

LDLs induce fibroblast spreading independently of the LDL receptor via activation of the p38 MAPK pathway[§]

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Abstract Because adventitial fibroblasts play an important role in the repair of blood vessels, we assessed whether elevation in LDL concentrations would affect fibroblast function and whether this depended on activation of intracellular signaling pathways. We show here that in primary human fibroblasts, LDLs induced transient activation of the p38 mitogen-activated protein kinase (MAPK) pathway, but not the c-Jun N-terminal kinase MAPK pathway. This activation did not require the recruitment of the LDL receptor (LDLR), because LDLs efficiently stimulated the p38 MAPK pathway in human and mouse fibroblasts lacking functional LDLR, and because receptor-associated protein, an LDLR family antagonist, did not block the LDL-induced p38 activation. LDL particles also induced lamellipodia formation and cell spreading. These effects were blocked by SB203580, a specific p38 inhibitor. Our data demonstrate that LDLs can regulate the shape of fibroblasts in a p38 MAPK-dependent manner, a mechanism that may participate in wound healing or vessel remodeling as in atherosclerosis.—Dobreva, I., G. Waeber, V. Mooser, R. W. James, and C. Widmann. LDLs induce fibroblast spreading independently of the LDL receptor via activation of the p38 MAPK pathway. *J. Lipid Res.* 2003. 44: 2382–2390.

Supplementary key words remodeling • cell spreading • lamellipodia • p38 mitogen-activated protein kinase • low density lipoprotein • lipoproteins • low density lipoprotein receptor • fibroblasts

Complications of atherosclerosis are the most common cause of death in Western societies. In addition to aging, elevated levels of lipoproteins are the major risk factor for the development of the disease, and they are sufficient to induce atherosclerosis in the absence of other risk factors (1). Among all types of lipoproteins circulating in the blood, LDLs are considered to be the main atherogenic class (2), but despite the indisputable epidemiologic association of LDLs and atherosclerosis, the mechanisms un-

derlying the development of the disease are still poorly understood.

The best-characterized function of LDLs is to deliver cholesterol to cells. They may, however, have functions in addition to transporting cholesterol. For example, they seem to produce a mitogenic effect on endothelial cells, smooth muscle cells, and fibroblasts, and induce growth-factor production, chemotaxis, cell proliferation, and cytotoxicity (3). Moreover, an increase of LDL plasma concentration, which is observed during the development of atherosclerosis, can activate various mitogen-activated protein kinase (MAPK) pathways, including the two stress-activated protein kinase (SAPK) pathways, the c-Jun N-terminal kinase (JNK) and the p38 MAPK pathways (3–5).

The functions of LDLs in blood vessels have been assessed mainly through studies of their effect in endothelial and smooth-muscle cells. There is a third cell type found in the adventitia of blood vessels that has been studied very little so far, the cells of the fibroblastic lineage. Because it appears that these cells play a more important role in blood vessels than initially anticipated (6), we assessed here the potential signaling properties of LDLs in a fibroblast cell model. Using LDL preparations that were thoroughly controlled and tested for their biological activity, we demonstrate that LDLs induce the activation of p38 MAPKs independently of LDL receptors (LDLRs). We also show that the activation of the p38 MAPKs is required for LDL-induced fibroblast spreading, a mechanism that could potentially participate in atherogenesis.

Abbreviations: FACS, fluorescent-activated cell sorter; FCS, fetal calf serum; FH, familial hypercholesterolemia; HS, human serum; JNK, c-Jun N-terminal kinase; LDLR, LDL receptor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; RAP, receptor-associated protein; SAPK, stress-activated protein kinase; SREBP, sterol-regulatory element binding protein.

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EXPERIMENTAL PROCEDURES

Materials

DL-[3-¹⁴C]HMG-CoA was purchased from Amersham Pharmacia. HMG-CoA, glucose-6-phosphate dehydrogenase, and β-NADP were from Sigma. Anti-phospho-p38 (Thr180/Tyr182) [catalog no. 9211] and anti-p38 MAPK antibodies [catalog no. 9212] were purchased from Cell Signaling Technology. SB203580 [4-(4'-fluorophenyl)-2-(4'-methylsulfinylphenyl)-5-(4'-pyridyl imidazole)] was from Promega (catalog no. V1161). Recombinant receptor-associated protein (RAP) was produced by Dr. Johannes Nimpf, University and Biocenter of Vienna, Austria.

