Characterization of Chinese Hamster Ovary Cells That Are Resistant to 3-β-[2-(Diethylamino)ethoxy]androst-5-en-17-one Inhibition of Low Density Lipoprotein-derived Cholesterol Metabolism*

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The pharmacological agent U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one) inhibits the intracellular transport of low density lipoprotein (LDL)-derived cholesterol in Chinese hamster ovary (CHO) cells. LDL-derived cholesterol accumulates in the lysosomes of U18666A-treated cells causing delayed LDL-mediated regulation of cellular cholesterol metabolism and impaired movement of LDL-derived cholesterol to other membranes. As a result of impaired LDL-derived cholesterol transport, LDL-dependent growth of CHO cells is also inhibited by U18666A. By selecting for cell growth in the presence of U18666A, we have identified a CHO cell line, designated U18R, that is resistant to U18666A-inhibition of LDL-derived cholesterol trafficking. Compared to parental CHO cells, U18R cells are relatively resistant to U18666A inhibition of LDL-derived cholesterol transport as well as LDL-mediated regulation of cellular cholesterol metabolism. In cell fusion experiments, the U18666A resistance observed in U18R cells displays a dominant phenotype. Identification of the U18666A-resistant factor may provide important insights toward the understanding of intracellular LDL-derived cholesterol regulation and trafficking.

The existence of specific lipid sorting and transport mechanisms has been evoked to explain the different phospholipid and sterol compositions of various cellular membranes (reviewed in Ref. 1). Determining the mechanism of intracellular cholesterol transport has been of particular interest because of cholesterol’s dual role as a structural component of the membrane and as a regulator of gene transcription (2), protein degradation (3), and enzyme activity (4). Possible methods of directed cholesterol movement include soluble carrier proteins or lipids, and vesicle budding and fusion. Gene products that facilitate cholesterol transport in vivo have yet to be described; however, the fact that cell lines have been identified that are resistant to U18666A inhibition of LDL-derived cholesterol trafficking observed in U18R cells displays a dominant phenotype. Identification of the U18666A-resistant factor may provide important insights toward the understanding of intracellular LDL-derived cholesterol regulation and trafficking.

Our goal is to identify cellular factors that facilitate cholesterol movement in vivo. Previously, we demonstrated that the pharmacological agent 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A) impairs the intracellular transport of low density lipoprotein (LDL)-derived cholesterol in cultured Chinese hamster ovary (CHO) cells (10). Upon receptor-mediated endocytosis, LDL is delivered to lysosomes, where LDL-derived cholesteryl esters are hydrolyzed to unesterified cholesterol (11). Normally, LDL-derived cholesterol is transferred from lysosomes to membranes throughout the cell; however, LDL-derived cholesterol accumulates in the lysosomes of U18666A-treated CHO cells (10). This result in impaired transfer of LDL-derived cholesterol to other cell membranes and delayed LDL-mediated regulation of cellular cholesterol metabolism (10). In addition, U18666A-treated cells are inefficient in utilizing LDL-derived cholesterol for growth. One explanation for these findings is that U18666A inhibits the activity or synthesis of a protein or lipid that facilitates cholesterol movement. Identification of the cellular process that is blocked by U18666A may yield insights into the mechanism of intracellular cholesterol trafficking and LDL-mediated regulation.

To identify the U18666A-inhibitable cellular component that modulates LDL-derived cholesterol movement, we have used a somatic cell genetic approach of identifying a drug-resistant CHO cell line. A CHO cell line has been selected for the ability to utilize LDL-derived cholesterol for growth in the presence of high concentrations of U18666A. The U18666A-resistant cell line, designated U18R, is resistant to U18666A inhibition of LDL stimulation of cholesterol esterification and LDL suppression of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity. U18R cells are also resistant to U18666A inhibition of the intracellular movement and efflux of LDL-derived cholesterol. However, U18R cells do not appear to be resistant to another inhibitory action of U18666A, which is the inhibition of cholesterol synthesis (12). U18R cells display a dominant phenotype when fused to parental CHO cells, and may be very useful in identifying the U18666A-inhibitable cellular component involved in intracellular cholesterol transport.

