Modulation of macrophage scavenger receptor transport by protein phosphorylation[1]

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Abstract The identification of three highly conserved phosphorylation sites in the cytoplasmic domain of each of the monomeric subunits of the macrophage scavenger receptor suggests that protein phosphorylation may regulate this receptor pathway. To investigate this, mouse peritoneal macrophages were pretreated with either the protein phosphatase inhibitor okadaic acid or the protein kinase inhibitor staurosporine to modulate cellular protein phosphorylation and their effects on the metabolism of acetyl-LDL were measured. Both okadaic acid and staurosporine inhibited the degradation of acetyl-low density lipoprotein (LDL) without affecting cellular lactic dehydrogenase (LDH) levels. The inhibition by okadaic acid was due to a 70% decrease in acetyl-LDL binding whereas post-receptor processing was minimally affected. Calyculin A, another serine/threonine phosphatase inhibitor, also reduced acetyl-LDL binding, whereas lithium chloride, an inositol phosphatase inhibitor, did not. Okadaic acid did not decrease steady state receptor mRNA levels nor decrease the number of total cellular receptors, consistent with a posttranslational mechanism of action. Interestingly, protease sensitivity studies showed that the receptors were still located on the cell surface. These studies suggest that okadaic acid inhibits acetyl-LDL binding by causing the redistribution of surface receptors into a sequestered compartment or inactivating the receptors. In contrast, staurosporine produced a paradoxical increase in receptor expression (30%) but slowed post-receptor processing (2.3-fold decrease). The latter was due to an inhibition of ligand internalization (2.9-fold decrease) via a protein kinase C-independent mechanism. Macrophage pinocytosis was also slowed by staurosporine (38% decrease); however, this does not appear to account for the inhibition of scavenger receptor internalization. Direct receptor phosphorylation was also investigated and it was established that the receptor can be phosphorylated; however, changes in receptor function did not correlate with changes in the degree of receptor phosphorylation. Together these studies demonstrate that changes in cellular protein phosphorylation affect the expression, surface transport, and internalization of the macrophage scavenger receptor and suggest that the regulated phosphorylation/dephosphorylation of cellular proteins may be an important biochemical mechanism that controls normal processing of ligands by this receptor pathway.—**Fong, L. G.** Modulation of macrophage scavenger receptor transport by protein phosphorylation. *J. Lipid Res.* 1996. **37:** 574–587.

Supplementary key words atherosclerosis • foam cell • receptor expression • endocytosis • internalization • okadaic acid • staurosporine

The scavenger receptor is a glycoprotein expressed on the cell surface of macrophages that mediates ligand internalization (1). The receptor exhibits features common to other receptors, such as saturable and high affinity binding, but it is unusual in that a diverse group of unrelated substances are recognized by the receptor. Based on competitive binding studies, modified forms of proteins, bacterial lipopolysaccharides, polysaccharides, asbestos, and ribonucleotides have all been shown to interact with this receptor (1, 2). This broad ligand specificity has been validated by studies of transfected COS cells or Chinese hamster ovary cells that express the cloned receptor (3–5). Unfortunately, this feature has made the definition of the receptor's physiological function difficult to determine definitively; however, recent studies provide evidence for its participation in host defense (6, 7) and macrophage adhesion (8, 9). A pathogenic role in the development of atherosclerosis has been suspected since the late seventies (1, 10). This was based on the demonstration that the metabolism of modified forms of low density lipoproteins (LDL) via the scavenger receptor led to the excessive accumulation of cholesteryl ester characteristic of foam cells (10) suggesting that this receptor pathway might contribute to fatty streak lesion formation. The documentation that the scavenger receptor is expressed by arterial wall foam cells (11, 12) and that modified forms of LDL recognized by the scavenger receptor are located in regions of

Abbreviations: LDL, low density lipoproteins; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; BHT, butylated hydroxytoluene; TCA, trichloroacetic acid; acetyl-LDL, acetylated low density lipoprotein; HRP, horseradish peroxidase; DMSO, dimethylsulfoxide; OKA, okadaic acid; STAUR, staurosporine; LDH, lactic dehydrogenase; PK, protein kinase; mBSA, maleylated bovine serum albumin; β-VLDL, beta-very low density lipoproteins.

[1]A preliminary report of this research has been published in abstract form (*Circulation.* 1994. **89:** Pt. 2, I-189).

atherosclerosis (13) further support this hypothesis. Thus, the metabolism of modified forms of LDL by this receptor pathway may affect the rate of cholesterol deposition within the developing atherosclerotic lesion.

The metabolism of modified LDL requires the coordination of multiple cellular processes. The cycle begins with the binding of modified LDL to scavenger receptors on the cell surface followed by their internalization at coated pits into endosomes (14–16). The internalized lipoprotein disassociates from the receptor and is transported to lysosomes where they are metabolized, while the unoccupied receptor recycles back to the cell surface (14–16). Studies based on the synchronized metabolism of surface bound lipoprotein estimate that the transit time from the cell surface to the lysosomal compartment requires less than 10 min (17). The efficiency of this process implies that there are control mechanisms that tightly regulate receptor trafficking during ligand processing. Recent studies have begun to provide insight into the determinants that govern receptor transport. Sprague and coworkers (18) investigated the role of N-glycosylation on receptor expression using the glycosylation processing inhibitor castanospermine. They determined that the maturation of N-linked glycosylated proteins is required for the directed transport of newly synthesized receptors from the endoplasmic reticulum to the Golgi, and eventually, to the plasma membrane. The recycling of internalized receptors also appears to be controlled by an intrinsic structural feature of the receptor. Doi and his colleagues (16) have demonstrated that substitution of a leucine for histidine at residue 260 within the α-helical coiled coil domain prevents the acid pH-induced dissociation of ligands from the scavenger receptor. When cells expressing this mutant receptor were incubated with ligand, the binding and internalization of ligand proceeded normally; however, after their internalization, the receptor-ligand complexes did not return to the cell surface but both were delivered to lysosomes.

Much less is understood of the mechanisms that control the transport of scavenger receptors on the cell surface or their internalization. For many receptors that are internalized, this is regulated by information expressed within their cytoplasmic domains in the form of either a signal sequence motif or a phosphorylation event (19–22). Internalization or clathrin-coated pit targeting sequences have not yet been identified in the cytoplasmic domain of the scavenger receptor; however, the deletion of amino acid residues 21–27 within the cytoplasmic domain has been reported in preliminary form to dramatically slow its rate of internalization (2). This supports the notion that the movement of surface scavenger receptors is regulated in part by a cytoplasmic domain signal-dependent mechanism. The nature of this mechanism is speculative; however, one possibility is receptor phosphorylation. The cytoplasmic domain of each of the three subunits of the mature receptor contains three serine/threonine phosphorylation sites that are conserved amongst all four species from which receptor cDNAs have been cloned (3, 5, 11); thus, each receptor trimer contains nine potential phosphorylation sites. To begin to examine the role of cellular protein phosphorylation in the function of the macrophage scavenger receptor pathway, the effects of the serine/threonine protein phosphatase inhibitor okadaic acid (23) and the protein kinase (PK) inhibitor staurosporine on lipoprotein processing, receptor transport, and receptor phosphorylation were studied.

MATERIALS AND METHODS

Materials

F-10 medium, Dulbecco's modified Eagle's medium (DMEM), methionine-deficient DMEM, and phosphate-deficient DMEM were purchased from GIBCO Laboratories (Grand Island, NY) and fetal calf serum (FCS) was obtained from Intergen Co. (Purchase, NY). Okadaic acid, calyculin A, 1-nor-okadaone, phorbol 12-myristate 13-acetate (PMA), and staurosporine were obtained from LC Laboratories (Woburn, MA). These were dissolved in DMSO and stored at -20°C until use. Pronase was from CalBiochem (San Diego, CA). $Na^{125}I$, [^{32}P]orthophosphate, L-[^{35}S]methionine, and [^{32}P]dATP were obtained from Amersham (Arlington Heights, IL). Protein A Sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden). Synthetic oligonucleotides and peptides were obtained from the Stanford University Digestive Diseases Center. Antisera directed against the mouse scavenger receptor was produced in rabbits by immunization with a 17 amino acid synthetic peptide (residues 1–17) coupled to keyhole limpet hemocyanin as described by Ashkenas and coworkers (5). The antibody titer was monitored by solid phase immunoassay.

