# Progesterone blocks intracellular translocation of free cholesterol derived from cholesteryl ester in macrophages

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**Abstract** Macrophage foam cells must accommodate continuing fluxes of free cholesterol in spite of a greatly expanded store of cholesteryl ester. Though endogenous free cholesterol synthesis is suppressed, free cholesterol continues to enter the cell via endocytosis of oxidized/modified lipoproteins. It has been shown previously that this free cholesterol is released into the lysosomal compartment and rapidly transported to the plasma membrane prior to its esterification. A substantial amount of free cholesterol is also presented via the continuous hydrolysis of cholesteryl ester during the cholesteryl ester cycle. We addressed the question of whether the intracellular free cholesterol derived from the hydrolysis of cholesteryl ester formed a protected pool for rapid re-esterification. Incubation of macrophage foam cells with cyclic AMP to enhance cholesteryl ester hydrolysis, and with S58035 to inhibit acyl-CoA:cholesterol acyltransferase (ACAT) activity, led to conversion of cellular cholesteryl ester to free cholesterol and transport of this free cholesterol to the plasma membrane. Addition of progesterone, previously demonstrated to be an inhibitor of free cholesterol transport in other cell types, also led to conversion of cholesteryl ester to free cholesterol even though progesterone was only a weak inhibitor of ACAT activity. Free cholesterol in the plasma membrane was an important source of ACAT substrate to balance the constitutive hydrolysis of cholesteryl ester in cholesterol-loaded macrophages. Treatment of cells with progesterone, however, prevented free cholesterol derived from cholesteryl ester hydrolysis from moving to the plasma membrane. The sequestration of free cholesterol by progesterone could be reversed by incubation with human $HDL_3$. Our data indicate that free cholesterol derived from cholesteryl ester hydrolysis requires translocation through the cell prior to becoming available for re-esterification. Disrupting free cholesterol transport to the plasma membrane by treatment with progesterone disrupts the cholesteryl ester cycle in cholesteryl ester-loaded macrophages.—**Mazzone, T., M. Krishna, and Y. Lange.** Progesterone blocks intracellular translocation of free cholesterol derived from cholesteryl ester in macrophages. *J. Lipid Res.* 1995. **36:** 544–551.

**Supplementary key words** macrophage foam cells • cellular cholesterol transport • cholesteryl ester cycle • acyl-CoA:cholesterol acyltransferase

Cholesterol is an important constituent of all eukaryotic cells and cellular free cholesterol (FC) balance is defended by multiple pathways including regulation of its synthesis, internalization, esterification, and efflux (1–5). The completion of cholesterol synthesis and its esterification occur in the endoplasmic reticulum; lipoprotein-derived free cholesterol becomes available in the lysosomal compartment; and cholesterol efflux utilizes the plasma membrane pool of free cholesterol as its substrate (6). Free cholesterol distribution in cell membranes is highly skewed with its highest content being found within the plasma membrane (7, 8). The transport mechanisms underlying the maintenance of this asymmetrical distribution of cholesterol among the various compartments noted above, however, have remained obscure. The approach to understanding these transport mechanisms has been facilitated by the study of models in which normal transport of free cholesterol is disrupted. For example, Niemann-Pick C cells display a defect of free cholesterol movement out of the lysosomes (9). This defect can be reproduced in fibroblasts by incubations with the inhibitor U18666A or with progesterone (10). Progesterone has also been used to study the transport of free cholesterol in rat hepatoma cells in which it inhibits the movement of free cholesterol from the plasma membrane to the endoplasmic reticulum (11).

Cholesterol metabolism in steroidogenic cells and in macrophages is unique in that each cell type is subject to large cholesterol fluxes and can maintain an expanded store of intracellular cholesterol as cholesteryl ester (CE) in cytoplasmic droplets. Cholesteryl ester droplets in these cells are metabolically active, undergoing a continuous cycle of hydrolysis and re-esterification; a process that has

Abbreviations: FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; ALDL, acetylated low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; FC, free cholesterol; CE, cholesteryl ester; BSA, bovine serum albumin; P, progesterone; ACAT, acyl-CoA:cholesterol acyltransferase.

