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Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland

(steroid synthesis/mitochondrial membrane fractionation/aminoglutethimide/cycloheximide/corticotropin)

CHRISTOPHER T. PRIVALLE, JOSEPH F. CRIVELLO*, AND COLIN R. JEFCOATE†

Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

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ABSTRACT **Rat adrenal mitochondria accumulated cholesterol during ether stress *in vivo* when side-chain cleavage was inhibited by aminoglutethimide (control = 14.6 vs. aminoglutethimide = 26.5 μg of cholesterol per mg of protein). This accumulation was insensitive to simultaneous administration of cycloheximide (24.2 μg/mg), but side chain cleavage in the mitochondria was greatly decreased. Outer and inner mitochondrial membrane fractions were separated by discontinuous Ficoll gradient centrifugation. Quantitation of marker enzymes for inner, outer, and microsomal enzymes indicated that outer membranes contained <5% inner membranes. The inner membrane fraction contained <7% outer membrane and included 90% of mitochondrial cytochrome P-450. Electron microscopy revealed outer membranes as circular intact ghosts, whereas inner membranes were largely intact and retained vesicular structure typical of intact adrenal cortex mitochondria. Administration of aminoglutethimide effected a 2-fold increase in inner membrane cholesterol (9.4 vs. 20.1 μg/mg) but simultaneous administration of cycloheximide completely blocked this increase (10.9 μg/mg). We conclude that: (*i*) in the presence of aminoglutethimide, stress stimulates accumulation of cholesterol in the inner membrane of adrenal mitochondria; and (*ii*) transfer of cholesterol from outer to inner membranes requires a cycloheximide-sensitive agent.**

The rate-limiting step in total steroid synthesis in steroidogenic tissues is the side-chain cleavage (SCC) of cholesterol to pregnenolone (1). This reaction is catalyzed by SCC cytochrome P-450 (cytochrome $P\text{-}450_{scc}$), which is located in the mitochondria of steroidogenic tissues (2). In adrenal mitochondria, this enzyme and the accompanying electron transport proteins have been located in the inner membrane fraction (3). Recent evidence suggests that the SCC system resides on the matrix side of the inner membrane (4). Cholesterol SCC is hormonally regulated in all steroidogenic tissues, and, in particular, by corticotropin (ACTH) in the adrenal gland (5). We have previously shown that ACTH stimulates SCC of cholesterol in rat adrenal mitochondria but that conversion of 25-hydroxycholesterol to pregnenolone remains high, irrespective of ACTH activation (6). This difference has been attributed to ACTH regulation of cholesterol transport rather than to direct activation of cytochrome $P\text{-}450_{scc}$ (7). Moreover, phospholipids modulate SCC by purified cytochrome $P\text{-}450_{scc}$ of cholesterol but not of 25-hydroxycholesterol (8). It has been proposed that ACTH modulates the immediate membrane environment of cytochrome $P\text{-}450_{scc}$, and recent evidence suggests that the most active phospholipid, cardiolipin, forms a 1:1 complex with the cytochrome (9).

Cholesterol transport to cytochrome $P\text{-}450_{scc}$ consists of several distinct phases: (*i*) receptor-mediated endocytosis of low density lipoprotein (or high density lipoprotein in rats) from plasma to lipid droplets (10), (*ii*) hydrolysis of cholesterol esters in lipid droplets (11), (*iii*) transfer of cholesterol to mitochondria, and (*iv*) intramitochondrial transfer of cholesterol to cytochrome $P\text{-}450_{scc}$ (12). ACTH regulates phase *i* via stimulation of the number of lipoprotein receptors (13) and phase *ii* via cAMP-dependent protein kinase activation of cholesterol esterases or suppression of cholesterol acyltransferase (14). In isolated adrenal cells in serum-free medium, ACTH stimulates cholesterol uptake by mitochondria (phases *ii* and *iii*) (12). This process is blocked by antimicrotubule and antimicrofilament inhibitors, suggesting that cellular ultrastructure is critical to this sequence (probably phase *iii*).

Protein synthesis inhibitors block hormonal activation of cholesterol SCC but not transfer of cholesterol to the mitochondria (12, 15) or SCC of 25-hydroxycholesterol (8). ACTH activation and cycloheximide inhibition *in vivo* are retained by the cholesterol SCC system in isolated mitochondria. This activation relates closely to the extent of cytochrome $P\text{-}450_{scc}$–cholesterol complex formation (16).

