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Evidence that the scavenger receptor is not involved in the uptake of negatively charged liposomes by cells

Kyung-Dall Lee [a], Robert E. Pitas [b] and Demetrios Papahadjopoulos [a]

[a] *Cancer Research Institute and Department of Pharmacology, University of California, San Francisco, CA (USA)* and [b] *The Gladstone Institute of Cardiovascular Disease, Cardiovascular Research Institute and Department of Pathology, University of California, San Francisco, CA (USA)*

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Scavenger receptors have a broad ligand specificity, ranging from modified low-density lipoproteins to a variety of high-molecular-weight poly-anions. A recent report by Nishikawa et al. (J. Biol. Chem. (1990) 265, 5226–5231) suggested that this receptor is also involved in the binding and endocytosis of liposomes containing negatively charged phospholipids. The mechanism by which liposomes are taken up by cells is of interest because liposomes are promising versatile carriers for macromolecules and drugs both in vitro and in vivo. In this report, we re-examine the role of the scavenger receptor in the uptake of liposomes using both Chinese hamster ovary cells transfected with the type I or type II bovine scavenger receptor, and smooth muscle cells induced to increase scavenger receptor expression by phorbol ester treatment. Expression of both types of scavenger receptors by Chinese hamster ovary cells induced an increase in the uptake of chemically modified low-density lipoproteins, but not the uptake of negatively charged liposomes. In smooth muscle cells treated with phorbol ester, scavenger receptor expression was upregulated and the uptake of chemically modified low-density lipoproteins was enhanced dramatically, but there was no effect on the uptake of negatively charged liposomes. We conclude that the existing evidence does not support the suggestion that the scavenger receptor is involved in the uptake of anionic liposomes by cells.

Introduction

It is well established that macrophages in vitro avidly internalize liposomes and accumulate them in low pH compartments [1]. Similarly, macrophages in the reticuloendothelial system take up more than 90% of intravenously administered negatively charged liposomes within 1 h of injection [2] This has been utilized to naturally target liposomes to the mononuclear phagocytic system, but has been an obstacle in targeting liposomes to other cells. Recent advances in the mouse model, however, show that intravenously injected liposomes of certain lipid compositions have a drastically reduced uptake by the reticuloendothelial system [3,4] and a consequent increase in uptake by tumors [4,5]. Understanding the mechanism by which cells bind and internalize liposomes will greatly facilitate the design of liposome carriers, which can deliver drugs preferentially to specific tissues such as tumors rather than to macrophages.

Although it is known that some cells take up liposomes through coated pit-mediated endocytosis [6], the exact mechanism by which liposomes bind to cells is not clear. We and others have shown that the uptake of liposomes by macrophages in vitro can be dramatically enhanced by including certain anionic phospholipids, such as phosphatidylserine (PS), phosphatidylglycerol, or phosphatidic acid [7,8]. The enhanced uptake of negatively charged liposomes compared with neutral liposomes suggests the existence of cell surface binding sites (i.e., receptors) that mediate the specific recognition of the negatively charged phospholipids and the avid internalization of the liposomes containing these lipids. A cell surface receptor that recognizes specific phospholipids, especially the negatively charged phospholipids that are not normally present on the

Correspondence to: K.-D. Lee, Cancer Research Institute, Box 0128, University of California, San Francisco, CA 94143, USA.

Abbreviations: LDL, low-density lipoprotein; Ac, acetyl; AcAc, acetoacetylated; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; HPTS, 1-hydroxypyrene 3,6,8-trisulfonate; PS, phosphatidylserine; PC, phosphatidylcholine; FACS, fluorescence-activated cell sorter; CHO, Chinese hamster ovary cells; CHO-SRI, Chinese hamster ovary cells transfected with type I bovine scavenger receptor; CHO-SRII, Chinese hamster ovary cells transfected with type II bovine scavenger receptor; SMC, smooth muscle cells; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate.

outer leaflet of cell membranes, would have important implications in cell biology. Increased expression of PS on senescent erythrocytes [9,10] and on tumor cells [11] has been proposed to be the signal for removal by macrophages, and therefore these cells are potential candidates for clearance via the scavenger receptor. Additionally, such a receptor would be of great interest to investigators using liposomes as model membranes [12] and as vehicles to deliver drugs or macromolecules to cells both in vitro and in vivo [13].

