Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 198-205

CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN THE OUTER AND INNER ZONES OF THE ADRENAL CORTEX

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Received November 29, 1983

Cholesterol side-chain cleavage activity in mitochondria isolated from the outer and inner zones of the guinea pig adrenal cortex was evaluated in order to clarify the role of the zona reticularis in steroidogenesis. It was found that side-chain cleavage activity was three times higher in the outer zone. In addition, ether stress increased side-chain cleavage activity in the outer zone but not the inner zone. The concentration of total and free cholesterol was also found to be higher in the outer zone. However, when exogenous cholesterol was added to mitochondria, there was no enhancement in side-chain cleavage activity in either zone.

Functional examination of the zona fasciculata and zona reticularis of the adrenal cortex has until recently been hampered by lack of an adequate animal model. The innermost zone of the adrenal cortex, the zona reticularis, is unusually large in the guinea pig enabling it to be separated from the fasciculata. Recently several reports have appeared in the literature describing marked functional differences between the outer (glomerulosa/fasciculata) and inner (reticularis) cortical zones of the guinea pig adrenal (1-4). It is now established that, in contrast to cells isolated from the fasciculata, cells isolated from the reticularis have a very low basal production of cortisol (5,6) and that there is no cortisol response to either ACTH (5,6) or cAMP (6). In addition, there is no detectable increase in the production of pregnenolone, progesterone, or androstenedione (6). Such results suggest that reticularis cells might lack the ability to convert cholesterol to pregnenolone. That is, the mitochondrial cholesterol side-chain cleavage (SCC) activity, the rate-limiting step in steroidogenesis, might be markedly reduced in the zona reticularis. A reduction in cholesterol

0006-291X/84 $1.50

SCC activity could be the result of either an alteration in the enzyme complex or in substrate availability or both. These considerations form the basis for this report.

MATERIALS AND METHODS

Male guinea pigs (NIH strain 2) weighing 700-900 g were killed by decapitation (within 10 sec upon handling) at 1000-1030 h. The adrenal glands were processed and the outer and inner cortical zones separated as previously reported (1). Mitochondria were isolated and partially purified as previously described (7). Morphological integrity of mitochondria prepared from the two zones was found to be excellent when examined by electron microscopy.

Mitochondrial incubations were carried out essentially as previously described (7). The incubation mixture (200 µl) contained 0.125 M sucrose, 30 mM Tris-HCl (pH 7.4), 20 mM KCl, 1 mM EDTA, 10 mM malate, 10 mM isocitrate, 10 µM trilostane, and the mitochondria (80-100 µg). When the effect of exogenous cholesterol or cholesterol sulfate was studied, the substrates (final concentration 100 µM) were added with ethanol (5% v:v for cholesterol and 1% v:v for cholesterol sulfate) and tween 80 (2 µg). Incubations were terminated by freezing in dry ice-acetone.

Pregnenolone was usually measured directly using a highly specific radioimmunoassay (8). When both pregnenolone and pregnenolone sulfate were assayed, the incubation mixture was extracted 3 times with ethyl acetate, and the two steroids isolated by thin-layer chromatography using the solvent system isooctane: ethyl acetate: t-butanol: methanol: 1 M NH_4OH (2:4:2:2:3). The recovery of pregnenolone was 86.2 ± 2.6% and pregnenolone sulfate 51.8 ± 7.3%. Pregnenolone sulfate was also measured using a radioimmunoassay. For both assays the intraassay CV was 4-8% while the interassay CV was about 10%.

Total and free cholesterol in mitochondrial preparations and whole homogenates were measured by the fluorescence-coupled cholesterol oxidase method of Gamble et al. (9) after extraction with chloroform-methanol. Protein was determined by the method of Lowry et al. (10) using BSA as the standard.

RESULTS

Cholesterol side-chain cleavage activity

The results of the time course and dose response studies are depicted in Fig. 1 and 2. Cholesterol SCC activity reached a plateau after about 5 min and demonstrated a linear dose response for mitochondria isolated from both zones. The SCC activity was approximately 3 times higher in mitochondria isolated from the outer zone. The pregnenolone concentration in the 0 min samples was 0.13-0.26 and 0.05-0.13 (nmol/mg protein) for the inner and outer mitochondria respectively.

