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Size-selective uptake of colloidal low density lipoprotein aggregates by cultured white blood cells

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Abstract

This paper illustrates how principles of colloid science are useful in studying atherosclerosis. Accumulation of foam cells in the arterial intima is a key step in atherogenesis. The extent of foam cell formation is enhanced by low density lipoprotein (LDL) aggregates, and we have previously shown that the size of sphingomyelinase (Smase)-hydrolysis-induced aggregates depends directly on the concentration of ceramide generated in the LDL phospholipid monolayer, mediated by the hydrophobic effect. Here, we focus on the effect of LDL aggregate particle sizes on their subsequent uptake by macrophages. Our data show the first direct measurement of uptake as a function of aggregate size and the first direct comparison of uptake after Smase-catalyzed and vortex-mixing-mediated aggregation. Vortex-mixed aggregates with radii 20–77 nm showed maximal uptake ~118 μg sterol/mg protein at a 53 nm intermediate size, consistent with a mathematical model describing competition between aggregate surface area and volume. Smase-treated aggregates with radii 25–211 nm also showed maximal uptake at an intermediate size, ~58 μg sterol/mg protein for 132 nm particles, and fit a modified model that incorporated ceramide concentration expressed as aggregate size. This study shows that particle size is significant and composition may also be a factor in LDL uptake.

Keywords

Aggregation; Ceramide; LDL; Size; Vortex; Uptake; Atherosclerosis

1. Introduction

Accumulation of foam cells within the arterial intima is a hallmark of early atherosclerotic lesional tissue [1–3]. Foam cells arise as a response to subendothelial retention of lipid-containing particles, especially low density lipoproteins (LDL), whereby monocyte-derived macrophages ingest the retained particles [4–6]. Chemical and physical modification of LDL results in its aggregation and subsequently in enhanced foam cell formation both *in vivo* and *in vitro* [1,7–12]. Examples of LDL modification include sphingomyelinase(Smase)-catalyzed hydrolysis of LDL-sphingomyelin [6,13], LDL self-aggregation [10], and oxidation of lipid and protein [7,14,15]. Smase-hydrolysis is of particular interest both because of its physiological relevance [12,16–18] and due to its synergy with lipoprotein lipase [19], which results in increased LDL concentration at the site of lesion formation in an *in vitro* model system.

Smase is secreted, in its Zn^{2+} -dependent form, by endothelial cells [16,18] in the vicinity of atherosclerotic lesion formation. The same form of Smase is also secreted by macrophages,

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which increases the enzymatic effect after these cells arrive at the site of the lesion [17]. Smase-catalyzed modification of LDL involves the cleavage of the phosphorylcholine headgroup of sphingomyelin, which is located in the LDL lipid monolayer and accounts for ~19% of the phospholipids contained therein, leaving hydrophobic ceramide in its place [20–22]. LDL from atherosclerotic lesional tissue contains 10–50-fold more ceramide than plasma LDL [13], reinforcing the importance of Smase in lesion formation.

Following the hydrolysis reaction, and the generation of ceramide, LDL forms aggregates of larger sized particles *in vitro*, which induce increased uptake of LDL by cultured macrophages [11,23–25]. These ceramide-containing aggregates are also found in excised atherosclerotic lesional tissue [11,13,26]. Colloid science can be used to study and explain some of the pathophysiological mechanisms in which LDL is involved. For example, a different set of investigations showed that apolipoprotein A-1 from high density lipoprotein inhibits LDL aggregation through a well-studied hydrophobic shielding mechanism [27,28]. Using colloid science techniques to study Smase-mediated LDL aggregation, we have found that LDL aggregate size is a sole function of ceramide concentration; that is, at any point in the hydrolysis reaction, sizes can be described by ceramide concentration, independent of Smase concentration. We note that the Mg^{2+} -dependent bacterial Smase used here has the same enzymatic effect on LDL-sphingomyelin as the Zn^{2+} -dependent secretory enzyme [1,29].

Though the role of macrophages in atherogenesis has been extensively studied [30–32], atherogenic mechanisms require further understanding. The size of single LDL particles has recently received attention as small, dense LDL have been identified as risk factors for atherosclerosis [33–35]. Here, instead of focusing on the size of single LDL particles, we focus on the size of LDL aggregates and aim to isolate the effect of aggregate size on uptake to evaluate its role in the pathogenesis of this disease. We control LDL aggregate size using both Smase-catalyzed ceramide generation and vortex-mixing, and incubate samples of identical total LDL concentration, but of different aggregate sizes, with macrophages.

2. Materials and methods

2.1. Materials

J774A.1 mouse macrophage cells were purchased from ATCC (Manassas, VA). LDL from human plasma was purchased from EMD Biosciences/Calbiochem (San Diego, CA). Dulbecco's Modification of Eagle's Medium (DMEM), heat-inactivated Fetal Bovine Serum (FBS), Penicillin–Streptomycin Solution, L-glutamine solution, and Dulbecco's Phosphate-Buffered Saline (DPBS) were purchased from Mediatech/Cellgro (Manassas, VA). Lyophilized sphingomyelinase, Lipoprotein Deficient Serum (LPDS), NaCl, HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), NaOH, Bovine Serum Albumin (BSA), Sodium Dodecyl Sulfate (SDS), oil red O, Phosphorylcholine chloride (calcium salt tetrahydrate), KH_2PO_4 , KI, NaN_3 , Triton X-100, benzalkonium chloride, ammonium molybdate, BHT, and EDTA were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Cell culture

J774A.1 Macrophage cells were cultured to ~70% confluence at 37 °C and 5% CO_2 in T75 Corning tissue culture flasks in DMEM, containing 8.85% (v/v) FBS, 88.5 IU penicillin, 88.5 IU streptomycin, and 4.5 g/L glutamine. Cells were transferred to BD Biosciences 6-well tissue culture plates, 35 mm well diameter. Cells were grown again to 50–70% confluence before starting experiments. Media was changed every 2–3 days. One day prior to the start of the experiment the media formulation was changed to include 8.85% LPDS [1,10,36] instead of FBS, all other media components remained the same.

