Oxidized LDL Inhibits Vascular Endothelial Growth Factor–Induced Endothelial Cell Migration by an Inhibitory Effect on the Akt/Endothelial Nitric Oxide Synthase Pathway

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**Background**—Oxidized LDL (oxLDL) inhibits endothelial cell (EC) migration. Stimulating ECs with vascular endothelial growth factor (VEGF) leads to the activation of Akt/protein kinase B, which in turn activates endothelial nitric oxide synthase (eNOS) by phosphorylation on serine 1177. VEGF-induced cell migration is dependent on the generation of nitric oxide (NO). Therefore, we investigated whether oxLDL affects EC migration by an inhibitory effect on the Akt/eNOS pathway.

**Methods and Results**—During an in vitro “scratched wound assay,” oxLDL dose-dependently inhibited the VEGF-induced migration of human umbilical vein endothelial cells. Western blot analysis revealed that oxLDL dose- and time-dependently led to dephosphorylation and thus deactivation of Akt. Moreover, oxLDL inhibited the VEGF-induced generation of NO, as detected and quantified using a fluorescent NO indicator, 4,5-diaminofluorescein diacetate. Overexpression of a constitutively active Akt construct (Akt T308D/S473D) or a phosphomimetic eNOS construct (eNOS S1177D) almost completely reversed the inhibitory effect of oxLDL on VEGF-induced EC migration and NO generation.

**Conclusions**—Our data indicate that oxLDL-induced dephosphorylation of Akt, followed by impaired eNOS activation, reduces the intracellular level of NO and thereby inhibits VEGF-induced EC migration. (Circulation. 2001;103:2102-2107.)

**Key Words** endothelium ■ lipoproteins ■ growth substances

Oxidized LDL (oxLDL) plays a major role during atherogenesis. OxLDL affects the endothelium in several ways by inducing the expression of adhesion molecules on endothelial cells (ECs), stimulating EC apoptosis, and impairing endothelial vasodilator function. In addition, oxLDL and its components, such as lysophosphatidylcholine, inhibit EC migration in a nontoxic manner. However, the precise mechanism of the migration-inhibitory effect remains unclear.

Stimulating ECs with specific growth factors like vascular endothelial growth factor (VEGF), angiopoietin-1, or fluid shear stress leads to the activation of the serine/threonine kinase Akt/protein kinase B in a phosphatidylinositol-3-kinase–dependent manner. The activation of Akt involves its phosphorylation on threonine 308 and on serine 473 by 3-phosphoinositide–dependent kinase-1 and -2, respectively. Besides mediating cell survival in ECs, Akt activates endothelial nitric oxide synthase (eNOS) by phosphorylation on serine 1177. Phosphorylation of eNOS on serine 1177 via the Akt/protein kinase B kinase is used by VEGF and shear stress to enhance NO generation in a calcium-independent manner.

Importantly, recent studies suggest that NO is an essential mediator of EC migration and VEGF-induced angiogenesis. Inhibiting NOS suppresses the mitogenic and migratory effects of VEGF on ECs in vitro and the VEGF-induced angiogenic response in vivo. Moreover, NO was absolutely required for ischemia-induced angiogenesis in a model of hindlimb ischemia. Finally, we and others have recently demonstrated that Akt mediates the VEGF-induced migration of ECs via activation of eNOS. Therefore, we hypothesized that oxLDL inhibits EC migration by affecting the Akt/eNOS pathway.

**Methods**

**Materials**

Human LDL was isolated by ultracentrifugation. LDL was oxidized by incubation for 6 hours with CuSO₄ (10 mmol/L). The oxidation...
was detected by the FOXII assay. Native LDL showed a concentration of 16 μmol of hydrogen peroxide per mg protein, whereas oxLDL generated a concentration of 578 μmol of hydrogen peroxide per mg protein. A monoclonal antibody against phospho-Akt (Ser 473) was purchased from New England Biolabs. Recombinant VEGF-165 was purchased from Biomol, N' mono-methyl-L-arginine (LNMA) was obtained from Alexis, and 4,5-diaminofluorescein diacetate (DAF-DA) was obtained from Calbiochem.

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Systems/Clonetics and cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/mL), bovine brain extract (3 μg/mL), gentamycin (30 μg/mL), amphotericin B (50 μg/mL), epidermal growth factor (10 μg/mL), and 10% FCS until the third passage. After detachment with trypsin, cells were grown on 6-cm dishes for at least 18 hours.

Assessment of Akt Phosphorylation
HUVECs were stimulated with several concentrations of oxLDL for 1 hour in the presence or absence of 10 ng/mL VEGF or with 5 μg/mL oxLDL for 1 to 3 hours. Cellular proteins were prepared and separated on SDSPAGE gels, as described previously.15,17 Immunoblotting was performed with a murine monoclonal phospho-Akt (Ser473) antibody (clone 4E2; 1:1000). Immunodetection was accomplished with an anti-mouse secondary antibody (1:4000) and the enhanced chemiluminescence kit (Amersham). The blots were re-probed with an anti-actin antibody (1:2000) and quantified by densitometry.

