Supplementation of Atherogenic Diet With B Vitamins Does Not Prevent Atherosclerosis or Vascular Dysfunction in Monkeys

Steven R. Lentz, MD, PhD; Donald J. Piegors, MBA; M. René Malinow, MD; Donald D. Heistad, MD

Background—Hyperhomocysteinemia is associated with increased risk of atherosclerotic and thrombotic vascular disease. In many patients, hyperhomocysteinemia can be treated or prevented by dietary supplementation with B vitamins, but the clinical benefit of B vitamins for the prevention of vascular disease has not been proven.

Methods and Results—Using an atherogenic diet that produces both hyperhomocysteinemia and hypercholesterolemia, we tested the hypothesis that dietary supplementation with B vitamins (folic acid, vitamin B₁₂, and vitamin B₆) would prevent hyperhomocysteinemia, vascular dysfunction, and atherosclerotic lesions in monkeys. After 17 months, plasma total homocysteine increased from 3.6±0.3 to 11.8±1.7 μmol/L in monkeys fed an unsupplemented atherogenic diet (P<0.01) but did not increase in monkeys fed an atherogenic diet supplemented with B vitamins (3.8±0.3 μmol/L). Serum cholesterol increased from 122±7 to 550±59 mg/dL in the unsupplemented group (P<0.001) and from 118±5 to 492±55 mg/dL in the supplemented group (P<0.001). Responses to endothelium-dependent vasodilators, both in resistance vessels in vivo and in the carotid artery ex vivo, were impaired to a similar extent in groups that did and did not receive vitamin supplements. Anticoagulant responses to the infusion of thrombin were also impaired to a similar extent in both groups. Vitamin supplementation failed to prevent intimal thickening in the carotid or iliac arteries.

Conclusions—These findings demonstrate that supplementation with B vitamins prevents hyperhomocysteinemia but is not sufficient to prevent the development of vascular dysfunction or atherosclerotic lesions in monkeys with marked hypercholesterolemia, even in the absence of preexisting atherosclerosis. (Circulation. 2001;103:1006-1011.)

Key Words: atherosclerosis • cholesterol • endothelium • homocysteine • thrombin

Hyperhomocysteinemia is associated with increased risk for stroke, myocardial infarction, and venous thrombosis. Hyperhomocysteinemia may be caused by a variety of factors, including renal insufficiency, genetic factors, medications, or deficiencies of B vitamins, such as folate, vitamin B₆, or vitamin B₁₂. Each of these B vitamins plays an essential role in homocysteine metabolism, and even subclinical deficiencies of them can elevate plasma total homocysteine (tHcy) to levels that are associated with vascular pathology.

With the exception of patients who have end-stage renal disease, plasma tHcy can be corrected to normal in most patients with hyperhomocysteinemia by oral administration of folic acid or combinations of B vitamins. The influence of dietary folate on plasma tHcy is illustrated by the significant decrease in mean plasma tHcy that was observed in the Framingham Study population after fortification of the US food supply with folic acid. However, the potential clinical benefit of B vitamins for prevention of cardiovascular events has not been established, although this approach is currently being evaluated in several prospective clinical trials. Because hyperhomocysteinemia often coexists with other risk factors, such as hypercholesterolemia, it will be important to determine whether interventions to decrease plasma tHcy will prevent vascular dysfunction in the presence of additional risk factors.

In previous studies, we have observed vasomotor dysfunction and impaired anticoagulant response to thrombin in monkeys with combined hypercholesterolemia and hyperhomocysteinemia. In monkeys with preexisting atherosclerosis, the addition of B vitamins to the atherogenic diet normalized plasma tHcy but did not restore normal vascular function or prevent the progression of atherosclerotic lesions and did not restore the normal anticoagulant responses to thrombin. However, it is not known whether prevention of hyperhomocysteinemia would attenuate the development of vascular dysfunction or atherosclerosis during hypercholesterolemia. Prevention of hyperhomocysteinemia may be protective, because impairment of endothelial function by hyperhomocysteinemia might contribute to the formation of early atherosclerotic lesions.
In the present study, we have tested the hypothesis that dietary supplementation with B vitamins attenuates the development of vascular dysfunction in monkeys without pre-existing atherosclerosis. Our results demonstrate that B vitamins prevented hyperhomocysteinemia but failed to avert the development of vascular dysfunction or atherosclerotic lesions in hypercholesterolemic monkeys.

