β-VLDL increases endothelial cell plasma membrane cholesterol

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Abstract In this study, the distribution of free cholesterol in cholesterol-loaded endothelial cells was examined. For these studies, cell fractionation methods were used to assess marker enzyme activity and cholesterol distribution. Treatment of rabbit aortic endothelial cells for 3 days with 50 μg/ml of β-very low density lipoprotein (β-VLDL) or malondialdehyde-low density lipoprotein (MDA-LDL) but not LDL caused a 50–100% increase in total cell unesterified cholesterol. The accumulation of free rather than esterified cholesterol in endothelial cells may be due to the ratio of hydrolysis to esterification, which we have shown in this study to be 10-fold higher in endothelial cells than in smooth muscle cells. This free cholesterol is found in the fractions enriched in plasma membrane markers and, to a lesser extent, in the Golgi-enriched fractions. The amount of cholesterol per mg of protein was increased approximately 50% in these fractions from cells treated for 3 days with 50 μg/ml of β-VLDL. These increases in cholesterol content were reversible upon incubation of cells for 3 days in medium containing 15% fetal bovine serum. Alterations in several membrane functions were also observed in cholesterol-loaded cells. The activity of alkaline phosphatase, an enzyme marker for plasma membranes, was decreased by 25% and an alteration in membrane-associated microfilaments was seen with phalloidin staining. This morphological change in microfilaments was reflected in a decrease in filament ends as shown by cytochalasin binding and occurred without a change in total actin or vinculin. These microfilament changes were reversible. The results show that increased free cholesterol in endothelial cells causes a change in plasma membrane cholesterol content and may contribute to alterations in membrane function. —**Kim, J. A., K. Maxwell, D. P. Hajar, and J. A. Berliner.** β-VLDL increases endothelial cell plasma membrane cholesterol. *J. Lipid Res.* 1991. **32:** 1125–1131.

Supplementary key words rabbit aortic endothelial cells • VLDL • LDL • free cholesterol

The function of a number of cell membrane proteins has been found to be regulated by changes in neighboring lipid domains (1). Alterations in enzyme activity (2, 3), in transport function (4), and in receptor function (5, 6) have been reported. Cholesterol is known to decrease the fluidity of the cell membrane and to decrease the movement of molecules within the lipid bilayer (7, 8). Previous studies have shown that treatment of endothelial cells with β-VLDL increases the total cell cholesterol content (9) and leads to changes in transport of proteins across the monolayer (9) and binding of monocytes (10). There are a number of possible causes of these functional alterations, one possible factor being a change in the plasma membrane lipid domains. All of the unesterified cholesterol in cells cultured in serum-containing medium is found in the cell membrane (11). We therefore hypothesized that in endothelial cells treated with β-VLDL there is an increase in free cholesterol and that this results in an increase in the cholesterol content of the plasma membrane contributing to alterations in the function of membrane-associated enzymes and proteins. We have tested this hypothesis by measuring the free cholesterol content of β-VLDL-treated endothelial cells, the cholesterol content of the plasma membrane, the function of the plasma membrane enzyme alkaline phosphatase, and the association of microfilaments with the membrane.

METHODS

Lipoprotein treatment of endothelial cells

Rabbit aortic endothelial cells were isolated and cultured as described previously (9). When they reached high density (approximately 2×10^5 cells/cm^2), they were treated for 3 days with or without rabbit β-VLDL (50 μg/ml), human LDL (50 μg/ml), or human LDL modified with malondialdehyde taken up by the scavenger receptor (25 μg/ml) in DME containing 10% serum (4:1, calf:fetal calf). β-VLDL was obtained from New Zealand White Rabbits fed a 0.1% cholesterol diet for 2 months. For cholesterol determination, cells were then rinsed four times with serum free medium and incubated for an additional hour in serum-free medium before harvesting.

Abbreviations: HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; PBS, phosphate-buffered saline; MDA, malondialdehyde; RAEC, rabbit aortic endothelial cells; EC, endothelial cells; CE, cholesteryl ester.