In rat adrenal cells, the effect of protein synthesis inhibitors on ACTH-stimulated steroidogenesis is remarkably rapid ($t_{1/2}$ = 3–4 min) (17). This has led to the hypothesis that a labile protein is required for ACTH action on the adrenal cortex. However, the shortest $t_{1/2}$ known for a mammalian protein is 12 min (ornithine decarboxylase), casting doubt on the role of a regulatory protein directly involved in the action of ACTH (18). However, recent evidence suggests that the cycloheximide block in steroidogenesis may derive from inhibition of the formation of polyphosphoinositides, which, in turn, can activate mitochondrial steroidogenesis (19).

The effect of protein synthesis inhibitors on ACTH-stimulated adrenal steroidogenesis is critical to the understanding of ACTH action. In this paper, we establish that inner and outer membranes of rat adrenal mitochondria can be separated and that differences in cholesterol distribution can be maintained during this separation. Furthermore, we demonstrate that aminoglutethimide treatment results in an accumulation of cholesterol in the inner mitochondrial membrane and that this increase is blocked by simultaneous administration of cycloheximide.

MATERIALS AND METHODS

***In Vivo* Treatments.** Rats were injected intraperitoneally with 10 mg of aminoglutethimide (CIBA Pharmaceutical) or 10 mg of cycloheximide in 1.0 ml of physiological saline, as indi-

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Abbreviations: SCC, side-chain cleavage; cytochrome $P\text{-}450_{scc}$, SCC cytochrome P-450; ACTH, corticotropin.

* Present address: Dept. of Medicine, Univ. of California at San Diego, La Jolla, CA 92093.
† To whom reprint requests should be addressed.

cated. Control groups received saline alone. Rats were then immediately exposed to anesthetizing concentrations of ether for 20 min. At the conclusion of the ether-stressing period, rats were killed by cervical dislocation and the adrenals were rapidly removed, trimmed of excess fat, and homogenized in 0.25 M sucrose/10 mM potassium phosphate, pH 7.2, containing 0.1 mM aminoglutethimide.

Mitochondrial Isolation and Fractionation. Cell debris and nuclei were removed from homogenates by centrifugation at 600 × *g* for 10 min. The supernatant fraction was centrifuged at 10,000 × *g* for 15 min to yield the mitochondrial pellet. The mitochondria were washed by resuspension in homogenization buffer and centrifugation at 10,000 × *g* for 15 min.

Rat adrenal mitochondria were fractionated into outer and inner membranes by a modification of the method described by Yago and Ichii for hog adrenal mitochondria (3). Isolated mitochondria were resuspended in hypoosmotic buffer consisting of 20 mM potassium phosphate (pH 7.2) and 0.02% bovine serum albumin. The mitochondrial membrane fraction was separated from matrix components by centrifugation at 17,000 × *g* for 20 min. The membrane fraction was resuspended in 0.25 M sucrose and layered over 7.5% (wt/vol) Ficoll in 0.25 M sucrose. Membrane separation was accomplished by centrifugation at 10,000 × *g* for 20 min. The pellet from this step was resuspended in 0.25 M sucrose.

Enzyme Assays. Malate dehydrogenase (EC 1.1.1.37) activity was assayed according to a modification of the method of Ochoa (20). Samples were sonically treated for 1 min prior to assay to ensure maximal activity, and assays were carried out in the presence of 2.1 mM sodium amytal. Succinate dehydrogenase (EC 1.3.99.1) activity was measured by the method of Singer (21). Assay mixtures contained 1.0 mM KCN and 200 μM antimycin A to prevent loss of succinate through the respiratory chain. Amytal-insensitive, NADH cytochrome *c* reductase (NADH dehydrogenase, EC 1.6.99.3) activity was measured by the method of Sottocasa *et al.* (22) in the presence of 2.1 mM sodium amytal. The cytochrome P-450 content in membrane fractions was quantified by measuring the reduced carbon monoxide minus the reduced difference spectrum by using a difference extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ for $A_{450-490}$ (23).

Cholesterol Analysis. Cholesterol was extracted from intact mitochondria and isolated membranes by the methanol/ethyl acetate method described by Simpson and Boyd (24). Cholesterol was quantitated by gas chromatography on a column packed with 3% (wt/vol) SP 2100 on 100/120-mesh Supelcoport (Supelco, Bellefonte, PA). Injection temperature, column temperature, and detector temperature were 275°C, 250°C, and 275°C, respectively. Cholesterol levels were determined from a standard curve with known ratios of cholesterol and 5α-cholestane were corrected for recovery.

