Chronic diet-induced hyperhomocysteinemia impairs eNOS regulation in mouse mesenteric arteries

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Looft-Wilson RC, Ashley BS, Billig JE, Wolfert MR, Ambrecht LA, Bearden SE. Chronic diet-induced hyperhomocysteinemia impairs eNOS regulation in mouse mesenteric arteries. Am J Physiol Regul Integr Comp Physiol 295: R59–R66, 2008. First published April 30, 2008; doi:10.1152/ajpregu.00833.2007.—Hyperhomocysteinemia (HHcy) impairs endothelium-dependent vasodilation by increasing reactive oxygen species, thereby reducing nitric oxide (NO) bioavailability. It is unclear whether reduced expression or function of the enzyme that produces NO, endothelial nitric oxide synthase (eNOS), also contributes. It is also unclear whether resistance vessels that utilize both NO- and non-NO-vasodilatory mechanisms, undergo alteration of non-NO-mechanisms in this condition. We tested these hypotheses in male C57BL/6 mice with chronic HHcy induced by 6 wk high methionine/low-B vitamin feeding (Hcy: 89.2 ± 49.0 μM) compared with age-matched controls (Hcy: 6.6 ± 1.9 μM), using first-order mesenteric arteries. Dilation to ACh (10−9–10−4 M) was measured in isolated, cannulated, and pressurized (75 mmHg) arteries with and without Nω-nitro-arginine methyl ester (l-NAME) (10−4 M) and/or indomethacin (10−5 M) to test endothelium-dependent dilation and non-NO-dependent dilation, respectively. The time course of dilation to ACh (10−4 M) was examined to compare the initial transient dilation due to non-NO- non-prostacyclin mechanism and the sustained dilation due to NO-. These experiments indicated that endothelium-dependent dilation was attenuated (P < 0.05) in HHcy arteries due to downregulation of only NO-dependent dilation. Western blot analysis indicated significantly less (P < 0.05) basal eNOS and phospho-S1179-eNOS/eNOS in mesenteric arteries from HHcy mice but no difference in phospho-T495-eNOS/eNOS, S1179 eNOS phosphorylation was also significantly less in these arteries when stimulated with ACh ex vivo or in situ. Real-time PCR indicated no difference in eNOS mRNA levels. In conclusion, chronic diet-induced HHcy in mice impairs eNOS protein expression and phosphorylation at S1179, coincident with impaired NO-dependent dilation, which implicates dysfunction in eNOS post-transcriptional regulation in the impaired endothelium-dependent vasodilation and microvascular disease that is common with HHcy.

HYPERHOMOCYSTEINEMIA (HHCY), the elevation of blood levels of homocysteine, is a risk factor for coronary artery disease and is associated with a negative prognosis in those with existing cardiovascular disease (22). While the mechanistic link between HHcy and vascular disease is not entirely understood, one consistent finding is that HHcy reduces endothelium-dependent vasodilation (13, 19, 20, 29, 31), which is likely to be an underlying mechanism for its contribution to the vascular disease process. Evidence indicates that HHcy increases superoxide levels, thereby reducing the bioavailability of nitric oxide and the ability of arteries to dilate (2, 8, 21, 28). It is unknown, however, whether chronic HHcy decreases vascular expression and activation of endothelial nitric oxide synthase (eNOS), the enzyme that produces nitric oxide. It is also unclear whether a general loss of endothelial-dependent mechanisms other than nitric oxide and prostacyclin, common in resistance arteries, also contributes to impaired endothelium-dependent dilation in general, as this has only been examined in the renal circulation (11).

