Dysfunction of Nitric Oxide Mediation in Isolated Rat Arterioles With Methionine Diet–Induced Hyperhomocysteinemia

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Abstract—In humans, increased plasma homocysteine (Hcy) has been shown to be correlated with occlusive arterial diseases and atherosclerosis. Studies of isolated conductance vessels of experimental animals suggest that Hcy may interfere with local vasoregulatory mechanisms, yet the effect of hyperhomocysteinemia (HHcy) on the function of microvessels, such as skeletal muscle arterioles, has not been investigated. Male Wistar rats were divided into 2 groups: control rats (C; plasma Hcy, 7.1 ± 0.3 μmol/L; n = 25), and rats made HHcy by 1 g/kg body weight daily intake of methionine in the drinking water for 4 weeks (plasma Hcy, 23.6 ± 2.9 μmol/L; P < 0.01 versus C; n = 25). First-order arterioles (≈130 μm in diameter) were isolated from gracilis muscle, cannulated, and pressurized (80 mm Hg, no-flow conditions). Changes in diameter were observed by videomicroscopy. Arteriolar constrictions to norepinephrine (NE; 3 × 10⁻⁷ mol/L) were significantly (P < 0.01) greater in HHcy compared with C rats (C, 37.7 ± 4.9%; HHcy, 59.5 ± 5.2%). Removal of the endothelium (-E) augmented NE-induced constrictions only in arterioles from C rats, whereas it had no effect on responses of arterioles from HHcy rats (C-E, 55.9 ± 6.9%; HHcy-E, 56.5 ± 7.0%). Dilations to cumulative doses of acetylcholine (ACH; 10⁻⁶ mol/L) were significantly reduced in arterioles from HHcy rats (C, 64.0 ± 5.2%; HHcy, 24.1 ± 6.8%). Inhibition of nitric oxide (NO) synthesis with Nω-nitro-L-arginine (L-NNA; 10⁻⁴ mol/L) significantly decreased ACh-induced dilations of C arterioles, whereas it did not affect HHcy arterioles. Similar alterations were found in arteriolar dilations to histamine, another known NO-dependent agonist. Endothelium-independent dilations to the NO donor sodium nitroprusside were not different in arterioles from C and HHcy rats, either in the presence or absence of L-NNA. Presence of superoxide dismutase and catalase (scavenger of reactive oxygen metabolites) did not affect HHcy-induced alterations in the ACh response. We conclude that hyperhomocysteinemia reduces rat skeletal muscle arteriolar dilations in response to ACh and histamine, and enhances constrictions to NE, alterations that are likely to be caused by the reduced mediation of these responses by NO. The reduced activity of NO in arterioles may contribute to the microvascular impairment described in HHcy. (Arterioscler Thromb Vasc Biol. 1999;19:1899-1904.)

Key Words: homocysteinemia ■ microcirculation ■ acetylcholine ■ norepinephrine ■ histamine ■ nitric oxide

An association between elevated plasma homocysteine (Hcy) concentrations and atherosclerotic vascular disease has been shown by several epidemiological studies.¹–⁴ The atherosclerotic and thrombotic complications of the severe inherited form of hyperhomocysteinemia (HHcy; plasma Hcy >100 μmol/L) was first described in the late sixties.⁵ By now it is well-documented that mild HHcy (plasma Hcy >16 μmol/L) occurs in up to 30% of patients with stroke, myocardial infarction, and peripheral vascular disease.⁶,⁷ Hcy is a sulfur-containing amino acid that is formed during methionine metabolism.⁸,⁹ Plasma Hcy concentrations may increase in different pathophysiological conditions, including deficiency of vitamins such as folic acid, cyanocobalamin, and pyridoxal phosphate, and in the presence of various enzyme (cystathionine β-synthetase, and temperature-sensitive methyltetrahydrofolate reductase) abnormalities (for further references see References 2 and 6), all of which participate in the metabolism of Hcy.⁷