Protein Determination. Protein content of the mitochondrial fractions was determined by Peterson's modification of the method of Lowry (25).

Electron Microscopy. Membrane fractions were fixed in 2.0% (vol/vol) glutaraldehyde in 100 mM phosphate buffer (pH 7.2). Samples were concentrated by centrifugation in a Beckman Microfuge for 30 sec. Pellets were resuspended in 3% agar at 70°C and centrifuged at 14,000 × *g* for 30 sec. The sectioned agar blocks were postfixed for 30 min in 1% osmium tetraoxide in 100 mM phosphate buffer (pH 7.2) and were washed for 10 min in the same buffer. The samples were dehydrated in acetone and embedded in Luft's epoxy (26). Thin sections were cut with a DuPont diamond knife on a Sorvall MT-2 ultramicrotome and were placed on 200-mesh copper grids. Sections were stained with uranyl acetate and lead citrate (27) and were examined with a Hitachi HU-12 electron microscope.

Table 1. Distribution of mitochondrial marker enzyme activities

Enzyme	Distribution, by fraction				
	1	2	3	4	5
Amytal-insensitive, NADH cytochrome *c* reductase	249	1,722	196	155	115
Succinate dehydrogenase	0	9	0	99	568
Cytochrome P-450	0	0.05	0.50	0.64	1.5
Malate dehydrogenase	1,808	645	368	58	1,913

Results are expressed as follows: malate dehydrogenase, milliunits/mg of protein (1 milliunit is defined as the amount oxidizing 1 nmol of NADH per min); amytal-insensitive, NADH cytochrome *c* reductase, milliunits/mg of protein (1 milliunit is defined as the amount reducing 1 nmol of cytochrome *c* per min); succinate dehydrogenase, milliunits/mg of protein (1 milliunit is defined as the amount reducing 1 nmol of dichloroindophenol per min); cytochrome P-450, nmol/mg of protein. Mitochondrial fractions were from control animals. Mitochondria from cycloheximide- and aminoglutethimide-treated animals gave similar distributions of enzymatic activities.

RESULTS

Fractionation of Mitochondria. Rat adrenal mitochondria have been fractionated by a modification of the procedure described by Yago and Ichii (3) for hog adrenal mitochondria. Osmotic shock of intact mitochondria, followed by discontinuous Ficoll gradient centrifugation, was effective in separating outer and inner membranes, as judged by assays of several marker enzymes. Table 1 shows the distribution of these enzyme activities between five fractions. Fraction 1 is the supernatant obtained from centrifugation of the hypotonically treated mitochondria. This fraction, based on malate dehydrogenase activity, consisted primarily of matrix proteins. The pellet from the "post shock" centrifugation was separated into four fractions on the discontinuous Ficoll gradient. Fraction 2 consisted of outer membrane (high amytal-insensitive, NADH cytochrome *c* reductase activity) with <5% contamination from inner membrane (succinate dehydrogenase and cytochrome P-450). Fraction 5 contained inner membranes that retained matrix enzymes and <7% contamination by outer membrane. Fraction 3 contained residual matrix protein not removed by the post shock centrifugation or matrix protein released from the inner mitochondria during the gradient centrifugation. Fraction 4 consisted predominantly of inner membrane detached from matrix, as judged by low malate dehydrogenase activity as compared to significant cytochrome P-450 and succinate dehydrogenase activity.

In Table 2, the distribution of protein between the various fractions is compared. Approximately half of the protein was located in fraction 5, whereas the remaining half was distributed equally between the other four fractions. Pretreatment with cycloheximide or aminoglutethimide did not affect the protein distribution.

Table 2. Protein distribution in mitochondrial fractions

Treatment	Distribution, %				
	1	2	3	4	5
Control	8 ± 2	11 ± 2	14 ± 2	11 ± 1	57 ± 5
Cycloheximide	8 ± 2	11 ± 3	13 ± 1	12 ± 2	56 ± 6
Aminoglutethimide	7 ± 1	10 ± 1	16 ± 2	10 ± 2	58 ± 5
Cycloheximide and aminoglutethimide	7 ± 2	12 ± 2	16 ± 2	10 ± 2	56 ± 6

Values (mean ± SD) are expressed as % of total protein. Protein content in mitochondrial fractions was determined by Peterson's modification of the method of Lowry (25).

