Pergamon

J. Steroid Biochem. Molec. Biol. Vol. 66, No. 5–6, pp. 335–345, 1998

Printed in Great Britain
PII: S0960-0760(98)00062-4
0960-0760/98 $19.00 + 0.00

Effects of Prolonged ACTH-stimulation on Adrenocortical Cholesterol Reserve and Apolipoprotein E Concentration in Young and Aged Fischer 344 Male Rats

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Changes in the morphology of rat adrenal cortex with age include increased accumulations of lipid droplets and lipofuscin granules. Because glandular concentrations of cholesteryl esters (CE) and apolipoprotein (apo) E are also increased in parallel, the utilization or metabolism of lipid-droplet stored CE for steroidogenesis might be altered in aging cells. To explore this possibility, adrenocortical cholesterol storage and utilization were studied in 3–6 months-old (mo) (Y) rats and 20–23 mo (O) Fischer 344 male rats. Both groups received either adrenocorticotropin (ACTH1-39, Acthar gel) or gelatin alone daily for seven consecutive days.

We found that: (a) the CE concentration in O rats, but not Y animals, was diminished by ACTH. The depleted CE in stimulated-O rats was replenished within five days post stimulation. Failure to deplete CE in stimulated-Y rats was not associated with an insufficient dose of the hormone, since stimulation of Y animals with higher doses of ACTH actually increased the CE concentration. In contrast, adrenocortical free cholesterol concentration remained constant during stimulation regardless of age. (b) The depleted CE in stimulated-O rats was principally comprised of cholesteryl adrenate, cholesteryl arachidonate and cholesteryl cervonate. The accumulated CE in stimulated-Y animals was primarily comprised of cholesteryl adrenate, cholesteryl arachidonate and cholesteryl oleate. (c) Whereas in stimulated-Y rats adrenal apoE concentration declined, the concentration in stimulated O animals was well maintained. (d) *In vitro*, adrenal homogenate or cytosolic fraction from stimulated-O rats displayed a higher capacity to hydrolyze exogenous CE than its Y counterpart. However, cholesterol esterification with external fatty acid substrates in adrenal homogenate or microsomal fraction was comparable in the two age-groups.

Our findings revealed altered adrenocortical cholesterol reserve in O rats to cope with prolonged ACTH-stimulation. Changes in apoE levels and CE hydrolysis activity may be factors associated with this alteration. Depletion and accumulation of adrenocortical CE are reflected in parallel changes in cholesteryl adrenate and cholesteryl arachidonate, suggesting physiologic importance of these polyunsaturated fatty acids during sustained steroidogenesis. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Although the adrenocortical cell is able to synthesize cholesterol, cellular cholesterol is principally derived from plasma lipoproteins. The internalized cholesterol is utilized as a precursor for steroid synthesis and as a membrane component. Excess cholesterol is esterified

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Received 19 Nov. 1997; accepted 20 Mar. 1998.

and stored in lipid droplets as a reserve for further needs. The adrenocortical cholesteryl esters are rich in polyunsaturated fatty acids, particularly, adrenic acid, which differ from those polyunsaturated fatty acids esterified with cholesterol in plasma lipoproteins. Thus, the presence of numerous lipid droplets, a relatively high cholesteryl ester content and unique cholesteryl ester composition are the characteristics of the adrenocortical cell in the human and rodents [1–3].

There is a great deal of evidence to indicate that short term stimulation of the rat adrenal cortex with ACTH for minutes or hours, *in vivo* or *in vitro*, results in depletion of stored adrenal cholesteryl esters [1]. The specific cholesteryl ester species and the mechanism involved in this depletion have been studied extensively [1, 4, 5]. In contrast, current knowledge regarding the effect of prolonged stress for days or longer on adrenal cholesterol metabolism *in vivo* is relatively limited and inconsistent [6, 7]. In fact, prolonged stimulation of the adrenal cortex frequently occurs during physical stress, severe illness, depression, and anxiety [8–11]. These conditions are all associated with change(s) in one or more adrenocortical characteristic(s): glandular size, zonal morphology, steroidogenic activity and steroid metabolic pathways. Thus, there is a need to further study adrenocortical cholesterol reserve in response to sustained stimulation.

Aging is associated with increased vulnerability to chronic stress, which has been linked to functional alterations in the hypothalamic–pituitary–adrenal axis [12]. Aging in rats is associated with a diminished maximal steroidogenic response to ACTH [13, 14], although differing opinions exist [15]. Nevertheless adrenocortical cholesteryl ester content in animals increases with age [3, 14], accompanied by massive deposition of lipofuscin, lipid droplets and lipofuscin–lipid droplet clusters [3]. Thus, age would be an additional factor influencing the adrenocortical cholesterol metabolic state, particularly if sustained glucocorticoid production is demanded.

The role of apolipoprotein (apo) E as a cholesterol transporter is well recognized [16]. The rat adrenal cortex synthesizes apoE, and has a high content of the apolipoprotein. Current evidence indicates that the rat adrenocortical zona fasciculata cell *in vivo* is encircled or covered with a "blanket" of apoE [17]. Prack *et al.* [18] have shown that total cholesterol content and apoE mRNA concentration in the rat adrenal are positively correlated, but are regulated independently by ACTH. The latter was based on the observation that stimulation of normal rats with ACTH in the presence of aminoglutethimide which blocks the mitochondrial cholesterol side-chain cleavage reaction, causes a diminution of adrenal apoE mRNA content despite an increase in total cholesterol content. As aforementioned, total adrenal cholesterol content in rats increases with age [3, 14], which is also positively correlated with an increased glandular concentration of apoE [19]. Whether the apoE concentration in aged animals would decrease under prolonged ACTH-stimulation is not known.

The following questions were addressed in the present work: (a) Does adrenal cholesterol concentration change with longer periods of stimulation with ACTH? (b) Is the change reversible after cessation of stimulation? (c) What types of cholesteryl esters are concomitantly altered in storage? (d) Is the change accompanied by an altered apoE concentration? (e) Is there an age-related difference associated with the change(s)?