To investigate the mechanisms for impaired endothelium-dependent vasodilation in resistance arteries with HHcy, we tested the hypotheses that chronic dietary-induced HHcy (1) decreases eNOS expression and phosphorylation on serine1179 (S1179), a key phosphorylation site involved in eNOS activation (14) and eNOS-dependent dilation of arteries (1), and (2) decreases only the nitric oxide-dependent component of dilation. These parameters were examined in first-order mouse mesenteric arteries, as a representative resistance artery, which controls the magnitude of blood flow to the intestines. Moreover, disease in resistance vessels, in addition to conduit arteries, is a feature of HHcy (18). This artery dilates to agonists through roughly equal contribution from nitric oxide and non-nitric oxide mechanisms (30). Thus, it provides a model for examining the correlation between nitric oxide-dependent function and eNOS expression and regulation, and the relative contribution of the various mechanisms of endothelium-dependent dilation with HHcy. The non-nitric oxide component of endothelium-dependent dilation, attributed to endothelium-dependent hyperpolarizing factor (EDHF) in these arteries (12), has recently been shown to be responsible for early transient peak dilation, while nitric oxide is responsible for long-lasting dilation in both rat and mouse mesenteric arteries (15). For this reason, we measured the time course of ACh-induced dilation to examine the various components of endothelium-dependent dilation.

METHODS

Chronic hyperhomocysteinemia. All experimental procedures were approved by the William & Mary Institutional Animal Care and Use Committee and are in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC). Chronic HHcy was induced in male C57BL/6 mice (n = 124, ~6 wk of age; Charles River, Wilmington, MA) by providing a diet high in methionine (1.2%) and deficient in folate, vitamin B6, and...
vitamin B<sub>12</sub> (Harlan Teklad, TD 97345) for 6 or 7 wk, while control mice (n = 119, ~6 wk of age) were given standard rodent chow for the same time period. Serum homocysteine concentrations were measured by stable isotope dilution and liquid chromatography/tandem mass spectrometry, performed at Yale Biochemical Disease Detection Laboratory (New Haven, CT). Serum nitrate/nitrite concentrations were measured as an index of in vivo nitric oxide levels, using a commercial colorimetric assay kit (Cayman Chemical, Ann Arbor, MI).

At ~12 wk of age, mice were anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg body mass ip), and blood and various tissues were collected to perform the experiments described below. Mice were euthanized through exsanguination.

**Mesenteric artery function.** The mesenteric arcade was removed and placed in cold physiological saline solution (PSS)-MOPS buffer (in mM: 145 NaCl, 4.7 KCl, 2.0 CaCl<sub>2</sub>, 1.17 MgSO<sub>4</sub>, 1.2 Na<sub>2</sub>PO<sub>4</sub>, 2.0 MOPS, 0.02 EDTA, 5.0 glucose, 2.0 pyruvate, pH 7.4). A first-order mesenteric artery was removed, cannulated in a vessel chamber (Danish Myo Technology, Aarhus, Denmark), filled with PSS-MOPS, secured with nylon suture (8–0 S & T, Neuhause, Switzerland), gently cleared of blood, and pressurized to 75 mmHg with PSS-MOPS + 1% BSA perfusion, with no subsequent flow through the lumen. The chamber was mounted on a microscope stage (Danish Myo Technology) and equilibrated at 37°C for 30 min, with constant superfusion before testing functional responses. Lumninal diameter was measured using a 10× objective, charge-coupled device camera, and VediView Software (Danish Myo Technology).

In the first experimental series, diameter changes were measured to cumulative doses of phenylephrine (10<sup>−9</sup>–10<sup>−5</sup> M, 3 min between doses), followed by 10-min equilibration to the highest dose of phenylephrine, then by measurement of diameter changes to cumulative doses of ACh (10<sup>−9</sup>–10<sup>−4</sup> M, 3 min between doses). Maximal diameter was measured after superfusion with Ca<sup>2+</sup>-free PSS-MOPS + 1 mM EGTA for at least 15 min.

In the second series of experiments, non-nitric oxide/non-prostaglandin-dependent dilation was measured. After the 30-min equilibration, arteries were constricted with phenylephrine (10<sup>−5</sup> M), then incubated with N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME, 10<sup>−4</sup> M, a nitric oxide synthase inhibitor) and indomethacin (10<sup>−5</sup> M, a cyclooxygenase inhibitor used to block prostacyclin formation) for 30 min. Dilation to cumulative doses of ACh (10<sup>−9</sup>–10<sup>−4</sup> M) was measured, followed by maximal diameter determination with Ca<sup>2+</sup>-free PSS-MOPS + 1 mM EGTA.