The nature of the receptor for negatively charged liposomes is not yet known. However, it has been recently suggested by Nishikawa et al. that the uptake of negatively charged liposomes is mediated via the scavenger receptor [7]. The scavenger receptor, or acetyl low-density lipoprotein (LDL) receptor, was originally described by Goldstein et al. on macrophages [14], and has subsequently been shown to be expressed by certain smooth muscle cells (SMC) and fibroblasts [15]. This receptor binds LDL that has been modified by acetylation (Ac), acetoacetylation (AcAc), or oxidation [14,16,17], all of which lead to an enhancement of the negative charge of the LDL. The uptake of oxidized forms of LDL by this receptor in vivo has been postulated to play an important role in atherogenesis [16,18]. The receptor has broad ligand specificity, binding modified LDL, maleylated albumin, and certain high-molecular-weight macromolecules such as polyinosinic acid, dextran sulfate, and fucoidan [14,16]. More recently, the scavenger receptor has been also reported to be involved in the uptake of lipopolysaccharide (LPS) [19]. Two forms of the bovine, human, and mouse scavenger receptor cDNA have now been cloned and the amino acid sequences deduced [20–23]. In all species the type I and the type II scavenger receptor differ only in their carboxy terminus.

The conclusion of Nishikawa et al. [7] that liposomes containing anionic phospholipids are taken up by the scavenger receptor was based on cross-competition between liposomes and Ac or oxidized LDL. However, the extreme conditions used in their competition experiments raise some doubts about whether the scavenger receptor indeed binds these liposomes. First, a high concentration of liposomes (a millimolar range of phospholipid concentration) was used to compete with modified LDL. The non-reciprocity observed between modified LDL and liposome particles was also much more pronounced than the non-reciprocal competition previously observed between oxidized LDL and Ac LDL [24]; modified LDL particles competed poorly with negatively charged liposomes, whereas liposomes competed with modified LDL significantly better. In addition, we have previously demonstrated that the uptake of liposomes by certain cells cannot be inhibited by a high concentration of ligands for the scavenger receptor, suggesting that scavenger receptor-mediated endocytosis is not the primary uptake mechanism in those cells [8]. For example, an African green monkey kidney cell line (CV1) internalizes PS-containing liposomes at a much higher rate than phosphatidylcholine (PC) liposomes. This interaction is not affected by poly(inosinic acid) or dextran sulfate, known competitors for binding of chemically modified LDL to the scavenger receptor. On the other hand, the uptake of PS-containing liposomes by a murine macrophage-like cell line (J774) could be competed by poly(inosinic acid) but not by poly(cytidylic acid), just as would be expected for a scavenger receptor ligand. In addition, it is established that Ca^{2+} is required for liposome uptake by macrophages [25] but is not required for the binding of Ac LDL to the scavenger receptor [26]. For these reasons, we have further assessed the role of the scavenger receptor in liposome uptake more directly both in Chinese hamster ovary cells (CHO) transfected to express the bovine scavenger receptor and in SMC in which scavenger receptor expression is up-regulated by phorbol esters.

Materials and Methods

The type I and type II bovine scavenger receptor expression vectors pXSR7 and pXSR3, respectively [20,21], were co-transfected with a neomycin resistance gene into CHO cells using a calcium phosphate precipitation method. Colonies resistant to the neomycin analogue G418 (0.4 mg/ml) were selected for high expression of the receptor by fluorescence-activated cell sorting (FACS) based on their uptake of AcAc LDL labeled with the fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) [27]. The cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's F12 medium (medium A) containing G418 and 10% fetal bovine serum. Control and transfected cells were plated at sub-confluent level 1 day before the experiment. Rabbit SMC were maintained in RPMI 1640 (medium B) containing 5% fetal bovine serum, and acetyl LDL receptor expression was up-regulated by pre-incubation for 2 days with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) as previously described [15]. Small unilamellar liposomes (approx. 85–100 nm in diameter) composed of PS/PC/cholesterol (1 : 1 : 1, mole ratio) were made by extrusion through polycarbonate membranes of 0.05 μm final pore size as described [28]. The liposomes were labeled either with DiI (0.2 mole% of phospholipids) by drying the DiI mixed with lipids in chloroform before hydration, or with the aqueous content fluorescent marker pyranine (1-hydroxypyrene 3,6,8-trisulfonate; HPTS) as described [1,8]. Unless otherwise noted, AcAc LDL (5 μg/ml, about $5 \cdot 10^{12}$ particles/ml) or liposomes (50 nmol of phospholipids/ml, about $2 \cdot 10^{11}$ particles/ml) were incubated with cells for the