MATERIALS AND METHODS

Materials

ACTH ($ACTH_{1-39}$, Acthar gel, Rhone-Poulenc Rorer Pharmaceuticals, Fort Washington, PA) was obtained from the pharmacy of this medical center. Cholesterol oxidase (Nocardia) was purchased from Calbiochem (San Diego, CA). Peroxidase (horseradish), cholesterol and cholesteryl esters were purchased from Sigma (St. Louis, MO). Goat anti-human-apoE polyclonal antiserum and peroxidase-conjugated rabbit anti-goat IgG antibody were purchased from Chemicon (Temecula, CA). Cholesteryl adrenate and cholesteryl cervonate were synthesized according to our previous report [2]. Silica gel-60 (230–400 Mesh) was purchased from EM Science (Gibbstown, NJ). Cholesteryl [^{14}C]oleate, [^{3}H]oleic acid, [^{3}H]oleyl-CoA, [^{14}C]cholesterol and [^{3}H]cholesteryl oleate were purchased from NEN (Boston, MA) and Amersham (Arlington Hts., IL).

Experimental conditions

Fischer 344 male rats were obtained from the National Institute on Aging U.S.A. The animals were divided into Y (3–6 months-old (mo)) and O (20–23 mo) groups. Prior to the studies all rats were kept in the Animal Facilities of this medical center and fed with Purina chow (#5001) containing 23% crude proteins and 4.5% fats. The protocol for stimulation of rats with ACTH was adopted from Rebuffat *et al.* [20] with slight modifications. Y and O rats were injected subcutaneously with ACTH (Acthar gel 6–36 U kg^{-1}) at 9:30–11:30 a.m. per day for seven consecutive days. Y and O rats injected subcutaneously with 16% gelatin served as unstimulated controls. Twenty-four hour urine was collected for determination of free corticosterone content which served as an index of stimulated steroidogenesis. To harvest adrenal glands rats were euthanized by guillotine 24 h after the last injection. Some stimulated rats were kept for five additional days without any treatment and then euthanized (post stimulation). Adrenal

weight was measured using a Mettler analytical balance. To isolate the adrenal cortex, each fresh adrenal gland was chilled on a glass dish set directly above dry ice in an ice bucket, and then quartered. The medulla was separated from the cortex in a semi-frozen adrenal quarter with a sharp scalpel. Whole adrenal glands and adrenal cortices were kept at −60°C prior to analysis.

Determination of cholesterol and cholesteryl ester content

Adrenal samples were extracted with a chloroform and methanol mixture according to a volume ratio of 3 (aqueous):8 (chloroform):4 (methanol) [21]. The protocol for cholesterol determination was reported [2, 3]. In brief, cholesterol was determined by a cholesterol oxidase–peroxidase coupling method before (unesterified) and after hydrolysis (total). The difference between these two determinations was designated as cholesteryl ester content. The enzymic reaction-induced fluorescence was recorded using an Aminco Fluorospectrophotometer. No cholesterol sulfate was detected under these conditions.

Specific cholesteryl ester content was quantified by a reversed-phase HPLC method with cholesteryl heptadecanoate as an internal standard [2, 3]. Briefly, adrenal homogenate was extracted after addition of cholesteryl heptadecanoate. Cholesteryl esters were separated from other major lipids of the extract with a silica gel-60 column. The eluted cholesteryl ester fraction was collected and subjected to analysis using a Waters HPLC system with a Beckman 5 μ Ultrasphere ODS column (25 cm × 4.6 mm i.d.), or with two columns in tandem with an additional Waters 10 μ Bondapak C18 column (30 cm × 3.9 mm i.d.) connected before the Beckman column. A mixture of acetonitrile and isopropanol (50:50, v/v) was employed as a mobile phase. The isocratic flow-rate was set at 1 ml min^{-1}. The separation was monitored at UV 210 nm. A coupled Shimadzu recorder/integrator (model CR3A, Japan) was used for data analysis.

Immunodetection of adrenal apoE

We have established a Western blotting method to evaluate adrenal apoE concentrations in rats [19, 22]. In brief, adrenal homogenates were boiled and then subjected to electrophoresis with a 10% discontinuous SDS-polyacrylamide gel in a Mini-Protean II Dual Slab Cell (Bio-Rad, Hercules, CA). Electrophoretic transfer of proteins from SDS-gels to nitrocellulose membranes (NC BA85, Schleicher and Schull, Keen, NH) was carried out at 4°C in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), under a constant voltage of 50 V for 24 h, and then 100 V for an additional 2 h in an ice-cooling system. The blots were then incubated with goat anti-human-apoE polyclonal antiserum at 4°C, overnight. Peroxidase-conjugated rabbit anti-goat IgG antibody served as the second antibody. Immunodetection was carried out by a luminographic method using a commercial kit (Amersham, Cat. No. RPN2109). SDS-gels were stained with Coomassie brilliant blue R-250. Luminograms were scanned by a densitometer (SciScan 5000) with a BioAnalysis software (United Stated Biochemical, Cleveland, OH) in the Molecular Biology Core Laboratory of the Case Western Reserve University School of Medicine.

Preparation of subcellular fractions and lipid droplet-depleted homogenates

The protocols for subcellular fractionation and lipid-droplet removal were reported previously [2, 23]. In brief, centrifugation procedures were carried out at 4°C. Decapsulated adrenal tissue was homogenized in a solution containing 8.6% (w/v) sucrose (8–10 mg ml^{-1}). The homogenate was centrifuged at 756 g for 10 min in a Beckman centrifuge Model J2-21 with a rotor JA-20 to remove cell debris and nuclear fraction. The resultant supernatant was then centrifuged at 12 100 g for 15 min to remove mitochondrial pellets. The post-mitochondrial supernatant was further centrifuged at 145 000 g for 1 h in a Backman ultracentrifuge model L5-75 with a rotor 50Ti to precipitate the microsomes. The post-microsomal supernatant was subjected to removal of its lipid droplets by a sucrose density gradient centrifugation at 145 000 g with a swinging bucket rotor SW50.1 for an additional 1 h. The supernatant after reduction of lipid droplets was designated as the cytosolic fraction. Independently, lipid droplets were removed directly from decapsulated adrenal homogenates without prior fractionation. After removal of lipid droplets, the remaining supernatant and pellet were re-homogenized, and the reconstituted homogenate was designated as "lipid droplet-depleted homogenate".

