

Expression of the human apolipoprotein E gene suppresses steroidogenesis in mouse Y1 adrenal cells

(lipoprotein/cholesterol transport/adrenal gland)

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Communicated by Richard J. Havel, December 27, 1990 (received for review June 16, 1990)

ABSTRACT The lipid transport protein, apolipoprotein E (apoE), is expressed in many peripheral tissues *in vivo* including the adrenal gland and testes. To investigate the role of apoE in adrenal cholesterol homeostasis, we have expressed a human apoE genomic clone in the Y1 mouse adrenocortical cell line. Y1 cells do not express endogenous apoE mRNA or protein. Expression of apoE in Y1 cells resulted in a dramatic decrease in basal steroidogenesis; secretion of fluorogenic steroid was reduced 7- to >100-fold relative to Y1 parent cells. Addition of 5-cholesten-3 β ,25-diol failed to overcome the suppression of steroidogenesis in these cells. Cholesterol esterification under basal conditions, as measured by the production of cholesteryl [¹⁴C]oleate, was similar in the Y1 parent and the apoE-transfected cell lines. Upon incubation with adrenocorticotropin or dibutyryl cAMP, production of cholesteryl [¹⁴C]oleate decreased 5-fold in the Y1 parent cells but was unchanged in the apoE-transfected cell lines. These results suggest that apoE may be an important modulator of cholesterol utilization and steroidogenesis in adrenal cells.

The lipid transport protein, apolipoprotein E (apoE), is an important modulator of cholesterol homeostasis *in vivo*. An integral component of very low density lipoproteins (VLDLs), chylomicron remnants, and some subclasses of high density lipoprotein, apoE functions as a ligand for the low density lipoprotein (LDL) receptor on peripheral cells and the LDL and remnant receptor on hepatocytes (1–4). Lipoprotein-supplied cholesterol is used for membrane synthesis in all cells and for hormone synthesis in steroidogenic cells. Unlike most apolipoproteins that are synthesized solely in the liver and intestine, apoE is synthesized in a number of peripheral tissues (5). The function of apoE in peripheral tissues is unclear. One hypothesis is that apoE is involved in transporting cholesterol from peripheral tissues to the liver for clearance, a process referred to as “reverse cholesterol transport” (5). apoE may also function in an autocrine or paracrine fashion to facilitate the local redistribution of cholesterol among cells within a tissue (5–7). apoE mRNA and protein synthesis are regulated by cell cholesterol content in macrophages (8, 9) and by changes in cholesterol biosynthesis in rat ovarian granulosa cells (10).

apoE is particularly abundant in steroidogenic tissues. In human or monkey adrenal tissue apoE is synthesized at a relative rate similar to, or greater than, that seen in the liver (5, 6). Adrenocortical cells *in vivo* and cultured adrenal cells *in vitro* synthesize steroid hormones from cholesterol in response to adrenocorticotropin (ACTH) stimulation. *In vivo* studies demonstrate a direct correlation between rat adrenal apoE expression and total cell cholesterol and an inverse

relationship between apoE expression and adrenal steroidogenesis (11).

The mouse Y1 adrenocortical cell line is functionally similar to adrenal cells *in vivo* (12, 13), although Y1 cells do not express detectable levels of apoE mRNA or protein. To investigate the relationship between adrenal apoE expression and cholesterol utilization we have transfected Y1 cells with a human genomic apoE expression vector. We report here that both basal and ACTH-stimulated steroidogenesis is dramatically reduced in the Y1 cell clones that express human apoE. These results provide evidence that apoE may play a role in regulating cholesterol utilization in adrenal cells and possibly other steroidogenic tissues.