TABLE I

Uptake of acetoacetylated low-density lipoproteins and liposomes by Chinese hamster ovary, CV1 and J774 cells

	Relative uptake per cell [a]		
	CHO	CV1	J774
AcAc LDL [b]	1 (±0.06)	19.8 (±1.13)	69 (±2.36)
Liposomes [c]	1 (±0.04)	6.5 (±0.23)	2.3 (±0.05)

[a] The experiments were done in triplicate and the standard deviations are shown in parentheses. The uptake of AcAc LDL and liposomes by CHO cells described in (b) and (c) below was normalized to the value of 1 and the uptake by CV1 and J774 cells was expressed as a multiple of that value. The concentrations of liposomes and Ac LDL were converted into molar concentrations of particles as follows: 1 μmole of lipid corresponds to about $4 \cdot 10^{12}$ liposome particles (assuming an average diameter of liposomes of about 100 nm), and 1 μg of Ac LDL protein (M_r = 600000) corresponds to about $1 \cdot 10^{12}$ particles.

[b] Acetoacetylated low-density-lipoproteins labeled with DiI (5 μg/ml) were incubated at 37°C with CHO, CV1 and J774 cells in medium A containing 10% fetal bovine serum. The CHO cells ($1 \cdot 10^6$) took up 34 ng of AcAc LDL (about $3.4 \cdot 10^{10}$ particles) during a 24-h incubation.

[c] For the uptake of DiI-labeled liposomes, 100 nmoles of total phospholipids (PS/PC/cholesterol, 1:1:1) per milliliter were incubated with the cells under the same conditions as above. The CHO cells ($1 \cdot 10^6$) took up 2 nmoles of liposomes (about $8 \cdot 10^9$ liposome particles) during a 24-h incubation.

times indicated in the figure legends. Cells were washed twice with medium and three times with phosphate-buffered saline, and solubilized with 0.2% Triton X-100 for the analysis by fluorometry. The DiI fluorescence associated with $1 \cdot 10^6$ cells was measured in a Spex fluorometer (λ_{ex} = 550 nm; λ_{em} = 575 nm). For analysis by FACS, the procedures were the same but the washed cells were suspended by trypsinization.

Results and Discussion

The wild-type native CHO cells have a relatively low level of liposome uptake by comparison with other cell lines, such as CV1 and J774 cells. The expression of scavenger receptors in wild-type CHO cells is also low. The relative uptake of AcAc LDL and PS-containing liposomes by CHO, CV1, and J774 cell lines is shown in Table I. To measure the uptake of AcAc LDL and liposomes, both were labeled with the fluorescent dye (DiI) and the association of the DiI fluorescence with cells was monitored. DiI-labeled AcAc LDL has been previously used to identify cells expressing the scavenger receptor both in vitro [27] and in vivo [29]. Because DiI is not degraded in the lysosomes, it accumulates in the cells, allowing an assessment of the total amount of uptake by cells [30]. As shown in Table I, CV1 cells take up approximately three times more liposomes than J774 cells, whereas CV1 cells take up only one-third as much AcAc LDL as J774 cells. In addition, the J774 cells, because of the high expression of the scavenger receptor, take up about 70 times as much AcAc LDL as CHO cells, while they are only 2.3-fold more effective in taking up anionic liposomes than CHO cells. This comparison shows that the relative activity of the scavenger receptor among these cells does not correlate with the extent of liposome uptake.

To address the question of whether an increase in scavenger receptor activity correlates with the level of uptake of anionic liposomes in a given type of cell, we used two cell systems, CHO and SMC, in which we can regulate and monitor the level of scavenger receptor activity. A straightforward examination was facilitated by the recent cloning of the bovine scavenger receptor, type I and II forms [20,21], and the successful expression of these receptors in CHO cells [24,31]. The type I receptor is identical to type II except that it has an extended cysteine-rich extracellular carboxy-terminal domain [20,21]. Both forms of the receptor bind chemically modified and oxidized LDL [24,31]. We transfected CHO cells with the expression vector pXSR7 and pXSR3, which contain the cDNA for the type I and type II bovine scavenger receptor, respectively, and investigated whether the expression of either type of scavenger receptor induces an enhanced uptake of liposomes in parallel to the enhanced uptake of AcAc LDL.

