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MODULATION OF MEMBRANE COMPOSITION OF SWINE VASCULAR SMOOTH MUSCLE CELLS BY HOMOLOGOUS LIPOPROTEINS IN CULTURE

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Summary

Swine vascular smooth muscle cells were exposed to homologous low-density or high-density lipoprotein fractions for 24 h. Total cell membranes were isolated from the post-nuclear supernatant of the cell homogenates, fractionated by sucrose density gradient centrifugation and characterized by enzyme assays. The membrane fraction with the lowest density was enriched in plasma membrane marker enzymes. Cholesterol analysis showed that cells exposed to low-density lipoprotein had higher cholesterol-to-protein ratios in total cells, total cell membranes and individual membrane fractions than had the cells exposed to high-density lipoproteins. Cholesterol-to-phospholipid ratios of the plasma membrane-enriched fraction from cells exposed to low-density lipoprotein were higher than the same membrane fraction of cells exposed to high-density lipoprotein. Studies with iodinated lipoproteins showed that these compositional changes could not be due to lipoprotein contamination. Membrane microviscosity was determined by fluorescence depolarization with diphenylhexatriene and the microviscosity of the plasma membrane-enriched fraction was different in the cells exposed to the two different lipoprotein fractions. This difference in membrane microviscosity was significant only when the medium

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Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; apoprotein E, the arginine-rich apoprotein of very low density and high-density lipoproteins of molecular weight approx. 37 000.

cholesterol content was 40 μg per ml or greater; cells exposed to low-density lipoprotein gave membranes with higher microviscosity.

These results demonstrate that the properties of vascular smooth muscle cell membranes are influenced by exposure of the cells to homologous lipoprotein fractions.

Introduction

In recent years it has become apparent that cell membranes are more than passive permeability barriers around and within the cell. This realization has fortunately coincided with the development of new techniques and hypotheses which can be applied to understanding membrane structure and function. Our views of membrane structure have gradually progressed in increasing degrees of complexity from the early bilayer model to the fluid mosaic model. It is implicit in this latter model that the membrane be in a fluid state and that constraints on fluidity of cell membranes are necessary for optimal cell function. Membrane fluidity can be estimated by a number of techniques and may be a useful monitor of membrane structure.

Studies with lipid vesicles [1] and red cell membranes [2] have shown correlations between microviscosity and lipid composition, particularly the cholesterol: phospholipid ratio. Borochov et al. [3] have shown that alterations in membrane fluidity, as monitored by changes in the fluorescence depolarization of diphenylhexatriene, cause changes in the amount of exposure of membrane proteins to the aqueous environment. Such changes in protein orientation could modulate enzymic properties of the membrane of the cell. Cooper [4] has postulated that alterations in membrane fluidity are associated with, and may possibly cause, the cell dysfunctions seen in disease states.

Numerous studies with cultured vascular smooth muscle cells have shown that plasma lipoproteins modulate cell lipid composition and cell lipid synthesis [5—7] but no detailed analysis has been made of the effect of lipoproteins on the composition of subcellular membranes of the vascular smooth muscle cell. Some lipoproteins are known to be taken up through a process of binding to high-affinity receptor sites on the cell surface; internalization and degradation of these particles leads to inhibition of hydroxymethylglutaryl CoA reductase activity, decreased synthesis of receptor sites, activation of cholesterol esterification, and an increased content of free cholesterol and cholesterol ester per cell. It is unclear whether this free cholesterol is present in the form of an increased pool of membranes, or in a pool of membranes of altered composition or in some non-membraneous pool. It is attractive to suggest that plasma lipoproteins could alter membrane composition of vascular smooth muscle cells in situ to bring about changes in membrane fluidity which could alter cellular metabolism [8].

In this report we have demonstrated that a plasma membrane-enriched fraction can be isolated from swine vascular smooth muscle cells, and that both composition and microviscosity are determined by the lipoprotein species in which the cells have been incubated.