MATERIALS AND METHODS

Cell Culture. The Y1 adrenal cell line was obtained from the American Type Culture Collection and maintained in Ham's F-10 medium supplemented with penicillin (100 units per ml), streptomycin sulfate (100 μ g/ml), 2 mM L-glutamine, 12.5% (vol/vol) heat-inactivated horse serum, and 2.5% (vol/vol) heat-inactivated fetal calf serum (FCS) (medium A). Ham's F-10 was obtained from Sigma; all additional cell culture reagents were obtained from GIBCO. For some experiments cells were cultured in medium B, which contained 5% horse serum and 2.5% FCS, or medium C, which contained 2% lipoprotein-poor FCS. Porcine ACTH (Parke-Davis), N⁶,O²-dibutyryl adenosine 3',5'-cyclic monophosphate [(Bt)₂cAMP] (Sigma) and 5-cholesten-3 β ,25-diol (25-OH-cholesterol) (Steraloids, Wilton, NH) were added to cultures as described in the figure legends.

Transfection. The human genomic apoE expression vector (pFE) was constructed from the plasmids pJS1 and pJS15, provided by J. Smith and J. Breslow (The Rockefeller University). The 2-kilobase (kb) *Nar* I-*Eco*RI fragment from pJS1 was inserted into vector pT7/T3 α -18 (BRL) to yield pFE1. The 2-kb *Eco*RI fragment from pJS15 was then inserted into pFE1 at the unique *Eco*RI site to yield pFE. pFE contains the entire genomic sequence of human apoE, including \approx 500 nucleotides (nt) 5' of the transcription start site and 628 nt 3' of the polyadenylation site. Calcium phosphate-mediated gene transfer was essentially as described (14). Y1 cells were cotransfected with 2 μ g of pSV2neo and 18 μ g of pFE or pUC8; transfected cells were selected in medium A containing G418 sulfate (active form) at 200 μ g/ml (Geneticin, GIBCO). Survivors were cloned and maintained

Abbreviations: apoE, apolipoprotein E; ACTH, adrenocorticotropin; FCS, fetal calf serum; (Bt)₂cAMP, N⁶,O²-dibutyryl adenosine 3',5'-cyclic monophosphate; 25-OH-cholesterol, 5-cholesten-3 β ,25-diol; nt, nucleotide(s); LDL, low density lipoprotein; VLDL, very low density lipoprotein.

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in medium A containing G418 sulfate (active form) at 100 $\mu\text{g}/\text{ml}$.

Quantification of apoE mRNA. For RNA blot analysis, 10 μg of total RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to Nytran (Schleicher & Schuell), crosslinked with UV light (Stratalinker), and hybridized to a single-stranded human apoE cDNA probe labeled with [α - ^{32}P]dCTP (Amersham; 800 Ci/mmol; 1 Ci = 37 GBq). As control for quantitative RNA recovery, the same blot was stripped by boiling in distilled H_2O for 15 min and reprobed with a rat glyceraldehyde phosphate dehydrogenase cDNA probe (from K. Marcu, State University of New York at Stony Brook) prepared by random priming with [α - ^{32}P]dCTP (Amersham; 800 Ci/mmol). DNA-excess solution hybridization assays for human apoE mRNA (15) and mouse apoE mRNA (16) were done as described.

Metabolic Labeling and Immunoprecipitation. Cells were incubated for 14 hr in 50% Ham's F-10 medium/50% methionine-free Dulbecco's modified Eagle's medium supplemented with [^{35}S]methionine (50 $\mu\text{Ci}/\text{ml}$; >800 Ci/mmol; Amersham), 5% dialyzed horse serum, and 2.5% dialyzed FCS. Culture medium was adjusted to aprotinin and leupeptin (Sigma) each at 12 $\mu\text{g}/\text{ml}$, centrifuged to remove cells, and analyzed by using a double-antibody procedure (17). Excess goat anti-human apoE antisera (Calbiochem) was used as primary antibody, and sheep anti-goat gamma globulin (Capel Laboratories) was used as secondary antibody. Control immunoprecipitates contained normal goat serum as primary antibody. Immunoprecipitates were resolved by NaDodSO₄/10% polyacrylamide gel electrophoresis followed by fluorography (17). To quantify the radioactivity incorporated into apoE protein, the apoE band was cut from the dried gel and solubilized; radioactivity was counted by liquid scintillation spectrometry (17).

