Sterol synthesis is up-regulated in cholesterol-loaded pigeon macrophages during induction of cholesterol efflux

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Abstract The extent to which cholesterol synthesis is modulated in macrophage foam cells by changes in cholesterol influx and efflux was determined using thioglycollate-elicited peritoneal macrophages from normal and cholesterol-fed White Carneau (WC) and Show Racer (SR) pigeons. In peritoneal macrophages from normocholesterolemic pigeons, sterol synthesis from [^{14}C]-acetate was down-regulated by more than 90% following incubation in vitro with β-VLDL. Sterol synthesis was increased when the cellular free cholesterol concentration was decreased in response to stimulation of cholesterol efflux with apoHDL/phosphatidylcholine vesicles and cyclodextrin. Peritoneal macrophages isolated from hypercholesterolemic pigeons were loaded with cholesterol to levels similar to foam cells from atherosclerotic plaques (375–614 μg/mg cell protein), and had an extremely low rate of sterol synthesis. When cholesterol efflux was stimulated in these cells, sterol synthesis increased 8 to 10-fold, even though the cells remained grossly loaded with cholesterol. Cholesterol efflux also stimulated HMG-CoA reductase activity and LDL receptor expression. This suggests that only a small portion of the total cholesterol pool in macrophage foam cells was responsible for regulation of sterol synthesis, and that cholesterol generated by hydrolysis of cholesteryl esters was directed away from the regulatory pool by efflux from the cells. When the increase in sterol synthesis was blocked with the HMG-CoA reductase inhibitor mevinolin, there was no difference in the cholesterol content of the cells, or in the mass efflux of cholesterol into the culture medium. Thus, under these conditions, the increase in cholesterol synthesis during stimulation of cholesterol efflux does not appear to contribute significantly to the mass of cholesterol in these macrophage foam cells. Whether a similar situation exists in vivo is unknown.—Nordskog, B. K., J. W. Reagan, Jr., and R. W. St. Clair. **Sterol synthesis is up-regulated in cholesterol-loaded pigeon macrophages during induction of cholesterol efflux.** ***J. Lipid Res.*** **1999.** 40: **1806–1817.**

Supplementary key words macrophages • foam cells • cholesterol efflux • White Carneau pigeons • Show Racer pigeons • cholesterol synthesis • HMG-CoA reductase

White Carneau (WC) pigeons develop atherosclerosis naturally and at an accelerated rate with cholesterol feeding, while Show Racer (SR) pigeons are resistant (1–4). A key cell in the pathogenesis of atherosclerosis in pigeons, as it is in other animal models and humans, is the macrophage foam cell (5–7). Like mammalian macrophages, pigeon macrophages possess a number of mechanisms for uptake of lipoproteins leading to the accumulation of esterified cholesterol. These include the uptake of abnormal lipoproteins via scavenger receptors (8), the uptake of β-VLDL by an LDL receptor-like mechanism (8, 9), and uptake by initial binding to cell surface heparan sulfate proteoglycans (10). Internalization of lipoproteins, with their accompanying cholesterol, stimulates cholesterol esterification (9) and down-regulates LDL receptor expression in pigeon macrophages (9), as it does in mammalian macrophages (11–19). The storage of excess cholesterol as cytoplasmic cholesteryl ester droplets and the down-regulation of LDL uptake and cholesterol synthesis protects the cell from the potentially toxic effects of elevated concentrations of free cholesterol (20). The delivery of cholesterol to cells by the receptor-mediated uptake of lipoproteins is thought to exert these effects by enriching a regulatory pool of cholesterol within the endoplasmic reticulum, which in turn begins a cascade of events resulting ultimately in the down-regulation of sterol synthesis and LDL receptor expression (21). There is, however, no information available on whether cholesterol synthesis also is down-regulated in pigeon macrophages under conditions of cholesterol loading. If there were differences in the extent to which cholesterol synthesis was down-regulated by cholesterol loading in macrophages from WC and SR pigeons, it could provide a possible mechanism for the susceptibility of WC pigeons to the development of atherosclerosis. In support of the possibility that cholesterol synthesis in pigeon cells may not be efficiently regulated is the observation that pigeon aortic smooth muscle cells

Abbreviations: WC, White Carneau; SR, Show Racer; β-VLDL, β-migrating very low density lipoprotein; apo, apoprotein; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methyglutaryl coenzyme A; LDL, low density lipoprotein; EC, esterified cholesterol; FC, free cholesterol; FBS, fetal bovine serum; CD, cyclodextrin; apoHDL/PC, apoHDL/phosphatidylcholine vesicles; LPDS, lipoprotein deficient serum.

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incubated in the presence of 10% fetal bovine serum (FBS) maintained a nearly 20-fold higher rate of cholesterol synthesis than mammalian cells incubated under the same conditions (22). Whether a similar situation exists in pigeon macrophages is unknown.

Cells can also rid themselves of excess cholesterol by transfer of free cholesterol to extracellular acceptors such as HDL. This is thought to represent a critical first step (23) in reverse cholesterol transport, the process by which cellular cholesterol is transported to the liver for excretion in the bile. While cholesterol efflux involves transfer of free cholesterol from the plasma membrane to extracellular acceptors, other intracellular pools of cholesterol are also affected by the loss of free cholesterol from the plasma membrane. This is illustrated by the observation that the cholesteryl ester content of macrophage foam cells is reduced after efflux of free cholesterol. This is the result of the hydrolysis of cytoplasmic cholesteryl esters, followed by efflux of the free cholesterol from the cell. Under these conditions there is net loss of cholesterol from the cells. We were interested in knowing whether this loss of cholesterol from macrophage foam cells during induced cholesterol efflux would result in a depletion of the putative regulatory pool of free cholesterol to an extent that there would be up-regulation of cholesterol synthesis, even while cells remained enriched in total cholesterol. If this occurred it might reduce the rate at which net cholesterol loss from macrophage foam cells could occur under conditions of plasma cholesterol lowering.

In the present study we determined the sensitivity of regulation of cholesterol synthesis by pigeon macrophages to the influx and efflux of lipoprotein cholesterol. In addition, we measured the rate of cholesterol synthesis in macrophages loaded in vivo with high concentrations of cholesterol, similar to macrophage foam cells of atherosclerotic plaques, to determine whether cholesterol synthesis could be up-regulated during periods of acute stimulation of efflux of cholesterol from the cells.

METHODS

Materials

Stigmasterol was purchased from Steroloids Inc., Wilton, NH. [1,2-^{3}H]cholesterol and [1-^{14}C]oleate were purchased from DuPont, Boston, MA. [1-^{14}C]acetic acid and [^{125}I]sodium iodide were purchased from Amersham Corporation, Arlington Heights, IL. dl-3-Hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A and dl-[5-^{3}H]mevalonic acid (*N,N'*-dibenzylenediamine salt) were purchased from NEN Life Science Products, Boston, MA. 3-Hydroxy-3-methylglutaryl coenzyme A, sodium was purchased from P-L Biochemicals Inc., Milwaukee, WI. Silica gel-thin-layer chromatography (TLC) plates and polysilicic acid gel-impregnated glass fiber sheets for TLC were purchased from Curtin-Matheson Scientific, Kennesaw, GA. Thioglycollate was obtained from Difco Laboratories, Detroit, MI. Eagle's minimal essential medium (MEM) and MEM vitamins were purchased from JRH Biosciences, Lenexa, KS. Penicillin–streptomycin solution and l-glutamine were purchased from Mediatech, Herndon, VA. Dimethyl sulfoxide (DMSO), ethylenediamine-tetra acetic acid (EDTA), HEPES buffer, egg phosphatidylcholine (PC), digitonin, bovine serum albumin, glucose-6-phosphate dehydrogenase, and mevalonic acid lactone were purchased from Sigma Chemical Company, St. Louis, MO. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Norcross, GA. Culture dishes (35 mm and 60 mm) were obtained from Becton Dickinson, Franklin Lakes, NJ. 2-Hydroxy-propyl-β-cyclodextrin (CD) was a gift from Dr. George Rothblat or was purchased from Sigma. Mevinolin was a gift from Merck & Co., Inc., Rathway, NJ.

Cells and cell culture

Thioglycollate-elicited peritoneal macrophages were obtained from WC and SR pigeons as described previously (24). Pigeons were obtained from our breeding colony and were fed either a cholesterol-free commercial diet of pigeon pellets or the same diet supplemented with 10% lard and 0.5% cholesterol for at least 6 months. Normal macrophages refer to macrophages isolated from pigeons fed the cholesterol-free pigeon diet, while in vivo cholesterol-loaded macrophages refer to macrophages isolated from pigeons fed the cholesterol-containing diet. Pigeon macrophages were incubated in 35- or 60-mm tissue culture dishes with 2 ml of MEM containing HEPES buffer (10 mm), 100 units penicillin/ml, 100 μg streptomycin/ml, Eagle's vitamins, and 2 mm l-glutamine (medium A). Peritoneal exudate cells were incubated initially in plastic tissue culture dishes for 4 h in medium A plus 10% FBS, after which nonadherent cells were discarded. Adherent cells were then incubated overnight in medium A plus 10% FBS. Under these conditions, greater than 95% of adherent cells are macrophages (25).

Normal macrophages were loaded with cholesterol in vitro by incubating them for up to 24 h with rabbit β-VLDL. Rabbit β-VLDL was used as previous studies have shown it to be more efficient than other lipoproteins, including acetyl LDL, in loading pigeon macrophages with cholesterol (8). These will be referred to as in vitro-loaded cells. To stimulate cholesterol efflux, in vitro or in vivo loaded macrophages were incubated for the indicated time in medium A containing 1% or 10% FBS or 2.5 mg/ml LPDS in the presence or absence of apoHDL/phosphatidylcholine vesicles (apoHDL/PC) (1 mg PC/0.4 mg apoHDL protein per ml) with or without cyclodextrin (CD) (2 mm). The specific conditions are indicated in the figure legends.

