EFFECT OF ACTH ON CHOLESTEROL SIDE-CHAIN CLEAVAGE IN RAT ADRENAL MITOCHONDRIA

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SUMMARY. The acute effect of ether stress on cholesterol side chain cleavage and cytochrome P-450 has been studied in intact rat adrenal mitochondria using light absorption and EPR spectroscopy. The effect of stress is to increase the ratio of high-spin to low-spin oxidized cytochrome P-450 and this is accompanied by an increase in the rate of pregnenolone synthesis from adrenal mitochondrial cholesterol. Cycloheximide pre-treatment prevented these effects of ether stress. The action of ACTH appears to involve an increase in the association of cholesterol with cholesterol side chain cleavage cytochrome P-450.

Side-chain cleavage (SCC) of cholesterol is the rate-limiting step in the production of corticosteroids in the adrenal cortex (1). The activation by ACTH is blocked by the inhibitors of protein synthesis, puromycin and cycloheximide (2, 3).

Studies involving the binding of steroids and selective inhibitors (4,5) have indicated that SCC of cholesterol and steroid 11β-hydroxylation occur at distinct P-450 entities within bovine adrenal cortical mitochondria. This has been confirmed by a partial separation of these cytochromes (6). Light

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absorption (7) and EPR spectra (8) of the specific SCC-cytochrome P-450 indicate that the cytochrome is predominantly in a high spin state at pH 7.4 in 10 mM phosphate buffer. Since the purified cytochrome P-450 which has been isolated from Pseudomonas putida exists in the high spin state only in the oxidized enzyme-camphor (substrate) complex (9), SCC-cytochrome P-450 is probably isolated as the enzyme-cholesterol complex. Support for this comes from the work of Harding (10) in which cholesterol SCC cytochrome P-450 has been isolated free from cholesterol and the addition of cholesterol then gives a type I light absorption difference spectrum with formation of a high spin complex.

An almost complete conversion of SCC-cytochrome P-450 from high to low spin state in the separated preparation and in intact mitochondria is associated with the provision of reducing equivalents (11). The major part of this change has been attributed to the rate of oxidation of cholesterol in the high spin complex exceeding the slow rate of cholesterol association with SCC-cytochrome P-450. This conclusion is supported by the kinetics of pregnenolone formation and oxygen utilization.

If such a situation occurs in vivo then the acute action of ACTH could result in an increase in the rate at which cholesterol is bound to the SCC-cytochrome P-450. The present communication describes experiments which support this concept.

Experimental Procedure

Female Sprague-Dawley rats (160-200 g) were used throughout. An acute increase in the blood level of ACTH was achieved by subjecting the rats to a 10-minute ether stress (12). Other rats were killed quickly with a minimum of stress (quiescent). Another group were given cycloheximide (10mg, I.P.) and 10 minutes later were killed. Still another group received cycloheximide and 10 minutes later were given the standard 10 minutes ether stress prior to killing. Adrenals were pooled by group, trimmed free of adhering fat and adrenal mitochondria prepared by conventional methods after homogenization in 0.25M sucrose.

Cholesterol SCC in rat adrenal mitochondria was determined as described previously (11). Cholesterol in mitochondrial samples was measured by gas liquid chromatography on 1% SE-30 with flame ionization detection. Optical spectra were obtained using an Aminco-Chance dual wavelength, split beam recording spectrophotometer. Mitochondrial protein was determined by the method of Lowry et al (13).

Adrenal mitochondrial samples for EPR measurements were prepared in duplicate at a concentration of approximately 30 mg protein per ml and frozen in matched quartz tubes. EPR spectroscopy was carried out as described (9). The temperature dependence of the high spin resonance at g = 8.2 was found not to be significantly different from that observed with the corresponding signal from P-450 of Ps. putida. The quantitative evaluation of the signal at g = 8.2 of adrenal mitochondria was therefore based on the previous work with bacterial P-450.