Animals

Female Swiss Webster mice (27–30 g) were purchased from Simonsen Laboratories (Gilroy, CA) or Banton and Kingman (Fremont, CA) and given standard mouse chow and water ad libitum.

Procedures

Lipoproteins. Human LDL (d 1.019–1.063 g/ml) was isolated from EDTA-treated plasma by density gradient ultracentrifugation (24) and dialyzed against PBS containing 0.01% EDTA. LDL was radiolabeled with $Na^{125}I$ using Iodogen beads (Pierce Chemical Co.).

Modification of LDL. LDL was acetylated essentially as described by Basu et al. (25) except that BHT (20 μM) was included during its preparation. LDL was oxidized in a cell-free system using 10 μM copper ion (26).

Macrophage lipoprotein metabolism. The degradation and binding of lipoproteins were measured as described elsewhere with slight modifications (17). To measure lipoprotein degradation, adherent macrophages in 24-well tissue culture plates were incubated with radiolabeled lipoproteins (5 μg/ml) in medium A at 37°C. The amount of trichloroacetic acid (TCA) and silver nitrate-soluble products in the incubation supernatant were then determined and the results were expressed as the amount of lipoprotein degraded in μg per mg cell protein. The amount of cell associated lipoprotein (degraded and nondegraded lipoprotein) was also measured and the results were expressed in the same manner. All values were corrected for lipoprotein incubated in the absence of cells. To measure lipoprotein binding, adherent macrophages in 24-well plates were incubated with ^{125}I-labeled acetyl-LDL (0.5–15 μg/ml) in 0.5 ml medium B (DMEM without $NaHCO_3$ supplemented with 10% FCS buffered with 10 mM HEPES; pH 7.4) in the presence or absence of fucoidan (200 μg/ml) at 4°C for 2 h. The cells were washed 3 times with ice-cold PBS and the amount of cell-associated radioactivity and cell protein was determined. The results were expressed as the ng amount of acetyl-LDL bound per mg cell protein. The B_{max} and K_d values were calculated using the specific binding results by Scatchard analysis.

Measurement of post-receptor processing of acetyl-LDL. Post-receptor processing of acetyl-LDL was measured as described previously (17). Briefly, adherent macrophages in 24-well plates were incubated with ^{125}I-labeled acetyl-LDL (15 μg/ml) in 0.5 ml medium B at 4°C for 2 h. The cells were then washed 3 times with ice-cold medium and then incubated in 0.5 ml medium (prewarmed to 37°C) at 37°C in a water bath for 0, 10, 20, 30, and 40 min. The amount of degradation products in the incubation medium and the amount of cell associated radioactivity were then measured as described above but the results were expressed as a percentage of the total amount of acetyl-LDL initially bound by the cells.

Measurement of the rate of internalization of acetyl-LDL. The disappearance of surface bound acetyl-LDL was measured as described previously (17). Briefly, macrophages in 15 × 85 mm glass tubes were incubated exactly as described above for the measurement of post-receptor processing except that the cells were incubated at 37°C for 0, 2.5, 5.0, and 7.5 min. The cells were immediately cooled to 4°C, the incubation media were removed and the amount of degradation products in the incubation medium was measured. The remaining cells were washed 3 times with ice-cold PBS and then incubated at 4°C with 0.5 mg/ml pronase dissolved in PBS for 2 h. The amount of lipoprotein released by pronase digestion was measured and expressed as a percentage of the total amount of lipoprotein initially bound. The results were normalized to the amount of lipoprotein released at time zero. The amount of lipoprotein released from control and from staurosporine-treated cells at time zero were not statistically different (see legend to Fig. 8A). The half-life of surface acetyl-LDL was calculated by least square analysis after log transformation of the data.

Measurement of scavenger receptors by ligand blotting. Macrophages in 6-well tissue culture plates were washed 3 times with ice-cold PBS and then incubated on ice with 0.5 ml lysis solution [1% Triton X-100, 50 mM Tris (pH 8 at 4°C), 100 mM KCl, 1 mM PMSF, 10 μg/ml aprotinin, 50 μM leupeptin] containing phosphatase inhibitors (50 mM NaF, 10 mM sodium pyrophosphate, 1 mM orthovanadate, and 0.8 μM okadaic acid) for 10 min. The cell lysate was centrifuged (10,000 *g;* 10 min at 4°C) and the soluble detergent extract was set aside. The sediment was resolubilized with lysis solution (0.1 ml) and the supernatant was combined with the first detergent extract. This was centrifuged (100,000 *g;* 1 h at 4°C) to sediment insoluble material and the supernatant was stored at -80°C until analysis. Equal amounts of protein (40–50 μg) were incubated in Laemelli sample buffer in the absence of reducing agents at 70°C for 2 min. The samples were then separated on 6% SDS-polyacrylamide gels and the proteins were transferred onto nitrocellulose membranes by electroblotting. The membranes were preincubated overnight in ligand binding buffer (90 mM NaCl, 50 mM Tris, 5% BSA; pH 7.4) at room temperature followed by the incubation with radiolabeled acetyl-LDL (5 μg/ml) at room temperature for 6 h. The membranes were washed with binding buffer 3 times each for 2 h and once with PBS for 5 min. The membranes were then exposed to X-ray film at -80°C and the autoradiograms were scanned by laser densitometry. Preliminary studies showed that the binding of acetyl-LDL was proportional to the amount of protein analyzed between 20 to 50 μg of solubilized protein (not shown).

Measurement of scavenger receptor mRNA levels by Northern blotting. Total cytoplasmic RNA from resident mouse peritoneal macrophages was isolated as described previously (26). Between 15 μg and 19 μg RNA was separated on a 1% agarose/formaldehyde gel, blotted overnight, and then probed using ^{32}P-labeled synthetic oligonucleotides specific for either type I (nucleotides 1189–1260) or both type I and II (nucleotides 25–96) scavenger receptor. Filters were then stripped and then reprobed using oligonucleotides specific for actin. Autoradiograms were scanned by laser densitometry.

Measurement of pinocytosis. The rate of horseradish peroxidase (HRP) uptake by macrophages was measured as described by Edelson, Zwiebel, and Cohn (27). Briefly, adherent macrophages in 12-well tissue culture plates were incubated with HRP (1 mg/ml) at 37°C for the times indicated. The cells were washed 3 times with serum-free medium and then incubated with medium containing serum for 30 min. The cells were washed with PBS and lysed with 0.1% Triton X-100 (0.2 ml). The lysate was cleared by centrifugation and the amount of HRP in a 10-μl aliquot was measured using *o*-diansidine. A second aliquot (40 μl) was removed to measure the amount of cell protein. The results were expressed as the amount of fluid ingested in nl per hour per mg cell protein.

Metabolic labeling of macrophages. Macrophage proteins were biosynthetically labeled with either [^{32}P]orthophosphate or [^{35}S]methionine. For the former, macrophages were preincubated with phosphate-deficient DMEM supplemented with 10% saline-dialyzed serum at 37°C for 30 min. This was discarded and the cells were incubated with fresh medium containing [^{32}P]orthophosphate (0.2 mCi/ml) for 4 h. Alternatively, macrophages were preincubated with methionine-deficient DMEM supplemented with 10% saline-dialyzed serum for 30 min. This was discarded and the cells were incubated with fresh medium (90% methionine-deficient DMEM, 10% DMEM, and 10% dialyzed serum) containing [^{35}S]methionine (0.2 mCi/ml) at 37°C overnight. The cells were washed 3 times with medium supplemented with 1 mM L-methionine and then incubated with the same medium for 4 h.

Immune precipitation of the scavenger receptor. Macrophages in 6-well tissue culture plates were washed with ice-cold PBS and the scavenger receptor was precipitated as described by Ashkenas et al. (5). Briefly, 0.2 ml lysis buffer (1% Triton X-100, 0.25% SDS, 10 mM iodoacetamide, 50 μg/ml leupeptin, and 1 mM PMSF in PBS) containing phosphatase inhibitors (see above) was added to each well, the cells were scraped and the lysates were transferred to microfuge tubes. They were boiled for 5 min, centrifuged (10,000 *g* for 10 min at 4°C) and the protein concentrations of the supernatants were measured. Equal amounts of solubilized protein were then subjected to immune precipitation. The samples were first incubated with non-immune rabbit serum and protein A Sepharose overnight at 4°C with constant rocking. The incubation supernatant was treated a second time with non-immune sera for 3 h and then incubated with the anti-scavenger receptor rabbit serum for 2 h. The protein A Sepharose sediment was then washed 6 times and resuspended with sample buffer in the absence of reducing agents. This was boiled for 3 min, centrifuged, and the supernatant was loaded onto a 6% polyacrylamide gel. The gel was dried and the labeled receptor was detected by autoradiography. Gels with samples from ^{35}S-labeled macrophages were incubated with 1 M sodium salicylate for 30 min before drying and fluorography.