Detection of Apoptosis and Necrosis
Cell apoptosis was detected morphologically by using DAPI staining of cell nuclei. Briefly, cells were centrifuged at 800 × g/mL for 20 minutes. Five hundred cells were counted, and the percentage of apoptotic cells was determined per total cells. To assess NO generation, transfected ECs were fixed in 4% paraformaldehyde, and incubated with the DAPI reagent for 10 minutes, which was significant at 5 g/mL, and 10% FCS until the third passage. After detachment with trypsin, cells were grown on 6-cm dishes for at least 18 hours.

Detection of NO Generation
To assess NO release from ECs, a membrane-permeable fluorescent NO indicator, DAF-DA, was used as described previously.26,27 For that purpose, DAF-DA (10 μmol/L) was added to the medium of the cells, and fluorescence images were taken after 5 minutes of incubation using a computer-assisted microscope (Zeiss) at 5 distinct positions (every 5 mm).

Detection of apoptosis, although 10 μg/mL and 50 μg/mL oxLDL did not increase the rate of EC apoptosis, suggesting a specific nontoxic effect of oxLDL on EC migration.

OxLDL Induces Deyphosphorylation of Akt
To test our hypothesis that oxLDL influences Akt activity, HUVECs were incubated with oxLDL for 1 hour, and immunoblots were performed with a phosphospecific Akt antibody directed at the Ser 473 phosphorylation site, which correlates with the activity of Akt.15,28 OxLDL induced a dose-dependent dephosphorylation of Akt, with a 51±8% reduction at a dose of 5 μg/mL (Figure 2A). Moreover, dephosphorylation of Akt by 5 μg/mL oxLDL was time-dependent, with a maximal 63±9% reduction occurring at 3 hours after stimulation (Figure 2B). Because VEGF signals via the Akt pathway,10 we tested whether oxLDL could reduce Akt phosphorylation in the presence of VEGF. For this purpose, HUVECs were stimulated for 1 hour with 10 ng/mL VEGF and several doses of oxLDL. Indeed, as illustrated in Figure 2C, oxLDL led to a dose-dependent dephosphorylation of Akt under simultaneous stimulation with VEGF, with a 45±9% reduction at 5 μg/mL oxLDL.
Effect of Phosphomimetic Akt and eNOS on oxLDL-Induced Inhibition of Cell Migration and NO Generation

The oxLDL-induced dephosphorylation of Akt associated with the inhibition of EC migration prompted us to investigate whether Akt dephosphorylation plays a causal role in the inhibitory effects of oxLDL on cell migration. Therefore, HUVECs were transfected with a phosphomimetic, constitutively active Akt mutant (T308D/S473D), which cannot become dephosphorylated and, therefore, inactivated. As illustrated in Figure 3A, overexpression of constitutively active Akt (T308D/S473D) was sufficient to stimulate EC migration, even in the absence of VEGF, to a level comparable to that seen on VEGF stimulation. In addition, VEGF stimulation of cells transfected with constitutively active Akt did not further increase cell migration (Figure 3A).

Most important, although in vector-transfected cells VEGF-stimulated EC migration was significantly inhibited by oxLDL, overexpression of active Akt (T308D/S473D) almost completely blocked the oxLDL-induced inhibition of EC migration (Figure 3A).

Serine 1177 of eNOS is the functionally relevant acceptor amino acid for Akt-induced eNOS phosphorylation and activation. On replacement of serine 1177 of eNOS by aspartate (S1177D), eNOS becomes resistant to dephosphorylation (inactivation) and is constitutively activated, resulting in continuous, calcium-independent NO generation. Therefore, we investigated whether a phosphomimetic (constitutively active) eNOS construct (S1177D) inhibits the oxLDL effects on EC migration. Overexpression of the phosphomimetic eNOS construct (S1177D) in the absence of oxLDL significantly increased EC migration to the extent observed after VEGF stimulation (Figure 3A). Moreover, overexpression of the phosphomimetic eNOS construct (S1177D) completely restored the impaired migratory capacity of ECs treated with oxLDL, indicating that the phosphorylation events of the Akt-dependent amino acid within eNOS is of fundamental importance for oxLDL-induced inhibition of cell migration (Figure 3A).

Because it is well established that VEGF-stimulated EC migration and angiogenesis is NO-dependent, we investigated the influence of transfection with the phosphomimetic Akt construct (T308D/S473D) or the phosphomimetic eNOS construct (S1177D) on the effects of oxLDL on NO generation. In vector-transfected cells, VEGF significantly increased NO generation, whereas oxLDL significantly inhibited both the basal and the VEGF-stimulated NO release (Figure 3B). Adding LNMA inhibited the basal and the VEGF-induced NO generation (Figure 3B). Overexpression of constitutively active Akt (T308D/S473D) was sufficient to stimulate NO generation in the absence of VEGF to the extent observed on VEGF stimulation (Figure 3B). Most important, transfection with the phosphomimetic Akt construct almost completely blocked the oxLDL-induced inhibition of NO generation (Figure 3B). Furthermore, overexpression of the phosphomimetic eNOS construct (S1177D) in the absence of oxLDL significantly increased the generation of NO in ECs to a level comparable to that observed on VEGF stimulation (Figure 3B). Moreover, overexpression of the phosphomimetic eNOS construct (S1177D) completely restored the capacity of ECs treated with oxLDL to generate NO.

