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Research Article

Elevated plasma homocyst(e)ine may predispose to complications of vascular disease. Homocysteine alters vasomotor regulatory and anticoagulant properties of cultured vascular endothelial cells, but little is known about effects of hyperhomocyst(e)inemia on vascular function in vivo. We tested the hypothesis that diet-induced moderate hyperhomocyst(e)inemia is associated with vascular dysfunction in cynomolgus monkeys. Plasma homocyst(e)ine increased from 4.O +/- O.2 microM when monkeys were fed normal diet to 10.6 +/- 2.6 microM when they were fed modified diet (mean +/- SE; P = 0.02). Vasomotor responses were assessed in vivo by quantitative angiography and Doppler measurement of blood flow velocity. In response to activation of platelets by intraarterial infusion of collagen, blood flow to the leg decreased by 42 +/- 9% in monkeys fed modified diet, compared with 14 +/- 11% in monkeys fed normal diet (P = 0.008), Responses of resistance vessels to the endothelium-dependent vasodilators acetylcholine and ADP were markedly impaired in hyperhomocyst(e)inemic monkeys, which suggests that increased vasoconstriction in response to collagen may be caused by decreased vasodilator responsiveness to platelet-generated ADP. Relaxation to acetylcholine and, to a lesser extent, nitroprusside, was impaired ex vivo in carotid arteries from monkeys fed modified diet. Thrombomodulin anticoagulant activity in aorta decreased by 34 +/- 15% in hyperhomocyst(e)inemic monkeys (P = 0.03). We conclude that diet-induced moderate hyperhomocyst(e)inemia is associated with altered […]

Vascular Dysfunction in Monkeys with Diet-induced Hyperhomocyst(e)inemia

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Abstract

Elevated plasma homocyst(e)ine may predispose to complications of vascular disease. Homocysteine alters vasomotor regulatory and anticoagulant properties of cultured vascular endothelial cells, but little is known about effects of hyperhomocyst(e)inemia on vascular function in vivo. We tested the hypothesis that diet-induced moderate hyperhomocyst(e)inemia is associated with vascular dysfunction in cynomolgus monkeys. Plasma homocyst(e)ine increased from 4.0±0.2 μM when monkeys were fed normal diet to 10.6±2.6 μM when they were fed modified diet (mean±SE; $P = 0.02$). Vasomotor responses were assessed in vivo by quantitative angiography and Doppler measurement of blood flow velocity. In response to activation of platelets by intraarterial infusion of collagen, blood flow to the leg decreased by 42±9% in monkeys fed modified diet, compared with 14±11% in monkeys fed normal diet ($P = 0.008$). Responses of resistance vessels to the endothelium-dependent vasodilators acetylcholine and ADP were markedly impaired in hyperhomocyst(e)inemic monkeys, which suggests that increased vasoconstriction in response to collagen may be caused by decreased vasodilator responsiveness to platelet-generated ADP. Relaxation to acetylcholine and, to a lesser extent, nitroprusside, was impaired ex vivo in carotid arteries from monkeys fed modified diet. Thrombomodulin anticoagulant activity in aorta decreased by 34±15% in hyperhomocyst(e)inemic monkeys ($P = 0.03$). We conclude that diet-induced moderate hyperhomocyst(e)inemia is associated with altered vascular function. (*J. Clin. Invest.* 1996. 98:24–29.) Key words: acetylcholine • atherosclerosis • endothelium • homocysteine • thrombomodulin

Introduction

Moderate elevation of plasma homocyst(e)ine[1] concentration is associated with stroke, peripheral vascular disease, and myocardial infarction (1). Like hypercholesterolemia, hyperhomocyst(e)inemia is caused by both genetic and dietary factors and may possibly contribute to vascular disease in a large number of patients (2). Unlike hypercholesterolemia, hyperhomocyst(e)inemia has not been demonstrated to be a sufficient stimulus for development of atherosclerosis per se, but it appears to predispose to complications and perhaps progression of atherosclerosis. Plasma homocyst(e)ine concentration can be decreased by dietary supplementation with folic acid, which suggests that hyperhomocyst(e)inemia may be a treatable risk factor for vascular disease (1, 2).