Determination of cholesterol esterification activity

The protocols for determination of cholesterol esterification activity were adopted from literature [24, 25] with minor modifications. Determination of the activity in lipid droplet-depleted homogenates was carried out in a buffered solution, pH 6.8, containing 24 mM potassium phosphate, 47 mM ATP, 0.2 mM CoA, 6 mM MgCl2, 24 mM glutathione, [^{3}H]oleic acid (186×10^3 dpm), and 200 μg homogenate protein, in a final volume of 0.85 ml. Incubation was performed at 37°C for 30 min. The resultant mixture was extracted after additions of cholesteryl [^{14}C]oleate (1.1×10^3 dpm) as an internal marker and 100 μg of authentic cholesteryl oleate as a carrier. The newly formed cholesteryl [^{3}H]oleate in the extract was separated from [^{3}H]oleic acid and other types of native lipids by a silica gel-60 column [2, 3], and counted. Determination of the activity in microsomal fractions was performed in a buffered solution, pH 7.4, con-

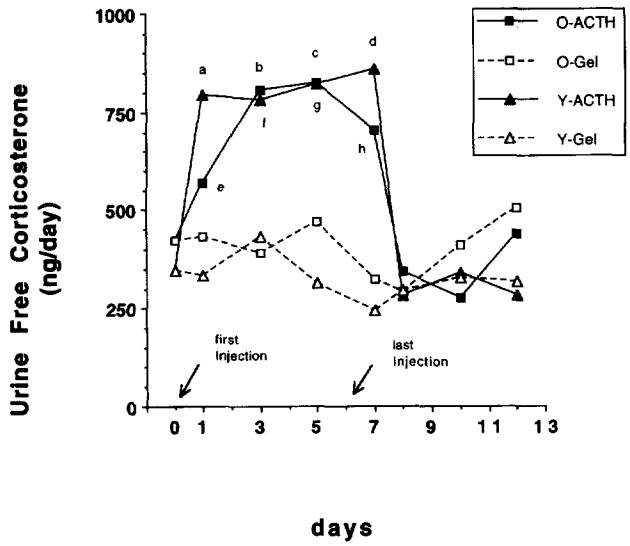

Fig. 1. Effects of prolonged stimulation on rat urinary free corticosterone content. Rats were divided into Y (3–6 mo) and O (20–23 mo) age groups. Animals were stimulated with ACTH (12 U kg^{-1}, sc) daily for seven consecutive days, and then were kept for five additional days without any treatment (post stimulation). Animals injected with 16% gelatin daily served as controls. 24 h urine was collected from individual rats for determination of free corticosterone content. Data presented are means; for clarity, standard deviations of the means are not shown. The data with footnotes (a–h) are statistically significant:

[a] $p < 0.001$,	Y-ACTH ($N = 10$)	vs.	Y-gel ($N = 9$)
[b] $p < 0.005$,	Y-ACTH ($N = 8$)	vs.	Y-gel ($N = 6$)
[c] $p < 0.01$,	Y-ACTH ($N = 8$)	vs.	Y-gel ($N = 6$)
[d] $p < 0.001$,	Y-ACTH ($N = 14$)	vs.	Y-gel ($N = 12$)
[e] $p < 0.05$,	O-ACTH ($N = 10$)	vs.	Y-ACTH ($N = 10$)
[f] $p < 0.005$,	O-ACTH ($N = 8$)	vs.	O-gel ($N = 6$)
[g] $p < 0.005$,	O-ACTH ($N = 8$)	vs.	O-gel ($N = 6$)
[h] $p < 0.001$,	O-ACTH ($N = 13$)	vs.	O-gel ($N = 12$)

taining 100 mM potassium phosphate, 1 mM glutathione, 1 mg BSA and 100 μg microsomal protein in a final volume of 0.2 ml. After preincubation for 30 min at 37°C, [^{14}C]oleoyl-CoA (103×10^3 dpm) was added to the mixture. Incubation was continued for an additional 15 min. Extraction and purification of the newly formed cholesteryl [^{14}C]oleate were carried out as previously described, except that [^{3}H]cholesteryl oleate (1.8×10^3 dpm) was used as an internal standard.

Determination of cholesteryl ester hydrolysis activity

Cholesteryl ester hydrolysis activity was determined in lipid droplet-depleted homogenates at 37°C for 15 min. The assay mixture was composed of 100 mM potassium phosphate (pH 7.4), 1 mM sodium EDTA, 0.1 mM digitonin, 2 mM taurocholic acid, [^{3}H]cholesteryl oleate ($180–190 \times 10^3$ dpm), and 50–80 μg sample protein, in a final volume of 0.35 ml. The same protocol was employed to determine the activity in cytosolic fractions, except that taurocholic acid was omitted from the assay solution, and the assay mixture was preincubated for 30 min prior to the addition of substrate. The resultant mixture was extracted in the presence of [^{14}C]cholesterol ($0.8–1.1 \times 10^3$ dpm) as an internal standard and 100 μg authentic cholesterol as a carrier. The newly released free [^{3}H]cholesterol in the extract was separated from [^{3}H]cholesteryl oleate and other types of native lipids with a silica gel-60 column. This was done by sequential flushing the column with three isooctane and ethyl acetate mixtures (40:1, 20:1 and then 3:1, v/v, 5 ml each). [^{3}H]cholesterol, was eluted in the 3:1 fraction, and counted. Under these conditions, free cholesterol was separated from cholesteryl ester and cholesterol sulfate [2, 26].

Other methods

Proteins were determined by a modified Lowry method [27]. Urine free corticosterone levels were determined in CH_2Cl_2 extracts with a radioimmunoassay kit (ICN Biomedical, Cat. No. 07120002). Radioactivity was determined in a Beckman scintillation counter (Model LS6000IC). The Student's *t*-test, Bonferroni's *t*-tests, and correlation coefficients were computed with the Glantz' primer biostatistics program, and the data were plotted with the cricket graph program using a Macintosh SE computer. The *Q*-test was performed according to Fritz and Schenk's quantitative analytical chemistry (Allyn and Bacon, 2nd ed., 1969, pp. 30–31).

