Cholesterol Accumulation in Adrenocortical Mitochondria after ACTH-stimulation

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Abstract

Rat adrenocortical cell suspensions (10^6 cells) were incubated with ACTH (40 nM) in 2 ml of Krebs-Ringer bicarbonate buffer for 90 min. About 42 nmol of corticosterone and 14 nmol of 18-hydroxydeoxycorticosterone were generated and released into the medium. Aminoglutethimide at 50 μ M inhibited the steroidogenesis to 16 %. Mitochondrial pellets were prepared from the cells incubated in the absence, or in the presence, of ACTH and aminoglutethimide, and cholesterol content was determined. The mitochondria of the cells incubated without the drugs contained 25.2 μ g cholesterol/mg protein. Cholesterol content increased by 10 % in the mitochondria of the ACTH-stimulated cells. The mitochondria of the cells incubated in the presence of both ACTH and aminoglutethimide contained 143 % of cholesterol compared to those of the nontreated cells. When rats were subjected to ether stress after aminoglutethimide pretreatment, cholesterol content of the mitochondrial fraction increased to about 200 % compared to that of the control rats. These results suggest that a cholesterol pool exists in the adrenocortical mitochondria and that the amount increases during the steroidogenic stimulation of the cells. The mitochondria were fixed with filipin-containing fixative and examined by freeze-fracture electron microscopy. Accumulations of filipin-cholesterol complexes were observed in the inner membrane of the mitochondria as protuberances or pits 25 nm in diameter.

Mitochondria of steroidogenic tissues contain a cholesterol side chain cleavage enzyme, cytochrome P-450_{scc}, in their inner membrane, and either a reaction catalyzed by this enzyme system or transport of cholesterol to the enzyme catalytic site through the mitochondrial membrane is believed to be a rate-determining step in steroidogenesis (Garren *et al.*, 1971; Kimura, 1981; Schuster, 1974; Simpson, 1979; Stone and

Hechter, 1954). This poses an interesting question about the status of cholesterol in the mitochondria of these tissues, because the mitochondria of nonsteroidogenic tissues are known not to contain much cholesterol in their membrane. Where cholesterol is localized in the subfraction of adrenocortical mitochondria and how it is transported to the catalytic site of cytochrome P-450_{scc} have been major questions inquired into by a number of biochemists and endocrinologists (Mahaffee *et al.*, 1974; Simpson *et al.*, 1978).

For instance, Privalle *et al.* (1983) observed that cholesterol accumulates in the mitochondrial fractions of rat adrenal glands during ether stress when the animal is pre-treated with aminoglutethimide. Kido and Kimura (1981) and Ohno *et al.* (1983) discussed the nature of a labile protein factor which could help cholesterol to move into the mitochondrial inner membrane and to place itself in the catalytic site of the enzyme. Crivello and Jefcoate (1980) reported participation of microtubules and microfilaments in achieving the appropriate transportation of cholesterol, while Hall *et al.* (1981) discussed a role of calmodulin in the same process.

It has been known that a polyene antibiotic, filipin, can specifically bind to membrane cholesterol and form filipin-sterol complexes (De Kruijff and Demel, 1974; Kinsky, 1970; Norman *et al.*, 1976). These complexes are easily recognized in freeze-fracture images as protuberances or pits 25–30 nm in diameter (Fujita *et al.*, 1981; Fujita *et al.*, 1982; Matsuda *et al.*, 1983; Montesano, 1979; Nakajima and Bridgman, 1981). Although a number of studies have recently been carried out on the distribution of cholesterol in plasma-and cyto-membranes of various organs using freeze-fracture images of filipin-treated tissues, no information is yet available about this subject of adrenocortical mitochondria.

In order to confirm the results of previous studies on cholesterol movement in adrenocortical cells and to obtain morphological information about the presence of cholesterol in the mitochondria, we have chemically analyzed the cholesterol content in the adrenocortical mitochondria during the steroidogenic stimulation of the cells *in vivo* and *in vitro*, and, at the same time, have microscopically observed the presence of filipin-cholesterol complexes in the mitochondria of these cells.

Materials and Methods

Animals and Chemicals

Female Sprague-Dawley rats, weighing 160–180 g, were used throughout this study. Synthetic ACTH_{1–24}, Cortrosyn, was obtained from Dai-ichi Seiyaku, Co. Aminoglutethimide was a gift from Ciba-Geigy, Co. Collagenase (Type I) and deoxyribonuclease were purchased from Worthington and Sigma, respectively. Steroids were products of either Makor Chemicals or Seraloids. Filipin was generously donated by the Upjohn Co., Kalamazoo, Mich. All other chemicals were from local sources and of the highest purity available commercially.