Mechanisms responsible for the association between hyperhomocyst(e)inemia and vascular disease are poorly understood. Results of studies with cultured endothelial cells suggest that homocysteine may impair vasomotor regulatory and antithrombotic properties of vascular endothelium. Exposure of cultured endothelial cells to homocysteine impairs nitric oxide–mediated inhibition of platelet aggregation (3) and inhibits thrombomodulin-dependent activation of protein C, a clinically important anticoagulant (4, 5). Homocysteine also induces cultured endothelial cells to express procoagulant molecules (6, 7) and alters binding of tissue plasminogen activator to endothelium (8). These effects of homocysteine in tissue culture suggest that endothelial dysfunction may be a plausible mechanism for predisposition to atherosclerotic vascular disease in hyperhomocyst(e)inemia.

Because most effects on cultured endothelial cells were observed at concentrations of homocyst(e)ine that are higher than plasma levels in patients with moderate hyperhomocyst(e)inemia, the clinical relevance of these findings in vitro has been questioned (9, 10). Effects of moderate hyperhomocyst(e)inemia on vascular function have not been examined in vivo, in part because an appropriate animal model of moderate hyperhomocyst(e)inemia has not been available (11).

The goal of this study was to test the hypothesis that hyperhomocyst(e)inemia is associated with vascular dysfunction in vivo and in blood vessels ex vivo. Moderate hyperhomocyst(e)inemia was induced in nonhuman primates by dietary modification, and vasomotor regulatory and anticoagulant functions were compared during periods of normal and moderately elevated plasma homocyst(e)ine concentration.

Methods

Animals. To induce moderate hyperhomocyst(e)inemia, adult cynomolgus monkeys (*Macaca fascicularis*) were fed modified diet that was enriched in methionine (1.0 gram/100 grams), relatively depleted in folic acid (0.15 mg/100 grams), and free of choline (Malinow, M.R., M. Axthelm, S. Kelley, and B. Upson, unpublished observations). In a randomized cross-over design, eight monkeys (weight, 5–8 kg) were

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1. The term "homocyst(e)ine" is used to indicate that plasma homocysteine assays measure the total concentration of thiol, disulfide, and mixed disulfide adducts of homocysteine (see reference 33).

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assigned to receive either normal diet (Purina Monkey Chow; Ralston-Purina, Richmond, VA) or modified diet for 4 wk, followed by the other diet for 4 wk.

Experimental protocol. After the first 4-wk period, animals were sedated with ketamine hydrochloride (25 mg/kg intramuscularly) and anesthetized with sodium pentobarbital (30 mg/kg intravenously). A tracheotomy was performed, and animals were intubated and ventilated with room air and supplemental oxygen. Heart rate, respirations and blood pressure were monitored continuously. A nonobstructive multiple sidehole catheter equipped with a Doppler transducer was inserted into the right femoral artery and positioned in the distal aorta, and the right femoral vein was cannulated for administration of supplemental anesthesia (pentobarbital 15 mg/kg intravenously as needed) and other drugs.

Changes in blood flow to the leg were measured in response to intraarterial infusion of collagen (150 mg/min for 10 min), or intraarterial injection of serotonin (100 μg), acetylcholine (1×10^{-7} and 3×10^{-7} mol), ADP (1×10^{-7} and 3×10^{-7} mol), or sodium nitroprusside (1×10^{-8} and 3×10^{-8} mol). Responses were monitored in vivo by quantitative angiography and Doppler measurement of hind limb blood flow velocity. Cineangiograms of the distal descending aorta and the left iliac arterial tree were obtained in an anterioposterior projection using standardized power injection of nonionic contrast (Iohexol; Sanofi-Winthrop Pharmaceuticals, New York) at a rate of 15 ml/s through the Doppler-tipped catheter. The target iliac and femoral arteries were not instrumented during angiography. Quantitation of arterial lumen diameter was performed using computerized arterial lumen edge detection software (12) as we have described previously (13, 14). Velocity of blood flow to the leg was measured using the Doppler transducer at the time of angiography. By measuring velocity of flow (by Doppler) and aortic mean diameter (by angiography), blood flow to the leg was calculated. At the end of the procedure, one common carotid artery was exposed, ligated proximally and distally with suture, and the isolated segment of artery was removed and placed into oxygenated Krebs solution. Removal of one carotid artery did not produce stroke or other adverse effects in any monkeys, and did not alter mean arterial blood pressure, which was 96±7 (mean±SE) mmHg after the first 4-wk period and 100±7 mmHg after the second 4-wk period.

