PMA activation of macrophages alters macrophage metabolism of aggregated LDL

Wei Huang, Itsuko Ishii, Wei-Yang Zhang, Miyahiko Sonobe, and Howard S. Kruth[1]

Section of Experimental Atherosclerosis, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 5N-113, 10 Center Drive MSC 1422, Bethesda, MD 20892

Abstract **Aggregation of LDL may contribute to its retention in atherosclerotic lesions. Previously, we showed that aggregated LDL induces and enters surface-connected compartments (SCCs) in human monocyte-derived macrophages by a process we have named patocytosis. Aggregated LDL was disaggregated and released from SCCs of macrophages when exposed to human lipoprotein-deficient serum. The serum factor that mediated aggregated LDL release and disaggregation was plasmin generated from plasminogen by macrophage urokinase plasminogen activator. We now show that activation of macrophages with PMA inhibits plasmin-mediated release of aggregated LDL from macrophages. With macrophage activation, plasminogen released about 60% less cholesterol and 63% less TCA-insoluble ^{125}I-aggregated LDL than when macrophages were not activated. Electron microscopy showed that PMA did not cause SCCs to close, which could have trapped aggregated LDL within the SCCs and limited protease access to aggregated LDL. Rather, PMA decreased macrophage generation of plasmin by 61%, and stimulated lysosomal degradation of aggregated LDL by more than 2-fold. Degradation was mediated by protein kinase C, shown by the finding that degradation was inhibited by the protein kinase C inhibitor Gö6976. PMA-stimulated degradation of aggregated LDL was associated with a 3-fold increase in cholesterol esterification, consistent with hydrolysis and re-esterification of aggregated LDL-derived cholesteryl ester. In conclusion, macrophage activation with PMA causes more of the aggregated LDL that enters macrophage SCCs to be metabolized by lysosomes. This results in more cholesterol to be stored in macrophages and less aggregated LDL to be available for plasmin-mediated release from macrophage SCCs.**—Huang, W., I. Ishii, W-Y. Zhang, M. Sonobe, and H. S. Kruth. **PMA activation of macrophages alters macrophage metabolism of aggregated LDL.** ***J. Lipid Res.*** **2002.** 43: **1275–1282.**

Supplementary key words acyl-CoA:cholesterol acyltransferase • atherosclerosis • cholesterol • endocytosis • plasmin • low density lipoprotein • surface-connected compartments • phorbol myristate acetate • protein kinase C • trichloroacetic acid • lysosomes • patocytosis

Focal vessel wall retention of plasma-derived LDL contributes to cholesterol buildup in vessel plaques. This leads to atherosclerosis, a disease that represents the response of the vessel wall to retained LDL (1). Evidence suggests that aggregation of LDL may contribute to its retention in developing atherosclerotic lesions. LDL recovered from lesions shows an increased tendency to aggregate, and LDL aggregates have been demonstrated in early developing lesions (2–4). Many modifications to LDL cause LDL aggregation. These include oxidation of LDL and treatment of LDL with certain lipases that are present within lesions (e.g., sphingomyelinase, phospholipases A2 and C) (5–7). Simple vortexing of LDL is a convenient way to produce aggregated LDL (AgLDL) for experimental studies (8).

Previously, we described an unusual endocytic pathway in macrophages through which AgLDL enters human monocyte-derived macrophages (9). In this pathway, which we call patocytosis, the AgLDL induces and enters surface-connected compartments (SCCs) where the AgLDL is stored. While some of the AgLDL undergoes lysosomal degradation in the macrophages, most of the AgLDL remains undegraded. In recent work, we showed that when macrophages are exposed to plasminogen, the macrophages release the stored AgLDL (10). This is the result of macrophage conversion of plasminogen to plasmin, mediated by macrophage urokinase plasminogen activator. The macrophage-generated plasmin disaggregates the AgLDL, allowing the unattached LDL particles to exit the open SCCs.

Macrophage activation influences many aspects of macrophage function and metabolism. In this study, we examined whether activation of macrophages would influence the fate of AgLDL that had entered macrophages by patocytosis and accumulated in SCCs. One commonly used ac-

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Abbreviations: AgLDL, aggregated LDL; DPBS, Dulbecco's phosphate-buffered saline; LPDS, lipoprotein-deficient serum; SCC, surface-connected compartment.

[1] To whom correspondence should be addressed.
e-mail: kruthh@nhlbi.nih.gov

tivator of macrophages is the phorbol ester, PMA. Here, we report that PMA activation of human monocyte-macrophages stimulates lysosomal degradation of AgLDL stored in SCCs. This promotes esterification of AgLDL cholesterol. At the same time, by removing AgLDL from SCCs, PMA activation of macrophages inhibits plasmin-mediated release of AgLDL from macrophage SCCs.

