Endothelial NOS-dependent activation of c-Jun NH$_2$-terminal kinase by oxidized low-density lipoprotein

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Go, Young-Mi, Anna-Liisa Levonen, Douglas Moellering, Anup Ramachandran, Rakesh P. Patel, Hanjoong Jo, and Victor M. Darley-Usmar. Endothelial NOS-dependent activation of c-Jun NH$_2$-terminal kinase by oxidized low-density lipoprotein. Am J Physiol Heart Circ Physiol 281: H2705–H2713, 2001.—Oxidized low-density lipoprotein (oxLDL) is known to activate a number of signal transduction pathways in endothelial cells. Among these are the c-Jun NH$_2$-terminal kinase (JNK), also known as stress-activated protein kinase, and extracellular signal-regulated kinase (ERK). These mitogen-activated protein kinases (MAP kinases) determine cell survival in response to environmental stress. Interestingly, JNK signaling involves redox-sensitive mechanisms and is activated by reactive oxygen and nitrogen species derived from both NADPH oxidases, nitric oxide synthases (NOS), peroxides, and oxidized low-density lipoprotein (oxLDL). The role of endothelial NOS (eNOS) in the activation of JNK in response to oxLDL has not been examined. Herein, we show that on exposure of endothelial cells to oxLDL, both ERK and JNK are activated through independent signal transduction pathways. A key role of eNOS activation through a phosphatidylinositol-3-kinase-dependent mechanism leading to phosphorylation of eNOS is demonstrated for oxLDL-dependent activation of JNK. Moreover, we show that activation of ERK by oxLDL is critical in protection against the cytotoxicity of oxLDL.

Nitric oxide synthase; NAD(P)H oxidase; nitric oxide; extracellular signal-regulated kinase

Redox cell signaling is now recognized as an important process controlling the response of a variety of cells to environmental stress (17, 20, 43, 49). The changes that occur in the early developing atherosclerotic lesion are of particular interest in this regard, because they involve a major change in the redox status of vascular cells to the more oxidized condition (6, 9). This appears to be driven by direct exposure to proinflammatory oxidants derived from a family of enzymes, including the lipoxygenases, myeloperoxidase, NAD(P)H oxidases, and nitric oxide synthase (NOS) (6, 7, 9, 20, 35, 44, 46). These in turn lead to the formation of second-ary signaling molecules derived from the oxidation of lipids, including the low level or “seeding” peroxides, oxysterols, and F$_2$-isoprostanes (6, 32). It is thus important to define the mechanisms of signal transduction initiated by such reactive oxygen (ROS) and reactive nitrogen species (RNS) and the downstream effects on cell growth, differentiation, and apoptosis.

In this respect those signaling pathways that lead to activation of the mitogen-activated protein (MAP) kinases are thought to be particularly important. The role of MAP kinases in linking extracellular stimuli to cellular responses such as cell growth, death, differentiation, and metabolic regulation is now well established (3, 12, 39, 43, 49). It has also been shown that peroxynitrite activates c-Jun NH$_2$-terminal kinase (JNK) in response to the laminar shear stress in endothelial cells (19). This physiological stimulus generates nitric oxide (NO) and ROS at extremely low levels (10–100 nM) and illustrates the potential for local intracellular production of reactive species to modulate redox-sensitive signal transduction pathways. However, the stimulus for the generation of ROS and RNS in a cellular setting, under both pathological and physiological, conditions remains uncertain. With respect to NO and the possible reactions with ROS, this has become an important problem in redox cell signaling particularly in the context of atherosclerosis and low-density lipoprotein (LDL) oxidation.

