Activation of MAPK by modified low-density lipoproteins in vascular smooth muscle cells

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Velarde, Victoria, Alicia J. Jenkins, Julie Christopher, Timothy J. Lyons, and Ayad A. Jaffa. Activation of MAPK by modified low-density lipoproteins in vascular smooth muscle cells. J Appl Physiol 91: 1412–1420, 2001.—A high concentration of circulating low-density lipoproteins (LDL) is a major risk factor for atherosclerosis. Native LDL and LDL modified by glycation and/or oxidation are increased in diabetic individuals. LDL directly stimulate vascular smooth muscle cell (VSMC) proliferation; however, the mechanisms remain undefined. The extracellular signal-regulated kinase (ERK) pathway mediates changes in cell function and growth. Therefore, we examined the cellular effects of native and modified LDL on ERK phosphorylation in VSMC. Addition of native, mildly modified (oxidized, glycoxidized) LDL at 25 μg/ml to rat VSMC for 5 min induced a fivefold increase in ERK phosphorylation. To elucidate the signal transduction pathway by which LDL phosphorylate ERK, we examined the roles of the Ca2+/calmodulin pathway, protein kinase C (PKC), src kinase, and mitogen-activated protein kinase kinase (MEK). Treatment of VSMC with the intracellular Ca2+ chelator EGTA-AM (50 μmol/l) significantly increased ERK phosphorylation induced by native and mildly modified LDL, whereas chelation of extracellular Ca2+ by EGTA (3 mmol/l) significantly reduced LDL-induced ERK phosphorylation. The calmodulin inhibitor N-(6-aminohexyl)-1-naphthalenesulfonamide (40 μmol/l) significantly decreased ERK phosphorylation induced by all types of LDL. Downregulation of PKC with phorbol myristate acetate (5 μmol/l) markedly reduced LDL-induced ERK phosphorylation. Pretreatment of VSMC with a cell-permeable MEK inhibitor (PD-98059, 40 μmol/l) significantly decreased ERK phosphorylation in response to native and modified LDL. These findings indicate that native and mildly and highly modified LDL utilize similar signaling pathways to phosphorylate ERK and implicate a role for Ca2+/calmodulin, PKC, and MEK. These results suggest a potential link between modified LDL, vascular function, and the development of atherosclerosis in diabetes.

VASCULAR INJURY IS CONSIDERED to be a critical event in the evolution and progression of atherosclerotic vascular disease. Chronic damage to arterial endothelium by increased hydraulic stress on the vessel wall or other undefined injuries causes functional alterations of endothelial cells, leading to accumulation of lipids within the subintima and migration and proliferation of smooth muscle cells (31). Support for endothelial injury or dysfunction as the initial lesion in atherosclerosis comes from the finding that experimental models of endothelial damage cause proliferation of the underlying smooth muscle cells (5, 6). Accumulation of such cells is established as a key event in the formation and progression of lesions of atherosclerosis (16).

The development of vascular lesions is accelerated in the diabetic state, and evidence indicates that diabetes mellitus per se is a powerful, independent risk factor for the development of atherosclerosis (18). The mechanisms by which diabetes accelerates vascular damage are not fully elucidated. Diabetes is associated with endothelial denudation, smooth muscle cell proliferation, basement membrane changes, and impaired endothelium-dependent relaxation of blood vessels (14, 27, 29, 38). Because atherosclerotic vascular disease is regarded as the major cause of morbidity and mortality in people with diabetes mellitus, epidemiologic studies have demonstrated an association between elevated cholesterol levels, the progression of renal damage, and atherosclerosis (1, 7, 28, 36). Increasing evidence indicates that circulating lipoproteins may exert cellular effects that are not directly related to their lipid-transporting functions. Increased infiltration of low-density lipoprotein (LDL) into the arterial wall may itself cause endothelial injury, which could lead to retention of LDL by extracellular matrix proteins, and this may in turn promote vascular smooth muscle cell (VSMC) migration and proliferation (11). In this regard, LDL has been shown to directly modify vascular tone and to increase VSMC proliferation (2, 33). Recent evidence indicates that modified forms of LDL are associated with increased atherogenicity (37). Of special interest is the possibility that the oxidative modifications of low-density lipoproteins; signal transduction; tyrosine and serine/threonine kinases; calcium/calmodulin; mitogen-activated protein kinase

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LDL that have been shown to occur in vivo may directly injure the endothelium and thus initiate proliferation of VSMC (9).

