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Effect of Hyperhomocystinemia and Hypertension on Endothelial Function in Methylenetetrahydrofolate Reductase–Deficient Mice

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Objective—We evaluated the effect of hyperhomocystinemia and angiotensin (Ang) II on vascular function and structure in methylenetetrahydrofolate reductase knockout mice (*Mthfr*$^{+/-}$).

Methods and Results—*Mthfr*$^{+/-}$ and controls (*Mthfr*$^{+/+}$) received Ang II (400 ng/kg per min SC) or saline (14 days). Blood pressure, similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$, was increased by Ang II. Acetylcholine- and bradykinin-induced relaxations were impaired in mesenteric resistance arteries (pressurized myograph) in *Mthfr*$^{+/-}$ and in Ang II–infused *Mthfr*$^{+/+}$ mice and additionally blunted in Ang II–infused *Mthfr*$^{+/-}$ mice. The inhibition by L-NAME on acetylcholine was reduced in *Mthfr*$^{+/-}$ and in Ang II–*Mthfr*$^{+/+}$ and absent in Ang II–*Mthfr*$^{+/-}$ mice. In these groups, vitamin C improved the response to acetylcholine and restored the inhibition by L-NAME. The media to lumen ratio of small arteries, similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$, was increased by Ang II. Vascular NADPH oxidase activity, similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$, increased after Ang II infusion. Vascular xanthine oxidase activity was also similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$. Superoxide production in the aorta was reduced by sepiapterin and by L-NAME, suggesting that reduced bioavailability of tetrahydrobiopterin and uncoupling of nitric oxide synthase were the origin of increased reactive oxygen species in this model.

Conclusions—*Mthfr*$^{+/-}$ mice show endothelial dysfunction of mesenteric vessels probably attributable to a reduced nitric oxide bioavailability caused by oxidative excess due to uncoupling of nitric oxide synthase without vascular structural alterations. Concurrent Ang II–induced hypertension additionally reduced nitric oxide, increased NADPH oxidase activity, and induced structural alterations. Our findings suggest additive adverse effect of Ang II–dependent hypertension and hyperhomocystinemia on endothelial function. **(*Arterioscler Thromb Vasc Biol*. 2003;23:1352-1357.)**

Key Words: angiotensin ■ endothelium ■ free radicals ■ nitric oxide ■ small arteries

Homocysteine (Hcy) is an intermediate amino acid derived from the metabolism of methionine. Mild hyperhomocystinemia (H-Hcy) has been associated with increased risk of vascular disease.[1] Studies in animal models and humans have demonstrated that mild H-Hcy induces endothelial dysfunction.[2–10] Reduced nitric oxide (NO) bioavailability and increased oxidative stress may play a role in its effects[3–5,8–10] and thus could be a mechanism whereby H-Hcy leads to vascular damage.

Genetic hypertension is a cardiovascular risk factor characterized by endothelial dysfunction and vascular remodeling of small resistance arteries. Angiotensin (Ang) II has been implicated in the development of vascular changes mainly via increased generation of reactive oxygen species (ROS) through NADPH oxidase activation.[11]

Methylenetetrahydrofolate reductase (MTHFR) plays a key role in the remethylation cycle, converting Hcy to methionine.[1] A common variant in *MTHFR*, 677C→T, is associated with decreased enzyme activity leading to mild H-Hcy in humans.[12,13] At the present time, the role of this genetic mutation on endothelial dysfunction remains to be determined. Recently, a new model of mild H-Hcy attributable to heterozygous deficiency of *Mthfr* was developed by gene inactivation in mice.[14] This is an interesting model with which to demonstrate the role of *MTHFR* deficiency in the pathogenesis of vascular alterations. Therefore, in the present study we assessed the impact of mild H-Hcy and hypertension on function and structure of mesenteric small arteries in heterozygous *Mthfr* knockout mice.

We also evaluated whether impaired endothelium-dependent relaxation secondary to H-Hcy was caused by reduced NO bioavailability attributable to increased ROS. The role of vascular NADPH oxidase and xanthine oxidase,

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as well as oxidation of tetrahydrobiopterin and uncoupling of NO synthase as possible sources of ROS, was also assessed.

