Regulation of Sterol Transport in Human Microvascular Endothelial Cells

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ABSTRACT   In cultured human dermal microvessel endothelial cells, the rate of efflux (about twofold greater than for fibroblasts under equivalent conditions) was coupled to an equivalent high rate of sterol net transport from the cells to the medium. This net transport was linked with esterification via lecithin:cholesterol acyltransferase. Since the use of free sterol by plasma transferase is constant, such increased net transport indicates that endothelial cells are highly efficient, in competition with plasma lipoproteins, in supplying free sterol for esterification.

These results indicate the marked ability of endothelial cells to regulate and maintain their sterol balance in the face of high sterol levels to which these cells are uniquely exposed in human plasma.

Cultures of endothelial cells from cutaneous microvessels, as do the endothelium from large vessels, form monolayers of polygonal cells of cobblestone appearance and exhibit contact-mediated inhibition of growth (1-3). Sterol metabolism in endothelial cells, and in particular its regulation by plasma lipoproteins, has attracted attention because of the uniquely high concentrations of sterol to which these cells are physiologically exposed in plasma (4-6). As shown for other cells, different fractions of plasma lipoproteins mediate the uptake and efflux of sterol. In cultured fibroblasts, a minor fraction of high density lipoprotein plays the major role in the promotion of efflux (7) When efflux is associated with net transport of sterol from cells to plasma, it is coupled to the activity of lecithin:cholesterol acyltransferase, which generates sterol esters and maintains plasma free sterol levels within well-defined limits.

In earlier work on the regulation of endothelial sterol metabolism, it was shown that contact-inhibition blocked the ability of endothelial cells to clear plasma lipoproteins by endocytosis (4, 8). This phenomenon, if cell sterol levels are maintained, must be associated with a comparable regulation of efflux in the absence of sterol demand for cell division. This kind of interaction between sterol balance and growth reflects the need to maintain cellular cholesterol homeostasis in the face of sudden metabolic demands on the cells, as in response to injury. The present research defines, for the first time, the factors involved in the regulation of sterol transport in human microvessel endothelial cells, and also demonstrates that endothelial cells have a highly efficient mechanism for donating cellular free cholesterol to the plasma for esterification.

MATERIALS AND METHODS

Isolation of Human Dermal Microvascular Endothelial Cells

Endothelial cells were isolated from the microvessels of the newborn human foreskin dermis as described earlier (3). Briefly, the tissue was trimmed of underlying fascia and the epidermis removed, using a Castroviejo keratotome set to cut at 0.1 mm, and discarded. The remaining dermis was cut into 5-mm square sections and incubated for 40 min at 37°C in a 0.3% trypsin solution in phosphate-buffered saline, (PBS; pH 7.4), containing 1.0% EDTA. The tissue was rinsed in 0.9% saline, and endothelial cells were squeezed from these dermal sections into Eagle's Minimal Essential Medium (MEM) containing 10% pooled human serum and antibiotics, as pressure was applied with a blunt scalpel blade in a sweeping motion from the center of each tissue piece outwards to the periphery. Cells obtained from several foreskins were pooled, collected by centrifugation, and resuspended in MEM supplemented with 50% pooled human serum, cholera enterotoxin (CT) 1×10^{-9} M and the phosphodiesterase inhibitor isobutyl methylxanthine (IMX) 3.3×10^{-5} M, penicillin 100 U/ml, streptomycin 50 μg/ml, and gentamycin 25 μg/ml. Freshly isolated cells were plated onto petri dishes (Lux 35×10 mm) coated with a solution of fibronectin (25 μg/ml in 0.9% saline) (3), at a seeding density of $1-3 \times 10^4$ cells/dish.

Endothelial Cell Culture

Cells were maintained at 37°C in a humidified atmosphere of 6% CO_2 and 94% air, and fed twice weekly in MEM with 50% pooled human serum containing the supplements and antibiotics as described above. At confluence (~10 d after isolation) the cells were serially passaged. Cells from several dishes were pooled after incubation for 4 min at 37°C in 0.1% trypsin solution with 0.33% EDTA in

PBS, collected by centrifugation, and resuspended in the feeding medium before plating onto fibronectin-coated dishes. Cells were seeded at densities 5–75 × 10⁴ cells/dish to yield cultures that ranged from sparse to confluent immediately after cell attachment. The feeding medium was replaced with labeled medium (MEM with 10% human serum containing [³H]cholesterol, see below) 48–72 h after serial cultivation. All studies on cholesterol efflux and net transport were performed using first-passage, serially cultured cells approximately 2 wk after their isolation. The endothelial origin of the cells cultured was confirmed by morphological (light microscopic), ultrastructural (Weibel-Phalade bodies), or indirect immunofluorescence (Factor VIII antigen) criteria, as described elsewhere (3).