Other procedures. Protein was measured by the method of Lowry et al. (28) or by the Pierce BCA protein assay method (Pierce; Rockford, IL). Lactic dehydrogenase activity was measured using Sigma kit DG134-UV (Sigma Chemical Co).

Statistics. Statistical analysis was done using nonpaired Student's *t*-test.

RESULTS

Changes in cellular protein phosphorylation modulate the macrophage scavenger receptor pathway

Macrophages were pretreated with either the protein phosphatase inhibitor okadaic acid or the protein kinase inhibitor staurosporine to modulate the level of cellular protein phosphorylation. The incorporation of [^{32}P]orthophosphate into acid-precipitable proteins confirmed that they produced the expected changes in protein phosphorylation; okadaic acid increased protein phosphorylation by 32% while staurosporine decreased it by 27%. Their effects on the metabolism of acetyl-LDL were then measured. Okadaic acid pretreatment inhibited both the degradation and cell association of acetyl-LDL in a concentration- and time-dependent manner, whereas the inactive structural analog 1-nor-okadaone had no effect. An 11% decrease (average of two experiments) was observed as early as 30 min after the addition of okadaic acid and a maximal inhibition of 74% was observed at 0.8 μM okadaic acid after a 2-h preincubation (**Fig. 1A**). Consistent with an inhibition of the scavenger receptor pathway, the metabolism of oxidized LDL was also suppressed (75.6 ± 6.9% inhibition; mean ± SEM of three experiments). Staurosporine pretreatment unexpectedly also inhibited the metabolism of acetyl-LDL (Fig. 1B). As an inhibition was produced by both treatments, it was possible that this represented drug-related toxicity; however, cellular lactic dehydrogenase (LDH) levels for okadaic acid- and staurosporine-treated cells were 104% ± 2 and 98% ± 4 of control cells, respectively. These studies show that changes in the level of cellular protein phosphorylation modulate the scavenger receptor pathway.

Identification of the cellular basis for the inhibition by okadaic acid and staurosporine

The metabolism of acetyl-LDL by macrophages can be divided into two sequential phases: the binding of

acetyl-LDL to surface receptors and the processing of surface bound acetyl-LDL. These were measured to locate the site of action for both okadaic acid and staurosporine. Okadaic acid inhibited the binding of acetyl-LDL by macrophages (**Fig. 2A**). The maximum amount bound decreased from 311.1 to 83.4 ng/mg cell protein, without a change in affinity (Fig. 2A, inset). The average decrease from three different experiments was 71.5 ± 4.4% (mean ± SEM). In contrast, okadaic acid produced only a minor slowing of acetyl-LDL processing as measured by the rate of degradation of surface bound acetyl-LDL (Fig. 2B). Thus the major effect of okadaic acid was to inhibit acetyl-LDL binding. The onset of the inhibition was rapid with a decrease (15%) detectable as early as 30 min after the addition of okadaic acid. Okadaic acid itself did not interfere with acetyl-LDL binding, as the coincubation of okadaic acid (0.8 μM) with acetyl-LDL and cells did not decrease the amount bound (not shown). Calyculin A (5 nM), another serine/threonine protein phosphatase inhibitor (29), also produced a significant inhibition (64.7 ± 4.4% inhibition; mean ± SEM of three experiments) while lithium chloride (20 mM), an inhibitor of inositol phosphatase (30), produced a slight increase.

Staurosporine inhibited the scavenger receptor pathway in a much different manner. The binding of acetyl-LDL actually increased from 297.4 to 389.4 ng/mg cell protein (**Fig. 3A**). This by itself would be expected to increase the rate of acetyl-LDL degradation; however, staurosporine also inhibited acetyl-LDL processing, which decreased from 4.2 to 1.8%/min (Fig. 3B). The effect on the latter most likely accounts for the overall inhibition of the receptor pathway.

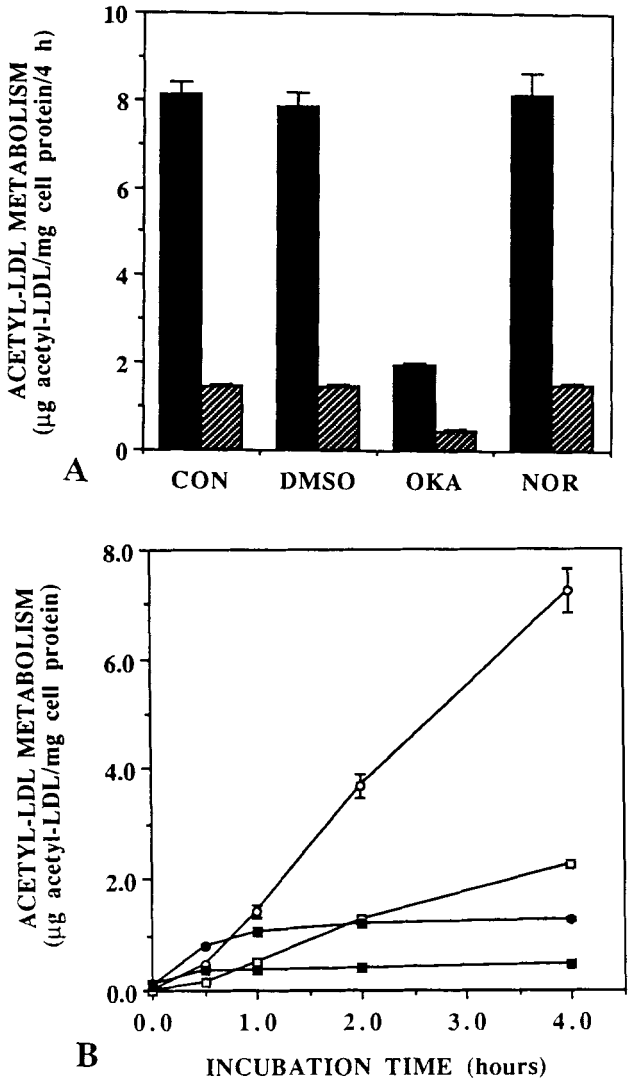


Fig. 1. Okadaic acid and staurosporine inhibit the metabolism of acetyl-LDL by macrophages. A. Macrophages were preincubated in medium (CON) or with 0.1% DMSO (DMSO), 0.8 μM okadaic acid (OKA) or 0.8 μM 1-nor-okadaone (NOR) at 37°C for 2 h. The cells were rinsed and incubated with 5 μg/ml ^{125}I-labeled acetyl-LDL at 37°C for 4 h and the amount of degradation products in the incubation medium (closed bars) and the amount of cell associated radioactivity (cross hatched bars) were measured as described under Materials and Methods. The results are expressed as the amount of lipoprotein in μg per mg cell protein per 4 h. The mean ± standard deviation of results from three replicate determinations in a representative experiment is shown. B. Macrophages were incubated with 0.05% DMSO (○, ●) or 0.4 μM staurosporine (□, ■) at 37°C for 1 h. The cells were rinsed and incubated with ^{125}I-labeled acetyl-LDL (5 μg/ml) at 37°C for the times indicated and the amount of degradation products (open symbols) and the amount of cell associated radioactivity (closed symbols) were measured as described under Materials and Methods. The mean ± standard deviation of results from three replicate determinations in a representative experiment is shown.

Scavenger receptor mRNA levels are not affected by okadaic acid

The transcriptional regulation of the macrophage scavenger receptor by growth factors and cytokines has been documented (31–34); however, the relatively rapid effects of okadaic acid and staurosporine on receptor expression are not consistent with regulation at the level of transcription. Nonetheless, steady state macrophage scavenger receptor mRNA levels were measured by Northern blotting. This was performed only for okadaic acid-treated cells as the dominant effect of staurosporine was to inhibit processing. Consistent with Ashkenas et al. (5), macrophages express one major type I mRNA of approximately 1.5 kb in length but three major classes of type II receptor mRNA ranging between 1.5 to 4 kb. Okadaic acid did not decrease type I or type II receptor mRNA levels (not shown). There was a slight increase (16%) but this did not reach statistical significance in three experiments. These studies suggest that okadaic acid reduces the number of scavenger receptors by a post-transcriptional mechanism and most probably by a post-translational mechanism.