EXPERIMENTAL PROCEDURES

Materials

Human LDL and human lipoprotein-deficient serum (LPDS) were obtained from Intracel; human ^{125}I-LDL from Biomedical Technologies; [9,10-^{3}H]oleic acid in ethanol from American Radiolabeled Chemicals; bovine lung aprotinin from ICN; human plasminogen and PMA from Calbiochem; human plasmin from American Diagnostica; D-Val-Leu-Lys-AFC plasmin substrate from Enzyme Systems Products; chloroquine, ε-amino-*N*-caproic acid, sodium oleate, and fatty acid-free BSA from Sigma; RPMI-1640 medium from Cellgro; pooled human AB, heat-inactivated serum from Pel-Freez; mouse monoclonal anti-cathepsin D (clone C5) from Biogenesis; mouse monoclonal anti-Aspergillus major glucose oxidase (clone DAK-G09) from Dako; Gö6976 and wortmannin from Biomol; polysilica acid gel-impregnated glass fiber sheets from Pall; Plastek C culture plates from MatTek; Lab-Tek chamber slides from Nalge-Nunc; and 20-nm BSA-gold from British Biocell.

Preparation of aggregated LDL

LDL was aggregated by vortexing (1 min at maximum setting with a VWR vortex mixer) 200 μl of ^{125}I-LDL (specific activity ranged from 70–200 μCi/mg of protein) in a 12 × 75 mm polypropylene microtube. The resulting aggregated fraction was collected by centrifugation at 12,000 *g* for 15 min. To increase the yield of aggregated ^{125}I-LDL to >90% of the original ^{125}I-LDL, the supernatant was subjected to vortexing and centrifugation an additional three times. All precipitates were then combined.

Assays of cell-association and degradation of ^{125}I-AgLDL

Human monocyte-derived macrophages were cultured as described previously except that 2 × 10^6 monocytes/well were initially seeded into 12-well (22 mm diameter) culture plates (11). Two-week-old monocyte-macrophage cultures were rinsed three times with RPMI-1640 medium and incubated at 37°C for the indicated times and concentrations of ^{125}I-AgLDL added to RPMI-1640 medium. The cell-association and degradation of ^{125}I-AgLDL by the macrophages were determined according to the methods of Goldstein et al. (12). Lipoprotein degradation was quantified by measurement of TCA-soluble organic iodide radioactivity in supernatants of media samples that were centrifuged at 15,000 *g* for 10 min. Values obtained in the absence of cells were <5% of those values obtained with cells. These control values were subtracted.

Cell-associated ^{125}I-AgLDL was determined by rinsing macrophages five times with Dulbecco's phosphate-buffered saline (DPBS) containing Ca^{2+}, Mg^{2+}, and 0.2% BSA (three quick rinses and two 10-min incubations, all on ice). After a final rinse with DPBS plus Ca^{2+} and Mg^{2+}, macrophages were dissolved overnight in 0.1 N NaOH. Aliquots of NaOH-solubilized cell samples were assayed for ^{125}I radioactivity. Values were subtracted for ^{125}I radioactivity determined for wells incubated with ^{125}I-AgLDL but without macrophages. These values were <1% of the cell-associated ^{125}I-AgLDL.

Assay of cholesterol and protein content of macrophages

Unesterified and esterified cholesterol contents of macrophages were determined according to the fluorometric method of Gamble et al. (13). For these assays, macrophages were harvested by scraping into distilled water and processed as described previously (11). Macrophage protein content was determined by the method of Lowry et al., using BSA as a standard (14). Protein contents of cultures generally ranged between 0.2 and 0.3 mg/well.

Assay of macrophage plasmin activity

Macrophages were incubated 24 h in RPMI-1640 medium (without phenol red) and 1 U/ml plasminogen without and with 0.1 μg/ml PMA. Macrophages were then rinsed three times with DPBS plus Ca^{2+}, Mg^{2+}, and 0.2% BSA, and three times with DPBS plus Ca^{2+} and Mg^{2+}. Next, membrane-bound plasmin was dissociated from cells with ε-amino-*N*-caproic acid in DPBS for 15 min at 4°C (15). Only membrane-bound plasmin is active in the presence of serum that contains natural inhibitors of plasmin activity. Plasmin activity was quantified essentially as described by Falcone et al. (16). Twenty-microliter aliquots of dissociation buffer were added to microtiter wells with 80 μl DPBS containing 0.05% Tween-20 and 0.0625 μg of the plasmin substrate, D-Val-Leu-Lys-AFC, and allowed to incubate for 30 min at 37°C. Cleavage of the plasmin substrate was monitored in a microplate reader with excitation of 360 nm and emission at 535 nm. Plasmin activity in the samples was calculated from a standard curve generated with 0–400 ng/ml purified human plasmin as a standard.

