LDLs Impair Vasomotor Function of the Coronary Microcirculation
Role of Superoxide Anions

Travis W. Hein, Lih Kuo

Abstract—Oxidized LDLs (Ox-LDLs) inhibit endothelium-dependent dilation of isolated conduit arteries in a manner comparable to the impairment demonstrated in atherosclerotic vessels. However, it is not known whether the microvessels, which do not develop atherosclerotic lesions, are susceptible to Ox-LDL. Since endothelial release of NO plays an important role in vasodilation and since its dysfunction associated with atherosclerosis has been shown to extend into the coronary microcirculation, we hypothesized that Ox-LDLs impair endothelium-dependent vasodilation of coronary arterioles by reducing the synthesis and/or release of NO. To test this hypothesis, porcine subepicardial vessels (50 to 100 μm) were isolated, cannulated, and pressurized to 60 cm H2O without flow for in vitro study. Isolated vessels developed basal tone and dilated in a dose-dependent manner to the endothelium-dependent vasodilators serotonin, ATP, and ionomycin. These vasodilatory responses were inhibited by the NO synthase inhibitor \( \text{N}^2\)-monomethyl-L-arginine and were subsequently reversed by extraluminal administration of the NO precursor L-arginine (3 mmol/L), suggesting the involvement of NO in these vasomotor responses. Intraluminal incubation of the vessels with native LDL (N-LDL) or Ox-LDL (1 mg protein/mL) significantly attenuated dilations to serotonin, ATP, and ionomycin. Ox-LDL produced more severe inhibition than did N-LDL, and the inhibitory effect was comparable to that of \( \text{N}^2\)-monomethyl-L-arginine. The inhibitory effects of N-LDL and Ox-LDL were reversed by exogenous L-arginine (3 mmol/L) and were prevented by sodium dihydroxybenzene disulfonate (Tiron), a cell-permeable superoxide scavenger. In contrast, administration of the cell-impermeable superoxide scavenger superoxide dismutase prevented the inhibitory effect of N-LDL but not of Ox-LDL. Neither N-LDL nor Ox-LDL altered endothelium-independent vasodilation to sodium nitroprusside. These results indicate that coronary arterioles are susceptible to LDLs that specifically impair endothelium-dependent vasodilation by reducing NO synthesis. It is suggested that the initiation of superoxide anion production and the subsequent L-arginine deficiency may be responsible for the detrimental effect of LDL. (Circ Res. 1998;83:404-414.)

Key Words: arteiole ■ atherosclerosis ■ endothelium ■ L-arginine ■ nitric oxide ■ superoxide anion

Low density lipoproteins at high plasma concentrations are a major risk factor for the development of atherosclerosis.1 Accumulating evidence suggests that Ox-LDL is closely linked to the atherosclerosis-related pathology.2 Ox-LDL may contribute to atherogenesis by a variety of mechanisms, such as being a chemoattractant for monocytes,3 enhancing lipid accumulation by monocytes,4 impairing metabolic activity of vascular cells,4 and altering endothelial function.5,6 It is generally accepted that the normal endothelium plays an important role in the regulation of vascular function through the release of vasoactive substances in response to various stimuli. One of the most important substances released from the endothelium is endothelium-derived relaxing factor, which has been identified chemically as NO7 or an NO-containing compound.8 In endothelial cells, NO is synthesized from the conversion of L-arginine to L-citrulline by constitutive NO synthase.9 The released NO subsequently activates soluble guanylyl cyclase in underlying vascular smooth muscle cells and thus produces vasodilation. In addition, NO has also been shown to inhibit platelet adherence and aggregation,10 smooth muscle proliferation,11 and endothelial cell–leukocyte interactions,12 all of which are key events in atherogenesis. Interestingly, endothelium-dependent vasodilation is impaired in arteries treated with Ox-LDL in animals5,6 and humans,13 and therefore it is speculated that a reduction in NO synthesis or release may be involved in vascular dysfunction and the development of atherosclerosis.

