L-4F, an Apolipoprotein A-1 Mimetic, Dramatically Improves Vasodilation in Hypercholesterolemia and Sickle Cell Disease

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Background—Hypercholesterolemia and sickle cell disease (SCD) impair endothelium-dependent vasodilation by dissimilar mechanisms. Hypercholesterolemia impairs vasodilation by a low-density lipoprotein (LDL)–dependent mechanism. SCD has been characterized as a chronic state of inflammation in which xanthine oxidase (XO) from ischemic tissues increases vascular superoxide anion ($O_2^-$) generation. Recent reports indicate that apolipoprotein (apo) A-1 mimetics inhibit atherosclerosis in LDL receptor–null (Ldlr$^{-/-}$) mice fed Western diets. Here we hypothesize that L-4F, an apoA-1 mimetic, preserves vasodilation in hypercholesterolemia and SCD by decreasing mechanisms that increase $O_2^-$ generation.

Methods and Results—Arterioles were isolated from hypercholesterolemic Ldlr$^{-/-}$ mice and from SCD mice that were treated with either saline or L-4F (1 mg/kg per day). Vasodilation in response to acetylcholine was determined by videomicroscopy. Effects of L-4F on LDL-induced increases in endothelium-dependent $O_2^-$ generation were determined on arterial segments via the hydroethidine assay and on stimulated endothelial cell cultures via superoxide dismutase–inhibitable ferricytochrome c reduction. Effects of L-4F on XO bound to pulmonary arterioles and content in livers of SCD mice were determined by immunofluorescence. Hypercholesterolemia impaired vasodilation in Ldlr$^{-/-}$ mice, which L-4F dramatically improved. L-4F inhibited LDL-induced increases in $O_2^-$ in arterial segments and in stimulated cultures. SCD impaired vasodilation, increased XO bound to pulmonary endothelium, and decreased liver XO content. L-4F dramatically improved vasodilation, decreased XO bound to pulmonary endothelium, and increased liver XO content compared with levels in untreated SCD mice.

Conclusions—These data show that L-4F protects endothelium-dependent vasodilation in hypercholesterolemia and SCD. Our findings suggest that L-4F restores vascular endothelial function in diverse models of disease and may be applicable to treating a variety of vascular diseases. (Circulation. 2003;107:2337-2341.)

Key Words: apolipoproteins ■ vasodilation ■ endothelium ■ hypercholesterolemia ■ anemia, sickle cell

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One of the earliest changes in vascular function in nearly all forms of vascular disease is impaired vasodilation. In hypercholesterolemia, low-density lipoprotein (LDL) is believed to impair vasodilation. In sickle cell disease (SCD), xanthine oxidase (XO) and free hemoglobin likely contribute to impaired vasodilation.

Recently, it was suggested that high-density lipoprotein (HDL) might be an important therapeutic target for atheroprotection. Apolipoprotein (apo) A-1 mimetics have been shown to increase HDL function to inhibit atherosclerosis and restore antiinflammatory and antioxidant properties to HDL that are lost during influenza infection. HDL serves many functions, eg, reverse cholesterol transport, removal of seeding molecules of oxidized lipids from LDL that inhibit subsequent oxidation, and inhibition of LDL-induced monocyte chemotactic protein-1 activity.
Clinical studies clearly indicate that HDL plays an important role in protecting vascular function against atherosclerosis. Transgenic expression of apoA-1, the major apolipoprotein of HDL, retards the progression of advanced lesions in transplanted aortas from apoE-null mice and remodels them to a more stable-appearing phenotype. Intraperitoneal injection of an apoA-1 mimetic (5F) and parental administration of another apoA-1 mimetic (D-4F) enhances the ability of HDL to inhibit LDL oxidation and to protect mice from diet-induced atherosclerosis without changing plasma cholesterol levels. Indeed, infusion of reconstituted HDL rapidly improves endothelium- and endothelial nitric oxide synthase (eNOS)–dependent forearm blood flow in hypercholesterolemic men, confirming that HDL plays a critical role in protecting endothelial cell function.

