ADRENAL RESPONSES TO CORTICOTROPHIN IN
THE PRESENCE OF AN INHIBITOR OF PROTEIN SYNTHESIS

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ABSTRACT

The effect of cycloheximide, an inhibitor of protein synthesis, on the response of the rat adrenal to ACTH was studied. Cycloheximide blocks corticosteroidogenesis in vivo and in vitro, but does not affect the increase in adrenal blood flow in vivo. When the corticosterone production of adrenal slices was studied after ACTH stimulation in vivo, it was found that adrenal slices from rats pre-treated with cycloheximide, secreted corticosterone just as efficiently as adrenal slices from control animals. It is concluded that cycloheximide does not block the primary action of ACTH but that it inhibits subsequent enzymatic processes taking place in the mitochondria. The hypothesis is put forward that the increase in adrenal blood flow induced by ACTH might be due to prostaglandins which could be formed from unsaturated fatty acids released by the cleavage of cholesterol esters.

Whether the steroidogenic action of adrenocorticotropic hormone (ACTH) is mediated via the synthesis of a specific protein is still a matter of controversy. Experiments with inhibitors of protein synthesis in vitro have indicated that active protein synthesis is required to initiate steroidogenesis. Thus the blocking effect of puromycin has been demonstrated by Ferguson (1963), that of chloramphenicol by Farese (1964), and that of cycloheximide by Garren et al. (1965). Inhibitors of protein synthesis suppress the steroidogenic action of ACTH in vivo when given before ACTH and block steroidogenesis when given after ACTH (Garren et al. 1965).

The corticosteroidogenic effect of corticotrophin in vivo is accompanied by

Dedicated to Professor Dr. A. Hottinger on the occasion of his 70th birthday.
an increase in adrenal blood flow (Holzbauer & Vogt 1957; Sapirstein & Goldman 1959; Staehelin et al. 1965) and a depletion of ascorbic acid (Sayers et al. 1948). These two effects occur independently of the synthesis of corticosterone (Staehelin et al. 1965). It therefore seemed interesting to examine the effects of an inhibitor of protein synthesis, administered either in vivo or in vitro, on the response of various measurements to corticotrophin.

METHODS AND MATERIALS

The ACTH preparation employed was $\beta^{1-24}$ corticotrophin (tetracosactide) (Schwyzter & Sieber 1963). The cycloheximide was kindly provided by Upjohn Company, Kalamazoo, through the courtesy of Dr. G. M. Savage.

Male rats were used which had been hypophysectomized 24 hours previously. Corticosterone was measured fluorimetrically using the procedure of Mattingly (1962). Cannulation of the adrenal vein was carried out as described previously (Staehelin et al. 1965).

Adrenal ascorbic acid depletion was determined according to the method of Sayers et al. (1948). 20 minutes after the injection of cycloheximide (50 mg/kg), one adrenal was removed as a control. Corticotrophin was then injected and the other adrenal removed an hour later. Plasma corticosterone was measured on these animals at sacrifice.

For the in vitro experiments, rats were hypophysectomized 2 hours before the experiments and adrenal slices were incubated according to the method of Saffran & Schally (1955). The four quarters of one adrenal usually served as a control for the other adrenal taken from the same animal.

RESULTS

a. Effects of cycloheximide added in vitro

Cycloheximide added to adrenal slices incubated in vitro in the presence of ACTH induces a dose-dependent inhibition of steroidogenesis (Fig. 1), a 50 % inhibition being obtained with a cycloheximide concentration of about 10 $\mu$g per ml. Like the effect of puromycin (Ferguson 1963), the inhibition caused by cycloheximide is time-dependent and is observed only when the cycloheximide is added together with or shortly after the addition of corticotrophin. When added 30 minutes later, cycloheximide exerts only a slight inhibitory effect, and after 60 minutes there is practically no inhibition (Fig. 2).

b. Effects of cycloheximide administered in vivo

In order to study the activity of cycloheximide in vivo, both the blood flow and steroidogenesis were examined by cannulation of the adrenal vein. Fig. 3 shows the parallel increase in blood flow and steroid production following an intravenous injection of $\beta^{1-24}$ corticotrophin (tetracosactide). The blood flow, which is initially enhanced by the administration of corticotrophin, subsequently
Inhibition of corticotrophin activity by cycloheximide in vitro.