It is important to note that previous in vitro studies involving Ox-LDL were performed with large-conduit arteries,5,6,13 which have been shown to be the primary site for the formation of atherosclerotic lesions.14 However, it is not clear...
These goals were accomplished by studying the vasodilatory mechanisms for NO deficiency during exposure to LDL. crovessels, (2) determine whether NO deficiency is involved in quantifying the effects of N-LDL and Ox-LDL on endothelium-function. Therefore, the goals of the present study were to (1) quantify the effects of N-LDL and Ox-LDL on endothelium-dependent and -independent vasodilation of coronary microvessels, (2) determine whether NO deficiency is involved in endothelial dysfunction, and (3) elucidate the vascular mechanisms for NO deficiency during exposure to LDL. These goals were accomplished by studying the vasodilatory response of isolated coronary arterioles (50 to 100 μm in diameter) before and after incubation with N-LDL or Ox-LDL, thereby eliminating the confounding influences from blood-borne substances and neurohumoral control mechanisms. Since the majority of coronary resistance (>60%) resides in arterioles <150 μm in diameter,17 it is important to understand the vasomotor regulation of these microvessels during exposure to atherogenic substances, ie, N-LDL and Ox-LDL.

### Materials and Methods

#### General Preparation

Pigs (8 to 12 weeks old of either sex) were sedated with an intramuscular injection of tiletamine and zolazepam (1:1, 4.4 mg/kg) and xylazine (2.2 mg/kg) and then anesthetized and heparinized by intravenous administration of pentobarbital sodium (20 mg/kg) and heparin (1000 U/kg), respectively, via the marginal ear vein. Pigs were intubated and ventilated with room air. After a left thoracotomy was performed, the heart was electrically fibrillated, excised, and immediately placed in cold (5°C) saline solution. The procedures followed were in accordance with guidelines set by the Laboratory Animal Care Committee at Texas A&M University.

#### Isolation and Cannulation of Microvessels

The techniques for identification and isolation of porcine coronary microvessels were described previously.18 In brief, a mixture of india ink and gelatin in PSS containing (in mmol/L) NaCl 145.0, KCl 4.7, CaCl₂ 2.0, MgSO₄ 1.17, NaH₂PO₄ 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, and MOPS 3.0 was perfused into the left anterior descending artery (0.3 mL) and the circumflex artery (0.4 mL) to enable visualization of coronary microvessels. Subepicardial arteriole branches (50- to 100-μm internal diameter and 0.6 to 1.0 mm long without branches) from the left anterior descending or circumflex arteries were selected and carefully dissected from the surrounding cardiac tissue under cold (5°C) PSS containing BSA (1%, Amersham) at pH 7.4. Each isolated arteriole was then transferred for cannulation to a Lucite vessel chamber containing PSS-albumin equilibrated with room air at ambient temperature. One end of the microvessel was cannulated with a glass micropipette (40 μm in tip diameter) filled with filtered PSS-albumin, and the outside of the microvessel was securely tied to the pipette with an 11-0 ophthalmic suture (Alcon). The ink-gelatin solution inside the vessel was flushed out at low perfusion pressure (<20 cm H₂O). Then the other end of the vessel was cannulated with a second micropipette and tied with a suture. We have previously shown that the ink-gelatin solution has no detectable detrimental effect on either endothelial or vascular smooth muscle function.19,20