Isolation of β-VLDL

Rabbit β-VLDL was isolated from the plasma of New Zealand White rabbits (Robinson Services, Inc., Winston-Salem, NC). Rabbits were fed a diet containing 0.5% cholesterol and 5% corn oil for at least 1 month. The pigeon β-VLDL was isolated from WC pigeons fed a diet supplemented with 0.5% cholesterol and 10% lard for at least 1 month. The β-VLDL was isolated by ultracentrifugation at $d < 1.006$ g/ml for 20 h at 36,000 rpm as previously described (25). The iodine monochloride method of MacFarlane as modified by Bilheimer, Eisenberg, and Levy (26) was used to label the β-VLDL with ^{125}I. After labeling, β-VLDL was dialyzed extensively against physiological saline containing EDTA (1 mg/ml) and sterilized by filtration through a Millipore filter (0.45 μm). Lipoproteins were added to macrophages in culture based on lipoprotein protein concentration. Cell and lipoprotein protein were measured by the method of Lowry et al. (27).

Apo HDL/PC vesicles

Phosphatidylcholine dissolved in chloroform was transferred to a glass tube and the solvent was evaporated at 37°C under a stream of nitrogen. Tris buffer (1.5–2.0 ml of 150 mm NaCl, 10 mm Tris-HCl, 1 mm EDTA, pH 7.6) was added to the tube and the mixture was sonicated in an ice bath for 10 min using a 3/8-in diameter sonicator tip on a Branson Sonifier Cell Disrupter (model W185) at setting 7. ApoHDL proteins were isolated from

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human HDL using the method of Scanu (28). HDL apoproteins were dissolved in a solution of 0.9% NaCl, 0.01% EDTA (pH 6.0) containing sodium azide (0.01%) and added to the sonicated PC while mixing gently on a vortex mixer to give a final concentration of 0.4 mg HDL protein/mg PC. The apoHDL/PC mixture was sonicated for an additional 10 sec. The apoHDL/PC vesicles were dialyzed for at least 3 h in medium A, sterilized by filtration through a Millipore filter (0.45 μm), and added to culture medium at a final concentration of 1 mg PC/0.4 mg apoHDL protein/ml. Cyclodextrin was added to the indicated dishes in the presence and absence of apoHDL/PC vesicles at a concentration of 2 mm CD. The CD was dissolved in culture medium and filter sterilized using a Millipore filter (0.45 μm).

Sterol mass, synthesis, and esterification

Macrophages were incubated as described in the figure legends. During the final 2 h of incubation [^{14}C]acetate or [^{14}C]oleate was added, as described previously (22, 29). Briefly, after the 2-h pulse with [^{14}C]acetate or [^{14}C]oleate, the medium was removed and the cells were washed 3 times with balanced salt solution (BSS) (137 mm sodium chloride, 2.7 mm potassium chloride, 1.5 mm potassium phosphate, and 8.1 mm sodium phosphate dibasic, pH 7.4). Cellular lipids were extracted directly from the tissue culture dishes with isopropanol (30). Lipids were extracted from the culture media using the method of Bligh and Dyer (31). Cholesterol mass was determined by gas–liquid chromatography (GLC) by the method of Ishikawa et al. (32) as modified by Klansek et al. (33) using stigmasterol as an internal standard. Results are expressed as μg/mg cell protein of free or esterified cholesterol. For measurement of sterol synthesis, an aliquot of the lipid extract was saponified in 1 N NaOH, 70% ethanol, for 2 h at 70°C. A [^{3}H]cholesterol internal standard was added and the nonsaponifiable lipids were extracted 3 times with hexane. The lipid extract was dried under nitrogen, resuspended in chloroform, and separated by TLC using a solvent system of hexane–ethyl ether–acetic acid 146:50:4 (v/v/v). The sterols that migrated with the authentic free cholesterol standard were isolated and counted in a Beckman LS 7800 liquid scintillation counter to determine sterol synthesis as measured by [^{14}C]acetate incorporated into total sterols. These methods have been described in detail elsewhere (22, 29). Mevinolin (5 μg/ml) was used in some experiments to inhibit sterol synthesis. Mevinolin was dissolved in DMSO prior to addition to the culture medium. Equivalent amounts of DMSO were used in control dishes. Preliminary experiments determined that mevinolin, at a concentration of 5 μg/ml, maximally inhibited sterol synthesis in pigeon macrophages (data not shown). For measurement of cholesterol esterification, an aliquot of the isopropanol lipid extract from cells was separated by TLC as described above. The cholesteryl ester band was counted to measure [^{14}C]oleate esterified to cholesterol. Purification of 3β-OH sterols using digitonin was performed using the method of Sperry (34) on the free cholesterol fraction after TLC of the saponified lipid extract from WC macrophages incubated for 2 h with [^{14}C]acetate. Prior to addition of digitonin, 100 mg of authentic cholesterol was added as carrier and [^{3}H]cholesterol was added as an internal standard. Cholesterol was recovered after treatment of the digitonide with pyridine (34) and reformed to constant specific activity.

HMG-CoA reductase activity

After incubation, as described in the figure legends, the culture medium was poured off and the cells were washed 2 times with buffer A (0.05 m Tris-HCl, 0.15 m NaCl, pH 7.4). Cells were scraped from the dish in the same buffer and transferred to a 12 ml centrifuge tube and pelleted by centrifugation. Cells were suspended in 1ml buffer A, and an aliquot was taken for protein determination. The remaining cells were transferred to microfuge tubes and pelleted by centrifugation. The supernatant fluid was removed and the cells were frozen at −70°C until the time of assay. HMG-CoA reductase activity was measured using the method of Brown, Dana, and Goldstein (35) as described previously (36).

Binding of ^{125}I-labeled pigeon β-VLDL to pigeon macrophages

LDL receptor expression on the surface of pigeon macrophages under various conditions of cholesterol loading or efflux was estimated from the binding at 4°C of ^{125}I-labeled pigeon β-VLDL as described previously (8–10). Briefly, cholesterol-loaded pigeon macrophages were incubated for 24 h as described in the figure legends. After incubation, the cells were washed three times with medium A and placed at 4°C for 30 min. Medium A, without FBS, containing ^{125}I-labeled pigeon β-VLDL, at a concentration of 5 μg protein/ml, in the presence or absence of a 20-fold excess of unlabeled homologous β-VLDL, was added to cells and incubated at 4°C. After 4 h the culture medium was removed and the cells were washed five times with BSS containing 2 mg/ml bovine serum albumin, then washed two times with BBS alone. The macrophage monolayer was allowed to air dry and 1 ml of 1 N NaOH was added to the dishes for at least 3 h to digest the cells. An aliquot was taken to determine both cell protein concentration and cell associated (Bound) ^{125}I radioactivity. Aliquots were counted for ^{125}I in a Beckman gamma counter (model 5500B). Specific binding was calculated as the difference between ^{125}I-labeled β-VLDL bound in the presence and absence of a 20-fold excess of unlabeled β-VLDL.

Statistics

Results of cell culture studies are expressed as the mean ± SEM of triplicate determinations for each data point. Where SEM bars are not seen they are contained within the symbol. Results of representative experiments are shown. Each experiment was repeated at least once with comparable results. Results were analyzed for statistically significant differences by one-way or two-way ANOVA using the SigmaStat statistical software from Jandel, San Rafael, CA. Values were considered statistically significant at $P < 0.05$. Differences between treatments were compared using the Student Newman-Keuls test.

RESULTS

Shown in **Fig. 1** are cholesterol mass and sterol synthesis in normal pigeon peritoneal macrophages incubated in media containing 1% FBS, 10% FBS, or 1% FBS plus apoHDL/PC vesicles. Cholesterol mass was significantly ($P < 0.05$) higher in both WC (Fig. 1A) and SR (Fig. 1C) macrophages incubated in medium containing 1% FBS than with 10% FBS or 1% FBS plus apoHDL/PC vesicles. This was due to increases in both free cholesterol and esterified cholesterol. Although the magnitude of this difference was not great (~20%), it was consistent among several experiments. When incubated in the presence of apoHDL/PC vesicles to stimulate efflux of cellular cholesterol, there was an approximate 10% decrease in cellular free cholesterol and a 50% decrease in esterified cholesterol in both WC and SR macrophages compared with cells incubated with 10% FBS. Sterol synthesis changed inversely with cholesterol mass and was highest when apoHDL/PC vesicles were present (Fig. 1B and Fig. 1D).

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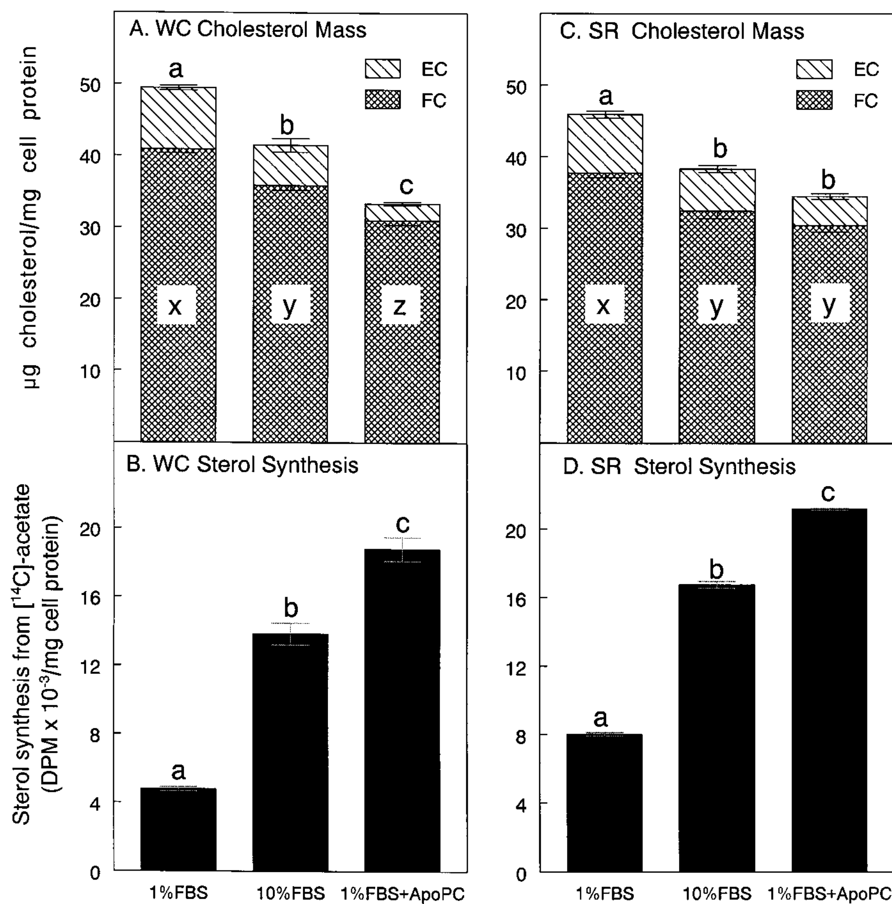

Fig. 1. Effect of culture medium (medium A) containing 1% or 10% FBS or apoHDL/PC vesicles on cholesterol mass and sterol synthesis in normal WC and SR pigeon macrophages. Normal pigeon macrophages were incubated in culture medium (medium A) containing 1% FBS, 10% FBS, or 1% FBS plus apoHDL/PC vesicles (ApoPC) (400 μg apoHDL and 1 mg PC/ml) for 24 h. [^{14}C]acetate was added in the final 2 h to measure sterol synthesis. Cholesterol mass was determined by GLC. Results are the mean ± SEM (n = 3). Significant differences ($P < 0.05$) between bars in the same panel are indicated by different letters (a, b, and c for EC and sterol synthesis, and x, y, and z for FC).

