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Regulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity in Cultured Swine Aortic Smooth Muscle Cells by Plasma Lipoproteins†

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ABSTRACT: 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity has been measured in cultured smooth muscle cells derived from swine aorta, and its regulation by serum lipoproteins from control and hypercholesterolemic swine has been determined. In confluent cells grown in the presence of 10% swine serum, 3-hydroxy-3-methylglutaryl coenzyme A reductase catalyzed the formation of 10 pmol of mevalonate/min per mg of protein (cell extract). Replacement of 10% swine serum in the growth medium at confluency for 18 hr by lipoprotein-deficient swine serum resulted in an eightfold increase of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. Addition of very low density and low density lipoproteins, however, led to a lipoprotein-cholesterol concentration-dependent suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity which was comparable for control and hyperlipidemic swine lipoproteins. A unique lipoprotein characteristic of experimentally induced hypercholesterolemia in the swine, which has been classified as high density lipoprotein (HDL_c) based on its migration properties on electrophoresis and its lack of the B apoprotein, exerted an inhibitory effect on reductase activity comparable to that of very low density and low density lipoproteins. However, typical high density lipoproteins from control and hypercholesterolemic swine did not suppress reductase activity. To confirm these results, selected cultures were grown in the presence of [^{3}H]acetate and [^{14}C]mevalonate. Lipid analysis was carried out by radiogas chromatography and mass spectrometry, and cholesterol, desmosterol, dihydrolanosterol, and lanosterol were identified as radioactive sterols. Measurements of the $^3H/^{14}C$ ratio in isolated sterols indicated a positive correlation with direct measurements of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity.

Biosynthesis of cholesterol from acetate and mevalonate in arterial tissue has been demonstrated in a number of species (Siperstein et al., 1951; Werthessen et al., 1954; Azernoff, 1958; Feller and Huff, 1955; Rao and Rao, 1968; Chobanian, 1968; Daly, 1971; Avigan et al., 1972). Studies of rate and control of cholesterol synthesis in arterial smooth muscle cells, which is the predominant cell type in the intima and media of large vessels, have not yet been performed. The present study demonstrates that the rate-limiting enzyme in cholesterol biosynthesis from acetyl-CoA, 3-hydroxy-3-methylglutaryl coenzyme A reductase (reductase; EC 1.1.1.34), is detectable in cultured smooth muscle cells from swine aorta. Its regulation by isolated serum lipoprotein fractions from normolipidemic (control) and hyperlipidemic swine has been determined.

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Experimental Section

Materials. (*R,S*)-3-Hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A (HMG-CoA,[1] 7.55 mCi/mmol), (*R,S*)-[2-^{14}C]mevalonic acid (dibenzylethylenediamine salt, 8.05 mCi/mmol), and (*R,S*)-[2-^{14}C]mevalonic acid lactone (7 mCi/mmol) were purchased from New England Nuclear; [^{3}H]acetic acid (sodium salt, 250 mCi/mmol) was obtained from Amersham/Searle. D-Glucose 6-phosphate (monosodium salt) and TPN were purchased from Boehringer Mannheim; glucose-6-phosphate dehydrogenase (201 TPN units/mg) was obtained from Worthington Biochemical Corp. Crystalline bovine serum albumin was purchased from Armour Pharmaceutical Co. Sterols used as reference compounds were obtained from Applied Science. Dithiothreitol (A grade) was obtained from Calbiochem. Anion exchange resin (AG3-X4A, 100–200 mesh, chloride form) and sodium dodecyl sulfate were purchased from Bio-Rad Laboratories, Richmond, Calif. Kyro EOB (a secondary alcohol where the average degree of ethoxylation is nine and the average chain length of the alkyl chain is 14) was a gift from Dr. D. H. Hughes (The Proctor & Gamble Co.). Millipore filters (type SXHA, 0.4 μm) were obtained from the Millipore Corp. All chemicals were analytical or certified grade.

