

Sitosterol-containing Lipoproteins Trigger Free Sterol-induced Caspase-independent Death in ACAT-competent Macrophages*

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Sitosterolemia is a disease characterized by very high levels of sitosterol and other plant sterols and premature atherothrombotic vascular disease. One theory holds that plant sterols can directly promote atherosclerosis, but the mechanism is not known. Unesterified, or “free,” cholesterol (FC) is a potent inducer of macrophage death, which causes plaque necrosis, a precursor to atherothrombosis. FC-induced macrophage death, however, requires dysfunction of the sterol esterifying enzyme acyl-coenzyme A-cholesterol acyltransferase (ACAT), which likely occurs slowly during lesion progression. In contrast, plant sterols are relatively poorly esterified by ACAT, and so they may cause macrophage death and plaque necrosis in an accelerated manner. In support of this hypothesis, we show here that macrophages incubated with sitosterol-containing lipoproteins accumulate free sterols and undergo death in the absence of an ACAT inhibitor. As with FC loading, sitosterol-induced macrophage death requires sterol trafficking to the endoplasmic reticulum, and sitosterol-enriched endoplasmic reticulum membranes show evidence of membrane protein dysfunction. However, whereas FC induces caspase-dependent apoptosis through activation of the unfolded protein response and JNK, sitosterol-induced death is caspase-independent and involves neither the unfolded protein response nor JNK. Rather, cell death shows signs of necroptosis and autophagy and is suppressed by inhibitors of both processes. These data establish two new concepts. First, a relatively subtle change in sterol structure fundamentally alters the type of death program triggered in macrophages. Understanding the basis of this alteration should provide new insights into the molecular basis of death pathway signaling. Second, sitosterol-induced macrophage death does not require ACAT dysfunction and so may occur in an accelerated fashion. Pending future *in vivo* studies, this concept may provide at least one mechanism for accelerated plaque necrosis and atherothrombotic disease in patients with sitosterolemia.

Plant sterols are abundant in human diets and are absorbed with cholesterol by intestinal epithelial enterocytes in a process involving the sterol transporter Niemann-Pick C1-like protein

1 (1). However, in most humans, plasma levels of intestinally derived plant sterols are much lower than those of intestinally derived cholesterol. The mechanism involves selective efflux of plant sterols into the intestinal lumen by the ABC² transporters ABCG5 and ABCG8 (1). Mutations in these ABC transporters give rise to a condition called sitosterolemia, in which plasma levels of plant sterols reach very high levels (2–4). Of great interest, patients with sitosterolemia have been observed to develop premature and severe coronary artery atherothrombotic disease (5–9).

The mechanism of accelerated coronary artery disease in sitosterolemic patients is unknown. One theory holds that these subjects have increased traditional atherosclerosis risk factors, such as elevated LDL cholesterol (10), but the correlation between high LDL and coronary events in sitosterolemia is not consistent (4). Another theory suggests that sitosterol and possibly other plant sterols have direct, pro-atherogenic effects in the arterial wall (11, 12). In support of this theory, plant sterol levels are found in atherosclerotic lesions or xanthomas of subjects with elevated plasma sitosterol levels (13–15). However, no clear mechanism of how lesional sitosterol might promote atherothrombosis, the cause of coronary artery disease, has been elucidated.

Atherothrombotic vascular disease occurs in two major stages. In the first stage, which evolves over years, atherogenic lipoproteins are internalized by arterial wall macrophages, leading to massive cholesteryl ester accumulation (foam cells) (16–18). The process involves transport of lipoprotein-derived cholesterol to the endoplasmic reticulum (ER), where it is esterified by acyl-CoA:cholesterol acyltransferase (ACAT) (19, 20). Early foam cell lesions are non-occlusive and are therefore largely asymptomatic (21, 22). In the second stage, macrophage-rich plaques rupture or erode, which exposes circulating platelets to thrombogenic material in the lesions (21–23). This process leads within minutes to acute arterial occlusion and tissue infarction.

A key event in plaque disruption is macrophage death, leading to generation of the necrotic or lipid core (24, 25). Necrotic

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² The abbreviations used are: ABC, ATP-binding cassette; ACAT, acyl-CoA:cholesterol acyltransferase; ER, endoplasmic reticulum; FC, free cholesterol; LDL, low density lipoprotein; Nec-1, necrostatin-1; rAcL-CO/SO, acetyl-LDL reconstituted with cholesteryl oleate or sitosteryl oleate; SERCA, sarcoplasmic-endoplasmic reticulum calcium ATPase; TUNEL, transferase-mediated dUTP nick-end labeling; UPR, unfolded protein response; JNK, c-Jun NH₂-terminal kinase; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MOPS, 4-morpholinopropanesulfonic acid; eIF, eukaryotic initiation factor.

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cores are thought to promote plaque disruption through release of proteases, inflammatory cytokines, and pro-coagulant/thrombotic molecules as well as through imparting physical stress on the arterial wall (23, 25–29). Because late lesional macrophages accumulate large amounts unesterified, or “free,” cholesterol (FC), which is cytotoxic, one proposed inducer of late lesional macrophage death is FC loading (20, 23, 30–40). Although the mechanism of FC accumulation is uncertain, we have *in vivo* evidence that events centered on the ER are involved (37, 38). The most likely explanation is that a combination of defective cholesterol esterification by ACAT and defective cholesterol efflux are involved. However, the actual extent of ACAT dysfunction or FC-induced macrophage apoptosis in lesions is not known.

If indeed late lesional macrophages acquire a defect in ACAT-mediated esterification, this process would likely occur gradually over a long period of time. However, plant sterols, including sitosterol, are incompletely esterified by macrophage ACAT (ACAT1), which may be due to a partial deficiency in their ability to activate the enzyme (41–44). Thus, if sitosterol-containing lipoproteins were internalized by macrophages, the accumulation of potentially cytotoxic free sterol might occur much earlier in the course of atherosclerosis progression. This mechanism could provide at least one explanation for the accelerated atherothrombosis in sitosterolemia.

To begin to test this concept, we have conducted a study to determine whether lipoprotein-derived sitosterol is cytotoxic in macrophages in the absence of an ACAT inhibitor (“ACAT-competent” macrophages). Our data show that such lipoproteins are robust inducers of cell death in ACAT-competent macrophages and that the mechanism is fundamentally different from that caused by free cholesterol. Whereas FC triggers caspase-dependent apoptosis through activation of a pro-apoptotic branch of the ER stress pathway known as the unfolded protein response (UPR) (37, 38), sitosterol-induced death involves neither caspases nor the UPR but rather has properties in common with a death process recently described as “necroptosis” (45). These findings not only suggest a possible mechanism of plant sterol-induced atherothrombotic disease but also reveal how a subtle change in sterol structure can have a dramatic effect on cell death pathways.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and reagents were from Invitrogen. HPLC grade organic solvents were from Fisher Scientific. LDL (d 1.020–1.063 g/ml) was isolated from fresh human plasma by preparative ultracentrifugation as previously described (46). Acetyl-LDL was prepared by reaction of LDL with acetic anhydride (47). Sitosterol and cholesteryl oleate, 3-methyladenine, and pepstatin A were from Sigma. Compound 58035 (3-[decyldimethylsilyl]-*N*-(2-(4-methylphenyl)-1-phenylethyl)propanamide), an inhibitor of ACAT, was generously provided by Dr. John Heider, formerly of Sandoz, Inc. (East Hanover, NJ) (48). A stock solution of 10 mg/ml was prepared in dimethyl sulfoxide and stored at -20°C . U18666A (3- β -[2-diethylaminoethoxy]androst-5-en-17-one hydrochloride) and necrostatin-1 (methylthiohydantoin-DL-tryptophan) were from Biomol Research laboratories, Inc.

(Plymouth Meeting, PA). SP600125 (anthra[1,9-*cd*]pyrazolo-6(2*H*)-one) was from BioSource International, Inc., and Z-DEVD-fmk was from R&D Systems, Inc. Antibodies against p38, phospho-p38, phospho-eIF-2 α , cleaved caspase-3, and cleaved caspase-9 were from Cell Signaling Technology. Antibodies against CHOP (GADD153), β -actin, rabbit anti-FasL IgG (C-178), and non-immune rabbit IgG were from Santa Cruz Biotechnologies, Inc. Anti-XBP-1 antibody was a gift from Dr. David Ron (New York University), and anti-LC3 antibody was kindly provided by Dr. Takahiro Kamimoto (National Institute of Genetics, Mishima, Japan).

Mice—Female mice, 8–10 weeks of age, were used in this study. C57BL/6J mice were from Jackson Laboratories. *Chop*^{-/-} mice on the C57BL/6 background were from Drs. David Ron (New York University) and Robert Burke (Columbia University). Macrophage-specific p38-deficient mice on the C57BL/6 background were created as previously described (49).

Eliciting, Culturing, and Incubations of Mouse Peritoneal Macrophage—Peritoneal macrophages were elicited by intraperitoneal injection of methyl-bovine serum albumin in mice previously immunized with this antigen (50, 51). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, and 20% L-cell-conditioned medium for 24–48 h to reach confluence.

Synthesis of Sitosteryl Oleate—Sitosterol (100 mg, 0.24 mmol) was dissolved in benzene (10 ml). To this solution, oleic anhydride (158 mg, 0.29 mmol) and 4-(dimethylamino)pyridine (5 mg, catalytic amount) were added. The reaction mixture was stirred overnight in the dark at room temperature and then washed twice with 5 ml of 1 *N* HCl and three times with 5 ml of saturated NaHCO₃ solution. The organic phase was dried with anhydrous Na₂SO₄ and filtered. The solvent was removed by evaporation under reduced pressure to provide crude product. Purification of the crude product by silica gel chromatography (petroleum ether/CH₂Cl₂, 10:1) gave sitosteryl oleate as a sticky oil in 95% yield. The structure of the product was confirmed by NMR.