#### Preparation of LDLs

Human LDLs (5 mg protein/mL) were obtained from Sigma Chemical Co. LDLs were oxidized by exposure to 10 μmol/L CuCl₂ for 8 to 24 hours at room temperature. The degree of LDL oxidation was measured by using a spectrophotometric method21 and TBARS assay.22 One characteristic of LDL oxidation involves the formation of conjugated dienes during the peroxidation of polyunsaturated fatty acids, which was monitored by UV absorption at 234 nm with a spectrophotometer (DU-65, Beckman Instruments Inc). Oxidation was stopped after 90% to 100% of maximal oxidation had been achieved by the addition of 1 mmol/L EDTA to the LDL. For TBARS analysis, LDL samples (10 to 100 μg) were mixed with 1 mL of trichloroacetic acid (20%) and 1 mL of thiobarbituric acid (1%) and heated at 100°C for 30 minutes. After being cooled in a water bath (22°C), the mixture was centrifuged at 12 000 g for 15 minutes, and the absorbance was measured at 550 nm with a microplate reader (Molecular Devices Corp). Serial dilutions of 1.1,3,3,4-tetramethoxypropane, which yields MDA, were used to construct the standard curve. TBARS data were expressed as nanomoles of MDA per milligram of LDL protein. N-LDL and Ox-LDL were dialyzed separately against Dulbecco’s PBS for 24 hours. The 2 forms of LDL were stored at 4°C and used within 2 weeks. Before each experiment, N-LDL and Ox-LDL were filtered with a 0.2-μm filter (Corning) and diluted to their final concentration (1 mg protein/mL) in PSS-albumin. The protein concentration of LDL was determined by using the modified Lowry assay.23 N-LDL used in this study exhibited only negligible oxidation levels (0.50 ± 0.08 nmol MDA/mg LDL protein, n = 6), whereas Ox-LDL presented extensive oxidation (13.40 ± 1.90 nmol MDA/mg LDL protein, n = 7). These initial levels were not significantly altered after 2 weeks.

#### Role of NO in Arteriolar Dilations to Serotonin, ATP, and Ionomycin

The following protocol was performed to determine the role of NO in receptor-dependent dilation to serotonin and ATP and in receptor-independent dilation to the calcium ionophore ionomycin.24 The cannulated arterioles were bathed in PSS-albumin and equilibrated with room air; the temperature was maintained at 36°C to 37°C by an external heat exchanger. The vessel was set to its in situ length 15 and allowed to develop basal tone at 60 cm H₂O intraluminal pressure without flow. This pressure has been demonstrated in coronary

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**Selected Abbreviations and Acronyms**

- L-NMMA = N⁵-monomethyl-L-arginine
- MDA = malondialdehyde
- N-LDL = native LDL
- Ox-LDL = oxidized LDL
- PSS = physiological salt solution
- SNP = sodium nitroprusside
- SOD = superoxide dismutase
- TBARS = thiobarbituric acid–reactive substances
arterioles of this size in vivo.17 After the vessels developed basal tone (30 to 40 minutes), the dose-response curves for serotonin (10^(-10) to 10^(-6) mol/L), ATP (10^(-8) to 10^(-5) mol/L), and ionomycin (10^(-9) to 3x10^(-7) mol/L) were examined before and after extraluminal incubation of the NO synthase inhibitor L-NMMA (10 μmol/L, Calbiochem)35 for 40 minutes. Subsequently, the effect of the NO precursor L-arginine (3 mmol/L, 20-minute incubation) on dose-dependent dilations to the aforementioned drugs was examined in the presence of L-NMMA.

Effect of LDL on Endothelium-Dependent, NO-Mediated Vasodilation

To study the effect of LDL (N-LDL and Ox-LDL) on endothelium-dependent vasodilation to serotonin, ATP, and ionomycin, dose-dependent dilations to these agonists were examined before and after replacing the solution inside the vessel with LDL (N-LDL or Ox-LDL, 60 minute-incubation) and after LDL removal. Subsequently, the effect of the NO precursor L-arginine (3 mmol/L, 20-minute incubation) on dose-dependent dilations to the aforementioned drugs was examined in the presence of L-NMMA.

Effect of Incubation Time on Endothelium-Dependent Vasodilation

To exclude the possibility that the observed vascular dysfunction was a result of nonspecific time-dependent deterioration of vasodilatory function during incubation with N-LDL or Ox-LDL, vessels were subjected to the same experimental interventions as described above except with a vehicle solution. The dose-dependent responses of isolated vessels to serotonin, ATP, and ionomycin were studied after incubating the vessels with vehicle for 60 minutes. In some experiments, 1 mg protein/mL albumin was added to the vehicle solution to examine whether the observed phenomenon was due to a nonspecific effect of increased luminal protein.