Cell culture

GM00316B and GM00488C human skin fibroblasts were purchased from Coriell Cell Repositories and maintained in DMEM supplemented with 15% nondepleted fetal calf serum (FCS) (Amimed) at 37°C and 5% CO₂. GM00316B cells were derived from a healthy 12-year-old Caucasian male subject and GM00488C cells were derived from an 11-year-old Caucasian male subject suffering from homozygous familial hypercholesterolemia (FH). Mouse embryonic fibroblasts (MEFs) lacking the LDLR were isolated from E12 embryos of the B6.129S7-*Ldl^{y⁰/Her}* mouse strain (The Jackson Laboratory, stock number 002207; background strain: C57BL/6J). Control MEFs were isolated from E12 C57BL/6J embryos. Briefly, 12 days-gestant females were killed by cervical dislocation, and the embryos were then removed from the placenta and placed in a 10 cm Petri dish containing 10 ml of ice-cold PBS [116 mM NaCl, 10.4 mM Na₂HPO₄, 3.2 mM KH₂PO₄ (pH 7.25)], where their livers were dissected out and discarded. The embryos were then minced and transferred to a bottle containing 200 ml of HBSS without calcium and magnesium (5.4 mM KCl, 0.44 mM KH₂PO₄, 137 mM NaCl, 3.4 mM Na₂HPO₄, and 4.2 mM NaHCO₃), 0.05% trypsin, 0.53 mM EDTA-4 Na, 5.5 mM glucose, and 28 μM phenolsulfonphthalein (phenol red) (Gibco; catalog no. 25300-062) for 2–3 h. The trypsinized cells were then collected by centrifugation, washed twice in cold PBS, resuspended in DMEM supplemented with 10% newborn calf serum, and 100 U/ml penicillin/streptomycin and plated in 10 cm Petri dishes (each dish containing the cells derived from one embryo).

LDL and lipoprotein-free serum preparation and purification

LDLs were isolated from human serum (HS) by sequential density ultracentrifugation as described previously (7, 8) and dialyzed for 48 h against PBS containing 100 μM EDTA. Human lipoprotein-free serum was prepared by removal of lipoproteins by ultracentrifugation at a density of 1.23 g/l (8). The protein concentration was measured by Bradford assay using BSA as a standard. The oxidation of the lipoproteins was measured by the lipoperoxide method (9). Analysis of lipopolysaccharide (LPS; endotoxin) contents was performed by the ENDOTELL AG company (Allschwil, Switzerland) using their kinetic turbidimetric Limulus amoebocyte lysate. LDL preparations used in the present study had undetectable LPS levels (<26 pg/ml). These LDL preparations had the expected biological activities, as demonstrated by their capacity for inhibiting the binding of fluorescent LDLs to normal human fibroblasts and by their ability to suppress the activity of the HMG-CoA reductase (data not shown).

Internalization of fluorescent LDLs

GM00316 and GM00488 cells were starved for 4 h in DMEM without serum and then treated with 10 μg/ml fluorescent human LDLs (LDL-BODIPY; Molecular Probes, catalog no. L-3483; excitation at 515 nm and emission at 522 nm) in the presence or

in the absence of 100 μg/ml unlabeled LDLs. The cells were washed once in cold PBS, and images were taken on live cells with an inverted Leica DMIRB microscope equipped for epifluorescence imaging using the Openlab software (Improvision). Quantitation of the cell-associated fluorescence was performed using a fluorescent-activated cell sorter (FACS) (Becton Dickinson FACSscan).

HMG-CoA-reductase assay

Cells were starved for 24 h in DMEM containing 2.5 mg/ml lipoprotein-free serum and incubated with 50 μg/ml LDL for 12 h. The HMG-CoA-reductase activity was measured according to Brown and Goldstein (10). Briefly, the cells were scraped in 1 ml of buffer A [0.05 M Tris-HCl (pH 7.4) and 0.15 M NaCl] and collected by centrifugation at 900 g for 3 min. The pellet was dissolved in 200 μl of buffer B [50 mM potassium phosphate (pH 7.4), 5 mM EDTA, 0.2 M KCl, and 0.25% Triton X-100]. After 10 min incubation at 37°C, the lysate was precleared by centrifugation at 12,000 g for 1 min. Protein concentration was measured by Bradford assay using BSA as a standard. Aliquots of the supernatant (20–100 μg protein) were incubated 2 h at 37°C in a final volume of 200 μl of 0.1 M potassium phosphate (pH 7.4), 20 mM glucose-6-phosphate, 2.5 mM β-nicotinamide adenine dinucleotide phosphate (triphosphopyridine nucleotide) 0.7 units glucose-6-phosphate-dehydrogenase, 5 mM DTT, and 3 × 10⁻⁵ M DL-[3-¹⁴C]HMG-CoA (5 Ci/mol). The enzymatic reaction was stopped with 20 μl 5 N HCl. Product marker (20 μl unlabeled mevalonolactone 5 mg/ml) and 0.5 g Na₂SO₄ were added, and the mixture was extracted twice with 10 ml diethylether. The extracts were pooled and dried, and the residue was dissolved in 60 μl acetone. The mevalonolactone was separated from the mixture by thin-layer chromatography in acetone-benzene (1:1; v/v) and counted in a liquid scintillation counter (Wallac).

p38 and JNK MAPK activity assays

The JNK activity was measured using a solid-phase kinase assay in which glutathione-S-transferase-c-Jun (1–79) bound to glutathione-Sepharose 4B beads was used to affinity purify JNK from cell lysates (11). The activation of p38 MAPKs was assessed by Western blot analysis using an antibody specific for the active form of p38 MAPK (Cell Signaling Technology, catalog no. 9211). Total levels of p38 MAPKs were visualized using a p38 MAPK-specific antibody from Cell Signaling Technology (catalog no. 9212). Western blotting was performed as described previously (12).