Methods

Cell Culture. Vascular smooth muscle cells were isolated by enzymatic dispersal of the aortic media of 30—40-kg white pigs according to the methods of Chamley-Campbell et al. [9]. Cells were inoculated at initial densities of $50 \cdot 10^3$ cells per cm^2 in M 199 medium (Grand Island Biological Co.) containing 10% fetal calf serum and were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. Upon reaching confluency the cells were treated with trypsin and subcultured. The swine vascular smooth muscle cells used in these experiments were passaged once.

Plasma lipoprotein preparation. Lipoproteins were isolated from swine plasma by isopycnic centrifugation according to the method of Havel et al. [10]. The fractions were desalted and purified by gel filtration on agarose 5 M (Biorad Inc.) in 5 mM sodium phosphate, 0.15 M NaCl containing 1 mg EDTA per ml (pH 7.4 at 4°C). The lipoprotein-free serum was desalted on Sephadex G-25 in this same buffer. When necessary, serum fractions were concentrated by ultrafiltration with a UM 10 membrane (Amicon Corp.).

Membrane preparation. Cells were incubated 48 h in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) containing lipoprotein-free serum at a concentration of 2.5 mg protein per ml. After removal of the medium, cells were incubated in Dulbecco's modified Eagle's medium containing low-density lipoprotein (LDL) or high-density lipoprotein (HDL), and lipoprotein-free serum at a concentration of 2.5 mg protein per ml. After a further 24 h the medium was replaced with Dulbecco's modified Eagle's medium for 1 h and the cells were then washed three times with Dulbecco's modified Eagle's medium containing 2 mg per ml of bovine serum albumin and once with Dulbecco's modified Eagle's medium alone. The cells were collected by scraping, washed, and resuspended in 1 mM triethanolamine hydrochloride, 0.15 M NaCl and disrupted by nitrogen cavitation at 800 $lb/inch^2$ for 20 min (Kontes Mini Bomb). The nuclei were removed by centrifugation at $1000 \times g$ for 5 min and the postnuclear supernatant was centrifuged at $205\,000 \times g$ for 1 h with the membranes pelleting onto a 0.1 ml layer of 55% sucrose in 1 mM triethanolamine hydrochloride/0.1 mM EDTA, pH 8.0. The membrane band was removed, dispersed in the same buffer without sucrose, and layered on a discontinuous sucrose gradient [11]. After centrifugation at 41 000 rev./min in an SW 41 rotor for 16 h the gradient was pumped off from the bottom to yield 25 to 30 fractions which were assayed for protein content with fluorescamine [12].

Enzyme and chemical assays. Ouabain-sensitive $(Na^+ + K^+)$-ATPase was assayed by the fluorimetric method of Takashi and Putnam [13] where the fractions were pre-incubated with 2.5 mM ouabain at room temperature for 5 min. 5'-Nucleotidase was assayed by using the radioactive procedure of Avruch and Wallach [14]. NADPH- and succinate- cytochrome *c* reductases were assayed spectrophotometrically according to the method of Sottocasa et al. [15] using an Aminco DW2 in the dual-wavelength mode. Lysosomal hexosaminidase was assayed with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide [16].

Free cholesterol content and fatty acid composition were determined by gas-liquid chromatography as described previously [17]. Total cholesterol was

determined after hydrolysis with 10% methanolic KOH. From the free cholesterol and fatty acid contents of the membrane fractions the cholesterol-to-phospholipid ratio was calculated by assuming that two fatty acid residues are equivalent to one phospholipid molecule. The validity of such an assumption has been confirmed in similar membrane systems [17]. Membrane microviscosity was determined according to the method of Shinitzky and Barenholz [18], utilizing the lipid-soluble fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene. All measurements were taken at 37°C in a Hitachi Perkin Elmer Spectrofluorimeter Model MPF-44A. Microviscosity values ($\overline{\eta}$) are calculated from the Perrin equation:

$$r_0/r = 1 + C(r)\, T\tau/\overline{\eta}$$

where r_0 = limiting anisotropy of the probe, taken as 0.362; r = measured anisotropy, defined as $(I_{/\!/}/I_{\perp} - 1)/(I_{/\!/}/I_{\perp} + 2)$. $I_{/\!/}$ and $I_{\perp}$ are the experimentally determined fluorescence intensities parallel and perpendicular, respectively, to the direction of polarization of the excitation beam. $C(r)$, a function of the molecular shape of the probe, is taken as $8.6 \cdot 10^5$ P · deg^{-1}; T = absolute temperature; τ = excited state lifetime, taken as 6 ns at 37°C, from studies with lecithin liposomes [18]. This latter parameter is assumed to remain constant with variations in membrane composition, and has in fact been shown to remain essentially constant in lipoprotein systems, where lipid: protein proportions vary widely [19]. Light scattering was less than 3% and fluorescence values were corrected accordingly. The phospholipid : diphenylhexatriene ratio was always maintained at greater than 200 : 1 (mol/mol) in order to minimize probe-probe interactions and/or perturbations of the membrane bilayer.

Results

Separation of membrane fractions

Discontinuous sucrose density gradient centrifugation of the membranes isolated from the post-nuclear supernatant of disrupted vascular smooth muscle cells gave the pattern shown in Fig. 1. The position of the peaks and the relative proportions of protein in the peaks are highly reproducible and are similar, as shown for experiments with several levels of either LDL or HDL. Peak fractions were pooled as shown and designated as fraction 1, 2 and 3. From these separations of swine vascular smooth muscle membranes 65 to 98% of the applied protein was recovered in these three membrane fractions. A very similar pattern was seen with rat vascular smooth muscle cells. From previous studies [11] these fractions should be enriched in mitochondria, microsomes and plasma membrane, respectively. This designation was essentially confirmed by enzyme marker assays shown in Table I. Compared to fraction 3; fraction 1 is enriched in succinate cytochrome *c* reductase, a mitochondrial marker, but has activities of 5′-nucleotidase and NADPH-cytochrome *c* reductase suggesting fraction 1 contains some aggregated total membranes. Compared to fraction 3, fraction 2 is enriched in NADPH-cytochrome *c* reductase, a marker for endoplasmic reticulum, but has some succinate-cytochrome *c* reductase and 5′-nucleotidase. Fraction 3 is enriched in 5′-nucleotidase and ouabain-sensitive ($Na^+ + K^+$)-ATPase, is free of succinate-cytochrome *c* reductase, and is mini-

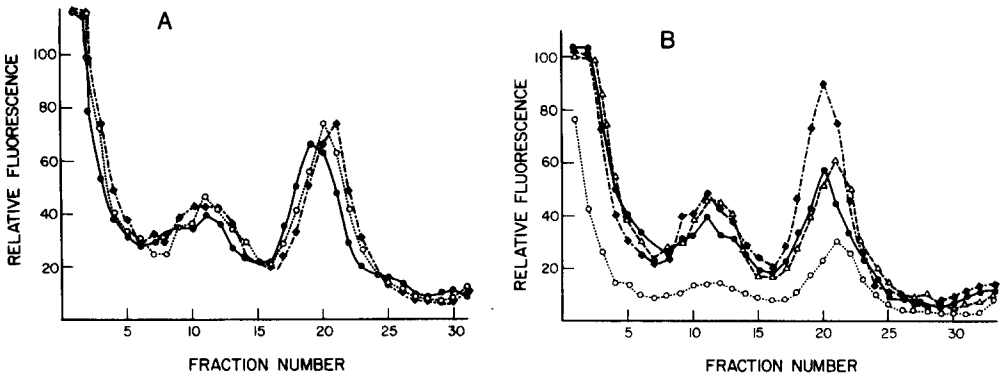

Fig. 1. Resolution of the total membrane fraction of vascular smooth muscle cell homogenates. The total membrane fraction was subjected to sucrose density gradient centrifugation as described in Methods and each fraction was assayed for protein by the fluorescamine assay. The relative fluorescence by this assay is shown for cells exposed to LDL (A) and HDL (B) at medium cholesterol concentrations of 10 (●———●), 50 (△———△), 100 (◆———◆) and 200 (○———○) μg cholesterol per ml. Fractions were pooled and designated as follows: 1—5 (fraction 1), 7—14 (fraction 2) and 17—25 (fraction 3). The total recovery of membrane protein from the gradient in these experiments was 76.8 ± 10.7%.

mally contaminated with NADP-cytochrome *c* reductase. Fraction 3 can be considered to be plasma membrane-enriched.