Specificity of L-Arginine

To examine whether the vasodilatory function of normal vessels was altered by L-arginine, vasodilations to ATP and serotonin were evaluated before and after treatment of the vessels with L-arginine (3 mmol/L) for 20 minutes. In addition, to determine whether the effect of L-arginine on the impaired vascular function was stereospecific, vasodilations of Ox-LDL–treated vessels to ATP and serotonin (10^(-7) mol/L) were examined in the presence of D-arginine (3 mmol/L) or L-arginine (3 mmol/L).

Effect of LDL Removal on Endothelium-Dependent Vasodilation

To determine whether the impaired vascular function was a result of extracellular scavenging of NO by LDL, the vasodilation induced by serotonin (10^(-7) mol/L) was examined in the presence of LDL (N-LDL or Ox-LDL, 60 minute-incubation) and after LDL removal by replacing the intraluminal LDL with vehicle solution. It should be noted that agonist-induced responses were examined within 30 minutes after LDL removal.

Contribution of Superoxide Anions to Vascular Dysfunction

To evaluate whether superoxide anions contributed to the vascular dysfunction elicited by LDL (N-LDL or Ox-LDL), coronary arteriolar dilations to serotonin (10^(-7) mol/L) and ATP (10^(-8) to 10^(-5) mol/L) were established before and after intraluminal administration of LDL (N-LDL or Ox-LDL, 1 mg protein/mL) or of LDL containing the superoxide anion scavengers SOD (100 U/mL, 60-minute incubation) or sodium dihydroxycinnamate disulfonate (Tiron, 1 μmol/L, 60-minute incubation). Exogenous SOD enzyme activity is primarily extracellular, whereas Tiron is capable of scavenging superoxide from both the intracellular and extracellular environment.26–28 In addition, to examine whether the vasodilatory function of normal vessels was altered by Tiron, vasodilations to ATP, serotonin, and ionomycin were evaluated before and after treatment of vessels with Tiron (1 mmol/L) for 60 minutes.

Results

Role of NO in Vasodilations to Serotonin, ATP, and Ionomycin

All vessels developed a similar level of basal tone (∼68±1% of their maximal diameter) within 40 minutes at a 36°C to 37°C bath temperature with 60 cm H2O intraluminal pressure. The average resting and maximal diameters of all vessels (n=109) studied were 77±2 and 114±2 μm, respectively. Under control conditions, serotonin, ATP, and ionomycin dilated the coronary arterioles in a dose-dependent manner and produced 85%, 90%, and 80% of maximal dilation, respectively, at their highest concentration studied (Figure 1). The NO synthase inhibitor L-NMMA (10 μmol/L) significantly attenuated these vasodilations by increasing the threshold concentration of each drug and by inhibiting the extent of vasodilation (Figure 1). Administration of L-arginine (3 mmol/L) to the L-NMMA–treated vessels subsequently restored the vasodilatory responses (Figure 1), suggesting that these agonists elicited NO-mediated dilation of coronary arterioles. The inhibitory effect of L-NMMA appears to be specific for NO because dilation of isolated vessels to the
endothelium-independent vasodilator SNP was not altered by L-NMMA (data not shown), as we have previously demonstrated in the same tissue.19

Effect of LDL on Endothelium-Dependent, NO-Mediated Vasodilation
Exposure of coronary arterioles to LDL (N-LDL or Ox-LDL, 1 mg protein/mL for 60 minutes) did not alter resting vascular tone but significantly attenuated dose-dependent dilations to serotonin, ATP, and ionomycin (Figures 2 and 3). The threshold concentration for dilation to each agonist was markedly increased by N-LDL and Ox-LDL. In fact, after Ox-LDL treatment, a slight but significant vasoconstriction was observed at the lower concentration of ATP (10^{-8} and 10^{-7} mol/L) (Figure 3B) and ionomycin (10^{-8} mol/L) (Figure 3C). The dilations of coronary arterioles to the highest concentrations of serotonin, ATP, and ionomycin were diminished to 55%, 60%, and 35%, respectively, after N-LDL treatment. In comparison with N-LDL, arteriolar dilations to the highest dose of the same agonists were reduced to a greater extent, to only 35%, 40%, and 20%, respectively, after Ox-LDL treatment (P<0.05 versus N-LDL). The vasodilatory responses were completely restored by subsequent incubation of LDL-treated vessels with L-arginine (3 mmol/L, 20 minutes) (Figures 2 and 3). Figure 4 shows that the inhibitory effect of LDL on vasodilation to serotonin was dose dependent. A lower concentration of N-LDL (0.3 mg protein/mL) did not have an inhibitory effect on serotonin-induced vasodilation (Figure 4A). However, the lower concentration of Ox-LDL produced a significant attenuation of vasodilation to serotonin. This inhibitory effect was enhanced by increasing the Ox-LDL concentration to 1 mg protein/mL (Figure 4B).