EXPERIMENTAL PROCEDURES

Materials—[9,10-3H]Oleic acid (10 Ci/mmol), cholesteryl [1-14C]oleate (57 mCi/mmol), 3-hydroxy-3-[3H]methylglutaryl CoA (10.2 Ci/mmol), (R)-[2-14C]mevalonolactone (48.6 mCi/mmol), [1,2,6,7-3H]cholesteryl linolate (82.9 Ci/mmol), [4-14C]cholesterol (53.2 mCi/1)

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mmol), and sodium [3H]acetate (86.4 mCi/mmol) were purchased from Du Pont-New England Nuclear. Mevinolin was a generous gift of A. Alberts (Merck Research Laboratory, Rahway, NJ). U18666A was kindly provided by The Upjohn Co. U18666A, ketoconazole, 25-hydroxycholesterol, cholesterol, colchicine, and daunomycin were added to media as ethanol solutions. Thioguanine and U18666A were dissolved in medium and dimethyl sulfoxide, respectively. Newborn calf serum and all other reagents were from Sigma or obtained as described previously (10).

**Preparation of LDL, Lipoprotein-deficient serum, [3H]CL-LDL, and [3H]CL-LDL-Mediated Lipoprotein-Deficient Serum** was prepared from fetal or newborn calf serum omitting the thrombin incubation (13). [3H]CL-LDL was prepared with an average specific activity of 17,000 cpm/nmol of total cholesteryl linoleate (14).

The following media were prepared: medium A (Ham's F-12 medium containing 5% (v/v) fetal or newborn calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES, pH 7.1); medium B (medium A in which 5% (v/v) calf serum was replaced with 5% (v/v) lipoprotein-deficient calf serum); medium C (medium B containing 20 μM mevinolin and 0.5 mM mevoleate); and medium D (medium C with 5% (v/v) lipoprotein-deficient calf serum replaced with 1% (v/v) calf serum and 4% (v/v) lipoprotein-deficient calf serum and containing 15 μg/ml of U18666A).

The following buffers were prepared: buffer A (150 mM NaCl, 50 mM Tris-chloride, pH 7.4) and buffer B (buffer A containing 2 mg/ml of bovine serum albumin).

Plating Efficiency and Cell Growth—On day 0, the U18R stock dish was washed with EBSS, refed 10 μl of medium A, and incubated for 15 min at 37℃. This refeeding and incubation was repeated twice to remove U18666A. CHO and U18R cells were then trypsinized and seeded onto 12-well plates (7,000-10,000 cells/22.6-mm well) in 2 ml of either medium C with 4 μg/ml of LDL and the indicated amount of U18666A, or medium A with the indicated amount of colchicine or daunomycin. On day 1, the monolayers were washed and the protein content of each well determined (8). Cell growth is defined as μg of cell protein per well.

Incorporation of [3H]Oleate into Cholesteryl [3H]Olate—On day 0, cells were seeded into 6-well plates (20,000-50,000 cells/35-mm well in 1.5 ml of medium A (CHO) or medium D (U18R)). On day 1, all monolayers were washed with 2 ml of EBSS. CHO cells were refed 2 ml of medium C containing 3 μg/ml cholesterol; U18R cells were refed 2 ml of medium C containing 3 μg/ml cholesterol and 15 μg/ml U18666. Experiments were initiated on day 2 by the removal of U18666A. CHO cells were washed once with 4 ml of EBSS, refed 4 ml of medium C, and incubated for 15 min at 37℃. The refeeding and incubation was repeated twice with which the experiment was initiated. Following incubations as indicated, monolayers were washed with 100 μl [3H]oleate (23,000 cpm/nmol) bound to albumin (13). After 1 h, cells were washed, and cholesterol esters in the medium and in the medium and in the aliquots removed for protein determination. Cholesterol esters were separated by thin-layer chromatography. Cholesterol esters were defined as nmol of cholesteryl [3H]oleate formed/h/mg of protein.

**Cell Hybridization—**Cells were fused in monolayer using polyethylene glycol 1000 (Baker) following the method of Davidson et al. (19, 20). Cells from each phenotype were plated together (400,000

**Incorporation of [3H]Acetate into [3H]Stereos—**Cells were seeded and grown as described for "Incorporation of [3H]Oleate into Cholesteryl [3H]Olate." U18666A was removed as described in "Incorporation of [3H]Oleate into Cholesteryl [3H]Olate." On day 3, cells were refed 1 ml of medium C. After the indicated incubation with 10 μg/ml [3H]-CL-LDL, cells were washed as described (8). Cellular [3H]cholesterol ester and [3H]cholesterol were quantitated as described (8), and are expressed as nmol per mg of cell protein.

