Mechanism of the defect in cholesteryl ester clearance from macrophages of atherosclerosis-susceptible White Carneau pigeons.

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Abstract Recent studies from our laboratory (Yancey, P. G., and R. W. St. Clair. 1992. *Arterioscler. Thromb.* **12:** 1291–1304) have shown that cultured peritoneal macrophages from White Carneau (WC) pigeons clear cholesteryl esters at a slower rate than do macrophages from Show Racer (SR) pigeons (9% per 24 h vs. 42% per 24 h, respectively) when incubated in the presence of a cholesterol acceptor apo high density lipoprotein/phosphatidylcholine (apoHDL/PC) at concentrations that are not rate-limiting for cholesterol efflux. In the present studies we have examined some potential mechanisms for this difference in cholesterol efflux. The desorption of [^{3}H]cholesterol from the plasma membranes of non-cholesterol-loaded cells was log-linear with half-times of 24–31 h, and was not different for WC and SR macrophages. As this rate of cholesterol desorption was 2- to 3-times faster than the rate of cholesteryl ester clearance, it is unlikely to be rate-limiting. In cells loaded with cholesteryl esters, the re-esterification of cholesterol was equally low in both WC and SR macrophages when incubated with apoHDL/PC. The addition of the acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Sandoz 58,035, reduced esterification approximately 50%, but did not affect the clearance of cholesteryl ester mass from either WC or SR macrophages. Thus, differences in cholesterol re-esterification could not explain the differences in cholesteryl ester clearance. The rate of hydrolysis of radiolabeled cellular cholesteryl esters was log-linear for up to 36 h in WC and SR macrophages. The half-time for hydrolysis of cholesteryl ester was 89 h in WC macrophages, compared to only 37 h in the SR macrophages, and paralleled the rate of clearance of cholesteryl esters in these cells (133 h and 51 h, respectively). Cyclic AMP stimulated cholesterol ester hydrolysis in WC macrophages and increased by 5-fold the clearance of cholesteryl esters in the presence of apoHDL/PC and Sandoz 58,035. These data are consistent with the conclusion that the rate-limiting step in the clearance of cholesteryl esters from pigeon macrophages is the hydrolysis of cholesteryl esters, and that the slow rate of cholesteryl ester clearance in WC macrophages is due, at least in part, to a defect in cholesteryl ester hydrolysis. It is tempting to speculate that this defect in cholesteryl ester hydrolysis may play a role in the difference in susceptibility to atherosclerosis between WC and SR pigeons.—**Yancey, P. G., and R. W. St. Clair.** Mechanisms of the defect in cholesteryl ester clearance from macrophages of atherosclerosis-susceptible White Carneau pigeons. *J. Lipid Res.* 1994. **35:** 2114–2129.

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White Carneau (WC) pigeons are susceptible to the development of both naturally occurring and cholesterol-aggravated aortic atherosclerosis while Show Racer (SR) pigeons are resistant (1, 2). This difference in atherosclerosis susceptibility appears to be genetically mediated as the F_1 offspring of WC and SR matings have an intermediate degree of atherosclerosis susceptibility (1, 2). As no differences have been found in the levels of known risk factors such as plasma cholesterol, lipoproteins, blood pressure, blood glucose, etc., the genetic control of susceptibility to atherosclerosis is most likely mediated at the level of the arterial wall (2–4).

As in humans (5) and a number of animal models (6–8), the appearance of cholesteryl ester-rich macrophage foam cells is an early event in the development of atherosclerosis in pigeons (9–11). Although specific mechanisms within the arterial wall by which macrophages become loaded with cholesteryl esters are unknown, the steady state concentration of cellular cholesteryl esters must be the sum of the processes that control influx and efflux of cholesterol by the cells. Cellular cholesterol synthesis probably is not a factor as synthesis is down-regulated in cells loaded with cholesterol (12). Previous studies from this laboratory have demonstrated several receptor-mediated mechanisms for the uptake of cholesterol by WC and SR pigeon peritoneal macrophages. These include the β-VLDL/LDL receptor and the

Abbreviations: β-VLDL, β-very low density lipoprotein; Ac-LDL, acetylated low density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; apoHDL/PC, apolipoprotein high density lipoprotein/phosphatidylcholine; BSA, bovine serum albumin; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GLC, gas–liquid chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein; nCEH, neutral cholesteryl ester hydrolase; PBS, phosphate-buffered saline; SR, Show Racer; TLC, thin-layer chromatography; WC, White Carneau.

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scavenger receptor (13, 14) that have properties similar to these same receptors on mammalian macrophages (15). Pigeon macrophages also have a "lipoprotein binding site" that takes up and degrades a number of normal and abnormal lipoproteins including pigeon and rabbit β-migrating very low density lipoproteins (β-VLDL), and acetylated low density lipoproteins (Ac-LDL) (13, 14). The "lipoprotein binding site" binds lipoproteins with low affinity but with high capacity and, like the scavenger receptor, is not down-regulated by cholesterol loading (14). Both WC and SR macrophages can take up lipoproteins and accumulate cholesteryl esters via each of these receptors, with the "lipoprotein binding site" being the most effective in loading pigeon cells with cholesteryl esters (13, 14). Thus far, however, no consistent differences have been observed between WC and SR macrophages in their ability to accumulate cholesteryl esters through any of these mechanisms (13, 14).

An alternative mechanism for the control of cellular cholesterol content is via regulation at the level of cholesterol efflux. The net loss of cholesteryl esters from cells requires that the cholesteryl esters first be hydrolyzed to free cholesterol prior to desorption of free cholesterol from the plasma membrane where it is ultimately taken up by a cholesterol acceptor (16–18). Although high density lipoproteins (HDL) promote the net efflux of cholesterol from mouse macrophage foam cells, HDL does not appear to stimulate net cholesterol efflux from other macrophage foam cells including J774 (19), p388D (19), rabbit (20, 21), and WC and SR pigeon macrophages (22). In contrast, net cholesterol efflux can be stimulated from most of these cells using a more efficient cholesterol acceptor such as apoHDL/phosphatidylcholine (apoHDL/PC) vesicles (19, 22).

Using high concentrations of apoHDL/PC vesicles with pigeon peritoneal macrophages loaded with cholesteryl esters, we have shown that the rate of cholesteryl ester clearance is log-linear for up to 48 h for both WC and SR macrophages, and that the rate of clearance is not dependent on the initial cholesteryl ester concentration of the cells (22). There were, however, significant differences in the ability of WC and SR macrophages to clear cholesteryl ester in the presence of apoHDL/PC. On average, SR macrophages cleared 42% of their cholesteryl esters in 24 h while the WC macrophages cleared only 9% (22). These studies suggest that, under certain conditions, the steady state cholesteryl ester content of macrophage foam cells may be mediated more by cholesterol efflux, as opposed to influx, and that differences in cholesterol efflux may help explain the differences in susceptibility to atherosclerosis between WC and SR pigeons.

There are a number of potential mechanisms by which cholesterol efflux could be regulated. The vast majority of the cholesteryl esters in pigeon macrophage foam cells (13, 14), as well as in mammalian macrophage foam cells (15, 23), are formed from the esterification of lipoprotein-derived cholesterol by the enzyme, acyl CoA:cholesterol acyltransferase (ACAT). The cholesteryl esters formed are stored within cytoplasmic droplets in the cell which are in a dynamic equilibrium by virtue of a continuous cycle of hydrolysis and re-esterification (17, 24–26). Hydrolysis of cholesteryl esters within lipid droplets is mediated by a neutral cholesteryl ester hydrolase (nCEH) (17, 26) and re-esterification is mediated by ACAT (17, 26). When an appropriate cholesterol acceptor is present in the medium surrounding the cells, there is net efflux of free cholesterol and transport of free cholesterol away from the cholesteryl ester cycle (17, 26). Thus, during net cholesteryl ester clearance, cellular cholesteryl ester hydrolysis remains constant and re-esterification by ACAT is inhibited by substrate removal (17, 25). Differences in cholesterol efflux could be regulated by a number of factors, among these are the rate of cholesteryl ester hydrolysis, cholesterol re-esterification, or cholesterol desorption from the plasma membrane. The goal of the present study was to determine whether one or more of these processes is responsible for the differences in rates of cholesterol efflux from WC and SR macrophage foam cells. The results suggest that, of these mechanisms, differences in the rate of hydrolysis of cholesteryl esters can explain the difference in rates of cholesteryl ester clearance between WC and SR macrophages.

METHODS

Materials

Egg phosphatidylcholine, bovine serum albumin (BSA) (fatty acid-free from fraction V), 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate (CPT-cAMP), and EDTA were purchased from Sigma, St. Louis, MO. Aprotinin and PMSF were purchased from Boehringer Mannhein Corp., Indianapolis, IN. D-Phenylalanyl-L-prolyl-arginine chlormethyl ketone (PPACK) was purchased from Calbiochem Corp., San Diego, CA. Stigmasterol was obtained from Steraloids, Wilton, NH. Penicillin, streptomycin, Eagle's vitamins, calf serum, and Eagle's Minimum Essential Medium (MEM) were purchased from Hazelton, Lenexa, KS. Fetal bovine serum (FBS) was purchased from ICN/Flow, Costa Mesa, CA and was heat-inactivated before use by incubation at 56°C for 1 h. All tissue culture plasticware was obtained from Corning. The ACAT inhibitor, compound 58,035, was kindly provided by Dr. David Weinstein of Sandoz, East Hanover, NJ. [1,2-^{3}H]cholesterol (46 Ci/mmol) and [4-^{14}C]cholesterol (50 mCi/mmol) were purchased from Amersham, Arlington Heights, IL. [1-^{14}C]oleic acid (56.0 nCi/mmol) and [9,10-^{3}H]oleic acid (10 Ci/mmol) were

purchased from New England Nuclear, Wilmington, DE and complexed to albumin as described (27).

