Hyperhomocysteinemia Impairs Angiogenesis in Response to Hindlimb Ischemia

Junli Duan, Toyooki Murohara, Hisao Ikeda, Ken-ichiro Sasaki, Satoshi Shintani, Takako Akita, Toshifumi Shimada, Tsutomu Imaizumi

Abstract—Hyperhomocysteinemia (HH) is an independent risk factor for atherosclerosis, including peripheral arterial occlusive disease (PAOD). Because angiogenesis and collateral vessel formation are important self-salvage mechanisms for ischemic PAOD, we examined whether HH modulates angiogenesis in vivo in a rat model of hindlimb ischemia. Rats were divided into 3 groups: the control group was given tap water, the HH group was given water containing L-methionine (1 g · kg⁻¹ · d⁻¹), and the HH+L-arg group was given water containing methionine (1 g · kg⁻¹ · d⁻¹) and L-arginine (2.25 vol%). At day 14 of the dietary modifications, the left femoral artery and vein were excised, and the extent of angiogenesis and collateral vessels in the ischemic limb were examined for 4 weeks. Plasma homocysteine levels significantly increased (P<0.001), and plasma and tissue contents of nitrite+nitrate as well as tissue cGMP levels significantly decreased in the HH group compared with the control group (P<0.01). Laser Doppler blood flowmetry (LDBF) revealed a significant decrease in the ischemic/normal limb LDBF ratio in the HH group at days 7, 14, 21, and 28 (P<0.01 versus control). Angiography revealed a significant decrease in the angiographic score in the HH group at day 14 (P<0.001 versus control). Immunohistochemistry of ischemic tissue sections showed a significant reduction in the capillary density in the HH group (P<0.001 versus control). Oral L-arginine supplementation in rats with HH (HH+L-arg) restored the decreased plasma and tissue nitrite+nitrate and cGMP contents (P<0.05) as well as angiogenesis, as assessed by LDBF (P<0.05 versus HH), angiographic score (P<0.01 versus HH), and capillary density (P<0.001 versus HH). In summary, HH impaired ischemia-induced angiogenesis and collateral vessel formation in a rat model of hindlimb ischemia in vivo. The mechanism of the HH-induced impairment of angiogenesis might be mediated in part by a reduced bioactivity of endogenous NO in the HH state. (Arterioscler Thromb Vasc Biol. 2000; 20:2579-2585.)

Key Words: endothelium ■ homocysteine ■ nitric oxide ■ peripheral artery disease ■ risk factors

Epidemiological studies have demonstrated an association between hyperhomocysteinemia (HH) and atherosclerotic vascular diseases.¹⁻⁵ Moreover, it has been established that modest HH occurs in up to 40% of patients with stroke, myocardial infarction, and/or peripheral arterial obstructive disease (PAOD).¹,² HH may exert multiple adverse effects on cells composing the vascular wall. In particular, homocysteine induces endothelial dysfunction and thereby accelerates atherosclerosis.²⁻⁶ Light- and electron-microscopic studies of arteries and arterioles from humans and animals have revealed that HH alters endothelial morphology.⁵⁻⁷⁻⁹ HH has also been shown to attenuate endothelium-dependent vasodilatation,⁸⁻¹⁰⁻¹¹ presumably because of the inactivation of endothelium-derived NO (EDNO).⁶ In addition, HH has been shown to produce oxygen-derived free radicals that further inactivate EDNO.¹²⁻¹³

We and others have recently reported that EDNO is an important endogenous modulator of angiogenesis.¹⁴⁻¹⁷ For example, angiogenesis induced by substance P, an endothelium-dependent vasodilator,¹⁴ or vascular endothelial growth factor,¹⁵⁻¹⁶ a potent angiogenic cytokine, was significantly attenuated by inhibitors of NO synthase (NOS). More recently, we showed that angiogenesis occurring after surgically induced hindlimb ischemia was severely impaired in mice lacking the gene for the endothelial constitutive NOS.¹⁷

Although such adverse effects of HH on endothelial cells (ECs) have been documented in numerous studies, the effects of HH on angiogenesis and collateral vessel formation in response to tissue ischemia have not been examined as yet. This issue is clinically relevant inasmuch as HH is an independent risk factor for PAOD. In patients with PAOD, regional angiogenesis and collateral vessel formation from surrounding tissues are critical self-defense mechanisms for tissue survival.¹⁸ Accordingly, we investigated the effects of HH on angiogenesis, collateral vessel formation, and regional blood flow in a rat model of surgically induced hindlimb ischemia. We also investigated whether oral supplementation of L-arginine, a substrate for NOS, had favorable effects on a potential HH-mediated impairment of angiogenesis.
Methods

Rat Model of Hindlimb Ischemia

All protocols were approved by the Institutional Animal Care and Use Committee. The rat model of hindlimb ischemia was produced as previously described.9,20 In brief, after male Sprague-Dawley rats (250 to 300 g) were anesthetized with pentobarbital sodium (50 mg/kg IP), the entire femoral artery and vein were excised. Consequently, the blood flow in the ischemic lower limb became completely dependent on collateral vessels issuing mainly from the internal iliac artery.