Measurement of Secreted Steroids. Fluorogenic steroids were measured as described (18) by using a modification of the method of Kowal and Fiedler (19). Individual steroid products were analyzed by HPLC on a reverse-phase C₁₈ column. Products were separated isocratically with a mobile phase of methanol/acetonitrile water (11:45:44) at a flow rate of 1.1 ml/min (20). HPLC standards, obtained from Sigma, were as follows: 18-hydroxy-11-deoxycorticosterone, corticosterone, 11-deoxycorticosterone, 20 α -hydroxypregn-4-en-3-one, and progesterone.

Cholesterol Esterification. [^{14}C]Oleate (ICN; 57.4 $\mu\text{Ci}/\text{mmol}$) was bound to albumin as described (21). Cells were cultured in the presence of [^{14}C]oleate for 6 hr. Lipids were extracted from labeled cells, and the amount of cholesteryl [^{14}C]oleate and [^{14}C]triglycerides was determined after thin-layer chromatography (21). [*cholesteryl*-1,2,6,7- $^3\text{H}(\text{N})$]-Oleate (NEN; 82.9 Ci/nmol) was added to each sample to determine recovery. After lipid extraction, cells were scraped into 1.0 M NaOH for protein determination (22).

Miscellaneous. RNA was prepared by the guanidine isothiocyanate/ethanol precipitation method (23). Cell protein concentration was determined by the method of Lowry *et al.* (22) by using bovine serum albumin (Sigma) as a standard. Lipoprotein-poor FCS was prepared as described (21). Human [^{14}C]apoE was prepared as described (24).

RESULTS

Expression of Human apoE mRNA and Protein. Y1 cells were transfected with pSV2neo plus the human genomic apoE expression vector pFE. Individual colonies of G418 sulfate-resistant cells were isolated and expanded into cell lines (Y1-E1–Y1-E16). As control for expression of the neomycin-resistance gene, Y1 cells were cotransfected with pSV2neo and pUC8. Approximately 200 G418-resistant clones were pooled; this cell line is referred to as Y1-neo. No

endogenous apoE mRNA was detected in either the Y1 parent or Y1-neo cell lines when assayed by DNA-excess solution hybridization with a mouse cDNA probe (data not shown). To screen clones for expression of human apoE mRNA, total RNA was prepared from Y1 parent cells and 16 Y1-E cell lines and subjected to Northern blot analysis. Fig. 1 shows that the human apoE mRNA expressed by Y1-E clonal cell lines is identical in size to apoE mRNA from the human liver cell line HepG2. The transcription start site of HepG2 apoE mRNA and the apoE mRNA expressed in the Y1-E clones are also identical, as determined by primer extension (data not shown). Human apoE mRNA was quantified by DNA-excess solution hybridization by using a 221-nt single-stranded probe, 160 nt of which are complementary to human apoE mRNA. Previous work has shown that human apoE mRNA completely protects the expected 160-nt probe fragment from digestion by S1 nuclease (15). No hybridization above the limit of detection (0.1 pg mRNA per μg of RNA) was seen with RNA from the Y1 parent or the Y1-neo cell lines. Among Y1-E clones examined, expression varied as much as 150-fold; representative values are shown in Table 1. Clone Y1-E15 expressed 0.8 pg of apoE mRNA per μg of total RNA, a level considerably lower than that seen in the rat adrenal gland *in vivo* (25). Clone Y1-E1 expressed 17 pg of apoE mRNA per μg of total RNA, a level within the range observed in rat adrenal gland *in vivo* (25) and comparable to the apoE mRNA concentration of the human liver cell line, HepG2 (22 pg per μg of total RNA) (26). The Y1-E12 cell clone expressed the highest amount of apoE mRNA, 120 pg of apoE mRNA per μg of total RNA. This level is similar to that seen in rat hepatocytes (25) and is approximately twice the maximal level seen in rat adrenal gland *in vivo* (11). No apparent toxicity resulted from expression of human apoE at this level; all Y1-E cell lines had growth rates similar to, or faster than, the Y1 parent or Y1-neo cell lines (data not shown). Secreted human apoE protein was detected by immunoprecipitation of medium from cells incubated with [^{35}S]methionine. Fig. 2 shows that the goat anti-human apoE antibody precipitated a protein from Y1-E1, Y1-E16, and Y1-E12 culture media identical in molecular weight to apoE precipitated from HepG2 culture medium. No apoE protein was detected in culture medium from the Y1 parent cells (lane 6) or in control immunoprecipitations that used nonimmune goat serum as the primary antibody (lane 1). The amount of secreted apoE protein correlated approximately with the mRNA content of the individual clonal lines analyzed. apoE represented $\approx 0.5\%$ of the total ^{35}S -labeled proteins secreted