RESULTS

Urinary corticosterone content and adrenal weight

Figure 1 presents the 24-h urine free corticosterone content in Y and O rats treated with ACTH or gelatin (unstimulated controls) daily, for seven consecutive days. A significant elevation of steroid production to near maximal levels occurred in stimulated-Y rats on the 1st day of stimulation, and was maintained throughout the entire seven days of stimulation, as compared to Y-controls (a–d, $p < 0.001–0.01$); but dissipated rapidly after cessation of the hormone injection. In contrast, the steroid output of stimulated-O rats increased slowly during the 1st day, reaching a maximum at the 3rd–5th days. The steroid production during the 3rd–7th days was significantly higher than O-controls (f–h, $p < 0.001–0.005$). After stopping the hormone injection, the content of stimulated-O rats fell to the O-control level. Noticeably, at the 1st day of stimulation, the steroid content of stimulated-O rats was significantly lower than that of stimulated-Y animals (e, $p < 0.05$); thereafter, no difference was observed between the two age groups. There was also no measurable difference in steroid production in the two age groups post stimulation.

To minimize ongoing acute effects of stimulation, adrenal glands used for the following studies were

Table 1. Effects of prolonged stimulation on absolute and relative adrenal weights

Experiment groups	Y rats	O rats	*t*-tests (O vs. Y)
	Weight in pair (mg)		
Gel	47.80 ± 4.30 (N = 16)	61.25 ± 10.90 (N = 15)	$p < 0.001$
ACTH	73.95 ± 6.00 (N = 14)	86.44 ± 14.20 (N = 14)	$p < 0.01$
Post ACTH	59.66 ± 4.48 (N = 6)	70.03 ± 12.10 (N = 6)	NS
Bonferroni's *t*-tests			
ACTH vs. Gel	$p < 0.05$	$p < 0.05$	
ACTH vs. Post ACTH	$p < 0.05$	$p < 0.05$	
Post ACTH vs. Gel	$p < 0.05$	NS	
	Relative to 100 g body weight		
Gel	16.0 ± 1.6 (N = 16)	14.5 ± 2.3 (N = 15)	NS
ACTH	25.5 ± 2.2 (N = 14)	20.9 ± 3.1 (N = 14)	$p < 0.001$
Post ACTH	19.8 ± 1.8 (N = 6)	16.9 ± 3.9 (N = 6)	NS
Bonferroni's *t*-tests			
ACTH vs. Gel	$p < 0.05$	$p < 0.05$	
ACTH vs. Post ACTH	$p < 0.05$	$p < 0.05$	
zPost ACTH vs. Gel	$p < 0.05$	NS	

Animal ages, hormone stimulation and post stimulation are described in Fig. 1. Rats were euthanized 24 h after the last injection of gelatin or ACTH and 5 days post stimulation. Data presented are means ± S.D. Bonferroni's *t*-test was used to test the significance among conditions within an age group. N = number of rats; NS = not significant.

harvested from rats 24 h after the last injection of ACTH (or gelatin), or five days post stimulation. Table 1 summarizes the changes of the absolute and relative adrenal weights in Y and O rats. Prolonged stimulation resulted in adrenal hypertrophy in both age groups; the hypertrophied adrenals atrophied post stimulation ($p < 0.05$, Bonferroni's *t*-tests). Although the absolute adrenal weight in stimulated-O animals was higher than that of their Y counterparts ($p < 0.01$), the relative adrenal weight (per 100 g body weight) of stimulated-O rats was actually lower than that of stimulated-Y animals ($p < 0.001$).

Adrenocortical cholesterol concentration

As shown in Fig. 2, the adrenocortical free cholesterol (FC) concentration in O rats did not differ from that in Y rats under all experimental conditions. However, the FC concentrations in both age groups decreased post stimulation as compared to their respective controls (a and b, $p < 0.05$). In parallel, the CE concentration in ACTH-stimulated Y rats increased by 12%, although this was not statistically significant. The concentration further increased post stimulation, which became significantly higher than the Y-controls (c, $p < 0.05$). The CE concentration in O-controls was higher than that of Y-controls (d, $p < 0.001$) as previously reported [3]. In contrast to their Y counterparts, the CE concentration in O animals decreased by 24% in response to ACTH (e, $p < 0.002$), but the diminution in O rats was reversed post stimulation (f, $p < 0.01$).

To determine whether stimulation of Y rats with higher doses of ACTH could deplete the adrenocortical CE as in the O animals, Y rats were treated with 0, 6, 12, 24 and 36 U ACTH kg^{-1} daily for seven consecutive days. As predicted, the stimulated adrenals became hypertrophic in a manner positively correlated with the dose of ACTH used ($r = 0.950$, $p < 0.001$). The glands obtained from rats stimulated with 36 U ACTH kg^{-1} showed almost no medullary tissue. There was no significant correlation between the doses of ACTH used for stimulation and the adrenocortical FC concentrations or lipid-phosphorus concentrations in Y animals (not shown). In contrast, there was a positive correlation between the dose of ACTH used for stimulation and the CE concentrations in the adrenal cortex of Y rats (Fig. 3). Consequently, the adrenocortical molar ratio of CE to FC was also positively correlated with the dose of ACTH used for stimulation ($r = 0.930$, $p < 0.001$).

