Apolipoprotein E Expression in Y1 Adrenal Cells Is Associated with Increased Intracellular Cholesterol Content and Reduced Free Cholesterol Efflux†

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ABSTRACT: The expression of apoE mRNA in the adrenal gland is inversely correlated to steroidogenesis and directly correlated to the level of cholesteryl ester stores. To further investigate the relationship between apoE and cellular cholesterol homeostasis, several parameters of cholesterol metabolism in the murine Y1 adrenal cell line engineered to constitutively express human apoE (Y1-E cells) have been studied. It is reported here that Y1-E cells have increased cellular cholesterol content and markedly reduced efflux of free cholesterol as compared to control Y1 cells that do not express apoE. Y1-E cells have increases in both free and esterified cholesterol. However, Y1 and Y1-E cells incorporate [^{14}C]oleate into cholesteryl ester at similar rates and have similar levels of maximal ACAT activity in isolated microsomes. Turnover of cholesteryl ester stores prelabeled with [^{14}C]oleate occurred at similar rates in Y1-E and control Y1 cells, suggesting that increased cholesteryl ester stores in Y1-E cells do not result from reduced cholesteryl ester hydrolysis. Y1-E cells showed reduced cholesterol efflux as compared to control Y1 cells with either native high-density lipoprotein or cholesterol-free reconstituted particles as extracellular acceptors. Cholesterol efflux was not altered by inhibition of ACAT, suggesting that cholesterol esterification in Y1-E cells is not inhibiting efflux. These results suggest that reduced cholesterol efflux is responsible, at least in part, for the cholesterol accumulation in Y1-E cells. In comparison to the rat adrenal gland *in vivo*, Y1-E cells resemble adrenocortical cells under conditions where steroidogenesis is suppressed and apoE expression and cholesteryl ester storage are increased. Thus, apoE expression in Y1 cells alters cholesterol metabolism to promote cholesteryl ester storage with reduced cholesterol utilization for either steroidogenesis or efflux from the cell.

Apolipoprotein E (apoE)[1] is a prominent component of several classes of plasma lipoproteins and functions in several ways to influence systemic lipoprotein and cholesterol metabolism. ApoE is known to facilitate removal of cholesteryl ester-rich remnant lipoproteins from plasma by hepatic receptors (Mahley et al., 1984). ApoE is also a ligand for the LDL receptor and may function in removal of cholesterol from extrahepatic tissues followed by delivery to the liver. The liver is the primary source of plasma apoE (Wu & Windmueller, 1979). However, in striking contrast to other apolipoproteins, apoE is expressed in a variety of peripheral tissues and cell types not involved in lipoprotein synthesis (Blue et al., 1983; Driscoll & Getz, 1984; Newman et al., 1985; Williams et al., 1985b; Elshourbagy et al., 1985; Lin et al., 1986). ApoE made in extrahepatic tissues is regulated in a manner distinct from the pattern of regulation in hepatocytes (Basu et al., 1981; Dawson et al., 1986; Prack et al., 1991). ApoE mRNA is particularly abundant in steroidogenic tissues (Newman et al., 1985; Driscoll & Getz, 1984; Prack et al., 1991). In adrenal glands isolated from human beings or monkeys, apoE, relative to total protein synthesis, is synthesized at rates similar to those measured in the liver (Williams et al., 1985b; Blue et al., 1983). In the rat adrenal gland apoE mRNA is expressed at the highest levels in cortical cells that synthesize glucocorticoids (Nicosia et al., 1992). Adrenal gland apoE expression declines in parallel with tissue cholesterol content when steroidogenesis is stimulated and increases when steroidogenesis is blocked by exogenous glucocorticoid (Prack et al., 1991; Nicosia et al., 1992). The function of apoE synthesized by the adrenal gland or other extrahepatic tissues is unclear; however, several possible roles in local cholesterol or phospholipid transport have been proposed (Blue et al., 1983; Dawson et al., 1986; Williams et al., 1985a; Mahley, 1988). Among the possibilities, apoE may facilitate lipoprotein uptake by cells, regulate cholesterol utilization, or facilitate cholesterol redistribution within a tissue. ApoE also may have autocrine or paracrine functions that are independent of the role of apoE in systemic lipid metabolism. Exogenous apoE can modify cell-specific responses in lymphocytes (Pepe & Curtiss, 1986; Avila et al., 1982) and ovarian theca/interstitial cells (Dyer & Curtiss, 1988).

To learn more about the potential role of apoE in adrenal cholesterol metabolism, the human apoE gene was stably and constitutively expressed in the murine Y1 adrenocortical cell line (Reyland et al., 1991). Y1 cells show increased steroid synthesis in response to ACTH or cAMP and utilize plasma lipoproteins for steroid synthesis (Faust et al., 1977). However,

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[1] Abbreviations: ACTH, adrenocorticotropin; ACAT, acylCoA: cholesterol acyltransferase; apoE, apolipoprotein E; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDS, lipoprotein-deficient serum.

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these cells have low cholesteryl ester content compared to primary adrenal cells (Pittman et al., 1987) and do not make apoE (Reyland et al., 1991). Expression of the human apoE gene in Y-1 cells (Y1-E cells) results in a dramatic decrease in both basal and ACTH-stimulated steroidogenesis (Reyland et al., 1991). Part of this decrease is due to a selective suppression of the cAMP-dependent protein kinase pathway and reduced expression of the mRNA for the P450-cholesterol side-chain cleavage enzyme (Reyland & Williams, 1991). However, the block in steroid production is more marked than the decrease in steroidogenic enzymes, suggesting that other pathways required for optimal steroid production are affected. One possibility is that cholesterol metabolism is altered in adrenal cells expressing apoE since cholesterol availability is critical for corticosteroid synthesis.