Lipoproteins and apoHDL/PC vesicles

Human HDL was isolated from plasma obtained from the Red Cross. Rabbit β-VLDL was obtained from the plasma of New Zealand White rabbits (Franklin Rabbitry, Wake Forest, NC) fed a commercial pelleted chow diet containing 0.5% cholesterol and 5% corn oil for at least 30 days. Pigeon LDL was isolated from the plasma of WC pigeons fed for at least 1 month with a diet of commercial pigeon pellets containing 0.5% cholesterol and 10% lard. For isolation of plasma lipoproteins, blood was collected into tubes containing the following agents at the indicated final concentration: 1 mg/ml EDTA, 1 μM PPACK, and 25-kallikrein inhibitory units/ml of apoprotinin. To inhibit lecithin:cholesterol acyltransferase activity and other proteases, PMSF at a final concentration of 0.5 mM was added to the plasma. The lipoproteins were isolated by sequential ultracentrifugation (28). Human HDL was isolated between d 1.063 g/ml and d 1.21 g/ml by ultracentrifugation for 48 h. Rabbit β-VLDL was isolated at d < 1.006 g/ml by ultracentrifugation for 20 h. Pigeon LDL was isolated between d 1.006 g/ml and d 1.063 g/ml by ultracentrifugation for 24 h. All lipoproteins were dialyzed extensively against a solution containing 0.9% NaCl and 0.01% EDTA. The isolated lipoproteins were sterilized by filtration through a Millipore filter (0.45 μm), and stored at 4°C for no longer than 4 weeks before use. Pigeon LDL was acetylated using the procedure of Basu et al. (29).

ApoHDL/PC vesicles were prepared as previously described (22) by following the procedure of Adelman et al. (30). Briefly, human HDL was delipidated using the diethyl ether–ethanol extraction procedure of Scanu (31). The HDL apolipoproteins were complexed to phospholipid by sonicating a mixture of 1 mg of phosphatidylcholine and 0.4 mg apoHDL protein per milliliter in 0.9% NaCl, 0.01% EDTA (pH 8.6) solution containing sodium azide (0.02% w/v). The apoHDL/PC vesicles were separated from multilamellar vesicles and metal from the sonicator probe by ultracentrifugation for 2 h at 4°C at 36,000 rpm. Prior to use, the apoHDL/PC vesicles were dialyzed extensively against 0.9% NaCl, 0.01% EDTA followed by dialysis for 12 h against 2 l of MEM-containing 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) (20 mM) buffer (pH 7.4), penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Cell culture

White Carneau and SR pigeons were obtained from our breeding colony and were fed a cholesterol-free pelleted grain diet. Elicited WC and SR pigeon peritoneal macrophages were obtained using thioglycolate as previously described (22). All incubations were carried out at 37°C in a humid atmosphere with 95% air and 5% CO_2. The exudate cells were suspended in MEM containing 20 mM HEPES and plated at a density of 6–10 × 10^6 cells in 0.5 ml in 35-mm dishes already containing 1.5 ml 10% FBS MEM supplemented with the following at the indicated concentrations: 20 mM HEPES, 23 mM bicarbonate, Eagle's vitamins, 200 mM L-glutamine, 1.5 mg/ml glucose, 100 μg/ml streptomycin, and 100 IU/ml penicillin. This supplemented MEM (medium B) was the working medium used for all of the experiments in this paper. Thioglycolate-elicited macrophages that were isolated from $B_6C_3F_1$ mice as described previously (22) were suspended in MEM containing 20 mM HEPES at a density of 6–8 × 10^6 cells per ml. One-half ml (3–4 × 10^6 cells) of this cell suspension was added to 35-mm dishes already containing 1.5 ml medium B. Both elicited pigeon and mouse peritoneal cells were allowed to adhere by incubation for 4–6 h at 37°C. The dishes were washed 3–5 times with phosphate-buffered saline (PBS) to remove nonadherent cells (32), and the remaining adherent cells were used for the following protocols.

Cholesterol efflux from non-cholesterol-loaded pigeon macrophages

For experiments to measure free cholesterol efflux from cells not loaded with cholesterol, elicited pigeon peritoneal macrophages were incubated in medium B with 1% FBS containing [1,2-^{3}H]cholesterol at a final concentration of 1 μCi/ml. The [^{3}H]cholesterol was dissolved in 500 μl ethanol, and then added to the medium B containing 1% FBS. The mixture was pre-incubated for 2 h at 37°C before addition to the cells. After 48 h, the cells were washed three times with PBS and incubated for an additional 24 h with medium B alone. The macrophages were washed 3 times with PBS and incubated for up to 8 h at 37°C with medium B containing 0.1% BSA alone or 0.1% BSA plus 50 μg apoHDL/PC vesicle protein/ml to measure cholesterol efflux. At each time point the medium was collected and centrifuged for 10 min at 500 *g* to pellet any cells released from the dish. The supernatant fluid was assayed for ^{3}H radioactivity as a measure of [^{3}H]cholesterol efflux. The remaining cells were washed 3 times with 2 ml each of PBS, and the cellular lipids extracted directly from the tissue culture dish by adding 2 ml isopropanol (22) containing stigmasterol and [^{14}C]cholesterol as internal standards. After an overnight incubation in a sealed chamber at room temperature, a portion of the isopropanol containing the extracted lipids was removed and used for cholesterol mass determination by gas–liquid chromatography (GLC). Other portions were used to measure total ^{3}H and ^{14}C radioactivity by liquid scintillation counting, and for determination of radioactivity in the free and esterified cholesterol fraction after separation by thin-layer chromatography (TLC).

Cholesterol efflux from cholesterol-loaded macrophages

For cholesterol efflux experiments from cells loaded with cholesteryl esters, WC and SR pigeon macrophages were loaded with cholesteryl esters by incubation for 24 to 48 h at 37°C in medium B containing 1% FBS and 100–150 μg/ml of unlabeled rabbit β-VLDL or rabbit β-VLDL labeled with [^{3}H]cholesterol following the procedure previously described (33, 34). Rabbit β-VLDL was used to load pigeon macrophages as previous studies have shown this to be the most effective method for loading these cells with cholesteryl esters (22). Elicited mouse peritoneal macrophages were loaded with cholesteryl ester by incubation for 48 h with medium B containing 1% FBS and 100 μg/ml of pigeon Ac-LDL. After the cholesteryl ester loading phase, the cells were washed 3 times with PBS and incubated at 37°C for 24–40 h in medium B containing 1% FBS. After this equilibration phase, the cells were washed 3 times with 2 ml each of PBS and incubated at 37°C for up to 36 h in medium B containing either 0.1% BSA or 1% FBS with or without one of the following additions: 5 μg/ml of Sandoz 58,035, 400 μg apoHDL/PC protein/ml, or 5 μg/ml Sandoz 58,035 + 400 μg apoHDL/PC protein/ml. The Sandoz 58,035 was added in dimethyl sulfoxide (DMSO) at a final concentration of 0.1%. The same amount of DMSO was added to control dishes. At the times indicated the cells were washed 3 times with PBS, and the cellular lipids were extracted with isopropanol as described above. Aliquots of the lipid extracts were used for cholesterol mass determination, total ^{3}H and ^{14}C radioactivity, and free and esterified cholesterol radioactivity after separation of lipid classes by TLC.

Cholesteryl ester hydrolysis

Two approaches were used to measure cholesteryl ester hydrolysis. In the first, WC and SR macrophages were loaded with cholesteryl ester by incubation with [^{3}H]cholesterol-labeled rabbit β-VLDL and equilibrated as described above. The macrophages were incubated for up to 24 h at 37°C with medium B containing 0.1% BSA alone or with 5 μg/ml of Sandoz 58,035 to reduce re-esterification of cholesterol. At the indicated times, the cells were washed three times with PBS and the lipids were extracted with isopropanol as described above. As net efflux of cholesterol does not occur under these conditions, the disappearance of the [^{3}H]cholesteryl ester was used as the measure of cholesteryl ester hydrolysis.

Cholesteryl ester hydrolysis also was measured as described by Brown, Ho, and Goldstein (17). Elicited WC and SR pigeon macrophages were loaded with cholesteryl ester by incubation at 37°C with medium B containing 100 μg/ml of rabbit β-VLDL in the presence of 0.2 mM [^{3}H]oleate. After 24 h, the macrophages were washed 3 times with 2 ml each of PBS and incubated with medium B containing only 0.2 mM [^{3}H]oleate. After 24 h (equilibration phase), the cells were washed 3 times with PBS and incubated for up to 36 h at 37°C with medium B containing 0.2 mM [^{14}]oleate alone or with the additions to the efflux medium indicated in the figure legends. At the end of the efflux phase of the experiment, the cells were washed 3 times with PBS, and 2 ml isopropanol containing the stigmasterol internal standard was added to the dishes containing the cells. After an incubation at room temperature for 30 min in a sealed chamber, aliquots of the isopropanol lipid extract were taken for ^{3}H and ^{14}C radioactivity in cholesteryl ester after separation by TLC, total ^{3}H and ^{14}C radioactivity determination, and cholesterol ester after separation by TLC, total ^{3}H and ^{14}C radioactivity determination, and cholesterol mass determination by GLC. The disappearance of cellular cholesteryl [^{3}H]oleate was used as the measure of hydrolysis and the appearance of cellular cholesteryl [^{14}C]oleate was used as the measure of cholesterol re-esterification.

