Acute Effect of Corticotropin on the Incorporation of ^3H -Choline into Phospholipid Fraction of Subcellular Components from Rat Adrenal Glands

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Synopsis

An injection of ACTH caused a remarkable stimulation in the rate of ^3H -choline incorporation into ethanol-ether extractable fraction of the subcellular components from rat adrenal glands as early as 3.5 hr after the injection. The rate of increase in the incorporation due to ACTH administration was most conspicuous in the inner membrane fraction of mitochondria; in this fraction almost 500% increase was observed. Administration of cycloheximide in amount that blocks protein synthesis prior to ACTH treatment inhibited the ACTH effect on the choline incorporation completely, while a large dose of Actinomycin D did not show any influence on the ACTH action.

In the previous work (Ichii *et al.* 1970), it was reported that the rate of ^3H -leucine incorporation into the inner membrane fraction from rat adrenal mitochondria was increased as early as 3.5 hr after the administration of ACTH. In the present work, the rate of the incorporation of ^3H -choline which was mainly incorporated into phospholipids of biological membranes (Pasternak and Bergeron, 1970) into the subcellular components of rat adrenal glands was determined after an acute administration of ACTH. The purpose of this experiment was to examine into whether or not the increase in the ^3H -leucine incorporation caused by ACTH was a concomitant result of new membrane synthesis. It was found that a conspicuous elevation of the rate of the choline incorporation took place in all the subcellular components 3.5 hr after ACTH injection, especially in the inner membrane fraction of mitochondria, almost 500% increase was noticed.

Materials and Methods

Animals

Male Wistar rats weighing 230–260 g were used throughout the experiment. They were fed *ad libitum* with balanced diet (Oriental NMF, Oriental Kobo Kogyo, Osaka, Japan) and kept in a room of constant temperature (25 °C) with 7 a.m.–7 p.m. daylight cycle. Each experimental group consisted of 6 rats. In all experiments, 12 adrenals from 6 rats of a group were pooled and processed simultaneously. ACTH (Cortrophine-Z, N.V. Organon, Oss, Holland) was injected subcutaneously with a dose of 6 units per rat 3.5 hr prior to the death of the animals. In each experiment, 20 μCi of methyl- ^3H -choline (specific activity, 550 mCi/mmol, New England Nuclear Corp., Boston, U.S.A.) per rat were intraperitoneally injected as 50% ethanol solution. Actinomycin D (Mann Research Lab., New York, U.S.A.) and cycloheximide (Nakarai Chem. Co., Kyoto, Japan) were dissolved in 0.5 ml saline and administered intraperitoneally 30 min before the ACTH injection with doses of 200 μg and 10 mg per rat, respectively. The animals were sacrificed by exsanguination 1.5 hr after the injection of the labeled compound.

Isolation of subcellular components

Isolation of the subcellular components was

performed by the method described previously (Yago and Ichii, 1969) with minor modifications. Pooled adrenals (approximately 270 mg) were homogenized in 9 volumes of ice-cold homogenizing medium which contained 210 mM manitol, 70 mM sucrose, 5 mM Tris-HCl buffer (pH 7.4) and 0.5 mg per ml of bovine serum albumin (crystalline, Sigma Chem. Co., St. Louis, Mo., U.S.A.). Homogenate was centrifuged at $600 \times g$ for 10 min. The supernatant was carefully separated and the precipitate was referred to as the "Crude Nuclear" fraction. The supernatant was centrifuged at $8,000 \times g$ for 15 min and the precipitate was resuspended in the homogenizing medium and the differential centrifugation ($600 \times g$, for 10 min and $8,000 \times g$, for 15 min) was repeated to minimize microsomal contaminations. The $8,000 \times g$ supernatant was referred to as the "Post-Mitochondrial" fraction. The mitochondrial fraction ($8,000 \times g$ precipitate) was washed once more and fractionated further into outer, inner membrane fractions and matrix fraction by the digitonine method described by Schnaitman and Greenwalt (1968). In this experiment, the purity of the separated fractions was not examined, since the purity of the membrane fractions separated by the same method had already been evaluated in the previous experiment (Ichii *et al.* 1970) using monoamine oxidase [EC 1.4.3.4.] for outer membrane and succinate-cytochrome c oxidoreductase [EC 1.3.99.1.] for inner membrane as the marker enzymes, respectively. Distribution of phosphorous in the organic solvent extractable fractions from three submit-

ochondrial fractions was 17.9% in the outer membrane 75.9% in the inner membrane and 8.2% in the matrix, respectively.

Extraction of phospholipids

For extraction of phospholipids from the subcellular components, trichloroacetic acid precipitates of each components were extracted twice with 5 ml of ethanol-ethylether mixture (3 : 1, v/v) at 60 C for 15 min. The organic solvent phase was combined and concentrated to 3 ml under a stream of nitrogen. An aliquot was transferred to a counting vial for assay of the radioactivity and another aliquot was set aside for the determination of phosphorous.

Analytical methods

Contents of phosphorous in the ethanol-ether extracts were determined by the method of Chen *et al.* (1956) after oxidation with perchloric acid. The radioactivity was determined in a Tri Carb liquid scintillation spectrometer with the automatic external standardization for quenching correction. A statistical counting accuracy (Loevinger and Berman, 1951) of 98% or more was attained of all samples.