The mechanism by which elevated Hcy impairs the vessel wall, and thereby promotes atherothrombosis, is still not clearly elucidated, and is likely to be multifactorial. It is thought that a high concentration of Hcy plays a crucial role by injuring the vascular endothelium.¹⁰,¹¹ Light- and electron-microscopic studies of arteries and arterioles from HHcy humans and animals revealed alterations in endothelial morphology.⁵,⁸–¹⁰ HHcy is also known to alter the expression of anticoagulant surface proteins in endothelial cells,¹¹,¹² but there are only a few functional studies extant. In conduit arteries of patients with HHcy, vasodilation associated with reactive hyperemia is impaired.¹³,¹⁴ Studies on conductance vessels isolated from HHcy monkeys¹⁵,¹⁶ suggest that Hcy may impair endothelial vasoregulatory mechanisms. The
adverse effect of Hcy was also confirmed by in vitro studies on cultured endothelial cells.\textsuperscript{17}

The effect of HHcy, and the possible changes in the endothelial function of microvessels regulating tissue blood flow, has not been investigated, yet the preponderance of vascular disturbances in HHcy occur in the peripheral circulation. The skeletal muscle microcirculation represents the major part of peripheral resistance; therefore its dysfunction can contribute to the development of peripheral vascular disease attendant with HHcy. We aimed to elucidate the effects of HHcy, induced by a methionine diet,\textsuperscript{18,19} on the vasomotor functions of rat skeletal muscle arterioles, by investigating their responses to vasoactive agents, the effects of which are known to be mediated by, or are independent of, nitric oxide (NO).\textsuperscript{20,21}

Methods

Moderate HHcy was induced in male Wistar rats (\textasciitilde 150 g; Charles River KFT, Budapest, Hungary) by administration of L-methionine (1 g/kg body weight per day) and succinylsulfathiazole (SS: 0.5 g/kg body weight per day) in the tap water for a period of 4 weeks (n = 25). SS was used to avoid bacterial proliferation and subsequent foetal production.\textsuperscript{15,16,19} The dosage administered per animal was based on average daily fluid intake. Control (C) animals (n = 25) had free access to tap water. Animals were housed separately, fed standard rat chow, and were weighed at the start and at the end of the 4-week period.

Determination of Serum Hcy

Blood was collected from the femoral artery of fasting rats. It was immediately cooled on ice and centrifuged at 3000 g for 20 minutes at 4°C to limit the release of Hcy from blood cells. Serum was then stored at \textasciitilde 20°C until assayed. Total Hcy concentrations were measured by high-performance liquid chromatography (HPLC) technique with fluorometric detection according to Ulbink et al.\textsuperscript{22} Briefly, 240 \textmu L of serum and 60 \textmu L of internal standard (N-acetyl-L-cysteine, 50 \mu mol/L final concentration) was reduced for 30 minutes at 4°C with 30 \textmu L of tri-n-butyl phosphine (10%). Deproteinization was performed with 300 \mu L of 10% trichloroacetic acid. After centrifugation, 100 \mu L of the clear supernatant was mixed with 20 \mu L of 1.55 mol/L NaOH, 250 \mu L of 0.125 mol/L borate buffer (pH 9.5), and 50 \mu L of 1 mg/mL 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. After derivatization at 60°C (1 hour), the sample was analyzed by HPLC (JASCO International Co Ltd), equipped with a fluorescence detector (LC 1255; GBC Scientific Equipment Pty Ltd). Separation was carried out on a 200 x 4.6 mm x 5 \mu mol/L Nucleosil C18 column. The eluant was 0.1 mol/L acetate buffer (pH 4.0) containing 2% methanol. The fluorescence intensities were measured with excitation at 386 nm and emission at 516 nm.