Fig. 1 shows the uptake of AcAc LDL and liposomes by CHO cells, CHO-SRI (CHO cells transfected with type I bovine scavenger receptor), and CHO-SRII (CHO cells transfected with type II bovine scavenger receptor). The CHO-SRI cells showed a 7-fold increase in the uptake of AcAc LDL relative to the wild-type

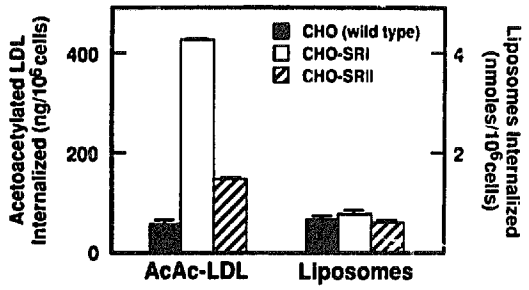

Fig. 1. Scavenger receptor expression by Chinese hamster ovary cells results in enhanced uptake of chemically modified LDL, but not of anionic liposomes. The uptake of AcAc LDL and liposomes (PS/PC/cholesterol) by CHO, CHO-SRI and CHO-SRII cells is reported. The uptake of DiI-labeled AcAc LDL (ng/$1 \cdot 10^6$ cells) and the uptake of DiI-labeled liposomes (nmol/$1 \cdot 10^6$ cells) by wild-type CHO cells (solid bars), by CHO-SRI cells (open bars) and by CHO-SRII cells (hatched bars) are shown. AcAc LDL (5 μg/ml) and liposomes (50 nmoles of phospholipids/ml) were incubated with cells in medium A for 8 h. The DiI fluorescence intensity associated with $1 \cdot 10^6$ cells was measured by fluorometry and it was divided by the fluorescence intensity per nanogram of DiI-labeled AcAc LDL or nanomole of DiI-labeled liposomes to express the uptake in nanograms or nanomoles per $1 \cdot 10^6$ cells.

(non-transfected control) CHO cells during an 8-h incubation at 37°C. A lower level of scavenger receptor expression was observed in CHO-SRII, in which the uptake of DiI-labeled AcAc LDL was increased by 2.4-fold over the control CHO cells. By contrast, the extent of liposome uptake by the CHO-SRI and CHO-SRII cell lines was the same as that by wild-type CHO cells. The results were similar in the presence or absence of 10% serum in the incubation medium (data not shown). If the extent of uptake is expressed as the average number of particles taken up per cell, $6.3 \cdot 10^4$ AcAc LDL particles were taken up by each CHO cell, $4.4 \cdot 10^5$ particles by each CHO-SRI cell, and $1.5 \cdot 10^5$ particles by each CHO-SRII cell, respectively. However, only about $3 \cdot 10^3$ liposome particles were taken up by each control CHO cell or each CHO cell expressing the scavenger receptor. These data suggest that the bovine scavenger receptor does not bind anionic liposomes.

A similar conclusion was suggested when the control and the transfected cells were incubated with DiI-labeled AcAc LDL or liposomes and analyzed by flow cytometry. A typical FACS analysis (Fig. 2) showed that CHO-SRI cells contained two populations of cells; the major population internalized about 10 times more DiI-labeled AcAc LDL than the other (apparently non-expressing cells), which had the same uptake of DiI-labeled AcAc LDL as the wild-type CHO cells. In contrast, both the wild-type CHO and the transfected CHO-SRI cells took up DiI-labeled liposomes to the same extent, with no indication of two populations that had different levels of scavenger receptor expression. The numerical results after two different incubation times are shown in Table II. The uptake of liposomes was also monitored by FACS using another fluorescent dye (HPTS) which was encapsulated within the internal aqueous space of the liposomes. This dye has been used previously for monitoring liposome uptake following the accumulation of the aqueous contents via endocytosis [1,32]. As with the DiI-labeled liposomes, the uptake of liposomes labeled with HPTS was not enhanced in the transfected cells. The data for these and other FACS analyses are summarized in Table II. Thus, the uptake of liposomes, documented by monitoring markers for both the lipid and aqueous contents, was shown not to depend on the level of scavenger receptor expression in CHO cells.