The mechanisms by which SCD have been shown to impair vasodilation at first glance appear distinctly different from those induced by hypercholesterolemia. In SCD, recent reports suggest that increased NO consumption is a primary mechanism for vascular dysfunction, not dyslipoproteinemia. Much of the morbidity and mortality in SCD is caused by alterations in vascular function that are secondary to red cell sickling. XO released from the liver, injured by acute episodic ischemic events, binds to endothelial cells in vascular beds distal from the original site of injury. Once bound, XO generates O$_2^-$, which inactivates NO produced locally by the endothelium.

Here we show that L-4F, an apoA-1 mimetic, dramatically improves vasodilation in murine models of hypercholesterolemia and SCD. Our findings demonstrate that L-4F is highly effective for restoring vascular function in these models and may provide a new therapeutic approach to improving vascular function regardless of the pathogenesis of disease.

**Methods**

**Mice**

Male, LDL receptor–deficient (Ldlr$^{-/-}$) mice on a C57BL/6 background were from Jackson Laboratory (Bar Harbor, Maine). Ldlr$^{-/-}$ mice were maintained on a high-fat, cholesterol diet (Teklad diet No. 88137) for 3 to 4 weeks. Male, LDL receptor–deficient Ldlr$^{-/-}$ mice were generously provided by Dr Mohandas Narla (New York Blood Center, New York, NY). A colony was established at the Medical College of Wisconsin Animal Research Care facility. SCD mice for this study (12 months of age; 5 males and 5 females) were generated in the Animal Research Care facility at Medical College of Wisconsin and fed a diet modified for SCD with 11% fat and 2.5% l-arginine supplementation (PMI No. 5740C-A; Purina Mills, Inc). SCD mice were treated with L-4F peptide by intraperitoneal injection (1 mg/kg per day) or saline (2% dimethyl sulfoxide for vehicle) for 30 days before studies. Mice were euthanized after anesthesia, and mandibular carotid arterioles were isolated and gently cleaned of nonvascular connective tissue by microdissection. The Animal Research Care Committee of Medical College of Wisconsin approved all animal protocols.

**Arteriolar Vasodilation**

Vasodilation of pressurized arterioles (180 to 250 μm) was determined as described with the exception that for this study, isolated, pressurized carotid arterioles were preconstricted with U46619 (10$^{-5}$ to 10$^{-9}$ mol/L). Endothelium- and eNOS-dependent responses were determined with acetylcholine (ACH) (10$^{-7}$ to 10$^{-4}$ mol/L) and L-nitroargininemethylester (L-NAME) (100 μmol/L), respectively. Endothelium-independent vasodilation was examined with papaverine (10$^{-2}$ mol/L), an endothelium-independent vasodilator that increases cyclic adenosine monophosphate in vascular smooth muscles to promote relaxation.

**Low-Density Lipoprotein**

Human plasma was obtained from the Blood Center of Southeastern Wisconsin, and LDL was isolated by sequential density ultracentrifugation (density=1.019 to 1.063 g/mL) as previously described.

**Carotid Artery and Endothelial Cell Culture Incubations**

Arterial segments from adult mongrel dogs were incubated with LDL (6.2 mmol/L, [240 mg cholesterol/dL]) ±L-4F (10 μg/mL) for 24 hours and assessed for increases in vascular O$_2^-$ by hydroethidine staining and confocal microscopy as described. Bovine aortic endothelial cell cultures were incubated in the absence and presence of LDL (6.2 mmol/L) ±L-4F (10 μg/mL) and assayed for changes in stimulated O$_2^-$ generation (calcium ionophore A23187—5 μmol/L, 30 minutes) by superoxide dismutase–inhibitable ferricytochrome c reduction as described.

**Immunohistochemistry of XO**

Fluorescent immunohistochemistry for XO was performed on sections of lung and liver tissue according to published protocols.

**Preparation of L-4F**

The apoA-1 mimetic was synthesized by the Protein & Nucleic Acid Shared Facility of Medical College of Wisconsin and The Blood Center of Southeastern Wisconsin.

**Other Methods**

Protein and cholesterol content of LDL preparations were determined as described. Differences among test groups were analyzed by ANOVA, with Bonferroni post hoc test (Prism v3.0 Macintosh, GraphPad Software, Inc) used to determine levels of significance among groups, which was set at $P<0.05$.

