Dietary Supplementation With Methionine and Homocysteine Promotes Early Atherosclerosis but Not Plaque Rupture in ApoE-Deficient Mice

Ji Zhou, Jan Møller, Carl C. Danielsen, Jacob Bentzon, Hanne B. Ravn, Richard C. Austin, Erling Falk

Abstract—Hyperhomocysteinemia is an independent risk factor for atherothrombosis. However, causality is unproven, and it remains unknown whether hyperhomocysteinemia promotes atherosclerosis, plaque rupture, and/or thrombosis. We evaluated the short- and long-term effects of hyperhomocysteinemia on plaque size and structure in 99 atherosclerosis-prone apolipoprotein E–deficient mice. Hyperhomocysteinemia was induced by methionine (Met) or homocysteine (HcyH) supplementation: low Met (+11 g Met/kg food), high Met (+33 g Met/kg food), low HcyH (0.9 g HcyH/L drinking water), and high HcyH (1.8 g HcyH/L drinking water). Met and HcyH supplementation significantly raised plasma total homocysteine levels by 4- to 16-fold above those observed in mice fed a control diet (up to 146.1 μmol/L). Compared with controls, aortic root plaque size was significantly larger in supplemented groups after 3 months (56% and 173% larger in high-Met and high-HcyH, respectively) but not after 12 months. Hyperhomocysteinemia was associated with an increase in the amount of collagen in plaques after both 3 and 12 months. Mechanical testing of the tail tendons revealed no weakening of collagen after 12 months of hyperhomocysteinemia. Many plaques in both control and supplemented mice appeared rupture prone morphologically, but all aortic root plaques and all but 1 coronary plaque had an intact surface without rupture or thrombosis. Thus, diet-induced hyperhomocysteinemia promotes early atherosclerosis and plaque fibrosis but does not, even in the long term, weaken collagen or induce plaque rupture.

Key Words: apoE-deficient mouse ■ homocysteine ■ atherosclerosis ■ plaque rupture

Numerous epidemiological studies have demonstrated that increased concentrations of plasma total homocysteine (tHcy) are an independent risk factor for atherothrombotic diseases such as ischemic heart disease, stroke, and peripheral vascular disease.1 Strong associations between plasma tHcy and atherothrombotic diseases have, however, predominantly been observed in cross-sectional and retrospective case-control studies, in contrast with a weak or no association in many nested case-control and prospective studies.2 These conflicting results may suggest that plasma tHcy could be a marker or a consequence of atherothrombotic diseases rather than a cause. In the case of causality, the epidemiological data suggest that homocysteine plays a more important role in patients with established disease than in persons without symptoms of vascular disease. That is, homocysteine might promote the complications of atherosclerosis rather than atherosclerosis itself.3

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The most frequent atherothrombotic disease is ischemic heart disease. Atherosclerosis of the coronary arteries may cause chronic stable angina, which in principle is a benign disease. If, however, atherosclerosis becomes complicated by luminal thrombosis, a life-threatening heart attack may supervene. About 75% of fatal coronary thrombi are precipitated by luminal rupture of an atherosclerotic vessel wall. However, not all atherosclerotic plaques appear to be equally vulnerable to rupture. On the basis of pathoanatomical studies, the following features are assumed to characterize rupture-prone plaques: (1) a large, lipid-rich core; (2) a thin and collagen-poor fibrous cap covering the core; and (3) inflammation and lack of smooth muscle cells at the site of rupture.4 Collagen confers mechanical strength and stability to tissues, and it is generally assumed that collagen, synthesized by the vascular smooth muscle cells, plays a critical stabilizing role during plaque development, protecting plaques against rupture and its severe consequences.5 Collagen molecules need to be cross-linked to obtain strength and stability. Cross-linking is a 2-step process. The key enzyme, lysyl oxidase, first generates highly reactive aldehyde groups that subsequently react spontaneously, form-

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ing intermolecular and intramolecular covalent bonds. Several clinical and experimental studies indicate that homocysteine may impair collagen cross-linking and thus, reduce the strength and stability of collagen fibers. First, 85% of patients with homocystinuria, an inborn error of metabolism associated with severe hyperhomocysteinemia, develop dislocation of the lens because of weakening of the crown of collagen fibers that holds the lens in place. Second, homocysteine thiolactone, a cyclic form of homocysteine, may inactivate lysyl oxidase by protein homocysteinylatation. Finally, homocysteine is structurally similar to o-phenicillamine (β,β-dimethylcysteine), a drug that interferes with collagen (and elastin) cross-linking by blocking the reactive aldehyde groups, and homocysteine, like penicillamine, has been reported to have lathyrogenic properties (inhibition of cross-linking) both in vitro and in vivo.