The concept that oxidized LDL (oxLDL) plays an important role in the etiologic basis of atherosclerosis is a well-established hypothesis. Early studies showed a functional interaction between NO production and oxLDL in control of vascular tone. For example, in intact aortic vessels NO-dependent relaxation is inhibited by oxLDL and in hypercholesterolemia. Surprisingly, atherosclerotic vessels were found to generate greater amounts of the stable metabolites of NO such as nitrate or nitrite (5, 20, 28, 33). These data are consistent with the loss of bioavailable NO through reaction with ROS. This response seems to arise from a combination

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of effects that include increased superoxide production from a variety of sources and decreased arginine availability (20, 26, 28, 29, 45, 47). It has recently been recognized that the NOS may become uncoupled such that the efficiency of electron transfer between the oxygenase and reductase domains of the enzyme become impaired (34, 44, 46). Under these conditions, some of the electrons are not transferred to arginine but to oxygen resulting in the formation of superoxide from the oxygenase domain of the enzyme that may then play a role in cell signaling (35, 44, 46, 48). The potential for the formation of a mixture of reactive oxygen and nitrogen species within the cell allows for the possibility of cross talk between nitrosative and oxidative cell signaling within the cell (19, 35, 46).

Interestingly, among the pathological stimuli thought to uncouple endothelial NOS (eNOS) is exposure to high concentrations of LDL (34, 35, 46). Chronic exposure to hydrogen peroxide and lipid peroxides have been shown to increase the expression of eNOS, again emphasizing the dynamic linkage between NOS activity and oxidative stress in the endothelial cells (15, 37, 41).

Recent endeavors in this area have focused on the potential of oxidized lipid products to initiate specific signal transduction pathways in the cell. Oxidized LDL (oxLDL) has differential effects on signal transduction pathways in the three cell types relevant to the development of atherosclerosis, namely, macrophages, smooth muscle, and endothelial cells (2, 3, 9, 15). Because the focus of this study is the effects of oxLDL on endothelial cells, key findings from this literature will be summarized. It has been shown that lysophosphatidylcholine, a component of both oxLDL and native LDL (nLDL) activates extracellular signal-regulated kinase (ERK) and JNK in bovine aortic endothelial cells (BAEC) through mechanisms that have yet to be defined in detail (15, 35, 50). In a recent study, oxLDL, whether mildly or extensively oxidized, induced endothelial cell apoptosis that was associated with activation of both ERK and JNK through mechanisms in which ROS were implicated (25, 50). Inhibition of either MAP kinase under these conditions decreased apoptosis through mechanisms in which ROS were implicated. The potential linkage between RNS generated from the interactions of eNOS with oxLDL and activation of MAP kinases in endothelial cells and key elements of the upstream signaling pathways have not been investigated and are the focus of the current study. The findings are placed in the context of the proapoptotic effects of oxLDL and the role of MAP kinases in controlling cell death under these conditions.

**MATERIALS AND METHODS**

**Isolation of LDL.** Human LDL was isolated from plasma of healthy donors by differential centrifugation using the method previously described (10). The LDL thus obtained was dialyzed against Ca\(^{2+}\)-Mg\(^{2+}\)-free phosphate-buffered saline (PBS) containing 10 μM EDTA and then sterilized by filtration through a 0.2-μm pore size filter and stored 4°C.

The concentration of LDL protein was determined using the BCA protein assay reagent.

**Oxidation of LDL.** Stock solutions of Cu\(^{2+}\) (10 mM) were made up in distilled water. LDL (1,000 μg/ml) was incubated with oxidant Cu\(^{2+}\) (25 μM) for 14–16 h. Reactions were stopped by addition of diethylenetriaminepentaacetic acid (40 μM). Oxidized LDL was also prepared by treatment with lipooxygenase, myoglobin, or hypochlorite as described previously (21, 27, 51). The relative electrophoretic mobility (REM) of LDL, typically 1.1–2.8, was determined on agarose gels using the lipoprotein electrophoresis system supplied by Beckman.

**Treatment of BAEC with oxLDL or nLDL.** Cell culture and treatment with oxLDL or nLDL BAEC harvested from descending thoracic aortas were maintained at 37°C, 5% CO\(_2\) in a growth medium (DMEM, 1 g/l glucose (GIBCO) containing 10% fetal calf serum (Atlanta Biologicals)). Cells used in this study were between passages 5 and 10. For nLDL or oxLDL treatment, 5 × 10\(^5\) cells were seeded in each well of six-well plates in the growth medium. On confluency, cells were used for LDL treatment (500 μg/ml) in a growth medium without previously exposing cells to serum starvation.