Isolation of Arterioles

Experiments were conducted on isolated first-order arterioles (\textasciitilde 130 \mu m active and \textasciitilde 180 \mu m passive diameter at 80 mm Hg) of rat gracilis muscle, as described previously.\textsuperscript{23} Briefly, in the fourth week, rats were fasted overnight, then were anesthetized with sodium pentobarbital (50 mg/kg; IP). Blood pressure was measured in the femoral artery, then blood was collected for measurement of Hcy concentration. The gracilis muscle was exposed and isolated from surrounding tissues. The muscle then was dissected out and placed in a silicone-lined Petri dish containing cold (0°C to 4°C) physiological salt (PS) solution composed of (in mmol/L) 110 NaCl, 5.0 KCl, 2.5 CaCl\textsubscript{2}, 1.0 MgSO\textsubscript{4}, 1.0 KH\textsubscript{2}PO\textsubscript{4}, 10.0 dextrose, and 24.0 NaHCO\textsubscript{3}; and was equilibrated with a gas mixture of 10% O\textsubscript{2}, 5% CO\textsubscript{2}, 85% nitrogen; pH 7.4. Then, using microsurgery instruments and an operating microscope, a 1.5- to 2-mm long segment of the first-order arteriole running intramuscularly was isolated and transferred to an organ chamber containing 2 glass micropipettes filled with PS solution. The vessel chamber (15 mL) was continuously supplied with fresh PS solution at a rate of 40 mL/min. After the vessel had been mounted on the proximal micropipette and was secured with sutures, the perfusion pressure was raised to 20 mm Hg to clear clotted blood from the lumen. The other end of the vessel was then mounted onto the distal pipette. Both micropipettes were connected with silicone tubing to an adjustable PS solution–reservoir. Pressure on both sides was measured by an electromanometer. The perfusion pressure was slowly (over \textasciitilde 1 minute) increased to 80 mm Hg. The temperature was set at 37°C by a temperature controller (Grant Instruments) and the vessel was allowed to equilibrate for \textasciitilde 1 hour.

Experimental Protocols

Only those vessels that developed spontaneous tone in response to perfusion pressure were used; thus no vasoactive agent was added to the PS solution to establish arteriolar tone. After the equilibration period, the diameter of arterioles was measured at 80 mm Hg perfusion pressure under zero-flow conditions. At the conclusion of each experiment, the suffusion solution was changed to a Ca\textsuperscript{2+}-free PS solution that contained sodium nitroprusside (SNP; 10\textsuperscript{2} mol/L) and EGTA (1.0 mmol/L). The vessel was incubated for 10 minutes, and the maximum passive diameter at 80 mm Hg pressure was obtained (passive diameter). The diameter was measured with a micromanometer and recorded with a chart recorder.

The constrictor responses of arterioles from C and HHcy rats to norepinephrine (NE; 10\textsuperscript{6} to 10\textsuperscript{3} mol/L) were compared before and after endothelium removal. The endothelium of arterioles was removed by perfusion of the vessel with air for \textasciitilde 1 minute at a perfusion pressure of 20 mm Hg. The arteriole was then perfused with PS solution to clear the debris. The perfusion pressure was raised to 80 mm Hg for 30 minutes to establish a stable tone. The efficacy of endothelial denudation was ascertained by arteriolar responses to acetylcholine (ACH; 10\textsuperscript{5} mol/L; an endothelium-dependent dilator agent), and SNP (10\textsuperscript{2} mol/L; an endothelium-independent agent) before and after the administration of the air bolus. The infusion of air resulted in loss of function of the endothelium, as indicated by the absence of dilation to ACH, whereas dilation to SNP remained intact.

In a second series of experiments, peak responses of arterioles to cumulative doses of ACh (10\textsuperscript{6} to 10\textsuperscript{5} mol/L) and SNP (10\textsuperscript{10} to 10\textsuperscript{8} mol/L) were obtained. The vessel was then incubated with L-NNA (10\textsuperscript{5} mol/L) for 30 minutes, and responses to ACh and SNP were reassessed.