To further investigate the relationship between apoE expression and cholesterol homeostasis in the Y1-E cells, we studied several aspects of intracellular cholesterol metabolism in Y1-E cell lines and a control Y1 cell line (Y1-neo). We report here that cellular cholesterol ester content is markedly increased and free cholesterol efflux is markedly reduced in Y1-E cells. These results suggest that apoE expression has the potential to alter intracellular cholesterol metabolism by promoting cholesterol storage and reducing cholesterol availability for steroidogenesis and efflux.

EXPERIMENTAL PROCEDURES

Cell Culture. The murine adrenal cell line, Y1, was obtained from American Type Tissue Collection (Rockville, MD). The preparation of human apoE transfected cell lines (Y1-E cells) and the Y1-neo cell line, a control cell line transfected only with the neomycin resistance gene, has been detailed previously (Reyland et al., 1991). Y1 cells defective in protein kinase A activity, Kin$^-$8 cells (Rak et al., 1979; Wong et al., 1989), and the stable Y1 subclone, BS-1 (Schimmer, 1979), were provided by Dr. Bernard Schimmer (University of Toronto, Toronto, Canada). The cell lines were maintained in Ham's F-10 medium supplemented with 12.5% (v/v) heat-inactivated horse serum and 2.5% (v/v) heat-inactivated fetal bovine serum with 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin sulfate (complete medium). Y1-E and Y1-neo cell lines were maintained in complete medium with 100 μg/mL (active form) G418 sulfate (geneticin, Gibco). Experiments were initiated 24 h after cells were plated at equal densities.

Cholesterol Esterification. [^{14}C]Oleate (NEN, 59 μCi/mmol) was bound to albumin with 0.1 mM oleic acid as previously described (Goldstein et al., 1983; Reyland et al., 1991). Cells were cultured in the presence of [^{14}C]oleate/albumin (specific activity 9000–10 000 dpm/nmol of oleate) as indicated under Results. Lipids were extracted from labeled cells with hexane/2-propanol (3:2), dried under nitrogen, and separated by thin-layer chromatography on silica gel plates (Kodak) in heptane/diethyl ether/acetic acid (90:30:1) (Goldstein et al., 1974). Cholesteryl 1,2,6,7-[^{3}H]oleate (NEN, 82.9 Ci/mmol) was added to each sample to determine recovery. The amount of cholesteryl [^{14}C]oleate, cholesteryl [^{3}H]oleate, and [^{14}C]triglycerides was determined by scintillation spectrometry following thin-layer chromatography. After lipid extraction, protein residue was scraped into 0.2 N sodium hydroxide for protein determination (Lowry et al., 1951). The ACAT activity in cellular homogenates and microsomal preparation was determined as described previously (Erickson & Fielding, 1986; Erickson et al., 1990).

Cholesterol Synthesis from Mevalonolactone. Cells were cultured for 48 h in Ham's F-10 containing 10% fetal bovine LPDS. Medium was removed and replaced with medium of the same composition containing 0.4 μCi/mL [^{14}C]mevalonolactone (DuPont/NEN) and incubated for an additional 6 h. Medium was removed, cell layers were washed with phosphate-buffered saline, and cells were harvested by scraping. Cellular lipids were extracted into chloroform (Bligh & Dyer, 1959) and separated by thin-layer chromatography, and the amount of radiolabel incorporated into free and esterified cholesterol was quantified by scintillation spectrometry.

Cholesteryl Ester Hydrolysis. Cells were incubated for 16 h in the presence of [^{14}C]oleate as described above, and the amount of label incorporated into intracellular cholesteryl esters was measured. After the labeling period, medium was removed and cell layers were washed with phosphate-buffered saline, and the incubation was continued as indicated under Results. At the end of the chase period, cellular lipids were extracted and cholesteryl [^{14}C]oleate was quantified as described above (Goldstein et al., 1983). Medium collected after the chase period was extracted into chloroform/methanol (Folch et al., 1925), and lipid classes were separated by thin-layer chromatography as described above.

Cholesterol Efflux. Cholesterol efflux was measured as detailed previously (Rothblat et al., 1986). In brief, cellular cholesterol pools were labeled with 4-[^{14}C]cholesterol or 1,2-[^{3}H]cholesterol (DuPont/NEN) in the presence of 5% fetal bovine serum for 1–5 days. Cells were washed three times with Ham's F-10, Ham's F-10 containing 0.2% bovine serum albumin was added, and cells were incubated for 18–24 h to allow equilibration of the label among intracellular pools. At the end of the equilibration period, cells were washed, and Ham's F-10 medium containing HDL (400 μg of lipid phosphorus/mL) or apoHDL/phosphatidylcholine vesicles (200–400 μg of lipid phosphorus/mL) prepared as described (Rothblat et al., 1986) as acceptor was added. The appearance of radiolabeled cholesterol in the media was measured at several times to determine efflux. The distribution between labeled free cholesterol and steroid at the end of the experiment was determined by thin-layer chromatography in benzene/acetone (120:30). Cholesterol efflux values were corrected for the amount of radiolabeled steroid in the medium, and half-life was calculated on the basis of total cellular radiolabeled cholesterol at the initial time point (t_0).