Other methods

After removal of the isopropanol containing the extracted cellular lipids, 1 ml 1 N NaOH was added to the dishes containing the fixed cell monolayers to digest the protein. Protein was determined on cells and lipoproteins by the method of Lowry et al. (35) using BSA as a standard. Turbid lipoprotein samples were cleared by extraction of the Lowry reaction mixture with chloroform before measurement of absorbance. Lipoprotein phosphorus was determined by the procedure of Fiske and SubbaRow (36). Lipids were extracted from lipoproteins and culture media by the method of Bligh and Dyer (37). Total and free cholesterol content of the cellular and lipoprotein lipid extracts were quantified by the procedure of Ishikawa et al. (38) by GLC using a Hewlett-Packard model 5890 gas chromatograph and autosampler. Separations were carried out as described (22). The distribution of [^{3}H]oleate and [^{14}C]oleate in cellular lipids was determined after TLC on Silica Gel 60 (EM Science) plates developed in hexane–diethyl ether–acetic acid 146:50:4 (vol/vol/vol). The distribution of [^{3}H]cholesterol in free and esterified form was determined either by TLC on Gelman Sciences ITLC SA glass fiber sheets using a solvent system of hexane–diethyl ether–acetic acid 90:10:1 (vol/vol/vol) or as described above. Bands were visualized using iodine and compared to authentic lipid standards. ^{3}H and ^{14}C were measured in a Beckman LS-230 liquid scintillation counter using Cytoscint (ICN Biochemicals, INC) as the scintillation fluid.

Statistics

Unless indicated otherwise, all data are expressed as means ± SD. When error bars are not displayed on the figures it indicates that they are smaller than the data

points. Individual comparisons were made using the Student's *t*-test. One-way analysis of variance (ANOVA) was used to identify statistically significant differences among multiple groups within the same experiment with comparisons between groups carried out using the Student-Neuman-Keuls test. Regression lines were compared using a *t*-test. All analyses were performed using the SigmaStat™ software program (Jandel Scientific, San Rafael, CA). Values were considered statistically significant when $P < 0.05$.

RESULTS

Differences in cholesterol efflux from WC and SR macrophages could be regulated by the rate of desorption of free cholesterol from the cells. To test this possibility, we compared the rate of efflux of [^{3}H]cholesterol from normal (e.g., not cholesteryl ester-loaded) WC and SR pigeon peritoneal macrophages that were pre-labeled by incubation with [^{3}H]cholesterol (**Fig. 1**). Prior to initiation of the efflux phase of the experiment, greater than 99% of the [^{3}H]cholesterol remained unesterified, and this percentage did not change in the 8 h over which efflux was measured. To measure efflux of cholesterol, WC and SR macrophages were incubated for up to 8 h with medium B containing 0.1% BSA alone or with apoHDL/PC vesicles (50 μg protein/ml). Previously, for cholesterol-loaded cells, we have shown that > 100 μg apoHDL/PC protein was not rate-limiting for cholesterol efflux (22). A lower concentration was used for the experiment shown in Fig. 1 as these cells were not loaded with cholesterol and incubations were carried out for only 8 h. Cells were harvested at the indicated times and the loss of [^{3}H]cholesterol from the cells was measured. The data are expressed as the fraction of the zero-time [^{3}H]cholesterol remaining, and are plotted with a log scale on the y axis. Data for experiment one (Expt. 1) are shown graphically with the half-times for efflux for three separate experiments shown in the insert. As there was no cholesterol in the apoHDL/PC vesicles, the results represent unidirectional net cholesterol efflux. There was little efflux of free cholesterol when the cells were incubated with medium containing 0.1% BSA alone. However, when apoHDL/PC vesicles were added, there was a log-linear rate of efflux of cholesterol from both WC and SR macrophages. As shown for three separate experiments, the half-times for free cholesterol efflux ranged from 23.5 to 31.2 h, and there was no significant difference between WC and SR macrophages. These results suggest that differences in the rate of free cholesterol desorption from the plasma membrane cannot explain the differences in the rates of cholesteryl ester clearance from WC and SR macrophages.

Next, we tested the possibility that the difference in cholesteryl ester clearance between WC and SR macrophages could be due to a more efficient re-esterification of cholesterol by ACAT in WC macrophages resulting in less intracellular cholesterol available for efflux. If such an hypothesis is valid, the rate of cholesteryl ester clearance should be equalized in WC and SR macrophages when ACAT is blocked. Prior to carrying out such a study, preliminary experiments were conducted to determine the concentration of Sandoz 58,035 that was required to in-

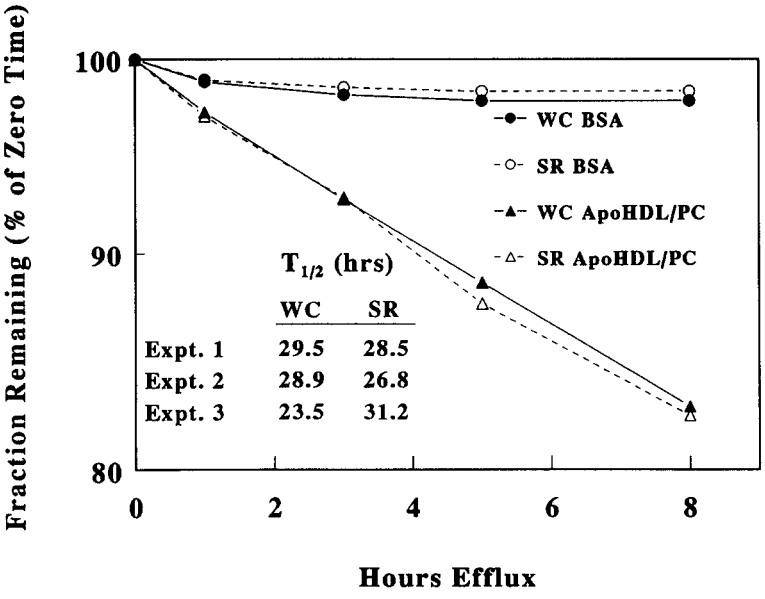

Fig. 1. Time course of the efflux of free [^{3}H]cholesterol from WC and SR macrophages not loaded with cholesterol. White Carneau and SR macrophages were isolated from pigeons fed a cholesterol-free diet. The cells were incubated at 37°C in medium B with 1% FBS containing [1,2-^{3}H]cholesterol at a final concentration of 1 μCi/ml. After 48 h, the cells were incubated for an additional 24 h at 37°C in medium B without [^{3}H]cholesterol to allow equilibration of [^{3}H]cholesterol with cellular pools of cholesterol. Three dishes of macrophages were harvested for zero-time values, and the remaining dishes of cells were incubated for the indicated times at 37°C with medium B containing 1% BSA alone or with apoHDL/PC at 50 μg protein/ml. At each time point, cells were harvested, and the cellular free [^{3}H]cholesterol radioactivity was determined as described in Methods. The results for Expt. 1 are shown graphically on a semilog plot as the fraction of the zero-time cellular [^{3}H]cholesterol remaining. All results are the mean of triplicate dishes at each point. Half-times for free [^{3}H]cholesterol efflux with apoHDL/PC were calculated from the slopes of the exponential curves and are shown in the insert for three separate experiments. The cholesterol content of the cells at zero time averaged 41.6 and 52.4 μg free cholesterol/mg cell protein and 4.6 and 8.5 μg esterified cholesterol/mg cell protein for WC and SR macrophages, respectively.

hibit cholesterol esterification in pigeon macrophages.

Due to the lipophilic nature of 58,035 it also was important to determine whether its ability to inhibit esterification could be influenced by its solubility in cellular lipids or in the apoHDL/PC used to promote efflux. Sandoz 58,035 was tested in pigeon macrophages at concentrations ranging from 0.62 to 20 μg/ml. Maximum inhibition of cholesterol esterification generally was achieved at a concentration < 2 μg/ml, but to be certain of achieving maximum inhibition, all of the studies in this paper used 58,035 at a concentration of 5 μg/ml. In **Table 1** is shown the effect of 58,035 on cholesterol esterification in WC, SR, and J774 macrophages that were normocholesterolemic or cholesterol-loaded. These data are representative of two similar experiments. In normal cells, 58,035 decreased cholesterol esterification in WC and SR cells by less than 50% and somewhat more, 74%, for J774 cells. In cells loaded with cholesterol the inhibition of cholesterol esterification was greater for SR cells (67%) than for WC cells (30%). This compares with a 97% reduction in cholesteryl ester-loaded J774 cells. The failure of 58,035 to completely inhibit cholesterol esterification in pigeon cells does not appear to be correlated with the extent of cholesterol-loading as inhibition was greater in SR cells that contained higher concentrations of cholesterol than did the WC cells. Furthermore, in J774 cells loaded with cholesterol to a level similar to WC cells, there was almost complete inhibition of cholesterol esterification. To determine whether the addition of apoHDL/PC reduced the effectiveness of 58,035 to inhibit cholesterol esterification, we incubated WC cells with or without apoHDL/PC in the presence or absence of 58,035 (**Table 2**). In the absence of apoHDL/PC, 58,035 decreased cholesterol esterification by 30% and caused a small increase in the free cholesterol content of the cells. A somewhat greater reduction in cholesterol esterification with 58,035 (48%) was seen in the presence of apoHDL/PC. Under these conditions the buildup in cellular free cholesterol was not seen, presumably due to efflux of some of the free cholesterol to the apoHDL/PC. As a result, the failure of 58,035 to more completely inhibit cholesterol esterification in pigeon cells cannot be explained by its solubility in either apoHDL/PC or cellular cholesterol, and thus appears to be a property of the pigeon macrophages themselves.