In other experiments, peak responses of arterioles to increasing doses of histamine (10\textsuperscript{5} to 10\textsuperscript{4} mol/L) were obtained in a noncumulative fashion. The vessel was then incubated with L-NNA (10\textsuperscript{4} mol/L) for 30 minutes, and responses to histamine were reassessed. The effect of removal of endothelium on histamine-induced responses was obtained.

Next, changes in diameter of arterioles to cumulative doses of ACh (10\textsuperscript{10} to 10\textsuperscript{8} mol/L) were obtained before and after intraluminal administration and incubation (15 minutes) of vessels with superoxide dismutase (SOD; 80 U/mL) and catalase (CAT; 120 U/mL), shown to be effective scavengers of superoxide and hydrogen peroxide, respectively, in the present experimental condition.\textsuperscript{24}

All drugs were added to the vessel chamber and final concentrations are reported. After responses to each drug subsided, the system was flushed with PS solution. All salts and chemicals were obtained from Sigma-Aldrich and were prepared on the day of the experiment. Dilations were expressed as a percentage of the maximal dilation of the vessel defined as the passive diameter at 80 mm Hg perfusion pressure in a Ca\textsuperscript{2+}-free medium containing 10\textsuperscript{2} mol/L EGTA and 10\textsuperscript{4} mol/L SNP. Constrictions were expressed as a percentage of baseline. From the cumulative dose-response curves of vasoactive agents the EC\textsubscript{50} were calculated. Data are expressed as means \textpm SEM. Statistical analyses were performed by ANOVA, followed by Tukey post hoc test or Student’s t test. P < 0.05 was considered statistically significant.

Results

A methionine-rich diet induced a significant (P < 0.01) 3-fold increase in plasma Hcy concentrations (Table). There was no significant difference between the systolic blood pressure, body weight, and plasma glucose concentration of C and
methionine-fed rats (Table). Isolated arterioles of gracilis muscle from both C and HHcy rats developed active tone in response to elevation of perfusion pressure to 80 mm Hg without the use of a vasoactive agent. There was no significant difference in pressure-induced diameter of arterioles from the 2 groups of rats (Table). In the absence of Ca2+ and presence of SNP (10⁻⁴ mol/L), at 80 mm Hg, the diameter of each arteriole was obtained. The normalized diameter, expressed as a percentage of the passive diameter, indicated no difference in spontaneous pressure-induced tone in the 2 groups of arterioles (Table).

### Arteriolar Responses to NE

In a dose-dependent manner, NE (10⁻⁹ to 10⁻⁵ mol/L) elicited significantly greater constrictions in arterioles from HHcy than that in those from C rats, with an EC₅₀ of 2.1±0.6×10⁻⁷ mol/L and 5.8±1.3×10⁻⁷ mol/L, respectively (P<0.05; Figure 1, upper panel). Removal of endothelium enhanced NE-induced constrictions of arterioles from C rats (EC₅₀, 2.3±0.7×10⁻⁷ mol/L; P<0.05; Figure 1, middle panel), whereas it had no significant effect on responses of arterioles from HHcy rats (EC₅₀, 1.8±0.5×10⁻⁷ mol/L; Figure 1, lower panel).

### Arteriolar Responses to ACh, Histamine, and SNP

In a dose-dependent manner, ACh (10⁻⁹ to 10⁻⁶ mol/L) elicited significantly greater peak dilations of arterioles from C than from HHcy rats (Figure 2A) with an EC₅₀ of 5.2±2.5×10⁻⁹ mol/L and 4.0±1.5×10⁻⁸ mol/L, respectively (P<0.01). After preincubation (for 30 minutes), and in the presence of the nitric oxide synthesis inhibitor L-NNA (10⁻⁴ mol/L), ACh-induced dilations of arterioles from C (EC₅₀, 1.8±0.7×10⁻⁸ mol/L; Figure 2B), but not from HHcy rats (EC₅₀, 5.1±1.1×10⁻⁸ mol/L; Figure 2C), decreased significantly. L-NNA caused a 8±5% and 3±3% decrease in diameter in arterioles from C and HHcy rats, respectively. The difference between the 2 groups of vessels was not significant.