Mitogen-activated proteins kinases (MAPKs; p42mapk and p44mapk), also known as extracellular signal-regulated kinases (ERK), belong to the group of serine/threonine kinases that are rapidly activated in response to growth factor stimulation. They integrate multiple signals from various upstream second messengers and regulate the expression of transcription factors leading to cellular proliferation or differentiation (30). Recently, it has been shown that MAPK activity is transiently activated after vessel wall injury, whereas MAPK phosphatase-1 is decreased (21).

Although several factors have been implicated in causing atherosclerosis, the underlying cellular mechanisms through which LDL promotes vascular injury are less understood. Because VSMC proliferation is a hallmark feature of atherosclerotic vascular disease, we initiated studies to determine the role and contribution of LDL to induce mediators of VSMC proliferation. In the present study, we studied the cellular mechanism leading to ERK phosphorylation in VSMC exposed to various types of LDL modified in vitro to resemble LDL that may be present in vivo in diabetic patients. We also examined whether there are differences in the activation profile of ERKs by native LDL and LDL modified by glycation and/or mild or heavy oxidation. A better understanding of the cellular signaling pathways through which LDL may influence vascular injury could result in the development of preventive measures to retard diabetic atherosclerosis.

METHODS

LDL preparation and modification. LDL was prepared by a modification of the method previously described (25). Blood was drawn from five to six healthy fasting nondiabetic normolipidemic subjects. Subjects were nonsmokers and were not taking supplemental vitamins. Written informed consent was obtained, and the Medical University of South Carolina Institutional Review Board approved the study. LDL (density 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation in a Beckman XL-90 ultracentrifuge using a Beckman Ti60 rotor and a Beckman SW41 rotor to wash and concentrate the LDL. LDL was dialyzed extensively against nitrogen-purged 0.15 mol/l NaCl, 0.3 mol/l EDTA, pH 7.4 at 4°C, in the dark. LDL was modified in vitro by glycation, minimal oxidation, or glycoxidation as previously described (33). Briefly, LDL was incubated at 37°C, pH 7.4, for 3 days in the absence or presence of freshly prepared 7.4 mmol/l D-glucose, 0.3 mmol/l EDTA, and, for native and glycated LDL, 1 mmol/l DTPA, under air (for oxidized or glycoxidated-LDL) or nitrogen (for native and glycated LDL). Modification was terminated by repeat extensive dialysis as described above. “Highly oxidized” LDL and “highly oxidized glycated” LDL were generated by buffer exchange of native and glycated LDL, respectively, into PBS, pH 7.4, dilution to 3 mg/ml protein, and addition of CuCl₂ to a final concentration of 10 μmol/l for 24 h at 37°C. Oxidation was terminated by the addition of EDTA to a final concentration of 100 μmol/l with extensive dialysis as described above. Thus six types of LDL were prepared: normal (N-LDL), glycated (G-LDL), mildly oxidized (mO-LDL), glycoxidized (GO-LDL), highly oxidized (HO-LDL), and highly oxidized glycated (HOG-LDL). LDL preparations were sterile filtered (0.22 μm), stored in the dark under nitrogen at 4°C, and used within 1 mo of preparation. Five different plasma pools were used in this series of experiments. The LDL pools were tested for endotoxin contamination by the Limulus amebocyte lysate (Bio Whittaker, Walkerville, MD) according to the manufacturer’s protocol.

