Progesterone Blocks Cholesterol Translocation from Lysosomes*

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Fluorescent microscopic examination of fibroblasts cultured with low density lipoprotein (LDL) and progesterone (10 μg/ml) for 24 h revealed extensive fli pin-cholesterol staining of perinuclear lysosomes. Levels of unesterified cholesterol were 2-fold greater than in fibroblasts cultured with LDL alone. Progesterone strongly blocked cholesteryl ester synthesis. When cellular uptake of LDL was monitored in the presence of 58035, a specific inhibitor of acyl-CoA:cholesterol acyltransferase, excess unesterified cholesterol was not stored in lysosomes. Discontinuation of LDL uptake in conjunction with progesterone washout markedly reversed the filipino-cholesterol staining of lysosomes. Reversal of the lysosomal cholesterol lipidosis was associated with a rapid burst of cholesteryl ester synthesis and a normalization of the cellular levels of free and esterified cholesterol. In contrast to normal cells, progesterone removal from Niemann-Pick C fibroblasts did not reverse the lysosomal cholesterol accumulation of these mutant cultures.

The metabolic precursor of progesterone, pregnenolone, also induced extensive accumulation of cholesterol in lysosomes. Other steroids induced less vacuolar cholesterol accumulation in the following decreasing order: corticosterone and testosterone, promegestone, RU 486. The relative inhibition of cellular cholesterol esterification by the steroids paralleled their respective abilities to sequester cholesterol in lysosomes rather than their inhibition of acyl-CoA:cholesterol acyltransferase activity in cell-free extracts.

The progesterone-related inhibition and restoration of lysosomal cholesterol trafficking is a useful experimental means of studying intracellular cholesterol transport. A particularly important feature of its utility is the facile reversibility of the steroid-induced block. The lysosomal cholesterol lipidosis established with a hydrophobic amine, U18686A, was not as readily reversed.

Cholesterol is an ubiquitous lipid of eukaryotic cells. Its cellular distribution in organellar membranes exhibits an increasing concentration gradient toward the exterior of the cell (1). The plasma membrane of cultured cells is reported to contain the highest percentage of cellular cholesterol (2). It is likely that the implementation and maintenance of this cellular cholesterol gradient depends on specific intracellular transport processes.

Cholesterol is provided to cells primarily through de novo synthesis and endocytic uptake of sterol-rich lipoproteins. The cellular mechanisms involved in the intracellular trafficking from these two cholesterol sources have recently been reviewed (1, 3, 4). Cholesterogenic enzymes located in the endoplasmic reticulum initiate the formation of de novo cholesterol, which appears to be transferred to the plasma membrane by a rapid temperature-sensitive, energy-dependent, vesicular-mediated process that appears to bypass the Golgi (5–7). The intracellular distribution of most exogenously derived cholesterol begins with its uptake through the LDL receptor pathway defined by Goldstein and Brown (8). Receptor-mediated uptake of cholesteryl ester-enriched LDL is followed by transfer of the lipoprotein to lysosomes. Lysosomal hydrolysis of cholesteryl esters frees cholesterol, which is then distributed to other organelles. The mechanisms that control the transport of cholesterol from lysosomes have begun to be characterized. Relocation to the plasma membrane has been reported to be too rapid to be accounted for by simple sterol diffusion (9). The rate of translocation from lysosomes was not dependent on the cellular cholesterol content or on the level of an acceptor lipoprotein in the medium, suggesting that the movement out of lysosomes is constitutive (10). The existence of animal (11) and human (12, 13) mutations that specifically block the relocation of cholesterol from lysosomes (Niemann-Pick C lesion) suggests that the pathway is protein-mediated.

Experimental opportunities to specifically trace the transport of cholesterol from lysosomes have advanced with the finding that a variety of hydrophobic amines can reversibly sequester LDL cholesterol in lysosomes (14). In studies pioneered by Liscum et al. (15) it has been shown that this chemically induced lysosomal cholesterol lipidosis is associated with delayed induction of homeostatic responses. Re-
The present report describes an additional means of modulating lysosomal cholesterol transport. Progesterone was shown to induce a reversible accumulation of cholesterol in lysosomes of fibroblasts during the endocytic processing of LDL. The potential biological and experimental implications of this finding are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fetal bovine serum was obtained from HyClone Laboratories, Inc., Logan, UT. Lipoprotein-deficient bovine serum (LPDS) and human low density lipoprotein (LDL) were prepared by Advanced Bioscience Laboratories, Rockville, MD. Plastic microscopic culture wells (Lab-Tek) were purchased from Thomas Scientific (9,10-14). Oleic acid (2-20 Ci/mmole) and [1-14C]cholesterol-coenzyme A (40-60 mCi/mmole) were purchased from Du Pont-New England Nuclear. Filipin was purchased from Polysciences, Warrington, PA. The following drugs were generously supplied as follows: U18666A, 3,5-2-(diethylaminoethoxy)-5-en-17β-acetate from Dr. W. Andrus, The Upjohn Company; 58035, (3-decylmethyl-silyl)-N(2-(1-4-methylphenyl)-1)-phenethyl) propanamide from Sandoz, Inc., East Hanover, NJ was a generous gift of Dr. L. Liscum, Tufts University, Boston, MA; RU 486 (mifepristone; 17β-hydroxy-11α-(4-dimethylaminophenyl)-17-(1-propynyl) estra-4,9-diene-3-one) from Roussel-UCLAF (Romainville, France) was a kind gift of Dr. G. Chrousos, National Institute of Child Health and Human Development, National Institutes of Health. Promegestone (R 5020; 17α, 21-dimethyl-19-norpregna-4,9-diene-3,20-dione) was purchased from Du Pont-New England Nuclear. Other steroids were from Sigma.