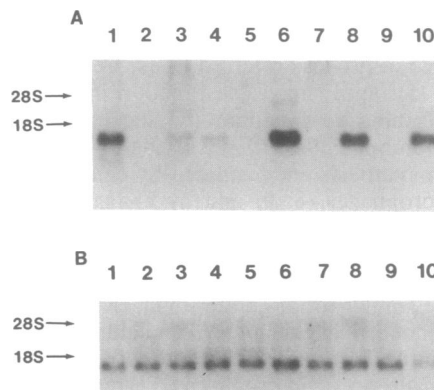


FIG. 1. Northern blot analysis of RNA from Y1 parent, Y1-E clones, and HepG2 cells. (A) Blot is probed with human apoE cDNA. (B) The same blot was stripped and reprobed with a rat glyceraldehyde phosphate dehydrogenase cDNA probe. Lanes: 1, Y1-E1; 2, Y1-E2; 3, Y1-E6; 4, Y1-E9; 5, Y1-E11; 6, Y1-E12; 7, Y1-E14; 8, Y1-E16; 9, Y1 parent; 10, HepG2.

Table 1. Quantification of fluorogenic steroid production and apoE mRNA

Cell line	apoE mRNA, pg/ μ g of RNA	Fluorogenic steroid, μ g/mg of cell protein		
		Basal	ACTH	(Bt) ₂ cAMP
Y1	<0.1	2.79	5.47	28.72
Y1-neo	<0.1	3.08	NT	26.33
Y1-E1	17.2	<0.01	<0.01	<0.01
Y1-E12	120.0	0.11	0.14	0.28
Y1-E15	0.8	0.38	NT	10.00
Y1-E16	57.2	0.05	0.06	0.17

Cells were plated on day 1 in medium A. On day 3, medium was replaced with medium B (basal conditions), medium B/ 10^{-7} M ACTH or medium B/1 mM (Bt)₂cAMP, as indicated. After an additional 24 hr, culture medium was assayed for fluorogenic steroids as described. apoE mRNA was assayed by hybridization in DNA-excess solution with a mouse cDNA probe (Y1 and Y1-neo cells) or a human cDNA probe (Y1-E cell lines). Numbers represent the average of three or more measurements. Variation between measurements was <10%. NT, not tested.

by Y1-E1 cells, a level comparable to the human liver cell line HepG2 (0.4%) and the human and monkey adrenal gland (0.5–1.2%) (5, 6). Thus, apoE mRNA and protein levels in the various Y1-E clones are within the range seen in liver and adrenal cells *in vivo* (15, 25).

Analysis of Steroid Production in Y1-E Cells. As an indicator of cholesterol utilization in the Y1-E cells, we measured secreted steroids by using the fluorometric assay of Kowal and Fiedler (19). Table 1 shows that human apoE expression in Y1 cells dramatically decreased the secretion of fluorogenic steroids. Secretion of fluorogenic steroid under basal conditions was reduced 7- to >100-fold in the Y1-E cell lines compared with the Y1 parent or the Y1-neo cells. Although basal steroidogenesis was suppressed in all Y1-E cell lines, the cell line that expresses the lowest level of apoE mRNA, Y1-E15, was the least affected. ACTH stimulated steroid secretion by the Y1 parent cells 2-fold but had no effect on steroid secretion by the Y1-E cell line examined. Conceivably, the lack of ACTH responsiveness in the Y1-E cell lines could reflect the loss of ACTH receptors or a block in the generation of the second messenger cAMP. To address these possibilities Y1 cells were treated with the cAMP analog, (Bt)₂cAMP, a potent stimulator of steroidogenesis. Incubation with 1 mM (Bt)₂cAMP increased secretion of fluorogenic steroid by Y1 parent and Y1-neo cells 8- to 10-fold (Table 1). Steroid production by the Y1-E12 and Y1-E16 cell lines was