Large Vessel Endothelium

For comparison, sterol efflux in human umbilical vein endothelium, isolated as described earlier (3), was studied. For these experiments human umbilical vein cells were maintained in vitro as described above for cutaneous microvessel endothelium, except that they were plated onto plain plastic dishes and CT and IMX were not included in the feeding medium (9).

Plasma Immunoaffinity Chromatography

Human blood from normal donors was collected into ice-cooled sodium citrate (pH 7.4) at a final concentration of 0.01 M. After centrifugation at 4°C (1,000 g, 20 min) to remove blood cells, the plasma was freed of fibrinogen by passage through a column (2.5 × 30 cm) of immobilized antibody to human fibrinogen (10), equilibrated with 0.15 M NaCl - 1 mM disodium EDTA (pH 7.4). Complete removal of fibrinogen (confirmed by radial immunoassay) was not accompanied by the loss of detectable free or ester sterol or lipoprotein apoproteins (10).

Apolipoproteins A-I, B, and E were isolated from plasma by ultracentrifugation, delipidation, and chromatography on Sephadex and DEAE-cellulose, according to methodology previously detailed (11–13). Recrystallized human serum albumin was dissolved in 0.15 M NaCl, pH 7.4, then passed through a column of immobilized antibody to apo A-I to remove any traces of this apoprotein. Antibodies were raised to the pure proteins in rabbits. 0.1–0.5 mg of antigen was injected into multiple lymphatic sites in Freund's complete antigen. The animals were boosted once or twice under the same conditions. The IgG fraction was purified from the antibody plasma by DEAE cellulose chromatography (14) and, in the case of the antialbumin antibody plasma, by passing the purified IgG fraction from chromatography down columns of immobilized albumin coupled to Sepharose 4B (15). The bound specific antibody was eluted with 3M NaCNS (pH 7.0), dialyzed and complexed with CNBr-Sepharose 4B. Immunoaffinity chromatography was carried out by passing plasma down columns of immobilized antibodies to individual apolipoproteins or to albumin. The complete removal of antigen was confirmed by radial immunoassays previously described (7, 10).

Determination of Sterol Efflux

Cells cultured in MEM with 50% vol/vol pooled human serum were transferred to MEM with 10% vol/vol serum containing [³H]cholesterol (1–2 × 10⁷ dpm/ml). The label (~40 Ci/mmol, New England Nuclear) was purified by thin-layer chromatography (16), eluted and dissolved in ethanol, and injected into 20 vol of plasma. After dialysis against 0.15 M NaCl, 1 mM disodium EDTA the serum was diluted with MEM (10% vol/vol). To label the cells, they were incubated for 48 h with labeled medium, then washed once with human serum albumin (3 mg/ml) in the MEM, and twice with MEM alone. Efflux was determined from the rate of appearance of radioactivity in the unlabeled culture medium. Samples of medium were taken at zero time and 15–60 min thereafter. After the final samples were taken, the remaining medium was removed, the cells were washed with MEM solubilized in 0.1 M NaOH, and cell sterol was extracted into chloroform and methanol mixture (17). Portions of the chloroform phase and of medium samples were taken for liquid scintillation counting. Sterol mass was determined with cholesterol oxidase and esterase (18).

Determination of Sterol Net Transport

Sterol net transport is defined as the net mass transport of sterol from cells to plasma medium. Such transport is coupled to the esterification of sterol in plasma by lecithin:cholesterol acyltransferase (LCAT) (7). Since LCAT activity is unchanged in the presence of cells, sterol net transport is determined as the difference in the decrement of medium free sterol in the presence and absence of cells. Portions of medium were taken as described for efflux, extracted into chloroform and methanol, and portions of the chloroform phase were taken for analysis of free and ester sterol mass, as described above.

Photography

Light micrographs of sparse and confluent cultures were made with a Nikon Inverted microscope with phase contrast optics.