Cholesterol concentration

The concentration of total and free cholesterol in whole homogenates and isolated mitochondria is shown in Fig. 3. As can be seen, both the total and free cholesterol are markedly reduced in the inner zone ($p < .001$). The proportion of free to total cholesterol in mitochondria was 67% for the inner zone

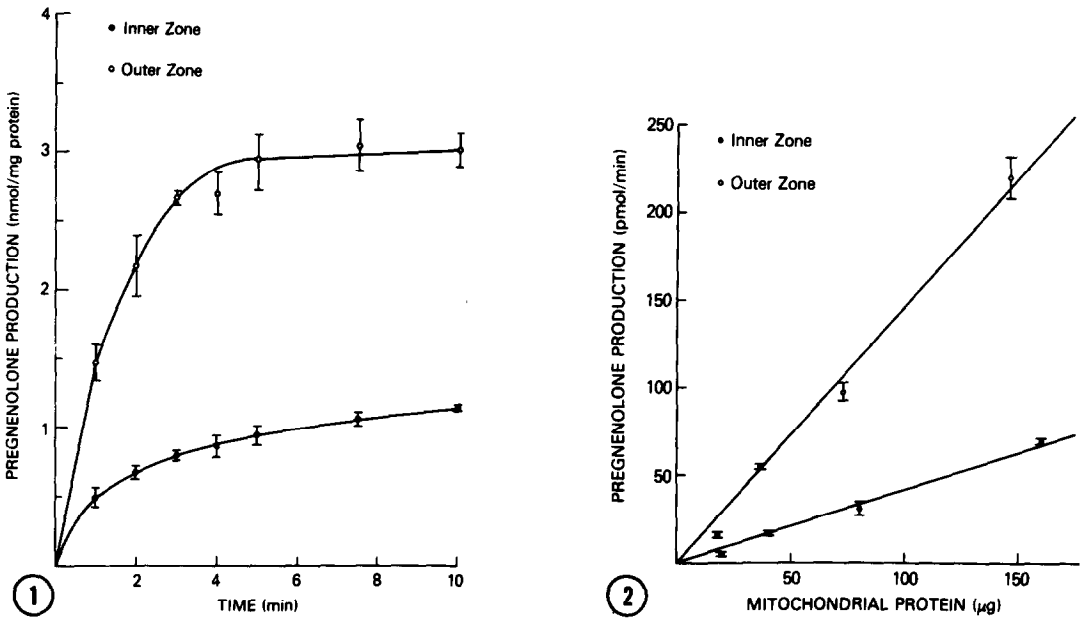

Fig. 1. Time course of pregnenolone production from endogenous cholesterol by mitochondria isolated from the inner and outer adrenocortical zones. Mitochondria were incubated at 37°C for the time indicated and pregnenolone was measured by radioimmunoassay. Data points and vertical bars represent the mean ± S.D. (n = 5).

Fig. 2. Dose response of pregnenolone production from endogenous cholesterol by mitochondria isolated from the inner and outer adrenocortical zones. Mitochondria were incubated at 37°C for 10 min and pregnenolone was measured by radioimmunoassay. Data points and vertical bars represent the Mean ± S.D. (n = 5).

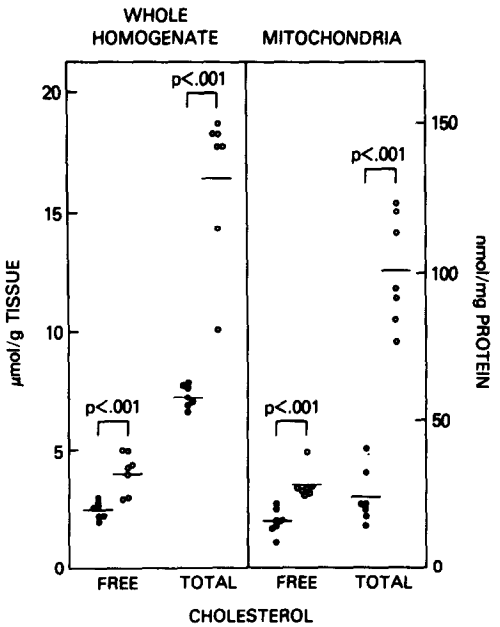

Fig. 3. Cholesterol concentration in whole homogenates and isolated mitochondria of the outer (O) and inner (●) adrenocortical zones. Cholesterol was measured by a fluorescence-coupled cholesterol oxidase method. The horizontal bars represent Mean values.

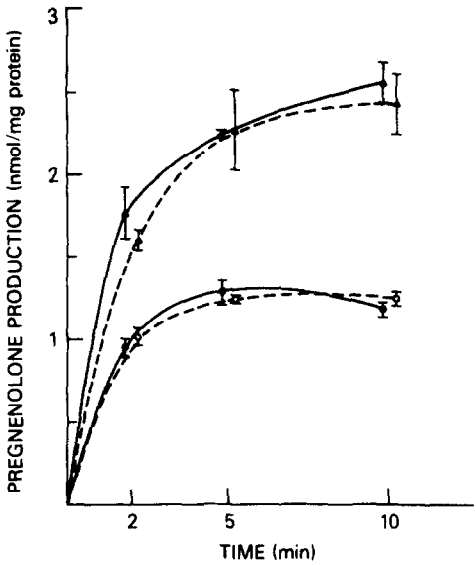

Fig. 4. Pregnenolone production from endogenous (solid lines) and exogenous (broken lines) cholesterol by mitochondria isolated from the inner (circles) and outer (triangles) adrenocortical zones. Mitochondria were incubated at 37°C for the times indicated and pregnenolone was measured by radioimmunoassay. Cholesterol (final concentration 100 μM) was added with ethanol (0.5% v:v) and Tween 80 (2 μg). Data points and vertical bars represent the Mean ± S.D. (n = 5).

and 24% for the outer zone, while in whole homogenates the values were nearly the same at 34 and 25%.