LDL characterization. Each LDL preparation, at a concentration of 100 μg/ml protein, was characterized as follows: Agarose gel electrophoresis (LIPOEPG, Beckman, Schaumburg, IL) was used to assess LDL purity and any alteration in negative charge (indicative of marked oxidation). The absorbance at 234 nm in a Beckman DU 650 spectrophotometer was used as a measure of conjugated diene formation in the lipid moiety. As a marker of protein modification, fluorescence at excitation wavelength 360 nm and emission wavelength 430 nm was measured in a Gilford Fluorimeter IV (Oberlin, OH). Cell culture. Rat aortic VSMC from 75- to 150-g male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were prepared by a modification of the method of Majack and Clowes (26). A 2-cm segment of artery cleaned of fat and adventitia was incubated in 1 mg/ml collagenase for 3 h at room temperature. The artery was then cut into small sections and fixed to a culture flask for explantation in MEM containing 10% FCS, 100 μU/ml of penicillin, and 100 μg/ml of streptomycin and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were identified as VSMC by their characteristic morphology and by their immunostaining to smooth muscle cell-specific α-actin. VSMC were used between passages 2 and 6 in all experiments. Quiescence was achieved by transferring semiconfluent (60–70%) cells to serum-free MEM media for 24–48 h before agonist stimulation. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cell extracts. VSMC stimulated with LDL were washed twice, scraped in PBS containing 2 mM sodium vanadate, and centrifuged at 3,000 g for 5 min. Pellets were resuspended in 100 μl of lysis buffer (20 mmol/l Tris, 130 mmol/l NaCl, 10% glycerol, 10 mmol/l 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate, 2 mmol/l sodium vanadate, 150 μM aprotinin, and 0.156 mg/ml benzamidine, pH 8.0), sonicated for 5 s, incubated on ice for 30 min, and centrifuged at maximum speed for 5 min. The supernatant was used as the protein source, and its concentration was determined by the method of Lowry et al. (23).

Western blotting of MAPK. To measure MAPK phosphorylation, 20–25 μg of soluble protein obtained as described above were separated by SDS-PAGE (12%) under reducing conditions and transferred to PVDF membranes at 300 mA for 2 h. The membranes were blocked for 30 min in 1% BSA-Tris-buffered saline (50 mmol/l Tris, pH 7.5, 150 mmol/l NaCl) at 37°C. The blocked membranes were immunoblotted with rabbit polyclonal tyrosine phospho-specific MAPK antibodies (14,000 dilution, New England Biolabs, Beverly, CA) that detect p42mapk and p44mapk. Total MAPK was measured in the same membranes by stripping the membrane and immunoblotting with anti-MAPK antibodies (16,000× dilution). The membranes were then incubated in a secondary antibody conjugated to horseradish peroxidase. The immuno-reactive tyrosine-phosphorylated MAPK and total MAPK bands were visualized by using the chemiluminescence reagent Renaissance (NEN Life Science Products, Boston, MA).
according to the procedure described by the supplier. Membranes were exposed to Kodak LS film, and bands were measured by densitometry and quantified by the NIH Image program.

Statistical analysis. Data are expressed as means ± SE and were analyzed by Student’s t-test for unpaired two-tailed analysis and by the Mann-Whitney nonparametric analysis. Differences are considered significant if P < 0.05.

RESULTS

LDL characterization. Agarose gel electrophoresis of LDL showed a single band, demonstrating lack of contamination by other lipoprotein subclasses. Results of the electrophoretic mobility, conjugated dienes and fluorescence are shown in Table 1. As expected, the electrophoretic mobility of HO-LDL and HOG-LDL was increased, but that of the N-, G-, mO-, and GO-LDL did not differ. Conjugated dienes and fluorescence were significantly higher in HO- and HOG-LDL vs. N-, G-, mO-, and GO-LDL. G-LDL, mO-LDL, and GO-LDL did not differ from each other. There was no significant difference in the extent of modification of HO- and HOG-LDL.

To determine whether the LDL pools we used in our studies were not contaminated with endotoxin, we measured the concentration of endotoxin in 25 µg/ml of N-LDL, G-LDL, mO-LDL, mO-G-LDL, HO-LDL, and HOG-LDL from three different pools of LDL prepared at different times. The results showed that all the LDL pools were negative for endotoxin contamination. This finding eliminates the possibility that the responses that we observed on MAPK activation are mediated via endotoxin contamination of LDL.

Phosphorylation of ERK by native, mildly modified, and highly modified LDL. To determine the appropriate dose at which LDLs would maximally phosphorylate ERK, VSMC were exposed to varying doses (0, 5, 25, 75, and 100 µg/ml) of LDL for 5 min. A representative of these experiments (shown in Fig. 1) demonstrates that 25 µg/ml is the minimal dose to produce a response in these cells. In preliminary studies, we have shown that all forms of modified LDLs maximally phosphorylate ERK at 5 min poststimulation. VSMC stimulated with 25 µg/ml N-LDL or mildly modified (G-LDL, mO-LDL, GO-LDL) and/or highly modified (HO-LDL or HOG-LDL) LDL for 5 min were able to phosphorylate ERK relative to the LDL-free control (Fig. 2). Mildly modified LDL induced a similar increase in ERK phosphorylation compared with N-LDL. G-LDL produced the highest increase in ERK 1/2 phosphorylation, but the difference did not reach statistical significance. On the other hand, the highly modified LDL (HO and HOG) were significantly less potent than native and mildly modified LDL to phosphorylate ERK (Fig. 2, P < 0.05). To place these results in perspective, the N-LDL-induced phosphorylation of ERK was compared with that of bradykinin, which is a known mitogen for MAPK in VSMC (39) (Fig. 3).