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been termed the cholesteryl ester cycle (12, 13). In both steroidogenic cells and macrophage cells, it has been shown that plasma membrane free cholesterol can serve as a substrate for acyl-CoA:cholesterol acyltransferase (ACAT) and thereby contribute to the intracellular cholesteryl ester pool (14, 15). It has been shown, for example, that newly internalized lipoprotein cholesterol and newly synthesized cholesterol are rapidly transported to the plasma membrane prior to esterification (15–17). Less information is available, however, regarding the fate of free cholesterol released from the hydrolysis of intracellular cholesteryl ester lipid droplets in macrophage foam cells. Detailed cytochemical and morphologic studies of these cells have provided evidence for a small pool of intracellular free cholesterol associated with intracytoplasmic cholesteryl ester lipid droplets (18). In these cells progesterone interrupts the cholesteryl ester cycle and stimulates the conversion of stored cholesteryl ester to free cholesterol. Morphologically, addition of progesterone for 48 h leads to disappearance of cholesteryl ester droplets and the appearance of filipin-staining material within an intracellular vesicular compartment. These effects of progesterone were thought to be related to inhibition of ACAT activity; however, recently it has been shown that progesterone is only a weak inhibitor of ACAT activity in cellular homogenates of rat hepatoma cells (11). In addition, the rapidity with which newly internalized or newly synthesized cholesterol mixes with plasma membrane cholesterol, and the observation that the plasma membrane free cholesterol pool can serve as substrate for ACAT, suggested that maintenance of overall cellular free cholesterol balance would be facilitated if free cholesterol derived from the hydrolysis of cholesteryl ester also rapidly mixed with plasma membrane free cholesterol prior to its re-esterification. In the studies reported in this manuscript, therefore, we further examine the mechanism for re-esterification of free cholesterol derived from the acute hydrolysis of cholesteryl ester in macrophage cells.

## METHODS

The macrophage-like J774 cell line was obtained from ATCC and maintained in 10% FBS in Dulbecco's modified Eagle's medium (DMEM) (19). Macrophages were enriched in cholesterol by incubation with acetylated low density lipoprotein (ALDL), which was prepared by the acetylation of human low density lipoprotein (LDL) as previously described (20). LDL (d 1.019–1.063 g/ml) and $HDL_3$ (d 1.125–1.210 g/ml) were prepared from human plasma by sequential density gradient ultracentrifugation as previously described (20). ALDL prepared from the plasma of different donors produced some variability in cholesterol loading from experiment to experiment.

Lipid dispersions with a free cholesterol–phosphatidylcholine molar ratio of >2 were prepared as described (21). Briefly, a cholesterol–phosphatidylcholine mixture was sonicated for 10 min twice, and recovered in the supernatant after a 30-min spin at 45,000 *g*. The dispersions were suspended in cell culture medium at 225 μg/ml of cholesterol and radiolabeled cholesterol was added to give a final concentration during incubation with the cells of 1 μCi/ml. This suspension was filter-sterilized and incubated at 37°C for 48 h prior to use. Cholesterol in cellular plasma membrane was radiolabeled with [$^3$H]cholesterol at 15°C as previously described (11).

Total cellular cholesterol and free cholesterol mass were measured in hexane–isopropyl alcohol extracts by gas–liquid chromatography (19, 20). Radioactivity in FC and CE present in these extracts was analyzed by thin-layer chromatography on silica gel G developed with petroleum ether–ethyl ether–acetic acid 75:25:1 (22). For the studies in this report, the plasma membrane free cholesterol pool was quantitated as cholestenone after its oxidation by exogenously added cholesterol oxidase as previously described in detail (7, 23). Cells in suspension were fixed with glutaraldehyde and treated with cholesterol oxidase; cholesterol and cholestenone were quantitated by high performance liquid chromatography. Cholesterol esterification in cell homogenates after addition of exogenous cholesterol substrate was measured as previously detailed (11, 24). Briefly, cell homogenates (10–15 μg cholesterol) were incubated for 30 min at 37°C with [$^3$H]cholesterol (~25 μg) in Triton WR-1339 (24). Bovine serum albumin (BSA) (1 mg/ml), dithiothreitol (1 mM), and oleoyl coenzyme A (24 μM) were added. After an additional 5-min incubation at 37°C, samples were extracted and analyzed for labeled cholesteryl esters. Lipoprotein and cellular protein mass was measured by the method of Lowry et al. using BSA as standard (19). Statistical comparisons were done using a two-tailed *t*-test. Progesterone was obtained from Sigma. All other materials were from previously detailed sources (11, 19–23). Results of representative experiments are shown, however, all results were confirmed in two or three comparable experiments.

## RESULTS

Macrophages were enriched with cholesterol by a preincubation in ALDL and then treated with cyclic AMP + S58035 in serum-free and lipoprotein-free medium in order to acutely increase cellular free cholesterol derived from the hydrolysis of cholesteryl ester (25). The cells were treated with cholesterol oxidase, a membrane-impermeable probe, to determine the distribution of the released free cholesterol between cell surface and interior (23). Compared to control incubations, 6 h in cyclic AMP + S58035 increased macrophage free

cholesterol by 6.2 μg/mg cell protein (**Table 1**). As shown here, the newly released free cholesterol achieves equilibrium distribution by 6 h such that 90% of it, an amount identical to control, becomes accessible to oxidation by cholesterol oxidase. No difference in free cholesterol oxidation could be detected compared to control cells as early as 3 h after addition of cAMP + S58035 (Table 1B), which was the earliest time point we could reproducibly detect an increase in cellular free cholesterol mass (not shown).

