Cholesterol metabolism in pigeon aortic smooth muscle cells lacking a functional low density lipoprotein receptor pathway

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Abstract Cholesterol metabolism was examined in aortic smooth muscle cells from atherosclerosis-susceptible White Carneau pigeons that have been shown to lack a functional LDL receptor pathway. In cells incubated in the presence of whole serum or low density lipoprotein (LDL) the rate of cholesterol synthesis from [1-^{14}C]acetate or of HMG-CoA reductase activity was 20–100 times greater than for mammalian cells incubated under the same conditions. Unexpectedly, cholesterol synthesis decreased by nearly 50% after preincubation for 24 hr with lipoprotein-deficient serum (LPDS). This occurred without a change in cellular cholesterol content. Neither the high rate of cholesterol synthesis nor the effect of LPDS could be accounted for by differences in cell turnover or state of growth. Cholesterol added in ethanol was ineffective in altering cellular cholesterol synthesis or esterification even though a near doubling in cellular free cholesterol content occurred. Cholesterol synthesis and esterification were, however, able to be regulated with 25-OH cholesterol and mevalonolactone, as indicated by their ability to suppress cholesterol synthesis and to stimulate cholesterol esterification. In spite of the high rate of endogenous cholesterol synthesis, cellular cholesterol content was maintained at a constant level by the efficient efflux of the newly synthesized cholesterol from the cell. Unlike mammalian cells that require a cholesterol acceptor in the medium for efflux to occur, cholesterol efflux from pigeon cells occurred in the absence of a cholesterol acceptor. This suggests either that pigeon cells utilize a different mechanism for cholesterol efflux or that they produce their own cholesterol acceptor. As a result of a lack of a functional LDL receptor pathway, pigeon smooth muscle cells do not maintain cholesterol homeostasis through the controlled uptake of exogenous LDL cholesterol, as do mammalian cells. Rather, pigeon smooth muscle cells would appear to regulate cholesterol concentrations at the level of either cholesterol synthesis or efflux.—**Randolph, R. K., B. P. Smith, and R. W. St. Clair.** Cholesterol metabolism in pigeon aortic smooth muscle cells lacking a functional low density lipoprotein receptor pathway. *J. Lipid Res.* 1984. **25:** 903–912.

Supplementary key words HMG-CoA reductase • acyl CoA:cholesterol acyltransferase • White Carneau pigeon • cholesterol synthesis • cholesterol efflux • 25-OH cholesterol

We have demonstrated in the preceding report that aortic smooth muscle cells from atherosclerosis-susceptible White Carneau (WC) pigeons fail to internalize low density lipoprotein (LDL) via the LDL receptor pathway (1). As a result exogenous LDL cholesterol is not efficiently delivered to these cells. In contrast, mammalian cells bind and internalize LDL with high affinity and specificity resulting in the efficient and regulated delivery of cholesterol to cells in response to changing cholesterol needs (2). Inasmuch as pigeon smooth muscle cells do not have such a mechanism for high affinity uptake of extracellular LDL cholesterol, they must possess other mechanisms for the regulation of cellular cholesterol metabolism.

The purpose of the present study was to examine cholesterol metabolism in pigeon smooth muscle cells in order to gain insight into possible mechanisms of regulation. Results suggest that in pigeon smooth muscle cells, endogenous cholesterol synthesis proceeds at a rate up to 100 times greater than in mammalian cells and that accumulation of excess cholesterol is prevented by a rapid efflux of endogenously synthesized cholesterol.

MATERIALS

Sodium [1-^{14}C]acetate (51.4 mCi/mmol) D,L-3-hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A (25 mCi/mmol), D,L-[5-^{3}H]mevalonic acid (N,N′-dibenzylethene diamine salt, 6.7 Ci/mmol), [1,2-^{3}H]cholesterol (40 Ci/mmol), [1-^{14}C]oleic acid (57 mCi/mmol), and [5′-

Abbreviations: LDL, low density lipoprotein; TLC, thin-layer chromatography; ACAT, acyl CoA:cholesterol acyltransferase; PBS, phosphate-buffered saline; LPDS, lipoprotein-deficient serum; FBS, fetal bovine serum.

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^{3}H]thymidine (10 Ci/mmol) were purchased from Amersham Corporation, Arlington Heights, IL. D,L-Mevalonolactone, D,L-3-hydroxy-3-methylglutaryl-coenzyme A,β-nicotinamide adenine dinucleotide phosphate, α-thymidine (crystalline), and 2′-deoxycytidine-5′-monophosphate (sodium salt) were purchased from Sigma Chemical Company, St. Louis, MO. Dithiothreitol was purchased from P-L Biochemicals, Inc.,, Milwaukee, WI. Oleic acid was purchased from Applied Science, State College, PA. Skellysolve B was purchased from Getty Oil Company, Tulsa, OK and purified by distillation. Kyro EOB was obtained from Proctor & Gamble Co., Cincinnati, OH. Stigmasterol was obtained from Analabs, Inc., North Haven, CT. Cholesterol was purchased from ICN Nutritional Biochemicals, Inc., Plainview, NY and was purified by recrystallization (three times) from ethanol. The 5-cholesten-3β-25-diol (25-OH cholesterol) was obtained from Steraloids, Inc., Wilton, NH. All other reagents and chemicals were analytical grade. Silica gel-coated glass plates for thin-layer chromatography (TLC) were purchased from American Scientific Products, McGaw Park, IL. All tissue culture supplies were obtained from Flow Laboratories, Rockville, MD.

METHODS

Pigeon and monkey cells were obtained and cultured as previously described (1). Protein was determined on cell extracts or sonicated mixtures by the method of Kashyap, Hynd, and Robinson (3) using bovine serum albumin as a standard.