Plasma membrane isolation

To isolate endothelial cell plasma membrane, we have used a previous method with slight modification (12). Medium was removed from five 100-mm culture dishes and cells were rinsed three times with ice-cold PBS. Then cells were scraped off with a rubber policeman into homogenization buffer (0.24 M sucrose, 5 mM Tris, pH 8.2, with 0.005% PMSF) and homogenized with 40 strokes of a loose-fitting Dounce homogenizer. The cells were examined to ensure rupture and then solutions were centrifuged at 12,000 *g*-min to obtain a nuclear pellet (N) and a post-nuclear supernatant. Post-nuclear supernatants were centrifuged at 121,000 *g*-min to obtain a mitochondrial pellet and supernatant. This supernatant and the upper white layer of the mitochondrial pellet were pooled. The remainder of the mitochondrial pellet was used as the mitochondrial fraction (M). The pool was centrifuged at 6.9×10^6 *g*-min to obtain a membrane-rich pellet and a supernatant (S). The pellet was resuspended in homogenization buffer and layered onto a linear sucrose gradient (10–60%) and centrifuged at 3.52×10^7 *g*-min in a Beckman SW41 rotor at 4°C. Gradients were collected in 19 1-ml or 28 0.5-ml fractions.

Biochemical assays on whole cells and fractions

Whole cells or aliquots of fractions were mixed with 0.4 N NaOH and assayed for protein content. An aliquot of resuspended whole cells or of fractions was also used to assay alkaline phosphatase, as a plasma membrane marker (12). Cytochrome C reductase, a marker of the smooth endoplasmic reticulum, succinic INT reductase as a marker of mitochondria, and galactosyl transferase were determined by previously described methods (12, 13). Cholesterol content was determined as follows. Cells or fractions were solubilized with 0.4 N NaOH and lipid was extracted with chloroform and methanol. The extract was dried and cholesterol content was determined enzymatically (13); both free and esterified cholesterol were determined.

Measurement of cholesteryl ester metabolism

Rabbit thoracic aortic endothelial cells (passages 4 to 6) and smooth muscle cells (passage 4) were used. At time 0, cells (5×10^5) were washed two times with PBS and then received 1.5 ml of 50 μg LDL containing [1-^{14}C]cholesterol oleate in RPMI-1640 to measure cholesteryl ester hydrolytic rates at pH 7. Unlabeled oleate (100 nmol) and 20 nmol of albumin were added to prevent re-esterification of released radiolabeled oleic acid from the hydrolyzed cholesteryl ester. For the cholesteryl ester synthetic experiment, at time 0, cells were washed with PBS and then pulsed with 1.5 ml of RPMI-1640 plus 100 nmol of [1-^{14}C]oleate, 20 nmol of albumin, and 50 μg of unlabeled LDL as a source of cholesterol. After 6 h at 37°C, cells were washed with dextran sulfate in PBS, then twice with PBS. Lipids were then extracted and radioactivity was assessed as described elsewhere (14).

Microfilament assay

Phalloidin staining. Confluent monolayers on 60-mm dishes were incubated with or without β-VLDL or MDA-LDL and stained with phalloidin using previously described methods with a slight modification (15). Cells were fixed for 20 min with 3% formaldehyde in PBS; after fixation, the cells were permeabilized with 0.1% Triton X, then rinsed with PBS for 1 h and stained with rhodamine-phallacidin for 20 min at room temperature.

[^{3}H]Cytochalasin B binding assay. To quantitate cytoskeletal actin microfilaments, radio-labeled cytochalasin B was used (16). Cells were pretreated with β-VLDL, rinsed 3 times with serum free medium, and 0.1 ml of medium containing [^{3}H]cytochalasin B in a 24-well dish with or without cytochalasin D was added. The cell layers were rinsed, dissolved in 0.4 N NaOH, and then used for determining protein concentration and bound [^{3}H]cytochalasin B by scintillation counting. Specific binding to filaments was determined as binding that could be eliminated by the presence of cytochalasin D in the medium.

Measurement of actin and vinculin content in cell lysates

To determine the total amount of actin (filamentous plus globular actin) and vinculin, Western blot analysis was used. Cells were removed from dishes by scraping with a rubber policeman. Cell suspensions were vortexed and mixed with 2 × SDS sample buffer and boiled. SDS sample buffer consisted of 2% SDS, 5% β-mercaptoethanol, 0.5 M Tris (pH 6.8), 10% glycerol, and 0.1% bromophenol blue. Proteins were separated on a slab gel (10% polyacrylamide) and transferrred to nitrocellulose. The blots were incubated with the primary antibody, washed, and then incubated in the buffer containing peroxidase-conjugated second antibody. Finally, quantitative densitometry of immunoblots was performed.