RESULTS

Characteristics of Sterol Efflux from Microvessel Endothelial Cells

In initial studies, the extent of equilibration of free sterol label with cellular cholesterol was determined. Labeled cells were incubated with lipoprotein-deficient serum ($d > 1.21$ g/cm³ infranatant solution) (4 mg/ml, <0.05 μg sterol/mg protein). Initial and final medium sterol mass and radioactivity were determined. The increment of medium sterol and radioactivity was determined under conditions where efflux of radioactivity was linear, and the ratio was compared with the specific activity of cell sterol, determined from its chloroform extract. The calculated specific activity of the increment of sterol in the medium in these experiments was $13,231 \pm 2,500$ dpm/μg sterol, compared with a cell specific activity in the same experiments of $11,504 \pm 1,497$ dpm/μg (eight determinations). These findings indicate an essentially complete equilibration of cell free sterol with the sterol efflux into the medium. Sterol ester was undetectable in endothelial cells cultured under the conditions described.

Efflux was determined as a function of time and plasma concentration. As shown in Fig. 1, the rate of appearance of cellular [³H]cholesterol radioactivity in the medium was linear for at least 60 min. As shown in Fig. 2, efflux was saturable with half-maximal efflux at $0.2 \pm 0.05\%$ vol/vol plasma. There was no further change in efflux rates as plasma concentration was increased to 100% vol/vol. When sterol net transport was assayed under the same conditions, in terms of the change in medium free sterol content, similar kinetics were obtained. Net transport was slightly lower than efflux at all concentrations.

Apoprotein Dependence of Efflux

In the case of cultured fibroblasts, efflux is in large part dependent upon the minor fraction of apo A-I (~0.05 of total plasma antigen) unassociated with other apolipoproteins (7). A smaller component of efflux is dependent upon albumin. The roles of individual apoproteins in the promotion of efflux were determined in human endothelial cells with plasma fractions prepared by immunoaffinity chromatography (Table I). Efflux from microvessel cells into human plasma medium was dependent almost entirely on the presence of plasma proteins.
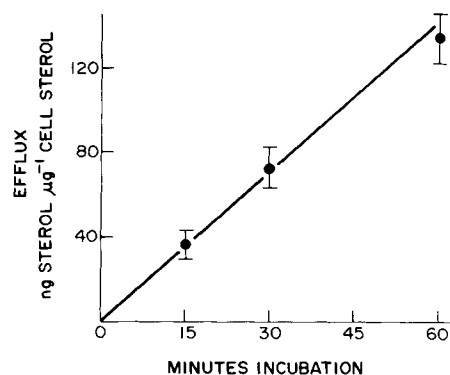

FIGURE 1 Time course of efflux of sterol from [³H]cholesterol-labeled microvessel endothelial cells. Four 3.5 cm dishes contained 0.90 ± .06 μg sterol and 1.5 ml of plasma (1.2% vol/vol) in minimal essential medium. After removal of an initial medium sample (0.1 ml), the cells were incubated at 37°C. At the intervals indicated, the same volume of medium was taken for analysis of sterol radioactivity. Values are means ± SD of efflux rates expressed in terms of the total original sample volume.

Removal of apo A-I removed ~60% of efflux-promoting activity from plasma. A smaller fraction (~30%) was lost when albumin was removed from the plasma. There was only minor decrease in efflux rates when apo B, the major protein of low density lipoprotein, or apo E, a protein implicated in sterol clearance by the liver (19), was removed. The dependence of efflux from umbilical vein endothelial cells was qualitatively similar but showed a greater dependence upon albumin and a correspondingly smalle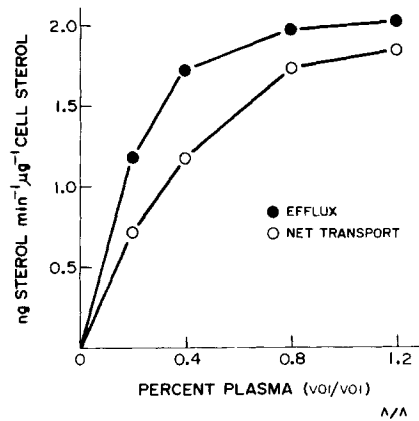r dependence upon apo A-I. The reason for this difference (Table I) is not known but may relate to differences in the architecture of the cell surface.

