The subendothelial aggregation and retention of low density lipoprotein (LDL) are key events in atherogenesis, but the mechanisms in vivo are not known. Previous studies have shown that treatment of LDL with bacterial sphingomyelinase (SMase) in vitro leads to the formation of lesion-like LDL aggregates that become retained on extracellular matrix and stimulate macrophage foam cell formation. In addition, aggregated human atherosclerotic LDL, but not unaggregated atherosclerotic LDL or plasma LDL, shows evidence of hydrolysis by an arterial wall SMase in vivo, and several arterial wall cell types secrete a SMase (S-SMase). S-SMase, however, has a sharp acid pH optimum using a standard in vitro SM-micelle assay. Thus, a critical issue regarding the potential role of S-SMase in atherogenesis is whether the enzyme can hydrolyze lipoprotein-SM, particularly at neutral pH. We now show that S-SMase can hydrolyze and aggregate native plasma LDL at pH 5.5 but not at pH 7.4. Remarkably, LDL modified by oxidation, treatment with phospholipase A2, or enrichment with apolipoprotein CIII, which are modifications associated with increased atherogenesis, is hydrolyzed readily by S-SMase at pH 7.4. In addition, lipoproteins from the plasma of apolipoprotein E knock-out mice, which develop extensive atherosclerosis, are highly susceptible to hydrolysis and aggregation by S-SMase at pH 7.4; a high SM:PC ratio in these lipoproteins appears to be an important factor in their susceptibility to S-SMase. Most importantly, LDL extracted from human atherosclerotic lesions, which is enriched in sphingomyelin compared with plasma LDL, is hydrolyzed by S-SMase at pH 7.4 10-fold more than more than donor plasma LDL, suggesting that LDL is modified in the arterial wall to increase its susceptibility to S-SMase. In summary, atherogenic lipoproteins are excellent substrates for S-SMase, even at neutral pH, making this enzyme a leading candidate for the arterial wall SMase that hydrolyzes LDL-SM and causes subendothelial LDL aggregation.

A critical event in early atherogenesis is the subendothelial retention of atherogenic lipoproteins, including LDL (1, 2), lipoprotein(a) (3), and triglyceride-rich lipoproteins (4). Retained lipoproteins likely trigger a series of biological responses, such as endothelial changes and recruitment of macrophages to the arterial wall, that are central to the initiation and progression of atherosclerosis (5).

Subendothelial lipoproteins are exposed to several modifying enzymes, including lipases (6–8), oxidizing enzymes (9), and proteases (10). The actions of these and other unknown factors lead to the several prominent lipoprotein modifications observed in vivo, including oxidation (11), enrichment with the phospholipid sphingomyelin (SM) (11–13), and self-aggregation (12, 14, 15). Lipoprotein aggregation is likely to be important in atherogenesis for at least two reasons. First, processes that promote lipoprotein aggregation before or during retention dramatically increase the amount of lipoprotein retained (16). Second, aggregated LDL, but not unaggregated LDL, is a potent inducer of macrophage foam cell formation (17–20).

Although the mechanism of lipoprotein aggregation in lesions is not known, several studies from our laboratories suggest a role for the enzyme sphingomyelinase (SMase). First, LDL treated with bacterial SMase forms lesion-like self-aggregates (20) due to enrichment in ceramide (7), the major product of SM hydrolysis; furthermore, these aggregates potentiate induction of macrophage foam cell formation in vitro (16, 20). Second, aggregated LDL from human atherosclerotic lesions, but not unaggregated atherosclerotic LDL or plasma LDL, shows evidence of hydrolysis by an extracellular SMase, and LDL retained in rabbit aortic strips ex vivo is hydrolyzed by an extracellular, cation-dependent SMase (7). Third, and most important, we have found that several cell types present in atherosclerotic lesions secrete a SMase (S-SMase).

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lesions, namely macrophages (21) and endothelial cells,2 secrete a Zn2+-activated SMase (S-SMase). The cellular origins, secretion, and cation dependence make S-SMase a leading candidate for the arterial wall SMase that acts on retained lipoproteins. Nonetheless, two major issues regarding the relevance of S-SMase to atherogenesis need to be addressed. First, mammalian SMases are much more selective than bacterial SMases in terms of the milieu in which the SM is presented to the enzyme (22). Second, studies on the molecular origin of S-SMase have revealed that it is a product of the same gene, the acid SMase (ASM) gene, that gives rise to lysosomal SMase (L-SMase) (21). Not surprisingly, therefore, S-SMase shares with L-SMase a sharp acid pH optimum when assayed under standard in vitro conditions using detergent-solubilized SM-micelles as a substrate (21, 23). Although it is possible that acidic enzymes are active in advanced atherosclerotic lesions, where local pockets of acidity may occur (24–28), a role for such enzymes in pre-lesional or early lesional events would require activity at neutral pH with physiologic substrates.

In this context, the goal of the current study was to test whether S-SMase can hydrolyze LDL-SM, particularly at neutral pH. Herein, we show that S-SMase can hydrolyze and aggregate native LDL at acid but not neutral pH. LDL modified by several means that have been shown to occur or might occur during atherogenesis, is an excellent substrate for S-SMase at pH 7.4. Most importantly, LDL extracted from human atherosclerotic lesions is efficiently hydrolyzed by S-SMase at neutral pH, suggesting that LDL is modified in the arterial wall to increase its susceptibility to S-SMase. Our results support a role for S-SMase in the subendothelial hydrolysis of LDL-SM, perhaps leading to lipoprotein aggregation and lesion initiation and progression.