Cell Culture. The technique for the growth of smooth muscle cells in culture was similar to that described by Ross (1971). A small piece of descending thoracic aorta was obtained under sterile conditions from each of three miniature swine which ranged from 1.5 to 4 months of age (Sinclair Research Farm, University of Missouri, Columbia, Mo.). By careful dissection, the intima and the outer two-thirds of the media were removed. The remaining inner media was minced into bits no greater than 0.5 mm greatest dimension. Five to ten of these were explanted under a sterile cover slip in a 35-mm Falcon plastic petri dish. These explants were incubated at 37° under 5% CO_2–95% air (pH 7.4) in 3 ml of modified Dulbecco–Vogt (D–V) medium (Ross, 1971) supplemented with 10% fetal calf serum and penicillin (50 μg/ml). Cells grew out of the explants within 3–5 days and reached sufficient numbers for transfer within 15–20 days. For transfer, the cells were suspended in medium after incubation for 6–8 min in 0.05% trypsin–0.02% Versene solution. This suspension was transferred to a 100-cm^2 Falcon plastic flask with approximately 60% plating efficiency. Cells grew to confluency within 5–8 days (logarithmic phase doubling time, 60 hr) depending on the initial plating density and the transfer generation. Contrary to adventitial cells and skin fibroblasts, aortic smooth muscle cells continue to grow after confluency and pile up into many cell layers in a characteristic multicentric proliferative pattern. As described elsewhere (Brown, B. G., Mahley, T. W., and Assmann, G., manuscript in preparation) smooth muscle cells thus obtained had distinct morphologic and electron microscopic characteristics (Figure 1) when compared to skin fibroblasts and to cells derived from adventitial explants. For example, smooth muscle cells were bipolar cells with a centrally located single nucleus, prominent nucleoli, dense cytoplasmic myofilaments, peripheral dense bodies, and longitudinal orientation of the cytoplasmic contents on light microscopy. Smooth muscle cells could be trypsinized and transferred at least ten times (approximately 40 cell generations) without loss of morphologic characteristics. However, with each succeeding transfer generation, the log phase growth rate slowed. This was pronounced after T_6; therefore, all studies were performed on primary cell lines in the T_3 to T_6 range.

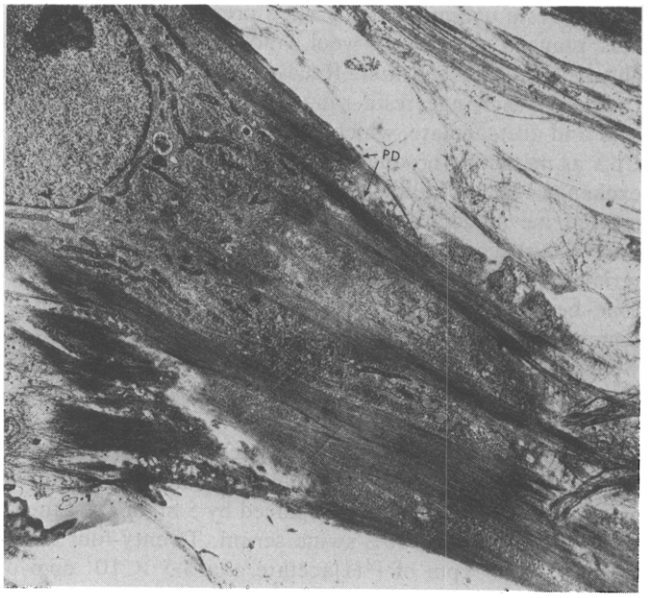

FIGURE 1: Electron micrograph of a smooth muscle cell derived from swine aorta and grown in tissue culture. Distinctive morphologic features included dense cytoplasmic myofilaments and peripheral dense bodies (PD); ×4348.

In order to determine the effect of various purified swine lipoproteins on HMG-CoA reductase activity, cells were grown to confluency in 100-cm^2 Falcon plastic flasks in D–V medium supplemented with 10% swine serum (obtained through clotting of blood). At confluency, the medium was aspirated, the cell monolayers were washed vigorously three times with phosphate-buffered saline (PBS), and additions were made as indicated in the figure captions. After incubation, the medium was aspirated and the cells were washed twice with PBS. The cells were removed from the flasks with a rubber policeman, suspended in 5 ml of chilled buffer containing 50 m*M* Tris-HCl (pH 7.4) and 0.15 *M* NaCl, and centrifuged (1500*g*, 5 min, 24°). The cell pellet was frozen in liquid nitrogen and stored at −196°.

HMG-CoA Reductase Assay. Cell extracts for measurements of HMG-CoA reductase activity were prepared as described previously (Brown et al., 1973). The extracts were obtained by dissolving the thawed pellet in 0.1 ml of buffer containing 50 m*M* potassium phosphate (pH 7.4), 5 m*M* dithiothreitol, 5 m*M* EDTA, 0.2 *M* KCl, and 0.25% Kyro EOB. After incubation for 10 min at 37°, the suspension was centrifuged for 5 min at 20,000*g*. The supernatant (50 μl) containing 50–150 μg of protein was incubated for 180 min at 37° in a total volume of 0.2 ml containing 0.1 *M* potassium phosphate (pH 7.5), 20 m*M* glucose 6-phosphate, 2.5 m*M* TPN, 0.7 unit of glucose-6-phosphate dehydrogenase, 5 m*M* dithiothreitol, and 70 μ*M* (*R,S*)-[3-^{14}C]-HMG-CoA. The reaction was stopped by the addition of 50 μl of 5 *N* HCl and the precipitate removed by centrifugation. Mevalonolactone was isolated from the supernatant by anion exchange chromatography in a modification of a previously described method (Huber et al., 1973). Anion ex-