2.3. LDL aggregate formation for pre-aggregated LDL

LDL aggregates were formed either by overnight treatment of $333.7 \pm 0.8 \mu\text{g}$ native LDL/mL with 0, 10, 100, 438, 938, 1875, and 3750 milliunits/mL neutral, Mg^{2+} -dependant Smase [1,19,25] (diluted LDL concentration after Smase addition $208.6 \pm 0.5 \mu\text{g}$ LDL/mL) or by vortex-mixing $332.2 \pm 1.6 \mu\text{g}$ native LDL/mL for 0, 15, 30, 60, 201, and 360 s (followed by dilution with deionized H_2O to $207.6 \pm 1.0 \mu\text{g}$ LDL/mL). In all cases aggregates were formed in LDL Stabilizing Buffer (5 mmol/L HEPES, 150 mmol/L NaCl, pH adjusted to 7.4 using NaOH) and diluted with deionized H_2O to equalize sample volumes. The next day an equal volume of DMEM (with 0.2% BSA, w/v, mg/mL) was added to all LDL aggregate samples, making the final LDL concentration $104.1 \pm 0.5 \mu\text{g}$ LDL/mL, and the mixture was either used as cell culture media or for particle size measurement. Aside from the presence of Phenol Red indicator, samples used in cell culture were of identical composition to samples prepared for DLS analysis.

2.4. Size-selective uptake experiments

Macrophages were incubated for 3 days [1,10,36] at 37°C and 5% CO_2 with culture media consisting of 50% DMEM (with 0.2% BSA, w/v, mg/mL) and 50% of the diluted LDL Stabilizing Buffer containing LDL aggregates created by treatment with Smase or vortex-mixing. Concentrations of 0, 46, 134, 361, 720, and 2870 milliunits Smase/mL were used to create aggregate particles with radii of 12, 25, 50, 100, 149, and 211 nm, and vortex times of 0, 13, 34, 64, 115, and 201 s were used to create aggregate particles with radii of 12, 20, 36, 52, 67, and 77 nm. The final LDL concentration in cell culture media for both enzyme-treated and vortexed samples was $104.1 \pm 0.4 \mu\text{g}$ /mL. All media was replaced with fresh material daily. At the end of the experiment, cultures were washed three times using 0.5% BSA (w/v, mg/mL) in DPBS. Washes were centrifuged at 1500 rpm for 10 min to recover any cellular material. Cells were removed from the culture wells by scraping and centrifuged under the same conditions. Cell pellets were treated with 1 mL of 3.5 mmol/L SDS in 0.3 mol/L NaOH and incubated overnight at room temperature before chemical analysis for cholesterol and protein concentrations. The presented data are the mean $\pm$ SD from three independent experiments, $n = 3$. Cell culture samples that were prepared for staining were washed twice with $1 \times$ PBS, fixed with 4% paraformaldehyde in 5 mmol/L PBS for 30 min at room temperature, again washed twice with $1 \times$ PBS, stained with 0.3% oil red O in 60% isopropanol for 30 min at room temperature, and finally washed three times with $1 \times$ PBS before observation with a Zeiss Axiovert 40 CFL. Measured uptake was reported as μg sterol/mg protein, and therefore a basis of 1 mg protein was assumed in the uptake model, which was calculated in terms of μg sterol and reported as μg sterol/mg protein. The value for the equivalent mass of cholesterol ester (CE) fed to each sample, representing the total mass of LDL, and calculated using the total sample volume of 3.2 mL, in model calculations was 359.77 μg .

2.5. Foam cell formation with in situ aggregated LDL

In some cases, macrophages were incubated with culture media in which LDL ($280 \pm 14 \mu\text{g}$ LDL/mL) and Smase (0, 9.82, and 90.25 milliunits Smase/mL) were introduced at the same time in a cell culture media consisting completely of DMEM (with 0.2% BSA, w/v, mg/mL), instead of performing the aggregation step in advance. In these cases, aggregation was induced during incubation with the cell culture and concurrently with uptake by macrophages. *In situ* uptake experiments were performed in duplicate as independent experiments, $n = 2$, and error bars represent the difference between the two values. All other aspects of the *in situ* aggregated LDL uptake experiment were identical to the pre-aggregated LDL uptake experiments.

2.6. Particle size measurement

LDL aggregate particle sizes were measured with dynamic light scattering (DLS) using a 90 Plus Particle Sizer (Brookhaven Instruments, Holtsville, NY) with a BI-9000AT digital autocorrelator. The equipment operates at 677 nm and contains a 15 mW solid-state laser. Apparent diffusivity and polydispersity were calculated using the method of cumulants (the first and second cumulants respectively, of a cumulant fit). Hydrodynamic radii of aggregates were calculated using the Stokes–Einstein equation [37] and are presented as LDL Aggregate Radius. The presented data are the mean $\pm$ SD from three independent experiments, $n = 3$.