Assay of cholesteryl ester synthesis

[^{3}H]oleic acid in ethanol (4.2 nmol with specific activity of 60 Ci/mmol) was evaporated to dryness under nitrogen gas and redissolved in a solution of nonradioactive sodium oleate complexed with BSA in 0.9% sodium chloride exactly as described by Goldstein et al. (12). After macrophages were loaded with AgLDL (200 μg/ml) for 5 h, they were rinsed three times with RPMI-1640, and then incubated 24 h without or with 0.1 μg/ml PMA and 533 μM [^{3}H]oleate-64 ug/ml albumin conjugate (specific activity of 2,300 dpm/nmol). Following incubations, macrophages were rinsed three times with DPBS plus Ca^{2+}, Mg^{2+}, and 0.2% BSA, and three times with DPBS plus Ca^{2+} and Mg^{2+}. Hexane-isopropanol (3:2, v/v) was then used to extract lipids from macrophages in the plates. Macrophages were scraped into 1 ml distilled water and their protein content was determined as described above. The lipid extract was separated by TLC on polysilica acid gel-impregnated glass fiber sheets. Cholesteryl ester spots were identified with iodine vapor, cut from the chromatogram, and counted in 10 ml of liquid scintillation fluid. Cholesteryl ester synthesis is expressed as nmoles of [^{3}H]oleate incorporated into cholesteryl ester per milligram of cell protein during 24 h.

Electron microscopic analysis of SCCs

Five × 10^6 mononuclear cells were seeded into each well of a two-well plastic chamber slide and cultured as described above. For assessment of the effect of PMA on SCCs, macrophages were rinsed three times with RPMI-1640 medium and incubated at 37°C with 200 μg/ml AgLDL for 5 h. Then, macrophages were rinsed and incubated 24 h without or with 0.1 μg/ml PMA. Following incubations, macrophages were rinsed three times with DPBS plus Ca^{2+} and Mg^{2+}, fixed at room temperature in glutaraldehyde, and labeled with ruthenium red as described previously (17). The number of macrophages with SCCs was determined by examining 120 macrophages in en face sections for each condition.

For assessment of lysosome-to-SCC transfer, macrophages were rinsed and incubated 24 h at 37°C with 250 μl of 20 nm BSA-coated gold particles (10^{12} particles/ml) to label macrophage lysosomes (18, 19). To prepare the gold particles, 1 ml of BSA-coated gold supplied in water by the manufacturer was dialyzed 16 h at 4°C against 1 l of RPMI-1640 medium. After incubation, macrophages were rinsed three times with RPMI-1640 medium to remove gold not taken up by the macrophages. Then, macrophages were incubated 5 h with 200 μg/ml AgLDL to induce AgLDL-containing SCCs. Macrophages were rinsed again, and incubated 24 h in RPMI-1640 medium plus 0.35% BSA without or with 0.1 μg/ml PMA. Following this incubation, the macrophage cultures were processed for electron microscopy as described above.

In addition, immunolabeling of the lysosomal marker, cathepsin D, was carried out as described previously (17) to determine whether PMA stimulated transfer of this soluble lysosomal enzyme to SCCs. In this case, macrophages were incubated with AgLDL as described above but without the preincubation of macrophages with gold particles.

Statistical analysis

All data are presented as means ± SE of the mean. The means were determined from three culture wells for each data point. Statistical comparisons of means were made using Student's *t* statistic (unpaired). A *P* value ≤0.05 was considered significant.

RESULTS

PMA inhibited plasminogen-mediated release of AgLDL from macrophages

We recently showed that macrophage conversion of serum plasminogen to the active serine-protease, plasmin, causes release of AgLDL from macrophage SCCs (10). In addition, the released AgLDL is substantially disaggregated by the macrophage-generated plasmin. Here we examined what would happen if macrophages with SCCs containing AgLDL were exposed to serum plasminogen and at the same time were activated with PMA. As shown previously, plasminogen, either as a purified protein or as a natural component of LPDS, caused release of AgLDL cholesterol from macrophage SCCs (10). However, PMA treatment of macrophages decreased LPDS- and plasminogen-induced release of AgLDL cholesterol from macrophage SCCs by 57% and 60%, respectively (**Fig. 1A**). PMA also reduced by 63% LPDS-induced release of TCA-insoluble ^{125}I-AgLDL (i.e., undegraded ^{125}I-AgLDL) (Fig. 1B).

