Objective.—We tested the hypothesis that hyperhomocysteinemia and hypercholesterolemia promote arterial thrombosis in mice.

Methods and Results.—Male apolipoprotein E (ApoE)-deficient mice were fed one of four diets: control, hyperhomocysteinemic (HH), high fat (HF), or high fat/hyperhomocysteinemic (HF/HH). Total cholesterol was elevated 2-fold with the HF or HF/HH diets compared with the control or HH diets (P<0.001). Plasma total homocysteine (tHcy) was elevated (12 to 15 μmol/L) with the HH or HF/HH diets compared with the control or HF diets (4 to 6 μmol/L; P<0.001). Aortic sinus lesion area correlated strongly with total cholesterol (P<0.001) but was independent of tHcy. At 12 weeks of age, the time to thrombotic occlusion of the carotid artery after photochemical injury was >50% shorter in mice fed the HF diets, with or without hyperhomocysteinemia, compared with the control diet (P<0.05). At 24 weeks of age, carotid artery thrombosis was also accelerated in mice fed the HH diet (P<0.05). Endothelium-dependent nitric oxide–mediated relaxation of carotid artery rings was impaired in mice fed the HF, HH, or HF/HH diets compared with the control diet (P<0.05).

Conclusions.—Hyperhomocysteinemia and hypercholesterolemia, alone or in combination, produce endothelial dysfunction and increased susceptibility to thrombosis in ApoE-deficient mice.

Key Words:

Hyperhomocysteinemia is a risk factor for stroke, cardiovascular disease, and venous thromboembolism. The association between hyperhomocysteinemia, defined as an elevation of plasma total homocysteine (tHcy), and atherosclerosis was first recognized in children with severe hyperhomocysteinemia (plasma tHcy ≥100 μmol/L) caused by inborn errors of the methionine cycle. It is now known that even mild elevation of plasma tHcy (10 to 20 μmol/L) is associated with increased cardiovascular risk. A meta-analysis of prospective studies concluded that a 3 μmol/L elevation in plasma total homocysteine is predictive of a 10% increased risk of myocardial infarction and a 20% increased risk of stroke, after adjustment for other known risk factors. Another recent meta-analysis found that a 5 μmol/L elevation of plasma tHcy was associated with a 27% higher risk of venous thromboembolism.

Considerable progress in defining the vascular effects of hyperhomocysteinemia has been achieved through the use of murine models. Endothelial dysfunction, caused by decreased bioavailability of endothelium-derived nitric oxide, has been detected in the aorta, mesenteric arterioles, and cerebral arterioles of mice with mild hyperhomocysteinemia (plasma tHcy 10 to 20 μmol/L). Elevation of plasma tHcy also has been shown to accelerate the development of atherosclerosis in the hypercholesterolemic ApoE-deficient (ApoE−/−) mouse, but only in the presence of moderate (plasma tHcy 25 to 50 μmol/L) or severe (plasma tHcy 90 to 210 μmol/L) hyperhomocysteinemia. It is not known whether mild elevation of plasma tHcy, which is present in the majority of human subjects with hyperhomocysteinemia-associated atherothrombosis, accelerates the development of atherosclerosis in susceptible animal models such as the ApoE−/− mouse. It also is not known whether hyperhomocysteinemia influences the susceptibility to thrombosis or endothelial dysfunction in ApoE−/− mice.

In the current study, we tested the hypothesis that mild hyperhomocysteinemia augments atherosclerosis and accelerates carotid artery thrombosis in ApoE−/− mice. Because the development of atherosclerotic lesions in ApoE−/− mice is highly dependent on sex, diet, and age, we chose to study...
the effects of diet-induced hyperhomocysteinemia in male Apoe−/− mice fed either a control diet or a high fat (Western) diet, and we assessed atherosclerotic lesion area at two time points (12 and 24 weeks of age). Our results demonstrate that mild hyperhomocysteinemia does not influence aortic lesion area but does cause increased susceptibility to thrombosis in an age-dependent manner. To begin to explore the mechanism of accelerated thrombosis, we examined the effects of hyperhomocysteinemia on platelet activation, aortic tissue factor expression, and carotid artery endothelial function in Apoe−/− mice.