2.7. Cholesterol and total protein assays

LDL uptake by macrophages was quantified by measuring cholesterol concentration in harvested cell pellets. The Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, California) was used in Nunc 96-well microplates in the Tecan Infinite 200™ (Männedorf, Switzerland) fluorescence microplate reader. Samples were treated with a standard assay composition according to vendor instructions. Samples were diluted 1:10 for analysis to avoid inner filter effects from excessive production of the assay's fluorescent product, resorufin. A calibration curve was made using 0, 0.5, 1.25, 2.50, 5.00, 7.50, and 10.0 μmol cholesterol/L. Cholesterol concentrations in cell pellets were normalized by total protein, measured with the Lowry Protein Assay Kit purchased from Bio-Rad (Hercules, CA). A calibration curve was made from BSA standards using 0.0, 0.2, 0.5, 0.8, 1.1, and 1.5 mg/mL protein.

2.8. Ceramide assay

Generation of ceramide was measured using Nunc 96-well microplates in the Tecan Infinite 200™ (Männedorf, Switzerland) fluorescence microplate reader. The Amplex Red-phosphorylcholine-coupled Smase fluorescence assay kit, purchased from Invitrogen (Carlsbad, CA), was used according to vendor instructions, and using HEPES buffer. As resorufin is produced in equimolar amounts with the phosphorylcholine and ceramide, the fluorescence is proportional to ceramide generation. A calibration curve for Smase kinetics measurements was made using fixed concentrations of phosphorylcholine in HEPES buffer (0, 0.025, 0.15, 0.25, 0.5, 1.5, 2.5, and 5 mmol/L) and by treatment with the Amplex Red standard fluorescence assay composition, excluding Smase, in order to measure resorufin fluorescence. The presented data are the mean $\pm$ SD from three independent experiments, $n = 3$.

2.9. LDL oxidation assays

Conjugated dienes were measured using absorbance spectroscopy at 234 nm with the Tecan Infinite 200™ (Männedorf, Switzerland). Lipid peroxides were measured using a modification of the method of El-Saadani et al. [22,38] whereby 9 μL of each sample was added to 90 μL of the color reagent, which consisted of 0.2 mol/L KH_2PO_4 , 0.12 mol/L KI, 0.15 mmol/L NaN_3 , 2 g of Triton X-100/L, 0.1 g of benzalkonium chloride/liter, 10 mmol/L ammonium molybdate, 20 mmol/L BHT, 25 mmol/L EDTA, and pH adjusted to 6.2 using NaOH, in 96-well UV Star Plates from Greiner Bio-One (Frickenhäusen, Germany). Calibration curves were made using H_2O_2 at various concentrations between 0 and 400 nmol/L. Absorbance was measured at 365 nm with the Tecan Infinite 200™ (Männedorf, Switzerland). The presented data are the mean $\pm$ SD from three independent experiments, $n = 3$.

3. Results

3.1. LDL aggregate size stability in culture media

Size-selective uptake of LDL aggregates by macrophages requires adding aggregates, which are formed separately the day before use to cell culture media, and incubating for a total of three days, while changing media with fresh material daily. Prior to the uptake studies, we therefore examined LDL aggregates in the presence of culture media by measuring aggregation kinetics, to confirm that aggregate sizes were stable in the culture media formulations. The ~12 nm measured radius of native, un-aggregated LDL used here is consistent with literature values [39,40].

Kinetics data was recorded for one day after enzyme addition. DMEM was then added and aggregate sizes were recorded for one additional day. Within a few hours after enzyme addition, aggregate sizes stabilized and remained constant (Fig. 1a, inset). The incubation with DMEM represents the same period of time and media formulation, without the Phenol Red indicator, as used in cell culture samples.

All data sets were fit with saturation curves of the form $y = y_0 + a(1 - \exp(-bx))$ to determine the plateau values. The final aggregate sizes and the enzyme concentrations associated with them were plotted (Fig. 1a) and used to determine the enzyme concentrations necessary for the production of aggregates of chosen sizes to be used in macrophage uptake experiments.

Since the size of Smase-treated LDL aggregates is dependent on the generation of ceramide, as shown in our previous work [25], at the end of the overnight aggregate-formation step, ceramide concentration was measured. For the enzyme concentrations 0, 46, 134, 361, 720, and 2870 milliunits Smase/mL, which correspond to aggregates with radii 12, 25, 50, 100, 149, and 211 nm, respectively, the following ceramide concentrations were observed: 0 ± 0.006 , 10.42 ± 0.08 , 11.4 ± 0.2 , 13.1 ± 0.6 , 13.5 ± 0.7 , and $21 \pm 2 \mu\text{mol/L}$, respectively. Using the measured bulk ceramide concentration, LDL concentration, molecular weight of LDL (2300 kDa [41]), total number of lipid molecules in an LDL particle (3070 [42]) the bulk solution ceramide concentrations are expressed as the mole percent of total LDL lipids as follows: $0 \pm 0\%$, $7.50 \pm 0.06\%$, $8.2 \pm 0.1\%$, $9.5 \pm 0.5\%$, $9.7 \pm 0.5\%$, $15 \pm 1\%$, respectively. The proportional increase in ceramide with both Smase concentration and aggregate size supports the idea of ceramide-drive aggregation for Smase-treated samples. The extent of ceramide generation as a function of enzyme concentration observed here is in agreement with data from previous work [25].