Activity of 3β-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) was assayed by a modification of the method of Brown, Dana, and Goldstein (4) as we have described previously (5). The reaction was linear to 120 min with up to 300 μg/tube of cell protein and 30 μg/tube of the D,L-3-hydroxy-3-methyl-[3-^{14}C]glutaryl coenzyme A substrate. The reaction mixture for the standard assay was incubated for 90 min with approximately 75 μg of cell protein/tube.

Sterol synthesis was measured as the incorporation of [1-^{14}C]acetate into cellular sterols during a 24-hr incubation as described previously (6). Final [1-^{14}C]acetate concentration was 0.5 mM and 1.2 μCi/ml. In some experiments endogenously synthesized ^{14}C-labeled sterols were isolated from the medium. This was accomplished by extracting total lipids from a 1.0-ml aliquot of culture medium by the method of Bligh and Dyer (7). A [1,2-^{3}H]cholesterol internal standard (~50,000 dpm) was added to each tube prior to extraction. The lipid extract was then subjected to TLC on silica gel G in a solvent system consisting of Skellysolve B–diethyl ether–acetic acid 146:50:4. An authentic cholesterol standard was co-chromatographed on each plate. Following chromatography, the cholesterol bands were visualized with iodine vapor, scraped into scintillation vials, and counted for ^{14}C radioactivity as described previously (6). Results were corrected for recovery of the [^{3}H]cholesterol internal standard and represent total sterol synthesis, as no attempt was made to further characterize the material that comigrated with cholesterol on TLC.

Incorporation of [^{3}H]thymidine into deoxyribonucleic acid (DNA) was estimated as described by Adams (8) utilizing a thymidine pool-flooding technique. The final [5′-^{3}H]thymidine concentration in tissue culture dishes was 10 μM and 5 μCi/ml. Deoxycytidine monophosphate (10 μM) was included during the incubation period so that ribonucleotide reductase activity would not be limiting for DNA synthesis (8). Results are expressed as dpm [^{3}H]thymidine incorporated/10^{6} cells. The total number of cells was counted using a hemocytometer. A vital dye such as trypan blue was not used in these experiments.

Cellular free cholesterol and cholesteryl ester mass were determined by gas–liquid chromatography as described previously (9).

Activity of acyl CoA:cholesterol acyltransferase (ACAT) was determined from the incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate. Preparation of the [1-^{14}C]oleate/albumin substrate was as described previously (10). Specific activities of substrate preparations ranged from 8,000 to 12,000 dpm/nmol. The substrate was added to dishes in a 20 μl volume that was sufficient to give a final [1-^{14}C]oleate concentration of 0.2 mM and 1 μCi/ml. For measurement of ACAT activity, the [1-^{14}C]oleate was added to the culture medium of each dish and incubation was continued for an additional 2 hr. The reaction was stopped by placing the cells on ice, followed by several washes with ice-cold phosphate-buffered saline (PBS) (9). Cells were harvested with trypsin-EDTA (9) and washed twice more by centrifugation. The cell pellet was disrupted by sonication (6), a [^{3}H]cholesterol internal standard (~50,000 dpm) was added, and lipids were extracted according to the method of Bligh and Dyer (7). The organic phase was dried under a stream of nitrogen, brought up in a small volume of chloroform, and lipids were spotted on TLC plates coated with silica gel G. Authentic cholesterol and cholesteryl ester standards were also plated. Chromatograms were developed as described above. Lipid bands were visualized with iodine vapor and scraped directly into scintillation vials. Scintillation counting was performed as described previously (1). Results were corrected for recovery of the [^{3}H]cholesterol internal standard.

Low density lipoproteins were isolated from normocholesterolemic grain-fed animals by previously described

methods (9). Medium containing lipoprotein-deficient serum (LPDS) was prepared as described in the preceding report (1).

RESULTS

Lipoprotein-deficient serum promotes the loss of cholesterol from mammalian cells with the resultant enhancement of endogenous cholesterol synthesis (4). To see if a similar effect occurred in pigeon cells, we grew both pigeon and monkey smooth muscle cells and monkey skin fibroblasts to confluence in medium containing 10% fetal bovine serum (FBS). A group of cells was harvested for HMG-CoA reductase activity and the remainder was incubated for up to 48 hr with LPDS, and HMG-CoA reductase activity was measured. As can be seen from the data in **Fig. 1,** HMG-CoA reductase activity was low in monkey cells incubated in medium containing FBS, but increased as expected after incubation with LPDS. Pigeon cells, however, had a rate of HMG-CoA reductase activity in the presence of medium containing 10% FBS that was approximately 20-fold higher than monkey cells incubated under the same conditions. When incubated with LPDS, there was a marked decrease in reductase activity after 24 hr with no further changes after 48 hr. This unexpected decrease in reductase activity was seen in cells from both White Carneau (WC) and Show Racer (SR) pigeons.

