Mode of Action of Glucocorticoids

I. STIMULATION OF GLUCONEOGENESIS INDEPENDENT OF SYNTHESIS DE NOVO OF ENZYMES*

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The adrenal glucocorticoids regulate carbohydrate formation and protein synthesis (1-6), but the mechanism by which they influence either of these processes remains to be elucidated.

The administration of glucocorticoids to rats is followed by increased formation of several liver enzymes that participate in amino acid degradation or gluconeogenesis; e.g., tyrosine-α-ketoglutarate transaminase (7), glutamate-pyruvate transaminase (8), glucose 6-phosphatase (9, 10), fructose 1,6-diphosphatase (10, 11), glycogen synthetase (12), tryptophan pyrrolase (13), phosphoenolpyruvate carboxykinase (14, 15), and pyruvic carboxylase (16). Karlson (17) has suggested that several hormones may function by stimulating enzyme synthesis and considerable evidence from the literature may be cited in support of this concept (18, 19). Greengard, Weber, and Singhal (20) have postulated that "the regulatory effect of glucocorticoids may be brought about by action on the cellular concentration of certain enzyme proteins." Their proposal was based on data showing that glycogen formation in rats given cortisone plus either actinomycin D or puromycin was less than the amount of glycogen formed by rats given cortisone alone. Weber, Singhal, and Stamm (21) and Kenny and Kull (22) have reiterated this postulate.

The concept that the primary effect of glucocorticoids is on enzyme induction is not in agreement with the previous findings of Kvam and Parks (10) that ethionine did not diminish the early effects of hydrocortisone on blood glucose and liver glycogen in fasted, adrenalectomized rats. Segal and Lopez (23) have questioned the proposal of Greengard et al. (20). We believe that the primary effect of glucocorticoids is on induction of enzyme formation, although the latter process may be required for the maximum expression of the hormones' activity.

EXPERIMENTAL PROCEDURE

Normal and adrenalectomized rats from the Badger Research Corporation, Madison, Wisconsin, and weighing from 150 to 220 g, were used in all experiments. Bilaterally adrenalectomized rats were used 8 to 10 days after the surgery. The absence of adrenal tissue was verified after death. Unless stated otherwise, rats were maintained on a commercial (Rockland) diet and water, or 1% sodium chloride if adrenalectomized.

Hydrocortisone was a gift from Drs. K. Folkers and M. Tishler of Merck and Company. Actinomycin D was obtained from Lederle Laboratories through the courtesy of Dr. N. Bohosno. Blood glucose was assayed by the glucose oxidase method (Glucostat, Worthington Biochemical Corporation). All blood samples were taken from the tail vein of nonanesthetized rats unless otherwise stated. Potassium oxalate was used as anti-coagulant. The animals were killed by decapitation, and 350 to 520 mg of liver for glycogen analysis were excised and quickly weighed on a Roller-Smith balance. Glycogen, isolated by the method of Good et al. (24), was measured by the anthrone procedure (25).

Since rat liver phosphoenolpyruvate carboxykinase is intimately involved in gluconeogenesis, is elevated by either fasting or the administration of hydrocortisone (14), and is rapidly responsive (16), the activity of this enzyme was used as a measure of responses and inhibition of the enzyme-forming systems. Phosphoenolpyruvate carboxykinase was assayed in the supernatant fraction of liver homogenized in 0.25 M sucrose and centrifuged at 105,000 × g for 1 hour. Assay conditions were as previously described (26) except for the determination of phosphoenolpyruvate and Pi. The enzyme was cleaved with mercuric ion (27) rather than hypochlorite and the Pi liberated was estimated by the method of Sumner (28). The use of mercuric ions gave more reproducible results, since the formation of iodoform in the hypochlorite method often interfered with colorimetric analysis.

In most experiments, data were collected from animals treated in the following ways: (a) normal or adrenalectomized rats receiving vehicle; (b) actinomycin D; (c) hydrocortisone; and (d) actinomycin D given ½ hour prior to hydrocortisone. Hydrocortisone, 10 mg per animal, was administered subcutaneously; actinomycin D, 0.15 mg per animal, was given intraperitoneally. NaCl solution, 0.9%, was used as a vehicle in all cases.