Specific adrenal cholesteryl esters

To identify the type of CE involved in the above outcomes, specific CE species were determined by HPLC. Figure 4 presents HPLC profiles of adrenal CE species of O rats (gelatin-treated, ACTH-stimulated, and post stimulation). The peak heights or areas of three polyunsaturated fatty acid-esterified CE species: cholesteryl cervonate (CE22:6), cholesteryl adrenate (CE22:4), cholesteryl arachidonate (CE20:4), were qualitatively decreased by the hormone action, with a subsequent increase post stimu-

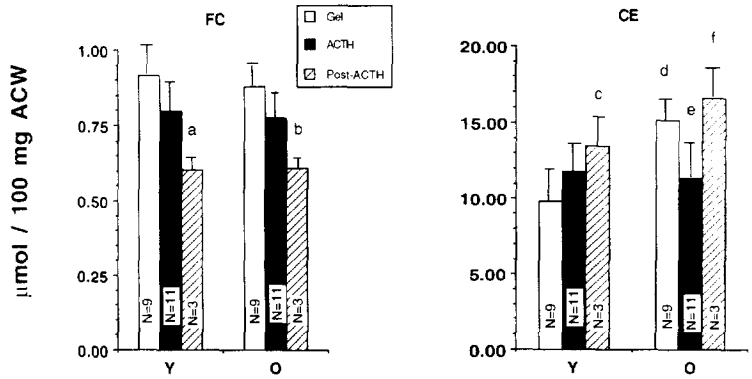

Fig. 2. Effects of prolonged stimulation on rat adrenocortical cholesterol concentrations. Left panel and right panel presented are free cholesterol (FC) and cholesteryl ester (CE) concentrations, respectively. Animal ages, hormone stimulation and post stimulation are described in Fig. 1. Rats were euthanized 24 h after the last injection of gelatin or ACTH and 5 days post stimulation. Data presented are means and standard deviations after normalization to 100 mg of adrenocortical weight (ACW). *N* = number of rats. The data with footnotes (a–f) are statistically significant:

FC:	[a]$p < 0.05$,	Y-post ACTH	vs.	Y-gel
	[b]$p < 0.05$,	O-post ACTH	vs.	O-gel
CE:	[c]$p < 0.05$,	Y-post ACTH	vs.	Y-gel
	[d]$p < 0.001$,	O-gel	vs.	Y-gel
	[e]$p < 0.002$,	O-ACTH	vs.	O-gel
	[f]$p < 0.01$,	O-post ACTH	vs.	O-ACTH

lation. The peak heights or areas of cholesteryl heptadecanoate, the internal standard (IS), were comparable among these three chromatograms. Table 2 presents the quantification of adrenocortical specific CE concentrations, based on HPLC analyses. Except for cholesteryl stearate (CE18:0), the adrenocortical concentrations of cholesteryl cervonate, cholesteryl arachidonate, cholesteryl adrenate, cholesteryl myristate (CE14:0), cholesteryl oleate (CE18:1), cholesteryl palmitate (CE16:0) of the O controls were all higher that of Y controls (a, $p < 0.001$–0.05). During the stimulation, the concentrations of cholesteryl cervonate, cholesteryl adrenate and cholesteryl arachidonate in O rats decreased, while the concentrations of cholesteryl adrenate, cholesteryl arachidonate and cholesteryl oleate in Y animals increased markedly ($p < 0.001$–0.02).

Apolipoprotein E

As shown in Fig. 5, the adrenal concentration of immunoreactive apoE in Y rats diminished upon prolonged stimulation with ACTH, despite an increase in total cholesterol concentration (Fig. 3). This phenomenon was expected [18]. Nevertheless, the decrease of apoE in Y animals was restored post stimulation. In contrast to the Y rats, prolonged stimulation of O animals with ACTH did not cause a diminution in adrenal apoE, which was unexpected under the conditions. To ascertain this difference, adrenal apoE was further studied by a semi-quantitative method. This was done by scanning the immunoblots of six pairs of adrenal samples with a densitometer [19, 22]. Each pair was composed of adrenal homogenates (or supernatants) derived from a control rat and a stimulated animal of the same age group. Stimulation of Y rats with ACTH resulted in a decrease of the adrenal apoE concentrations by 32–50% (two pairs). In contrast, stimulation of O rats with ACTH increased the adrenal apoE concentrations by 20–127% (four pairs).

Cholesteryl ester metabolic activity

To shed light on the age-difference of adrenal CE concentration in rats with prolonged ACTH-stimulation, CE metabolic activity was determined. To minimize a possible interference of endogenous CE, native lipid droplets were therefore removed from the homogenates prior to the present study. Independently, the activity was also determined in adrenal subcellular fractions as an alternative approach. As shown in Table 3, we found that both adrenal homogenate and cytosolic samples from ACTH-stimulated O rats displayed a higher activity in hydrolysis of exogenous cholesteryl oleate than their stimulated-Y counterparts ($p < 0.02$ and $p < 0.001$, respectively). In contrast, the activity in esterification of endogenous cholesterol with externally added oleic acid or oleyl-CoA in both adrenal homogenate and microsomal samples of stimulated-O rats was not different from that of stimulated-Y animals.

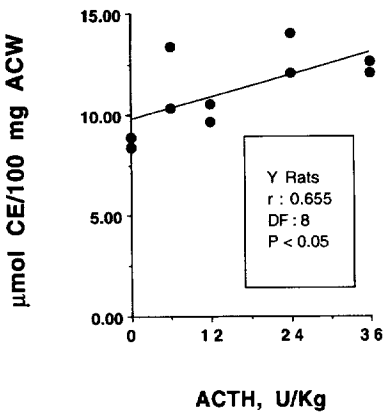

Fig. 3. Effects of prolonged stimulation with varied ACTH doses on Y rat adrenocortical cholesteryl ester (CE) concentrations. A total of eight Y rats were stimulated with ACTH containing 6, 12, 24 or 36 U kg^{-1} daily for seven consecutive days (two rats per dose). Two Y rats injected with gelatin daily served as controls. The adrenocortical CE content of rats were plotted against the doses of ACTH received for stimulation. ACW = adrenocortical weight.

DISCUSSION

Adrenal cholesterol reserve in ACTH-stimulated young rats

As mentioned, the data dealing with the effect of prolonged ACTH-stimulation for days on the adrenal cholesterol content *in vivo* are limited, and lack a consistent pattern: Lehoux *et al.* [6] reported that stimulation of rats with ACTH for nine consecutive days results in decreases of both free cholesterol and cholesteryl ester content in the adrenal cortex. In contrast, Andersen and Dietschy [7] reported that stimulation of rats with ACTH for four consecutive days causes an increase in adrenal cholesteryl ester content to nearly double that of controls; adrenal free cholesterol content is not affected. Neither study dealt with specific cholesteryl esters. The present data indicate that prolonged stimulation of rats with ACTH for seven consecutive days is associated with an increased cholesteryl ester concentration while maintaining a constant free cholesterol concentration in the adrenal cortex, which is consistent with the finding of Andersen and Dietschy [7]. Our data further show that prolonged ACTH-stimulation causes increases in adrenocortical cholesteryl adrenate, cholesteryl arachidonate and cholesteryl oleate concentrations.

