Hypercholesterolemia Inhibits Angiogenesis in Response to Hindlimb Ischemia
Nitric Oxide–Dependent Mechanism

Junli Duan, MD; Toyoaki Murohara, MD; Hisao Ikeda, MD; Atsushi Katoh, MD; Satoshi Shintani, MD; Ken-ichiro Sasaki, MD; Hidemichi Kawata, BS; Naoki Yamamoto, BS; Tsutomu Imaizumi, MD

Background—Endothelium-derived nitric oxide (EDNO) plays an important role in the regulation of angiogenesis, whereas hypercholesterolemia (HC) impairs EDNO release. We examined the hypothesis that HC may inhibit ischemia-induced angiogenesis by inhibition of EDNO in a rat model of unilateral hindlimb ischemia and that oral L-arginine supplementation, a substrate for NO synthase, may prevent HC-related impairment of angiogenesis.

Methods and Results—Male Sprague-Dawley rats were fed (A) standard diet (control), (B) 2% high-cholesterol diet (HC group), or (C) high-cholesterol diet with oral L-arginine (2.25% in drinking water) (HC + L-arg group). At 2 weeks of the dietary intervention, unilateral limb ischemia was surgically induced in all animals. Dietary HC groups (B and C) revealed elevated total and LDL cholesterol levels compared with control animals. Laser Doppler blood flow analyses showed significant decreases in the ischemic/normal limb blood flow ratio in the HC group compared with controls (P < 0.05) when followed up until 4 weeks after surgery. Selective angiography and immunohistochemical analyses in the ischemic limb at postoperative day 14 revealed significantly lower angiographic scores (P < 0.01) and capillary densities (P < 0.01) in the HC group than controls, which were associated with decreased tissue contents of NOx and cGMP. Oral L-arginine supplementation (HC + L-arg) significantly improved all parameters of the laser Doppler blood perfusion ratio, angiographic scores, and capillary densities (P < 0.01 versus HC group), which were accompanied by significant elevations in serum L-arginine levels and tissue NOx and cGMP contents.

Conclusions—Collateral vessel formation and angiogenesis in response to hindlimb ischemia were significantly attenuated in rats with dietary HC. The mechanism may be related to the reduced NO bioactivity in the ischemic tissues. Augmentation of the tissue NO activity by oral L-arginine supplementation restored the impaired angiogenesis in HC. (Circulation. 2000;102[suppl III]:III-370-III-376.)

Key Words: peripheral vascular disease • hypercholesterolemia • angiogenesis • nitric oxide • endothelium
hypothesis that dietary supplementation of L-arginine, a substrate for NO synthesis, may have beneficial effects on the ischemia-induced angiogenesis in this rat model.

**Methods**

**Animal Model**

All animal protocols were approved by the Institutional Animal Care and Use Committee. A rat model of hindlimb ischemia was induced by a previously described method with slight modification. Male Sprague-Dawley rats (250 to 300 g) were anesthetized with intraperitoneal pentobarbital (50 mg/kg). After skin incision, the entire femoral artery and vein were excised after all of their major branches were tied. Consequently, the blood flow to the ischemic lower limb became completely dependent on collateral vessels issuing from the internal iliac artery and its branches.

**Study Protocol**

Rats (n=53) were divided into 3 groups (Figure 1). Rats in the control group (n=18) were fed a standard diet throughout the experiment. Rats in the HC group (n=18) were fed a 2% cholesterol diet, and rats in the HC+L-arg group (n=17) were fed an HC diet and given oral L-arginine in the drinking water (2.25%). At 2 weeks of the dietary modification, all rats were subjected to unilateral hindlimb ischemia as described above. On the day of surgery, rats in the HC+L-arg group started to receive L-arginine until the end of the protocol. The oral dose of L-arginine was chosen according to a previous study, and this regimen of L-arginine administration effectively increased serum levels of L-arginine and tissue contents of stable NO metabolites [nitrite (NO2)+nitrate (NO3)] in rats. Before surgery and at postoperative days 7, 14, and 28, a tail-cuff method was applied for systemic blood pressure measurements in the conscious state.