Cell spreading measurements

Human fibroblasts were plated on uncoated 12 mm-diameter glass coverslips (VWR International; catalog no. 6012401) in 10 cm dishes supplemented with DMEM containing 15% FCS. On the following day, the medium was replaced with DMEM containing 2.5 mg/ml lipoprotein-free serum, and the cells were starved for 24 h. The cells were then pretreated or not with 10 μM SB203580 for 30 min and incubated for 24 h with 200 μg/ml LDL. After various periods of time, cells on coverslips were washed in PBS and fixed in 4% paraformaldehyde/PBS for 30 min. The coverslips were then mounted in Vectashield mounting medium (Vector Lab.; catalog no. H-1000), and pictures were taken with a Zeiss Axioplan 2 microscope. Cell area was measured on digital pictures of the cells using the Object-Image 2.08 software (Norbert Vischer, University of Amsterdam; <http://simon.bio.uva.nl>).

Statistics

Comparisons between groups were made by homoscedastic two-tailed *t*-test analysis using the Excel 2000 software.

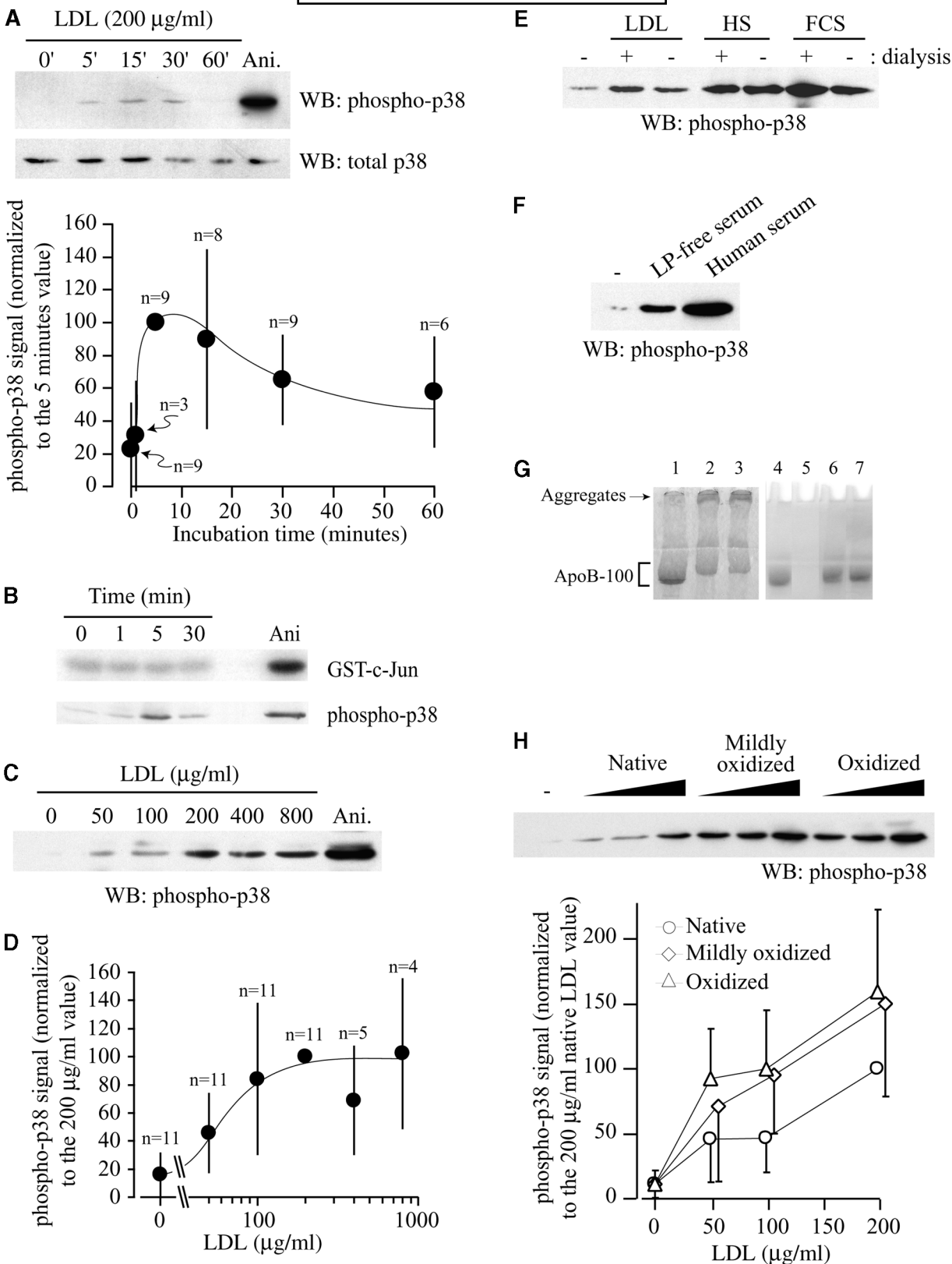


Fig. 1. LDLs activate the p38 mitogen-activated protein kinase (MAPK) pathway but not the c-Jun N-terminal kinase (JNK) MAPK pathway in human fibroblasts. Human fibroblasts (GM00316B) were starved 24 h in lipoprotein-free medium. Anisomycin (Ani; 10 $\mu\text{g/ml}$ for 30 min) was used as a positive control for p38 and JNK activation. **A:** Cells were stimulated with 200 $\mu\text{g/ml}$ of LDLs for the indicated periods of time. The activated and total p38 MAPK levels were assessed by Western blot analysis using phospho-specific and total anti-p38 MAPK antibodies, respectively. The graph represents the quantitation of the Western blot band detected by the phospho-specific antibody (mean \pm SD) for the indicated numbers of independent experiments. **B:** Cells were incubated with 200 $\mu\text{g/ml}$ of LDLs for the indicated periods of time. The activation of JNK was measured using an in vitro kinase assay. The activation of p38 MAPK was performed as described in **A**. This experiment was performed three times with similar results. **C, D:** Cells were incubated with increasing concentrations of LDLs for 10 min.