The aim of the present study was to first develop a model of hyperhomocysteinemia in the atherosclerosis-proneapolipoprotein E–deficient (apoE−/−) mouse and then test the following 3 hypotheses: hyperhomocysteinemia (1) accelerates atherogenesis, (2) weakens collagen, and (3) promotes plaque rupture.

Methods

Mice, Diets, and Supplementation

Ninety-nine female homozygous apoE−/− mice, back-crossed 9 generations onto the C57BL/6 background, were obtained from Transgenic Alliance (Lyon, France) and fed a high-fat, Western-type diet (Altromin C1057-157d) containing 21% vegetable fat, 0.15% cholesterol, 1.1% methionine, and 0.001% folic acid (all weight per weight) at 6 weeks of age. A week later, the mice were divided into a control group (Western-type diet alone) and 4 groups that were supplemented with methionine (Met) or homocysteine (HcyH): low-HcyH group (0.9 g DL-HcyH/L drinking water), high-HcyH group (1.8 g DL-HcyH/L drinking water), low-Met group (additional 1.1% L-Met [Sigma M-2893] in the diet, giving a total of 2.2% Met), and a high-Met group (additional 3.3% L-Met, giving a total of 4.4% Met). The low-HcyH and low-Met doses were selected based on previous studies.20 Tendons from ventral fascicles were teased from a 16-mm tail segment obtained just distal to the fur border and kept immersed in a 50 mmol/L Tris-HCl (pH 7.4) buffer during the mechanical analysis. Load and deformation were recorded, and the load-deformation curve was converted to a normalized load-strain curve. From this curve, normalized maximum load (N×mm/mg), normalized maximum slope of the load-strain curve (N×mm/mg, equivalent to the stiffness of the collagen), and normalized energy absorption for deformation until maximum load (mJ/mg) were derived. The average of 3 tendon specimens analyzed for each mouse was used in the calculation of the group mean.

Statistical Analyses

Results were expressed as mean±SEM for each group of mice. One-way ANOVA and unpaired Student’s t test were used to examine the differences among or between groups. Correlation studies were done with the Pearson product-moment correlation test or the Spearman rank-order correlation test according to the distribution of the data. Linear regression was also used to test the correlation between two variables, if one variable was dependent on another. Probability values <0.05 were considered significant, unless otherwise stated.

Results

Ninety-nine mice entered the study at 11/2 months of age, and 18 mice were humanely killed as planned 3 months later (short-term study). Initial body weights were similar in all groups, and weight gains during the study were similar in control and HcyH-supplemented groups. Methionine is, however, the most toxic of all amino acids, and mice in the high-Met group failed to gain weight normally; in fact, mice in both Met-supplemented groups started to lose weight and died prematurely (19 at 8 months of age; Figure 1). Therefore,
we decided to euthanize the remaining 13 Met-supplemented mice at 81/2 months of age. No control mice were killed at that time and no blood was obtained for analyses, but microscopic examination of the aortic root revealed a larger mean plaque size in the high-Met group than in the low-Met group (0.84 ± 0.08 vs 0.66 ± 0.03 mm², P = 0.05). The myocardium, lungs, spleen, and intestine were normal by microscopic examination, and only minor and nonspecific hepatic changes, including mild steatosis, were present in the high-Met group. In the control and HcyH-supplemented groups, 39 mice survived until the end of the study and were humanely killed as planned at 131/2 months of age (long-term study).

**Plasma tHcy and Lipids**

Met and HcyH supplementation significantly elevated plasma tHcy levels in both the short and long term (the Table). In the control group, mean plasma TC was 21.5 and 17.6 mmol/L (short and long term, respectively), mean plasma TG was 0.9 and 0.6 mmol/L (short and long term, respectively), and mean plasma HDL-C was 2.8 mmol/L (long term only). Plasma TC was similar in all groups, but TG was mildly reduced in the short-term, high-Met group (0.5 mmol/L, P < 0.05), and HDL-C was moderately elevated in the long-term, low-HcyH group (4.1 mmol/L, P < 0.01).