**Tissue Culture**—Normal and NP-C fibroblasts were derived from volunteers and confirmed patients of the Developmental and Metabolic Neurology Branch under the guidelines approved by clinical research committees of the National Institutes of Health. Fibroblasts (3–15 passages) were cultured in Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM glutamine, and 100 units of penicillin-streptomycin/ml in humidified 95% air and 5% CO2 at 37 °C.

**Lipid Analyses**—Cell monolayers were washed directly in their plastic 35-mm wells twice with chilled phosphate-buffered saline (PBS) and then extracted with 0.5 mL of isopropanol by gentle rocking for 30 min at room temperature in sealed plates. The isopropanol-extracted lipids were stored at −20 °C in sealed tubes until analyzed. The lipid-free cell residues remaining in the culture wells were taken up in 0.5 mL of 0.5 N NaOH and protein measured by the method of Lowry (17). Unesterified and esterified cholesterol levels were measured in aliquots of the isopropanol extract corresponding to approximately 10 μg of protein with a sensitive fluorescently linked assay employing cholesteryl oxidase and cholesteryl esterase as previously described (18). Cellular cholesterol esterification was measured by the incorporation of [3H]oleate into newly synthesized sterol esters as previously described (11) utilizing thin layer chromatography to separate the free oleate-[3H]oleate. In *in vitro* assay of acyl-CoA:cholesterol acyltransferase (ACAT) was measured in cell free extracts of fibroblasts cultured with LDL (50 μg/ml) for 24 h. Aliquots of total cell extract (100 μg of protein) were incubated for 1 h at 37 °C with [1-14C]cholesterol-CoA (45 dpm/pmol) as described (19).

**Cytochemical Analyses**—Cells in plastic slide chambers were fixed and treated with filipin- and rhodamine-tagged antisyalosidase antibody to concurrently label cholesterol and lysosomes as previously described (20). Fluorescent cytochemical and immunocytochemical staining of cholesterol and lysosomes was viewed with a Leitz fluorescent microscope using an excitation filter of 350–410 nm for filipin and 530–560 nm for rhodamine.

**RESULTS**

**Cytocchemical Evaluation of the Effect of Steroids, U18666A, and 58035 on the Intracellular Distribution of Cholesterol in Fibroblasts Cultured with LDL**—Fluorescence microscopic examination of normal fibroblasts cultured with LDL and progesterone (10 μg/ml) for 24 h revealed intense filipin-cholesterol staining of perinuclear vacuoles that were immunofluorescently identified as lysosomes (Fig. 1, A and B). A conspicuous paranuclear area of lighter filipin staining could be noted which was essentially free of any lysosomal immunofluorescence. This non-lysosomal filipin cholesterol staining has been shown to correspond to the Golgi complex (20). Following removal of LDL and progesterone from the media for 24 h, the intense filipin-cholesterol staining of lysosomes was lost (Fig. 1, C and D). Concentrations of progesterone below 1 μg/ml caused no detectable lysosomal cholesterol accumulation (data not shown). No prominent filipin staining was noted in cells treated with progesterone alone; and filipin-cholesterol staining was not shifted from lysosomes when only LDL was removed from the incubations (data not shown).

The effects of several agents were compared with the lysosomal sequestration of cholesterol induced by progesterone. Cells cultured with LDL alone revealed little perinuclear filipin-cholesterol staining (Fig. 2A). Pregnenolone (Fig. 2B) as well as progesterone (Fig. 2C) induced extensive accumulation of cholesterol in perinuclear vacuoles. The filipin-fluorescent staining noted with corticosterone (Fig. 2D) or testosterone (Fig. 2E) was notably less. A synthetic progesterin agonist, promegestone (R 5020) (21) (Fig. 2F), and antagonist, RU 486 (22) (Fig. 2G), induced little perinuclear filipin cholesterol staining. Sandoz compound 58035, a well recognized blocker of cellular cholesterol esterification through its specific inhibition of ACAT (23) (Fig. 2H), did not induce filipin-cholesterol staining of lysosomes. U18666A caused very strong filipin-cholesterol staining of perinuclear vacuoles (Fig. 2F). U18666A, is one of a number of hydrophobic amines that previously were reported to induce accumulation of LDL-cholesterol in lysosomes (14, 15).