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RESULTS

It has been shown that puromycin, which inhibits synthesis of protein (29, 30), also causes a depletion of hepatic glycogen (31). The rapidity of this depletion makes puromycin entirely unacceptable as a tool in studies (e.g. Greengard et al. (20)) where glycogen formation is to be measured. Therefore, prior to the use of actinomycin D to block enzyme synthesis, it was necessary to investigate the possibility that this antibiotic might exhibit a similar effect on glycogen.

In preliminary experiments, a depletion of hepatic glycogen was observed in adrenalectomized male rats fed ad libitum and treated with actinomycin D for periods up to 7 hours. Unlike the depletion of hepatic glycogen by puromycin, depletion caused by actinomycin D in these experiments was slower and was accompanied by a slight decrease of blood glucose. These data suggested that the animals had undergone a self-imposed fast under the influence of the antibiotic. Therefore, it was necessary to exclude any effect of actinomycin D on food consumption.

Adrenalectomized male rats were fed Wayne Lab Blox for 5 days prior to experimental use. Food was removed from all animals just prior to treatment with either actinomycin D or 0.9% NaCl solution. Blood for glucose analyses was collected after decapitation of the rats; samples of liver for glycogen analyses were taken as described above. Fig. 1 indicates that actinomycin D has no significant effect on the levels of either blood glucose or hepatic glycogen of fasting rats. It is therefore a suitable agent for blocking induced enzyme formation in studies of the primary function of glucocorticoids.

It is difficult to demonstrate maximal effects of glucocorticoids on both carbohydrate formation and enzyme formation in the same experiment. The former can be demonstrated most readily in animals whose carbohydrate reserves have been depleted by fasting. However, fasting induces the formation of many enzymes involved in gluconeogenesis (8, 9, 14, 16), including phosphoenolpyruvate carboxykinase in both normal and adrenalectomized rats (14).

The experiment summarized in Fig. 2 was designed to measure the influence of hydrocortisone on carbohydrate formation in adrenalectomized rats whose liver phosphoenolpyruvate carboxykinase was held at normal levels by administration of actinomycin D prior to and during the fasting period.

Fed, adrenalectomized female rats were grouped and treated as described under “Experimental Procedure,” except that the initial dose of actinomycin D was 88 μg per rat; food was removed from their cages immediately after treatment. Since this experiment was run over a period of 12 hours, an additional 175 μg of actinomycin D were given midway through the test period. The hepatic supernatant fractions of 12 adrenalectomized rats, killed at zero time before treatment or fasting, formed 53.9 ± 11.4 mmoles of phosphoenolpyruvate per minute per mg of protein. It is apparent from Fig. 2 that depletion of hepatic glycogen and lowering of blood glucose by fasting is sufficient stimulus to cause nearly a 2-fold increase in phosphoenolpyruvate carboxykinase activity. Hydrocortisone administration followed by fasting resulted in a 1.5-fold increase in enzyme concentration. 3 The activity of phosphoenolpyruvate carboxykinase in rats treated with actinomycin D prior to fast. Data were collected from adrenalectomized female rats, fasted for 12 hours after treatment. Standard deviations are represented by the vertical lines. The number of rats contributing to each value is given above the standard deviation.

2 In these experiments, a high concentration of liver glycogen was desirable in order to study its rate of depletion for as long as possible. Past experience indicated that our rats maintained higher concentrations of hepatic glycogen when fed Wayne Lab Blox rather than Rockland chow.

3 Unpublished data from this laboratory indicate that the extent of rapid induction of phosphoenolpyruvate carboxykinase following hydrocortisone administration is inversely related to carbohydrate reserves at the time of treatment. In animals treated with hydrocortisone, carbohydrate reserves are maintained near normal levels during fasting and thus induction of the enzyme is partially suppressed.
whose carbohydrate reserves had been depleted by fasting for 12 to 24 hours prior to treatment. In such fasted animals, the administration of glucocorticoids results in levels of phosphoenolpyruvate carboxykinase which are enhanced only slightly beyond the levels induced by fasting alone. However, this slight increase was found to be consistent throughout the entire series of experiments.