[1] Abbreviations used are: HMG-CoA, (*R,S*)-3-hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A; PBS, phosphate-buffered saline; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

change resin columns (1 ml) were prepared in Pasteur pipets plugged with glass wool, and mevalonolactone was eluted with 3 ml of water. Recoveries of mevalonate from the anion exchange resin ranged from 63 to 65%. HMG acid and acetoacetate, which might be formed from HMG-CoA as metabolic products, were not eluted from the anion exchange resin. The identity and radioactive purity of the labeled mevalonolactone formed from [3-^{14}C]HMG-CoA were established by cochromatography with authentic material (silica gel G; layer thickness, 2 mm) using 1:1 acetone–benzene as a solvent system. Radioactive mevalonolactone moved as a single band. Appropriate corrections were made for blank values. The mean variation in HMG-CoA reductase activity between duplicate dishes was less than ±5%.

[^{3}H]Acetate-[^{14}C]Mevalonate Incorporation into Cellular Lipids. At confluency, the medium in two sets of five Falcon flasks (100 cm^2) was replaced by 5 ml of D–V medium with or without 10% swine serum. Twenty-four hours later, 5 × 10^8 cpm of [^{3}H]acetate and 3.5 × 10^7 cpm of (*R,S*)-[2-^{14}C]mevalonic acid were added in 50 μl of PBS and incubation was continued for 6 hr at 37°. Then the medium was removed. The cells were washed several times with PBS and transferred to a centrifuge tube using a rubber policeman. After centrifugation, the cell pellet was extracted with 20 vol of 2:1 chloroform–methanol. Ultrasonication at 5 kHz for 5 min using a Bronson sonifier (Heat Systems, Ultrasonics, Inc., Plainview, N.Y., Model 185 W) was applied to ensure complete dispersion of the cell material during the extraction procedure. Lipids were extracted by the method of Bligh and Dyer (1959). The lipids of the organic phase were taken to dryness under nitrogen and fractionated by thin-layer chromatography, using 65:25:4 chloroform–methanol–H_2O or 70:30:1 petroleum ether–ethyl ether–acetic acid as the solvent system. Thin-layer chromatograms were scanned in a radiochromatogram scanner (Packard Model 7201), and the radioactive bands were isolated according to Goldrick and Hirsch (1963), eluted with 2:1 chloroform–methanol, concentrated, and assayed for radioactivity on a Beckman gas chromatograph (Model 65) equipped with a splitting device to collect aliquots for determination of radioactivity (column, 1% OV-17 on Chromosorb Q; carrier gas, helium; detector, FID). Sterols were further analyzed by combined gas chromatography (1% OV-17)–mass spectrometry (LKB 9000).

Lipoproteins. Swine plasma lipoproteins were isolated and purified using the combination of sequential flotation in the ultracentrifuge and Geon-Pevikon block electrophoresis (Mahley and Weisgraber, 1974). The procedure used for the isolation of plasma lipoproteins from control swine fasted overnight included an initial centrifugation of plasma at d = 1.006 to obtain very low density lipoproteins (VLDL). Low density lipoproteins (LDL) were isolated from d = 1.006 to 1.063 and high density lipoproteins (HDL) from d = 1.10 to 1.21. The isolated fractions were washed at the corresponding densities by ultracentrifugation.

Feeding the miniature swine a diet composed of 15% lard and 1.5% cholesterol resulted in a marked increase in plasma cholesterol from a normal of ~90 mg/100 ml to levels ranging from 200 to 600 mg/100 ml and a profound change in the lipoprotein pattern (Mahley and Weisgraber, 1974). Lipoproteins isolated by ultracentrifugation and washed at d = 1.006 were referred to as VLDL. The low density fraction (d = 1.02–1.063) contained LDL and an α-migrating lipoprotein (HDL_c). LDL and HDL_c were purified by Geon-Pevikon block electrophoresis (Mahley and Weisgraber, 1974). The HDL from cholesterol-fed swine were isolated by ultracentrifugation at d = 1.10 to 1.21 and washed at d = 1.21 by ultracentrifugation (Mahley and Weisgraber, 1974).

Isolated fractions were dialyzed at least 48 hr at 4° against several changes of at least 100 vol of PBS. All lipoprotein fractions were adjusted by Diaflo concentration or dilution with PBS to a final cholesterol or triglyceride concentration of 100 mg %.

Lipoprotein-free plasma was prepared by removal of lipoproteins from swine plasma in the ultracentrifuge (1.21 g/ml KBr, 214,000g_{av}, 4°, 48 hr), dialyzed, and adjusted to its original plasma volume by dilution with D–V medium. Chemical determinations revealed the complete absence of cholesterol and triglyceride in the lipoprotein-free plasma. All materials, including medium, swine serum, and isolated swine lipoprotein fractions, were sterilized by Millipore filtration before addition to the culture flasks.

