Moderate Hyperhomocysteinemia Decreases Endothelial-Dependent Vasorelaxation in Pregnant But Not Nonpregnant Mice

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Abstract—Increased homocysteine is associated with the pregnancy complication preeclampsia and with later-life cardiovascular disease. Although elevated homocysteine persists after pregnancy, the vascular changes of preeclampsia abate with delivery, and cardiovascular disease occurs decades later. This suggests the vasculature during pregnancy may manifest increased sensitivity to homocysteine. We used the cystathionine-β synthase (CBS)–deficient transgenic mouse to investigate whether hyperhomocysteinemia would differentially affect vascular function in nonpregnant and pregnant animals. Mesenteric arteries from nonpregnant and midpregnant (14 to 16 days) wild-type, heterozygous, and homozygous CBS-deficient transgenic mice were investigated for their response to vasoconstriction, endothelial-dependent, and endothelial-independent relaxation using an isometric wire myograph system. Endothelial-dependent vasodilation was similar in arteries from nonpregnant heterozygous and wild-type mice. In contrast, endothelial-dependent relaxation was reduced significantly in arteries from pregnant heterozygous animals compared with wild-type mice. Inhibition of NO synthesis blunted relaxation in arteries from pregnant wild-type but not pregnant heterozygous mice. Endothelial-dependent relaxation was restored by in vitro pretreatment with the tetrahydrobiopterin precursor sepiapterin. These data indicate that in pregnant mice, endothelial-dependent vasodilation is more sensitive to the effect of increased homocysteine than arteries from nonpregnant mice. This effect appears to result from a loss in NO-mediated relaxation that may be mediated by the oxidative inactivation of the NO synthase cofactor tetrahydrobiopterin. (Hypertension. 2004;44:327-333.)

Key Words: endothelium ■ nitric oxide ■ pregnancy

Increased homocysteine is associated with an increased risk of stroke, peripheral vascular disease, and coronary artery disease. Recent studies demonstrate that moderate hyperhomocysteinemia in experimental models is associated with impaired endothelial-dependent vasodilation. Several mechanisms have been proposed to explain the homocysteine-mediated loss in endothelial-dependent vasodilation, including oxidative stress and altered cellular methylation patterns. However, the precise mechanism(s) by which increased homocysteine alters endothelial function are not understood.

Preeclampsia is a specific hypertensive disorder of pregnancy and is a leading cause of maternal and fetal mortality. Endothelial dysfunction is proposed to be a central feature of preeclampsia pathophysiology resulting in altered vascular reactivity, activation of the coagulation cascade, and loss of vascular integrity. Several studies report that moderate hyperhomocysteinemia is more common in women with preeclampsia (20% to 30%) compared with normal pregnant women (2% to 3%). Women with a history of preeclampsia also have an increased prevalence of moderate hyperhomocysteinemia. This raises the question: If homocysteine remains elevated (and actually increases) after pregnancy, why does the vascular dysfunction present in preeclampsia resolve with delivery? Therefore, we asked whether pregnancy might increase vascular sensitivity to perturbing agents such as homocysteine.

A transgenic mouse model of hyperhomocysteinemia was developed by Watanabe et al in 1995 by targeted disruption of the gene for cystathionine-β-synthase (CBS; Figure 1). This model has been used by other investigators to study homocysteine-mediated changes in vascular function and the possible mechanism(s) involved in alterations in vascular function. However, none of these studies assessed the effect of pregnancy on homocysteine sensitivity, and none investigated the function of the vasculature from the homozygous CBS knockout (KO) mice, which have 20-fold greater plasma homocysteine concentrations compared with wild-type (WT) mice. Therefore, the focus of this study was to investigate the effect of increased homocysteine on vascular...
function in nonpregnant and midpregnant female CBS transgenic mice. We posited that although large increases in homocysteine present in homozygous mutant animals would alter vascular function to a greater extent in pregnant than in nonpregnant CBS transgenic mice. Finally, we attempted to begin to discern the mechanism by which increased homocysteine affects vascular function in pregnancy.