In a dose-dependent manner, SNP elicited similar dilation of arterioles from C and HHcy rats with an EC₅₀ of 1.5±0.7×10⁻⁸ mol/L and 1.3±0.5×10⁻⁸ mol/L, respectively. L-NNA did not significantly affect the dilations to SNP in either group (EC₅₀ for C, 1.5±0.8×10⁻⁸ mol/L; EC₅₀ for HHcy, 1.2±0.6×10⁻⁸ mol/L; Figure 2D).

In a dose-dependent manner, histamine (10⁻⁶ to 10⁻⁴ mol/L) elicited significantly greater peak dilations of arterioles from C than from HHcy rats (Figure 3, upper panel) with an EC₅₀ of 9.2±1.0×10⁻⁶ mol/L and 4.0±1.2×10⁻⁵ mol/L, respectively (P<0.01). After preincubation, and in the presence of L-NNA (10⁻⁴ mol/L), histamine-induced dilations of arterioles from C (EC₅₀, 4.2±0.5×10⁻⁵ mol/L; Figure 3, middle panel) but not from HHcy rats (EC₅₀, 3.0±1.0×10⁻⁵ mol/L; Figure 3, lower panel) decreased significantly. Removal of endothelium decreased histamine-induced dilations of arterioles from C (EC₅₀, 3.7±0.5×10⁻⁵ mol/L; Figure 3, middle panel), whereas it had no significant effect on responses of arterioles from HHcy rats (EC₅₀, 3.2±0.9×10⁻⁵ mol/L; Figure 3, lower panel). Responses of arterioles from C and HHcy rats did not differ significantly after removal of endothelium.

Changes in diameter to cumulative doses of ACh (10⁻¹⁰ to 10⁻⁶ mol/L) were also obtained before and after intraluminal administration and incubation (15 minutes) of arterioles with SOD (80 U/mL) and CAT (120 U/mL), shown to be effective scavengers of superoxide and hydrogen peroxide in the present experimental condition. Incubation of arterioles with SOD and CAT did not significantly affect ACh-induced dilations in arterioles from C or HHcy rats (Figure 4).

### Discussion

The major findings of this study are that elevated plasma Hcy concentration induced by a methionine-rich diet is associated with increased NE-induced constrictions and reduced endo-
thelium-dependent dilations of isolated rat skeletal muscle arterioles. The underlying mechanism of these changes is most likely an impairment in endothelial NO mediation of the responses.

Epidemiological studies indicate that even a mild elevation of plasma Hcy concentration (plasma Hcy $16 \mu$mol/L) is an independent risk factor for atherothrombotic diseases. In the general human population mild HHcy has proven to be rather common (1:70) and is found in $\approx 30\%$ of individuals with coronary, cerebrovascular, and peripheral atherosclerotic disease. Common reasons for increased plasma Hcy concentrations are the inadequate concentrations of vitamins (folic acid or pyridoxal phosphate) required for Hcy metabolism. In addition to low supply, an increased requirement for folic acid, or interference of drugs with folate metabolism, can cause a folate deficiency, as observed in elderly people, pregnant women, smokers, alcoholics, and users of contraceptive medication.

Several mechanisms have been proposed to explain how elevated plasma Hcy might promote atherothrombotic vascular disease. Adverse effects of HHcy can alter vessel wall morphology, increase platelet activity, stimulate smooth muscle cell proliferation, and promote LDL oxidation. In addition, several microscopic studies of arteries and arterioles from HHcy humans and animals revealed alterations in endothelial morphology. Only a few studies have reported changes in the endothelial function in HHcy. There is an impaired dilation in conduit arteries of patients with HHcy after release of an occlusion, a response thought to be associated with flow-dependent endothelium-mediated dilation. Also, the endothelium-dependent relaxation of carotid artery, and an increase in hindlimb circulation to ACh, is reduced in monkeys with diet-induced homocysteinemia. These studies suggest that one of the possible mechanisms causing these alterations is that high concentration of Hcy impairs the function of vascular endothelium, yet the effect of elevated Hcy on the function of microvessels, primarily responsible for local regulation of blood flow, have not been