Miscellaneous. Lipoproteins were prepared from human plasma by sequential ultracentrifugation (Havel et al., 1955) using a 60 Ti rotor (Beckman) at 50 000 rpm. Ethylenediaminetetraacetic acid (final concentration, 0.1%), dithiobis-(nitrobenzoic acid) (final concentration, 0.1%), and phenylmethanesulfonyl fluoride (0.08 μg/mL) were added to plasma prior to isolation of lipoproteins. Potassium bromide was added to adjust the plasma density. LPDS was prepared from fetal bovine serum (Gibco/BRL) by ultracentrifugation after the density of the serum was adjusted to 1.225 g/mL with potassium bromide. Lipoproteins and LPDS were dialyzed against phosphate-buffered saline and filter sterilized prior to addition to cell culture medium. Lipoprotein cholesterol was measured using an enzymatic kit (Boehringer-Mannheim (Allain et al., 1974)).

Free cholesterol and total cholesterol in cellular lipid extracts were assayed by gas chromatography on Hewlett-Packard gas chromatograph Model 5890 using a 30-m DB-5 silica column (J&W Scientific) with an oven temperature of 300 °C. Stigmasterol (Maytreya) was added to each sample as

Table 1: Relative Increase in Free and Esterified Cholesterol Content in Y1-E Cell Lines[a]

cell line (n)	total C	free C	esterified C
Y1-neo (8)	1.0	1.0	1.0
Y1-E1 (3)	2.35 ± 0.1[b]	1.56 ± 0.2[b]	5.6 ± 1.5[b]
Y1-E2 (6)	1.72 ± 0.2[b]	1.41 ± 0.1[b]	2.83 ± 0.7
Y1-E10 (3)	2.00 ± 0.7	1.60 ± 0.5	2.97 ± 1.9
Y1-E12 (8)	1.75 ± 0.2[b]	1.31 ± 0.08[b]	3.04 ± 0.6[b]
Y1-E15 (7)	1.63 ± 0.5[b]	1.26 ± 0.2	2.80 ± 0.6

[a] Cells were grown to confluence in complete medium. Medium was removed, cell layers were washed three times with phosphate-buffered saline, and lipids were extracted into chloroform. Free and total cholesterol (C) were measured as described under Experimental Procedures. Esterified cholesterol was determined by difference between free and total cholesterol. Data shown were obtained from two–six independent experiments. In each set of experiments, the values for the Y1-E cell lines were normalized to the Y1-neo values as 1.0. Average values for Y1-neo were 66.3 ± 8.8 μg of total cholesterol/mg of protein, 44.7 ± 6.1 μg of free cholesterol/mg of cell protein, and 21.3 ± 3.4 μg of esterified cholesterol/mg of protein. *n* indicates the number of measurements obtained for each cell line. [b] $p < 0.05$ by ANOVA corrected for multiple comparisons by the method of Bonferroni.

an internal standard. Protein was measured according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Three ACAT inhibitors were used. Sandoz 58-035 was a gift of the Sandoz Pharmaceutical Co. (Rahway, NJ). Cl976 (PD128042) and Cl984 (PD132301) were gifts of the Parke-Davis Pharmaceutical Division of the Warner-Lambert Co. (Ann Arbor, MI). ACAT inhibitors were added to cell cultures in dimethyl sulfoxide (Fisher Scientific). An equal volume of solvent was added to control cell dishes.

Statistics. Data were tested for differences between groups by analysis of variance using a commercially available statistics package, GraphPad version 2.0 (Instat). The level of significance was corrected for multiple comparisons by the method of Bonferroni (Daniel, 1983).

RESULTS

Cholesterol Content of Cells. The reduction of steroid production in the Y1-E cells may reflect reduced intracellular cholesterol or cholesteryl ester content. To determine the level of cholesterol stores, the free and total cholesterol concentrations of several apoE-secreting cell lines were measured and compared to that in the Y1-neo cell line. Table 1 shows data collected in many experiments over a 3-year period in which the values for the Y1-E cell lines were normalized to the free and esterified cholesterol content of the Y1-neo control line within each experiment. These data show increased total cholesterol concentration in apoE-expressing Y1 cell lines. Y1-E cell lines show a modest increase in free cholesterol ranging from 1.2- to 1.6-fold relative to the Y1-neo cell line and have increased cholesteryl ester content ranging from 1.6- to 5.6-fold relative to the Y1-neo cell line. Y1-E cell lines have a significantly ($p < 0.01$) higher fraction of total cholesterol in cholesteryl esters. The fraction of cholesterol mass in cholesteryl ester was 32.4 ± 2% in Y1-neo cells and averaged 57.5% (range 47.7–76.0) in the apoE-expressing cells. These results indicate that cholesteryl ester storage is enhanced in adrenal cells that express the human apoE gene.

