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p38 Mitogen-activated Protein Kinase (MAPK) Promotes Cholesterol Ester Accumulation in Macrophages through Inhibition of Macroautophagy*[S]

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Background: The direct role for p38 MAPK in foam cell formation has not been investigated.
Results: Inhibition and activation of p38 MAPK alter levels of autophagy activity and cholesterol ester accumulation in macrophages.
Conclusion: p38 MAPK promotes cholesterol ester accumulation and foam cell formation through inhibition of autophagy.
Significance: Results from this study provide brand new understanding of the role for p38 MAPK in the development of atherosclerosis.

p38 MAPK has been strongly implicated in the development of atherosclerosis, but its role in cholesterol ester accumulation in macrophages and formation of foam cells, an early step in the development of atherosclerosis, has not been investigated. We addressed this issue and made some brand new observations. First, elevated intracellular cholesterol level induced by the exposure to LDL-activated p38 MAPK and activation of p38 MAPK with anisomycin increased the ratio of cholesterol esters over free cholesterol, whereas inhibition of p38 MAPK with SB203580 or siRNA reduced the LDL loading-induced intracellular accumulation of free cholesterol and cholesterol esters in macrophages. Second, exposure to LDL cholesterol inhibited autophagy in macrophages, and inhibition of autophagy with 3-methyladenine increased intracellular accumulation of cholesterol (free cholesterol and cholesterol esters), whereas activation of autophagy with rapamycin decreased intracellular accumulation of free cholesterol and cholesterol esters induced by the exposure to LDL cholesterol. Third, LDL cholesterol loading-induced inhibition of autophagy was prevented by blockade of p38 MAPK with SB203580 or siRNA. Neutral cholesterol ester hydrolase was co-localized with autophagosomes. Finally, LDL cholesterol loading and p38 activation suppressed expression of the key autophagy gene, *ulk1*, in macrophages. Together, our results provide brand new insight about cholesterol ester accumulation in macrophages and foam cell formation.

Accumulation of free cholesterol and cholesterol esters in monocytes/macrophages is an essential step in the development of foam cells and atherosclerotic plaques (1, 2). Free cholesterol can go through plasma membrane through either diffusion or transporter (ABCA1, ABCG1, and Apo-E)-mediated efflux (2, 3). Thus, the accumulation of cholesterol esters (free cholesterol + free fatty acids) in macrophages is necessary for foam cell formation. Cholesterol ester accumulation is mainly balanced by two independent processes: conversion of free cholesterol into cholesterol esters by acyl-CoA-cholesterol transferase 1 (ACAT1) and degradation of cholesterol esters into free cholesterol and free fatty acids by the neutral cholesterol ester hydrolase (CEH)[2] (2–6). It is known that overexpression of CEH cloned by Ghosh *et al.* (2, 4) in macrophage alone can degrade cholesterol esters and reduce foam cell formation and atherosclerosis. The diet-induced atherosclerosis is increased in $ApoE^{-/-}$ mice when the cholesterol ester hydrolase (nCEH1/nCEH) cloned by Ishibashi *et al.* (7) is knocked out. However, regulation of the cholesterol ester hydrolase-mediated degradation of cholesterol esters remains unestablished currently.

Macroautophagy (referred to as "autophagy" hereafter) is an essential process of breaking down macromolecules and aged/damaged cellular organelles for providing a fuel source or maintaining cellular health (8, 9). Autophagy has been shown to be activated in advanced (late stage) atherosclerotic plaques by many (10, 11). Both protective and detrimental effects of autophagy have been described in atherosclerosis (10, 11). However, the potential role of autophagy in the formation of foam cells from macrophages, an early event in the development of atherosclerosis, has not been established. It has recently been shown that autophagy is required for breaking down triglyceride into glycerol and free fatty acids in hepatocytes and adipocytes (12, 13) and involved in cholesterol efflux

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[2] The abbreviations used are: CEH, cholesterol ester hydrolase; nCEH, neutral CEH; ANOVA, analysis of variance.

from macrophages (14). It is noteworthy that triglyceride (glycerol + free fatty acids) and cholesterol esters (free cholesterol + free fatty acids) are both a form of fat storage and share similar components. It is currently unknown whether or not degradation of cholesterol esters also depends on autophagy.

p38 MAPK has been strongly implicated in the development of atherosclerosis. It can promote atherosclerosis in many different ways. For example, p38 MAPK can stimulate secretion of MCP-1 and IL-8, which attract monocytes to vascular endothelial cells (15–22). p38 MAPK mediates the MCP-1-dependent transendothelial migration, integrin activation, and chemotaxis (23–26). p38 MAPK promotes differentiation of human monocytes into macrophages (27), inhibits proliferation while inducing apoptosis of endothelial cells (28–30), stimulates endothelial migration (30), down-regulates endothelial progenitor cells (31), and accelerates endothelial progenitor cell senescence (32). p38 MAPK can be activated in monocytes/macrophages, vascular endothelial cells, and vascular smooth muscle cells by a variety of stimulants, including reactive oxygen species (18, 29); high level of glucose (21, 28, 32); chylomicron remnants (19); free fatty acids (33); cholesterol (34); proinflammatory cytokines, such as TNF-α (35); and growth factors, such as PDGF (36–39). Finally, it is known that p38 MAPK can inhibit autophagy (40). Nevertheless, it is currently unknown whether or not p38 MAPK inhibition of autophagy is involved in cholesterol ester accumulation within macrophages and foam cell formation, an early event in the development of atherosclerosis. In this study, we investigated the potential roles of p38 MAPK and autophagy in cholesterol ester accumulation in macrophages and defined the relationship between p38 MAPK and autophagy in the process.

