Native and Modified LDL Activate Extracellular Signal–Regulated Kinases in Mesangial Cells

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Glycation and/or oxidation of LDL may promote diabetic nephropathy. The mitogen-activated protein kinase (MAPK) cascade, which includes extracellular signal–regulated protein kinases (ERKs), modulates cell function. Therefore, we examined the effects of LDL on ERK phosphorylation in cultured rat mesangial cells. In cells exposed to 100 µg/ml native LDL or LDL modified by glycation, and/or mild or marked (copper-mediated) oxidation, ERK activation peaked at 5 min. Five minutes of exposure to 10–100 µg/ml native or modified LDL produced a concentration-dependent (up to sevenfold) increase in ERK activity. Also, 10 µg/ml native LDL and mildly modified LDL (glycated and/or mildly oxidized) produced significantly greater ERK activation than that induced by copper-oxidized LDL ± glycation ($P < 0.05$). Pretreatment of cells with Src kinase and MAPK kinase inhibitors blocked ERK activation by 50–80% ($P < 0.05$). Native and mildly modified LDL, which are recognized by the native LDL receptor, induced a transient spike of intracellular calcium. Copper-oxidized (± glycation) LDL, recognized by the scavenger receptor, induced a sustained rise in intracellular calcium. The intracellular calcium chelator (EGTA/AM) further increased ERK activation by native and mildly modified LDL ($P < 0.05$). These findings demonstrate that native and modified LDL activate ERKs 1 and 2, an early mitogenic signal, in mesangial cells and provide evidence for a potential link between modified LDL and the development of glomerular injury in diabetes. *Diabetes* 49:2160–2169, 2000

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Ac-LDL, acetylated LDL; AGE, advanced glycation end product; BSA, bovine serum albumin, ERK, extracellular signal–regulated protein kinase; FLIPR, fluorimetric imaging plate reader; G-LDL, glycated LDL; GO-LDL, glycoxidized LDL; HO-LDL, highly oxidized LDL; HOG-LDL, highly oxidized glycated LDL; JDFI, Juvenile Diabetes Foundation International; LDLr, receptors for native LDL; LPC, lysophosphatidylcholine; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; mO-LDL, mildly oxidized LDL; N-LDL, native LDL; PBS, phosphate-buffered saline; PKC, protein kinase C; SFM, serum-free media; TBS, Tris-buffered saline; TGF, transforming growth factor.

Diabetes is the most common cause of end-stage renal disease in the Western world (1). Features of diabetic nephropathy include an alteration in glomerular cellularity, with early hypercellularity and late hypocellularity, thickened basement membranes, and increased mesangial matrix (2). Associations between elevated cholesterol levels, atherosclerosis, and the progression of renal damage are recognized (3–7). There is an extensive amount of literature suggesting that qualitative as well as quantitative abnormalities of the major cholesterol carrier LDL may contribute to its atherogenicity (7–11). More recently, LDL has been implicated in diabetic microvascular complications (12,13). Qualitative abnormalities of LDL occurring in diabetes include enhanced glycation (9,14), oxidation (14,15), and glycoxidation (combined glycation and oxidation) (9,11).

Modified lipoproteins may be involved in nephrosclerosis and glomerulosclerosis (15–23). Oxidized LDL has been demonstrated in renal glomeruli in vivo, and intravenous administration of in vitro–oxidized LDL results in renal injury and LDL accumulation (23). Diabetic nephropathy-like lesions have also been induced in a rat model by glycated and glycoxidized albumin (24–26). With use of cultured mesangial cells, cytotoxicity (23,27) and enhanced production of mesangial matrix (23) in response to LDL have been demonstrated. We previously demonstrated that a noncytotoxic dose of glycated LDL increases transforming growth factor (TGF)-β mRNA expression (28): TGF-β is a modulator of mesangial cell survival and matrix production (29,30).

Mitogen-activated protein kinases (MAPKs) are serine threonine–specific kinases that are activated in response to extracellular stimuli by dual phosphorylation at conserved tyrosine and threonine residues. Activation of the MAPK signaling pathways has been associated with effects relevant to vascular pathology in cultured cells implicated in macrovascular disease (31–33) and, more recently, in renal disease (34–36). Effects include stimulation or inhibition of cell proliferation (34,35), induction of apoptosis (37), and matrix production (36). There are at least three parallel series of MAPK cascades: *1*) p38 MAPK; *2*) stress-activated protein kinase/c-*jun*–NH_2-terminal kinase (both associated with apoptosis); and *3*) extracellular signal–regulated protein kinases (ERKs) 1 and 2 (subsequently referred to as ERK 1/2), which are associated with proliferative and protein synthetic responses (34).

Osmotic stress, nonenzymatic glycation, oxidative stress, and perhaps other hyperglycemia-induced changes thought to contribute to the chronic complications of diabetes share the capacity to activate MAPK (38). Cell signaling effects of

a broad spectrum of LDL types that may occur in vivo in diabetes have not been studied extensively. There is much "cross-talk" between the numerous cell signaling pathways, including between the MAPK and protein kinase C (PKC) pathways (34). The MAPK cascades are downstream to PKC, and activation of PKC by hyperglycemia is a well-recognized feature of diabetes (39,40).

Because mesangial cell activation and glomerulosclerosis are features of diabetic renal damage, we studied ERK phosphorylation in cultured rat mesangial cells exposed to LDL modified in vitro to resemble LDL that may be present in vivo in diabetes. A better understanding of the cellular signaling involved and the responses induced by the different types of LDL may provide a rationale for the development of preventive measures to retard diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Materials. Unless stated otherwise, materials were obtained from Sigma (St. Louis, MO).

LDL preparation and modification. LDL was prepared by a modification of the method previously described (12). Briefly, blood was drawn from five to six healthy fasting nondiabetic normolipidemic subjects. Subjects were nonsmokers who 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 was used to wash and concentrate the LDL. LDL was dialyzed extensively against nitrogen-purged 0.15 mol/l NaCl, 0.3 mmol/l EDTA, pH 7.4 at 4°C, in the dark. LDL was modified in vitro by glycation, by minimal oxidation, or by glycoxidation as previously described (12). Briefly, LDL was incubated with 0.3 mmol/l EDTA at 37°C, pH 7.4 for 3 days in the absence or presence of freshly prepared 50 mmol/l D-glucose. For normal and glycated LDL, 1 mmol/l *N*,*N*-bis[2-(bis[carboxymethyl]-amino)ethyl]glycine (DTPA) was also added and incubations were under nitrogen. For oxidized or glycoxidized LDL, incubations were under air. Modification was terminated by repeat extensive dialysis as described above. Markedly (i.e., highly) oxidized LDL and highly oxidized glycated LDL (HOG-LDL) were generated by buffer exchange of native LDL (N-LDL) and glycated LDL (G-LDL) into phosphate-buffered saline (PBS), pH 7.4, dilution to 3 mg/ml protein, and addition of $CuCl_2$ to a final concentration of 10 μmol/l for 24 h, under air at 37°C. Oxidation was terminated by the addition of EDTA (to a final concentration of 100 μmol/l) and extensive dialysis as described above. Thus, six types of LDL were prepared: N-LDL, G-LDL, mildly oxidized LDL (mO-LDL), glycoxidized LDL (GO-LDL), highly oxidized LDL (HO-LDL), and HOG-LDL. LDL preparations were sterile filtered (0.22 μm), stored in the dark under nitrogen at 4°C, and used within 1 day to 1 month of preparation. Seven 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, Walkersville, MD) according to the manufacturer's suggestion.