After the second 4-wk period, animals were anesthetized again and measurements of vasomotor responses in vivo to collagen, serotonin, acetylcholine, ADP, and nitroprusside were repeated. Segments of thoracic aorta and the remaining common carotid artery were removed and placed into oxygenated Krebs solution. Then, animals were killed by administration of sodium pentobarbital (200 mg/kg intravenously) followed by exsanguination while under deep anesthesia. The protocol was approved by the University of Iowa Animal Care and Use Committee.

Carotid artery vasomotor responses. After removal of loose connective tissue, the common carotid artery was cut into multiple 5-mm rings. Carotid artery rings were suspended in an organ chamber containing oxygenated Krebs buffer maintained at 37°C, and connected to a force transducer to measure changes in isometric tension (contraction and relaxation). Rings were precontracted to a tension of 1.0 *g* by stepwise addition of prostaglandin $F_{2\alpha}$ (1–3 μM), and relaxation dose–response curves were generated by cumulative addition of acetylcholine (10^{-9} to 10^{-4} M) or sodium nitroprusside (10^{-9} to 10^{-5} M). In other rings, contraction dose–response curves were generated by cumulative addition of the thromboxane A_2 analogue U46619 (10^{-9} to 10^{-6} M).

Thrombomodulin-dependent protein C activation. Thrombomodulin activity was measured by modification of an assay described previously (15). After removal of adventitia and loose connective tissue, segments of thoracic aorta or common carotid artery were rinsed in Krebs solution, and cut into multiple discs of 3 mm in diameter (corresponding to an endothelial surface area of ~ 7 mm^2). Arterial discs were incubated for 60 min at 37°C in 50 μl of assay buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2.0 mM $CaCl_2$, 1% bovine serum albumin) containing 2.6 nM human thrombin (Enzyme Research Laboratories, South Bend, IN) and 0.84 μM human protein C (a generous gift of Dr. Hans Peter Schwarz, Immuno AG, Vienna, Austria). The reaction was stopped by addition of a mixture of 25 μg/ml antithrombin III and 25 U/ml heparin, and the amidolytic activity of activated protein C was measured spectrophotometrically using the chromogenic substrate S-2366 (Kabi Pharmacia Hepar, Inc., Franklin, OH). Reference curves were generated using rabbit lung thrombomodulin (American Diagnostica Inc., Greenwich, CT). One unit of activity was defined as the amount of activated protein C generated in the presence of 1.0 nM rabbit thrombomodulin.

Replicate assays were performed with two discs from carotid artery or three discs from aorta. Endothelial dependence of protein C activation was determined by performing assays with arterial discs from which endothelium had been denuded. Paired data for thrombomodulin activity in carotid artery were obtained by removing one carotid artery after monkeys were fed each diet for 4 wk. Because it is not feasible to obtain paired data for thrombomodulin activity in aorta, unpaired data were obtained in aorta after the second 4-wk diet, and additional assays were performed using aorta from two other monkeys fed either normal or modified diet for 4 wk.

Other assays. Frozen plasmas were coded and air shipped under dry ice to Dr. Malinow's laboratory in Beaverton, OR. Fasting plasma homocyst(e)ine concentration was measured by high-performance liquid chromatography and electrochemical detection, based on the method of Smolin and Schneider (16), as described previously (17, 18). Fasting plasma folate concentration was measured by radioimmunoassay (Quantaphase II; Bio-Rad Diagnostics, Hercules, CA).