MATERIALS AND METHODS

Reagents and Antibodies—THP-1 cells were obtained from the American Type Culture Collection (ATCC). Primary human $CD14^+$ monocytes were obtained from Sanguine (catalog no. PBMC-005a). Low density lipoproteins (LDLs), anisomycin, SB203580, rapamycin, and 3-methyladenine were from Sigma. GFP-LC3-expressing plasmids (pEGFP-LC3) were kind gifts from Dr. Tamotsu Yoshimori (Osaka University, Osaka, Japan). Antibodies against LC3, phosphorylated p38 MAPK, total p38 MAPK, Ulk1, phospho-$Ulk1^{Ser\text{-}317}$, phospho-$Ulk1^{Ser\text{-}757}$, or LAMP-1 were from Cell Signaling Technologies Inc. (Beverly, MA). Antibody against neutral cholesterol ester hydrolase 1 (nCEH1) was from Sigma (catalog no. HPA026888). Antibodies against β-actin, mouse IgG, and rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). BODIPY493/503 and the Amplex Red cholesterol assay kit were obtained from Invitrogen. The apoptosis assay kit was from Roche Applied Science (catalog no. 11774425001). Other materials were all obtained commercially and are of analytical quality.

Cell Cultures—The human THP-1 cell line was obtained from the ATCC and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Invitrogen). THP-1 cells were cultured at 37 °C, 100% humidity, and 5% CO_2 at 5×10^5 cells/ml density. Primary human $CD14^+$ monocytes were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin at a density of 1–2 million cells/ml.

Immunoblotting—THP-1 cells (5×10^5/ml) were pretreated with 1640 medium with PMA (100 ng/ml) for 3 days, stimulated as noted in the figures for 24 h, and then lysed on ice for 30 min in 35 μl of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF, and proteinase inhibitor mixture (Roche Applied Science)). Cell debris was pelleted by centrifugation, and supernatants were collected and stored at −80 °C until assayed. Thirty micrograms of total cellular protein were denatured at 95 °C for 5 min in the loading buffer (60 mM Tris, 2.5% SDS, 10% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue) and subjected to 10% SDS-PAGE. For detection of LC3I and -II, the 16% SDS-PAGE straight gels were used. Proteins in the gels were transferred to PVDF membranes and blocked with TBS containing 0.05% Tween 20 (TBS-T) and 5% nonfat milk powder for 1 h. After being washed in TBS-T, membranes were probed with specific antibodies (1:1000) overnight at 4 °C and were then washed with TBS-T and incubated with a polyclonal secondary goat anti-rabbit antibody or rabbit anti-mouse antibody (1:5000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Following three washes in TBS-T, membranes were incubated with the ECF substrate solution (GE Healthcare) according to the manufacturer's protocol (Thermo Scientific). Fluorescent bands were visualized with a Molecular Imager VersaDoc MP 4000 System (Bio-Rad) and quantified by using densitometry analyses with ImageQuant version 5.2 software from GE Healthcare.

Immunoprecipitation—Cytosolic extracts (1000 μg) were used to perform immunoprecipitation assays according to the manufacturer's instructions (Pierce). Rabbit polyclonal anti-LC3b or rabbit IgG (10 μg/sample) was used for immunoprecipitating nCEH overnight at 4 °C. Resins (Pierce) were added then, and the samples were agitated for an additional 2 h at 4 °C and centrifuged at $1000 \times g$ for 1 min at 4 °C six times. The resulting pellets were washed three times with lysis buffer (Pierce), suspended in 5× sample buffer, and heated to 95 °C for 5 min. The resulting mixtures were subjected to immunoblotting as described above with rabbit monoclonal antibody against nCEH as the primary antibodies and goat anti-rabbit IgG as the secondary antibodies. LC3b or IgG was detected as loading control.

Transfection—THP-1 cells (2×10^5 cells in 12-well plates) were transiently transfected with 2 μg of purified recombinant plasmid, pEGFP-LC3, using Lipofectamine™ 2000 (Invitrogen) overnight in Opti-MEM medium following the manufacturer's instructions. Before they were treated, THP-1 cells were maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Invitrogen). THP-1 cells were cultured at 37 °C, 100% humidity, and 5% CO_2 at 5×10^5 cells/ml density.

Measurement of Cholesterol—After cells were treated as noted, cells were washed with PBS at room temperature three

times. Cells were scraped off in ice-old lysis buffer (18 mM Tris-HCl, 300 mM mannitol, 50 mM EGTA, pH 7.6, with protease inhibitors). The cell suspension was sonicated for 10 1-s pulses with a microtip. Lipid was extracted from cells by using a method described by Folch *et al.* (41). Briefly, methanol (2 ml) and chloroform (4 ml) were added into cell lysate and mixed by vortex, followed by a 2-h violent shaking. KCl (0.8 ml, 0.15 M) was added and mixed by vortex. These mixtures were centrifuged for 5 min at 750 r.p.m (3000 × *g*) for the phase separation. The top aqueous phase was removed with a Pasteur pipette. The bottom phase (organic solvent) was evaporated under vacuum to achieve lipid pellet. Isopropyl alcohol with 10% Triton X-100 was used to dissolve lipids (100–200 μl in total) containing cholesterol methyl ether. Total and free cholesterol was quantified with the Amplex Red cholesterol assay kit in a 96-well format. Meanwhile, protein levels in the same samples were measured as a normalization standard.