**MAP kinase assays.** After exposure to oxLDL or nLDL, BAEC were washed in ice-cold PBS, and cell lysates were obtained by lysis in 0.2 ml of buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1mM Na\(_3\)VO\(_4\), 10% glycerol, and 1% Triton X-100). ERK activation in the cell lysates (20 μg) was examined by Western blot analysis using an antibody specific to the active, phosphorylated form of ERK (phospho-ERK) (New England Biolabs) as described previously (19). The JNK assay was carried out using an antibody specific for JNK1 (Pharmergen). The antibody was incubated with the soluble lysates (100 μg) for 1 h at 4°C, followed by an additional 1-h incubation with Protein G-agarose beads. The immune complex was washed three times in lysis buffer, followed by additional wash in kinase assay buffer (20 mM HEPES, pH 7.6, 20 mM MgCl\(_2\), 20 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM Na\(_3\)VO\(_4\), and 2 mM dithiothreitol) and incubated in the presence of c-Jun (amino acids 5–89) fused to glutathione S-transferase (GST-cJun) and [\(^{32}\)P]ATP as described previously (19). The reaction products were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane, and the autoradiogram was obtained and radioactivity incorporated into each band quantified by scintillation counting. The membrane was then probed with a polyclonal antibody (Santa Cruz) to JNK to monitor the total amount of immunoprecipitated JNK in each experiment.

**Western blot analysis of Akt and eNOS phosphorylation.** To determine the phosphorylation status of Akt and eNOS, aliquots (30–40 μg protein) of the lysates were resolved on a 7.5% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). The PVDF membrane was probed with Akt or eNOS antibodies specific for phosphorylated Ser\(^{473}\) (New England Biolabs) or phosphorylated Ser\(^{1177}\) (New England Biolabs), respectively. An Akt antibody (New England Biolabs) or eNOS antibody (Transduction laboratory) detecting the total amount of Akt or eNOS, respectively, was also used as a control. Goat anti-rabbit- or mouse IgG conjugated to alkaline phosphatase was used as secondary antibody and developed by a chemiluminescence detection method.

**l-Citrulline assay.** Confluent BAECs plated in a six-well plate were used for measuring NOS activity using NOS assay kit (Calbiochem). Briefly, the cells were harvested in PBS containing 1 mM EDTA and were spun at 13,500 rpm for 2 min. The proteins were then extracted by disrupting the
pelleted cells by repeated pipetting in 100 μl of homogenization buffer (25 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM EGTA). The homogenates were centrifuged at 13,500 rpm for 5 min, and then the pellet was used for NOS assay. The reaction buffer (25 mM Tris, pH 7.4, 3 mM TH, 1 μM flavin adenine dinucleotide, 1 μM flavin mononucleotide, 8 mM NADPH, 1 μCi [3H]arginine, and 0.6 mM CaCl₂), 40 μl, was added to each pelleted cells, resuspended, and incubated at 30°C for 30 min. The reaction was stopped by adding 400 μl of stop buffer (50 mM HEPES, pH 5.5, 5 mM EDTA). With the use of a 100-μl sample, citrulline, being neutral at pH 5.5, was separated from arginine bound to resin and was then quantified by counting radioactivity to determine NOS activity.

Cytotoxicity assays. Cytotoxicity was measured by labeling the cells with annexin V-FITC and propidium iodide (PI) and subsequent fluorescence-activated cell sorting analysis (FACS). BAEC (5 × 10⁵ cells/well) were plated in six-well (35-mm²) plates (Costar; Cambridge, MA) and grown to confluency. After treatment, the cells were detached using trypsin-EDTA, washed with PBS, and resuspended in 100 μl of annexin V binding buffer, with 0.5 ng annexin V-FITC and 2.5 ng PI (Clontech). Cells (10⁶) were analyzed on a FACScan (Becton-Dickinson; Mountain View, CA) using WINMDI 2.8 software (The Scripps Research Institute Cytometry Software Page) within 30 min after staining (40).