increased 2- to 4-fold under these conditions. Steroid secretion by Y1-E15 cells was increased 26-fold by (Bt)₂cAMP, although this level of steroid production was still 3-fold lower than that seen for Y1 parent cells under identical conditions. ACTH or (Bt)₂cAMP had no effect on apoE mRNA or protein synthesis in the Y1-E cell lines (data not shown).

In rat ovarian theca/interstitial cells, exogenous apoE alters the profile of secreted steroids by inhibiting the conversion of progesterone to androgen (27). The major steroid products of Y1 cells, 20 α -dihydroprogesterone and 11 β -hydroxy, 20 α -dihydroprogesterone (12, 13) can be detected with the fluorogenic steroid assay used above. To determine whether the Y1-E cells produce nonfluorogenic steroids, we analyzed culture medium from Y1 parent and Y1-E12 cells by HPLC; Fig. 3 shows an HPLC profile of culture medium from these cells incubated for 24 hr in medium A/ 10^{-7} M ACTH. At 254 nm two steroid products were detected in medium from the Y1 parent cells. Peak A, the minor product, is presumably 11 β -hydroxy, 20 α -dihydroprogesterone, whereas peak B has a retention time identical to the standard 20 α -hydroxy-pregn-4-en-3-one (20 α -dihydroprogesterone). No steroids were detected in medium from the Y1-E12 cell line, even when 10 times more medium was analyzed. Progesterone and its hydroxylated derivatives, but not pregnenolone, can be detected by absorption at 254 nm, whereas pregnenolone can be detected at 210 nm. When media extracts were separated by HPLC and analyzed at 210 nm, no pregnenolone could be found in either the Y1 parent or Y1-E12 cell lines. The absence of pregnenolone from the parent line presumably reflects its rapid conversion to other steroids. In contrast, the absence of pregnenolone and distal steroid products from the Y1-E12 line appears to reflect suppression at, or before, pregnenolone formation.

When available, Y1 cells preferentially utilize lipoprotein cholesterol for steroid hormone synthesis (12). To determine whether the Y1-E cell lines can utilize a nonlipoprotein substrate for steroid production, ACTH-stimulated Y1 parent, Y1-E1, and Y1-E12 cells were incubated in lipoprotein-poor medium (medium C) with or without 25-OH-cholesterol at 15 μ g/ml. As seen in Fig. 4, the parent line effectively converted 25-OH-cholesterol to fluorogenic steroid. In contrast, production of fluorogenic steroid from 25-OH-cholesterol was reduced 41- and 4-fold, respectively, in the

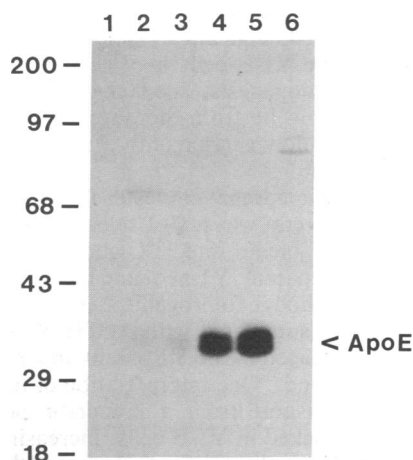


FIG. 2. Immunoprecipitation of human apoE. Trichloroacetic acid-precipitable counts (200,000) from each sample were analyzed. Lanes: 1, nonimmune goat serum control; 2, HepG2; 3, Y1-E1; 4, Y1-E16; 5, Y1-E12; 6, Y1 parent. $M_r \times 10^{-3}$ is at left.