Current knowledge indicates that the cholesteryl esters in lipid droplets are continually being turned over [28], thus their composition would also constantly be re-configured to meet the needs of a cell. Cholesterol esterified with polyunsaturated fatty acids composing more than twenty carbons and four or more double bonds would display a liquid physical state [29]. Thus, the observed increases in the cholesteryl adrenate and cholesteryl arachidonate after prolonged stimulation could serve to increase the fluidity of lipid droplet-containing lipids. In addition, adrenic acid serves as a precursor for the synthesis of adrenoyl phosphatidylethanolamine which acts as a stimulator of the mitochondrial cholesterol side-chain cleavage (SCC) reaction [30], whereas arachidonic acid serves as a precursor for the synthesis of prostaglandins and arachidonyl phospholipids. Boyd and Trzeciak [4] reported that stressing rats with ether for 10 min resulted primarily in depletion of adrenal cholesteryl

Table 2. Effects of prolonged stimulation on adrenocortical specific cholesteryl ester concentrations (µmol 100 mg^{-1})

Age groups	CE species	Gel	ACTH	*t*-test	
Y rats	CE22:6	0.66 ± 0.09	0.53 ± 0.03	NS	
	CE22:4	2.05 ± 0.24	2.70 ± 0.17	$p < 0.02$	(+)
	CE20:4	1.30 ± 0.01	1.45 ± 0.03	$p < 0.001$	(+)
	CE18:1	0.67 ± 0.02	1.07 ± 0.04	$p < 0.001$	(+)
	CE18:0	0.20 ± 0.05	0.32 ± 0.03	NS	
	CE16:0	0.58 ± 0.11	0.68 ± 0.13	NS	
	CE14:0	0.28 ± 0.03	0.31 ± 0.02	NS	
O rats	CE22:6	0.94 ± 0.04†	0.54 (0.56, 0.52)		(−)
	CE22:4	3.59 ± 0.28†	2.54 (3.04, 2.06)		(−)
	CE20:4	1.73 ± 0.08†	1.22 (1.52, 0.93)		(−)
	CE18:1	1.08 ± 0.08†	1.16 (1.32, 1.01)		
	CE18:0	0.29 ± 0.05	0.27 (0.36, 0.19)		
	CE16:0	0.98 ± 0.22†	0.74 (0.91, 0.57)		
	CE14:0	0.50 ± 0.07†	0.43 (0.42, 0.43)		

Data are presented are means ± S.D. ($N = 3$) or averages ($N = 2$) from HPLC analyses as described in Fig. 4. Animal ages, hormone stimulation, post stimulation and normalization of the data to 100 mg adrenocortical tissue weight are described in Fig. 1 and 2. The abbreviations for specific cholesteryl esters (CE) are described in Fig. 4. " + " increase; " − " decrease; N = number of rats; NS = not significant.

†O-Gel vs. Y-Gel, increases ($p < 0.001$–0.05). [Note: A set of data from a stimulated-O rat were excluded for presentation: This rat exhibited unusually high content of CE14:0 (1.11 µmol) and CE18:0 (4.46 µmol) as compared to normal ranges of CE14:0 (0.30–0.57 µmol) and CE18:0 (0.15–0.39 µmol) detected under varying experimental conditions. Both unusual data were rejected by Q-test.]

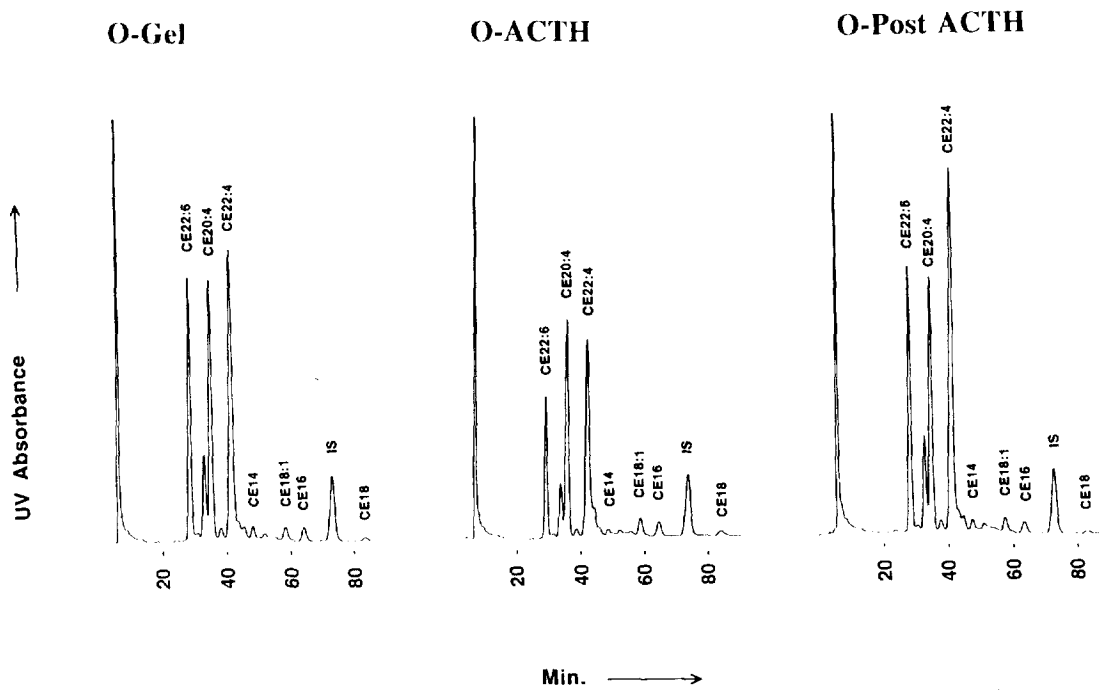

Fig. 4. The adrenal cholesteryl ester (CE) profiles of O rats. Animal ages, hormone stimulation and post stimulation are described in Figs 1 and 2. Prior to extraction, the adrenal homogenate was mixed with cholesteryl heptadecanoate, an internal standard (IS). Adrenal CE fractions were first separated from other native lipids by silica gel-60 column chromatography, and then analyzed by reversed phase HPLC. Seven major CE peaks shown in the profiles were identified (see below). At least six minor peaks were not identified:

CE22:6	**cholesteryl cervonate**
CE20:4	**cholesteryl arachidonate**
CE22:4	**cholesteryl adrenate**
CE14	**cholesteryl myristate**
CE18:1	**cholesteryl oleate**
CE16	**cholesteryl palmitate**
CE18	**cholesteryl stearate**

Table 3. Effects of prolonged stimulation on adrenal cholesteryl ester metabolic activity in vitro

	ACTH-Y rats	ACTH-O rats	*t*-test
	I. Cholesterol esterification		
	a. Incorporation of [^{3}H]oleic acid into CE in LD-depleted homogenates		
CE recovery (%)	84.7 ± 1.2	84.0 ± 2.7	NS
SA (dpm/30 min mg^{-1})	4066 ± 811	4631 ± 623	NS
	b. Incorporation of [^{14}C]oleyl-CoA into CE in microsomal fractions		
CE recovery (%)	89.0 ± 2.0	86.7 ± 3.2	NS
SA (dpm/15 min mg^{-1})	9102 ± 433	12 175 ± 1922	NS†
	II. Cholesteryl ester hydrolysis		
	a. Hydrolysis of [^{3}H]Cholesteryl oleate in LD-depleted homogenates		
FC recovery (%)	79.3 ± 4.7	80.3 ± 2.1	NS
SA (dpm/15 min mg^{-1})	36 609 ± 4361	51 658 ± 3689	$p < 0.02$
	b. Hydrolysis of [^{3}H]cholesteryl oleate in cytosolic fractions		
FC recovery (%)	86.7 ± 3.8	84.0 ± 6.2	NS
SA (dpm/15 min mg^{-1})	107 830 ± 12247	317 085 ± 34310	$p < 0.001$

Animal ages, hormone stimulation, and cholesterol metabolic determinations are detailed in the section *Materials and methods*. In brief, determinations were independently carried out in lipid droplet (LD)-depleted homogenates, microsomal fractions and cytosolic fractions prepared from pooled adrenal glands. [^{14}C]cholesteryl oleate, [^{3}H]cholesteryl oleate or [^{14}C]cholesterol were used as internal markers for calculation of recovery. The data presented as means ± S.D. from triplicated samples. The specific activity (SA) has been normalized with technical loss. CE, cholesteryl ester; FC, free cholesterol; NS, not significance. $^{\dagger}p = 0.05$.

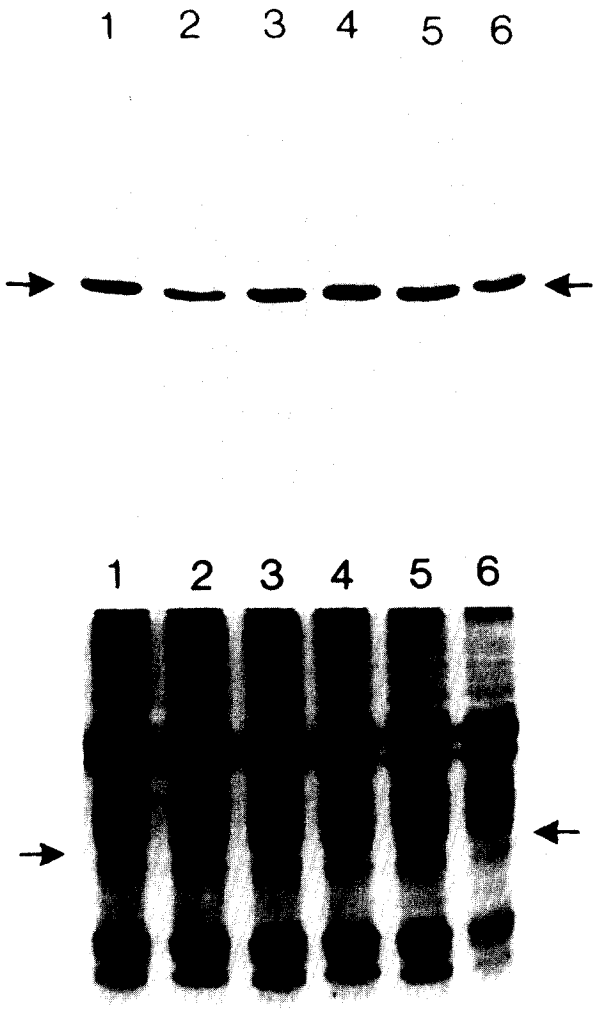

Fig. 5. Effects of prolonged stimulation on adrenal apolipoprotein E concentrations. Animal ages, ACTH stimulation, and post stimulation are described in Figs 1 and 2. ACTH at the dose of 24 U kg^{-1} was employed. Adrenal homogenates were prepared from rats accordingly. 50 μg protein from each homogenate sample was used for analysis. Upper and lower panels are immunodetection (luminography) and SDS-PAGE (stained with R-250) after electrophoretic transfers, respectively. The "arrow" indicates the apoE protein bands. The lanes and treatments are described below:

	Gel	*ACTH*	*Post-ACTH*
Y	1	2	3
O	4	5	6

oleate content. Therefore, the ACTH-induced accumulation of these cholesteryl esters observed here may serve multiple purposes: (a) preserving cholesterol, (b) controlling key polyunsaturated fatty acids, and (c) promoting the liquid physical state as "standby" for mobilization if the demand is further sustained. Our data may explain why chronic stimulation of young rats with ACTH for 3–12 days not only increases adrenocortical lipid droplets *in vivo*, but also enhances subsequent adrenocortical steroidogenesis in response to acute ACTH-stimulation *in vitro* for 90 min, as reported by Andreis *et al.* [31].