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). LDL degradation studies were also performed (details in RESULTS). We have previously demonstrated in other LDL preparations using this protocol that the early glycation product fructoselysine is increased severalfold in the glucose-exposed preparations, and that the glycoxidation/lipoxidation product carboxymethyllysine is increased in the glycoxidized LDL, and to a much greater extent, in copper-oxidized (± glycation) LDL preparations (41–43).

Mesangial cell culture. Rat mesangial cells were isolated and cultured as previously described (44). Briefly, glomerular cores were isolated from male 4-week-old nondiabetic Sprague-Dawley rats by differential sieving, and then they were cultured in plastic flasks (Costar; Corning, Corning, NY) in growth media consisting of RPMI 1640 (Cellgro; Mediatech, Herndon, VA), 17% (v/v) fetal calf serum (Bio-Whittaker), 1% (v/v) insulin-transferrin-selenite (Sigma) and 1% (v/v) penicillin-streptomycin (Cellgro). Mesangial cells were characterized by their typical stellate morphology on light microscopy, ability to grow in multilayers and hillocks, sensitivity to mitomycin C (10 μg/ml), positive staining for smooth muscle–specific actin, negative staining for factor VIII antigen (excluding endothelial cells), and for cytokeratin (excluding epithelial cells). Cells of passages three to six, from seven different cell lines, were used.

LDL signaling studies.

Time course. Cells were grown to confluence in six-well tissue culture plates (Costar; Corning), rendered quiescent by 24-h exposure to serum-free media (SFM), and then exposed to LDL at 100 μg/ml in SFM for 1–60 min. Three experiments were performed, with each condition being studied in duplicate.

Dose response. Cells prepared as above were exposed to 10–100 μg/ml LDL for 5 min. This concentration range was chosen as representative of LDL concentrations that mesangial cells may be exposed to in vivo in diabetes. A concentration of 100 μg/ml LDL protein corresponds to 10–20% of the normal circulating level of LDL. It is estimated that between 5 and 25% of circulating LDL may be nonenzymatically glycosylated in vivo (45). The concentrations of extravasated and extensively oxidized LDL in vivo are unknown. Three experiments were performed, with each condition being studied in duplicate.

Effects of Src kinase and MAPK kinase inhibitors on LDL-induced ERK activation. Cells prepared as above were preincubated with inhibitor for 30 or 60 min (as described in RESULTS) before the addition of LDL. An LDL dose of 25 μg/ml was chosen, because this concentration is the lowest within the plateau region of the dose response for ERK activation, and hence more likely to be representative of concentrations of modified LDL found in vivo in glomeruli. Three experiments were performed, with each condition being studied in duplicate.

Western blot. Cells were washed twice, scraped in PBS containing 2 mmol/l sodium vanadate, and centrifuged at 3,000*g* for 5 min. Pellets were resuspended in 100 μl lysis buffer (20 mmol/l Tris, 130 mmol/l NaCl, 10% glycerol, 10 mmol/l (3-[3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), 1 mmol/l phenylmethylsulfonyl fluoride, 2 mmol/l Na vanadate, 100 mU/ml aprotinin, 0.156 mg/ml benzamidin, 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. (46). Proteins were separated by SDS-PAGE (12%) under reducing conditions (47) and transferred to 0.45-mm polyvinylidene fluoride membranes at 300 mA for 2 h. The membranes were blocked for 30 min in 1% bovine serum albumin (BSA)–Tris-buffered saline ([TBS], 50 mmol/l Tris, 150 mmol/l NaCl, pH 7.5) at 37°C. The blocked membranes were blotted with antibodies against the phosphorylated form of ERK 1/2 (1:6,000) or total ERK 1/2 (1:4,000), diluted in TBS containing 1% BSA and 0.05% Tween 20 overnight, and then washed in TBS 0.05% Tween 20. The membranes were incubated in a secondary antibody conjugated to alkaline phosphatase. Immunoreactive bands were visualized using the chemiluminiscence reagent CDP Star (NEN Life Science Products, Boston, MA), according to the manufacturer's instructions. Membranes were exposed to Kodak LS film and bands measured by densitometry and quantified by NIH Image.

Intracellular calcium determination. Intracellular calcium release was assessed using a fluorimetric imaging plate reader (FLIPR) system (Molecular Devices, Sunnyvale, CA), a high-throughput optical screening system for cell-based fluorometric assays (48). Mesangial cells were grown to confluence in 96-well clear-bottom black microplates (Corning Costar Corp., Cambridge, MA) and rendered quiescent by 24-h exposure to SFM. Eight wells were used for each condition evaluated. Cells were dye loaded with 4 μmol/l Fluo-3 a.m. ester (excitation 488 nm, emission 540 nm; Molecular Probes, Eugene, OR) in a loading buffer (1 × Hanks' balanced salt solution buffer, 20 mmol/l HEPES, 2.5 mmol/l probenecid, pH 7.4) for 1 h at 37°C. After washing four times with loading buffer, cells were exposed in the FLIPR by the automatic simultaneous addition of 25 μg LDL. Intracellular calcium release was monitored over 4 min. The experiment was performed three times using different cell lines and LDL preparations.

LDL degradation studies. Aliquots of N-LDL, G-LDL, mO-LDL, GO-LDL, HO-LDL, or HOG-LDL were radiolabeled with ^{125}I-iodine as described previously (45). Mesangial cells were grown to confluence in 12-well culture plates. The growth medium was then removed, the cells were washed with 1 ml basal medium, and the cells were cultured for an additional 24 h in SFM. At the end of this incubation, the medium was replaced with 1 ml SFM containing ^{125}I-labeled N-LDL, G-LDL, mO-LDL, GO-LDL, HO-LDL, or HOG-LDL (10 μg/ml) and nonradiolabelled N-LDL or acetylated LDL (Ac-LDL) (250 μg/ml). The cultures were then incubated for 20 h at 37°C. After the incubation, the proteolytic degradation by mesangial cells of each ^{125}I-labeled LDL preparation was measured by assaying the amount of ^{125}I-labeled trichloroacetic-soluble (noniodide) material formed by the cells and excreted into the culture medium as described previously (45). Rates of total degradation were determined in incubations containing only ^{125}I-labeled LDL preparations and the rates of nonspecific degradation were determined in parallel incubations containing a 25-fold excess of nonradiolabeled N-LDL or Ac-LDL. Receptor-mediated degradation rates were calculated as the difference between total and nonspecific degradation rates. At the conclusion of each experiment,

TABLE 1
LDL characterization

Parameter	N-LDL	G-LDL	mO-LDL	GO-LDL	HO-LDL	HOG-LDL
Absorbance	1.0	0.9 (0.2)	1.2 (0.1)	1.1 (0.1)	2.3* (0.3)	2.4* (0.2)
Fluorescence	1.0	1.0 (0.1)	1.3 (0.1)	1.2 (0.1)	7.1* (0.7)	6.8* (0.5)
Electrophoretic mobility	1.0	1.0 (0.1)	1.0 (0.1)	1.0 (0.1)	3.5* (0.2)	3.7* (0.3)