EXPERIMENTAL PROCEDURES

Materials—sn-1,2-Diacylglycerol kinase (from Escherichia coli) was purchased from Calbiochem. Cardiolipin and 1,2-diacylglycerol were purchased from Avanti Polar Lipids (Alabaster, AL). [9,10-3H]palmitic acid and [γ-32P]ATP were obtained from NEN Life Science Products. Tissue culture media and reagents were purchased from Life Technologies, Inc., and fetal bovine serum was from Gemini Bioproducts (Calabasas, CA). Human native apoCIII (29), human recombinant nonpancreatic soluble PLA2 (sPLA2) (30), and partially purified phospholipid transfer protein (31) were prepared as described previously. Soybean lipoxygenase and all other reagents were from Sigma.

Lipoprotein Isolation and Modification—Human and murine LDL (density, 1.020–1.063 g/ml) were isolated from fresh plasma by preparative ultracentrifugation as described previously (36). LDL (5 mg/ml) was oxidized by dialysis against 150 mM NaCl, 6 mM FeSO4, 0.04% azide for 24 h at room temperature followed by addition of EDTA (1 mM) and BHT (150 μM) and then dialysis against 150 mM NaCl, 0.3 mM EDTA (37). Alternatively, LDL (1 mg) was incubated with 275 units soybean lipoygenase/ml and 60 μg of lipoileic acid/ml in 50 mM Tris-HCl, pH 7.4, 0.04% azide for 24 h at 37 °C (38); LDL was re-isolated using a G-200 molecular weight cut-off gel filtration column and then concentrated using a Centricon 30 (molecular weight cut-off = 30,000) ultrafiltration device. For modification by sPLA2, LDL (5 mg/ml) was incubated with 15 μg of pure human recombinant sPLA2/ml in 0.12 M Tris-HCl, pH 8.0, 12 mM CaCl2, 0.1 mM EDTA, 10 μM butylated hydroxytoluene for 14 h at 37 °C; control and modified LDL (50 μg protein) were then treated directly with S-SMase as described below. Acetylated-LDL was prepared by acetylation of LDL with acetic anhydride (39).

Isolation of LDL from Human Lesions—LDL was extracted from abdominal aortic aneurysm plaque material as described previously (4). Briefly, aortic plaque was removed from individuals as part of the San Francisco Veterans Affairs Medical Center. Plaque material, which ranged in weight from 2 to 12 g, was obtained in the operating room and immediately placed into ice-cold citrate buffer containing 15 mM NaCl, 3 mM EDTA, 0.5 mM butyrylhydroxyluteone, 1 mM phenylmethylsulfonil fluoride, 1.5 mM aprotinin/ml, 2 mM benzamidene, and 0.08 mg of gentamicin sulfate/ml. Blood and adherent thrombus were removed by blotting with absorbent gauge, scrubbing with a small brush, and sharp dissection as necessary. Loosely retained lipoproteins were removed by mincing the plaque into 0.5–1.0-mm2 pieces and incubating them overnight on a Labquake shaker at 4 °C in a non-denaturing buffer (0.1 M citrate, pH 7.4, with 1 mg of EDTA/ml, 0.3 mg of benzamidene/ml, 0.08 mg of gentamicin sulfate/ml, 10 μg of aprotinin/ml, 10 μg of Trollox (an anti-oxidant)/ml, and 20 μg of phenylmethylsulfonil fluoride/ml). The extracted material was cleared of particulate matter by centrifuging at 800 × g for 10 min, and 1.019 < d < 1.063/g/ml lipoproteins were isolated by sequential density ultracentrifugation (4, 36).

Synthesis of 3H-labeled [N-palmitoyl-9,10-3H]HM was synthesized as follows (7, 40, 41): [9,10-3H]palmitic acid (25 mCi, 450 nmol) was stirred for 12 h at room temperature with an equimolar equivalent of N-hydroxysuccinimide and with 3 mM eq of 1,3-dicyclohexylcarbodiimide in N,N-dimethylformamide. The reaction was run under dry argon in the dark. Sphingosylphosphorylcholine (300 nmol) and N,N-diisooctylethylamine (10 μl) were then added, and the reaction was stirred another 12 h at room temperature. The reaction was stopped by evaporating the N,N-dimethylformamide under a stream of N2. [N-palmitoyl-9,10-3H]HM was purified by preparative thin layer chromatography of the reaction products three consecutive times in chloroform:methanol:water (50:25:8). More than 95% of the [N-palmitoyl-9,10-3H]HM was converted to [N-palmitoyl-9,10-3H]ceramide after treatment with 10 millunits of SMase/ml (Bacillus cereus) for 1 h at 37 °C, as assayed by TLC, indicating a pure, functional substrate.