Results

In order to prevent further metabolism of pregnenolone, adrenal mitochondrial incubations were carried out in the presence of 4 μM 2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androstene-3-one (cyanoketone), an inhibitor of steroid 3β-ol-dehydrogenase (14). Figure 1 shows the total pregnenolone formation from endogenous cholesterol in intact mitochondria from rats which were ether-stressed and rats which were treated with cycloheximide. Addition of isocitrate to the mitochondria effected a rapid phase of pregnenolone formation, followed by a much slower phase. The effect of ether stress was to increase the initial rate of pregnenolone formation by 2-3 times.

Similar kinetics were observed for the depletion of endogenous cholesterol in these incubations. The adrenal mitochondria from stressed rats were depleted of cholesterol faster after addition of isocitrate than adrenal mitochondria from cycloheximide-treated rats. However, the initial cholesterol level was not significantly different in the mitochondria from the two groups.

The total cytochrome P-450 content and the 11β-hydroxylase activity were similar in rat adrenal mitochondria from both groups. In order to examine SCC-

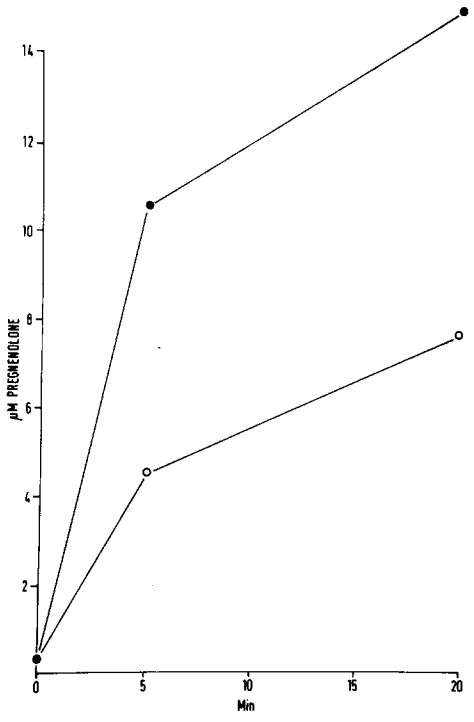

Figure 1. Total pregnenolone formation in intact adrenal mitochondria from rats treated with cycloheximide (o) and rats given an ether stress (•). Pregnenolone formation was initiated by addition of 9 mM D,L-isocitrate. Cyanoketone was present at a concentration of 4 μM and the mitochondrial protein concentrations were: 1.7 mg/ml (o); 1.4 mg/ml (•). The incubation temperature was 28°C.

cytochrome P-450, advantage was taken of the fact that pregnenolone in high concentrations binds to high spin SCC-cytochrome P-450 with complete conversion to the low spin state. A type II difference spectrum is produced which is directly related to the amount of the high spin complex (7). The magnitude of the type II difference spectrum induced by pregnenolone was at least twice as large in adrenal mitochondria from ether-stressed rats as compared to cycloheximide-treated rats (Table 1). When reducing equivalents were added to rat adrenal mitochondria, a type II spectral change was observed similar to that reported for bovine adrenal mitochondria (11). This spectral change again appears to measure the amount of high spin SCC-cytochrome P-450. In adrenal mitochondria from ether-stressed rats this change was about 2-3 times greater than in mitochondria from cycloheximide-treated rats (Table 1).

Table I

Type II difference spectra in intact rat adrenal mitochondria ($\Delta A_{420-390\ nm}$/mg protein x 10^3)

	Isocitrate[a]	Pregnenolone[b]
cycloheximide-treated	3.0	6.0
		7.0
stressed	10.0	15.0
		15.0

a. D,L - Isocitrate concentration was 2 mM with 2µg/ml rotenone present.

b. Pregnenolone concentration was 13 µM with 4 µM cyanoketone present.