Taken together, these results demonstrate that the oxLDL-induced inhibition of VEGF-stimulated cell migration and NO generation requires the dephosphorylation of Akt and...
eNOS on Ser1177, suggesting that the inhibitory effect of oxLDL on EC migration is mediated by the inactivation of Akt and of eNOS, leading to reduced generation of NO.

Discussion

EC migration is crucial for angiogenesis and reendothelialization after denuding injuries of arteries. VEGF signaling stimulates the generation of NO, which is essential for VEGF-induced angiogenesis and EC migration.19–21 We24 and others22,23 demonstrated that VEGF-induced cell migration is mediated by the Akt pathway and is dependent on the phosphorylation of Akt on Thr308/Ser473.24 Moreover, Akt-mediated EC migration is dependent on subsequent phosphorylation of eNOS on Ser1177 and on NO generation.24 However, a study by Morales-Ruiz et al23 in another model of cell migration suggested that constitutively active Akt may affect additional NO-independent pathways to regulate cell migration.

In the present study, we provide evidence that oxLDL inhibits VEGF-induced cell migration through an effect on the Akt/eNOS pathway. In detail, we demonstrate that oxLDL induced the dephosphorylation of the Akt kinase on Ser473. Dephosphorylation of Akt on Ser473 leads to inactivation of Akt16 and may affect eNOS activity, because eNOS is a downstream target of Akt.17,18 Functionally, oxLDL completely inhibited the stimulatory effect of VEGF on EC migration, without inducing apoptosis or necrosis. These results are consistent with the observation of Murugesan et al8 that oxLDL inhibits EC migration in the presence of basic fibroblast growth factor in a nontoxic manner. Lyso phosphatidylcholine, a component of oxLDL, also blocks the motility of ECs,9 and it inhibits basic fibroblast growth factor–induced EC motility by inhibiting the Ras/
extracellular signal–regulated kinase pathway.\textsuperscript{29} Moreover, oxLDL inhibits angiogenesis.\textsuperscript{30} Interestingly, simvastatin, a lipid-lowering drug, was recently shown to activate the Akt/eNOS pathway and to promote angiogenesis.\textsuperscript{31} The scratched wound assay predominantly assesses EC migration, not proliferation, because BrdU-staining reveals only a very low proliferation rate in the migration zone.\textsuperscript{24} Consequently, the effect of oxLDL in this assay can be mainly classified as an effect on EC migration. Furthermore, our results demonstrate that overexpression of a constitutively active, non-dephosphorylatable Akt construct (Akt T308D/S473D) or a phosphomimetic, non-dephosphorylatable eNOS construct (eNOS S1177D) completely reversed the oxLDL-induced inhibition of reendothelialization to the level observed under VEGF stimulation, establishing causality between the oxLDL-induced dephosphorylation of Akt and the oxLDL-induced inhibition of EC migration. Both phosphomimetic constructs could also reverse the inhibitory effect of oxLDL on VEGF-induced NO generation. Taken together, these results, in association with the NO dependence of the signaling pathways mediating the migratory effects of VEGF\textsuperscript{19–21} and Akt,\textsuperscript{24} indicate that the oxLDL-induced dephosphorylation/inactivation of Akt on Ser473 and of eNOS on Ser1177 reduce the intracellular level of NO, thereby inhibiting VEGF-induced EC migration.

The mechanism by which oxLDL induces Akt dephosphorylation is unclear. Osmotic stress\textsuperscript{32} and ceramide\textsuperscript{33} have been found to lead to Akt-dephosphorylation and inactivation by stimulating protein phosphatase 2A (PP2A)-like activity. Moreover, other stimuli such as tumor necrosis factor-α and angiotensin II are also capable of leading to Akt-dephosphorylation in ECs.\textsuperscript{34} It is possible that oxLDL leads to the activation of a serine/threonine phosphatase, which subsequently dephosphorylates Akt. Because it is well established that oxLDL leads to increased intracellular ceramide generation,\textsuperscript{3} it is conceivable that oxLDL could activate, via ceramide, a protein phosphatase with PP2A-like activity, which dephosphorylates Akt. Calyculin A (a PPI/PP2A inhibitor) and okadaic acid (a PP2A inhibitor) both induced maximal phosphorylation of Akt on serine 473 in HUVECs, suggesting that PP2A-like activity is involved in the dephosphorylation and inactivation of Akt in ECs (data not shown). Furthermore, it is possible that oxLDL activates a lipid phosphatase such as phosphatase and tensin homologue deleted on chromosome 10 (PTEN) or SH2-domain–containing inositol 5-phosphatase-2 (SHIP-2), which inactivate the phospholipid phosphatase 3′-kinase/Akt signal transduction pathway: requirement for Flik-1/KDR activation. J Biol Chem 1998;273:30336–30343.

Acknowledgments

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