Methods

Mice and Experimental Protocol
Animal use protocols were approved by the University of Iowa and Veterans Affairs Animal Care and Use Committees. Apoe−/− mice19 on the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, Me), and a breeding colony was maintained at the University of Iowa. At three weeks of age, mice were fed one of four diets: (1) control (LM 485, Harlan Teklad, Madison, Wis), which contains 4.0 g/Kg methionine, 6.7 mg/Kg folic acid, and 5.5% fat; (2) hyperhomocysteinemic (HH; TD00205, Harlan Teklad), which contains 8.2 g/Kg methionine, 0.2 mg/Kg folic acid, and 5.5% fat; (3) high fat (HF; TD03159, Harlan Teklad), which contains 4.5 g/Kg methionine, 6.7 mg/Kg folic acid, and 20.3% fat; or (4) high fat (TD03066, Harlan Teklad), which contains 13.5 g/Kg methionine, 6.7 mg/Kg folic acid, and 20.3% fat. Mice heterozygous for disruption of the Apoe gene (Apoe+/−) were fed the control diet and served as an additional control group. Experimental procedures were performed on male mice at either 12 or 24 weeks of age.

Plasma tHcy, Methionine, and Cholesterol
Blood samples were collected from anesthetized mice by cardiac puncture into EDTA (final concentration 5 mmol/L) for measurement of plasma tHcy, methionine, and total cholesterol. Plasma tHcy, the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds,2 was measured by high-performance liquid chromatography (HPLC) and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBDF) fluorescence detection.19 Plasma methionine was measured by HPLC coupled to fluorescence detection after precolumn derivatization using o-phthalaldehyde as described previously.20 Plasma total cholesterol was measured enzymatically using the Infinity Cholesterol Reagent (Thermo Electron Corporation).

Morphometric Analysis of Atherosclerotic Lesions
The heart and common carotid arteries were removed, washed in PBS, and immersed in Penfix (Richard Allen Scientific) for at least 48 hours. The heart was then cut transversely and embedded in paraffin, and 8-μm serial sections were cut through the entire aortic sinus and stained with Verhoeff-van Geison. Lesion area was measured using NIH Image J 1.29x, and calculated as the mean of 5 sections, each 80 μm apart, spanning 320 μm of the aortic sinus, beginning at the aortic valve leaflets.21 Sections (10 μm) of the right common carotid artery were cut every 200 μm from the innominate artery to the carotid bifurcation and stained with Verhoeff-van Geison.

Immunohistochemistry
Immunohistochemical staining was performed using the VEC-TASTAIN ABC System (Vector Laboratories) as described previously.14 Paraffin-embedded sections (4 μm) of the aortic sinus were deparaffinized and the endogenous peroxidase activity blocked with 0.5% hydrogen peroxide in methanol for 10 minutes. After blocking with 5% normal goat or rabbit serum, sections were incubated with rabbit anti-phospho-PERK (Cell Signaling Technology, Beverly, Mass) or sheep anti-human tissue factor antibody (SATF-IG, Affinity Biologicals Inc, Ancaster, ON) for 1 hour, followed by goat anti-rabbit or rabbit anti-sheep biotinylated secondary antibodies (Vector Laboratories) for 30 minutes, and streptavidin-peroxidase (Zymed Laboratories, San Francisco, Calif) for 5 minutes. Sections were developed in Nova Red peroxidase substrate (Vector Laboratories) and counterstained with hematoxylin.

Carotid Artery Thrombosis
Carotid artery thrombosis was induced by photochemical injury as described previously.22 Mice were anesthetized with sodium pentobarbital (70 to 90 mg/kg intraperitoneally) and ventilated mechanically with room air and supplemental oxygen using a Harvard rodent respirator. The left femoral vein was cannulated for the administration of rose bengal. Carotid artery blood flow was measured with a 0.5 PSB Doppler flow probe (Transonic Systems Inc) and digital recording system (Gould Ponemah Physiology Platform, version 3.33). The right common carotid artery was transilluminated immediately proximal to the flow probe with a 1.5-mV, 540-nm green laser (Melles Griot) from a distance of 6 cm, and rose bengal (50 mg/kg for mice at 12 weeks of age and 35 mg/kg for mice at 24 weeks of age) was injected via a femoral vein catheter. The time to first occlusion was defined as the time at which blood flow first decreased to zero for ≥10 seconds, and the time to stable occlusion was defined as the time at which blood flow remained absent for ≥10 minutes.