**Methods**

**Animals**

Sixteen adult cynomolgus monkeys (Macaca fascicularis) were fed a control diet (Harlan Teklad) for at least 4 months and then fed an atherogenic diet that was either unsupplemented (n=8) or supplemented with B vitamins (n=8) for 13 to 26 months. The unsupplemented atherogenic diet contained 43% of total calories as fat, 0.7% as cholesterol, and small amounts of B vitamins (≈1.0 μg vitamin B12, 0.75 mg vitamin B6, and <25 μg folic acid daily).11,12 The B vitamin supplementation group received 5 mg folic acid, 400 μg cyanocobalamin, and 20 mg pyridoxine daily. All diets contained approximately 0.4 g methionine per 100 g. The weights of the monkeys increased from 6.1±0.5 to 7.2±0.4 kg on the unsupplemented atherogenic diet and from 6.0±0.6 to 8.3±0.5 kg on the atherogenic diet supplemented with B vitamins. The amount of weight gained did not differ significantly between the 2 groups of monkeys (P>0.05).

**Experimental Protocol**

Before beginning the atherogenic diet, all animals were studied on 2 days separated by a recovery period of at least 1 week. On the first study day, animals were sedated with ketamine hydrochloride (20 mg/kg IM) and anesthetized with sodium pentobarbital (20 mg/kg IP) and positioned in the distal aorta, and the right femoral vein was cut into multiple side-hole 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, 5 mg/kg per hour) and other drugs. Changes in blood flow velocity to the leg were measured in response to intra-arterial injection of acetylcholine (3×10⁻⁶, 1×10⁻⁵, and 3×10⁻⁶ moles), ADP (3×10⁻⁶, 1×10⁻⁵, and 3×10⁻⁶ moles), and sodium nitroprusside (1×10⁻⁸, 3×10⁻⁸, and 1×10⁻⁷ moles). Responses were monitored in vivo by Doppler measurement of hindlimb blood flow velocity as described previously.8,10 At the end of the procedure, 1 common carotid artery was exposed and ligated proximally and distally with sutures, and the isolated segment of artery was removed and placed in oxygenated Krebs solution as described previously.8,10

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. Rings were precontracted by stepwise addition of prostaglandin F₂α (1 to 3 μmol/L), and relaxation dose-response curves were generated by cumulative addition of acetylcholine or nitroprusside as described previously.10

**Histology and Morphometric Analysis**

Sections of carotid artery or iliac artery were fixed in formalin, embedded in paraffin, and stained with Verhoeff–van Gieson’s stain. Intimal, medial, and luminal areas were measured as described previously.11 The luminal area was corrected for the absence of pressure by measuring the length of the internal elastic lamina.

**Other Assays**

Fasting plasma tHcy, defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds,13 was measured by high-performance liquid chromatography and electrochemical detection as described previously.14,15 Serum total cholesterol was measured enzymatically (Cholesterol/HP, Boehringer-Mannheim). Serum levels of folate and vitamin B12 were measured by use of an isotopic assay (Quantaphase II, Bio-Rad Diagnostics). Serum levels of biologically active vitamin B6 (pyridoxal 5-phosphate) were measured by use of a radioenzymatic assay (American Laboratory Products Co) as described by Shin et al.16 The activated partial thromboplastin time (APTT) was measured in an ACL-300+ coagulometer (Instrumentation Laboratory) with use of the Platelin L reagent (Organon Teknika Corp).

**Statistical Analysis**

A paired 2-tailed Student t test was used to compare baseline values with those obtained after the experimental diet within each group of monkeys. An unpaired 2-tailed Student t test was used to compare values between groups. Responses to vasodilators in the carotid artery were analyzed by 2-way repeated-measures ANOVA with Bonferroni multiple comparison analysis at specific concentrations of vasodilator. The Wilcoxon rank sum test was used to compare nonparametric APTT data after infusion of thrombin. Correlation coefficients were calculated by the Pearson method. A value of P<0.05 was used to define statistical significance. Values are reported as mean±SE.