[3H]SM Labeling of LDL—Plasma LDL was labeled with [N-palmitoyl-9,10-3H]HM as follows (7).—3.5 mCi (85 nmol) of [N-palmitoyl-9,10-3H]HM and 13 nmol of phosphatidylcholine (PC) were mixed in chloroform, and the solvent was removed first under a stream of nitrogen and then by lyophilization. The dried lipids were resuspended in 1 ml of 150 mM NaCl, 1 mM EDTA, 10 mM Tris-Hcl, pH 7.5, and, to prepare [3H]SM/PC liposomes, sonicated for three 50-s pulses at 4 °C using a tapered microtip on a Branson 450 sonicator (setting 3). The liposomes were then incubated with 30 mg (by protein mass) of LDL, 50 μg of partially purified phospholipid transfer protein, 100 units of penicillin, and 100 μg of streptomycin for 18 h at 37 °C under argon. LDL was then separated from the liposomes after phospholipid transfer by centrifuging the mixture at density = 1.006/g/ml for 8 h at 35,000 rpm in a Beckman 50.3 rotor; the supernatant containing the liposomes was removed, and the LDL band at the bottom of the tube was harvested. The LDL solution was washed with buffer containing 0.12 M NaCl, 0.3 mM EDTA, pH 7.4, and centrifuged as before. This wash procedure was performed a total of four times, resulting in the removal of 95% of the unreacted [3H]SM/PC liposomes. All lipoproteins were stored under argon at 4 °C and were used within 2 weeks of preparation.

[3H]SM-Emulsions—[3H]SM-emulsions with a lipid composition similar to human LDL were prepared as follows: 5.4 mg of cholesterol olate, 0.48 mg of triolein, 1.08 mg of free cholesterol, 2.04 mg of phosphatidylcholine, 0.96 mg of sphingomyelin, and 50 μCi of [N-palmitoyl-9,10-3H]HM and 50 μCi of [N-palmitoyl-9,10-3H]HM, both in chloroform to a sonication vial, and the solvent was completely evaporated by exposure to a stream of nitrogen, followed by the high vacuum of a lyophilizer. The dried lipids were resuspended in 5 ml of buffer containing 150 mM NaCl, 0.3 mM EDTA, pH 7.4, and sonicated under a stream of argon at 40 °C until translucent (approximately 90 min). The sonicated material was then centrifuged twice at 15,000 × g to pellet any titanium shed from the microtip and the resulting supernatant was used as the starting material. SM-rich emulsions were prepared exactly as above except that 1.2 mg of phosphatidylcholine and 1.8 mg of SM were used, and sonication time was increased to 120 min. [3H]SM-emulsions were emulsified with apoCIII using the method of Ahmad et al. (42). Briefly, [3H]SM-emulsions (0.4 ml; 182 μCi of SM) were incubated with 200 μl of apoCIII (22.5 nmol) for 2 h at 40 °C. A portion of the emulsion was re-isolated from free apoCIII using ultracentrifugation (29) as follows: the crude emulsion/apoCIII mixture was diluted to 2 ml with buffer containing 150 mM NaCl, 0.3 mM EDTA, pH 7.4, and ultrafiltered and concentrated to 0.2 ml using a Centricon 30 (molecular weight cut-off = 30,000); the concentrated emulsions were then diluted to 2 ml, and the...
process was repeated 5 times. [3H]SM-emulsions run through the same enrichment and re-isolation protocols in the absence of apoCIII served as the control for the experiments in Fig. 5A.

Ceramide Assay—Ceramide was measured from LDL lipid extracts using the method described by Schneider and Kennedy (45) and adapted by Preiss et al. (44). In this method, diacylglycerol (DAG) kinase phosphorylates ceramide and DAG using \( \gamma^{32P} \)ATP. For ceramide measurement, the lipids were first incubated with 0.1x KOH in methanol for 1h at 37°C, which hydrolyzes DAG but not ceramide. The extracted lipids were dried under nitrogen and then solubilized in 5mL cardiolipin, 7.5% octyl-glucopyranoside, and 1mM diethylenetriaminepentaaetic acid by bath sonication. This solution was then added to reaction buffer (50mM imidazole HCl, pH 6.6, 50mM NaCl, 12.5mM MgCl₂, 1mM EGTA) containing sn-1,2-DAG kinase (0.7 units/mL). The reaction was initiated by the addition of \( \gamma^{32P} \)ATP (final concentration = 10mM). After incubation at room temperature for 60min, the reaction was stopped by lipid extraction with chloroform:methanol:HCl (100:100:1, v/v/v) and 10mL EDTA. Ceramide 1-\( ^{32P} \)phosphate in the organic phase was separated by TLC using chloroform:methanol:acetic acid (65:15:5, v/v/v) and visualized with autoradiography and identified by comparing with standards. The spots corresponding to ceramide 1-\( ^{32P} \)phosphate were scraped and counted, and the mass was calculated by comparison with a ceramide standard curve. To rule out changes in the DAG kinase enzyme itself (cf. Ref. 45), C20 ceramide was added to some of the lipid extracts and shown not to undergo increased phosphorylation in samples in which LDL ceramide was found to be elevated.

Sphingomyelin (SM) and Phosphatidylcholine (PC) Assays—Lipid extracts (46) of lipoproteins were chromatographed by TLC using chloroform:methanol:acetic acid (65:15:5, v/v/v) and visualized with autoradiography and identified by comparing with standards. The spots were extracted, scraped twice with chloroform:methanol (2:1), and assayed for phosphate content by the method of Bartlett (47).