Platelet Activation
Washed platelets were isolated from male mice aged 15 to 21 weeks and resuspended in modified Tyrode buffer as described previously.23 Platelet procoagulant activity was measured in a prothrombinase assay.23 Briefly, washed platelets were either left unstimulated or stimulated with the collagen receptor agonist convulxin (250 ng/ml; Centerchem), bovine α-thrombin (0.5 U/ml; Hematologic Technologies), or convulxin plus thrombin. After 5 minutes, bovine factor Xa (3.0 nmol/L) and bovine factor Va (6.0 nmol/L) were added, followed one minute later by bovine prothrombin (4.0 μmol/L). The rate of thrombin generation was determined by subsampling the reaction mixture and measuring thrombin activity using the chromogenic substrate Chromozym TH (1.9 mmol/L; Roche Diagnostics). For platelet flow cytometry, washed platelets (3.6×10^6 per reaction) were either left unstimulated or stimulated with convulxin (250 ng/ml) plus thrombin (0.5 U/ml) for 5 minutes at 37°C without stirring. Platelets were then incubated for 10 minutes at 23°C with fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Pharmingen), FITC-conjugated sheep anti-human fibrinogen antibody (Novus Biologicals), or FITC-conjugated anti-murine CD62P (BD Pharmingen). Platelets were then fixed in 1% paraformaldehyde, diluted, and analyzed on a Becton Dickinson FACScan flow cytometer as described previously.23

Vasomotor Responses
The right and left carotid arteries were removed from mice at 12 weeks of age and cleaned of loose connective tissue. Each artery was cut into two equal rings of 5 to 6 mm and suspended in an organ chamber containing Krebs oxygenated buffer at 37°C. Rings were contracted submaximally using the thromboxane analogue U46619, and relaxation dose-response curves were generated by cumulative additions of the endothelium-dependent vasodilator acetylcholine (10^-6 to 10^-4 mol/L), or the endothelium-independent vasodilator nitroprusside (10^-7 to 10^-4 mol/L), as described previously.8 Some rings were preincubated with indomethacin (10 μmol/L) or Nω-nitro-l-arginine (L-NNA; 100 μmol/L) for 30 minutes prior to addition of acetylcholine.

Statistical Analysis
One-way and two-way analysis of variance (ANOVA), with the Bonferroni post-hoc t test for multiple comparisons, was used to analyze effects of age and diet on body weight, total cholesterol, tHcy, aortic sinus lesion area, and platelet activation responses.
24 weeks of age, plasma tHcy levels had fallen to aortic sinus of
At 12 weeks of age, atherosclerotic lesions were seen in the
Atherosclerosis
Body Weight, Cholesterol, Homocysteine, and Methionine
Body weights were age-dependent, and were similar in male Apoe+/− and Apoe−/− mice fed either the control or HH diets (Table). Apoe−/− mice fed the HF or HF/HH diets tended to be heavier than those fed the control or HH diets, a difference that reached statistical significance only for the HF/HH diet at 24 weeks of age (P<0.05). As expected, plasma levels of total cholesterol were strongly influenced by diet, and the effects of diet on total cholesterol were similar at 12 weeks and 24 weeks of age (Table). Compared with Apoe+/− mice fed the control diet, total cholesterol was elevated over 5-fold in Apoe−/− mice fed the control diet, and over 10-fold in Apoe−/− mice fed the HF or HF/HH diets (P<0.001). Levels of total cholesterol in Apoe−/− mice fed the HH diet were slightly lower than those in Apoe−/− mice fed the control diet, an effect that was statistically significant at 12 weeks of age (6.5±0.6 versus 9.0±0.4 mmol/L; P<0.01) but not at 24 weeks of age (8.0±0.6 versus 9.3±0.4 mmol/L; P>0.1). Plasma levels of tHcy were elevated significantly in Apoe−/− mice fed the HH or HF/HH diets (P<0.01; Table). The degree of elevation of plasma tHcy at 12 weeks was in the range of mild hyperhomocysteinemia (12 to 15 μmol/L). Interestingly, at 24 weeks of age, plasma tHcy levels had fallen to 8.8±0.4 μmol/L in Apoe−/− fed the HH diet, and to 5.9±0.4 μmol/L in Apoe−/− mice fed the HF/HH diet. Plasma methionine levels did not differ significantly between any groups.