3.2. Cholesterol ester accumulation with Smase-induced aggregation

CE accumulation was observed in all samples incubated with LDL, and began to increase with higher aggregate size before reaching a maximum at particle radius ~132 nm and corresponding CE concentration ~58 μg sterol/mg protein, and then decreasing (Fig. 1b). Controls (not shown) showed no significant CE accumulation. Unesterified cholesterol was also measured and is roughly constant across all samples analyzed. Micrograph images of cells during incubation revealed the presence of a number of small dark spots inside the macrophages which was proportional to the measured CE concentrations. Staining these cells with oil red O confirmed the presence of foam cells (Fig. 2c and d). Analysis for lipid oxidation showed a slight saturation effect for Smase treatment (Fig. 3a and b, closed circles) and a ~5-fold increase resulting from overnight incubation with macrophages.

3.3. Cholesterol ester accumulation with vortex-mixing-induced aggregation

Smase is a likely candidate for the cause of LDL aggregation *in vivo* due to its presence in the vicinity of arterial lesions [16,17]. However, since modification of LDL with Smase causes ceramide generation [25], higher aggregate size in Fig. 1b also represents higher concentration of both ceramide and Smase. To separate aggregate size from the other two factors, it was important to evaluate CE accumulation using another aggregation method such as vortex-mixing, which did not induce elevation of the ceramide content in LDL. Aggregate sizes were measured before (at ~24 h) and after DMEM dilution (at ~48 h) and the averages of these values were plotted against vortex-mixing time (Fig. 4a). Our data show that vortex-mixing of LDL for up to 360 s resulted in the formation of aggregates of radii up to ~80 nm. The fit of vortexed LDL particle size data shown in Fig. 4a is not ideal. This may be due to the amorphous, and non-spherical nature of vortexed-mixed aggregates [10,43]. Though it is not possible to conclude the shape and dimensions of the vortexing-mediated aggregates using our experimental techniques, we can conclude that increased vortexing causes increased aggregate size as per the relationship shown in Fig. 4a, and thus present the aggregate hydrodynamic radii without specifying a shape for these aggregates.

Similar in trend to Smase-treated samples, though different in aggregate size and CE content, the CE accumulation in vortex-mixed samples also showed a maximum, here at a particle radius of ~53 nm and a corresponding CE concentration ~118 μg sterol/mg protein (Fig. 4b). The presence of a maximum in CE accumulation as a function of vortex time is consistent with a previous study of the uptake of aggregated LDL [10]. Micrograph images of cells showed a trend similar to cell samples treated with Smase, and foam cells were observed using oil red O staining (Fig. 2e and f). Analysis for lipid peroxides and conjugated dienes showed mild oxidation after the vortex-mixing step, followed by increased oxidation after incubation with macrophages; however, there was no significant difference between samples of different vortex times (Fig. 3c and d).

In order to confirm the observed differences in CE accumulation between Smase-treated and vortexed aggregates, one additional 'follow-up' sample was prepared. LDL was treated with 134 milliunits Smase/mL, creating aggregates with 50 nm radii. After incubation with macrophages the CE concentration in this sample was 31.4 μg sterol/mg protein, which matches the Smase-derived aggregate curve (Fig. 1b) but not the vortex-mixing-derived aggregate curve (Fig. 4b), and which showed CE concentrations ~3.6-fold greater at this aggregate size value. This comparison confirms the observed differences in the two data sets (from Figs. 1b and 4b).

3.4. Cholesterol ester accumulation with *in situ* aggregation

In each experiment discussed thus far, LDL was modified, either enzymatically or by vortex-mixing, prior to incubation in cell culture media. This approach offered control of aggregate size and allowed for the investigation of size-selective uptake. A more physiologically relevant experimental setup would allow for uptake to occur simultaneously with aggregation. An additional study was therefore done, in which LDL and Smase were added to cell culture without pre-aggregating. A drawback of this *in situ* approach is that it precludes measurement of LDL aggregation sizes with DLS. On the other hand, it does permit semi-quantitative assessment of uptake while maintaining physiological relevance.

During a 3-day incubation period, foam cells were observed (Fig. 2g). Measured total cholesterol accumulation increased with respect to Smase concentration (Fig. 5), which is consistent with previously reported experiments [1] and also with the size-controlled experiments reported here (Figs. 1b and 4b).

4. Discussion and conclusions

This study showed that CE accumulation resulting from the uptake of LDL aggregates by cultured mouse macrophages depends on aggregate size. The size dependence is not due to trivial LDL valence effects. For a given number of uptake events, a larger aggregate would deliver a greater payload than a smaller aggregate simply due to a larger LDL aggregation number, or the number of LDL monomers contained in the aggregate. We compensated for this trivial effect by maintaining a constant total LDL concentration in all samples. That is, we fed an identical number of LDL monomers to each sample such that any difference in LDL aggregation number was offset by a corresponding change in LDL aggregate concentration (as depicted in Fig. 6a). Thus, the results observed in Figs. 1b and 4b are truly size dependent phenomena. Moreover, the size dependence exhibits an optimum, which leads to maximal uptake, irrespective of the mechanism that causes aggregation. An interesting question naturally arises, namely, what factor(s) determine the optimal size? We believe the answer is a competition between surface area and volume effects (as illustrated in Scheme 1). On the one hand, uptake increases with increasing surface area due to a greater number of exposed apoB molecules, since macrophage-accessible apoB moieties facilitate aggregate uptake [10,44] or due to other receptor-mediated processes that require an interaction with the lipoprotein surface [7,8]. On the other hand, uptake decreases with increasing volume due to spatial constraints at the site of ingestion.