LDL Oxidation Assays—LDL lipid peroxides were measured using the method of El-Saadani et al. (48). LDL (50–150μg of protein), in a volume of no more than 100μL, was added to 1mL of color reagent (0.2M KH₂PO₄, 0.12mM KI, 0.15mM NaN₃, 2g of Triton X-100/filtrer, 0.1g of benzalkonium chloride/liter, 10μM ammonium molybdate, 20μM BHT, 25μM EDTA, pH 6.2) and incubated in the dark for 30min at room temperature; light absorbance at 365nm was then measured and lipid peroxides were quantified by comparison with a H₂O₂ standard curve. Thiobarbituric acid-reactive substances (TBARS) were measured using a standard method (49). Briefly, LDL (100μg, 100–200μg of protein) was mixed with 1mL of 20% trichloroacetic acid and incubated on ice for 30min. Following precipitation, 1mL of 1% thiobarbituric acid was added, and the samples were heated at 95°C for 45min. After cooling, the samples were centrifuged at 1000×g for 20min, and the light absorbance at 532nm was measured. TBARS were quantified by comparison with a malonaldehyde standard curve prepared using tetramethoxynaphtalene. LDL electrophoretic mobility was assayed by adding 30μg LDL protein onto a polyacrylamide 0.75–27% gradient gel (Lipo-gel; Zaxis, Hudson, OH) and electrophoresing in 0.1M Tris base, 0.1M boric acid, 20mM EDTA (upper chamber, pH 8.7; lower chamber, pH 8.3) for 12h at 100V. The gel was then stained with Sudan black and the bands visualized by counter-staining with methanol:acetic acid:water (10:7:33, v/v/v).

S-SMase—The source of S-SMase was serum-free conditioned media from DG44 Chinese hamster ovary cells stably transfected with the human acid SMase cDNA (21, 50). Our previous work demonstrated that S-SMase is the only detectable SMase secreted into the culture medium (21). Cells were plated in 100-mm dishes and cultured for 48h in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 292μg of glutamine/ml, 100 units penicillin/ml, and 100μg of streptomycin/ml. The cells were then challenged to low protein serum-free media for 12h, washed 3times with phosphate-buffered saline, and finally incubated for 18h in fresh serum-free media (6mL per 100-mm dish). The "18-h conditioned medium" was then collected, centrifuged at 1000×g to pellet any cells, and, except where indicated, ZnCl₂ (final concentration = 100μM) was added to fully activate and stabilize S-SMase; this S-SMase-containing conditioned medium was then used fresh to treat LDL and lipid emulsions.

S-SMase Treatment of LDL and Emulsions—The standard incubation mixture consisted of up to 50μL of sample (LDL or emulsions), 25μL of S-SMase-containing conditioned media (see above), and a volume of assay buffer (0.1M Tris-HCl, pH 7.4, 0.04% azide; or, where indicated, 0.1M sodium acetate, pH 5.5, 0.04% azide) to bring the final volume to 200μL. The reactions were incubated at 37°C for no longer than 16h and then extracted by the method of Bligh and Dyer (46). For the samples containing \( {\text{[N-palmitoyl-9–10-}^{3} \text{H}]SM-LDL or [N-palmitoyl-9–10-}^{3} \text{H]} \)SM-emulsions, the lower organic phase was harvested, evaporated under N₂, and fractionated by TLC using chloroform:methanol (95:5). The ceramide spots were scraped and directly counted to quantify \( ^{3} \)Hceramide. In all other samples ceramide was determined as described above.

Statistics—Unless otherwise indicated, results are given as means ± S.D. (n = 3); absent error bars in the figures signify S.D. values smaller than the graphic symbols.

RESULTS

Hydrolysis and Aggregation of Native, Oxidized, and sPLA₂-treated LDL by S-SMase—We first sought to determine whether S-SMase could act on intact LDL and, if so, whether it would cause LDL aggregation. Although treatment of native LDL with S-SMase at pH 7.4 caused very little SM hydrolysis, S-SMase hydrolyzed significant amounts (nearly 90%) of LDL-SM at pH 5.5 (Fig. 1A), leading to LDL aggregation (Fig. 1B). Thus, S-SMase can hydrolyze the SM in intact LDL without the need for detergent, leading to LDL aggregation. Prior data from Callahan et al. (51) suggested to us that S-SMase might require low pH only for binding of SM to the enzyme and not for the hydrolysis process per se. We reasoned, therefore, that changes in the surface of lipoproteins might favor the binding of lipoprotein-SM to S-SMase, which might allow the enzyme to function at neutral pH. In particular, several modifications of LDL relevant to atherosclerosis, including oxidation (52), hydrolysis with phospholipase A₂ (8, 30, 53), and sphingomyelin enrichment, alter the structure of the lipoprotein surface. In this context, we first examined S-SMase.
hydrolysis of [3H]SM-labeled LDL oxidized by two independent methods, FeSO₄ and lipoxygenase. Whereas oxidation alone caused no artifactual LDL-[3H]SM hydrolysis (Fig. 2A, open bars), oxidized [3H]SM-LDL was hydrolyzed 5–6-fold more than native [3H]SM-LDL by S-SMase at pH 7.4 (Fig. 2A, solid bars). Similar results were obtained when LDL-SM hydrolysis was assayed by measuring generation of ceramide mass (data not shown). Note that FeSO₄ and lipoxygenase caused similar degrees of LDL oxidation as indicated by the levels of thiobarbituric acid-reactive substances (TBARS) (Fig. 2B) and lipid peroxides (LPO) (C) in LDL.