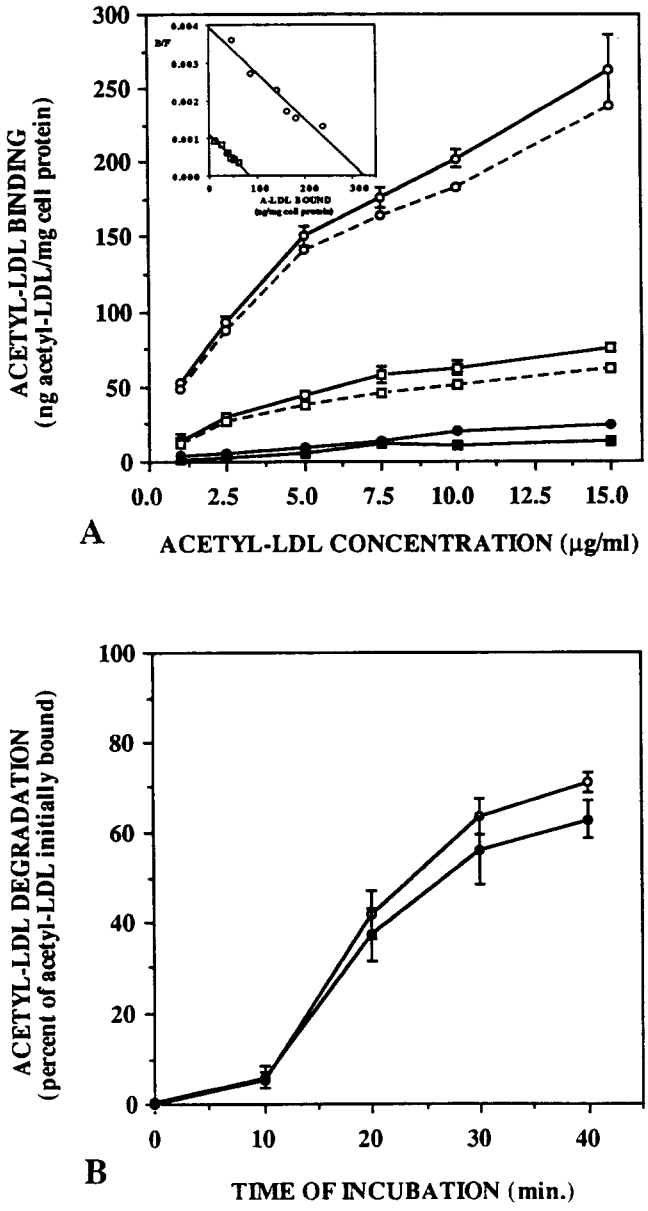
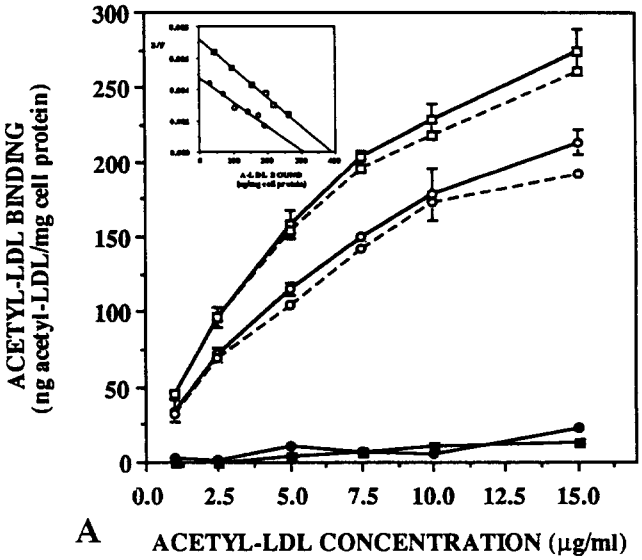


Fig. 2. Okadaic acid inhibits the binding but not post-receptor processing of acetyl-LDL. A. Macrophages were incubated with 0.1% DMSO (○, ●) or 0.8 μM okadaic acid (□, ■) at 37°C for 2 h. The cells were rinsed and incubated with increasing concentrations of ^{125}I-labeled acetyl-LDL in the presence (closed symbols) or absence (open symbols) of fucoidan (200 μg/ml) at 4°C for 2 h. The amount of specific binding (dashed line) was calculated as described under Materials and Methods. The mean ± standard deviation of results from three replicate determinations in a representative experiment is shown. Shown in the inset is the Scatchard transformation of the specific binding data. B. Macrophages were incubated with 0.1% DMSO (○) or 0.8 μM okadaic acid (●) at 37°C for 2 h. The cells were rinsed and incubated with ^{125}I-labeled acetyl-LDL (15 μg/ml) at 4°C for 2 h. The cells were rinsed a second time and incubated at 37°C for 0, 10, 20, 30, and 40 min. The amount of degradation products in the incubation medium was measured and the results are expressed as a percentage of the total amount of acetyl-LDL initially bound by the cells. The mean ± standard error of results from three different experiments is shown.



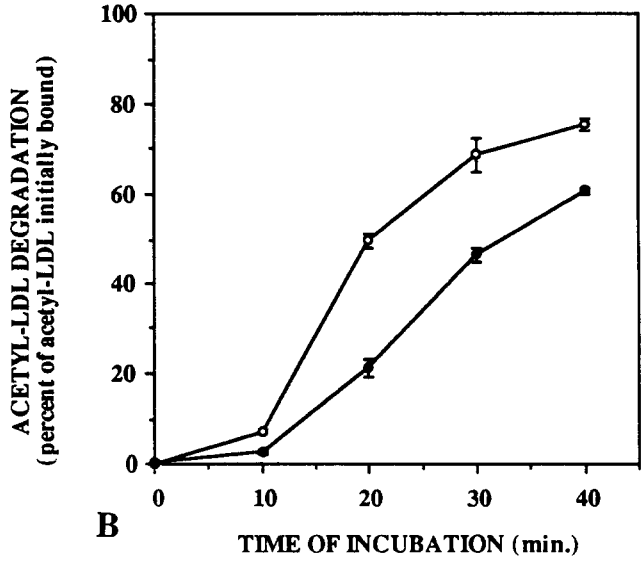


Fig. 3. Staurosporine increases the binding but inhibits post-receptor processing of acetyl-LDL. A. Macrophages were incubated with 0.05% DMSO (○, ●) or 0.4 μM staurosporine (□, ■) at 37°C for 1 h. The cells were rinsed and the binding of ^{125}I-labeled acetyl-LDL in the presence (closed symbols) or absence (open symbols) of fucoidan (200 μg/ml) was measured as described in the legend to Fig. 2A. The mean ± standard deviation of results from three replicate determinations in a representative experiment is shown. Shown in the inset is the Scatchard transformation of the specific binding data. B. Macrophages were incubated with 0.05% DMSO (○) or 0.4 μM staurosporine (●) at 37°C for 1 h. The cells were rinsed and the degradation of surface bound ^{125}I-labeled acetyl-LDL was measured as described in the legend to Fig. 2B. The mean ± standard deviation of results from three replicate determinations in a representative experiment is shown.

Okadaic acid stimulates receptor sequestration on the cell surface

To begin to identify the mechanism of regulation by okadaic acid, total cellular receptor numbers were measured by ligand blotting using radiolabeled acetyl-LDL.