Study Protocol

Rats (n = 54) were randomly divided into 3 groups (Figure I, which can be accessed online at http://atvb.ahajournals.org). The control group (n = 18) was given tap water. The HH group (n = 18) was given tap water containing methionine (1 g · kg⁻¹ · d⁻¹), and the HH + L-arg group was given tap water containing methionine (1 g · kg⁻¹ · d⁻¹) and L-arginine (2.25%). Rats were started on methionine and L-arginine 2 weeks before the induction of ischemia, and administration was continued until the end of the protocol. At 2 weeks after the induction of ischemia, animals were euthanized by an overdose of sodium pentobarbital. Medial thigh adductor muscles of the ischemic (left) and nonischemic (right) limbs were harvested and fixed in methanol. Tissues were embedded in paraffin, and 5-μm-thick sections were prepared. We used a monoclonal antibody (MAB) against von Willebrand factor (vWF; clone F8/86, DAKO) as a marker for ECs because this molecule is constitutively expressed on all ECs and because its expression does not depend on phenotypic changes. ECs positively stained by this method were counted under light microscopy, and capillary densities of ischemic and nonischemic hindlimb muscles were analyzed for specific evidence of vascularity at the microcirculation level. Ten different microscopic fields from at least 3 different sections from each animal were counted, and the capillary density was expressed as the number of capillaries/field (magnification ×400). Nonimmune mouse IgG (Sigma Chemical Co) was used instead of the anti-vWF MAB as the negative control.

cGMP Contents in Ischemic Hindlimb Tissues

Four skeletal muscle samples were harvested from the medial thigh of the ischemic hindlimb of 6 rats in each group at postoperative day 14. The tissue samples were weighed, snap-frozen in liquid N₂, and stored at −80°C until analysis. Tissue cGMP was assayed as previously described.17 In brief, tissues were homogenized in 10 vol of 6% trichloroacetic acid in polypropylene tubes at 4°C and centrifuged at 2000g for 15 minutes. The supernatant was collected and washed 4 times with 5 vol of water-saturated diethyl ether. The liquid samples were then frozen in liquid N₂ and lyophilized. The product of lyophilization was dissolved in 1 mL of 0.05 mol/L sodium acetate buffer (pH 5.8). cGMP was measured by use of a cGMP enzyme-immunoassay kit (Biotrak, Amersham Life Sciences). Values for cGMP were standardized by tissue weight (in grams).

Plasma and Tissue Biochemical Measurements

Plasma levels of homocysteine, folate, and vitamin B₁₂ were measured by high-performance liquid chromatography (HPLC) as described previously.22–25 Plasma and tissue levels of total nitrite (NO₂⁻) plus nitrate (NO₃⁻), known as NOx, were measured by HPLC after reduction of total NOx to NO₂⁻, as described previously.26 Plasma levels of asymmetrical dimethyl arginine (ADMA), an endogenous NOS inhibitor, were determined by using HPLC as described previously.27 Serum levels of total cholesterol and triglycerides were determined enzymatically by using commercially available kits (Boehringer Diagnostica and Wako Chemicals).

Reagents

All reagents were purchased from Sigma unless otherwise specified. The immunostaining kit (VECTASTAIN, Elite) including the secondary anti-mouse IgG antibody was purchased from Vector Laboratories.

Statistical Analysis

Data are expressed as mean±SE. Comparisons among the 3 groups were performed by ANOVA followed by the Fisher t test for comparison between 2 means. Statistical significance was assumed at P<0.05.

Results

Table 1 summarizes mean body weight, heart rate, and systolic blood pressure in the 3 experimental groups measured before surgery and at postoperative days 7, 14, and 28. There were no significant differences in any of these parameters among the 3 groups at any determination points.