Consistent with our experience with pigeon smooth muscle cells (22), macrophages incubated in 10% FBS had higher rates of sterol synthesis than those incubated with 1% FBS, and the rate of sterol synthesis with 10% FBS approached that found for cells incubated with apoHDL/PC vesicles. These data are consistent with the conclusion that 10% FBS and apoHDL/PC vesicles promote net cholesterol efflux, thus stimulating sterol synthesis, while 1% FBS is a poor promoter of cholesterol efflux in these unloaded cells, which results in the build-up or maintenance of cellular cholesterol mass and the ultimate down-regulation of sterol synthesis. The remainder of the studies in this paper were designed to test the relationship between cellular cholesterol mass and sterol synthesis in WC and SR macrophages and their modulation by the influx or efflux of cholesterol.

To determine the relative sensitivity of regulation of sterol synthesis to increasing cellular cholesterol concentrations, normal WC and SR macrophages were incubated for 24 h with rabbit β-VLDL at concentrations from 0 to 50 μg β-VLDL protein/ml (**Fig. 2**). Rabbit β-VLDL was used as previous studies had shown that it is unusually effective in increasing the cholesterol content of pigeon macrophages (9). As expected, there was an increase in both the free and esterified cholesterol content of macrophages with increasing β-VLDL concentrations in the culture medium (Fig. 2A) in both WC and SR macrophages. Esterified cholesterol concentrations increased more steeply with increasing β-VLDL concentrations particularly at concentrations above 6.25 μg/ml. Sterol synthesis (Fig. 2B) was exquisitely sensitive to cellular cholesterol concentrations as seen by the nearly 50% down-regulation of sterol synthesis with the lowest concentration of β-VLDL used (0.78 μg/ml). At this concentration there was a statistically insignificant change in esterified cholesterol concentration (5.86 ± 0.23 to 7.96 ± 1.34 μg esterified cholesterol/mg cell protein for WC pigeon macrophages and 8.84 ± 0.64 to 8.14 ± 0.37 μg esterified cholesterol/mg cell protein for SR pigeon macrophages), but a small increase in free cholesterol concentration (41.21 ± 0.16 to 44.13 ± 0.29 μg free cholesterol/mg cell protein for WC pigeon macrophages and 38.83 ± 0.55 to 40.67 ± 1.1 μg free cholesterol/mg cell protein for SR pigeon macro-

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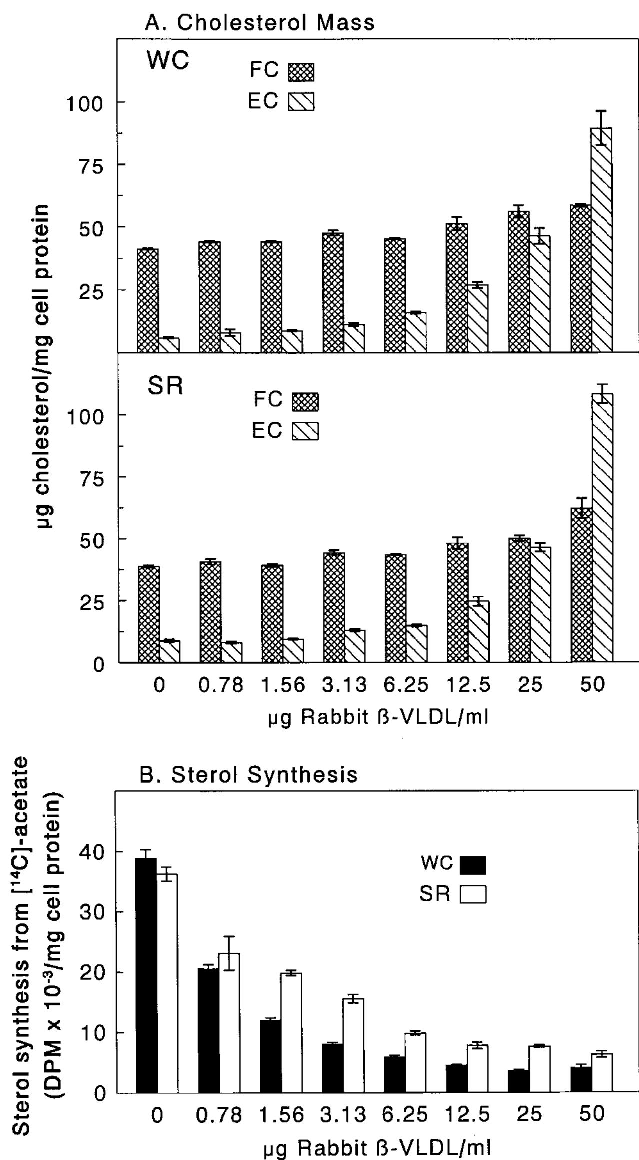

Fig. 2. Effect of in vitro cholesterol loading on cholesterol mass and sterol synthesis in WC and SR macrophages. Normal pigeon macrophages were incubated for 24 h in culture medium (medium A) containing 10% FBS and the indicated amounts of rabbit β-VLDL. [^{14}C]acetate was added in the final 2 h to measure sterol synthesis. Cholesterol mass was determined by GLC. Results are the mean ± SEM (n = 3).

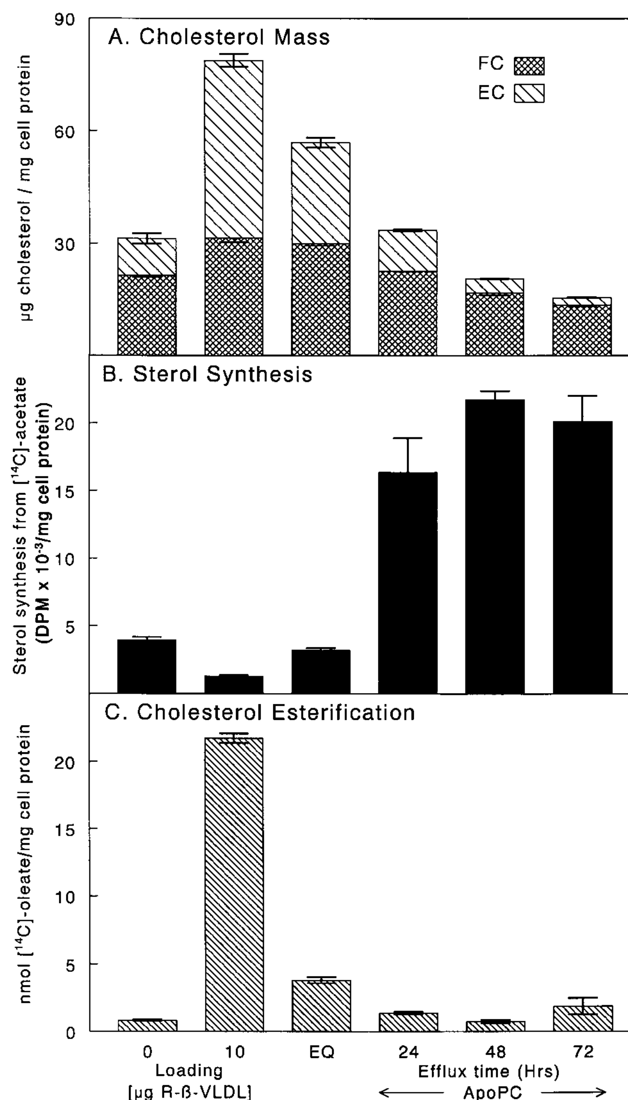

Fig. 3. Regulation of sterol synthesis and cholesterol esterification during cholesterol efflux from WC pigeon peritoneal macrophages loaded with cholesterol in vitro. WC macrophages were incubated for 24 h with culture medium (medium A plus 1% FBS) containing 0 or 10 μg/ml of rabbit β-VLDL (loading). The cells then were incubated with medium A containing 1% FBS for a 24-h equilibration period (EQ). After the equilibration period, the cells were incubated with medium A containing 1% FBS with apoHDL/PC vesicles (ApoPC) for up to 72 hours. [^{14}C]acetate or [^{14}C]oleate were added for the final 2 h of incubation to measure sterol synthesis and cholesterol esterification respectively. Cholesterol mass was measured by GLC. Results are the mean ± SEM (n = 3).

phages) that was significant ($P < 0.001$) for WC but not for SR cells. At the highest concentration of β-VLDL, sterol synthesis was reduced by 87% for WC macrophages and 80% for SR macrophages. Thus, pigeon macrophages have a sensitive mechanism for the down-regulation of sterol synthesis that correlates best with increases in cellular free cholesterol concentration.