To determine whether the increased cholesteryl ester content of the Y1-E cells was due to an extracellular action of apoE in facilitating uptake and esterification of lipoprotein cholesterol, Y1-neo cells were cultured in medium conditioned by the highest apoE expressor, Y1-E12 (Reyland et al., 1991) or medium conditioned by Y1-neo cells. Medium for conditioned medium experiments was comprised of a 50/50 (v/v) mix of complete F-10 medium and complete F-10 conditioned for 24 h by Y1-E12, the cell line expressing the highest amount of apoE (E12-conditioned medium) or conditioned for 24 h by Y1-neo cells (Neo-conditioned medium). The apoE content of this medium was estimated by western blotting using authentic human apoE (a gift of A. Attie, University of Wisconsin, Milwaukee, WI) as a standard. The E12-conditioned medium contained 6 μg/mL apoE and 27.3 μg/mL cholesterol. Neo-conditioned medium contained 26.6 μg/mL cholesterol and no detectable apoE. Y1-neo cells were grown in each type of conditioned medium, changed daily, for 5 days. On the fifth day, cells were harvested, lipids were extracted, and total cholesterol was measured (Allain et al., 1974). Y1-neo grown in medium conditioned by Y1-neo cells had 32.5 ± 0.1 μg of cholesterol/mg of cell protein (average ± SEM; $n = 4$), and Y1-neo cells grown in medium conditioned by Y1-E12 contained 32.8 ± 0.1 μg of cholesterol/mg of cell protein (average ± SEM; $n = 4$). Y1-E12 cells harvested at the same time contained 74 μg of cholesterol/mg of cell protein. These results suggest that cholesterol accumulation in Y1-E cell lines is not solely attributable to apoE in the culture medium.

To examine whether the increased cholesteryl ester content of the Y1-E cells was due secondarily to the inability to use cholesterol for steroidogenesis, we measured the cholesterol content of a cell line that is deficient in the ability to activate protein kinase A, Y1-kin$^-$8 (Williams & Schimmer, 1983). Similar to Y1-E cells, Y1-kin$^-$8 cells have a low level of P450 cholesterol side-chain cleavage enzyme and make very little steroid. Total cholesterol in Y1-kin$^-$8 cells was no higher than that measured in its parent cell line, Y1-BS1 (49 ± 8 μg of cholesterol/mg of protein vs 50 ± 4; $n = 3$ for each line). The same fraction of total cholesterol was found in cholesteryl esters in each cell line (32.5 ± 1.5% for Y1-BS1 and 34.9 ± 3% for Y1-kin$^-$8 cells). In contrast, in measurements made at the same time, the Y1-E12 cell line had 2-fold higher total cholesterol content than Y1-neo cells (155 ± 6 vs 78 ± 9 μg of cholesterol/mg of protein, $n = 4$ for each cell line). In Y1-E12 56.8 ± 0.8% of the total cholesterol was esterified compared to 29.1 ± 1.4% in the Y1-neo cell line. This suggests that changes in the cholesteryl ester content in Y1-E cells do not result simply from an inability to utilize cholesterol for steroid production or the inability to activate the protein kinase A pathway.

Cholesterol Esterification. To test whether the increased cholesteryl ester content of Y1-E cells was attributable to a higher basal rate of cholesterol esterification compared to that of the Y1-neo cells, the incorporation of [^{14}C]oleic acid into cholesterol oleate was measured in cells grown in medium containing either fetal bovine serum or LPDS. There were no apoE-related differences between Y1 and Y1-E cells in cholesterol esterification rates either in medium containing serum or in medium containing LPDS (Figure 1). Cholesterol esterification appears similarly responsive to exogenous lipoprotein cholesterol in Y1-neo and Y1-E cell lines as judged by the increased oleate incorporation in the presence of serum as compared to LPDS. To determine if there was a difference in the ability of different lipoproteins to promote cholesterol esterification in the Y1-E compared to Y1-neo cells, cholesterol esterification was measured in medium containing LPDS or LPDS with LDL or HDL. Cholesterol esterification rates were increased, compared to LPDS, in the presence of LDL but not HDL in each cell line examined (Figure 2). There

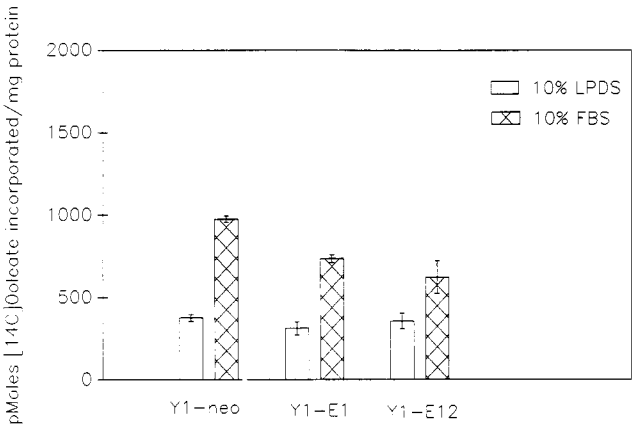


FIGURE 1: Oleate incorporation into cholesteryl esters in Y1-neo and Y1-E cell lines. Y1 cells grown in 12 well plates were incubated for 24 h in Ham's F-10 medium containing either 10% fetal bovine serum (10% FBS) or 10% lipoprotein-deficient fetal bovine serum (10% LPDS). Medium was replaced with fresh medium of the same composition containing [^{14}C]oleate on albumin (10 000 dpm/nmol) and incubation continued for 2 h. Lipids were extracted and analyzed by thin-layer chromatography as described under Experimental Procedures. Data shown are the averages ± SEM of triplicate wells and are representative of three independent experiments.