**RESULTS**

Activation of MAP kinases by oxLDL. In the first series of experiments it was determined whether oxLDL is capable of activating JNK or ERK in endothelial cells. In this study nLDL from the same plasma donor was used as a control. After a period of 15–20 min, a robust activation of JNK occurred with oxLDL as measured by the in vitro phosphorylation of GST-cJun that essentially returned to basal levels after 2 h. In contrast nLDL had a more modest effect over the same time period with twofold activation evident at between 30 and 60 min (Fig. 1A). To determine whether JNK activation resulted in phosphorylation of c-Jun, which is a well-known endogenous substrate of JNK, the cell lysates were analyzed by Western blot using an antibody specific for c-Jun phosphorylated at Ser⁶³ (pSer⁶³-cJun). In this case only with oxLDL was significant phosphorylation of c-Jun evident, whereas nLDL had little effect on the endogenous substrate for JNK (Fig. 1B).

In the second series of experiments the effects of oxLDL on activation of ERK was determined. The activation time course of ERK differed from that of JNK in that maximal and robust activation was evident at only 5 min with oxLDL and persisted for ~30 min before declining to basal levels. Again, nLDL had detectable but minimal effects on ERK activation (Fig. 1C). In subsequent experiments the effects of oxLDL on the activation of MAP kinases was investigated in more detail.

Activation of Akt and eNOS by oxLDL. The activation of JNK in endothelial cells can be elicited by ROS and RNS. The activity of eNOS, a potential source of both ROS and RNS, has been shown to be associated

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Fig. 1. Oxidized low-density lipoprotein (oxLDL) induces activation of extracellular signal-regulated kinase (ERK), NH₄₇ terminal c-Jun kinase (JNK), and c-Jun. Bovine aortic endothelial cells (BAEC) were exposed to oxLDL or native LDL (nLDL) (150 μg/ml) for the indicated times. A: lysates (100 μg/ml) from cells treated with oxLDL (top) or nLDL (bottom) were incubated with a JNK-1 antibody (Pharmingen) and protein G agarose. Immune complex was then used to phosphorylate c-Jun fused to glutathione S-transferase (GST-cJun) to examine activity of JNK. Phosphorylated proteins were resolved by 10% SDS-PAGE and followed by transfer to polyvinylidene (PVDF) membrane and autoradiography. Phosphorylated GST-cJun bands were excised from the membrane and were quantified by counting. Fold stimulation from 15 to 60 min after treatment had a P value <0.05 compared with basal. B: BAECs incubated with oxLDL (top) or nLDL (bottom) for the indicated times were lysed. Protein (50 μg) was resolved by SDS-PAGE and Western blotted with antibody specific to phosphorylated c-Jun at Ser⁶³ to determine the activity of c-Jun. The line graph shows the fold stimulation (means ± SE, n = 3) compared with the basal (time 0). *P < 0.01. C: cell lysates that were incubated with oxLDL (top) or nLDL (bottom) were (20 μg cell protein) resolved by 10% SDS-PAGE and Western blotted with antibodies specific to phospho-ERK1/2 (pERK1/2). Line graph shows means ± SE (n = 3) obtained from densitometric quantitation of ERK 1 band. P < 0.05 for fold stimulation between 5 and 15 min compared with basal.
with a signaling pathway involving Akt phosphorylation of eNOS (13, 16, 18). Thus to further identify potential upstream elements of the signal transduction pathways that lead to JNK activation, the phosphorylation of Akt by oxLDL was examined. Exposure of BAEC to 150 μg/ml oxLDL transiently stimulated Akt reaching a maximum by 5 min as determined by Western blot using the antibody specific to the Akt phosphorylated at Ser473 (Fig. 2A). In a parallel experiment the phosphorylation of eNOS at Ser1177 was determined. In this case the time course for eNOS phosphorylation was persistent between 15 and 30 min before returning to basal levels (Fig. 2B). The levels of both Akt and eNOS proteins remained essentially constant throughout this experiment as determined by using antibodies detecting the total amount of Akt and eNOS, respectively (Fig. 2, A and B). To determine whether phosphorylation of eNOS under these conditions resulted in active enzyme, the conversion of arginine to citrulline was determined. The results of this experiment (Fig. 3) indicate a threefold stimulation of eNOS activity over a similar time course shown for phosphorylation of eNOS. The medium used in these experiments contains low levels of fetal calf serum. As a control, the phosphorylation of eNOS was also determined in serum-free media, and the oxLDL-dependent level of activation was essentially the same (3.28 ± 0.33-fold after treatment for 15 min, means ± SE, n = 3) as found in the presence of complete media.