Fluorescence Microscopy—For fluorescence microscopy, THP-1 cells and primary human CD14$^+$ monocytes were cultured on microscopic coverglasses in 12-well plates overnight and were then treated as noted for 24 h. After the noted treatments, cells were fixed with 4% paraformaldehyde in PBS for 15 min. All of the cellular images were obtained using an inverted Nikon Eclipse 200 fluorescence microscope. For quantification of autophagic cells, GFP-LC3 punctated dots were determined from triplicates by counting a total of more than 60 cells. Human CD14$^+$ monocytes were fixed in 4% paraformaldehyde, blocked/permeabilized in 2.5% BSA, 0.1% Triton X-100 for 1 h at room temperature, and stained with the LAMP-1 primary antibodies overnight at 4 °C. Fluorophore-conjugated secondary antibodies were incubated in the presence of BODIPY493/503 (0.1 μM) to stain neutral lipids at room temperature. Apoptotic cell death was determined by nuclear staining with Hoechst 33342 (5 μg/ml) for fragmented and condensed nuclei for 15 min followed by fluorescence microscopy.

Introduction of Small Interfering RNA (siRNA)—THP-1 cells were transiently transfected with 100 nM siRNA against p38 MAPKα (Santa Cruz Biotechnology, Inc.) with Lipofectamine 2000 transfection reagents (Invitrogen) according to instructions from the manufacturer. Transfection mixtures were added to cells in Opti-MEM medium for 16 h before media were replaced with the regular RPMI 1640 medium supplemented with 10% FBS.

Oil Red O Staining—THP-1 cells were cultured at an initial density of 2 × 10^5 cells/well in 12-well plates and treated with the noted reagents for 24 h. Cells were then washed three times with cold PBS and fixed with 4% paraformaldehyde for 30 min. Cells were then washed three times with cold PBS and stained with the Oil Red O solution (0.5 g of Oil Red O powder dissolved in 60% ethanol) for 15 min at room temperature. Cells were subsequently washed again with PBS to remove the unbound dye and visualized by using a light microscopy.

RESULTS

Cholesterol Loading with LDL Activates p38 MAPK in Macrophages—To examine the effect of the exposure to a high level of cholesterol on p38 MAPK activity, primary human CD14$^+$ monocytes were incubated with LDL aggregates for a different time (1, 3, 6, 12, or 24 h) as described previously (42) or for 24 h with a different amount of LDL aggregates (10–50 μg/ml), followed by evaluations of total and phosphorylated p38 MAPK as we described previously (43–47). As shown in Fig. 1, exposure to LDL stimulated phosphorylation of p38 MAPK in a time- and dose-dependent manner. Similar results were observed in human THP-1 macrophages (data not shown).

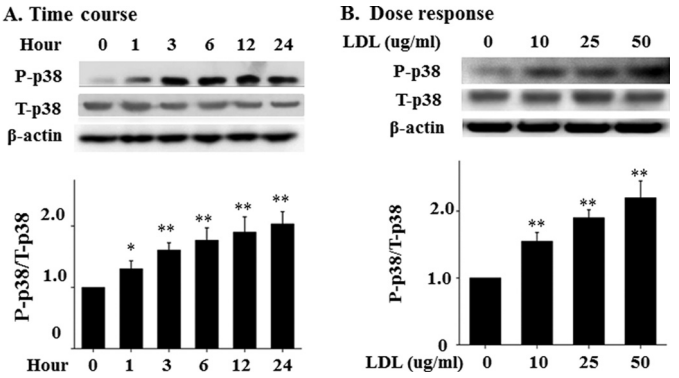

FIGURE 1. **Cholesterol loading with LDL activates p38 MAPK in human CD14$^+$ monocytes.** Primary Human CD14$^+$ monocytes were incubated with LDL aggregates with for a different time (1, 3, 6, 12, or 24 h) (*A*) or for 24 h with different amounts of LDL aggregates (10–50 μg/ml) (*B*), followed by evaluations of total and phosphorylated p38 MAPK as indicated ($n = 3$) by using immunoblotting with specific antibodies. Levels of β-actin in the same blots were measured by immunoblotting. Levels of phosphorylated p38 MAPK (*P-p38*) and total p38 MAPK (*T-p38*) were quantified by densitometry and normalized to β-actin. Results were presented as mean ± S.E. (*error bars*) of three independent experiments. **, $p < 0.01$ *versus* control.