Preparation of Adrenocortical Cell Suspensions

The cell suspensions were prepared from rat adrenal glands as described by Haning *et al.* (1970) with some modification. At a time between 8 and 10 am, rats were sacrificed by decapitation. The adrenal glands were quickly excised and placed in Krebs-Ringer bicarbonate buffer (120 mM NaCl, 1.5 mM CaCl₂, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 22 mM NaHCO₃ and 0.2 % glucose, pH 7.4) containing 1 % bovine serum albumin (KRBGA). Fat tissues around the gland were trimmed off. The glands were cut into small pieces with a razor blade, and the fragments obtained from 30 adrenal glands were suspended in 10 ml of KRBGA containing 2 mg/ml collagenase and 0.05 mg/ml deoxyribonuclease and incubated in an atmosphere of 95 % air and 5 % CO₂ for 45 min at 37°C. The digested fragments were further dispersed by pipetting. The cell suspensions were centrifuged at 400×g for 7 min. The pellets were washed once by Krebs-Ringer bicarbonate buffer containing 4 % bovine serum albumin and the recovered suspensions were filtered through a Nylon mesh to remove tissue debris. The free cell suspensions thus obtained were preincubated for 60 min at 37°C in an atmosphere of 95 % air and 5 % CO₂, then washed once, and resuspended in KRBGA. Viability of the cells, estimated in 0.04 % Trypan-blue solution, was usually higher than 90 %. According to these procedures, approximately 3 × 10⁵ viable cells were obtained from one adrenal gland.

Measurement of Steroidogenesis by Adrenocortical Cells and Determination of Cholesterol

In a typical experiment, 5×10^5 cells were incubated with, or without, ACTH (40 nM) in 1 ml of KRBGA in an atmosphere of 95 % air and 5 % CO_2 for 60 min. The incubation was terminated by centrifugation at $1,100 \times g$ for 10 min. The supernatants were measured for steroid content by high performance liquid chromatography (HPLC) as described previously (Kim *et al.*, 1983; Momoi *et al.*, 1983; Yagi *et al.*, 1983). The pellets were homogenized in 0.25 M sucrose containing 10 mM Tris-HCl and 1 mM EDTA, pH 7.4, using a glass homogenizer with a teflon pestle. The homogenate was centrifuged at $900 \times g$ for 10 min and the supernatant fraction obtained was again centrifuged at $8,000 \times g$ for 15 min. The pellets recovered from the second centrifugation were used as the mitochondrial fraction. The mitochondrial pellets were frozen and thawed to disrupt the membrane. To the pellets 5α -cholestane was added as an internal standard, and cholesterol was extracted with dichloromethane. Cholesterol was determined by gas chromatography using an OV-1 column (3 mm $\times$ 2 m) at a column temperature of 250°C and an injector temperature of 270°C . The content of the ester form of cholesterol was estimated as the difference between the cholesterol content after alkaline digestion (1 M KOH) of the sample and that before the saponification. The experiments were performed at least three times under the same incubation conditions. The average content of the mitochondrial cholesterol in the non-treated cells was $25.2 \pm 2.2 \mu\text{g}/\text{mg}$ protein.

Preparation of Adrenocortical Mitochondria from Rats Suffering from Ether Stress

This experiment was carried out in essentially the same way as described by Privalle *et al.* (1983). Six rats were injected intraperitoneally with 10 mg of aminoglutethimide dissolved in 1 ml of saline. Control groups received saline alone. The animals were immediately transferred into a container saturated with ether gas, and maintained in anesthetic conditions for 20 min. After the ether stress, the animals were killed and the adrenals were excised. The mitochondrial fractions were prepared from the whole adrenal glands in the manner described above.

Freeze Fracture Electron Microscopy

The mitochondrial pellets were fixed with 2 % glutaraldehyde solution buffered at pH 7.4 with 0.1 M cacodylate for 5–10 min, and treated with

0.02–0.05 % filipin contained in the same fixative for 12–24 h at room temperature. The samples were soaked in 30 % glycerol solution for 20 min and rapidly frozen in melting nitrogen slush. The specimens were cleaved, shadowed with platinum-palladium, and stabilized with carbon in a JEOL-7000 type universal freeze specimen preparation device. The replicas made were examined in a Hitachi H-500 type electron microscope.

Protein Determination

Protein concentration was determined in the manner described by Lowry *et al.* (1951) using bovine serum albumin as the standard.