Atherosclerosis
At 12 weeks of age, atherosclerotic lesions were seen in the aortic sinus of Apoe−/− mice fed the HF or HF/HH diets. Aortic sinus lesion area was increased over 10-fold in Apoe−/− mice fed the HF or HF/HH diets compared with Apoe−/− mice fed the control diet (P<0.001; Figure 1). Apoe−/− mice fed the control or HH diets exhibited only scattered foam cells or early fatty streaks in the aortic sinus, with many mice having no detectable lesions. No atherosclerotic lesions were seen in the common carotid artery in any group of mice at 12 weeks of age. At 24 weeks of age, advanced atherosclerotic lesions were present in the aortic sinus of all four groups of Apoe−/− mice. Aortic sinus lesion area was significantly larger in Apoe−/− mice fed the HF or HH diets compared with Apoe−/− mice fed the control diet (P<0.001; Figure 1). Atherosclerotic lesions were also present in the carotid bifurcation of several Apoe−/− mice fed the HF or HH diets, but not in Apoe−/− mice fed the control or HF/HH diets. By linear regression analysis, aortic sinus lesion area correlated directly with plasma total cholesterol at both 12 weeks (R=0.80, P<0.001) and 24 weeks (R=0.89, P<0.001), but was independent of plasma tHcy.

<table>
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<tr>
<th></th>
<th>Apoe+/− Control diet</th>
<th>Apoe−/− Control Diet</th>
<th>Apoe−/− HH Diet</th>
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<td>9.0±0.4</td>
<td>6.5±0.6*</td>
<td>18.3±0.7*</td>
<td>17.2±0.8*</td>
</tr>
<tr>
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<td>6.0±0.4</td>
<td>15.3±1.0*</td>
<td>4.2±0.4</td>
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</tr>
<tr>
<td>Met, μmol/L</td>
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<td>33.0±4.9 (n=8)</td>
<td>28.0±2.7</td>
<td>24.1±1.5</td>
</tr>
</tbody>
</table>

*P<0.05 vs Apoe−/− mice fed the control diet by one-way ANOVA.
nd indicates not determined

Figure 1. Atherosclerotic lesion area in the aortic sinus of male Apoe−/− mice fed the control diet and male Apoe−/− mice fed the control, hyperhomocysteinemic (HH), high fat (HF), or high fat/hyperhomocysteinemic (HF/HH) diets. Black bars, 12 weeks of age (n=11 to 18); gray bars, 24 weeks of age (n=4 to 9). Values are mean±SE. *P<0.01 vs Apoe−/− mice fed the control diet.

Table: Body Weight, Plasma tHcy, and Total Cholesterol.
Aortic sinus lesion histology is shown in Figure 2. Compared with Apoe−/− mice fed the HF diet, Apoe−/− mice fed the HF/HH diet often had more complex atherosclerotic lesions, with prominent cholesterol clefts (Figure 2, D versus A) and strong immunohistochemical staining for phospho-PERK (Figure 2, E versus B), a marker of endoplasmic reticulum stress. Tissue factor was detected by immunohistochemistry at similar levels in the atherosclerotic lesions of Apoe−/− mice fed either the HF or HF/HH diets (Figure 2C and 2F).

Carotid Artery Thrombosis
Experimental thrombosis of the carotid artery was induced by photochemical injury in mice that were anesthetized and mechanically ventilated to prevent respiratory acidosis. At 12 weeks of age, thrombotic occlusion of the carotid artery after photochemical injury occurred 50% faster in Apoe−/− mice fed the HF or HF/HH diets compared with Apoe−/− mice fed the control or HH diets (P<0.05; Figure 3A). In Apoe−/− mice fed the HF or HF/HH diets, 50% of animals exhibited stable occlusion of the carotid artery within 17 minutes of photochemical injury, compared with 38 minutes in Apoe−/− mice fed the control diet and 26 minutes in Apoe−/− mice fed the HH diet (Figure 3B). No significant differences in thrombosis were observed between Apoe+/− and Apoe−/− mice fed the control diet (31±8 versus 32±5 minutes for first occlusion and 41±8 versus 46±7 minute for stable occlusion, respectively). These findings indicate that severe hypercholesterolemia, with or without mild hyperhomocysteinemia, causes accelerated thrombosis, even in the absence of histologically discernable atherosclerosis of the carotid artery.