Data are means ± SE of seven LCL preparations. The absorbance (234 nm), fluorescence (ex. 360, em. 430), and electrophoretic mobility of N-LDL and modified LDL at 100 µg/ml protein are shown and expressed as the ratio to N-LDL. The mean value of N-LDL absorbance was 0.94 U, fluorescence was 19.0 U, and the distance migrated from the origin on agarose gel electrophoresis was 7 mm. The absorbance, fluorescence, and electrophoretic mobility of HO- and HOG-LDL was significantly greater than that of N-LDL and mildly modified LDL, but that of N-, G-, mO-, and GO-LDL did not differ from each other. There was no significant difference in the extent of modification of HO- and HOG-LDL. *$P < 0.05$.

each culture was washed with twice with 1 ml PBS containing 1% (w/v) BSA and twice with PBS. The cells were then dissolved in 1 ml 0.2 mol/l NaOH for determination of cellular protein content (49).

Statistical analysis. Results were expressed as the mean ± SE, and statistical significance was assessed by one-way analysis of variance according to the method of Fisher.

RESULTS

LDL characterization. Agarose gel electrophoresis of LDL showed a single band, demonstrating lack of contamination by other lipoprotein subclasses. Results of the conjugated dienes (absorbance 234 nm), fluorescence, and electrophoretic mobility are shown in Table 1. As expected, the electrophoretic mobility of highly modified LDL (HO-LDL and HOG-LDL) was increased, but that of mildly modified LDL (G-LDL, mO-LDL, and GO-LDL) did not differ from N-LDL. Conjugated dienes and fluorescence were significantly higher in HO- and HOG-LDL versus N-LDL, G-LDL, mO-LDL, and GO-LDL. In RESULTS to follow, LDL degradation studies provide further LDL characterization.

To determine whether the LDL pools that 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,

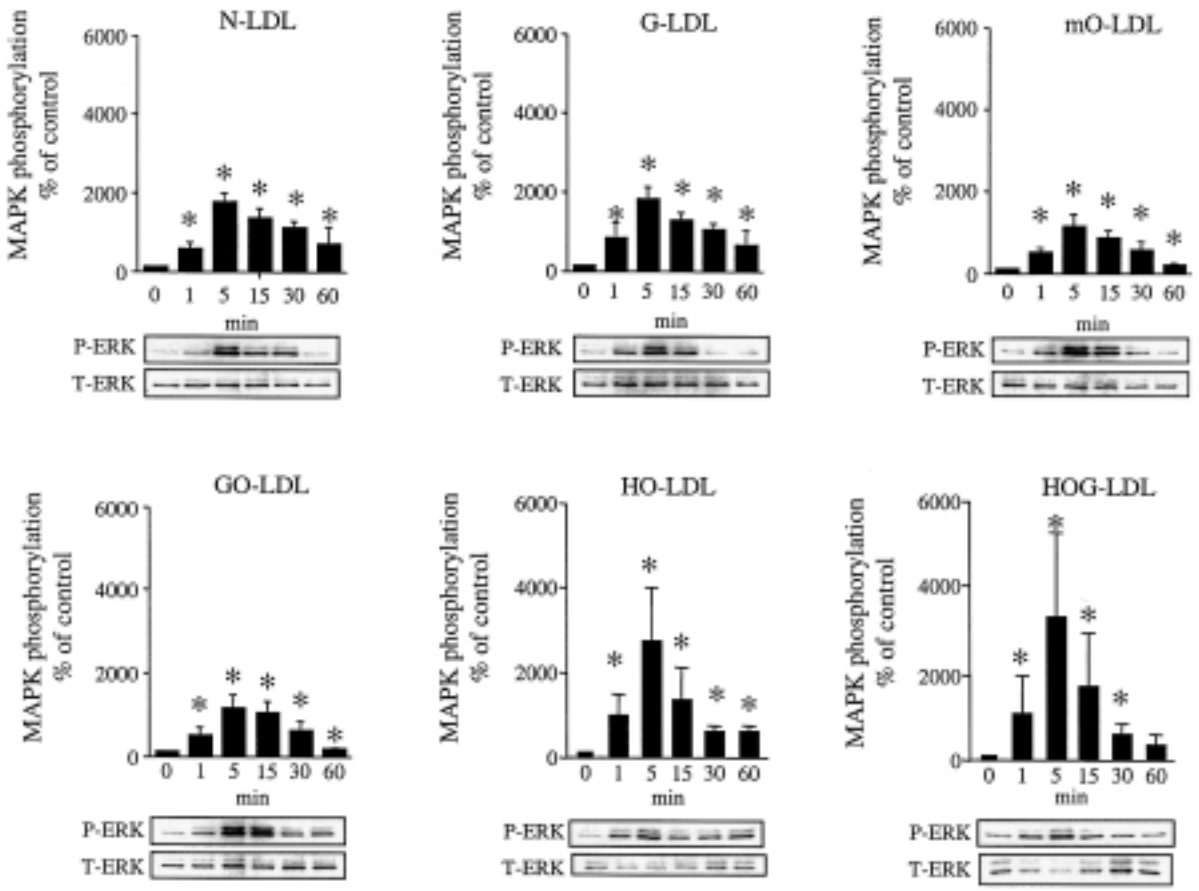

FIG. 1. Time course of LDL induction of MAPK phosphorylation. Mesangial cells were incubated for 1–60 min with 100 µg/ml LDL protein. Cell proteins were separated by SDS-PAGE (12%) and immunoblotted with a polyclonal antibody against phosphorylated (P-) ERK (1:6,000) and total (T-) ERK (1:4,000). Bars represent means ± SE of three separate experiments. Densitometric calculations of P-ERK are corrected for T-ERK. *$P < 0.05$ vs. control (0 min).