Results

To establish the optimal conditions for ACTH-stimulation of the adrenocortical cell suspensions, various doses of ACTH were added to the cells, and steroids released in the medium were determined. The amount of corticosterone and 18-hydroxydeoxycorticosterone released was dependent on the concentration of ACTH in the incubation medium. Forty nM of ACTH was chosen as the optimal concentration for steroidogenesis. The time course of steroidogenesis was investigated. When the optimal dose of ACTH was added to the adrenocortical cell suspension, corticosterone release began after a time lag of 10–15 min, continued linearly for the next 50 min, and then gradually slowed down. From these results, the cells after the 90 min-incubation with ACTH were taken for analysis of cholesterol movement in the cells.

Aminoglutethimide is a well-known inhibitor of cytochrome P-450_{sc}. The effect of aminoglutethimide on the ACTH-stimulated steroidogenesis was examined. As shown in Table 1, 50 μM of the drug seemed to be enough to inhibit the steroidogenesis caused by the optimal dose of ACTH.

Figure 1 shows the relative contents of the mitochondrial cholesterol under various incubation conditions of the adrenocortical

Table 1. Effect of aminoglutethimide on steroidogenesis.*

ACTH nM	Aminoglutethimide μ M	Steroid (nmol/90 min)	
		Corticosterone	18-Hydroxydeoxycorticosterone
40	0	42.0	14.0
40	50	6.8	1.6
40	100	3.3	1.0

* The cells (5×10^5) were incubated with ACTH (40 nM) in the presence, or absence, of aminoglutethimide for 90 min, and the medium was analyzed for steroids as described in Materials and Methods.

Fig. 1. Relative contents of cholesterol in adrenocortical mitochondria under various incubation conditions. The cells (5×10^6) were incubated with, or without, ACTH (40 nM) and aminoglutethimide (50 μ M) in 10 ml of KRBGA for 90 min, and the mitochondrial cholesterol contents were determined as described in Materials and Methods. The results are the means for three experiments and the bars represent standard deviations of the means.

cells. The mitochondrial fraction of the rat adrenocortical cells in a resting state contained an appreciable amount of free cholesterol (25.2 μ g/mg). When the cells were stimulated by ACTH under the optimal conditions, only a 10% increase in cholesterol content was observed. This result suggests that the cholesterol content in the mitochondria is in a nearly steady state during the ACTH-mediated steroidogenesis. If aminoglutethimide, an inhibitor of cholesterol side chain cleavage, was present in the incubation so that the steroidogenesis was prevented, a greater accumulation of cholesterol was observed in the mitochondria of ACTH-stimulated cells than in those of nonstimulated cells. The increase in cholesterol was about 43% compared to the cells without both ACTH and aminoglutethimide and about 23% compared to the

cells treated with aminoglutethimide alone. Although a variety of the incubation times other than 90 min were tested, we could not find an accumulation of cholesterol greater than 150% in the stimulated cells when compared to the control cells.

The cholesterol ester content in the mitochondrial fraction was about 25 μ g/mg protein, and no variation was observed under a variety of incubation conditions (data not shown).

Next, we studied the intramitochondrial accumulation of cholesterol during ether stress applied to the animal. Rats were pretreated by injecting aminoglutethimide and immediately transferred into a container saturated with ether gas. The animals were maintained under anesthetic conditions for 20 min, and then the adrenal glands were excised and analyzed for cholesterol in the

Table 2. Cholesterol content in the mitochondrial fraction of the adrenal cortex during ether stress of the animal.*

Treatment		Cholesterol content $\mu\text{g}/\text{mg}$ protein	
Injection	Anesthesia		
Saline	Ether	Experiment I 18.5 (100)	Experiment II 25.5 (100)
Aminogluthethimide	Ether	41.2 (222)	45.0 (177)

* Six rats were injected with aminogluthethimide, and the animals were subjected to ether stress for 20 min. Control rats received a saline injection and were exposed to ether. The mitochondrial pellets were prepared from adrenal glands and analyzed for cholesterol as described in Materials and Methods. The numbers in parentheses represent the percentages of the control.

Fig. 2. Freeze-fracture image of mitochondria (M) of adrenocortical cells treated with filipin. Note the numerous filipin-cholesterol-complexes (C) in the inner mitochondrial membrane. $\times 37,500$

Fig. 3. Freeze-fracture image of mitochondria of rat liver cells treated with filipin under the same conditions as the treatment for adrenocortical mitochondria as described in Materials and Methods. $\times 37,500$

mitochondrial fractions. The results of two experiments are shown in Table 2. The pretreatment with aminogluthethimide caused the accumulation of cholesterol in the mitochondrial fraction during the ether stress. The increase was almost 100 % compared to the non-treated control group.