Methods

Animals
Female littermates of the CBS-deficient transgenic mouse model were used for all experiments. Mice were all between 3 and 4 months old and after weaning, were fed a standard rodent chow diet (Prolab RMH 2000, Purina Mills). Methionine-treated mice were provided drinking water that contained 0.5% L-methionine for 4 weeks before experimentation. Females were mated with heterozygous (HT) CBS-deficient males, and day 1 of gestation was considered to be the morning on which a copulation plug was found. Between days 14 and 16 of gestation, mice were anesthetized with 2.5 mg of sodium pentobarbital administered intraperitoneally (Abbott Laboratories), and blood was collected by cardiac puncture and tubes containing EDTA (final concentration 5 to 10 mmol/L) for homocysteine measurement. Mice were euthanized by cervical dislocation, and the mesentery was dissected and placed in ice-cold HEPES-buffered physiological saline. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Magee-Women’s Research Institute.

Homocysteine Determination
Plasma homocysteine was measured by high-performance liquid chromatography with fluorescent detection. The methodology was a modification of the published method of L.J. Fortin and J. Genest Jr.19 A quality-control plasma sample was included in each analysis, and the interassay variability is <5%.

Vascular Responses
Mesenteric arteries (=190 μm in diameter) were dissected from the surrounding adipose tissue and mounted in a 5-mL water-jacketed organ bath kept at 37°C. As reported previously, arteries were kept in a HEPES-buffered physiological saline solution (142 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH2PO4, 1.17 mmol/L MgSO4, 7H2O, 2.5 mmol/L CaCl2, 2H2O, 10 mmol/L HEPES, and 5.5 mmol/L dextrose, pH 7.4) and were mounted on 15-μm wires in a Mulvany myograph system (details available in an online supplement at http://www.hypertensionaha.org).

Dose-response curves were generated by cumulative addition of the α-adrenergic agonist phenylephrine (10-4 to 10-3 mol/L). Arteries were then washed with several changes of HEPES buffer and were contracted to ~50% of maximum with phenylephrine, and relaxation dose responses were generated by cumulative additions of the endothelium-dependent vasodilator methacholine (10-6 to 10-3 mol/L) or the endothelium-independent vasodilator sodium nitroprusside (SNP; 10-10 to 3×10-5 mol/L). Arteries were also preincubated for 30 minutes with 10-4 mol/L Nω-nitro-L-arginine methyl ester (L-NAME) to inhibit NO synthase (NOS) or 10-6 mol/L sepiapterin in dimethyl sulfoxide (DMSO) as vehicle and evaluated for their response to agonist-stimulated contraction and relaxation as described above. All chemicals were purchased from Sigma.

Nitrotyrosine Western Blots
Mouse plasma samples were diluted 1:10 in extraction buffer (100 μL of 1 mol/L Tris, pH 6.8, 2 mL of glycerol, 1 mL of 10% sodium dodecyl sulfate, and 6.9 mL of distilled water). Ten microliters of each diluted plasma sample was loaded per lane onto Carbix PAGEr Tris-glucine 10% gel and run at 80 V. The primary antibody was a mouse monoclonal anti-nitrotyrosine antibody from Calbiochem diluted 1:1000, and the secondary antibody was a preabsorbed anti-mouse antibody conjugated to alkaline phosphatase from the Jackson Laboratory. Both antibodies were diluted in 5% (wt/vol) nonfat milk in tris-buffered saline tween 20. Protein bands were imaged using an enhanced chemiluminescence kit (Amersham Pharmacia), and films were electronically scanned and band densities quantified.

Statistical Analysis
Comparisons of homocysteine concentrations, vessel wall thickness, and diameter were done by 1-way ANOVA with Bonferroni–Dunn post hoc testing. Comparisons of vascular responses between groups were analyzed by ANOVA with repeated measures, and comparisons between effects of individual agonist concentrations between groups were performed using unpaired 2-tailed Student t tests. Values are reported as mean±SE. Significance was accepted at P<0.05.

Results

Body Weight, Vessel Measurements, and Experimental Homocysteine Concentrations
CBS KO mice had a significant decrease in body weight at weaning that persisted into adulthood compared with WT and HT mice (Table). However, there were no significant differences in litter size, wall thickness, or luminal diameter between the different mice genotypes. Specifically, wall thickness of arteries from the 3 mice genotypes were WT 15.2±0.8 μm, HT 16.6±2.1 μm, and KO 15.9±2.2 μm (P=0.64; Table). Luminal diameters of the mesenteric arteries among the 3 genotypes of nonpregnant mice were WT 188.7±17 μm, HT 179.2±13 μm, and KO 196.8±18 μm (P=0.21; Table). There was also no difference in luminal diameters of the arteries from pregnant mice (pregnant WT 197.0±15 μm versus pregnant HT 197.1±25 μm; P=0.99; Table). And there was no difference in luminal diameters between arteries from nonpregnant and pregnant mice (P=0.13).