**Results**

It is well established that hypercholesterolemia impairs endothelium- and eNOS-dependent vasodilation. To determine if L-4F protected vasodilation during hypercholesterolemia, we extended a previous report by Navab and associates, who showed that D-4F, a form of L-4F that can be taken orally, prevented atherosclerosis in Ldlr$^{-/-}$ mice. We found that diet-induced hypercholesterolemia significantly impairs ACh-induced vasodilation of isolated, pressurized arterioles from Ldlr$^{-/-}$ mice (Figure 1A, △) compared with controls (Figure 1A, ○). More importantly, L-4F (1 mg/kg per day) improved vasodilation of arterioles from hypercholesterolemic Ldlr$^{-/-}$ mice (Figure 1A, ●) to the point that it was indistinguishable from controls. Papaverine (10$^{-4}$ mol/L) dilated the arterioles from the mice to 85% to 95% of their original maximal diameters, and L-NAME blocked vasodilation of arterioles from controls and hypercholesterolemic Ldlr$^{-/-}$ mice treated without and with L-4F (see Data Supplement). These data show that L-4F dramatically improved endothelium-dependent and eNOS-dependent mechanisms of vasodilation that are lost during hypercholesterolemia.

Previously it was shown that oxidized lipids in LDL impaired ACh-induced vasodilation of arterial rings. Recent...
reports have shown that apoA-1 mimetics can remove oxidized lipids from LDL, rendering the lipoprotein highly resistant to oxidation.6,19 In vitro, we recently found that L-4F inhibits LDL oxidation,20 consistent with previous reports.6,19 To determine if L-4F could prevent LDL-induced decreases in vasodilation, we incubated isolated, pressurized murine carotid arterioles with LDL or L-4F that was pretreated with L-4F. As seen in Figure 1B, LDL (●) inhibited ACh-induced vasodilation compared with controls (○). More importantly, pretreatment of LDL with L-4F partially protected vasodilation by blocking the inhibitory effects of LDL (Figure 1B, ▲). As above, papaverine dilated the arterioles incubated with LDL or with LDL+L-4F to 85% to 95% of their original maximal diameters, while L-NAME inhibited vasodilation of arterioles incubated with LDL+L-4F (data not shown). These data demonstrate that pretreatment of LDL with L-4F affords partial protection of endothelium- and eNOS-dependent mechanisms of vasodilation.

One mechanism by which hypercholesterolemia induces vascular dysfunction is by increasing endothelial cell generation of O2−, which scavenges NO to inhibit vasodilation. Incubation of arterial segments with LDL increases O2− generation in native endothelial cells on isolated arterial segments.15 To determine if L-4F blocks LDL-induced increases in endothelial cell O2− generation, we incubated canine arterial segments with LDL+L-4F overnight as before.15 As seen in Figure 2A, LDL increased ethidine fluorescence, an index of intracellular O2− generation15 in native endothelial cells on carotid arteries (center image) compared with controls (left image). Pretreatment of LDL with L-4F reduced ethidine fluorescence (Figure 2A, right image) to levels that were no different than controls. This protective effect of L-4F on LDL-induced decreases in vascular endothelial cell O2− generation was further confirmed in cultured endothelial cells. Figure 2B shows that LDL increased stimulated O2− generation, which could be prevented simply by pretreating the LDL with L-4F. Thus, L-4F protects endothelial cell function, at least in part, by preventing LDL-induced increases in endothelial cell O2− generation.

To determine if L-4F can favorably modify the vasculopathy of SCD, we treated SCD mice of advanced age (12 months) with L-4F for 30 days. Consistent with a previous report,2 pressurized arterioles from SCD mice contract when stimulated with ACh (Figure 3A, ●). However, arterioles from SCD mice treated with L-4F (Figure 3A, ▲) vasodilate to ∼70% of C57BL6 controls (Figure 3A, ○), a remarkable improvement in endothelium-dependent vasodilation considering that arterioles from untreated SCD mice contract when stimulated with ACh. As with the Ldlr−/− mice on L-4F, L-NAME blocked vasodilation of arterioles from the SCD mice that were treated with L-4F (see Data Supplement). Once again, papaverine diluted the arterioles from SCD mice and SCD mice treated with L-4F to 85% to 95% of their original maximal diameters.
original maximal diameters (data not shown). These data demonstrate that L-4F dramatically improves endothelium-dependent vasodilation in SCD mice. This represents a second, distinct murine model of vascular disease in which L-4F is able to increase endothelium-dependent vasodilation of isolated, pressurized arterioles.