**Incorporation of [3H]Acetate into [3H]Sterols—**Cells were seeded and grown as described for "Incorporation of [3H]Oleate into Cholesteryl [3H]Olate." U18666A was removed as described in "Incorporation of [3H]Oleate into Cholesteryl [3H]Olate." CHO cells were incubated with [3H]-CL-LDL (10-20 pg/ml) was added and the incubation continued for 2 h. Monolayers were then washed with 3 ml of EBSS and refed 1 ml of medium C with 25 μl of small unilamellar vesicles (cholesterol and 1 μmol of cholesterol, 5% (v/v) of L-α-phosphatidylcholine), and the indicated amount of U18666A or ketoconazole. Cells were harvested after 2 or 5 h as described (8). Cellular [3H]cholesterol ester and [3H]cholesterol and monomer [3H]cholesterol were quantitated as described (8), and are expressed as nmol per mg of cell protein.

**Incorporation of [3H]VLDL into [3H]Sterols—**Cells were seeded and grown as described for "Incorporation of [3H]Oleate into Cholesteryl [3H]Olate." U18666A was removed as described in "Incorporation of [3H]Oleate into Cholesteryl [3H]Olate." On day 3, cells were refed 1 ml of medium C containing the indicated amount of U18666A or ketoconazole. After 15 min, [3H]CL-LDL (10-20 μg/ml) was added and the incubation continued for 2 h. Monolayers were then washed with 3 ml of EBSS and refed 1 ml of medium C with 25 μl of small unilamellar vesicles (cholesterol and 1 μmol of cholesterol, 5% (v/v) of L-α-phosphatidylcholine), and the indicated amount of U18666A or ketoconazole. Cells were harvested after 2 or 5 h as described (8). Cellular [3H]cholesterol ester and [3H]cholesterol and monomer [3H]cholesterol were quantitated as described (8), and are expressed as nmol per mg of cell protein.

**Lipid Metabolism—**Small unilamellar vesicles with a cholesterol:phosphatidylycerine molar ratio of 0.7 were prepared as described (8). Cells were seeded, grown, and U18666A was removed as described for "Incorporation of [3H]Oleate into Cholesteryl [3H]Olate." On day 3, cells were refed 1 ml of medium C. After the indicated incubation with 10 μg/ml [3H]-CL-LDL, cells were washed as described (8). Cellular [3H]cholesterol ester and [3H]cholesterol were quantitated as described (8), and are expressed as nmol per mg of cell protein.
total) in 60-mm tissue culture dishes. After 1 day, monolayers were washed with EBSS and treated for 60 min with 50% polyethylene glycol 1000 in 75 mM HEPES, pH 7.4. Cells were then washed 3 times with 3 ml of EBSS and cultured in medium A. The next day, cells were trypsinized and replated in 100-mm dishes in medium A containing 5 µg/ml thioguanine and HAT. Monolayers were refed the above medium twice a week for 2 weeks. Cells were then seeded and grown as described for "Cell Growth."

**RESULTS**

Selection of U18666A-resistant CHO Cells—Mammalian cells require an exogenous source of cholesterol for growth when endogenous cholesterol synthesis is inhibited by culturing in the presence of mevinolin (21). The addition of LDL restores the growth of mevinolin-treated cells. The cell's ability to utilize LDL-derived cholesterol is dependent upon the efficient transport of LDL-derived cholesterol from lysosomes to sites of membrane biogenesis. Previously, we have shown that U18666A blocks the transport of LDL-derived cholesterol from lysosomes and impairs LDL-sustained growth (10). A CHO cell line that is resistant to U18666A inhibition of LDL-derived cholesterol transport was developed by taking advantage of this finding. U18666A-resistant cells were selected using a multistep strategy in which cells were cultured in increasing concentrations of drug (22). The selected colonies contained 1% whole newborn calf serum, 4% lipoprotein-deficient serum, 20 µM mevinolin, 0.5 mM mevalonate, and increasing concentrations of U18666A. The 1% newborn calf serum supplied the equivalent of 3–4 µg/ml LDL, which provided for maximal cell growth when endogenous cholesterol synthesis was inhibited by mevinolin in the absence of U18666A (data not shown). Over a 2-month period, cells were exposed to increasing concentrations of U18666A (1, 2, 3, 4, 5 µg/ml) after which a colony was picked. Preliminary experiments were performed with this cell line to demonstrate resistance to U18666A after which the concentration of U18666A was raised further (7, 10, 12, 15 µg/ml) over a 5-month period. At each step, the U18666A concentration was raised to a level sufficient to kill approximately 80% of the cells. The U18666A-resistant cells, designated U18R, have been maintained in medium D which contains 15 µg/ml of U18666A since May, 1990.