In **Fig. 2** is shown the effect of Sandoz 58,035 on the clearance of esterified cholesterol from WC and SR macrophages in the presence of apoHDL/PC. The cells initially were loaded with cholesteryl ester by incubation for 48 h with rabbit β-VLDL. After an equilibration phase,

TABLE 1. Effect of 58,035 on cholesterol esterification in normal and cholesterol-loaded macrophages

Macrophages	Cholesterol Esterification	Cell Cholesterol		
		Total	Free	Esterified
	nmol/mg cell protein	*μg/mg cell protein*		*%*
Normal				
White Carneau				
− 58,035	2.78 ± 1.9	52 ± 0.1	88	12
+ 58,035	1.50 ± 0.7 (−46%)	48 ± 0.8	90	10
Show Racer				
− 58,035	2.25 ± 0.25	79 ± 2.7	63	37
+ 58,035	1.29 ± 0.9 (−43%)	79 ± 1.6	62	38
J774				
− 58,035	9.42 ± 0.82	27.9 ± 1.3	82	18
+ 58,035	2.41 ± 0.61 (−74%)	27.1 ± 0.8	93	7
Cholesterol-loaded				
White Carneau				
− 58,035	14.2 ± 10	473 ± 12	16	84
+ 58,035	9.9 ± 2.0 (−30%)	407 ± 11	28	72
Show Racer				
− 58,035	45.7 ± 4.9	1308 ± 41	12	88
+ 58,035	14.9 ± 1.7 (−67%)	1071 ± 11	30	77
J774				
− 58,035	70.7 ± 7.3	411 ± 20	26	74
+ 58,035	2.32 ± 0.18 (−97%)	300 ± 16	71	29

Normal macrophages were isolated from pigeons consuming a cholesterol-free diet. Cholesterol-loaded macrophages were isolated from cholesterol-fed pigeons. Cells were plated into tissue culture dishes in MEM with 10% FBS and, after 4 h, nonadherent cells were washed off and the adherent cells were incubated overnight with MEM/10% FBS. The cells were again washed and incubated for 24 h in MEM/1% FBS ± 5 μg/ml 58,035. During the final 2 h of this incubation, [^{14}C]oleate was added in order to measure esterification to cholesterol. Free, esterified, and total cholesterol were measured on the total lipid extract from the same cells. Results are the mean ± SD of triplicate cultures from a single experiment. Similar results were obtained in a second experiment. Values in parentheses represent the % decrease in cholesterol esterification due to 58,035 for each cell type.

TABLE 2. Effect of apoHDL/PC on inhibition of cholesterol esterification by 58,035 in cholesterol-loaded WC peritoneal macrophages

Treatment			Cell Cholesterol		
58,035	ApoHDL/PC	Cholesterol Esterification	Total	Free	Esterified
		nmol/mg cell protein	*μg/mg cell protein*	*%*	
–	–	14.2 ± 10	473 ± 12	16	84
+	–	9.9 ± 2.0 (–30%)	407 ± 11	28	72
–	+	14.3 ± 1.3	347 ± 5	17	83
+	+	7.6 ± 2.6 (–48%)	346 ± 16	19	81

White Carneau (WC) pigeon peritoneal macrophages were isolated from cholesterol-fed pigeons and cultured as described in Table 1. Where indicated, apoHDL/PC (400 μg protein, 1 mg PC/ml) and 58,035 (5 μg/ml) were added to the MEM/1% FBS medium and incubated with the cells for 24 h. In the final 2 h of this incubation [^{14}C]oleate was added to measure cholesterol esterification. Results are mean ± SD of triplicate cultures. The same control cells (– apoHDL/PC) were used for this experiment as for the cholesterol-loaded, White Carneau group shown in Table 1. Values in parentheses represent the % decrease in cholesterol esterification due to 58,035.

the cells were incubated for up to 24 h with medium B containing 1% FBS and apoHDL/PC (400 μg protein/ml) or with the combination of apoHDL/PC and Sandoz 58,035. Cells were harvested at the indicated times and the results are expressed as a percent of the 1% FBS controls. Similar to previous studies (22), the WC macrophages did not reduce their esterified cholesterol stores significantly when incubated for 24 h in the presence of apoHDL/PC (Fig. 2A). Addition of the ACAT inhibitor did not alter the rate of clearance of esterified cholesterol from the WC macrophages. The SR cells cleared 33% of their initial esterified cholesterol content in 24 h when incubated with apoHDL/PC. This was significantly different from WC cells at both 12 ($P < 0.01$) and 24 h ($P < 0.01$). As with the WC macrophages, the mass of esterified cholesterol cleared by the SR macrophages was not influenced by the addition of Sandoz 58,035. The results suggest that in the presence of HDL/PC, reduction in re-esterification of cholesterol with 58,035 does not alter the rate of cholesteryl ester clearance in either WC or SR macrophages, and the slow rate of cholesteryl ester clearance from WC macrophages cannot be explained by more efficient re-esterification of cholesterol.

Mouse peritoneal macrophages have been used extensively for studies on cholesteryl ester clearance as they readily efflux cholesterol to a variety of acceptors (16, 26). In order to directly compare pigeon macrophages with mouse macrophages, we carried out parallel studies of the clearance of free and esterified cholesterol under identical conditions (**Fig. 3**). Mouse and WC peritoneal macrophages were loaded with cholesterol and after an equilibration phase were incubated for 24 h with medium B containing 1% FBS with or without Sandoz 58,035, apoHDL/PC, or Sandoz 58,035 + apoHDL/PC. At the end of the initial equilibration phase, the mouse macrophages contained 312 μg total cholesterol, 251 μg esterified cholesterol, and 61 μg free cholesterol/mg cell protein, and did not reduce their cholesterol content significantly when incubated with 1% FBS alone. With the addition of Sandoz 58,035, there was significant net hydrolysis of cholesteryl esters as shown by the 60% decrease in esterified cholesterol and the increase in free cholesterol. The total cholesterol content of the mouse cells also decreased significantly when Sandoz 58,035 was added to the medium suggesting that these cells are capable of the efflux of a larger net amount of cholesterol to 1% FBS when excess free cholesterol is generated through the inhibition of ACAT. When the mouse cells were incubated with either apoHDL/PC alone or apoHDL/PC plus Sandoz 58,035, the amount of cholesteryl ester clearance was similar to that observed with 1% FBS and Sandoz 58,035, but excess free cholesterol was not generated when the apoHDL/PC vesicles were present. In contrast to the mouse cells, the WC macrophages hydrolyzed only about 20% of their esterified cholesterol when incubated with 1% FBS and Sandoz 58,035. This was accompanied by a small but significant increase in the free cholesterol content of the cells. The amount of cholesteryl ester cleared from the WC macrophages was the same with apoHDL/PC and apoHDL/PC + Sandoz 58,035. There was no increase in the free cholesterol content of the cells when apoHDL/PC alone was present. Thus, the pattern of cholesteryl ester hydrolysis and clearance was qualitatively similar for WC and mouse macrophages, but the rates were much lower in WC macrophages.

The results shown in Figs. 2 and 3 imply that the rate of hydrolysis of cholesteryl esters plays an important role in regulating the rate of net loss of cholesteryl esters from pigeon cells when the acceptor is present in excess. To measure hydrolysis of cholesteryl esters more directly, the time course for hydrolysis of cellular [^{3}H]cholesteryl esters was determined in WC and SR macrophages incubated with 0.1% BSA and Sandoz 58,035 (**Fig. 4A**). Under these conditions there is no net cholesterol efflux and re-

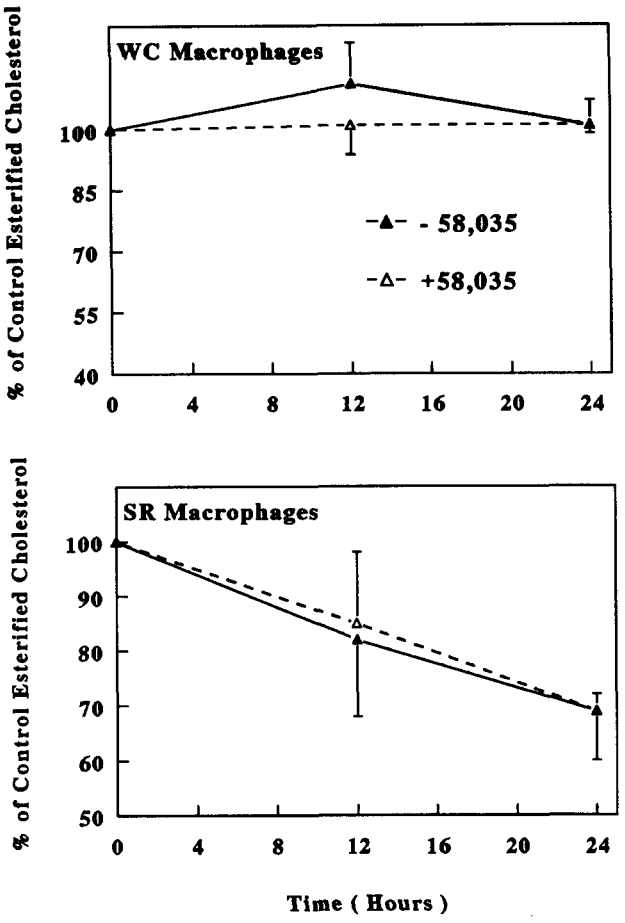

Fig. 2. Effect of Sandoz 58,035 on the clearance of esterified cholesterol from WC and SR macrophages. White Carneau and SR pigeon macrophages isolated from animals consuming a cholesterol-free diet were loaded with cholesterol in vitro by incubation for 48 h at 37°C with rabbit β-VLDL (150 μg protein/ml) in medium B containing 1% FBS. After removal of β-VLDL and equilibration of the cells in medium B containing 1% FBS for 16 h at 37°C, the cells were incubated for up to 24 h with medium B containing 1% FBS, 0.1% DMSO, and apoHDL/PC (400 μg protein/ml) alone or with 5 μg/ml of Sandoz 58,035 in DMSO (0.1% final concentration). At the indicated times, cells were harvested and the cellular esterified cholesterol concentrations were determined by GLC. The results are expressed as a percent of controls incubated with medium B containing 1% FBS and 0.1% DMSO, and are the mean ± SD for triplicate dishes at each point. The zero-time esterified cholesterol concentrations were 972 ± 21 and 235 ± 39 for SR and WC cells, respectively. The absolute amount of cholesterol effluxed can be calculated by multiplying the initial cholesteryl ester content by the % remaining at a given time. As there was no difference in cholesterol efflux in the presence or absence of 58,035, these groups were combined for statistical evaluation (*t*-test) of WC versus SR cells. The percent of control esterified cholesterol content was significantly less in SR cells versus WC cells after both 12 ($P < 0.01$) and 24 h ($P < 0.001$).