Progesterone has been shown to inhibit the cholesteryl ester cycle in macrophage foam cells (12). It has also been recently reported to be an inhibitor of the transport of newly internalized cholesterol (10) as well as newly synthesized sterol precursors (Y. Lange, unpublished observation) in fibroblasts and hepatoma cells. We therefore undertook a series of studies designed to probe a relationship between progesterone-related inhibition of sterol transport in macrophage foam cells and its inhibition of the cholesteryl ester cycle. The data in **Table 2A** show that incubation with progesterone, along with cyclic AMP and S58035, further decreased cellular cholesteryl ester and increased cellular free cholesterol mass during a 6-h incubation. It also can be seen that progesterone decreased the fraction of free cholesterol found in the plasma membrane compartment from 91.8 ± 0.6% to 87.4 ± 1.2% ($P < 0.01$). After cholesterol oxidase treatment, cells incubated with cAMP + S58035 contained 6.8 μg/mg of unoxidized free cholesterol while cells incubated with these agents plus progesterone contained 11.8 μg/mg. Therefore, of the 10.5 μg/mg increase of total cellular free cholesterol due to progesterone treatment, 5 μg (48%) is in a non-oxidizable pool. Table 2B shows the results of continuing the experiment shown in 2A such that cells incubated for 6 h with cyclic AMP + S58035 + progesterone were extensively washed and placed in serum-free medium alone or this medium containing only progesterone for an additional 4 h. The cells incubated with serum-free medium alone rapidly re-esterify cholesterol. After 4 h, cellular free cholesterol has fallen from 93.5 to 73.5 μg/mg and cholesteryl ester has risen from 32.4 to 50.5 μg/mg. The inclusion of progesterone in this incubation, however, completely prevents re-esterification such that free cholesterol and cholesteryl ester remain essentially unchanged.

**Table 3** shows the results of experiments in which

TABLE 1. Free cholesterol (FC) derived from cholesteryl ester (CE) is rapidly mobilized to the plasma membrane

| A | FC | CE | FC Oxidation |
|---|---|---|---|
| | *μg/mg* | | % |
| Control | 45.8 | 15.4 | 90.2 |
| cAMP + S | 52.0 | 7.2 | 90.0 |

| B | FC Oxidation | | | |
|---|---|---|---|---|
| | 3 h | 4 h | 5 h | 6 h |
| | % | | | |
| Control | 87.2 | 87.5 | 87.7 | 87.8 |
| cAMP + S | 88.8 | 87.0 | 89.1 | 88.8 |

J774 cells (1 × $10^6$ per 35 mm dish) were seeded in 10% FBS/DMEM. After 3 days, fresh medium containing 50 μg/ml of ALDL was added to enrich cells with cholesterol. After 48 h in this medium, cells were incubated for 16 h in 0.2% BSA/DMEM. At that time ethanol vehicle or cAMP (1 mM) with S58035 (1 μg/ml) was added. A. Cells were incubated for an additional 6 h and harvested for measurement of FC and CE or treated with cholesterol oxidase. B. Cells were incubated in ethanol or cAMP + S58035 for the indicated times and treated with cholesterol oxidase. Values shown are averages of measurements from duplicate dishes.

TABLE 2. Progesterone sequesters FC in a non-oxidizable pool and prevents its re-esterification

| A | Addition | FC | CE | FC Oxidation |
|---|---|---|---|---|
| | | | *μg/mg* | % |
| | cAMP + S | 83.0 ± 1.4 | 38.9 ± 2.9 | 91.8 ± 0.6 |
| | cAMP + S + P | 93.5 ± 1.7 | 32.4 ± 3.2 | 87.4 ± 1.2 |

| B | After removal of cAMP + S + P | | |
|---|---|---|---|
| | | FC | CE |
| | | *μg/mg* | |
| | None | 73.5 ± 0.8 | 50.5 ± 3.6 |
| | P | 92.6 ± 2.1 | 27.8 ± 6.9 |

J774 cells were plated and enriched with cholesterol as described in the legend to Table 1. At the start of the experiment cAMP (1 mM) with S58035 (1 μg/ml) were added with or without progesterone (10 μg/ml). A. After 6 h cells were harvested for measurement of FC and CE, or treated with cholesterol oxidase. B. After a 6-h incubation with cAMP + S58035 + progesterone, cells were washed twice with 0.2% BSA/DMEM and placed in this medium containing ethanol vehicle or progesterone (10 μg/ml). After an additional 4 h cells were harvested for measurement of FC and CE. Values shown are the mean ± SD from triplicate cultures. The difference in FC oxidation (91.1 ± 0.6 vs. 87.4 ± 1.2%) is significant at the $P < 0.01$ level.