With regard to these results obtained from the experiments conducted *in vitro* as well as *in vivo* on the mitochondrial fraction of rat adrenal glands, the following two points should be noted: (1) the mitochondria of rat adrenal cortex contained an appreciable amount of cholesterol, and (2) the cholesterol

content in the mitochondria increased after the adrenocortical cells were stimulated with ACTH *in vitro* or by anesthetic stress *in vivo*, when aminogluthethimide, an inhibitor of side chain cleavage of cholesterol, was present.

To further confirm the presence of cholesterol in the adrenocortical mitochondria and to get more insight into the status of cholesterol in the mitochondrial membrane, the mitochondrial pellets were treated with filipin-containing fixing reagent and the samples were examined by means of a freeze-fracture method. A similar filipin-treatment was conducted on the rat liver mitochondrial

pellets for the purpose of comparison. As shown in Fig. 2, the accumulations of numerous filipin-cholesterol complexes were found mainly in the inner membrane of about one third of the adrenocortical mitochondria as protuberances or pits of 25 to 30 nm in diameter. On the other hand, the rat liver mitochondria did not reveal any filipin-cholesterol complexes at all (Fig. 3).

Discussion

In this paper we first described the optimal conditions for ACTH-stimulated steroidogenesis of the rat adrenocortical cell suspensions. By conducting the incubation of 5×10^5 cells with 40 nM ACTH (Cortrosyn) in 1 ml KRBGA for 90 min, we were able to obtain the release from the cells of substantial amounts of corticosterone and 18-hydroxydeoxycorticosterone, which could be measured by HPLC. A use of HPLC for the measurement of corticosteroids would be easier than a cumbersome conventional radioimmunoassay.

After establishing the optimal conditions, we asked two questions concerning the behavior of cholesterol in the adrenocortical cells. The first question was whether cholesterol, the primary substrate of corticosteroids, moved into the mitochondria, the site of cholesterol side chain cleavage, during the steroidogenesis, and if so, whether or not its movement was regulated by ACTH. Our results suggested that an appreciable amount of cholesterol existed in the mitochondrial fractions of adrenocortical cells in a resting state, and the ACTH-stimulation in the presence of aminoglutethimide caused about a 40 % of increase in cholesterol content in the mitochondria (Fig. 1). Jefcoate and colleagues, using the *in vitro* superfusion of rat adrenal cells (Crivello and Jefcoate, 1980) or the *in vivo* anesthetic stress of rats (Privalle *et al.*, 1983), have reported that the cholesterol content in the mitochondrial

fraction increased to about 180 % in the steroid-producing adrenal glands compared to the non-producing glands. We also performed the experiments on the animals under the ether stress, and obtained similar results to those of Jefcoate *et al.* (Table 2). A discrepancy between the degree of accumulation of cholesterol on *in vitro* (140 %) and *in vivo* (200 %) stimulation should be explored in the future. A possible explanation may be that the adrenocortical cells in KRBGA were under the unphysiological conditions which could not supply cholesterol from the extracellular source, while the adrenal glands of the animal under the ether stress could obtain cholesterol from the circulation via a low-density lipoprotein pathway.

The second question we asked about the cholesterol in the adrenocortical mitochondria concerned the localization of free cholesterol in the submitochondrial fraction. This question was asked, because it is generally known that mitochondria of the non-steroidogenic tissues do not contain much cholesterol in their membrane. To answer this question, the mitochondrial pellets were treated with filipin-containing fixative, and examined by freeze fracture electron microscopy. The results clearly indicated the presence of filipin-cholesterol complexes in the inner membrane of mitochondria of the adrenocortical cells, which is the site of the rate-determining reaction of steroidogenesis. Unfortunately, however, we could not find a significant morphological difference in the amount of the complexes between the mitochondria of the ACTH-stimulated cells and those of the non-stimulated cells. In contrast with these results, the liver mitochondria which had been treated with filipin under the same conditions as those for the adrenocortical mitochondria did not reveal the filipin-cholesterol complexes. These results are consistent with our previous observations obtained on other cells such as adrenal medullary cells, anterior pituitary cells,

thyroid follicle cells, pancreatic exocrine cells and ovarian duct cells (Fujita *et al.*, 1981; Fujita *et al.*, 1982; Matsuda *et al.*, 1983).

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