To more precisely define the relationship between LDL oxidation and its susceptibility to S-SMase hydrolysis, we incubated [3H]SM-LDL with FeSO₄ for increasing amounts of time and compared its extent of oxidation to its hydrolysis by S-SMase at pH 7.4. [3H]SM-LDL oxidation, assayed by measuring lipid peroxides (Fig. 3, open circles) increased almost linearly with increasing time of incubation with FeSO₄. Electrophoretic mobility of LDL as assessed by native polyacrylamide gradient gel electrophoresis, indicating addition of negative charge, also increased with time of oxidation (not shown). Most importantly, the susceptibility of [3H]SM-LDL to S-SMase hydrolysis at neutral pH was, after an initial threshold level of oxidation, closely correlated with the extent of [3H]SM-LDL oxidation (Fig. 3, closed squares). Note that oxidation of [3H]SM vesicles that contained only SM did not lead to a substantial increase in susceptibility to S-SMase at neutral pH (data not shown). Moreover, acetylated LDL, another negatively charged form of LDL as assessed by native polyacrylamide gradient gel electrophoresis, indicating addition of negative charge, also increased with time of oxidation (not shown).

Lysophosphatidylcholine enrichment of LDL occurs during oxidation due to activation of an apparently latent phospholipase A₂ activity (54), and recent in vitro and in vivo data suggest that hydrolysis of lesional LDL by nonpancreatic secretory phospholipase A₂ type II (sPLA₂) is an atherogenic event (8, 30, 53). We therefore asked whether LDL treated with

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**Fig. 2.** Oxidized LDL is susceptible to hydrolysis by S-SMase at neutral pH. [3H]SM-LDL (Native) was oxidized with 6 μM ferrous sulfate (FeSO₄) or with soybean lipoxygenase (S-LO) as described under “Experimental Procedures.” A, LDL particles (250 μg protein/ml) were then incubated in the absence (open bars) or presence (solid bars) of S-SMase-containing conditioned media (125 μl/ml) at pH 7.4 for 14 h at 37 °C. LDL-[3H]SM hydrolysis was assayed by measuring [3H]ceramide. LDL oxidation was assayed by measuring the content of TBARS (B) and lipid peroxides (LPO) (C) in LDL.

**Fig. 3.** Relationship between LDL oxidation and susceptibility to hydrolysis by S-SMase at neutral pH. [3H]SM-LDL was oxidized with 6 μM FeSO₄ as described under “Experimental Procedures,” and at the indicated time points, oxidation was terminated by the addition of 150 μM butylated hydroxytoluene and 1 mM EDTA followed by dialysis against 150 mM NaCl, 0.3 mM EDTA for 36 h at 4 °C. [3H]SM-LDL samples (250 μg of protein/ml) were then incubated with S-SMase-containing conditioned media (125 μl/ml) at pH 7.4 for 14 h at 37 °C. LDL-[3H]SM hydrolysis was assayed by measuring [3H]ceramide (closed squares). [3H]SM-LDL oxidation was assayed by measuring lipid peroxides (open circles).
sPLA₂ was more susceptible than native LDL to S-SMase hydrolysis at neutral pH. Whereas sPLA₂ treatment alone caused no [³H]SM-LDL hydrolysis, S-SMase hydrolysis of [³H]SM-LDL at pH 7.4 was markedly enhanced by treating the LDL with sPLA₂ (Fig. 4). Furthermore, exogenously added lysophosphatidylcholine stimulated S-SMase hydrolysis of [³H]SM-LDL 2–3-fold at pH 7.4 (data not shown). Thus, while it is possible that several oxidation products are acting together, lysophosphatidylcholine appears to be particularly effective in stimulating S-SMase hydrolysis of LDL.

Lipoproteins Enriched in ApoCIII Are Susceptible to S-SMase-induced Hydrolysis and Aggregation at Neutral pH—Recent data suggest that apoCIII enrichment of lipoproteins promotes atherogenesis (35, 55, 56), although the mechanism is not known. Moreover, Ahmad et al. (42) have shown that apoCIII, but not other apolipoproteins, stimulates L-SMase hydrolysis of SM-liposomes. Given that L-SMase and S-SMase are such similar enzymes (21), we asked whether apoCIII could stimulate S-SMase hydrolysis of lipoprotein-SM at neutral pH. Because enriching native LDL with apoCIII in vitro proved extremely difficult, we first examined the effect of apoCIII on S-SMase hydrolysis of [³H]SM-labeled emulsions containing lipids in the same proportions as in LDL. For this experiment we treated three different types of emulsions with S-SMase at pH 7.4: control emulsions not enriched with apoCIII (control), emulsions mixed with apoCIII (+ CIII, emulsion-bound + free CIII), and emulsions mixed with apoCIII followed by re-isolation from free apoCIII (+ CIII, emulsion-bound only). Although S-SMase hydrolysis of the emulsions with bound plus free CIII was 2-fold greater than hydrolysis of the control emulsions, emulsions with only bound CIII were hydrolyzed approximately 10-fold more than control emulsions (Fig. 5A). Thus, apoCIII stimulates S-SMase hydrolysis of LDL-like emulsions, particularly in the absence of free apoCIII (see Ref. 42 and “Discussion”).