TABLE 3. Progesterone alone sequesters FC and interrupts re-esterification

| Addition | Unoxidized FC | Total FC |
|---|---|---|
| | % | *μg/mg* |
| S58035 | 10.4 ± 1.0 | 42.0 ± 1.2 |
| P | 16.4 ± 1.6 | 45.0 ± 0.5 |
| P + cAMP | 14.8 ± 1.3 | 49.2 ± 1.3 |
| P + S58035 | 16.1 ± 0.6 | 49.3 ± 4.0 |
| P + cAMP + S | 13.8 ± 1.6 | 55.8 ± 2.4 |

J774 cells were plated and enriched with cholesterol as described in the legend to Table 1. At the start of the experiment S58035 (1 μg/ml), cAMP (1 mM), or progesterone (10 μg/ml) was added as indicated in the table for an additional 6 h. Values shown are the mean ± SD from triplicate cultures. The difference in unoxidized cholesterol for S58035 vs. P is significant at $P < 0.01$; S58035 vs. P + cAMP, $P < 0.01$; S58035 vs. P + S58035, $P < 0.01$; S58035 vs. P + cAMP + S, $P < 0.05$.

progesterone alone or progesterone in combination with cAMP and/or S58035 was compared to the effect of S58035 alone on cellular free cholesterol content and its accessibility to oxidation by cholesterol oxidase. Progesterone alone leads to a free cholesterol mass higher than that produced by S58035 alone and alters the accessibility of free cholesterol to cholesterol oxidase compared to S58035 treatment (unoxidized cholesterol 16.4 ± 1.6% vs. 10.4 ± 1%, $P < 0.01$). All combinations that include progesterone have an altered accessibility of free cholesterol to cholesterol oxidase that is significantly different from S58035 alone (see Table 3 legend).

Because progesterone expands cellular free cholesterol content in macrophage foam cells more effectively than the ACAT inhibitor S58035 (Table 3) and completely suppresses re-esterification of a grossly expanded free cholesterol pool (Table 2B), its potency as an inhibitor of ACAT in macrophage cells was evaluated. **Table 4** shows the results of two experiments in which the effect of progesterone on ACAT activity was measured in macrophage homogenates. Exogenous cholesterol substrate, in the benign vehicle WR-1339, was added to cell extracts. For experiment 1, progesterone (10 μg/ml) was also added to the cell extract. For experiment 2, progesterone (10 μg/ml) or S58035 (1 μg/ml) was incubated with cells for 6 h prior to cell homogenization and was also added during the in vitro incubation with labeled substrate. As shown, ACAT activity is reduced by 24% by 10 μg/ml of progesterone added directly to cell homogenates and by 29% when the drug was also present during a 6-h preincubation. This small degree of inhibition is similar in magnitude to what was observed in homogenates of rat hepatoma cells (11) and indicates that the results shown in Tables 2 and 3 cannot be completely accounted for by inhibition of ACAT enzyme activity by progesterone. ACAT inhibition produced by S58035 (76%) is substantially greater than that produced by progesterone.

TABLE 4. Progesterone is a weak inhibitor of ACAT activity

| Addition | CE Synthesized | Inhibition |
|---|---|---|
| | *μg/h/mg cell cholesterol* | % |
| Experiment 1 | | |
| None | 80.8 | |
| Progesterone, 10 μg/ml | 61.2 | 24% |
| Experiment 2 | | |
| None | 35.0 | |
| Progesterone, 10 μg/ml | 24.7 | 29% |
| S58035, 1 μg/ml | 8.5 | 76% |

J774 cells were prepared and enriched with cholesterol as described in the legend for Table 1. Homogenates of macrophage cells were incubated with cholesterol (~25 μg) in Triton WR-1339, BSA (1 mg/ml), DDT (1 mM), and [$^{14}$C]oleoyl-CoA (24 μM). After a 5-minute incubation at 37° with the indicated addition, samples were extracted and analyzed for labeled CE. For experiment 2, cells were incubated in vehicle or the indicated concentration of progesterone or S58035 for an additional 6 h before harvest. Values shown are the means of duplicate determinations which agreed to within 5%.