**Laser Doppler Blood Perfusion Analysis**

We measured the ischemic/normal hindlimb blood perfusion ratio using a laser Doppler perfusion image (LDPI) analyzer (moorLDI, Moor Instrument) as described previously. In this method, a color-coded image representing blood flow distribution is displayed. Low or no blood perfusion is displayed as dark blue, and the highest perfusion level is displayed as red to white colors. At 7 predetermined time points (before and immediately after surgery and at postoperative days 3, 7, 14, 21, and 28) (Figure 1), we performed 2 consecutive LDPI scans over the same region of interest. After 2 scans, the average perfusion values of the ischemic and nonischemic (normal) hindlimbs were computed from histograms of the colored pixels. To minimize variations due to ambient light, calculated blood perfusion (relative units) was expressed as the ischemic (left)/normal (right) limb blood perfusion ratio.

**Angiographic Score**

At postoperative day 14, under pentobarbital anesthesia (50 mg/kg), a 22-gauge soft-tip catheter was inserted into the abdominal aorta of control (n=6), HC (n=6), and HC+L-arg (n=5) rats. Both hindlimbs were perfused with 10 mL of saline containing heparin (10 U/mL). Postmortem angiography was then performed by injection of 3 mL of contrast medium through the catheter inserted into the abdominal aorta. X-ray films were recorded by mammography (Senographe 500T, General Electric), and the extent of collateral vessels was calculated by the angiographic score. For calculation of angiographic score, a composite of 2-mm2 grids printed on a transparent sheet was placed over the thigh area of each film. The score was determined by calculating intersections crossed by opacified arteries divided by the total number of grid intersections within the area of interest.

**Immunohistochemistry and Determination of the Capillary Density**

Six animals in each group were euthanized at postoperative day 14 with an overdose of sodium pentobarbital. Ischemic (left) and nonischemic (right) hindlimb skeletal muscles were harvested and fixed in methylsalicylate. Tissues were embedded in paraffin, and 5-μm-thick histological sections were prepared. We used a monoclonal antibody (MAb) directed against von Willebrand factor (vWF) (DAKO) as a marker for ECs because this molecule is constitutively expressed in all ECs and its expression does not depend on either phenotypic changes or activation states of ECs. The vWF-bound MAb was detected by an avidin-biotin-peroxidase method from a commercially available kit (Vector Laboratories). Positively stained ECs were counted, and the capillary densities of both ischemic and nonischemic limb muscles were analyzed for specific evidence of the vascularity at the microcirculation. Ten different microscopic fields from the 3 different sections from each animal were counted, and capillary density was expressed as number of capillaries/field (×400).

**Measurements of Tissue cGMP Contents**

Four tissue blocks (1 to 1.2 g) from the thigh adductor muscle were harvested from the ischemic hindlimb of 6 rats in each group at postoperative day 14. The tissue samples were weighed, snap-frozen in liquid nitrogen, and stored at −80°C. The assay for tissue cGMP contents was performed as previously described. Serum levels of total, LDL, and HDL cholesterols and of triglyceride were determined enzymatically with commercially available kits (Boehringer Diagnostica and Wako Chemicals). Serum L-arginine levels, tissue contents of NOx (stable metabolites of NO), and asymmetrical dimethyl arginine (ADMA), an endogenous NOS inhibitor, were also measured by high-performance liquid chromatography as described previously.

**Statistical Analysis**

Results are all expressed as mean±SEM. Comparisons were performed by use of ANOVA followed by Fisher’s test for comparisons between any 2 groups. Statistical significance was assumed at P<0.05.
TABLE 1. Basal Data of Animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HC</th>
<th>HC+L-Arg</th>
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<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Before surgery</td>
<td>323±3</td>
<td>330±5</td>
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<tr>
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<td>330±7</td>
<td>341±3*</td>
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<td>Day 14</td>
<td>332±5</td>
<td>350±6*</td>
<td>350±3*</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>379±3</td>
<td>442±6*</td>
<td>437±5*</td>
<td></td>
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<tr>
<td>Heart rate, bpm</td>
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<td>371±15</td>
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<tr>
<td>Day 14</td>
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<td>Day 28</td>
<td>367±11</td>
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<tr>
<td>SBP, mm Hg</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Before surgery</td>
<td>120±5</td>
<td>117±4</td>
<td>116±3</td>
<td></td>
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<tr>
<td>Day 7</td>
<td>132±7</td>
<td>117±8</td>
<td>115±4</td>
<td></td>
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<tr>
<td>Day 14</td>
<td>119±2</td>
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<td>Day 28</td>
<td>138±10</td>
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<tr>
<td>DBP, mm Hg</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Before surgery</td>
<td>79±4</td>
<td>75±4</td>
<td>77±5</td>
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<tr>
<td>Day 28</td>
<td>71±4</td>
<td>82±5</td>
<td>78±2</td>
<td></td>
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</table>

SBP indicates systolic blood pressure; DBP, diastolic blood pressure. *P<0.05 vs control.