Time-Dependent Effect on Arteriolar Function
Since impaired vasodilation was observed after LDL (N-LDL or Ox-LDL) treatment for 60 minutes, it is possible that the altered vascular response was a result of time-dependent...
deterioration of endothelial function rather than the specific action of LDL. To address this issue, another set of experiments was performed in isolated coronary arterioles treated intraluminally with a vehicle solution for 60 minutes. As shown in the Table, dose-dependent dilations of coronary arterioles in response to serotonin, ATP, and ionomycin were not altered after this treatment. It should be noted that these vasodilatory responses were also not altered by excess albumin in the vehicle solution.

Specificity of L-Arginine

Pretreatment of coronary arterioles with L-arginine (3 mmol/L) for 20 minutes did not alter the vasodilatory response to ATP (Figure 5A) and serotonin (data not shown). To determine whether L-arginine was stereospecific for the restoration of vascular function impaired by LDL, dilation of isolated vessels to serotonin (10^{-7} mol/L) was examined in the presence of D-arginine (3 mmol/L) or L-arginine (3 mmol/L). Figure 5B shows that the impaired vasodilation to serotonin by Ox-LDL was not affected by D-arginine but was completely reversed by L-arginine.

Endothelium-Dependent Vasodilations to Pharmacological Agonists Before and After Administration of Vehicle

<table>
<thead>
<tr>
<th>Dose, log (mol/L)</th>
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<tr>
<td>-9</td>
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Serotonin (n=4)  
Control: 15±8 56±17 88±3 93±2  
Vehicle: 18±6 67±13 89±4 93±3  

ATP (n=4)  
Control: 2±1 20±3 70±6 88±2  
Vehicle: 2±1 17±8 76±5 87±5  

Ionomycin (n=3)  
Control: 4±1 27±7 83±5  
Vehicle: 4±1 32±5 78±3  

n indicates sample size.  
All values are mean±SEM expressed as percent of maximal dilation.  
Dose-dependent dilations of isolated coronary arterioles to serotonin, ATP, and ionomycin were studied before and after intraluminal incubation of the vessels with vehicle for 60 minutes.
Effect of LDL Removal on Serotonin-Induced Vasodilation

Coronary arteriolar dilation to serotonin (10^{-7} mol/L) was examined in the LDL-treated vessels after the intraluminal LDL (N-LDL or Ox-LDL) had been replaced with vehicle solution. Both N-LDL and Ox-LDL impaired vasodilation to serotonin, which is in agreement with the results shown in Figures 2A and 3A. After removal of intraluminal LDL (N-LDL or Ox-LDL), vasodilation to serotonin was still attenuated (Figure 6), and this inhibitory effect was not different from that observed in the presence of LDL.