Eq. (1) describes this competition between surface area and volume quantitatively,

$$\kappa_{\text{size}} = \alpha_{\text{vortex}}(4\pi r^2) - \beta_{\text{vortex}}\left(\frac{4}{3}\pi r^3\right) \quad (1)$$

where κ_{size} is a dimensionless quantity that is tantamount to a constant of proportionality between uptake and the total mass of LDL monomers, in terms of the equivalent mass of CE in the monomers ($[LDL]$), fed to macrophages in each sample (Eq. (2)).

$$\text{Uptake} = (\kappa_{\text{size}})[LDL] \quad (2)$$

Accordingly, κ_{size} may also be viewed as the fraction of LDL monomers fed to macrophages that are subsequently ingested, plus the effects of cellular cholesterol efflux (Fig. 6b). The parameters α_{vortex} and β_{vortex} quantify the relative importance of surface area and volume effects, respectively.

A fit of Eq. (2) to the data from vortex-mixed samples is shown in Fig. 6c (using $\alpha_{\text{vortex}} = 2.53\text{e-}5 \text{ nm}^{-2}$ and $\beta_{\text{vortex}} = 9.17\text{e-}7 \text{ nm}^{-3}$). While vortexed aggregates are shown to be non-spherical [10,43], a shape analysis with information on aggregate length or aspect ratio has not been performed. Here, due to the spherical shape of the more physiologically relevant Smase-induced aggregates [1], we have used a spherical shape as a first approximation to represent vortex-mixing-mediated aggregates. The parameters α_{vortex} and β_{vortex} are then lumped parameters that account for both fitting the relative effects of surface area and volume, and also accounting for an adjustment to a non-spherical shape. The accurate fit resulting from this size-selective model supports the idea that competition between surface area and volume, that is aggregate size alone, determines the uptake of vortex-mixed LDL aggregates; however there is more to the story than size alone.

When applied to Smase-treated aggregates, the size-selective model does not provide an accurate fit (Fig. 6d, solid line). Unlike vortex-mixing, which involves physical modification of LDL monomers, Smase-mediated aggregation results from a change in LDL composition,

namely, the formation of ceramide in the phospholipid monolayer due to Smase-hydrolysis of LDL-sphingomyelin. The generation of hydrophobic moieties on the phospholipid monolayer leads not only to aggregation but also to fusion of the LDL monomers [23,45]. As a result, the physical properties of an Smase-induced LDL aggregate are also likely to be different than those of a vortex-mixed aggregate. Due to these differences we modified Eq. (2) to account for composition (Eq. (3)).

$$\text{Uptake} = \frac{\kappa_{\text{size}}}{\kappa_{\text{cer}}} [\text{LDL}] \quad (3)$$

It is possible to fit the uptake data for Smase-treated aggregates with a modified version of this model if κ_{cer} is assigned a value of ' r ' (Eq. (4)). This 'reduction in size-dependence' due to ceramide could be described by two different changes to the physical system, both caused by ceramide, including a reduction of unmodified surface area in which apoB resides and a reduction of the rigidity in aggregate volume due to the fusion of native LDL particles through ceramide-rich regions. Both effects could occur in Smase-induced aggregates due to the presence of ceramide, but since they deal with changes in aggregate functionality based on aggregate geometry, they are modeled with a dependence on aggregate radius and not on ceramide concentration. Combining this with Eqs. (1) and (3) gives a new model which also accounts for changes in ceramide concentration (Eq. (5)).

$$\kappa_{\text{cer}} = r \quad (4)$$

$$\text{Uptake} = \left((\alpha'_{\text{Smase}})(4\pi r) - (\beta'_{\text{Smase}}) \left(\frac{4}{3}\pi r^2 \right) \right) [\text{LDL}] \quad (5)$$

Fitting Smase-treated experimental data with the size-and-composition-dependent model (Eq. (5), using $\alpha'_{\text{Smase}} = 2.04e - 4 \text{ nm}^{-2}$ and $\beta'_{\text{Smase}} = 2.32e - 6 \text{ nm}^{-3}$ supports the idea that both size and composition affect uptake (Fig. 6d, dashed line). Since α_{vortex} and β_{vortex} account for surface area versus volume effects and also for a correction for unknown aggregate shape, and α'_{Smase} and β'_{Smase} account for surface area versus volume effects and also for the effects of ceramide, these two sets of parameters are inherently different and are fitted separately. The size-and-composition-dependent model produces a rounded peak and appears flattened when compared to the size-dependent model. The locations of maximal uptake (r^*) observed in Fig. 6c and d (55.2 nm and 132 nm, for Eqs. (6) and (7), respectively) are both functions of the alpha and beta values.

$$r^*_{\text{vortex}} \Big|_{\frac{d(\text{Uptake})}{dr}=0} = 2 \left(\frac{\alpha_{\text{vortex}}}{\beta_{\text{vortex}}} \right) \quad (6)$$

$$r^*_{\text{Smase}} \Big|_{\frac{d(\text{Uptake})}{dr}=0} = \left(\frac{3}{2} \right) \left(\frac{\alpha'_{\text{Smase}}}{\beta'_{\text{Smase}}} \right) \quad (7)$$

Since uptake of LDL aggregates can occur by various mechanisms such as scavenger receptor-mediated processes [8,46,47], pinocytosis [48,49], phagocytosis [10,50,51], and patocytosis [48,52,53], we make no conclusions about the actual mechanism or mechanisms of uptake here. Since oxidation did not differ significantly between samples subjected to vortex-mixing for different lengths of time (Fig. 3c and d), and showed a slight saturation

effect for Smase-treated samples (Fig. 3a and b), it was not considered as a cause for the observed maxima in CE accumulation. Lipid oxidation may, however, change the structure of the LDL surface as per the Lipid Whisker Model, and this surface alteration could play a role in enhanced macrophage recognition [54,55]. Also, it is possible that additional negative charge on the LDL surface could inhibit LDL aggregation through electrostatic repulsion, which would affect its uptake via phagocytosis and the scavenger receptor mechanism [7,8,10].