Reconstitution of Acetyl-LDL—Acetyl-LDL was reconstituted with defined neutral lipids as described (52). Briefly, 1.9 mg of acetyl-LDL and 25 mg of potato starch were frozen and lyophilized in a 15-ml glass tube. The endogenous core lipids were then extracted three times with 5 ml of heptane, and then 6 mg of the indicated steryl oleate (dissolved in 200 μ l of heptane) were added to the tube. This mixture was then dried, and the sample was resuspended in 1 ml of 10 mM Tricine (pH 8.4). This suspension was incubated for 10–24 h. The solubilized reconstituted lipoprotein was separated from the bulk of the starch and excess lipid by low-speed centrifugation (2,000 \times *g*). The specimen was further clarified by two additional centrifugations at 10,000 \times *g* for 10 min and stored under argon at 4 $^{\circ}\text{C}$. Aliquots were assayed for protein concentration by the method of Lowry *et al.* (53).

Lipid Mass Analysis—Cell monolayers were extracted twice with hexane/isopropyl alcohol (3:2, v/v), and the lipid extracts were assayed for free and total sterol mass by gas-liquid chromatography as described (54). 5 α -Cholestane served as an internal standard to correct for losses during the extraction

procedure. The cell monolayers remaining after lipid extraction were dissolved in 0.1 N NaOH, and aliquots were assayed for protein concentration by the method of Lowry (53).

Measurements of Ca^{2+} -dependent ATPase Activity—Sarcoplasmic-endoplasmic reticulum calcium-dependent ATPase (SERCA) activity in control or sterol-enriched microsomal membranes was measured as previously described (55). Briefly, 100 μ g of microsomal membranes isolated from wild type or SERCA2b-overexpressing HEK293 cells were incubated with various amounts of sterols in complex with methyl- β -cyclodextrin at 25 °C for 30 min. The microsomes were recovered by centrifugation, and SERCA ATPase activity was measured using an enzyme-coupled spectrophotometric assay in which hydrolysis of ATP is coupled to the oxidation of NADH (55). The depletion of NADH was then detected by a decrease in absorption at 340 nm using a Spectra Max 190 fluorospectrometer (Molecular Devices) maintained at 30 °C. The assay buffer contained 120 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 1.5 mM phosphoenolpyruvate, 1 mM dithiothreitol, 0.45 mM $CaCl_2$, 0.5 mM EGTA, 25 mM MOPS/KOH, 0.32 mM NADH, 5 units/ml pyruvate kinase, 10 units/ml lactate dehydrogenase, and 2 μ M of the calcium ionophore A23187. The pH was adjusted to 7.0 with KOH before addition of the enzymes, and the free Ca^{2+} concentration in the solution was determined to be ~ 3 μ M. The reaction was started by adding 10 μ g of the membranes to 200 μ l of the assay buffer in wells of a 96-well UV-visible transparent bottom plate, followed by rapid mixing. The absorption at 340 nm was recorded at 30-s intervals for 30 min using the SOFTmax PRO 3.0 program.

Annexin V-Propidium Iodide Staining—Annexin V staining was performed using the Vybrant Apoptosis Assay kit 2 (Molecular Probes). At the end of incubation, cells were gently washed twice with phosphate-buffered saline, and then incubated in 100 μ l of annexin-binding buffer (25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% bovine serum albumin) containing 5 μ l of Alexa Fluor 488 annexin V, and 1 μ l of 100 μ g/ml propidium iodide for 15 min at room temperature. Cells were immediately viewed using an Olympus IX-70 inverted fluorescence microscope equipped with filters appropriate for fluorescein and rhodamine. For quantification, three fields of cells for each condition (~ 1500 cells) were counted.

Hoechst Staining—Cells were washed with phosphate-buffered saline 3 times, incubated with 100 ng/ml Hoechst 33258 for 30 min at room temperature, and then washed with phosphate-buffered saline. Cells were viewed by fluorescence microscopy using a UV filter.

TUNEL Assay—Macrophages were fixed with 1% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 10 min at room temperature, and then post-fixed in pre-cooled ethanol/acetic acid (2:1) for 5 min at -20 °C. The cells were then stained using ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon International). In this assay, the incorporated dNTP is modified by digoxigenin, which in turn is recognized by a fluorescein-labeled antibody. A propidium iodide counterstain, which is taken up by fixed cells, was used to stain all of the cells on the dish. The fluorescence microscopy and quantification procedures were similar to that described above for the

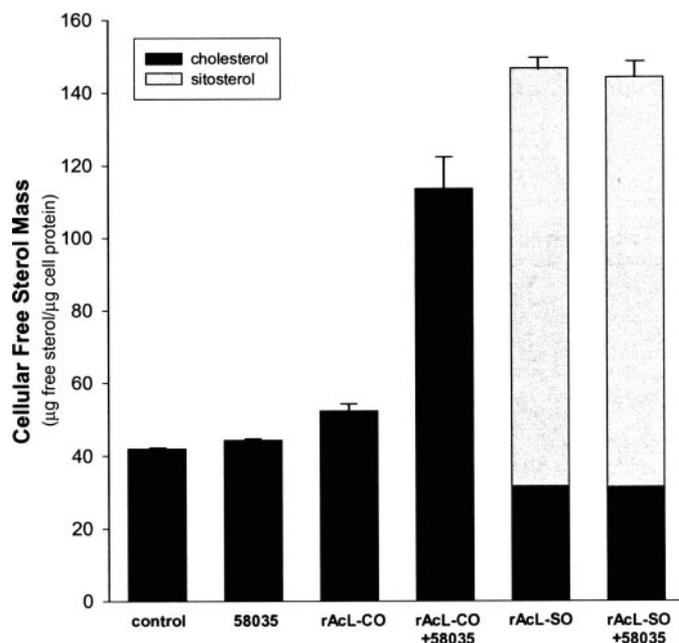


FIGURE 1. Macrophages incubated with rAcLDL-sitosteryl oleate in the absence of an ACAT inhibitor accumulate large amounts of free sitosterol. Macrophages were incubated for 15 h with medium alone (*control*) or with medium containing 10 μ g/ml 58035 (ACAT inhibitor), 100 μ g/ml rAcLDL-cholesteryl oleate (*rAcL-CO*) \pm 58035, or 100 μ g/ml rAcLDL-sitosteryl oleate (*rAcL-SO*) \pm 58035. Then cells were then rinsed, incubated with medium alone for 30 min, and rinsed again. Cellular lipids were extracted and assayed for free cholesterol and free sitosterol, and the lipid-extracted cell residues were assayed for protein. The data are displayed as micrograms of free cholesterol (*solid bars*) or sitosterol (*gray bars*) per μ g of cell protein.

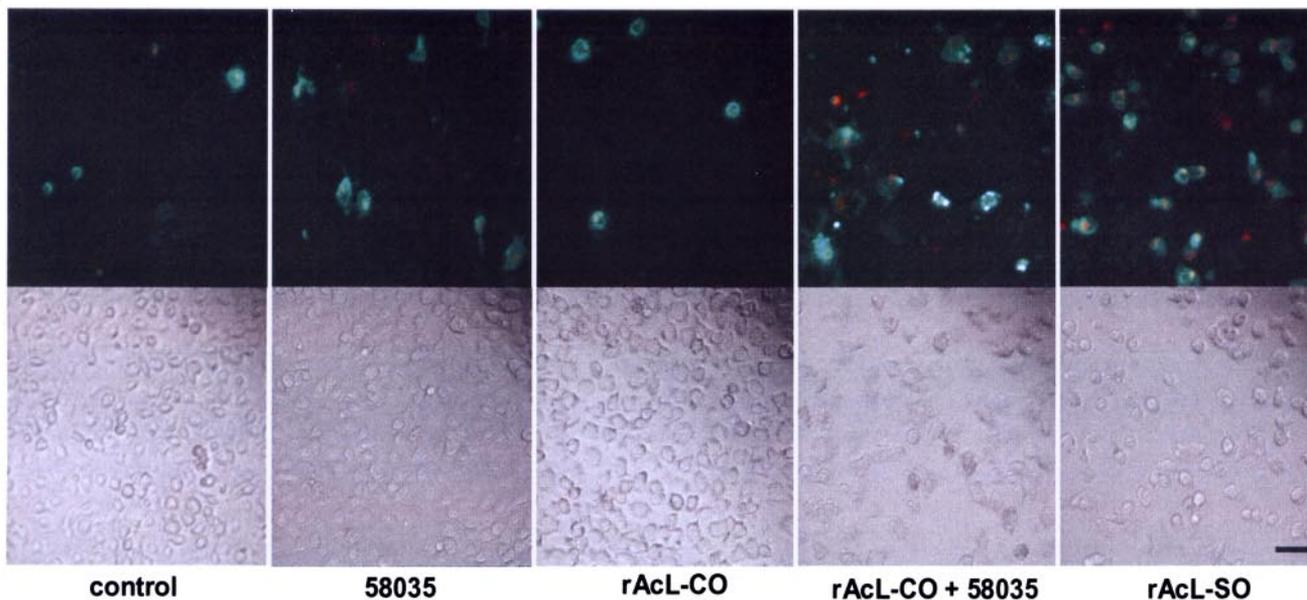
annexin assay. Apoptosis is defined as those cells that stain with the fluorescein-labeled anti-digoxigenin-dNTP antibody.

Mitotracker Red Staining—Macrophages on coverslips were incubated in media containing 300 nM Mitotracker Red (Molecular Probes) for 30 min at 37 °C. The cells were washed three times with phosphate-buffered saline, fixed with 2% paraformaldehyde for 15 min at room temperature, and viewed by fluorescence microscopy. The fluorescent images were collected using a Zeiss LSM 510 confocal microscope.