To determine whether apoCIII stimulates S-SMase hydrolysis of lipoprotein-SM, we compared the hydrolysis of LDL from LDLr0 mice (32) with LDL from LDLr0 mice expressing a human apoCIII transgene (LDLr0/CIII) (35); as expected, LDL from the LDLr0/CIII mouse was enriched in apoCIII, whereas LDL from the LDLr0 mouse was not (data not shown). The data in Fig. 5B clearly show that LDL from LDLr0/CIII mice was significantly hydrolyzed by S-SMase at pH 7.4, whereas LDL from LDLr0 mice was hydrolyzed very little. Importantly, the turbidity at A₄₃₀ nm of LDLr0/CIII LDL, but not of LDLr0 LDL, increased 1.5-fold following treatment with S-SMase, indicating particle aggregation. Lipoprotein enrichment with apoCIII, therefore, increases its susceptibility to hydrolysis and aggregation induced by S-SMase.

**Fig. 4.** LDL treated with sPLA₂ is susceptible to hydrolysis by S-SMase at neutral pH. [³H]SM-LDL (5 mg/ml) was incubated in the absence (−) or presence (+) of sPLA₂, for 14 h at 37 °C. Samples of control and treated [³H]SM-LDL (250 μg/ml) were then incubated in the absence (−) or presence (+) of S-SMase-containing conditioned media (125 μl/ml) at pH 7.4 for 14 h at 37 °C. LDL-[³H]SM hydrolysis was assayed by measuring [³H]ceramide.

**Fig. 5.** ApoCIII enhances the susceptibility of [³H]SM-emulsions and murine plasma LDL to hydrolysis by S-SMase at neutral pH. A, LDL-like [³H]SM-emulsions alone (Control), emulsions mixed with 1 mol of apoCIII, 8 mol of emulsion-SM (+ CIII, emulsion-bound + free CIII), and emulsions mixed with apoCIII followed by separation from free apoCIII (+ CIII, Emulsion-bound only) were prepared as described under “Experimental Procedures.” [³H]SM-emulsions were incubated at a concentration of 325 μg of cholesteryl ester (CE/ml) in the absence (hatched bars) or presence (solid bars) of S-SMase-containing conditioned media (125 μl/ml) at pH 7.4 for 6 h at 37 °C, and [³H]SM hydrolysis was assayed by measuring [³H]ceramide. B, plasma LDL (100 μg of protein/ml) from LDL receptor-deficient mice (LDLr0) and LDL receptor-deficient mice expressing a human apoCIII transgene (LDLr0/CIII) were incubated in the absence (hatched bars) or presence (solid bars) of S-SMase-containing conditioned media (125 μl/ml) at pH 7.4 for 14 h at 37 °C. Lipids were then extracted and assayed for ceramide mass. In data not displayed in this figure, we found that the turbidity at A₄₃₀ nm of LDLr0/CIII LDL, but not of LDLr0 LDL, increased 1.5-fold following treatment with S-SMase, indicating particle aggregation.
**Lipoprotein Hydrolysis by S-SMase**

**Plasma LDL from E0 Mice Is Efficiently Hydrolyzed by S-SMase at Neutral pH** — The E0 mouse develops widely distributed and complex atherosclerotic lesions like those seen in humans and thus has become a widely used model of atherosclerosis (33, 34). A likely factor contributing to the extensive atherosclerosis in these mice is the atherogenicity of E0 lipoproteins, but the mechanisms whereby apoE-deficient lipoproteins lead to foam cell formation and other lesional events is not known. In the context of our current study, we tested whether plasma LDL from these mice was susceptible to S-SMase at neutral pH; as a comparison, we used plasma LDL from LDLr0 mice, which also develop atherosclerosis, although to a lesser degree than E0 mice (32–34). Although the basal ceramide contents of native plasma LDL from these two strains of mice are equivalent (Fig. 6A, hatched bars), treatment with S-SMase at pH 7.4 resulted in generation of 4–5-fold more ceramide in LDL from E0 mice than in LDL from LDLr0 mice (Fig. 6A, solid bars), which resulted in aggregation of E0 LDL.

Jeong et al. have recently shown that E0 LDL has a high SM:PC molar ratio (0.36) compared with LDLr0 LDL, which has a SM:PC ratio of 0.19. Since SM enrichment of lipoproteins may increase their substrate availability to S-SMase, one factor contributing to the increased susceptibility of E0 LDL to S-SMase might be its relatively high SM:PC ratio. To test the importance of the SM:PC ratio on S-SMase activity in a controlled system, [3H]SM-labeled emulsions were prepared containing lipids similar to those in human LDL with a SM:PC molar ratio of 0.5 (control) or similar emulsions except with a SM:PC molar ratio of 1.5 (SM-rich) were incubated at a concentration of 450 μmol of cholesteryl ester CE/ml in the absence or presence of S-SMase-containing conditioned media (125 μl/ml) at pH 7.4 for 12 h at 37 °C ([3H]SM hydrolysis was assayed by measuring [3H]ceramide. Note that S-SMase treatment of E0 LDL at neutral pH lead to lipoprotein aggregation.4