It is known that principle mechanisms of uptake for aggregated, oxidized LDL involve the scavenger receptor and phagocytosis [7,8,10]. We therefore consider that these could be the relevant uptake mechanisms involved here. Additionally, increased oxidation and aggregation were shown to decrease the effect of the scavenger receptor mechanism [8] and enhance uptake by phagocytosis [7]. Finally, in the case where no LDL oxidation occurred, the scavenger receptor was shown to be inactive in LDL uptake, further supporting the uptake, mechanisms considered here [10].

In conclusion, size-selectivity was observed in the uptake of LDL aggregates by macrophages, both when aggregates were formed by Smase-mediated ceramide generation and also by vortex-mixing. The presence of maximal uptake values, which was independent of the method of aggregation, indicated a competition between different factors that influence uptake, namely: surface area and volume, representing the number of macrophage-accessible apoB molecules and total LDL mass per aggregate, respectively. Additionally, in the case of Smase-treated aggregates, we suspect that LDL composition, namely ceramide concentration, reduced but did not eliminate this size-selective effect. Our study shows that aggregation of colloidal LDL is a significant factor in its size-and-ceramide-selective uptake by macrophages and subsequently in the progression of atherosclerotic lesions.

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References

1. Xu XX, Tabas I. *J Biol Chem.* 1991; 266:24849. [PubMed: 1761578]
2. Schaffner T, Taylor K, Bartucci EJ, Fischer-Dzoga K, Beeson JH, Glagov S, Wissler RW. *Am J Pathol.* 1980; 100:57. [PubMed: 6772035]
3. Hoff HF, O'Neil J, Pepin JM, Cole TB. *Eur Heart J.* 1990; 11(Suppl E):105. [PubMed: 2226518]
4. Williams KJ, Tabas I. *Arterioscler, Thromb, Vasc Biol.* 1995; 15:551. [PubMed: 7749869]
5. Tabas I, Weiland DA, Tall AR. *Proc Natl Acad Sci U S A.* 1985; 82:416. [PubMed: 3855559]
6. Williams KJ, Tabas I. *Curr Opin Lipidol.* 1998; 9:471. [PubMed: 9812202]
7. Hoff HF, O'Neil J. *Arterioscler Thromb.* 1991; 11:1209. [PubMed: 1911707]
8. Hoff HF, Whitaker TE, O'Neil J. *J Biol Chem.* 1992; 267:602. [PubMed: 1730620]
9. Hoff HF, O'Neil J, Chisolm GM 3rd, Cole TB, Quehenberger O, Esterbauer H, Jurgens G. *Arteriosclerosis.* 1989; 9:538. [PubMed: 2751482]
10. Khoo JC, Miller E, McLoughlin P, Steinberg D. *Arteriosclerosis.* 1988; 8:348. [PubMed: 3395271]
11. Tabas I. *Annu Rev Nutr.* 1999; 19:123. [PubMed: 10448519]
12. Devlin CM, Leventhal AR, Kuriakose G, Schuchman EH, Williams KJ, Tabas I. *Arterioscler, Thromb, Vasc Biol.* 2008; 28:1723. [PubMed: 18669882]
13. Schissel SL, Tweedie-Hardman J, Rapp JH, Graham G, Williams KJ, Tabas I. *J Clin Invest.* 1996; 98:1455. [PubMed: 8823312]
14. Steinberg D. *J Biol Chem.* 1997; 272:20963. [PubMed: 9261091]
15. Steinbrecher UP. *J Biol Chem.* 1987; 262:3603. [PubMed: 3102491]