Effect of lipoproteins on cell and membrane cholesterol content

When the effect of homologous lipoproteins on vascular smooth muscle cells was examined, it was found that LDL produced higher total cholesterol-to-protein ratios in both intact cells and isolated membranes (Table II). Analysis of the free cholesterol content of the three membrane fractions were made (Table II). Under all conditions, fraction 3 has the highest cholesterol-to-protein ratio supporting our contention that this fraction is predominantly plasma membrane. The cholesterol content of fraction 3 is significantly higher when cells have been incubated with LDL than with HDL. However, all membrane fractions were similarly affected. The increments in cholesterol content of fractions 1, 2 and 3 were 34, 41, and 37%, respectively.

TABLE I

MEMBRANE MARKER ENZYMES IN MEMBRANE FRACTIONS RESOLVED BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

The pooled membrane fractions isolated by sucrose density gradient centrifugation as described in Methods were assayed for the membrane marker enzymes as described in Methods. The data shown are the means (±S.D.) of four determinations and are expressed as the specific activity relative to the membrane homogenate.

Sucrose gradient fraction	Succinate-cytochrome *c* reductase	NADPH-cytochrome *c* reductase	5′-Nucleotidase	Ouabain-sensitive ATPase
3	0.17 ± 0.03	0.46 ± 0.09	1.21 ± 0.06	21.25 ± 2.86
2	0.55 ± 0.08	0.62 ± 0.04	0.67 ± 0.06	6.78 ± 4.29
1	0.92 ± 0.33	0.85 ± 0.11	0.34 ± 0.04	0.71 ± 0.54

TABLE II

EFFECT OF LIPOPROTEINS ON THE CHOLESTEROL CONTENT OF SWINE VSM CELLS AND MEMBRANES

Swine vascular smooth muscle cells were exposed for 24 h to homologous lipoprotein and the cells were harvested as described in Methods. Cholesterol content of cells and membranes was determined as described in Methods. For cells and total membranes the data shown are the total (free plus esterified) cholesterol. For the fractions the data are for free cholesterol, esterified cholesterol content was less than 5% of these values. For experiments where cells and total membranes were assayed the lipoprotein concentrations were 100 μg cholesterol per ml, in other experiments data are combined from experiments where the cholesterol concentrations were either 100 or 200 μg per ml. Total membranes were isolated from the post-nuclear supernatant of the cell homogeneate. Fractions 1 to 3 were obtained by sucrose density gradient centrifugation as described in Methods.

Lipoprotein	Cholesterol concentration (μg/mg protein)				
	Cells	Total membranes	Fraction 1	Fraction 2	Fraction 3
HDL	18.3 ± 2.1	36.6 ± 2.2	19.0 ± 0.1	15.5 ± 1.1	47.4 ± 0.4
LDL	25.6 ± 1.1	45.7 ± 1.8	23.9 ± 1.6	21.5 ± 0.1	66.6 ± 1.3

Effect of lipoprotein on microviscosity and composition of fraction 3 (plasma membrane enriched)

Two experiments are reported in Table III which gave qualitatively similar results but differ somewhat in quantitative analysis. The data in experiment A show that cells exposed to LDL have higher membrane microviscosity, cholesterol-to-protein ratios and cholesterol-to-phospholipid ratios, compared to HDL exposed cells. There is no significant difference in the phospholipid-to-protein ratio or in the double bond index-saturated fatty acid ratio. There was the expected correlation between mean membrane microviscosity and mean cholesterol-to-phospholipid ratio, but no concentration-dependence was seen with medium cholesterol content. In experiment B, where more data for microviscosity were obtained, a correlation between membrane microviscosity and medium cholesterol concentration was seen. The difference in microviscosity of membrane fraction 3 for LDL-exposed compared to HDL-exposed cells is not significant at low concentrations of cholesterol but increases in significance as the cholesterol concentration increases. Similarly, the cholesterol-to-phospholipid ratios are identical, in experiment B (Table III), at low incubating cholesterol concentrations but appear to diverge at medium concentrations of 80 and 190 μg cholesterol per ml. It is of interest that the data in Table IIIB suggest that the effect of increasing lipoprotein concentration is to produce a lowering of cholesterol-to-phospholipid ratio and microviscosity brought about by HDL rather than increase in these parameters by LDL.