What are the critical modifications to lesional LDL that stimulate its hydrolysis by S-SMase? To begin addressing this question, we examined lesional LDL for the presence of the LDL modifications that we identified above as increasing susceptibility to hydrolysis by S-SMase at neutral pH *in vitro*. For oxidation, we compared the levels of TBARS and lipid peroxides in the lesional and plasma LDL samples from Fig. 7; levels in both the plasma and lesional samples were below the detection limit of our assay (<0.5 nmol/mg protein and <4 nmol/mg protein for TBARS and lipid peroxides, respectively). Similarly, we were unable to detect apoCIII in our two samples of lesional LDL (data not shown). We next asked whether the ratio of SM to PC was different for lesional and plasma LDL. The data in Table I show that whereas the PC level in lesional LDL was modestly reduced compared with plasma LDL, the SM content of lesional LDL was nearly 3-fold higher than that in plasma LDL (cf. Refs. 11–13). As a result, the SM:PC ratio for lesional LDL was 3–4-fold higher than the ratio for plasma LDL. Thus, lesional LDL is highly enriched in SM, a modification that stimulates S-SMase hydrolysis of LDL at neutral pH *in vitro* (see Fig. 6).

**DISCUSSION**

Aggregated LDL retained *in vivo* shows evidence of hydrolysis by a SMase in the subendothelium (7). Although the mammalian SMase responsible for this extracellular process has yet to be identified, S-SMase is a leading candidate since it is the only SMase that we have been able to detect in the conditioned media of cultured arterial wall cells (7). Furthermore, prelim-

5 Note that lesional LDL is already in an aggregated form when isolated (7), and so the effect of S-SMase treatment on aggregation could not be assessed.
plasma LDL but only at acid pH (Fig. 1). Although S-SMase-
carrying conditioned media (125 μg/ml) at pH 7.4 for 14 h at
37 °C. Lipids were then extracted and assayed for ceramide mass.
Incubation at 37 °C in the absence of S-SMase did not affect the
ceramide contents of plasma and lesional LDL.

TABLE I
Sphingomyelin and phosphatidylcholine contents
of plasma and lesional LDL

| Sample          | Sphingomyelin content | Phosphatidylcholine content | SM:PC molar ratio |
|-----------------|-----------------------|----------------------------|------------------|
| Plasma LDL 1    | 376 ± 20              | 785 ± 13                   | 0.478 ± 0.022    |
| Plasma LDL 2    | 352 ± 10              | 727 ± 6                    | 0.473 ± 0.025    |
| Lesion LDL 1    | 858 ± 19              | 710 ± 12                   | 1.203 ± 0.040    |
| Lesion LDL 2    | 902 ± 50              | 516 ± 22                   | 1.740 ± 0.170    |

Binary immunohistochemistry studies indicate that S-SMase is
present in both the pre-lesional artery and in atherosclerotic lesions.6
Macrophage-derived S-SMase (7) may be a source of the
enzyme in established atherosclerotic lesions. Most
recently, we have found that cultured human coronary artery
divertorial cells secrete abundant amounts of S-SMase bosa-
laterally,2 making the endothelium a potential source of the
enzyme in the pre-lesional artery.

S-SMase, however, has an acid pH optimum using a stand-
and in vitro, detergent-based SM-micelle assay (21, 23). Indeed,
we found that S-SMase can hydrolyze and aggregate native
plasma LDL but only at acid pH (Fig. 1). Although S-SMase-
induced LDL aggregation under these conditions may be rele-
vant in more advanced lesions, where an acidic environment
may exist in the vicinity of lesion macrophages (27, 28) or as a
result of hypoxia-induced metabolic acidosis (57), early lipopro-
tein aggregation likely occurs at a more neutral, physiologic pH. Thus, our finding that certain types of atherogenic lipopro-
teins are substrates for S-SMase at neutral pH may have relevance
to early atherogenesis. Callahan et al. (51) demonstrated
that for L-SMase, which is closely related to S-SMase (21),
only the affinity for SM in detergent micelles (i.e., \( K_m \)) is
highly sensitive to changes in pH, whereas the maximal veloc-
ity (\( V_{max} \)) for SM hydrolysis is pH-independent. Thus, a plau-
sible explanation for our findings is that changes in the lipid
surface of the various forms of modified lipoproteins increased
the accessibility of their SM to S-SMase. The overall concept
that “acid” S-Mase can function at neutral pH may also have
relevance to ceramide-mediated cell-signaling processes, such
as apoptosis, that are triggered by the hydrolysis of cellular SM
by a product or products of the acid SMase gene (58–62).

What is the physiologic significance of subendothelial lipoprotein
SM hydrolysis? One theoretical possibility is that
SMase-treated lipoproteins may be able to deliver ceramide to
cells and induce certain ceramide-mediated signal transduction
events (63–66). In fact, oxidized LDL-induced stimulation of
smooth muscle cell proliferation may involve the ceramide
signaling pathway (67). The major physiological link that we
have been studying, however, is SMase-induced lipoprotein
self-aggregation (16, 20). In this regard, we have found three
examples in which S-SMase hydrolysis led to lipoprotein ag-
gregation as follows: native LDL at acid pH (Fig. 1B), LDL from
LDLr0/CIII mice at neutral pH (see “Results”), and VLDL +
LDL and LDL from E0 mice at neutral pH.4 In contrast, two
of the lipoproteins that were hydrolyzed by S-SMase at neutral
pH, namely oxidized LDL and sPLA2-treated LDL, did not
show visible aggregation. In these two cases, the level of
ceramide generated (−0.05 and 0.013 nmol of ceramide/μg
of protein, respectively) was below the threshold level for lipopro-
tein aggregation in vitro (−0.08 nmol of ceramide/μg of protein)
(7). Thus, more extensive exposure of these lipoproteins to
SMase at neutral pH would likely be required for aggregation
to occur. Furthermore, it is possible that other arterial wall
factors, such as proteoglycans, collagen, and lipoprotein lipase
(5, 68, 69), lower the threshold for ceramide-mediated lipopro-
tein aggregation in vivo. Interestingly, oxidized LDL treated
with S-SMase at neutral pH contained similar levels of ceram-
ide as aggregated lesional LDL (7) (also, compare Fig. 2A with
Fig. 7), consistent with the idea that the threshold for ceram-
ide-mediated aggregation may be lowered in vivo.