esterification of cholesterol is minimized. Thus, the loss of cellular [^{3}H]cholesteryl esters is a measure of net cholesteryl ester hydrolysis. Results are expressed as a percent of zero-time cellular [^{3}H]cholesteryl ester hydrolyzed over time. Over the initial 4 h of the experiment, there was an increase in the amount of [^{3}H]cholesteryl esters in the cells which is seen in Fig. 4A as a negative change in [^{3}H]cholesteryl ester hydrolyzed. This increase in [^{3}H]cholesteryl esters probably is the result of continued esterification of the [^{3}H]cholesterol from the loading and equilibration phases of the experiment. After the initial 4 h, there was a linear rate of hydrolysis of

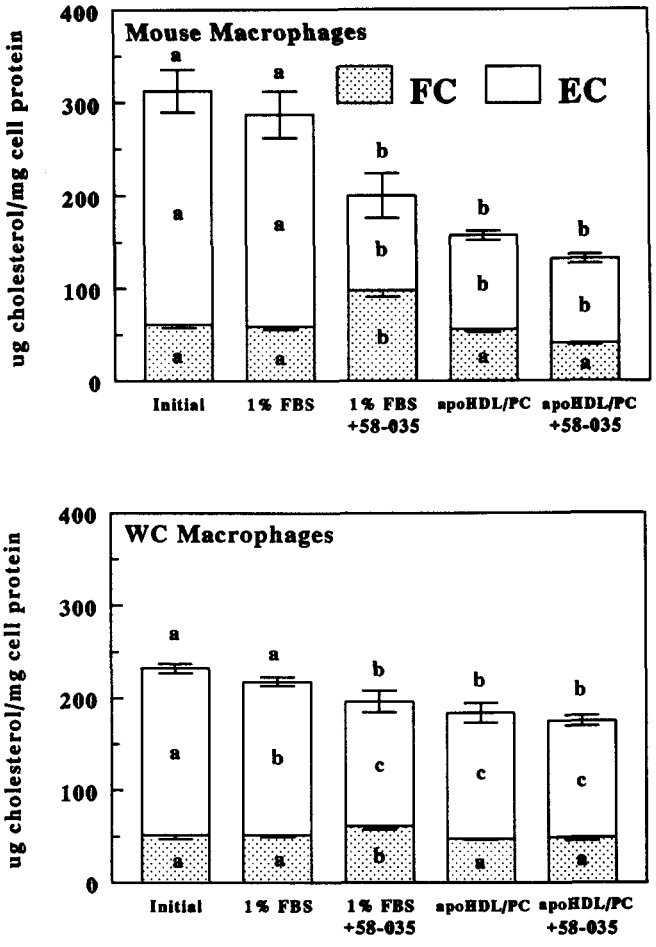

Fig. 3. Effect of Sandoz 58,035, apoHDL/PC vesicles, and Sandoz 58,035 + apoHDL/PC on the distribution of cellular free and esterified cholesterol in mouse and WC pigeon peritoneal macrophages. Elicited mouse peritoneal macrophages were loaded with cholesterol by incubation for 48 h at 37°C with pigeon Ac-LDL (100 μg protein/ml) in medium B containing 1% FBS. White Carneau macrophages isolated from birds consuming a cholesterol-free diet were loaded with cholesterol by incubation for 48 h at 37°C in medium B containing 1% FBS and 150 μg rabbit β-VLDL protein/ml. Both types of macrophages were incubated for an additional 16 h at 37°C in medium B containing 1% FBS to allow cholesterol pools to equilibrate. After the equilibration phase, the cells were incubated for 24 h with medium B containing 1% FBS and 0.1% DMSO alone or with Sandoz 58,035 (5 μg/ml), apoHDL/PC vesicles (400 μg protein/ml), or apoHDL/PC + Sandoz 58,035. Cells were harvested, and the cellular free and esterified cholesterol contents were measured by GLC. The data are expressed as the mean ± SD for three to five replicate determinations. By one-way ANOVA there was a significant difference ($P < 0.001$) among the five groups for total, free, and esterified cholesterol. Significant ($P < 0.05$) comparisons among groups by the Neumann-Keuls test are indicated by different letters for total cholesterol (above the error bar) and for free (FC) and esterified (EC) cholesterol within the bars. Comparisons among groups with the same letters are not significantly different.

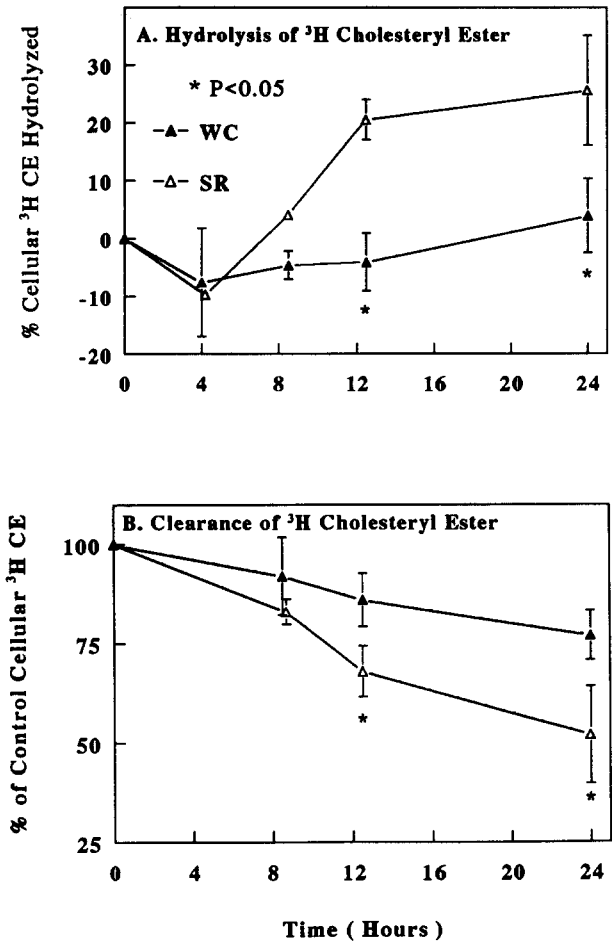

Fig. 4. Time course of cholesteryl ester hydrolysis and clearance of esterified cholesterol in WC and SR macrophages. White Carneau and SR peritoneal macrophages were isolated from pigeons fed a cholesterol-free diet and were loaded with [^{3}H]cholesteryl esters by incubation at 37°C with medium B containing 1% FBS and rabbit β-VLDL (100 μg protein/ml) labeled with [^{3}H]cholesterol. After 24 h, the cells were incubated for 40 h at 37°C with medium B containing 1% FBS in order to allow [^{3}H]cholesteryl esters to equilibrate with cellular pools of cholesterol. A group of cells was harvested for zero-time values. Half of the remaining dishes were used for measurement of cholesteryl ester hydrolysis by incubation at 37°C for up to 24 h with medium B containing 0.1% BSA alone or 0.1% BSA plus 5 μg/ml of Sandoz 58,035 to inhibit re-esterification of cholesterol (Fig. 4A). The remaining half of the dishes of cells were incubated for the indicated times with medium B containing 0.1% BSA alone or with apoHDL/PC at 400 μg protein/ml. As these dishes contained the cholesterol acceptor (apoHDL/PC) they were used to measure clearance of [^{3}H]cholesteryl esters (Fig. 4B). At each time point, cells were harvested, and the content of cellular [^{3}H]cholesteryl ester was measured as described in Methods. The percent hydrolysis was calculated as (% of cellular [^{3}H]cholesteryl ester in cells incubated with 0.1% BSA alone) − (% of cellular [^{3}H]cholesteryl ester in cells incubated with 0.1% BSA and Sandoz 58,035)/(% of cellular [^{3}H]cholesteryl ester that was esterified in cells incubated with 0.1% BSA) × 100%. Fig. 4B shows the changes in cellular [^{3}H]cholesteryl ester for cells incubated with apoHDL/PC, and the data are expressed as the percent of controls incubated with medium B containing 0.1% BSA. The results are the average ± SD for triplicate dishes for all points. The asterisks indicate significant differences (t-test) between WC and SR cells at $P < 0.05$.

cholesteryl esters in SR macrophages up to 12 h with a slower rate from 12 to 24 h. The rate of hydrolysis of cholesteryl esters was much slower for WC than SR macrophages. The WC cells hydrolyzed only 4% of their [^{3}H]cholesteryl esters in 24 h compared with 25% for the SR macrophages. The data in Fig. 4B show the clearance of [^{3}H]cholesteryl ester to apoHDL/PC from the same two batches of cells used to measure cholesteryl ester hydrolysis in Fig. 4A. The SR macrophages cleared over 48% of their [^{3}H]cholesteryl ester content in 24 h compared to only 23% cleared by the WC macrophages. Unlike the hydrolysis of cholesteryl esters in SR macrophages, the clearance of labeled cholesteryl esters from these cells was linear for up to 24 h. The results suggest that the rate of cholesteryl ester hydrolysis, measured under conditions where efflux of cholesterol could not occur (Fig. 4A), may be down-regulated by the build up of free cholesterol in these cells.