Methods

The study protocol was approved by the Animal Care Committee of the Clinical Research Institute of Montreal. Mice heterozygous for disruption of the *Mthfr* gene were generated at the Montreal Children's Hospital Research Institute by Dr R. Rozen.

Generation of Mice With Targeted Disruption of the *Mthfr* Gene

The generation of mice with targeted disruption of the *Mthfr* gene has already been reported.[14] Briefly, to inactivate the mouse *Mthfr* gene in embryonic stem (ES) cells, an insertion type of targeting vector was constructed in which exon 3 of the mouse *Mthfr* gene was interrupted by the *neo*^{r} gene. After transfection of the construct to ES cells, 5 of 150 doubly resistant ES clones were identified by Southern blot analysis to have undergone the expected homologous recombination event. Three of the positive clones were injected into blastocysts, and chimeric mice from 1 cell line successfully transmitted the modified *Mthfr* gene to the next generation. Heterozygous mice were generated through breeding of chimeric mice with BALB/cAnNCrlBR mice (Charles River Canada).

Animal Experiments

Male heterozygous (+/−) *Mthfr*-deficient mice and littermate wild-type (+/+) control mice aged 12 to 14 weeks were studied. Under anesthesia with methoxyflurane, mice were implanted subcutaneously with osmotic minipumps (Alza Corp) that infused 400 ng/kg per min Ile^5-Ang II (Peninsula) or saline for 14 days. Systolic blood pressure (SBP) was measured by the tail-cuff method. The average of 3 pressure readings was obtained. Mice were killed by decapitation.

Preparation and Study of Small Arteries

Second-order superior mesenteric arteries (≈ 2 mm in length) were placed in cold physiological salt solution containing (in mmol/L) NaCl 120, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 2.5, EDTA 0.026, and glucose 5.5. They were mounted on 2-glass microcannulae in a pressurized myograph, as previously described.[15] Endothelium-dependent relaxations were assessed by measuring the dilatory responses to acetylcholine (Sigma, 10^{-9} to 10^{-4} mol/L) in vessels precontracted with norepinephrine (5×10^{-5} mol/L). To exclude the possibility that impaired relaxation in response to acetylcholine resulted from a muscarinic receptor defect, in 4 additional groups of mice (n=4 each group), a dose-response curve to bradykinin (Sigma, 10^{-10} to 10^{-6} mol/L), an endothelial agonist acting through a different receptor and signal transduction pathway,[16,17] was evaluated. Endothelium-independent relaxation was assessed by the dilatory response to sodium nitroprusside (10^{-8} to 10^{-4} mol/L).

To evaluate NO availability, in a different set of experiments (n=6 each group) the dose-response curve to acetylcholine was determined before and after 30-minute preincubation with the NO synthase inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME, Sigma, 100 μmol/L). Moreover, to assess production of ROS, acetylcholine administration was repeated in the presence of an antioxidant, vitamin C (Sigma, 100 μmol/L, 30-minute preincubation). To evaluate whether oxidative stress could influence NO bioavailability, another dose-response curve to acetylcholine was repeated under simultaneous administration of L-NAME and vitamin C.

To ensure that the magnitude of the dose-response curve to acetylcholine was stable when repeated several times on the same vessel, we performed preliminary studies where the dose-response curve to acetylcholine was repeated consecutively 4 times on control vessels precontracted with norepinephrine. The results obtained were identical, excluding tachyphylaxis to acetylcholine (data not shown).

