Evidence for Irreversible, Actinomycin D-sensitive, and Temperature-sensitive Steps following Binding of Cortisol to Glucocorticoid Receptors and Preceding Effects on Glucose Metabolism in Rat Thymus Cells*

Kathleen Mazur Mosher, Donald A. Young, and Allan Munck†

From the Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire 03755

SUMMARY

Following cortisol binding to the specific glucocorticoid receptors of thymus cells at 37° (a process which takes about 7 min), there is a 5- to 10-min lag period before a cortisol effect can be observed on glucose 6-phosphate accumulation after a glucose pulse. Evidence is presented that during this period the signal initiated by cortisol binding traverses an irreversible, an actinomycin D-sensitive, and a temperature-sensitive step.

The irreversible step is shown by the fact that removal of cortisol from the glucocorticoid receptors (by displacement with cortexolone, a metabolically inactive glucocorticoid competitor, or by washing) well before any metabolic effect has appeared does not prevent the subsequent appearance of the cortisol effect.

The actinomycin D-sensitive step is shown by the fact that, whereas addition of actinomycin D to thymus cells together with cortisol prevents the cortisol effect from developing subsequently, addition of actinomycin D 5 min after cortisol does not prevent the cortisol effect. To produce these actions, actinomycin D must be used at 100 μg per ml. This same concentration is necessary to inhibit RNA synthesis rapidly in thymus cells by about 80%.

The temperature-sensitive step is shown by the fact that the duration of the lag period preceding the appearance of a cortisol effect increases markedly at temperatures below 37° to more than 120 min at 20°. The duration of the lag period at 37° can be shortened if cells are first incubated with cortisol at 20°.

The temperature dependence of the irreversible step is such that it cannot be identical with, but must precede, the temperature-dependent step. The actinomycin D-sensitive step may or may not be identical with one of the other two steps.

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METHODS

Male Sprague-Dawley rats (Charles River Breeding Laboratories) were adrenalectomized and given 1% NaCl drinking...
water and fed Labena (Ralston-Purina Company). The animals were used 4 to 15 days postadrenalectomy when they weighed between 150 and 250 g. No differences were noted in the responsiveness of the thymus cells to cortisol with time after adrenalectomy. Thymus cell suspensions were prepared and incubated in Krebs-Ringer bicarbonate buffer as described previously (2). Incubation temperatures were controlled to ±0.5°C. In experiments involving temperature changes, separate Dubnoff incubators were used and flasks were transferred quickly from one incubator to another. Except for the experiments in Table I, cell concentrations, V (milliliters of packed cells per ml of suspension), measured by microhematocrit (2), were between 0.12 and 0.30.

For measurement of glucose 6-phosphate, incubations were stopped by adding 1 ml of 12% cold perchloric acid per ml of incubation mixture. The perchloric acid suspensions were treated as described previously (2). Values of glucose 6-phosphate are referred to 1-ml packed cell volume (2).

Steroid concentrations (in water or buffer) were determined spectrophotometrically (12). Except when noted, cortisol was present from the start of the incubation as a concentration in the cell suspension of 10⁻⁴ M. Actinomycin D was added in aqueous solution (2.5 mg per ml) to give a concentration in the cell suspension of 1, 10, or 100 μg per ml. Steroids were obtained from Calbiochem, and enzymes and cofactors from Boehringer. Actinomycin D was a gift from Merck, Sharp & Dohme. Uridine-5-³H (20 Ci per mmole), thymidine methyl-³H (19.9 Ci per mmole), and generally labeled L-valine-³H (2.97 Ci per mmole), and generally labeled L-valine-³H (2.97 Ci per mmole) were purchased from New England Nuclear.