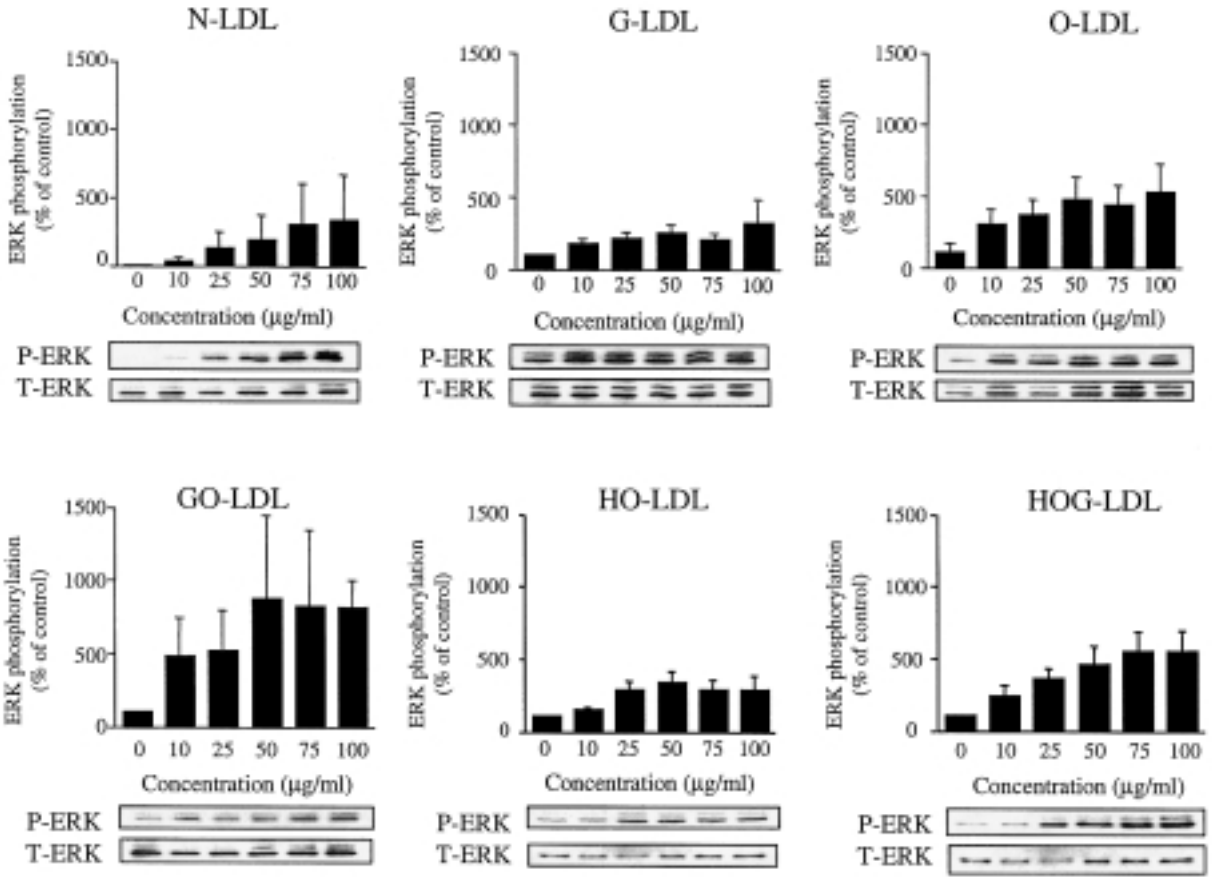

FIG. 2. LDL induces a dose-dependent increase in MAPK phosphorylation. N-LDL and modified LDL differ in their ability to induce ERK 1/2 phosphorylation at low concentrations. Mesangial cells were incubated for 5 min with 10–100 μg/ml of N-LDL, G-LDL, mO-LDL, GO-LDL, HO-LDL, or HOG-LDL. Cell proteins were separated by SDS-PAGE (12%) and immunoblotted with a polyclonal antibody against phosphorylated (P-) ERK (1:6,000) and total (T-) ERK (1:4,000). Bars represent means ± SE of three separate experiments. Densitometric calculations of P-ERK are corrected for T-ERK. *P < 0.05 vs. control (0 min).

mO-LDL, GO-LDL, HO-LDL, and HOG-LDL from three different pools of LDL prepared at different times. The results showed that all LDL pools were negative for endotoxin contamination. This finding eliminates the possibility that the responses we observed on ERK activation are mediated by endotoxin contamination of LDL.

Native and modified LDL induce ERK 1/2 phosphorylation in a time-dependent manner. Quiescent mesangial cells were incubated with 100 μg/ml of normal (N-LDL) or modified (G-LDL, mO-LDL, GO-LDL, HO-LDL, or HOG-LDL) for 1, 5, 15, 30, and 60 min. For each LDL preparation (Fig. 1), ERK 1/2 phosphorylation increased at 1 min, peaked at 5 min, and subsequently decreased, but did not reach baseline by 60 min. In addition, the magnitude of the peak activation in ERK was greater in response to highly modified LDLs (Ho-LDL and HOG-LDL) compared with minimally modified LDLs (G-LDL, mO-LDL, and GO-LDL). In all subsequent experiments, 5-min stimulation was used.

Native and modified LDLs differ in their ability to induce ERK 1/2 phosphorylation at low concentrations. Mesangial cells were incubated for 5 min with 10, 25, 50, 75, and 100 μg/ml of normal LDL (N-LDL) or modified LDL (G-LDL, mO-LDL, GO-LDL, HO-LDL, or HOG-LDL). Dose-response curves (Fig. 2) showed that all LDLs induced a dose-dependent increase in ERK 1/2 tyrosine phosphorylation. At 100 μg/ml, the effect of all LDLs on ERK 1/2 phosphorylation was similar, inducing a three- to sevenfold increase in ERK 1/2 phosphorylation versus unstimulated cells. At 10 μg/ml, mO-LDL and GO-LDL tended to induce a higher increase in ERK phosphorylation than N-LDL and G-LDL, but the differences did not reach statistical significance. At 10 μg/ml, the highly modified LDLs (HO-LDL and HOG-LDL) were significantly less potent than native and mildly modified LDLs, $P < 0.05$, inducing a 1.3- to 2.5-fold increase in ERK phosphorylation. These results suggest a higher sensitivity of the receptor and associated MAPK cascade for native and mildly modified LDLs than for highly modified LDL, and that glycation per se does not alter ERK 1/2 phosphorylation.

Time and dose-response effects did not differ among LDL pools according to the time elapsed between LDL preparation and cell exposure.

ERK phosphorylation induced by highly modified LDLs involves both Src kinase and MAPK kinase. Mesangial cells were preincubated with either the cell-permeable Src kinase inhibitor PP1 (10 μmol/l, 1 h) or the cell-permeable MAPK kinase (MEK) inhibitor PD 98059 (PD, 40 μmol/l, 30 min) followed by stimulation for 5 min with 25 μg/ml of each LDL. ERK 1/2 phosphorylation was significantly inhibited by PP1 only when cells were incubated with the highly modified LDLs (HO-LDL and HOG-LDL) (Fig. 3). ERK 1/2 activation by