Results

Table 1 summarizes body weight, heart rate, and systolic and diastolic blood pressures of each group measured before surgery and at postoperative days 7, 14, and 28. Mean body weights of rats in the HC and HC+L-arg groups were heavier than those of control animals at days 7, 14, and 28. However, there were no significant differences in body weight between the HC and HC+L-arg groups at any time points. Thus, the HC diet increased body weight but supplemental oral L-arginine did not affect the body weight in the HC state. There were no differences in heart rate or systolic and diastolic blood pressures among the 3 experimental groups at any time points (Table 1).

Serum Lipid Levels

Table 2 summarizes serum lipid levels of each group examined at postoperative day 14 (ie, 28 days after dietary modification). Serum total and LDL cholesterol levels were significantly but modestly increased in the HC and HC+L-arg groups compared with controls. Serum HDL cholesterol levels decreased significantly in the HC and HC+L-arg groups compared with the controls. Serum triglyceride levels did not differ among the 3 groups. There were no significant differences in total, LDL, and HDL cholesterol levels between HC and HC+L-arg groups, indicating that supplemental oral L-arginine did not affect the serum lipid profiles in the HC state.

Laser Doppler Analysis for Hindlimb Blood Perfusion

Before surgery, the left/right hindlimb blood perfusion ratio was 1.0 in all groups. Immediately after operative induction of the left hindlimb ischemia, the ratios of ischemic/normal blood perfusion markedly decreased, ranging from 0.37 to 0.41. These ratios immediately after induction of unilateral limb ischemia did not differ among the 3 groups, indicating that the severity of the ischemia created was comparable among the 3 groups.

Representative images of the hindlimb blood perfusion before and immediately after surgery and at postoperative days 7, 14, and 28 are shown in Figure 2A. LDPI analyses disclosed progressive recovery of the blood perfusion within 28 days after surgery in controls (Figure 2B). However, the blood perfusion ratios in the HC group were significantly smaller than those of controls at postoperative days 7, 14, 21, and 28. In contrast, the blood perfusion ratio improved significantly in the HC+L-arg group compared with the HC group at days 7, 14, 21, and 28 (Figure 2B).

Angiographic Score

To further examine whether the altered blood perfusion detected by the LDPI is associated with changes in collateral vessel formation, we performed iliac angiography. Figure 3A shows representative angiograms taken at postoperative day 14. There are numerous collateral vessels in the ischemic thigh muscle area in a rat from the control group. However, there are fewer collateral vessels issuing from the internal iliac artery in a rat from the HC group. Oral L-arginine supplementation, however, markedly increased the number of collaterals.

Collateral vessels in the medial thigh area were quantitatively assessed by calculation of the angiographic score. The angiographic score was significantly lower in the HC group than in controls. However, the angiographic score was restored to a level comparable to that of the control group by oral L-arginine supplementation (Figure 3B).

Immunohistochemical Identification of ECs and Capillary Density

To examine whether the changes in the hindlimb blood perfusion (by LDPI) and collateral vessel formation (by angiographic score) are associated with changes in capillary EC formation at the microcirculation level, we measured capillary densities in histological sections harvested from ischemic and nonischemic hindlimbs at postoperative day 14. Immunohistochemical staining by an anti-vWF MAb identified capillary ECs in the skeletal muscle tissues. Representative photomicrographs of histological sections are shown in

TABLE 2. Serum Lipid Levels

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HC</th>
<th>HC+L-Arg</th>
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</thead>
<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>54±6</td>
<td>74±10*</td>
<td>68±5*</td>
<td></td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>8.3±1.5</td>
<td>15±4†</td>
<td>13±1†</td>
<td></td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>22±1</td>
<td>18±1*</td>
<td>18±1*</td>
<td></td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>91±10</td>
<td>96±20</td>
<td>93±21</td>
<td></td>
</tr>
</tbody>
</table>

C indicates cholesterol; TG, triglyceride. *P<0.05, †P<0.01 vs control.
Figure 4A. The number of capillary ECs in the ischemic limb of an HC rat is decreased compared with a control rat, which was restored by oral L-arginine supplementation.