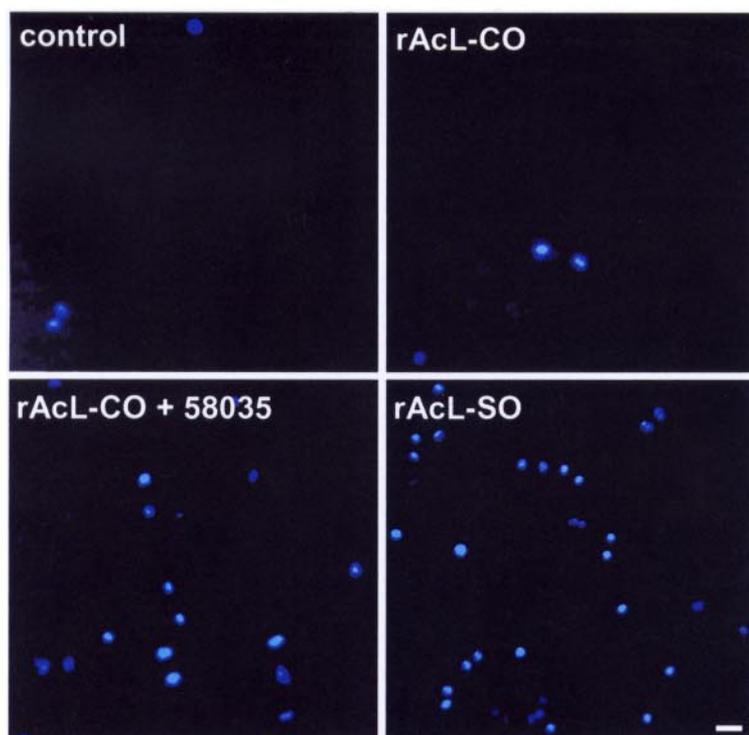
Immunoblotting—Whole cell lysates were prepared by homogenizing the cells into 1 \times Sample Loading buffer from Bio-Rad. Nuclear extracts were prepared using the Nuclear Extraction kit from Panomics, Inc. Cell extracts were electrophoresed on 4–20% gradient SDS-PAGE gels and transferred to 0.45- μ m nitrocellulose membranes. For caspase-3 and caspase-9 immunoblots, 15 and 12.5% SDS-PAGE gels and 0.2- μ m nitrocellulose membranes were used. The membrane was blocked in Tris-buffered saline, 0.1% Tween 20 (TBST) containing 5% (w/v) nonfat milk at room temperature for 1 h, then incubated with the primary antibody in TBST containing 5% (w/v) nonfat milk or 5% bovine serum albumin at 4 °C overnight, followed by incubation with the appropriate secondary antibody coupled to horseradish peroxidase. Proteins were detected by ECL chemiluminescence (Pierce).

Statistics—Data are presented as mean \pm S.E. of triplicate experiments unless stated otherwise. Absent error bars in the bar graphs signify S.E. values smaller than the graphic symbols.

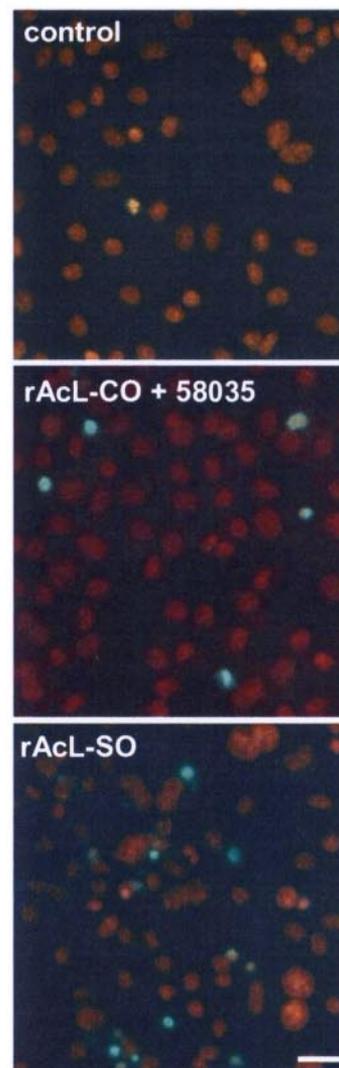
A



B



C



RESULTS

Incubation of Macrophages with Sitosteryl Ester-containing Lipoproteins Leads to Free Sitosterol Accumulation in the Absence of an ACAT Inhibitor—When lipoprotein-cholesterol is delivered to macrophages, the cholesterol is efficiently esterified by the ER enzyme ACAT, thus preventing FC accumulation and FC-induced macrophage death (20, 56, 57). Thus, lipoprotein-cholesterol accumulation induces death only when ACAT-mediated esterification is inhibited *in vitro* or becomes dysfunctional *in vivo*, which we propose occurs in a gradual manner during the course of advanced atherosclerosis (20). Previous work from our group and others has shown that a number of plant sterols, including sitosterol, is less efficiently esterified by ACAT than cholesterol (41–44). If so, free sitosterol levels should accumulate in macrophages even in the absence of an ACAT inhibitor. To test this idea using a physiologic delivery system, we synthesized the oleate ester of sitosterol and packaged it in reconstituted acetyl-LDL lipoprotein particles (rAcL-SO). We then compared free sterol accumulation in control and ACAT-inhibited macrophages incubated with these particles, as well as acetyl-LDL reconstituted with cholesteryl oleate (rAcL-CO). As expected, excess FC accumulated only when rAcL-CO was incubated with ACAT-inhibited macrophages (Fig. 1, *first four bars*). In striking contrast, free sitosterol accumulated when rAcL-SO was incubated with macrophages in the absence of an ACAT inhibitor (Fig. 1, *last two bars*). As predicted from the recent findings of Rudel and colleagues (44) that ACAT1, the predominant form of ACAT in macrophages, shows partial activity with sitosterol, some sitosterol esterification did occur in the macrophages without the ACAT inhibitor (data not shown). However, the proportion of sitosterol esterified by ACAT was substantially less than that of cholesterol: 42.8% sitosterol esterified *versus* 71.6% cholesterol esterified at similar levels of cellular sterol loading. Thus, macrophages accumulate large amounts of free sitosterol when exposed to sitosterol-containing lipoproteins even in the absence of an ACAT inhibitor.

Sitosterol-containing Lipoproteins Are Potent Inducers of Cell Death in ACAT-competent Macrophages—The data in Fig. 1, previous work demonstrating the cytotoxicity of free cholesterol, and the structural similarity between sitosterol and cholesterol provide the basis for our hypothesis that sitosterol-containing lipoproteins would be potent inducers of macrophage death even in the absence of an ACAT inhibitor. To directly test this hypothesis, macrophages were incubated with the aforementioned reconstituted lipoproteins in the absence or presence of an ACAT inhibitor. As shown in Fig. 2 using three different cell death assays: annexin V-propidium iodide staining, Hoechst nuclear staining, and TUNEL assay, rAcL-CO induced macrophage death only in the presence of an ACAT inhibitor, which is consistent with previous data (57, 58). In

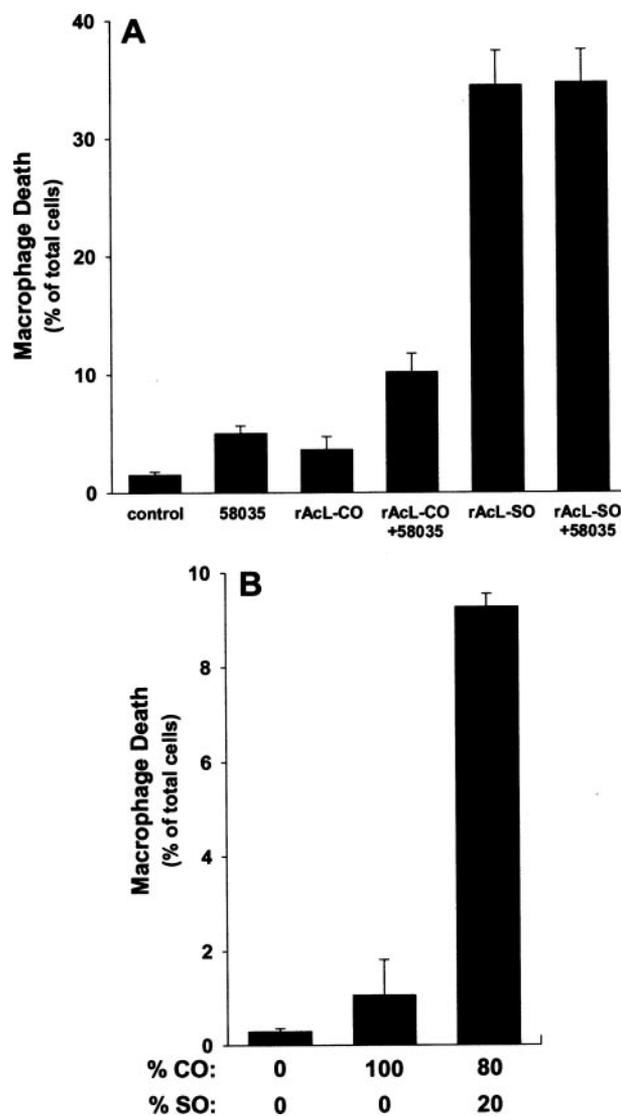


FIGURE 3. rAcLDL-sitosteryl oleate is a potent inducer of macrophage death in the absence of an ACAT inhibitor: quantitative data. *A*, macrophages from the experiment described in the legend to Fig. 2A were quantified as percentage of total cells that showed annexin V staining. *B*, macrophages were incubated for 71 h with medium alone or medium containing 100 $\mu\text{g}/\text{ml}$ rAcLDL (rAcL) containing the indicated percentages of cholesteryl oleate (CO) and sitosteryl oleate (SO). The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as in panel A.

contrast, rAcL-SO was a potent inducer of macrophage death even in the absence of an ACAT inhibitor. Quantitative annexin V data from a repeat experiment with additional controls are displayed in Fig. 3A. Consistent with the data in Fig. 2, rAcL-SO was a relatively potent inducer of macrophage death, and this effect was not dependent on ACAT inhibition. Whereas the percent rAcL-SO-induced macrophage death varied from experiment to experiment (range ~ 10 –35%),

FIGURE 2. rAcLDL-sitosteryl oleate is a potent inducer of macrophage death in the absence of an ACAT inhibitor: microscopy images. Macrophages were incubated for 26 h with medium alone (*control*) or with medium containing 10 $\mu\text{g}/\text{ml}$ 58035 (ACAT inhibitor), 100 $\mu\text{g}/\text{ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) \pm 58035, or 100 $\mu\text{g}/\text{ml}$ rAcLDL-sitosteryl oleate (rAcL-SO) \pm 58035. In *A*, the cells were stained with fluorescent annexin V (*green*) and propidium iodide (*red*) and then viewed by fluorescence (*top images*) and phase (*bottom images*) microscopy. In *B*, cells from the indicated conditions were stained with Hoechst nuclear stain and viewed by fluorescence microscopy. In *C*, cells from the indicated conditions were stained by the TUNEL method (*green*) and with a nuclear counterstain (*orange*). Bar, 10 μm .