In a further series of experiments the effect of increasing concentrations of oxLDL on eNOS phosphorylation was determined. Phosphorylation of eNOS increased with increasing concentrations of oxLDL up to 600 μg/ml (Fig. 4A). With copper-oxidized LDL, the activation correlated with the REM (an index of protein modification) of the oxLDL, with maximum activation seen with oxLDL with REMs of 2–3 (data not shown). To confirm that this effect of oxLDL was not restricted to LDL modified with copper, experiments were also undertaken with LDL oxidized by myoglobin, hypochlorite, and lipoxygenase. As seen in Fig. 4B, LDL modified by any one of these methods was capable of inducing significant eNOS phosphorylation. However, REM of the LDL modified by these methods (nLDL 1, Cu oxLDL 1.84, myoglobin 1.95, HOCl 3.26, and lipoxygenase 1.08) was not correlated with the capacity to induce eNOS phosphorylation. For example, myoglobin modified LDL with an REM of ~2 had approximately the same capacity to induced eNOS phosphorylation as lipoxygenase modified LDL with an REM essentially the same as nLDL. Lipid oxidation as measured by lipid peroxides (nLDL 2.8 mmol/mg protein, Cu oxLDL 62 nmol/mg protein, myoglobin 242 nmol/mg protein, HOCl 45 nmol/mg protein, and lipoxygenase 66 nmol/mg protein) also varied between the different methods for oxidation but did indicate that lipid peroxidation was a consistent feature of all the oxidation methods used. These data indicate that the stimulus for eNOS phosphorylation resides in an oxidized lipid component.

Identification of the upstream signaling pathway leading to eNOS phosphorylation. In the next series of experiments further examination of the pathways leading to oxLDL-dependent eNOS phosphorylation was undertaken. For this study, cells were pretreated with or without selective inhibitors of the ERK pathway the MAPK/ERK kinase 1 and 2 (MEKI/2 inhibitor PD-98059), NOS [N<sup>G</sup>-nitro-L-arginine (L-NNA)], and phosphatidylinositol-3-kinase (PI3 kinase) (wortmannin) before adding oxLDL. The MEK inhibitor and the NOS inhibitor l-NNA had no effect on Akt phosphorylation, whereas wortmannin completely prevented Akt phosphorylation as expected (Fig. 5A). Furthermore, it is clear that phosphorylation of eNOS induced by oxLDL is dependent on PI3 kinase because it is inhibited by wortmannin (Fig. 5B).

Role of eNOS in JNK and ERK activation. To test for a role of RNS derived from eNOS in JNK activation, the effects of l-NNA and wortmannin were determined on phosphorylation of GST-cJun. These data (Fig. 6) indicate that JNK activation by oxLDL is prevented by
the NOS inhibitor, whereas wortmannin has a significant but partial effect. Although ERK can be activated by ROS and RNS under some conditions, the lack of an effect of the MEK inhibitor (Fig. 5A) suggests that the ERK pathway is not an upstream regulator of Akt in response to oxLDL. As a further test of this concept, the effects of the MEK1/2, NOS, and PI3 kinase inhibitor on ERK activation were determined. Only the MEK1/2 inhibitor prevented ERK activation by oxLDL, indicating that activation of this MAP kinase is distinct from that of JNK in its lack of requirement for either PI3 kinase or NOS activity (Fig. 6B). Effects of ERK and JNK activation on the cytotoxicity of oxLDL. The previous data indicate that an early response of endothelial cells to oxLDL is the activation of at least two independent signal transduction pathways leading to activation of the ERK and JNK. Both pathways have been implicated in controlling cellular responses to environmental stress. Because PD-98059 prevents ERK activation induced by oxLDL, it was then used to determine the effects of this MAP kinase pathway on oxLDL cytotoxicity. Under these conditions, where confluent cells are being used in the presence of serum, no cytotoxicity of oxLDL is evident unless concentrations exceed 500–750 μg/ml. This is evident from the data shown in Fig. 7 in which it is clear that neither oxLDL or nLDL induce cell death as assessed by FACs analysis using annexin V antibody and PI staining. In contrast, treatment with the MEK inhibitor results in pronounced apoptosis on exposure to oxLDL, whereas the compound itself has little or no effect in the control or nLDL-treated cells. Although eNOS activation on shear stress as well as the NO donor detaNONOate have been reported to ameliorate oxLDL cytotoxicity in low serum conditions (16, 22), inhibition of endogenous NO production by L-NNA had no effect on the cytotoxic effects of oxLDL in conditions used (results not shown).

