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Effects of chloramphenicol on the long term trophic action of ACTH on rat adrenocortical cells: a combined stereological and enzymological study*

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INTRODUCTION

Recently it has been shown that the increase in the surface area of agranular endoplasmic reticulum membranes and mitochondrial cristae per cell, induced in the rat zona fasciculata by chronic ACTH administration (Nussdorfer, Mazzocchi & Meneghelli, 1978), is tightly coupled with an enhancement in the activity per cell of 3β hydroxysteroid dehydrogenase (3βHSD) and 11β-hydroxylase (11βOH) (Nussdorfer & Mazzocchi, 1983). These two enzymes of steroid synthesis are contained in the agranular reticulum and mitochondria, respectively (Tamaoki, 1973). Since there is general agreement that the long term action of ACTH involves stimulation of protein synthesis (Nussdorfer *et al.* 1978), stereological and enzymological techniques have been used to study the effects of a prolonged administration of chloramphenicol on the zona fasciculata cells of chronically ACTH-treated rats. In fact, chloramphenicol is known to inhibit mitochondrial DNA-dependent protein synthesis (Beattie, Basford & Koritz, 1967).

MATERIALS AND METHODS

Forty two male adult albino rats of the Wistar strain, about 200 g in weight, were divided into seven equal groups. Six groups were given intraperitoneal injections of 0·5 ml 0·85 % saline containing 12·5 IU/kg ACTH (Sigma Chemical Co., St. Louis, U.S.A.) or 12·5 IU/kg ACTH plus 50 mg/kg CAP (Synthomycetin, Lepetit, Milan, Italy) every 12 hours, for 3, 6 or 12 consecutive days. The seventh (the control) group received only intraperitoneal injections of 0·5 ml normal saline twice a day for 12 consecutive days. All the animals were killed by cervical dislocation at 10 am, 12 hours after the last injection.

The left adrenal gland of each rat was cleaned of all adherent fat, bisected, demedullated under the dissecting microscope and weighed. One half of the specimen was fixed in 10 % formalin, embedded in paraffin wax for optical microscopy, and serially cut at a thickness of 7 μm. Sliced pieces of the other half were processed for electron microscopy: they were fixed in 3 % glutaraldehyde, postfixed in 1 % osmium tetroxide and embedded in an epoxy resin (Nussdorfer, Mazzocchi & Rebuffat, 1973). Thick sections of the three adrenal zones were cut with an LKB III ultramicrotome and photographed at a magnification of × 1250. Thin sections were taken at the level of the zona fasciculata and examined in a Hitachi HS-9 electron micro-

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scope at low magnification (×500). Only parenchymal cells located in the middle portion of the zona fasciculata were photographed and recorded for stereologic evaluation.

Sampling procedure for morphometry was the same as detailed in earlier work (Nussdorfer *et al.* 1973). The average volume of the three adrenal zones and the percentage volume occupied by the extraparenchymal space were estimated on paraffin sections, 7 μm thick (Nussdorfer *et al.* 1973). The mean volume of zona glomerulosa, zona fasciculata and zona reticularis cells was calculated using light micrographs at ×1250 (Nussdorfer, 1970). The average number of parenchymal cells per mg of adrenocortical tissue was evaluated roughly according to the procedure of Neri, Gambino, Mazzocchi & Nussdorfer (1978). The adrenocortical halves were weighed and the average number of adrenocortical cells in each zone was estimated; this last parameter was obtained by dividing the volume of each zone (minus that occupied by the extraparenchymal space) by the average cell volume. The surface area per zona fasciculata cell of agranular endoplasmic reticulum membranes and mitochondrial cristae was measured on electron micrographs at a final magnification of ×42000, by estimating the surface concentration of these two classes of membrane (i.e. μm^2 of agranular reticulum membranes and mitochondrial cristae per μm^3 of cell; Loud, 1962) and then multiplying this parameter by the mean, zona fasciculata, cell volume.