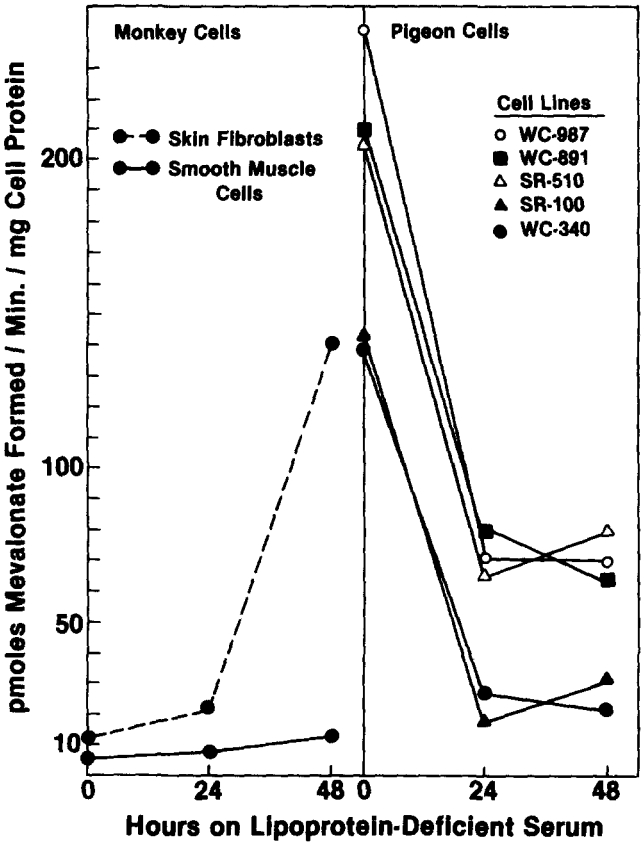

Fig. 1. Effect of incubation with LPDS on HMG-CoA reductase activity in monkey and pigeon cells. Cells were grown to confluence in medium containing FBS, washed with PBS, and incubated with medium containing LPDS. After 0, 24, or 48 hours, cells were harvested by scraping, washed, and HMG-CoA reductase activity was determined. Results are the average of duplicate dishes.

In order to determine whether this change in HMG-CoA reductase activity with LPDS was correlated to changes in cellular cholesterol content, cholesterol synthesis and cellular cholesterol content were measured in pigeon and monkey cells grown to confluence in medium containing FBS and after 24 hr of incubation with medium containing LPDS (**Table 1**). Consistent with the data for HMG-CoA reductase in Fig. 1, sterol synthesis from [^{14}C]acetate in monkey cells was suppressed when incubated in medium containing FBS and increased when incubated with LPDS. This stimulation of cholesterol synthesis was accompanied by a decrease in cellular cholesterol content. Sterol synthesis, in contrast, was 70-fold higher in pigeon cells incubated with medium containing FBS and markedly decreased in cells incubated with LPDS. Moreover, the changes in sterol synthesis in pigeon cells occurred without changes in the cellular cholesterol concentration.

In order to determine whether the rate of sterol synthesis in pigeon cells and the response to LPDS varied with the stage of cell growth, the experiment shown in **Fig. 2** was performed. The rate of sterol synthesis in monkey cells was initially low and increased throughout the log phase of growth. As the cells approached confluence, the rate of sterol synthesis tended to decline toward initial levels. As expected, sterol synthesis increased when the monkey cells were switched to medium containing LPDS. Initially sterol synthesis in pigeon cells was approximately 20-fold higher than in monkey cells. Sterol synthesis increased through the log phase of growth and decreased to initial levels at confluence. The addition of LPDS medium to pigeon cells again resulted in a reduction in the rate of sterol synthesis that was not associated with a decrease in the number of cells per dish or with large changes in [^{3}H]thymidine incorporation. The pattern of [^{3}H]-thymidine incorporation was similar for both monkey and pigeon cells with maximum incorporation occurring early during the log phase of growth. The effect of LPDS on cell turnover, as determined by [^{3}H]thymidine incorporation, was similar for monkey and pigeon cells. LPDS produced only slight reductions in DNA synthesis rates and in cell numbers in both cell types. We conclude from these experiments that the higher rate of sterol synthesis in pigeon cells incubated with serum-containing medium and the lower rate when incubated with LPDS

TABLE 1. Effect of incubation with medium containing whole or lipoprotein-deficient serum on cholesterol mass and incorporation of [^{14}C]acetate into sterols in pigeon and monkey smooth muscle cells

Cell Line	[^{14}C]Acetate → [^{14}C]Sterols		Cholesterol Mass[a]	
	FBS[b]	LPDS[c]	FBS[b]	LPDS[c]
	dpm × 10^{-3}/mg cell protein		*μg/mg cell protein*	
Monkey (n = 2)	3.8	16.2	34.5 (23)	29.1 (8)
Pigeon WC65 (n = 5)	229 ± 8.7	132 ± 30	23.4 ± 1.7 (<2)	24.5 ± 1.4 (<2)
WC645 (n = 5)	282 ± 39	152 ± 22	21.6 ± 2.5 (<2)	21.6 ± 1.3 (<2)

Results are the mean ± SD for the number of replicates indicated.

[a] Represents total cholesterol mass. Values in parentheses are percent of total cholesterol mass that is cholesteryl ester.

[b] Medium contained 10% fetal bovine serum and was incubated for 24 hr with confluent cells.

[c] Medium contained lipoprotein-deficient calf serum and was incubated for 24 hr with confluent cells.

were not the result of differences in cell death and turnover. Had there been a much higher rate of turnover of pigeon cells, there would have been a much higher rate of [^{3}H]thymidine incorporation in pigeon cells than monkey cells when incubated in serum-containing medium in order to maintain a constant number of cells per dish.