As one of the mechanisms for impairing vasodilation in SCD is increased XO-dependent O$_2^-$ generation,$^2$ we examined pulmonary vascular and liver sections for changes in XO content due to treatment with L-4F. As seen in Figure 3B, XO staining of the endothelium lining small blood vessels in lung sections from SCD mice (top middle image) was increased compared with controls (top left image), confirming a previous report.$^2$ Treatment of SCD mice with L-4F for 30 days decreased XO staining (top right image). In liver sections, we see that XO content in SCD mice (bottom middle image) compared with C57BL6 controls (bottom left image) is decreased, confirming a previous report.$^2$ L-4F increased XO content in the liver sections of SCD mice (bottom right image). These findings indicate that L-4F improves vasodilation in SCD, at least in part, by decreasing XO release from livers in SCD mice.

We also observed marked differences in the behavior and activity of the mice. Untreated SCD mice appeared lethargic and made little attempt to avoid handling. In contrast, SCD mice treated with L-4F were mobile and actively avoided handling. Such subjective observations suggest that L-4F increases the overall well-being of SCD mice, an added benefit from increasing HDL function to prevent endothelial dysfunction.

**Discussion**

On the basis that oxidized lipids in LDL are proinflammatory$^{19}$ and accelerate vascular lesion formation$^{19}$ and that L-4F not only inhibits LDL oxidation but also formation of vascular lesions during hypercholesterolemia,$^6$ findings here indicate that L-4F protects vasodilation, in part, by inhibiting the formation of oxidized lipids in the LDL particle in situ. Comparing data in Figure 1A and 1B, it is clear that L-4F affords greater protection in vivo than in vitro. Some comparisons support the concept that the mechanisms by which L-4F increases HDL function are dynamic and that they rely on participation of other systems to achieve maximal protection.$^6$ Accordingly, apoA-1 mimetics that are resistant to metabolism, such as D-4F,$^6$ may provide a practical means for increasing HDL function and improving vasodilation during hypercholesterolemia.

Findings here show that L-4F not only protects vascular function in an established murine model of atherosclerosis$^6$ but also in a transgenic murine model of SCD.$^{23}$ Although appearing mechanistically diverse, the fact that L-4F protects endothelium-dependent vasodilation in both models suggests that a common mechanism is involved. This common mechanism is most likely oxidative stress, which is well recognized for inducing chronic states of inflammation.$^{19,22}$ Oxidative stress increases the formation of proinflammatory lipids$^{23,24}$ and isoprostanes$^{21,25}$ that are key elements of coronary artery disease$^{19}$ and impaired vasodilation.$^{21,25}$ In this regard, it is interesting to note that systemic concentrations of isoprostanes are increased in SCD.$^{23}$ Whether or not L-4F decreases the formation of proinflammatory lipids in SCD to protect endothelium-dependent vasodilation remains to be determined in future studies.

Studies here show that L-4F preserves endothelial cell function by preventing eNOS dysfunction, a finding that is consistent with a recent study by our laboratory showing that L-4F restores NO and O$_2^-$ balance to LDL-treated endothelial cell cultures by preventing uncoupled eNOS activity.$^{20}$ Whether L-4F prevents the formation of proinflammatory lipids or limits their access to the endothelium, findings here show that by decreasing O$_2^-$ generation, L-4F preserves endothelial cell function. Thus, apoA-1 mimetics may be able to protect endothelial function against vascular disease driven by chronic states of inflammation. Finally, as apoA-1 mimetics are designed to increase HDL function,$^{6,7,10,26}$ our findings support the concept that HDL plays a critical role in maintaining vascular function$^{6,5}$ in diseases that are as distinct as hypercholesterolemia and sickle cell.

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