To determine whether sterol synthesis could be upregulated by cholesterol efflux, we initially loaded WC pigeon macrophages in vitro with cholesterol by incubation with rabbit β-VLDL (10 μg/ml) for 24 h and then measured cellular cholesterol mass (**Fig. 3A**), sterol synthesis (Fig. 3B), and cholesterol esterification (Fig. 3C). Under these conditions, incubation with β-VLDL increased cell cholesterol mass to levels about 2.5-times higher than in non-loaded cells. There was a significant increase in both free and esterified cholesterol, a marked inhibition of sterol synthesis, and stimulation of cholesterol esterification. These cholesterol-loaded cells then were allowed to equilibrate for 24 h in medium containing 1% FBS. During the equilibration period, the macrophages lost approximately 50% of their esterified cholesterol but little free cholesterol. Sterol synthesis returned to initial levels

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and cholesterol esterification decreased by about 80%, even though the cells contained nearly twice as much cholesterol as was present before loading. Cells then were incubated in medium containing apoHDL/PC vesicles to promote cholesterol efflux. After 72 h, 50% of the total cellular cholesterol was lost, and free cholesterol concentrations decreased to levels less than control cells before loading with β-VLDL. At the same time, sterol synthesis rose nearly 5-fold and cholesterol esterification was reduced to control levels. Sterol synthesis and cholesterol esterification were maximumly stimulated and inhibited, respectively, by 24 h, even though cellular cholesterol mass continued to decrease through 72 h.

To determine whether sterol synthesis was stimulated during cholesterol efflux, but before cellular cholesterol approached normal concentrations, we used WC macrophages that had been loaded in vivo with cholesterol to levels typically found in macrophage foam cells from atherosclerotic plaques (37). These cholesterol-loaded WC macrophages were incubated in media containing 1% FBS (control) or 1% FBS plus apoHDL/PC vesicles, CD or CD plus apoHDL/PC vesicles (**Fig. 4**). Cyclodextrin (2 mm) by itself had only a modest effect on free and esterified cholesterol mass (Figs. 4A and 4B) and no effect on sterol synthesis (Fig. 4C). When apoHDL/PC vesicles were added to the culture medium, the free cholesterol mass was reduced by 25% and esterified cholesterol mass was reduced by 50% at 48 h. Sterol synthesis was increased about 8-fold. The combination of 2 mm CD and apoHDL/PC vesicles further depleted the cells of cholesterol, primarily free cholesterol, and markedly enhanced sterol synthesis. The increase in sterol synthesis with apoHDL/PC vesicles with or without CD was somewhat unexpected considering these macrophages still contained abnormally high concentrations of cholesterol (>200 μg/mg protein). The data in Fig. 4 were obtained using WC macrophages, but similar results also were obtained with SR macrophages (data not shown).

In preliminary experiments we determined the extent to which the ^{14}C-labeled sterols in the free cholesterol band after incubation of WC macrophages for 2 h with [^{14}C]acetate represented 3β-hydroxy sterols such as cholesterol. Unlabeled authentic cholesterol was added to the ^{14}C-labeled FC fraction after separation of the nonsaponifiable lipids by TLC, and specific activity was determined. After precipitation with digitonin, the cholesterol specific activity decreased by approximately 50% suggesting that about one-half of the ^{14}C-labeled sterols separated by TLC represented high specific activity precursors of cholesterol that were not yet 3β-hydroxy sterols.

To be certain that the increases in ^{14}C-labeled sterols from [^{14}C]acetate truly represented an up-regulation of the cholesterol synthetic pathway, we stimulated cholesterol efflux with apoHDL/PC vesicles in the presence or absence of CD and measured HMG-CoA reductase activity and cholesterol mass. As shown in **Fig. 5**, conditions that stimulated efflux also resulted in stimulation of HMG-CoA reductase activity. The data from Figs. 4 and 5 are also consistent with the conclusion that during active cholesterol efflux, the pool of cholesterol responsible for regulation of sterol synthesis was depleted, even while total cell cholesterol concentrations remained high.

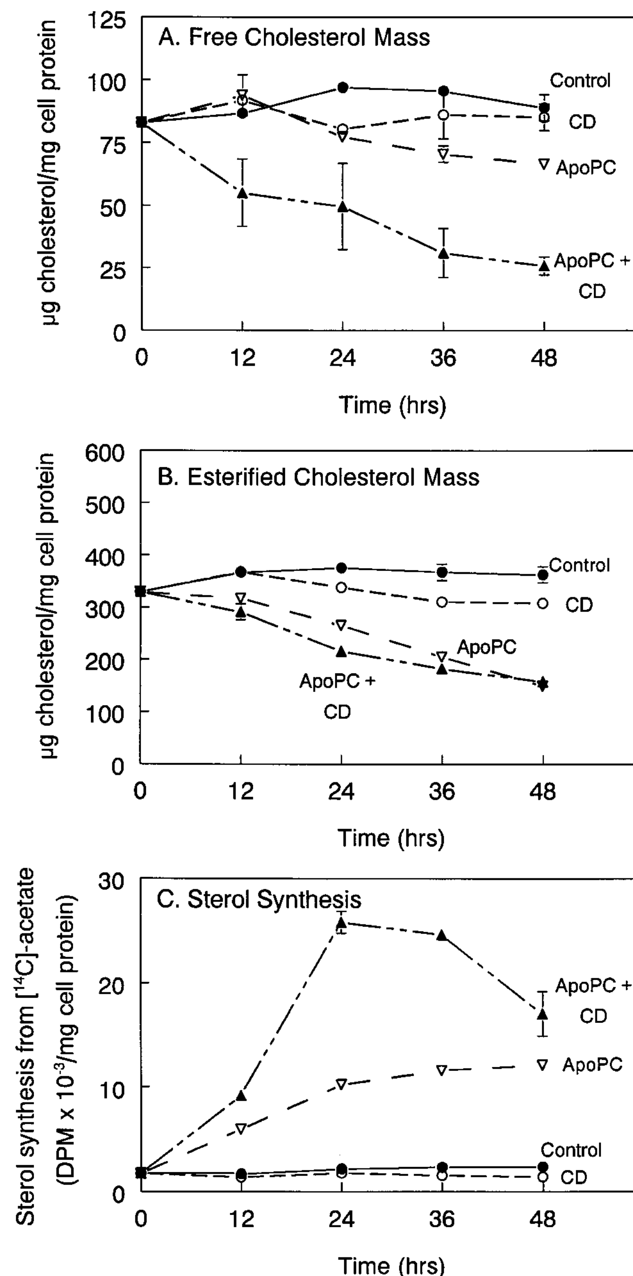

Fig. 4. Sterol synthesis is up-regulated during cholesterol mobilization (efflux) in peritoneal macrophages isolated from cholesterol-fed pigeons. In vivo loaded WC peritoneal macrophages were incubated for up to 48 h in medium A containing 1% FBS or with medium A containing 1% FBS plus CD (2 mm) and/or apoHDL/PC vesicles (ApoPC) to promote cholesterol efflux. Sterol synthesis was measured in the final 2 h from [^{14}C]acetate. Cholesterol mass was measured by GLC. Results are the mean ± SEM (n = 3).

As sterol synthesis and LDL receptor expression are coordinately regulated (11, 38), we also determined the effect on LDL receptor binding as a result of stimulating cholesterol efflux by apoHDL/PC vesicles plus CD in heavily cholesterol-loaded WC macrophages. The ligand

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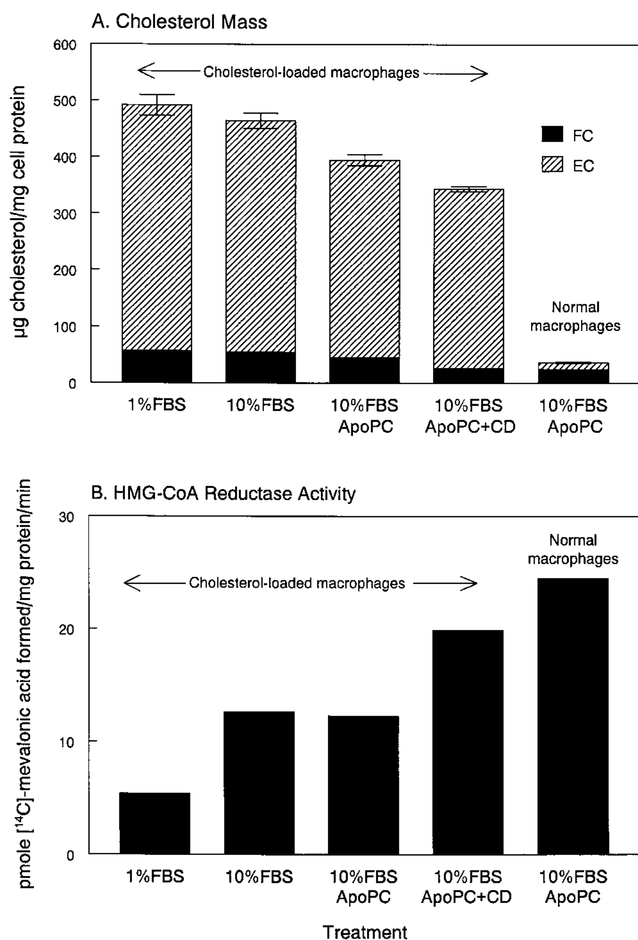

Fig. 5. HMG-CoA reductase activity in normal pigeon macrophages and during cholesterol efflux in cholesterol loaded cells. Normal and in vivo cholesterol-loaded WC peritoneal macrophages were incubated for 24 h in medium A containing the indicated treatments to promote cholesterol efflux. Cell cholesterol mass was measured in parallel treated dishes by GLC. Results are the mean ± SEM (n = 3). HMG-CoA reductase activity was measured in cells at the end of the indicated treatment period. For these analyses, cells from each treatment were scraped from two 60-mm culture dishes and pooled. The HMG-CoA reductase assay was run in triplicate from the pooled samples and results are expressed as the mean of these triplicates. Thus, error bars are not shown in panel B. ApoPC is apoHDL/PC vesicles.

for these binding studies was ^{125}I-labeled pigeon β-VLDL. Previous studies have shown that pigeon β-VLDL binds to an LDL-like receptor on pigeon macrophages (8, 9). As shown in **Fig. 6**, apoHDL/PC vesicles plus CD stimulated cholesterol efflux (Fig. 6A) and sterol synthesis (Fig. 6B) from [^{14}C]acetate, and in the same cells stimulated specific binding of ^{125}I-labeled pigeon β-VLDL, without affecting nonspecific binding (Fig. 6C). This provides further evidence that when net cholesterol efflux is stimulated there is a reduction in the regulatory pool of cellular cholesterol resulting in the coordinate stimulation of sterol synthesis and LDL receptor expression.