**Aortic Atherosclerosis**

All mice had severe atherosclerosis. Early as well as advanced lesions were found in both control and supplemented groups in the short-term study (Figures 2A through 2C). Only advanced and much larger lesions were found in the long-term study (Figure 2D, the Table). Many plaques appeared vulnerable to rupture, with a large, lipid-rich core covered by a thin, fibrous cap. Beneath advanced plaques, the media was frequently destroyed, with disrupted elastic membranes. All plaques had, however, an intact surface without rupture or luminal thrombosis. A small, superficial hemorrhage (extravasated erythrocytes) was seen in 2 plaques of HcyH-supplemented mice, but the origin could not be traced.

Compared with control mice, significantly more atherosclerosis was observed in mice fed hyperhomocysteinemic diets for 3 months (the Table). After 12 months, however, the amount of atherosclerosis was similar in all groups, regardless of the method used for quantification. Aortic root plaque size (assessed microscopically) and the surface area covered by plaques in the aorta (assessed in en face specimens) were not correlated.

Hyperhomocysteinemia was associated with an increase in the relative amount of collagen in plaques after both 3 and 12 months, but the increase was not statistically significant in all groups (the Table, Figure 3). Collagen-rich areas within the plaque increased significantly by 81% in the short-term, high-HcyH group and by 73% in the long-term, low-HcyH group.

**Homocysteine, Lipids, and Atherosclerosis**

In the short term, mice on hyperhomocysteinemic diets developed more atherosclerosis than did control mice (the Table). A positive correlation between plasma tHcy levels and plaque size was present in the control group (ie, in mice with plasma tHcy levels within the normal range) but not in the supplemented groups (Figure 4). In the long term, no

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Plasma tHcy, μmol/L</th>
<th>Lesion Size in Aortic Root, mm²</th>
<th>Collagen in Root Lesion, %</th>
<th>En Face Lesion Area, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short term: 3 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (6)</td>
<td>7.9 ± 1.3</td>
<td>0.18 ± 0.04</td>
<td>21 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>High HcyH (6)</td>
<td>35.6 ± 8.9*</td>
<td>0.50 ± 0.07*</td>
<td>38 ± 4.2†</td>
<td></td>
</tr>
<tr>
<td>High Met (6)</td>
<td>44.4 ± 9.6*</td>
<td>0.29 ± 0.01†</td>
<td>27 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Long term: 12 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (11)</td>
<td>9.1 ± 0.9</td>
<td>1.08 ± 0.05</td>
<td>22 ± 2.0</td>
<td>21.1</td>
</tr>
<tr>
<td>Low HcyH (16)</td>
<td>67.0 ± 9.9*</td>
<td>1.11 ± 0.06</td>
<td>38 ± 2.1*</td>
<td>18.6</td>
</tr>
<tr>
<td>High HcyH (12)</td>
<td>146.1 ± 16.7*</td>
<td>1.25 ± 0.07</td>
<td>30 ± 3.5</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

*P < 0.01, †P < 0.05 vs control group.
correlation was found between plasma tHcy and atherosclerosis.

Plasma lipid levels (TC, HDL-C, and TGs) and the amount of atherosclerosis were not correlated in either the short term (aortic root plaque size) or the long term (aortic root plaque size and en face evaluation). Furthermore, there was no correlation between plasma lipids and plasma tHcy.

**Coronary Atherosclerosis**
Both the coronary ostia and the proximal parts of the coronary arteries were automatically revealed by the aortic root step-sectioning technique used in the present study. Severe atherosclerosis, often causing total coronary occlusion, was present in virtually all long-term mice. In a single mouse belonging to the high-Met, short-term group, a ruptured plaque with superimposed thrombus was found proximally in the coronary artery (Figure 5).

**Quality of Collagen (Tail Tendons)**
No significant difference was observed in the mechanical properties (maximum load, maximum slope, and energy absorption) of tail tendons between groups in the long-term study (data not shown).

