Arginine Restores Cholinergic Relaxation of Hypercholesterolemic Rabbit Thoracic Aorta

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Background. Reduced synthesis of endothelium-derived relaxing factor (EDRF) may explain impaired endothelium-dependent vasodilation in hypercholesterolemia. Accordingly, we designed studies to determine if endothelium-dependent relaxation in hypercholesterolemic rabbits may be restored by supplying L-arginine, the precursor of EDRF.

Methods and Results. Normal or hypercholesterolemic rabbits received intravenous L-arginine (10 mg/kg/min) or vehicle for 70 minutes. Subsequently, animals were killed, thoracic aortas were harvested, and vascular rings were studied in vitro. Rings were contracted by norepinephrine and relaxed by acetylcholine chloride or sodium nitroprusside. Vasorelaxation was quantified by determining the maximal response (expressed as percent relaxation of the contraction) and the ED₅₀ (dose of drug inducing 50% relaxation; expressed as -log M). In vessels from hypercholesterolemic animals receiving vehicle, there was a fivefold rightward shift in sensitivity to acetylcholine compared with normal animals (p=0.05, n=5 in each group). In vessels from hypercholesterolemic animals, L-arginine augmented the maximal response to acetylcholine (83±16% versus 60±15%, p=0.04 versus vehicle) and increased the sensitivity to acetylcholine (ED₅₀ value: 6.7±0.2 versus 6.2±0.2, p<0.05 versus vehicle). Arginine did not affect maximal and ED₅₀ responses to acetylcholine in vessels from normal animals. Arginine did not potentiate endothelium-independent responses in either group.

Conclusions. We conclude that the endothelium-dependent relaxation is normalized in hypercholesterolemic rabbit thoracic aorta by in vivo exposure to L-arginine, the precursor for EDRF. (Circulation 1991;83:1057–1062)

In animal models and in humans, hypercholesterolemia causes endothelial dysfunction that is manifested as an attenuation of endothelium-dependent vasorelaxation.¹⁻捌 This abnormality may be due to reduced synthesis and release of endothelium-derived relaxing factor (EDRF).⁹⁻¹⁰ EDRF is nitric oxide or a labile nitroso compound that liberates nitric oxide¹¹⁻¹³ and is derived from the metabolism of L-arginine.¹３⁻¹⁶

We postulated that endothelium-dependent vasorelaxation in hypercholesterolemic animals could be restored by administering the substrate for EDRF synthesis. Accordingly, we examined the effect of L-arginine administration on endothelial function in hypercholesterolemic rabbits.

Methods

Animals

New Zealand White rabbits weighing 2.1–2.7 kg were housed individually with free access to water. Thirteen animals were fed regular rabbit chow, and 15 were fed chow containing 2% cholesterol for 8 weeks.

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(Farmer’s Exchange, Framingham, Mass.). These protocols were approved by the Harvard University Standing Committee on Animals and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Experimental Protocols

Animals were anesthetized with chlorpromazine hydrochloride (15 mg/kg i.m.) and sodium pentobar-
bital (8–12 mg/kg via the marginal ear vein). Normal saline was administered intravenously (0.2 ml/min for 70 minutes) to 10 animals (five normal and five cholesterol-fed rabbits). L-Arginine (10 mg/kg/min, dissolved in normal saline and infused at 0.2 ml/min for 70 minutes) was administered intravenously to 15 animals (eight normal and seven cholesterol-fed rabbits). This dose of L-arginine was found to increase serum arginine levels from 0.1 to 3 mM when measured after 70 minutes of the L-arginine infusion.17 In three hypercholesterolemic animals, the inactive enantiomer, D-arginine, was substituted for L-arginine in the infusate.

In some animals, the responsiveness of the hind limb resistance vessels to intra-arterial administration of acetylcholine or sodium nitroprusside was studied in vivo; these data form the basis of another report.17 The animals were then killed, and the thoracic aortas were removed for in vitro studies of vascular reactivity. These tissues were not exposed to L-arginine in vitro.