To determine whether the stimulation in sterol synthesis during cholesterol efflux from macrophages contributes to the mass of cholesterol in the cell, cholesterol efflux

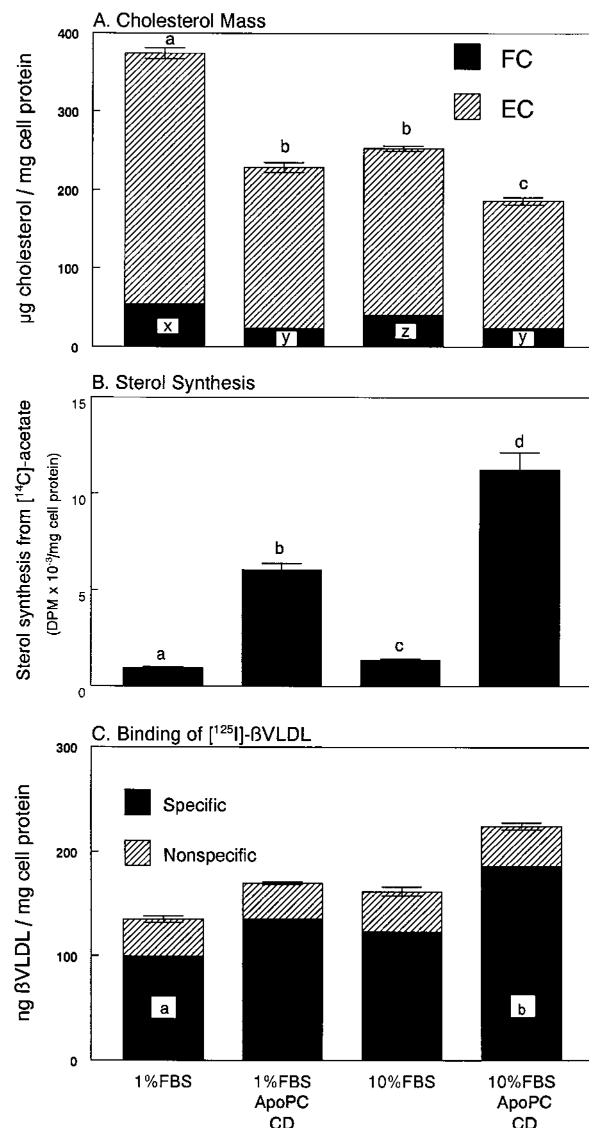

Fig. 6. Specific binding of ^{125}I-labeled pigeon β-VLDL to pigeon peritoneal macrophages is up-regulated during cholesterol efflux. Peritoneal macrophages from WC pigeons loaded with cholesterol in vivo were incubated for 24 h in medium containing the indicated additions. Sterol synthesis and cholesterol mass were measured on the same cells. For this, [^{14}C]acetate was added for the final 2 h of the 24-h incubation. Cells were washed, lipids were extracted and processed for mass and sterol synthesis from [^{14}C]acetate. Cholesterol mass was determined by GLC. Binding of ^{125}I-labeled pigeon β-VLDL was measured in a second set of dishes using the same cells, prepared at the same time, as those used for cholesterol mass and sterol synthesis. Binding was determined by incubation for 4 h at 4°C with 5 μg/ml of ^{125}I-labeled pigeon β-VLDL in serum-free medium, in the presence or absence of a 20-fold excess of unlabeled pigeon β-VLDL. Specific binding was calculated as the difference between total and nonspecific binding. Results are the mean ± SEM (n = 3). Significant differences ($P < 0.05$) are indicated by different letters above or within each bar. CD, cyclodextrin at 2 mm; ApoPC, apoHDL/PC vesicles.

was stimulated with apoHDL/PC vesicles in the presence or absence of the HMG-CoA reductase inhibitor mevinolin, at a concentration sufficient to completely block sterol synthesis (5 μg/ml) (**Fig. 7**). The culture medium for

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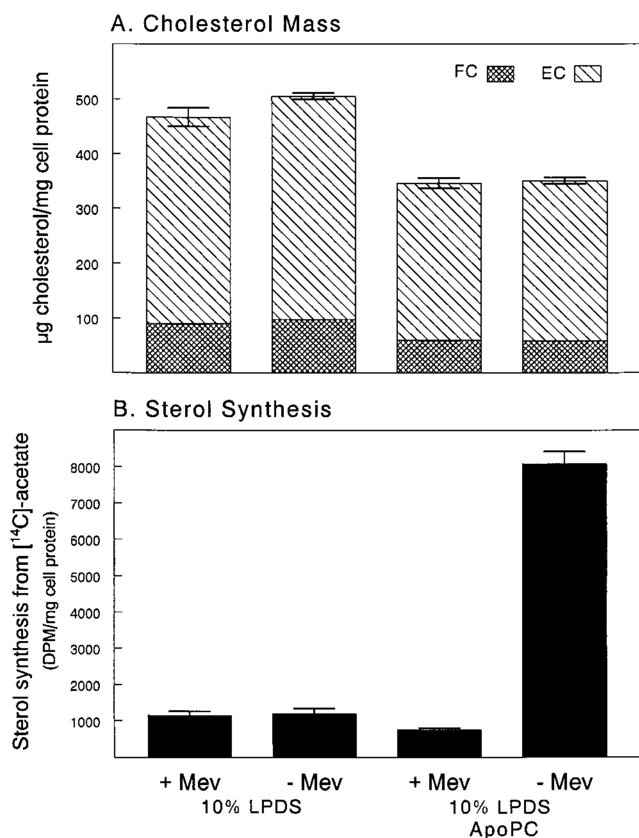

Fig. 7. Effect of mevinolin on cholesterol mass and sterol synthesis in WC pigeon peritoneal macrophages loaded in vivo with cholesterol. Peritoneal macrophages loaded in vivo from WC pigeons were incubated for 24 h in medium A containing the indicated treatments in the presence (+ Mev) or absence (− Mev) of 5 μg/ml mevinolin. Sterol synthesis was measured in the final 2 h from [^{14}C]acetate. Cholesterol mass was measured by GLC. Results are the mean ± SEM (n = 3). Apo PC is apoHDL/PC vesicles.

these studies contained 10% LPDS (2.5 mg/ml) rather than 10% FBS, in order to eliminate a source of cholesterol in the medium. With LPDS medium alone, cellular cholesterol mass ranged from 470 to 500 μg/mg cell protein. When apoHDL/PC vesicles were added, cell cholesterol concentrations decreased to approximately 350 μg/mg cell protein (Fig. 7A) and sterol synthesis was increased over 10-fold (Fig. 7B). When this increase in sterol synthesis was prevented with mevinolin (Fig. 7B), there was no effect on cellular cholesterol mass. This suggests that, over the 24 h of this experiment, either the mass of cholesterol represented by this amount of sterol synthesis is small relative to the mass of cholesterol in the cell or that newly synthesized sterols efflux preferentially from the cell.

To determine the fate of newly synthesized cholesterol in cholesterol-loaded macrophages, we incubated WC macrophages in medium containing 10% LPDS, apoHDL/PC vesicles and CD (to induce cholesterol efflux and up-regulate sterol synthesis), in the presence or absence of mevinolin (to inhibit endogenous cholesterol synthesis). Cholesterol mass and ^{14}C-labeled newly synthesized sterols were measured in the cells and the culture medium. Consistent with the results shown in Fig. 7, there was no significant difference in the loss of cellular cholesterol in the presence or absence of mevinolin (**Fig. 8A**). This also was true for the mass of cholesterol secreted into the culture medium in the presence or absence of mevinolin (Fig. 8C). After 18 h the majority of the cholesterol in the medium was free cholesterol, but surprisingly, there were also detectable amounts of esterified cholesterol, which, by 36 h, was equivalent to the amount of free cholesterol that was lost by cholesterol efflux into the medium. Mevinolin inhibited the synthesis of sterols by greater than 99% (Fig. 8B), and virtually no ^{14}C-labeled sterols were detected in the culture medium in mevinolin-treated cells (Fig. 8D). In cells incubated without mevinolin, greater than 90% of newly synthesized sterols were found in the culture medium at 36 h, compared with about 70% of the mass of free cholesterol and 46% of the mass of esterified cholesterol over the same time period. Thus, these data suggest that much of the newly synthesized sterols efflux preferentially from the cells.

DISCUSSION

The results of this and previous studies (8) show that pigeon peritoneal macrophages, like mammalian cells (16, 17, 35, 39, 40), respond to an increase in cellular cholesterol content by the coordinate down-regulation of endogenous cholesterol synthesis and LDL receptor binding, and the up-regulation of cholesteryl ester synthesis. Down-regulation of sterol synthesis was sensitive to small changes in cellular cholesterol as seen by the 40–50% reduction in sterol synthesis in cells incubated with concentrations of rabbit β-VLDL as low as 0.78 μg/ml. At this concentration there was only a 2–3 μg/mg cell protein increase in the free cholesterol content of the cells, and little consistent change in esterified cholesterol content. As in other cell types (41) the changes in sterol synthesis were most highly associated with changes in cellular free cholesterol concentration, although under certain conditions that stimulate cholesterol efflux, changes in sterol synthesis occurred without detectable changes in total cellular free cholesterol content. The maximum reduction in sterol synthesis of 80–90% occurred in pigeon macrophages incubated with β-VLDL at concentrations of 12.5 μg/ml or greater. Not only was sterol synthesis down-regulated by increases in cellular free cholesterol concentration, but cholesterol esterification was up-regulated. Down-regulation of cholesterol esterification appeared to be more sensitive to depletion of cellular free cholesterol than was up-regulation of sterol synthesis (Fig. 3). This may reflect the different mechanisms by which these two processes are regulated. Cholesterol synthesis is regulated at many levels including transcriptional, translational, and protein degradation (11, 42, 43), while cholesterol esterification is regulated primarily by free cholesterol substrate concentration (44–46).