To confirm that the dilation remaining after blockade of nitric oxide synhase and cyclooxygenase was due to a mechanism consistent with EDHF-dependent dilation, a separate group of mesenteric arteries was treated with high K<sup>+</sup> (35 mM)-PSS-MOPS (equimolar substitution of NaCl with KCl), to abolish K<sup>+</sup> channel-mediated hyperpolarization, a required event for EDHF-mediated dilation. This was followed by treatment with L-NAME (10<sup>−4</sup> M) and indomethacin (10<sup>−5</sup> M) for 30 min. To confirm that all three pathways were effectively blocked, a separate group of mesenteric arteries was treated with high K<sup>+</sup> (35 mM)-PSS-MOPS (equimolar substitution of NaCl with KCl), followed by dilation to cumulative doses of ACh (10<sup>−9</sup>–10<sup>−4</sup> M), which produced a similar constriction.

In the third series of experiments, the time course of dilation to ACh was examined with and without L-NAME (10<sup>−4</sup> M) and/or indomethacin (10<sup>−5</sup> M) or 35 mM K<sup>+</sup> treatment for 30 min. Vessels were constricted with phenylephrine (10<sup>−5</sup> M) for 10 min (except when preconstricted with 35 mM K<sup>+</sup>, which produced a similar magnitude of constriction), and dilation to a single dose of ACh (10<sup>−4</sup> M) was measured every minute for 30 min, followed by maximal diameter determination with Ca<sup>2+</sup>-free PSS-MOPS + 1 mM EGTA.

The ability of the smooth muscle to respond to nitric oxide was tested in a separate series of experiments by constriction with phenylephrine (10<sup>−5</sup> M) for 10 min, followed by dilation to cumulative addition of sodium nitroprusside (SNP, 10<sup>−2</sup>–10<sup>−4</sup> M), a nitric oxide donor, and maximal diameter determination with Ca<sup>2+</sup>-free PSS-MOPS + 1 mM EGTA. Vessel responses to SNP were tested with or without 30 min of pretreatment with L-NAME to eliminate the contribution of endogenous nitric oxide.

Dilation responses were calculated by % dilation = (diameter with ACh or SNP – preconstricted diameter)/(maximal diameter – preconstricted diameter)·100. Constriction responses to phenylephrine were calculated by diameter with phenylephrine/baseline diameter. Vessel function experiments were generally performed in one artery from individual mice.

**Agonist-induced phosphorylation of eNOS on S1179 in mesenteric arteries.** Control and HHcy mice were anesthetized and the entire intestinal tract and associated mesentery was stimulated with ACh either ex vivo, to examine phosphorylation in response to agonist stimulation, or in situ, to examine phosphorylation to both agonist stimulation and endogenous flow. For ex vivo stimulation, the tissue was tied off and removed, bathed in a Petri dish filled with PSS-MOPS (37°C) or PSS-MOPS + 10<sup>−4</sup> M ACh for 5 min; then first-order mesenteric arteries (5 or 6 arteries) were dissected (3–4 min) and flash frozen in liquid nitrogen. In two ex vivo experiments, dissections were performed in buffer plus phosphatase inhibitors (1 mM Na<sub>2</sub>VO<sub>4</sub>, 20 mM NaF, 1 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>) but yielded similar results to those without inhibitors; therefore, the data were pooled. For in situ stimulation, a midline incision was made to exteriorize the intestines, which were then bathed in PSS-MOPS (37°C) or PSS-MOPS + 10<sup>−4</sup> M ACh for 4 min. The intestine and mesentery were then rapidly tied off and removed and placed in ice-cold buffer; first- and second-order arteries and veins were removed and frozen within 30 s. The proportion of eNOS phosphorylated on S1179 (normalized to total eNOS) was compared in these tissues between each group using Western blot analysis, as described subsequently.