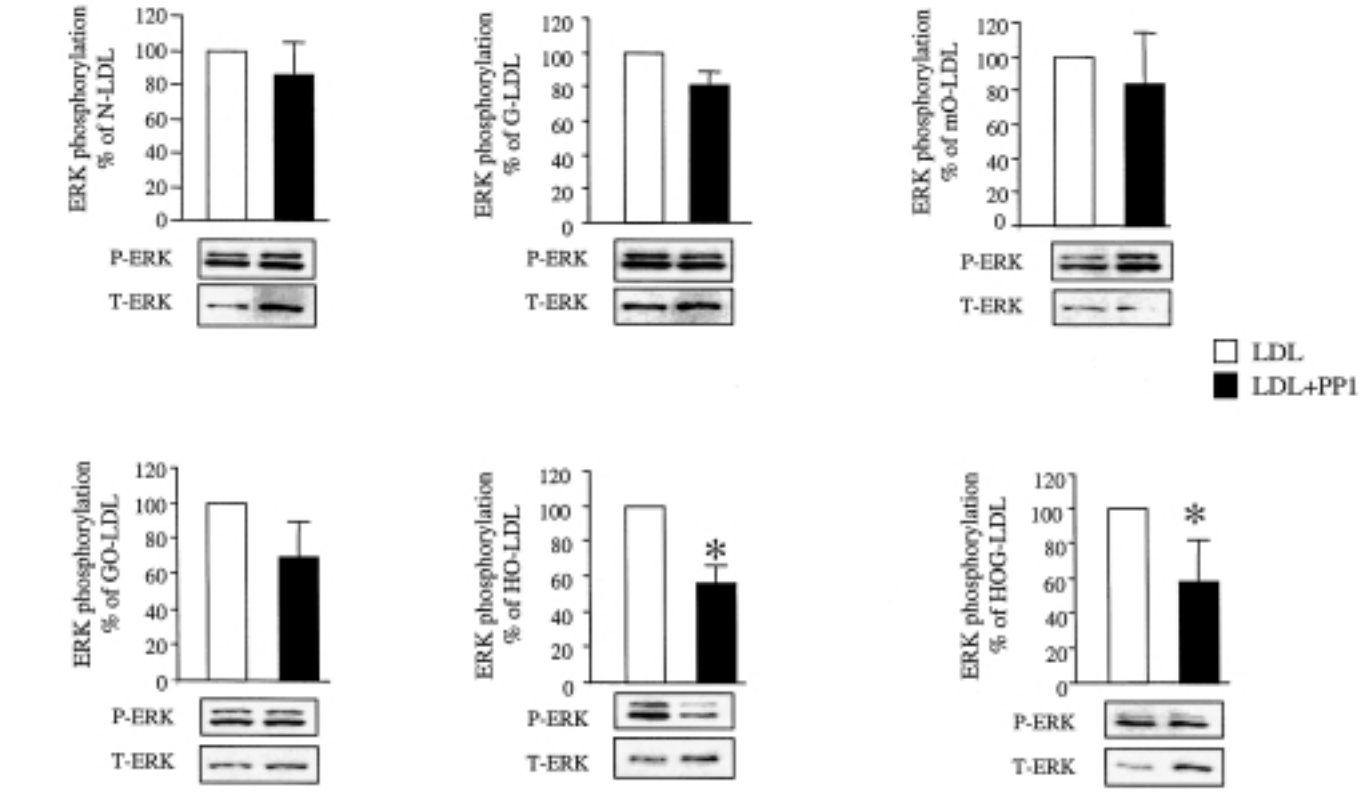

FIG. 3. ERK 1/2 phosphorylation induced by highly modified LDL is significantly inhibited by a Src kinase inhibitor. Mesangial cells were preincubated with the Src kinase inhibitor PP1 (10 µmol/l, 1 h) and incubated for 5 min with 25 µg/ml of each LDL. Cell proteins were separated by SDS-PAGE (12%) and immunoblotted with a polyclonal antibody against phosphorylated (P-) ERK (1:6,000) and total (T-) ERK (1:4,000). Bars represent means ± SE of three separate experiments. Densitometric calculations of P-ERK are corrected for T-ERK. *P < 0.05 vs. LDL.

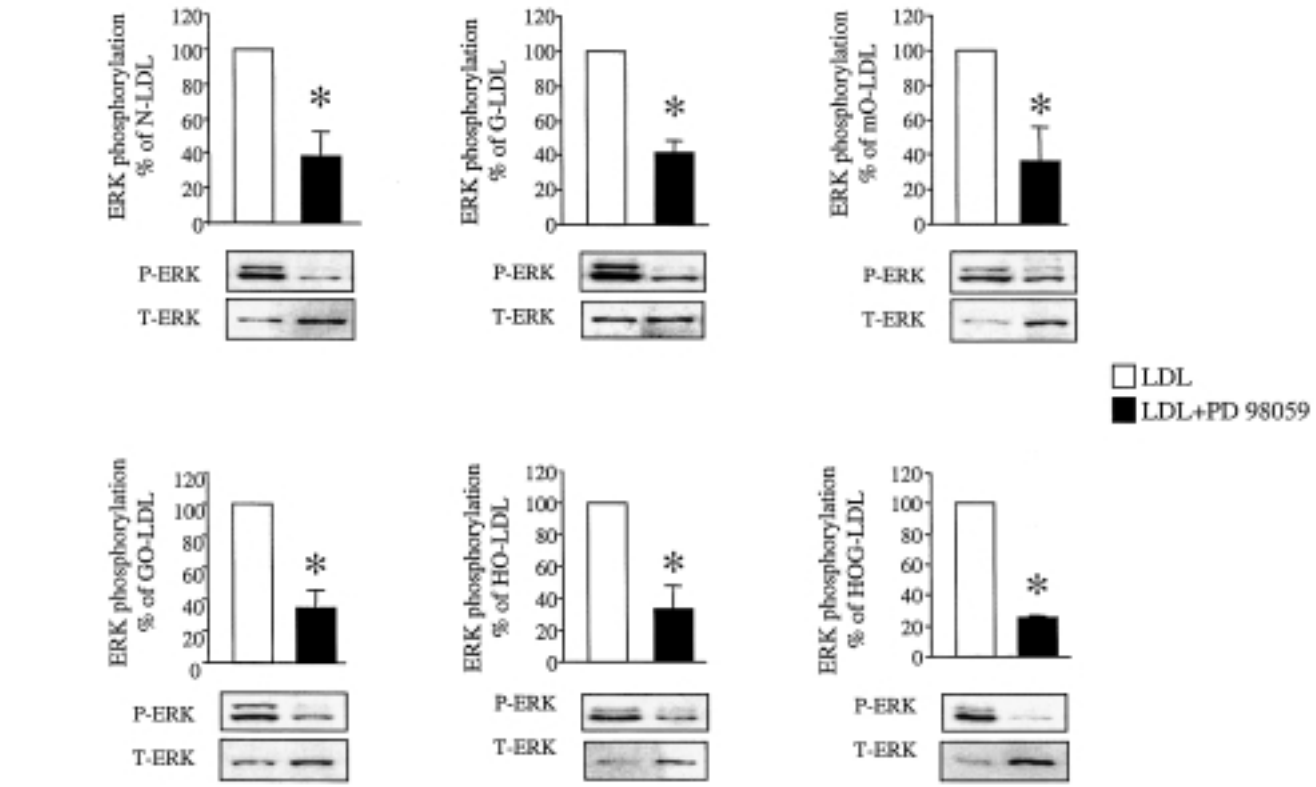

FIG. 4. LDL-induced ERK 1/2 phosphorylation is significantly inhibited by an MEK inhibitor. Mesangial cells were preincubated with the MEK inhibitor PD98059 (PD, 40 µmol/l, 30 min) and incubated for 5 min with 25 µg/ml of each LDL. Cell proteins were separated by SDS-PAGE (12%) and immunoblotted with a polyclonal antibody against phosphorylated (P-) ERK (1:6,000) and total (T-) ERK (1:4,000). Bars represent means ± SE of three separate experiments. Densitometric calculations of P-ERK are corrected for T-ERK. *P < 0.05 vs. LDL.