RESULTS

Treating cells with β-VLDL increased the total cell cholesterol content approximately twofold; the increased total cell cholesterol was essentially all in the form of free cholesterol (**Fig. 1**). **Table 1** compares LDL cholesteryl ester metabolism in rabbit thoracic aortic endothelial cells and smooth muscle cells. Endothelial cells had a higher hydrolysis rate and lower synthetic rate than those of smooth muscle cells (160% of hydrolytic rate of smooth muscle cells and 15% of synthetic rate of smooth muscle cells).

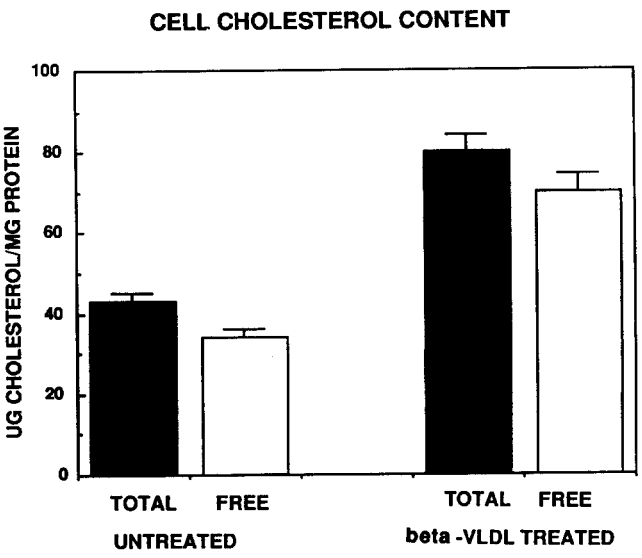

Fig. 1. Increased cell cholesterol content of rabbit aortic endothelial cells (RAEC) exposed to β-VLDL. Confluent RAEC were either (1) untreated or (2) treated for 3 days with β-VLDL (50 μg/ml). Free and total cholesterol content was determined enzymatically. Values are mean ± SD; n = 5.

The distribution of free cholesterol in the cell was examined using differential sucrose gradient fractionation. The specific amounts of cholesterol in nuclear (N) and mitochondrial (M) pellets, pooled upper gradient fractions (PM), pooled fractions from the lower gradient (LG) and the postmembrane supernatant (S) were determined. In separate aliquots of each fraction, the distribution of marker enzymes was examined. Alkaline phosphatase, previously shown to be entirely restricted to the plasma membrane in endothelial cells (12), was taken as a marker of the plasma membrane, succinic reductase as a mitochondrial marker, cytochrome C reductase as a marker of SER, and galactosyl transferase for Golgi. Marker distributions were similar to those previously seen in endothelium (**Fig. 2** and ref 12). The distribution of cholesterol was similar to that of alkaline phosphatase: the specific amount was highest in the upper gradient peak, while a lower but significant amount was found in the lower gradient which contained the Golgi marker and some of the SER marker (Fig. 2). Seventy to 85% of the total cell cholesterol and total cell alkaline phosphatase was recovered in these fractions with 80–90% of recovered cholesterol in the plasma membrane pool.

We next examined the effect of β-VLDL on cellular and plasma membrane content of cholesterol and alkaline phosphatase (**Fig. 3**). In this series of experiments the mean cholesterol contents of β-VLDL-treated cells and plasma membrane fractions were increased by 50%; and, the activity of plasma membrane-bound enzyme, alkaline phosphatase, was decreased by 25% (Fig. 3). There was also an increase in the specific amount of cholesterol in the lower gradient fractions from cells treated with β-VLDL (61%). In two experiments, similar increases in cell and membrane cholesterol content were seen when cells were treated for 3 days with 25 μg/ml MDA-LDL (64% ± 6, n = 4) but not LDL (4% ± 3); and, the activity of 5' nucleotidase, another plasma membrane marker, was also slightly decreased (MDA-LDL-treated: 3.4 ± 0.3 nU/μg; untreated: 4.3 ± 0.5 nU/μg). No further studies were performed with this enzyme since it showed extremely low activity in endothelial cells as has been previously observed (12).