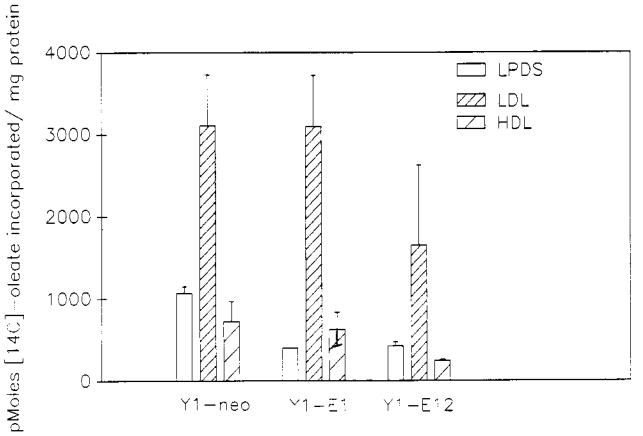


FIGURE 2: LDL but not HDL supports cholesterol esterification in Y1-neo and Y1-E cell lines. Y1 cells were grown in 12 well plates in complete medium. Prior to the experiment, medium was removed and cell layers were washed with Ham's F-10. Medium was replaced with Ham's F-10 containing 10% LPDS alone or with LDL or HDL added at 50 μg of cholesterol/mL. After 24 h, medium was removed and replaced with fresh medium of identical composition containing [^{14}C]oleate on albumin (10 000 dpm/nmol) and incubation continued at 37 °C for 2 h. Data shown are the averages ± SEM of triplicate wells.

were no significant differences between cell lines. This preference for LDL cholesterol is consistent with the findings of Faust and co-workers (Faust et al., 1977) for the parent Y1 cell line.

These data indicate that cholesteryl ester formation in Y1-E cells is regulated by exogenous cholesterol in a manner similar to that in Y1-neo and the parent Y1 cells (Faust et al., 1977). These data also suggest that the increased cholesteryl ester stores in Y1-E cells do not result from an increased basal rate of cholesteryl ester formation as judged by [^{14}C]oleate incorporation.

To confirm that cholesteryl oleate formation was due to ACAT, Y1-neo and Y1-E cells were treated with the ACAT inhibitor Sandoz 58-035. [^{14}C]Oleate incorporation into cholesteryl esters was inhibited 85% in both Y1-neo and Y1-E cell lines by 58-035 at 20 μg/mL (data not shown). Similarly, the ACAT inhibitors C1976 (PD 128042) (Bocan et al., 1991; Field et al., 1991) and CI984 (PD132301) at 0.5 μmol/mL inhibited oleate incorporation by 95% in both Y1-neo and Y1-E cell lines (data not shown). Direct measurements of ACAT activity in microsomal fractions were performed without and with the addition of cholesterol substrate in liposomes. When ACAT activity in Y1-E10 and Y1-E12 cells was normalized to that in the parental Y1 line in each experiment, there was no significant difference in maximal ACAT activity (Table 2; assay with liposomes). This result is consistent with the oleate incorporation studies performed in intact cells (Figures 1 and 2). In the absence of liposomes, ACAT activity is higher in the Y1-E cell lines compared to the parent cell line (Table 2; endogenous). This result is consistent with the increased cholesterol concentration measured in microsomes isolated from Y1-E cells (Table 2, footnote) and may indicate a greater availability of endogenous cholesterol substrate to ACAT in apoE-expressing cells.

To determine whether a higher proportion of newly synthesized cholesterol was esterified in the Y1-E cells, cells were incubated with [^{14}C]mevalonolactone for 6 h. There was a lower incorporation of mevalonolactone into free cholesterol in the Y1-E cell lines compared to Y1-neo cells (Table 3). However, there was no difference between cell lines in incorporation into esterified cholesterol expressed per milligram of protein or as the ratio of label in esterified to total newly synthesized cholesterol. These data suggest that there is no preferential esterification of newly synthesized cholesterol in Y1-E cells compared to the control cell line.

Table 2: Microsomal ACAT Activity in Y1 Cell Lines[a]

cell line	endogenous	+ liposomes
Y1	1.00	1.00
Y1-E10	1.89 ± 0.4[b]	0.89 ± 0.3
Y1-E12	1.55 ± 0.1[c]	0.79 ± 0.2

[a] Cells from 10 T-162 mm^2 flasks were grown to confluence in complete medium for each assay. Homogenates and microsomal fractions were prepared (Erickson & Fielding, 1986) and assayed for ACAT activity in the absence (endogenous) or presence of cholesterol:egg lecithin liposomes (Erickson et al., 1990). Data shown are the average ± SEM of three (Y1-E10) or four (Y1-E12) independent experiments. In each set of experiments, the values for the Y1-E10 and Y1-E12 cells were normalized to the Y1 values as 1.00. Y1 average values were 5.7 ± 1.7 pmol of cholesteryl ester min^{-1} (mg of protein)$^{-1}$ for activity from endogenous substrate and 22.8 ± 11.8 pmol of cholesteryl ester min^{-1} (mg of protein)$^{-1}$ for maximal activity assayed in the presence of liposomes (+ liposomes). Average total cholesterol concentrations in microsomal fractions were 37.5 μg of cholesterol/mg of protein, 58.3 ± 6[b] μg of cholesterol/mg of protein, and 75.0 ± 8[b] μg of cholesterol/mg of protein for Y1-neo, Y1-E10, and Y1-E12, respectively. [b] Different from Y1 ($p < 0.01$) by paired t-test. [c] Different from Y1 ($p < 0.05$) by paired t-test.