Fig. 5. Activation of Akt and phosphorylation of eNOS by oxLDL is regulated by propidium iodide (PI)-3-kinase. BAEC were pretreated with or without inhibitors, including PD-98059 (10 μM for 30 min), NG-nitro-l-arginine (L-NNA, 250 μM for 30 min), or wortmannin (100 nM for 20 min) before incubating cells with oxLDL for 5 min (A) or 15 min (B). Cell lysates were used to examine Akt phosphorylation (A) and eNOS phosphorylation (B) following the methods described in Fig. 2. *P < 0.05.

Fig. 6. OxLDL-dependent activation of JNK but not that of ERK is regulated by AKT and eNOS. Cells were pretreated with or without inhibitors, including PD-98059 (10 μM for 30 min), L-NNA (250 μM for 30 min), or wortmannin (100 nM for 20 min) before oxLDL treatment for 5 min for extracellular signal-regulated kinase (ERK) activation (A) or 15 min for JNK activation (B). Cell lysates were used to examine the activation of ERK or JNK following the method described in Fig. 1. *P < 0.05.
DISCUSSION

A number of studies have investigated the effects of oxLDL on eNOS in endothelial cells. It has been shown that high concentrations of nLDL induce eNOS to generate both superoxide and NO resulting in the formation of peroxynitrite (34). Functional studies in vessel preparations and in vivo data are consistent with oxLDL inducing loss of bioavailable NO from the endothelium (28, 33, 47). Indeed, pretreatment of endothelial cells with oxLDL for 1 h results in the inhibition of acetylcholine-induced NO formation, intracellular redistribution of the enzyme, and no change in phosphorylation (4, 45). However, the effects on eNOS on immediate exposure to oxLDL have not been examined. It is thus somewhat surprising to find that oxLDL induced the phosphorylation of eNOS at Ser1177 because this site is associated with activation of eNOS stimulated by fluid shear stress and vascular endothelial growth factor (VEGF) (13, 24). That this resulted in active enzyme is supported by the finding that oxLDL resulted in the increased conversion of arginine to citrulline.

It has been shown by other investigators that exposure of endothelial cells to oxLDL results in the formation of ROS, although the source of these species remains an area of uncertainty (11). Recent studies have shown that eNOS is capable of generating superoxide under conditions that include depletion of substrates such as arginine or tetrahydrobiopterin (23, 35, 44, 48). Such conditions are thought to occur on exposure of endothelial cells to oxLDL. However, the lack of a specific inhibitor for superoxide generation from eNOS...
makes probing this aspect of the hypothesis difficult in a cellular setting. The data demonstrate that an early event on exposure to oxLDL is the initiation of PI3 kinase-dependent pathway leading to activation of eNOS, and this in turn leads to activation of JNK. Although nLDL has a similar effect, this is much less marked and did not result in significant activation of JNK pathway at these concentrations. These data are, however, consistent with recent studies, suggesting a role for high concentrations of nLDL in modulation of eNOS function in the endothelium (23, 34). An interesting aspect of these data is that they serve to emphasize the current view that modification of LDL is a continuous process in a biological setting. Circulating plasma LDL clearly contains low levels of oxidized lipids capable of initiating cell signaling events. However, it is only the more oxidized forms of LDL that are thought to be generated within the vasculature that are thought to contribute to apoptosis in the atherosclerotic lesion and are the focus of the current study.