The right adrenal gland of each rat was obtained in the same way as the left one and homogenised in 0·25 M buffered sucrose. 3βHSD activity was estimated on the whole homogenate (Salomon & Sherman, 1976). Adrenal homogenates, each equivalent to 8 mg of tissue, were centrifuged at 90000 *g* for one hour and incubated in glass tubes in 0·2 ml Tris-hydrochloride buffer, pH 8·3, containing 0·16 mg NAD and 1 μg pregnenolone (Sigma) in 0·01 ml ethanol. Incubation was performed at 37 °C for two hours with agitation and stopped by placing the tubes in a dry ice acetone bath. Progesterone was extracted with ether and the ether extracts were desiccated at 37 °C. Dried samples were then dissolved in cyclohexane-benzene-methanol (60:40:10, by volume) and chromatographed on Sephadex LH-20 columns, using the same solvent as the mobile phase. Purified progesterone was estimated by radioimmunoassay, using standard kits (Progesterone-3H RIA Pak, New England Nuclear Corporation, Frankfurt/M, W. Germany). Results were expressed as ng of progesterone formed by 1 mg of adrenal in 1 minute.

Activity of 11βOH was assayed according to Greiner, Kramer & Colby (1976), on a mitochondrial fraction obtained by differential centrifugation at 10000 *g* for 15 minutes (Péron & McCarthy, 1968). Mitochondrial pellets, each equivalent to 10 mg of tissue, were incubated in glass tubes in 0·5 ml of Tris-hydrochloride buffer, pH 7·4, containing 0·9 mg KCl, 0·11 mg $MgCl_2$, 1·1 mg NaCl, 8 mg sucrose, 1·2 mg sodium isocitrate and 10 μg 11-deoxycorticosterone (Sigma) in 0·01 ml ethanol. Incubation was carried out at 37 °C for 12 minutes and stopped by adding 0·3 ml 0·5 % mercuric chloride. Corticosterone was extracted and purified as previously described and quantitated according to Spät & Jozan (1972), using mouse serum as a source of binding protein. Results were expressed as ng of corticosterone formed by a mitochondrial equivalent to 1 mg of adrenal tissue in 1 minute.

By knowing the average number of parenchymal cells per mg of adrenocortical tissue, it was possible to express the enzymatic activities as ng product/10^6 cells/minute.

The data obtained from each rat were averaged for each experimental group and

Table 1. *Effects of ACTH and CAP on the zona fasciculata cells and the activity of some enzymes of steroid synthesis of the rat adrenal cortex*

Experimental group	Volume of cells (μm^3)	Surface of agranular endoplasmic reticulum (μm^2/cell)	3βHSD activity (ng/10^6 cells/minute)	Surface of mitochondrial cristae (μm^2/cell)	11βOH activity (ng/10^6 cells/minute)
1. Controls (6)	1790·8±232·5	11604·4±1508·5	603·2±77·2	10046·4±1316·1	1518·2±197·3
2. ACTH 3 days (6)	2293·7±275·2 $p_1 < 0{\cdot}01$	14277·9±1962·1 $p_1 < 0{\cdot}01$	766·1±98·4 $p_1 < 0{\cdot}01$	12798·8±1638·8 $p_1 < 0{\cdot}01$	1988·8±245·1 $p_1 < 0{\cdot}01$
3. ACTH/CAP 3 days (6)	2111·1±280·8 $p_1 < 0{\cdot}02$ p_2 NS	14123·5±1823·5 $p_1 < 0{\cdot}01$ p_2 NS	754·6±90·1 $p_1 < 0{\cdot}01$ p_2 NS	9723·4±1254·2 p_1 NS $p_2 < 0{\cdot}01$	1460·3±170·1 p_1 NS $p_2 < 0{\cdot}01$
4. ACTH 6 days (6)	2412·9±320·9 $p_1 < 0{\cdot}01$	15942·6±2005·4 $p_1 < 0{\cdot}01$	833·0±107·5 $p_1 < 0{\cdot}01$	13096·6±1728·7 $p_1 < 0{\cdot}01$	2064·7±265·3 $p_1 < 0{\cdot}01$
5. ACTH/CAP 6 days (6)	2266·0±297·5 $p_1 < 0{\cdot}01$ p_4 NS	15023·8±1984·7 $p_1 < 0{\cdot}01$ p_4 NS	806·9±99·4 $p_1 < 0{\cdot}01$ p_4 NS	9318·0±1138·2 p_1 NS $p_4 < 0{\cdot}01$	1369·6±178·0 p_1 NS $p_4 < 0{\cdot}01$
6. ACTH 12 days (6)	2870·5±361·6 $p_1 < 0{\cdot}01$	19318·5±2414·8 $p_1 < 0{\cdot}01$	980·3±118·4 $p_1 < 0{\cdot}01$	15414·6±1926·7 $p_1 < 0{\cdot}01$	2353·2±305·8 $p_1 < 0{\cdot}01$
7. ACTH/CAP 12 days (6)	2697·7±334·1 $p_1 < 0{\cdot}01$ p_6 NS	18425·3±2231·5 $p_1 < 0{\cdot}01$ p_6 NS	1000·4±132·1 $p_1 < 0{\cdot}01$ p_6 NS	8557·8±1093·2 $p_1 < 0{\cdot}05$ $p_6 < 0{\cdot}01$	1273·2±152·7 $p_1 < 0{\cdot}02$ $p_6 < 0{\cdot}01$