Activation of p38 MAPK Is Associated with Increased Accumulation of Cholesterol Esters in Macrophages Exposed to LDL—To determine the role of p38 MAPK activation in cholesterol accumulation, activity of p38 MAPK was either stimulated or inhibited in THP-1 macrophages that were treated with LDL as noted, followed by visualization of cholesterol accumulation in macrophages with Oil Red O staining. As shown in Fig. 2*A*, lipid droplets were significantly increased in macrophages exposed to LDL. Treatment with anisomycin, a p38 MAPK activator, alone increased the accumulation of lipid droplets and further enhanced the LDL-mediated accumulation of lipid droplets in macrophages. In contrast, treatment with SB203580, a specific inhibitor of p38 MAPK, completely prevented the LDL-mediated accumulation of lipid droplets (Fig. 2*A*). Similarly, inhibition of p38 MAPK with siRNA against p38 MAPKα prevented the LDL-induced accumulation of lipid droplets, whereas the scrambled siRNA had no effect. To determine the components of the accumulated lipid droplets, levels of free cholesterol or cholesterol esters in the macrophages that were similarly treated as described in the legend to Fig. 2*A* were quantified. As shown in Fig. 2*B*, levels of both free cholesterol and cholesterol esters were increased by the exposure to LDL in macrophages. Treatment with LDL together with p38 MAPK activator, anisomycin, significantly increased the level of cholesterol esters while decreasing the level of free cholesterol in macrophages. As a result, the ratio of cholesterol esters over free cholesterol was dramatically increased by anisomycin (Fig. 2*B*). Treatment of macrophages with anisomycin alone mainly increased the level of free cholesterol (Fig. 2*B*). This was prob-

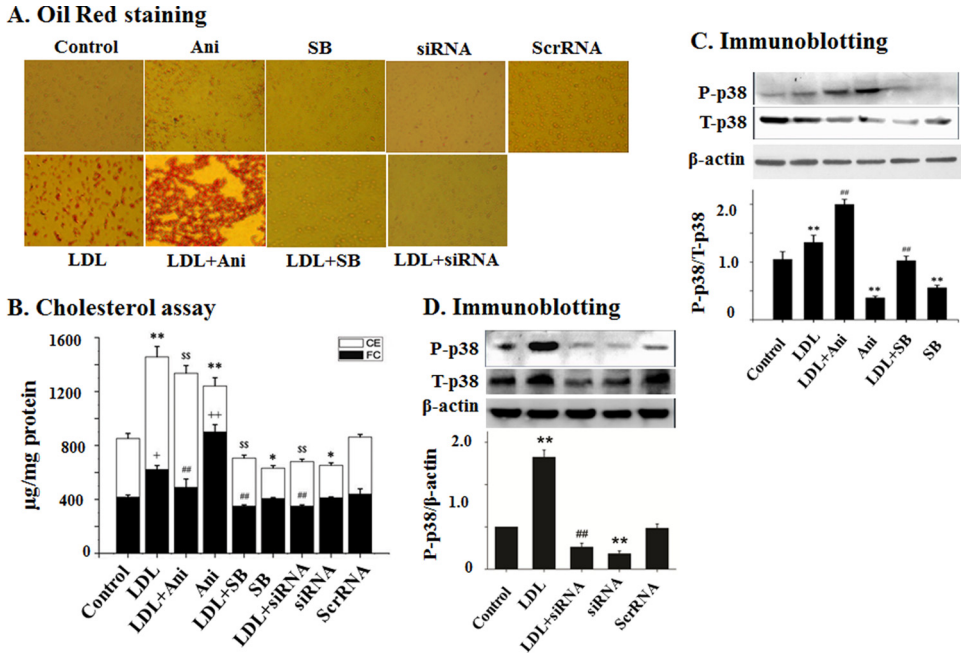

FIGURE 2. **Accumulation of cholesterol esters induced by cholesterol loading with LDL in macrophages is p38 MAPK-dependent.** *A*, THP-1 cells were treated by using the vehicle solution, LDL (25 μg/ml), LDL plus anisomycin (*Ani*; a p38 MAPK activator, 10 μM), anisomycin alone, LDL plus SB203580 (*SB*) (a p38 MAPK inhibitor, 10 μM), SB203580 alone, LDL plus siRNA against p38 MAPKα (*siRNA*), siRNA against p38 MAPKα alone, or scrambled siRNA (*ScrRNA*) for 24 h as indicated ($n = 3$). Lipid droplets were visualized by using Oil Red O staining (magnification $\times$10). *B*, levels of free cholesterols and cholesterol esters were evaluated by using the Amplex Red cholesterol assay kit in the THP-1 cells similarly treated as described in *A*. Results were presented as mean $\pm$ S.E. of three independent experiments, each in triplicate. +, $p < 0.05$ *versus* free cholesterol in control; ++, $p < 0.01$ *versus* free cholesterol in control; ##, $p < 0.01$ *versus* free cholesterol in cells loaded with LDL; *, $p < 0.05$ *versus* cholesterol esters in control; **, $p < 0.01$ *versus* cholesterol esters in control; $$, $p < 0.01$ *versus* cholesterol esters in cells treated with LDL (one-way ANOVA with Scheffe's post hoc test). *C* and *D*, levels of phosphorylated and total p38 MAPK and β-actin in the cells described in *A* and *B* were detected by using immunoblotting with specific antibodies and quantified. Results represent mean $\pm$ S.E. (*error bars*) of three independent experiments. **, $p < 0.01$ *versus* control; ##, $p < 0.01$ *versus* LDL alone (one-way ANOVA with Scheffe's post hoc test).