Vessels were then deactivated by perfusion with Ca^{2+}-free physiological salt solution containing 10 mmol/L EGTA for 30 minutes. Lumen and media were measured with intraluminal pressure at 45 mm Hg, as previously described.[18]

Measurement of Plasma Hcy

Blood samples from the mice were collected in tubes containing EDTA. Plasma was separated by centrifugation and stored at −80°C. Total plasma Hcy was measured by a high-pressure liquid chromatography.[19]

Measurement of NADPH Oxidase and Xanthine Oxidase Activity

Activity of NADPH oxidase and of xanthine oxidase was measured in mesenteric vessels by chemiluminescence assay using 5 μmol/L lucigenin as the electron acceptor and 100 μmol/L NADPH or xanthine as the substrate. The reaction was initiated by the addition of NADPH or xanthine to tissue sample. Luminescence was measured every 1.8 seconds for 3 minutes in a luminometer (AutoLumat LB 953, Berthold). A buffer blank was subtracted from each reading. Activity was expressed as counts per minute per milligram of dry tissue weight.[20]

Determination of Superoxide Anion Production in the Aorta

Unfixed frozen ring segments of aorta were cut into 10-μm-thick sections and placed on a glass slide. Two sections per slide were analyzed simultaneously as follows: 1 section was preincubated in presence of either NO synthase inhibitor L-NAME (10^{-5} mol/L) or sepiapterin (10^{-4} mol/L) at 37°C for 30 minutes, whereas the other section was preincubated in fresh Krebs solution. Then both sections were incubated in the presence of 100 μL dihydroethidine (2×10^{-6} mol/L) in a dark incubator at 37°C for 30 minutes. Ethidium fluorescence (red signal) was detected with a 563-nm long-pass filter after excitation at 543 nm, and images were obtained with a Zeiss LSM 510 confocal microscope equipped with a krypton/argon laser. A second acquisition was performed using a 505- to 535-nm band-pass filter after excitation at 488 nm to detect the autofluorescence of elastin. Four aortas of each group were studied, and 2 to 3 areas per aortic ring were analyzed. Experiments were performed on duplicate.

Data Analysis

Results are presented as mean±SEM. Comparisons between groups were made by repeated-measures ANOVA or by one-way ANOVA followed by a Student Newman-Keuls test, where appropriate. $P<0.05$ was considered statistically significant.

Results

Mthfr$^{+/+}$ and *Mthfr*$^{+/-}$ mice had similar SBP and body weight (Table). Ang II infusion significantly increased SBP similarly in both groups. Plasma total Hcy was significantly increased in *Mthfr*$^{+/-}$ compared with *Mthfr*$^{+/+}$ mice and unaffected by Ang II (Table).

Vasodilation of resistance arteries to acetylcholine was attenuated in *Mthfr*$^{+/-}$ compared with *Mthfr*$^{+/+}$ mice (highest dose, 79.0±2.6% versus 96.6±0.8%, respectively; $P<0.01$). Endothelium-dependent vasodilation was also blunted in Ang II–infused *Mthfr*$^{+/+}$ mice (65.2±2.2%, $P<0.01$ versus *Mthfr*$^{+/+}$) and additionally reduced in Ang II–infused *Mthfr*$^{+/-}$ mice (56.6±0.9%, $P<0.01$, versus Ang II–infused *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice) (Figure 1A). Similarly, endothelium-dependent relaxation to bradykinin (Figure 1B) was significantly ($P<0.01$) blunted in *Mthfr*$^{+/-}$ (highest dose, 79.3±1.0%) compared with *Mthfr*$^{+/+}$ mice (95.4±0.7%). Vasodilation to bradykinin was also reduced in Ang II–infused *Mthfr*$^{+/+}$ (73.6±5.4%, $P<0.01$ versus *Mthfr*$^{+/+}$) and additionally attenuated in Ang II–infused *Mthfr*$^{+/-}$ mice (57.7±1.8%, $P<0.01$

Physiological and Morphological Characteristics and NADPH Oxidase Activity of Resistance Arteries From *Mthfr*$^{+/+}$ or *Mthfr*$^{+/-}$ Mice±Ang II Infusion