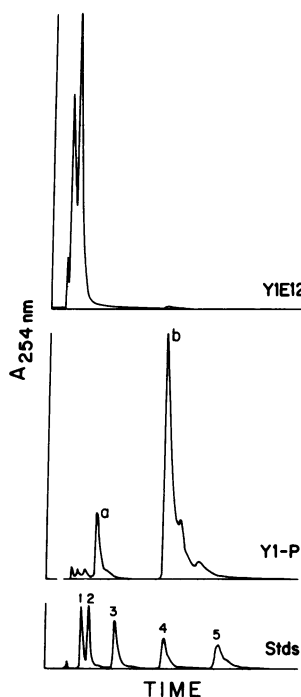


FIG. 3. HPLC analysis of secreted steroids. Y1 parent and Y1-E12 cells were grown to confluence in 60-mm dishes and then incubated for 25 hr with ACTH (10^{-7} M) in medium A. The medium was then extracted with 8 ml of methylene chloride, and 5 ml of the extract was dried under nitrogen and resolubilized in 150 μ l of methanol. Fifteen microliters of the Y1 parent product and 150 μ l of the Y1-E12 product were analyzed by HPLC. Standards are as follows: 1, 18-hydroxy-11-deoxycorticosterone; 2, corticosterone; 3, 11-deoxycorticosterone; 4, 20 α -hydroxy-pregn-4-en-3-one; and 5, progesterone.

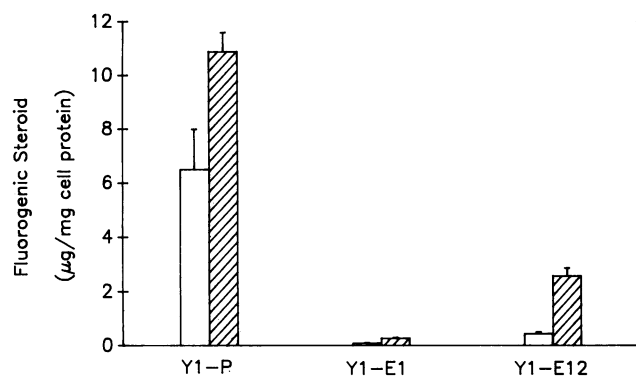


FIG. 4. Cells were plated on day 1 in medium B/ACTH (10^{-7} M). On day 2, medium was replaced with medium C (open bars) or medium C/25-OH-cholesterol at $15 \mu\text{g/ml}$ (hatched bars) for an additional 24 hr. Fluorogenic steroid in the culture medium was measured as described.

Y1-E1 and Y1-E12 cell lines. These results suggest that the suppression of steroidogenesis in the Y1-E cells may occur, at least in part, at the mitochondrion level.

Incorporation of [^{14}C]Oleate into [^{14}C]Cholesteryl Oleate. To determine whether expression of apoE in Y1 cells alters regulation of cholesterol esterification, we measured the incorporation of [^{14}C]oleate into [^{14}C]cholesteryl oleate in the Y1 parent, the Y1-neo cells, and three Y1-E cell lines with and without ACTH or (Bt) $_2$ cAMP stimulation. As seen in Table 2, in the absence of ACTH, synthesis of [^{14}C]cholesteryl oleate is comparable in all cell lines analyzed, suggesting that apoE expression has no effect on esterification rate. Incorporation of [^{14}C]oleate into triglyceride was equivalent in all cell lines tested, indicating that uptake of [^{14}C]oleate on albumin did not vary (data not shown). Upon incubation with 10^{-7} M ACTH, incorporation of [^{14}C]oleate into [^{14}C]cholesteryl oleate decreased 5- to 6-fold in the Y1 parent and Y1-neo cells, whereas no decrease was seen in the three Y1-E cell lines examined. Similarly, stimulation of steroidogenesis with (Bt) $_2$ cAMP reduced [^{14}C]cholesteryl oleate formation in the Y1 parent and Y1-neo cell lines but was without effect on Y1-E cell lines. These results suggest that apoE may affect availability of cholesterol for steroid synthesis.