Since the LPDS in these studies was derived from calf serum and since FBS was used as the serum source in the growth medium, it was necessary to determine

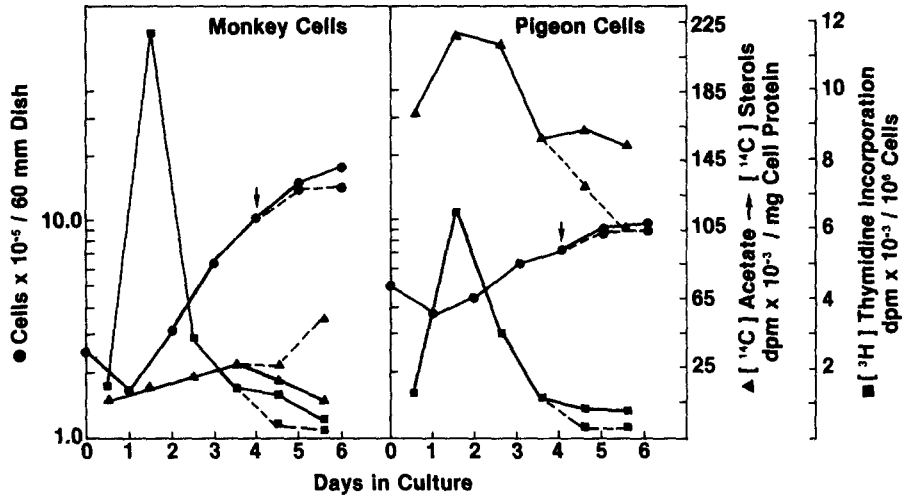

Fig. 2. Effect of cell growth on sterol synthesis from [^{14}C]acetate and on [^{3}H]thymidine incorporation in monkey and WC pigeon smooth muscle cells. Monkey and pigeon cells were plated in medium containing FBS at densities of 0.2 and 0.5 × 10^{-5} cells/60-mm dish, respectively. Dishes were assigned to one of three groups. The first group received (at plating) medium containing [^{14}C]acetate (0.5 mM and 2.44 μCi/μmole) (▲). On each subsequent day, two dishes in this group received [^{14}C]acetate. Each day, following addition of [^{14}C]acetate, incubations were continued for an additional 24 hr before cells were harvested, washed, and ^{14}C-labeled sterol synthesis from [^{14}C]acetate was determined. These data were plotted at the midpoint of each 24-hr period. The second group of dishes was utilized to assess cell growth as the number of viable cells/dish (●). Cell number data was plotted to reflect the day that the particular cell count was done. The third group of cells was utilized to determine rate of cell division as [^{3}H]thymidine incorporation (■). These data are plotted at the midpoint of each 24-hr period as this was when the assay was performed. All dishes received fresh medium containing FBS on day 3, and on day 4 (arrow) each of the three groups was divided into two subgroups each. One subgroup received fresh medium containing FBS (solid line) and the other subgroup received medium containing LPDS (dashed line). The data shown for each determination are the average of duplicate dishes.

whether the effect of LPDS on cholesterol synthesis in pigeon cells was due to some component of the calf serum. **Fig. 3** shows that the high level of cholesterol synthesis in pigeon cells incubated with FBS was suppressed by incubation with LPDS derived from both FBS and calf serum. Although the relative degree of suppression was similar between days 10 and 12, sterol synthesis in cells incubated with calf LPDS was suppressed to a greater extent (25% decrease) on day 10 than in cells incubated with fetal bovine LPDS (7% decrease). Again, these changes were not accompanied by large changes in the number of cells per dish. In addition, the rate of sterol synthesis (although suppressed) was not further decreased in cells incubated for up to 4 days in LPDS. Taken together, these data (Figs. 2 and 3) suggested that the effect of LPDS on sterol synthesis in pigeon cells was not the result of a general toxic effect on the cells.

The effect of different sources of exogenous sterols on HMG-CoA reductase and ACAT activity is shown in **Fig. 4.** While both lipoprotein (LDL) and non-lipoprotein sterol (cholesterol added in ethanol) effectively altered the activities of HMG-CoA reductase and ACAT in monkey cells, as has been described for human cells (11, 12), neither LDL nor cholesterol added in ethanol were able to change the activity of these enzymes in pigeon

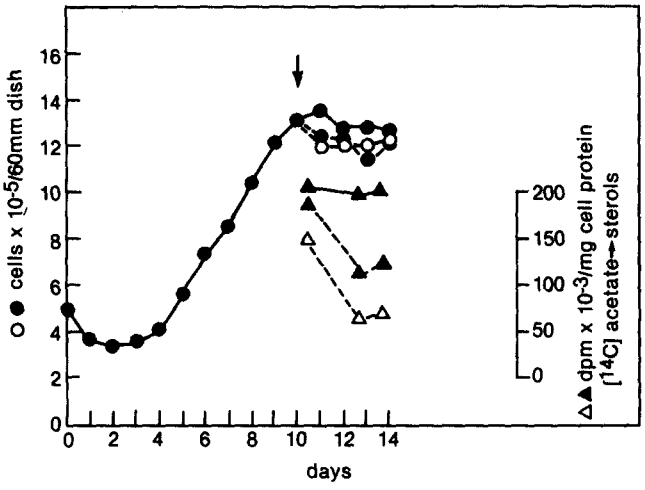

Fig. 3. Effect of LPDS derived from fetal bovine or calf serum on cell growth and rate of sterol synthesis in WC pigeon smooth muscle cells. Cells were plated at a density of 0.5 × 10^5 cells/dish. Every third day dishes received fresh medium containing FBS until day 10 when dishes were divided into three groups. One group received fresh medium containing FBS (● —— ●) and the other two groups received medium containing LPDS (● - - - ●, fetal bovine LPDS; O - - - O, calf LPDS). The ^{14}C-labeled sterol synthesis was determined as described under Methods in control cells by incubating them on days 10, 13, and 14 with medium containing FBS (▲ —— ▲) or LPDS derived from fetal bovine serum (▲ - - - ▲) or calf serum (△ - - - △). Cells were counted on the day of plating and on each day thereafter throughout the experiment. Values are the average of duplicate cultures.