Plasma homocysteine concentrations were significantly different among the 3 genotypes of nonpregnant CBS transgenic mice. Values were highest in the homozygous KO (361.9±146 μmol/L), next highest in the HTs (106.5±4 μmol/L), and lowest in WTs (2.9±2 μmol/L; P<0.001 all; Table).
The difference by genotype persisted in pregnancy with HTs (14.4±8 μmol/L) and WTs (6.1±3 μmol/L, P=0.03; Table). Although there was a modest increase in plasma homocysteine concentrations in the pregnant compared with the nonpregnant mice, this increase was not statistically significant within the individual mouse genotypes. Methionine treatment also increased plasma homocysteine in the nonpregnant mice, this increase was not statistically significant within the individual mouse genotypes (data not shown). There was also no difference in phenylephrine sensitivity similarly in arteries from both genotypes of pregnant mice (Figure 3A). However, arteries from KO mice exhibited a significant decrease in endothelium-dependent vasorelaxation in response to methacholine stimulation compared with arteries from WT and HT mice (Figure 3B). Methacholine-stimulated maximal relaxation in arteries from KO mice was significantly less than in arteries from WT and HT mice (50±7%, 85±4%, and 90±2% respectively; P<0.01). There was no difference in sensitivity of WT or HT mice to methacholine. l-NAME had no effect on the methacholine-stimulated relaxation response in arteries from KO mice (Figure 4A); however, l-NAME significantly blunted relaxation of arteries from WT and HT mice similar to that of untreated vessels from KO mice (Figure 4B and 4C).

### Vascular Responses in Pregnant Mice

Dose-response curves were generated to phenylephrine, methacholine, and SNP in mesenteric arteries from midpregnant WT and HT mice (the female CBS KO mice failed to breed and therefore could not be investigated). Dose-response curves to phenylephrine were similar in arteries from pregnant WT and HT CBS mice (EC50=3.2±1.0 μmol/L WT and 3.5±1.6 μmol/L HT; P=0.09). As described previously, arteries from pregnant mice were significantly less sensitive to phenylephrine than arteries from nonpregnant mice (EC50=3.2±1.0 μmol/L versus 2.0±1.7 μmol/L respectively; P<0.0001). Pretreatment with l-NAME increased sensitivity to phenylephrine similarly in arteries from both genotypes of pregnant mice (EC50=1.8±0.3 μmol/L WT versus 1.6±0.2 μmol/L HT; P=0.52), and there was no difference in phenylephrine sensitivity in endothelium-

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**Figure 2.** Increased homocysteine does not affect phenylephrine-induced contraction. Dose-response curves to phenylephrine for mesenteric arteries from nonpregnant CBS transgenic mice (circles represent WTs n=6; squares, HTs n=7; triangles, KOs n=6) in the absence (○, □, and △) or presence of l-NAME (●, ■, and ▲). Data are expressed as the percentage of maximum phenylephrine response. Data represent mean±SE.
denuded arteries between pregnant WT and HT mice (data not shown).

Similar to arteries from nonpregnant mice, there was no difference in relaxation response to the endothelium-independent vasodilator SNP in arteries from pregnant mice (data not shown). However, in contrast to arteries from nonpregnant mice, the methacholine-stimulated endothelial-dependent relaxation response in arteries from the pregnant HT CBS mice was significantly blunted compared with arteries from pregnant WT mice (59±16% versus 93±2% respectively; \( P<0.01 \); Figure 5A). L-NAME treatment blunted the relaxation response of arteries from pregnant WT mice and had no effect on arteries from pregnant HT mice (53±12% L-NAME–treated pregnant WT and 58±7% L-NAME–treated pregnant HT versus 59±16% untreated pregnant HT; \( P=0.95 \); Figure 5B).