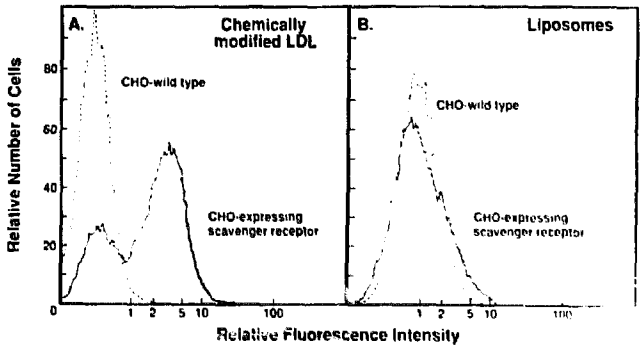

Fig. 2. Fluorescence-activated cell sorter analysis showing that the uptake of chemically modified LDL is enhanced in CHO-SRI cells expressing the type I bovine scavenger receptor, whereas the uptake of liposomes is not. This representative FACS scan shows the uptake of DiI-labeled AcAc LDL (panel A) and DiI-labeled liposomes (PS/PC/cholesterol) (panel B) by wild-type CHO cells (dotted lines) and by CHO-SRI cells expressing the type I bovine scavenger receptor (solid lines). The incubation condition was the same as in Fig. 1. The mean background relative fluorescence intensity for the control cells without incubation with either DiI-labeled AcAc LDL or liposomes was 0.32.

The recent observation [15] that rabbit SMC express the scavenger receptor, and that receptor expression in these cells is readily regulated by phorbol esters, provided another system in which to evaluate the role of the scavenger receptor in the uptake of liposomes. The uptake of DiI-labeled AcAc LDL was enhanced, as expected, by pre-incubation of the SMC with PMA, whereas PMA treatment of the SMC had little, if any,

TABLE II

Uptake of acetoacetylated low-density lipoproteins and liposomes by control Chinese hamster ovary cells and by cells transfected to express the type I bovine scavenger receptor

After the indicated period of incubation with DiI-labeled AcAc LDL (5 μg/ml) or DiI-labeled liposomes (50 nmol/ml), cells were washed and suspended as single cells for FACS analysis. The relative fluorescence intensity associated with the cells is shown and the ratio of the relative fluorescence intensity (CHO-SRI/CHO) is reported in each case. The 6-h and 24-h incubations are two independent experiments performed on different days. The 24-h experiment was repeated with essentially similar results (data not shown). The average background relative fluorescence intensity for control cells (cells without incubation with DiI-labeled AcAc LDL or liposomes) was 0.32 (6 h) and 0.24 (24 h). The values shown in parentheses for liposomes represent the amount of uptake monitored using liposomes labeled with HPTS instead of DiI.

		Uptake (relative fluorescence intensity)		
		control CHO	transfected CHO-SRI	ratio (CHO-SRI/CHO)
AcAc LDL	6-h incubation	0.29	1.56	5.4
	24-h incubation	0.95	14.8	15.6
Liposomes	6-h incubation	0.94 (0.86)	0.97 (0.73)	1.03 (0.85)
	24-h incubation	6.6	3.3	0.5

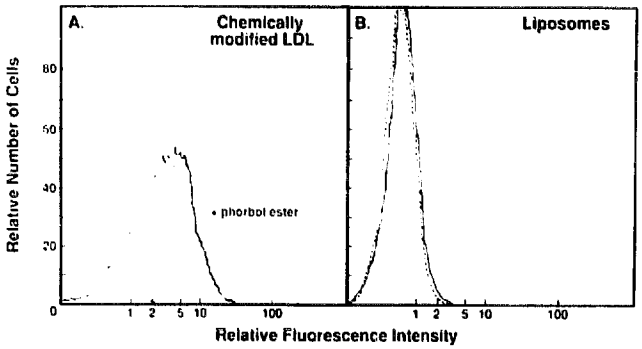

Fig. 3. Fluorescence-activated cell sorter analysis showing that phorbol ester treatment of smooth muscle cells lead to enhanced uptake of chemically modified LDL, but not the uptake of liposomes. This representative FACS scan shows the uptake of DiI-labeled AcAc LDL (panel A) and DiI-labeled liposomes (PS/PC/cholesterol) (panel B) by control rabbit SMC (dotted lines) and by rabbit SMC incubated with phorbol ester to up-regulate the scavenger receptor (solid lines). The incubation condition was the same as in Fig. 1. The background relative fluorescence intensity for the cells without incubation with either DiI-labeled AcAc LDL or liposomes was 0.38.

effect on the uptake of liposomes (Fig. 3 and Table III).