**Effect of U18666A on Cell Growth—**U18R cells were selected for their ability to overcome U18666A inhibition in LDL-mediated cell growth. The relative drug sensitivity of parental CHO cells and U18R cells is illustrated in Fig. 1. CHO and U18R cells grew equally well when cultured in medium C with 4 µg/ml LDL and no U18666A, and the protein content in wells of CHO and U18R cells was 46 and 47 µg, respectively (100% of control). The addition of low concentrations of U18666A sharply inhibited the growth of CHO cells with 50% inhibition at 0.7 µg/ml and complete inhibition at 1 µg/ml U18666A. However, U18R cells were able to grow in 7–10-fold higher concentrations of U18666A, with 50% inhibition at 4.5 µg/ml and complete inhibition at 11 µg/ml.

We observed that U18R cells cultured in medium with 1% whole newborn calf serum and 4% lipoprotein-deficient serum were able to grow in higher levels of U18666A than U18R cells cultured in medium with 5% lipoprotein-deficient serum and 4 µg/ml LDL. The difference in U18666A sensitivity between the two mediums may be due to variation in the amount of lipoproteins or to other factors that are removed during the delipidation.

**Effect of U18666A, Colchicine, and Daunomycin on Plating Efficiency and Cell Growth—**Drug-resistant cultured cells often express a multidrug-resistant phenotype due to the expression of a 170-kDa plasma membrane protein, P-glycoprotein (23). Expression of P-glycoprotein results in lower intracellular concentration of a diverse group of structurally and functionally distinct cytotoxic drugs (23). To determine if U18R cells express a multidrug resistant phenotype, we tested for cross-resistance to colchicine and daunomycin, two characteristic substrates for P-glycoprotein.

CHO and U18R cells were plated in medium containing various concentrations of U18666A, colchicine, and daunomycin and allowed to grow for 4 days. Fig. 2 shows that a 13-fold higher concentration of U18666A was required to inhibit the plating and growth of U18R cells when compared with CHO cells, with 50% inhibition at 11.6 and 0.3 µg/ml of U18666A, respectively. However, U18R and CHO cells were equally sensitive to colchicine and daunomycin.

**Effect of U18666A on LDL-stimulated Cholesterol Esterification—**Previously, we showed that U18666A inhibits several aspects of LDL-mediated regulation of cellular cholesterol metabolism (10). The LDL-mediated regulatory response that was most sensitive to U18666A inhibition was the LDL stimulation of cholesterol esterification, catalyzed by the endoplasmic reticulum enzyme, acylCoA:cholesterol acyltransferase. Fig. 3 shows that LDL stimulation of cholesterol esterification also exhibits the highest resistance to U18666A inhibition in U18R cells.

The concentration dependence of U18666A inhibition of LDL-stimulated cholesterol esterification was measured in CHO and U18R cells. In CHO cells, incubation with 30 µg/ml LDL for 7 h stimulated cholesterol esterification 3.3-fold, to 2.66 nmol/h/mg (100% of control). LDL stimulation of cholesterol esterification was sensitive to low concentrations of U18666A such that 0.02 µg/ml U18666A inhibited cholesterol esterification to 50% of control. In U18R cells, incubation with 30 µg/ml LDL for 7 h stimulated cholesterol esterification 3.2-fold, to 3.67 nmol/h/mg (100% of control). However, the concentration dependence of U18666A inhibition was shifted 100-fold such that 2 µg/ml was required to inhibit LDL stimulation of cholesterol esterification to 50% of control. In the same experiment, 25-hydroxycholesterol (1 µg/ml) stimulated cholesterol esterification to 0.99 and 2.12 nmol/h/mg in CHO and U18R cells, respectively. This was unaffected by concentrations of U18666A to 2 µg/ml in either cell type (data not shown).

To assess if CHO and U18R cells differ in acyl...
Resistance to U18666A Inhibition of Cholesterol Metabolism

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FIG. 2. Effect of U18666A, colchicine, and daunomycin on CHO and U18R cell plating efficiency and growth. Cells were grown as described under "Experimental Procedures." CHO (○) and U18R (■) cells were plated in 2 ml of medium C with 4 μg/ml LDL and the indicated amount of U18666A (panel A), or medium A with the indicated amount of colchicine (panel B) or daunomycin (panel C). On day 4, the protein content of each well was measured as described under "Experimental Procedures." Each data point represents the average of two wells. The data are expressed as a percentage of the cell growth in control incubations that received no drug. These control values were 43 and 75 μg (panel A) and 69 and 78 μg (panels B and C) for CHO and U18R cells, respectively.

Fig. 3. Effect of U18666A on LDL stimulation of cholesterol esterification. Cells were grown as described under "Experimental Procedures." On day 3, following the removal of U18666A, CHO (○) and U18R (■) monolayers received 1 ml of medium C or medium C containing 30 μg/ml LDL plus the indicated concentration of U18666A. After 6 h of incubation, each monolayer was pulsed with [3H]oleate for 1 h. The cellular content of cholesteryl [3H]oleate was determined as described under "Experimental Procedures." Each data point represents the average of two wells. The data are expressed as a percentage of the cholesteryl [3H]oleate formed in control incubations that received LDL in the absence of U18666A. These control values were 2.66 and 3.67 nmol/h/mg for CHO and U18R cells, respectively.