To determine whether the difference in relaxation response to methacholine during pregnancy compared with arteries from nonpregnant mice was mediated by the moderate increase in plasma homocysteine observed in the pregnant mice, we compared the methacholine-mediated relaxation response of arteries from nonpregnant mice and methionine-treated nonpregnant mice. Methionine treatment of nonpregnant mice increased plasma homocysteine to concentrations

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**Figure 3.** Increased homocysteine does not affect endothelial-independent relaxation but does affect endothelial-dependent relaxation. A, Dose-response curves to SNP for mesenteric arteries from nonpregnant CBS transgenic mice. B, Dose-response curves to methacholine for mesenteric arteries from nonpregnant CBS transgenic mice. ○ represents WTs \( n=6 \); □, HTs \( n=7 \); △, KOs \( n=6 \). Responses are expressed as a percentage of relaxation from phenylephrine (PE)-preconstricted levels. Data represent mean±SE. *\( P<0.05 \) compared with WT.

**Figure 4.** Inhibition of NOS does not affect endothelial-dependent relaxation in arteries from severe hyperhomocysteinemic mice. Dose-response curves to methacholine for mesenteric arteries from nonpregnant CBS transgenic mice. A, Arteries from CBS KO mice not treated with L-NAME (○ represents \( n=6 \)) or treated with L-NAME (●, \( n=6 \)). B, Arteries from WT mice not treated with L-NAME (○, \( n=6 \)) or treated with L-NAME (●, \( n=6 \)). C, Arteries from CBS HT mice not treated with L-NAME (○, \( n=7 \)) or treated with L-NAME (●, \( n=7 \)). PE indicates phenylephrine. Data represent mean±SE. *\( P<0.05 \) compared with WT.
comparable to those present in pregnant mice (Table). Despite this increase in plasma homocysteine, the methacholine-mediated relaxation response of the arteries from nonpregnant methionine-treated WT and HT mice was not significantly different from that of arteries from untreated nonpregnant mice (methionine-treated nonpregnant WTs 79 ± 3% versus nonpregnant WT 85 ± 4%; P = 0.09; and methionine-treated nonpregnant HTs 88 ± 6% versus nonpregnant HTs 90 ± 2%; P = 0.23).

We also investigated whether there was a significant increase in oxidative stress as assessed by increased circulating nitrotyrosine. There was no significant difference in nitrotyrosine-modified plasma proteins as assessed by Western blot between any of the mice genotypes during pregnancy or in nonpregnant animals (data not shown).

We further investigated the altered methacholine-mediated relaxation response of arteries from pregnant hyperhomocysteinemic HT mice by testing whether the tetrahydrobiopterin precursor sepiapterin was capable of restoring the endothelial-dependent relaxation response. Arteries from pregnant WT and hyperhomocysteinemic HT CBS mice were preincubated with sepiapterin in vitro (1 μmol/L final concentration) or vehicle. In vitro pretreatment with the tetrahydrobiopterin precursor sepiapterin improved the endothelial-dependent relaxation response of arteries from pregnant HT mice compared with vehicle-treated HT controls (88.9 ± 6.7% versus 56.2 ± 6.7%, respectively; P < 0.01; Figure 6A), but sepiapterin had no effect on arteries from pregnant WT mice when compared with vehicle-treated WT controls (86.6 ± 5.2% versus 81.2 ± 7.8%; P = 56; Figure 6B).

**Discussion**

The focus of this study was to determine the effect of increased homocysteine on vascular function during preg-
nancy and to begin to elucidate mechanisms responsible for these homocysteine-mediated changes in vascular function. The main finding is that a moderate increase of homocysteine is associated with blunted endothelial-dependent relaxation in arteries from pregnant but not in nonpregnant CBS transgenic mice. This loss of endothelial-dependent relaxation appears to be mediated by a loss of NO-mediated response. Although homocysteine concentrations were slightly but not significantly higher in pregnant mice, this did not appear to explain the differences in response because increasing homocysteine concentrations in nonpregnant animals by methionine loading did not reduce endothelial-dependent relaxation. However, the decreased endothelial-mediated relaxation was improved by providing exogenous sepiapterin, a substrate for the synthesis of tetrahydrobiopterin, an important cofactor for NOS function. Finally, large increases in homocysteine significantly decreased endothelial-dependent relaxation in arteries from nonpregnant CBS KO female mice. The absence of modification of vascular function by the NOS inhibitor l-NAME indicates that this decrease in endothelial-dependent relaxation also appears to be mediated by a loss in NO.