None of the agents tested induced filipin staining in cells cultured in the absence of LDL. RU 486 (10 μg/ml) did not block the ability of progesterone to induce strong filipin fluorescent staining of lysosomes in LDL-cultured cells. Inhibition of protein synthesis with 125 μM cycloheximide did not block progesterone-induced lysosomal cholesterol accumulation (data not shown).

**Comparative Effects of Steroids, U18666A, and 58035 on Cholesterol Esterification in Cells and in Cell-Free Extracts**—The extensible lysosomal cholesterol sequestration induced by progesterone or U18666A treatment suggests the mechanism by which these agents dramatically restrict the cellular processing of endocytosed cholesterol. It was, however, surprising to find that corticosterone and testosterone treatment did induce some lysosomal cholesterol accumulation since earlier studies reported that these particular steroids did not affect the ability of LDL to stimulate cholesteryl ester synthesis during the latter stages (after 24 h) of lipoprotein processing (19). In NP-C fibroblasts, lysosomal cholesterol sequestration is associated with a notable block in esterification during the early rather than latter stages of LDL uptake (24). This suggested a "leaky" sequestration and partial access of lysosomes which caused extensive lysosomal cholesterol accumulation during the latter stages of LDL uptake (24). This suggested a "leaky" sequestration and partial access of lysosomes which caused extensive lysosomal cholesterol accumulation during the latter stages of LDL uptake (24). This suggested a "leaky" sequestration and partial access of lysosomes which caused extensive lysosomal cholesterol accumulation during the latter stages of LDL uptake (24).
Progestosterone Blocks Cholesterol Translocation from Lysosomes

**FIG. 1.** Cytochemical assessment of the effect of progesterone on intracellular trafficking of cholesterol in fibroblasts cultured with LDL. Normal human fibroblasts were cultured with McCoy’s medium supplemented with 5% LPDS, 2 mM glutamine, 100 units of penicillin, and 100 μg of streptomycin/ml for 7 days. Cultures were trypsinized and seeded at a density of 20,000 cells in 9.5-cm plastic microscopic wells coated with fibronectin. Cells were cultured in the above described medium for 4 days and then incubated in fresh medium containing LDL (50 μg/ml) and progesterone (10 μg/ml) for 24 h. A portion of the cultures were then washed with PBS, fixed, and cytochemically stained with filipin for unesterified cholesterol and immunocytochemically stained with rhodamine-labeled antibodies for lysosomes as described under “Experimental Procedures.” The remaining cultures were incubated 3 times with 2 ml of McCoy’s medium and 5% LPDS for 15 min at 37 °C to initiate a washout of progesterone. These cultures were subsequently incubated in fresh McCoy’s, 5% LPDS medium for 24 h and then examined cytologically as described. A, LDL + progesterone with filipin-cholesterol stain; B, LDL + progesterone with lysosome immunostain; C, LDL and progesterone washout with filipin-cholesterol stain; D, LDL and progesterone washout with lysosome immunostain. L, lysosomes; N, nucleus. Magnification, × 320.

The mechanism responsible for the suppression of cellular cholesteryl ester synthesis by steroids may largely represent lysosomal cholesterol sequestration. A strong correlation could be traced between the cellular esterification block and the ability of the steroids to induce cholesterol accumulation in lysosomes. No consistent relation could be found in comparing the ability of the various steroids to inhibit cholesterol esterification in cells and in cell-free extracts. Pregnenolone and testosterone, for example, at 10 μg/ml inhibited ACAT equally in cell-free extracts (50% of normal), whereas the inhibition of cholesteryl ester synthesis in intact cells was notably more severe with pregnenolone (5% of normal) than with testosterone (42% of normal). The means by which U18666A and 58035 inhibit cellular cholesteryl ester synthesis clearly represent opposing mechanisms. Inhibition by the hydrophobic amine must largely reflect its strong induction of lysosomal cholesterol sequestration since no direct inhibition of ACAT was noted in cell extracts. On the other hand, the effects of 58035 must largely represent direct ACAT inhibition since no lysosomal cholesterol accumulation could be noted with this agent.

