L-4F, an Apolipoprotein A-1 Mimetic, Restores Nitric Oxide and Superoxide Anion Balance in Low-Density Lipoprotein–Treated Endothelial Cells

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**Background**—Low-density lipoprotein (LDL) impairs endothelial cell function by uncoupling endothelial nitric oxide synthase (eNOS) activity, which allows superoxide anion (O$_2^-$) to be generated rather than nitric oxide (·NO). Recent reports indicate that apolipoprotein (apo) A-1 mimetics inhibit the development of atherosclerotic lesions in LDL receptor–null mice. Here we hypothesize that L-4F, an apoA-1 mimic that inhibits atherosclerosis induced by hypercholesterolemia, protects endothelial cell function by preventing LDL from uncoupling eNOS activity.

**Methods and Results**—Bovine aortic endothelial cells were incubated with LDL±L-4F, and changes in A23187-stimulated ·NO and O$_2^-$ generation were determined by ozone chemiluminescence and superoxide dismutase–inhibitable ferricytochrome c reduction, respectively. Western analysis of eNOS immunoprecipitates was used to determine effects of LDL and L-4F on heat shock protein 90 (hsp90) interactions with eNOS. LDL decreased ·NO production and increased eNOS-dependent O$_2^-$ generation. Pretreatment of LDL with L-4F increased ·NO and decreased O$_2^-$ generation. By itself, L-4F had no effect on O$_2^-$ but did increase ·NO generation. Stimulation of endothelial cells incubated with LDL decreased the association of hsp90 with eNOS. Pretreatment of LDL with L-4F prevented a decrease in hsp90 association with eNOS and often enhanced association on stimulation.

**Conclusions**—These data demonstrate that L-4F protects endothelial cell function by preventing LDL from uncoupling eNOS activity. L-4F allows endothelial cell to maintain coupled eNOS activity to generate ·NO even in the face of atherogenic concentrations of LDL. (*Circulation. 2003;107:1520-1524.)*

**Key Words:** apolipoproteins ■ cells, endothelial ■ lipoproteins ■ nitric oxide ■ nitric oxide synthase

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mimetic, might protect endothelial cell function from the atherogenic effects of LDL.

Here we investigate whether L-4F protects against LDL-induced increases in endothelial cell O$_2^-$ generation. We find that L-4F inhibits LDL-induced increases in stimulated O$_2^-$ production and restores coupled eNOS activity to increase ·NO production, at least in part by restoring and increasing heat shock protein 90 (hsp90) interactions with eNOS.

**Methods**

**Synthesis of L-4F**
L-4F (Ac-DWFKAFYDKVAEFKEAFNH$_2$) was synthesized by the Protein & Nucleic Acid Share Facility of the Medical College of Wisconsin in Milwaukee. Purity (typically >98%) and structure were assessed by GC/MS.

**Endothelial Cells**
Bovine aortic endothelial cells (BAECs) were purchased from VEC Technologies (Rensselaer, NY). BAECs were initially cultured and expanded in MCDB-131C from VEC Technologies (passage 0 to 1) and gradually conditioned over 4 days so that they could be expanded and maintained in RPMI 1640 media containing 20% FBS (SH30070, HyClone).

**LDL**
LDL (1.019 to 1.063 g/mL) was isolated by sequential density ultracentrifugation with density adjustments made by the addition of solid KBr as described. For the incubations here, LDL was used at 6.2 mmol/L (240 mg/dL), and pretreatment of LDL was made by adding L-4F (10 µg/mL, final concentration) to the media for 30 minutes before incubating with endothelial cell cultures.

**Stimulated Endothelial Cell O$_2^-$ Generation**
Superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction based on molar extinction coefficient (ε = 21 000 mol/L$^{-1}$ cm$^{-1}$) was used to calculate stimulated endothelial cell O$_2^-$ generation. After experimental treatments with LDL (6.2 mmol/L [240 mg/dL]) and/or L-4F (10 µg/mL) for 24 hours, endothelial cell cultures were washed 3 times with Hanks Balanced Salts Solution (HBSS) and stimulated in HBSS containing A23187 (5 µmol/L). To quantify eNOS-dependent O$_2^-$ generation, cultures were incubated with L-nitroargininemethylester (L-NAME, 1 mmol/L), 30 minutes before and during incubation with ferricytochrome c (50 µmol/L). Measurements of ferricytochrome c reduction were performed in triplicate ±SOD (1000 U/mL) and cell proteins determined in duplicate as described.