TABLE 5. Effect of progesterone on distribution of radioactivity in free cholesterol

| | Cholestenone | Cholesterol | Oxidation |
|---|---|---|---|
| | *dpm/mg cell protein* | | % |
| Control | 16,432 ± 1,200 | 3,841 ± 395 | 81.1 ± 1.3 |
| Progesterone | 17,191 ± 1,366 | 5,278 ± 122 | 76.4 ± 1.0 |
| Change (with P) | +5% | +37% | |

J774 cells were seeded at 1 × $10^6$ cells per 35 mm dish and grown for 3 days. At that time cells were placed in 0.2% BSA containing 50 μg/ml of ALDL and 225 μg/ml of free cholesterol in lipid dispersions. This concentration of lipid dispersions gave a final concentration of 1 μCi/ml of [$^3$H]cholesterol contained in the dispersions. After 48 h cells were washed and placed in 0.2% BSA/DMEM for 16 h. At that time progesterone (10 μg/ml) or ethanol vehicle was added. After an additional 6 h cells were harvested for analysis. Values shown are mean ± SD from triplicate cultures. The statistical significance of differences for comparison of control versus progesterone-treated cells is as follows: FC oxidation, $P < 0.01$; radioactivity in cholesterol, $P < 0.005$; radioactivity in cholestenone, $P$ = NS.

The effect of progesterone on increasing free cholesterol in macrophage foam cells while inhibiting its transport to the plasma membrane can also be demonstrated in cells labeled to equilibrium with [$^3$H]cholesterol. **Table 5** shows the results of such an experiment. As shown, after 6 h treatment with progesterone, there is a statistically significant difference in oxidation of the labeled free cholesterol compared to control. The increase in labeled cholestenone of 5% during incubation with progesterone is not significant but the increase in labeled intracellular cholesterol of 37% during incubation with progesterone is highly significant ($P < 0.005$). Of the 2196 dpm/mg increase in free cholesterol radioactivity produced by the progesterone incubation, 1437 dpm/mg or 65% is in the unoxidized cholesterol fraction. This result is in good agreement with that obtained from measurement of free cholesterol mass after incubation in progesterone shown in Table 2 (see above).

The data thus far indicate that progesterone alters the movement of free cholesterol derived from cholesteryl ester and prevents it from mixing with plasma membrane free cholesterol. The largest pool of free cholesterol is in the plasma membrane and it has been shown that progesterone blocks movement of free cholesterol out of the plasma membrane in rat hepatoma cells (11). In order to examine whether progesterone produces a bi-directional block of sterol movement in macrophage foam cells, plasma membrane cholesterol was labeled at 15°C and the effect of progesterone over a subsequent 6-h incubation was determined. As shown in **Table 6,** inclusion of progesterone when cells were warmed to 37°C almost completely blocked esterification of labeled plasma membrane cholesterol (<1%) while control cells esterified 12%

TABLE 6. Effect of progesterone on the distribution of radiolabeled cholesterol internalized from the plasma membrane

| | FC | CE | FC Oxidation |
|---|---|---|---|
| | *dpm/mg cell protein* | | *%* |
| Control | 88,837 ± 1,176 | 12,150 ± 1,056 | 84.7 ± 1.1 |
| Progesterone | 108,327 ± 2,964 | 289 ± 37 | 80.3 ± 1.2 |

J774 cells were prepared as described in the legend to Table 1. Prior to starting the experimental incubations cells were labeled with exogenous [$^3$H]cholesterol (2 $\mu$Ci/ml) for 30 min at 15°C. Cells were then washed and medium at 37°C containing ethanol vehicle or progesterone (10 $\mu$g/ml) was added. After an additional 6 h cells were harvested for analysis. Values shown are mean ± SD from triplicate cultures. The difference in FC oxidation for control versus progesterone is significant at the $P < 0.01$ level.

of plasma membrane cholesterol radioactivity within 6 h. Even though progesterone-treated cells have an expanded pool of free cholesterol compared to control cells, less free cholesterol is accessible to oxidation by cholesterol oxidase (80.3 ± 1.2% vs. 84.7 ± 1.1%, $P < 0.01$). These data indicate that 40% of the extra free cholesterol radioactivity that accumulated during progesterone treatment is not in the plasma membrane, which was the source of the label, but was also not available as substrate for esterification by ACAT.