Density Dependence of Efflux

Endothelial cells were cultured to produce a range of cell densities from sparse to confluent (Fig. 3). Even at low seeding densities, serially passaged cutaneous microvascular endothelial cells retain the polygonal shape, the close apposition of cytoplasmic membranes, and the cobblestone appearance characteristic of bovine aortic and human umbilical vein endothelium at confluence (Fig. 3). The rate of efflux was highly dependent upon cell density, with an approximately fourfold

FIGURE 2   Efflux (●——●) and sterol net transport (O——O) from cultured microvessel endothelial cells as a function of medium plasma concentration. Incubation conditions were as in the legend to Fig. 1. Initial and final (60 min) samples of medium were taken for analysis of sterol radioactivity.

TABLE I

Apoprotein Dependence of Efflux of Cholesterol From Microvessel and Umbilical Vein Endothelial Cells

Plasma fraction	Microvessel cells	Umbilical vein cells
Whole plasma	1.0	1.0
Plasma – apo A-I	0.42 ± 0.01	0.68 ± 0.05
Plasma – apo B	0.88 ± 0.02	0.94 ± 0.02
Plasma – apo E	0.84 ± 0.07	0.97 ± 0.07
Plasma – albumin	0.67 ± 0.06	0.58 ± 0.03
MEM	0.03 ± 0.02	0.04 ± 0.03

Values are the means ± SD of triplicate determinations of the proportion of efflux from plasma (1.2% vol/vol in minimal essential medium) remaining after removal of the indicated protein antigen by immunoaffinity chromatography. Complete removal of antigen was confirmed immunologically in each case. Efflux was measured with cells labeled by preincubation (48 h, 37°C) with [^{3}H]cholesterol. Efflux was measured over a 60 min incubation period under the same conditions as described in Methods.
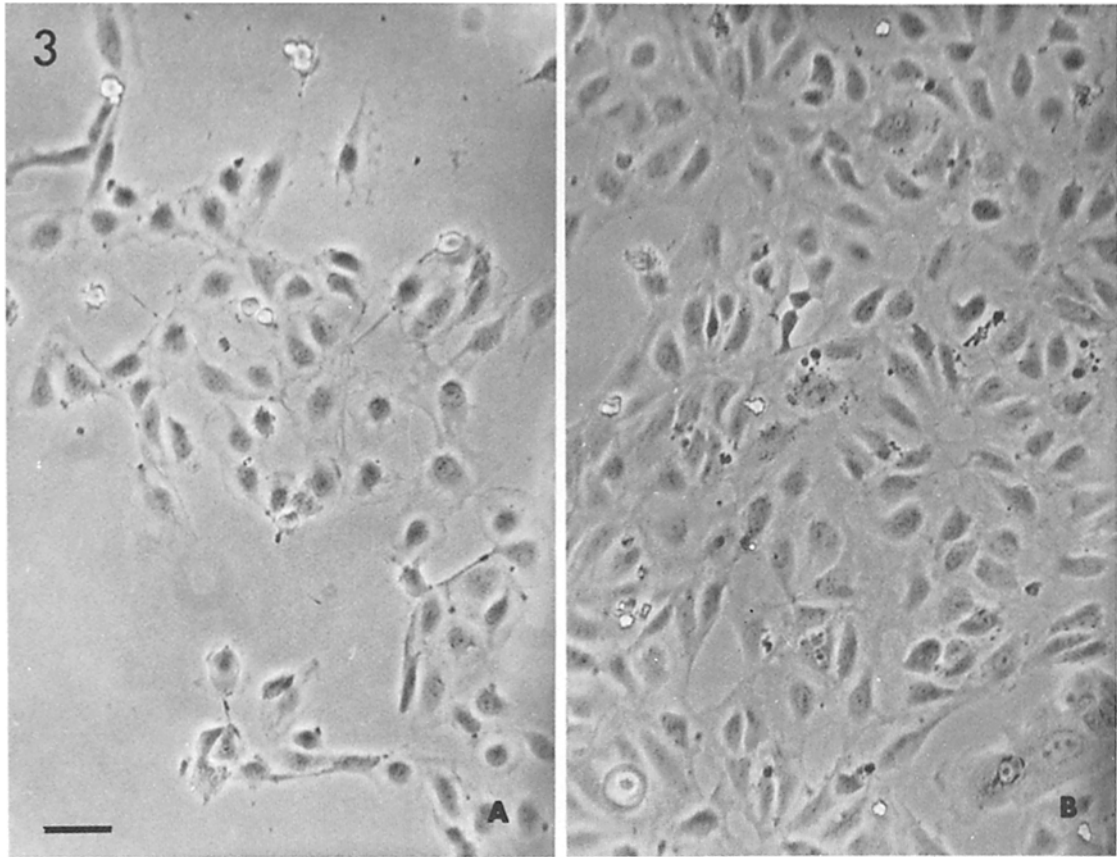
FIGURE 3   First-passage serially cultivated cutaneous microvascular endothelial cells, 48 h after transfer to 10% serum supplemented medium containing [^{3}H]cholesterol. These cells retain the characteristically endothelial polygonal morphology, have well-defined nuclei, and form colonies of cells with closely apposed cytoplasmic membranes in both sparsely (A) and more densely (B) seeded cultures. Bar, 50 μm.
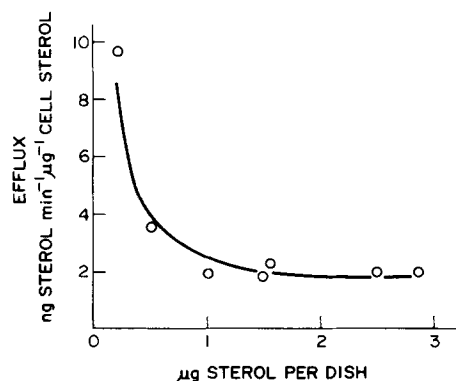
FIGURE 4 Dependence of efflux rate from cultured microvessel endothelial cells on cell density. Initial and final (60 min) samples of medium were collected from ^{3}H-labeled sparse, intermediate, or confluent cell cultures. Cell sterol specific activity was determined at each cell density and efflux calculated as described in Materials and Methods.
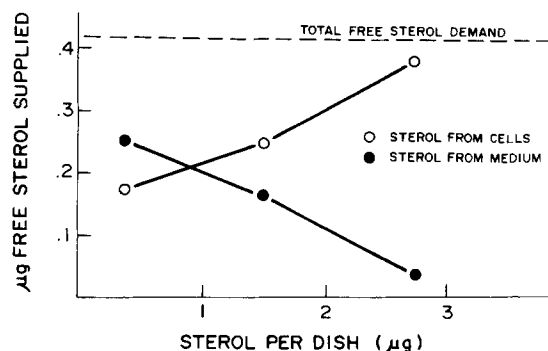