Assays of plasma fibrinogen (von Clauss method [19]) and von Willebrand factor (functional assay of ristocetin cofactor activity) were performed in the hemostasis laboratory of the University of Iowa Hospitals. Plasma levels of thrombin–antithrombin III complexes and prothrombin fragment F1+2 were measured by enzyme immunoassay (Behring Diagnostics Inc., Westwood, MA). Reference curves were generated using pooled normal human plasma.

Statistical analysis. Statistical comparisons were performed using the paired, one-tailed Student's *t* test for sequential measurements in monkeys fed normal or modified diet. The unpaired Student's *t* test was used for comparison of aortic thrombomodulin activity in separate groups of monkeys. A value of $P < 0.05$ was used to define statistical significance.

Results

Plasma homocyst(e)ine and folate. To induce moderate hyperhomocyst(e)inemia, eight monkeys were fed diet that was enriched in methionine, relatively depleted in folic acid, and free of choline. Each monkey received either normal or modified diet for 4 wk, followed by the other diet for 4 wk. In seven of the eight monkeys, plasma homocyst(e)ine concentration was higher during the modified diet than during the normal diet. In one monkey, plasma homocyst(e)ine did not increase during the modified diet, and values obtained from this monkey were excluded from analysis. Plasma homocyst(e)ine increased from 4.0±0.2 μM when monkeys were fed normal diet to 10.6±2.6 μM when they were fed modified diet ($n = 7$; mean±SE; $P = 0.02$) (Fig. 1 *A*). As expected, plasma folate decreased when monkeys were fed modified diet ($P = 0.004$) (Fig. 1 *B*).

Vasomotor responses in the leg. Intraarterial infusion of collagen, which activates platelet aggregation in vivo (13), decreased hind limb blood flow by 42±9% when monkeys were fed modified diet, compared with 14±11% when monkeys were fed normal diet ($P = 0.008$) (Fig. 2). After infusion of collagen, platelet count in venous blood from the leg decreased

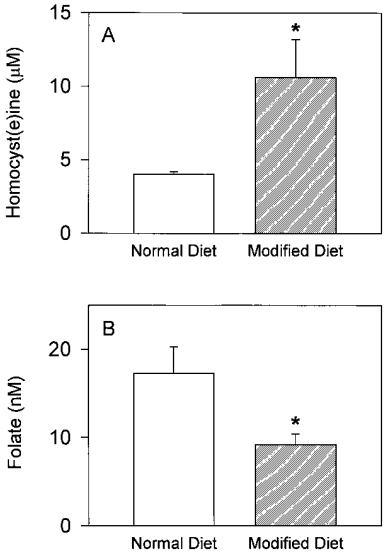

Figure 1. Plasma levels of homocyst(e)ine and folate. Fasting plasma levels of (*A*) homocyst(e)ine and (*B*) folate in monkeys fed either normal diet (*open bars*) or modified diet (*filled bars*) for 4 wk. $n = 7$; mean±SE; $^*P < 0.05$ vs. normal diet.

by 43±3% in monkeys that were fed normal diet, and by 36±5% in monkeys that were fed modified diet ($n = 6$; $P > 0.1$). Intraarterial administration of serotonin produced minimal reduction in hind limb blood flow in monkeys that were fed either normal or modified diet (Fig. 2).

Compared with monkeys fed normal diet, monkeys that were fed modified diet exhibited smaller increases in blood flow in response to the endothelium-dependent vasodilators acetylcholine ($P = 0.02$) (Fig. 3 *A*) and ADP ($P = 0.003$) (Fig. 3 *B*). Monkeys that were fed modified diet also had decreased responses to the highest dose of nitroprusside, an endothelium-independent vasodilator ($P = 0.04$) (Fig. 3 *C*).

Carotid artery vasomotor responses. Acetylcholine and nitroprusside each produced dose-dependent relaxation of carotid artery rings from monkeys fed either normal or modified diet. However, carotid arteries were markedly less responsive to acetylcholine when monkeys were fed modified diet than when they were fed normal diet (Fig. 4 *A*). The highest dose of acetylcholine (3×10^{-5} M) relaxed carotid artery rings by 82±8% when monkeys were fed normal diet, but only by 51±12% when monkeys were fed modified diet ($P = 0.03$). Carotid arteries also were slightly less responsive to nitroprusside when monkeys were fed modified diet ($P = 0.03$), although the decrease in response to nitroprusside was less than observed with acetylcholine (Fig. 4 *B*). No differences in carotid artery contraction in response to the thromboxane A_2 analogue U46619 were observed in monkeys that were fed normal or modified diet (Fig. 4 *C*).