Our finding that S-SMase can hydrolyze and cause the ag-
gregation of apoCIII-enriched lipoproteins at neutral pH is of
particular interest since, through unknown mechanisms, apo-
CIII has been linked to certain forms of coronary heart disease
(55). In addition, overexpression of apoCIII in mice signifi-
cantly increases atherosclerosis (35). Although our samples of
lesional LDL (1.019 < \( d < 1.063 \) g/ml) did not contain apoCIII,
it is possible that less dense lesional lipoproteins or lipopro-
teins isolated from other individuals or from earlier lesions
might have shown evidence of apoCIII. A possible mechanism
for the apoCIII effect described herein may be gleaned from the
finding that S-SMase-mediated hydrolysis of apoCIII-containing
SM-emulsions was greater in the absence of free apoCIII
(Fig. 5A). These data are consistent with the idea that apoCIII
may act as a bridge between S-SMase and membrane SM,
thereby stimulating SM hydrolysis.

A property of E0 LDL that likely plays a role in its suscep-
tibility to S-SMase is the high SM:PC ratio in these particles
(Fig. 6B). The mechanism of lipoprotein-SM enrichment ap-
pears to be a combination of increased SM synthesis and de-
creased plasma SM clearance in the E0 mice.4 There is evidence,
however, that other factors may also be involved in the
increased susceptibility of E0 lipoproteins. In particular, native
human plasma LDL has an SM:PC ratio that is greater than

6 S. Marathe and I. Tabas, unpublished data.
that in E0 LDL and equal to that in the "control" emulsions in Fig. 6B and yet is substantially less susceptible to hydrolysis by S-SMase at neutral pH than these particles. Since E0 LDL contains mostly apoB48 (33, 34) and the emulsions contain no protein, it is possible that apoB100, the protein in human native plasma LDL, confers relatively resistance to hydrolysis by S-SMase. If so, the degradation of apoB100 that is known to occur with LDL oxidation (52) may contribute to the enhanced susceptibility of oxidized LDL to hydrolysis by S-SMase. It is also possible that apoE itself may be an inhibitor of S-SMase hydrolysis, although we have recently found that apoE enrichment of the E0 lipoproteins does not block S-SMase-induced aggregation.4

A critically important finding in this report was that LDL extracted from human atherosclerotic lesions was hydrolyzed 10-fold more than the same donor plasma LDL by S-SMase at neutral pH (Fig. 7). These data indicate that lesional LDL is modified in vivo, either before or after retention in lesions (see below), to a form that renders it more susceptible to hydrolysis by S-SMase. Although it is difficult to determine the specific characteristics of lesional LDL that increase its susceptibility to S-SMase, we have observed, as have others (11–13), that lesional LDL is enriched in SM (Table I), which we have shown is a potent stimulator of S-SMase hydrolysis of LDL (Fig. 6B).

The mechanism of SM enrichment of lesional LDL is not known. The possibilities include SM enrichment of LDL retained in the subendothelium (e.g. by increased arterial wall synthesis of SM (70) followed by transfer of the SM to retained LDL) or selective uptake of SM-rich LDL from the plasma. Another property of lesional LDL that may increase its susceptibility to S-SMase is oxidation. Although we did not find evidence of TBARS or lipid peroxides in our samples of lesional LDL, more sensitive and/or specific techniques, such as immunostaining for oxidized epitopes (11) or evidence of myeloperoxidase-induced oxidation (9), may be required.

In summary, S-SMase, a product of the acid SMase gene (21), can efficiently hydrolyze and aggregate modified LDL at neutral pH. Most importantly, LDL extracted from atherosclerotic lesions, but not plasma LDL, is significantly hydrolyzed by S-SMase at neutral pH, indicating that modifications present in vivo can stimulate S-SMase hydrolysis of LDL. In previous reports, we have shown that SMase treatment of lipoproteins promotes lipoprotein aggregation and retention to matrix, which induces macrophage foam cell formation (16, 20), that lipoprotein-SM hydrolysis occurs in the subendothelium in vivo (7), and that S-SMase is secreted by both macrophages (21) and endothelial cells.2 Based on the sum of the data, we propose a model in which S-SMase secreted by arterial wall cells hydrolyzes lipoproteins retained in the subendothelium, which, in turn, promotes lesion initiation and progression.
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Secretory Sphingomyelinase, a Product of the Acid Sphingomyelinase Gene, Can Hydrolyze Atherogenic Lipoproteins at Neutral pH: IMPLICATIONS FOR ATHEROSCLEROTIC LESION DEVELOPMENT

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