The results in Table 6 implicate the plasma membrane as an important source of free cholesterol substrate to balance the constitutive cholesteryl ester hydrolysis during the cholesteryl ester cycle in macrophages. The importance of plasma membrane free cholesterol as ACAT substrate during this cycle could help to explain two seemingly divergent observations as follows. In Tables 2 and 3, it can be seen that progesterone is at least as effective as S58035 in elevating cellular free cholesterol in cholesteryl ester-loaded macrophages. On the other hand, in Table 4, it can be seen that progesterone is a much weaker inhibitor of ACAT activity than S58035. In **Figure 1,** the results of an experiment are shown in which plasma membrane free cholesterol was labeled at 15°C and then progesterone or S58035 was added at the start of a 37°C incubation. As shown, progesterone was much more effective than S58035 at preventing the esterification of plasma membrane cholesterol. In this experiment, which monitors only plasma membrane free cholesterol as substrate for esterification, the addition of S58035 to progesterone produces no additional effect. We conclude from this experiment that progesterone is more effective than S58035 at interrupting the cholesteryl ester cycle in macrophage foam cells due to its inhibition of substrate free cholesterol transport to an ACAT accessible pool, and further, that plasma membrane is the likely source of this substrate.

We also used the same approach, i.e., labeling plasma membrane cholesterol, to assess the rapidity with which free cholesterol can move between the plasma membrane and ACAT substrate pool (**Fig. 2**). After labeling plasma membrane free cholesterol at 15°C, cells were warmed to 37°C in the presence or absence of progesterone. As shown, by 15 min there was significant movement of plasma membrane free cholesterol to the ACAT substrate pool. Also, as shown, progesterone inhibition of this movement is also evident by 15 min.

The preceding observations indicate that progesterone blocks movement of FC derived from cholesteryl ester hydrolysis to the plasma membrane and thereby prevents its re-esterification. We next considered whether the sequestered pool of free cholesterol could be mobilized by extracellular acceptors of cholesterol. It has previously been shown, for example, that human $HDL_3$ induces the

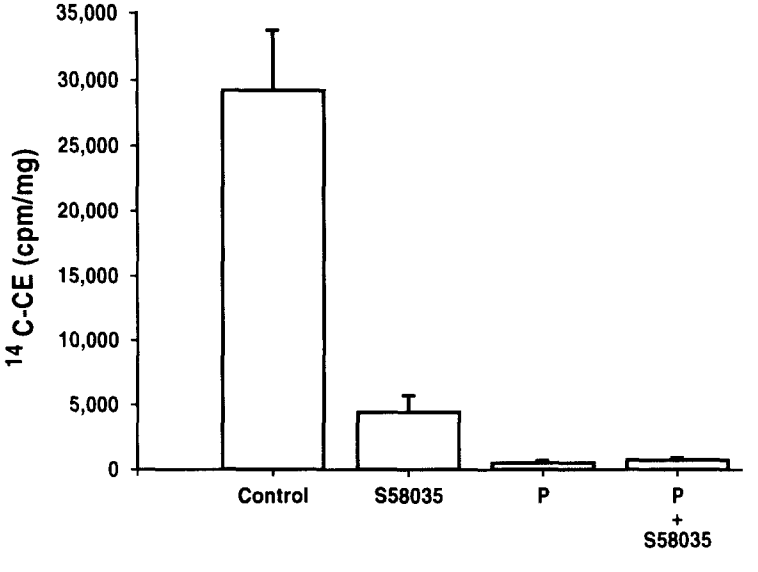


**Fig. 1.** Effect of progesterone versus S58035 for inhibiting esterification of plasma membrane free cholesterol. Cells were prepared and plasma membrane free cholesterol was labeled as described in the legend to Table 6. At the start of the 37°C incubation, S58035 (1 $\mu$g/ml) or progesterone (10 $\mu$g/ml) was added as indicated. After 6 h, cultures were harvested for measurement of radioactive cholesterol incorporated into cholesteryl ester. Values shown are the mean ± standard deviation from triplicate cultures.