To measure the hydrolysis of cholesteryl esters directly during cholesteryl ester clearance, the cellular pools of cholesteryl ester were labeled by allowing WC and SR macrophages to esterify [^{3}H]oleate during the cholesteryl ester loading and equilibration phases of the experiment described in **Fig. 5.** These cells then were incubated for up to 36 h in the presence of [^{14}C]oleate and apoHDL/PC with or without Sandoz 58,035. The disappearance of cholesteryl [^{3}H]oleate and the appearance of cholesteryl [^{14}C]oleate were used as measures of cholesteryl ester hydrolysis and cholesterol re-esterification, respectively. Shown in Fig. 5A are cholesteryl ester hydrolysis and cholesterol re-esterification data for WC and SR macrophages in the presence and absence of Sandoz 58,035. In the absence of Sandoz 58,035, the rates of re-esterification of cholesterol in both WC and SR macrophages were low. This is consistent with the conclusion that in the presence of apoHDL/PC, the free cholesterol generated by hydrolysis of cholesteryl esters is preferentially directed to efflux rather than re-esterification. Cholesterol re-esterification was not zero, however, as seen from the significantly lower rates of re-esterification at all time points in both WC and SR macrophages when Sandoz 58,035 was added to the clearance medium. Nevertheless, the magnitude of the difference was small. Hydrolysis of cellular [^{3}H]cholesteryl esters proceeded at a faster rate than re-esterification of cholesterol in both WC and SR cells. As seen from the slopes of the lines, the rate of hydrolysis was faster in SR versus WC macrophages. The addition of Sandoz 58,035 did not affect the rate of cholesteryl ester hydrolysis in either WC or SR macrophages. The free and esterified cholesterol content of the same cells also was measured and the data are shown in Fig. 5B. Consistent with previous studies (22), the clearance of esterified cholesterol mass in the presence of apoHDL/PC occurred at a

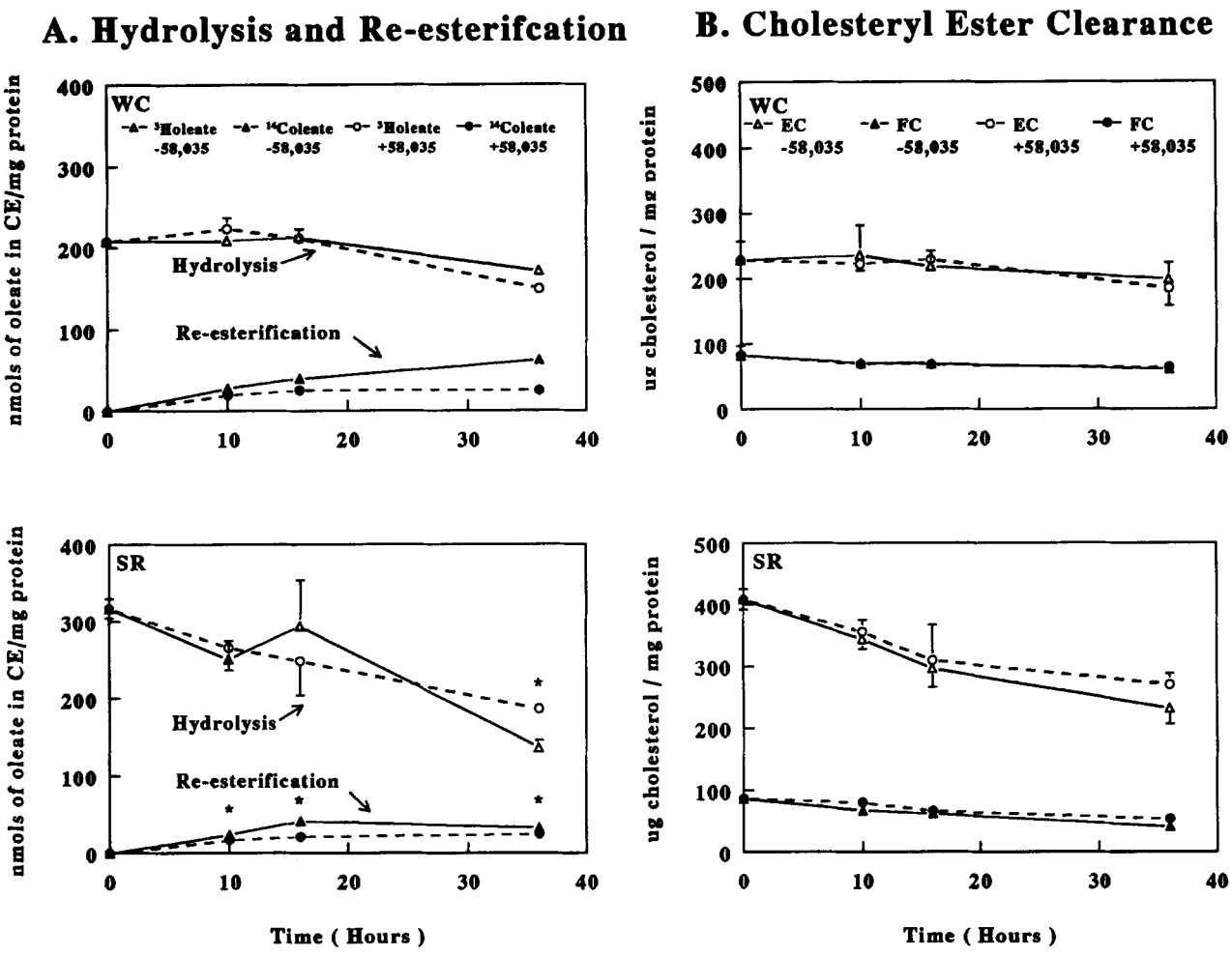

Fig. 5. Time course of cellular cholesteryl ester hydrolysis, cholesterol re-esterification, and cholesterol clearance in WC and SR macrophages in the presence of apoHDL/PC with or without Sandoz 58,035. White Carneau and SR macrophages isolated from pigeons fed a cholesterol-free diet were loaded with cholesterol by incubation at 37°C with medium B containing rabbit β-VLDL (100 μg protein/ml) and 0.2 mM [^{3}H]oleate (208,749 dpm/nmol). After 24 h, the cells were incubated for an additional 24 h at 37°C with medium B containing 0.2 mM [^{3}H]oleate, but no β-VLDL. Cells were harvested for zero-time values, and the remaining dishes of cells were incubated for up to 36 h at 37°C with medium B containing 0.2 mM [^{14}C]oleate (11,462 dpm/nmol) and apoHDL/PC (400 μg protein/ml) with or without Sandoz 58,035 (5 μg/ml). At the indicated times cells were harvested, and cellular cholesteryl [^{3}H]oleate and cholesteryl [^{14}C]oleate (panel 5A), and cholesterol mass (panel 5B) were measured as described in Methods. The disappearance of cholesteryl [^{3}H]oleate and the appearance of cholesteryl [^{14}C]oleate in the cells over time were used as measures of cholesteryl ester hydrolysis and cholesterol re-esterification, respectively. The results for each point are the mean $\pm$ SD of triplicate dishes. Differences in cholesteryl ester hydrolysis and cholesterol re-esterification with 58,035 were tested at each time point using the *t*-test. Those values that are significantly different ($P < 0.05$) are indicated with asterisks. The slopes of the lines for hydrolysis of cholesteryl [^{3}H]oleate between WC and SR cells were tested using linear regression of the semilog plots shown in Fig. 6. Significant differences between slopes were tested using a *t*-test. The rate of hydrolysis of cholesteryl [^{3}H]oleate and the clearance of cholesteryl esters were significantly ($P < 0.05$) greater in SR cells.

significantly greater rate in SR macrophages compared with WC cells, and the addition of the ACAT inhibitor did not affect the clearance of free or esterified cholesterol mass from either cell type.

By plotting the data in Fig. 5 for hydrolysis and cholesteryl ester clearance on a log scale it was possible to calculate half-times for these parameters (**Fig. 6**). As expected, both the clearance and hydrolysis of cholesteryl esters were log-linear for up to 36 h. Half-times were calculated from the slopes of the lines. The half-times for hydrolysis of cholesteryl esters were 89 h for WC macrophages and 37 h for SR macrophages (Fig. 6A). This compares with the half-time for clearance of esterified cholesterol from WC macrophages of 133 h versus 51 h for the SR cells (Fig. 6B). This is consistent with the conclusion that a defect in the hydrolysis of cholesteryl esters accounts, at least in part, for the slow rate of clearance of cholesteryl esters from WC macrophages.