ably caused by the reduced efflux of free cholesterol from macrophages by activation of p38 MAPK because it has previously been shown that p38 MAPK can inhibit the ABCG1-dependent efflux of free cholesterol (48). Incubation of macrophages with LDL together with p38 MAPK inhibitor, SB203580, decreased the level of total cholesterol and the ratio between cholesterol esters over free cholesterol (Fig. 2*B*). Incubation of macrophages with SB203580 alone decreased the level of total cholesterol and the cholesterol esters/free cholesterol ratio (Fig. 2*B*). Similarly, knockdown of the p38 MAPKα gene with the specific siRNA decreased the ratio of cholesterol esters over free cholesterol in the presence or absence of LDL (Fig. 2*B*). As shown in Fig. 2, *C* and *D*, p38 MAPK phosphorylation was stimulated by the exposure to LDL and further enhanced by anisomycin but was inhibited by SB203580 in the macrophages. It should be noted that treatment with anisomycin did not cause obvious apoptosis (supplemental Fig. 1). Together, these results indicate that activation of p38 MAPK promotes cholesterol ester accumulation, whereas inhibition of p38 MAPK decreases cholesterol ester accumulation in macrophages.

Modulation of Autophagy Activity Can Alter Accumulation of Cholesterol Esters Induced by Exposure to LDL in Macrophages—To examine the effect of autophagy in cholesterol accumulation in macrophages, autophagy activity in human THP-1 macrophages was either stimulated with rapamycin, an inhibitor of mTOR, or inhibited by using 3-methyladenine, an inhibitor of autophagy. As shown in Fig. 3*A*, lipid accumulation was increased by the exposure to LDL, but the increase was prevented by rapamycin. In contrast, inhibition of autophagy with 3-methyladenine enhanced the basal or the LDL-induced lipid accumulation. Levels of both free cholesterol and cholesterol esters were increased by the exposure to LDL (Fig. 3*B*). Stimulation of autophagy with rapamycin decreased the LDL-induced accumulation of cholesterol and cholesterol esters (Fig. 3*B*). The cholesterol esters/cholesterol ratio was also significantly decreased by rapamycin (Fig. 3*B*). Rapamycin alone did not alter the level of total cholesterol but decreased the cholesterol esters/cholesterol ratio significantly (Fig. 3*B*). In contrast, inhibition of autophagy with 3-methyladenine increased both total level of cholesterol and the ratio of cholesterol esters/cholesterol in macrophages that were either treated with LDL or not (Fig. 3*B*). Together, these results show that autophagy plays an important role in determining the accumulation of cholesterol and cholesterol esters in macrophages.

Exposure to LDL Inhibits Autophagy in Macrophages—To examine the effect of exposure to LDL on autophagy, autophagy activity was examined in THP-1 macrophages in two different methods: measurements of LC3II-LC3I ratio and visualization of GFP-labeled autophagosomes. As shown in Fig. 4*A*, exposure to LDL decreased the ratio of LC3II/LC3I. Treatment with rapamycin increased the LC3II/LC3I ratio, whereas 3-methyladenine decreased the LC3II/LC3I ratio. Similarly, exposure of macrophages to LDL decreased the GFP-labeled autophagosomes (Fig. 4*B*). Treatment with rapamycin increased autopha-

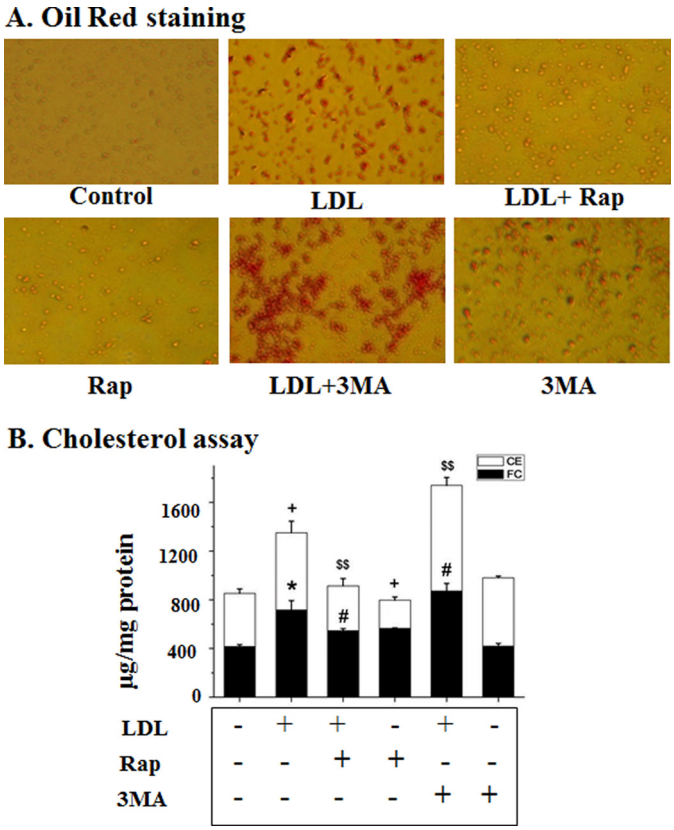

FIGURE 3. **Accumulation of cholesterol esters induced by cholesterol loading with LDL in macrophages is regulated by autophagy.** *A*, THP-1 cells were treated by using the vehicle solution, LDL (25 μg/ml), LDL plus rapamycin (*Rap*) (an autophagy activator, 10 μM), rapamycin alone, LDL plus 3-methyladenine (*3-MA*; an autophagy inhibitor, 10 μM), or 3-methyladenine alone for 24 h as indicated ($n = 3$). Lipid droplets were visualized with Oil Red O staining (magnification ×10). *B*, levels of free cholesterol and cholesterol esters in similarly treated cells were quantified by using the Amplex Red cholesterol assay kit. Results were presented as mean ± S.E. (*error bars*) of three independent experiments, each in triplicate. *, $p < 0.05$ *versus* free cholesterol in control; #, $p < 0.05$ *versus* cells treated with LDL alone; +, $p < 0.05$ *versus* cholesterol esters in control; $$, $p < 0.01$ *versus* cholesterol esters in cells treated with LDL alone (one-way ANOVA with Scheffe's post hoc test).