Adrenal slices were incubated for 2 hours, according to the method of Saffran & Schally (1955), in the presence of 10 μg tetracosactide (β1-24 corticotrophin). One adrenal was used as control, whereas the other adrenal from the same animal was incubated with cycloheximide. The corticosterone in the medium was determined fluorimetrically. The ordinate represents the amount of corticosterone produced per adrenal. Control values are indicated by the hatched area. Each dose is the mean of 3 animals ± S. E. M.

gradually diminishes. This is probably due to the constant loss of blood from the circulation during cannulation. The fact that after 80 minutes it is still possible to collect roughly the same amount of blood as at the beginning of the experiment, indicates that corticotrophin continues to enhance adrenal blood flow, since the blood flow gradually decreases in control animals.

The effect of pre-treatment with cycloheximide on adrenal blood flow and corticosterone secretion is shown in Fig. 4. Whereas cycloheximide completely abolishes the rise in steroid excretion, it does not prevent the increase in blood flow caused by corticotrophin.

It has been reported by Garren et al. (1965) that cycloheximide not only abolishes the steroidogenesis action of ACTH but also blocks the steroid secretion once this has been initiated. It was confirmed that cycloheximide, administered at the beginning of the plateau of steroid secretion, rapidly blocks steroid production (Fig. 5). We also investigated whether cycloheximide has an effect on adrenals which have been stimulated by ACTH for prolonged periods and in which any possible enzyme synthesis must have proceeded extensively. But it was found that even 8 hours after the administration of a long-acting
Adrenal slices were incubated as in Fig. 1 in the presence of 10 μg tetracosactide (β¹⁻²⁴ corticotrophin). The medium (2 ml) was removed at 60, 30, or 10 min, respectively, and fresh medium with or without cycloheximide (1 mg/ml) was then added. The corticosterone values represent the total quantity of corticosterone produced per flask from the beginning of the experiment. The unbroken lines indicate the time during which tetracosactide was present. At the end of this period the medium was changed. To one adrenal from the same animal fresh medium containing no cycloheximide (dashed line) and to the other fresh medium containing cycloheximide (dotted line) was added. Between 6 and 10 animals were used per group ± S. E. M.

ACTH preparation (Cortrophin-Z®), which induces high plasma corticosterone values for as long as 24 hours, steroid secretion into the adrenal vein was blocked by cycloheximide within 15 minutes.

In another experiment the effect of cycloheximide on adrenal ascorbic acid depletion was measured in hypophysectomized rats. The injection of cycloheximide caused no change in the adrenal ascorbic acid values after 20 min.
Fig. 3.

Time course of corticosterone secretion and adrenal blood flow after corticotrophin. The adrenal vein of one rat was cannulated as previously described (Staehelin et al. 1965). Blood was collected at 3-minute intervals into pre-weighed tubes containing 1 ml saline. At the time indicated by the arrow, 6 µg tetracosactide ($\beta^{1-24}$ corticotrophin) was injected intravenously. The blood (dashed line) was determined by weight, corticosterone was determined fluorimetrically.

The injection of 0.1 µg tetracosactide, however, caused a decrease in ascorbic acid (-48 mg/100 ml) as well as an increase in plasma corticosterone (+36 µg/100 ml) in control animals, both effects being completely abolished in the animals treated with cycloheximide.

c. Corticosteroidogenesis by adrenal slices after stimulation with corticotrophin in vivo

The finding that cycloheximide does not block the action of ACTH on the adrenal blood flow, raises the question whether the inhibitory action on steroidogenesis is exerted on the initial changes occurring in adrenal metabolism due to ACTH, or whether it might act on some subsequent enzymatic reactions which may be involved in the production of corticosteroids from steroid precursors. In order to separate the initial effects of ACTH from the subsequent corticosteroid production, the following experiments were designed. Rats were injected with ACTH, and after a short interval the adrenals were removed and adrenal slices were incubated in vitro without ACTH. From the data presented in Fig. 6, it is apparent that after 1 and 2 minutes of cortico-
Effect of pre-treatment with cycloheximide on corticosterone secretion and adrenal blood flow.