**Endothelial Cell ·NO Generation**
Endothelial cell nitrite + nitrate production was determined by ozone chemiluminescence with VCl$_3$ as described. After the endothelial cell cultures were incubated with L-4F (10 µg/mL) for either 30 minutes or 24 hours, they were washed (3 ×) with HBSS and then incubated with HBSS containing l-arginine (25 µmol/L, basal activity) or with HBSS containing l-arginine (25 µmol/L) and A23187 (5 µmol/L) for 30 minutes. Each experiment was performed in triplicate; NO measurements were made in duplicate, and cell proteins determined in duplicate as described.

**Western Analysis**
Hsp90 interactions with eNOS and phosphorylation of eNOS (S1179) were determined as described. Briefly, after experimental treatments, cultures were washed with HBSS and cells lysed in modified RIPA buffer. eNOS was immunoprecipitated with H32 (SH-258, BioMol) 1-µg antibody per 100-µg cell lysate (300 to 500 µg total) as described. Immunoblots were visualized by enhanced chemiluminescence with Immunostar reagents (Biorad).

**Statistical Analysis**
Data were analyzed by Student’s t test for experiments with 2 groups and ANOVA with a Newman-Keuls’ test as a post-hoc test for experiments with more than 2 groups for determining levels of significance. Minimum levels of significance were set at P<0.05.

**Results**
Incubation of BAEC cultures with L-4F (30 minutes) enhanced stimulated ·NO generation (Figure 1A). Incubation of cultures with L-4F (24 hours) enhanced basal and stimulated ·NO generation (Figure 1B) without altering stimulated O$_2^-$ generation (Figure 1C). Short-term incubation with LDL (6.2 mmol/L, [240 mg cholesterol/dL], 24 h) attenuated basal and stimulated NO generation (Figure 2). Pretreatment of LDL with L-4F (10 µg/mL, 30 minutes) before incubation with the cultures, however, enhanced basal and stimulated NO generation (Figure 3A) and attenuated stimulated O$_2^-$ generation (Figure 3B). Previous studies showed that short-term incubation (24 hours) of isolated arterial segments with LDL increased endothelial O$_2^-$ generation by a predominantly eNOS-dependent mechanism. L-NAME inhibition of stimulated O$_2^-$ generation in LDL-treated cultures confirms that LDL uncouples eNOS activity (Figure 3B). Pretreatment of LDL with L-4F markedly decreased stimulated O$_2^-$ generation (Figure 3B). The observation that L-NAME did not further decrease O$_2^-$ in LDL + L-4F cultures is consistent with the idea that eNOS activity in the LDL + L-4F cultures is coupled.

Numerous reports indicate that an increase in the association of hsp90 with eNOS plays an important role in increasing ·NO generation. L-4F had modest effects on the levels of
phospho-eNOS (S1179) on eNOS isolated by immunoprecipitation from control cultures under basal and stimulated conditions (Figure 4, top panel, lane 2 versus lane 1 and lane 4 versus lane 3, respectively). L-4F appears to increase hsp90 association with eNOS under basal conditions (Figure 4, bottom panel, lane 2 versus lane 1). Stimulation markedly increases hsp90 association with eNOS (Figure 4, bottom panel, lane 3 versus lane 1), which was slightly reduced in cultures incubated with L-4F (Figure 4, bottom panel, lane 4 versus lane 3).

Previously, we reported that under basal conditions, long-term LDL exposure (4 days) increases uncoupled eNOS activity by increasing phospho-eNOS (S1179) levels on eNOS and decreasing hsp90 interactions on stimulation.5 The salient features of the blots in Figure 4 are that A23187 stimulation increases phospho-eNOS (S1179) and hsp90 association with eNOS (lane 3 versus lane 1); that LDL decreases hsp90 association with eNOS in A23187-stimulated cultures (bottom panel, lane 7 versus lane 3) without inhibiting phosphorylation (top panel, lane 7 versus lane 3); and that pretreatment of LDL with L-4F restores hsp90 interactions with eNOS (bottom panel, lane 8 versus lane 3) in addition to slightly increasing phosphorylation of eNOS (top panel, lane 8 versus lane 3). These data show that stimulation of LDL-treated cultures decreased hsp90 interactions with eNOS compared with A23187-stimulated control cultures, confirming previous findings.4 The marked increase in hsp90 association with eNOS in the LDL + L-4F-treated cultures suggest that L-4F helps endothelial cells maintain coupled eNOS activity by enhancing hsp90 interactions in the face of an LDL-induced atherogenic challenge.