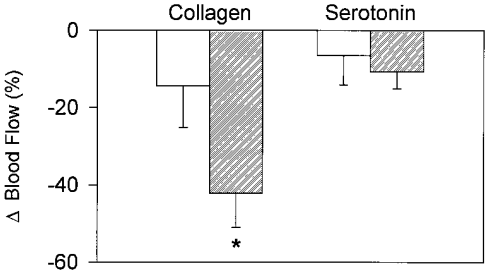

Figure 2. Vasoconstrictor responses in the leg in vivo to intraarterial administration of collagen (150 mg/min for 10 min) or serotonin (100-μg bolus) in monkeys fed either normal diet (*open bars*) or modified diet (*filled bars*). $n = 7$; mean±SE; $^*P < 0.05$ vs. normal diet.

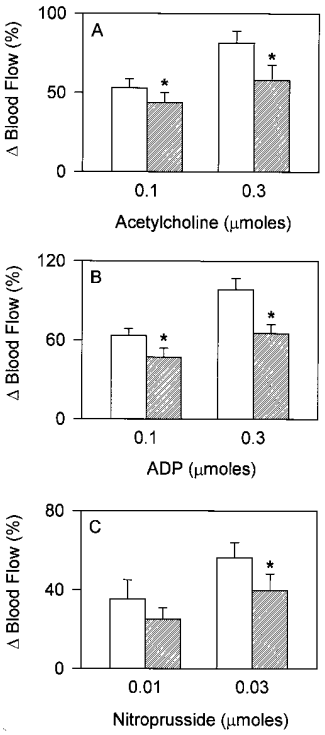

Figure 3. Vasodilator responses in the leg in vivo to intraarterial administration of (*A*) acetylcholine, (*B*) ADP, or (*C*) sodium nitroprusside in monkeys fed either normal diet (*open bars*) or modified diet (*filled bars*). $n = 7$; mean±SE; $^*P < 0.05$ vs. normal diet.

Hemostatic parameters. Monkeys that were fed normal or modified diet for 4 wk had similar baseline platelet counts and similar plasma levels of fibrinogen and von Willebrand factor (Table I). No diet-induced differences in plasma markers of

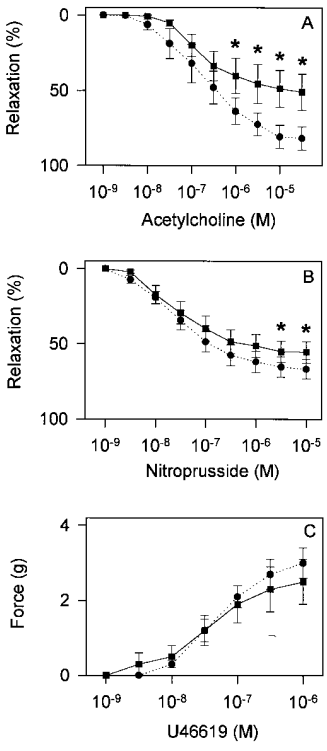

Figure 4. Carotid artery vasomotor responses ex vivo in monkeys fed normal diet (*circles*) or modified diet (*squares*). (*A*) Relaxation in response to acetylcholine. (*B*) Relaxation in response to sodium nitroprusside. (*C*) Contraction in response to the thromboxane A_2 analogue U46619. $n = 7$; mean±SE; $^*P < 0.05$ vs. normal diet.

Table I. Hemostatic Parameters in Monkeys Fed Normal and Modified Diet

	Normal diet	Modified diet
	$n = 7$	$n = 7$
Platelet count ($\times 10^{-3}/\mu l$)	325±35	315±28
Fibrinogen (mg/dl)	171±11	181±20
von Willebrand factor (%)	236±19	242±31
Thrombin–antithrombin III complexes (ng/ml)	6.8±1.6	6.0±1.8
Prothrombin fragment F1+2 (nM)	1.5±0.3	1.9±0.4

Values are mean±SE.