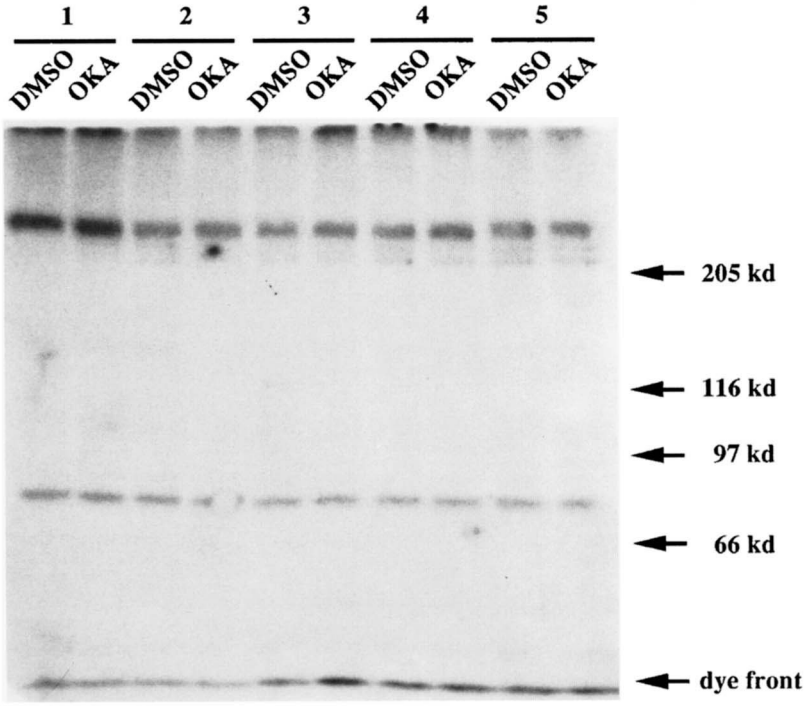


Fig. 4. Okadaic acid does not decrease total cellular scavenger receptor numbers. Macrophages were incubated with 0.1% DMSO or 0.8 μM okadaic acid at 37°C for 2 h. The cells were rinsed and solubilized cellular extracts were prepared in the presence of phosphatase inhibitors. Equivalent amounts of protein (40 μg) were separated by 6% SDS-PAGE, transferred to nitrocellulose membranes, and the scavenger receptor protein was detected by ligand blotting using ^{125}I-labeled acetyl-LDL as described under Materials and Methods. Arrows identify the location of the molecular weight standards myosin, β-galactosidase, phosphorylase b, and bovine serum albumin. An autoradiogram comparing samples from five different experiments is shown.

This would distinguish between a change in receptor turnover from a redistribution of receptors. Two proteins with approximate molecular sizes of 240 kD and 80 kD were detected that bound acetyl-LDL (**Fig. 4**); however, binding to only the larger protein was blocked by the competitive inhibitor fucoidan (not shown). When okadaic acid-treated cells were examined, the binding to the scavenger receptor did not decrease. This was consistently observed in eight separate experiments [111 ± 3% of DMSO-treated cells (mean ± SEM)]. As the size of the receptor pool did not decrease, a likely explanation for the inhibition of surface binding was a redistribution of receptors.

To test whether they might have been internalized, the distribution of receptors between the cell surface and inside the cell was measured by protease digestion of intact cells. Cells were digested with pronase at 4°C to remove surface receptors and then the number of receptors that remained was measured by ligand blotting. Preliminary studies showed that this was able to decrease acetyl-LDL binding by cells by 69% without affecting cellular LDH levels (95.3 ± 3.5% of control). Further studies showed that the preincubation of cells with unlabeled acetyl-LDL, to promote ligand-induced receptor internalization, resulted in a greater proportion of receptors that were protected from protease digestion (**Fig. 5A**). Thus protease treatment was able to distinguish cell surface and internalized receptors. When DMSO-treated cells were analyzed, there was a substantial decrease in the number of cellular receptors (66%, average of three experiments), as expected (Fig. 5B). This is consistent with the location of the majority of cellular receptors on the cell surface. When okadaic acid-pretreated cells were tested, there also was a large decrease that was not statistically different from that of control cells (61%, average of three experiments). These studies show that okadaic acid does not change the distribution of receptors between the cell surface and inside the cell.

Staurosporine inhibits acetyl-LDL internalization and pinocytosis by a protein kinase C-independent mechanism

The inhibition of acetyl-LDL post-receptor processing by staurosporine could be due to a slowing of one or more of the multiple steps involved in ligand processing. To examine whether ligand internalization was affected, the rate of disappearance of acetyl-LDL from the cell surface was measured. Consistent with previous measurements (17), acetyl-LDL was internalized at a rapid rate by control cells; it required only 2.8 ± 0.2 min (mean ± SEM of three experiments) to internalize 50% of the surface associated acetyl-LDL (**Fig. 6A**). This was slowed substantially by staurosporine, increasing the half-life to 7.9 ± 0.2 min (mean ± SEM of three experiments). To determine whether the inhibition was specific for the scavenger receptor pathway, the effect of staurosporine on fluid phase endocytosis was determined. This was assessed by measuring the uptake of horseradish peroxidase (HRP) (27). The accumulation of HRP by both

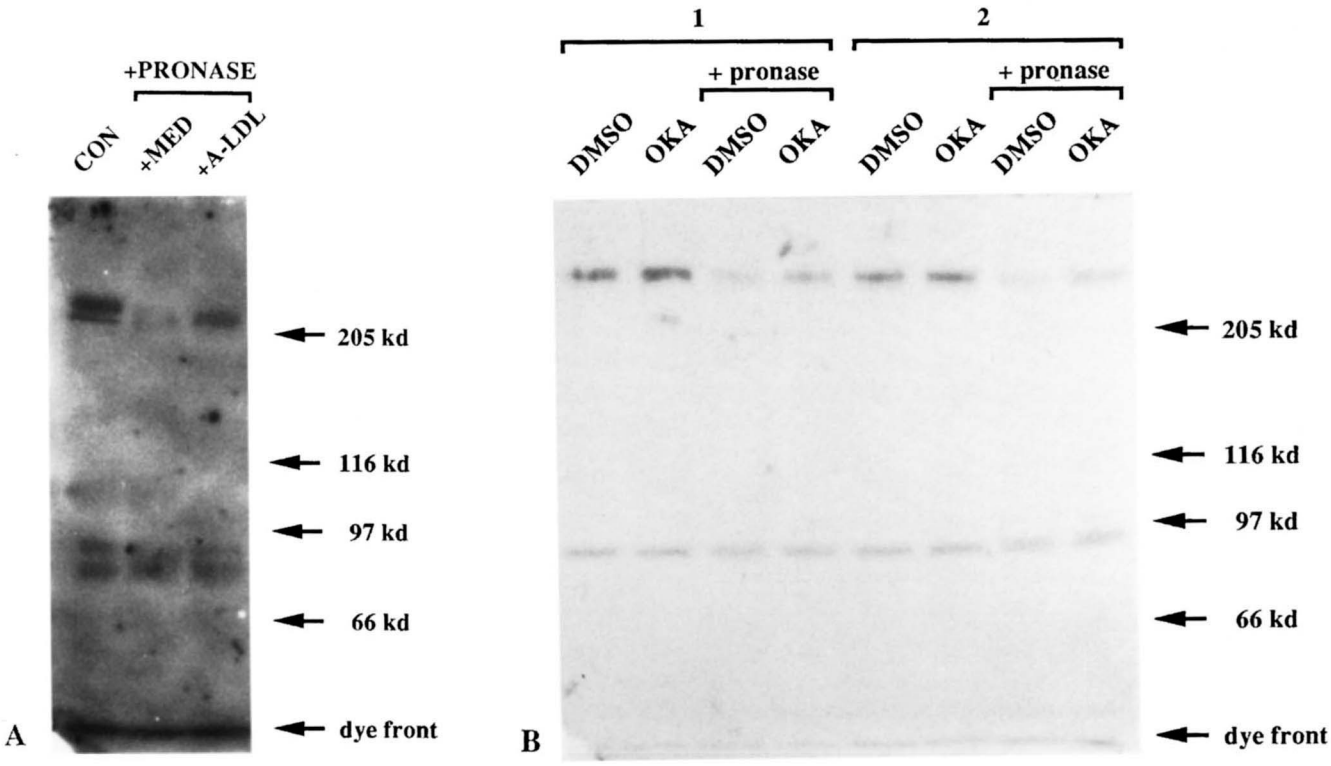


Fig. 5. Okadaic acid does not change the distribution of receptors between the cell surface and inside the cell. A. Macrophages were preincubated with buffer (+MED) or 10 μg/ml acetyl-LDL (+A-LDL) for 5 min at 37°C, washed to remove non-receptor bound lipoprotein, and then incubated for an additional 5 min to promote receptor internalization. The cells were digested with pronase at 4°C and the number of cellular receptors was measured by ligand blotting as described in the legend to Fig. 4. Macrophages preincubated with buffer but not digested with pronase were included as a control (CON). B. Solubilized cellular extracts from DMSO (DMSO) and okadaic acid (OKA) pretreated macrophages were prepared and analyzed exactly as described in the legend to Fig. 4 except that a portion of the cells from each treatment group was digested with pronase as described under Materials and Methods to remove cell surface scavenger receptors. An autoradiogram comparing samples from two different experiments is shown.

control and staurosporine-treated cells was time- and temperature-dependent; however, the rate of uptake was slower for staurosporine-treated cells (Fig. 6B). In a representative experiment, the rate of pinocytosis decreased from 528 to 292 nl/h per mg cell protein. Based on four different experiments, the average inhibition was 38.5% ± 10.8. These studies show that staurosporine inhibits both acetyl-LDL internalization and fluid phase endocytosis and suggest that these two processes are both dependent upon a staurosporine-sensitive protein kinase (PK).