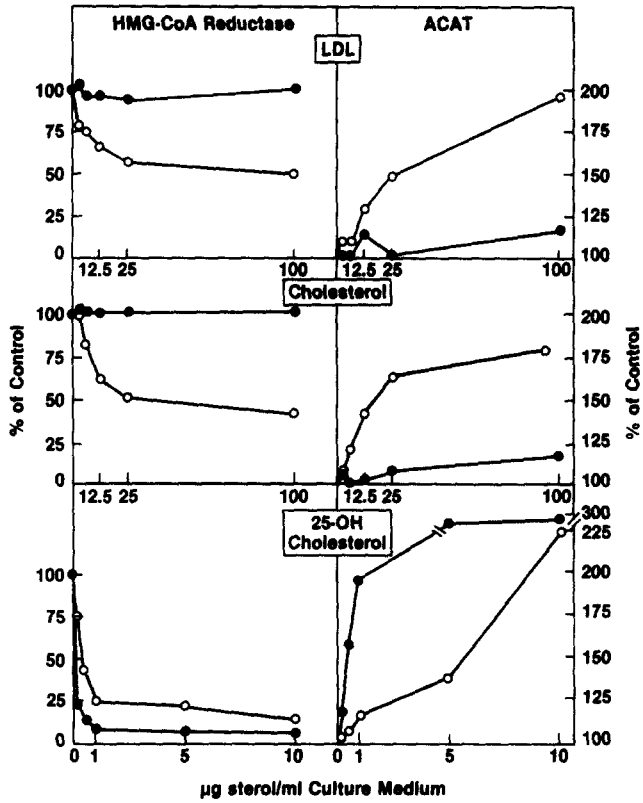

Fig. 4. Effect of LDL, cholesterol, and 25-OH cholesterol on HMG-CoA reductase and ACAT activity in monkey and WC pigeon smooth muscle cells. Cells were grown to confluence in medium containing FBS. Monkey cells were incubated with medium containing LPDS for an additional 24 hr. Monkey (O) and pigeon (●) cells were then incubated with medium containing LPDS or FBS, respectively, plus the indicated concentrations of sterol for 5 hr (note difference in concentration scale for 25-OH cholesterol as compared to LDL and cholesterol). Cells to be assayed for HMG-CoA reductase activity were harvested and those to be assayed for ACAT activity were incubated for an additional 2 hr with [^{14}C]oleate (0.2 mM and 1 µCi/ml). Control HMG-CoA reductase activities for pigeon and monkey cells were 222 and 31 pmol of mevalonate formed/min per mg, respectively. Control ACAT activities were 4.1 and 5.0 nmol/mg for monkey and pigeon cells, respectively. Note the different "% of control" scales for HMG-CoA reductase and ACAT. Values are the average of duplicate dishes.

cells. The failure of cholesterol in ethanol to inhibit HMG-CoA reductase or stimulate ACAT was surprising inasmuch as 25-OH cholesterol modulated the activity of these enzymes in both pigeon and monkey cells, indicating that these enzymes had the potential for regulation in a manner similar to mammalian cells. In fact, these enzymes appeared to be more sensitive to 25-OH cholesterol in pigeon cells than in monkey cells.

The failure of LDL and of cholesterol added in ethanol to modulate the activity of ACAT and of HMG-CoA reductase in pigeon cells occurred despite the fact that cellular free cholesterol content was substantially increased (**Table 2**) by both of these treatments. Cholesteryl ester content was increased in monkey cells

TABLE 2. Effect of FBS, LPDS, LDL, and cholesterol on cholesterol mass in monkey and pigeon smooth muscle cells

Treatment	Monkey Cells		Pigeon Cells	
	FC	CE	FC	CE
	μg/mg cell protein			
FBS control	28.1	6.4	15.4	0.6
LPDS control	26.9	2.2	16.0	0.4
LDL	35.9	10.9	25.5	0.9
Cholesterol	47.8	34.7	33.6	0.7

WC pigeon and monkey cells were grown to confluence in medium containing FBS. Cells were then divided into two groups. The first group (controls) received fresh medium containing FBS or LPDS. To pigeon and monkey cells in the second group were added fresh medium containing FBS or LPDS, respectively, plus monkey LDL (50 μg LDL protein/ml) or cholesterol (added in 20 μl ethanol) giving a final added cholesterol concentration of 100 μg/ml. A 20-μl aliquot of ethanol alone was added to control dishes and dishes with LDL. All dishes were incubated at 37°C for 24 hr, cells were removed with trypsin-EDTA, and cholesterol mass was determined. Results are the average of duplicate dishes.

incubated with LDL and with cholesterol added in ethanol, whereas there was no change in the cholesteryl ester content of pigeon cells. These differences are consistent with the stimulation of ACAT activity in monkey cells but not in pigeon cells (Fig. 4). Pigeon cells were also found to be resistant to depletion of cellular cholesterol content when incubated with LPDS relative to medium containing FBS (Table 2).

The addition of mevalonolactone to cells in culture has been shown by Edwards et al. (13) to decrease the activity of HMG-CoA reductase. To determine if mevalonolactone had a similar effect in pigeon cells, and also to determine if mevalonolactone influenced ACAT activity, we carried out the experiment shown in **Fig. 5.** Mevalonolactone effectively decreased HMG-CoA reductase activity and stimulated ACAT activity in both pigeon and monkey cells. This occurred without a consistent change in cellular cholesterol content (data not shown).