Table 3: Incorporation of Mevalonolactone into Free and Esterified Cholesterol in Y1 Cell Lines[a]

cell line	free C (dpm/mg of protein)	esterified C (dpm/mg of protein)	esterified C/total C (dpm)
Y1-neo	5688 ± 619	316 ± 59	5.3 ± 0.8
Y1-E10	3311 ± 509[b]	208 ± 64	6.8 ± 0.4
Y1-E12	3578 ± 209[b]	205 ± 33	5.4 ± 0.8

[a] Cells were cultured for 24 h in Ham's F-10 supplemented with 10% fetal bovine serum LPDS. After 48 h, medium was replaced with the same medium containing 0.4 μCi/well [^{14}C]mevalonolactone. Incubation was continued for 6 h, after which time medium was removed, cell layers were washed with phosphate-buffered saline, and lipids were extracted. The amount of mevalonate incorporated into free and esterified cholesterol (C) was quantified as described under Experimental Procedures. Data shown are average ± SEM of triplicate measurements expressed as dpm/mg of cell protein. [b] Different from neo ($p < 0.05$) by one-way analysis of variance.

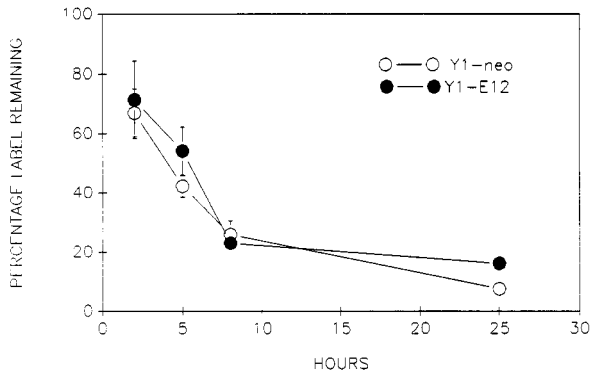
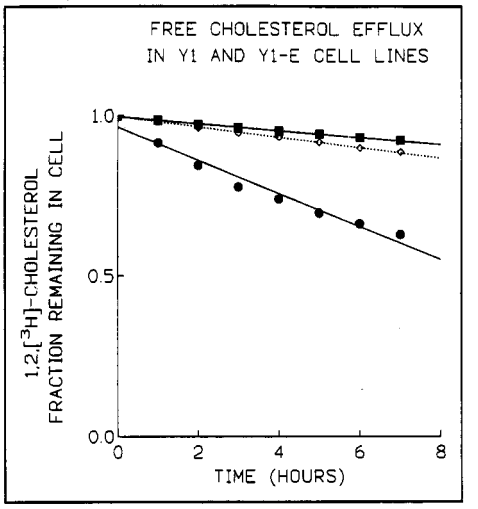


FIGURE 3: Cholesteryl [^{14}C]oleate turnover in Y1 cells in the presence of an ACAT inhibitor. Confluent Y1 cells in 12 well plates were incubated for 16 h in complete media containing 0.1 mM [^{14}C]oleate on albumin as described under Experimental Procedures. Labeled medium was removed, and cell layers were washed twice with Ham's F-10. Medium was replaced with Ham's F-10 containing 10% FBS-LPDS and 20 μg/mL Sandoz 58-035. Cells were harvested at the indicated times, lipids were extracted, and radiolabel remaining in cholesteryl ester was quantified. Data shown are the averages ± SEM of triplicate wells and are representative of two independent experiments.

Hydrolysis of Cholesteryl Esters. To test for differences in cholesteryl ester turnover between Y1-neo and Y1-E cells, the disappearance of prelabeled cholesteryl [^{14}C]oleate was measured in Y1-E or Y1-neo in the presence of the ACAT inhibitor Sandoz 58-035. Radiolabel was not recovered in cellular cholesterol, and no radiolabeled triglyceride or cholesteryl esters accumulated in the media over 24 h (data not shown), confirming that these lipids were not secreted by Y1-cells. The time course of cholesteryl [^{14}C]oleate turnover for Y1-E12 and Y1-neo cell lines (Figure 3) showed no substantial difference. In other experiments, the extent of cholesteryl [^{14}C]oleate hydrolysis after 24 h was 80–93% and showed no difference between Y1-neo and several Y1-E cell lines (data not shown). This suggests that cholesteryl ester hydrolysis occurs at similar rates in Y1-neo and Y1-E cells.

Cellular Cholesterol Efflux. To determine whether apoE expression resulted in an alteration in cholesterol efflux, Y1-E and Y1-neo cells were grown in medium containing 5% fetal bovine serum containing 4-[^{14}C]cholesterol or 1,2-[^{3}H]-cholesterol (Rothblat et al., 1986). After the cells were washed to remove extracellular radiolabeled cholesterol, cells were incubated with medium containing HDL or apoHDL/PC disks. The rate of appearance of radiolabeled free cholesterol in the medium was used to assess cholesterol efflux. Free cholesterol efflux from the Y1-neo cell line to HDL occurred twice as fast ($t_{1/2}$ = 15.1 h) as from Y1-E12 ($t_{1/2}$ = 27.9 h) or Y1-E1 ($t_{1/2}$ = 29.5 h) cell lines (data not shown). To exclude that these results were due to exchange of cholesterol from the HDL particle to the cells, the efflux experiments were also performed using a cholesterol-free reconstituted particle containing the apolipoproteins of HDL and phosphatidylcholine (apoHDL/PC) as the acceptor. Figure 4 presents the data from experiments comparing two Y1-E cell lines, E1 and E12, to Y1-neo and shows that efflux occurred 4 times faster from Y1-neo cells than from either Y1-E cell line. In another experiment employing apoHDL/PC acceptor particles, efflux from the Y1-neo cell lines was 2.1-fold faster than from Y1-E12 or Y1-E1 cell lines (data not shown). These results indicate that the reduced efflux in Y1-E cells does not reflect a difference in cholesterol exchange between cells and acceptor particles. In a separate experiment, this difference was confirmed by measuring cellular cholesterol content before and after an 8-h efflux period to apoHDL/PC. In this