Measurement of Cortisol Effect by Glucose Pulse—In all experiments considered here the method for testing for the presence of a cortisol effect on glucose metabolism is to add glucose (as a 2% solution in buffer to give a concentration of 5.5 mM) to control and cortisol-treated cell suspensions that up to that point have not had substrate, and then 5 or 6 min later to stop the incubations and analyze for glucose 6-phosphate. Previous studies (2) have shown that under these conditions a glucose pulse at 37°C raises glucose 6-phosphate to levels that remain fairly constant between 5 and 40 min. The early time course of such pulses, at 37°C and 20°C, is shown in Fig. 1. At 37°C about 5 min is required to reach a constant glucose 6-phosphate level. It should be noted that the cortisol effect is evident from the 1st min. At 20°C, by contrast, the level of glucose 6-phosphate continues to rise for at least 30 min, and is distinctly higher than at 37°C. In other experiments we have found that higher concentrations of glucose do not lead to higher levels of glucose 6-phosphate and do not alter the cortisol effect.

The magnitude of the cortisol effect given in subsequent figures, referred to as “per cent inhibition,” represents the difference between glucose 6-phosphate levels of control and cortisol-treated cells 5 or 6 min after addition of glucose, expressed as per cent of the control level. Control and cortisol-treated samples are paired (12), per cent inhibition is calculated for each pair, and means and standard errors are calculated from replicate pairs.

RESULTS AND DISCUSSION

Temperature-sensitive Step—The cytolytic effects that cortisol produces after several hours of incubation with thymus cells at 37°C (13) do not take place at 20°C. This observation, and our own finding of a temperature-induced artifact in glucose uptake (2), has led us to study the time and temperature dependence of the early cortisol effect on glucose metabolism.

Fig. 2 illustrates the magnitude of the hormonal inhibition of glucose metabolism when cells are incubated with cortisol for
suspensions were first incubated with or without cortisol \((10^{-6} \text{ M})\), and without substrate, at 20° for the periods indicated. At zero time the suspensions were warmed to 37° and the incubations continued. At various times after warming to 37° the presence of a cortisol effect was tested for by adding glucose and measuring glucose 6-phosphate 5 min later (see “Methods” for details). The data for 10- and 120-min preliminary incubation with cortisol are from a single experiment in which all cells were first incubated at 20° for 120 min, but cortisol was added to the 10-min flasks 10 min before warming to 37°. For the other data cortisol was present for the full preliminary incubation period. Values represent means \(\pm 1\) S.E. for three pairs of flasks.

several different time periods and at various temperatures. The solid symbols on the solid curve show that the magnitude of the cortisol effect after a 25-min incubation at various temperatures diminishes sharply as the temperature is reduced below 37°, disappearing altogether at 25°. As can be seen from the upper curve of Fig. 1, this diminution as the temperature is lowered is not due to a lack of accumulation of glucose 6-phosphate at low temperatures; nor can the diminution be accounted for by slow binding of cortisol to the specific glucocorticoid receptors of thymus cells, since the rate of specific binding is almost the same at 22° as at 37° (10), and at both of these temperatures most of the specific binding is accounted for by a nuclear cortisol receptor complex (11). Finally, the diminution is not due to the inability of cells at low temperatures to exhibit a cortisol effect (owing to a change in rate-limiting step, for example) since after a 210-min incubation a distinct cortisol effect appears even at 20° (Fig. 2) and since (as can be seen from the 60-min incubation, broken curve in Fig. 2) at all temperatures the magnitude of the effect increases with time.

Fig. 3 illustrates the progress of the action of cortisol at 20°. All glucose pulses in these experiments were at 37°. The data show that the time that it takes for a cortisol effect to appear at 37° is progressively shortened by progressively longer periods of preliminary incubation with cortisol at 20°. The time course at 37° after 10-min preliminary incubation at 20° (top row) is very similar to the time course determined previously (2) without any prior incubation; in both instances the earliest significant cortisol effect appears with a glucose pulse at 15 min.

Our interpretation of these results is that on the path between the initiating events of cortisol action—binding to glucocorticoid receptors—and the effect on glucose metabolism that appears 5 to 10 min after binding is complete there is a temperature-sensitive step. At temperatures below 37°, this step (or steps) can still proceed, but at a slower rate. Judging from the results in Fig. 3, at 20° the rate is roughly 10 times smaller than at 37°.

