Evidence for Induction by Cortisol In Vitro of a Protein Inhibitor of Transport and Phosphorylation Processes in Rat Thymocytes

(messenger RNA/rubidium transport/hexose transport)

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ABSTRACT Studies of intact rat thymocytes incubated in vitro with cortisol, actinomycin D, puromycin, and cycloheximide indicate that distinct inhibitory effects of cortisol on transport and phosphorylation are due to an action on mRNA synthesis with consequent induction of synthesis of protein(s) with inhibitory influence. Incubation of thymocytes with cortisol results in inhibition of the rate of labeled orthophosphate incorporation into ATP and the entry of rubidium ion and hexoses into the cells. Continuing protein synthesis is required for the progressive and persistent manifestation of the inhibitory effects of the steroid. RNA synthesis is also required during the initial phase of incubation of cells with cortisol, but significant inhibitory effects of cortisol, once initiated, are evident for at least 60-120 min after addition of actinomycin D. In contrast, addition of cycloheximide sometime after cortisol results in prevention or reversal of the effects of the steroid. In the absence of cortisol, the antibiotics exert relatively little effect on orthophosphate incorporation and on the transport processes studied. It is suggested that the sequence of events leading to dissolution of thymocytes exposed to cortisol is initiated by the synthesis of mRNA coding for inhibitory protein(s) with more rapid turnover rates than that of the mRNA, and that these events are modulated by the relative sensitivity of different cellular processes to the protein inhibitor(s).

Since the first demonstration of the lymphocytolytic action of glucocorticoids (1), the biochemical basis for this effect has been explored by many investigators, but remains as yet an unsolved problem. The problem is of particular significance because of the roles of lymphoid cells in antibody synthesis (2-4) and in host cell-mediated immunological competence (4-6).

The study of the mechanism of lymphocytolysis has been facilitated by the development of an in vitro system in which biochemical alterations were discernible in lymphoid cells that were exposed to physiological concentrations of thymic steroids either in vivo or in vitro (7). Utilizing this in vitro model, we have shown that cortisol acts on rat thymocytes at three distinct loci, each reflecting an inhibitory influence of the steroid: (a) the transport of small molecules (8); (b) phosphorylation (9); and (c) the DNA-dependent RNA polymerase of thymocyte nuclei (10). The last two of these effects are also seen in broken-cell preparations of thymocytes previously exposed to cortisol in vitro (9, 10).

The inhibitory action of cortisol on amino acid transport and on nucleoside transport and (or) phosphorylation by lymphoid cells in vitro required continuing RNA and protein synthesis (9-11). In contrast, the decreased activity of the nuclear DNA-dependent RNA polymerase of rat thymocytes exposed to cortisol was not dependent upon continuing protein synthesis (10). Also, cortisol inhibited the transport of glucose, 2-deoxyglucose, and 3-O-methylglucose into thymocytes and the incorporation of orthophosphate into ATP by thymocytes or lysates of these cells; these effects required RNA and protein synthesis (9, 10). Recently, the inhibition of glucose accumulation by cortisol was shown to be no longer evident when a high dose of actinomycin D was added together with cortisol, but the cortisol effect was still evident when actinomycin D was added shortly after the steroid (12). The role of RNA and protein synthesis in processes influenced by cortisol was not clearly defined in these previous studies. However, an induction of inhibitors by cortisol was suggested by the prevention of steroid effects on α-aminoisobutyric acid transport by actinomycin D (10) and on phosphorylation in cell lysates by cycloheximide (9) since these processes were not altered by the antibiotics in the absence of cortisol.

In the present study, cortisol is shown to inhibit transport of rubidium ion. Additional data are presented concerning the inhibitory influence of steroid on transport of 3-O-methylglucose (which is actively transported but not further metabolized by the cell) and other hexoses and on the conversion of orthophosphate to ATP by thymic cells. Under the conditions studied, none of these processes is appreciably influenced by the presence of antibiotic inhibitors of RNA and protein synthesis in the absence of cortisol. In contrast, these steroid effects can be entirely prevented by the simultaneous addition of actinomycin D with the steroid or by inhibitors of protein synthesis. An even more striking finding is that these steroid effects may be completely reversed by late addition of cycloheximide, whereas the steroid inhibitory actions are only slightly diminished by a similar late addition of actinomycin D.

METHODS Suspensions of rat thymocytes (2 X 10⁷ cells/ml) were prepared, equilibrated, and incubated with or without steroids as described previously (8). Concentrations of cycloheximide, puromycin, and actinomycin D were based upon previous studies (10).