In these studies, sterol synthesis was measured from [^{14}C]acetate incorporation into compounds that migrated with free cholesterol on TLC. Previous studies (47, 48) in-

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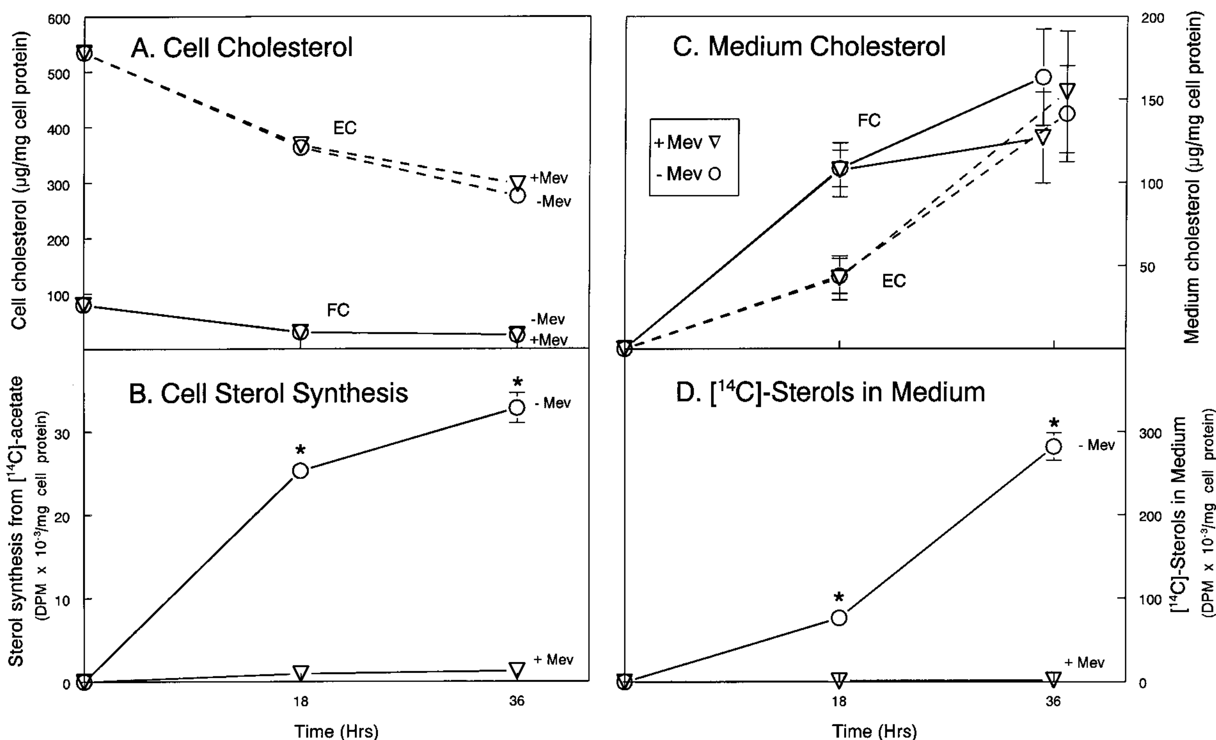

Fig. 8. Effect of inhibition of cholesterol synthesis during cholesterol efflux from in vivo cholesterol loaded WC macrophages on the concentration of cholesterol and newly synthesized sterols in cells and culture medium. Peritoneal macrophages loaded in vivo from WC pigeons were incubated for up to 36 h in medium A containing 10% LPDS, apoHDL/PC vesicles, and CD (2 mm) in the presence or absence of mevinolin (5 μg/ml). Sterol synthesis was measured in the final 2 h from [^{14}C]acetate. The appearance of ^{14}C-labeled sterols was measured in cells and culture medium. Free (FC) and esterified cholesterol (EC) mass were measured in cells and culture medium by GLC. Results are the mean ± SEM (n = 3). Significant differences at $P < 0.05$ were detected by two way ANOVA. Significant differences between treatments (± mevinolin) are indicated by the asterisk (*).

cluding our own (49), have shown that in most nonhepatic cells in culture and in normal and atherosclerotic arterial tissue, only a small percentage of ^{14}C-labeled sterols from [^{14}C]acetate, mevalonate, or squalene is converted to authentic cholesterol in a 2-h incubation period. This was the case in the present study as well. In control experiments we found that less than 50% of the ^{14}C-labeled sterols separated by TLC were precipitable with digitonin, and thus represented 3β-OH-sterols. As the digitonin-precipitable fraction was not further characterized, we cannot say what proportion represented cholesterol. From previous studies of nonhepatic cells, including macrophages, much of this would be expected to be precursors of cholesterol such as desmosterol (50–52) or zymosterol (53). This is particularly true for short-term labeling experiments such as these (47). As a result, it is likely that [^{14}C]acetate incorporation into ^{14}C-labeled sterols overestimates the actual rate of cholesterol synthesis, as it is not known what proportion of these precursors eventually will be converted to cholesterol. However, the fact that they represent products that are beyond the rate-limiting step in sterol synthesis means that they still can provide a useful measure of relative rates of total sterol synthesis. In the present study, changes in [^{14}C]acetate incorporation into ^{14}C-labeled sterols with cholesterol loading or efflux could not be attributed to changes in [^{14}C]acetate precursor pool specific activity, as there was little change in [^{14}C]acetate incorporation into ^{14}C-labeled fatty acids under the same conditions where sterol synthesis increased by greater than 7-fold (data not shown).

Previous studies with pigeon smooth muscle cells suggested that they may not regulate cellular cholesterol homeostasis in the same way as mammalian cells. In mammalian cells 10% FBS down-regulates cholesterol synthesis relative to LPDS, due to the net uptake of LDL cholesterol from the FBS compared with the net efflux of cholesterol to LPDS. In pigeon smooth muscle cells the opposite occurs (22). In the present study, like pigeon smooth muscle cells, cholesterol synthesis was up-regulated when pigeon macrophages were incubated in medium containing 10% FBS compared with 1% FBS (Fig. 1) or 10% LPDS (Fig. 7). This does not appear to be the result of any fundamental difference in regulation of cholesterol homeostasis by pigeon cells, however. Instead, it can be explained by differences in the ability of FBS and LPDS to induce cholesterol accumulation by pigeon cells. Unlike mammalian cells, pigeon macrophages had a higher cholesterol content when incubated with 1% FBS (Fig. 1) or 10% LPDS (data not shown), than cells incubated with 10% FBS. This appears to be the result of two factors. Mammalian LDL binds with low affinity to pigeon LDL receptors (8, 54) resulting in little delivery of cholesterol to pigeon cells

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from FBS. FBS instead, promotes cholesterol efflux by acting as an effective acceptor of cholesterol, with 10% FBS being more effective than 1% FBS or LPDS. As a result, 10% FBS promotes net cholesterol efflux from pigeon cells thus up-regulating sterol synthesis, while in mammalian cells 10% FBS promotes net cholesterol accumulation and down-regulates sterol synthesis.

Although there are numerous reports showing regulation of cholesterol synthesis in a variety of cells with changes in cellular cholesterol concentration, there are relatively few reports with macrophages and, to our knowledge, this is the first report using avian macrophages. These studies also are unique in that they have used macrophages isolated from hypercholesterolemic pigeons that are loaded in vivo with cholesterol (350–600 μg/mg cell protein) to levels typical of macrophage foam cells from atherosclerotic arteries (37, 55). When these macrophage foam cells were stimulated to efflux cholesterol by incubation with a variety of cholesterol acceptors, sterol synthesis was up-regulated to levels approaching the maximum rate seen in cholesterol-depleted cells. This occurred even though the cells remained grossly loaded (>200 μg/mg cell protein) with cholesterol. The maximum rate of sterol synthesis in cholesterol-loaded cells was achieved under conditions where there was the greatest rate of loss of cellular cholesterol (e.g., in those cells incubated in the presence of apoHDL/PC vesicles or apoHDL/PC vesicles+CD). The most effective promoter of cholesterol efflux from macrophage foam cells was apoHDL/PC vesicles+CD. As described by Atger et al. (56), CD alone at a concentration of 2 mm acts primarily as a "shuttle" to move cholesterol from the plasma membrane to apoHDL/PC vesicles which acts as a "sink" for the cholesterol. The enhanced mobilization of free cholesterol with apoHDL/PC vesicles+CD is evident from the marked decrease in cellular free cholesterol, compared with apoHDL/PC vesicles alone (Fig. 4). During the first 24 h of incubation with apoHDL/PC vesicles alone, cellular free cholesterol concentrations did not change. At the same time there was a nearly 40% decrease in cellular esterified cholesterol content. As free cholesterol is the major form to appear in the culture medium in this time frame, the hydrolysis of cholesteryl esters must be the ultimate source of the bulk of free cholesterol for efflux.

Associated with the greater loss of free cholesterol with apoHDL/PC vesicles+CD, was a much greater increase in sterol synthesis than with apoHDL/PC vesicles alone. The esterified cholesterol content of the cells declined at a similar rate in cells treated with apoHDL/PC vesicles with or without CD (Fig. 4). This suggests that the rate of hydrolysis of esterified cholesterol was maximized with apoHDL/PC vesicles, and that this rate did not increase with addition of CD, even though with CD there was a more than doubling of the rate of loss of FC from the cells. This implies that the rate of hydrolysis of esterified cholesterol in macrophage foam cells is not greatly influenced by the rate of free cholesterol efflux from the cells. This is in contrast to a recent report using mouse macrophages (57).