Irreversible Step—It is well known that in vivo some of the effects of an injected dose of cortisol may not appear until well after the hormone has practically disappeared from the blood. In vitro, with incubated thymus cells, the continued presence of hormone does not appear to be necessary either, since the slow effects of cortisol on cytolysis (13) and on incorporation of uridine into RNA (8) are not removed by washing cells free of cortisol after 1- or 2-hour incubation.

In preliminary experiments in which cortisol added initially at 10^{-6} \text{ M} was removed by repeated washing (reducing the cortisol concentration to less than 5 \times 10^{-5} \text{ M}) we found that even a few minutes exposure to cortisol at either 37° or 20° was sufficient to produce a subsequent cortisol effect on a glucose pulse. Because of the unwieldiness of the washing procedure, for more
precise studies we made use of cortexolone, a steroid that has been shown to compete with cortisol for binding to the specific glucocorticoid receptors of thymus cells, and thereby to reduce or abolish the effects of cortisol on glucose uptake (10).

In Fig. 4A the first three bars (0 min) illustrate, respectively, the inhibitory effect of cortisol at $10^{-8}$ M, of cortexolone at $10^{-4}$ M, and of both these steroids when added simultaneously. These data, in which inhibition is determined with respect to glucose 6-phosphate accumulation following a 6-min glucose pulse, agree with our earlier results (10) showing that cortexolone drastically reduces the effect of cortisol on inhibition of glucose uptake. The magnitude of the reduction is about what would be expected from the dose-response curve for cortisol (12) and the relative binding affinities of cortisol and cortexolone for glucocorticoid receptors (10).

The next pair of bars in Fig. 4A (5 min) show that if instead of being added together with cortisol cortexolone is added 5 min later, it hardly reduces the effect of cortisol at all. By 5 min the binding of cortisol to glucocorticoid receptors is approaching completion (10). Cortexolone displaces cortisol from the glucocorticoid receptors within approximately another 5 min, well before a cortisol effect on glucose metabolism can be detected (2). The subsequent appearance of a cortisol effect, which we know from other measurements persists for at least 40 min, therefore shows that specific cortisol binding is rapidly followed, or is accompanied, by an irreversible step—i.e. a step that results in a change that is not reversed by removing cortisol.

Fig. 4B shows that this irreversible step probably proceeds more slowly at 20°. The experimental design here is similar to that in Fig. 4A, except that the initial period of exposure to cortisol alone was at 20°; after addition of cortexolone the incubations were continued at 20° for 20 min, sufficient time for cortexolone to displace cortisol almost completely. Then, in order to allow the temperature-sensitive step to proceed, all flasks were rewarmed to 37° for 20 min, and finally the presence of a cortisol effect was tested for with a glucose pulse. Since the only difference between the various experiments in which cortisol and cortexolone were both added was the amount of time that the cells were exposed to 20° cortisol alone, it may reasonably be concluded that the increase in the cortisol effect with time of exposure to cortisol represents the rate at which the irreversible step proceeds at 20°. Part of the decrease in rate at 20° (as compared to 37°) may be ascribed to a slight decrease in rate of formation of the nuclear cortisol-receptor complex (10, 11), so that if anything the rate of the irreversible step is greater at 20° than would appear from Fig. 4B.

The main conclusion from these experiments is that, although the rate of the irreversible step at 20° is slower than at 37°, it is not nearly slow enough to account for the temperature-sensitive step discussed above (at 20° the cortisol effect takes more than 130 min to appear). Consequently, the irreversible and the temperature-sensitive steps must be separate steps. Furthermore, since the irreversible step can be initiated rapidly by adding cortisol at 20°, it is clear that the irreversible step must precede the temperature-sensitive step.