Role of the calcium/calmodulin pathway on ERK phosphorylation by native and modified lipoproteins. It has recently been shown that native LDL evokes a rise in intracellular calcium concentration (intracellular calcium) in VSMC (33). To determine whether this rise in intracellular calcium is required for the activation of ERKs by LDLs, quiescent VSMC were pretreated with 50 µmol/l of the intracellular calcium chelator EGTA-AM for 30 min followed by stimulation with 25 µg/ml of normal or modified LDL for 5 min. As shown in Fig. 4, in the absence of EGTA-AM, native and all

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<tr>
<td>Absorbance</td>
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<td>0.85 ± 0.22</td>
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<td>1.05 ± 0.05</td>
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<td>1.24 ± 0.08</td>
<td>7.20 ± 0.70*</td>
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<td>Electrophoretic mobility</td>
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*Vvalues are means ± SE of 5 low-density lipoprotein (LDL) preparations. The absorbance (234 nm, fluorescence at excitation 360 nm, emission 430 nm), and electrophoretic mobility of native (N) and modified LDL at 100 µg/ml protein are shown, expressed as ratio to N-LDL. The absolute value of N-LDL absorbance, fluorescence, and electrophoretic mobility of highly oxidized (HO-) and highly oxidized glycated (HOG) LDL was significantly greater than that of N-LDL and mildly modified LDL. *P < 0.05, but that of N-, glycated (G-), mildly oxidized (mO), and glycoxidized (GO) LDL did not differ from each other. There was no significant difference in the extent of modification of HO- and HOG-LDL.

Fig. 1. Extracellular signal-regulated kinase (ERK) phosphorylation response in these cells. In preliminary studies, we have shown that all forms of modified LDLs maximally phosphorylate ERK at 5 min poststimulation. VSMC stimulated with 25 µg/ml N-LDL or mildly modified (G-LDL, mO-LDL, GO-LDL) and/or highly modified (HO-LDL or HOG-LDL) LDL for 5 min were able to phosphorylate ERK relative to the LDL-free control (Fig. 2). Mildly modified LDL induced a similar increase in ERK phosphorylation compared with N-LDL. G-LDL produced the highest increase in ERK 1/2 phosphorylation, but the difference did not reach statistical significance. On the other hand, the highly modified LDL (HO and HOG) were significantly less potent than native and mildly modified LDL to phosphorylate ERK (Fig. 2, P < 0.05). To place these results in perspective, the N-LDL-induced phosphorylation of ERK was compared with that of bradykinin, which is a known mitogen for MAPK in VSMC (39) (Fig. 3).

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forms of modified LDL produced a significant increase in MAPK phosphorylation compared with unstimulated cells. Pretreatment of VSMC with EGTA-AM resulted in a further increase in ERK phosphorylation in response to N-LDL, O-LDL, GO-LDL, and HOG-LDL (Fig. 4, \( P < 0.04 \) compared with LDL, respectively), and slight nonsignificant increases were observed with G-LDL and HO-LDL. These data rule out a role for increased intracellular calcium in ERK phosphorylation by native and modified LDL.

To rule out a role for influx of calcium and/or requirement for extracellular calcium in regulating MAPK phosphorylation, VSMC were stimulated with LDL in the presence and absence of EGTA (3 mmol/l) in the extracellular medium. Addition of EGTA to the extracellular media significantly reduced MAPK activation in response to N-LDL, mO-LDL, and GO-LDL but had no significant effect on MAPK in response to G-LDL, HO-LDL, and HOG-LDL (Fig. 4). Overall, the findings indicate that the rise in intracellular calcium is not required for ERK phosphorylation, whereas the influx of extracellular calcium into the cell may modulate ERK activity in response to native and modified LDL but seems to have little effect on responses to highly modified LDL.