At 24 weeks of age, thrombotic occlusion of the carotid artery was accelerated not only in Apoe−/− mice fed the HF/HH and HF diets, but also in Apoe−/− mice fed the HH diet (Figure 3C). Fifty percent of the animals fed the HH or HF/HH diets exhibited stable occlusion of the carotid within 11 minutes of photochemical injury, compared with 49 minutes in Apoe−/− mice fed the control diet and 22 minutes in Apoe−/− mice fed the HF diet (Figure 3B). These findings suggest that hyperhomocysteinemia itself is prothrombotic in Apoe−/− mice.

Platelet Activation
To begin to explore possible mechanisms of accelerated thrombosis, we examined the effects of hypercholesterolemia and hyperhomocysteinemia on the activation of platelets isolated from Apoe−/− mice. Platelets were either left unstimulated, or stimulated with the collagen receptor agonist convulxin (250 ng/mL), thrombin (0.5 U/mL), or convulxin plus thrombin. The extent of platelet activation was assessed by measuring the procoagulant activity of activated platelets in a thrombin generation assay and by flow cytometric measurement of annexin V binding and surface fibrinogen (Figure 4). As expected, dual stimulation with thrombin and convulxin produced greater levels of thrombin generation, annexin V binding, and fibrinogen surface expression than stimulation with either convulxin or thrombin alone, but no significant effects of Apoe genotype or diet were observed for any of the platelet activation responses. We also did not observe any differences in the surface expression of P-selectin, a marker of platelet alpha granule release (not shown).

Endothelial Function
To further explore the mechanism of accelerated thrombosis, we examined endothelium-dependent and -independent vaso-motor responses in common carotid artery rings. No differences in the relaxation dose-response curves to the endothelium-independent vasodilator, nitroprusside, were seen...
between Apoe−/− mice fed the control, HH, HF, or HF/HH diets (Figure 5A). Relaxation responses to the endothelium-dependent vasodilator, acetylcholine, also were similar in Apoe−/− mice fed the four diets, except for a small decrease in relaxation to the highest dose of acetylcholine in Apoe−/− mice fed the HF/HH diet (54±3% versus 65±3%; P<0.05; Figure 5B). Larger differences in response to acetylcholine were seen after preincubation of carotid artery rings with 10 μmol/L indomethacin, an inhibitor of prostaglandin (PG)-mediated vasodilatation (Figure 5C). After preincubation with indomethacin, significant impairment of relaxation responses to acetylcholine was seen in Apoe−/− mice fed either the HF, HH, or HF/HH diets (38±8%, 49±6%, 51±4%, respectively) compared with Apoe−/− mice fed the control diet (68±5%; P<0.05). Preincubation of carotid artery rings with 0.1 mmol/L L-NNA, an inhibitor of nitric oxide synthase, decreased maximal relaxation responses to acetylcholine to less than 20% in all groups of mice (Figure 5D). These findings suggest that endothelium-derived nitric oxide is a major mediator of acetylcholine-induced vasodilation in all groups of Apoe−/− mice, and that a prostanoid vasodilator partially compensates for loss of endothelium-derived nitric oxide in mice fed the HH, HF, and HF/HH diets.

**Discussion**

The major findings of this study are that Apoe-deficient mice exhibit accelerated arterial thrombosis and endothelial dysfunction when fed high fat and/or hyperhomocysteinemic diets. The high fat or hyperhomocysteinemic diets did not affect platelet activation responses. These findings suggest that the mechanism of accelerated thrombosis is unlikely to be attributable to effects of hypercholesterolemia or hyperhomocysteinemia on platelet activation, but instead may be related to diminished production of endothelium-derived nitric oxide or other prothrombotic factors.