**Actinomycin D-sensitive Step**—The data in Fig. 5, obtained with incubations at 37°, show that if actinomycin D is added to cells at the same time as cortisol (0) no cortisol effect is observed on a glucose pulse 20 min later; if it is added 5 min after cortisol (5) a normal cortisol effect develops. These experiments have been repeated many times, with completely consistent results. In other experiments we have found that the same dose of actinomycin D has no effect on the rate or magnitude of specific binding of cortisol to whole thymus cells or to the nuclear receptors, so we conclude from these results that specific cortisol binding is rapidly followed by an actinomycin D-sensitive step.

Values in Fig. 5 are in absolute amounts of glucose 6-phosphate rather than per cent inhibition, to show that actinomycin D by itself increases glucose 6-phosphate levels. From other evidence, it is likely that this action of actinomycin D is due to a decreased rate of utilization of glucose 6-phosphate. It can be seen to be separate from the action that leads to elimination of the cortisol effect, because when actinomycin D is added 5 min after cortisol a conspicuous cortisol effect develops despite the elevation of glucose 6-phosphate levels.

Using an approach similar to that which allows us to distinguish between the temperature-sensitive and the irreversible step, we have attempted, unsuccessfully, to see whether the actinomycin D-sensitive step can be distinguished from the irreversible or the temperature-sensitive step. A series of experiments in which cortisol and actinomycin D were added at various times relative to one another at various temperatures has shown that actinomycin D at 20°C fails to block the cortisol effect. These results can be explained by supposing that actinomycin D has no metabolic effect on thymus cells at 20°, an explanation which is supported by separate experiments in which we have found that: (a) in incubations at 20°C actinomycin D has no effect on RNA synthesis at least during the first 30 min, and (b) after preliminary incubation with actinomycin for 10 min at 37° the antibiotic subsequently inhibits the incorporation of uridine into RNA at 20°.

High concentrations of actinomycin D (100 μg per ml) are essential in order to produce the results in Fig. 5; 10 μg per ml

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2 The trivial name used is: cortexolone, 17,21-dihydroxyprogren-4-ene-3,20-dione.

2 A. Munck, unpublished results.
Early Steps in Glucocorticoid Actions on Glucose Metabolism

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Duplicate 1-ml aliquots of thymus cell suspension (cell concentration V = 0.064) were incubated for 5 min at 37°C with actino-
mycin D at the concentration indicated; a mixture of unlabeled uridine (2.2 μg), thymidine (2.2 μg), and valine (4.3 μg); and
weightless amounts of either [H]-uridine (2.2 μCi), [H]-thymidine (2.2 μCi), or [H]-valine (2.2 μCi). Incubation volume was 1.16 ml.
Incubations were terminated by adding the wash solution—insoluble precipitates in 1 N NaOH for 18 hours at 37°C. After reprecipitation of the
DNA and protein with 10% trichloroacetic acid, counts in RNA were determined after adding duplicate 0.5-ml aliquots of the supernatant
to scintillation vials with Bray's fluid. After recounting the activity from [H]-valine in the RNA fraction represents
virtually no incorporation, since

| Radioactive precursor | Actinomycin D concentration | Radioactivity in macromolecular fractions | Protein |
|-----------------------|-----------------------------|-----------------------------------------|---------|
| %H-Uridine | μg/ml | RNA | DNA | Protein |
| 0 | 174 (167-177) | 4 (2-5) | 26 (23-22) |
| 1 | 178 (152-205) | 3 (2-3) | 30 (28-31) |
| 10 | 150 (147-164) | 5 (3-8) | 25 (23-29) |
| 100 | 38 (39-44) | 3 (2-3) | 28 (26-33) |
| %H-Thymi-
dine | μg/ml | RNA | DNA | Protein |
| 0 | 26 (22-31) | 252 (232-271) | 14 (9-17) |
| 1 | 26 (24-29) | 244 (222-284) | 7 (6-9) |
| 10 | 24 (16-30) | 230 (192-255) | 12 (7-13) |
| 100 | 26 (31-39) | 245 (235-259) | 8 (5-14) |
| %H-Valine | μg/ml | RNA | DNA | Protein |
| 0 | 415 (403-449) | 25 (17-34) | 378 (339-381) |
| 1 | 400 (410-494) | 24 (11-33) | 388 (325-434) |
| 10 | 378 (309-443) | 26 (13-35) | 388 (355-396) |
| 100 | 381 (371-402) | 22 (11-31) | 396 (375-368) |