Quantitative analyses counting the vWF-positive capillary ECs under light microscopy (×400) revealed that the capillary densities were significantly lower in the HC group than controls in the ischemic limb (P<0.001) at postoperative day 14 (Figure 4B). However, capillary densities were restored in the HC+L-arg group (P<0.001 versus HC group). In the contralateral nonischemic right limb, capillary densities were not different among the 3 groups (Figure 4B). Therefore, oral L-arginine increased ischemia-induced angiogenesis without affecting capillary densities of nonischemic tissues.

Serum Levels of L-Arginine and Tissue Contents of NOx and cGMP
The serum L-arginine levels at postoperative day 14 were not significantly different between the control and HC groups. Oral L-arginine supplementation significantly increased that level (Table 3). To evaluate whether HC and L-arginine supplementation altered NO formation in the ischemic tissues, we analyzed the contents of NOx and cGMP in the ischemic hindlimb tissues. Both tissue NOx and cGMP contents were significantly lower in the HC rats than controls (Table 3). Oral L-arginine supplementation, however, significantly restored both tissue NOx and cGMP contents (P<0.05) compared with the HC group (Table 3).

Tissue Contents of ADMA in the Ischemic Hindlimb
Accumulation of ADMA, an endogenous inhibitor of NOS, in vascular tissue has been implicated as a risk factor for atherogenesis. We therefore measured tissue contents of ADMA in the ischemic hindlimb. Tissue ADMA levels were significantly greater in the HC group than the control group. Oral L-arginine supplementation restored tissue ADMA contents (P<0.05) to a level similar to that of the control group (Figure 5).

Discussion
Major findings of the present study are that (1) ischemia-induced angiogenesis was impaired in rats with dietary HC; (2) the HC-related impairment of angiogenesis was associated with decreased NO and cGMP productions in the ischemic hindlimb tissues; and (3) oral L-arginine supplementation, a substrate for NOS, rescued the HC-related impairment of ischemia-induced angiogenesis, accompanied by improved NO/cGMP production in the ischemic tissues. Therefore, HC impairs angiogenesis and collateral vessel formation in response to regional tissue ischemia, possibly through decreased tissue NO bioactivity. HC, however, does not preclude augmentation of collateral vessel development by means of oral L-arginine supplementation, a precursor of NO.

Rat Model of HC
In the present study, rats with HC revealed mild increases (by 37%) in total cholesterol levels compared with control rats.
One may speculate that animals with such modest HC may not be a proper model to test the effects of HC in vivo. However, serum LDL cholesterol levels were almost doubled in HC rats compared with controls in the present study. Moreover, previous studies showed that rat models of HC elicit impaired vascular functions. For example, Nunnari et al. showed that dietary HC resulted in significant deposition of oil red O-positive lipids on the rat arterial wall. We previously showed that HC attenuated endothelium-dependent relaxation to acetylcholine in the isolated rat aorta ex vivo, and HC enhanced leukocyte-endothelium interactions in the mesenteric microvasculature in vivo. Furthermore, alterations in microvascular reactivity to vasoactive substances have been identified in a rat model of HC before any histological changes of atherosclerosis. Taken together, the rat model of HC, despite the modest increase in serum cholesterol levels, exhibits vascular dysfunction regardless of morphological atherosclerotic changes and thus seems to be a proper model to analyze the effects of HC on angiogenesis in vivo.

**HC Impaired Ischemia-Induced Angiogenesis in Vivo**

HC is one of the established risk factors for atherosclerotic vascular diseases. A recent report by van Belle et al. showed

**TABLE 3. Serum and Tissue Biochemical Markers**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HC</th>
<th>HC+L-Arginine</th>
</tr>
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<tbody>
<tr>
<td>Serum L-arginine, nmol/mL</td>
<td>129±10</td>
<td>111±9</td>
<td>188±25*†</td>
</tr>
<tr>
<td>Tissue NOx, nmol/g tissue</td>
<td>18±2.5</td>
<td>12±3*</td>
<td>31±5†</td>
</tr>
<tr>
<td>Tissue cGMP, fmol/g tissue</td>
<td>43±5</td>
<td>34±3*</td>
<td>72±8‡</td>
</tr>
</tbody>
</table>

*P<0.05 vs control; †P<0.05, ‡P<0.01 vs HC.
that angiogenesis in an ischemic limb was severely impaired in Watanabe heritable hyperlipidemic (WHHL) rabbits. The WHHL rabbit is a genetic model of HC, however, and has extremely high concentrations of serum cholesterol levels (ie, 600 to 900 mg/dL). Therefore, the WHHL rabbit may mimic familial HC but may not represent the modest HC commonly observed in humans. In this regard, the effects of diet-induced modest HC on in vivo angiogenesis were little known.