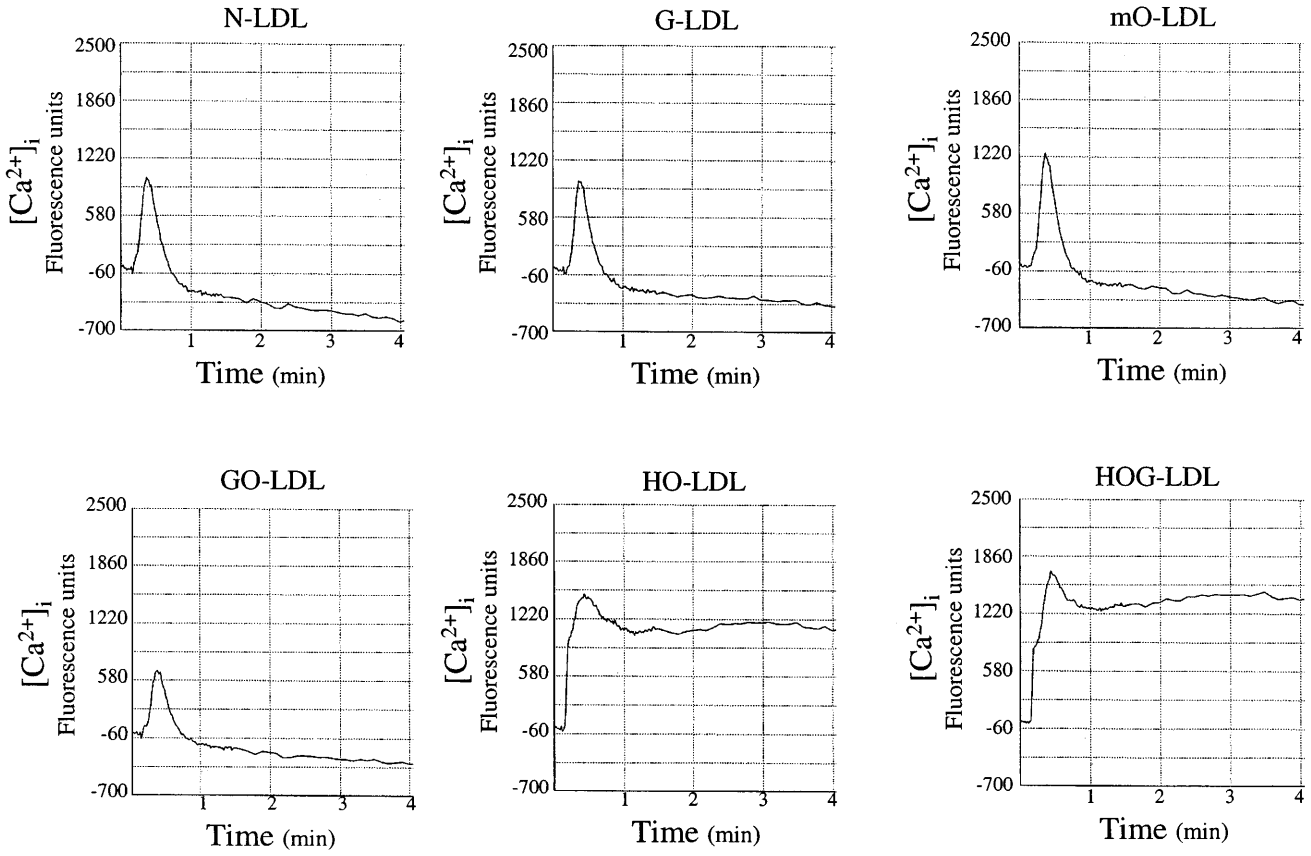

FIG. 5. Different patterns of intracellular calcium release in response to N-LDL and mildly modified LDL versus highly modified LDL. Confluent quiescent mesangial cells in a 96-well plate were dye loaded with 4 µmol/l Fluo-3 a.m. ester and exposed to 25 µg of each LDL preparation. Intracellular calcium release was monitored in the FLIPR over 4 min. Eight wells were used for each condition evaluated. The experiment was performed three times using different cell lines and LDL preparations. A representative tracing is shown.

N-LDL and mildly modified LDL was not blocked. However, PD 98059 blocked ERK 1/2 phosphorylation induced by all six LDL preparations (Fig. 4), implicating a role for MEK in activating ERK. These results suggest that highly oxidized LDLs activate ERK through a Src kinase–dependent pathway, whereas N-LDL and mildly modified LDL activate ERK via a Src kinase–independent mechanism.

Role of calcium in LDL-induced ERK phosphorylation. N-LDL and mildly modified LDL induced a transient spike of intracellular calcium, whereas highly modified LDL induced a sustained rise (Fig. 5). It has been shown that G-protein–coupled receptors activate MAPK in cultured aortic smooth muscle cells via rises in intracellular calcium (50). To explore the potential role for calcium in LDL-induced ERK activation, mesangial cells were preincubated with the intracellular calcium chelator EGTA/AM (50 µmol/l, 30 min) and then exposed to 25 µg/ml LDL for 5 min. In the absence of chelator, N-LDL and mildly modified LDL increased ERK phoshorylation fivefold above untreated cells. However, in the presence of EGTA/AM, ERK activation was increased to a significantly greater extent by N-LDL and mildly modified LDL (Fig. 6). Intracellular calcium chelation also tended to increase ERK activation by highly modified LDL, but these changes did not reach statistical significance (Fig. 6).

Receptor uptake. The extent of modification of each LDL preparation was also characterized by determining its uptake and degradation via receptors for N-LDL (LDLr) compared with scavenger receptors. As shown in Fig. 7*A*, a majority of the degradation of N-LDL, G-LDL, mO-LDL, and GO-LDL was via LDLr, and rates of receptor-mediated degradation were similar for N-LDL, G-LDL, mO-LDL, and GO-LDL. This finding suggests that the extent of modification of G-LDL, mO-LDL, and GO-LDL was not sufficient to reduce its recognition by LDLr by mesangial cells. In contrast, HO-LDL and HOG-LDL were not recognized by LDLr (Fig. 7*A*). As shown in Fig. 7*B*, the scavenger receptor accounted for very little of the degradation of N-LDL and the mildly modified preparations but was responsible for most of the degradation of the two heavily modified preparations. This finding confirms the much more severe modification of HO-LDL and HOG-LDL.

DISCUSSION

The receptor and signaling pathways through which LDL, as modified in diabetes, can produce effects on mesangial cells are not fully elucidated. In the present manuscript, we demonstrate that N-LDL and LDL modified by glycation and/or oxidation induce ERK 1/2 activation in cultured mesangial cells in a time- and concentration-dependent manner. We demonstrate that N-LDL and mildly modified LDL differ from highly modified (copper-oxidized) LDL in several ways: capacity for ERK activation at low concentrations, effects on proximal signaling pathways, patterns of intracellular calcium release, and receptor uptake.