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rAcL-SO always caused more death than rAcL-CO + 58035 whenever the two conditions were compared directly in the same experiment.

We next determined whether rAcLDL containing the proportion of sitosterol that can be seen in patients with sitosterolemia is able to cause apoptosis in the absence of an ACAT inhibitor. In a recent study from the Multicenter Sitosterolemia Study Group by Salen *et al.* (59), 37 patients with sitosterolemia had an average of ~34% plant sterols and 66% cholesterol in their LDL, and approximately two-thirds of these plant sterols were sitosterol (*i.e.* ~22% sitosterol in LDL). As shown in Fig. 3B, significant macrophage death occurred when the lipoprotein contained as little as 20% sitosteryl oleate.

Sitosterol-induced Macrophage Death Requires Sterol Trafficking to the ER, and Sitosterol Enrichment of ER Membranes Inhibits SERCA Activity—For FC loading to induce macrophage death, cholesterol trafficking to the ER is absolutely necessary (38). To test this point with sitosterol-induced death, macrophages were incubated with rAcL-SO in the absence or presence of the cholesterol trafficking inhibitor, U18666A. In a pilot experiment (not displayed), we showed that U18666A was an effective inhibitor of sitosterol trafficking to the ER. As shown in Fig. 4A, U18666A was a potent inhibitor of sitosterol-induced macrophage death. Similar results were obtained when sterol trafficking was inhibited by the heterozygous Niemann-Pick C1 mutation (data not shown; *cf.* Ref. 37). Thus, lipoprotein-derived sitosterol, like lipoprotein-derived cholesterol, requires trafficking to the ER to induce death.

Our previous work provided evidence that free cholesterol exerts its cytotoxicity at least in part by altering the structure and function of ER membranes, which normally have a low cholesterol:phospholipid ratio (38, 55). In particular, we showed that an integral membrane ER enzyme involved in UPR activation, SERCA, was inhibited when membranes containing this enzyme were enriched with cholesterol *in vitro* (55). Enzyme inhibition occurred at levels of cholesterol that corresponded to cholesterol-induced ER membrane-phase transition and to cholesterol enrichment of the ER in FC-loaded macrophages (55). To determine whether sitosterol enrichment of ER membranes was also able to inhibit SERCA activity, SERCA2b-containing membranes were assayed in the absence of sterol enrichment or after enrichment with cholesterol or sitosterol. As shown in Fig. 4B, addition of 1.5 μg of sitosterol inhibited SERCA activity, albeit somewhat less than 1.5 μg of cholesterol. At 15 μg of sterols, sitosterol inhibited SERCA to the same degree as cholesterol. These data are consistent with prior work (60–65) showing that sitosterol can affect the structure of biological membranes in a manner similar, although not identical, to cholesterol.

Sitosterol-induced Macrophage Death Does Not Depend upon the UPR Effector CHOP or JNK but Requires p38 MAP Kinase—FC trafficking to the ER triggers the ER stress pathway known as the UPR (38). Importantly, one of the UPR effectors induced by FC loading, CHOP (GADD153), is necessary for the full apoptotic response (38). Given the similar effects of cholesterol and sitosterol on SERCA activity and the requirement for sitosterol trafficking to the ER (above), we predicted that the UPR would be activated by sitosterol loading and involved in

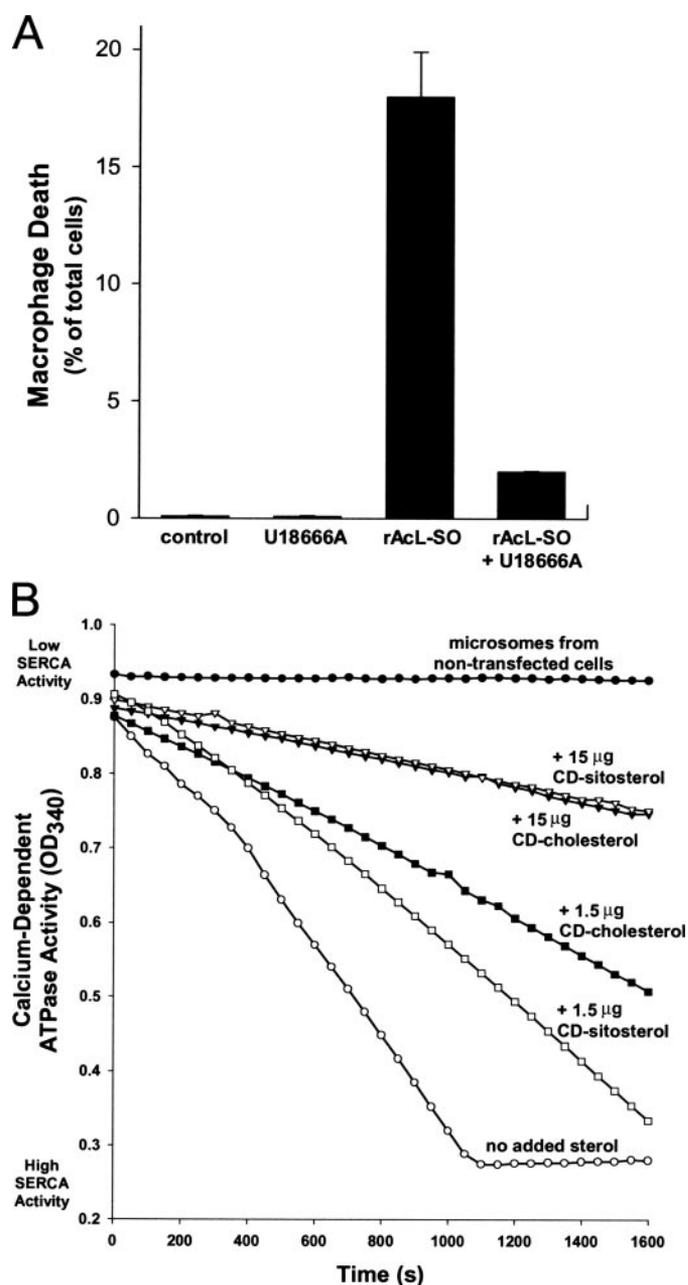


FIGURE 4. rAcLDL-sitosteryl oleate-induced macrophage death is blocked by U18666A, an inhibitor of sterol trafficking to the ER, and sitosterol, like cholesterol, inhibits SERCA2b activity when added to SERCA2b-containing microsomes. *A*, macrophages were incubated for 23 h with medium alone (*control*) or with medium containing 1 μM U18666A, 100 $\mu\text{g}/\text{ml}$ rAcLDL-sitosteryl oleate (*rAcL-SO*), or with both rAcLDL-sitosteryl oleate + U18666A. The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3. *B*, microsomal membranes (100 μg of protein) from SERCA2b-transfected HEK293 cells were incubated with either 1.5 or 15 μg of cholesterol or sitosterol complexed with methyl- β -cyclodextrin (*CD*). Controls were SERCA2b-containing membranes incubated with no added sterols and microsomes from non-transfected controls. The membranes were re-isolated by centrifugation and assayed for calcium-dependent ATPase activity, as measured by a decrease in absorption at 340 nm, indicative of consumption of NADH in this enzyme-coupled assay. In this assay, increasing SERCA activity is associated with lower OD₃₄₀. In additional controls, not shown here, ATPase activity was dependent on calcium, inhibited by the SERCA inhibitor thapsigargin, and not affected by incubation with methyl- β -cyclodextrin alone.