**mRNA expression in mesenteric arteries.** Real-time PCR was performed to quantify eNOS mRNA in mesenteric arteries (>5 pooled first-order arteries from the mesenteric bed of individual mice), normalized to smooth muscle alpha actin (SMAA) mRNA using primers and TaqMan probes previously reported (3).

Mesenteric arteries were dissected and immediately placed in RNALater (Ambion, Austin, TX) to preserve RNA integrity. RNA was isolated using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA), with the following cycling conditions: 50°C (10 min), 95°C (15 s), and 60°C (1 min). Relative standard curves were generated for each assay using serial-diluted RNA from pooled control mesenteric artery RNA. The presence of only one PCR product of the correct size was confirmed for each set of assay conditions by gel electrophoresis. Expression of each gene in each sample was calculated based on the arbitrary values of the standard curve, and this value was normalized to the expression of the control gene (SMAA).

**eNOS protein expression in mesenteric arteries.** Western blot analysis was performed to quantify eNOS and phosphorylation of eNOS on S1179 or T495, a phosphorylation site known to inhibit eNOS activity (26), in pooled mesenteric arteries (~5 pooled first-order arteries from the mesenteric bed of individual mice), which were collected from the mesentery of the anesthetized mice and immediately frozen in liquid nitrogen and stored at −80°C. Tissues were homogenized in lysis buffer [50 mM Tris·HCl, 100 mM NaF, 15 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1% Triton X-100, and 1:200 protease inhibitor cocktail solution (#P2714; Sigma, St. Louis, MO)], and centrifuged (14,000 rpm, 10 min) to remove insoluble material. Total protein was measured by colorimetric assay (DC Protein Assay, Bio-Rad), and proteins were separated by 10% SDS-PAGE (4% stacking gel) using 50 μg of protein, unless otherwise noted. Proteins were transferred to a nitrocellulose membrane, labeled for total
Data using Ponceau-S or SYPRO Rose (Invitrogen, Carlsbad, CA), then cut in half just above the 50 kDa marker. The top portion of the membrane was immunolabeled for pS1179-eNOS or pT495-eNOS (1:300–1:500; #612393, #612706; BD Transduction Laboratories, San Diego, CA), stripped for 15–20 min (Restore Western blot analysis stripping buffer; Pierce Biotechnology, Rockford, IL), then reprobed for eNOS (1:1,000, #610297; BD Transduction Laboratories), unless otherwise noted. We found no residual labeling of phosphorylated eNOS with this stripping procedure. The bottom half of the membrane was probed for GAPDH in those membranes used to assess total basal eNOS (1:1,000, #MAB374; Chemicon International, Temecula, CA). Following incubation with each primary antibody, the membranes were incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5,000, Pierce Biotechnology, Rockford, IL), and protein bands were visualized with enhanced chemiluminescence (Pierce Biotechnology). Densities of the protein bands were quantified using Image J software (National Institutes of Health, Bethesda, MD).

**RESULTS**

**Dietary treatment.** After the 6-wk dietary treatment, moderate-severe hyperhomocysteinemia was induced to a similar magnitude as described previously in rats (11), and average serum concentration was 13-fold greater ($P < 0.05$) than in control mice (Table 1). Serum nitrate+nitrite concentration and body mass were significantly less ($P < 0.05$) in the HHcy mice (Table 1).

**Mesenteric artery function.** Maximal diameters of first-order mesenteric arteries utilized in functional experiments were slightly, but significantly greater in the control group (228 ± 25 μm, $n = 70$) compared with the HHcy group (211 ± 28 μm, $n = 77$). Constriction to phenylephrine (Fig. 1A) and dilation to SNP (Fig. 1B) were not different between the groups, indicating well-preserved smooth muscle cell function with HHcy. SNP responses were not affected by 1-NAME treatment in either group. Dilation to cumulative addition of ACh was significantly less in HHcy mesenteric arteries. Treatment with high K+ (10 mM), 1-NAME, and indomethacin resulted in no dilation in either group (Fig. 2), which indicates that all dilation mechanisms were effectively blocked and that the non-nitric oxide, non-prostacyclin portion of the dilation is due to a mechanism consistent with EDHF. There were no significant differences in EC-50 values for any of the preceding pharmacological treatments between the groups.