RESULTS

LDLs activate the p38 but not the JNK MAPK pathway in human fibroblasts

To determine whether LDLs could activate the p38 MAPK pathway in fibroblasts, the human cell line GM00316C was incubated with 200 $\mu\text{g}/\text{ml}$ LDL for up to 60 min. Western blot detection of the active form of p38 MAPKs demonstrated that the LDL treatment resulted in a transient p38 activation peaking between 5 min and 15 min of stimulation (Fig. 1A). This p38 MAPK activation was not a consequence of increased protein expression, because the levels of total p38 MAPK remained constant during the LDL incubation (Fig. 1A).

In contrast to the p38 MAPKs, the JNK MAPKs were not activated by LDLs (Fig. 1B). The absence of JNK activation was not due to a nonfunctional JNK MAPK pathway in GM00316C cells, because anisomycin, a known SAPK activator (13), potently stimulated the JNKs in these cells (Fig. 1B).

To assess at which LDL concentrations human fibroblasts activated the p38 MAPK pathway, GM00316C cells were stimulated with increasing concentrations of LDLs for 10 min. Figures 1C and D show that p38 MAPK activation started to be detected when cells were incubated with 50–100 $\mu\text{g}/\text{ml}$ of LDLs. The maximal stimulation was observed when LDL concentration reached 200 $\mu\text{g}/\text{ml}$ and higher. Because the extravascular LDL concentration is ~ 100 $\mu\text{g}/\text{ml}$ (14), these results indicate that the p38 MAPK pathway can be activated by physiological concentrations of LDLs in fibroblasts. The maximal p38 MAPK response is, however, observed at higher, pathological, extravascular concentrations of these lipoproteins.

A series of experiments were performed to rule out that the p38 activation induced by LDLs was due to serum contaminants. First, sera or LDLs were dialyzed against membranes with a 100 kDa cutoff. This procedure removes molecules with molecular mass lower than 100 kDa, including hormones such as insulin, epidermal growth factor, and estrogens, known to potently activate the p38 MAPK pathway in various cell types (15, 16). This procedure will not eliminate LDLs weighing ~ 3 million Da.

The dialyzed sera or LDL preparations had similar abilities to activate the p38 MAPK pathway compared with the nondialyzed preparations (Fig. 1E), indicating that the observed response is not due to small-to-medium molecular weight contaminants. Second, LPS, which is a possible contaminant known to induce the p38 MAPK pathway (17), was not detected in our LDL preparations (see Experimental Procedures). Finally, while normal HS efficiently stimulated the p38 MAPK pathway in human fibroblasts, the serum depleted from lipoproteins was less potent in activating the p38 MAPK pathway (Fig. 1F). This indicates that among the various proteins and particles found in blood, lipoproteins are major stimulators of the p38 MAPK pathway.

Oxidation may be important for the signaling properties of LDLs (5, 18). Using the lipoperoxide method, we found that no oxidation could be detected in our LDL preparations (see Experimental Procedures). Moreover, incubating LDLs with cells for the time required to induce the p38 MAPK pathway (10 min) did not lead to detectable modifications of the migration pattern of the LDLs in SDS-PAGE, such as those observed for mildly and strongly oxidized LDLs (Fig. 1G). However, mildly and strongly oxidized LDLs had a tendency to stimulate the p38 MAPKs in fibroblasts more strongly than did native LDLs (Fig. 1H). These results indicate that oxidation of LDLs, even though it is not a prerequisite for the stimulation of the p38 MAPK pathway, may enhance this response.

LDL-induced p38 activation is not mediated by the LDLR

We next assessed whether the LDL-induced p38 activation requires the recruitment of the LDLR. Fibroblasts from a control subject or fibroblasts from an FH patient lacking functional LDLRs were stimulated with 200 $\mu\text{g}/\text{ml}$ LDL. As shown in Fig. 2A and B, this resulted in transient p38 activation in both types of fibroblasts. There was a tendency for the FH-derived fibroblasts to generate a more sustained p38 MAPK activation in response to LDLs, compared with control cells, but this did not reach statistical significance (Fig. 2B). Similarly, MEFs derived from wild-type mice or from LDLR knockout mice both activated the p38 MAPK pathway in response to LDLs (Fig. 2C).