From the data presented here it is not clear whether the effects of the NOS inhibitor l-NNA can be interpreted solely in the context of a role of NO in the activation of JNK by oxLDL. Because this arginine analog in a cellular context could allow both generation of superoxide and NO from eNOS, a role for reactive species such as peroxynitrite or S-nitrosylation can be postulated (44). In a separate study, Jo and colleagues (19) have demonstrated that NO alone at concentrations that could be derived from eNOS does not activate JNK. These data are again consistent with the hypothesis that exposure of oxLDL induce production of both ROS and RNS. The coproduction of these species results in redirection of NO signaling away from the soluble guanylate cyclase pathways to redox signaling dependent on the activation of JNK. It is interesting to note that if the intracellular signaling molecule is hydrogen peroxide, then the sensitivity of the oxLDL-dependent activation of JNK may be modulated by the intracellular concentration of catalase. It has been reported that the concentration of this antioxidant enzyme varies between endothelial cells and is in fact higher in the BAEC used in this study relative to human aortic endothelial cells (38, 42). This could result in a lower threshold for activation for hydrogen peroxide-dependent signaling pathways in human endothelial cells.

It is interesting to note that at least two other pathways are activated by oxLDL. Activation of ERK is apparently distinct from JNK, because it is not modified by either wortmannin or NOS inhibitors. The situation with Akt is more complex. It has no requirement for NO formation but is PI3 kinase dependent. Previous studies showed that phosphorylation of eNOS is PI3 kinase dependent and that Akt may be the upstream kinase, which directly phosphorylates Ser1179 in response to shear stress (13). Whether Akt is also responsible for phosphorylation of eNOS is not clear from the present data and requires further investigation. However, it is important to note that these experiments follow a much shorter time course than that reported in a recent study where it was shown that a 1-h exposure of endothelial cells to oxLDL results in inhibition of Akt phosphorylation initiated by VEGF (8).

It has been recognized by a number of investigators (1, 9) that oxLDL can exert both growth stimulatory effects and apoptosis in smooth muscle cells dependent on concentration and conditions of exposure. The responses in endothelial cells have not been investigated in detail with respect to the potential role of eNOS in this process. In other contexts and cell types it has been shown that JNK and ERK exert contrasting functions in controlling apoptosis and survival (17, 43, 49). As demonstrated here exposure of oxLDL to endothelial cells is an interesting example of a cellular response to stress, because it activates both MAP kinases and through induction of both ROS and RNS has the potential to initiate redox cell signaling. Clearly, the net response of the cell to oxLDL as a stimulus of cell signaling is important in the vasculature where low levels of lipid oxidation products may exert cytoprotection in response to chronic exercise, whereas in the atherosclerotic lesion apoptosis and cell death are thought to occur (31). It is then important to understand the mechanisms and factors that control activation of signal transduction pathways by oxLDL.

At present, what component of the oxLDL molecule mediates the activation of eNOS is not known. The fact that the level of eNOS phosphorylation is associated with lipid hydroperoxides measured in oxLDL but not with REM suggests that lipid peroxidation products could be the initiators of this pathway. For example, 13-hydroperoxy-octadecadienoate (13-HPODE), a major lipid hydroperoxide present in oxLDL, has specific signaling functions, which include transcriptional up-regulation of eNOS (37). However, it is possible that other lipid oxidation products such as lyso-PC or 4-hydroxynonenal are also involved. The role of eNOS in regulation of apoptosis from the data presented here and in the literature appears to be highly dependent on the conditions under which it is activated. For example, it is clear that activation of eNOS occurs in response to laminar flow, or shear stress, over the endothelium, and in this case is antiapoptotic (14). As in the case of exposure to oxLDL, both ERK and JNK are activated by shear stress, and under these conditions of low concentration and serum supplemented medium, this is not associated with apoptosis or necrosis. However, if ERK activation is prevented on exposure to oxLDL by the MEK inhibitor PD-98059, extensive cell death ensues. Because the MEK inhibitor has no effect on the phosphorylation of Akt on which eNOS activation is dependent, these data suggest that under conditions where NOS is active it alone is not sufficient to prevent apoptosis.

In summary, this study develops the concept that oxLDL can initiate diverse signal transduction events in endothelial cells. An interesting aspect revealed here is an unexpected role for eNOS in redox-dependent cell signaling, leading to activation of JNK but not ERK. The impact of the relative activation of these
MAP kinases appears critical in controlling whether oxLDL is proapoptotic.

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REFERENCES