Fig. 6 shows the rate of increase in cellular sterol specific activity resulting from incubation of pigeon and monkey cells with [^{14}C]acetate (panel A) and the rate of decrease in cellular sterol specific activity upon removal of [^{14}C]acetate (panel B). Cells were plated into dishes and incubated with [^{14}C]acetate in serum-containing medium for 6 days. Sterol specific activity increased rapidly in pigeon cells, consistent with a high rate of cholesterol synthesis, and plateaued at a steady state by day 3. In monkey cells, however, there was a much slower rate of increase in cellular sterol specific activity that may not have achieved a steady state even by 6 days. This was consistent with a low rate of endogenous

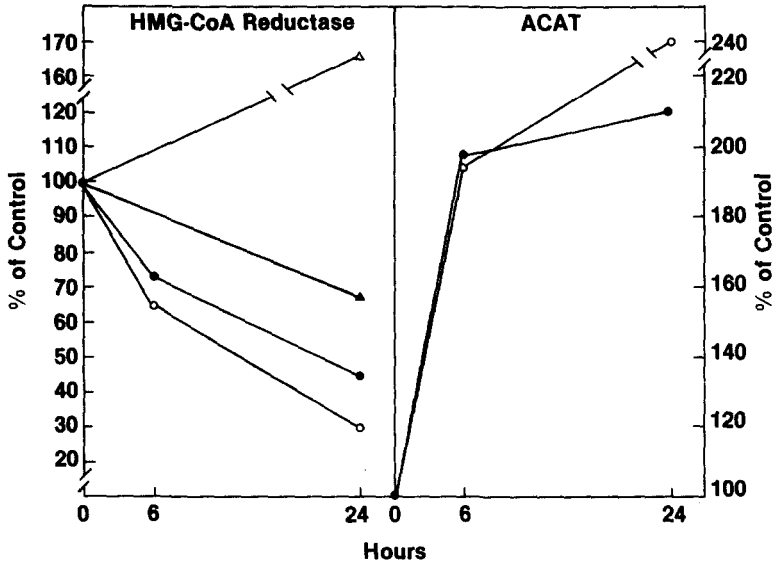

Fig. 5. Effect of mevalonolactone on HMG-CoA reductase and ACAT activity in monkey and WC pigeon smooth muscle cells. Monkey (open symbols) and WC pigeon (closed symbols) smooth muscle cells were grown to confluence in medium containing FBS. Monkey cells were incubated with medium containing LPDS for an additional 12 hr. The experiment was started with the addition of LPDS alone (△, ▲) or LPDS plus mevalonolactone (○, ●) which was added to each dish in a volume of 20 μl ethanol giving a final concentration of 10 mM. At the indicated times, cells were harvested and HMG-CoA reductase activity was determined. Cells that were to be assayed for ACAT activity were incubated for an additional 2 hr with [^{14}C]oleate (0.2 mM and 1 μCi/ml) at the indicated times. HMG-CoA reductase activities at zero time were 261 and 26 pmoles/min per mg for pigeon and monkey cells, respectively. Control ACAT activities were 3.1 and 2.6 nmoles/mg for monkey and pigeon cells, respectively. Results are the average of duplicate dishes.

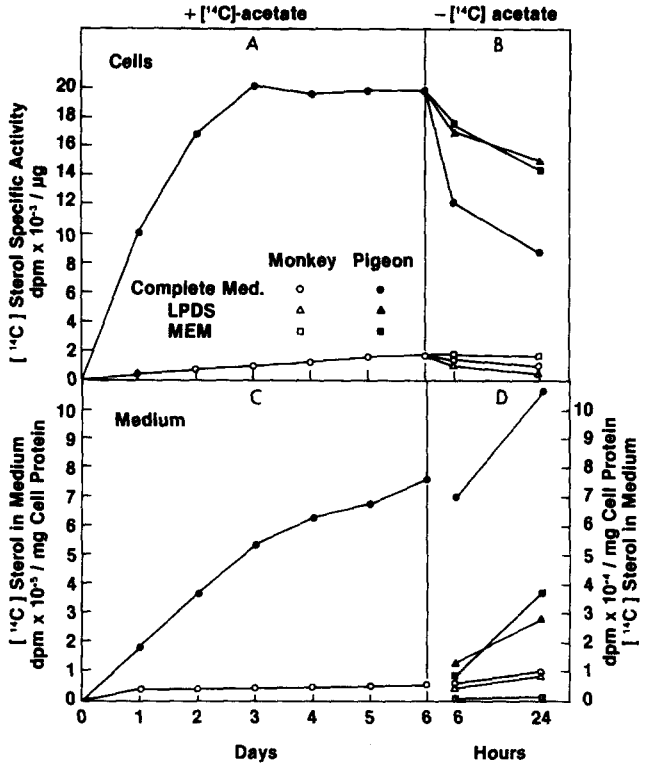

Fig. 6. Specific activity of endogenously synthesized ^{14}C-labeled sterols and their efflux into medium containing FBS, LPDS, or MEM by WC pigeon and monkey smooth muscle cells. Monkey (open symbols) and pigeon cells (closed symbols) were plated at densities of 2.5×10^5 and 5.0×10^5 cells/60 mm dish, respectively, in 4 ml of medium containing FBS and 0.5 mM [^{14}C]acetate (2.44 μCi/μmole). Cells were harvested each 24-hr period and total cholesterol mass and cellular ^{14}C-labeled sterol content were measured as described in Methods (panel A). All dishes received fresh medium containing the same concentration of [^{14}C]acetate on day 3. On day 6 (panel B) dishes were washed extensively with PBS and divided into three groups in which the [^{14}C]acetate was replaced with 0.5 mM sodium acetate plus the following constituents. The first group received medium containing FBS (○, ●); the second group received medium containing LPDS (△, ▲); and the third group received medium (MEM) alone (□, ■). At 6 and 24 hr, cellular cholesterol mass and the content of ^{14}C-labeled sterol were determined. Panels C and D show the amount of ^{14}C-labeled sterol isolated from the medium. These data represent the cumulative ^{14}C-labeled sterol in the medium at each time. Results are the average of duplicate dishes.

cholesterol synthesis in monkey cells incubated with serum-containing medium. Upon removal of [^{14}C]acetate (panel B) there was a rapid reduction in cellular sterol specific activity in pigeon cells consistent with dilution by newly synthesized, nonradioactive cholesterol. The rate of reduction in specific activity was greatest in pigeon cells incubated with medium containing FBS and less in cells incubated with LPDS and serum-free medium (MEM). This was consistent with the conclusion that cholesterol synthesis was highest in cells incubated with medium containing FBS, and decreased by LPDS. The reduction in specific activity in monkey cells was greatest in LPDS followed by medium containing FBS. There was no change with MEM alone in monkey cells. This suggests that, in monkey cells, cholesterol synthesis was highest with LPDS and completely inhibited in MEM.