The activation of p38 MAPKs was visualized on Western blot (C) and quantitated (D) as described in A. E: Cells were stimulated for 10 min with 200 $\mu\text{g}/\text{ml}$ LDLs or with 15% human serum (HS) or fetal calf serum (FCS) that had been dialyzed or not against 100 kDa cutoff membranes. Removal of molecules with an apparent molecular mass lower than 100 kDa did not affect the capacity of LDL preparations or human and calf sera to stimulate the p38 MAPK pathway. This blot is a representative example of five independent experiments. F: Cells were left untreated (–) or incubated with 5.5 mg protein/ml (the concentration found in 15% HS) of either lipoprotein-free serum or normal HS for 10 min. The activation of p38 MAPKs was measured as in A. Depletion of lipoproteins substantially reduced the ability of the HS to stimulate the p38 MAPKs. This experiment was performed seven times, and similar results were obtained in each case. G: Left panel, native (lane 1), mildly oxidized (lane 2), and strongly oxidized (lane 3) LDLs (5 μg) were resolved on a 3.6–15% SDS-PAGE and visualized by Coomassie blue staining. Oxidation of LDLs generated a slower-migrating form of apolipoprotein B-100 and the appearance of high-molecular-weight aggregates. Right panel, human fibroblasts starved for 24 h in serum-free medium were incubated or not with 200 $\mu\text{g}/\text{ml}$ LDLs for 10 min at 37°C. Volumes of the culture medium containing 6 μg of LDLs (or an equivalent volume in the case of the cells cultured in LDL-free medium) were analyzed as described for the left panel. Lane 4, native, untreated LDLs; lane 5, LDL-free medium; lane 6, LDLs in culture medium in the absence of cells; lane 7, LDLs in culture medium in the presence of human fibroblasts. Incubation of LDLs with cells did not lead to the changes observed in oxidized LDLs. H: Cells were incubated for 10 min with 0 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, 100 $\mu\text{g}/\text{ml}$, and 200 $\mu\text{g}/\text{ml}$ of native, mildly oxidized, or oxidized LDLs. Activation of p38 MAPKs was measured as described in A. The results in the graph correspond to the mean \pm SD of three (mildly oxidized) or six (native or oxidized) independent measurements (points are offset for clarity). The differences between the various forms of LDLs did not reach statistical significance.