Apoprotein Analysis. Isolated lipoproteins were analyzed by polyacrylamide gel electrophoresis following delipidation with 2:1 chloroform–methanol (Lux et al., 1972). The amount of protein applied to each gel was 5–50 μg. Gels were composed of 10% acrylamide in 0.1 *M* Tris-HCl buffer (pH 8.2) in 0.1% sodium dodecyl sulfate (Weber and Osborn, 1969).

Chemical Determinations. Protein was determined by the method of Lowry et al. (1951) using a bovine albumin standard. Lipid analyses included triglyceride (Fletcher, 1968) and total cholesterol (Abell et al., 1952).

Results

In the standard assay system containing 50–150 μg of protein per tube, the rate of formation of mevalonolactone was linear with protein concentration and time during a 3-hr incubation period. As determined in preliminary experiments, the enzyme required a substrate concentration of 30 μmol/l. of (*S*)-[3-^{14}C]HMG-CoA for maximal velocity.

In smooth muscle cells grown to confluence in media containing 10% swine serum, the reductase activity averaged 10 ± 2 pmol/min per mg of protein (Table I). Removal of swine serum lipoproteins from the medium resulted in a progressive increase of reductase activity, reaching maximum levels at 14–18 hr (Figure 2). During this time period, both the removal of swine serum and the removal of lipoproteins from the medium (replacement by 10% $d > 1.21$ lipoprotein-free plasma) resulted in a similar enhancement of enzyme activity (Table I), which suggested that the observed increase was mediated through the absence of plasma lipoproteins. In eight different experiments, the induction of reductase activity in the absence of serum lipoproteins varied between 7.5- and 15-fold.

The addition of various isolated plasma lipoproteins to D–V medium supplemented with 10% swine $d > 1.21$ lipoprotein-free plasma resulted in a lipoprotein-specific and lipoprotein-cholesterol concentration-dependent suppression of reductase activity in smooth muscle cells. The various lipoprotein classes from four control and four hypercholesterolemic swine were studied using the smooth muscle cells derived from aortic explants from three swine with comparable results. The data from two separate experiments are presented (Table I).

The addition of VLDL and LDL from normocholesterolemic (control) swine to the cultured smooth muscle cells led to a significant reduction of HMG-CoA reductase activ-

Table I: Effect of Swine Lipoproteins on 3-Hydroxy-3-methylglutaryl Coenzyme A Activity.[a]

	Experiment A					Experiment B				
	Concn in Medium (μg/ml)			HMG-CoA Reductase Act.		Concn in Medium (μg/ml)			HMG-CoA Reductase Act.	
	Protein	Cholesterol	Triglyceride	pmol/min per mg of Protein	% of Max. Act.	Protein	Cholesterol	Triglyceride	pmol/min per mg of Protein	% of Max. Act.
				Normocholesterolemic						
VLDL	38.0	31.8	222.1	15	17	12.0	21.8	175	49	54
	24.4	21.2	148.1	17	19	6.0	10.9	87.5	64	70
	12.2	10.6	74.0	30	34	3.0	5.4	43.7	58	64
	6.1	5.3	37.0	44	50	0.6	1.1	8.7	67	74
	2.5	2.1	14.8	47	53	0.3	0.5	4.3	95	105
	1.2	1.0	7.4	70	80	0.06	0.1	0.9	90	100
	0.6	0.5	3.7	74	84					
	0.3	0.2	1.5	85	97					
	0.06	0.04	0.3	87	100					
LDL	43.1	74.0	4.7	3	3	67.1	94.9	n.d.	19	27
	21.6	37.0	2.4	8	7	33.6	47.5		25	35
	10.8	18.5	1.2	9	8	6.7	9.5		38	55
	4.3	7.4	0.5	22	21	3.4	4.8		59	85
	2.2	3.7	0.2	83	77	1.7	2.4		62	90
	1.1	1.9	0.1	102	95	0.7	1.2		69	100
	0.4	0.7	0.04	105	98					
	0.08	0.1	0.008	107	100					
HDL	97.9	74.0	0.8	75	72	380	203.6	n.d.	50	70
	49.0	37.0	0.4	78	75	285	152.7		57	79
	24.5	18.5	0.2	84	81	190	101.8		66	91
	9.8	7.4	0.07	102	98	95	50.9		62	86
	4.9	3.7	0.03	102	98	47.5	25.5		60	83
	2.4	1.9	0.01	103	100	19	10.2		72	100
	0.96	0.7	0.007	103	100					
	0.14	0.1	0.001	103	100					
				Hypercholesterolemic						
VLDL	20.6	31.7	148.1	10	12	45.6	200	198.4	5	15
	10.3	15.8	74.0	12	14	22.8	100	99.2	5	16
	5.1	7.9	37.0	30	34	11.4	50	49.6	5	15
	2.1	3.1	14.8	53	60	2.3	10	9.9	15	47
	1.0	1.6	7.4	78	89	0.23	1	1	31	100
	0.5	0.8	3.7	83	94					
	0.2	0.3	1.5	86	98					
	0.04	0.06	0.3	87	100					
LDL	31.8	74.0	n.d.	6	12	16.7	100	3.0	2	7
	15.9	37.0		10	20	8.3	50	1.5	2	8
	7.9	18.5				1.7	10	0.3	10	39
	3.2	7.4		20	40	0.8	5	0.15	20	81
	1.6	3.7		33	65	0.4	2.5	0.07	24	96
	0.8	1.9		45	87	0.2	1	0.03	25	100
	0.3	0.7		51	100					
HDL	119.1	74.0	0.40	60	62	276	200	17.2	28	89
	59.5	37	0.20	68	70	207	150	12.9	27	87
	29.7	18.5	0.10	72	73	138	100	8.6	23	74
	11.9	7.4	0.04	73	75	34.5	25	2.1	31	98
	5.9	3.7	0.02	94	96	13.8	10	0.9	31	100
	2.9	1.9	0.01	97	100					
	1.2	0.7	0.004	97	100					
	0.2	0.1	0.0008	97	100					
HDL_c	94.1	74.0	n.d.	4	3	71.0	204	34	4	5
	47.0	37.0		12	11	35.5	102	17	5	6
	23.5	18.5		25	24	17.8	51	8.5	7	9
	9.4	7.4		68	64	3.6	10.2	1.7	28	36
	4.7	3.7		97	92	1.8	5.1	0.8	77	100
	2.4	1.9		102	96					
	0.9	0.7		104	98					
	0.13	0.1		105	100					
10% control swine plasma	n.d.	76.6	24	12		n.d.	50	98	8	
10% $d > 1.21$ (lipoprotein-free plasma)	%	%	%	91		%	%	%	81	
Buffer	%	%	%	92		%	%	%	82	