As expected,17 the HF diet had a profound influence on plasma total cholesterol (Table) and atherosclerotic lesion area (Figure 1). We also found that Apoe−/− mice fed the HF diet exhibited enhanced susceptibility to carotid artery thrombosis, particularly at 12 weeks of age (Figure 3). A previous report by Eitzman et al demonstrated that short-term (5 days) feeding of a high fat diet to Apoe−/− mice resulted in accelerated carotid artery thrombosis.24 Our data confirm and extend the findings of Eitzman et al in a model of chronic hypercholesterolemia.

The effect of hyperhomocysteinemia on atherosclerosis in Apoe−/− mice has been investigated in several prior studies that used either dietary or genetic approaches to elevate plasma tHcy.12–16 Many of these studies found that moderate or severe hyperhomocysteinemia (plasma tHcy concentrations of 25 to 50 μmol/L or higher) leads to accelerated development of atherosclerosis. The atherogenic effect of hyperhomocysteinemia was quite variable, however, perhaps because of factors such as diet, sex, and the severity and
duration of hyperhomocysteinemia.\textsuperscript{6} None of these previous studies investigated the effects of hyperhomocysteinemia on the susceptibility to thrombosis or endothelial dysfunction in Apoe\textsuperscript{−/−} mice. We chose to use HH and HF/HH diets that produce plasma tHcy concentrations of 12 to 15 μmol/L, which are comparable to the levels of tHcy seen in most patients with mild hyperhomocysteinemia and atherothrombotic disease.\textsuperscript{1}

In contrast to previous findings in Apoe\textsuperscript{−/−} mice with more severe hyperhomocysteinemia,\textsuperscript{12–16} we found that mild hyperhomocysteinemia did not influence atherosclerotic lesion area in the aortic sinus (Figure 1). However, Apoe\textsuperscript{−/−} mice fed the HF/HH diet did have more complex atherosclerotic lesions and exhibited stronger immunohistochemical staining for phospho-PERK, compared with Apoe\textsuperscript{−/−} mice fed the HF diet (Figure 2). Increased phospho-PERK staining is consistent with previous observations that hyperhomocysteinemia leads to endoplasmic reticulum (ER) stress in Apoe-deficient mice,\textsuperscript{14} perhaps through a peroxynitrite-mediated mechanism.\textsuperscript{25} Phospho-PERK is a key mediator of the unfolded protein response (UPR), an integrated intracellular signaling pathway that responds to ER stress by increasing the expression of ER-resident chaperones, attenuating general protein synthesis, and degrading misfolded ER proteins.\textsuperscript{26} Activation of the UPR also can trigger apoptotic and inflammatory pathways, and homocysteine has been reported to induce programmed cell death in endothelial cells through activation of the UPR.\textsuperscript{27} This mechanism may contribute to the prothrombotic effects of hyperhomocysteinemia.

The most novel finding of this study is that Apoe\textsuperscript{−/−} mice with mild hyperhomocysteinemia had markedly enhanced susceptibility to carotid artery thrombosis (Figure 3). The prothrombotic phenotype of Apoe\textsuperscript{−/−} mice fed the HH diet was more evident at 24 weeks of age than at 12 weeks of age, which suggests that prolonged hyperhomocysteinemia may be necessary to induce the prothrombotic phenotype. Apoe\textsuperscript{−/−} mice fed the HH diet, which produced both severe hypercholesterolemia and mild hyperhomocysteinemia, exhibited accelerated thrombosis of the carotid artery at both 12 and 24 weeks of age. To begin to explore possible mechanisms of accelerated thrombosis, we performed immunohistochemical staining for tissue factor in atherosclerotic lesions and measured platelet activation responses to thrombin and convulxin. In agreement with prior studies performed with LDL receptor-deficient mice,\textsuperscript{28} tissue factor was highly expressed in lesion-resident cells as well as the fibrous cap, but there was no apparent difference in tissue factor expression between Apoe\textsuperscript{−/−} mice with or without hyperhomocysteinemia (Figure 2). We also found no significant differences in platelet activation responses between any of the groups of mice (Figure 4). These findings argue against a major prothrombotic effect of the high fat or hyperhomocysteinemic diets on platelet activation or arterial tissue-factor expression. We cannot, however, exclude the possibility that tissue factor activity may have been altered in the carotid artery or in circulating vascular cells or microparticles.