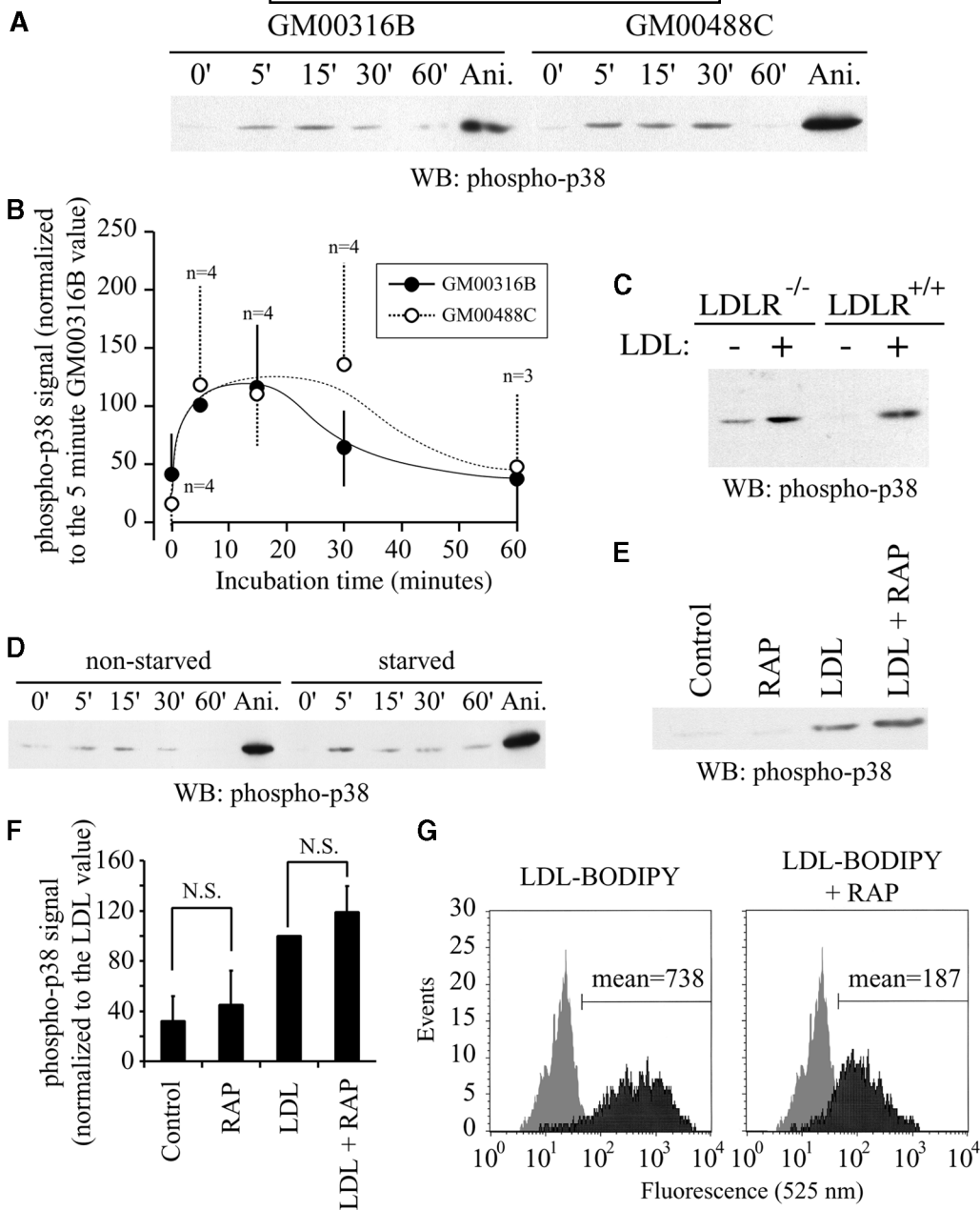


Fig. 2. The LDL receptor (LDLR) is not involved in LDL-induced p38 activation. A, B: Normal human fibroblasts (GM00316B) and fibroblasts derived from a familial hypercholesterolemia subject (GM00488C) were starved as described in Fig. 1. The cells were incubated with 200 $\mu\text{g}/\text{ml}$ of LDLs for the indicated periods of time. Anisomycin (Ani; 10 $\mu\text{g}/\text{ml}$ for 30 min) was used as a positive control for p38 MAPK activation. Western blotting was performed with an anti-phospho p38 antibody (A). B: Represents the quantitation of the Western blot band detected by the phospho-specific antibody (mean \pm SD) for the indicated numbers of independent experiments. The absence of functional LDLR in GM00488C cells did not prevent efficient stimulation of the p38 MAPK by LDLs. C: Starved mouse embryonic fibroblasts derived from control mice (LDLR^{+/+}) or from LDLR knockout mice (LDLR^{-/-}) were stimulated for 10 min with 200 $\mu\text{g}/\text{ml}$ of purified LDLs. The activation of the p38 MAPK pathway was assessed as described above. The absence of the LDLR did not hamper LDL-induced p38 MAPK activation. This experiment was performed three times, and similar results were obtained in each case. D: Normal human fibroblasts were starved or not as described above and stimulated with 200 $\mu\text{g}/\text{ml}$ of LDLs for the indicated periods of time before p38 MAPK activation was assessed. Starvation, which results in modulation of LDLR levels, did not affect the overall p38 MAPK response induced by LDLs. This experiment was performed three times with similar results. E, F: Starved human fibroblasts were left untreated or incubated with 1 μM receptor-associated protein (RAP) at 37°C for 15 min. The cells were further stimulated for 10 min in the presence or in the absence of 200 $\mu\text{g}/\text{ml}$ of purified LDLs. The extent of p38 MAPK activation was visualized by Western blotting (E) and quantitated (F; mean \pm SD of three independent experiments) as described in Fig. 2A, B. G: Starved human fibroblasts were incubated or not with RAP as described above, followed by a 2 h incubation with or without 10 $\mu\text{g}/\text{ml}$ of fluorescent LDLs (LDL-BODIPY). The amount of cell-associated fluorescent LDLs was then assessed using a fluorescent-activated cell sorter apparatus. This experiment was performed twice with similar results.

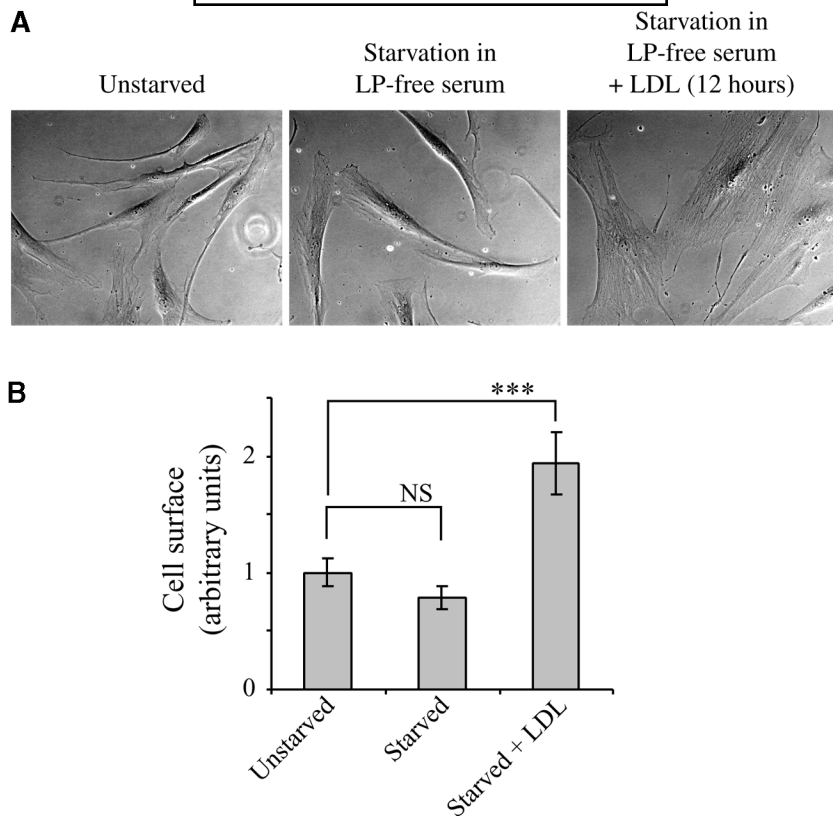


Fig. 3. LDLs induce cell spreading and lamellipodia formation. A: Human fibroblasts were incubated 24 h in lipoprotein-free medium and stimulated with 200 $\mu\text{g}/\text{ml}$ LDLs for an additional 12 h. Shown are images of the cells before the incubation in lipoprotein-free medium (unstarved), at the end of the starvation period (starvation in lipoprotein-free medium) and 12 h after the addition of LDLs (right panel). B: Human fibroblasts plated on coverslips were treated as above and their cell surface measured. At least 20 cells per condition were analyzed. The results correspond to the mean \pm SE. This experiment was performed four times with similar results.