The studies presented above demonstrate that the type I and type II scavenger receptors expressed in CHO cells do not induce receptor-mediated endocytosis of liposomes, while they do cause an increase in the uptake of a known ligand, AcAc LDL. This result is most likely due to the lack of binding of liposomes to this receptor. A recent report by Hampton et al. [19] suggested the involvement of scavenger receptors in LPS uptake by showing a 2–4-fold increase of LPS uptake in CHO cells transfected with the bovine scavenger receptors relative to wild-type CHO cells, which is essentially the same cell system as the one discussed in this report. Therefore, an increased expression of scavenger receptors in CHO cells leads to an increase in the uptake of chemically modified LDL [24,31] and LPS [19], but not the uptake of liposomes as we have demonstrated here. However, we cannot yet eliminate the possibility that even though the scavenger receptors bind liposomes, subsequent endocytosis of liposomes might require additional machinery in CHO cells that is not needed for the uptake of AcAc LDL or LPS. It is also conceivable that the density of scavenger receptors on the cell surface required for successful endocytosis of liposomes may be higher than that for chemically modified LDL, as could be the case if the successful uptake of a liposome requires clustering of more than one receptor. Further studies are needed in order to clarify these possibilities. However, we have also demonstrated a lack of correlation between scavenger receptor activity and liposome uptake in rabbit SMC treated with phorbol ester, which up-regulates receptor expression and leads to the enhanced binding and degradation of Ac and oxidized LDL [15,31]. Based on the results in these two systems, we conclude that the existing evidence does not support the suggestion that the scavenger receptor is responsible for liposome uptake by cells. Rather, it suggests that the presence of the scavenger receptors does not affect liposome uptake, at least by the cells under study.

Our work does not rule out the possibility that other scavenger receptors exist that do bind liposomes, just as there may in fact be more than one receptor that binds modified forms of LDL. In mouse peritoneal macrophages, for example, cross-competition studies with Ac LDL and oxidized LDL have demonstrated that neither ligand can completely compete for the binding or degradation of the other [33,34]. These studies have led to the hypothesis that there may be at least three different receptors for modified LDL: one receptor that binds Ac LDL, one that binds oxidized LDL, and one that recognizes both ligands. However, this does not appear to be true in all species. In rabbit SMC, oxidized LDL and chemically modified LDL bind to the same receptor and there is no evidence for an independent receptor for oxidized LDL [31]. This also appears to be true for human endothelial cells [35]. In addition, it has been demonstrated that both oxidized LDL and chemically modified LDL bind to the bovine scavenger receptor [24,31]. Although it is still not clear how anionic liposomes and modified LDL (or large poly-anions) cross-compete for their uptake by cells [7,8], we recommend caution in the interpretation of competition studies involving large macro-molecular particles in such a complicated process as binding at the cell surface and internalization. Additional caution would be also needed to extrapolate from in vitro study to the uptake of liposomes by

TABLE III

The effect of phorbol ester treatment of smooth muscle cells on the uptake of acetoacetylated low-density lipoproteins and liposomes

The conditions and the presentations are the same as in Table II. The 6-h and 24-h incubations are two independent experiments performed on different days. The average background relative fluorescence intensity for control cells (cells without incubation with DiI-labeled AcAc LDL or liposomes) was 0.38 (6 h) and 1.3 (24 h). The values shown in parentheses for liposomes represent the amount of uptake monitored using liposomes labeled with HPTS instead of DiI.

	Uptake (relative fluorescence intensity)		
	(−)PMA	(+)PMA	ratio ((+)PMA/(−)PMA)
AcAc LDL			
6-h incubation	0.43	2.66	6.2
24-h incubation	3.47	14.8	4.2
Liposomes			
6-h incubation	0.56 (0.41)	0.58 (0.26)	1.04 (0.63)
24-h incubation	6.14	2.39	0.4

cells in vivo where other complicated factors such as plasma proteins may play significant roles.

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