Recent studies by Bernard et al. (39) have shown that incubation of J774 macrophages with cAMP analogues stimulated hydrolysis of cholesteryl esters and promoted cholesteryl ester clearance to HDL. To determine whether cholesteryl ester hydrolysis could be stimulated in WC pigeon macrophages and its effect on clearance of

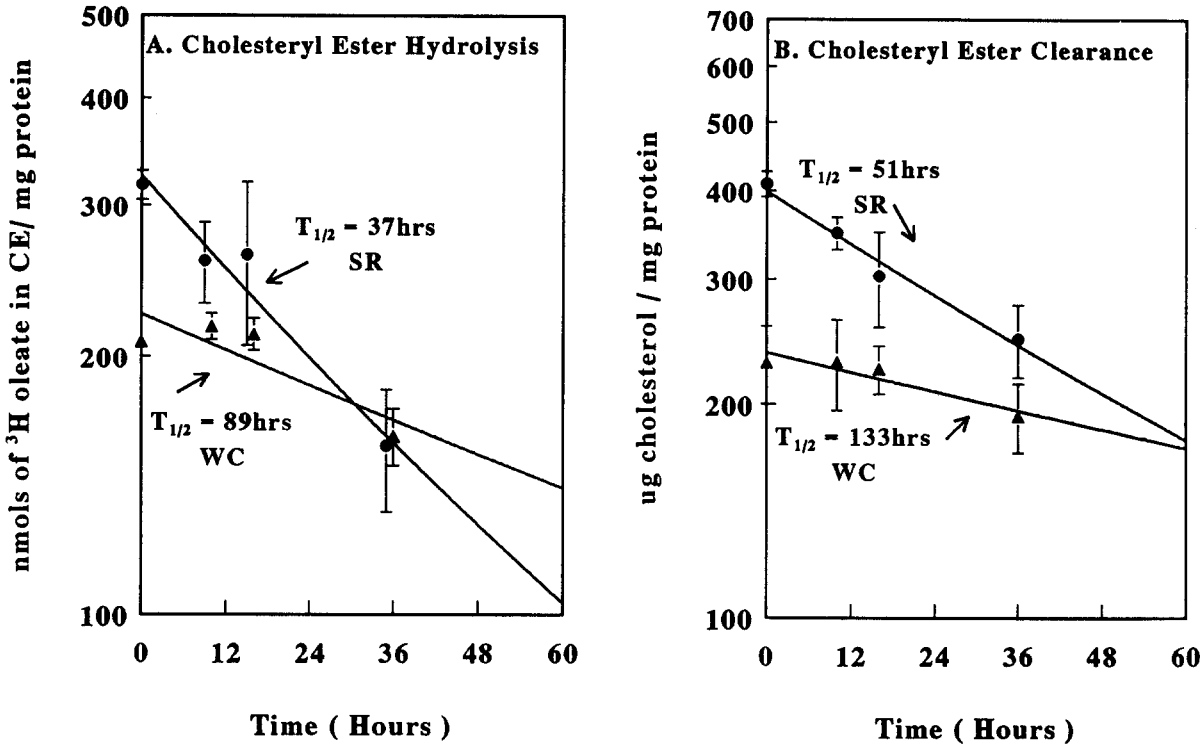

Fig. 6. Rates of cholesteryl ester hydrolysis and cholesteryl ester clearance in WC and SR pigeon macrophages in the presence of apoHDL/PC vesicles. The data from Fig. 5 for cholesteryl ester hydrolysis (panel A) and cellular esterified cholesterol clearance (panel B) in WC and SR macrophages in the presence of apoHDL/PC are plotted with a log scale on the y-axis. Shown are the half-times that were calculated from the slopes of the exponential curves. As the addition of Sandoz 58,035 to the medium containing apoHDL/PC did not affect hydrolysis of cholesteryl esters or clearance of esterified cholesterol in either WC or SR macrophages (Fig. 5), the data for the two experimental treatments were averaged, and the results are the mean ± SD for six dishes at each point. As indicated in the legend to Fig. 5, the differences in the slopes of the lines between WC and SR cells for both hydrolysis and clearance were significantly different ($P < 0.05$).

cholesteryl ester mass, we incubated WC macrophages with apoHDL/PC in the presence or absence of CPT-cAMP, with or without Sandoz 58,035 to inhibit cholesterol re-esterification. WC macrophages were first loaded with cholesteryl [^{3}H]oleate as described for Fig. 5, and cholesteryl ester hydrolysis, cholesterol re-esterification, and esterified cholesterol mass were measured over a 24-h efflux period in the presence of apoHDL/PC (**Fig. 7**). As seen previously, there was only a small decrease (approximately 5%) in the esterified cholesterol content in WC macrophages incubated for 24 h with apoHDL/PC vesicles. Addition of Sandoz 58,035 had no influence on cholesteryl ester hydrolysis, cellular cholesteryl ester content, or cholesterol re-esterification. The addition of CPT-cAMP increased cholesterol re-esterification by 221% and esterified cholesterol mass by 15%, and reduced cholesteryl ester hydrolysis by 15%. In the presence of Sandoz 58,035, where cholesterol re-esterification was inhibited, addition of CPT-cAMP produced a 41% increase in cholesteryl ester hydrolysis and a decrease in esterified cholesterol content of 23%. Thus, cholesteryl ester hydrolysis can be stimulated with CPT-cAMP resulting in increased clearance of esterified cholesterol mass from WC macrophages, but these effects of cAMP only occur if cholesterol re-esterification is reduced.

DISCUSSION

Previous studies from our laboratory have shown that when WC macrophages are incubated for 24 h with apoHDL/PC, they clear cholesteryl esters at a significantly slower rate than SR macrophages (22). These studies were done under conditions where the apoHDL/PC was present in excess such that the acceptor was not rate-limiting for cholesterol efflux. This suggested that the difference in cholesteryl ester clearance between WC and SR macrophages was a cell-mediated process. Therefore, the purpose of the present study was to identify the cellular mechanisms responsible for mediating the difference in cholesteryl ester clearance rates between WC and SR macrophages. Three potential mechanisms were investigated.

The first was suggested by the studies of Bellini et al. (40) showing that in the presence of excess cholesterol acceptor there can be large differences in the rate of free

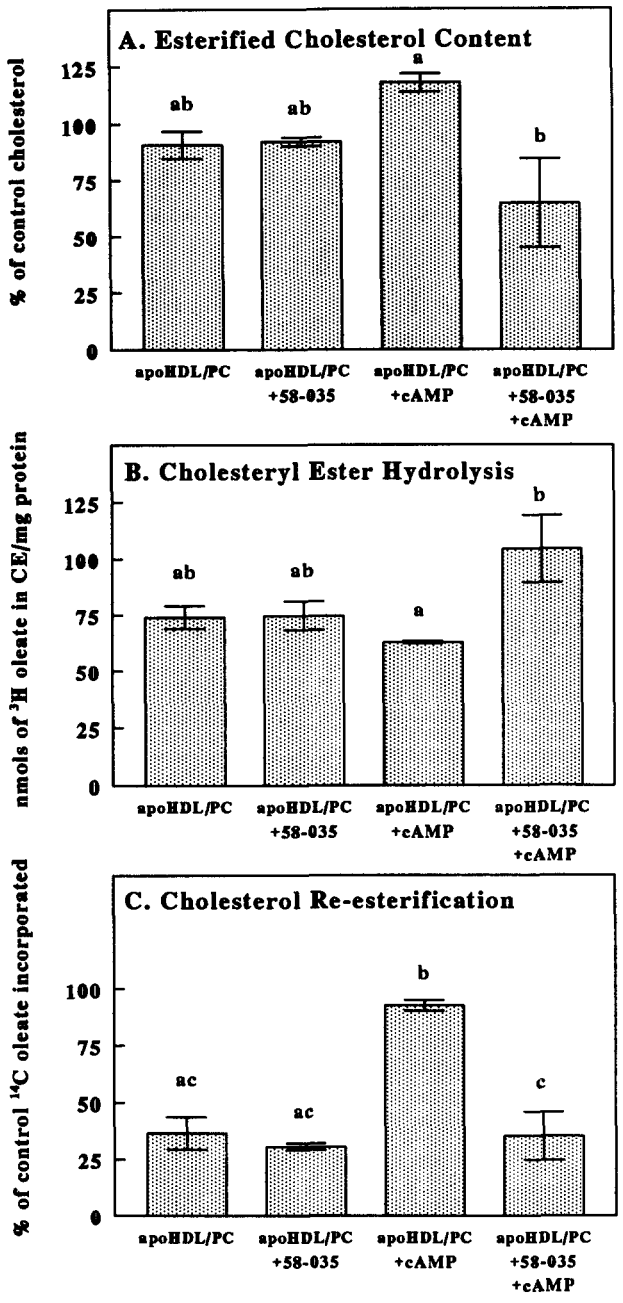

Fig. 7. The effect of CPT-cAMP on esterified cholesterol clearance, cholesteryl ester hydrolysis, and cholesterol re-esterification in WC pigeon macrophages incubated with apoHDL/PC. White Carneau pigeon macrophages were isolated from birds fed a cholesterol-free diet and were loaded with cholesteryl esters by incubation at 37°C with medium B containing 0.2 mM [^{3}H]oleate (259,904 dpm/nmol) and rabbit β-VLDL (100 μg protein/ml). After 24 h, the cells were incubated for an additional 24 h at 37°C in medium B containing 0.2 mM [^{3}H]oleate. Cells were harvested for zero-time values, and the remaining dishes were incubated for 24 h at 37°C with medium B containing 0.2 mM [^{14}C]oleate (12,780 dpm/nmol) and apoHDL/PC (400 μg protein/ml) with or without Sandoz 58,035 (5 μg/ml), CPT-cAMP (1000 μM), or Sandoz 58,035 + CPT-cAMP. Cells were harvested, and cellular esterified cholesterol mass, cholesteryl [^{3}H]oleate, and cholesteryl [^{14}C]oleate were determined as described in Methods. The esterified cholesterol content (panel A) of the cells is expressed as the % of the controls incubated with medium B and [^{14}C]oleate. The esterified cholesterol content of the control cells was 102 ± 1 μg/mg cell protein. Cholesteryl ester hydrolysis (panel B) was calculated as (nmols [^{3}H]oleate in cellular CE/mg protein at zero-time) − (nmols [^{3}H]oleate in cellular CE after 24 h incubation with indicated treatment). Cholesterol re-esterification (panel C) is expressed as the percent of control [^{14}C]oleate incorporated into CE for cells incubated with medium containing 0.2 mM [^{14}C]oleate. The control value was 67 ± 5 nmols of [^{14}C]oleate/mg cell protein. The results represent the means ± SD for triplicate dishes. By one-way ANOVA there were significant differences among the four groups for esterified cholesterol content ($P < 0.008$), cholesteryl ester hydrolysis ($P < 0.03$), and cholesterol re-esterification ($P < 0.01$). Significant ($P < 0.05$) comparisons among the groups by the Neumann-Keuls test are indicated by different letters above the error bars. Comparisons among groups with the same letters are not significantly different.

cholesterol efflux from different cell types. Much of this difference was attributable to intrinsic properties of the plasma membrane resulting in different rates of cholesterol desorption (40, 41). We investigated the possibility that there were differences in the rates of cholesterol desorption from WC and SR macrophages by measuring the rate of unidirectional efflux of [^{3}H]cholesterol from macrophages that had not been loaded with cholesterol. Under these conditions, greater than 90% of cellular free cholesterol is thought to reside in the plasma membrane (42). The efflux of free cholesterol from both WC and SR macrophages was log-linear for up to 8 h, consistent with it being derived from a single kinetic pool of cholesterol that either represented the cholesterol of the plasma membrane or was in equilibrium with it. There were no differences in the rate of cholesterol efflux between WC and SR macrophages, suggesting that the slow rate of cholesteryl ester clearance from the WC cells is not due to an inherent difference in their plasma membranes that limits the desorption of cholesterol and its ultimate efflux from the cell. Consistent with this conclusion is the fact that the half-times of 23.5 to 29.5 h for free cholesterol efflux from

WC macrophages were much faster than the 88 to 133 h for the half-times of cholesteryl ester clearance. It must be kept in mind, however, that these studies were done with cells that were not loaded with cholesteryl esters. It is not difficult to envision that differences in the packing of free cholesterol molecules in plasma membranes of cells loaded with cholesteryl esters could affect their rate of desorption. Thus, the possibility cannot be ruled out that the absolute rates of desorption of free cholesterol may be different between WC and SR macrophages loaded with cholesteryl esters.