16. Marathe S, Schissel SL, Yellin MJ, Beatini N, Mintzer R, Williams KJ, Tabas I. *J Biol Chem.* 1998; 273:4081. [PubMed: 9461601]
17. Schissel SL, Schuchman EH, Williams KJ, Tabas I. *J Biol Chem.* 1996; 271:18431. [PubMed: 8702487]
18. Tabas I. *Chem Phys Lipids.* 1999; 102:123. [PubMed: 11001566]
19. Tabas I, Li Y, Brocia RW, Xu SW, Swenson TL, Williams KJ. *J Biol Chem.* 1993; 268:20419. [PubMed: 8376399]
20. Nelson GJ, Freeman NK. *J Biol Chem.* 1960; 235:578. [PubMed: 14426450]
21. Kinnunen PK, Holopainen JM. *Trends Cardiovasc Med.* 2002; 12:37. [PubMed: 11796243]
22. Schissel SL, Jiang X, Tweedie-Hardman J, Jeong T, Camejo EH, Najib J, Rapp JH, Williams KJ, Tabas I. *J Biol Chem.* 1998; 273:2738. [PubMed: 9446580]
23. Oorni K, Hakala JK, Annala A, Ala-Korpela M, Kovanen PT. *J Biol Chem.* 1998; 273:29127. [PubMed: 9786921]
24. Pentikainen MO, Lehtonen EM, Kovanen PT. *J Lipid Res.* 1996; 37:2638. [PubMed: 9017515]
25. Walters MJ, Wrenn SP. *Langmuir.* 2008; 24:9642. [PubMed: 18671414]
26. Guyton JR, Klemp KF. *Arterioscler, Thromb, Vasc Biol.* 1996; 16:4. [PubMed: 8548424]
27. Khoo JC, Miller E, McLoughlin P, Steinberg D. *J Lipid Res.* 1990; 31:645. [PubMed: 2112580]
28. Gudheti MV, Lee SP, Danino D, Wrenn SP. *Biochemistry.* 2005; 44:7294. [PubMed: 15882068]
29. Marathe S, Choi Y, Leventhal AR, Tabas I. *Arterioscler, Thromb, Vasc Biol.* 2000; 20:2607. [PubMed: 11116060]
30. Maor I, Hayek T, Hirsh M, Iancu TC, Aviram M. *Atherosclerosis.* 2000; 150:91. [PubMed: 10781639]
31. Tabas I. *Arterioscler, Thromb, Vasc Biol.* 2005; 25:2255. [PubMed: 16141399]
32. Brown MS, Goldstein JL. *Annu Rev Biochem.* 1983; 52:223. [PubMed: 6311077]
33. Ellington AA, Kullo IJ. *Adv Clin Chem.* 2008; 46:295. [PubMed: 19004193]
34. Superko HR, Gadesam RR. *Curr Atheroscler Rep.* 2008; 10:377. [PubMed: 18706278]
35. Williams KJ, Tabas I. *Arterioscler, Thromb, Vasc Biol.* 2005; 25:1536. [PubMed: 16055756]
36. Steinbrecher UP, Loughheed M. *Arterioscler Thromb.* 1992; 12:608. [PubMed: 1576122]
37. Evans DF, Wennerström H. *The Colloidal Domain: where Physics, Chemistry, Biology, and Technology Meet (second).* 1999
38. El-Saadani M, Esterbauer H, El-Sayed M, Goher M, Nassar AY, Jurgens G. *J Lipid Res.* 1989; 30:627. [PubMed: 2754343]
39. O'Neal D, Harrip P, Dragicevic G, Rae D, Best JD. *J Lipid Res.* 1998; 39:2086. [PubMed: 9788255]
40. DeBlois RW, Uzgiris EE, Devi SK, Gotto AM Jr. *Biochemistry.* 1973; 12:2645. [PubMed: 4351205]
41. Lodish, H., et al. *Molecular Cell Biology.* 5th. W.H. Freeman; 2003.
42. Esterbauer H, Wag G, Puhl H. *Br Med Bull.* 1993; 49:566. [PubMed: 8221023]
43. Guyton JR, Klemp KF, Mims MP. *J Lipid Res.* 1991; 32:953. [PubMed: 1940627]
44. Skalen K, Gustafsson M, Rydberg EK, Hulten LM, Wiklund O, Innerarity TL. *J Boren, Nature.* 2002; 417:750.
45. Oorni K, Posio P, Ala-Korpela M, Jauhiainen M, Kovanen PT. *Arterioscler, Thromb, Vasc Biol.* 2005; 25:1678. [PubMed: 15879301]
46. Steinbrecher UP, Loughheed M, Kwan WC, Dirks M. *J Biol Chem.* 1989; 264:15216. [PubMed: 2768257]
47. Greaves DR, Gordon S. *J Lipid Res.* 2009; 50(Suppl):S282. [PubMed: 19074372]
48. Kruth HS. *Curr Opin Lipidol.* 2002; 13:483. [PubMed: 12352011]
49. Haberland ME, Mottino G, Le M, Frank JS. *J Lipid Res.* 2001; 42:605. [PubMed: 11290833]
50. Suits AG, Chait A, Aviram M, Heinecke JW. *Proc Natl Acad Sci U S A.* 1989; 86:2713. [PubMed: 2704743]

51. Aviram M, Maor I, Keidar S, Hayek T, Oiknine J, Bar-El Y, Adler Z, Kertzman V, Milo S. *Biochem Biophys Res Commun.* 1995; 216:501. [PubMed: 7488140]
52. Zhang WY, Gaynor PM, Kruth HS. *J Biol Chem.* 1997; 272:31700. [PubMed: 9395512]
53. Sakr SW, Eddy RJ, Barth H, Wang F, Greenberg S, Maxfield FR, Tabas I. *J Biol Chem.* 2001; 276:37649. [PubMed: 11477084]
54. Hazen SL. *J Biol Chem.* 2008; 283:15527. [PubMed: 18285328]
55. Greenberg ME, Li XM, Gugiu BG, Gu X, Qin J, Salomon RG, Hazen SL. *J Biol Chem.* 2008; 283:2385. [PubMed: 18045864]

Fig. 1. Smase-mediated aggregation of LDL and subsequent CE accumulation in macrophages: *Panel a*, Inset plot: Experimental data points and smoothed mathematical fit of LDL aggregation kinetics after Smase addition and followed by dilution with DMEM. The data show that LDL aggregates have a stable, constant size up to at least 1 day after the addition of DMEM. Enzyme concentrations of 0($\bullet$), 10($\blacktriangledown$), 100($\blacksquare$), 438($\blacklozenge$), 938($\blacktriangle$), 1875(hexagon), and 3750 ($\circ$) milliunits Smase/mL were used. *Panel a*, Large plot: experimental data points and smoothed mathematical fit of the final, equilibrium LDL aggregate sizes (the plateau value for each curve in the inset plot) at each enzyme concentration. *Panel b*, LDL was incubated with various concentrations of Smase (0, 46, 134, 361, 720, and 2870 milliunits/mL) to form aggregates of various sizes (12, 25, 50, 100, 149, and 211 nm) and added to cell culture media. Total LDL concentration was identical in all samples ($104.1 \pm 0.5 \mu\text{g LDL/mL}$). CE accumulation was selective based on aggregate

radius, with a maximum near 132 nm ($\sim 58 \mu\text{g}$ sterol/mg protein). Experimental data is shown (bars) along with an empirical fit of CE concentration (curve). Unesterified cholesterol (UC) concentration was also measured and was roughly constant in all samples. All data presented here are the mean $\pm$ SD from three independent experiments, $n = 3$.