[a] Aortic smooth muscle cells were grown in Falcon flasks containing 10% swine serum as described in the Experimental Section. At confluency, the medium was replaced with swine lipoprotein-free plasma (6 mg/ml protein) containing the indicated fractions. After 18 hr cells were harvested, and extracts from single flasks were assayed for reductase activity. Lipoproteins in swine plasma were fractionated, and their contents of protein, cholesterol, and triglyceride were measured. The lipoprotein fractions from normocholesterolemic swine included VLDL ($d < 1.006$), LDL (1.006–1.063), and HDL (1.10–1.21). The lipoprotein fractions from hypercholesterolemic swine included VLDL ($d < 1.006$), LDL (1.02–1.063 plus purification by Geon-Pevikon electrophoresis), HDL (1.10–1.21), and HDL_c (1.02–1.063 plus purification by Geon-Pevikon electrophoresis). The plasma cholesterol concentration of the donor swine was 200 mg/100 ml in experiment A and 450 mg/100 ml in experiment B.

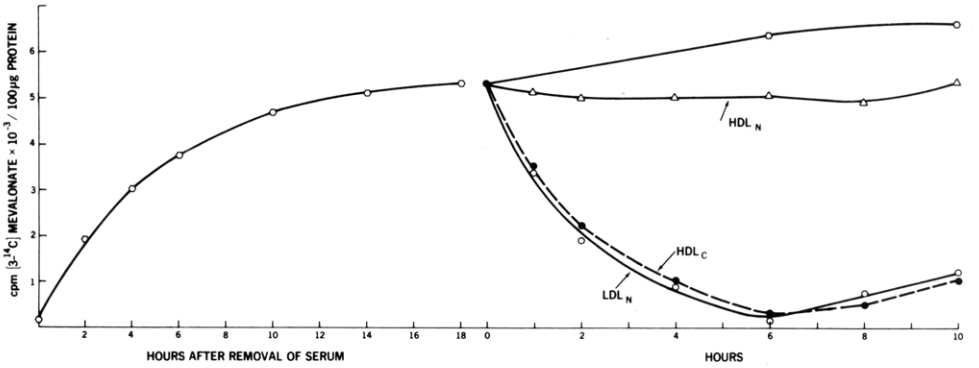

FIGURE 2: Time course of induction and suppression of 3-hydroxy-3-methylglutaryl coenzyme A activity by serum lipoproteins. Aortic smooth muscle cells were grown in Falcon flasks containing 10% swine serum. At confluency the medium was replaced with 9 ml of fresh medium containing swine lipoprotein-deficient serum (left). At the indicated intervals, cells were harvested from a single flask, and reductase activity was determined as described in the Experimental Section. After 18 hr of incubation in 10% lipoprotein-deficient swine serum, 1 ml of lipoprotein fraction in D–V medium was added to give a final cholesterol concentration of 100 μg/ml of medium: (O—O) LDL_N; (●- - -●) HDL_c; (Δ—Δ) HDL_N; (○— ○) no additions. Subscripts N (LDL_N, HDL_N) were made to indicate that LDL and HDL were derived from normocholesterolemic (control) swine plasma. HDL_c (subscript c) refers to the α-migrating lipoprotein induced by cholesterol feeding (see text).