Parameter	*Mthfr*$^{+/+}$	*Mthfr*$^{+/-}$	*Mthfr*$^{+/+}$ With Ang II	*Mthfr*$^{+/-}$ With Ang II
Body weight, g	25.4±1.6	26.7±0.9	24.3±1.1	26.3±1.2
SBP, mm Hg	116.6±1.7	116.2±1.0	171.1±1.8*	169.4±2.5†
Plasma total Hcy, mmol/L	4.3±0.3	7.7±0.5*	5.3±0.7	8.2±0.4‡
Lumen diameter, μm	215.8±9.8	205.8±13.7	200.7±8.6	208.1±14.8
Media thickness, μm	12.2±0.5	12.6±0.7	14.2±0.4§	14.4±0.3‖
Media to lumen, %	5.7±0.2	6.1±0.2	7.1±0.2§	6.8±0.3‖
Media CSA, $10^3\times\mu m^2$	8.7±0.6	8.7±1.0	9.6±0.6	10.0±0.6
NADPH oxidase activity, 10^5 cpm/mg dry tissue weight	138±28	143±38	469±130§	382±41‖
Xanthine oxidase activity, cpm/mg dry tissue weight	60697±8518	54037±5710	Not done	Not done

N=7 per group; results are mean±SEM.

*$P<0.01$ vs *Mthfr*$^{+/+}$; †$P<0.01$ vs *Mthfr*$^{+/-}$; ‡$P<0.01$ vs *Mthfr*$^{+/+}$ with Ang II; §$P<0.05$ vs *Mthfr*$^{+/+}$; ‖$P<0.05$ vs *Mthfr*$^{+/-}$.

versus Ang II–infused *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice). Endothelium-independent relaxation by sodium nitroprusside was similar in all groups (Figure 1C).

In *Mthfr*$^{+/-}$ mice, the inhibitory effect exerted by L-NAME on acetylcholine (highest dose acetylcholine, 74.7±1.0%; with L-NAME, 60.8±1.2%; inhibition, 13.9±0.9%) was significantly lower compared with that in *Mthfr*$^{+/+}$ mice (acetylcholine, 93.0±1.9%; with L-NAME, 59.8±3.7%; inhibition, 33.2±2.6%) (Figures 2A, 2B, and 2E). In *Mthfr*$^{+/-}$ mice, vitamin C administration significantly improved the response to acetylcholine (87.3±2.1%) and restored the inhibitory effect of L-NAME (55.2±2.9%; inhibition, 32.1±2.9%) (Figures 2B and 2E). In contrast, in control *Mthfr*$^{+/+}$ mice, the antioxidant failed to modify either the relaxing effect of acetylcholine (93.8±1.9%) or the inhibitory effect of L-NAME (62.2±3.3%; inhibition, 31.7±2.7%) (Figures 2A and 2E). In the presence of vitamin C, the relaxing effect of acetylcholine and the inhibitory effect of L-NAME were no longer different between *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice (Figure 2E).

In Ang II–infused *Mthfr*$^{+/+}$ mice, the inhibitory effect of L-NAME on acetylcholine (highest-dose acetylcholine, 65.2±4.4%; with L-NAME, 54.7±0.5%; inhibition, 10.4±4.1%) was significantly reduced compared with that in *Mthfr*$^{+/+}$ mice. In the presence of vitamin C, the relaxing effect of acetylcholine was significantly improved (88.7±4.9%) and the inhibitory effect of L-NAME was restored (56.0±5.7%; inhibition, 32.7±1.7%) (Figures 2C and 2F).

In Ang II–*Mthfr*$^{+/-}$ mice, the inhibitory effect of L-NAME on acetylcholine was absent (acetylcholine, 54.6±0.8%; with L-NAME, 50.8±0.9%; inhibition, 2.3±0.5%) (Figures 2D and 2F). Vitamin C significantly enhanced the response to acetylcholine (86.6±1.1%) and restored the inhibitory effect of L-NAME (56.4±2.0%; inhibition, 30.2±2.4%) (Figures 2D and 2F).

Media thickness, similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice, was significantly increased after Ang II infusion in both groups (Table). Media to lumen ratio was similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice (6.2±0.2% and 5.7±0.2%, respectively) and significantly ($P<0.05$) increased by Ang II (*Mthfr*$^{+/-}$, 7.1±0.5%; *Mthfr*$^{+/+}$, 7.1±0.2%) (Table). Media cross-sectional area of resistance arteries was similar in all groups (Table).