The mechanism of the PMA decrease in plasminogen-induced release of AgLDL from macrophage SCCs was investigated. It is possible that PMA-activated macrophages generated less plasmin than did unstimulated macrophages. This was the case as PMA decreased by 61% the amount of plasmin associated with macrophage plasma membranes (566 ± 68 ng plasmin/mg cell protein without PMA and 222 ± 3 ng plasmin/mg cell protein with PMA). However, PMA inhibition of plasminogen-induced AgLDL release was not only due to a decrease in plasmin activity. This was shown by the observation that PMA also inhibited trypsin-induced release of AgLDL from macrophages. Immediately following a 1-h PMA treatment, trypsin-induced AgLDL cholesterol release from macrophages was reduced by 32% compared with AgLDL cholesterol release induced by trypsin without PMA treatment (**Table 1**). However, 24 h following PMA treatment, trypsin-induced release of AgLDL cholesterol was completely inhibited. Thus, after sufficient time, even an exogenously supplied active protease could not cause release of AgLDL from SCCs.

This finding suggested that protease no longer was accessible to the AgLDL within macrophages. This could have occurred by two mechanisms. First, PMA could have caused macrophage SCCs to close and lose their connection to the extracellular space. However, this was not the case. Electron microscopic analysis of SCCs in macro-

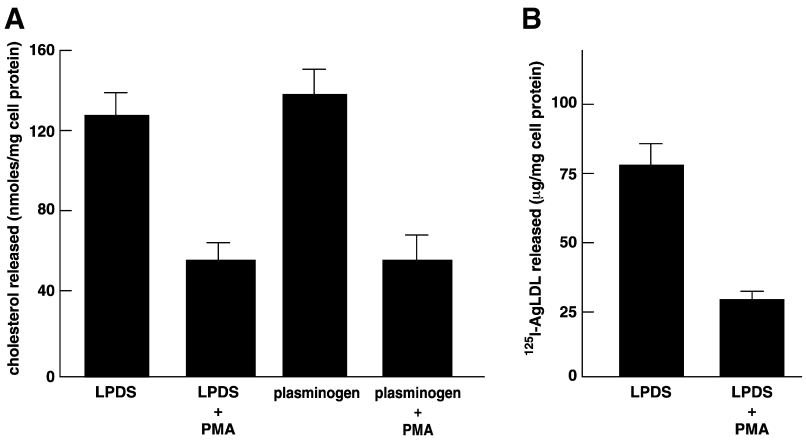

Fig. 1. PMA inhibited plasminogen-induced release of ^{125}I-aggregated LDL (AgLDL) and cholesterol from macrophages. Macrophage cultures were incubated with 200 μg/ml AgLDL (A) or 50 μg/ml ^{125}I-AgLDL (B) for 5 h to allow AgLDL to accumulate within surface-connected compartments (SCCs). Then, macrophages were rinsed and exposed for 24 h to either 1 U/ml purified plasminogen or 10% lipoprotein-deficient serum (LPDS) as a source of plasminogen in the absence and presence of 0.1 μg/ml PMA. Next, the macrophage content of cholesterol (A) and the medium content of TCA-insoluble ^{125}I-AgLDL (B) were analyzed to determine the amounts of released cholesterol and ^{125}I-AgLDL, respectively.

TABLE 1. Effect of PMA on trypsin release of AgLDL cholesterol from macrophages

Condition	Total Cholesterol Content
	nmoles/mg cell protein
No addition 0 h	99 ± 3
AgLDL 5 h	315 ± 17
AgLDL 5 h/RPMI 1 h/trypsin 30 m	134 ± 6
AgLDL 5 h/PMA 1 h/trypsin 30 m	192 ± 9
AgLDL 5 h/RPMI 1 h/RPMI 24 h/trypsin 30 m	190 ± 18
AgLDL 5 h/PMA 1 h/RPMI 24 h/trypsin 30 m	343 ± 36

Macrophage cultures were incubated with 200 μg/ml AgLDL for 5 h, and then incubated 1 h without or with 0.1 μg/ml PMA to activate macrophages. Following either immediately or after a 24-h incubation with serum-free media, macrophage cultures were exposed to 50 μg/ml trypsin for 30 min. Then, cholesterol contents of macrophage cultures were analyzed to determine the amount of cholesterol released from macrophages by each treatment. AgLDL, aggregated LDL.

phages following incubation with PMA showed that PMA treatment did not significantly change the number of macrophages with SCCs (62 ± 5% of macrophages showed SCCs with PMA treatment and 52 ± 6% of macrophages showed SCCs without PMA treatment). Second, SCCs could have remained open but the AgLDL they contained could have been transferred from the open SCCs into the macrophage cytoplasm as discussed below.