**Discussion**

In this report we show that L-4F increases endothelial cell 
\[ \text{NO} \] generation under basal and stimulated conditions and decreases LDL-induced increases in endothelial cell \[ \text{O}_2^- \] generation. Western analysis of eNOS immunoprecipitates reveals that one of the mechanisms by which this apoA-1 mimetic increases 
\[ \text{NO} \] generation is by restoring hsp90 interactions with eNOS in LDL-treated endothelial cells. Stimulation of LDL-treated endothelial cell cultures decreased hsp90 association with eNOS compared with stimulated controls (Figure 4, lane 7 versus lane 3), confirming previous reports.4 However, when LDL was pretreated with L-4F and then incubated with cultures, hsp90 interactions with eNOS were maintained under basal conditions and enhanced under stimulated conditions. Thus, L-4F protects endothelial cell function against LDL, at least in part, by increasing hsp90 interactions with eNOS, an essential step in the mechanisms by which endothelial cells generate \[ \text{NO} \].19–26 In spite of the fact that L-4F increases 
\[ \text{NO} \] and decreases \[ \text{O}_2^- \] generation in LDL-treated cultures, the mechanisms by which this apoA-1 mimetic restores eNOS function remain unclear. For example, when control cultures are stimulated with A23187, L-4F slightly decreases the association of hsp90 with eNOS, yet increases stimulated 
\[ \text{NO} \] generation. Such discrepancies suggest that alternative mechanisms are governing eNOS activity and function beyond hsp90. As L-4F was designed to mimic apoA-1 and increase HDL function, it is possible that this mimetic may be activating eNOS by the scavenger receptor, a ceramide-dependent and calcium-independent mechanism.27 Regardless, these data demonstrate that L-4F is highly effective at protecting endothelial cell function against LDL-induced shifts in 
\[ \text{NO} \] and \[ \text{O}_2^- \] balance.

One of the earliest events in vascular disease is a loss in 
\[ \text{NO} \] activity. Decreases in 
\[ \text{NO} \] activity appear to develop before structural changes in the vessel wall.28 On the basis that \[ \text{NO} \] is a chain-breaking antioxidant that inhibits LDL oxidation,29 platelet aggregation,30 and smooth muscle cell proliferation,31 it is easy to understand how a loss in 
\[ \text{NO} \] activity increases atherosclerosis. If the underlying atherogenic mechanisms cannot be corrected, then logically providing 
\[ \text{NO} \] may help prevent vascular disease. Indeed, this approach is effective based on recent reports showing that
NO donors inhibited atherosclerosis in hypercholesterolemic mice and decreased inflammation of the gastrointestinal tract in a murine model of inflammatory bowel disease. However, if the underlying defect in endothelial cell function could be corrected, then the atherogenic effects of LDL should be eliminated or at least minimized. If LDL induces endothelial cell dysfunction by increasing $\cdot$$\text{NO}/\text{H}_{2}\text{O}_{2}$ generation, which inactivates $\cdot$$\text{NO}$ and HDL improves endothelial- and eNOS-dependent forearm blood flow, then L-4F, an apoA-1 mimic, should prevent LDL-induced increases in endothelial cell $\cdot$$\text{NO}$ generation. Our findings that L-4F preserves $\cdot$$\text{NO}$ balance by preventing LDL-induced increases in uncoupled eNOS activity are consistent with the notion that HDL function is essential for protecting endothelial cell function and that apoA-1 mimetics can be used to enhance HDL function to increase atheroprotection, as was shown earlier. Although L-4F prevented LDL from uncoupling eNOS, we also observed that it markedly increased $\cdot$$\text{NO}$ production in control cultures after just 30 minutes incubation, with no other intervention (Figure 1A). Mechanistically, we find this effect of L-4F on eNOS $\cdot$$\text{NO}$ generation in control cultures to be as exciting as the observation that L-4F restores $\cdot$$\text{NO}$ balance in LDL-treated endothelial cell cultures, in that it suggests that even under the best culture conditions, negative regulators of eNOS $\cdot$$\text{NO}$ generation are present. This idea is reinforced by findings that L-4F increased $\cdot$$\text{NO}$ but had no effect on $\cdot$$\text{O}_{2}/\text{H}_{2}\text{O}_{2}$ generation in control cultures in contrast to the effects of the mimetic on LDL-treated cultures in which L-4F increased $\cdot$$\text{NO}$ and decreased $\cdot$$\text{O}_{2}/\text{H}_{2}\text{O}_{2}$ generation.

The mechanisms by which L-4F improves endothelial cell $\cdot$$\text{NO}$ generation remain unclear at this time. L-4F inhibits LDL oxidation, confirming previous reports with D-4F (Data Supplement Figure). Yet, in vitro, its ability to inhibit LDL oxidation is modest compared with the antioxidant properties of probucol or BHT. Such differences suggest that although oxidative stress induces endothelial cell dysfunction, L-4F is doing more than simply inhibiting LDL oxidation.
oxidation. Possibly, L-4F partitions or removes proinflammatory oxidized lipids such that they are no longer available to negatively regulate eNOS activity. Future studies are required to determine whether such mechanisms are involved in how L-4F increases coupled eNOS activity to shift the balance of NO and O$_2^-$ generation toward NO.

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