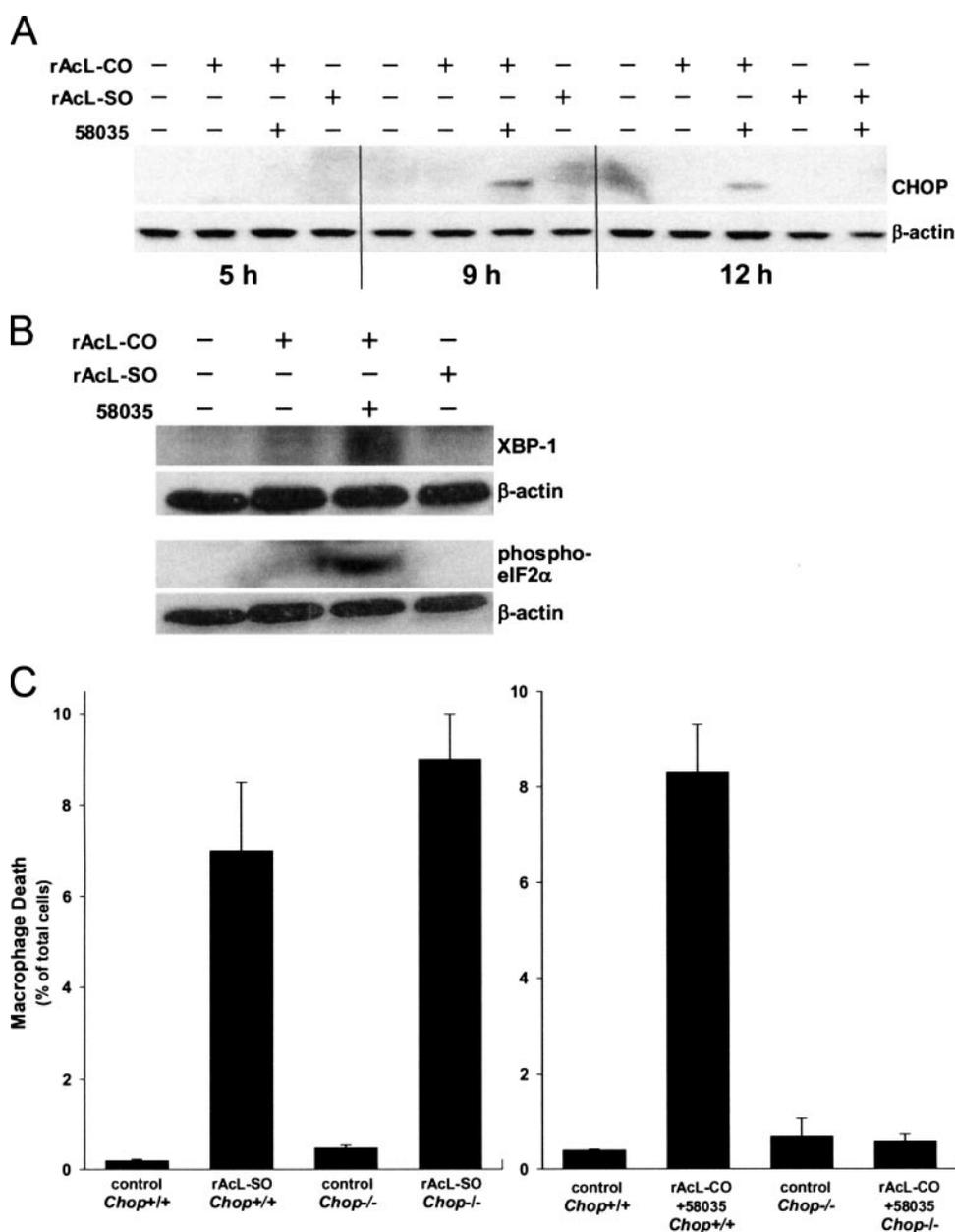


FIGURE 5. CHOP is not involved in rAcLDL-sitosterol-induced macrophage death. *A*, macrophages were incubated for the indicated times with medium alone or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) \pm 58035 or 100 $\mu\text{g/ml}$ rAcLDL-sitosterol (rAcL-SO) \pm 58035. Cell extracts were probed for CHOP and actin by immunoblotting. The doublet bands migrating above CHOP in the rAcL-SO lane at 9 h and control lane at 12 h were not consistently observed in repeat experiments. *B*, macrophages were incubated with medium alone or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) \pm 58035 or 100 $\mu\text{g/ml}$ rAcLDL-sitosterol (rAcL-SO). Cell extracts were probed for XBP-1, phosphorylated eIF2 α (P-eIF2 α), and actin by immunoblotting. The incubation time was 9 h for the XBP-1 experiment and 5 h for the eIF2 α experiment. *C*, macrophages from *Chop*^{+/+} or *Chop*^{-/-} mice were incubated for 24 h with medium alone (control) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-sitosterol (rAcL-SO) + 58035. The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3.

sitosterol-induced macrophage death. To test this prediction, we assayed CHOP in macrophages loaded with FC or sitosterol. As expected, FC loading induced CHOP, but, surprisingly, sitosterol loading of macrophages did not induce CHOP at the time points tested (Fig. 5A) and only minimally induced CHOP at 17 h of incubation (not shown). Moreover, two other makers of UPR activation induced by FC loading, expression of XBP-1 and

phosphorylation of eIF2 α , were also not found with sitosterol loading (Fig. 5B). Most importantly, deficiency of CHOP, which blocks FC-induced apoptosis (38), did not block sitosterol-induced macrophage death (Fig. 5C). These surprising data indicate that sitosterol-induced macrophage death does not involve UPR activation.

In FC-loaded macrophages, p38 mitogen-activated protein kinase is activated, which is necessary for UPR induction and UPR-induced macrophage death (49, 51). Despite the lack of a role for the UPR in sitosterol-induced death, we determined whether p38 was activated by sitosterol loading. As shown in Fig. 6A, sitosterol loading of macrophages was an extremely potent inducer of p38 phosphorylation. Moreover, we found that p38 played a major role in sitosterol-induced death, as demonstrated by the marked inhibition of death in macrophages lacking p38 (Fig. 6B). In contrast, JNK, another mitogen-activated protein kinase that is critically involved in FC-induced macrophage death (49) and that is activated by sitosterol loading of macrophages (data not shown), was not involved in sitosterol-induced death (Fig. 6C). In FC-loaded macrophages, p38 activation requires FC trafficking to the ER (51). A similar result with sitosterol could explain the importance of sitosterol trafficking to the ER in macrophage death. However, sitosterol-induced p38 phosphorylation was completely intact in the presence of the sterol trafficking inhibitor, U18666A (Fig. 6D). These data indicate the critical role of p38 in sitosterol-induced macrophage death and further highlight how the mechanism differs from FC-induced macrophage death. Moreover, the data with U18666A suggest that two independent pathways are necessary for sitosterol-induced death: one involving sitosterol trafficking to the ER and the other involving p38.

Sitosterol-induced Macrophage Death Does Not Involve Activation of Caspases-3 and -9, Fas Ligand, or Loss of Mitochondrial Membrane Potential—FC-induced macrophage death requires both the Fas and mitochondrial/caspase-9 pathways of

Sitosterol-induced Macrophage Death

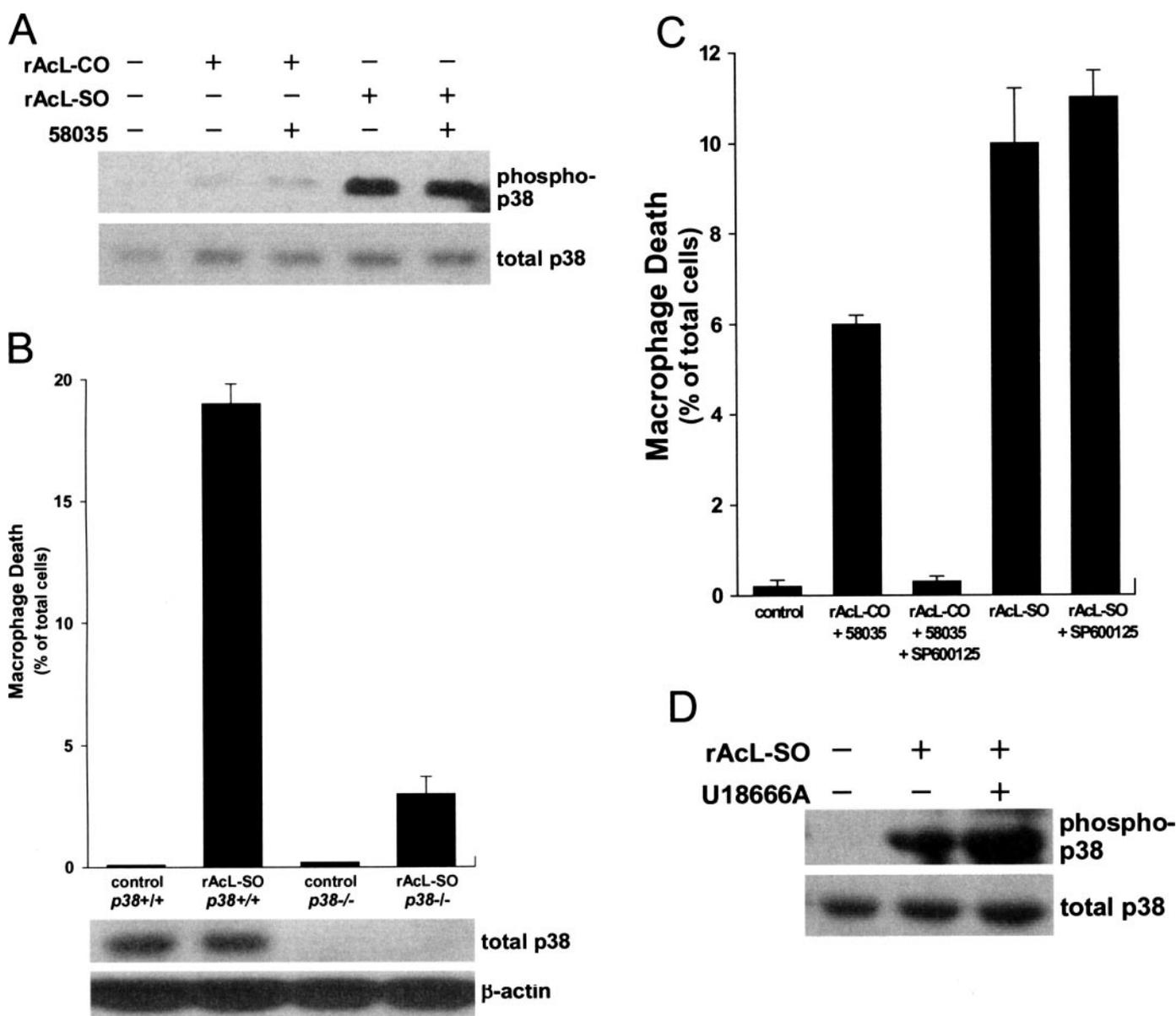


FIGURE 6. p38 is activated by rAcLDL-sitosteryl oleate and is necessary for rAcLDL-sitosteryl oleate-induced macrophage death. *A*, macrophages were incubated for 12 h with medium alone or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) \pm 58035, or 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (rAcL-SO) \pm 58035. Cell extracts were probed by immunoblotting for phosphorylated (P) and total p38. *B*, wild-type (p38^{+/+}) or p38-deficient (p38^{-/-}) macrophages were incubated for 17 h with medium alone (control) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (rAcL-SO). The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3. Shown below the graph are total p38 and actin immunoblots of samples from these macrophages. Consistent with our previous data (49), apoptosis induced by rAcLDL-cholesteryl oleate + 58035 was also lower in the p38-deficient macrophages (data not shown). *C*, macrophages were incubated for 23 h with medium alone (control) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) + 58035 \pm 20 μM SP600125 or 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (rAcL-SO) \pm 20 μM SP600125. The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3. As reported previously, SP600125 alone has no effect on cell viability (49). *D*, macrophages were incubated for 7 h with medium alone or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) \pm 1 μM U18666A. Cell extracts were probed by immunoblotting for phosphorylated and total p38.

apoptosis, leading to activation of the downstream apoptosis effector caspase-3 (58, 66). To test these pathways in sitosterol-induced macrophage death, macrophages incubated with rAcL-CO + 58035 (FC loading) were compared with those incubated with rAcL-SO for caspase-9 and caspase-3 activation. As expected, both caspases were activated (cleaved) in FC-loaded macrophages, but neither were activated by sitosterol loading (Figs. 7, *A* and *B*). Most importantly, the caspase-3 inhibitor Z-DEVD-fmk, which inhibits FC-induced apoptosis, did not inhibit sitosterol-induced apoptosis (Fig. 7*C*). We next

examined the Fas and mitochondrial pathways in more detail. Although blocking Fas ligand with an antibody inhibits ~60–70% of the FC-induced apoptosis (58), the same antibody did not block sitosterol-induced apoptosis (Fig. 7*D*). Similarly, FC loading decreases the mitochondrial membrane potential, consistent with FC-induced cytochrome *c* release and caspase-9 activation (66). Using uptake of Mitotracker Red as an indicator of mitochondrial membrane potential, we found that loading macrophages with sitosterol did not substantially decrease mitochondrial uptake of the dye under conditions in which FC