In the present study, angiogenesis in response to hindlimb ischemia was significantly attenuated by diet-induced modest HC in rats. There are several possible mechanisms for the impaired angiogenesis in the HC state. First, HC-related EC dysfunction and decreased EDNO formation may account for the impaired angiogenesis, because proliferation and migration of ECs are essential processes for angiogenesis. HC impairs EDNO formation not only in large conduit arteries but also in microvessels. Moreover, angiogenesis in response to hindlimb ischemia in rats with HC. The improved angiogenesis by l-arginine was documented by the increased ischemic/normal blood perfusion ratio by the LDPI analysis, increased angiographic score, and increased capillary density compared with the HC group without l-arginine. Moreover, oral l-arginine supplementation significantly increased the contents of NOX and cGMP in the ischemic tissues (Table 3). L-Arginine did not alter the serum levels of total, LDL, and HDL cholesterol and triglyceride, indicating that the effects of l-arginine on angiogenesis were not due to the changes in serum lipid profiles. Because oral l-arginine did not affect systemic blood pressure, the beneficial effects of l-arginine on angiogenesis and collateral vessel formation probably are not due to blood pressure changes. On the basis of these findings, oral l-arginine administration seems to have improved ischemia-induced angiogenesis in HC rats, possibly by augmenting the NO bioactivity in the ischemic tissues.

The precise mechanisms by which l-arginine improved tissue NO activity and angiogenesis in the ischemic limb are still enigmatic. Previous studies have documented that regenerating ECs in ischemic tissues are generally dysfunctional. It is possible that the availability of l-arginine by ecNOS in regenerating ECs may be impaired. Alternatively, endogenous antagonists of NOS, such as ADMA, may accumulate in regenerating ECs. In the present study, HC significantly elevated tissue contents of ADMA. Therefore, elevated ADMA may in part account for the decreased tissue NOX contents and impaired angiogenesis in the ischemic limb in the HC state. Administration of l-arginine might have favorably changed the enzymatic kinetics of ecNOS by competitive inhibition of endogenous antagonists of NOS, such as ADMA.

**Study Limitations**

In our rat model of hindlimb ischemia, angiogenesis in the HC rats kept up with the extent of angiogenesis seen in the control animals in the chronic phase (at postoperative day 35 and thereafter). Thus, we could not determine the effects of l-arginine on angiogenesis in the chronic phase. In this sense, our rat model of limb ischemia is an acute model of ischemia and therefore may not mimic chronic arterial occlusive diseases or critical limb ischemia observed in humans. Nevertheless, supplemental oral l-arginine significantly im-

**Figure 5.** Tissue contents of ADMA in ischemic hindlimb at postoperative day 14. Tissue contents of ADMA were significantly greater in HC group than controls. However, oral l-arginine supplementation in HC state significantly decreased tissue ADMA contents, which were comparable to values of control group. n=6 in each group. *P<0.05.
proved angiogenesis at postoperative days 7 through 28, which is a critical period for endothelial regeneration in the ischemic tissues in these animal models.\(^5,9\)

We started administration of oral \(L\)-arginine on the day of surgery in the HC+L-arg group in the present study. At postoperative day 3, the HC+L-arg group tended to have better blood perfusion than the HC group despite there being no significant difference between them. It then may be clinically relevant to examine the effects of oral \(L\)-arginine starting at a later time point (several days) after surgery on angiogenesis. This issue is now under investigation in our laboratory.

Conclusions and Clinical Implications

Our findings provide evidence that HC impairs angiogenesis in response to regional tissue ischemia, presumably by the decreased activity of the \(L\)-arginine/NO pathway in the ischemic tissues. Therefore, augmentation of endogenous NO bioactivity (eg, by means of \(L\)-arginine, tetrahydrobiopterin, or ecNOS gene transfer) may deserve further consideration as a therapeutic strategy for patients with peripheral arterial occlusive diseases associated with lipid disorders, who often have impaired endothelial functions and decreased EDNO release.

Acknowledgments

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References

1. Isner JM, Pieczek A, Schanfeld R, et al. Clinical evidence of angiogenesis at postoperative days 7 through 28, which is a critical period for endothelial regeneration in the ischemic tissues in these animal models.\(^5,9\)


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