After harvesting the thoracic aortas, the tissues were placed into cold physiological saline solution (PSS) of the following composition (mM): NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, calcium disodium edetate 0.026, and glucose 11.1. The vessels were cleaned of adherent connective tissue, and the aortas were cut into rings (5 mm in length) for study in the organ chamber. The vascular rings were then suspended in organ chambers filled with 25 ml PSS (37°C aerated with 95% O2-5% CO2). Rings were connected to force transducers (model FTO3C, Grass), and changes in isometric force were recorded continuously (polygraph 79B, Grass). During a 90-minute period, the vascular rings were stretched to the optimal point of their length-tension relation (determined by periodically stimulating the tissue with 10^-6 M norepinephrine). Subsequently, in all rings, the ED50 (concentration of drug inducing the half-maximal response to norepinephrine) was determined by exposing the tissues to increasing concentrations of norepinephrine added to the organ chamber in half-log increments in a cumulative manner. After a maximal response to norepinephrine was obtained, the vascular rings were washed repeatedly with fresh PSS until tension returned to the previous baseline value. To study vasodilating agents, rings were then contracted by the ED90 concentration of norepinephrine. After a stable contraction was obtained, the tissue was exposed to cumulative increases in concentration of the vasodilator in half-log increments.

**Drugs**

The drugs used were acetylcholine chloride, L-arginine hydrochloride, D-arginine hydrochloride, norepinephrine bitartrate, and sodium nitroprusside (Sigma Chemical Co., St. Louis, Mo.). All agents were soluble in distilled water or PSS. Stock solutions of hygroscopic acetylcholine chloride were prepared and stored frozen. All other drug solu-

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**Figure 1.** Plot of attenuated endothelium-dependent relaxation in thoracic aorta of hypercholesterolemic rabbit (2% CHOL). Vascular rings of rabbit thoracic aorta were harvested from animals receiving a saline infusion in vivo. Rings were contracted by norepinephrine and then relaxed by increasing concentrations of acetylcholine (ACH). Rings from normal animals were significantly more sensitive to ACH than were those from hypercholesterolemic rabbits.

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**Results**

**Hypercholesterolemic Animals**

Endothelium-dependent relaxations were impaired in vessels harvested from hypercholesterolemic animals that received vehicle. In these animals, there was a fivefold rightward shift in the sensitivity to acetylcholine compared with normal animals receiving vehicle (p=0.05, n=5 in each group; Figure 1). L-Arginine improved endothelium-dependent relaxation in hypercholesterolemic animals. Vascular rings from hypercholesterolemic animals exposed to L-arginine were threefold more sensitive to acetylcholine than were those exposed to vehicle (Table 1). Furthermore, L-arginine potentiated the maximal relaxation induced by acetylcholine (Table 1). In fact, endothelium-dependent relaxations in vessels from hypercholesterolemic animals that received arginine were not different from those that occurred in normal animals (p=NS, n=8 in the normal group, n=7 in the cholesterol-fed group; Figure 2). When the inactive enantiomer, D-arginine, was substituted for L-arginine in the saline infusion,
no improvement was observed in endothelium-dependent relaxation (Figure 3).

L-Arginine did not alter endothelium-independent relaxation to sodium nitroprusside, nor did it affect contraction to norepinephrine in vessels from hypercholesterolemic animals (Table 1).

Normal Animals

L-Arginine did not alter endothelium-dependent relaxation in vascular rings from normal animals, nor did it affect contractions to norepinephrine (Table 1). L-Arginine did not affect maximal relaxation to sodium nitroprusside, but it did induce a rightward shift in the ED$_{50}$ (Table 1).

Discussion

The salient finding of our investigation is that an in vivo infusion of L-arginine (but not its enantiomer, D-arginine) potentiated endothelium-dependent relaxation in vascular rings from hypercholesterolemic rabbits. L-Arginine did not potentiate endothelium-dependent relaxation in normal animals, nor did it improve endothelium-independent relaxation in either group. These findings suggest that the endothelial impairment in hypercholesterolemia may be caused by a reversible reduction in intracellular arginine availability or metabolism.