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Panel A, Effect of cumulative doses of ACh on normalized diameter of arterioles isolated from C (C, $n=10$) and HHcy (C, $n=9$) rats. Panels B and C, Effect of L-NNA (C, $n=9$), a nitric oxide synthase inhibitor on ACh-induced responses in arterioles isolated from C and HHcy rats, respectively. Panel D, The effect of cumulative doses of SNP on normalized diameter of arterioles from C and HHcy rats in the presence and absence of L-NNA Data are mean±SEM. *Indicates significant ($P<0.01$) differences from C.

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Upper panel, Effect of histamine on normalized diameter of arterioles isolated from C (C, $n=10$) and HHcy (C, $n=9$) rats. Effect of L-NNA (C, $n=9$), a nitric oxide synthase inhibitor, and endothelium removal (-E, M, lower panel, $n=9$) rats, respectively. Data are mean±SEM. *Indicates significant ($P<0.01$) differences from C.

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Effect of cumulative doses of ACh on normalized diameter of skeletal muscle arterioles isolated from C (C) and HHcy (C) rats in the absence (C) and presence (C+SOD/CAT) of SOD (80 U/mL) and CAT (120 U/mL). Data are mean±SEM ($n=4$).
characterized. Several animal models of HHcy,9,10,15,19,20,25–27 were developed to investigate the underlying mechanisms eliciting adverse changes in the cardiovascular system. In the present study methionine (the precursor of Hcy) and SST (an inhibitor of bacterial folate synthesis) were administered in the drinking water of rats for 4 weeks, a method similar to that used previously by others.9,18,19 Plasma Hcy increased by $\approx3$-fold and reached a concentration similar to levels that are associated with an increased risk of vascular disease in humans.1–4 Although plasma methionine levels are likely to be increased, there are no reports to indicate that a high level of methionine or other amino acids would elicit adverse vascular changes.28 It is an intriguing question whether SST alone would elevate serum Hcy concentrations to a level that would have an affect on vascular function or, itself, would have effects unrelated to Hcy. Studies, showing that prolonged infusion of Hcy alone elicits endothelial impairment,10 however, strongly suggest that HHcy is responsible for the observed changes in arteriolar responses in the present study.

It has recently been demonstrated that several risk factors elicit endothelial dysfunction in the arterioles, vessels in which overt atherosclerosis does not develop.28–30 Because the skeletal muscle microcirculation represents a major part of peripheral resistance, its dysfunction may contribute to the development of peripheral vascular disease in HHcy. Therefore, to examine changes in vasoregulatory function of the endothelium and smooth muscle we used isolated gracilis muscle arterioles from normal and methionine diet–induced HHcy rats.

First we compared the constrictor function of arterioles isolated from C with those from HHcy rats in response to 80 mm Hg pressure, a pressure to which these arterioles are exposed in vivo. We found no significant differences between the pressured-induced tone of the 2 groups of arterioles, suggesting that the pressure-induced contractile activity of arteriolar smooth muscle is not affected by high levels of Hcy. In contrast, we found that HHcy enhanced arteriolar constrictions to NE. Previous studies in skeletal muscle arterioles demonstrated that, upon administration of NE, there is a concomitant release of NO from the endothelium, modulating the magnitude of NE-induced constriction.33 We assumed that impaired endothelial NO synthesis is responsible for the increased responsiveness of arterioles to NE in HHcy rats. Indeed, in arterioles from C rats, removal of the endothelium enhanced constrictions to NE, whereas NE-induced responses of arterioles from HHcy rats remained unchanged, supporting our hypothesis.