Results and Discussion

Effect of ACTH acutely administered 3.5 hr prior to sacrificing the animals on the rate of 1.5 hr-incorporation of ^3H -choline into the

Table 1. Effect of ACTH on the incorporation of ^3H -choline into the ethanol-ether extracted fractions of subcellular components from rat adrenal glands

Exp. No.	Fraction	Specific Activity (dpm/ μgP)		Increase (%) due to ACTH Treatment
		Control	ACTH-Treated	
1	Homogenate	64	202	215
	Crude Nuclear	64	148	130
	Post-Mitochondrial	116	316	170
	Mitochondria			
	Inner Membrane	33	189	480
	Outer Membrane	70	299	330
	Matrix	82	271	233
2	Homogenate	55	213	296
	Crude Nuclear	56	196	250
	Post-Mitochondrial	85	324	283
	Mitochondria			
	Inner Membrane	28	168	508
	Outer Membrane	47	240	407
	Matrix	53	197	268

7 units of ACTH per rat were injected subcutaneously 3.5 hr before the death of animals. For details of the experimental conditions, see text.

ethanol-ether extracted fractions of the subcellular components from rat adrenal glands was summarized in Table 1. Two experiments were carried out independently and almost identical results were obtained. Incorporation of ^3H was highest in the post-mitochondrial fraction, while the incorporation into the inner membrane of mitochondria was about 1/3 of that of the post-mitochondrial fraction. This might imply that phospholipids of inner membrane of mitochondria are most stable metabolically in adrenal glands. Increased uptake of the radioactivity was noticed in all the subcellular components of adrenal from the ACTH-treated animals. A most remarkable increase in the incorporation was noticed in the inner membrane fraction of mitochondria. In this fraction, almost 500% stimulation was observed. The inner membrane of adrenal mitochondria has been considered to be a site of the steroidogenic action of ACTH and increased uptake of ^3H -leucine was also observed in this fraction after the same treatment of animals (Ichii *et al.* 1970). While the rate of stimulation of choline incorporation by ACTH was moderate in the crude nuclear and post-mitochondrial fractions. The rate of incorporation into the outer membrane and matrix fraction was significantly higher than that into the inner membrane fraction, however there might be a possibility that the outer membrane fraction was contaminated with microsomes. The rate of microsomal contamination in the outer membrane fraction could not be determined in this experiment, since a proper marker enzyme for adrenal microsomes was not detected. This conspicuous stimulation in the incorporation of ^3H -choline into adrenal after ACTH administration seems to be quite a great contrast to the results reported by Redmond and Kostyo (1970). These authors observed no significant changes in phospholipid content in rat adrenal 3 hr after administration of a large dose (10 units per rat) of ACTH. They used oxycel-purified ACTH and a relatively crude preparation of

long-acting type ACTH was used in this experiment. However, the difference of ACTH preparations used for the experiments does not seem to explain these different results. It is unlikely that the rate of choline incorporation does not reflect the activity of membrane biosynthesis, since injected choline is mainly incorporated into phospholipids of biological membranes in rat liver (Pasternak and Bergerone, 1970). One possibility which must be examined to explain these different results is that the increased ^3H -choline incorporation is the result of the accelerated rate of membrane turnover which might induce increased exchange of membrane components. An experiment is in progress to examine this possibility. Increased phospholipid biosynthesis has been reported in rat liver after thyroxine treatment (Tata, 1967) and in rat uterus after administration of estradiol (Aizawa and Mueller, 1961, Ui and Mueller, 1963).

Administration of cycloheximide in that block protein biosynthesis in the adrenal (Davis and Garren, 1968) 30 min before ACTH injection inhibited the increase in the

Table 2. Effect of Cycloheximide and Actinomycin D on the ACTH-induced incorporation of ^3H -choline into the ethanol-ether extracted fractions of subcellular components from rat adrenal glands

Treatment	Specific Activity (dpm/ μgP)			
	Homogenate	Crude Nuclear	Mitochondr.	Post-Mitochondr.
None	70	67	52	114
Cyclohex.	64	60	49	98
Act. D	75	69	58	115
ACTH	267	229	232	383
ACTH + Cyclohex.	68	74	55	129
ACTH + Act. D	245	198	236	323

7 units of ACTH per rat were administered subcutaneously 3.5 hr before the death of animals. 10 mg of cycloheximide and 200 μg of actinomycin D per rat were injected intraperitoneally 30 min before treatment of animals with ACTH. For other experimental conditions, see text.

^3H -choline incorporation induced by ACTH completely, while a large dose of Actinomycin D did not influence the stimulating effect of ACTH (Table 2). Inhibitory effect of cycloheximide was observed in all the sub-cellular fractions examined. Either cycloheximide or Actinomycin D alone did not influence the basal rate of the incorporation. These results seem to be quite meaningful from the view that administration of cycloheximide inhibited the stimulation of corticosterone production by ACTH (Ferguson, 1963, Davis and Garren, 1968), while administration of Actinomycin D did not influence the steroidogenic effect of ACTH (Ferguson and Morita, 1964). On the other hand, a rapid transformation of tubular to vesicular structure of mitochondrial cristae in cultured rat adrenal cells by supplementing ACTH to the culture medium was reported by Kahri (1968) and Actinomycin D and puromycin inhibited this transformation of mitochondrial cristae if they were added with ACTH. In this experiment, Actinomycin D had no influence on the rate of ACTH-induced ^3H -choline incorporation into membrane systems. However, the relationship between the ultrastructural changes of mitochondrial cristae and the activity of choline incorporation is not clear at this moment. Furthermore, the physiological relationship between the steroidogenic effect and the stimulation of choline incorporation into membrane systems induced by ACTH must be also clarified to throw insight into the action mechanism of ACTH.

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