Table I

|                  | CHO                   | U18R                  |
|------------------|-----------------------|-----------------------|
| No addition      | 0.21                  | 0.11                  |
| LDL              | 1.01                  | 0.92                  |
| 25-Hydroxycholesterol | 1.00                 | 0.64                  |

LDL Receptor Activity in CHO and U18R Cells—LDL receptor activity was assessed in CHO and U18R cells by performing a time course of incubation with [3H]CL-LDL. The total cellular content of [3H]cholesteryl ester and [3H]cholesterol reflects the amount of [3H]CL-LDL internalized and hydrolyzed by the cells during the incubation period. Fig. 4 shows that U18R cells had slightly higher LDL receptor activity compared with CHO cells. The difference between the cell

CoA:cholesterol acyltransferase activity, noninduced cholesterol esterification was measured in addition to LDL- and 25-hydroxycholesterol-stimulated cholesterol esterification. The results shown in Table I, which are the average of three separate experiments, indicate that nonstimulated and stimulated acyl CoA:cholesterol acyltransferase activities are comparable in the two cell lines. Some variation is seen between experiments, as indicated by the range of values in Table I.

LDL Receptor Activity in CHO and U18R Cells—LDL receptor activity was assessed in CHO and U18R cells by performing a time course of incubation with [3H]CL-LDL. The total cellular content of [3H]cholesteryl ester and [3H]cholesterol reflects the amount of [3H]CL-LDL internalized and hydrolyzed by the cells during the incubation period. Fig. 4 shows that U18R cells had slightly higher LDL receptor activity compared with CHO cells. The difference between the cell
lines was variable, and can be up to 2-3-fold (see Table III).

Given that U18R cells exhibited higher LDL receptor activity, could this contribute to the resistance to U18666A inhibition of LDL-stimulated cholesterol esterification? To test this possibility, CHO cells were incubated with increasing amounts of LDL along with several concentrations of U18666A. The amount of U18666A required to inhibit the LDL-stimulated cholesterol esterification by 50% (IC50) was calculated for each level of LDL. In the absence of U18666A, the rate of cholesterol esterification increased as cells were incubated with higher concentrations of LDL (Table II). If increased uptake of LDL results in relative resistance to U18666A, we would expect that the IC50 for U18666A would increase as cells took up greater amounts of LDL. However, Table II shows that the IC50 did not change significantly, even with a 4.5-fold increase in the amount of LDL-derived cholesterol delivered to the cells.

Effect of U18666A on LDL Suppression of HMG-CoA Reductase Activity—Are other regulatory responses elicited by LDL also resistant to inhibition by U18666A in U18R cells? The rate limiting step in cholesterol biosynthesis is the conversion of HMG-CoA to mevalonate, which is catalyzed by the endoplasmic reticulum enzyme, HMG-CoA reductase (24). Normally, LDL suppresses the activity of HMG-CoA reductase; however, LDL-mediated suppression of this enzyme is impaired by co-cultivation with U18666A (10). Fig. 5 shows the effect of U18666A on LDL suppression of HMG-CoA reductase in CHO and U18R cells. When CHO and U18R cells were cultured in medium C (with no LDL or U18666A), HMG-CoA reductase activities were 1.38 and 2.41 units/mg, respectively (100% of control). Incubation with 30 µg/ml LDL in the absence of U18666A suppressed HMG-CoA reductase activity to 15.9 and 17.2% of control values in CHO and U18R cells, respectively. In CHO cells, low concentrations of U18666A blocked the LDL-mediated down-regulation of HMG-CoA reductase, such that suppression to 50% of control was seen with 0.2 µg/ml U18666A. In U18R cells, however, higher concentrations of U18666A were required to block the effects of LDL, and suppression to 50% of control was achieved with 1.3 µg/ml U18666A. The addition of U18666A up to 3 µg/ml had no effect on 25-hydroxycholesterol suppression of HMG-CoA reductase activity in either cell type (data not shown).