NADPH oxidase activity in mesenteric arteries was similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice and significantly ($P<0.05$) increased by Ang II infusion, independently of Hcy plasma levels (Table). Xanthine oxidase activity was similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice. Figure 3 shows representative confocal fluorescent microscopy sections of aortic rings from wild-type and *Mthfr*$^{+/-}$ mice incubated with dihydroethidine for detection of superoxide production. In *Mthfr*$^{+/-}$ mice (upper right), the intensity of the fluorescent signal was higher than that observed in *Mthfr*$^{+/+}$ (upper left) and detected mainly in the media. Preincubation of the same vessel with L-NAME (10^{-5} mol/L, lower left) or sepiapterin (10^{-4} mol/L, lower right), a precursor of tetrahydrobiopterin, decreased superoxide production. This suggests that free radical formation in this model may be dependent on decreased bioavailability of tetrahydrobiopterin and uncoupling of NO synthase.

Discussion

This new murine model of mild H-Hcy, attributable to heterozygous deficiency of *Mthfr* that mimics mild MTHFR deficiency in humans, is characterized by reduced endothelium-dependent vasodilation. This alteration is caused by reduced NO bioavailability attributable to increased oxidative stress as demonstrated by the results with L-NAME and vitamin C. Our finding that mild hyperhomocystinemic *Mthfr*$^{+/-}$ mice are characterized by small artery endothelial dysfunction agrees with and extends previous studies in different animal models and in humans. Indeed, rats with diet-induced mild H-Hcy showed an impaired endothelium-dependent vasodilation of skeletal muscle arterioles.[3] In mice heterozygous for a deletion in the cystathionine β-synthase (CBS) gene, reduced endothelium-dependent vasodilation in conduit and resistance vessels was observed.[5] In humans, either acutely after methionine administration or chronically in mild H-Hcy, endothelium-dependent vasodilation is impaired in the peripheral macrocirculation and microcircula-

tion.[6–10] The reduced endothelial function observed in *Mthfr*$^{+/-}$ mice in the present study was obtained with both acetylcholine and bradykinin. These 2 agonists act on different receptor and signal transduction pathways involving a G_i protein that is sensitive and insensitive to pertussis toxin, respectively.[16,17] Therefore, our findings indicate that endothelial dysfunction is not related to a specific defect of the muscarinic receptor for acetylcholine or to an abnormality of a single intracellular signal-transduction pathway but to a more generalized abnormality of endothelial vasodilator function in this model of mild H-Hcy, which seems to depend on enhanced production of reactive oxidative species. Our measurement of NADPH oxidase and xanthine oxidase activity shows that neither of these potential sources of increased oxidative excess contributes to increased reactive oxygen species formation in this model of mild H-Hcy, because their activity was similar in *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice. However, the results with sepiapterin, a precursor of tetrahydrobiopterin that is a cofactor for NO synthesis and may be oxidized by free radicals[21] and L-NAME, strongly implicate uncoupling of NO synthase with production of free radicals as the possible source of oxidant excess,[22,23] associated with decreased bioavailability of NO as shown in the study of endothelium-dependent relaxation of intact resistance arteries.