Rises in intracellular calcium result in a reversible formation of a calcium/calmodulin complex. To evaluate the requirement for calmodulin in the phosphorylation of MAPK in response to LDL, VSMC were pretreated for 45 min with the calmodulin inhibitor \( \text{N}-(6\text{-aminohexyl})\text{-1-naphthalenesulfonamide (W-7)} \) (30 \( \mu \)mol/l). The inhibitor was used at a concentration that achieved half-maximal inhibition of calmodulin (13). Once again, native, mildly modified, and/or highly modified LDL produced a marked increase in tyrosine phosphorylation of MAPK. This was inhibited by W-7 to a level not significantly different from unstimulated control cells (Fig. 5). Another study was also carried out to assess the effects of a second calmodulin inhibitor, calmidazolium, on LDL-induced MAPK phosphorylation. Treatment of VSMC with native, mildly modified, and heavily modified LDL resulted in a sevenfold increase in MAPK phosphorylation compared with unstimulated controls (\( P < 0.005 \)). However, in the presence of calmidazolium (10 \( \mu \)g), the percent increase in MAPK phosphorylation in response to native, mildly modified, and/or heavily modified LDL was reduced by 25–30% (\( P < 0.05, n = 3 \) experiments, data not shown).

**ERK phosphorylation induced by mildly and highly modified LDL is mediated by PKC.** To evaluate whether protein kinase C (PKC) is essential for LDL-induced ERK phosphorylation, VSMC were treated...
with native and modified LDL for 5 min after down-regulation of PKC by 24-h pretreatment with phorbol myristate acetate (PMA; 5 μmol/l). As shown in Fig. 6, PKC depletion by PMA reduced LDL-induced ERK phosphorylation by more than 45% compared with LDL-treated cells. The magnitude of this effect was similar for all LDL preparations. These findings indicate that one or more isoforms of PKC is activated by native and modified LDL, which in turn results in MAPK phosphorylation.

Fig. 4. Calcium modulates ERK phosphorylation induced by mildly and highly modified LDL. VSMC were preincubated with the extracellular calcium chelator EGTA (10 μmol/l, 60 min) and/or with the cell-permeable intracellular calcium chelator EGTA-AM (50 μmol/l, 30 min) followed by stimulation for 5 min with 25 μg/ml of each LDL. Cell proteins were separated by SDS-PAGE (12%) and immunoblotted with a polyclonal antibody against P-ERK 1/2 (1:6,000) and T-ERK 1/2 (1:4,000). Blots shown are representative of at least 3 experiments. Bar graphs represent intensities of both ERK 1 and ERK 2 bands relative to total ERK and expressed as percent phosphorylation relative to corresponding LDL. Bars represent means ± SE. *P < 0.05 vs. corresponding LDL.

Fig. 5. Calmodulin mediates ERK phosphorylation induced by native, mildly modified, and heavily modified LDL. Vascular SMC were preincubated with the calmodulin inhibitor N-(6-aminohexyl)-1-naphthalenesulfonamide (W-7; 35 μmol/l, 30 min) followed by incubation for 5 min with 25 μg/ml of each LDL. Cell proteins were separated by SDS-PAGE (12%) and immunoblotted with a polyclonal antibody against P-ERK 1/2 (1:6,000) and T-ERK 1/2 (1:4,000). Blots shown are representative of at least 3 experiments. Bar graphs represent intensities of both ERK 1 and ERK 2 bands relative to total ERK and expressed as percent phosphorylation relative to corresponding LDL. Bars represent means ± SE. *P < 0.01 vs. corresponding LDL.
Role of cytoplasmic kinases in LDL-induced ERK phosphorylation. To identify the cytoplasmic kinases involved in LDL-induced ERK phosphorylation, we examined the effects of the specific cell-permeable kinase inhibitors, PP1 (Biomol Research Laboratories, Plymouth, PA), which inhibits the src family tyrosine kinases, and PD-98059 (NEN-Biolabs, Beverly, MA), which specifically inhibits the MAPK activator MAPK kinase (MEK; Refs. 8 and 12). VSMC were pretreated with either PP1 (10 μmol/l) for 2 h or PD-98059 (40 μmol/l) for 30 min, followed by LDL stimulation for 5 min. Inhibition of src kinase by PP1 reduced ERK phosphorylation in response to native and modified LDL by ~25%, but this reduction did not reach statistical significance (6.4 ± 2.25, 7.38 ± 1.33, 6.58 ± 1.88, 7.08 ± 1.05, 4.03 ± 0.67, 4.34 ± 0.27 arbitrary units for N-LDL, O-LDL, G-LDL, GO-LDL, HO-LDL, and HOG-LDL, respectively, vs. 3.79 ± 2.9, 4.78 ± 2.35, 3.88 ± 2.34, 4.60 ± 2.01, 3.13 ± 0.22, and 2.55 ± 0.96 in response to LDL+PP1, respectively). On the other hand, phosphorylation of ERK in response to native and modified LDL was significantly reduced (80%) by the MEK inhibitor (Fig. 7).
DISCUSSION