Experimental details as in Fig. 3. 30-min intervals were used. Circles: corticosterone in the adrenal venous blood per 30 min. Triangles: amount of blood collected from the adrenal vein in µl per 30 min. The upper portion of the figure represents control animals which received tetracosactide (β1-24 corticotrophin) only in a dose of 30 µg/kg. The lower portion shows data from rats which had received 10 mg cycloheximide 15 min before the tetracosactide (indicated by the first arrow). The data represent the average values of three separate experiments ± S. E. M.

In order to study the effect of cycloheximide under these experimental conditions rats were pre-treated with cycloheximide. 5 minutes after the injection of ACTH, the adrenals were removed and incubated in vitro without cycloheximide or ACTH. In the experiment outlined in Fig. 7 A, rats were pre-treated with doses of cycloheximide ranging from 1 to 10 mg/kg 20 minutes
Effect of cycloheximide on adrenal corticosterone secretion and adrenal blood flow. Adrenal venous blood was collected as in Fig. 3. 10-minute intervals were used. Tetracosactide (\(\beta^{1-24}\) corticotrophin) was given in a dose of 6 \(\mu g/kg\) after the first collection period. In the lower figure, cycloheximide (10 mg) was administered intraperitoneally at the time indicated by the second arrow. Each point represents the average of 9 separate experiments ± S.E.M.

before the administration of corticotrophin. From these data it is apparent that in adrenal slices from rats pre-treated with cycloheximide, steroidogenesis attains a level of 4–5 \(\mu g\) per adrenal, which corresponds closely to that of slices from corticotrophin-treated control rats (Fig. 6). To ensure that steroidogenesis in the animals pre-treated with cycloheximide is indeed due to the effect of corticotrophin, the experiment shown in Fig. 7 B was performed. Twenty minutes after the injection of cycloheximide, one adrenal was removed to serve as a control. Corticotrophin was then injected intravenously, and 5 minutes later the other adrenal was removed. Both adrenals were incubated for 2 hours immediately after removal. The data obtained confirm that the steroido-
Fig. 6.

Time course of the response to ACTH, added in vivo before incubation of adrenal slices.

One adrenal was removed from anaesthetized rat and incubated immediately as in Fig. 1. After an intravenous injection of tetracosactide (30 μg/kg into the vena cava), the other adrenal was removed and similarly incubated at the times indicated. The triangles represent values from single animals. Corticosterone production from the control adrenal is indicated by the hatched area.

genesis is in fact attributable to the activity of corticotrophin and that it shows a similar pattern after the three different doses of cycloheximide.

**DISCUSSION**

In view of the different effects of cycloheximide on the various parameters of ACTH action it seems of value to subdivide the observed phenomena into those related to the primary events elicited by ACTH, i.e. the mechanism of adrenal steroidogenesis, and the effects on adrenal blood flow.

1) **Primary events of ACTH action:** The data on corticosterone production in adrenal slices reported in this paper where ACTH had been administered to rats pre-treated with cycloheximide, show that cycloheximide did not inhibit under these conditions. If the assumption is made that ACTH as well as cycloheximide are washed out by the incubation of the slices, the results must reflect the events which occur during the short period of ACTH action in vivo. The
Fig. 7.

Effect of cycloheximide, given in vivo, on corticosterone production by adrenal slices 20 minutes after cycloheximide had been administered. 

$\beta^{1-24}$ corticotrophin (30 $\mu$g/kg) was injected intravenously.

A) 5 minutes later the adrenals were removed and incubated as in Fig. 1.

B) Before the injection of ACTH one adrenal was removed as a control. The other adrenal was removed 5 min after the injection of ACTH. Adrenal slices from controls receiving tetracosactide and no cycloheximide produced about 5 $\mu$g of corticosterone (see Fig. 6).