Effect of U18666A and Ketoconazole on the Movement of Cholesterol to the Plasma Membrane—Previously, we showed that U18666A and ketoconazole inhibit the movement of LDL-derived cholesterol from lysosomes to plasma membranes (10, 25). To examine the movement of LDL-derived [3H]cholesterol from lysosomes to plasma membranes, CHO and U18R monolayers were pulsed with [3H]CL-LDL for 2 h in the absence and presence of 1.5 µg/ml U18666A or 20 µM ketoconazole. Monolayers were then washed and subjected to chase incubations in the same media containing small unilamellar vesicles to trap the desorbed [3H]cholesterol. The amount of cellular [3H]cholesterol ester, [3H]cholesterol (derived from hydrolysis of [3H]CL-LDL), and medium [3H]cholesterol (derived from movement of LDL-[3H]cholesterol from lysosomes to the plasma membrane and subsequent desorption into the medium) were quantitated after chase incubations.

Table III shows the effects of U18666A and ketoconazole on the movement of LDL-derived [3H]cholesterol in CHO and U18R cells after 2 h (Experiment 1) and 5 h (Experiment 2) of chase. The data show that U18R cells had higher LDL receptor activity than CHO cells such that about 2.5 times more [3H]cholesterol was released from [3H]CL-LDL internalization and hydrolysis. In addition, U18R cells desorbed a 2.3-fold higher percentage of the LDL-derived [3H]cholesterol than CHO cells. Efflux of LDL-derived [3H]cholesterol from CHO cells was inhibited by U18666A and ketoconazole; however, efflux from U18R cells was inhibited by ketoconazole and not significantly inhibited by U18666A.

Since developing this assay for intracellular cholesterol movement (8), we have found that [3H]cholesterol efflux from cells varied greatly with different types and lots of serum used to culture the cells before and during the experiment. In addition, the degree of U18666A inhibition of LDL-derived [3H]cholesterol efflux varied greatly with types of serum. The variation in this assay may be related to lipid acceptors in the serum, although U18666A inhibition of LDL-stimulated cholesterol esterification remained invariant.

U18666A Inhibition of Cholesterol Synthesis—Rudney and colleagues (12) have demonstrated U18666A inhibition of cholesterol synthesis in rat intestinal epithelial cells. They propose that U18666A blocks 2,3-oxidosqualene:lanosterol cyclo-

![FIG. 5. Effect of U18666A on LDL suppression of HMG-CoA reductase activity.](image-url)
Control values were 474 and 166 cpm/pg for CHO and U18R cells, respectively. Heterokaryons expressed as a percentage of the acetate incorporated into [3H]cholesterol was determined as described under "Experimental Procedures." Each data point represents the average of two wells.

| Experiment 1 | Cell [3H]cholesteryl ester (nmol/mg) | Medium [3H]cholesterol (nmol/mg) | Experiment 2 | Cell [3H]cholesteryl ester (nmol/mg) | Medium [3H]cholesterol (nmol/mg) |
|--------------|--------------------------------------|----------------------------------|--------------|--------------------------------------|----------------------------------|
| No additions | 0.37                                 | 0.74                             | No additions | 0.52                                 | 0.57                             |
| U1866A       | 0.74                                 | 0.51                             | U18R         | 0.45                                 |                                   |
| Ketoconazole |                                      |                                  |              |                                      |                                  |

Fig. 6. Effect of U18666A on cellular cholesterol synthesis. Cells were grown as described under "Experimental Procedures." On day 3, following the removal of U18666A and mevinolin, CHO (□) and U18R (■) monolayers received 1 ml of medium B containing the indicated amount of U18666A. After 2 h, monolayers were pulsed with [3H]acetate for 4 h. [3H]Acetate incorporation into [3H]cholesterol was determined as described under "Experimental Procedures." Each data point represents the average of two wells. The data are expressed as a percentage of the [3H]acetate incorporated into [3H]cholesterol in control incubations that received no U18666A. These control values were 474 and 166 cpm/μg for CHO and U18R cells, respectively.

LDL-derived cholesterol transport is not clear. One approach to assessing if a common mechanism underlies the two inhibitory actions of U18666A is to determine if U18R cells are resistant to U18666A inhibition of cholesterol synthesis. CHO and U18R cells were pulse-labeled with [3H]acetate for 4 h in the presence of various concentrations of U18666A. Fig. 6 shows that the concentration-dependent inhibition of [3H]acetate incorporation into [3H]cholesterol was not significantly different in CHO and U18R cells. Inhibition of cholesterol synthesis to 50% of control values was achieved by 0.07 and 0.20 μg/ml U18666A in CHO and U18R cells, respectively.