While dose-response curves to ACh suggest that there is only a minor attenuation in functional responses in HHcy, this measure only compares the peak response to ACh at 3 min. The response to ACh is much more dynamic in this sized artery because of the contribution from both nitric oxide and non-nitric oxide sources. The dynamics of ACh responses are shown in Fig. 3A and indicate that ACh induces an initial, transient peak dilation, followed by a

**Table 1. Body mass and serum parameters**

<table>
<thead>
<tr>
<th>Control</th>
<th>HHcy</th>
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<tr>
<td>Body mass, g</td>
<td>26.6 (2.0)</td>
</tr>
<tr>
<td>Serum homocysteine, μM</td>
<td>6.6 (1.9) ($n = 13$)</td>
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<tr>
<td>Serum nitrate+nitrite, μM</td>
<td>19.0 (9.5) ($n = 18$)</td>
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Values are presented as means (SD). *$P < 0.05$ compared to control.

13.2% in control arteries (Fig. 2), indicating that non-nitric oxide, non-prostacyclin-dependent dilation was well preserved in HHcy mesenteric arteries. Treatment with high K+, 1-NAME, and indomethacin resulted in no dilation in either group (Fig. 2), which indicates that all dilation mechanisms were effectively blocked and that the non-nitric oxide, non-prostacyclin portion of the dilation is due to a mechanism consistent with EDHF. There were no significant differences in EC-50 values for any of the preceding pharmacological treatments between the groups.

![Fig. 1](http://ajpregu.physiology.org/)

**Fig. 1. Diameter changes (means ± SE) to phenylephrine (A) and sodium nitroprusside (B) in isolated mesenteric arteries from control and hyperhomocysteinemia (HHcy) mice. Responses to sodium nitroprusside were measured with or without 30 min Nω-nitro-arginine methyl ester (1-NAME) pretreatment, followed by preconstriction with phenylephrine (10−5 M).**

![Fig. 2](http://ajpregu.physiology.org/)

**Fig. 2. Dilation to ACh (means ± SE) in isolated mesenteric arteries from control and HHcy mice. Arteries were preconstricted with phenylephrine (10−5 M), and changes in diameter to cumulative addition of ACh (upper two curves; *$P < 0.05$ at highest dose) were measured. In separate groups of vessels, these responses were measured in the presence of 1-NAME (10−4 M) and indomethacin (10−5 M) [LNAME, INDO], and in the presence of high K+ (35 mM), L-NAME, and indomethacin [Hi K+, LNAME, INDO].**
reduced, sustained dilation, as has been recently reported (15). The peak dilation to a single high dose of ACh \(10^{-4}\) M occurred during the first 4 min (time of peak dilation varied between arteries) and was significantly reduced in HHcy (Table 2). The sustained dilation was significantly reduced in HHcy at all times from 6 to 30 min (Fig. 3A).

The addition of \(\text{l}-\text{NAME}\) significantly reduced the peak dilation to the same magnitude in both groups (Fig. 3B, Table 2). The sustained dilation was nearly eliminated by \(\text{l}-\text{NAME}\) in control arteries (Fig. 3B), indicating that this portion of the dilation response is nitric oxide dependent. Dilation in the presence of \(\text{l}-\text{NAME}\) (Fig. 3B) was significantly less than the dilation with ACh alone (Fig. 3A) at all time points in control arteries but only during the transient dilation (minutes 3–7) in HHcy arteries. The addition of both \(\text{l}-\text{NAME}\) and indomethacin (Fig. 3C) had no effect on the dynamics of the response in control arteries compared with \(\text{l}-\text{NAME}\) (all points were, however, significantly less than ACh alone), indicating that prostacyclin is not prominent in the response to ACh. However, in HHcy arteries, the transient dilation was significantly greater than control arteries at 1 to 4 min and was greater than HHcy with \(\text{l}-\text{NAME}\) at minute 3. Collectively, these data indicate that the nitric oxide component of ACh-induced dilation is attenuated with HHcy, but the non-nitric oxide, non-prostacyclin component, attributed to a mechanism consistent with EDHF, is enhanced. Both the transient response attributed to EDHF and the sustained response attributed to nitric oxide were eliminated by 35 mM KCl and \(\text{l}-\text{NAME}\) pretreatment (Fig. 3D).