The results are expressed as the mean value of 5 to 10 animals ± S. E. M.

fact that cycloheximide does not block the subsequent corticosteroidogenesis in vitro indicates that the primary events caused by ACTH are not affected by cycloheximide. This is consistent with the observation reported by Davis & Garren (1966), that cycloheximide does not prevent the decrease in esterified cholesterol caused by ACTH, but leads to an accumulation of free cholesterol. This assumption is further substantiated by our results which show that ACTH-induced adrenal hyperaemia is not prevented by cycloheximide. The results support the hypothesis that ACTH first causes the production of $3',5'$ cyclic AMP, which activates cholesterol esterase, and that this is not inhibited by cycloheximide. In a very recent paper Grahame-Smith et al. (1967) have actually demonstrated that the production of $3',5'$ cyclic AMP is not inhibited by cycloheximide.

2) Steroidogenesis: To understand the inhibitory effect of antibiotics on the action of ACTH it must be borne in mind that steroidogenesis can also be triggered off by $3',5'$ cyclic AMP which is known to have effect on pre-existing cytoplasmic enzymes (Krebs et al. 1966). The action of this nucleotide
is also blocked by inhibitors of protein synthesis (Ferguson 1963). On the other hand, it should be noted that steroidogenesis can be blocked not only by cycloheximide and puromycin, but also by chloramphenicol. It has been found that this latter antibiotic has no effect on mammalian cytoplasmic protein synthesis, but that it inhibits protein synthesis in mitochondria and in the nucleus (Rendi 1959). The adrenal mitochondria have been shown to be the sites of side-chain cleavage of cholesterol (Constantopoulos & Chen 1961) as well as of 11β-hydroxylation (Sharma et al. 1962).

All these facts seem to suggest that inhibitors of protein synthesis interfere with reactions on the adrenal cells which occur in the mitochondria, the highly specialised structures of which might easily suffer if mitochondrial protein synthesis is blocked. Electron-micrographic studies carried out by Dr. Suter and Dr. Stäubli in our laboratories have indeed demonstrated that the structure of adrenal mitochondria is altered by cycloheximide.

This second step is not only dependent on mitochondrial protein synthesis but also on the TPNH source available for steroidal hydroxylation. The depletion of ascorbic acid is probably also involved in this step since cycloheximide blocks ascorbic acid depletion as well as steroidogenesis.

3) Adrenal blood flow: The increase in adrenal blood flow can be either an independent effect of ACTH on the adrenal vascular system or, alternatively, an effect linked to the enzymatic reactions taking place in the cytoplasm (e.g. cleavage of cholesterol esters), which still continue despite inhibition by cycloheximide. Dr. J. Grant has drawn our attention to a hypothesis, put forward by Bergström (1967), that ACTH causes the formation of prostaglandins via the liberation of unsaturated fatty acids (Bergström 1967; Shaw & Ramwell 1967). Cleavage of the cholesterol esters of the adrenal, which are particularly rich in tetraenoic acids (Eberhagen 1966) results in release of considerable amounts of unsaturated fatty acids. Since these can easily be converted to prostaglandins, the latter might constitute the link between the action of ACTH in cholesterol-ester cleavage and its action on the adrenal blood flow.

An increase in blood supply leads to a more extensive oxygen supply of the tissue. The fact that not only 3',5' cyclic AMP but even TPNH (Koritz & Peron 1958) can stimulate steroidogenesis in vitro, suggests that an activation of cytoplasmic energy production alone might suffice to stimulate steroid production. Whether such a mechanism is operative in vivo is not known at present. The fact that the increase in blood flow parallels the steroid secretion might, however, be related to the extremely rapid onset of steroidogenesis in vivo, already reported by Lipscomb & Nelson (1960). It is therefore conceivable that an increase in blood flow caused by ACTH in vivo could activate adrenal energy metabolism which in turn might potentiate the effect of 3',5' cyclic AMP on steroidogenesis and thus account for the very rapid response to ACTH in vivo.
REFERENCES


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