**Results**

**Effects of Diet on Cholesterol, tHcy, and B Vitamins**

Eight monkeys were fed an unsupplemented atherogenic diet, which produces both hypercholesterolemia and hyperhomocysteinemia,8 for 17.2±1.5 months. The other 8 monkeys were fed an atherogenic diet supplemented with B vitamins for 17.6±1.6 months. At the time of the terminal study, serum total cholesterol was elevated compared with baseline values in both groups (P<0.001), but plasma tHcy was elevated only in the unsupplemented group (P<0.01) (Table). Elevation of tHcy was associated with decreases in plasma folate (P<0.01), vitamin B12 (P<0.01), and vitamin B6 (P<0.05) (Table). The elevation of plasma tHcy occurred gradually and progressively, as did the decline in plasma folate, vitamin B12, and vitamin B6 (Figure 1). Regression analysis of data obtained at all time points demonstrated strong inverse correlations between plasma tHcy and folate...
Effect of Diet on Total Homocysteine in Plasma and Total Cholesterol and B Vitamins in Serum

<table>
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<tr>
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<th>Unsupplemented (n=8)</th>
<th>Vitamin Supplemented (n=8)</th>
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<td></td>
<td>Baseline</td>
<td>Terminal</td>
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<td>tHcy, μmol/L</td>
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<td>11.8±1.7*</td>
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<td>Total cholesterol, mg/dL</td>
<td>122±7</td>
<td>550±59*</td>
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<td>Folate, nmol/L</td>
<td>17.2±1.0</td>
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<td>Vitamin B_{12}, nmol/L</td>
<td>335±26</td>
<td>195±46*</td>
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<tr>
<td>Vitamin B_{6}, pmol/L</td>
<td>908±141</td>
<td>165±38*</td>
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Values are mean±SE.
*P<0.05 vs baseline.

(r=−0.70, P<0.001), plasma tHcy and vitamin B_{12} (r=−0.68, P<0.001), and plasma tHcy and vitamin B_{6} (r=−0.72, P<0.001).

Effects of Diet on Vasomotor Responses In Vivo

Relaxation of resistance vessels in response to intra-arterial injection of vasodilators was measured by monitoring blood flow velocity to the leg. Compared with responses obtained at baseline, responses to acetylcholine and ADP were impaired in monkeys fed an atherogenic diet either with or without supplemental B vitamins, and the degree of impairment was similar in both groups (Figure 2). In response to the highest dose of acetylcholine, blood flow velocity increased 79±5% at baseline, 40±5% in monkeys fed an atherogenic diet without vitamins (P<0.001), and 43±3% in monkeys fed an atherogenic diet with vitamins (P<0.001). In response to the highest dose of ADP, blood flow velocity increased 91±7% at baseline, 49±7% in monkeys fed an atherogenic diet without vitamins (P<0.001), and 40±4% in monkeys fed an atherogenic diet with vitamins (P<0.01). The responses to acetylcholine or ADP did not differ between the unsupplemented and vitamin-supplemented groups (P>0.05). Vasodilator responses to nitroprusside were also impaired to a similar extent in monkeys fed an atherogenic diet either with or without supplemental B vitamins (Figure 2).

Effects of Diet on Relaxation of Carotid Artery

Acetylcholine and nitroprusside each produced dose-dependent relaxation of the common carotid artery ex vivo (Figure 3). Relaxation to acetylcholine was impaired after the atherogenic diet in both the unsupplemented and vitamin-supplemented groups. Maximal relaxation to the highest dose of acetylcholine (1×10^{-7} mol/L) was 80±5% at baseline, 53±11% after the unsupplemented atherogenic diet (P<0.05), and 52±11% after the atherogenic diet supplemented with B vitamins (P<0.05). Compared with responses measured at baseline, carotid artery responses to low doses of nitroprusside were decreased after the atherogenic diets, but relaxation responses to the highest doses of nitroprusside were similar in each group. There were no differences in responses to nitroprusside between monkeys fed unsupplemented and vitamin-supplemented diets.