PMA stimulated degradation of AgLDL

The fact that PMA decreased release of AgLDL by exogenously supplied protease even though SCCs remained open suggested that AgLDL was transferred out of SCCs into the macrophage. This possibility was examined by determining whether PMA stimulated degradation of AgLDL, and if so, where did the degradation occur. Macrophages were incubated with 50 μg/ml ^{125}I-AgLDL for 5 h to accumulate the ^{125}I-AgLDL in macrophage SCCs. Then, macrophages were rinsed and incubated without and with PMA for 24 h. PMA treatment decreased cell-associated ^{125}I-AgLDL by 65% and increased degraded ^{125}I-AgLDL by 2.5-fold (**Fig. 2**). PMA stimulation of ^{125}I-AgLDL degradation was mediated by protein kinase C because the indolocarbazole protein kinase C inhibitor, Gö6976, strongly inhibited spontaneous and PMA-stimulated ^{125}I-AgLDL degradation (**Fig. 3**). PMA-stimulated ^{125}I-AgLDL degradation was also inhibited by wortmannin and chloroquine, an indication that phosphatidylinositide 3-kinase and lysosomes, respectively, functioned in ^{125}I-AgLDL degradation (**Fig. 4**). The fact that chloroquine decreased ^{125}I-AgLDL degradation below the level of spontaneous degradation (i.e., degradation without added PMA) is consistent with our previous finding that this degradation, like PMA-stimulated degradation, is also inhibited by chloroquine (9).

PMA-stimulated degradation of cell-associated ^{125}I-AgLDL contributed to the decreased plasminogen-induced release of ^{125}I-AgLDL from PMA-activated macrophages. Exposure of macrophages to LPDS or LPDS with PMA caused similar decreases in cell-associated ^{125}I-AgLDL (70.5 ±1.8 μg/mg cell protein decreased to 11.4 ± 0.9

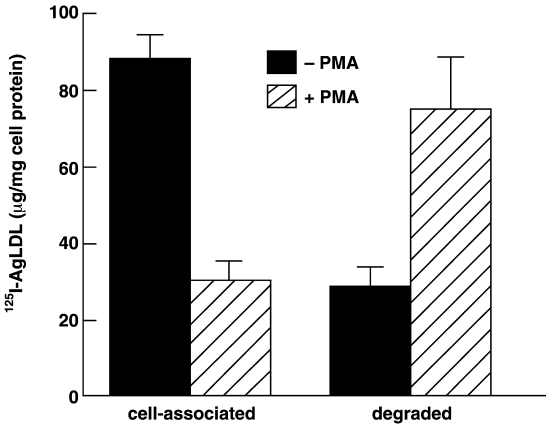

Fig. 2. PMA-stimulated degradation of ^{125}I-AgLDL accumulated in macrophage SCCs. Macrophages were incubated 5 h with 50 μg/ml ^{125}I-AgLDL added to RPMI-1640 culture medium in order to accumulate ^{125}I-AgLDL in macrophage SCCs. Then, macrophages were rinsed and incubated 24 h in RPMI-1640 culture medium containing 0.35% BSA without and with 0.1 μg/ml PMA. Following incubations, cell-associated ^{125}I-AgLDL and degraded ^{125}I-AgLDL (i.e., medium TCA-soluble ^{125}I) were determined.

μg/mg cell protein with LPDS and to 12.4 ± 0.6 μg/mg cell protein with LPDS containing PMA). However, LPDS released 43.0 ± 3.7 μg/mg cell protein of TCA-insoluble ^{125}I-AgLDL into the medium (i.e., undegraded ^{125}I-AgLDL), while LPDS + PMA released only 27.5 ± 2.0 μg/mg cell protein of TCA-insoluble ^{125}I-AgLDL into the medium. Simultaneously, addition of PMA to LPDS increased degradation of ^{125}I-AgLDL (i.e., TCA-soluble ^{125}I-

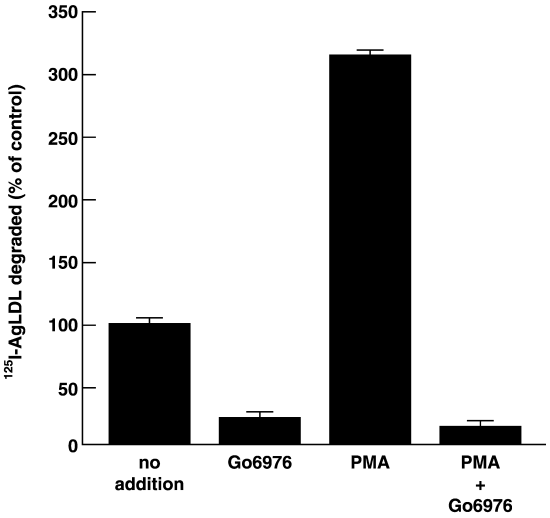

Fig. 3. Effect of a protein kinase C inhibitor on spontaneous and PMA-stimulated macrophage degradation of accumulated ^{125}I-AgLDL. Macrophages were incubated 5 h in RPMI-1640 culture medium containing 0.35% BSA and 50 μg/ml ^{125}I-AgLDL, rinsed, and then incubated 24 h in RPMI-1640 culture medium and 0.35% BSA either with no addition, 3 μM Gö6976, 0.1 μg/ml PMA, or PMA plus Gö6976. Following incubations, the amount of degraded ^{125}I-AgLDL was determined and expressed as a percentage relative to the incubation without any additions.