FIGURE 5 Dependence of endothelial cell contribution to sterol demand by lecithin:cholesterol acyltransferase as a function of cell density. Sterol net transport was determined from the initial and final medium sterol mass measurements of microvessel endothelial cells incubated in 1.2% vol/vol plasma in minimal essential medium for 60 min at 37°C. Total sterol demand by transferase (-----) was determined from the decrease in cell sterol in empty dishes incubated simultaneously with the same plasma medium. Cellular sterol transport (O-----O) was obtained from the incubation measurements from dishes containing endothelial cells and the medium sterol contribution (●——●) by difference.

difference between sparsely and densely packed cultures (Fig. 4). Efflux declined as cell density increased, reaching a minimum when less than half the area of the dish was covered. A similar relationship between efflux rate and cell density was observed with umbilical vein endothelial cells (unpublished observations). The rate of efflux from endothelial cells was twofold higher than from fibroblasts into the same plasma medium (7) at the same cellular cholesterol concentration.

Sterol net transport was also strongly dependent upon cell density. Net transport per cell decreased approximately fourfold over the indicated density range. However, the major effect of cell density was upon the contribution of cell membrane sterol to lecithin:cholesterol acyltransferase (Fig. 5). Confluent endothelial cells contributed a mass of sterol to the medium almost equivalent to the total required for esterification. By comparison, under equivalent conditions, cultured fibroblasts contributed less than half of sterol used for transferase activity (7).