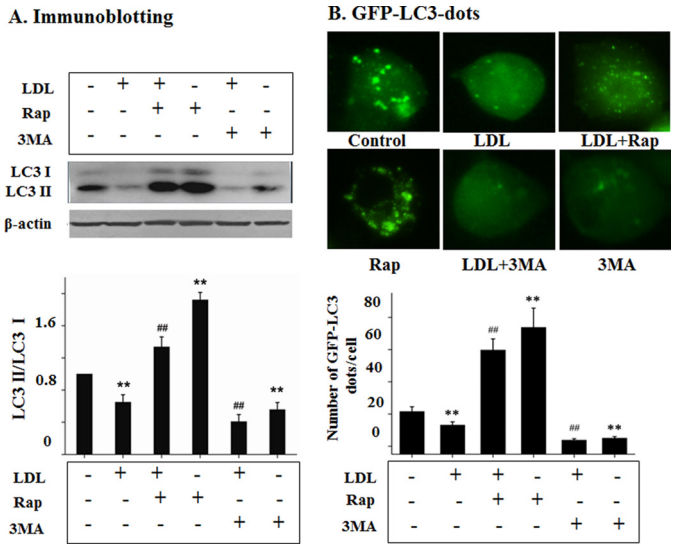

FIGURE 4. **Cholesterol loading with LDL inhibits autophagy in macrophages.** *A*, THP-1 cells were treated with either the vehicle solution or LDL (25 μg/ml) in the presence of rapamycin (*Rap*; 10 μM) or 3-methyladenine (*3MA*; 10 μM), rapamycin alone, or 3-methyladenine alone as indicated for 24 h ($n = 3$). Levels of LC3II and -I were detected by using immunoblotting with specific antibodies and quantified by using densitometry analysis. The ratio of the LC3II/LC3I band was subsequently calculated. β-Actin in the same samples was detected by immunoblotting. **, $p < 0.01$ *versus* control. ##, $p < 0.01$ *versus* LDL alone (one-way ANOVA). *B*, GFP-tagged LC3 was introduced into THP-1 cells via transient transfection overnight prior to the treatment with LDL, rapamycin, and 3-methyladenine similarly as described in *A*. The GFP-tagged LC3 dots were visualized by a fluorescence microscope (magnification ×40) and quantified in at least 20 cells/group and presented as mean ± S.E. (*error bars*) of three independent experiments. **, $p < 0.01$ *versus* control; ##, $p < 0.01$ *versus* LDL alone (one-way ANOVA).

gosomes, whereas 3-methyladenine decreased autophagosomes in macrophages (Fig. 4*B*).

p38 MAPK Mediates LDL-induced Inhibition of Autophagy in Macrophages—To determine the role of p38 MAPK in LDL-induced inhibition of autophagy, activators and inhibitors of p38 MAPK were applied. As shown in Fig. 5*A*, exposure of THP-1 macrophages to LDL decreased the LC3-GFP-labeled autophagosomes. Activation of p38MAPK with anisomycin also reduced the LC3-GFP-labeled autophagosomes. In contrast, inhibition of p38 MAPK with either a chemical (SB203580; *SB*) or siRNA against p38MAPKα increased LC3II-GFP-labeled autophagosomes in the presence or absence of LDL (Fig. 5*A*). To determine the connection between autophagy and cholesterol ester degradation, immunoprecipitation assays were applied with antibodies against LC3, nCEH, or species-specific IgG. As shown in Fig. 5*B*, LDL loading decreased the co-localization of LC3 and nCEH, but inhibition of p38 MAPK with SB203580 increased the co-localization of LC3 and nCEH. Together, these results demonstrate that autophagosomes co-localize with nCEH, and the LDL-induced inhibition of autophagy is mediated by p38 MAPK.

Localization of Neutral Lipids and Lysosomes—Because the autophagy-mediated degradation of macromolecules has to go through lysosomes and autophagolysosomes (49), we attempted to localize the lipid droplets and lysosomes by staining neutral lipids with fluorescent dye BODIPY493/503 and lysosomes with antibodies against the marker protein of lysosomes, LAMP-1, as described (14). As shown in supplemental Fig. 2, the level of neutral lipids was increased, whereas the number of lysosomes was reduced by the treatment with LDL aggregates or anisomycin. Most lipid droplets were not co-localized with lysosomes. In contrast, inhibition of p38 MAPK with SB203580 decreased the number of lipid droplets while increasing the number of lysosomes. Inhibition of p38 MAPK with SB203580 increased the merging of lipid droplets and lysosomes. Together, these results indicate that p38 MAPK plays a critical role in regulating accumulation of neutral lipids in macrophages through the lysosome/autophagolysosome-mediated degradation of neutral lipids.