Even though there was no reduction in total cellular free cholesterol concentration with apoHDL/PC vesicles alone over the first 24 h, there must have been some depletion of the regulatory pool of free cholesterol in the cells to account for the up-regulation of sterol synthesis. This suggests that the regulatory pool of free cholesterol in the cells is small relative to the total cell cholesterol content. In addition, the results imply that the free cholesterol generated from hydrolysis of esterified cholesterol must not be in rapid equilibrium with the regulatory pool of free cholesterol, otherwise there should have been no stimulation of sterol synthesis with apoHDL/PC vesicles alone. The reason CD alone did not stimulate sterol synthesis is probably because the absence of a "sink" reduces the capacity of 2 mm CD to solubilize and remove sufficient amounts of cholesterol to deplete the regulatory pool in the presence of the rapid generation of free cholesterol from the hydrolysis of esterified cholesterol. Consistent with the interpretation that incubation of macrophage foam cells in the presence of apoHDL/PC vesicles depletes the regulatory pool of free cholesterol is the coordinate up-regulation of sterol synthesis, HMG-CoA reductase activity, and LDL receptor binding. An additional possible regulatory process, which we did not explore, is the role of second messengers like ceramide in the activation of sterol regulatory element binding proteins (SREBPs) as suggested by Lawler et al. (58).

A surprising finding of this study was the apparent efflux of esterified cholesterol into the culture medium. In virtually all published studies it has been shown that free cholesterol is the primary form of cholesterol lost from a variety of cell types in culture under many different conditions that favor cholesterol efflux (59). However, most studies have used cells either not loaded with cholesterol or loaded in vitro to relatively low levels. In contrast, the macrophages used in these studies were loaded in vivo with cholesterol to high levels, resembling that seen in foam cells from atherosclerotic arteries. There are several possible explanations for the apparent efflux of esterified cholesterol from these pigeon macrophage foam cells. Cell death, rather than an active efflux process is one possibility. This would result in the nonspecific release of cellular cholesteryl esters into the culture medium. Although this possibility cannot be completely eliminated, the culture medium was initially centrifuged to remove cells and cell debris before analysis, and there was no change in cell protein per dish during the 36-h efflux phase of the experiment that would be expected if there had been significant cell death. A more likely alternative is that either a few cells with very high esterified cholesterol to protein ratios died, or that macrophages highly enriched in esterified cholesterol can secrete esterified cholesterol either by a vesicular exocytosis process or by another yet-to-be identified mechanism. The answer to this will have to await future studies.

As sterol synthesis is increased in macrophage foam cells during rapid depletion of cholesterol, the question arises as to whether this would result in the production of sufficient new cholesterol to contribute significantly to the total mass of cholesterol in the cell, thus reducing the rate at which macrophage foam cells can be depleted of cholesterol. To test this possibility we inhibited sterol synthesis

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with mevinolin and determined whether the rate of cholesterol depletion from macrophage foam cells was slowed. As shown in Figs. 7 and 8, inhibiting the more than 10-fold increase in sterol synthesis upon addition of a cholesterol acceptor had no effect on the loss of cellular cholesterol mass from macrophage foam cells or the appearance of cholesterol in the culture medium. This would indicate that even under conditions of maximum stimulation of cholesterol synthesis, the contribution of newly synthesized cholesterol to the total cellular cholesterol pool is probably small. As there were no differences between macrophages of WC and SR pigeons in their ability to up-regulate sterol synthesis upon stimulation of cholesterol efflux or for sterol synthesis to contribute to the sterol content of the cells, it is unlikely that this process is responsible for the differences in susceptibility or resistance to atherosclerosis between these breeds of pigeons. The above interpretation must be taken with some caution because of the difficulties in measuring absolute rates of cholesterol synthesis from short-term incubations with [^{14}C]acetate (47). For example, newly synthesized ^{14}C-labeled sterols effluxed from the cells at a greater rate than cholesterol mass, and after a 2-h incubation there was more than 10-times more ^{14}C-labeled sterols in the culture medium than in the cells (Fig. 8). This is consistent with reports by others (51–53), but not all (50), that sterol intermediates such as desmosterol and zymasterol efflux from cells more rapidly than cholesterol. Thus, we cannot eliminate the possibility that more authentic cholesterol is synthesized, and less effluxes from cells in vivo, compared to cells maintained in culture. If so, this may imply that HMG-CoA reductase inhibitors that are used to lower plasma cholesterol concentrations may have an additional effect to inhibit the stimulation of cholesterol synthesis in macrophage foam cells that are undergoing rapid efflux of cholesterol secondary to the lowering of plasma cholesterol concentrations. The loss of macrophage foam cells from atherosclerotic plaques is believed to be a critical component of plaque remodeling that significantly reduces the risk of plaque rupture and thrombosis (60). Consistent with this concept are reports that HMG-CoA reductase inhibitors have beneficial effects on the arterial wall that are greater than their ability to lower plasma cholesterol levels (61–65).

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REFERENCES

1. Clarkson, T. B., R. W. Prichard, M. G. Netsky, and H. B. Lofland. 1959. Atherosclerosis in pigeons: its spontaneous occurrence and resemblance to human atherosclerosis. *Arch. Pathol.* **68:** 143.
2. St. Clair, R. W., H. B. Lofland, and T. B. Clarkson. 1970. Influence of duration of cholesterol feeding on esterification of fatty acids by cell-free preparation of pigeon aorta. Studies on the mechanism of cholesterol esterification. *Circ. Res.* **27:** 213–225.
3. Prichard, R. W., T. B. Clarkson, H. O. Goodman, and H. B. Lofland. 1966. Coronary atherosclerosis. A study of coronary disease occurring naturally in White Carneau pigeons. *Arch. Pathol.* **81:** 292–301.
4. Clarkson, T. B., and H. B. Lofland. 1967. Response of pigeon arteries to cholesterol as a function of time. *Arch. Pathol.* **84:** 513–516.
5. Lewis, J. C., R. G. Taylor, and W. G. Jerome. 1985. Foam cell characteristics in coronary arteries and aortas of White Carneau pigeons with moderate hypercholesterolemia. *Ann. NY Acad. Sci.* **454:** 91–100.
6. Lewis, J. C., R. G. Taylor, N. D. Jones, and D. Grimes. 1979. Vascular endothelium in pigeon coronary atherosclerosis. *Scan. Electron. Microsc.* **III:** 823–828.
7. Lewis, J. C., R. G. Taylor, N. D. Jones, R. W. St. Clair, and J. F. Cornhill. 1982. Endothelial surface characteristics in pigeon coronary artery atherosclerosis. I. Cellular alterations during the initial stages of dietary cholesterol challenge. *Lab. Invest.* **46:** 123–138.
8. Adelman, S. J., and R. W. St. Clair. 1988. Lipoprotein metabolism by macrophages from atherosclerosis-susceptible White Carneau and resistant Show Racer pigeons. *J. Lipid Res.* **29:** 643–656.
9. Adelman, S. J., and R. W. St. Clair. 1989. β-VLDL metabolism by pigeon macrophages. Evidence for two binding sites with different potentials for promoting cholesterol accumulation. *Arteriosclerosis.* **9:** 673–683.
10. Seo, T., and R. W. St. Clair. 1997. Heparan sulfate proteoglycans mediate internalization and degradation of β-VLDL and promote cholesterol accumulation by pigeon macrophages. *J. Lipid Res.* **38:** 765–779.
11. Brown, M. S., and J. L. Goldstein. 1985. The LDL receptor and HMG-CoA reductase-two membrane molecules that regulate cholesterol homeostasis. *Curr. Top. Cell. Regul.* **26:** 3–15.
12. Brown, M. S., J. L. Goldstein, M. Krieger, Y. K. Ho, and R. G. W. Anderson. 1979. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J. Cell Biol.* **82:** 597–613.
13. Ho, Y. K., J. R. Faust, D. W. Bilheimer, M. S. Brown, and J. L. Goldstein. 1977. Regulation of cholesterol synthesis by low density lipoprotein in isolated human lymphocytes. Comparison of cells from normal subjects and patients with homozygous familial hypercholesterolemia and abetalipoproteinemia. *J. Exp. Med.* **145:** 1531–1549.
14. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage foam cells. *J. Biol. Chem.* **255:** 9344–9352.
15. Ponec, M., J. A. Kempenaar, L. Havekes, J. G. Van der Schroeff, J. J. Emeis, and B. J. Vermeer. 1981. Effects of LDL, HDL and their combination on the endogenous cholesterol synthesis in monocyte-macrophages. *Biochim. Biophys. Acta.* **666:** 405–410.
16. Traber, M. G., V. Defendi, and H. J. Kayden. 1981. Receptor activities for low-density lipoprotein and acetylated low-density lipoprotein in a mouse macrophage cell line (IC21) and in human monocyte-derived macrophages. *J. Exp. Med.* **154:** 1852–1867.
17. Knight, B. L., D. D. Patel, and A. K. Soutar. 1983. The regulation of 3-hydroxy-3-methylglutaryl-CoA reductase activity, cholesterol esterification and the expression of low-density lipoprotein receptors in cultured monocyte-derived macrophages. *Biochem. J.* **210:** 523–532.
18. Knight, B. L., A. K. Soutar, and D. D. Patel. 1987. Non-saturable degradation of LDL by monocyte-derived macrophages leads to a reduction in HMG-CoA reductase activity with little synthesis of cholesteryl esters. *Atherosclerosis.* **64:** 131–138.
19. Angelin, B. 1988. Regulation of 3-hydroxy-3-methyglutaryl-coenzyme A reductase activity in mouse peritoneal macrophages. *Biochem. J.* **255:** 529–534.
20. Warner, G. J., G. Stoudt, M. Bamberger, W. J. Johnson, and G. H. Rothblat. 1995. Cell toxicity induced by inhibition of acyl coenzyme A: cholesterol acyltransferase and accumulation of unesterified cholesterol. *J. Biol. Chem.* **270:** 5772–5778.
21. Edwards, P. A., and J. Ericsson. 1998. Signaling molecules derived from the cholesterol biosynthetic pathway: mechanisms of action and possible roles in human disease. *Curr. Opin. Lipidol.* **9:** 433–440.
22. Randolph, R. K., B. P. Smith, and R. W. St. Clair. 1984. Cholesterol