**Specific Biochemical Quantitation of the Effects of Progesterone, 58035, and U18666A on the Cellular Processing of Cholesterol**—The cytochemical studies also showed that progesterone could induce an accumulation of LDL cholesterol in lysosomes that was reversible upon removal of the steroid. Direct measurements of cellular cholesterol levels and rates of cholesteryl ester synthesis provided biochemical support.
TABLE 1
Cholesteryl ester synthesis in cultured fibroblasts and cell-free extracts

Semiconfluent normal fibroblast cultures were depleted of cholesterol by incubation in LPDS media for 4 days. A portion of the cultures were then enriched in cellular cholesterol by incubation with LDL (50 μg/ml) for 24 h. Cells were harvested by trypsinization, washed, and frozen at -70 °C. Cell pellets were subsequently thawed and suspended with a small glass-fritted hand homogenizer in distilled H2O (5 mg/ml), and 20 μl of total cell extract was assayed for ACAT activity with 80 μM [3H]oleyl-CoA for 1 h at 37 °C as described under "Experimental Procedures." All drugs were added to 0.200 ml of the assay buffer as 2-μl aliquots of stock acetone solutions prior to addition of cell extract. The final concentrations were: steroids (10 μg/ml), U18666A (2 μg/ml), and 58035 (1.8 μg/ml). Other cholesterol-depleted cultures were prepared in 35-mm wells as described in Table II. These cultures were incubated for 5 and 24 h with 100 μM [3H]oleate in the absence or presence of LDL (50 μg/ml). Some of the lipoprotein-enriched cultures were incubated in the presence of the various listed drugs at the same concentration as those employed for the cell-free extract assays. Cell monolayers were subsequently washed and extracted in the dishes with isopropanol. Levels of cholesteryl-[3H]oleate formed in situ and [3H]oleate in cell-free extracts was determined by thin layer chromatography as described under "Experimental Procedures." The data are presented as the average value ± S.D. obtained with these separate cultures or with triplicate cell-free assays. Cholesteryl ester synthesis in non-LDL-treated preparations was less than 1% of that observed with LDL.

| Additions of drugs to culture media or in vitro assay | Cholesteryl-oleate synthesis |
|------------------------------------------------------|-------------------------------|
|                                                      | Cultured cells                | Cell extract                  |
|                                                      | 0-5 h                         | 0-24 h                        |
| None                                                 | 6.0 ± 0.5 (100%)              | 95 ± 5 (100%)                 |
| Pregnenolone                                          | 0.1 ± 0.0 (2%)                | 4 ± 1 (4%)                    |
| Progestosterone                                       | 0.5 ± 0.0 (5%)                | 5 ± 1 (5%)                    |
| Corticosterone                                        | 1.8 ± 0.2 (30%)               | 39 ± 5 (41%)                  |
| Testosterone                                          | 1.3 ± 0.2 (22%)               | 55 ± 4 (58%)                  |
| RU 486                                               | 38 ± 11 (40%)                 | 130 ± 4 (42%)                 |
| U18666A                                               | 5 (1%)                        | 460 ± 20 (94%)                |
| 58035                                                 | 2 ± 5 (2%)                    | 20 ± 4 (4%)                   |

for these cytochemical observations. When cholesterol-depleted cells were incubated with LDL (50 μg/ml) for 24 h, unesterified cholesterol levels doubled and cholesteryl esters accumulated. Based on the incorporation of [3H]oleate this increase in cellular cholesteryl esters could be accounted for by de novo synthesis (Table II). LDL uptake in the presence of progestrone, 58035 or U18666A induced a significantly greater accumulation of unesterified cholesterol, which was accompanied by depressed incorporation of [3H]oleate into cholesteryl esters (Table II). These findings are in accordance with previous studies reporting the inhibition of cellular cholesterol esterification by progestrone (19), 58035 (23), and U18666A (15). Inhibition of cholesteryl ester synthesis by these agents did not prevent an accumulation of cholesteryl esters, which was notably greater than the rate of depressed ester synthesis. The cellular basis for this apparent accumulation of LDL-derived cholesteryl esters is not known but may in part be associated with the excess accumulation of unesterified cholesterol in lysosomes since similar lipoprotein-derived cholesteryl ester accumulation was noted in NP-C fibroblasts (24).

Cellular responses were also monitored during a secondary phase of incubation (24–48 h) where further exposure to the agents and/or LDL was discontinued (Table II). Discontinuation of LDL uptake lowered cellular levels of both free and esterified cholesterol and diminished further cholesteryl ester synthesis to 39% of the total cellular cholesteryl ester mass. Continued cholesteryl esterification in the absence of further LDL uptake may in large part represent turnover of an existing cholesteryl ester pool. Such a cholesteryl ester cycle with a similar rate of turnover (50%–24 h) has been described in cultured macrophages (26). Removal of both progestrone, 58035, or U18666A and LDL restored active cholesteryl ester synthesis which now accounted for more than 80% of the total cellular cholesteryl ester mass. The cholesteryl ester synthesis induced upon progestrone and 58035 removal was associated with an expected reduction of free cholesterol. The induction of cholesteryl ester synthesis with U18666A wash-out was unexpectedly accompanied by an increase and decrease of unesterified and esterified cholesterol, respectively. These apparent discordant responses may reflect a more complete restoration of LDL cholesteryl ester hydrolysis than cholesterol mobilization from lysosomes. Cytochemical observations have shown that filipin-cholesterol staining of lysosomes is not substantially diminished during the first 24 h of U18666A washout (data not shown).