To examine the effect of β-VLDL on microfilament distribution, we examined the cytoplasmic microfilament arrangement with phalloidin staining. In untreated cells, microfilaments appeared to be dense bundles of filaments around the cytoplasm running parallel to each other. However, in β-VLDL-treated cells, there was a general decrease in filaments especially apparent in the central bundles (**Fig. 4 a, b**). To quantitatively determine the numbers of filaments, the binding of [^{3}H]cytochalasin B to the untreated and β-VLDL-treated cells was determined at concentrations below 80 nM cytochalasin B. The [^{3}H]cytochalasin B binding to filament ends that could be eliminated with excess cytochalasin D was higher for unteated cells than for treated ones at concentrations below 50 nM (**Fig. 5**). At 80 nM, differences were abolished (as has been previously observed, ref. 16) due to nonspecific binding. After pooling the data from several experiments, a Scatchard plot was constructed and the number of binding sites in untreated cells was found to be approximately twofold that of untreated cells. The total amount of actin and vinculin examined by quantitative Western blotting showed no significant differences between the untreated and treated cells (**Table 2**). When β-VLDL-treated cells were washed and transferred to fresh medium containing 15% fetal bovine serum for 3 days, the total cell and membrane cholesterol content decreased to control levels (plasma membrane cholesterol: 103 ± 4% of control value) as did the microfilament distribution (Fig. 4 c).

TABLE 1. Cholesteryl ester metabolic rate in endothelial cells

Cells	Hydrolysis	Synthesis
	nmol/mg protein/6 h	
Endothelial	6.2 ± 1.7*	0.9 ± 0.1**
Smooth muscle	3.9 ± 1.2*	5.7 ± 0.4**

Cells were grown to confluency in RPMI-1640 containing 20% fetal calf serum. At time 0, cells received 50 μg LDL containing [1-^{14}C]cholesteryl oleate for measurement of CE hydrolytic rate. To prevent reesterification, unlabeled 100 nmol of oleate and 20 nmol of albumin were added to the cells. For the measurement of the synthetic rate, cells were pulsed with 100 nmol of [1-^{14}C]oleate, 20 nmol of albumin, and 50 μg of unlabeled LDL. After 6 h at 37°C, lipids were extracted and radioactivity was determined. Values are given as mean ± SD; n = four separate experiments. Values with the same superscript are significantly different ($P < 0.05$).