Sterol-regulatory element binding proteins (SREBPs) mediate the transcription of both the LDLR and the HMG-CoA reductase genes (19). When cells are cultured in lipoprotein-containing medium (e.g., serum-containing medium), SREBPs remain membrane associated and do not translocate to the nucleus; hence, the LDLR gene and the HMG-CoA reductase gene are not transcribed, and therefore their cellular levels decrease. Conversely, in lipoprotein-free medium, LDLR levels and HMG-CoA reductase activity increase [(19) and data not shown]. Figure 2D shows that LDLs stimulated the p38 MAPK pathway to a similar extent in cells cultured in serum-containing medium or in starved cells. This indicates that variations in HMG-CoA reductase activities and LDLR levels do not affect the LDL-induced p38 MAPK response. Figure 2D also shows that fibroblasts cultured in 15% FCS-containing medium that contains LDLs do not have a sustained p38 MAPK activation (first lane of Fig. 2D). This is likely a consequence of the fact that LDL-induced p38 MAPK activation is quickly desensitized (Fig. 1A). As exogenous addition of 200 $\mu\text{g}/\text{ml}$ human LDLs in 15% FCS-containing medium increases by about eight times the LDL concentration in the cell culture medium (data not shown), this may be sufficient, however, to override the desensitization

and induce p38 MAPK activation (Fig. 2D, left part). Altogether, the experiments presented in Fig. 2A–D demonstrate that LDL-induced p38 MAPK activation does not involve the LDLR.

To assess whether other members of the LDLR family are required for the LDL-induced p38 MAPK activation, we investigated whether the 39 kDa RAP that functions as a general LDLR family antagonist could block the activation of p38 in human fibroblasts. RAP did not block the LDL-induced p38 MAPK activation but rather had a marginal potentiation effect on the LDL-induced p38 phosphorylation that did not, however, reach statistical significance (Fig. 2E, F). RAP was functional in these experiments because it could efficiently reduce the internalization of fluorescent LDLs (LDL-BODIPY), as measured by FACS analysis (Fig. 2G). Therefore, antagonizing LDLR family members did not inhibit the ability of LDLs to stimulate the p38 MAPKs.

LDLs induce lamellipodia formation and cell spreading in a p38 MAPK-dependent manner

Activation of the p38 MAPK pathway can regulate the dynamic of actin polymerization and therefore cell morphology (20). Because LDLs can stimulate p38 MAPKs, we tested whether LDLs could modulate cell shape. Human

fibroblasts were incubated in lipoprotein-free medium. Under these conditions, the fibroblasts remained elongated and developed few lamellipodias (Fig. 3A, left panel). Twenty-four hours later, 200 $\mu\text{g}/\text{ml}$ of LDLs were added. This treatment favored the development of numerous lamellipodias, which resulted in a dramatic cell spreading (Fig. 3A, right panel; see also the movie in the supplemental data). Quantitation of the cell area prior to and after the LDL stimulation indicated that LDLs induced a 2- to 3-fold increase in cell surface (Fig. 3B). To assess whether LDL-induced cell increase depended on the activation of the p38 MAPK pathway, cells were pre-treated or not with the specific p38 MAPK inhibitor SB203580 before being subjected to LDL stimulation. The SB compound efficiently blocked the increase in cell size stimulated by the LDLs (Fig. 4). Altogether, our results indicate that LDLs induce cell spreading via activation of the p38 MAPK pathway in an LDLR-independent manner.

DISCUSSION

The p38 MAPK pathway can be stimulated by cytokines via G protein-coupled receptors or in response to various stress conditions, such as increase in osmolarity (21) or, as shown here in fibroblasts and by others in other cell types, by LDLs (18, 22–25). How LDLs activate p38 MAPKs is, however, unclear. The LDLR is not involved because the p38 MAPK pathway is activated by LDLs, both in normal and LDLR-deficient human and mouse fibroblasts, and because RAP, an LDLR antagonist, does not block LDL-induced p38 activation (Fig. 2). The LDLR seems also not to be involved in situations in which LDLs stimulate the extracellular signal-regulated kinase or the JNK MAPK pathways (5, 26). Receptors belonging to the family of G protein-coupled receptors are potential mediators of LDL-induced MAPK pathway activation (3). In particular, it has been suggested that the presence of lysophosphatidic acid, platelet-activating factor, or lysophosphatidylcholine in LDL particles could induce signals in cells by activating the Edg G protein-coupled receptors (3). Because the first two of these compounds are, in principle, only found in oxidized LDLs (3) and because oxidation seems not to be a prerequisite for LDL stimulation of p38 MAPKs (Fig. 1), it is unlikely that they mediate the activation of the p38 MAPK in fibroblasts in response to native LDLs. However, we cannot rule out that incubation with fibroblasts could have caused a low level of oxidation of LDLs, as reported by previous studies (27). These studies revealed, however, that LDLs thus modified did not display the pathological characteristics of “minimally modified LDL” (that is, no proinflammatory activity such as monocyte activation, migration, and adhesion) (28). In contrast, fibroblasts that were transfected to overexpress 15-lipoxygenase, leading to increased seeding of LDLs with peroxidized lipids, converted LDLs to proinflammatory particles with properties typical of minimally modified LDLs. This suggests that normal fibroblasts should not modify LDLs to the extent that they adopt pathological characteristics.