Fig. 3 depicts data obtained from adrenalectomized female rats killed at 2, 3, 4, 6, and 8 hours after treatment as defined in “Experimental Procedure.” It is apparent that rats receiving hydrocortisone either alone or in conjunction with actinomycin D had levels of blood glucose above control values at each time examined. Blood glucose in rats receiving hydrocortisone plus actinomycin D was elevated to almost the same extent as in rats getting hormone alone.

Although rats given hydrocortisone or hydrocortisone plus actinomycin D had significantly elevated blood glucose at 2 hours, neither group showed any increase in liver glycogen at this time. In the group given only hydrocortisone, glycogen levels increased significantly by 3 hours and were still rising at 8 hours. In the animals receiving actinomycin D plus hydrocortisone, liver glycogen was significantly increased after 4 hours and was still increasing rapidly at 8 hours. It should be emphasized that the amount of glycogen synthesized by rats receiving hydrocortisone plus actinomycin D increased from 15% of the amount formed by rats given hydrocortisone alone after 4 hours, to 40% after 8 hours. It is apparent that the activity of phosphoenolpyruvate carboxykinase found in rats given actinomycin or actinomycin plus hydrocortisone was well below that present in rats given hydrocortisone alone, and was also less than that found in the fasted controls.

Similar experiments were conducted with female rats with intact adrenal glands, treated for 6 hours after a 24-hour fast. Qualitatively, the data obtained with the normal rats (Fig. 4) were similar to those shown above for the adrenalectomized rats. Those given hydrocortisone plus actinomycin D made 43% as much liver glycogen as rats receiving hormone alone. Assays for phosphoenolpyruvate carboxykinase in the liver from 1 animal in each group verified that actinomycin suppressed the elevation of this enzyme after treatment with hydrocortisone.

In another experiment (Fig. 5), fasted, adrenalectomized female rats were given 540 mg (3 mmol) of glucose intraperitoneally in addition to the usual treatments, and were killed after 4 hours. It is evident that blood glucose was elevated in all of these animals as compared to those reported in Fig. 3. Blood glucose appeared to be slightly higher in the rats getting hydrocortisone or hydrocortisone plus actinomycin D. All groups of animals given glucose made significantly greater amounts of glycogen than comparable groups not given glucose (Fig. 3), and hydrocortisone greatly enhanced glycogen deposition. Actinomycin D did not suppress glycogen formation in rats given glucose and hydrocortisone. Again, it can be seen...
that actinomycin D inhibited any elevation of phosphoenolpyruvate carboxykinase following administration of hydrocortisone.

**DISCUSSION**

A great variety of tools is available to study the question whether induction of enzyme formation is the primary mode of action of glucocorticoids. In selecting bioantagonists for use in such studies it is essential to establish that their inhibitory effect is limited to protein synthesis. Puromycin is unsatisfactory for such studies for reasons described earlier in this paper. Ethionine, which had been used by Kvam and Parks (10) as an antagonist of methionine, has since been found to deplete tissues of adenosine triphosphate (32). Despite the complexity of its effects, ethionine in doses sufficient to block induction of glucose 6-phosphatase and fructose 1,6-diphosphatase formation following treatment with hydrocortisone did not block the gluconeogenic effect (10).