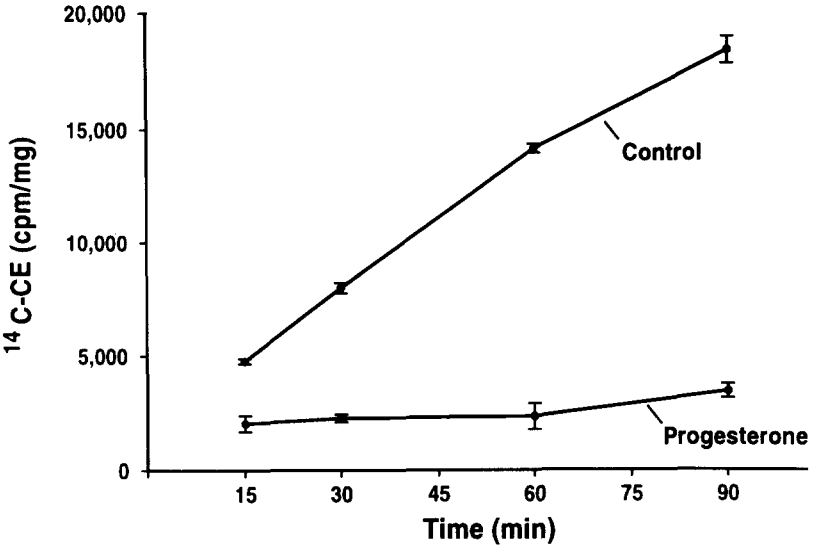


**Fig. 2.** Time course for incorporation of plasma membrane free cholesterol into cholesterol ester and its inhibition by progesterone. Cells were prepared and plasma membrane free cholesterol was labeled as described in the legend to Table 6. At the start of the 37°C incubation (0 time) progesterone (10 μg/ml) or ethanol vehicle was added and cells were harvested at the times indicated for measurement of incorporation of radioactive cholesterol into cholesteryl ester. Values shown are mean ± standard deviation of triplicate cultures.

translocation of intracellular free cholesterol to the plasma membrane (26, 27). **Table 7** shows the results of two experiments performed in cells that were enriched in free cholesterol to different degrees. Cells were incubated with progesterone, $HDL_3$, or a combination of these agents for 6 h. Analysis of cells treated with cholesterol oxidase showed the expected increase of non-oxidizable cholesterol (75% in experiment 1 and 69% in experiment 2) after treatment with progesterone alone. Addition of $HDL_3$, however, substantially reduced the mass of sequestered free cholesterol produced by progesterone. In this experiment, the percentage decrease in total cellular cholesterol induced by $HDL_3$ was not significantly different between cells treated with $HDL_3$ alone and cells treated with progesterone and $HDL_3$ (10% and 12%, respectively). Thus, the progesterone block of free cholesterol movement to the plasma membrane is reversible, even in the continued presence of progesterone, by extracellular cholesterol acceptors.

TABLE 7. Effect of $HDL_3$ on sequestration of FC by progesterone

| | Unoxidized FC | |
|---|---|---|
| | Experiment 1 | Experiment 2 |
| | *μg/mg* | |
| Control | 2.8 ± 0.1 | 10.8 ± 1.5 |
| P | 4.9 ± 0.4 | 18.2 ± 1.6 |
| $HDL_3$ | 2.3 ± 0.1 | 8.2 ± 0.8 |
| P + $HDL_3$ | 3.5 ± 0.1 | 12.2 ± 0.5 |

J774 cells were prepared as described in the legend to Table 1. At the start of the experiment ethanol vehicle (control) or the incubated additions were made. After an additional 6 h cells were treated with cholesterol oxidase and the remaining free cholesterol was quantitated by high performance liquid chromatography. Values shown are mean ± SD from triplicate cultures. Statistical significance of differences are as follows. Experiment 1: control versus P, $P < 0.001$; control versus $HDL_3$, $P < 0.001$; P versus P + $HDL_3$, $P < 0.005$. Experiment 2: control versus P, $P < 0.005$; control versus $HDL_3$, $P$ = NS; P versus P + $HDL_3$, $P < 0.005$.

## DISCUSSION

Macrophages with greatly expanded stores of cholesteryl ester have been identified and isolated from the diseased arterial vessel wall (28). Large deposits of macrophage intracellular cholesterol can arise from ingestion of oxidized lipoproteins or the membranes of degenerating cells. Under these conditions cholesterol synthesis is suppressed and would not therefore continue to contribute to cellular free cholesterol flux. However, FC continues to arise from ongoing endocytosis of modified lipoproteins and the continuous hydrolysis of cholesteryl ester in cytoplasmic droplets (12, 29). Excess cholesterol remains within the macrophage cell and may eventually contribute to cell death unless it can be returned to the extracellular space. Cholesterol efflux is dependent on the presence of extracellular acceptor particles for cholesterol that utilize the plasma membrane free cholesterol pool as the donor site (6). Arterial wall macrophage foam cell free cholesterol metabolism must therefore account for that internalized via the scavenger receptor/endocytosis, and that derived from cholesteryl ester hydrolysis during the cholesteryl ester cycle. All of this free cholesterol must remain in equilibrium with the free cholesterol utilized as the substrate pool for ACAT, and with that pool which will serve as substrate for cholesterol efflux, i.e., the plasma membrane which contains the largest pool of cellular free cholesterol.