Plasma lipoproteins

The lipoproteins used in these studies were obtained from normal chow-fed animals. Microviscosities were obtained for the two species of lipoprotein as follows (in P at 37°C) LDL, 2.30 and HDL, 2.24. These may be compared to values obtained for human LDL and HDL of 3.16 and 2.69 P, respectively [19]. Polyacrylamide gel electrophoresis [20] of the lipoproteins showed no cross contamination between the LDL and the HDL fractions.

TABLE III

EFFECT OF LIPOPROTEINS ON THE PROPERTIES OF MEMBRANE FRACTION 3 OF SWINE VASCULAR SMOOTH MUSCLE

Membrane fraction 3, obtained by sucrose density gradient centrifugation of the vascular smooth muscle cell membranes, was analyzed for phospholipid content (PL) and free cholesterol content (CHOL). The membrane microviscosity ($\bar{\eta}$) was determined with diphenylhexatriene as described in Methods. The double bond index-saturated fatty acid ratio (DBI) was calculated from the fatty acid analysis data and is equal to the number of moles of double bonds divided by the number of moles of saturated fatty acid residues. Statistical significance of the difference between the mean values for HDL-exposed cells vs. LDL-exposed cells (Students *t*-test). n.d. not determined: protein content of these membranes was not determined but cholesterol and phospholipid were determined on the same sample hence the CHOL/PL ratios are valid.

Expt.	Lipoprotein (Cholesterol, μg/ml)	$\bar{\eta}$ (P)	Content		CHOL/PL	DBI
			PL (μ mol/mg protein)	CHOL		
(A)	HDL 10		0.25	0.11	0.44	0.72
	40		0.30	0.12	0.40	0.91
	80		0.28	0.12	0.43	0.94
	200		0.25	0.12	0.48	0.59
	Mean ± S.D.	1.44 ± 0.11	0.27 ± 0.02	0.12 ± 0.01	0.44 ± 0.03	0.79 ± 0.14
	LDL 10		0.28	0.15	0.54	0.61
	40		0.30	0.16	0.53	0.55
	80		0.33	0.18	0.55	0.86
	200		0.29	0.17	0.59	0.50
	Mean ± S.D.	1.60 ± 0.12	0.30 ± 0.02	0.17 ± 0.01	0.55 ± 0.02	0.63 ± 0.14
		$P = 0.001$	P not significant	$P < 0.02$	$P < 0.05$	P not significant
(B)	HDL 8	2.15 ± 0.15 [a]	n.d.	n.d.	0.46	1.47
	40	2.13 ± 0.07 [b]	n.d.	n.d.	0.55	1.28
	80	2.07 ± 0.09 [c]	n.d.	n.d.	0.30	1.30
	190	1.91 ± 0.13 [d]	n.d.	n.d.	0.30	1.34
	LDL 8	2.19 ± 0.10 [a]	n.d.	n.d.	0.47	0.71
	40	2.29 ± 0.10 [b]	n.d.	n.d.	0.56	0.94
	80	2.22 ± 0.07 [c]	n.d.	n.d.	0.45	1.64
	190	2.14 ± 0.09 [d]	n.d.	n.d.	0.50	1.13

The statistical significance between the HDL-exposed vs. the LDL-exposed cells for membrane fractions having the same superscript (a—d) by Student's *t*-test were: [a] $P > 0.5$, [b] $P < 0.002$, [c] $P < 0.002$ and [d] $P < 0.001$.