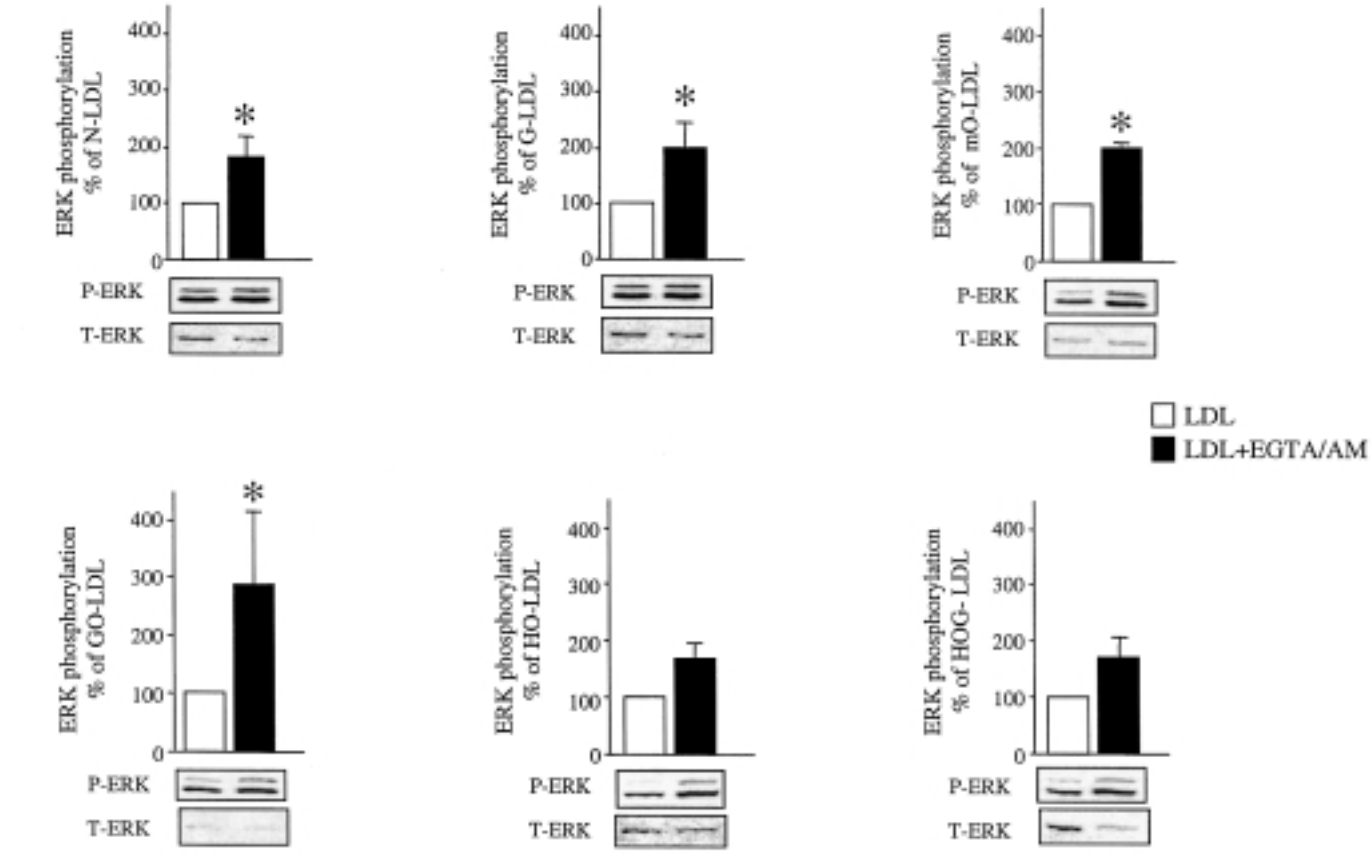

FIG. 6. LDL-induced ERK 1/2 phosphorylation is modulated via a calcium-dependent pathway. Mesangial cells were preincubated with the intracellular calcium chelator EGTA/AM (50 µmol/l, 30 min) and incubated for 5 min with 25 µg/ml of each LDL. Cell proteins were separated by SDS-PAGE (12%) and immunoblotted with a polyclonal antibody against phosphorylated (P-) ERK (1:6,000) and total (T-) ERK (1:4,000). Bars represent means ± SE of three separate experiments. Densitometric calculations of P-ERK are corrected for T-ERK. *P < 0.05 vs. LDL.

In agreement with Bassa et al. (35), we demonstrated ERK activation by N-LDL and metal-oxidized LDL in a time- and dose-dependent manner. We studied a broader range of LDL modifications of potential relevance to diabetic renal damage, including glycated LDL ± mild and marked oxidation. Our times of peak ERK activation differed from those of Bassa et al., as did our pattern of response to increasing doses of oxidized LDL. We demonstrated that all LDL types show the same time course of ERK activation in mesangial cells, peaking at 5 min. The earliest time point evaluated by Bassa et al. was 15 min (35). Bassa et al. found that LDL-induced ERK activation was less at higher concentrations (50 µg/ml) than at at 10 and 25 µg/ml for all preparations of LDL (35). In doses up to 100 µg/ml, we did not find any decrease in ERK 1/2 activation. We also demonstrated that at low doses (10 µg/ml), the potency of N-LDL and mildly modified LDL was equal, and greater than that of highly modified LDL. In contrast, Bassa et al. (35) showed that relative to N-LDL, mildly ($FeSO_4$) oxidized and highly ($CuSO_4$ and Hams media) oxidized LDL were more potent activators of ERK 2 in transformed murine mesangial cells (35). The differences may reflect the different mesangial cells used, different methods of LDL oxidation, and different methods of assessing ERK activation. Bassa et al. also demonstrated MAPK activation after 5- to 30-min exposure to the lipid oxidation product lysophosphatidylcholine (LPC) (35). We did not measure LPC levels in our LDL preparations, but we would anticipate them to be much higher in the highly oxidized LDL preparations compared with N-LDL, G-LDL, and mO-LDL based on the results of other groups using similar oxidation protocols (51). Glycation of LDL by our protocol is not associated with significant oxidation, as demonstrated by a lack of increase in levels of conjugated dienes, fluorescence, and electrophoretic mobility in this study (Table 1) or by a decrease in levels of lipid-soluble antioxidants, or increase in thiobarbituric acid reducing substances (TBARS) or carboxymethyllysine in our other research ([12,41–43]; T.J.L., unpublished observations). Our results are in keeping with the presence of ERK-activating moities in both N-LDL and modified LDL.

Relatively small amounts of N-LDL and various types of modified LDL may trigger mesangial cell signaling cascades pertinent to renal damage. It has been suggested that oxidative stress, protein modification by glycation, advanced glycation end products (AGEs), and binding to the receptor for AGEs trigger MAPK activity (52–54). In our LDL-mesangial cell system, glycation of LDL alone or in combination with mild or marked oxidation did not increase ERK 1/2 phosphorylation versus N-LDL or HO-LDL. LDL glycation may still have deleterious effects on mesangial cell function. Mesangial cell responses of relevance to diabetic renal damage include increased TGF-β expression and glomerulosclerosis (2,29). We have previously demonstrated increased rat mesangial cell TGF-β expression in response to glycated LDL versus N-LDL and the other types of LDL evaluated in this manuscript (28), but the cell signaling pathways involved have not been fully elucidated (13,28).