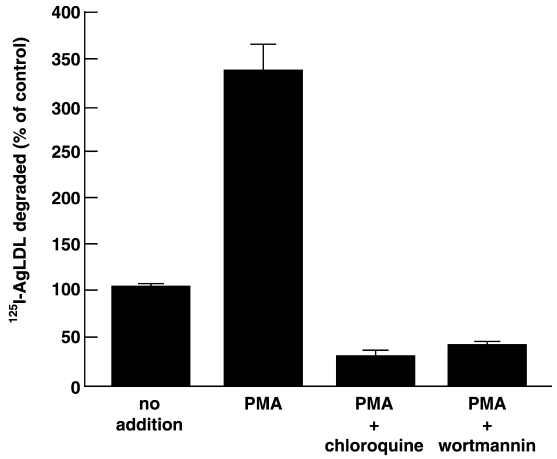

Fig. 4. Chloroquine and wortmannin inhibition of PMA-stimulated ^{125}I-AgLDL degradation. Macrophages were incubated 5 h with 50 μg/ml ^{125}I-AgLDL, rinsed, and incubated 24 h in RPMI-1640 culture medium with 0.35% BSA containing no addition, 0.1 μg/ml PMA, 0.1 μg/ml PMA plus 100 μM chloroquine, or 0.1 μg/ml PMA plus 0.1 μM wortmannin. Following incubations, the amount of degraded ^{125}I-AgLDL was determined and expressed as a percentage relative to the incubation without any additions.

AgLDL) by an amount equivalent to the amount that PMA decreased LPDS-induced release of TCA-insoluble ^{125}I-AgLDL into the medium (**Table 2**).

PMA did not stimulate transfer of lysosomal contents into SCCs

Given that PMA stimulated lysosomal-mediated degradation of AgLDL, we next sought to determine where this degradation occurred in SCCs or lysosomes. To investigate this point, we examined whether lysosomal contents were delivered to SCCs, and if so, whether PMA stimulated lysosome-to-SCC transfer. Previously, we showed the absence of cathepsin D, a soluble lysosomal enzyme, in SCCs (17). Here, we observed that PMA did not cause the appearance of this lysosomal marker in SCCs (data not shown). However, a soluble lysosomal enzyme transferred from lysosomes to SCCs might diffuse out of the open SCCs. Thus, we examined whether a much larger lysosomal marker, gold particles accumulated within lysosomes, showed evidence of transfer to SCCs. This was done by pre-loading macrophage lysosomes with gold, then inducing macrophage SCCs with AgLDL, and finally exposing macrophages to PMA. Although lysosomes were heavily labeled with gold, no gold transferred to SCCs during post-incubation of macrophages without or with PMA (**Fig. 5**).

PMA stimulation of AgLDL degradation also stimulated cholesterol esterification

Macrophages were incubated with 200 μg/ml AgLDL for 5 h, rinsed, and then incubated 24 h without and with PMA in the presence of [^{3}H]oleate to determine the synthesis of cholesteryl [^{3}H]oleate. PMA stimulated cholesteryl [^{3}H]oleate synthesis 3.2-fold with 38 ± 6 nmoles cholesteryl [^{3}H]oleate/mg cell protein synthesized without PMA and 122 ± 6 nmoles cholesteryl [^{3}H]oleate/mg cell protein synthesized with PMA.

DISCUSSION

We have shown that macrophage activation with PMA affects how macrophages metabolize AgLDL. Macrophage activation causes an increase in degradation of AgLDL contained in macrophage SCCs. Previously, we showed that AgLDL in these SCCs could be released from the macrophage in a disaggregated state when macrophages were exposed to plasminogen (10). The macrophages convert plasminogen to the active serine protease plasmin, which is sufficient to cause the release and disaggregation of AgLDL through limited proteolysis of the AgLDL. Disaggregation of AgLDL may promote removal of LDL from atherosclerotic lesions, because efflux of lipoproteins from atherosclerotic lesions is inversely proportional to their size (20). However, the lipid core of atherosclerotic lesions is filled with cholesteryl ester-rich lipid particles that resemble the disaggregated lipid particles released from macrophages (21, 22). Thus, the rate of removal of these particles from the lipid core is not sufficient to prevent them from accumulating.