The cell signaling pathways by which LDL induces smooth muscle cell proliferation, a feature of atherosclerotic lesions, are not yet fully elucidated. In the present study, we identified several second messenger systems that are generated on activation of LDL receptors in response to native, mildly modified, and heavily modified lipoproteins that converge to activate the MAPK pathway in VSMC. We have shown that native, mildly modified (G-, mO-, and GO-), and heavily modified (HO- and HOG-) LDL stimulate ERK phosphorylation via activation of the calcium/calmodulin pathway, PKC, and MEK. These findings demonstrate that native, mildly modified, and highly modified lipoproteins play a role in several signaling pathways that stimulate early mitogenic signals associated with phosphorylation of ERK in VSMC.

It is generally accepted that ERK activation plays a principal role in cell proliferation and differentiation (30). In the present study, we showed that native and modified lipoproteins induce a rapid phosphorylation of ERK in VSMC. Our data are in general agreement with previous studies showing that native and copper-oxidized LDL stimulate MAPK activation in VSMC (20, 33). However, our study differs from those reported in the literature in that we studied a broader range of LDL modifications, including five forms of glycated LDL and/or oxidized LDL. Minimally oxidized LDL is likely to circulate in vivo in diabetic patients, whereas highly oxidized LDL is likely to form in the subendothelial space. Our findings also indicate that native and mildly modified LDL are more potent activators of ERK in VSMC than heavily modified LDL. Once activated, ERK can translocate to the nucleus, thus providing a link in the signal transduction pathway from the cytoplasm to the nucleus. Once in the nucleus, ERK can phosphorylate and activate transcription factors that may alter gene regulation (10).

Nonenzymatic glycation of circulating LDL occurs in both diabetic and nondiabetic subjects, correlating with other indexes of glycemic control, such as hemoglobin A1 glycosylate. Glycated LDL from diabetic subjects has adverse effects on cultured cells relevant to atherosclerosis, including cholesterol ester accumulation in monocyte-derived macrophages and procoagulant effects on endothelial cells (19, 41). To our knowledge, there are no published studies on the effects of glycated LDL with or without oxidation on cell signaling pathways leading to proliferation of VSMC. Glycation of LDL by our protocol is not associated with significant oxidation, as demonstrated by a lack of an increase in levels of conjugated dienes, fluorescence, and electrophoretic mobility (Table 1). Our results are in keeping with the presence of ERK-activating moiety in native LDL, which is not affected by glycation or mild oxidation.

The initiating and sustaining signals that link LDL receptor activation to MAPK regulation are not defined. On binding to its receptors in VSMC, native LDL induces a marked increase in inositol 1,4,5-triphosphate and also elicits a rise in intracellular calcium (33). A role for calcium as an intracellular signal mediating proliferation induced by various growth factors in different cell types has been established (3, 32). In VSMC, a rise in intracellular calcium has been implicated in proliferation and migration after endothelial injury; however, the mechanism through which calcium results in VSMC proliferation is as yet undefined (15). In the present study, we examined whether the rise in intracellular calcium and/or the influx of extracellular calcium evoked by LDL contributes to MAPK activation. Addition of EGTA to the extracellular medium significantly reduced the response of N-LDL, O-LDL, and GO-LDL to stimulate ERK phosphorylation, indicating that transmembrane influx of calcium contributes to ERK phosphorylation in response to LDL. However, when the rise in intracellular calcium was blocked by EGTA-AM, a further increase in MAPK phosphorylation was observed in response to N-LDL, O-LDL, GO-LDL, and HOG-LDL. This interesting finding was somewhat surprising because rises in intracellular calcium have been linked to activation of MAPK (4). In support of our findings, a recent study by Sachinidis et al. (34) has also shown that chelation of intracellular calcium resulted in a twofold increase in MAPK activation in response to native LDL. The authors speculate that the LDL-induced rise in intracellular calcium is implicated in the regulation of a MAPK phosphatase-1. Furthermore, a recent study by Vichi et al. (40) showed that endothelin-stimulated ERK activation in airway smooth muscle cells requires calcium influx, because chelation of extracellular calcium or preventing calcium influx inhibited ERK activation in response to endothelin, whereas inhibition of rises in intracellular calcium did not alter ERK activation in response to endothelin. Taken together, these findings suggest that both extracellular and rises in intracellular calcium modulate ERK activation in response to native and modified LDL. Although calcium influx contributes in the LDL signaling pathway leading to ERK activation, it is unlikely to be the only requirement for LDL to activate ERK.