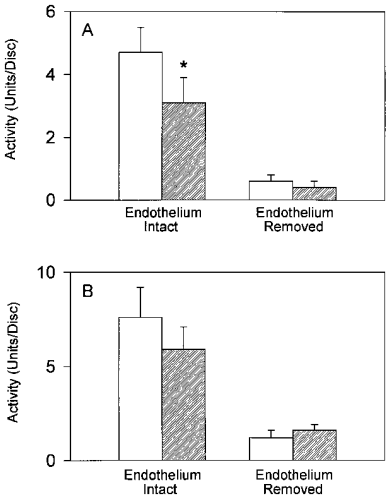

Figure 5. Thrombomodulin-dependent protein C activation in monkeys fed normal diet (*open bars*) or modified diet (*filled bars*). (*A*) Aortic thrombomodulin activity. $n = 5$; mean±SE; $^*P < 0.05$ vs. normal diet. (*B*) Carotid artery thrombomodulin activity. $n = 7$; mean±SE.

coagulation system activation (thrombin–antithrombin III complexes and prothrombin fragment F1+2) were observed (Table I).

Discs of thoracic aorta or common carotid artery were assayed for thrombomodulin-dependent protein C activation. Aortic thrombomodulin activity was 34±15% lower in monkeys that were fed modified diet than in monkeys that were fed normal diet ($P = 0.03$) (Fig. 5 *A*). Carotid artery thrombomodulin activity did not differ significantly when monkeys were fed normal or modified diet (Fig. 5 *B*). When endothelium was denuded before the assay, thrombomodulin activity decreased by 80–90% in monkeys that were fed either normal or modified diet (Fig. 5, *A* and *B*).

Discussion

The major findings of this study are that plasma homocyst(e)ine concentration can be elevated by dietary modification in primates, and that diet-induced moderate hyperhomocyst(e)inemia is associated with altered vasomotor regulatory function and endothelial anticoagulant function in vivo. Vessels from monkeys with moderate hyperhomocyst(e)inemia exhibited increased platelet-mediated vasoconstriction, impaired endothelium-dependent vasodilation, and decreased thrombomodulin-dependent activation of protein C. These alterations occurred at concentrations of homocyst(e)ine that were approximately twofold higher than those in monkeys fed normal diet. These concentrations of homocyst(e)ine are similar to levels that are associated with increased risk of vascular disease in humans (2).

Because this study used a dietary model of hyperhomocyst(e)inemia, it is not possible to determine whether vascular dysfunction was caused by hyperhomocyst(e)inemia or by other abnormalities induced by the modified diet. For example, in addition to moderate hyperhomocyst(e)inemia, monkeys that were fed modified diet had decreased plasma levels of folate (9.2±1.2 nM) compared with monkeys that were fed normal diet (17.3±3.0 nM). Concentrations of plasma folate > 4 nM are considered to be normal in humans (20). However, low-normal levels of folate (< 10 nM) are associated with both moderate hyperhomocyst(e)inemia and carotid artery stenosis in humans, particularly in elderly populations (21, 22). Thus, this animal model appears to mimic a combination of laboratory findings that are observed commonly in humans with atherosclerotic vascular disease. It is also possible that other features of the modified diet (such as methionine enrichment) may be important in altering vascular function.

There is substantial evidence for impairment of endothelial function in human and animal models of atherosclerosis (23–25). Endothelial dysfunction appears to be a sensitive indicator of the atherosclerotic process, since impairment of endothelium-dependent vasodilation occurs early during progression of atherosclerosis and improves early during regression of the atherosclerotic lesion (14, 26). In this study, we observed impairment of endothelium-dependent vasodilation in hyperhomocyst(e)inemic monkeys that is comparable in magnitude with that seen previously in our laboratory in monkeys with moderately severe atherosclerosis (23, 26). These findings are consistent with the observation that prolonged exposure to homocysteine decreases the activity of nitric oxide in cultured endothelial cells (3), and with the observation that flow-mediated dilation of systemic arteries is impaired in children with severe hyperhomocyst(e)inemia due to homozygous cystathionine β-synthase deficiency (27). In the latter study, flow-mediated vasodilation was not impaired in heterozygous adults who may have had moderate hyperhomocyst(e)inemia, although total plasma homocyst(e)ine was not measured.