Staurosporine is a potent inhibitor of PKC activity, thus the role of PKC in the staurosporine inhibition of acetyl-LDL internalization was further probed. Cells were pretreated with PMA to down-regulate cellular PKC activity and then the effect of staurosporine on acetyl-LDL internalization was measured. An overnight incubation with PMA was found to be sufficient to down-regulate macrophage PKC activity. This was determined by measuring the effects of PMA on pinocytosis. Macrophages incubated with PMA for 1 h increased the rate of fluid phase pinocytosis by 1.7 times (**Table 1**). This is consistent with previous studies indicating that PMA stimulates macrophage pinocytosis (35); however, when PMA was added to cells that were first preincubated overnight with PMA, pinocytosis was not affected. Using the same preincubation conditions, the effect of staurosporine on the internalization of acetyl-LDL was determined. If the inhibition by staurosporine is dependent upon PKC activity then staurosporine should not affect acetyl-LDL internalization in PKC-depleted cells; however, when staurosporine was added to PKC-depleted cells, there was a 3-fold inhibition of acetyl-LDL internalization ($t_{1/2}$ values; 2.9 min vs. 9.3 min) (**Fig. 7**). The extent of the inhibition was similar to that observed for non-PMA-treated cells (see Fig. 6A). These studies indicate that staurosporine inhibits acetyl-LDL internalization by a PKC-independent mechanism.

Scavenger receptor is phosphorylated in situ and the level can be modulated

The metabolic studies demonstrate that changes in cellular protein phosphorylation can modulate the function of the scavenger receptor pathway. Whether this is

related to changes in scavenger receptor phosphorylation or to changes in phosphorylation of other proteins

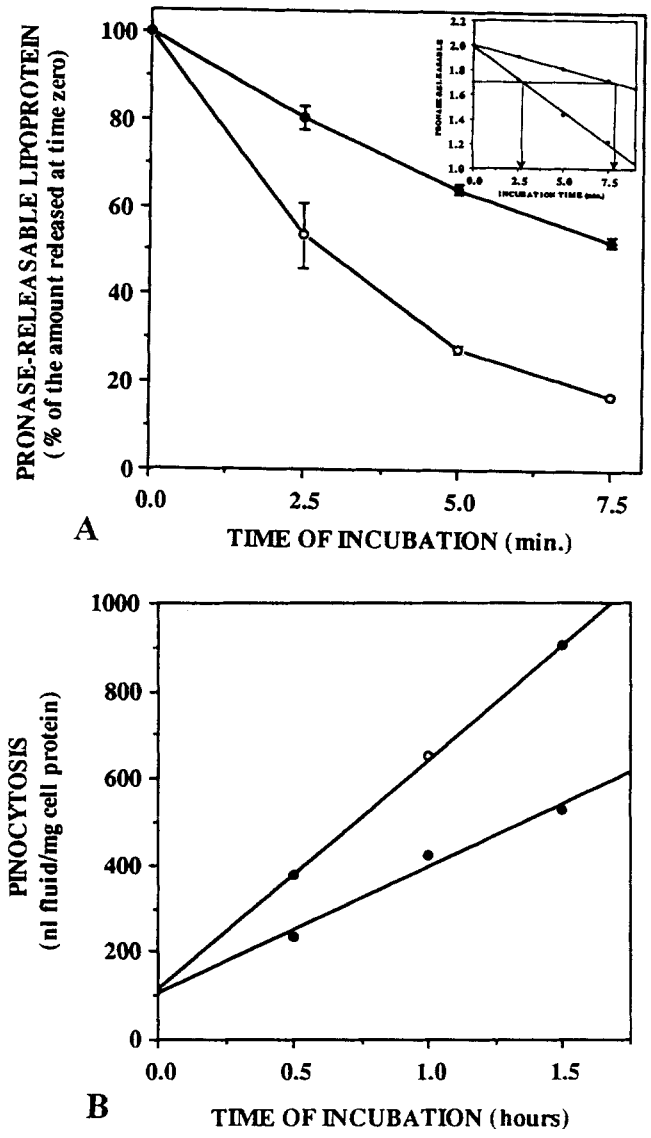


Fig. 6. Staurosporine slows acetyl-LDL internalization and pinocytosis. A. Macrophages were incubated with 0.05% DMSO (○) or 0.4 μM staurosporine (●) at 37°C for 1 h. The cells were rinsed and incubated with ^{125}I-labeled acetyl-LDL (15 μg/ml) at 4°C for 2 h to allow lipoprotein binding. The cells were rinsed a second time and incubated at 37°C for 0, 2.5, 5, and 7.5 min. The amount of lipoprotein remaining on the cell surface was measured by pronase digestion as described under Materials and Methods. The results are expressed as a percentage of the amount releasable at time zero. Based on three different experiments, the amounts released from DMSO- and staurosporine-treated cells at time zero were 82.6 ± 2.2% and 85.1 ± 0.6%, respectively. The amounts of degradation products in the medium after 7.5 min were 1.6 ± 0.1% and 1.3 ± 0.1% for DMSO- and staurosporine-treated cells, respectively. The mean ± standard error of results from three different experiments is shown. Shown in the inset is the log transformation of the data. B. Macrophages were incubated with 0.05% DMSO (○) or 0.4 μM staurosporine (●) at 37°C for 1 h. The cells were rinsed, incubated with HRP (1 mg/ml) for the times indicated, and the amount of cell associated HRP was measured as described under Materials and Methods. The results are expressed as the amount of fluid ingested in nl per mg cell protein.

TABLE 1. Effect of PMA on macrophage pinocytosis

Incubation Conditions	Pinocytosis
	nl fluid/mg cell protein
DMSO (0.05%) overnight	
+0.05% DMSO/1 h	126.6 ± 14.3
+0.5 μM PMA/1 h	217.4 ± 11.8
PMA (0.5 μM) overnight	
+0.05% DMSO/1 h	226.7 ± 11.8
+0.5 μM PMA/1 h	229.4 ± 6.9

Macrophages were preincubated with 0.05% DMSO or 0.5 μM PMA at 37°C overnight. The cells were washed and then incubated with either DMSO or PMA for 1 h at the concentrations shown. The uptake of HRP was measured after a 1-h incubation and the amount of cell-associated HRP measured as described under Materials and Methods. The results are expressed as the amount of fluid ingested in nl per mg cell protein. The mean ± standard deviation of results from three replicate determinations in a representative experiment is shown.

was next studied. To address this, the effects of okadaic acid and staurosporine on scavenger receptor phosphorylation were determined. The phosphorylation of the receptor was examined by incubating cells with [^{32}P]phosphoric acid and measuring the incorporation of radiolabel into the scavenger receptor by immune precipitation and autoradiography. A receptor-specific antibody was generated in rabbits immunized with a synthetic peptide corresponding to the amino terminus of the murine scavenger receptor. This has been shown by Ashkenas et al. (5) to generate an antisera that recognizes the mature receptor. This was confirmed by the precipitation of a [^{35}S]methionine-labeled protein from macrophages that was identical in size to that precipitated by an anti-scavenger receptor antisera kindly provided by Dr. Monty Krieger and his colleagues (not shown). When the effects of okadaic acid and staurosporine were examined, there was an unexpected result. At baseline conditions (medium control and DMSO treatment), the receptor was in a phosphorylated state and this was reduced by both okadaic acid and staurosporine (**Fig. 8A**). This was not due to the loading of different amounts of receptor protein onto the gel as the amounts of ^{35}S-labeled receptor protein precipitated from the four groups were similar (Fig. 8B). These studies document that the receptor can be phosphorylated and that the level is susceptible to regulation; however, they suggest that the change in receptor function observed in the metabolic studies could not be directly due to altered phosphorylation of the scavenger receptor.