**Discussion**
The present study is the first to report both short- and long-term effects of hyperhomocysteinemia on the develop-
ment of atherosclerosis in an animal that develops human-like atherosclerosis spontaneously, the apoE−/− mouse. Both HcyH and Met supplementation induced hyperhomocysteinemia, which accelerated the development of plaques in young mice (<4 1/2 months of age) without influencing plaque burden later in life (at 13 1/2 months of age). Plaque composition was, however, changed at both the short and long term: hyperhomocysteinemia was associated with enhanced collagen deposition in plaques. Only a single ruptured plaque was identified, and prolonged hyperhomocysteinemia did not weaken the collagen, as hypothesized.

**Dietary Hyperhomocysteinemia in Atherosclerosis-Prone Mice**

The influences of hyperhomocysteinemia on vascular biology have been studied in different animal models, but the effect of hyperhomocysteinemia on the development of atherosclerosis has not been reported until recently. Hofmann et al. studied the influence of dietary hyperhomocysteinemia on early lesion formation in apoE−/− male mice. At 12 weeks of age, only fatty streaks were present in control mice fed standard rodent chow; however, in mice fed a diet high in Met and low in B vitamins (folate, B6, and B12), the mean plasma tHcy level was increased 19-fold, mean aortic root lesion size was enlarged 2-fold, and complex lesions had developed, associated with an enhanced proinflammatory response in the vessel wall. ApoE−/− mice develop atherosclerosis spontaneously on normal chow, but the process is accelerated by a high-fat, Western-type diet. On such a diet, advanced plaques usually are present at multiple locations, including the aortic root, at 20 weeks of age, which is why we decided to use a Western-type diet. This protocol allowed us to study the effects of hyperhomocysteinemia on the development of mature plaques, not fatty streaks, at both the short and long term, and advanced plaques were indeed already present in the short-term mice.

**Methods of Inducing Hyperhomocysteinemia**

In humans, homocysteine is produced by demethylation of the essential amino acid methionine. Thus, dietary methionine plays an important role in generating homocysteine. Folate, vitamin B12, and vitamin B6 are crucial cofactors in the metabolism of methionine and homocysteine and constitute the major determinants of plasma tHcy level. One of the most sensitive markers of folate and vitamin B12 deficiency is, in fact, an elevated plasma tHcy level. In the present study, a vitamin deficiency was highly unlikely, because the amounts of vitamins in the diet, according to the supplier (Altromin GmbH), exceeded by far the minimum nutrient requirements of laboratory mice, as estimated by the National (US) Research Council (actual diet vs National Research Council requirements: 10.0 vs 0.5 mg/kg for folate; 41 vs 10 μg/kg for vitamin B12; 15 vs 8 mg/kg for vitamin B6).

We induced hyperhomocysteinemia by adding L-methionine to the food or DL-homocysteine to the drinking water. The former is a well-established method of inducing hyperhomocysteinemia in both humans (oral methionine load test) and experimental animals. The latter method, supplementation with DL-homocysteine, has previously been used in rats to induce hyperhomocysteinemia and in cell culture studies. Although the fate of the D-enantiomer in the body is unknown, the fact that similar results were obtained in the 2 supplemented groups indicates that hyperhomocysteinemia induced by dietary DL-homocysteine may generate reliable results. An advantage of adding homocysteine (vs methionine) to the diet is that severe and prolonged hyperhomocysteinemia may be induced without concomitant growth retardation.

**Hyperhomocysteinemia Accelerates Atherosclerosis**

Hyperhomocysteinemia was associated with a significant increase in plaque size (only in the short term), and the relative amount of collagen in plaques was increased in both the short and long term. Because a concomitant vitamin deficiency is highly unlikely, the present study and the study by Hofmann et al. indicate that homocysteine is atherogenic in the apoE−/− mouse. This in vivo observation is, in fact, consistent with a recent in vitro study in which homocysteine promoted the proliferation of cultured arterial smooth muscle cells and enhanced the synthesis and accumulation of collagen. The underlying molecular mechanisms are, however, unknown, and it should be stressed that hyperhomocysteinemia may promote both atherosclerosis and thrombosis by...
numerous other mechanisms, of which endothelial toxicity, resulting in endothelial dysfunction and documented both experimentally and in humans, appears plausible.\textsuperscript{20–22,31} However, although dietary supplementation with B vitamins (folic acid, B\textsubscript{6}, and B\textsubscript{12}) prevented the development of hyperhomocysteinemia in monkeys fed a lipid-rich, cholesteryl-elevating diet, it failed to prevent the development of endothelial dysfunction and intimal thickening in carotid and iliac arteries, suggesting that interventions to lower plasma tHcy may have limited clinical benefit unless other risk factors are also controlled.\textsuperscript{35} In cultured endothelial cells, high levels of methionine and, to a lesser extent, homocysteine enhanced the methylation of L-arginine to asymmetric dimethylarginine, an endogenous competitive inhibitor of NO synthase.\textsuperscript{34} Folate/B\textsubscript{12} supplementation increases the overall methylation capacity, and if methylation of L-arginine constitutes the link between hyperhomocysteinemia and endothelial dysfunction (caused by reduced NO production) as recently suggested,\textsuperscript{34} then treatment with these vitamins will not eliminate the proatherogenic effect associated with hyperhomocysteinemia.