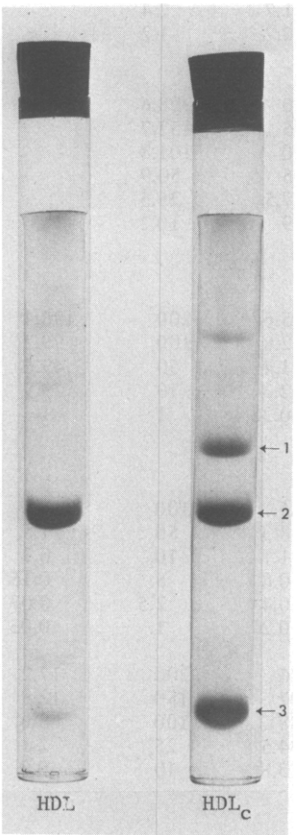

FIGURE 3: Disc electrophoresis patterns of the apolipoproteins of HDL (d = 1.1 to 1.21) and HDL_c (d = 1.02 to 1.063, plus Geon-Pevikon electrophoresis) in 0.1% polyacrylamide gels containing 10% sodium dodecyl sulfate (pH 8.2) and stained with Coomassie Brilliant Blue.

ity. However, control swine HDL at concentrations of 20 mg % cholesterol in the medium failed to suppress the enzyme activity below 70% of the maximal activity (Table I).

The addition of VLDL and LDL from the hypercholesterolemic swine to the cultured smooth muscle cells also resulted in a marked reduction of enzyme activity. It should be noted that the VLDL fraction (d < 1.006) from the hypercholesterolemic swine contained β-migrating lipoproteins (β-VLDL) in addition to pre-β lipoproteins. The HDL (d = 1.10 to 1.21) from the hypercholesterolemic swine were much less potent inhibitors of reductase activity and gave results similar to those of the HDL from control animals. However, the lipoprotein class referred to as HDL_c (cholesterol) was as potent an inhibitor of reductase activity as LDL at comparable cholesterol concentrations (Figure 2). It should be noted that at low levels of lipoprotein cholesterol in the medium (<10 μg/ml), LDL appear to be more effective than HDL_c in preventing the increase in reductase activity (Table I). The HDL_c have previously been described as a class of lipoproteins induced by cholesterol feeding in dogs (Mahley et al., 1974) and miniature swine (Mahley and Weisgraber, 1974). They are cholesterol-rich lipoproteins which have α mobility on paper and Geon-Pevikon electrophoresis and resemble high density lipoproteins with respect to apoprotein content. The apoprotein pattern of swine HDL_c on polyacrylamide gel electrophoresis (Figure 3) revealed the presence of apoproteins equivalent to apoA-I (band 2), a high molecular weight (35,000) protein (band 1), and the "C" peptides (band 3). The "B" apoprotein was absent, a fact confirmed by immunochemistry (Mahley et al., 1975).

The time course of suppression of reductase activity was determined by addition of LDL, HDL_c, and HDL to smooth muscle cells in which activity had been increased by prior incubation for 18 hr in D–V medium supplemented with 10% d > 1.21 fraction. Whereas LDL and HDL_c led to maximal suppression of reductase activity within 6 hr, HDL was almost ineffective (Figure 2).

In order to further assess the effect of lipoproteins in the medium on the sterol metabolism of smooth muscle cells, the incorporation of [^{3}H]acetate and [2-^{14}C]mevalonate was monitored by incubation of cells in the absence and presence of lipoproteins (Figure 4). There was little enhancement of incorporation of [2-^{14}C]mevalonate into sterols (fraction 4, Figure 4) after a 6-hr incubation in a lipoprotein-free medium, while [^{3}H]acetate incorporation into sterols and fatty acids of phosphatidylcholine and phosphatidylethanolamine (fractions 1 and 2, Figure 4) was greatly stimulated. Sterol synthesis from acetate increased by about eightfold when swine serum was replaced by lipoprotein-deficient medium. More than 80% of the radioactivity in neutral lipids (fraction 4, Figure 4) was associated with 3-hydroxysterols, as demonstrated by preparative thin-layer chromatography (solvent system 70:30:1 petroleum ether–ether–acetic acid; fraction 2, Figure 5, left) and radiogas