We also considered the possibility that endothelial dysfunction may contribute to accelerated carotid artery thrombosis. We found clear evidence of endothelial dysfunction in carotid artery rings from Apoe\textsuperscript{−/−} mice fed either the HH, HF, or HF/HH diets (Figure 5). Preincubation experiments with the PG synthase inhibitor, indomethacin, or the nitric oxide synthase inhibitor, L-NNA, suggested that nitric oxide was the major mediator of acetylcholine-induced relaxation in all groups of mice and that a PG-dependent mechanism partially compensated for loss of nitric oxide in Apoe\textsuperscript{−/−} mice fed the HH and/or HF diets. Loss of endothelium-
derived nitric oxide is a plausible mechanism for accelerated thrombosis, but two groups have reported recently that mice deficient in endothelial nitric oxide synthase (Nos3) do not have accelerated thrombosis in response to carotid artery injury induced by ferric chloride.\textsuperscript{29,30} We have recently confirmed this finding in Nos3-deficient mice using the rose bengal photochemical injury model.\textsuperscript{31} These observations suggest that loss of endothelial nitric oxide synthase activity is not sufficient to accelerate arterial thrombosis in mice, but do not completely exclude the possibility that decreased nitric oxide contributes to accelerated thrombosis in mice with hypercholesterolemia or hyperhomocysteinemia. The precise role of diminished nitric oxide bioavailability in the prothrombotic effects of hypercholesterolemia and hyperhomocysteinemia is yet to be determined. Other prothrombotic mechanisms, such as diminished activation of anticoagulant protein C,\textsuperscript{31} altered fibrinogen structure, or decreased annexin 2 activity, also may play a role.\textsuperscript{6}

Finally, we observed an interesting influence of age on plasma tHcy levels in Apoe\textsuperscript{−/−} mice fed the HH or HF/HH diets. Plasma tHcy levels in these groups of mice were higher at 12 weeks of age than at 24 weeks of age (Table). We have observed a similar age-dependent decline in plasma tHcy associated with an increase in plasma methionine and a decrease in hepatic levels of S-adenosylmethionine in some cystathionine β-synthase–deficient mice fed high methionine diets (Dayal and Lentz, unpublished observations). These observations suggest that the age-dependent decline in plasma tHcy may be related to altered hepatic methionine adenosyltransferase activity. No age-dependent changes in methionine levels were seen in Apoe\textsuperscript{−/−} mice fed the HH or HF/HH diets (Table), however, which argues that metabolic effects other than loss of methionine adenosyltransferase activity may be responsible for the age-related decline in plasma tHcy. Confounding effects of altered methionine metabolism may shed light on the surprising results of a previous study in which plasma tHcy levels were lower than expected and did not correlate with atherosclerosis in Apoe\textsuperscript{−/−} mice fed a high methionine diet.\textsuperscript{16}

Although there is convincing evidence from epidemiological studies that mild elevation of plasma tHcy is associated with an increased risk of vascular events, it is not yet known whether treatment to lower homocysteine improves vascular outcomes. Several secondary prevention trials of homocysteine-lowering therapy (folic acid, vitamin B12, and/or pyridoxine) in subjects with stroke, coronary heart disease, venous thromboembolism, or renal disease have been initiated.\textsuperscript{1} Many of these trials are still ongoing, but a majority of those that have been completed have failed to demonstrate a clinical benefit of homocysteine-lowering therapy for the secondary prevention of vascular events.\textsuperscript{32} The negative results of these trials may be related to inadequate sample size and lack of adequate statistical power to demonstrate the 10% to 20% improvement in clinical outcome that might be expected from homocysteine-lowering treatment,\textsuperscript{1} or to confounding attributable to effects of folate fortification and inadequate vitamin B12 replacement.\textsuperscript{3,32,33} It also is possible that homocysteine-lowering therapy maybe more beneficial for the primary prevention of vascular disease rather than the secondary prevention of vascular events in subjects who already have advanced atherosclerosis.\textsuperscript{34} Until more clinical data become available, murine models will continue to be useful to define the mechanisms of enhanced thrombotic susceptibility in hyperhomocysteinemia and hypercholesterolemia.

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Disclosures

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Prothrombotic Effects of Hyperhomocysteinemia and Hypercholesterolemia in ApoE-Deficient Mice
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