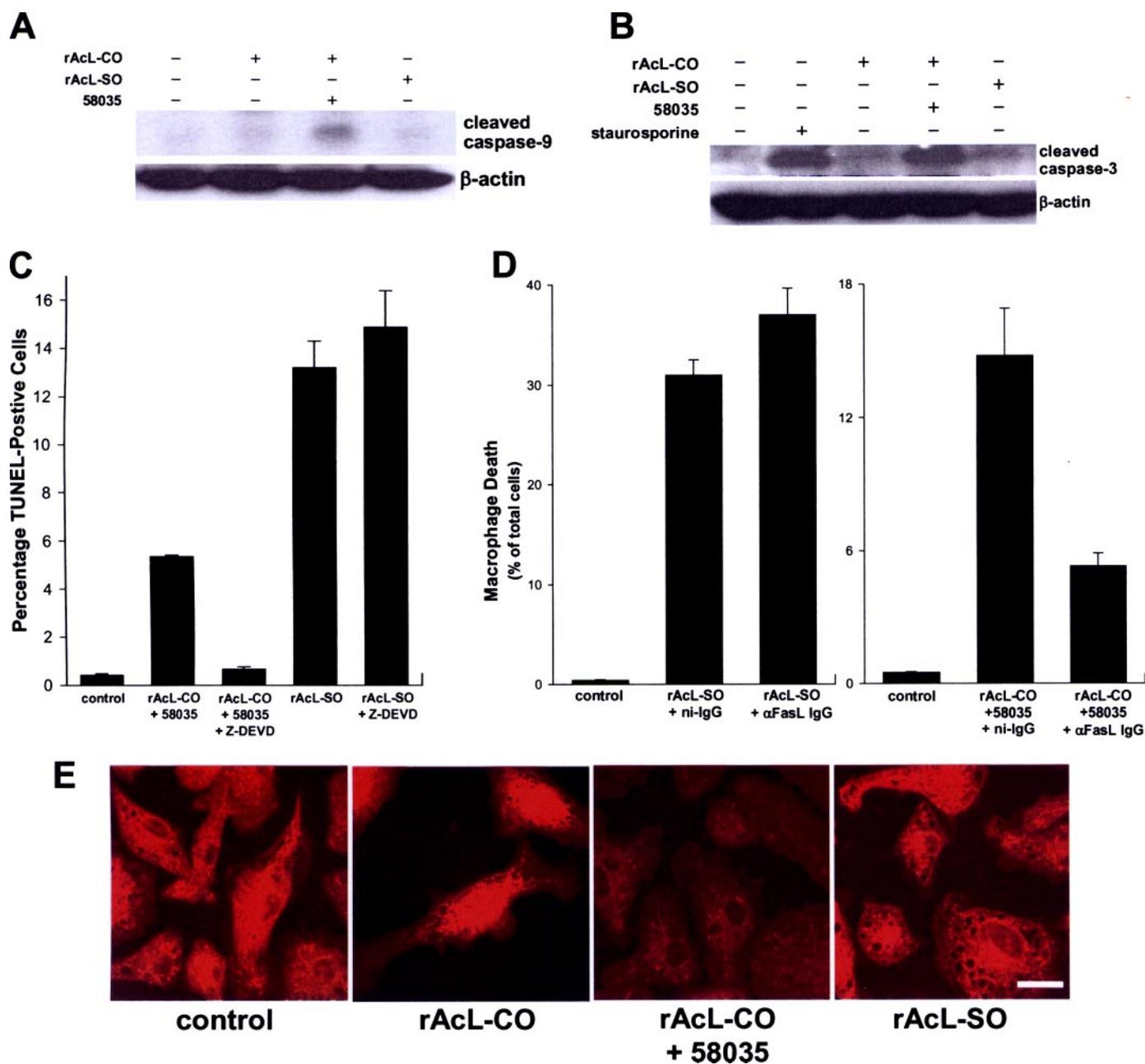


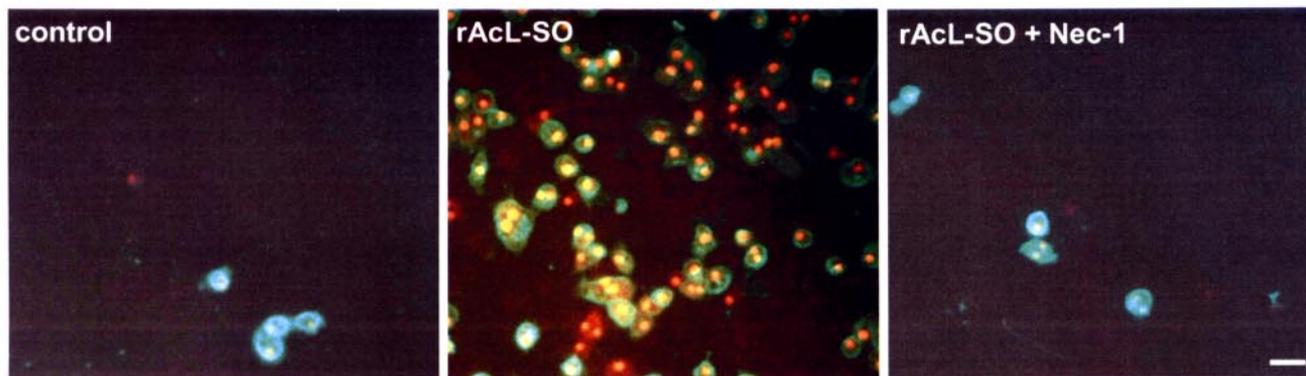
FIGURE 7. rAcLDL-sitosteryl oleate-induced macrophage death does not involve activation of caspases-9 and -3, Fas ligand, or loss of mitochondrial membrane potential. *A*, macrophages were incubated for 16 h with medium alone or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) \pm 58035, or 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (rAcL-SO). Cell extracts were probed for cleaved caspase-9 and actin by immunoblotting. *B*, macrophages were incubated for 18 h with medium alone or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) \pm 58035, or 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (rAcL-SO). Another set of macrophages was incubated with 50 nM staurosporine as an additional positive control. Cell extracts were probed for cleaved caspase-3 by immunoblotting. *C*, macrophages were incubated for 18 h with medium alone (control) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) + 58035 \pm 20 μM Z-DEVD-fmk or 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (rAcL-SO) \pm 20 μM Z-DEVD-fmk. The cells were assayed for TUNEL staining and quantified. *D*, macrophages were incubated for 24 h with medium alone (control) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (rAcL-SO) \pm 25 $\mu\text{g/ml}$ non-immune rabbit IgG (ni-IgG) or rabbit anti-FasL IgG (α FasL IgG). A similar experiment was conducted with rAcLDL-cholesteryl oleate (rAcL-CO) + 58035 (right graph). The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3. *E*, macrophages were incubated for 15 h with medium alone (control) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (rAcL-CO) \pm 58035, or 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (rAcL-SO). The cells were then stained with MitoTracker Red and viewed by confocal microscopy. Bar, 5 μm .

loading caused a marked decrease (Fig. 7E). Together, these data clearly indicate that sitosterol-induced macrophage death, in stark contrast to FC loading-induced macrophage death, does not involve classic Fas, mitochondrial, or caspase pathways.

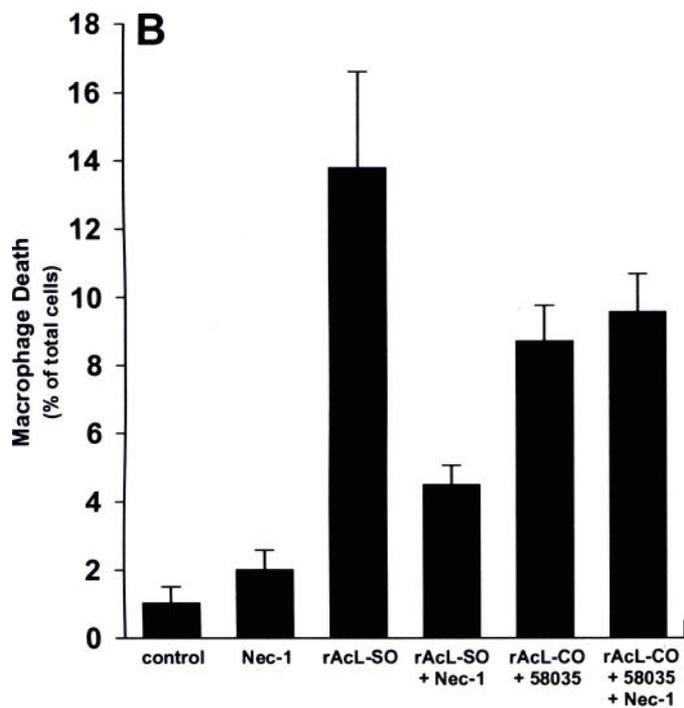
Sitosterol-induced Macrophage Death Is Inhibited by Necrostatin-1 and Leads to the Accumulation of Autophagic

Vacuoles—A recent study has shown that certain types of caspase-independent cell death with features of necrosis can be inhibited by methylthiohydantoin-DL-tryptophan (necrostatin-1 or Nec-1) (45). Examples include cultured cells treated with the combination of an apoptosis inducer plus a caspase inhibitor, cells treated with an activator of receptor-interacting protein, and neurons *in vivo* after ischemic brain injury (45).