Newly synthesized ^{14}C-labeled sterols were excreted into the culture medium from both pigeon and monkey cells, with greater amounts of total radioactivity excreted from pigeon cells (Fig. 6, panels C and D). The continued increase in ^{14}C-labeled sterols in the medium of pigeon cells during a time when the cells had achieved a steady state (panel A, days 3–6) indicates that the pigeon cells were continuing to synthesize and efflux cholesterol into the medium.

Previous studies with mammalian cells have shown that the loss of free cholesterol from cells requires the presence of an extracellular cholesterol acceptor (14–16). To test this requirement in pigeon cells, we removed the [^{14}C]acetate from the medium and added fresh medium containing FBS, LPDS, or MEM alone. MEM does not promote cholesterol efflux in mammalian cells (14–16). As shown in Fig. 6 (panel B), there were only trace amounts of ^{14}C-labeled sterols lost from monkey cells into MEM alone, but there was efflux into medium containing FBS or LPDS. In contrast, pigeon cells lost considerably more ^{14}C-labeled sterol with medium containing FBS than with LPDS, consistent with the greater rate of sterol synthesis in cells incubated in medium containing FBS. A major difference between the pigeon and monkey cells, however, was the observation that as much ^{14}C-labeled sterol was lost from pigeon cells in the presence of MEM alone as in the presence of LPDS, while in monkey cells there were only trace amounts of ^{14}C-labeled sterol lost with MEM alone.

The quantitative amounts of sterol synthesis and efflux from the experiment shown in Fig. 6 are sum-

TABLE 3. Rates of cholesterol synthesis and efflux in White Carneau pigeon and monkey smooth muscle cells

	Pigeon	Monkey
	µg/day per mg cell protein	
Cholesterol synthesis (in FBS medium)		
Steady state method[a]	3.5	
Isotopic method[b]	2.3	0.05
Cholesterol efflux[c]		
FBS	3.94	1.01
LPDS	1.01	1.28
MEM	2.05	0.11

For the conditions of this experiment see the legend for Fig. 6.

[a] Steady state method = slope of [^{14}C]sterol appearance in culture medium ÷ average cell cholesterol specific activity. This method was utilized only during time when cellular cholesterol specific activity was constant and there was no change in cellular cholesterol content.

[b] Isotopic method = [^{14}C]acetate incorporated into cholesterol (based on [^{14}C]acetate specific activity) ÷ mole ratio of acetate into cholesterol.

[c] Efflux = [^{14}C]sterol in medium ÷ cellular cholesterol specific activity.

marized in **Table 3.** Based on the steady state rate of appearance of ^{14}C-labeled sterol in the culture medium of pigeon cells, the rate of cholesterol synthesis was calculated to be 3.5 μg/mg cell protein per day. A similar calculation was not done for monkey cells since it was unclear whether the isotopic steady state was achieved. Cholesterol synthesis was also estimated from the number of moles of [1-^{14}C]acetate incorporated into sterols. Using this method, the rate of cholesterol synthesis was calculated to be 0.05 μg/mg per day and 2.3 μg/mg per day for monkey and pigeon cells, respectively. In the same culture medium (FBS medium) the measured rate of cholesterol efflux was similar to the steady state rate of synthesis. This provides further evidence that the bulk of cholesterol in pigeon cells is derived from synthesis. In monkey cells the total rate of cholesterol efflux exceeded the rate of synthesis by approximately 20-fold, indicating that most of the cholesterol lost by monkey cells was originally derived from exogenous sources (presumably the LDL of FBS). Sterol efflux in pigeon cells was reduced by LPDS, while in monkey cells efflux was greater relative to FBS medium. The most surprising finding was that the efflux of ^{14}C-labeled sterol continued at a substantial rate in pigeon cells incubated in MEM alone, while in monkey cells there was virtually no efflux into MEM, as expected from previous studies (14).

DISCUSSION

The results of these studies suggest that pigeon smooth muscle cells, in contrast to mammalian cells, do not regulate cholesterol metabolism by a mechanism involving the influx of exogenous cholesterol. In support of this conclusion is the fact that the rate of cholesterol synthesis, as measured from [^{14}C]acetate or by HMG-CoA reductase activity, in pigeon cells incubated with medium containing whole serum or isolated LDL, was up to 100-fold greater than in monkey cells incubated under identical conditions. Moreover, this difference persisted regardless of the growth phase of the cells and could not be explained by differences in the rate of cell turnover.

In spite of this high rate of cholesterol synthesis the rate of cholesterol esterification (ACAT activity) was low in pigeon cells incubated with either whole serum, as reported here and previously (10), or with isolated LDL. The low rate of cholesterol esterification was consistent with low concentrations of cholesteryl esters in the pigeon cells.

A further difference in pigeon cells relative to mammalian cells is the failure of cholesterol added in ethanol to regulate either HMG-CoA reductase or ACAT activity. This treatment has been shown to suppress HMG-CoA reductase activity and to stimulate ACAT activity in normal mammalian cells and in cells from patients with either the receptor-negative or the internalization-defective forms of familial hypercholesterolemia (17, 18). The failure to regulate these enzymes in pigeon cells occurred despite the near doubling of the cellular free cholesterol content.