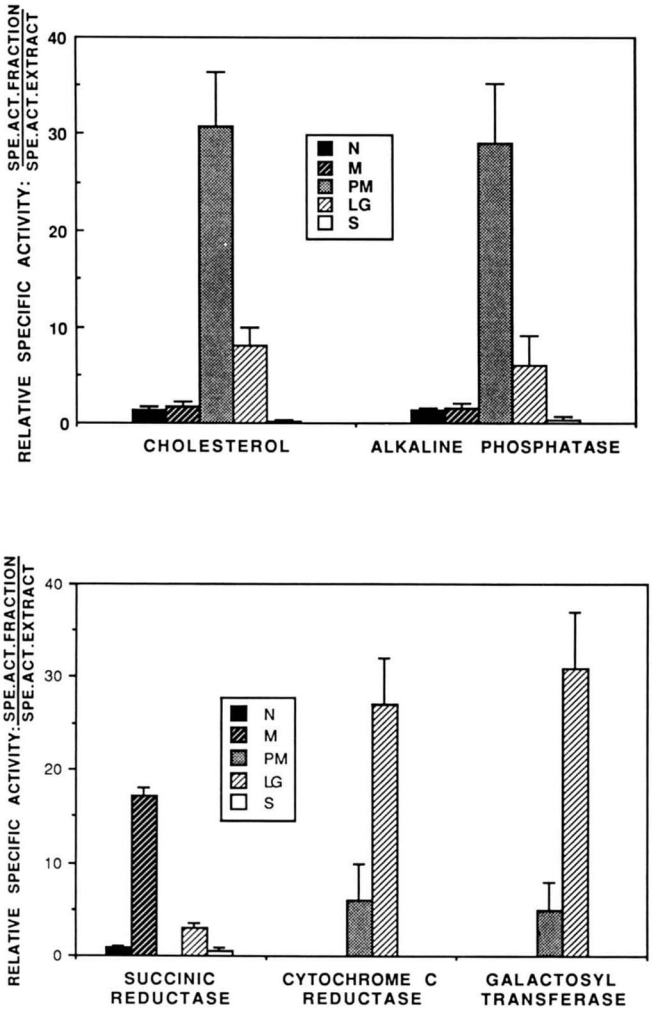

Fig. 2. The specific activity of enzymes in the different cell fractions: (1) nuclear fraction (N); (2) mitochondrial fraction (M); (3) plasma membrane fraction (PM); (4) lower gradient fraction (LG); and (5) soluble fraction (S). Each fraction was collected and subjected to determination of the activity of cholesterol, alkaline phosphatase, succinic reductase, cytochrome c reductase, and galactosyl transferase. Values represent mean ± SD; n = 4.

DISCUSSION

Endothelial cells in vivo are exposed to varying levels of plasma cholesterol; in cholesterol-fed animals, this may reach as high as 1500 mg/dl. The question of how endothelial cells handle cholesterol could have important implications for atherogenesis in which there is evidence of altered endothelial function (17). The present study has addressed this question using an in vitro model system.

It has been previously noted that endothelial cells in vivo contain few lipid droplets compared to macrophages and smooth muscle cells in cholesterol-fed animals. The present study has shown a possible cause of this difference. Endothelial cells, unlike smooth muscle cells and macrophages, accumulate free cholesterol rather than esterified cholesterol (Fig. 1), and free cholesterol is more easily removed from the cells by HDL. The cholesterol accumulated in lipid droplets in foam cells derived from smooth muscle cells or macrophages is mainly in the form of esterified cholesterol. The high cholesteryl ester hydrolase/esterase ratio in endothelial cells (Table 1) may contribute to the mechanism for accumulation of free rather than esterified cholesterol.

A major issue addressed by these studies focused on the intracellular location of free cholesterol in endothelial cells. In fibroblasts not subjected to cholesterol loading, most of the free cholesterol is found in the plasma membrane (11). The compartmentation of free cholesterol in cholesterol-loaded macrophages has been studied in several laboratories (18, 19). Using inhibitors of cholesterol esterification, vesicles containing free cholesterol and phospholipid as membrane whorls accumulated in cells treated with acetyl-LDL. When HDL was added to these cells the vesicles disappeared. From these studies and those of others, it was clear that under non-loading conditions cholesterol is mainly found in the cell membrane (11); however, under cholesterol loading conditions in cells where the cholesterol content is increased 4- to 6-fold, free cholesterol accumulates in vesicles as well as in the plasma membrane. In the present studies we have shown that in endothelial cells most cholesterol is found in the plasma membrane fraction (Fig. 2) though some is found in a Golgi-enriched fraction. In cells treated with β-VLDL where the cholesterol content is increased 50–100%, the cholesterol/protein ratio in the plasma membrane as well as the Golgi-enriched fraction is increased (Fig. 3). This latter fraction may represent the membrane whorls seen by others; however, no obvious whorls were seen in electron micrographs (data not shown).

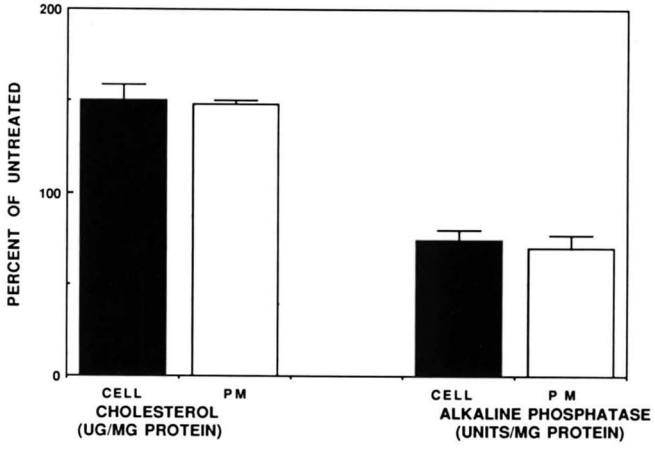

Fig. 3. Effect of β-VLDL on plasma membrane (PM) composition. RAEC were treated with β-VLDL (50 μg/ml) for 3 days and the extracts of whole cells or aliquots of plasma membrane fractions were used for determination of cholesterol and alkaline phosphatase activity. Values are mean ± SD; n = 8.

