantly different from MSHR(+/H11001 Met) at an ACh concentration of 10⁻⁸ mol/L, and the difference became significant (P<0.008 or better) when ACh concentration exceeded 10⁻⁷ mol/L (up to 10⁻⁵ mol/L). However, the SBP at week 14 (201.8±2.5), week 15 (206.0±4.4), or week 18 (213.5±4.7) of MSHR(+E₂+Met) was not significantly different from MSHR(+/H11001 Met) groups (Figure 2B).

Direct Homocysteine Administration in Female SHR
To test the gender-dependent effect of Hcy on BP directly, female SHR with established hypertension (216±5 mm Hg) were treated with drinking water containing Hcy. We found that SBP slightly increased at week 2 but gradually decreased in the following 2 weeks (Figure 5). The SBP at week 3 (after Hcy treatment) was significantly lower than that at week 0 (P=0.046). The SBP of control group was not significantly different from that of the Met-administered group (Figure 4).
altered (data not shown). The vascular response was not determined in this group of animals.

**Discussion**

When elevation of [Hcy] (HHcy) occurred, we found that development of SBP was accelerated in male SHR (Figure 2) but significantly reduced in female SHR. An increase of ACh-induced relaxation (EDR) and an enhanced sensitivity (to ACh) of isolated aortic rings derived from FSHR (+Met) were also discovered (Figure 3 and Table 1), and that similar improvement of EDR was observed in estrogen-treated MSHR (+Met) (Figure 4) as well. Taken together, the present results indicate a gender-dependent response to HHcy in both SBP development and vascular functions. There was therefore an association between HHcy and improved EDR in female SHRs.

Positive association between [Hcy] and both SBP/diastolic BP has been reported in humans. Elevated [Hcy] is considered to cause isolated systolic hypertension in some individuals. Acute Met loading induces an increase in [Hcy] and impairs flow-mediated endothelium-dependent vasodilation but does not alter SBP in healthy adults. In animal models, 4 months of a Met-rich diet induces mild HHcy in male minipigs and causes significant increase in SBP/diastolic BP with vascular abnormalities. Matthias et al reported that with similar Met feeding, male SHR have a higher [Hcy] than that of normotensive rats and exacerbated vascular damages including endothelium loss. However, Ungvari et al found no significant effect of HHcy on BP after 4-week Met loading in male rats. In anesthetized male rats, superfusion with Hcy (1 mmol/L) and Cu on the parietal cortex did not alter BP, either. The present results show that HHcy exerted an accelerating effect on the development of SBP and adverse effects on vascular responses in male rats but not in female rats. In fact, the development of hypertension in

**TABLE 1. ACh-Induced EDR in Aortic Rings of SHR**

<table>
<thead>
<tr>
<th></th>
<th>Rmax (%)</th>
<th>ED50 (10⁻⁸ mol/L)</th>
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<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>−Met</td>
<td>72.8±4.0</td>
<td>71.5±2.6</td>
</tr>
<tr>
<td>+Met</td>
<td>69.4±2.7</td>
<td>91.2±2.1*†</td>
</tr>
</tbody>
</table>

*P<0.05 between without (−Met) and with (+Met) methionine-loading in the same gender.
†P<0.05 between genders in the presence of +Met.
TABLE 2. PE-Induced Contractile Response in Aortic Rings of SHR

<table>
<thead>
<tr>
<th></th>
<th>Tmax (g/mg tissue)</th>
<th>ED₅₀ (10⁻⁸ mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>−Met</td>
<td>0.29±0.02</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>+Met</td>
<td>0.30±0.02</td>
<td>0.28±0.03</td>
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*P<0.05 between without (−Met) and with (+Met) methionine-loading in the same gender.
†P<0.05 between genders in the presence of +Met.

Figure 4. Acetylcholine-induced relaxation in MSHR (+Met) without (open column) or with (hatch column) estrogen coadministration, that is, MSHR (+Met+E₂). Data are mean±SEM (n=6). *P<0.05 between MSHR (+Met) without and with estrogen coadministration.

Figure 5. Change of SBP in female SHR with direct Hcy administration (300 μmol/L) in drinking water. Hcy treatment began at week 0. Data are mean±SEM (n=6). *P<0.05 between weeks 0 and 3.

FSHR was reduced in conjunction with moderate HHcy (Figure 2).

Previous morphological study suggests that aortic endothelium shows exacerbated damage in Met-loaded SHR; we thus further investigated the aortic contractile and relaxant properties. HHcy enhanced sensitivity to the vasoconstrictor in MSHR (Table 2). This is consistent with previous findings in monkeys after diet-induced HHcy. However, there was no effect on FSHR. In FSHR (+Met), ACh-induced relaxation of aortic rings was enhanced (Figure 3B), with a 28% increase (Table 1). In addition, sensitivity to ACh in aortic rings of FSHR (+Met) was significantly enhanced after Met loading, which resulted in a significantly stronger EDR than that in MSHR (+Met).