Recent investigations suggest that increased homocysteine impairs endothelial-dependent vasorelaxation by promoting increased oxidative stress.\textsuperscript{20,21} For several years, increased homocysteine has been linked to increased oxidative stress. The exact mechanism by which homocysteine leads to oxidative stress has been debated, but the most likely explanation is that homocysteine itself forms oxygen-free radicals as it becomes oxidized and forms homocysteine and other oxidized thiols. The alternative answer is that increased homocysteine impairs activity of oxygen-free radical-scavenging systems such as glutathione peroxidase.\textsuperscript{22} This homocysteine-mediated increased oxidative stress could affect NO-mediated vascular function through at least 2 mechanisms. First, increased superoxide can reduce NO by converting it to the pro-oxidant peroxynitrite. However, in this study, we did not observe an increase in nitrotyrosine-modified plasma proteins between these groups. Importantly, this negative result does not exclude oxidative stress as contributing to changes in vascular function because this measure may be more related to whole-body oxidative stress. A second possibility is that reactive oxygen species and localized oxidative stress can oxidize and inactivate NOS cofactors such as tetrahydrobiopterin. Tetrahydrobiopterin is required to maintain the coupled status of NOS and promote NO production.\textsuperscript{23} In conditions in which tetrahydrobiopterin is limited because of oxidation or reduced synthesis, NOS activity is changed from producing NO to producing superoxide.\textsuperscript{24,25} In addition, treatment with tetrahydrobiopterin or sepiapterin has been shown to improve vascular function and restore NOS coupling and activity in subjects with cardiovascular disease, diabetes, hypercholesterolemia, and those who smoke.\textsuperscript{26–29} Exogenous tetrahydrobiopterin has also been shown to restore NO synthesis in cultured endothelial cells treated with homocysteine.\textsuperscript{30} Our data indicate that moderate increases in homocysteine during pregnancy may have a more pronounced effect on NOS coupling, possibly via tetrahydrobiopterin oxidation. This pregnancy-mediated effect of moderate hyperhomocysteinemia on vascular function is particularly intriguing in light of several other recent studies that report a similar significant pregnancy-specific blunting of NO-mediated relaxation in arteries from pregnant animals compared with nonpregnant animals when both groups of animals are subjected to the same insult (ethanol, tumor necrosis factor-\(\alpha\), or l-NAME).\textsuperscript{31–33} These insults also affect NOS coupling, particularly l-NAME, and therefore support the concept that the maternal vasculature during pregnancy may be more susceptible to agents that mediate NO uncoupling.

Previous studies of vascular function involving these same CBS-deficient mice have observed similar results. Lentz et al reported no difference in endothelial-dependent relaxation in aortas from WT and HT mice but a significant loss of acetylcholine-mediated relaxation in aortas from hyperhomocysteinemic HT mice compared with WT mice when both were placed on a low-folate diet.\textsuperscript{15} In contrast, Eberhardt et al reported a paradoxical acetylcholine-mediated vasoconstriction of superfused mesenteric arteries and a reduction in endothelial-mediated relaxation in aortas from hyperhomocysteinemic HT mice compared with WT mice.\textsuperscript{14} Additional studies have reported that this endothelial dysfunction can be restored by overexpressing the antioxidant enzyme glutathione peroxidase, suggesting that the altered vascular function is at least partly mediated by increased oxidative stress.\textsuperscript{18,22} These data do not conflict with our results and suggest that increased homocysteine can disrupt NO-mediated vascular function via an oxidative stress mechanism in pregnant and nonpregnant animals.

Perspectives
Moderate increases in homocysteine is a common condition (\(\approx\)5% of the population). These moderate increases in homocysteine have been associated with vascular dysfunction and several pregnancy complications, including neural tube defects, spontaneous abortion, preterm birth, low birth weight, and preeclampsia.\textsuperscript{34,35} Our data suggest that the maternal vasculature during pregnancy is particularly sensitive to moderate increases in homocysteine, leading to impaired maternal endothelial-dependent vascular function, and that in vitro treatment with the tetrahydrobiopterin precursor sepiapterin is capable of restoring this decreased endothelial-dependent relaxation. These data may help explain why the maternal vascular dysfunction of preeclampsia abates after delivery and why these same women have a significantly increased risk of future vascular disease. Finally, It is worth further exploring whether simple interventions such as increased folic acid intake (which is already recommended and has been demonstrated to reduce the incidence of neural tube defects) may reduce the risk of these pregnancy complications.

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References


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