A positive correlation between plasma tHcy and aortic root plaque size was found in the short-term control group (ie, in mice with plasma tHcy levels within the normal range) but not in the 2 supplemented groups (Figure 4). We measured nonfasting plasma tHcy levels because mice with free access to food and water, as in the present study, spend more of their time in the nonfasted than in the fasted state. Nevertheless, the measured values probably reflect the 24-hour plasma tHcy burden more accurately in the chow-fed mice than in the supplemented mice, because the circadian variation in plasma tHcy levels was probably more pronounced in the latter mice.\textsuperscript{31} This may explain the lack of a correlation between the measured plasma tHcy levels and aortic root plaque size in the supplemented groups.

In the long term, all 3 groups of mice developed or reached the same amount of atherosclerosis, indicating that hyperhomocysteinemia affects the development of early lesions more than it does the progression of established plaques. This observation, that an intervention has a greater impact on early lesion development than on late plaque progression, is far from unique.\textsuperscript{36,37} Atherosclerosis appears to be much more dynamic, with greater potential for both progression and regression, in the early vs late stage. This result implies that it may be more difficult to identify an effect of intervention on plaque burden if mice are allowed to survive into middle age, when they reach a more stable and quiescent phase of the disease. If enhanced vascular inflammation\textsuperscript{26} plays a more important role in the initiation and early development of atherosclerosis than in its gradual, nonrupture-related progression, it could offer a likely explanation for the present finding of an early, but not a late, atherogenic effect of hyperhomocysteinemia.

**Homocysteine, Collagen, and Plaque Rupture**

The tensile strength of collagen fibers is drastically reduced when the collagen molecules are not properly cross-linked. Homocysteine has been reported to interfere with this process by inactivating the cross-linking enzyme lysyl oxidase (homocysteinylation) or by blocking the lysyl oxidase-generated reactive aldehyde groups that subsequently mediate cross-linking (penicillamine-like effect). If such effects of homocysteine exist in vivo, they might result in the development of fragile and rupture-prone plaques, resulting in an increased risk of a rupture/thrombus-mediated heart attack. However, our studies failed to confirm this hypothesis. Despite long-term hyperhomocysteinemia, no qualitative collagen defects could be detected by mechanical testing, and only a single ruptured plaque was identified. This is, however, one of the first well-documented cases of spontaneous rupture of a coronary plaque with superimposed thrombosis in experimental atherosclerosis research. In middle-aged apoE\textsuperscript{−/−} mice, extravasated erythrocytes are a rather frequent finding in plaques located in the brachiocephalic trunk, but frank rupture of the plaque surface appears to be rare,\textsuperscript{38} although conflicting observations have recently been published.\textsuperscript{39}

**Limitations**

The atherogenic effect of hyperhomocysteinemia was observed in a relatively small short-term study (n=3×6), and the positive correlation between plasma tHcy and plaque size was seen only in the control group (n=6). These results need to be confirmed in larger groups of mice, and it would also be interesting to know whether hyperhomocysteinemia induced by vitamin deficiency alone, the most frequent cause of hyperhomocysteinemia in humans, has a similar effect.

**Conclusions**

Dietary hyperhomocysteinemia induced by methionine or HcyH supplementation, accelerated early plaque development and enhanced plaque fibrosis in apoE\textsuperscript{−/−} mice. The plaques did not appear more vulnerable morphologically, and plaque rupture was rare, even after 12 months of severe hyperhomocysteinemia. Although collagen-rich areas were increased in the plaques of hyperhomocysteinemic mice, no effect of prolonged hyperhomocysteinemia on collagen strength could be detected by mechanical testing.

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