Contribution of Superoxide Anions to Vascular Dysfunction

Under control conditions, serotonin (10^{-7} mol/L) produced \( \approx 80\% \) of maximal dilation of coronary arterioles. This dilation was significantly attenuated by intraluminal LDL (N-LDL or Ox-LDL, Figure 7). On administration of N-LDL with SOD (100 U/mL), impairment of vasodilation to serotonin (10^{-7} mol/L) was not observed (Figure 7A). However, coadministration of Ox-LDL and SOD did not influence the inhibitory effect of Ox-LDL on serotonin-induced dilation (Figure 7B). L-Arginine (3 mmol/L) administered extraluminally to these vessels completely restored the vasodilation in response to serotonin as shown in Figure 7B. In another series of experiments, intraluminal administration of LDL (N-LDL or Ox-LDL) with Tiron (1 mmol/L), a cell-permeable superoxide scavenger, eliminated the inhibitory action of N-LDL and Ox-LDL on vasodilations to ATP (Figure 8A and 8B), serotonin, and ionomycin (data not shown). Treatment of coronary arterioles with Tiron (1 mmol/L) for 60 minutes did not alter the resting diameter (76 \pm 10 \mu m; maximal diameter, 117 \pm 8 \mu m; n=3). *P<0.05 vs control.

Discussion

The major findings of the present study are that LDLs (N-LDL or Ox-LDL) inhibit endothelium-dependent dilations of isolated coronary arterioles to serotonin, ATP, and ionomycin in a manner similar to that under NO synthase inhibition. The inhibitory effects of LDL on endothelium-dependent vasodilation were reversed by subsequent administration of \( \alpha \)-arginine and were prevented by the cell-permeable superoxide scavenger Tiron. In contrast, administration of the cell-impermeable superoxide scavenger SOD only prevented the inhibitory effect elicited by N-LDL. This is the first study to report the direct impairment of endothelial function by LDL in the coronary microcirculation. Since N-LDL and Ox-LDL impair both receptor-
non–receptor-mediated vasodilations and since application of l-arginine or Tiron preserved vascular function, it is suggested that the impairment of endothelium-dependent vasodilation results from the deficiency of NO release associated with the cellular production of superoxide anions by LDL. To provide a perspective for our observations and conclusions, methodological considerations such as isolated-vessel preparations and the treatment of LDL will be discussed. In addition, the effect of LDL on vascular function and the possible mechanism involved will be addressed.

Methodological Considerations
In the present study, use of the isolated-vessel technique allowed us to directly examine the effect of LDL on microvascular function without confounding influences from the interaction of vascular cells with either blood-borne substances or circulating cells. Since the effect of LDL was examined after incubation of arterioles in the bath solution without l-arginine for 60 minutes, it is possible that the observed vascular dysfunction resulted from nonspecific depletion of vascular l-arginine or from the time-dependent deterioration of vasomotor function. However, in the time-control study without l-arginine, vascular function remained intact after a 60-minute incubation with vehicle solution (the Table). This finding argues against the idea that the observed vascular dysfunction was a result of time-dependent deterioration of endothelial function or nonspecific depletion of l-arginine during the course of LDL incubation. Furthermore, vasodilatory function was not altered by an increase of inert protein in the lumen, suggesting the specific inhibitory effect of LDL. Therefore, the impaired vasodilations appear to be a direct effect of LDL rather than a nonspecific effect from experimental interventions.

Recent studies have demonstrated that oxidation of LDL occurs in vivo,29,30 and oxidatively modified LDLs have been detected in both plasma30 and atherosclerotic lesions of various species, including humans.31 Although the plasma concentration of Ox-LDL in vivo is not known, it has been predicted to be 0.5 to 2 mg protein/mL in human atherosclerotic lesions.5 These concentrations of Ox-LDL have been shown to inhibit vascular relaxation of large-conduit arteries in vitro.5,13,32 A recent clinical study has demonstrated increased plasma levels of autoantibodies against Ox-LDL in hypercholesterolemic patients.33 Interestingly, these patients also exhibited impaired endothelial function of forearm resistance vessels. In the present study, the microvascular dilations to endothelium-dependent agonists were impaired by 0.3 and 1 mg protein/mL of Ox-LDL, concentrations that

![Figure 7. Effects of SOD and l-arginine on vascular dysfunction caused by LDL. A, Serotonin-induced dilation was inhibited by N-LDL. Co-administration of N-LDL with SOD (100 U/mL) prevented the inhibitory effect of N-LDL (resting diameter, 67±12 μm; maximal diameter, 101±16 μm; n=3). B, Serotonin-induced dilation was inhibited by Ox-LDL (resting diameter, 74±4 μm; maximal diameter, 107±8 μm; n=4). Inhibitory effect of Ox-LDL was not altered by adding SOD (100 U/mL) to the vessels. Administration of l-arginine (3 mmol/L) to Ox-LDL–treated vessels with SOD completely restored vasodilation to serotonin. *P<0.05 vs control.](http://circres.ahajournals.org/)