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metabolism in pigeon aortic smooth muscle cells lacking a functional low density lipoprotein receptor pathway. *J. Lipid Res.* **25:** 903–912.
23. Phillips, M. C., K. L. Gillotte, M. P. Haynes, W. J. Johnson, S. Lund-Katz, and G. H. Rothblat. 1998. Mechanisms of high density lipoprotein-mediated efflux of cholesterol from cell plasma membranes. *Atherosclerosis.* **137 (Suppl.):** S13–S17.
24. Yancey, P. G., and R. W. St. Clair. 1992. Cholesterol efflux is defective in macrophages from atherosclerosis-susceptible White Carneau pigeons relative to resistant Show Racer pigeons. *Arterioscler. Thromb.* **12:** 1291–1304.
25. St. Clair, R. W., R. K. Randolph, M. P. Jokinen, T. B. Clarkson, and H. A. Barakat. 1986. Relationship of plasma lipoproteins and the monocyte-macrophage system to atherosclerosis severity in cholesterol-fed pigeons. *Arteriosclerosis.* **6:** 614–626.
26. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
28. Scanu, A. 1966. Forms of human serum high density lipoprotein protein. *J. Lipid Res.* **7:** 295–306.
29. Guertler, L. S., and R. W. St. Clair. 1977. In vitro regulation of cholesterol metabolism by low density lipoproteins in skin fibroblasts from hypo- and hyperresponding squirrel monkeys. *Biochim. Biophys. Acta.* **487:** 458–471.
30. Bernard, D. W., A. Rodriguez, G. H. Rothblat, and J. M. Glick. 1990. Influence of high density lipoprotein on esterified cholesterol stores in macrophages and hematoma cells. *Arteriosclerosis.* **10:** 135–144.
31. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
32. Ishikawa, T. T., J. MacGee, J. A. Morrison, and C. J. Glueck. 1974. Quantitative analysis of cholesterol in 5 to 20 μl of plasma. *J. Lipid Res.* **15:** 286–291.
33. Klansek, J. J., P. Yancey, R. W. St. Clair, R. T. Fischer, W. J. Johnson, and J. M. Glick. 1995. Cholesterol quantitation by GLC: artifactual formation of short-chain steryl esters. *J. Lipid Res.* **36:** 2261–2266.
34. Sperry, W. M. 1963. Quantitative isolation of sterols. *J. Lipid Res.* **4:** 221–225.
35. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1973. Regulation of 3-hydroxy-3methylglutaryl coenzyme A reductase activity in human fibroblasts by lipoproteins. *Proc. Natl. Acad. Sci. USA.* **70:** 2162–2166.
36. Guertler, L. S., and R. W. St. Clair. 1980. Low density lipoprotein receptor activity on skin fibroblasts from rhesus monkeys with diet-induced or spontaneous hypercholesterolemia. *J. Biol. Chem.* **255:** 92–99.
37. Rosenfeld, M. E., J. C. Khoo, E. Miller, S. Parthasarathy, W. Palinski, and J. Witztum. 1991. Macrophage-derived foam cells freshly isolated from rabbit atherosclerotic lesions degrade modified lipoproteins, promote oxidation of low-density lipoproteins, and contain oxidation-specific lipid-protein adducts. *J. Clin. Invest.* **87:** 90–99.
38. Brown, M. S., and J. L. Goldstein. 1990. Regulation of the mevalonate pathway. *Nature.* **343:** 425–430.
39. Bilheimer, D. W., Y. K. Ho, M. S. Brown, R. G. W. Anderson, and J. L. Goldstein. 1978. Genetics of the low density lipoprotein receptor. Diminished receptor activity in lymphocytes from heterozygotes with familial hypercholesterolemia. *J. Clin. Invest.* **61:** 678–696.
40. Fogelman, A. M., J. Seager, M. Hokom, and P. A. Edwards. 1979. Separation of and cholesterol synthesis by human lymphocytes and monocytes. *J. Lipid Res.* **20:** 379–388.
41. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
42. Luskey, K. L. 1988. Regulation of cholesterol synthesis: mechanism for control of HMG CoA reductase. *In* Recent Progress in Hormone Research. J. H. Clark, editor. Academic Press, Inc., San Diego. 35–51.
43. Rudney, H., and S. R. Panini. 1993. Cholesterol biosynthesis. *Curr. Opin. Lipidol.* **4:** 230–237.
44. Cheng, D., C. C. Y. Chang, X. Qu, and T. Y. Chang. 1995. Activation of acyl-coenzyme A:cholesterol acyltransferase by cholesterol or by oxysterol in a cell-free system. *J. Biol. Chem.* **270:** 685–695.
45. Chang, C. C. Y., J. Chen, M. A. Thomas, D. Cheng, V. A. Del Priore, R. S. Newton, M. E. Pape, and T. Y. Chang. 1995. Regulation and immunolocalization of acyl-coenzyme A:cholesterol acyltransferase in mammalian cells as studied with specific antibodies. *J. Biol. Chem.* **270:** 29532–29540.
46. Synouri-Vrettakou, S., and K. A. Mitropoulos. 1983. Acylcoenzyme A:cholesterol acyltransferase. Transfer of cholesterol to its substrate pool and modulation of activity. *Eur. J. Biochem.* **133:** 299–307.
47. Burki, E., J. Logel, and M. Sinensky. 1987. Endogenous sterol synthesis is not required for regulation of 3-hydroxy-3methylglutaryl coenzyme A reductase by low density lipoprotein. *J. Lipid Res.* **28:** 1199–1205.
48. Pinkerton, F. D., A. Izumi, C. M. Anderson, L. R. Miller, A. Kisic, and G. J. Schroepfer, Jr. 1982. 14α-Ethyl-5α-cholest-7-ene-3β,15α-diol, a potent inhibitor of sterol biosynthesis, has two sites of action in cultured mammalian cells. *J. Biol. Chem.* **257:** 1929–1936.
49. St. Clair, R. W., H. B. Lofland, R. W. Prichard, and T. B. Clarkson. 1968. Synthesis of squalene and sterols by isolated segments of human and pigeon arteries. *Exp. Mol. Pathol.* **8:** 201–215.
50. Aviram, M., E. L. Bierman, and J. F. Oram. 1989. High density lipoprotein stimulates sterol translocation between intracellular and plasma membrane pools in human monocyte-derived macrophages. *J. Lipid Res.* **30:** 65–76.
51. Johnson, W. J., R. T. Fischer, M. C. Phillips, and G. H. Rothblat. 1995. Efflux of newly synthesized cholesterol and biosynthetic sterol intermediates from cells. Dependence on acceptor type and on enrichment of cells with cholesterol. *J. Biol. Chem.* **270:** 25037–23046.
52. Phillips, J. P., W. V. Rodrigueza, and W. J. Johnson. 1998. Basis for rapid efflux of biosynthetic desmosterol from cells. *J. Lipid Res.* **39:** 2459–2470.
53. Echevarria, F., R. A. Norton, W. D. Nes, and Y. Lange. 1990. Zymosterol is located in the plasma membrane of cultured human fibroblasts. *J. Biol. Chem.* **265:** 8484–8489.
54. St. Clair, R. W., M. A. Leight, and H. A. Barakat. 1986. Metabolism of low density lipoproteins by pigeon skin fibroblasts and aortic smooth muscle cells. Comparison of cells from atherosclerosis-susceptible and atherosclerosis-resistant pigeons. *Arteriosclerosis.* **6:** 170–177.
55. Naito, M., H. Nomura, T. Esaki, and A. Iguchi. 1997. Characteristics of macrophage-derived foam cells isolated from atherosclerotic lesions of rabbits. *Atherosclerosis.* **135:** 241–247.
56. Atger, V. M., M. de la Llera Moya, G. W. Stoudt, W. V. Rodrigueza, M. C. Phillips, and G. H. Rothblat. 1997. Cyclodextrins as catalysts for the removal of cholesterol from macrophage foam cells. *J. Clin. Invest.* **99:** 773-780.
57. Miura, S., T. Chiba, N. Mochizuki, H. Nagura, K. Nemota, I. Tomita, M. Ikeda, and T. Tomita. 1997. Cholesterol-mediated changes in neutral cholesterol esterase activity in macrophages. *Arterioscler. Thromb. Vasc. Biol.* **17:** 3033–3040.
58. Lawler, J. F., Jr., M. Yin, A. M. Diehl, E. Roberts, and S. Chatterjee. 1998. Tumor necrosis factor-α stimulates the maturation of sterol regulatory element binding protein-1 in human hepatocytes through the action of neutral sphingomyelinase. *J. Biol. Chem.* **273:** 5053–5059.
59. Phillips, M. C., W. J. Johnson, and G. H. Rothblat. 1987. Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim. Biophys. Acta.* **906:** 223–276.
60. Libby, P., Y. J. Geng, Y. J. Aikawa, U. Schoenbeck, F. Mach, S. K. Clinton, G. K. Sukhova, and R. T. Lee. 1996. Macrophages and atherosclerotic plaque stability. *Curr. Opin. Lipidol.* **7:** 330–335.
61. Corsini, A., F. Bernini, P. Quarato, E. Donetti, S. Bellosta, R. Fumagalli, R. Paoletti, and V. M. R. Soma. 1996. Non-lipid-regulated effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Cardiology.* **87:** 458–468.
62. Zhu, B. Q., R. E. Sievers, Y. P. Sun, W. M. Isenberg, and W. W. Parmley. 1992. Effect of lovastatin on suppression and regression of atherosclerosis in lipid-fed rabbits. *J. Cardiovasc. Pharmacol.* **19:** 246–255.
63. Harsch, M., J. H. Braesen, and A. Niendorf. 1997. Effects of low-dose pravastatin sodium on plasma cholesterol levels and aortic atherosclerosis of heterozygous WHHL rabbits fed a low cholesterol (0.03%) enriched diet for one year. *Atherosclerosis.* **128:** 139–147.
64. Jukema, J. W., A. V. G. Bruschke, A. J. van Boven, J. H. C. Reiber, E. T. Bal, A. H. Zwinderman, H. Jansen, G. J. M. Boerma, F. M. van Rappard, and K. I. Lie. 1995. Effects of lipid lowering by pravastatin on progression and regression of coronary artery disease in symptomatic men with normal to moderately elevated serum cholesterol levels. The regression growth evaluation statin study (REGRESS). *Circulation.* **91:** 2528–2540.
65. MAAS Investigators, 1994. Effect of simvastatin on coronary atheroma: the multicentre anti-atheroma study (MAAS). *Lancet.* **344:** 633–638.

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