The cytochemical studies (Fig. 2) also showed that accumulation of unesterified cholesterol can occur in separate intracellular compartments. LDL uptake in the presence of progestrone or U18666A induced cholesterol accumulation in lysosomes, whereas LDL alone or with 58035 induced accumulation in a nonlysosomal compartment not yet identified. The effect of U18666A, a strong inhibitor of lysosomal cholesterol trafficking, on the mobilization of cholesterol from lysosomal and non-lysosomal compartments was compared by adding the hydrophobic amine during a secondary washout phase in which the steroid and ACAT inhibitor were removed and further incubation with LDL was discontinued (24–48 h) (Table III). Addition of U18666A to cultures for 24 h after the completion of only LDL uptake led to increased unester-

**Fig. 2.** Cytochemical examination of the effects of various drugs on the intracellular deposition of cholesterol in fibroblasts cultured with LDL. Normal human fibroblasts were depleted of cholesterol and cultured in microscopic chamber wells as described in Fig. 1. Cells were cultured with LDL (50 μg/ml), LDL + steroids (10 μg/ml), LDL + 58035 (1 μg/ml), or LDL + U18666A (2 μg/ml) in McCoy's, 5% LPDS medium for 24 h at 37 °C. Cells were subsequently washed with PBS 3 times, fixed, and cytochemically stained with filipin to fluorescently located intracellular distribution of cholesterol. A, LDL alone; B, LDL + pregnenolone; C, LDL + progestrone; D, LDL + corticosterone; E, LDL + testosterone; F, LDL + promegestone; G, LDL + RU 486; H, LDL + 58035; I, LDL + U18666A. Magnification, x 185.
Stock fibroblasts were incubated in standard EMEM culture medium containing 10% LPDS for 3 days. These LPDS-conditioned cells were harvested by trypsinization and were plated at a density of 100,000 cells into 35-mm wells. Cells were incubated for 4 days and the protocol initiated by the addition of fresh LPDS media. Cells were cultured for 0–24 h with LPDS, LDL (50 μg/ml), LDL + progesterone (PRG) (10 μg/ml), LDL + 58035 (0.01 μg/ml), and LDL + U18666A (2 μg/ml) in the presence or absence of 100 μM [3H]oleate (200 dpm/nmol). Some of the cell cultures were washed 3 times with PBS at room temperature and subsequently treated with isopropanol. The remaining cultures were incubated in fresh LPDS medium 3 times for 10 min at 4°C. These cultures were subsequently incubated with LPDS for 24–48 h in the presence of 100 μM [3H]oleate. Stock solutions of drugs in ethanol were actively mixed with culture medium by vortexing for 1 min to provide the designated final concentrations (0.1% ethanol). The final concentration of 58035 in these experiments, 0.01 μg/ml, was lower than that normally prescribed to block cellular cholesterol esterification (23). These suboptimal concentrations were chosen to allow reversal of the cellular block to occur upon initiation of drug washout. Higher concentrations of 58035 were refractory to reversal by washout as previously reported (25). The values represent the average ± S.D. of three separate cultures.

| Additions to media during the incubation intervals | Time of [3H]oleate addition | Cholesterol mass | Cholesteryl-[3H]oleate synthesis |
|--------------------------------------------------|----------------------------|------------------|----------------------------------|
|                                                   | 0–24  | 24–48 h  | nmoles/mg protein | nmol/mg protein | % of total mass |
| LPDS                                             | 53 ± 5 | 0       | 1                  |
| LPDS                                             | 56 ± 2 | 0       | 91                 |
| LDL                                              | 98 ± 4 | 65 ± 4  | 62 ± 1             |
| LDL                                              | 71 ± 2 | 33 ± 4  | 13 ± 2             |
| LDL + PRG                                        | 142 ± 3| 30 ± 10 | 1                  |
| LDL + PRG                                        | 79 ± 1 | 43 ± 4  | 34 ± 1             |
| LDL + 58035                                      | 159 ± 3| 34 ± 3  | 14 ± 1             |
| LDL + 58035                                      | 89 ± 3 | 49 ± 5  | 48 ± 3             |
| LDL + U18666A                                    | 114 ± 5| 80 ± 10 | 1                  |
| LDL + U18666A                                    | 187 ± 15| 20 ± 5 | 22 ± 0             |