Effect of exogenous cholesterol

When cholesterol was added to mitochondrial incubations to a final concentration of 100 μM, the results obtained are depicted in Fig. 4. As can be seen, the cholesterol SCC activity for the inner and outer zones was essentially the same in the presence and absence of exogenous cholesterol. We also tested adding cholesterol in 0.1% BSA with and without tween 80 but could detect no enhanced activity of cholesterol SCC in the two zones.

Effect of ether stress and dexamethasone suppression

Plasma cortisol was monitored to evaluate the efficacy of procedures used to alter adrenal cortisol secretion. The mean serum cortisol values (μg/dl) were (n=5): control 32.9, ether stress 58.2, dexamethasone suppression 4.1.

Cholesterol SCC activity in the three groups is shown in Fig. 5. Stress caused a significant increase ($p<.005$) in SCC activity in the outer zone but

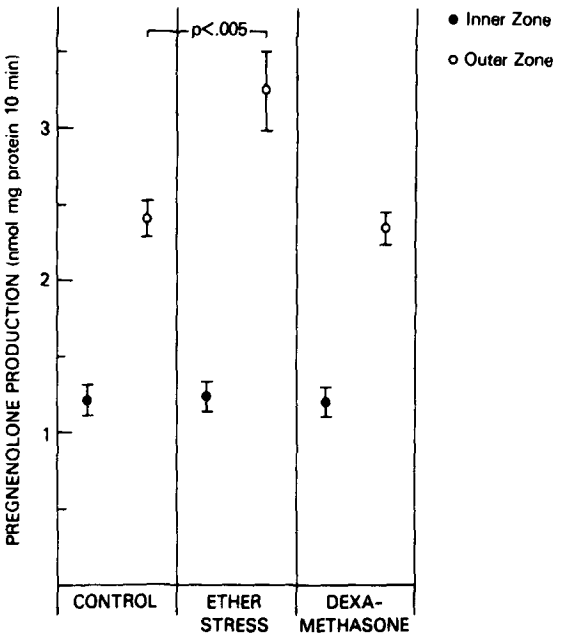

Fig. 5. Pregnenolone production from endogenous cholesterol by mitochondria isolated from the inner and outer adrenocortical zones in response to ether stress and dexamethasone suppression. Mitochondria were incubated at 37°C for 10 min and pregnenolone was measured by radioimmunoassay. For ether stress animals were exposed twice to ether vapor for 1 min in a jar at an interval of 5 min and killed 15 min after the final exposure. For dexamethasone suppression, dexamethasone sodium phosphate was dissolved in normal saline and injected i.p. in a dose of 1.0 mg/Kg BW at 0600 h. The control animals were not manipulated. All animals were killed at 1200 h. The data points and vertical bars represent the Mean ± S.D. (n=6).

had no effect in the inner zone. Acute dexamethasone suppression did not alter SCC activity in either zone. Using mitochondrial preparations from stressed animals, the effect of exogenous cholesterol (100 μM) on SCC activity was examined. But, as with control animals, no enhancement in SCC activity was noted in either zone (data not shown).

Cholesterol sulfate side-chain cleavage activity

Data on the production of pregnenolone sulfate from endogenous substrate and in the presence of 100 μM cholesterol sulfate are given in Table 1. From endogenous substrate, the amount of pregnenolone sulfate formed was very low and there was no difference between mitochondria isolated from the outer and inner zones. In the presence of exogenous cholesterol sulfate, however, pregnenolone sulfate formation increased 10 to 30-fold and the cholesterol sulfate SCC activity was greater in the inner zone ($p < .02$). It should be noted that the production of pregnenolone sulfate was only one-tenth the

Table 1. Cholesterol Sulfate Side-Chain Cleavage Activity by Isolated Mitochondria [a]

Cholesterol Sulfate (100 μM)	Inner Zone	Outer Zone
-	0.016 ± 0.014[b]	0.021 ± 0.021[c]
+	0.418 ± 0.104	0.246 ± 0.031[d]

a) nmol/mg protein/10 min
b) Mean ± S.D. (n=4)
c) Not significant vs. inner zone
d) $p<0.02$ vs. inner zone

amount of pregnenolone formed in the outer zone, while in the inner zone the proportion was approximately 40%.