Our data indicate that progesterone blocks re-esterification of the free cholesterol derived from cholesteryl ester

hydrolysis in macrophages by blocking its cellular translocation and not by directly inhibiting ACAT activity. The argument that movement through the plasma membrane is required prior to esterification is supported by the observations that *a)* inhibition of ACAT activity (by S58035) leads to accumulation of free cholesterol in the plasma membrane (Table 1); *b)* progesterone is only a weak inhibitor of ACAT activity compared to S58035 but is more effective at interrupting the cholesteryl ester cycle in macrophages (Tables 2–4); *c)* progesterone blocks cholesterol translocation to and from the plasma membrane (Tables 3, 5, 6 and Figs. 1, 2); and *d)* plasma membrane free cholesterol rapidly moves to the ACAT substrate pool and is converted to CE in cells in which the only significant source of free cholesterol is the hydrolysis of cholesteryl ester (Fig. 2). These observations support the importance of the plasma membrane in allowing various sources of free cholesterol to be accommodated while maintaining cellular free cholesterol/cholesteryl ester equilibrium in cholesterol-loaded macrophages.

In attempting to address the rapidity with which free cholesterol could move to the plasma membrane after its release from cholesteryl ester, we noted that as early as we could detect an increase in cellular free cholesterol mass, this cholesterol could be detected in the plasma membrane (by 3 h). Utilization of isotopically labeled cholesterol would allow more sensitivity in detecting the subcellular movement of such cholesterol but there is no means of selectively labeling the free cholesterol in cholesteryl ester droplets. However, the observation that plasma membrane free cholesterol was rapidly esterified in macrophages, under conditions in which the only source of free cholesterol is the hydrolysis of cholesteryl ester, suggested that movement of newly released free cholesterol from CE droplets to the plasma membrane was also rapid. In these cells, free cholesterol movement from the plasma membrane to ACAT, and subsequent deposition as CE, should be balanced by movement in the opposite direction in order to maintain equilibrium of free cholesterol distribution. The data in Fig. 1, therefore, suggest significant bidirectional movement between CE droplets and the plasma membrane within 15 min. In addition, this rapid movement is inhibitable by progesterone.

It has been shown previously that the growth state of cells can acutely modify the distribution of free cholesterol between plasma membrane and intracellular pools (30). These studies were performed using fibroblast cells which do not usually store large amounts of CE. However, the ongoing growth of the J774 cells, used for these studies, may have influenced the movement of cholesterol between intracellular pools and the plasma membrane. On the other hand, the data in Table 6 and Figs. 1 and 2 indicate that movement of free cholesterol to the plasma membrane in J774 cells is not solely related to cell growth because plasma membrane free cholesterol also serves as a major source of free cholesterol substrate for re-esterification.

The data in Table 7 indicate that free cholesterol sequestered by progesterone can be mobilized by extracellular $HDL_3$. Addition of cyclic AMP, like $HDL_3$, has also been reported to increase the movement of intracellular free cholesterol to the plasma membrane in human skin fibroblasts (31). Cyclic AMP may not, however, be able to overcome the block of sterol movement produced by progesterone treatment in macrophages. The data in Table 3 indicate that non-oxidizable free cholesterol mass, in cells treated with progesterone compared to cyclic AMP + progesterone, is not significantly different. Also, in our studies, cyclic AMP added alone did not increase free cholesterol mass, though its stimulation of cholesteryl ester hydrolysis in macrophage foam cells has been well documented (25, 32). Presumably this can be accounted for by rapid re-esterification of newly liberated free cholesterol, and further underscores the rapidity with which FC is transported between its site of hydrolysis in CE droplets and the plasma membrane.

Progesterone inhibits the release of lysosomal free cholesterol in fibroblasts and blocks the esterification of free cholesterol derived from the lysosomal hydrolysis of internalized lipoprotein. This accumulated free cholesterol has been shown to accumulate in perinuclear lysosomes (10). Treatment of fibroblasts with S58035, an ACAT inhibitor that also blocks esterification, does not lead to accumulation in these structures (10). As noted above, detailed morphologic and cytochemical studies have been reported for macrophage foam cells (18). In these cells, an intracellular pool of free cholesterol has been identified, by filipin staining, associated with cytoplasmic cholesteryl ester droplets. Our data indicate that this free cholesterol requires transport to the plasma membrane prior to re-esterification but the site of the progesterone-induced block of sterol movement in macrophages remains to be identified. Progesterone treatment of macrophage foam cells for 48 h produced free cholesterol accumulation in myelin-like vesicular structures (18). If these can be identified as lysosomal in origin, it would implicate the lysosome as an important intermediary structure for the transport of all free cholesterol between plasma membrane and intracellular pools.

In summary, our data assign an important role to the constitutive hydrolysis of cholesteryl ester in macrophage foam cells. Rapid transport of the free cholesterol derived from this ongoing hydrolysis to the plasma membrane may allow for the size of the cholesteryl ester pool to be monitored and remain in rapid equilibrium with other sources of free cholesterol. Further, our data indicate that the composition of the plasma membrane will be a prime determinant of the fraction of FC derived from CE hydrolysis that will be re-esterified.

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