Table III
Effect of hydrophobic amine U18666A on recovery of cellular cholesterol processing following removal of progesterone or compound 58035

Cholesterol-depleted normal fibroblast cultures were prepared in 35-mm wells as described in Table II. Separate cultures were incubated for 0–24 h under the following conditions: LDL (50 μg/ml), LDL + progesterone (PRG) (10 μg/ml), LDL + 58035 (0.01 μg/ml). All cultures were subsequently washed 3 times with fresh LPDS medium with 100 μM [3H]oleate (200 dpm/nmol) in the presence or absence of U18666A (2 μg/ml). Cell monolayers were washed 3 times with cold PBS and extracted with isopropanol. Levels of unesterified and esterified cholesterol and cholesteryl-[3H]oleate formation were determined as described under "Experimental Procedures." The results represent the average ± S.D. of three separate cultures.

| Additions to culture media | Cholesterol levels | Cholesteryl-[3H]oleate synthesis |
|---------------------------|--------------------|----------------------------------|
|                          | nmol/mg protein    |                                  |
| 0–24 h                   | 24–48 h            | Free                            |
| LDL                       | 74 ± 1             | 17 ± 1                           |
| LDL                       | 92 ± 7             | 6 ± 0                            |
| LDL + U18666A            | 83 ± 6             | 31 ± 2                           |
| LDL + U18666A            | 123 ± 4            | 1                               |
| LDL + 58035              | 88 ± 5             | 46 ± 11                          |
| LDL + 58035              | 145 ± 5            | 11 ± 1                           |

The ability of U18666A to block restoration of cellular cholesterol processing following progesterone washout was expected since both drugs were shown to sequester LDL-cholesterol in lysosomes (Fig. 2). The ability of U18666A to also partially block cellular cholesterol mobilization from largely non-lysosomal compartments (cells cultured with LDL in absence or presence of 58035) suggests that: (a) various cellular cholesterol pools may cycle through lysosomes such that they become trapped in lysosomes in the presence of the hydrophobic amine, or (b) U18666A can block cellular cholesteryl trafficking not only from lysosomes but also through other cellular organelles.

The Relative Mobilization of Cholesterol from Lysosomes of Normal and NP-C Cells—The cellular and biochemical consequences of the progesterone-induced block in lysosomal cholesterol transport strongly resemble the phenotypic manifestations of the NP-C mutation (27, 28). A direct comparison of the mutant and steroid-induced blocks in cellular cholesterol processing were carried out. LDL uptake (0–24 h) by NP-C fibroblasts was characterized by excess accumulation of free cholesterol and extensive suppression of cholesteryl ester synthesis (Table IV). In the 24 h following LDL uptake (24–48 h), unesterified cholesterol levels did not appreciably normalize in the NP-C cells and cholesterol esterification remained suppressed (Table IV). When progesterone (10 μg/ml) was added to normal cells during LDL uptake, cholesterol processing was essentially indistinguishable from that observed in NP-C cultures treated with or without the steroid. Such cultures were all marked by excess unesterified cholesterol accumulation and blocked cholesteryl ester synthesis. During a subsequent 24-h period of progesterone washout, the response of normal and mutant cultures clearly differed. In normal cells progesterone removal induced a rapid burst of esterification, which was accompanied by a notable decrease in unesterified cholesterol. These recovery responses remained deficient in NP-C cultures (Table IV).

The efficiency with which cholesterol esterification could
Progesterone Blocks Cholesterol Translocation from Lysosomes

TABLE IV

| Cell culture | Additions to culture media | [3H]oleate addition | Cholesterol levels | Cholesteryl-[3H]oleate synthesis |
|--------------|----------------------------|---------------------|-------------------|----------------------------------|
|              | 0-24 h                     | 24-48 h             |                   | Free Ester                      |
| Normal LDL   | 0-24                        | 190 ± 2 107 ± 2     | 99 ± 10           |
| NP-C LDL     | 0-24                        | 180 ± 5 20 ± 3      | 4 ± 1             |
| Normal LDL + PRG | 24-48              | 76 ± 5 61 ± 3       | 20 ± 3            |
| NP-C LDL + PRG | 24-48               | 172 ± 8 35 ± 2      | 9 ± 1             |
| Normal LDL + PRG LDPS | 24-48       | 192 ± 6 31 ± 4     | 4 ± 1             |
| NP-C LDL + PRG LDPS | 24-48        | 130 ± 5 25 ± 5     | 1 ± 1             |
| Normal LDL + PRG LDPS | 24-48       | 86 ± 7 77 ± 7      | 81 ± 3            |
| NP-C LDL + PRG LDPS | 24-48       | 115 ± 2 17 ± 1     | 7 ± 0             |