DISCUSSION

The data presented in this report clearly demonstrate an important functional difference between the zona reticularis and zona fasciculata. The finding that cholesterol SCC activity is markedly reduced in the reticularis is consistent with the finding that cells isolated from the zona reticularis fail to respond to either ACTH or cAMP with an increase in steroid synthesis (6). The reason for the reduced cholesterol SCC activity in the reticularis is not totally clear. Certainly the reduction is due in part to the reduced concentration of cholesterol in mitochondria isolated from the inner zone, but there may be alterations in the enzyme complex as well. Incubating mitochondria with exogenous cholesterol was an attempt to distinguish between these two possibilities. The results, however, were not conclusive. It is known that mitochondria isolated from the human adrenal (11) and from the adrenal of quiescent rats (12) will not increase cholesterol SCC activity when incubated with exogenous cholesterol, a finding which is similar to our results. On the other hand, rats given ACTH or subjected to ether stress will increase cholesterol SCC activity over that achieved with endogenous cholesterol, when exogenous cholesterol is added to the incubation medium (12, 13). In our studies with ether-stressed animals, exogenous cholesterol failed to enhance cholesterol SCC activity in either zone.

Ether-induced stress did cause a significant increase in cholesterol SCC activity from endogenous cholesterol in outer zone mitochondria, but not in the inner zone (Fig. 5). Studies in rats have also demonstrated that ether-induced stress will increase adrenal cholesterol SCC activity from endogenous substrate (13). This is an important in vivo study demonstrating that reticularis cells, in contrast to fasciculata cells, fail to respond to endogenous ACTH just as they fail to respond to exogenous ACTH in vitro (6).

The cholesterol sulfate SCC activity studies were of considerable interest. It has long been known that cholesterol sulfate can serve as a substrate for mitochondrial SCC without removal of the sulfate moiety (14). It has been suggested that cholesterol and cholesterol sulfate are cleaved by separate enzymes (15). It has also been reported that the human fetal adrenal can actively cleave cholesterol sulfate while this activity is absent in the adult adrenal (11). In fact, SCC activity in the human fetal adrenal when cholesterol sulfate was used as substrate was markedly greater than when cholesterol was used as substrate (16). The fetal adrenal in the guinea pig, unlike the human fetal adrenal, does not atrophy after birth and disappear (17). It has previously been suggested that the zona reticularis of the guinea pig adrenal cortex may form from the fetal cortex which failed to involute (1). In this regard it is interesting that cholesterol sulfate SCC activity was greater in the reticularis than in the fasciculata (Table 1).

In conclusion, there is now substantial evidence that the zona reticularis of the guinea pig adrenal cortex is not actively engaged in steroidogenesis, and in this regard is unresponsive to the physiological regulator, ACTH. Basic questions concerning the development of the zona reticularis and differential regulation are currently being pursued.

REFERENCES

1. Strott, C.A., Goff, A.K., and Lyons, C.D. (1981) Endocrinology 109, 2249-2251.
2. Martin, K.O. and Black, V.H. (1982) Endocrinology 110, 1749-1757.
3. Strott, C.A., Goff, A.K., and Lyons, C.D. (1983) J. Steroid Biochem. 18, 489-498.
4. Martin, K.O. and Black, V.H. (1983) Endocrinology 112, 573-579.
5. Eacho, P.I. and Colby, H.D. (1983) Life Sciences 32, 1119-1127.

6. Nishikawa, T. and Strott, C.A. Endocrinology (in press).
7. Ray, P. and Strott, C.A. (1981) Life Sciences 28, 1529-1534.
8. Di Pietro, D.L., Brown, R.D., and Strott, C.A. (1972) J. Clin. Endocr. Metab. 35, 729-735.
9. Gamble, W., Vaughan, M., Kruth, H.S., and Avigan, J. (1978) J. Lipid Res. 19, 1068-10790.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, J. (1951) J. Biol. Chem. 193, 265-275.
11. Mason, J.I. and Hemsell, P.G. (1982) Endocrinology 111, 208-213.
12. Simpson, E.R., McCarthy, J.L., and Peterson, J.A. (1978) J. Biol. Chem. 253, 3135-3139.
13. Mason, J.I., Arthur, J.R., and Boyd, G.S. (1978) Biochem. J. 173, 1045-1051.
14. Roberts, K.D., Bandy, L., and Lieberman, S. (1967) Biochem. and Biophys. Res. Commun. 29, 741-746.
15. Young, D.G. and Hall, P.F. (1969) Biochemistry 8, 2987-2996.
16. Korte, K., Hemsell, P.G., and Mason, J.I. (1982) J. Clin. Endocr. Metab. 55, 671-675.
17. Idelman, S. (1978) The Structure of the Mammalian Adrenal Cortex, pp. 1-199, Academic Press, New York.