Laser Doppler Blood Flow

Figure 1A shows representative pictures of LDBF at postoperative days 3, 7, 14, and 28. Serial LDBF revealed progressive recovery of the blood flow within 28 days after induction of left hindlimb ischemia in the control group. However, the recovery of the limb blood flow was impaired in the HH group. Oral L-arginine supplementation restored the blood flow in the ischemic limb in the HH + L-arg group. Figure 1B summarizes the calculated ratio of ischemic to normal limb blood flow. Before surgery, the blood flow ratio was ~1.0 in all 3 groups. Imme-

Immunohistochemical Analysis and Determination of Capillary Density

Six animals in each group were euthanized at postoperative day 14 with an overdose of sodium pentobarbital. Medial thigh adductor muscles of the ischemic (left) and nonischemic (right) limbs were harvested and fixed in methanol. Tissues were embedded in paraffin, and 5-μm-thick sections were prepared. We used a monoclonal antibody (MAB) against von Willebrand factor (vWF; clone F8/86, DAKO) as a marker for ECs because this molecule is constitutively expressed on all ECs and because its expression does not depend on phenotypic changes. ECs positively stained by this method were counted under light microscopy, and capillary densities of ischemic and nonischemic hindlimb muscles were analyzed for specific evidence of vascularity at the microcirculation level. Ten different microscopic fields from at least 3 different sections from each animal were counted, and the capillary density was expressed as the number of capillaries/field (magnification ×400). Nonimmune mouse IgG (Sigma Chemical Co) was used instead of the anti-vWF MAB as the negative control.
Immediately after operative induction of left limb ischemia, the blood flow ratio decreased to almost 0.4 in all 3 groups, showing no differences among the 3 groups. Therefore, the severity of induced limb ischemia was comparable among the 3 groups. The ratios of the ischemic to normal blood flow at postoperative days 7, 14, 21, and 28 were significantly smaller in the HH group than in the control group. The ratios were significantly restored in the HH+L-arg group at postoperative days 7, 14, 21, and 28 compared with the HH group.

Angiographic Score
Figure 2A shows representative angiographic films. There were numerous collateral vessels in the ischemic medial thigh

<table>
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<th>TABLE 1. Basal Data on Animals</th>
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<td>Group</td>
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<tr>
<td>Control</td>
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<td>HH</td>
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<td>HH+L-arg</td>
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Values are mean±SE. BW indicates body weight; HR, heart rate; and SBP, systolic blood pressure.

Figure 1. A, Representative pictures of LDBF are shown. The left hindlimb was subjected to surgically induced ischemia. Blood flow is displayed by color-coded pixels. B, The ratio of ischemic to normal limb blood flow decreased from 1.0 to ~0.4 after surgery in all groups, showing no differences among the groups. Progressive recovery of blood flow within 28 days after surgery was observed in the control group, whereas the blood flow ratios remained impaired in the HH group compared with the control group at postoperative days 7, 14, 21, and 28. Oral L-arginine supplementation restored blood flow ratios at these determination points (n=6 in each group): *P<0.01 and **P<0.001 vs control; †P<0.05 vs HH.

Figure 2. A, Representative angiograms taken at postoperative day 14. One animal from each group is shown. B, Angiographic score was significantly lower in HH group than in control group. Oral L-arginine supplementation significantly restored angiographic score compared with HH with no L-arginine (n=6 in each group).
area in the control group. In contrast, the HH group had less collateral vessels issuing from the internal iliac artery. Oral l-arginine increased the angiographically visible collateral vessels in the HH state. The development of collateral vessels in the ischemic medial thigh area was quantified by using the angiographic score (Figure 2B). The angiographic score at postoperative day 14 was significantly lower in the HH group than in the control group. However, oral L-arginine supplementation restored the angiographic score (P < 0.01 versus HH).

Capillary Density
Immunohistochemical staining with use of an anti-vWF MAb identified capillary ECs in the isolated skeletal muscles. Representative photomicrographs of histological sections are shown in Figure 3A. There were fewer capillary ECs in the ischemic limb in the HH group. Higher numbers of capillary ECs were seen in the HH + L-arg group. There was virtually no staining in a slide incubated with nonimmune IgG. Figure 3B shows quantitative data of the vWF-positive capillary ECs counted under light microscopy (magnification ×400). The capillary density was significantly lower in the HH group than in the control group in the ischemic limb (P < 0.001).

However, in the group receiving oral l-arginine supplementation, the number of capillary ECs was significantly increased compared with the number in the HH group (Figure 3B). In contralateral nonischemic hindlimbs, there were no significant differences in capillary density among the 3 groups (Figure 3B).

Plasma Biochemical Markers
At postoperative day 14, plasma homocysteine concentrations of the rats given methionine were significantly higher than those of the control rats (P < 0.001) (Figure 4). Plasma homocysteine levels did not differ between the HH and the HH + L-arg groups, indicating that oral l-arginine did not affect oral methionine–induced HH levels. Plasma NOx levels decreased in rats with HH (P < 0.01 versus control), but oral L-arginine completely restored the plasma NOx levels (P < 0.01 versus HH) (Figure 4). Plasma levels of ADMA, folate, and vitamin B12 did not differ significantly among the 3 groups (Table 2). Serum levels of total cholesterol and triglycerides did not differ either (Table 2).