U18666A Resistance in U18R Cells Is a Dominant Pheno-type—To determine if resistance to U18666A in U18R cells is a dominant or recessive phenotype, somatic cell hybrids were formed between CHO and U18R cells. Heterokaryons were selected, and drug resistance was tested in the hybrid cells (18). To facilitate selection of heterokaryons, a CHO cell line that is resistant to 1 μM ouabain and sensitive to HAT medium was selected from parental CHO cells. This cell line, designated CHO(HAT' oua'), was fused separately to parental CHO and U18R cells, both of which are ouabain-sensitive and HAT-resistant. Heterokaryons were selected in medium containing ouabain and HAT, which killed unfused CHO, U18R, and CHO(HAT' oua') cells as well as fused homokaryons. The CHO × CHO(HAT' oua') and U18R × CHO(HAT' oua') heterokaryons were then tested for U18666A inhibition of LDL-dependent cell growth. Both types of heterokaryons grew equally well in medium C with 4 μg/ml LDL. The protein content of CHO × CHO(HAT' oua') and U18R × CHO(HAT' oua') heterokaryons was 47 and 41 μg, respectively (100% of control). The U18R × CHO(HAT' oua') heterokaryons showed resistance to U18666A inhibition of cell growth similar to that observed in unfused U18R cells (compare Figs. 1 and 7). Fifty percent inhibition of cell growth was seen with 0.85 and 7 μg/ml U18666A in CHO × CHO(HAT' oua') and U18R × CHO(HAT' oua') heterokaryons, respectively. This result indicates that U18666A resistance in U18R cells is due to a genetic factor.

Fig. 7. Effect of U18666A on LDL-mediated cell growth in CHO × CHO(HAT' oua') and U18R × CHO(HAT' oua') heterokaryons. Cells were grown as described under "Experimental Procedures." On day 3, following the removal of U18666A, CHO × CHO(HAT' oua') (□) and U18R × CHO(HAT' oua') (■) monolayers received 1 ml of medium C containing 4 μg/ml LDL plus the indicated amount of U18666A. On day 6, the protein content of each well was measured as described under "Experimental Procedures." Each data point represents the average of two wells. The data are expressed as a percentage of the cell growth in control incubations that received no U18666A. These control values were 47 and 41 μg for CHO × CHO(HAT' oua') and U18R × CHO(HAT' oua') cells, respectively. In the figure, CHO(HAT' oua') cells are indicated by CHO*.
DISCUSSION

U18666A is a pharmacological agent that affects cellular cholesterol metabolism at two loci. First, U18666A blocks the intracellular transport of LDL-derived cholesterol from lysosomes to other cellular sites (10). The impaired movement of LDL-derived cholesterol from lysosomes causes delays in LDL-mediated regulation of cellular cholesterol metabolism in CHO and rat intestinal epithelial cells (10, 12). Second, U18666A inhibits cellular cholesterol synthesis (12). Cells incubated with U18666A accumulate squalene 2,3-oxide and squalene 2,3,22,23-dioxide indicating that U18666A inhibits 2,3-oxidosqualene-lanosterol cyclase activity (12). The impaired intracellular LDL-derived cholesterol transport described in U18666A-treated CHO cells is phenotypically similar to the defective LDL-derived cholesterol transport observed in fibroblasts from individuals with Niemann-Pick disease, type C (5–8). Both the primary defect in Niemann-Pick type C and the cellular target(s) of U18666A are unknown.

We plan to use a somatic cell genetic approach to identify the target of U18666A inhibition of cholesterol trafficking in cultured cells. The first step in our approach was to isolate a U18666A-resistant CHO line, designated U18R, using a multistep selection strategy. Due to impaired LDL-derived cholesterol transport, CHO cells acutely treated with U18666A are unable to grow with LDL as the sole cholesterol source. U18R cells were selected for the ability to grow in increasing concentrations of U18666A and a limited supply of LDL. They are also resistant to U18666A inhibition of intracellular LDL-derived [3H]cholesterol transport to the plasma membrane and LDL-mediated regulation of cholesterol esterification and HMG-CoA reductase activity. However, U18R cells are not as resistant to the second action of U18666A, that is inhibition of cholesterol synthesis. This result suggests that U18666A may exert two independent inhibitory effects on cholesterol metabolism.

There are several possible explanations for U18666A resistance. The selection protocol that we employed renders cells cholesterol-deficient and thus incapable of growth. Our goal is to identify a cell line that achieves resistance by overexpressing the cellular target for U18666A inhibition. Our selective pressure precludes cells from acquiring additional cholesterol by de novo synthesis because of the acute inhibition of HMG-CoA reductase by mevinolin. However, increased LDL receptor activity would deliver more LDL-derived cholesterol and could account for some degree of U18666A resistance. Although LDL receptor activity is fairly comparable in CHO and U18R cells, we occasionally observe that 2–3-fold more cholesterol is derived from LDL hydrolysis in U18R cells (see Table III). This increased cholesterol delivery may account for a small part of the resistance to U18666A inhibition of LDL-derived cholesterol transport; however, our results suggest that the 100-fold resistance of U18R cells to U18666A inhibition of LDL stimulation of cholesterol esterification cannot be explained by 2–3-fold greater LDL-derived cholesterol delivery.