Contamination of cell membranes:

Contamination of membranes, particularly plasma membrane, by absorbed lipoprotein might be predicted to alter membrane microviscosity and membrane lipid composition. The data presented previously provide some indirect evidence that this is not occurring. In Table III, experiment B, the decrease in microviscosity and cholesterol-to-phospholipid ratio as the HDL concentration is raised could not be due to an increase in the adherent HDL as this could not lower the microviscosity (membrane 2.15 to 1.91; HDL 2.24) and cholesterol-to-phospholipid ratio. In addition we directly evaluated the possibility of con-

TABLE IV

ESTIMATION OF CONTAMINATION OF CELL MEMBRANES BY ADHERENT LIPOPROTEINS

Cells were incubated with ^{125}I-labeled lipoproteins (LDL and HDL at 2.8 and 4.8 cpm per ng protein, respectively) at media cholesterol concentrations of 10—200 μg cholesterol per ml. The cell membranes were isolated and fractionated as described in Methods and the membrane-associated radioactivity was determined. The same cell membranes were assayed for total cholesterol content (Memb. Chol.). If it is assumed that the ^{125}I represents intact LDL or HDL the associated protein (LP Protein) and cholesterol (LP Chol.) can be calculated. The ratio of the latter to the actual total membrane cholesterol (Memb. Chol.) gives the contamination. The data shown are from incubations at a cholesterol concentration of 190 μg per ml of incubation medium.

		Radioactivity (cpm × 10^{-3})	LP Protein (μg)	LP Chol. (μg)	Memb. Chol. (μg)	% contamination (based on Chol.)
LDL	Total Mb.	12	4.30	6.51	94.5	6.9
	fraction 3	2.78	0.99	1.50	25.5	5.9
	fraction 2	1.03	0.37	0.55	4.6	12.0
	fraction 1	1.08	0.39	0.58	19.0	3.1
HDL	Total Mb.	24	5.04	2.67	70.0	3.8
	fraction 3	5.51	1.16	0.61	23.4	2.6
	fraction 2	2.66	0.56	0.30	6.8	4.4
	fraction 1	4.62	0.97	0.51	10.7	4.8

tamination as follows: ^{125}I-labeled lipoproteins were incubated with swine vascular smooth muscle cells under the normal conditions and the amount of ^{125}I associated with each cell fraction was estimated. The results are shown in Table IV. Of the intracellular ^{125}I less than 20% sediments with the membrane fraction with either LDL or HDL. The remaining 80% of the radioactivity remains in the post-membrane supernatant. All the cholesteryl ester in these cells was also in the supernatant fraction. Of the radioactivity which is associated with the membrane fraction, there is no specificity of the distribution when the membranes are subsequently fractionated by sucrose density gradient centrifugation (Table IV). Likewise, there is no lipoprotein specificity. The radioactivity from either LDL or HDL is distributed throughout the gradient in proportion to the membrane protein. If it is assumed that radioactivity in each membrane fraction represents native lipoprotein then the amount of cholesterol associated with this quantity of lipoprotein can be calculated. Knowing the actual cholesterol content of the individual fractions when the concentration of the cholesterol in the medium was 190 μg per ml (the maximum used) the maximum possible contamination of fraction 3 with cholesterol from absorbed LDL and HDL would be 5.9 and 2.6%, respectively. This is clearly less than the differences seen in fraction 3 cholesterol between cells exposed to LDL compared to those exposed to HDL.

Estimation of lysosomal contamination of plasma membranes

The possibility that alterations in the cholesterol content of the membrane fractions might be due to lysosomally contained cholesterol was also examined by considering the known distribution of the lysosomal enzyme, hexosaminidase. Of the activity of this enzyme present in the original cell homogenate 86% (84—88%) remained in the supernatant when total cell membranes were isolated. Of the membrane-associated hexosaminidase, 6% or less was associated

with fraction 3. Thus, intact lysosomes are not significant contaminants of this membrane fraction.

Discussion

The data presented indicate that the cholesterol contents of vascular smooth muscle cells and a vascular smooth muscle plasma membrane-enriched fraction are influenced by exposure of the cells to homologous lipoproteins. Several workers have previously demonstrated an increase in total cholesterol or cholesteryl ester content of cells and a stimulation of cholesterol esterification by cells upon their exposure to LDL or apoprotein E-containing HDL. [5—7]. It has not previously been determined how, if at all, this increase in cell cholesterol content affects the composition or properties of subcellular membrane fractions. To answer this question it was necessary to isolate and characterize the membranes of vascular smooth muscle cells.