Values are group means ± S.D. The number of rats in each group is indicated in parentheses. The degree of variability in the intra-animal determinations as compared to the intra-group means was evaluated by the analysis of variance and found to be not significant ($P > 0{\cdot}6$–$0{\cdot}7$). p, level of significance of the difference from the group indicated by the subscript; NS, not significant; CAP, chloramphenicol.

the standard deviation (± S.D.) was calculated. Student's *t*-test was used for statistical comparison of the data.

RESULTS

Stereological and enzymological data are shown in Table 1. The average volume of rat zona fasciculata cells increased as a function of the duration of ACTH treatment, and the cell hypertrophy was associated with a time-dependent increase in the surface area per cell of agranular endoplasmic reticulum membranes and mitochondrial cristae. The average activity per cell of 3βHSD and 11βOH was found to increase as a function of the number of days of ACTH administration.

Chloramphenicol partially blocked the ACTH-elicited cell hypertrophy, while it did not affect agranular reticulum membrane proliferation. Conversely, chloramphenicol treatment not only annulled the ACTH-caused rise in the surface area per cell of mitochondrial inner membranes, but at the twelfth day provoked a significant decrease in the surface area per cell of cristae, compared with the control value. The ACTH-induced increase in the activity per cell of 3βHSD was not affected by chloramphenicol, whereas that of 11βOH was completely reversed.

Figures 1 and 2 illustrate how the changes in the surface area per cell of agranular endoplasmic reticulum tubules and mitochondrial cristae in the treated rats were strictly similar to those in the activity per cell of 3βHSD and 11βOH, respectively.

DISCUSSION

The present results confirm the view that the ACTH-induced hypertrophy of rat zona fasciculata cells is coupled with an enhanced *de novo* synthesis of the enzymes of steroid synthesis and that a close interrelationship exists between the surface area of agranular endoplasmic reticulum membranes and mitochondrial cristae and the activity of these enzymes (Nussdorfer *et al.* 1978; Nussdorter & Mazzocchi, 1983). It may be conceived that, like the classic respiratory enzymes, those of steroid synthesis also require an adequate steric arrangement for their complete activity. Therefore, it is proposed that the ACTH-elicited increase in the surface area of agranular reticulum tubules and mitochondrial cristae provides the increased framework of basic membrane into which the newly synthesised steroidogenic enzymes can be inserted.

It is generally agreed that the mechanism underlying the ACTH-induced enhancement of the growth and steroidogenic capacity of adrenocortical cells involves stimulation of nuclear DNA-dependent protein synthesis at the transcriptional and /or translational level (for review, see Nussdorfer *et al.* 1978). The present findings indicate that stimulation of mitochondrial protein synthesis is also required. In fact, chloramphenicol, an antibiotic which specifically inhibits mitochondrial ribosomal translation (Beattie *et al.* 1967), administered at a dosage which is found to persistently block *in vivo* rat adrenocortical mitochondrial protein synthesis without affecting microsomal protein synthesis (Mazzochi *et al.* 1978), is able to reverse the ACTH trophic action on zona fasciculata mitochondria. The present results fit well with the previous demonstration that chloramphenicol blocks the ACTH-induced rise in mitochondrial cytochrome P450, but not that in microsomal cytochrome P450 (Purvis *et al.* 1973).