Fig. 2.

Micrograph images of macrophage cells: after 3 days of incubation with LDL aggregates formed either by Smase-mediated hydrolysis of LDL-sphingomyelin, vortex-mixing, or by *in situ* aggregation. Images include: (a) the control sample with no LDL and no Smase before staining with oil red O, (b) the control sample with no LDL and no Smase after staining, (c) cells incubated with medium-sized, 149 nm, Smase-treated LDL aggregates, (d) cells incubated with large-sized, 211 nm, Smase-treated LDL aggregates, (e) cells incubated with medium-sized, 52 nm, vortex-mixed LDL aggregates, (f) cells incubated with large-sized, 77 nm, vortex-mixed LDL aggregates, and (g) cells incubated with *in situ*-aggregated LDL, with the maximum Smase concentration, 90.25 milliunits/mL. Scale bars = 20 μm .

Fig. 3. Lipid peroxidation: evaluated for Smase-treated (a and b) and vortex-mixed (c and d) samples by measuring lipid peroxide concentration (a and c) and absorbance of conjugated dienes at 234 nm (b and d). Measurements were made after LDL modification (●) and after incubation of the modified LDL aggregates with macrophages (○). All data presented here are the mean $\pm$ SD from three independent experiments, $n = 3$.

Fig. 4.

Vortex-mixing-mediated aggregation of LDL and subsequent CE accumulation in macrophages: *Panel a*, LDL samples were vortexed for various periods of time (0, 15, 30, 60, 201, and 360 s). Equilibrium aggregate sizes (similar to the plateau values in the Fig. 1 inset plot for Smase-treated samples) were calculated as averages of size measurements at ~24 h and ~48 h (before and after addition to cell culture media) and were plotted (shown as data points) versus vortex time along with a smoothed mathematical fit. Data show that LDL aggregates can be formed with radii ranging from 20 to ~80 nm. *Panel b*, LDL was vortexed for various times (0, 13, 34, 64, 115, and 201 s), to form aggregates of various sizes (12, 20, 36, 52, 67, and 77 nm), and added to cell culture media. Total LDL concentration was identical in all samples ($104.1 \pm 0.5 \mu\text{g LDL/mL}$). CE accumulation was selective based on aggregate radius, with a maximum near 53 nm ($\sim 118 \mu\text{g sterol/mg protein}$). Experimental data is shown (bars) along with an empirical fit of CE concentration (curve). Unesterified cholesterol (UC) concentration was also measured and was roughly constant in all samples. All data presented here are the mean $\pm$ SD from three independent experiments, $n = 3$.

Fig. 5.

In situ uptake: LDL (280 ± 14 µg LDL/mL) and Smase (0, 9.82, and 90.25 milliunits Smase/mL) were added to cell culture media. Figure shows total cholesterol accumulation (including CE and unesterified cholesterol) minus the cholesterol concentration in the control sample (23 µg sterol/mg protein), which consisted only of macrophage cells without LDL or Smase. Data presented here are the mean of two independent experiments and error bars were calculated as the difference between the two values, $n = 2$.

Fig. 6.

Size- and composition-dependent model for the uptake of LDL aggregates by macrophages: *Panel a*, LDL aggregate concentration and aggregation number calculations, showing that an increasing LDL aggregation number is accompanied by a decreasing aggregate concentration. LDL aggregate concentration was calculated by dividing the total LDL concentration ($104.1 \pm 0.5 \mu\text{g LDL/mL}$, which was converted to $2.72\text{e}+13$ LDL particles/mL using $\text{MW}_{\text{LDL}} = 2.3\text{e}+3$ kDa) by the LDL aggregation number (assuming an aggregate particle packing parameter of 0.25). *Panel b*, Fractional uptake curves, including the surface area and volume effects, which compose κ_{size} . Plot was constructed using model-fit variables (Eqs. (1) and (2)) for uptake of vortex-mixed aggregates. *Panel c*, model fit (curve) of LDL uptake experimental data (bars) from vortex-mixed samples, showing the competition between aggregate surface area and volume in size-selective uptake. Uptake was calculated using the model constants $\alpha_{\text{vortex}} = 2.53\text{e} - 5 \text{ nm}^{-2}$ and $\beta_{\text{vortex}} = 9.17\text{e} - 7 \text{ nm}^{-3}$. *Panel d*, Size-dependent model fit (solid curve) and size- and composition-dependent model fit (dashed curve) of LDL uptake experimental data (bars) from Smase-treated samples, showing the competition between aggregate surface area, volume, and ceramide concentration in size- and composition-selective uptake. Uptake was calculated using the model constants $\alpha'_{\text{Smase}} = 2.04\text{e} - 4 \text{ nm}^{-2}$ and $\beta'_{\text{Smase}} = 2.32\text{e} - 6 \text{ nm}^{-3}$.

Cross-Sections of LDL Aggregates

Scheme 1.

Increasing aggregate size decreases the surface area-to-volume ratio. Surface area is helpful to uptake because apoB is accessible at the aggregate surface. Volume is obstructive to uptake due to spatial constraints.