Methods for isolation of specific cell membranes from vascular smooth muscle cells have not been extensively described. One recent procedure [21] using borate to stabilize the plasma membrane would be, we felt, unsuitable for our studies as the membrane fluidity might be severely altered by this procedure. We chose to use the procedure developed by Kent et al. [11] for isolation of membranes from chick muscle cells in culture. This discontinuous gradient reproducibly gave three protein fractions when total membranes from the postnuclear supernatant were applied. In contrast to the results of Fowler et al. [22], we found the equilibrium positions of these three membrane fractions were uninfluenced by the nature or concentration of the lipoprotein to which the cells were exposed. Chick muscle cell membranes when subjected to this procedure yield fractions which are enriched in mitochondria, endoplasmic reticulum and plasma membrane. Based on enzyme markers for these membranes we found the same general separation although only one fraction was appreciably enriched in marker enzymes. The fraction with the lowest density, fraction 3, was enriched in plasma membrane marker enzymes and has been most extensively studied in this report.

In a cultured cell system such as that described in the present report it is difficult to choose a control culture medium to which other culture conditions may be compared. We chose instead to compare the effects produced by LDL to those produced by (HDL). These two lipoproteins would be expected to, respectively, increase (LDL) and either decrease or not change HDL cholesterol compared to incubation of cells in lipoprotein-free serum. In an effort to measure a general membrane property we monitored membrane microviscosity as reported by the fluorescent probe, diphenylhexatriene. Fluorescence depolarization has been previously utilized to assess the fluidity of biological membranes in terms of a microviscosity ($\overline{\eta}$) which can be calculated from the Perrin equation. However, the results obtained with this probe should be evaluated with certain qualifications. Initially, it must be considered that microviscosity parameters so obtained do not represent absolute values, since this will in general depend on the choice of reference standard. Although natural membranes are inherently anisotropic, the Perrin equation assumes that the probe is subject to rotational diffusion in an isotropic medium. Thus, microviscosity cannot

be considered an exact analogue of bulk viscosity. The recent publication of Lakowicz et al. [23], although critical of the use of fluorescence techniques to measure microviscosity, did demonstrate that the relative microviscosities of two model membrane systems was relatively independent of the molecular process used in the determinations. The absolute microviscosity of any one membrane could, however, vary by 100-fold depending on the method used.

Our data demonstrate, for the first time, that membrane cholesterol-to-protein and cholesterol-to-phospholipid ratios are elevated in cells exposed to LDL relative to those exposed to HDL. Following membrane fractionation by sucrose density gradient centrifugation the fraction with the lowest density, fraction 3, had the highest cholesterol-to-protein ratio. This ratio in this fraction was greatly influenced by LDL compared to HDL. The high cholesterol-to-phospholipid ratio seen in fraction 3 also indicates that this fraction is enriched in plasma membrane. When membrane microviscosity was determined upon fraction 3 there was a correlation between cholesterol-to-phospholipid ratio and microviscosity. No significant difference was found, in fraction 3 from LDL- compared to HDL-exposed cells, in phospholipid-to-protein ratio or double bond index-saturated fatty acid ratio. Previous studies with lipid vesicles [1] and red cell membranes [2], have shown a correlation of cholesterol-to-phospholipid ratio with microviscosity. On the other hand, studies with liver endoplasmic reticulum from estrogenized roosters [17] show a clear correlation between double bond index-saturated fatty acid ratio and microviscosity. We do not think the double bond index-saturated fatty acid ratio is without influence in this system. The experiment which showed a concentration dependence upon lipoprotein cholesterol of microviscosity and cholesterol-to-phospholipid ratio (Table III, expt. B) demonstrates that changes in these parameters (LDL compared to HDL) are seen at 40—80 μg cholesterol per ml. In similar experiments to those reported here, we have investigated the modulation of microviscosity of rat vascular smooth muscle cells by homologous rat lipoproteins. In contrast to the results with swine cells and lipoproteins, no increase in microviscosity is seen in the rat cells unless very high concentrations of LDL cholesterol are included in the incubating medium (350 μg cholesterol/ml), despite the fact that lower concentrations of lipoprotein do increase the cholesterol-to-protein ratio of the plasma membrane of the rat cells, as these lower concentrations do in the experiments with swine. A striking difference between the swine and rat cells is the high double bond index-saturated fatty acid ratio of the rat plasma membrane fraction, approx. 4-fold more unsaturated than the corresponding value for the swine cells. This may permit incorporation of cholesterol without a rise in microviscosity and may have relevance to the difference in the propensity of these two species to develop atherosclerosis.