A major novel finding of our study concerns the mechanisms responsible for endothelial dysfunction in this model. To assess NO availability and oxidative stress, we used L-NAME and vitamin C, respectively. In *Mthfr*$^{+/-}$ mice, the inhibitory effect exerted by L-NAME on acetylcholine responses was significantly lower compared with that observed in controls, indicating reduced NO bioavailability. Vitamin C improved the response to acetylcholine, demonstrating the presence of decreased bioavailability of ROS in H-Hcy mice. The finding that in the presence of the antioxidant the inhibitory effect of L-NAME on acetylcholine was restored suggests that reduced NO bioavailability was caused by increased production of ROS. These findings agree with previous studies proposing a major role of ROS in the mechanisms responsible for Hcy-induced endothelial dysfunction. An in vitro study from rabbit aorta showed that the inhibitory effect of homocysteine on acetylcholine-dependent relaxation was attributable to an increase in the endothelial cell intracellular levels of superoxide anion.[4] Accordingly, Eberhardt et al[5] demonstrated a greater superoxide production in aortic tissue from heterozygous CBS-deficient mice. In human studies, Chambers et al[8] and Kanani et al[9] found that in healthy subjects, oral administration of vitamin C prevented the decrease in flow-mediated vasodilation after methionine loading in peripheral macrocirculation. More recently, it has been demonstrated that in the forearm microcirculation of normotensive subjects, mild chronic H-Hcy impairs endothelial function by producing oxidative stress that reduced NO bioavailability.[10] As mentioned above, our results suggest uncoupling of NO synthase that results in generation of free radicals that inactivate tetrahydrobiopterin, which aggravates the deficient generation of NO and impaired endothelial function.

Vitamin C is considered an extremely effective antioxidant. However, it should be pointed out that the very low rate constant of reaction between this compound and superoxide ($k=3\times10^5$ mol/L per s) requires providing vitamin C at very high concentrations (10 to 100 mmol/L) to successfully compete with NO for superoxide anion.[24] The dose of vitamin C used in the present study (100 μmol/L) is lower than that necessary to scavenge superoxide. Therefore, in our experimental conditions, we cannot exclude that the beneficial effect of vitamin C on NO availability might depend on other mechanisms, such as stimulation of NO production, reduced lipid peroxidation, or increased production of intracellular tetrahydrobiopterin.[25]

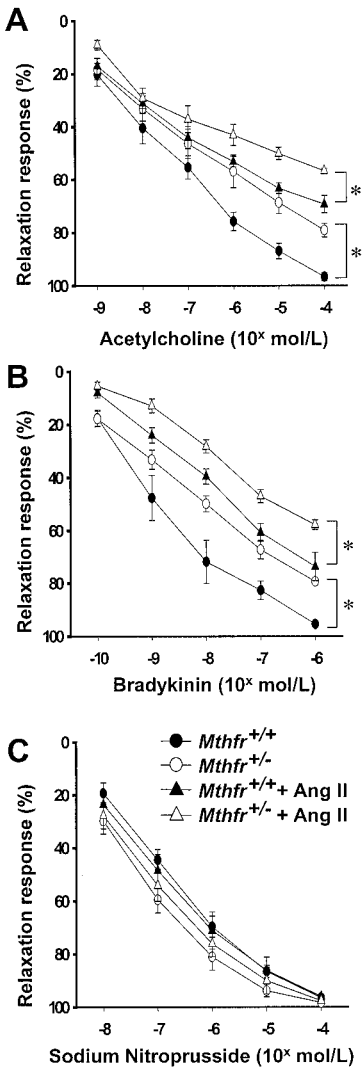

Figure 1. Endothelium-dependent relaxation of mesenteric resistance arteries from *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice±Ang II to acetylcholine (A) and bradykinin (B) and endothelium-independent relaxation to sodium nitroprusside (C). Results are mean±SEM. *$P<0.01$.

Another major finding of our study is that the concurrent presence of hypertension as observed in Ang II–infused *Mthfr*$^{+/-}$ mice additionally reduced the endothelium-dependent relaxation, showing additive adverse effects of these 2 risk factors on endothelial function. Our data agree with a recent human study indicating that essential hypertension potentiates endothelial dysfunction of forearm resistance vessels in patients with chronic mild H-Hcy.[10] With regard to the impact of hypertension, we observed that the inhibitory effect of L-NAME on