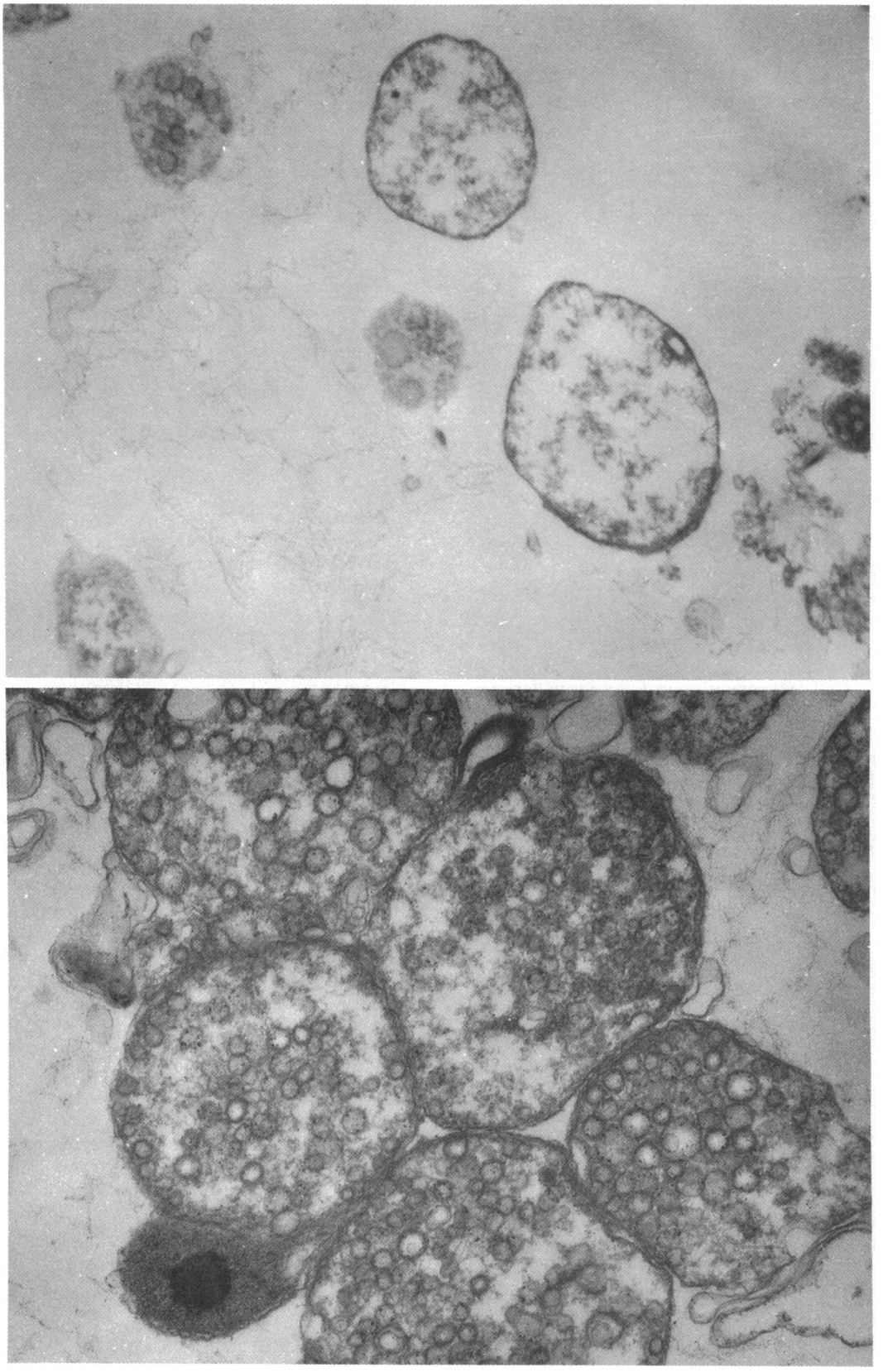

FIG. 1. Transmission electron micrographs of rat adrenal mitochondria. (*Upper*) Outer membranes. The small vesicle fraction recovered from the interface of the Ficoll gradient consists of intact membrane ghosts that lack extensive internal vesicular structure. (×27,000.) (*Lower*) Inner membranes. This large vesicle fraction is recovered as the pellet from the Ficoll gradient and contains extensive internal vesiculation. (×27,000.)