Hyperhomocyst(e)inemic monkeys also had slightly impaired responses to the endothelium-independent vasodilator nitroprusside, an effect that we have observed previously in carotid arteries from atherosclerotic monkeys (28). From these data, we cannot determine whether decreased responsiveness to nitroprusside was caused by decreased vascular smooth muscle responses to nitric oxide or by inactivation of nitric oxide generated from nitroprusside. This question presumably could be addressed by administration of a vasodilator that does not act through production of cGMP.

Hyperhomocyst(e)inemic monkeys exhibited augmented vasoconstrictor responses to intraarterial infusion of collagen. At doses used in this study, collagen has no direct vasomotor effects but it is a potent activator of platelets in vivo (13). The venous platelet count decreased by ~ 30–40% after collagen infusion on both diets, which suggests that increased collagen-induced vasoconstriction in monkeys that were fed modified diet was not caused by increased platelet activation. Activated platelets release ADP, which is a vasodilator, and serotonin and thromboxane A_2, which are vasoconstrictors (29, 30). In

contrast to atherosclerotic monkeys, which exhibit enhanced vasoconstrictor responses to both collagen and serotonin (13, 14), we did not observe augmented vasoconstrictor responses to serotonin in hyperhomocyst(e)inemic monkeys. Similarly, we observed no augmentation of carotid artery contraction in response to the thromboxane A_2 analogue U46619 in monkeys that were fed modified diet. Based on these findings, it is likely that augmented vasoconstrictor responses to collagen in hyperhomocyst(e)inemic monkeys were caused by decreased vasodilator responses to platelet-generated ADP rather than by increased sensitivity to platelet-generated vasoconstrictors.

Homocysteine inhibits thrombomodulin-dependent protein C activation in vitro (4, 5) and also decreases expression of thrombomodulin on the surface of cultured endothelial cells (5). In this study, we observed a modest 34% decrease in thrombomodulin activity of thoracic aorta from monkeys with moderate hyperhomocyst(e)inemia. Thrombomodulin activity of carotid artery tended to decrease in monkeys fed modified diet, but it did not achieve statistical significance. No diet-induced differences in plasma levels of fibrinogen, thrombin–antithrombin III complexes, or prothrombin fragment F1+2 were observed, which indicates that monkeys with moderate hyperhomocyst(e)inemia did not have a detectable increase in systemic activation of the coagulation system. These findings are not surprising, because inhibition of protein C activation in vitro requires homocyst(e)ine concentrations that are 10- to 100-fold higher than those produced in plasma by the modified diet (5). Impairment of the protein C anticoagulant pathway may be more prominent in severe hyperhomocyst(e)inemia (for example, in homozygous cystathionine β-synthase deficiency [31]) than in moderate hyperhomocyst(e)inemia.

Many of the effects of homocysteine on endothelial function in vitro are dependent on the free thiol group of homocysteine, which may function as a reducing agent toward disulfide bonds in thrombomodulin, protein C, or other endothelial cell molecules (5, 32). However, because only a small fraction of plasma homocyst(e)ine contains a free thiol (33), it is likely that vascular function in hyperhomocyst(e)inemia is impaired through other mechanisms, such as generation of hydrogen peroxide or other reactive oxygen species (3, 34).

In summary, this study demonstrates that vascular function in primates is altered by diet-induced elevation of plasma homocyst(e)ine to levels comparable with those that are associated with predisposition to atherosclerotic and thrombotic vascular disease in humans. Monkeys fed hyperhomocyst(e)inemic diet for 4 wk developed vascular dysfunction that was similar in severity to that seen in monkeys fed atherogenic diet for 18 mo (13, 26). We speculate that altered vascular function may contribute to vasospasm, thrombosis, and progression of atherosclerosis in hyperhomocyst(e)inemia.

Acknowledgments

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