PMA activation of macrophages inhibited plasminogen-stimulated release of aggregated LDL from macrophages in part by decreasing macrophage generation of plasmin. Simultaneously, PMA stimulated lysosomal degradation of AgLDL. Lysosomal degradation of LDL leads to storage of LDL cholesterol in lipid droplets (23), a process often considered to promote retention of cholesterol in atherosclerotic plaques. However, it is possible that storage of cholesterol within macrophages facilitates cholesterol removal from atherosclerotic plaques. This is because macrophages can excrete this cholesterol in discoidal particles produced by the macrophages when either macrophage-produced apolipoprotein E (apoE) or exogenously supplied amphipathic HDL apolipoproteins (e.g., apoA-I) complex with macrophage-derived phospholipid (11, 24, 25). In this regard, activation of protein kinase C stimulates cholesterol efflux from human skin fibroblasts, rat vascular smooth muscle cells, and mouse peritoneal macrophages induced by exogenously supplied apoA-I (26–28), but does not stimulate cholesterol efflux from the human monocyte THP-1 cell line (29). Not only do macrophages repackage their stored cholesterol for excretion, macrophages also can directly transport their accumulated cholesterol out of plaques when macrophage foam cells emigrate from plaques (30). Thus, it is not clear whether it is better for macrophage processing of AgLDL to result in release of disaggregated LDL into the extracellular space, or retention of AgLDL-derived cholesterol within macrophages stored in lipid droplets.

Besides variably affecting macrophage cholesterol efflux, PMA also variably affects other aspects of macrophage metabolism of lipoproteins and cholesterol, depending on the particular macrophage model being

TABLE 2. Effect of LPDS on PMA stimulation of ^{125}I-AgLDL metabolism

Condition	Cell-Associated ^{125}I-AgLDL	TCA-Soluble ^{125}I-AgLDL in Medium	TCA-Insoluble ^{125}I-AgLDL in Medium	Total ^{125}I
		μg/mg cell protein		
^{125}I-AgLDL 5 h	70.5 ± 1.8	—	—	70.5 ± 1.8
^{125}I-AgLDL 5 h/LPDS 24 h	11.4 ± 0.9	14.6 ± 0.8	43.0 ± 3.7	69.0 ± 5.1
^{125}I-AgLDL 5 h/LPDS + PMA 24 h	12.4 ± 0.6	29.5 ± 0.6	27.5 ± 2.0	69.3 ± 1.2

Macrophages were either incubated 5 h with 50 μg/ml ^{125}I-AgLDL and then harvested, or were rinsed and incubated 24 h with 10% LPDS without or with 0.1 μg/ml PMA before harvest. Following incubations, media were collected and analyzed for their content of TCA-soluble and -insoluble ^{125}I to provide measurement of ^{125}I-AgLDL that had been degraded and ^{125}I-AgLDL that had been released from macrophages undegraded. In addition, undegraded ^{125}I-AgLDL that remained cell-associated was determined.

studied. For example, PMA decreases the number of cell-surface receptors for and uptake of acetyl-LDL by mouse resident peritoneal macrophages (31), but increases scavenger receptor concentration in U937 human macrophages (32). PMA increases cholesterol esterification in the presence of exogenously supplied oleic acid in IC-21 mouse macrophages (33), but does not stimulate cholesterol esterification in THP-1 human macrophages (34). In the current study, PMA stimulated cholesterol esterification, probably because of increased availability of cholesterol substrate for the ACAT enzyme secondary to the PMA-induced increase in AgLDL degradation.

Chloroquine inhibition of PMA-stimulated AgLDL degradation suggests that degradation depended on lysosomal function. The increased degradation of AgLDL that had accumulated in macrophage SCCs could have occurred either in the SCCs following transfer of lysosomal enzymes to the SCCs, or after transfer of AgLDL from SCCs to lysosomes. We conclude that the latter occurred because no transfer of lysosomal contents (i.e., cathepsin D or gold particles) to SCCs occurred without or with PMA treatment of macrophages. Although we did not observe transfer of AgLDL to lysosomes by electron microscopy, wortmannin, a phosphoinositide 3-kinase inhibitor, decreased PMA-stimulated degradation of AgLDL. Wortmannin has been shown to inhibit both macropinocytosis and phagocytosis in macrophages, suggesting that transfer of AgLDL from SCCs to lysosomes may involve one of these two endocytic processes (35). Both processes mediate uptake of large particles through formation of vacuoles. If vacuoles form by pinching off from SCC membranes, they could accommodate the large size of AgLDL and transport the AgLDL to lysosomes. However, because vacuole formation was not observed by electron micros-