The second potential mechanism by which cholesteryl ester clearance could be regulated is by differences in the efficiency of re-esterification of free cholesterol derived from cellular cholesteryl ester hydrolysis. As only free cholesterol can efflux from cells, re-esterification of cholesterol would effectively reduce the amount of free cholesterol available for efflux. Several pieces of data suggest that a difference in cholesterol re-esterification is not the major factor responsible for the differences in cholesteryl ester clearance between WC and SR macrophages. The rates of cholesterol re-esterification in cholesteryl ester-loaded cells incubated in the presence of apoHDL/PC were low and similar for WC and SR macrophages. In the presence of Sandoz 58,035 and apoHDL/PC, where cholesterol re-esterification rates were even lower, the clearance of esterified cholesterol was not affected in WC, SR, or mouse macrophages, suggesting that cholesterol re-esterification is not rate-limiting for cholesterol efflux. In all macrophages in which apoHDL/PC stimulated cholesterol efflux, cholesterol re-esterification was found to be down-regulated when compared to incubation in medium without a cholesterol acceptor. These data are consistent with studies by others using a number of cell types including the FU5AH hepatoma cell line (25) and mouse peritoneal macrophages (17), showing that cholesterol re-esterification is inhibited during cholesteryl ester clearance in the presence of an appropriate cholesterol acceptor.

The fact that cholesterol re-esterification is inhibited in the presence of a cholesterol acceptor also suggests that the rate-limiting step in cholesteryl ester clearance is not the transport of free cholesterol from the site of cholesteryl ester hydrolysis to the plasma membrane. If this step were rate-limiting we would expect to see either a buildup of free cholesterol or high levels of cholesterol re-esterification in cells that were actively clearing cholesteryl esters. Instead, the data suggest that, in the presence of apoHDL/PC, the free cholesterol generated from hydrolysis of cholesteryl esters is directed away from the pool of cholesterol in the cell that regulates ACAT and to the plasma membrane for efflux.

The third potential mechanism for regulation of cholesteryl ester clearance is cholesteryl ester hydrolysis. Results from this study suggest that differences in the rate of cholesteryl ester hydrolysis can best explain the differences in cholesteryl ester clearance between WC and SR macrophages. Two approaches were used to measure the rate of cholesteryl ester hydrolysis. In the first, the generation of free cholesterol from cholesteryl esters was measured in the presence of 58,035 to reduce the re-esterification of cholesterol. Under these conditions the SR macrophages hydrolyzed 25% of their cholesteryl esters in 24 h while the WC cells hydrolyzed only 4%. The rate of cholesteryl ester hydrolysis also was found to be slower for WC than for SR macrophages when it was measured directly during cholesteryl ester clearance by using cells whose cholesteryl esters were metabolically labeled with cholesteryl [^{3}H]oleate. Under these conditions the half-time for cholesteryl ester hydrolysis in WC macrophages was found to be 89 h compared to 37 h for the SR cells. To our knowledge, the 89 h for WC cells is the longest half-time for cholesteryl ester hydrolysis to be reported for any cell type. In the same cells the half-time for cholesteryl ester clearance was 133 h for WC cells and 51 h for SR cells.

Regardless of the method used to measure cholesteryl ester hydrolysis, the SR macrophages hydrolyzed cholesteryl esters at a faster rate than did the WC macrophages. However, when cholesteryl ester hydrolysis was measured in the presence of Sandoz 58,035 in the absence of a cholesterol acceptor, the rate of hydrolysis was reduced relative to the same cells incubated in the presence of apoHDL/PC. Without a cholesterol acceptor there is the generation of excess free cholesterol, but no net efflux of cholesterol from the cells. The data suggest that this build-up of free cholesterol may be able to down-regulate cholesteryl ester hydrolysis. Consistent with this possibility is the observation that the rate of cholesteryl ester hydrolysis plateaued in SR cells after about 12 h of incubation under experimental conditions where free cholesterol would be expected to increase. Studies by Li and Sawyer (43) have shown that addition of free cholesterol to lipid emulsions causes cholesteryl ester to be less soluble in the phospholipid surface monolayer. This could affect the ability of cholesteryl esters to be hydrolyzed, as has been shown by Fielding (44), in which addition of free cholesterol to a triglyceride-rich emulsion decreased the ability of lipoprotein lipase to hydrolyze triglycerides.

Hydrolysis of accumulated cholesteryl esters in macrophages is catalyzed by a neutral pH cholesteryl ester hydrolase (nCEH) (17), which has biochemical (45) and immunological characteristics (46) identical to hormone-sensitive lipase. Our data in pigeon macrophages also is consistent with this conclusion as cholesteryl ester hydrolysis was increased by cAMP, a known stimulator of hormone-sensitive lipase (39). Net cholesteryl ester hydrolysis was stimulated, however, by only about 40% which is less than the 300% increase seen in J774 macro-

phages (39). The reason for this difference is unknown, but is consistent with low levels of nCEH activity in WC macrophages. In pigeon macrophages the effect of cAMP on cholesterol re-esterification was much greater than that seen in J774 macrophages (39) even when incubated with a potent cholesterol acceptor such as apoHDL/PC. The most likely explanation is that the translocation of free cholesterol to the plasma membrane for efflux was overwhelmed in WC macrophages resulting in the build-up of free cholesterol in the cellular pool that regulates ACAT activity. The possibility cannot be discounted, however, that cAMP also may stimulate ACAT directly.

For our studies reported in this and previous papers (22, 32) pigeon peritoneal macrophages have been loaded with cholesterol either in vivo by isolation of macrophages from cholesterol-fed animals or in vitro by incubation with lipoproteins such as rabbit β-VLDL. This results in cells having a wide range of cholesterol concentrations, from unloaded cells with about 50 μg total cholesterol/mg protein to values approaching 1500 μg/mg cell protein. The latter is in the upper range of cholesterol concentrations reported for foam cells isolated from atherosclerotic rabbit aortas (47). In most of the experiments in the present study, the cholesterol content of SR macrophages was higher than WC macrophages for both in vivo and in vitro loaded cells. In our experience, however, this does not reflect a consistently greater ability of SR macrophages to accumulate cholesterol (22). Instead, this is a reflection of the large individual variability seen with in vivo loaded cells. This is due, in part, to a positive correlation of macrophage cholesterol content with the plasma β-VLDL concentration in the pigeons from which the cells were isolated (32). As we do not routinely screen birds for plasma β-VLDL concentrations prior to isolation of macrophages, there is the potential for large differences in cholesterol content from macrophages isolated from individual birds. This will not affect the measurement of the rate of cholesteryl ester clearance, however, as clearance rate is an exponential function, independent of initial cholesteryl ester content (22).

If the steady state cholesteryl ester content of macrophages can be regulated by cholesteryl ester hydrolysis and efflux, as suggested by our studies, why is it that SR macrophages with greater rates of cholesterol efflux have similar, or at times, greater concentrations of cellular cholesterol when isolated from hypercholesterolemic pigeons (Table 1), or after incubation with rabbit β-VLDL (Fig. 5)? The explanation almost certainly lies in the fact that cellular cholesteryl ester content represents the balance between processes that deliver cholesterol to the cells and efflux of cholesterol to an acceptor in the fluid surrounding the cells (18). Under conditions where cells are incubated in culture with β-VLDL in the absence of a cholesterol acceptor, the accumulation of cholesterol must be controlled largely by β-VLDL uptake and not efflux. In in vivo loaded cells the conditions surrounding the macrophages in the peritoneal cavity are not known, but even if there was HDL present, pigeon plasma HDL stimulates cholesterol efflux from pigeon macrophages poorly or not at all (32). Thus, under these conditions of cholesterol loading, even if a cholesterol acceptor was present, the extent of cholesterol influx would be so great that any difference in efflux probably would be overwhelmed. The conditions in the arterial wall may be quite different, however. The endothelium normally provides a barrier to the uncontrolled influx of plasma lipoproteins. Thus, the concentration of abnormal lipoproteins in the arterial wall probably is much less than that used in vitro to load cells. In the arterial wall this may make it possible for SR macrophages to efflux cholesterol at a rate sufficient to keep up with influx, while in WC macrophages that efflux cholesterol poorly, the same rate of cholesterol influx may exceed the ability of cholesterol efflux to keep up, resulting in an increase in the steady state concentration of cholesteryl esters in the cells. If such a scenario is correct than, overall, WC pigeons would be expected to have greater atherosclerosis than SR birds.

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