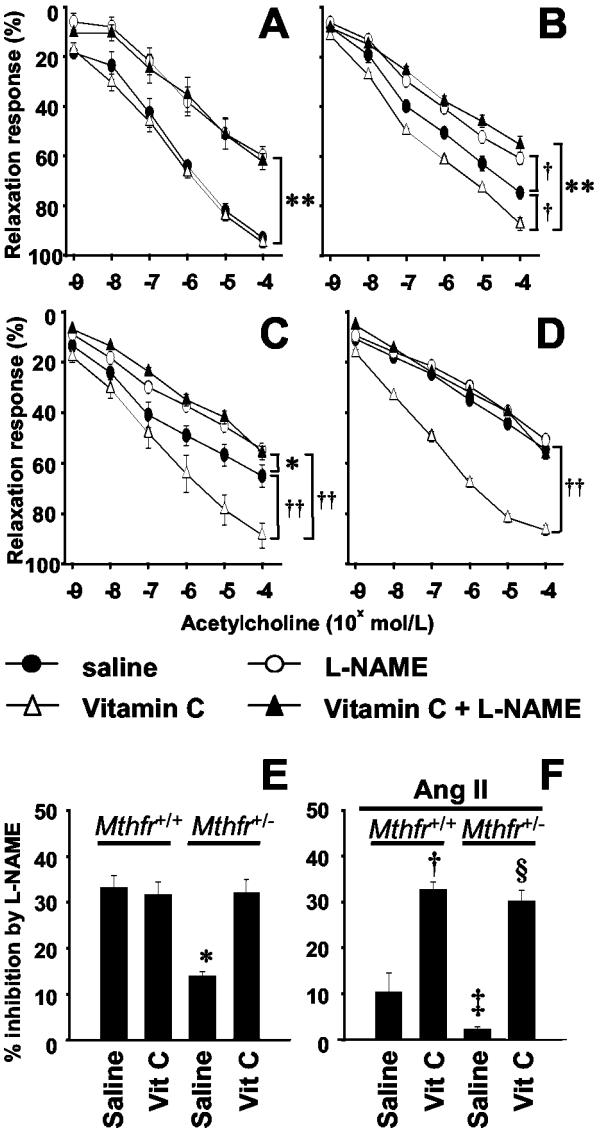

Figure 2. Endothelium-dependent relaxation of mesenteric resistance arteries from *Mthfr*$^{+/+}$ (A) and *Mthfr*$^{+/-}$ (B) mice to acetylcholine in the absence (saline) and in the presence of L-NAME, vitamin C, or both. Endothelium-dependent relaxation of mesenteric resistance arteries from Ang II–infused *Mthfr*$^{+/+}$ (C) and Ang II–infused *Mthfr*$^{+/-}$ mice (D) to acetylcholine in the absence (saline) and in the presence of L-NAME, vitamin C, or both. Inhibition exerted by L-NAME on maximal relaxing response of mesenteric resistance arteries from *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice (E) and from Ang II–infused *Mthfr*$^{+/+}$ and Ang II–infused *Mthfr*$^{+/-}$ mice (F) to acetylcholine in the absence (saline) and in the presence of vitamin (Vit) C. Results are mean±SEM. A, B, C, and D, **P*<0.01; ***P*<0.001; †*P*<0.01; ††*P*<0.001. E and F, **P*<0.001 vs other groups; †*P*<0.001 vs saline Ang II–*Mthfr*$^{+/+}$; ‡*P*<0.05 vs saline Ang II–*Mthfr*$^{+/+}$; §*P*<0.001 vs saline Ang II–*Mthfr*$^{+/-}$.

acetylcholine was reduced in Ang II–*Mthfr*$^{+/+}$ mice. This alteration was reversed by vitamin C, confirming that Ang II–induced hypertension is characterized by an increased production of ROS.[11,26] Importantly, in Ang II–*Mthfr*$^{+/-}$ mice the response to acetylcholine was totally resistant to L-NAME, indicating that NO bioavailability was dramatically decreased. Vitamin C restored the response to acetylcholine and the inhibitory effect of L-NAME. Taken together, these findings