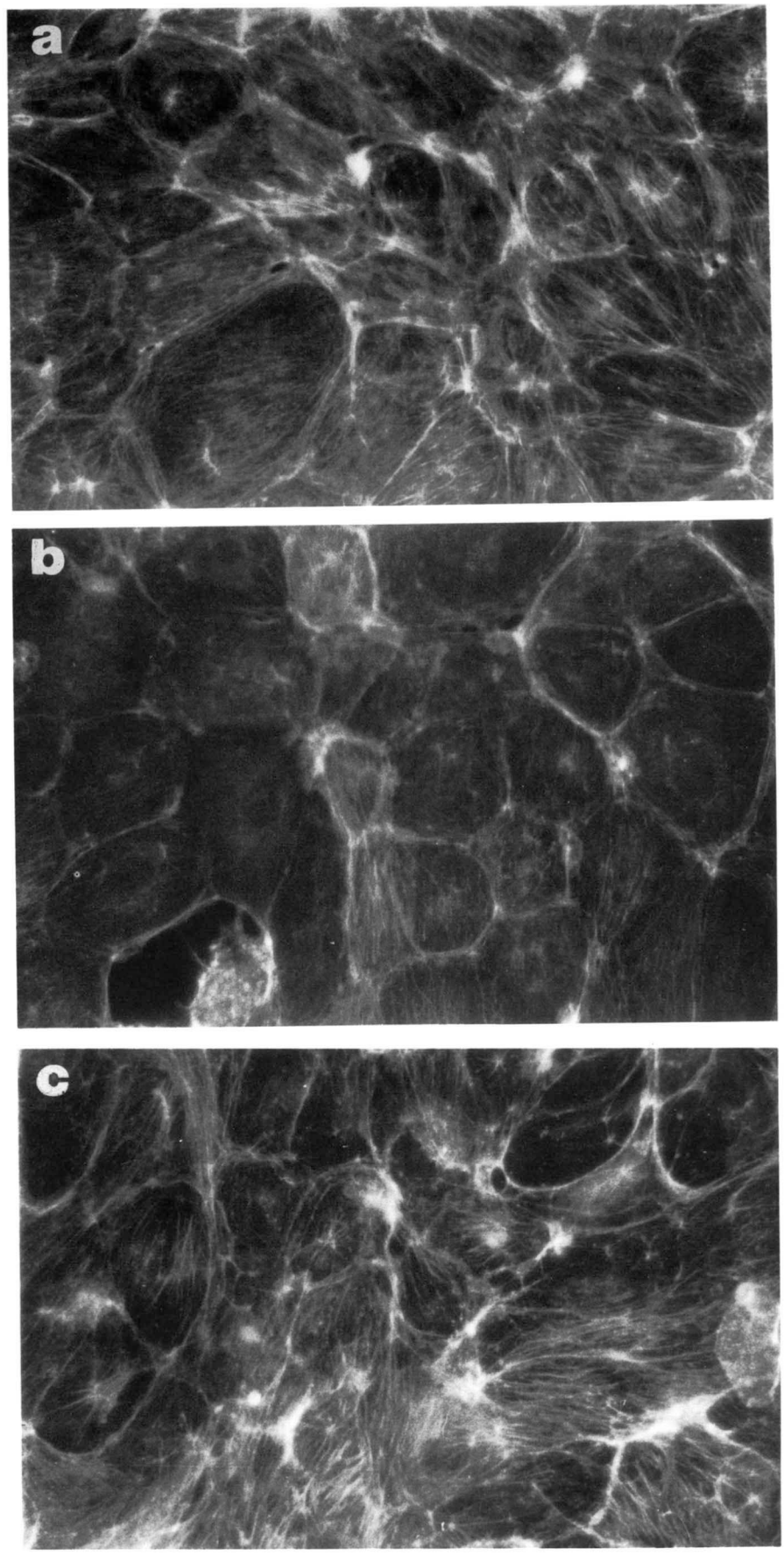

Fig. 4. Phalloidin staining of RAEC monolayer. Confluent monolayers of endothelial cells on 60-mm dishes were exposed to β-VLDL (50 μg/ml) for 3 days and fixed with 3% formaldehyde for 20 min. After permeabilization with 0.1% Triton X, cells were washed with PBS and stained with rhodamine-phallacidin for 20 min. (a): Untreated RAEC; (b): β-VLDL-treated RAEC; (c): after washing and transferring (b) to fresh medium containing 15% fetal bovine serum for 3 days.

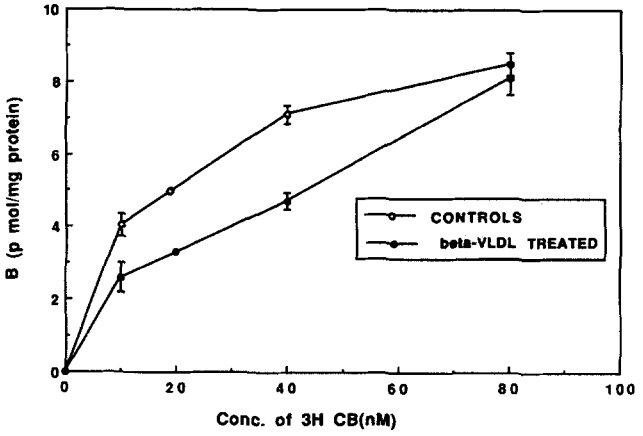

Fig. 5. Binding of cytochalasin B (CB) to RAEC. Untreated monolayers of RAEC (○) and β-VLDL-treated (50 μg/ml for 3 days) RAEC (●) were assayed for CB binding. Specific binding, B, defined as the amount of [^{3}H]CB binding that is eliminated by excess unlabeled CD in the medium, was measured at concentrations below 80 nM.

Indirect evidence that the plasma membrane lipid domains are altered in β-VLDL-treated cells is derived from the measurements of membrane-associated functions. The activity of alkaline phosphatase was slightly reduced in β-VLDL-treated cells. In addition, the number of microfilaments associated with the plasma membrane was strongly decreased (Fig. 5), despite the lack of change in amounts of actin and vinculin in the cells (Table 2). It is clear from previous studies with transforming viruses that membrane associations of actin can be easily disrupted leading to microfilament breakdown (16, 20) without a change in the levels of associated proteins. Breakdown may be due to post-translation modification of proteins such as through phosphorylation or acylation mechanisms (21, 