DISCUSSION

The Y1 adrenal cell line has been used extensively as a model for adrenal cholesterol metabolism and steroidogenesis *in vitro*. Although functionally similar to adrenal cells *in vivo*, Y1 cells do not have detectable levels of apoE mRNA or

Table 2. Cholesterol esterification

Cell line	Production of [^{14}C]cholesteryl oleate, pM/mg of cell protein		
	Basal	ACTH	(Bt) $_2$ cAMP
Y1	1683	546	331
Y1-neo	2982	563	367
Y1-E1	2765	3164	2957
Y1-E16	2145	2483	1860
Y1-E12	2511	2484	3251

Cells were plated on day 1 in medium A. On day 2 the medium was replaced with medium B, and on day 3 the medium was again replaced with medium B (basal conditions), medium B/ 10^{-7} M ACTH or medium B/1 mM (Bt) $_2$ cAMP, as indicated. After 24 hr, medium was replaced with fresh medium containing [^{14}C]oleate bound to albumin ($10,000 \text{ dpm/nmol}$) for 6 hr. Lipids were analyzed as described. Numbers represent the average of two measurements; variation between measurements was $<15\%$.

protein, a normal product of adrenal glands and other steroidogenic tissues. As a model to study the role of apoE in adrenal cholesterol homeostasis, we introduced the human genomic apoE gene into Y1 cells by transfection and isolated Y1 clonal cell lines that express human apoE mRNA and protein. Steroid hormone production was analyzed in these cell lines to indicate cholesterol availability and utilization. These experiments show that apoE expression in Y1 cells markedly decreases both basal and ACTH- or (Bt) $_2$ cAMP-stimulated steroidogenesis.

Although apoE is commonly thought of as a plasma cholesterol transport protein, expression of apoE in many peripheral tissues suggests it may also function in cellular cholesterol metabolism. In recent studies (11), treatment of rats with ACTH or 4-aminopyrazolopyrimidine was shown to decrease adrenal gland cholesterol and apoE mRNA levels and to increase plasma corticosterone levels. In contrast, when rats were treated with dexamethasone to suppress ACTH release, adrenal cholesterol content, apoE mRNA, and apoE synthesis increased, whereas plasma corticosterone decreased. Thus, *in vivo*, apoE expression correlates positively with adrenal cholesterol content and negatively with adrenal steroidogenesis. Taken together, the *in vivo* results discussed above and the results presented here are consistent with a role for apoE in regulating availability and/or utilization of cholesterol for steroidogenesis.

Although steroidogenesis was suppressed in all Y1-E cell lines examined, clone Y1-E15, which expresses the lowest level of apoE mRNA, was the least affected. The level of apoE mRNA expressed by this cell line is about 10-fold lower than that seen in adrenal glands from rats treated with ACTH or 4-aminopyrazolopyrimidine (11). If expression of physiological levels of apoE mRNA completely suppresses steroidogenesis in adrenal cells, how is it that adrenal cells *in vivo* express apoE and produce steroid hormones? One explanation is suggested by *in situ* hybridization studies in the rat adrenal gland (M. Nicosia and D.L.W., unpublished data). These studies show that apoE mRNA levels vary substantially among cortical zones and are regulated in a zone-specific manner in response to agents that alter steroidogenesis. This cellular heterogeneity in apoE expression may permit steroid production to occur in some cells that express little or no apoE, whereas apoE expression is high in other cells that are not producing steroids.

Secondly, down-regulation of apoE expression may be required for steroidogenesis in adrenal cells. Although apoE expression is decreased by ACTH and conditions that deplete adrenal cholesterol stores in the rat adrenal gland, apoE mRNA and protein synthesis were not altered by ACTH or (Bt) $_2$ cAMP in the Y1-E cell lines. Failure of apoE to be down-regulated in the Y1-E cells may account for the observed block in steroidogenesis. The lack of apoE regulation in the Y1-E cells could be from the absence of regulatory elements upstream or downstream of the genomic DNA used in the expression vector.