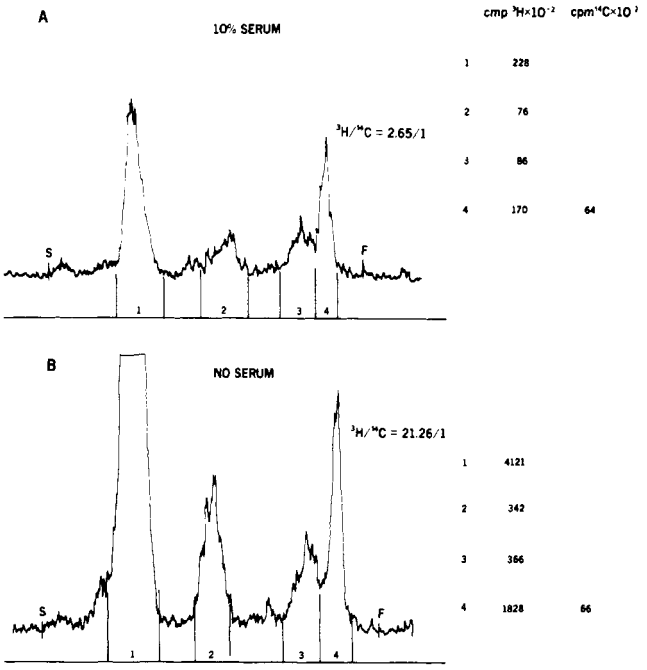

FIGURE 4: Radio-thin-layer chromatography of lipid extracts of aortic smooth muscle cells. Cells were grown in Falcon flasks containing 10% swine serum. At confluency the medium was replaced with fresh medium containing 10% swine serum (A) or lipid-free D-V medium (B). After 18 hr [^{3}H]acetate and [2-^{14}C]mevalonate were added, and the incubation was continued for 6 hr (see Experimental Section). After extraction of lipids and Folch partition, lipids were fractionated by thin-layer chromatography using 65:25:4 chloroform-methanol-water as solvent system and eluted from the silica gel. The main lipid fractions were: fraction 1, phosphatidylcholine; fraction 2, phosphatidylethanolamine; fraction 3, cardiolipin; and fraction 4, neutral lipids. Radioactivity data represent 1% of the recovered amount.

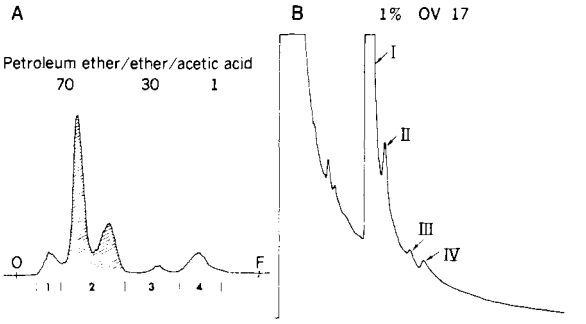

FIGURE 5: Radio-thin-layer chromatography (left) and gas chromatography (right) of 3-hydroxysterols. Neutral lipids of fraction 4 (Figure 4B) were rechromatographed in 70:30:1 petroleum ether-ether-acetic acid and sterols of fraction 2 subjected to radiogas chromatography (right, 1% OV-17) and mass spectrometry (not shown). Cholesterol (I), desmosterol (II), dihydrolanosterol (III), and lanosterol (IV) were identified by gas chromatographic retention time and their characteristic mass fragment pattern and contained 5, 33, 21, and 35% of the recovered radioactivity (68%), respectively.

chromatography. The radioactivity in the 3-hydroxysterols (fraction 2, Figure 5A) was mostly associated with cholesterol precursors (33% desmosterol, 35% lanosterol, 21% dihydrolanosterol) rather than cholesterol (5%) itself (Figure 5B). Most of the acetate label incorporated into phosphatidylcholine and phosphatidylethanolamine (fractions 1 and 2, Figure 4) was recovered in the fatty acid moiety.

Discussion

These investigations were carried out particularly to explore the mechanisms of regulation of HMG-CoA reductase activity in swine aortic smooth muscle cells by swine plasma lipoproteins. The method of Ross (1971) was adapted to obtain pure cultures of medial smooth muscle cells from swine aorta. Lipoproteins were from donors of the same strain of miniature swine used to establish the cultured cells.

Our data indicate that cholesterol synthesis in these cells in culture is regulated by the level of HMG-CoA reductase activity by specific lipoproteins in the medium. The addition of VLDL and LDL significantly suppressed reductase activity of cells grown in lipoprotein-deficient medium, while with HDL concentrations in the medium as high as 20 mg % cholesterol only partial suppression could be obtained. Similar data have been previously reported for the regulation of cholesterol synthesis in human fibroblasts (Brown et al., 1973).