Actinomycin D blocks messenger RNA formation (33) but does not prevent existing messenger from acting as a template for protein synthesis. Its effect on messenger RNA formation does not preclude enhancement of enzyme activities by other means such as allosteric effects, conversions of inactive to active molecular forms, or increased synthetic ability of pre-existing messenger. It has been shown that actinomycin D, in smaller doses than used in the present work, inhibits the enhancement of glucose 6-phosphatase (21), fructose 1,6-diphosphatase (21), lactate dehydrogenase (21), tryptophan pyrrolase (34), and tyrosine-α-ketoglutarate transaminase (34) following gluco- corticoid administration. The enhancement of phosphoenolpyruvate carboxykinase activity following hydrocortisone treatment (15) or fasting (Fig. 2) is completely abolished by prior treatment with larger doses of actinomycin D. In this laboratory we have found no evidence for latent forms of phosphoenolpyruvate carboxykinase in the soluble fraction of liver homogenates nor have any in vitro effects of glucocorticoids on this enzyme been detected. Thus the enhancement of several gluconeogenic enzymes following fasting or glucocorticoid administration seems to be mediated via messenger RNA. These facts, together with the finding (Fig. 1) that actinomycin D does not cause glycogenolysis, indicate that actinomycin D seems to be a suitable agent for testing whether glucocorticoids cause gluconeogenesis by inducing enzyme formation or by a process independent of such induction.

It is now evident that enzyme formation under the influence of hydrocortisone is neither a sufficient nor a necessary condition for the expression of the gluconeogenic effects of this hormone. As cited above, fasting induces the formation of greatly increased amounts of several key enzymes of the gluconeogenic pathway, yet the fasted animal does not utilize protein for glycogen formation. Therefore, whatever the effect of glucocorticoids on RNA formation (22) or total enzyme activity, these phenomena are not sufficient to induce gluconeogenesis. It should be emphasized that at least one enzyme that is responsive to glucocorticoid administration (phosphoenolpyruvate carboxykinase) responds equally well to fasting in the normal or adrenalectomized rat (14). That actinomycin D, in doses sufficient to block all messenger RNA formation, does not abolish the gluconeogenic effect of hydrocortisone demonstrates that enzyme synthesis de novo is not necessary for that effect.

If the primary action of glucocorticoids is to establish conditions which direct metabolites (i.e. amino acids, pyruvate, etc.) toward carbohydrate formation, it would be reasonable to expect secondary, induced elevations of enzymes that participate in that process. This response could occur as a result of derepression of enzyme-forming systems by altered concentrations of intermediates on the path to carbohydrate. The elevated levels of key enzymes would be expected to enhance the rate of carbohydrate formation above that which occurs without enzyme induction (cf. Fig. 3).

This concept is in keeping with the finding (Fig. 5) that actinomycin administration did not depress the effect of hydrocortisone on glycogen formation from a glucose load. In this case, glycogen synthesis does not require the complement of enzymes necessary to mobilize tissue protein to carbohydrate.

Weber et al. (21) have shown with normal rats, fed ad libitum, that administration of cortisone for 5 days results in increased deposition of hepatic glycogen and increased activities of several hepatic enzymes, but that when the elevation of enzyme levels is blocked by actinomycin D, glycogen formation is below normal. These authors conclude that the stimulation of gluconeogenesis by glucocorticoids entails an increased rate of synthesis of gluconeogenic enzymes. Experiments previously mentioned demonstrated that actinomycin D caused depletion of liver glycogen of rats fed ad libitum. These observations agree with the findings of Tata (35), who has shown that actinomycin D causes an immediate fall in basal metabolic rate and a corresponding loss of body weight. The loss of body weight, according to Tata, is due at least partly to a decreased food intake. This effect of the antibiotic might account for the low concentration of hepatic glycogen observed by Weber et al. (21).

**SUMMARY**

In contrast to some inhibitors of protein synthesis, e.g. puromycin, actinomycin D was found to have no glycogenolytic activity in rats. This agent, which inhibits induced formation of enzymes by blocking messenger ribonucleic acid synthesis,
is therefore suitable for investigating whether the primary function of glucocorticoids is on induction of enzyme synthesis.

Hydrocortisone stimulated gluconeogenesis in both normal and adrenalectomized rats even though they had been treated with sufficient actinomycin D to block induced synthesis of enzymes that participate in gluconeogenesis.

The data indicate that the primary effect of glucocorticoids is exerted on existing enzyme systems so as to direct metabolites toward carbohydrate formation and that the induction of enzyme synthesis is a secondary effect.

REFERENCES

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