Response to Infusion of Thrombin

Infusion of thrombin in monkeys or baboons produces prolongation of the APTT that is caused mainly by activation of anticoagulant protein C. This anticoagulant response to thrombin is impaired in atherosclerotic monkeys. To determine whether dietary supplementation with B vitamins pro-
tects from impairment of thrombin-dependent anticoagulation, we measured prolongation of the APTT after the infusion of human thrombin at baseline and after the atherogenic diet in both groups of monkeys. Thirty minutes after a 10-minute infusion of thrombin, the APTT increased by 45 ± 6 seconds at baseline, by 12 ± 6 seconds after the atherogenic diet without supplemental vitamins (P < 0.01), and by 15 ± 6 seconds after the atherogenic diet supplemented with B vitamins (P < 0.01) (Figure 4). No differences in response to thrombin were observed between unsupplemented and vitamin-supplemented animals.

Morphometry of Carotid and Iliac Arteries
At the time of the terminal study, the common carotid artery from both groups of monkeys exhibited moderate intimal thickening with no change in luminal area compared with baseline measurements (Figure 5). These findings indicate that vascular remodeling occurred in both groups. Compared with measurements performed at baseline, the medial areas increased slightly in monkeys fed the unsupplemented atherogenic diet, but no significant differences were observed in the intimal, medial, or luminal areas between monkeys fed the unsupplemented atherogenic diet and monkeys fed the atherogenic diet supplemented with B vitamins. Intimal thickening was also observed in the iliac artery at the time of the terminal study in both the unsupplemented (1.4 ± 0.6 mm²) and vitamin-supplemented (1.0 ± 0.5 mm²) groups (P > 0.05).

Discussion
The major finding of the present study is that dietary supplementation with B vitamins failed to attenuate the development of vascular dysfunction or atherosclerosis in hypercholesterolemic monkeys. As found previously,8,9 monkeys fed an atherogenic diet without supplemental B vitamins developed both hypercholesterolemia and hyperhomocysteinemia. Supplementation of the atherogenic diet with B vitamins completely prevented hyperhomocysteinemia but did not alter the development of hypercholesterolemia. Vasomotor and anticoagulant responses were impaired to an almost identical extent in monkeys fed an atherogenic diet with or without supplemental B vitamins. Both groups of monkeys developed atherosclerotic lesions in large arteries, and vitamin supplementation did not prevent vascular remodeling of the common carotid artery.

In monkeys fed an unsupplemented atherogenic diet, plasma tHcy increased progressively over several months (Figure 1). The increase in plasma tHcy was strongly associated with decreases in plasma folate, vitamin B12, and vitamin B6. Each of these B vitamins is essential for normal homocysteine metabolism, and deficiencies of these vitamins are associated with hyperhomocysteinemia in humans.5 Elevation of plasma tHcy was completely prevented in the group of monkeys that received vitamin supplementation, which indicates, as suggested previously,8 that the likely cause of hyperhomocysteinemia in these monkeys was a deficiency of B vitamins in the atherogenic diet. We cannot completely exclude the possibility that the bioavailability of B vitamins was decreased by the atherogenic diet. If so, the effect of decreased bioavailability was clearly overcome by dietary supplementation with large doses of B vitamins, inasmuch as metabolically adequate levels of all 3 vitamins were achieved in the group that received supplementation. Vitamin supplementation did not influence serum cholesterol concentration (Table).
Both groups of monkeys exhibited impaired vasomotor responses to acetylcholine and ADP in resistance vessels in vivo and in rings of carotid artery ex vivo. Responses to nitroprusside, an endothelium-independent vasodilator, were also impaired. We have observed similar impairment of vasodilator responses to nitroprusside in previous studies of atherosclerotic or hyperhomocysteinemic monkeys.8,10,20 Because nitroprusside is a nitrovasodilator, these observations are consistent with the concept that the impairment of vasomotor responses is caused by oxidative inactivation of NO derived from either endogenous or exogenous sources.21 Alternatively, impaired responsiveness to nitroprusside may be caused by other abnormalities of the vascular wall that lead to decreased relaxation of vascular smooth muscle, such as decreased expression or activity of soluble guanylyl cyclase. In either case, it is clear that supplementation of the atherogenic diet with B vitamins did not influence these vasodilator responses.