Tissue Contents of NOx and cGMP in the Ischemic Hindlimb
To examine whether HH altered NO production in the ischemic tissues, we examined the contents of NOx and cGMP in ischemic hindlimb tissues. Both tissue NOx and cGMP levels were significantly lower in the HH group than in the control group (P < 0.01) (Figure 5). However, oral l-arginine supplementation restored the tissue contents of both NOx (P < 0.05 versus HH) and cGMP (P < 0.01 versus HH) (Figure 5).

<table>
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<th>Table 2. Biochemical Parameters</th>
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<td>Group</td>
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<tr>
<td>ADMA, μmol/L</td>
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<tr>
<td>Folate, ng/mL</td>
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<tr>
<td>Vitamin B12, ng/mL</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
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<td>Triglycerides, mg/dL</td>
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Values are mean ± SE. Biochemical markers did not differ significantly among the 3 experimental groups.
Folate and vitamin B12 levels were not altered by oral methionine.21 In the present study, rats were given L-methionine via drinking water at a dose (1 g·kg\(^{-2}\)·d\(^{-1}\)) via drinking water. In their study, first-order arterioles (\(\approx 130 \, \mu m\) in diameter) isolated from rats with chronic HH showed impaired endothelium-dependent relaxations to acetylcholine and histamine.21 In the present study, rats were given L-methionine via drinking water at a dose (1 g·kg\(^{-1}\)·d\(^{-1}\)) that was identical to the dose of methionine used by Ungvari et al. Because plasma homocysteine levels were elevated by 2-fold compared with those in the control group, our rat model of HH mimicked the moderate HH commonly observed in humans.21,28,29 Durand et al29 demonstrated that the decreased blood flow was associated with increased plasma NOx and tissue NOx and cGMP contents in the ischemic tissues; and (4) the HH-induced impairment of angiogenesis was restored by oral l-arginine supplementation, which was associated with increased plasma NOx and tissue NOx and cGMP levels.

**Rat Model of Dietary HH and Angiogenesis**

Dietary methionine load has been shown to elevate plasma concentrations of homocysteine and thereby induce vascular dysfunction in animals and humans.21,28,29,31 Ungvari et al21 produced a rat model of chronic HH (4 weeks) by giving methionine (1 g·kg\(^{-2}\)·d\(^{-1}\)) via drinking water. In their study, first-order arterioles (\(\approx 130 \, \mu m\) in diameter) isolated from rats with chronic HH showed impaired endothelium-dependent relaxations to acetylcholine and histamine.21 In the present study, rats were given L-methionine via drinking water at a dose (1 g·kg\(^{-1}\)·d\(^{-1}\)) that was identical to the dose of methionine used by Ungvari et al. Because plasma homocysteine levels were elevated by 2-fold compared with those in the control group, our rat model of HH mimicked the moderate HH commonly observed in humans. Taken together, the rat model of dietary HH seemed to be an appropriate model to test the effects of HH on vascular functions and morphological changes, including angiogenesis, in vivo.

Serial LDBF demonstrated that the recovery of the regional blood flow in the ischemic limb was significantly attenuated in the HH group compared with the control group. We further demonstrated that the decreased blood flow was associated with reduced vascularity at macrovascular (angiographic score) and microvascular (capillary density) levels. Importantly, LDBF values have been shown to correlate well with tissue capillary density.30 Therefore, to our knowledge, the present study is the first study that provides evidence that HH inhibits ischemia-induced angiogenesis in vivo. Although several vitamins have been shown to participate in the metabolic process of methionine as coenzymes,31 plasma folate and vitamin B12 levels were not altered by oral methionine–induced HH in the present study. These results suggest that the impaired angiogenesis observed in the HH group was not likely due to changes in folate or vitamin B12 status.