Another possible explanation for U18666A resistance is that U18R cells have developed the capability to efflux or metabolize the drug. The selection of some drug-resistant cultured cells has been correlated with the expression of P-glycoprotein, a plasma membrane protein thought to mediate the efflux of cytotoxic drugs (23). To assess the possibility that U18R cells are expressing P-glycoprotein and achieving resistance by U18666A efflux, we determined the following. (i) P-glycoprotein is not expressed in detectable levels in U18R cells. This was tested by immunoblotting using monoclonal antibody C219 (Cenotec, Malvern, PA). (ii) The dose dependence of U18666A inhibition of LDL-stimulated cholesterol esterification is identical in wild type CHO cells and CH3+C5 cells, which are multidrug-resistant CHO cells expressing P-glycoprotein. This indicates that U18666A is not a substrate for P-glycoprotein. (iii) The plating efficiency of CHO and U18R cells in U18666A, colchicine, and daunomycin shows that U18R cells are not cross-resistant to the cytotoxic drugs most frequently associated with multidrug resistance. (iv) U18R cells largely retain their sensitivity to U18666A inhibition of cholesterol synthesis. If U18666A was being effluxed or metabolized to an inactive compound, we would expect the dose dependence of all inhibitory actions to be affected to the same extent.

In several studies, we have noted a distinction between LDL stimulation of cholesterol esterification and the other LDL-mediated regulatory responses. In Niemann-Pick type C fibroblasts, LDL stimulation of cholesterol esterification is more profoundly defective than the other regulatory responses (10). Similarly, LDL stimulation of cholesterol esterification in CHO cells is the regulatory response most sensitive to inhibition by U18666A (10). It appears as though any perturbation in LDL-derived cholesterol metabolism most dramatically influences the LDL stimulation of acyl CoA:cholesterol acyltransferase. Here we show that U18R cells are 100-fold resistant to U18666A inhibition of LDL stimulation of cholesterol esterification, whereas other LDL-mediated regulatory responses and LDL-derived [3H]cholesterol transport exhibit a 7–10-fold resistance. Once again, an alteration in LDL-derived cholesterol metabolism has most dramatically affected LDL stimulation of cholesterol esterification.

The mechanism by which LDL-derived cholesterol is released from lysosomes and transported throughout the cell to membranes and regulatory sites is unknown but may involve soluble carrier proteins or lipids, or vesicular transporters. In CHO cells, the movement of LDL-derived cholesterol from lysosomes to plasma membranes does not appear to be altered by agents that affect lysosomal function or cytoskeletal organization, as well as energy poisons or cycloheximide (25). Movement of LDL-derived cholesterol to the plasma membrane is extremely rapid when measured using cholesterol oxidase as a probe of plasma membrane cholesterol (26). Although the precise pathway has yet to be elucidated, there is evidence that LDL-derived cholesterol does not pass through the endoplasmic reticulum during transport to the cell surface (27). Two systems have been described in which LDL-derived cholesterol transport is perturbed: NPC fibroblasts with an inherited metabolic defect (5–8) and cultured cells treated with U18666A, ketoconazole, and imipramine (10, 25, 28). The mechanisms of these disruptions in LDL-derived cholesterol transport are unknown, but may involve the activity or synthesis of a protein or lipid that facilitates LDL-derived cholesterol movement.

Drug-resistant cells have proved to be a powerful tool for the isolation and study of genes to which drugs are specifically targeted. Several cell lines have been developed that have overcome selection constraints by amplifying or mutating the target gene for the drug (18, 22). Examples include cells that have developed resistance to methotrexate, colcemid, and cadmium by amplification of the genes for dihydrofolate reductase, HMG-CoA reductase, and metallothionein II, respectively (29–32). In future studies, we hope to use U18R cells to identify the target for U18666A inhibition of LDL-derived cholesterol metabolism.

2 H. Shu, L. Liscum, and I. M. Arias, unpublished observation.
3 L. Liscum and I. M. Arias, unpublished observation.
cholesterol transport. Since U18R cells express a dominant phenotype, the gene(s) conferring U18666A resistance can be
isolated by transfer of U18R genomic DNA into U18666A-sensitive cells. Identification of the cellular target of U18666A
may provide important information on the mechanisms of LDL-derived cholesterol trafficking and regulation.

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