Our data also suggest the differences seen between the effect of LDL and HDL are due to a lowering of both microviscosity and cholesterol-to-phospholipid ratio with incubation in increasing concentrations of HDL. Glomset's [24] hypothesis that HDL functions to scavenge cholesterol from cells would suggest that cells lose cholesterol from the plasma membrane to HDL. Cholesterol exchange to HDL apoproteins complexed to phospholipids has been demonstrated by Stein et al. [25]. Our data directly demonstrate a concentration-dependent effect of HDL on plasma membrane microviscosity and cho-

lesterol-to-phospholipid ratio. This may reflect loss of plasma membrane to HDL in the medium rather than any intracellular effect of HDL. However, loss of cholesterol from all subcellular membranes must also occur, perhaps via exchange to the plasma membrane.

In reporting these changes in composition and microviscosity following exposure of vascular smooth muscle cells to lipoproteins it is necessary to establish that the changes seen are in the membrane and are not due to contaminating lipoprotein particles. The plasma membrane in particular could be contaminated with adherent lipoprotein particles. Several lines of evidence suggest such contamination does not occur. First, plasma membrane microviscosity does not directly parallel lipoprotein microviscosity. In experiments parallel to those reported here, plasma membranes from rat cells have a microviscosity very close to that of LDL (1.25 P) when exposed to low levels of LDL but their microviscosity is much higher at higher concentrations of LDL. Similarly, in unpublished experiments, we fould that very low-density lipoprotein which has the lowest microviscosity (0.8 P) does not produce membranes with the lowest microviscosity. More direct approaches to estimation of lipoprotein contamination made use of ^{125}I-labeled lipoproteins. Under our normal incubation conditions, the amount of ^{125}I-labeled lipoproteins present in fraction 3 was determined. From a knowledge of the cholesterol-to-protein ratio of the original lipoprotein the maximum amount of lipoprotein-cholesterol contamination was calculated. In all cases the amount of cholesterol which could be accounted for in this manner was an insignificant percentage of the cholesterol content of fraction 3 determined chemically.

One other possible source of contamination, that of intact lipid-rich lysosomes, was also examined. Upon examination of the distribution of the lysosomal enzyme hexosaminidase, we found that 86% of this lysosomal marker does not sediment with our total membrane pellet. The co-occurence of ^{125}I label, cholesteryl ester and hexosaminidase in the supernatant suggests either that these are associated perhaps as light lysosomes or as contents of ruptured lysosomes.

The changes in membrane cholesterol content and membrane microviscosity we have reported are small but consistently observable. Based upon early studies with bacterial cells, it was postulated that alterations in membrane fluidity of animal cells would alter transport processes associated with these membranes. Studies of Baldassare and Silbert [26] have shown cells with altered cholesterol contents (but with unchanged membrane fluidity) have altered rates of sugar transport; an increase in membrane cholesterol content of 30% (in the same range as we report for the difference between HDL and LDL exposure) produces a doubling in the rate of sugar transport. The publications of Amatruda and Finch [27] and Luly and Shinitzky [28] demonstrate that insulin modulates both membrane fluidity and sugar transport. The latter paper showed that insulin binding to liver membranes in vitro produced small (10—20%) increases in membrane microviscosity and alterations in exposure of membrane proteins.

This report of changes in the properties of vascular smooth muscle membranes suggests that such changes could occur in vivo and reinforces the suggestion of Jackson and Gotto [8] that changes in vascular smooth muscle mem-

brane microviscosity may be pathogenetic in the development of atherosclerosis. The fact that our data show similar changes in cholesterol-to-protein ratios in all cell membranes would suggest that many membrane-bound enzymes in the cell may be influenced by exposure of the cell to various plasma lipoproteins.

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