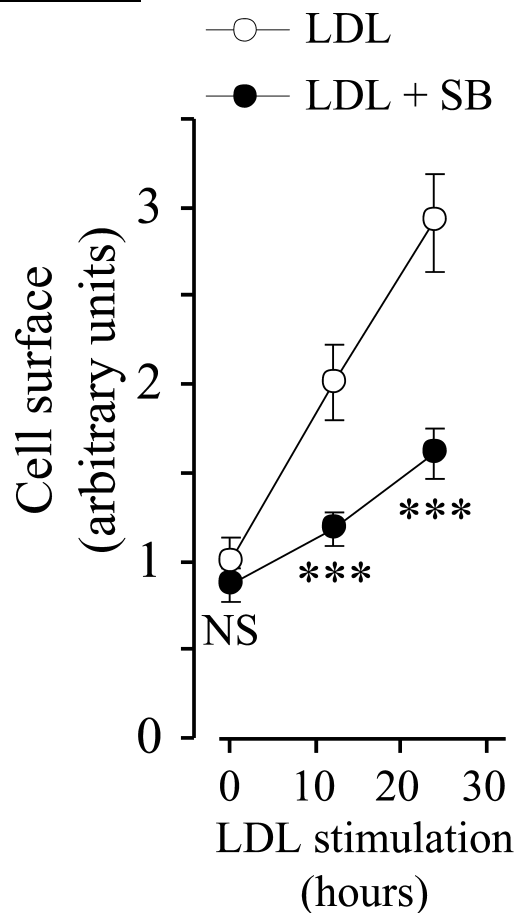


Fig. 4. LDL-induced cell spreading requires p38 activation. Human fibroblasts plated on coverslips were starved as above, pre-treated or not with 10 μM SB203580 for 30 min, and incubated for 24 h with 200 $\mu\text{g}/\text{ml}$ LDL. Cell surface was then measured. The results correspond to the mean \pm SE. This experiment was performed three times with similar results.

Phospholipids in LDLs may be involved in the activation of the p38 MAPK. For example, lysophosphatidylcholine that is present in native LDLs (3) has been shown to activate the p38 MAPK pathway (29, 30). It could therefore be a critical LDL component mediating the activation of the p38 MAPK. Consistent with this possibility is the observation that oxidation, which is known to increase the content of lysophosphatidylcholine in LDL particles, seems to enhance the ability of LDLs to stimulate the p38 MAPK pathway (Fig. 1).

LDLs function as cargo particles for delivering cholesterol to cells (31), but they also participate in the development of atherosclerosis when their blood levels increase (1). It is not known whether the capacity of LDL to promote the development of atherogenic plaques is related to their cargo function or to their ability to transduce intracellular signals such as the activation of the p38 MAPK pathway. The observation that the thickening of blood vessels can be inhibited by p38 MAPK inhibitors (32) supports, however, the notion that activation of the p38 MAPK pathway by LDLs could participate, possibly by pro-

moting lamellipodia formation and cell size increase, in the thickening of the walls of blood vessels observed during atherogenesis.

In the human fibroblasts used in this study, the p38 MAPK pathway was activated at LDL concentrations of 100 $\mu\text{g}/\text{ml}$ and above. In healthy subjects, the plasmatic concentration of LDL cholesterol is $\sim 2\text{--}4$ mM, corresponding to a protein concentration of $\sim 350\text{--}700$ $\mu\text{g}/\text{ml}$. The extravascular LDL concentration is much lower, however. Measured in human synovial fluid samples, the extravascular LDL concentration was found to be about five times lower than the blood concentration (14). Therefore, leakage of LDLs from the vascular space (as a result of blood vessel microhemorrhages for example) would induce a local increase in the LDL concentration in the connective tissue surrounding the blood vessels. If this increase reaches the 100 $\mu\text{g}/\text{ml}$ threshold, fibroblasts in the connective tissue would activate their p38 MAPK pathway. The activation of the p38 MAPK pathway in these fibroblasts would then promote an increase in their cell surface, which could prevent excessive LDL leakage from the blood vessel and facilitate closure of the wound. In this case, activation of the p38 MAPK pathway in the fibroblasts found in the conjunctive tissue of blood vessels would have beneficial effects. On the other hand, if there is a continuous activation of this pathway, caused by an increase in LDL blood levels for example, chronic hyperplasia of cells that could ensue may participate in thickening of the blood vessels and contribute to the atherogenic process (see above). Activation of the p38 MAPK pathway by LDLs could therefore induce a beneficial or detrimental response, depending on whether vascular lipoprotein concentrations are elevated. **■**

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