![Figure 8. Effect of Tiron on vascular dysfunction caused by LDL. N-LDL (A; resting diameter, 104±9 μm; maximal diameter, 140±11 μm; n=5) and Ox-LDL (B; resting diameter, 71±7 μm; maximal diameter, 99±8 μm; n=5) significantly attenuated dilation of vessels to ATP. In the presence of Tiron (1 mmol/L) intraluminally, inhibitory effects of N-LDL and Ox-LDL were prevented. Treatment of normal coronary arterioles with Tiron (1 mmol/L) did not affect dose-dependent dilation to ATP, demonstrating that vasodilatory function of these vessels was not influenced by Tiron (C; resting diameter, 76±10 μm; maximal diameter, 117±13 μm; n=5). *P<0.05 between groups.](http://circres.ahajournals.org/)

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Mechanism of LDL-Induced Vascular Dysfunction

There are several proposed mechanisms that may explain the observed vascular dysfunction elicited by LDL. First, a selective loss of receptor-mediated, endothelium-dependent vasodilation has been described in various animal models of atherosclerosis, including human. In addition, Flavahan suggested that endothelium-dependent vasodilation mediated by receptor-incorporated pertussis toxin–sensitive G proteins may be selectively affected by an early stage of atherosclerosis or by a low concentration of Ox-LDL (≤50 μg protein/mL). However, the present study indicated that impaired vasodilation to both receptor-dependent and receptor-independent agonists occurred after a 60-minute exposure of the vessel to LDL. It is likely that the high concentration of LDL (1 mg protein/mL) used in our study may have produced a general inhibitory effect on vascular function beyond the receptor level.

Second, it has been demonstrated that endothelium-derived relaxing factor released from cultured endothelial cells is inactivated by both N-LDL and Ox-LDL in a bioassay system, suggesting that LDL may directly contribute to the degradation of NO and thus attenuate vasodilation to agonists. In this cultured cell study, the investigators used the acetyltransferase inhibitor thimerosal to stimulate endothelium-derived relaxing factor release and assumed that the released factor was NO. However, this assumption is weak, since thimerosal has recently been shown to stimulate the release of endothelium-derived hyperpolarizing factor rather than NO in both cultured endothelial cell and intact-vessel prepara-
tions. Therefore, these investigators might have studied the effect of LDL on endothelium-derived hyperpolarizing factor instead of NO. Furthermore, in our intact-microvessel study, we found that the impaired vasodilation was still present within 30 minutes of LDL removal (Figure 6). Therefore, this result does not favor the idea of degradation of NO by LDL. Nevertheless, it remains to be elucidated whether the impaired vascular function is reversible beyond 30 minutes of LDL removal, since endothelium-dependent function has been shown to be partially restored after correction of plasma lipid concentrations in hypercholesterolemic animals and humans.

Third, the deficiency of NO production or release associated with hypercholesterolemia and atherosclerosis has generally been proposed as a primary mechanism for vascular dysfunction in various animal models and in humans. This contention is based on the fact that impaired endothelium-dependent vasodilation can be normalized by administration of the NO precursor L-arginine. It is believed that the increased NO production from exogenous L-arginine reverses this aberrant response. This idea may hold true only under conditions with unsaturated NO synthase. Normally, intracellular levels of arginine (≈0.1 mmol/L) are high enough to saturate NO synthase, whose $K_m$ has been determined to be in the micromolar range ($≈2.9 \mu$mol/L). In this regard, it is expected that excess L-arginine would not enhance NO-dependent relaxation of normal vessels, as evident in the present study (Figure 5A) and in other studies. However, if L-arginine availability were reduced to a level where NO synthase was no longer saturated, this effect could limit the stimulated production of NO. Thus, it may be the case in the presence of LDL, since exogenous L-arginine could then restore NO-dependent vasodilation (Figures 2 and 3). Interestingly, reduced levels of L-arginine have been shown to enhance the generation of superoxide anions from constitutive NO synthase by uncoupling the L-arginine/NO pathway. Perturbation could further decrease functional levels of NO through direct inactivation of the synthesized NO by superoxide.