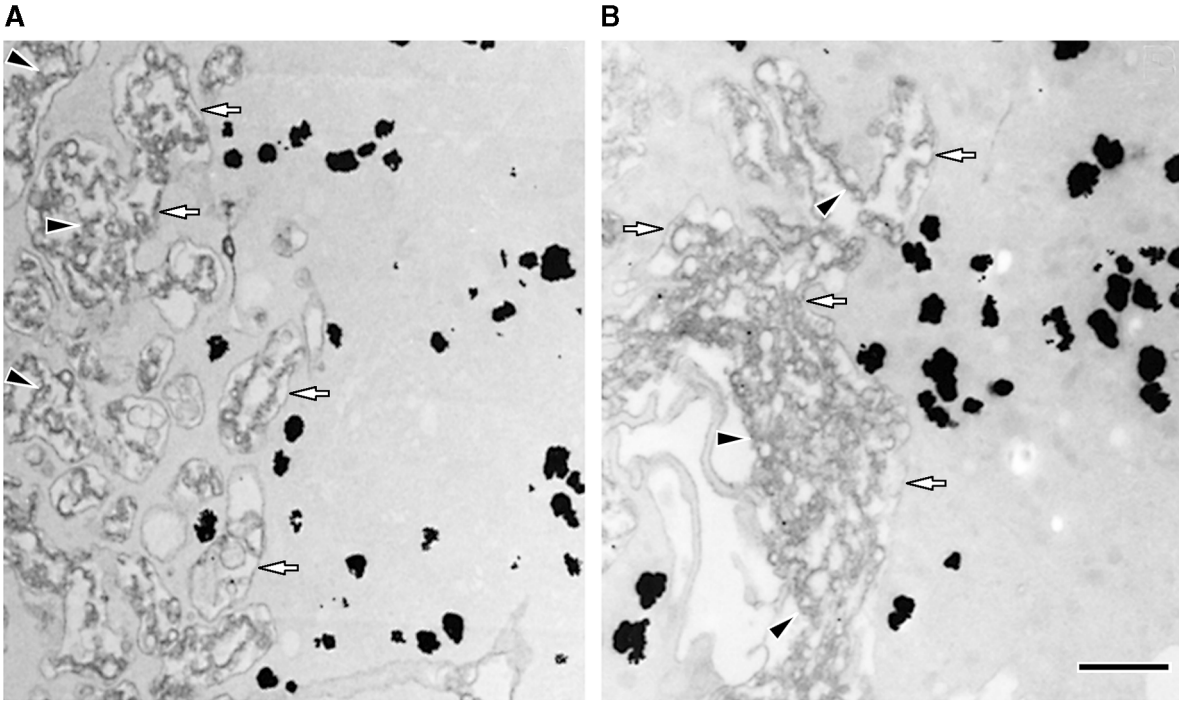

Fig. 5. Electron microscopic analysis of lysosome-to-SCC transfer. Macrophages were incubated with BSA-gold for 24 h to label lysosomes. Then, the macrophages were rinsed and incubated with 200 μg/ml AgLDL for 5 h to cause formation of SCCs containing AgLDL. After rinsing, macrophages were incubated 24 h without (A) or with (B) 0.1 μg/ml PMA. Macrophages were fixed and SCCs were labeled with ruthenium red before cultures were prepared for electron microscopic analysis. The AgLDL appears as chains of particles (arrowheads) within the SCCs (arrows). The lysosomes appear black due to their loading with gold particles. Bar in B is 1 μm and also applies to A.

copy, possibly AgLDL transfers from SCCs to lysosomes during transient connection of these two compartments. Human monocyte-macrophages incubated with AgLDL have recently been shown to have an extensive array of tubules that show connections with spherical lysosomal-like structures (36). Although we observed that PMA stimulated accumulation of similar tubules in our experiments (unpublished data), a role for these tubules in transport of AgLDL to lysosomes remains to be shown.

In conclusion, PMA activation of human monocyte-macrophages alters how they metabolize AgLDL. Without this activation, most AgLDL remains within macrophage SCCs, with only a small amount of AgLDL undergoing lysosomal degradation. In this case, the macrophages can disaggregate and release AgLDL from these compartments when the macrophages encounter plasminogen and convert this plasminogen to plasmin. On the other hand, PMA activation of macrophages increases lysosomal degradation of AgLDL. This releases cholesterol from the degraded AgLDL that is then available to be esterified and stored in macrophage lipid droplets. At the same time, lysosomal degradation of AgLDL that was previously stored in macrophage SCCs means that less AgLDL is available for disaggregation and release when macrophages are exposed to plasminogen. It remains to be determined whether it is better for AgLDL to be degraded, its cholesterol stored in macrophage lipid droplets, or to be disaggregated and its cholesterol released as partially degraded LDL particles into the extracellular space.

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