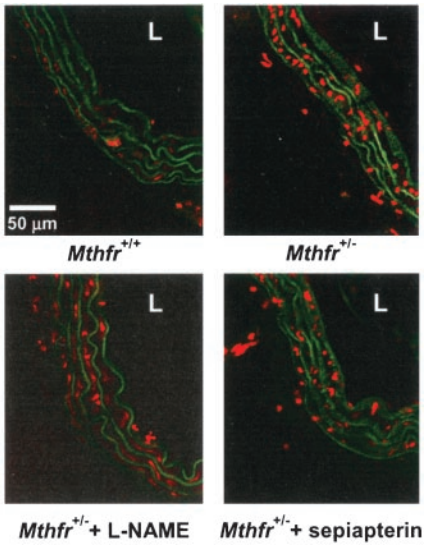

Figure 3. Representative confocal fluorescent microscopy sections of aortic rings from wild-type and *Mthfr*$^{+/-}$ mice incubated with dihydroethidine for detection of superoxide. In *Mthfr*$^{+/-}$ mice (upper right), the intensity of the fluorescent signal was higher than that observed in *Mthfr*$^{+/+}$ (upper left) and detected mainly in the media. Preincubation of the same vessel with L-NAME (10^{-5} mol/L, lower left) or sepiapterin (10^{-4} mol/L, lower right), a precursor of tetrahydrobiopterin, decreased superoxide production. Original magnification is ×320. L indicates lumen.

allow us to conclude that the additive adverse effect of mild H-Hcy and hypertension on endothelial function depends on reduction of NO bioactivity. The greater impairment of endothelial function seen in Ang II–infused *Mthfr*$^{+/-}$ mice is caused by an exacerbated production of NADPH-derived ROS induced by Ang II, likely leading to decreased NO bioavailability. Our results agree with a recent human study showing that in essential hypertensive patients the presence of H-Hcy led to an additional reduction in endothelial function by enhanced oxidative stress.[10] In this experimental paradigm, production of endothelium-dependent hyperpolarizing factor did not seem to be affected, because bradykinin responses were conserved, and there did not seem to be a compensatory increase of endothelium-dependent hyperpolarizing factor in the presence of decreased bioavailability of NO,[27] because acetylcholine responses in *Mthfr*$^{+/-}$ mice infused or not with Ang II were abnormal.

The structure of mesenteric vessels was unaltered in *Mthfr*$^{+/-}$ mice. This agrees with results from heterozygous CBS-deficient mice characterized by endothelial dysfunction in the absence of frank atherosclerotic lesions.[5] A likely explanation of this finding may be the young age of the mice. Vascular structural changes consequent to chronic exposure to H-Hcy may be a later manifestation compared with functional alterations. As expected, the presence of Ang II produced structural alterations in mesenteric resistance arteries in this study. This effect occurred independently of Hcy plasma levels.

The association of homocysteine with endothelial dysfunction has been documented in a large number of animal and human studies.[3–10] The originality of the present study relates to the fact that for the first time a reduced NO availability has been described in this new murine of mild hyperhomocystinemia. With respect to the clinical relevance of our findings, it is

noteworthy that this model mimics a common cause of mild hyperhomocystinemia in humans. Indeed, mild MTHFR deficiency occurs in humans as a result of the common mutation of *MTHFR* (677C→T) that decreases the enzyme activity to a similar extent as that observed in these *Mthfr*$^{+/-}$ mice,[12,13] resulting in comparable increases in plasma Hcy levels.[13,28] This mutation is present in the homozygous state in 10% to 15% of North American and European populations.[13,28] Present and future work with this mouse model will allow us to clarify the role of *MTHFR* deficiency in the pathogenesis of vascular disease.

In conclusion, mild H-Hcy attributable to heterozygous *Mthfr* deficiency impairs endothelium-dependent vasodilation of small mesenteric vessels in the absence of structural alterations. Attenuated endothelium-dependent vasodilation is caused by a reduced NO availability that could be secondary to enhanced oxidative stress. NADPH and xanthine oxidase do not seem to be major sources of ROS involved in Hcy-induced functional alterations. Reduced bioavailability of tetrahydrobiopterin and uncoupling of NO synthase may contribute to the oxidant excess, as evidenced by our findings with sepiapterin and L-NAME. The concurrent presence of Ang II–induced hypertension additionally reduced endothelial function, possibly by exacerbating the production of free radicals, suggesting an additive adverse effect of Ang II, hypertension, and mild H-Hcy on endothelial function.

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