Direct confirmation of the increase in high-spin SCC-cytochrome P-450 in adrenal mitochondria from ether-stressed rats was obtained by EPR measurements. Figure 2 shows an X-band EPR spectrum from 200 to 6000 gauss. The amplitude of the center of the spectrum is reduced by a factor of approximately 30. From Figure 3 it can be seen that there was about a three-fold increase in the high-spin signal (g = 8.2) in adrenal mitochondria from stressed rats when compared to adrenal mitochondria from rats which had been given cycloheximide, or had been given cycloheximide and then an ether stress, or were quiescent. The concentration of P-450 in the low spin form was 2.8 ± 0.2 nmoles/mg protein whereas the high spin form maximally amounted to a concentration of 0.19 ± 0.05 nmoles/mg protein (Fig. 3, stressed). Consequently no significant differences in the concentration of the low spin form could be seen in the various types of samples. It is noteworthy that the concentration of reduced adrenodoxin, measured after the addition of methyl viologen and an excess of dithionite, was 2.0 ± 0.2 nmoles/mg protein, i.e., of approximately the same magnitude as that of total P-450. The changes in the signals at g = 6 which are presumably due to other high spin heme compounds are not understood.

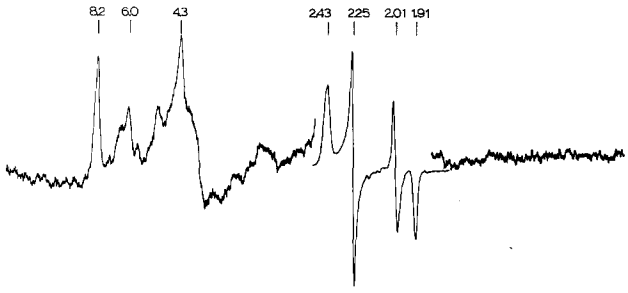

Figure 2. EPR spectrum (first derivative) of adrenal mitochondria from stressed rats. The conditions of EPR spectroscopy were: Microwave power 30 mwatt; frequency 9.17 GHz; modulation amplitude 10 gauss; temperature 12.3°K; scanning rate 500 gauss/min and time constant 1 sec. For recording the center, the amplification, power and time constant were reduced 3.1-, 100- and 4-fold, respectively.

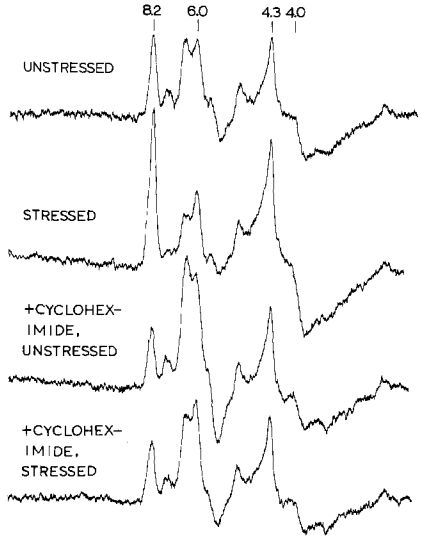

Figure 3. Low field portion of EPR spectra of adrenal mitochondria obtained from rats under different conditions. The conditions of EPR spectroscopy were: Microwave power 10 mwatt; temperature 8°K; time constant 0.5 sec. and other conditions as for Fig. 2.

Discussion

The time course for the conversion of cholesterol to pregnenolone and the effect of ether stress upon the initial phase of this conversion are similar to those reported for the conversion of cholesterol to corticosterone in a

combined system of mitochondria and microsomes (15). Since ether stress increases blood ACTH levels in the rat 3-4 fold (12) these results are in keeping with ACTH action being expressed by an increase in cholesterol SCC activity.

From the measurements of pregnenolone and isocitrate-induced spectral changes it is clear that adrenal mitochondria from ether-stressed rats have a 2-3 fold increase in the high spin cholesterol SCC cytochrome P-450 compared to adrenal mitochondria from rats which have been treated with cycloheximide to inhibit ACTH action. The EPR measurements confirm that there is indeed a significant increase in high spin cytochrome P-450, and as this high spin form is in all probability cytochrome P-450 combined with cholesterol, the effect of ACTH is apparently to increase the amount of this cholesterol-cytochrome P-450 complex. It seems likely that the 2-3 fold increase in the initial rate of pregnenolone formation induced by ether stress is related to this increase in the initial concentration of enzyme-substrate complex.

Treatment of rats with cycloheximide prevented the increase in high spin SCC cytochrome P-450 suggesting that the ACTH initiated process is dependent upon protein synthesis. It is apparent that ACTH effects a redistribution of cholesterol within adrenal mitochondria and a labile protein may be involved in this redistribution.

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