To further ascertain that HHcy affects endothelial NO mediation we have tested the responses of arterioles to ACh and histamine, which are known to elicit the release of NO. We found that both ACh- and histamine-induced dilations were reduced in arterioles isolated from the skeletal muscle of HHcy rats compared with rats with normal serum levels of Hcy. Furthermore, inhibition of NO synthesis with L-NNA decreased dilations to ACh and histamine only in arterioles from C rats, whereas L-NNA had no effect on responses from HHcy rats. In addition, endothelium removal eliminated the differences in histamine-induced dilations between arterioles from C and HHcy rats. The sensitivity of vascular smooth muscle to NO, however, was not affected by HHcy, as indicated by the unaltered arteriolar responses to the NO donor SNP. Collectively, these results indicate that agonist-induced synthesis, release, or bioavailability of endothelial NO is impaired in HHcy.

Previous reports suggested that the synthesis and/or the release of endothelium-derived relaxing factor (presumably NO) is impaired in large vessels from HHcy animals.14–16 Our results are consistent with these reports, and extend the findings to the level of arterioles. It has been suggested by other studies that pathophysiological conditions, such as hypercholesterolemia, which predispose conduit vessels to atherosclerosis, can cause dysfunction of the endothelium in the microcirculation.29,30,32 Small coronary arteries and arterioles that do not exhibit atherosclerotic lesions show altered responses to endothelium-dependent dilators, caused by impaired NO synthesis.30 In addition, elevated levels of Hcy have been shown to lead to increased levels of plasma triglycerides.33 Similarly, hypertension, another known risk factor for atherosclerosis, can further aggravate the impairment of endothelial function in skeletal muscle arterioles.54 Taken together, endothelial dysfunction in the microvasculature seems to be an early, general feature, associated with several vascular diseases. Because NO, in addition to eliciting vasodilation, exerts anticoagulant and antithrombotic effects, endothelial dysfunction of microvessels may be an important link between HHcy and atherothrombotic diseases. The lack of endothelial NO may also promote the smooth muscle proliferation observed in HHcy.2 Impaired endothelial function would also enhance the adhesion/attachment of leukocytes and platelets to the vessel wall.

One of the mechanisms by which NO synthesis, release, or action might be impaired is an increased formation of reactive oxygen metabolites.35–38 It has been shown that during the autoxidation of the sulfhydryl group of biological thiols, hydrogen peroxide is generated.36 Methionine does not have a free thiol group, further suggesting a primary role for HHcy in the observed arteriolar endothelial impairment. Elevated levels of oxygen free radicals can react with NO, thus decreasing its bioavailability by producing peroxynitrite (and other NO radicals). Hcy was also reported to decrease intracellular glutathione and glutathione peroxidase, which are responsible for the elimination of oxygen free radicals.39,40 Free radical–mediated cytotoxic effects of Hcy on cultured endothelial cells have already been demonstrated,17 and HHcy may sensitized endothelial cells to oxidative stress by affecting cysteine-requiring reactions, thereby reducing endothelial cell glutathione levels.40 Nevertheless, it seems that the HHcy-induced impairment is irreversible in the present study because SOD and CAT, scavengers of reactive oxygen metabolites, did not restore the NO mediation of arteriolar responses. One can also speculate that HHcy affects the endothelial receptors of the agonists investigated, but it seems unlikely that both muscarinic and histaminergic receptor–mediated dilations would be affected.

Although the exact mechanisms responsible for the impairment of NO-mediated arteriolar responses in HHcy still need to be further investigated, the present study provides the first evidence that the function of microvessels are importantly affected in HHcy, and may explain the widespread changes observed in the circulation.

In summary, our findings suggest that a diet-induced, moderate elevation of plasma Hcy concentrations is associ-
ated with increased NE-induced constrictions and reduced ACh- and histamine-induced dilations of rat skeletal muscle arterioles. These alterations are likely to be caused by dysfunction of NO mediation of arteriolar vasomotor responses, and could constitute an important early step in the development of vascular diseases associated with HHcy.

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