However, many lines of evidence suggest that the bulk of the enzymatic mitochondrial proteins are synthesised in the microsomes and then transferred to the mitochondria (Tzagoloff, Rubin & Sierra, 1973; Fridman, Foglia & DeNicola, 1975).

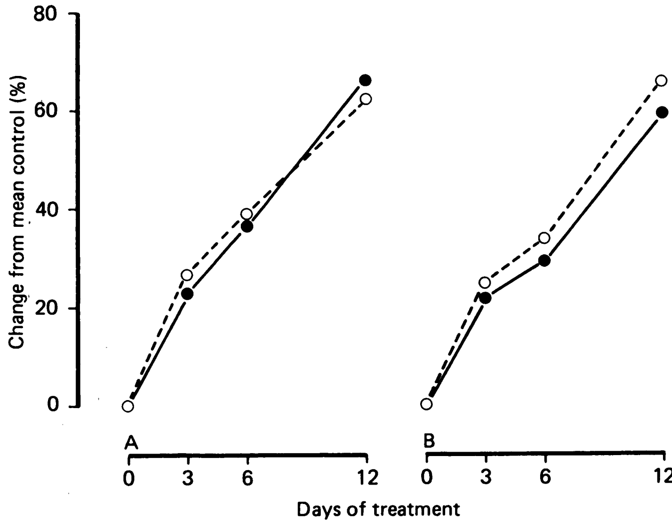

Fig. 1. Changes in the surface area of SER (●—●) and in the activity of 3βHSD (○----○) in adrenocortical cells of rats administered ACTH (A) and ACTH/chloramphenicol (B).

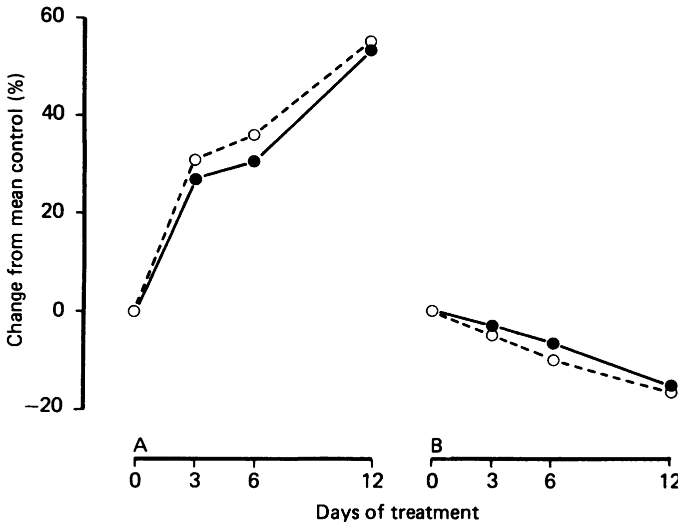

Fig. 2. Changes in the surface area of mitochondrial cristae (●—●) and in the activity of 11βOH (○----○) in adrenocortical cells of rats administered ACTH (A) and ACTH/chloramphenicol (B).

Therefore, the data in the present study concerning 11βOH may be explained solely by assuming that assembly into mitochondrial cristae of this enzyme, synthesised at the microsomal level, is possible only in the presence of proteins produced by the mitochondrial DNA-dependent enzymatic machinery.

Further studies are in progress to verify the effects of agents exclusively blocking nuclear DNA-dependent protein synthesis on the ACTH trophic action on rat zona fasciculata cells.