p38 MAPK Mediates LDL Cholesterol Loading-inhibited Expression of Key Autophagy Gene, ulk1, in Macrophages—To investigate the mechanism by which LDL cholesterol loading and p38 MAPK inhibit autophagy in macrophages, the effects of LDL cholesterol loading and p38 MAPK on phosphorylation and expression of *ulk1*, the key autophagy initiation gene, were examined. As shown in Fig. 6, LDL loading did not alter Ulk1 phosphorylation but obviously decreased Ulk1 protein level and the LC3II/LC3I ratio. Activation of p38 MAPK with anisomycin also decreased expression of Ulk1 and the LC3II/LC3I

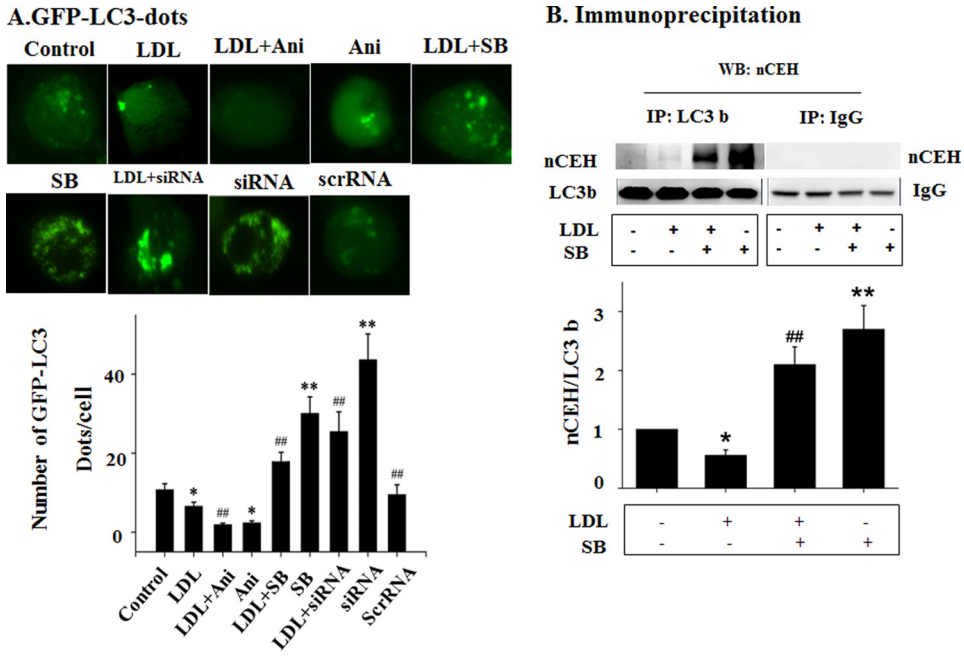

FIGURE 5. **The LDL-induced inhibition of autophagy is p38 MAPK-dependent in macrophages.** *A*, GFP-LC3 was introduced into THP-1 cells via transient transfection overnight prior to treatments with LDL (25 μg/ml), anisomycin (*Ani*; 10 μM), SB203580 (*SB*; 10 μM), siRNA against p38 MAPKα (*siRNA*), or scrambled siRNA (*ScrRNA*) for 24 h ($n = 3$). The GFP-LC3 dots were visualized by a fluorescence microscope (magnification $\times$40) and quantified. *B*, THP-1 cells were treated with LDL (25 μg/ml) in the presence or absence of SB203580 for 24 h prior to the immunoprecipitation (*IP*) assays. Antibodies against LC3b were used to precipitate nCEH, followed by detection of nCEH in the precipitates with antibodies against either nCEH or LC3. As a control, nonspecific IgG was used to precipitate nCEH. The ratio of precipitated nCEH over LC3b was quantified and presented as mean $\pm$ S.E. of three independent experiments. *, $p < 0.05$ *versus* control; **, $p < 0.01$ *versus* control; ##, $p < 0.01$ *versus* LDL alone.