The calcium-binding protein calmodulin is implicated in the regulation of many cellular pathways triggered by calcium, including progression of cell cycle and regulation of cell proliferation (17, 24). Calmodulin binds to and activates several cellular proteins in response to a rise in intracellular calcium. The results of the present study indicate that phosphorylation of MAPK in response to mildly and highly modified LDL is mediated through a calmodulin-dependent pathway. Support for this pathway comes from the findings that inhibition of calmodulin with cell-permeable inhibitors W-7 and/or calmidazolium significantly reduced ERK activation in response to native, mildly modified, and highly modified LDL.

In addition to calcium binding, phosphorylation of calmodulin may regulate its mode of action. Tyrosine phosphorylation of calmodulin by src kinases or the insulin receptor kinase was shown to enhance biological activity, whereas serine and threonine phosphory-
loration decreased calmodulin activity (35). In this regard, LDL could activate calmodulin by two distinct regulatory pathways, one through an increase in calcium influx and the other via activation of cytoplasmic tyrosine kinases such as protein tyrosine kinase 2 or focal adhesion kinase (22, 40).

Rises in intracellular calcium have also been shown to result in PKC activation. We reasoned that native and modified LDL through activation of their receptors would stimulate ERK phosphorylation via activation of PKC. In support of our notion, we observed that pretreatment of VSMC with PMA to downregulate PKC activity significantly decreased the increase in ERK phosphorylation in response to native, mildly modified, and highly modified LDL. This finding suggests that native, mildly modified, and highly modified LDL stimulate MAPK phosphorylation via a PKC-dependent mechanism and supports earlier findings that native and copper-oxidized LDL activate MAPK via PKC (20). The specific PKC isoform that is responsible for MAPK activation by LDL and/or the upstream second messengers that result in PKC activation by LDL are as yet undefined.

Tyrosine phosphorylation is an early signal required for growth responses that have intrinsic tyrosine kinase activity. Although the LDL receptor lacks an intrinsic tyrosine kinase domain, our findings indicate that LDL can recruit and activate cytoplasmic tyrosine kinases, which in turn stimulate tyrosine phosphorylation of a number of cytoplasmic proteins, leading to MAPK phosphorylation. To delineate the role of cytoplasmic tyrosine kinases in LDL-induced MAPK activation, we examined the effects of cell-permeable specific inhibitors of p60src kinase and MEK. Pretreatment of VSMC with the src kinase inhibitor did not significantly alter the phosphorylation of ERK in response to native, mildly modified, and heavily modified LDL, suggesting that src kinases are not involved in ERK activation in response to LDL stimulation. On the other hand, the MEK inhibitor PD-98059 completely blocked ERK phosphorylation induced by all six LDL preparations, implicating a role for MEK in ERK phosphorylation by all forms of LDL.

In summary, we report that native and modified LDL phosphorylate ERK in cultured aortic smooth muscle cells. The cellular mechanisms by which LDL stimulates ERK phosphorylation involve the calcium/calmodulin-dependent pathway, PKC, and MEK. Various components of the diabetic milieu, such as hyperglycemia, glycoxidation products, and, as we now demonstrate, LDL and its modification forms, may promote their deleterious effects via activation of the MAPK cascade. Better understanding of the molecular and cellular mechanisms by which LDL stimulate VSMC proliferation in vivo, a process obligatory to the development of vascular injury, could lead to the development of new strategies for intervention and treatment of atherosclerotic vascular disease.

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