**DISCUSSION**

Certain steroid hormones were shown to cause accumulation of cholesterol in lysosomes when added to the medium of fibroblasts cultured with LDL. The major findings derived from this observation can be summarized as follows. 1) The steroid-induced sequestration of cholesterol in lysosomes appears to represent a structurally specific effect since progesterone and pregnenolone caused appreciably more cholesterol accumulation than other steroids such as corticosterone or testosterone (Fig. 2). 2) The specific ACAT inhibitor 58035 caused as much accumulation of excess unesterified cholesterol as progesterone (Table II) without specific storage in lysosomes (Fig. 2). 3) The progesterone-induced block in lysosomal cholesterol trafficking is readily reversible through steroid washout and can be conveniently monitored cytochemically through a notable loss of filipin-cholesterol staining from lysosomes (Fig. 2) or biochemically by the induction of a rapid burst of cellular cholesteryl ester synthesis (Fig. 3). 4) The cellular inhibition of cholesteryl ester synthesis by progesterone or pregnenolone appears more strongly linked to lysosomal cholesterol sequestration than to direct inhibition of ACAT (Table I). 5) The sequestration and accumulation of LDL-cholesterol in lysosomes of NP-C fibroblasts closely correspond to the cytochemical and biochemical phenotype induced by progesterone but is not reversed in the mutant cells by steroid washout (Fig. 3 and Table IV).

A similar inhibition of lysosomal cholesterol trafficking has been reported with another natural metabolite, sphinganine (14). This lipid belongs to a diverse group of hydrophobic amines including U18666A (15), ketoconazole (16), imipramine (29), and stearlyamine (14), which all induce accumulation of cholesterol in lysosomes of cultured cells. The cellular and molecular bases by which progesterone and hydrophobic amines trap cholesterol in lysosomes are not known. A broad spectrum of secondary cellular perturbations do not affect the ability of cells to restore normal cholesterol trafficking from lysosomes following the initiation of U18666A washout (16). Thus transport of cholesterol from lysosomes to the plasma membrane has been reported to be substantially retarded when cells are treated secondarily with brefeldin A, energy poisons, NH₄Cl, leupeptin, and agents that disrupt normal cytoskeletal architecture (16). The altered cellular states under which cholesterol trafficking could be restored suggested that these particular cellular perturbations are not the major means by which hydrophobic
amines induced accumulation of cholesterol in lysosomes. The common cationic amphiphilic nature of the hydrophobic amines may be the principal determinant by which these agents block lysosomal cholesterol trafficking. Their most immediate and direct interdiction may be through hydrophobic and hydrophilic interactions with phospholipids (30).

The effects of steroids and hydrophobic amines are not mutually exclusive since progesterone reversal was effectively blocked by secondary U18666A addition (Table III). The structural specificity of the studied steroids appears more rigid than that of hydrophobic amines since only progesterone and its precursor, pregnenolone, induced extensive and sustained accumulation of cholesterol in lysosomes. The implied specificity in the steroid-mediated block may reflect interactions with specific cellular proteins. These effects of progesterone clearly do not represent hormone-mediated responses for the following reasons: (a) the concentration of progesterone (10 μg/ml, 10^{-8} M) is several orders of magnitude higher than needed for normal receptor-mediated hormone responses (10^{-6} M) (31, 32); (b) other progestins (promegestone/agonist and RU 486/antagonist) do not mimic or block the response to progesterone; (c) classic cytosolic hormone receptors have not been reported in human cultured fibroblasts; (d) cycloheximide does not block the effects of progesterone (data not shown). Many nongenomic interactions of steroids have been documented (33). Progesterone has been shown to bind to hepatic microsomal membranes in a specific and saturable manner suggestive of receptor-mediated interactions (34).

Binding of progesterone to oocytes (35, 36) and spermatozoa (37) induces a very rapid cellular influx of calcium ions. Treatment of human monocytic leukemic cells with calcium ionophore has been reported to suppress sterol-mediated homoeostatic responses (38). If such imbalances in cellular calcium ions occur in progesterone-treated fibroblasts their potential effects on cellular cholesterol trafficking need to be examined. Progesterone can interact with the GABA receptor (39) and the multiple drug-resistant protein (40). In addition to these interactions with membrane proteins, progesterone has also been shown to bind to circulating proteins such as steroid-binding globulin (41), orosomucoid (42), and apoprotein-D (43). Apoprotein-D and orosomucoid are members of the lipocalin superfamily of proteins that feature a unique binding cavity suitable for effective interactions with small molecular weight hydrophobic molecules (44, 45).