Adrenal cholesterol reserve in ACTH-stimulated aged rats

The urinary free corticosterone content in aged rats is lower than that in Y animals only during the first day of stimulation, but not during the subsequent period of stimulation or post stimulation. However, the measurements were not normalized for body weight. Conjugated corticosterone and corticosterone metabolites were not measured. Therefore, the urine steroid data reported here do not reflect the total adrenal steroidogenic capacity, but serve as a reliable index of stimulation. Nevertheless, there is a diminished capability to maintain adrenocortical cholesteryl ester levels in aged rats during prolonged stimulation as compared to the younger animals. This decline is associated with reduced cholesteryl adrenate, cholesteryl arachidonate and cholesteryl cervonate content. It is conceivable that the physical property of adrenal lipid droplets in stimulated aged rats would be different from that of their young counterparts, as previously discussed. Since male rats were studied in the present work, our results may serve as a reference as why aged male rats have lower steroidogenic responses to acute ACTH-stimulation (60–70 min) than young male rats do, when animals of both age groups have previously been subjected to chronic ACTH-stimulation for 2–6 weeks, as reported by Hess and Riegle [32].

Aging in rats does not seem to be accompanied by reduced adrenal ACTH receptors and cAMP production [14]. At least two possibilities may be considered for the cause of the decline in cholesterol reserve in stimulated aged rats: (a) the ability to acquire cholesterol from plasma lipoproteins is impaired; (b) cholesteryl ester metabolic activity favors hydrolysis rather synthesis. Azhar and Reaven [33] have shown that adrenal cells isolated from aged rats have reduced steroidogenic activity, but their ability to acquire cholesterol from extracellular lipoproteins is comparable to the cells isolated from young animals. If this occurs, then the first possibility (a) is incorrect. The cholesteryl ester hydrolysis activity (but not the cholesterol esterification activity) in ACTH-stimulated aged rats observed here appears to be higher than that in younger rats, which supports consideration of (b). Adrenal cholesteryl esters are rich in adrenic acid which is a polyunsaturated fatty acid with four double bonds. The unavailability of radiolabeled adrenic acid, adrenyl-CoA and cholesteryl adrenate, limited the present studies to the use of radiolabeled oleic acid, oleyl-CoA and cholesteryl oleate; oleic acid is monounsaturated.

Adrenal apolipoprotein E in both ACTH-stimulated young and aged rats

Majack *et al.* [34] reported that expression of apoE in cultured vascular smooth muscle cells is negatively controlled by the growth state. Schreiber *et al.* [35]

also showed that the addition of βVLDL to cultured smooth muscle cells increases DNA synthesis and cell numbers, but concomitantly diminishes apoE mRNA and protein secreted into the medium. Thus, the diminished adrenal apoE concentration observed in ACTH-stimulated young rats might be associated with hypertrophy of the gland. Our data are consistent with the reported effect of ACTH on apoE mRNA content [18]. However, this hormone effect at the apoE protein level is absent in older animals, despite the occurrence of glandular hypertrophy. We propose the following explanation:

Cellular free cholesterol can be regulated at the levels of both influx and efflux [36]. In rat adrenocortical fasciculata cells, apoE is localized in both Golgi region and multivascular body [17]. Thus, apoE may be involved in controlling the flux of free cholesterol in and out of rat adrenal cells. In young rats, adrenal absorption of plasma cholesterol is increased during prolonged stimulation, with a concomitant reduction in apoE. This concurrence would serve to minimize cholesterol efflux, ensuring a sufficient amount of free cholesterol for synthesis of steroids and new membranes, while excess free cholesterol is converted to cholesteryl ester for storage. In aged rats, the adrenal cortex contains numerous degenerating cells and accumulates clusters of lipofuscin–lipid inclusions; cell debris is even seen in the extracellular spaces and intercellular canaliculus [3]. A relatively higher adrenal apoE concentration observed in aged rats [19] could serve to redistribute cholesterol and other lipids from degenerating cells to "healthy" cells for storage or to the circulation for disposal. Thus, adrenal apoE level in aged rats could be maintained or preserved for this purpose during prolonged stimulation. Therefore, free cholesterol derived from internalized plasma lipoproteins during prolonged stimulation may partially be excreted by this process. Consequently, the hydrolysis of adrenal cholesterol ester should be increased to maintain a sufficient amount of free cholesterol for steroid and new membrane syntheses. To support this speculation, apoE has been shown to play a role in the mobilization and re-utilization of lipid for repair, growth and maintenance of myelin and axonal membranes after injury [16].

Adrenal cholesterol reserve in both young and aged rats post stimulation

The elevated adrenocortical cholesteryl ester concentration post stimulation are accompanied by decreases in glandular size (atrophy) and free cholesterol concentrations in both young rats and aged rats, and a restoration of apoE levels in young rats. During this transition, the removal of free cholesterol from steroidogenic pools or from unwanted cellular and subcellular membranes in the adrenal cortex should be activated. This would promote the esterification of free cholesterol inside cells and the efflux of free cholesterol into the circulation for disposal.

CONCLUSION

Using Fischer 344 male rats as a model, our previous work showed that the adrenal cortex of aged rats accumulates and forms clusters of lipid droplets and lipofuscin granules to a greater extent than young rats. The present work further demonstrates that, in the aged animal, there is an altered adrenocortical cholesterol reserve in response to prolonged ACTH-stimulation. Although the mechanism that underlies this alteration is not known, changes in apoE concentration and cholesteryl ester hydrolysis activity may be involved. The data also suggest that metabolism of adrenic acid and arachidonic acid in the adrenal gland may be important for steroidogenesis in the rat to cope with sustained stress regardless of age. We feel that this and the metabolism of other polyunsaturated fatty acids during and post prolonged ACTH-stimulation are deserving of further attention.

Acknowledgements—The present data have been presented in part as abstracts to the Programs of 74th and 75th Annual Meetings of the Endocrine Society. This research was supported by a research grant (RO1 AG-08332), and a geriatric medicine research training grant (AG-0014407) from the National Institute on Aging, U.S.A.

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