ApoE may influence adrenal cholesterol homeostasis and steroidogenesis in several ways. (i) Under basal conditions, incorporation of [^{14}C]oleate into [^{14}C]cholesteryl oleate is comparable in the Y1 parent, Y1-neo, and the three Y1-E cell lines analyzed, even though fluorogenic steroid output varied by >100 -fold. When stimulated with ACTH or (Bt) $_2$ cAMP, esterification is decreased in the Y1 parent and Y1-neo lines but not in the Y1-E lines. One interpretation of this result is that cholesterol transport from a precursor pool to the mitochondrion is blocked in Y1-E cells, increasing the substrate level for cholesterol esterification even with ACTH or (Bt) $_2$ cAMP. Although the rate-limiting step in steroidogenesis is generally recognized to be cholesterol side-chain cleavage, the true rate-limiting step in steroidogenesis is probably delivery of cholesterol to the inner mitochondrial membrane,

the site of side-chain cleavage. A number of adrenal and liver proteins have been identified that may be involved in intracellular transport and mitochondrial cholesterol transport (28–30).

(ii) A specific precursor pool of unesterified cholesterol could be depleted in the Y1-E cells, perhaps because of enhanced apoE-mediated cholesterol efflux from the cell membrane. In the mouse Leydig cell line MA-10 Freeman (31) has shown that the major pool of unesterified cholesterol for steroidogenesis comes from the plasma membrane. If this is the case, the alteration in Y1-E cells may be quite specific for a distinct precursor pool because basal esterification rates were not substantially different among examined Y1-E lines.

(iii) Enhanced cholesterol esterification from a distinct precursor pool, or decreased cholesteryl ester hydrolysis, could reduce the unesterified cholesterol available for steroidogenesis in the Y1-E cells. This result is consistent with the sustained level of [¹⁴C]oleate incorporation into cholesteryl ester in ACTH-treated Y1-E cells. Because we have seen no major differences in cholesterol or cholesteryl ester content between the Y1 parent, Y1-neo, and Y1-E cell lines (data not shown), such enhanced esterification must reflect a distinct and small cholesterol pool. Consistent with this possibility is a recent report from Adelman and St. Clair (32) showing that the addition of apoE to pigeon β -VLDL, normally devoid of apoE, stimulated cholesterol esterification in mouse peritoneal macrophages with no change in cholesteryl ester accumulation. Similarly, Kowal *et al.* (33) showed that apoE-enriched rabbit β -VLDL stimulated cholesterol esterification in human skin fibroblasts via the LDL-receptor-related protein without detectable uptake of β -VLDL.

The results of experiments with 25-OH-cholesterol may indicate alterations at other points in the steroidogenic pathway in the Y1-E cells. The mitochondrial side-chain cleavage enzyme can convert 25-OH-cholesterol to pregnenolone and thereby stimulate the production of fluorogenic steroids in Y1 cells. In contrast to the Y1 parent line, fluorogenic steroid production from precursor 25-OH-cholesterol is reduced 41-fold in the Y1-E1 cells and 4-fold in the Y1-E12 cells. These results suggest a block or inhibition in Y1-E cells in cholesterol side-chain cleavage activity or a restriction in the access of the precursor to the mitochondrial enzyme. Whether this effect is distinct from, or related to, the sustained level of cholesterol esterification in Y1-E cells treated with ACTH or (Bt)₂cAMP is unclear. Previous studies have shown that inhibition of cholesterol side-chain cleavage with aminoglutethimide prevents the ACTH-stimulated decrease in cholesterol esterification in Y1 cells (12). Thus, it is possible that the primary block in the Y1-E cells is at the mitochondrion level. Future experiments with isolated mitochondria should allow uncoupling of cholesterol esterification, intracellular transport, and mitochondrial events. Such studies will be necessary to explore the mechanism by which apoE expression suppresses steroidogenesis in Y1-E cells.

We are grateful to K. Woodward, D. Mahaffee, and Q.-Y. Zhao for expert technical assistance and to S. M. Anderson for helpful comments on the manuscript. This work was supported by National Institutes of Health Grants HL32868 (D.L.W.) and HL28306 (J.T.G.)

and by an American Cancer Society Institutional Research Grant (M.E.R.). M.M.P. is supported by a National Research Service Award (DK07521), and P.F. is a scholar of the Fogarty International Center.

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