DISCUSSION

Ultrastructural and biochemical studies have provided a relatively complete understanding of the se-

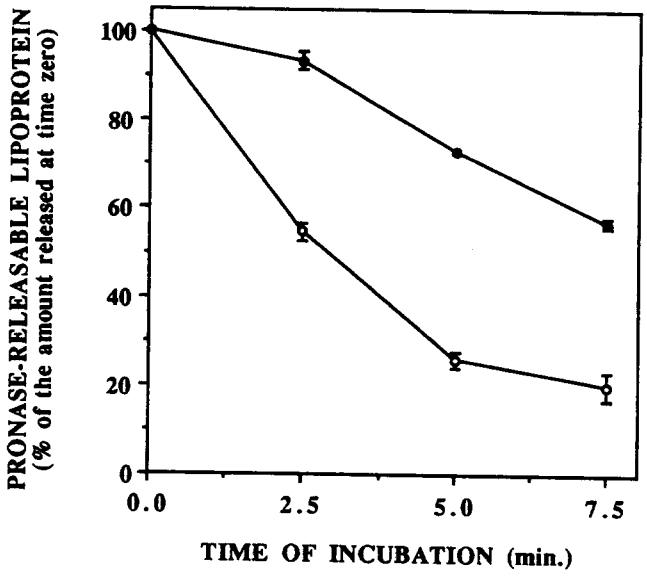


Fig. 7. Staurosporine inhibits acetyl-LDL internalization by a protein kinase C-independent mechanism. Macrophages were preincubated with PMA (0.5 μM) overnight to deplete PKC activity. The cells were rinsed and incubated with 0.05% DMSO (○) or 0.4 μM staurosporine (●) at 37°C for 1 h. The cells were rinsed a second time and the internalization of surface bound ^{125}I-labeled acetyl-LDL was measured as described in the legend to Fig. 6A. The mean ± standard deviation of results from three replicate determinations in a representative experiment is shown.

quence of cellular events that mediate the scavenger receptor-dependent metabolism of ligands by macrophages (1, 14–18, 36); however, the mechanisms that regulate the individual steps are just now being explored. Ligand binding, the first step in the process, is governed primarily by receptor expression. Most regulation has been demonstrated to occur at this level and correlates for the most part with gene transcription and receptor synthesis (31–34). Modulation of ligand binding by changes in receptor affinity has also been documented (16); however, its role during normal lipoprotein processing has not yet been established. After the internalization of the ligand, its dissociation from the receptor is dependent upon endosomal acidification and a pH-induced change in the conformation of the receptor ligand binding domain (16). Interestingly, site-directed mutations that prevent ligand dissociation also inhibit receptor recycling, suggesting that receptor occupancy within the sorting compartment is one of the determinants that govern the intracellular fate of internalized receptors. Much less is understood of the mechanisms that control or signal the surface transport and internalization of receptor-ligand complexes. For several other receptors that mediate internalization, this information is encoded within the cytoplasmic domains either as an internalization sequence motif or a phosphorylation event. Preliminary studies from Freeman (2) suggest that a similar control mechanism also applies to the scavenger receptor. They deleted a small stretch of amino acids (residues 21–27; bovine sequence) within the cytoplasmic domain of the receptor that flank the aromatic amino acid phenylalanine. This mutation produced a dramatic decrease in receptor internalization, suggesting that this may represent a consensus internalization sequence, analogous to the NPXY or YXRF sequence utilized by other receptors (19, 20); however, it is interesting to note that the deletion also eliminated two conserved phosphorylation sites.

We initiated studies to examine whether protein phosphorylation might control scavenger receptor transport. The cytoplasmic domain of each monomer of the mature receptor contains three highly conserved serine/threonine phosphorylation sites (3, 5, 11). Although phosphorylation of the scavenger receptor had not been previously documented, its central importance to the transport of other receptors suggested that one or more of these potential phosphorylation sites may be involved in a similar capacity. Cellular protein phosphorylation was modulated pharmacologically and the effects on the metabolism of acetyl-LDL were determined. Cellular protein phosphorylation was increased by inhibiting serine/threonine protein phosphatases with okadaic acid. This approach was taken as opposed to directly stimulating cellular PK activity, because of the potential for selectively increasing the phosphorylation of proteins that contain serine/threonine phosphorylation sites. Although okadaic acid did increase the average level of cellular phosphorylation, it is evident that not all proteins with such sites were affected similarly as scavenger receptor phosphorylation was not increased. To reduce protein phosphorylation, cells were treated with staurosporine, a broad spectrum PK inhibitor. This particular inhibitor was chosen based on studies that showed that it was able to completely block the effects of okadaic acid on acetyl-LDL binding, whereas inhibitors of tyrosine kinase (20 μM genistein) and PKA (100 μM HA1004) were ineffective (not shown). Unexpectedly, both okadaic acid and staurosporine inhibited acetyl-LDL metabolism despite producing opposite effects on global cellular protein phosphorylation. To determine the basis for this, their mechanisms of action were investigated and specific steps of the scavenger receptor pathway that were affected by changes in protein phosphorylation were identified.

Okadaic acid inhibited scavenger receptor expression and acetyl-LDL metabolism. This is in concert with previous studies that have shown that phorbol ester treatment of macrophages inhibits acetyl-LDL binding (37) and degradation (17). Although several mechanisms could account for the inhibition induced by okadaic acid, such as an alteration of receptor confor-

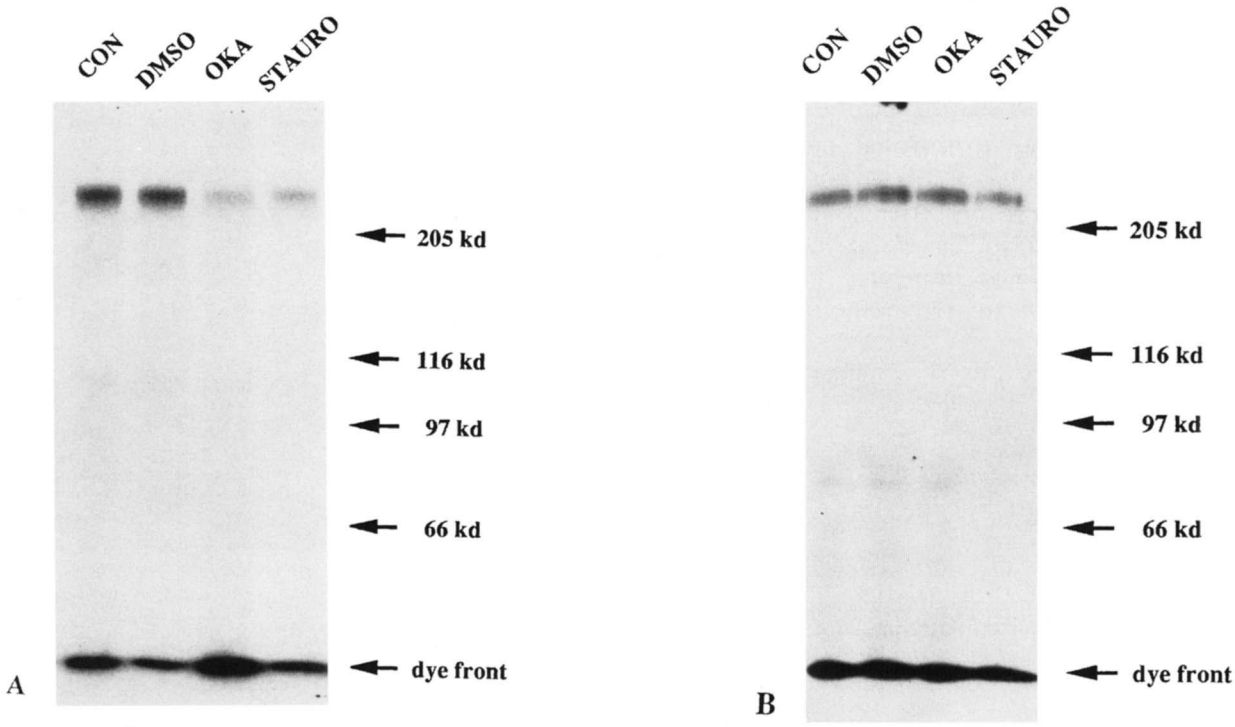


Fig. 8. The scavenger receptor is phosphorylated in situ. A. Macrophages were preincubated with phosphate-deficient DMEM containing 10% dialyzed FCS supplemented with [^{32}P]orthophosphate (0.2 mCi/ml) for 4 h. During the final 2 h of incubation, medium (CON), 0.05% DMSO (DMSO), 0.7 μM okadaic acid (OKA), or 0.4 μM staurosporine (STAURO) were added. The cells were washed, solubilized in the presence of phosphatase inhibitors, and the scavenger receptor was immune precipitated as described under Materials and Methods. The precipitate was separated on 6% polyacrylamide gel under nonreducing conditions and phosphoproteins were detected by autoradiography. Arrows identify the location of the molecular weight standards myosin, β-galactosidase, phosphorylase b, and bovine serum albumin. An autoradiogram from a representative experiment is shown. B. Macrophages were preincubated with methionine-deficient DMEM containing 10% dialyzed FCS supplemented with [^{35}S]methionine (0.2 mCi/ml) at 37°C overnight. The incubation medium were removed and the cells were washed and incubated for 4 h in complete medium. The cells were then treated as described in section A and the radiolabeled receptor was precipitated and detected by fluorography.