are ineffective. There is therefore a distinct possibility that we may be dealing with nonspecific effects of actinomycin D, rather than solely with an effect on RNA synthesis. However, as shown by the data in Table I, in a 5-min incubation under comparable conditions the same high concentration of actinomycin D is necessary to reduce RNA synthesis (measured by uridine incorporation into RNA) by 78%. In other experiments (data not presented) we have found that this inhibition increases to 90 to 95% by 10 min, whereas with 10 μg of the antibiotic the inhibition is only 40 to 50% by 15 min. Moreover, as also shown in Table I, actinomycin D at 100 μg per ml does not affect DNA synthesis (measured by thymidine incorporation into DNA) and has only a slight effect on protein synthesis (here reduced by about 14%). The radioactivity from [H]-uridine in the DNA fraction represents virtually no incorporation, since

The requirement for high concentrations of actinomycin D in order to inhibit RNA synthesis specifically at 37°C, as well as our failure to find any effect of actinomycin D on RNA synthesis at 20°C, may both be indicative of poor penetration of intact thymus cells by the antibiotic.

General Discussion—Up to now, the assumption that the effect of cortisol on glucose metabolism in thymus cells is not

affected by phenol extraction) with incorporation into protein. We accordingly assume that, with this short incubation time,

the radioactivity from [H]-valine in the RNA fraction represents

only 4 to 8 cpn above background. The relative amounts of valine incorporated into protein with respect to RNA agree well with the results of similar experiments in which we have compared the incorporation of [H]-valine into Val-tRNA (iso-

lated by phenol extraction) with incorporation into protein. The results leave open the possibility that either the irreversible and temperature-sensitive step may be sensitive to actinomycin D.

These steps are the most rapid events (aside from formation of hormone receptor complexes) that have so far been detected

after addition of a steroid hormone to an isolated tissue. Al-

though our evidence for the steps is still somewhat indirect, the sensitivity of one of them to actinomycin D clearly suggests the

possibility that RNA synthesis plays a role in the earliest actions of the hormone. This possibility, which is in accord with our

separate observation that under the conditions of the experiments described here the cortisol-receptor complex is largely in the

nucleus (11), has of course been considered frequently in connection with the mechanism of action of glucocorticoids and other

steroid hormones, but in no case that we are aware of has actino-

mycin D sensitivity been shown to be confined to a very brief,

early period. There have been numerous reports of slow inhibi-

tory effects of glucocorticoids on uridine incorporation into RNA (5, 6, 8), as well as on amino acid incorporation into protein (3, 8, 14). With thymus cells it is clear that these effects have little to do with the primary actions of the hormones, but rather are probably secondary to preceding effects on glucose metabo-

lism (4-6, 15).

Our results leave open the possibility that either the irreversible or the temperature-sensitive step may be sensitive to actino-

mycin D (preliminary results with inhibitors of protein syn-

thesis suggest that there is also a cycloheximide-sensitive step; it ocurs at about the time of the appearance of the cortisol effect on glucose metabolism (10)). It is in any case not unreason-

able to suppose that these steps involve macromolecular metab-

olism, which could well account for their irreversible and tem-

perature-dependent characteristics. There is of course no

reason to assume that the steps that we have detected are the

only ones that occur during the latent period, since undoubtedly

D. Young, unpublished results.

C. Hallahan, D. A. Young, and A. Munck, unpublished results.
many complex events take place in the 5 to 10 min between the initial actions of the hormone and the ultimate effect on glucose transport.

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