Figure 3E indicates that there was some decay of the phenylephrine-induced preconstriction over the 30-min time course, which was not different between the groups and may account for the upward drift of the sustained dilation during the later minutes of the time course in Fig. 3, A–C.

**Basal eNOS expression and phosphorylation.** Total eNOS protein (normalized to GAPDH or total protein), as well as the ratio of phosphorylated S1179-eNOS/eNOS was significantly less in HHcy arteries \(P < 0.05\) (Fig. 4, A, C, D). However, the ratio of phosphorylated T495-eNOS/eNOS was not different between the groups (Fig. 4, B and D). There was a trend for decreased expression of eNOS mRNA in HHcy, which was nearly significant \(P = 0.06\) (Fig. 4E). Expression of eNOS...
mRNA was normalized to SMAA because it is highly expressed and specific to smooth muscle cells, the predominant cell type in the intact artery, and unlikely to be altered with the HHcy treatment because contractile function, which relies on this gene, was similar between the groups.

Agonist-induced eNOS phosphorylation. Treatment of mesenteric arteries with ACh ex vivo resulted in greater phosphorylation of eNOS on S1179 compared with vehicle treatment in arteries from control mice (Fig. 5). This response was significantly attenuated in arteries from HHcy mice. Similarly, treatment of the mesenteric vasculature with ACh in situ resulted in significantly greater S1179 phosphorylation in control than in HHcy vessels (Fig. 6).

DISCUSSION

The key findings of this study are that in mesenteric arteries of chronically moderate-severe HHcy mice both total eNOS and the ratio of eNOS phosphorylated on serine 1179 to total eNOS are downregulated in vivo, and agonist-induced phosphorylation of eNOS is significantly attenuated. This is the first study to show that both eNOS expression and regulation by phosphorylation on S1179 is impaired with chronic HHcy. S1179 is a key regulatory site on eNOS, which has recently been shown, using transgenic mice, to be necessary for ACh-induced nitric oxide-dependent vasodilation (1). Thus, it is reasonable to conclude that the impaired ACh-induced vasodilation observed in this study is at least partly due to the impaired ability to activate eNOS. However, the contribution of other factors, including oxidative stress or dysfunction of other NOS isoforms, cannot be ruled out.

Other studies have indicated a decrease in the bioavailability of nitric oxide, a consequence of increased superoxide, as the

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<th>Table 2. Peak dilation to a single dose of ACh (10^{-4} M)</th>
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<td>ACh only</td>
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<td>ACh + l-NAME</td>
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<td>ACh + l-NAME + INDO</td>
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<td>ACh + l-NAME + INDO + L-NAME</td>
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Values are presented as means (SD). Arteries were preconstricted with phenylephrine (10^{-5} M) for 10 min before ACh addition. *P < 0.05 vs. control. †P < 0.05 vs. control ACh only.
mechanism for endothelium dysfunction in HHcy (2, 8, 21, 28). This study reveals decreased eNOS expression and decreased ability to phosphorylate eNOS at S1179 with HHcy. These findings indicate that interventions aimed solely at reversing oxidative stress may prove insufficient to restore function. This idea is supported by a previous study that suggested there may be other mechanisms involved in HHcy-induced endothelial dysfunction besides elevated superoxide, because it reported that the addition of antioxidants SOD and catalase did not correct impaired endothelium-dependent dilation in aortas of mice with severe HHcy (17).