SUMMARY

Chronic chloramphenicol administration was found to block the ACTH-induced increase in both the surface area of mitochondrial cristae and the activity of 11β-hydroxylase in zona fasciculata cells of the rat adrenal cortex. The surface area of agranular endoplasmic reticulum membranes and the activity of 3β-hydroxysteroid dehydrogenase were not affected by treatment with chloramphenicol. These findings suggest that the mechanism of the ACTH-induced enhancement of the growth and steroidogenic capacity of rat adrenocortical cells involves stimulation of mitochondrial DNA-dependent protein synthesis.

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REFERENCES

Beattie, D. S., Basford, R. E. & Koritz, S. S. (1967). The inner membrane as the site of the *in vitro* incorporation of L(14C)-leucine into mitochondrial protein. *Biochemistry* **6**, 3099–3106.

Fridman, O., Foglia, V. G. & de Nicola, A. F. (1975). Translocation of proteins to adrenal mitochondria: a specific effect of ACTH. *Acta physiologica latinamericana* **25**, 141–143.

Greiner, J. W., Kramer, R. E. & Colby, H. D. (1976). Changes in adrenocortical function in male and female guinea pigs during maturation. *Journal of Endocrinology* **70**, 127–134.

Loud, A. V. (1962). A method for the quantitative estimation of cytoplasmic structures. *Journal of Cell Biology* **15**, 481–487.

Mazzocchi, G., Neri, G., Robba, C., Belloni, A. S. & Nussdorfer, G. G. (1978). Investigations on the turnover of adrenocortical mitochondria. X. A correlated biochemical and stereological study of the effects of chronic treatment with chloramphenicol on the mitochondria of the rat zona fasciculata. *American Journal of Anatomy* **153**, 67–80.

Neri, G., Gambino, A. M., Mazzocchi, G. & Nussdorfer, G. G. (1978). Effects of chronic treatment with ACTH on the intracellular levels of cyclic-AMP and cyclic-GMP in the rat adrenal cortex. *Experientia* **34**, 815–816.

Nussdorfer, G. G. (1970). Effects of corticosteroid hormones on the smooth endoplasmic reticulum of the rat adrenocortical cells. *Zeitschrift für Zellforschung und mikroskopische Anatomie* **106**, 143–154.

Nussdorfer, G. G. & Mazzocchi, G. (1983). Long-term effects of ACTH on rat adrenocortical cells: a coupled stereological and enzymological study. *Journal of Steroid Biochemistry* **19**, 1753–1756.

Nussdorfer, G. G., Mazzocchi, G. & Rebuffat, P. (1973). An ultrastructural stereologic study of the effects of ACTH and adenosine 3′:5′-cyclic monophosphate on the zona glomerulosa of rat adrenal cortex. *Endocrinology* **92**, 141–151.

Nussdorfer, G. G., Mazzocchi, G. & Meneghelli, V. (1978). Cytophysiology of the rat adrenal zona fasciculata. *International Review of Cytology* **55**, 291–365.

Péron, F. G. & McCarthy, J. L. (1968). Corticosteroidogenesis in the rat adrenal gland. In *Functions of the Adrenal Cortex* (ed. K. W. McKerns), vol. 2, pp. 261–337. Amsterdam: North Holland Publishing Co.

Purvis, J. L., Canick, J. A., Mason, R. W., Estabrook, R. W. & McCarthy, J. L. (1973). Lifetime of adrenal cytochrome P-450 as influenced by ACTH. *Annals of the New York Academy of Sciences* **212**, 319–342.

Salomon, D. S. & Sherman, M. I. (1976). Gonadotrophin stimulation of progesterone synthesis by midpregnancy mouse ovarian cells *in vitro*. *Endocrinology* **99**, 800–808.

Spät, A. & Jozan, S. (1972). Competitive protein binding assay of corticosterone. *Journal of Steroid Biochemistry* **3**, 755–759.

Tamaoki, B. I. (1973). Steroidogenesis and cell structure. Biochemical pursuit of sites of steroid biosynthesis. *Journal of Steroid Biochemistry* **4**, 89–118.

Tzagoloff, A., Rubin, M. S. & Sierra, M. F. (1973). Biosynthesis of mitochondrial enzymes. *Biochimica et biophysica acta* **301**, 71–104.