Cholesterol Distribution. The distribution of cholesterol in intact mitochondria and isolated inner membrane (fraction 5) is shown in Table 3. *In vivo* pretreatment of stressed rats with either aminoglutethimide or cycloheximide resulted in a doubling of mitochondrial cholesterol, as previously reported (15). However, these treatments resulted in distinct differences in

Table 3. Cholesterol concentrations in intact adrenal mitochondrial and inner membrane fractions

Treatment	Cholesterol, μg/mg	
	Inner membrane	Total mitochondria
Control	9.4 ± 0.4	14.6 ± 1.7
Cycloheximide	11.3 ± 0.5	25.8 ± 3.0
Aminoglutethimide	20.1 ± 1.2	26.5 ± 3.3
Cycloheximide and aminoglutethimide	10.9 ± 0.7	24.2 ± 3.1

Values (mean ± SD) are expressed as μg of cholesterol per mg of mitochondrial protein.

cholesterol distribution. After aminoglutethimide treatment, cholesterol accumulated in the inner membrane, which became the dominant location of cholesterol. Administration of cycloheximide simultaneous with aminoglutethimide prevented the accumulation of cholesterol in the inner mitochondrial membrane. Cycloheximide treatment alone had similar effects on mitochondrial cholesterol accumulation and distribution. The cholesterol contents of fractions 3 and 4, which also contained 10% of mitochondrial cytochrome P-450, were insensitive to *in vivo* pretreatment.

Electron Microscopy. Electron microscopy reveals fraction 2 as intact outer membrane ghosts that retained only traces of vesicular structures within the ghosts (Fig. 1 *Upper*). Fraction 5 appears to consist of intact inner membranes (Fig. 1 *Lower*) that retained the full complement of vesicular structures that also typify intact bovine adrenal mitochondria (28).

DISCUSSION

In previous studies, we have shown that although ACTH activation of the transport of cholesterol to cytochrome P-450$_{scc}$ and of SCC is retained by isolated mitochondria, the activation is dependent on mitochondrial integrity (7). The low extent of combination of cholesterol with cytochrome P-450$_{scc}$ in adrenal mitochondria, from cycloheximide-treated animals or hypophysectomized animals, is increased after ultrasonic disruption to the level found in mitochondria from stressed or ACTH-treated animals. This has led to the hypothesis that ACTH action overcomes a normal restraint to the mobility of mitochondrial cholesterol (7).

In this report, we have established that when SCC of cholesterol is blocked by formation of an aminoglutethimide–cytochrome P-450$_{scc}$ complex (29), the full accumulation of cholesterol is found in the inner mitochondrial membrane. We have reported previously that the cholesterol that accumulates in mitochondria of ACTH-stimulated rat adrenal cells during inhibition by aminoglutethimide is completely converted to pregnenolone within 3–4 min upon removal of the inhibitor (12). Localization of cytochrome P-450$_{scc}$ in the inner mitochondrial membrane provides a ready explanation for this reactivity.

In vivo cycloheximide treatment, while permitting cholesterol accumulation in the mitochondria in the presence of aminoglutethimide, almost completely blocks movement to the inner mitochondrial membrane. Although we do not demonstrate activation of this step by ACTH in this study, cholesterol added to intact mitochondria will not combine with cytochrome P-450$_{scc}$ without *in vivo* ACTH treatment (15). Therefore, ACTH facilitates cholesterol transport to the inner membrane by a cycloheximide-sensitive process. The limiting and controlled step could be either transit from the outside to the inside of the outer membrane or transport between outer and inner membranes. Highly variable rates have been reported for both types of processes, depending on the membrane and lipid content. Exchange times vary from seconds to hours for transmembrane exchange (30) and from minutes to hours for intermembrane exchange (31). The removal of the restraint by mitochondrial disruption suggests that the main limitation to cholesterol mobility is the aqueous space between the outer and inner membrane, which probably collapses in the disruption process. The separation technique described here occurs without substantial intermembrane cholesterol movement, probably because the cholesterol is "frozen" in the membranes at the low temperature of separation (4°C). Previous work has shown that Ca^{2+} activates cholesterol movement in intact adrenal mitochondria (15), possibly by facilitating contacts between outer and inner membrane.

Farese and Sabir have shown that phosphoinositides and cardiolipin activate the combination of cholesterol with cytochrome P-450$_{scc}$ in rat adrenal mitochondria (32). They propose that phosphoinositides are the natural mediators of cholesterol movement in adrenal mitochondria. Consistent with this suggestion is the rapid stimulation of synthesis of phosphoinositides by ACTH, the sensitivity of this synthesis to cytohexamide, and the stimulation of phosphatidylinositol kinase in adrenal mitochondria. Phosphoinositides could facilitate intermembrane transport of cholesterol through mechanisms such as modification of membrane fluidity or increased contacts between the membranes or through phosphoinositide exchange in conjunction with cholesterol exchange.

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