Phosphorylation of eNOS at S1179 participates in the activation of eNOS with both agonist stimulation and shear stress (5, 14) and has been found to be important for agonist-induced nitric oxide-dependent dilation (1). In the present study, both basal eNOS expression and the proportion of S1179 phosphorylation were downregulated in HHcy mesenteric arteries in vivo, suggesting that the basal ability to produce nitric oxide and the response to basal shear stress may be impaired. The basal phosphorylation state of the negative regulatory site T495 (26), however, was not affected in vivo. The ability to increase S1179 phosphorylation with an agonist was impaired with HHcy, both in the absence of shear stress (ex vivo stimulation, Fig. 5) and in the presence of shear stress (in situ stimulation, Fig. 6), suggesting that an upstream signaling event common to both shear stress and agonist stimulation is altered with HHcy.

These biochemical changes correlate with a loss of the sustained dilation due to nitric oxide in these arteries (Fig. 3). A previous study in cultured endothelial cells reported that acute homocysteine treatment had no effect on agonist-induced S1179 phosphorylation, but impaired dephosphorylation of the inhibitory site, T495. Our results are in contrast, with impaired S1179 phosphorylation as a notable effect of chronic HHcy. These differences may be due to intrinsic differences between cultured cells and vascular cells in vivo or due to a secondary effect of the chronic HHcy condition.

The mesenteric arteries used in the present study rely on both nitric oxide and EDHF for agonist-induced endothelium-dependent dilation (12, 15, 30). Studies in which nitric oxide has been blocked pharmacologically or by genetic knockout of eNOS have shown a general compensation for the loss of nitric oxide by upregulation of EDHF or prostacyclin (4, 6, 16, 27). General compensation by combined non-nitric oxide mechanisms is not indicated in the present study, because there is no difference in dilation between control and HHcy arteries with 1-NAME (Fig. 3B). However, in the presence of both 1-NAME and indomethacin, there is enhanced transient dilation in HHcy vessels (Fig. 3C). This suggests that the presence of prostacyclin masks the upregulation of the non-nitric oxide, nonprostacyclin (EDHF) component.

This preservation, and apparent enhancement, of non-nitric oxide, nonprostacyclin (EDHF)-dependent dilation in HHcy mice is contrary to the attenuation of this response found in vivo in the renal circulation of rats (11). The identical dietary intervention was used in this study, and similar plasma
homocysteine levels were induced. The differences may be due to intrinsic differences in these vascular beds, species, or vessel preparations. For example, the in vivo preparation used by De Vriese et al. (11) incorporates both responses to flow and responses to the endothelium-dependent dilator, and the in vitro preparation in the present study has no luminal flow and thus isolates the response to the endothelium-dependent dilator. Whatever the differences may be, the present study demonstrates that high chronic levels of homocysteine do not impair this mechanism of dilation in murine mesenteric arteries. Moreover, other studies report that nitric oxide blockade impairs this mechanism of dilation in murine mesenteric arteries from HHcy mice. This type of reduction in eNOS function, along with the previously reported increases in oxidant stress, may be the mechanism of the decreased bioavailability of nitric oxide and the vascular disease observed in small blood vessels with HHcy (18). This study thus supports the idea that it is primarily the nitric oxide system that is impaired in arteries with HHcy and indicates that therapeutic approaches to rescuing vascular dysfunction in HHcy should target eNOS expression and activation, in addition to reducing the influence of oxidative stress on nitric oxide bioavailability.

Perspectives and Significance

This study reports that chronic, moderate-severe HHcy impairs basal and agonist-induced eNOS phosphorylation (on S1179) and agonist-induced nitric oxide-dependent vasodilation, without impairment of non-nitric oxide-dependent vasodilation, and also provides evidence that the non-nitric oxide, nonprostacyclin (EDHF) component is upregulated in mesenteric arteries from HHcy mice. This type of reduction in eNOS function, along with the previously reported increases in oxidative stress, may be the mechanism of the decreased bioavailability of nitric oxide and the vascular disease observed in small blood vessels with HHcy (18). This study thus supports the idea that it is primarily the nitric oxide system that is impaired in arteries with HHcy and indicates that therapeutic approaches to rescuing vascular dysfunction in HHcy should target eNOS expression and activation, in addition to reducing the influence of oxidative stress on nitric oxide bioavailability.

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REFERENCES