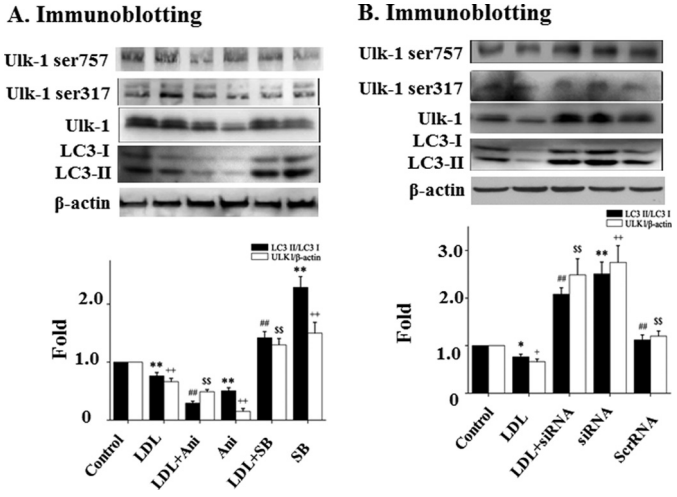

FIGURE 6. **LDL cholesterol loading and p38 MAPK inhibits expression of the key autophagy initiation gene, *ulk1*, in macrophages.** *A* and *B*, THP-1 cells were treated with LDL (25 μg/ml) in the presence or absence of anisomycin (*Ani*; 10 μM), SB203590 (*SB*; 10 μM), or siRNA against p38 MAPKα as noted for 24 h. Levels of phosphorylated Ulk1 ($Ulk1^{Ser\text{-}317}$ and $Ulk1^{Ser\text{-}757}$), total Ulk1, LC3I/LC3II, and β-actin were detected by using immunoblotting with specific antibodies. Levels of LC3II, LC3I, and Ulk1 were quantified. The LC3II/LC3I ratio and Ulk1/β-actin ratio were subsequently calculated. Results were presented as mean $\pm$ S.E. (*error bars*) of three independent experiments. *, $p < 0.05$ *versus* control. **, $p < 0.01$ *versus* control; +, $p < 0.05$ *versus* control; ++, $p < 0.01$ *versus* control; ##, $p < 0.01$ *versus* LDL alone; $$, $p < 0.01$ *versus* LDL alone.

ratio in the presence or absence of LDL loading. In contrast, inhibition of p38 MAPK with either SB203580 or specific siRNA against p38 MAPKα increased expression of Ulk1 and the LC3II/LC3I ratio in the presence or absence of LDL loading. Together, these results indicate that LDL cholesterol loading inhibits autophagy and expression of the key autophagy gene (*ulk1*) in a p38 MAPK-dependent manner.

DISCUSSION

p38 MAPK has been strongly implicated in the development of atherosclerosis (50). However, its direct role in foam cell formation, an early event in the development of atherosclerosis, has not been investigated. We addressed this issue in this study and made several key findings.

First, p38 MAPK regulates the level of cholesterol ester accumulation in macrophages. Cholesterol ester accumulation in macrophages is an essential step of foam cell formation and development of atherosclerosis. The level of cholesterol esters in vascular macrophages is determined by the conversion of free cholesterol and fatty acids into cholesterol esters catalyzed by ACAT1 and the degradation of cholesterol esters back to free cholesterol and fatty acids catalyzed by cholesterol ester hydrolase. Transgenic overexpression of cholesterol ester hydrolase has been shown to prevent/reduce atherosclerosis (2, 4). Although p38 MAPK has been shown to be involved in the development of atherosclerosis in many different ways, its direct role in foam cell formation has not been described. For the first time, we show here that cholesterol loading via LDL can activate p38 MAPK, and activation of p38 MAPK is involved in elevated accumulation of cholesterol esters in macrophages (Figs. 1 and 2). This finding will provide a more specific target to prevent and reverse foam cell formation and atherosclerosis through p38 MAPK.

Second, autophagy regulates the level of cholesterol ester accumulation in macrophages. Autophagy is a housekeeping process to maintain cellular health by removing aged/damaged

large and long living molecules and cellular organelles (11, 51). Its activation may be required for maintaining the health of vascular wall cells. That may be why some studies have shown that autophagy is protective against development of atherosclerosis (10, 11). However, some have suggested that overactivation of autophagy may destabilize existing atherosclerotic plaques, leading to acute cardiovascular events (10, 11). A recent study has shown that autophagy regulates cholesterol efflux from macrophage foam cells via lysosomal acid lipase (14). However, the direct role for autophagy in cholesterol ester accumulation in macrophages, foam cell formation, and atherosclerosis has not been established. Results from this study clearly show that activation of autophagy can reduce levels of total cholesterol and cholesterol esters in macrophages exposed to LDL cholesterol, whereas inhibition of autophagy can increase intracellular accumulation of total cholesterol and cholesterol esters in macrophages (Figs. 3 and 4). Furthermore, our results show that the enzyme that degrades cholesterol esters, nCEH, is co-localized with autophagosomes (Fig. 5). To our knowledge, this is the first evidence showing that the nCEH-mediated degradation of cholesterol esters involves autophagy and will provide new and more effective methods to prevent and reverse atherosclerosis through modulating autophagy.

Third, p38 MAPK regulates cholesterol ester accumulation in macrophages through autophagy. Atherosclerosis is considered an inflammatory disorder of the arterial intima (11). It is well known that p38 MAPK can activate the inflammatory program through various mechanisms, and inflammation in turn can also activate p38 MAPK (50). Thus, p38 MAPK and inflammation are inseparable, and the cause and effect relationship between them is not established. It is noteworthy that increased intracellular cholesterol level can activate p38 MAPK in macrophages as shown in this study. Activation of p38 MAPK then leads to increased accumulation of cholesterol esters in macrophages. Therefore, it appears that activation of p38 MAPK plays a critical role in cholesterol ester accumulation in macrophages and formation of foam cells, an early step in the development of atherosclerosis no matter whether p38 MAPK is activated by either cytokines or elevated intracellular cholesterol level. It has previously shown that p38 MAPK can inhibit autophagy in hepatocytes (40). In this study, we show that activation of p38 MAPK leads to inhibition of autophagy in macrophages, probably via inhibition of the key autophagy gene, *ulk1* (Figs. 5 and 6). Most importantly, LDL cholesterol loading-induced cholesterol ester accumulation and inhibition of autophagy in macrophages are p38 MAPK-dependent. All of these observations are brand new and provide a deeper level of understanding about mechanisms of foam cell formation and atherosclerosis. Therefore, results from this study will provide new and more specific targets to prevent and treat atherosclerosis and its many associated major health problems.

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