Earlier works demonstrate that in normotensive subjects and (male) rats, HHcy depresses flow-induced or ACh-induced EDR. On the one hand, our findings for SHRs confirmed that HHcy exerted negative effect in male rats; on the other hand, our results showed significant improvements in female rats. The reasons for these differences are not clearly known, but existing evidence supports that these observations are related to the characteristics of female sex and hypertension. First, Chao et al. reported that Met loading–induced HHcy decreases the level of p-selectin (taken as index of oxidative stress) in young female subjects but not in male adults, which suggests a gender-dependent response to HHcy. Second, SHRs are found to express a higher level of NO than their normotensive counterpart. Because NO can detoxify Hcy by forming vasodilatory S-nitroso-homocysteine (S-NO-Hcy), both the elevated NO level and S-NO-Hcy in SHR could contribute to ACh-induced EDR. Moreover, E₂ can reduce reactive oxygen species generation in endothelial cells, suggesting a potential mechanism in female protection. Third, we observed a significantly higher (24%) ACh-induced relaxation in MSHR (+E₂+Met) compared with MSHR (+Met) (Figure 4), suggesting that E₂ can mimic the observed female-specific enhancement of EDR induced by HHcy. Therefore, the gender-dependent vascular response in SHR appeared to be estrogen-related.

Although female-specific enhancement of EDR induced by HHcy could be mimicked by exogenous E₂ administration in MSHR (Figure 4), the reduced SBP development was not observed in MSHR (+E₂+Met) compared with MSHR (+Met). Previous studies also showed that changes in BP did not always correlate with vascular responses in postmenopausal women receiving estrogen.

Interestingly, in the presence of an eNOS agonist such as bradykinin, HHcy causes an increase of S-nitrosothiol production concomitantly with increased eNOS activity and mRNA level. This effect is potentially protective as the result of the endothelium-derived relaxing factor–like properties of S-nitrosothiol and is not shared by cysteine or glutathione. Because E₂ is known to stimulate eNOS, resulting in beneficial vascular effects, our findings (HHcy enhanced EDR in female rats and that such effect could be mimicked by E₂) are consistent with the view that stimulation of endogenous NO may ameliorate endothelial injury, which is otherwise induced by HHcy. Whether the HHcy-stimulated EDR was connected to change of BP is unknown. However, we found that female SHR with established hypertension had a reduction of SBP when Hcy was added in drinking water for 3 weeks (Figure 5). Because HHcy was achieved by directly adding Hcy, the SBP-lowering effect probably was not caused by a higher Met level. Nevertheless, the role of E₂ in SHRs is more complicated. Superoxide production in isolated aortic segments of SHRs after ovari-
ecstasy (5 weeks) was significantly higher than that of sham SHRs, whereas carbachol-induced NO release was not different. Similar experiments with SHR with HHcy should provide further testing for the E2-HHcy interactions in SHRs.

In conclusion, we found that methionine loading for 6 weeks caused a moderate increase (3-fold) in Hcy level (HHcy) in serum in each gender. The treatment attenuated the development of hypertension and improved endothelial functions (both EDR and endothelium suppression) in FSHRs but not in MSHRs. Estrogen administration in MSHRs mimicked the gender difference for endothelial improvement but not the effect on BP. Direct feeding of Hcy to FSHRs reduced SBP; taken together, these data suggest that HHcy in FSHRs could be beneficial.

Perspectives

A moderately elevated plasma level of homocysteine (HHcy) is an independent risk factor for atherosclerosis and atherothrombosis and is associated with an increased risks for ischemic stroke. It is also an independent risk factor for vascular balance. Hypertension enhances production of both agonists for eNOS. Our results that estrogen could be beneficial.

Acknowledgments

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References


15. Hayakawa H, Rais L. The link among nitric oxide synthase activity, endothelial function, and NO production.45 Apparently, many factors enter the above equation and that both estrogen and HHcy are important components when considered individually. Hypertension is a compounded factor for the equation, and its potential interactions with gender difference and hyperhomocysteinemia are unknown. Our results may reflect the complex interactions among these protective and adverse factors of vascular balance. Hypertension enhances production of both NO4.45 and superoxide48 in SHRs; estrogen regulates NO production both transcriptionally and posttranslationally49, whereas HHcy may increase or decrease NO, depending on the duration of HHcy exposure17 and the presence or absence of agonists for eNOS.37 Our results that estrogen could normalize and even enhance EDR in hypertensive rats illustrate that complex interactions among estrogen, HHcy, and hypertension could provide paradoxical protection in female subjects with HHcy. Both epidemiologic investigation and mechanistic study are required to further delineate this situation.


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