mation that disrupts the ligand binding domain or the inactivation of accessory molecules that are required for receptor function, it is our hypothesis that okadaic acid inhibits acetyl-LDL binding by stimulating the redistribution of surface scavenger receptors into a sequestered compartment located on the cell surface. This is supported by two observations. First, the total number of cellular receptors measured by ligand blotting did not decrease, in fact, there was a small but statistically significant increase. This established that the receptors remained cell associated and were not degraded or converted to an irreversibly inactivated state. It is possible that a decrease could have been overlooked if the number of receptors on the cell surface was small compared to the total, in which case, a loss of surface receptors may have produced a decrease too small to detect by ligand blotting; however, the pronase digestion studies demonstrated that the opposite was the case, that most cellular receptors reside on the cell surface. Second, the distribution of receptors between the cell surface and inside the cell did not significantly change, which argues against their internalization and sequestration in an intracellular compartment. This was concluded from the protease digestion studies which are based on the assumption that the enzyme does not cross the plasma membrane. This was evaluated in two ways. First, LDH levels were measured to assess whether the permeability of the plasma membrane was compromised. Consistent with an unchanged permeability, the levels did not decrease. Second, it was verified that receptors located intracellular were protected from protease digestion. The simplest explanation for the results is that there is a redistribution of surface receptors into a plasma membrane invagination or pocket that is open to the extracellular medium but not accessible to the larger molecule acetyl-LDL.

The precise location of the sequestered surface receptors is unclear. They may congregate to one specific region analogous to "capping" or coalesce in multiple patches. Fluorescence microscopy using DiI-labeled acetyl-LDL and the cell-impermeable fluorescent quencher trinitrobenzene sulfonic acid would under normal circumstances be able to address this issue; however, the inability of acetyl-LDL to gain access to the receptors

after okadaic acid treatment precludes their use. If it is assumed that entry is limited by size, the approach would be amenable to using a ligand that is both specific for the scavenger receptor and small in size. Unfortunately, a ligand that satisfies these requirements has not been identified by the author. An alternative approach was considered that combined the use of radiolabeled maleylated albumin (mBSA) as a ligand for the scavenger receptor with an inhibitor to block non-scavenger receptor mBSA binding sites (e.g., casein); however, preliminary studies were unable to identify an inhibitor that blocked only mBSA binding. Antibodies that recognize the extracellular portion of the receptor are now being generated that will be used in future studies to determine the distribution of surface receptors. Currently, the localization of the receptors can only be described in biochemical terms.

The characteristics of the compartment are strikingly similar to those exhibited by the surface-connected tubules that were described by Myers et al. (38) and Zha, Tabas, and Maxfield (39). Using video intensified fluorescence microscopy, they followed the transport of surface bound β-VLDL (38) and more recently acetyl-LDL in a preliminary study (39) by macrophages, and observed that prior to their internalization they enter a network of tubular-like structures that are open to the extracellular medium. Access to the tubules was limited by size; a dye added to the medium was able to enter the tubules while intact immunoglobulin was not. Although the requirements for access into the okadaic acid-generated compartment has not yet been defined (e.g., size, charge), it is interesting to speculate that the compartments are identical and that the entry of scavenger receptors into these tubules after ligand binding is dependent upon protein phosphorylation. Consistent with this was the inhibition of scavenger receptor internalization by the protein kinase inhibitor staurosporine. Further studies will be required to evaluate the relation, if any, of the tubules with that of the okadaic acid-generated compartment.

After the binding of acetyl-LDL to surface scavenger receptors, the receptor-ligand complexes are rapidly internalized, disappearing from the cell surface with a half-life of 2.8 min. The ligand is delivered to lysosomes and degradation products are detectable between 7.5 to 10 min later. These biochemical measurements closely approximate the kinetics of acetyl-LDL transport based on the ultrastructural studies of Mori et al. (15) in which they identified acetyl-LDL within endosomes of bovine alveolar macrophages after 5 min and localized with lysosomal enzymes after 10 min. The inhibition of acetyl-LDL internalization by staurosporine identified this as another step in the metabolic pathway affected by a change in protein phosphorylation; however, there also was a parallel inhibition of the uptake of HRP, a measure of fluid phase pinocytosis, which suggested that the inhibition of the scavenger receptor pathway may be secondary to an affect on endocytosis per se. The studies of Racoosin and Swanson (40) though have demonstrated that solute pinocytosis and receptor-mediated endocytosis are not necessarily coupled. Similarly, our studies also provide another example of the uncoupling of these two processes; the stimulation of HRP uptake by PMA did not increase the rate of acetyl-LDL internalization. These studies instead favor a mechanism where staurosporine inhibits both fluid phase pinocytosis and scavenger receptor internalization independent of the other. The inhibition of receptor internalization was also accompanied by an inhibition of receptor phosphorylation but it is too early to conclude whether there is a connection. It is clear though that an inhibition of receptor phosphorylation is not sufficient to cause a slower internalization inasmuch as okadaic acid, which also inhibited receptor phosphorylation, did not inhibit post-receptor processing. Thus, the internalization of the receptor may be unrelated to its phosphorylation or if it is, it may be the phosphorylation of a specific amino acid and not the total extent of receptor phosphorylation that is the critical determinant. Another alternative is that receptor phosphorylation and a second event together govern its internalization. Future studies of mutant receptors will address this issue more precisely.

The increase in surface receptors after staurosporine treatment was unexpected. Analogous to the effects of okadaic acid, it appears that this is due to a shift in the distribution of receptors as staurosporine did not increase the total number of cellular receptors (Fig. 8B). It is interesting to speculate that the slower rate of receptor internalization is indirectly responsible for this by increasing the residence time that receptors spend on the cell surface during their normal recycling. An analogous proposal has been suggested for the asialoglycoprotein receptor of HepG2 cells (41). This assumes that the scavenger receptor normally recycles between the cell surface and an intracellular compartment in the absence of ligand and that the return rate of internalized receptors back to the cell surface is not affected by staurosporine. Further studies will be required to determine the precise mechanism.

In summary, the present studies demonstrate that the transport of the scavenger receptor can be modulated by changes in cellular protein phosphorylation. Its stimulation by okadaic acid resulted in a redistribution of surface receptors into a compartment that was accessible to pronase but not acetyl-LDL. This led to a functional down-regulation of surface receptors even though the total number of cellular receptors and the propor-

tion of receptors on the cell surface did not decrease. As expected, the inhibition of acetyl-LDL binding inhibited its metabolism. Despite this, we speculate that this actually mimics one of the early steps of acetyl-LDL metabolism; it is our hypothesis that after the binding of acetyl-LDL to surface receptors, the complexes are transported to regions of endocytosis by a phosphorylation-dependent mechanism. This is analogous to the phosphorylation-dependent transport of insulin receptors prior to their internalization (22) and would be consistent with the entry of acetyl-LDL into a network of size-limited surface-connected tubules during its processing (39). The inhibition of cellular PK activity by staurosporine also had profound effects on the scavenger receptor pathway. Staurosporine increased the number of surface receptors but inhibited their internalization with the overall effect of inhibiting acetyl-LDL metabolism. The inhibition of internalization was not affected by PKC-depletion and appears to be independent of staurosporine's effect on fluid phase pinocytosis. Staurosporine also inhibited receptor phosphorylation but a relationship between receptor internalization and its phosphorylation will require further study to document. These studies establish that changes in macrophage protein phosphorylation modulate the expression, transport, and the internalization of scavenger receptors. They also document for the first time that the receptor can be phosphorylated in situ and that this level can be altered. Together they suggest that the phosphorylation and dephosphorylation of specific cellular proteins may be an important biochemical mechanism that governs the transport of receptors during the normal processing of ligands by the scavenger receptor pathway.

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