Half-times for efflux of free cholesterol from Y1 cells

$t_{½}$ (hours)

Cell	Apo HDL/PC	Apo HDL/PC + 58-035
Y1-neo	10.6	9.4
Y1-E1	40.0	41.4
Y1-E12	48.4	43.3

FIGURE 4: Efflux of free cholesterol from Y1 cells. Y1 cells, grown in 35-mm dishes, were incubated for 24 h in Ham's F-10 medium containing 5% fetal bovine serum and 2 μCi/mL 1,2-[^{3}H]cholesterol (NEN). Cells were washed three time with Ham's F-10, Ham's F-10 containing 0.2% bovine serum albumin was added, and cells were incubated for 18 h to allow equilibration of the label among intracellular pools. During the labeling and equilibration, Sandoz 58-035 (1 μg/mL) was included with cells as indicated. At the end of the equilibration period, cells were washed three times, and Ham's F-10 containing apoHDL/PC (200 μg/mL phospholipid) with or without 58-035 was added. One set of cells was harvested immediately for lipid analysis. The remaining cells were incubated at 37 °C, and media were sampled at the indicated times. Lipids were extracted from the media samples and subjected to thin-layer chromatography to separate free cholesterol from steroids. The fraction of cholesterol that had moved to the medium compartment at each time point was calculated. Data points are the average of samples from triplicate plates. These data are representative of two independent experiments. (●) Y1-neo; (◇) Y1-E1; (■) Y1-E12.

experiment cellular total cholesterol decreased 15% in Y1-neo (40 ± 1 vs 33 ± 1 μg of cholesterol/mg of protein; n = 3, $p < 0.01$), while there was no decrease in the cellular cholesterol content of Y1-E12 cells (85 ± 10 vs 104 ± 8 μg of cholesterol/mg of protein). Thus, Y1-E cells show reduced efflux when this is assessed by measuring radiolabeled cholesterol efflux to HDL or apoHDL/PC or by determining loss of cellular cholesterol mass.

Under conditions used to label cellular cholesterol prior to efflux measurements, incorporation of radiolabeled cholesterol into cholesteryl esters was low, presumably reflecting reduced ACAT activity under these low serum conditions. Nevertheless, to ensure that the presence of a labeled cholesteryl ester pool or ongoing cholesterol esterification was not confounding these studies of free cholesterol efflux, the experiment was repeated with the ACAT inhibitor Sandoz 58-035 present during the labeling, equilibration, and efflux periods (Figure

4, bottom). In the presence of the ACAT inhibitor, free cholesterol efflux was unchanged in both Y1-neo and Y1-E cells. This result suggests that differences in ongoing cholesterol esterification are not responsible for the difference in efflux between Y1-neo and Y1-E cell lines.

These observations strongly suggest that there is a sequestered cholesterol pool in Y1-E cells that is not available for cholesterol efflux. These results suggest that one function of apoE expression in adrenocortical cells is to promote cholesterol storage at the expense of cholesterol availability for efflux.

DISCUSSION

To explore the potential role of apoE in adrenal cholesterol homeostasis, the human apoE gene was expressed in the mouse Y1 adrenocortical cell line. In the present paper, Y1-E cells are shown to have increased cholesteryl ester content as compared to the control Y1 cell line (Table 1). Accompanying the increased cholesteryl ester content is decreased efflux of free cholesterol. It is likely that the 2–4-fold reduced cholesterol efflux in Y1-E cell lines contributes significantly to the increased cholesterol and cholesteryl ester content of Y1-E cells. Expression of apoE in Y1 cells was previously found to dramatically reduce basal or ACTH stimulated steroidogenesis. Taken together, the results suggest that apoE expression in Y1 adrenocortical cells promotes cholesterol storage at the expense of cholesterol availability for steroidogenesis or efflux from the cell.

Although apoE is expressed by a wide variety of cell types, this is the first study in which expression of this apolipoprotein has altered cellular cholesterol homeostasis. The changes in cholesterol homeostasis seen in the Y1-E cells are consistent with the pattern of apoE expression in rat adrenal cortex *in vivo*. In this case, high apoE expression is associated with cholesteryl ester accumulation and reduced steroidogenesis, whereas apoE expression is reduced when steroid production is increased by ACTH stimulation ((Prack et al., 1991; Nicosia et al., 1992). In Y1-E cells, in contrast to the *in vivo* situation, the transfected apoE gene is expressed constitutively and its expression is not reduced by ACTH (Reyland et al., 1991; Reyland & Williams, 1991). The constitutive nature of apoE expression in Y1-E cells may have disrupted normal cholesterol utilization in favor of cholesteryl ester storage. We interpret these findings as suggestive of a potential function for apoE in adrenal cells. However, additional studies are required to test the hypothesis that high apoE expression facilitates cholesteryl ester accumulation in normal adrenocortical cells *in vivo*.