The degree of suppression obtained appeared not to be dependent on whether these lipoproteins were derived from normo- or hypercholesterolemic animals; rather, the regulation appeared to be mediated by the concentration of lipoprotein cholesterol in the medium. An intriguing finding was the observation that HDL_c, in contrast to HDL, suppressed HMG-CoA reductase to a similar extent as VLDL and LDL. HDL_c have been described as unique lipoproteins induced by cholesterol feeding in dogs (Mahley et al., 1974) and swine (Mahley and Weisgraber, 1974). In the swine, HDL_c are isolated by a combination of ultracentrifugation (d = 1.02 to 1.06 g/ml KBr) and Geon-Pevikon electrophoresis (Mahley and Weisgraber, 1974). The particle size of HDL_c (diameter 150–250 Å) is larger than that of HDL (diameter 70–110 Å); both HDL and HDL_c share apoA-I as the major apoprotein and are free of immunochemically detectable apolipoprotein B (Mahley et al., 1975). HDL_c are relatively enriched in "C" peptides compared with HDL and possess an additional high molecular weight protein (approximately 35,000) as a structural component which appears to be equivalent to the human "arginine-rich" apoprotein (Mahley et al., 1975). On sodium dodecyl sulfate gel electrophoresis this protein has the same migration properties as the arginine-rich peptide described for human VLDL (Shelburne and Quarfordt, 1974) and the β-VLDL of human type III hyperlipoproteinemia (Havel and Kane, 1973; Mahley et al., 1975).

Brown et al. (1974) have demonstrated that in human fibroblasts apolipoprotein B is specifically involved in the suppression of HMG-CoA reductase activity. They have provided experimental evidence that, in order to suppress cholesterol synthesis, LDL must first bind to a specific high-affinity receptor on the cell surface. We have shown that both the time course and the degree of suppression of reductase obtained with LDL and with HDL_c (a lipoprotein devoid of apolipoprotein B) are similar for aortic smooth muscle cells. These data, however, do not necessarily contradict the findings of Brown et al. (1973); it needs yet to be demonstrated that LDL and HDL_c compete for the same binding mechanism at the cell surface site. Thus, the precise mechanism by which the delivery of cholesterol and the consecutive suppression of reductase activity occurs is not yet known. It has been shown in other laboratories that cholesterol itself, even when bound to a nonspecific carrier such as albumin, will also suppress cholesterol synthesis in all cell types studied so far (Bailey, 1973; Brown, et al., 1974). The question of whether HDL_c exerts its effect on reductase activity by specific interaction with a cell surface receptor

or nonspecific delivery of cholesterol to the cell remains to be further investigated.

We have investigated both the inhibition of previously "induced" reductase activity (Figure 2) and the ability of lipoproteins to prevent the increase in reductase activity that occurs when swine serum is replaced with lipoprotein-deficient swine serum (Table I). Though the prevention of the increase of reductase activity may be partly caused by nonspecific factors (Bailey, 1973), the opposite response of HDL and HDL_c in these experiments suggests a different mechanism of interaction of lipoprotein cholesterol with arterial smooth muscle cells. The observation that HDL_c, in contrast to HDL, at the concentrations studied suppresses reductase activity may imply that only the former delivers cholesterol to the cell. This may cause cellular cholesterol dysbalance and contribute to the development of atherosclerosis.

Enhancement of the rate of acetate incorporation in lipid-deficient medium into fatty acids and cholesterol of mammalian cells has been previously observed by several investigators (Avigan et al., 1972; Bailey, 1966; Rothblat, 1969; Lengle and Smith, 1969; Williams and Avigan, 1972; Jacobs et al., 1973; Goldstein and Brown, 1973). The marked rise in total fatty acid and phospholipid synthesis is consistent with the recent finding that acetyl-CoA synthetase activity in fibroblasts is enhanced when serum is removed from the medium (Howard et al., 1974). It cannot be excluded, however, that the apparent increase in synthesis rates from radioactive acetate may be due to a decrease in the size of the unlabeled precursor pool in the cell. Our results further confirm that a substantial proportion of radioactivity within a 6-hr incubation period is associated with cholesterol precursors, a finding previously obtained with rabbit aortic preparations (Avigan et al., 1972).

The smooth muscle cell is the predominant cell type in the intima and media of large arteries and is known to proliferate early in the development of atheroma. It is interesting to speculate that in familial hypercholesterolemia, as demonstrated for human fibroblasts (Brown et al., 1974), feedback suppression in arterial tissue of reductase activity mediated by serum lipoproteins might not exist. Thus, arterial cholesterol derived from synthesis in situ could accumulate and become an important pathologic factor underlying the rapidly progressive vascular occlusive disease in childhood.

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