Cellular free cholesterol content was also increased when pigeon cells were incubated with LDL. Since LDL was not internalized (1), this increase in cellular free cholesterol content probably resulted from the surface transfer of free cholesterol from the LDL to the plasma membrane. Regardless of the mechanism, the magnitude of the increase in the free cholesterol content would be expected to cause some down-regulation of cellular cholesterol synthesis and perhaps some stimulation of cholesterol esterification. In this regard, pigeon cells are clearly different from mammalian cells in which enrichment with cholesterol, independent of the uptake of intact lipoprotein particles, results in the same metabolic responses that are elicited by the uptake of LDL via the LDL receptor pathway (19, 20). The most plausable explanation of these findings is that the enrichment in cellular free cholesterol content occurs in a cellular compartment that does not participate in regulation of cholesterol synthesis or esterification. An example would be the plasma membrane with failure to transfer this excesss cholesterol to the endoplasmic reticulum where HMG-CoA reductase (21) and ACAT (22) are located.

In contrast to the failure of cholesterol in ethanol or of LDL cholesterol to regulate cellular cholesterol metabolism, 25-OH cholesterol was even more effective in pigeon cells than monkey cells in reducing HMG-CoA reductase activity and in stimulating ACAT activity. There are at least two possible explanations for these seemingly contradictory findings. First, regulation of cholesterol metabolism in pigeon cells may not be influenced by the cellular cholesterol concentrations achieved in these studies. That is, in order for the appropriate regulatory responses to be elicited, it may be necessary that a certain threshold of cellular cholesterol concentration be reached. It is possible that, in these studies, this threshold was not achieved.

Second, and perhaps most likely, is the possible intracellular compartmentalization of cholesterol in such a manner that the added cholesterol may not have access to the "regulatory cholesterol pool." In contrast, oxygenated sterols such as 25-OH cholesterol may be capable of accessing the "regulatory pool" of cholesterol within the cell as Field and Mathur (23) have suggested. Consequently, it is uncertain from the available data the extent to which pigeon cells are capable of regulation of cholesterol metabolism by cholesterol per se. It is

clear from the experiments with 25-OH cholesterol and mevalovolactone that these enzymes are capable of being regulated in pigeon cells. Whether this occurs in vivo, however, is uncertain.

Since cholesterol synthesis is high in pigeon cells under conditions in which cellular cholesterol content remains unchanged, the cells must be in a dynamic steady state in which efflux equals synthesis. Pigeon cells do not possess a functional LDL receptor pathway (1) and cannot regulate cholesterol metabolism at the level of cholesterol influx. Thus, cholesterol homeostasis must be controlled either at the level of cholesterol synthesis or efflux. Although from the present study it is not possible to select from these two possibilities, there is evidence to indicate that the mechanism of cholesterol efflux may be different in pigeon cells relative to mammalian cells. In mammalian cells cholesterol efflux has an absolute requirement for the presence of a cholesterol acceptor in the medium (e.g., phospholipid vesicles, LPDS, apoproteins such as A-I, etc.) (14, 15). The requirement for a cholesterol acceptor in the culture medium is supported by the data from this study as well, in which there was little efflux of cholesterol from monkey cells into MEM alone (Fig. 6). Pigeon cells, however, lost considerable amounts of cholesterol into MEM alone, suggesting that a preexisting cholesterol acceptor was not required for cholesterol efflux from pigeon cells.

There are several possible explanations for this observation. Pigeon cells may be capable of producing their own cholesterol acceptor that is secreted into the medium either independently or as a complex with cholesterol. Blue and co-workers (24) for example have shown that cultured chick embryo muscle cells, as well as a number of extrahepatic tissues from chickens, synthesize and secrete apoA-I. The ability of chick muscle cells to synthesize and secrete apoA-I has recently been confirmed by Shackelford and Lebherz (25). ApoA-I, particularly when it is complexed with phospholipids, is one of the most efficient cholesterol acceptors that has been described, (26, 27). Preliminary unpublished data from our laboratory indicate that pigeon smooth muscle cells also produce small amounts of apoA-I, but whether the apoA-I is functioning to solubilize cholesterol effluxed from the cells is unknown. The synthesis of apoA-I by tissues other than liver, intestine, and stomach may be unique to birds, since others have failed to demonstrate apoA-I synthesis in a variety of mammalian tissues (28, 29). It is also possible, of course, that a new yet-to-be-identified material is being produced and secreted by the pigeon cells that acts to solubilize the cholesterol lost from the cells.

Another mechanism whereby cholesterol could be lost from the cell without the presence of a preexisting cholesterol acceptor is by membrane shedding (30). This proccess has been described in several cell types, but we have no evidence for or against its existence in pigeon cells.

The observation that the rate of cholesterol efflux parallels the rate of cholesterol synthesis by pigeon cells suggests that synthesis and efflux may be tightly coupled. As a result, it is difficult to know whether this relationship between synthesis and efflux is due to a primary effect on synthesis (resulting in an altered rate of efflux) or to a primary effect on efflux (resulting in an altered rate of synthesis).

Thus far we have carried out only a minimum number of studies with arterial smooth muscle cells from the atherosclerosis-resistant SR pigeon. In all of these studies a pattern of cholesterol metabolism similar to that seen in WC pigeon cells has been observed (Fig. 1). This does not exclude the possibility, however, that there may be differences in other parameters of cholesterol metabolism, or that quantitative differences in cholesterol synthesis or efflux may exist that might be important in understanding the mechanism of the difference in susceptibility to atherosclerosis between White Carneau and Show Racer pigeons.

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