We hypothesize that reduced efflux of free cholesterol from Y1-E cells reflects an alteration in cholesterol trafficking within the cell. We make this suggestion for two reasons. First, it is unlikely that enhanced esterification of free cholesterol in the Y1-E cells is removing cholesterol from the efflux pathway since maximal ACAT activity in isolated microsomes and oleate incorporation into cholesteryl ester in intact cells are not increased in Y1-E cells. Furthermore, when cells were labeled with cholesterol and efflux was examined in the presence of an ACAT inhibitor, efflux was identical to that in cells without the inhibitor and the difference between Y1-neo and Y1-E cell lines was retained. Another explanation for reduced efflux of radiolabeled cholesterol in Y1-E cells may be that unlabeled cholesterol derived from the expanded cholesteryl ester pool reduced the specific activity of free cholesterol leaving the cells. This does not appear to be the case since cholesterol mass measurements confirmed the difference in cholesterol removal from Y1-E12 cells under efflux conditions. Furthermore, if this were occurring, the appearance of radiolabeled cholesterol in culture medium should be reduced further in Y1-E cells when ACAT is inhibited. However, there was no difference from uninhibited controls seen in efflux of radiolabeled cholesterol in Y1-E or Y1-neo cells when ACAT was inhibited. These results suggest that some cholesterol in Y1-E cells is somehow isolated or sequestered from the free cholesterol pool that leaves the cell during the efflux experiments. Second, since it is the plasma membrane pool of cholesterol that is available for efflux (Johnson et al., 1991; Mahlberg & Rothblat, 1992), the reduced efflux from Y1-E cells suggests that some component of the free cholesterol pool in Y1-E cells does not equilibrate with the plasma membrane. This may also be relevant to the reduced utilization of cholesterol for steroid production in Y1-E cells (Reyland et al., 1991). Studies with the Leydig cell line MA-10 implicate the plasma membrane cholesterol pool as the immediate precursor for steroid synthesis (Freeman & Ascoli, 1982) with cholesterol from cholesteryl ester stores first mobilized to the plasma membrane and then to the mitochondrion (Nagy & Freeman, 1990; Freeman, 1987). This has also recently been reported to be the case in the Y1 cell (Gocze & Freeman, 1993). ApoE expression in Y1 cells may inhibit cholesterol trafficking to the plasma membrane, thereby reducing cholesterol efflux as well as cholesterol availability for steroidogenesis.

Increased cholesteryl ester accumulation in Y1-E cell lines does not appear to reflect increased ACAT activity or decreased cholesteryl ester turnover as monitored by [^{14}C]-oleate incorporation studies in intact cells (Figures 1–3). However, microsomal ACAT activity assayed in the absence of excess substrate cholesterol was elevated 1.5–1.9-fold in Y1-E cells, and the free cholesterol content of the isolated microsomes was also increased (Table 2). This suggests that the substrate pool available to ACAT in microsomal preparations is expanded in the Y1-E cells, although this is not evident from the measurement of [^{14}C]oleate incorporation into cholesteryl ester in intact cells.

One factor that may contribute to increased accumulation of cholesteryl ester in Y1-E cells is an apoE-mediated alteration in lipoprotein uptake or trafficking in the cell. For example, the increased cholesteryl ester stores in Y1-E cells may arise via alterations in one or both of the two major pathways through which Y1 cells acquire lipoprotein cholesterol. Y1 cells have an active LDL receptor pathway (Brown et al., 1979) which would be expected to internalize apoE-containing particles as well as LDL. One possibility is that apoE secreted by Y1-E cells enhances particle uptake by this pathway either by binding to lipoprotein particles or by facilitating the interaction of particles with the cell surface. If apoE acts in this way, the intracellular itinerary of particles or of the cholesterol derived therefrom also must be altered in Y1-E cells since these cells do not show enhanced cholesterol esterification in response to LDL. Y1 cells also acquire cholesteryl ester via the selective uptake pathway without the necessity to internalize lipoprotein particles (Pittman et al., 1987). Although lipoprotein-associated apoE is not required for selective uptake of cholesteryl ester, recent studies in HepG2 cells suggest that apoE localized on the cell surface may participate in the uptake of HDL cholesteryl esters (Leblond & Marcel, 1991). It is possible that apoE facilitates or enhances this process in Y1 cells when present at high concentrations near or on the cell surface. In either case, direct analyses of lipoprotein binding and uptake will be required to assess the role of apoE expression on these processes.

The mechanism through which apoE expression alters cellular cholesterol metabolism is not known. Experiments employing prolonged culture of parental Y1 cells in medium conditioned by the highest apoE-producing Y1-E cell line failed to increase cellular cholesterol content. This raises the possibility that apoE acts intracellularly to produce its effects. Although apoE is considered a typical secretory protein, an intracellular pool of apoE has been identified in rat liver peroxisomes and other cytoplasmic sites in addition to the expected localization in the secretory and endocytic pathways (Hamilton et al., 1990). It is interesting to speculate that apoE functions intracellularly to alter cholesterol homeostasis in adrenocortical cells.

ApoE might alter cholesterol metabolism in Y1-E cells by directly participating in cholesterol trafficking within cells or by indirectly modulating signal transduction pathways that regulate cholesterol trafficking. Alterations in both the protein kinase A and protein kinase C pathways occur in Y1-E cells. These include a chronic increase in protein kinase C activity, elevated *sn*-1,2-diacylglycerol concentrations (Reyland et al., 1992), and reduced responsiveness to cAMP (Reyland & Williams, 1991). Since the Y1-kin$^-$8 cell line that lacks functional protein kinase A activity does not have increased cholesteryl ester stores, the suppression of this signaling pathway does not appear to be responsible for increased cholesteryl ester stores in Y1-E cells. However, the roles of the protein kinase A and C pathways in regulating cholesterol efflux and cholesterol storage, two processes that are altered in Y1-E cells, are not known and will require further study. Since little is yet known about how signal transduction pathways alter cholesterol trafficking in most cell types, Y1 and Y1-E cells provide a useful model for defining these relationships.

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