The mechanism by which hypercholesterolemia impairs endothelium-dependent relaxation is not known. Intimal thickening associated with hypercholesterolemia could represent a physical barrier to the diffusion of EDRF from the endothelium to the vascular smooth muscle. However, this cannot be the only mechanism for the reduction in endothelium-dependent relaxation because endothelial dysfunction may occur before the onset of morphological changes in the vessel wall. In the hypercholesterolemic swine coronary artery, endothelium-dependent relaxation is attenuated in the absence of gross structural changes. Resistance vessels do not develop atheromas, so a physical barrier does not explain the blunted blood flow response to cholinergic agonists in the hypercholesterolemic rabbit hind limb or human forearm. Indeed, endothelium-dependent responses in vessels from normal animals are attenuated within minutes by in vitro exposure to low density lipoprotein cholesterol. Therefore, intimal thickening cannot be invoked as the only mechanism for the impairment of endothelium-dependent relaxation in hypercholesterolemic animals.

Reduced responsiveness of the vascular smooth muscle to EDRF can be excluded as an explanation for the impairment of endothelium-dependent relaxation because most investigators have found that the response to exogenous nitrovasodilators is unaffected. A more likely explanation is that exposure to elevated levels of cholesterol reduces the release and/or synthesis of EDRF just as it interferes with other synthetic functions of the endothelial cell.

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**TABLE 1. Effects of Arginine Versus Saline Infusions In Vivo on Endothelium-Dependent and Endothelium-Independent Responses In Vitro**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Contraction (g)</th>
<th>ED$_{50}$ (− log M)</th>
<th>Maximal response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypercholesterolemic animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (n=5)</td>
<td>2.8±0.2</td>
<td>6.2±0.2</td>
<td>61±6.8</td>
</tr>
<tr>
<td>L-Arginine (n=7)</td>
<td>3.2±0.4</td>
<td>6.7±0.2*</td>
<td>83±6.2*</td>
</tr>
<tr>
<td>Saline (n=5)</td>
<td>2.7±0.4</td>
<td>6.5±0.1</td>
<td>100</td>
</tr>
<tr>
<td>L-Arginine (n=7)</td>
<td>2.7±0.3</td>
<td>6.9±0.2</td>
<td>100</td>
</tr>
<tr>
<td>Normal animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (n=5)</td>
<td>2.7±0.3</td>
<td>6.9±0.2</td>
<td>79±8.2</td>
</tr>
<tr>
<td>L-Arginine (n=8)</td>
<td>4.0±0.2</td>
<td>6.8±0.1</td>
<td>72±5.7</td>
</tr>
<tr>
<td>Saline (n=5)</td>
<td>2.8±0.1</td>
<td>7.0±0.2</td>
<td>100</td>
</tr>
<tr>
<td>L-Arginine (n=8)</td>
<td>2.1±0.4</td>
<td>6.5±0.2*</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM. *p<0.05.
Reduced synthesis and release of EDRF would explain the observation that bioassay rings relax less when exposed to perfusate from hypercholesterolemic rabbit thoracic aorta.\(^9,^{10}\)

If reduced synthesis of EDRF underlies the abnormal vascular response in hypercholesterolemia, it may be possible to normalize vascular reactivity by providing increased amounts of the substrate, L-arginine. In normal vessels, there appears to be sufficient endogenous L-arginine to saturate the nitric oxide-forming enzyme, as the addition of L-arginine does not enhance endothelium-dependent relaxation in this circumstance.\(^{23-26}\) However, under certain experimental conditions, exogenous substrate is critical to restore this endothelial response. Endothelial cells cultured in arginine-deficient medium release little EDRF until L-arginine is added to the medium.\(^{13}\) Structural analogues of L-arginine inhibit endothelium-dependent relaxation; this inhibition is reversed by exogenous L-arginine.\(^{14-16}\) In rings of bovine pulmonary artery, prolonged stimulation by calcium ionophore renders the vessel refractory to agonists of endothelium-dependent relaxation; normal responses are restored by administration of L-arginine.\(^{27}\) In vivo administration of L-N-monomethyl-arginine (the competitive antagonist of arginine metabolism) reduces in vitro endothelium-dependent relaxation and release of nitric oxide from rabbit thoracic aorta; both responses are restored by administration of L-arginine.\(^{26}\)

The serum level of L-arginine in our rabbits was increased 30-fold by the L-arginine infusion (from 0.1 to 3 mM).\(^{17}\) We used this dose of L-arginine because it is the standard dose used in stimulation tests for growth hormone release, has been safely administered to humans, and results in significant increases in serum arginine levels.\(^{28}\) We find that this dose of L-arginine normalizes endothelium-dependent relaxation in both resistance and conduit vessels. The hind limb blood flow response to intra-arterial acetylcholine that received a saline infusion alone, whereas the response to sodium nitroprusside was unaffected.\(^{17}\) In animals that received an infusion of L-arginine, the