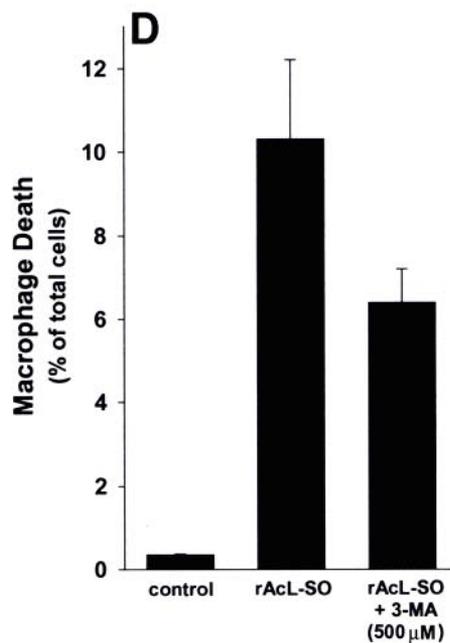
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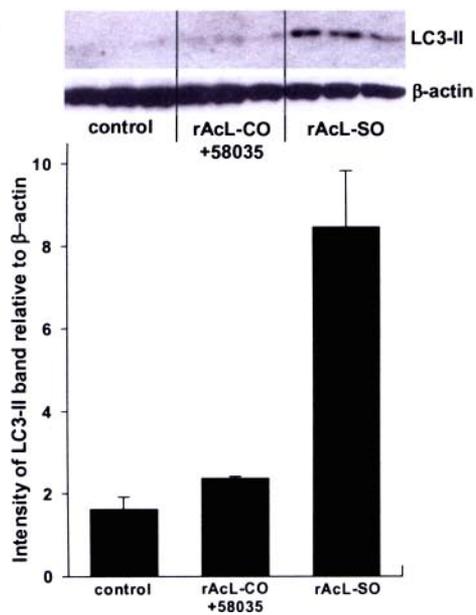
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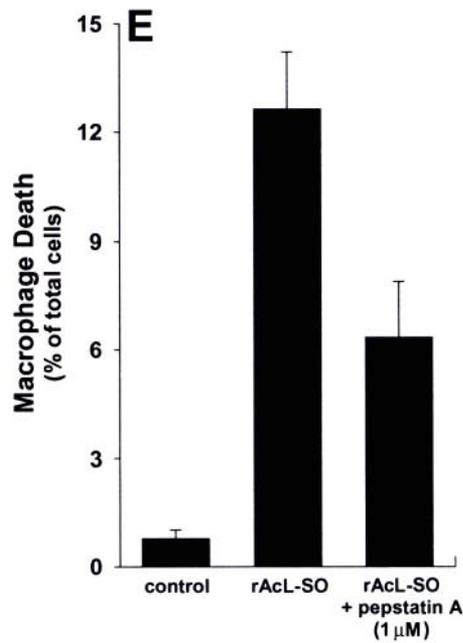
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The death-inhibiting effect of Nec-1 was highly specific for this form of death, which the authors termed necroptosis (45). Given the fact that caspases are not activated during sitosterol-induced death, we considered that a process similar to necroptosis may be involved. As shown by the images and quantified data in Fig. 8, *A* and *B*, Nec-1 almost completely inhibited sitosterol-induced macrophage death. In contrast, Nec-1 did not inhibit FC-induced macrophage apoptosis (Fig. 8*B*). Consistent with the lack of an effect of Nec-1 on FC-induced apoptosis, we showed that Nec-1 was not an inhibitor of sterol trafficking to the ER (data not shown). Moreover, Nec-1 did not block activation of p38 by sitosterol (data not shown). Therefore, Nec-1 most likely inhibits sitosterol-induced death downstream of sterol-trafficking to the ER and p38 activation (see "Discussion").

The aforementioned study with caspase-inhibited cells showed that autophagy was associated with necroptosis (45). To begin to explore the involvement of autophagy in sitosterol-induced macrophage death, control macrophages and those exposed to either FC-loading conditions or lipoprotein-sitosterol were immunoblotted for the autophagy marker, LC3-II (67). As shown in Fig. 8*C*, there was a clear increase in LC3-II in macrophages incubated with lipoprotein-sitosterol compared with control or FC-loaded macrophages. Although autophagy was downstream of cell death in the necroptosis study referred to above (45), we found that two autophagy inhibitors, 3-methyladenine and the cathepsin D inhibitor pepstatin A (68, 69), partially inhibited sitosterol-induced macrophage death (Fig. 8, *D* and *E*). Neither compound blocked FC-induced macrophage apoptosis (data not shown). Thus, sitosterol-induced macrophage death is associated with both necroptosis and autophagy in a causative manner, which remarkably distinguishes death by this sterol from death by cholesterol.

DISCUSSION

The findings in this study have revealed two new concepts that have potential implications for both pathophysiology and fundamental aspects of cellular death signaling (Fig. 9). First, incubation of macrophages with lipoproteins whose steryl esters are composed of as little as one-fifth sitosteryl ester leads to macrophage death even when ACAT activity is not inhibited. Because free sterol-induced macrophage death likely contributes to necrotic core formation in vulnerable atherosclerotic plaques (29), this finding may provide at least one mechanism for accelerated atherothrombosis in patients with very high levels of plant sterols. Second, whereas FC induces macrophage death by a pathway dependent on the UPR, JNK activation, and

caspases, sitosterol-induced death involves none of these factors. Rather, sitosterol-induced death has features similar to those of a caspase-independent cell death process referred to as necroptosis: marked suppression by necrostatin-1 and evidence of autophagy (45). Moreover, in this system, there is evidence that autophagy itself partially contributes to the death process. Thus, the addition of one extra β -ethyl group on C-24 of the sterol side chain fundamentally changes the mechanism of sterol-induced death signaling.

Regarding the first concept, the association between elevated plant sterols and accelerated atherothrombosis has been the subject of recent interest. Whereas it appears clear that premature coronary artery disease occurs in patients with sitosterolemia in a manner that cannot always be explained by other risk factors (5–9), a mechanism is lacking. In terms of macrophage biology, Nguyen *et al.* (11) showed that sitosterol suppressed hydroxymethylglutaryl-CoA reductase activity less than cholesterol in human monocyte-derived macrophages, but how this feature would promote atherogenesis is not clear. Awad *et al.* (70) found that phytosterols decreased the release of the prostaglandins E₂ and I₂ from a murine macrophage cell line, but the net effect of these effects on specific atherogenic processes was not reported. Other cells might also explain the potential atherogenicity of high plant sterol levels. For example, Boberg *et al.* (12) found that sitosterol was cytotoxic to cultures of human umbilical vein endothelial cells. The mechanism of cytotoxicity in this study was not reported. We suggest an alternative mechanism: the accumulation of sitosterol in advanced lesional macrophages leads to cell death in an accelerated manner, because the delay associated with ACAT becoming dysfunctional would not be needed. Accelerated macrophage death, in the face of relatively inefficient phagocytic clearance of dead cells (Ref. 29, and see below), would lead to faster progression to plaque necrosis, plaque disruption, and thrombo-occlusive vascular events. This hypothesis is based on the findings here together with previous work showing that (*a*) lesional necrosis consists of dead macrophages (24, 25, 27); (*b*) a genetic alteration in mice that decreased advanced lesional macrophage death *in vivo* decreased plaque necrosis (37); (*c*) necrotic areas in plaques likely promote plaque disruption (23, 26, 28, 29); and (*d*) large areas of necrosis characterize the coronary artery plaques of sitosterolemic patients (5–9).

It should be noted that the concept of accelerated macrophage death by sitosterol raises, at least in theory, the possibility of a very different scenario. Recent studies have shown that macrophage apoptosis in early atherosclerotic lesions,

FIGURE 8. rAcLDL-sitosteryl oleate-induced macrophage death is inhibited by necrostatin-1 and has characteristics of autophagy. *A*, macrophages were incubated for 23 h with medium alone (*control*) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (*rAcL-SO*) \pm 30 μM necrostatin-1 (*Nec-1*). The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3. *Bar*, 10 μm . *B*, macrophages were incubated for 18 h with medium alone (*control*), 30 μM necrostatin-1 (*Nec-1*) alone, rAcLDL-sitosteryl oleate (*rAcL-SO*) \pm 30 μM necrostatin-1, or rAcLDL-cholesteryl oleate (*rAcL-CO*) \pm 30 μM necrostatin-1. The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3. *C*, macrophages were incubated for 13 h with medium alone (*control*) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-cholesteryl oleate (*rAcL-CO*) + 58035 or 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (*rAcL-SO*). Cell extracts in triplicate were immunoblotted using antibodies against LC3-II and actin. The densitometry data for LC3-II relative to β -actin are quantified in the graph. *D*, macrophages were incubated for 31 h with medium alone (*control*) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (*rAcL-SO*) \pm 500 μM 3-methyladenine (3-*MA*). The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3. *E*, macrophages were incubated for 22 h with medium alone (*control*) or with medium containing 100 $\mu\text{g/ml}$ rAcLDL-sitosteryl oleate (*rAcL-SO*) \pm 1 μM pepstatin A. The cells were assayed for externalized phosphatidylserine by fluorescent annexin V staining and quantified as described in the legend to Fig. 3.