**Mechanisms by Which HH Impairs Angiogenesis**

There may be several possible mechanisms by which HH impairs angiogenesis. First, HH-induced endothelial dysfunction may account for the impaired angiogenesis. A previous study reported that prolonged exposure to homocysteine decreased the bioactivity of NO in cultured ECs.32 HH impaired EDNO formation not only in large conduit arteries but also in microvessels in vivo.9,21,33 Especially, EDNO formation in arterioles located in skeletal muscles in rats was impaired by HH,21 which is directly relevant for the present study. Consistent with these previous reports, plasma NOx levels and tissue contents of NOx and cGMP were significantly reduced in the HH group compared with the control group. In this regard, we and others14–17 have reported that EDNO is an important regulator of angiogenesis. For example, EDNO maintains EC integrity34 and the expression of integrin \(\alpha \beta \)35 and thus promotes endothelial podokinesis and migration.35,36 Angiogenesis induced by substance P or vascular endothelial growth factor was attenuated by inhibitors of NOS.14–16 We also showed that angiogenesis occurring in the ischemic hindlimb was severely impaired in mice lacking the gene for endothelial NOS.17 Therefore, the endothelial dysfunction and decreased bioactivity of NO may in part account for the impaired angiogenesis in the HH state.

Second, HH-induced production of reactive oxygen radicals may contribute to further impairment of angiogenesis.12,13 Homocysteine undergoes oxidation to homocystine during metabolism, and in this process, hydrogen peroxide is released.12 Moreover, homocysteine decreases the ability of endothelial cells to detoxify hydrogen peroxide by reducing intracellular antioxidant enzymes, especially glutathione peroxidase37 and superoxide dismutase.38 Thus, enhanced generation of oxygen radicals in the HH state might further degrade NO, which may in part explain the impaired ischemia-induced angiogenesis in the HH rats.

Third, HH itself might directly inhibit EC proliferation and/or migration. Outiline et al39 recently demonstrated that homocysteine induced growth arrest in human ECs in vitro. However, these biological actions were elicited by very high concentrations of homocysteine (0.2 to 5 mmol/L) that were much higher than those observed in the present study and in HH in humans. Therefore, this issue (ie, EC growth arrest by HH in vivo) should be further investigated. Taken together, endothelial dysfunction, decreased NO bioactivity, and increased oxidative stress seem to account for impaired angiogenesis in the HH state in vivo.

There may be a possibility that oral methionine–induced HH affected the expression of inducible NOS in vascular smooth muscle cells and macrophages. However, inducible NOS generally produces a massive amount of NO; thus, this possibility may be less likely, at least in the ischemic tissues in the present study, because tissue contents of NOx and cGMP were decreased in the HH group compared with the control group. We and others40 have recently reported that hypercholesterolemia reduced plasma and tissue NOx levels while it increased ADMA contents and thereby inhibited...
ischemia-induced angiogenesis in rats. However, we did not find any differences in serum total cholesterol, triglyceride, or plasma ADMA levels among the 3 groups. These results suggest that HH likely attenuated angiogenesis independently of ADMA or serum lipid profile.

L-Arginine Rescued the HH-Induced Impairment of Angiogenesis

To further clarify the above issues, we examined the effects of oral supplementation of L-arginine, a substrate for NO, on angiogenesis in response to hindlimb ischemia in rats with HH. In previous studies, oral L-arginine improved endothelium-dependent relaxation in humans and experimental animals without changing systemic hemodynamics. Oral L-arginine improved EC function not only in large conduit arteries but also in microvessels. In the present study, oral L-arginine restored the impaired angiogenesis in rats with HH. Compared with HH without L-arginine, the restoration of angiogenesis by oral L-arginine was documented by the increased serial ischemic/normal LDBF ratios, angiographic score, and capillary density. Moreover, oral L-arginine significantly increased the plasma NOx and tissue contents of NOx and cGMP. L-Arginine did not alter the serum levels of total cholesterol or triglycerides, indicating that the effects of L-arginine on angiogenesis were not due to the changes in the serum lipid profile. Because oral L-arginine did not affect systemic blood pressure, the beneficial effects of L-arginine on angiogenesis were not likely mediated by blood pressure changes. On the basis of these findings, oral L-arginine supplementation seemed to restore the ischemia-induced angiogenesis in the HH state, possibly by augmenting endogenous NO bioactivity.

Conclusions and Clinical Implications

In summary, our findings suggest that angiogenesis and collateral vessel formation were impaired by methionine-induced HH, which was almost comparable to clinical HH in humans. Rats with HH showed decreased plasma NOx levels and tissue contents of NOx and cGMP. Moreover, oral L-arginine supplementation restored NO formation in the ischemic tissues as well as angiogenesis in the HH state. Thus, the mechanism of the HH-mediated impairment of angiogenesis is likely due to the reduced bioactivity of endogenous NO in the HH state.

The present findings may have important clinical implications. First, impaired collateral vessel formation in patients with ischemic heart disease or PAOD may be in part related to HH because up to 40% of patients with atherosclerotic diseases have HH. Second, in PAOD patients with HH, dietary intervention to reduce plasma homocysteine levels may facilitate angiogenesis and collateral vessel formation.

Acknowledgments

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References


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