22). There is previous evidence that changes in membrane lipid composition may predispose to microfilament alterations from studies with erythrocytes where changes in the spectrin network are correlated with an alteration in lipid organization (23). Therefore, changes in lipid composition might affect the acylation of the "capping" proteins. Or, changes in fluidity may inhibit assembly of microfilament-associated proteins. Our hypothesis that increased cholesterol is responsible for these changes in cellular function is supported by the fact that MDA-LDL, which also causes cholesterol loading, can alter the activity of a plasma membrane enzyme. In addition, the change in microfilaments as well as cholesterol loading are reversible when cells are returned to medium without added lipoprotein.

TABLE 2. Effect of β-VLDL on actin and binding protein

Cells	Actin	Vinculin
	μg/mg protein	*unit/mg protein*
Untreated	150 ± 3	61 ± 5
β-VLDL treated	152 ± 3	54 ± 5

Confluent RAEC monolayers were incubated with β-VLDL at the concentration of 50 μg/ml for 3 days. Cells were washed with PBS and subjected to Western blot analysis. Values are the mean ± SD of two separate experiments.

Our findings suggest that endothelial cells process cholesterol differently from the well-documented mechanisms of cholesterol trafficking in macrophages and smooth muscle cells. The change in the total cell cholesterol content of endothelial cells exposed to β-VLDL or MDA-LDL, though small compared to the other cell types, can alter the cholesterol content of the plasma membrane and consequently alter membrane function.

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