DISCUSSION

Sterol efflux rates from microvessel endothelial cells have not been previously reported, and the present study demonstrates that these cells are highly active in this regard. At cellular cholesterol concentrations comparable to those used in earlier studies of fibroblasts (0.3–0.35 μg sterol cm^{-2} cell surface), efflux from the endothelial cells was ~2.0 ng sterol/min per μg sterol, double the rate for fibroblasts under these conditions (7). Hepatocytes, also exposed to high plasma sterol concentrations via fenestrated endothelium also show high rates of sterol efflux (20). It is notable that even in endothelial cells cultured in 50% human serum, there was no accumulation of sterol ester in the cells. This finding illustrates the ability of these cells to maintain sterol balance in the face of high medium sterol concentration to which they are exposed under normal physiological conditions in human plasma. Rapid efflux may therefore represent an adaptation of the cells to this environment. Efflux is saturable with endothelial cells, as with other cultured cell systems (6, 20–22). Because of the low apparent K_m for efflux into plasma (~0.25% vol/vol), the cells in vivo will express maximal efflux rates. Several times greater efflux rates are found with sparse as compared to more dense cultures, with the decrease in efflux essentially complete when the cultures are ~50% confluent. Previous research has demonstrated that endothelial cells bind and interiorize low density lipoprotein (LDL) in cultures of actively dividing endothelial cells. This occurs at cell concentrations where the rate of efflux of cholesterol, as shown in the present study, has already reached its minimum. At much higher cell density, where the endothelial monolayer becomes contact-inhibited, the internalization of LDL is blocked. Therefore, it is apparent that while both of these phenomena are density dependent and serve to modulate cellular cholesterol content, their regulation occurs at very different cell concentrations and it is unlikely that they are related.

Efflux is only significant metabolically in terms of cellular sterol regulation if associated with net mass transport of sterol between cells and their medium. The requirement for free sterol in human plasma is represented essentially by the rate of lecithin:cholesterol acyltransferase. As previously reported, when cell membranes and lipoproteins are both present, both can contribute to provide the sterol needed for esterification. Confluent cultures of human fibroblasts under the conditions used here provided about one-half of sterol needed by the LCAT reaction (7). In confluent microvessel cells, where efflux was double the rate found with fibroblasts, sterol mass transport coupled to LCAT was proportionately increased. Since the total demand for sterol is unchanged by the presence of cells, these findings, taken together, indicate that endothelial cells are very efficient in donating sterol for esterification. This question was addressed directly by the experiments on cell density, in which cell membrane sterol was varied at a constant level of sterol in plasma lipoprotein form. As shown in Results, in confluent endothelial cells almost the whole of sterol required for the LCAT reaction was provided not by plasma lipoproteins, but by the cells.

It could be argued that while the predominance of cellular sources of sterol might be significant at low plasma concentrations, in undiluted plasma the reverse might be the case. However, while plasma concentrations used in this study were dilute, calculation suggests they are quite appropriate for the study of endothelial sterol metabolism in vivo. The data in this

study indicate that increasing availability of cell sterol can limit the contribution of the plasma lipoproteins to the LCAT reaction. In a microvessel of 6 μM lumen diameter, each milliliter of plasma will be associated with 600 cm^2 of endothelial surface. For arterioles or venules of 25-μM diameter, the corresponding value is 150 cm^2. In the present experiments where plasma (1.2% vol/vol) was incubated with endothelial cells at confluence in 3.5-cm dishes, 1 ml of medium was exposed to 9.6 cm^2 endothelial surface. The equivalent surface area, in terms of the sterol used by LCAT in undiluted plasma, is (9.6/0.012) cm or 800 cm^2 of endothelial surface per milliliter of plasma. The data from this study therefore are consistent with the suggestion that in microvessels in vivo, the major part of sterol required by the LCAT reaction could be provided not by plasma lipoproteins, but by cell membranes.

In microvascular endothelium, as in fibroblasts, efflux was largely dependent upon the presence of apolipoprotein A-I. Apo A-I unassociated with other apoproteins has been shown in an earlier study to function as a major sterol carrier in plasma. A smaller proportion of efflux is dependent upon the presence of albumin. Efflux was minimal in the absence of plasma, discounting a significant contribution of apolipoproteins secreted from these cells to efflux. In neither case did apo B, the protein of LDL, the major plasma sterol carrier, contribute significantly to efflux. Apo E, an apoprotein associated in plasma with high free sterol levels, did not contribute substantially to efflux in these studies. The results suggest that the characteristics of sterol metabolism in plasma after the efflux step of net transport, are largely independent of the cells present. The rapid efflux in microvascular endothelium, coupled to a more effective mass transport of sterol in competition with plasma lipoproteins, may provide a specific and efficient mechanism by which these endothelial cells protect themselves against the high levels of circulating plasma sterol to which they are at all times exposed.

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