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**FIGURE 3.** Tracings of original records demonstrating that endothelium-dependent relaxation in isolated thoracic aortas from hypercholesterolemic rabbits is normalized by L-arginine but not its enantiomer, D-arginine. Panel A: Vascular ring from a normal rabbit is contracted by norepinephrine (NE) and then relaxed with increasing concentrations of acetylcholine (ACH). w/o, washout (replacement of bathing solution with fresh physiological saline solution). Panel B: Vascular ring from a hypercholesterolemic rabbit that had received an intravenous infusion of D-arginine is exposed to protocol described in panel A. Note impaired relaxation to ACH. Panel C: Vascular ring from a hypercholesterolemic rabbit that had received an intravenous infusion of L-arginine is exposed to protocol described in panel A. Note normalized response to ACH.
response to acetylcholine was normalized, whereas that to sodium nitroprusside was unaffected.\textsuperscript{17} The effects of L-arginine are stereospecific because D-arginine has no effect on vascular reactivity of the hind limb vessels or thoracic aorta.

The most likely explanation for the improvement of endothelium-dependent relaxation is an effect of L-arginine on the endothelial synthesis and release of EDRF. An alternative explanation is that L-arginine may have physicochemical actions on the vascular smooth muscle to augment its responsiveness to EDRF. This is unlikely because the response to sodium nitroprusside was unaffected in the vessels from hypercholesterolemic animals. Furthermore, any physicochemical effects should be shared by D-arginine, but the enantiomer did not augment endothelium-dependent relaxation.

Another possible explanation for the improvement in the response to acetylcholine could be that L-arginine enhances muscarinic receptor efficacy, affinity, or signal transduction mechanisms. However, in the perfused basilar artery from hypercholesterolemic rabbits, we have observed that the attenuated vasodilation to acetylcholine and the augmented vasoconstrictions to serotonin or endothelin are all corrected by L-arginine.\textsuperscript{29} Because these agents are known to release EDRF,\textsuperscript{30,31} the effect of L-arginine on the response to these agents probably involves the increased release of EDRF.

In our normal animals, the infusion of L-arginine did not affect endothelium-dependent relaxation. There was a reduction in sensitivity to sodium nitroprusside, but maximal endothelium-independent relaxation was unaffected. Previous studies have shown that sensitivity to sodium nitroprusside is reduced in vessels with intact endothelium; it is believed that basal release of EDRF may desensitize the underlying smooth muscle to exogenous nitrovasodilators.\textsuperscript{32–35} Removal of the endothelium augments the response to sodium nitroprusside and sodium nitrite in the isolated rat aorta.\textsuperscript{35} In vitro exposure to norepinephrine or removal of the endothelium augments the response of the isolated rabbit aorta to nitrovasodilators; conversely, stimulation of EDRF release or exposure of a denuded ring to EDRF from a donor vessel segment reduces the response to nitrovasodilators.\textsuperscript{34} Therefore, the reduced sensitivity to sodium nitroprusside in the normal rings from rabbits exposed to arginine may be a result of increased basal release of EDRF during the in vivo infusion. In some vascular beds, in vivo infusion of L-arginine may enhance the basal release of EDRF\textsuperscript{36} and might thereby reduce sensitivity to exogenous nitrovasodilators.

Many strategies have been used to reverse the endothelial dysfunction induced by elevated levels of cholesterol. Chronic administration of fish oil or hydroxymethylglutaryl coenzyme A reductase inhibitors prevents the attenuation of endothelium-dependent relaxation in hypercholesterolemic animals.\textsuperscript{5,7} Similarly, a low-cholesterol diet normalizes endothelium-dependent relaxations in hypercholesterolemic monkeys.\textsuperscript{8} We now report that administration of L-arginine (the precursor of EDRF) acutely normalizes endothelium-dependent relaxation to acetylcholine in hypercholesterolemic animals. This observation suggests that hypercholesterolemia induces a reversible reduction in intracellular arginine availability or metabolism.

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