Sitosterol-induced Macrophage Death

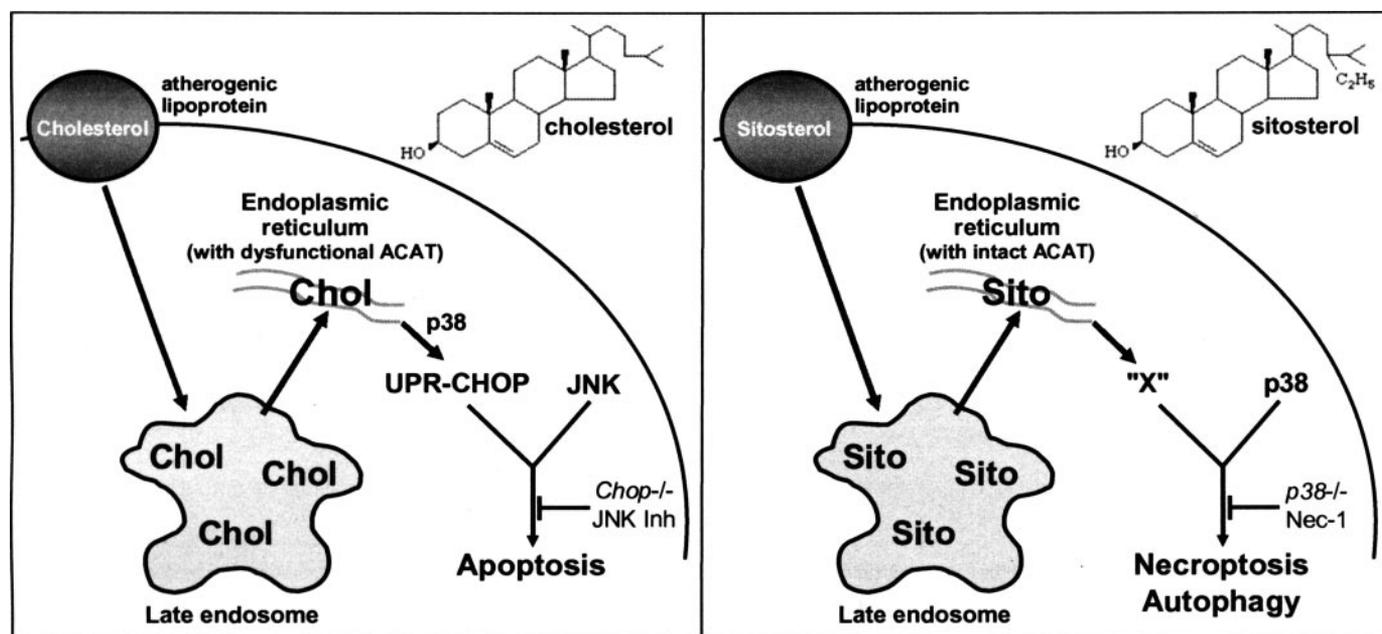


FIGURE 9. Summary scheme of how lipoprotein-derived cholesterol and sitosterol trigger different death pathways in macrophages. See text for details. In the *right panel*, "X" refers to a non-CHOP process that requires sitosterol trafficking to the ER and that, together with activated p38, is necessary for sitosterol-induced necroptosis. This 2-branch model is based on the findings that both the sterol trafficking blocker U18666A and p38 deficiency block sitosterol-induced necroptosis, but U18666A does not block p38 activation, and p38 deficiency does not block sterol trafficking to the ER. Note the inhibitory action of Nec-1 is placed downstream of both pathways because Nec-1 does not inhibit either sterol trafficking to the ER or p38 activation.

unlike that in advanced lesions, is accompanied by rapid phagocytic clearance of the dying cells. This efficient phagocytic clearance prevents inflammation and post-apoptotic necrosis, and so the net effect of early lesional macrophage death is decreased in lesional macrophage cellularity, which retards plaque progression (29). Therefore, if macrophages killed by sitosterol were phagocytosed in an efficient and non-inflammatory manner, sitosterol-induced death in early, ACAT-competent macrophages may actually retard lesion progression. Whereas this hypothesis remains a formal possibility pending additional *in vivo* studies, we found that macrophages killed by sitosterol are relatively poorly ingested by phagocytic macrophages (data not shown). Moreover, the association of sitosterolemia with premature coronary artery disease (above) and the finding that foam cell lesions in Western diet-fed sitosterolemic *Abcg5g8*^{-/-}; *Ldlr*^{-/-} mice are not significantly smaller than those in *Abcg5g8*^{+/+}; *Ldlr*^{-/-} mice (10) are consistent with inefficient phagocytic clearance of sitosterol-killed macrophages.

Despite the close association between sitosterolemia and coronary artery disease, a number of seemingly contradictory findings have appeared in the literature. For example, a recent study using a mouse model of sitosterolemia reported no increase in aortic atherosclerosis (10). However, atherosclerosis was assayed by percent *en face* lipid staining, not by morphological analyses of advanced lesions that would be needed to detect lesional macrophage death and plaque necrosis (10, 71), which is the most relevant parameter related to the findings and hypothesis here. Another area of recent interest is whether humans without sitosterolemia *per se* but with levels of plasma plant sterols in the upper percentiles of the normal population are at risk for coronary artery disease (6, 71). Such individuals, perhaps due to poly-

morphisms in ABCG5/G8 or secondary to high dietary plant sterol consumption, have plant sterol plasma levels that are relatively elevated but substantially less than that seen in patients with sitosterolemia (71). Whereas some reports have suggested that these individuals have increased risk for heart disease (72–76), other studies have suggested otherwise (10, 77). Indeed, dietary plant sterol supplements in normal individuals can lower plasma LDL by blocking intestinal cholesterol absorption and may be atheroprotective (78). The mechanism explored herein, in which lipoproteins with a sitosteryl ester:cholesteryl ester ratio in the ~20% range is needed to induce death in ACAT-competent macrophages, would be most applicable to individuals with very high levels of sitosterol.

The finding that free cholesterol and free sitosterol trigger macrophage death by fundamentally different mechanisms is instructive. Sterols can trigger processes in cells through direct effects, for example, by direct interaction with proteins, or by affecting cell membrane structure, which secondarily affects the function of proteins (79, 80). In the case of cholesterol loading of macrophages, the latter mechanism is suggested (55). In particular, FC loading increases the order of ER membrane phospholipids, which is directly correlated with inhibition of the activity of an integral ER membrane protein, SERCA, known to play an important role in UPR activation (55). Because sitosterol can also increase the order of phospholipids in membranes (62, 64, 65), inhibit SERCA (Fig. 4), and activate p38 (Fig. 6A; *cf.* Ref. 49), we expected the UPR to be activated as it is with cholesterol. The surprising result that this did not occur suggested the possibility that sitosterol may suppress UPR activation. However, we were able to show that CHOP expression induced by the UPR activator tunicamycin was not sup-

pressed by loading macrophages with sitosterol.³ Thus, the explanation must lie elsewhere. Whatever the mechanism, our data indicate that the presence of a 24- β -ethyl group on the sterol side chain can have a critical effect on cellular physiology. Indeed, the effect of sitosterol on membrane structure is not identical to cholesterol (63), and a recent study showed that sitosterol cannot substitute for cholesterol to restore growth in Chinese hamster ovary cells in which cholesterol synthesis was completely inhibited (81).

Categorizing cellular death as "apoptotic" or "necrotic" can be challenging, because biochemical and morphological features of these and other death processes may co-exist under certain conditions (82–87). Nonetheless, we can be confident in concluding that the death process induced by sitosterol in macrophages is different from that induced by cholesterol, which has features most in common with caspase-dependent apoptosis (57, 58, 66). The salient features of sitosterol-induced death include loss of plasma membrane integrity (*e.g.* propidium iodide staining and Hoechst staining), caspase independence, suppression by necrostatin-1, and increase in an autophagy marker plus partial suppression of death by autophagy inhibitors. These features are remarkably similar to those described for a type of death in other cells, termed necroptosis, which is caused by the combination of an apoptosis stimulus plus a caspase inhibitor or by activation of receptor-interacting protein, a death receptor-interacting kinase (45, 88). A similar form of cell death also occurs in neurons *in vivo* as a consequence of ischemic stroke (45). However, in the study of Yuan and colleagues (45), in which non-macrophage were killed by the combination of an apoptosis inducer plus a caspase inhibitor, autophagy was proposed to be downstream of necroptosis, because death was not blocked by autophagy inhibitors. Sitosterol-loaded macrophages also undergo phosphatidylserine externalization, and they stain positively for TUNEL. Whereas these features are often associated with caspase-dependent apoptosis, they can also be seen with necrotic forms of death including necroptosis (88, 89). Indeed, in sitosterol-induced death, unlike cholesterol-induced death, TUNEL staining was resistant to caspase inhibition (Fig. 7C). Sitosterol-loaded macrophages also show much less mitochondrial membrane depolarization than cholesterol-loaded macrophages (Fig. 7E), which can also be found with necroptotic-like death (88) and may be due to absence of caspase-mediated amplification of this process (90).

Why the presence of an ethyl group on C24 of the sterol side chain would trigger necroptotic/autophagic-like death instead of apoptosis is a fascinating question that arises from this study, particularly because sterol trafficking to the ER is necessary for both processes. One possibility is that sitosterol accumulation in macrophages somehow affects molecules in a manner that would suppress apoptosis and/or promote necroptotic-like death. For example, sitosterol accumulation may somehow inhibit caspases or activate receptor-interacting protein (above). Of interest, it is possible that the type of death triggered by sitosterol may be unique to macrophages or at least to non-

cancerous cells, because a number of studies have shown that sitosterol can cause apoptosis in cancer cell lines, and, when reported, apoptosis was shown to be caspase-dependent (91, 92).

More important than categorizing the death pathway is elucidating its biological consequences. In general, necrotic forms of death tend to induce more inflammation than apoptotic forms of death, usually in response to phagocytic clearance (93), but exceptions occur (94). How macrophages killed by sitosterol fit into this spectrum, and whether they elicit biological responses different from cholesterol-killed macrophages, represent critical areas of future investigation that are highly relevant to atherosclerosis (above). In this context, we have found that exposure of macrophage phagocytes to macrophages killed by lipoprotein-sitosterol induces both tumor necrosis factor- α and interleukin-1 β mRNA in the phagocytes (data not shown).

Sitosterol loading of macrophages, like cholesterol loading, activates p38, and activated p38 is necessary for cell death in both scenarios (Fig. 7 and Ref. 49). In the case of cholesterol loading, cholesterol trafficking to the ER is necessary for p38 activation, and p38 activation is necessary for pro-apoptotic CHOP expression (49). With sitosterol loading, however, the scenario is very different: p38 activation is not downstream of sitosterol trafficking to the ER, and it does not result in CHOP induction. Thus, we propose that two parallel pathways, one involving sitosterol trafficking to the ER and the other involving p38 activation, conspire to cause necroptotic-like death in macrophages (Fig. 9). Of interest, there are a number of reports that have implicated p38 in necroptotic-like death processes (95, 96), and the necroptosis-inducing receptor-interacting protein has been shown to activate p38 (97).

In summary, we have shown that macrophages exposed to sitosterol ester-containing lipoproteins accumulate large amounts of free sitosterol even in the absence of ACAT inhibition, resulting in robust macrophage death. Given the role of advanced lesion macrophage death in plaque necrosis, this finding may provide at least one cellular mechanism for the association of sitosterolemia with accelerated atherothrombotic vascular disease. Moreover, the death process induced by sitosterol is remarkably different from that induced by free cholesterol, despite the fact that the two sterols are structurally and functionally very similar and that sterol to ER trafficking is necessary for death in both cases. These findings may lead to new insights into the general issue of how cellular death in the necrosis-apoptosis continuum is triggered and into the specific question of how subtle changes in sterol structure, perhaps by differentially altering ER membrane function, can affect these death processes.

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