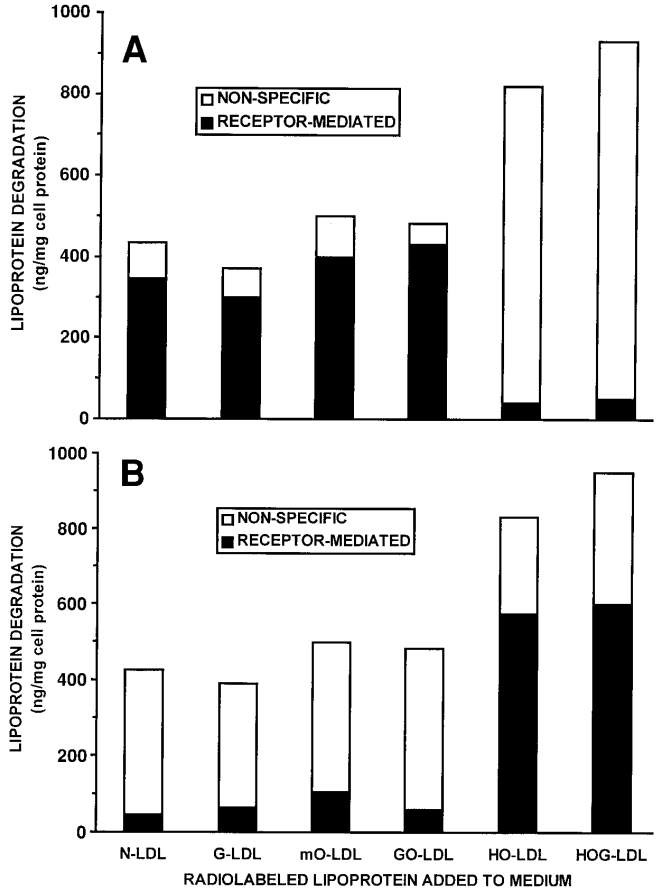

FIG. 7. Rates of receptor-mediated and nonspecific degradation of ^{125}I-labeled N-LDL, G-LDL, mO-LDL, GO-LDL, HO-LDL, or HOG-LDL via LDLr-mediated (*A*) and scavenger receptor–mediated (*B*) pathways in rat mesangial cells. Each radiolabeled LDL preparation (10 µg/ml) was incubated alone for 20 h with rat mesangial cells and with a 25-fold excess of nonradiolabeled N-LDL (*A*) or Ac-LDL (*B*). The rates of receptor-mediated and nonspecific LDL degradation were determined as detailed in RESEARCH DESIGN AND METHODS. Experiments were performed in triplicate. The mean results are shown for two studies using two different LDL preparations and two different mesangial cell lines.

These results suggest that our earlier finding of TGF-β induction by G-LDL may not be mediated by ERK activation. We have evidence to suggest that increased TGF-β expression induced by bradykinin is linked to MAPK activation (A.A.J., unpublished observations). In ongoing studies, we are evaluating the role of MAPK cascades on gene expression in cultured mesangial cells exposed to N-LDL and modified LDL.

The cellular mechanisms by which N-LDL and modified LDL activate ERK 1/2 appear to involve both Src kinases and MEK, because pretreatment with inhibitors to these signaling molecules can block LDL-induced ERK activation. The MEK inhibitor PD98059, which acts distally in the ERK cascade, mitigated the effect of all six types of LDL studied. For PP1, an inhibitor of Src kinase, which is upstream of ERK and MEK, there were divergent responses between the HO-LDL and N-LDL or mildly modified LDL. PP1 only inhibited ERK activation by HO-LDL and HOG-LDL, suggesting that highly modified LDLs signal via Src, but N-LDL and G-LDL and/or mO-LDL do not. Confirmation is required by direct examination of the effects of LDL on these signaling molecules.

The intracellular calcium chelator EGTA/AM potentiated ERK activation by N-LDL and mildly modified LDL, with less potent effects on activation by highly modified LDL. This study investigated effects of a broader spectrum of LDL types than has previously been studied. The data suggest that an increase in intracellular calcium reduces ERK activation in mesangial cells. In contrast, Stiko et al. (55) observed that oxidized LDL activated phospholipase C, increased intracellular calcium, and activated MAPK in vascular smooth muscle cells. Our results suggest that calcium-dependent pathways are activated in response to LDL exposure, and that a rise in intracellular calcium may activate tyrosine phosphatases in addition to ERK.

N-LDL and mildly modified LDL induced transient rises in intracellular calcium levels, whereas HO-LDL with or without glycation induced a sustained rise in intracellular calcium. Using similar concentrations of N-LDL (only), Pahl et al. (56) demonstrated a similar transient increase in intracellular calcium in rat mesangial cells. This was predominantly due to influx of extracellular calcium (56). We have evaluated intracellular responses to a wider range of LDL types and demonstrated different responses induced by N-LDL and mildly modified LDL versus highly modified LDL. Calcium influx into cells has been associated with apoptosis (57). Using similar LDL preparations, we previously demonstrated that 3 days' exposure of cultured rat mesangial cells to N-LDL and mildly modified LDL does not affect cell viability, but HO-LDL (with and without glycation) are toxic, with cell death occurring by apoptosis ([43]; T.J.L., unpublished observations). The observed differences in calcium responses to short-term LDL exposure (in this series) may be in keeping with the divergent effects on cell viability with longer-term LDL exposure (43).

Based on responses to signaling inhibitors, N-LDL and LDL modified by glycation and/or mild oxidation activate ERK 1/2 via the same proximal pathways, but the two highly modified LDL preparations HO-LDL and HOG-LDL signal via distinct proximal MAPK cascades. Recent studies have provided evidence for several different types of receptors on mesangial cells for N-LDL and modified LDL. These include the LDLr, the scavenger or Ac-LDL receptor (58), and a receptor for AGEs (59). We have demonstrated that in rat mesangial cells, N-LDL and mildly modified LDL are taken up by LDLr, and HO-LDL and HOG-LDL are taken up by the Ac-LDL receptor. The observed pattern of response to the normal and mildly modified LDL versus the highly modified LDL is in keeping with linkage of LDLr with ERK via MEK, but not Src kinase, whereas the Ac-LDL receptor may be linked with ERK via Src kinase and MEK. LDL signaling via LDLr is associated with a transient rise in intracellular calcium, whereas LDL taken up by the Ac-LDL receptor is associated with a sustained rise in intracellular calcium.

In summary, we report that N-LDL and modified LDL activate ERK 1/2, an early mitogenic signal, in cultured mesangial cells, in a time- and concentration-dependent manner. There are divergent effects of N-LDL and mildly modified LDL versus highly modified LDL on the potency of ERK activation at low LDL concentrations, on proximal signaling pathways, on the pattern of intracellular calcium flux, and on LDL receptor interaction. LDL may potentiate renal damage in both diabetic and nondiabetic subjects. MAPK inhibitors have been suggested as potential therapeutic agents for renal disease, and for mitigating the widespread chronic complications of diabetes (34,38). Various components of the dia-

betic milieu, such as hyperglycemia, AGEs, and—as we now demonstrate—N-LDL and modified LDL, may promote their deleterious effects via activation of the MAPK cascades. However, we find no evidence of a specific defect relating to LDL glycation in the cell-signaling parameters evaluated. These findings and further studies should be taken into consideration when targeting cell-signaling responses as a potential means of inhibiting diabetic nephropathy.

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