**Superoxide Anions and Microvascular Dysfunction**

Several in vitro models have demonstrated an increase in superoxide production by endothelial cells during hypercholesterolemia. Similarly, stimulation of superoxide production from endothelial cells and neutrophils by N-LDL and Ox-LDL was also reported. Since the superoxide anion inactivates NO and has been implicated in the alteration of endothelium-dependent relaxation in hypercholesterolemia and atherosclerosis, its contribution to LDL-induced vascular dysfunction should be considered in the present study. Our results show that administration of Tiron, an antioxidant that is capable of scavenging superoxide from both the intracellular and extracellular environment, prevented the inhibitory action of both N-LDL (Figure 8A) and Ox-LDL (Figure 8B) on vasodilation. However, the salutary effect of Tiron was not evident in control vessels (Figure 8C). These results indicated that LDL-induced vascular dysfunction is associated with the production of superoxide anions.

In contrast to the Tiron study, treatment of the vessels with SOD for 60 minutes prevented the inhibitory action of N-LDL but not of Ox-LDL (Figure 7). It is possible that the oxidation of N-LDL during this incubation period is responsible for the observed vascular dysfunction, since SOD has been shown to prevent oxidation of N-LDL in vitro. At this time, it is unclear where the LDL is oxidized, in terms of either the intracellular space or at the cell membrane. However, our SOD data suggest that oxidation is likely to take place at the cell membrane, since SOD is rather impermeable. A recent study on isolated coronary arteries indicated that endothelial dysfunction induced by N-LDL (0.2 mg protein/mL) was time dependent, since the inhibitory effect was observed only after a longer period (4 hours versus 20 minutes) of incubation. In a similar manner, we noted that vessels treated with N-LDL for 2 hours exhibited impairment of vasodilation in a manner comparable to that of Ox-LDL–treated vessels for 60 minutes (n=3, data not shown). It appears that time-dependent oxidation of N-LDL is likely involved in the initiation of vascular impairment.

Taken together, the ability of superoxide scavengers to prevent LDL-induced vascular dysfunction and of excess L-arginine to restore impaired vascular function suggests that the initiation of superoxide production and the subsequent reduced intracellular L-arginine for NO synthesis are responsible for the inhibitory effect of LDL. However, the intracellular pathway involved in the L-arginine deficiency remains unclear. Endogenous levels of L-arginine in endothelial cells have been proposed to be maintained in part by the recycling of L-citrulline to L-arginine. It is possible that the initial production of superoxide by LDL inhibits this pathway and thus reduces the availability of cellular L-arginine for NO synthesis. A decrease in L-arginine levels may also enhance superoxide anion production and consequently further aggravate this detrimental process. In this respect, it is conceivable that excess L-arginine would not only overcome the reduction in L-arginine and replenish NO for normal vasodilation but also restore vascular function by reducing superoxide generation. The results of the present study are consistent with recent studies suggesting that supplementation of hypercholesterolemic animals and humans with L-arginine or antioxidants decreases the vascular release of superoxide anion and partly restores NO production.

In summary, the findings of the present study indicate that isolated coronary arterioles are susceptible to an oxidized form of LDL that specifically impairs endothelium-dependent vasodilation by reducing NO synthesis. This deleterious effect may result from a reduction in the cellular level of L-arginine after the enhanced production of superoxide anions. We speculate that the impaired coronary flow regulation observed in patients and animals with hypercholesterolemia or atherosclerosis may be due in part to LDL-induced microvascular dysfunction that is associated with superoxide anion–mediated NO deficiency. In this regard, antioxidants and L-arginine may be beneficial not only in the prevention of LDL oxidation and oxygen-derived free-radical formation but also in amelioration of vasomotor function in the microcirculation.
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