Progesterone appears able to sequester in lysosomes cholesterol derived not only directly from endocytosed LDL but also from other existing intracellular sterol pools. Interruption of the cholesteryl ester synthesis in cultured macrophages with progesterone has been shown to translocate cholesterol from intracellular cholesteryl ester droplets to unesterified cholesterol located in perinuclear filipin-positive vacuoles (46). Although these cellular organelles were not identified, their structural similarities to the cholesterol-filled lysosomes of NP-C fibroblasts suggest that they may well have been cholesterol-loaded lysosomes. The apparent ability of progesterone to trap in lysosomes cholesterol released from cytoplasmic cholesteryl ester droplets by neutral cholesteryl esterase suggests that the continuous turnover of the cholesteryl ester cycle may in part represent a transient passage of free cholesterol through lysosomes. The routing of at least a portion of the cholesterol through lysosomes is supported also by the current findings that U18666A blocked cellular cholesterol processing and esterification when added subsequently to the completion of LDL uptake (Table II). This secondary block was associated with the appearance of cholesterol-filled lysomes (data not shown).

The intracellular route taken by cholesterol for reesterification by microsomal ACAT may also involve passage through the plasma membrane. Cytosolic cholesteryl esters mobilized for steroidogenesis have been suggested to pass through a large pool of plasma membrane cholesterol (47). The expanded cellular pool of cholesterol that serves as substrate for ACAT during lipoprotein uptake by cultured macrophages is not drawn directly from an enriched lysosomal pool of exogenously derived cholesterol but rather from an enriched plasma membrane sterol pool (48, 49). Plasma membrane destabilization resulting from cellular exposure to neutral spingomyelinase induces internalization and esterification by ACAT of cholesterol specifically derived from the plasma membrane (50). This cellular routing of plasma membrane cholesterol for esterification has been shown to be delayed (51) or defective (52) in NP-C fibroblasts and can be blocked in normal cells both by hydrophobic amines and progesterone (data not shown).

Although the mechanisms responsible for lysosomal cholesterol trapping by progesterone remain to be elucidated, its effect may be considered in the context of other cellular responses that have been observed with this steroid. The present studies showed that progesterone at 10 μg/ml (33 μM) induced pronounced sequestration of cholesterol in lysosomes (Figs. 1 and 2). Such levels of the steroid can be found in steroidogenic tissues. In rabbit ovarian interstitial tissue, progesterone levels have been reported to be in the range of 30-60 μg/ml tissue water (53). Progesterone content of human placenta was shown to be 7 μg/g wet tissue, and its concentration in the endoplasmic reticulum may reach 30-40 μM (54). Thus the progesterone concentrations reported to block lysosomal cholesterol trafficking in tissue culture may have their physiological correlates. Progesterone has been reported to block the ability of LDL to down regulate 3-hydroxy-3-methylglutaryl-coenzyme A reductase in cultured cells (55) and to increase basal levels of the enzyme 3-fold in rat intestinal epithelial cells (56). Such cellular responses have generally been attributed to a specific inhibition by progesterone of 450-mediated oxyter formation thought to be needed for the down-regulation of this enzyme (24, 55-57). It should, however, also be considered that progesterone-induced lysosomal sterol sequestration may neutralize the ability of enriched cellular cholesterol to regulate 3-hydroxy-3-methylglutaryl-CoA reductase levels.

Irrespective of the potential biological significance of the presently reported effect of progesterone, the phenomenon provides a fertile experimental opportunity to study the mechanisms that regulate intracellular cholesterol transport. Progesterone effectively and reversibly blocks lysosomal cholesterol transport. In the presence of this steroid, large amounts of LDL cholesterol accumulate in lysosomes. Upon progesterone removal, sequestered lysosomal cholesterol is rapidly redistributed and mobilized for further cellular processing. This facile redistribution can be monitored cytochemically by a reduction in filipin cholesterol staining of lysosomes (Fig. 1). Mobilization of cholesterol from lysosomes may also be conveniently monitored indirectly by measuring the induction of cholesteryl ester synthesis (Fig. 3). Such readily discernable responses reflecting the rapid intracellular relocation of a large bolus of cholesterol directly from lysosomes appear entirely amenable to further cytochemical and biochemical study.

Progesterone can also be used to probe the molecular lesion responsible for deficient lysosomal cholesterol transport in NP-C disease. Cytochemically the intracellular accumulation of cholesterol in progesterone-treated cells is so far indistin-
guishable from the lysosomal cholesterol storage noted in cultured NP-C fibroblasts (20). The exact relationship between the NP-C lesion and the progesterone-induced block remains to be established. The ω-unsaturated ketone function of progesterone is photo-labile (58), and this inherent property of the steroid has been employed to photo-label cellular proteins with [3H]progesterone (40). A comparative survey of ω-steroid-protein conjugates in normal and NP-C cells may provide an opportune strategy to identify possible relevant protein lesions. Selection of amplification mutants resistant to the steroid-induced block in lysosomal cholesterol transport may provide an additional strategy for identifying relevant proteins.

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