Monkeys fed the atherogenic diet supplemented with B vitamins tended to have less impairment of relaxation responses in the carotid artery and smaller intimal area than did the monkeys fed the unsupplemented atherogenic diet (Figures 3 and 5). Although these trends were not significant, these observations suggest the possibility that a significant benefit from B vitamins might have been detected if the hypercholesterolemia had been less severe or if the duration of supplementation had been longer. Additional studies will be necessary to address these questions.

Anticoagulant responses to thrombin can be measured by the infusion of thrombin and measurement of the APTT, which becomes prolonged in response to activation of the endogenous anticoagulant protein C.9,17–19 After infusion, thrombin quickly binds to a high-affinity receptor, thrombomodulin, which is expressed on the luminal surface of the endothelium. When bound to thrombomodulin, the ability of thrombin to activate protein C is increased several thousandfold.22 Abnormalities of the protein C anticoagulant pathway are found commonly in patients with thromboembolism.23 Thrombomodulin-dependent activation of protein C can be inhibited by the addition of exogenous homocysteine to cultured human endothelial cells,24–26 and experimental hyperhomocysteinemia in monkeys is associated with decreased thrombomodulin anticoagulant activity.10 However, abnormalities of protein C activation have not been observed consistently in association with hyperhomocysteinemia in humans.27

In a previous study, we found that atherosclerotic monkeys had impaired prolongation of the APTT and decreased expression or activity of soluble guanylyl cyclase. In monkeys with preexisting atherosclerosis,9 our findings in the present study not restore these responses to normal in monkeys with hyperhomocysteinemia.9,10 Moreover, we also found that dietary supplementation with B vitamins did not restore these responses to normal in monkeys with preexisting atherosclerosis.9 Our findings in the present study are concordant: dietary supplementation with B vitamins did not attenuate the impairment of anticoagulant responses to thrombin in monkeys without preexisting atherosclerosis. These observations suggest that impairment of the anticoagulant activity of thrombin is mediated by the effects of hypercholesterolemia rather than hyperhomocysteinemia. It cannot be determined from these data whether the blunted anticoagulant response to thrombin was caused by hypercholesterolemia itself or perhaps by an abnormality of the atherosclerotic vessel wall.

Hyperhomocysteinemia is recognized as a clinical risk factor for vascular events, and the potential beneficial role of treatment with B vitamins to lower plasma tHcy is being actively investigated in clinical trials.2 Although mechanisms responsible for vascular pathology in hyperhomocysteinemia are still poorly understood, abnormal responses to endothelium-dependent vasodilators have been observed consistently in studies of experimental hyperhomocysteinemia in animals and humans.28 In previous studies, we observed a similar degree of impairment of vascular function in monkeys with isolated hyperhomocysteinemia10 and in those with combined hyperhomocysteinemia and hypercholesterolemia,8 which suggested that hyperhomocysteinemia may be a contributing factor to dysfunction in atherosclerotic animals. However, our present results clearly demonstrate that prevention of hyperhomocysteinemia by dietary supplementation with B vitamins is not sufficient to attenuate the development of abnormal vascular responses in monkeys with marked hypercholesterolemia, even in the absence of preexisting atherosclerosis. These results imply that interventions to lower plasma tHcy may have limited clinical benefit unless other risk factors are also controlled.

Acknowledgments

This work was supported by the Office of Research and Development, Department of Veterans Affairs, and National Institutes of Health grants HL-63943, DK-25295, NS-24621, and HL-16066. We thank Rochelle Erger, Lorie Leo, and Robert M. Brooks II for technical assistance.

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*Circulation.* 2001;103:1006-1011
doi: 10.1161/01.CIR.103.7.1006

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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