For studies employing ⁸⁶RbCl or NaH₂³⁵PO₄, the cells were first incubated in minimal essential medium for sus-
pension culture (13) with 10% CO₂ in air as gas phase. Immediately prior to labeling, cells were centrifuged, washed, and resuspended in a medium containing additional NaCl to replace all but 1 mM of KCl for studies with ³²RbCl, or in a phosphate-free medium in experiments with NaH₂³²PO₄. In some experiments cells were both incubated and labeled in Dulbecco's modified medium (14). The medium used for studies with labeled hexoses was a balanced salt solution with 0.03% of glucose present, unless otherwise indicated. At specified times, 1-ml aliquots of cell suspensions were pipetted into 15-m1 conical centrifuge tubes containing either 0.005-0.015 µCi (about 1 Ci/g) of ³²RbCl (Abbott Laboratories), 0.075-0.30 µCi (0.2-2.0 Ci/g) of NaH₂³²PO₄, 0.025-0.150 µCi (0.2-2.0 Ci/g) of NaH₂¹⁷PO₄, 0.34 µCi of [U-¹⁴C]glucose (16 Ci/mol) or 0.10 µCi of [¹³C]2-deoxyglucose (10.6 Ci/mol) (New England Nuclear Corp.) or 0.5 µCi of 3-O-methyl-[U-¹⁴C]glucose (4.6 Ci/mol) (International Chemical and Nuclear Corp.). After being labeled for specified periods, the samples were chilled and centrifuged at 160 X g for 5 min. and the cell pellets were washed in cold incubation medium (3 ml X 3 ml). The washed cell pellets were then resuspended in 0.5 ml of 5% trichloroacetic acid (TCA) for separation and recovery of both the total intracellular TCA-soluble and TCA-insoluble fractions, or in 0.5 ml of water for recovery of the total intracellular radioactivity.

Radioactivity was measured by liquid scintillation spectrometry. Counting efficiencies for ¹H and ¹⁴C have been reported (8). Efficiencies for ³²Rb, ³¹P, and ³²P were, respectively, 86, 84, and 87%.

For charcoal adsorption, aliquots of the TCA-soluble fractions were mixed with a slurry of activated charcoal (Norit A; Matheson, Coleman and Bell) with a final concentration of 10 mg/ml charcoal and 1% TCA. After 10 min of standing on ice, the samples were centrifuged and the supernatant fractions were used for determination of radioactivity. The TCA-soluble fraction was extracted with butanol in the presence of carrier orthophosphate for quantitative recovery of labeled orthophosphate in the butanol phase (15). Ion-exchange column chromatography on Dowex AGI-X8 resin (Bio-Rad) for separation of free hexoses from metabolites was conducted as described by Bartlett (16).

### RESULTS AND DISCUSSION

**Influence of cortisol on hexose and rubidium ion transport and on orthophosphate incorporation**

Incubation of thymocytes for 1 hr with cortisol inhibited the rate of accumulation of labeled glucose, 2-deoxyglucose, and 3-O-methylglucose in the cells (Table 1). Both 2-deoxyglucose and 3-O-methylglucose have been shown to share a common system with glucose for transport into lymph node cells (17). The intracellular metabolism of 2-deoxyglucose is limited primarily to the hexokinase reaction (18). However, the rate of phosphorylation of 3-O-methylglucose by a mammalian hexokinase is too low to be detected (19). Therefore, the rate of intracellular accumulation of radioactivity in cells incubated with 3-O-methylglucose should reflect the rate of entry of this sugar into the cell. When 3-O-methylglucose was used as labeled precursor and the intracellular contents were fractionated by chromatography, essentially all the radioactivity was recovered as the free hexose. With either glucose or 2-deoxyglucose as precursor the expected evidence for intracellular phosphorylation was obtained. Thus, decreased hexose transport accounts for the inhibition of glucose utilization produced by cortisol (20, 21).

In contrast to the effects of cortisol, progesterone (1 or 20 µM), estradiol (1 µM), and 17 α-ethyl-19-nortestosterone (1 µM) did not alter the rate of uptake of 3-O-methylglucose by thymocytes (data not presented). The rate of egress of hexose from prelabeled and washed thymocytes was greater for 3-O-methylglucose than for the phosphorylated sugars and was not altered by cortisol (data not presented). These latter results resemble the lack of effect of cortisol on the rate of egress of α-aminoisobutyrate from thymocytes in vitro (11).

Hechter and coworkers (22) reported a marked decrease in the potassium/sodium ratio in thymus after repeated injection of cortisol into adrenalectomized rats. An inhibitory influence of cortisol added directly in vitro on the net uptake of rubidium ions by thymocytes is shown in Table 1. The uptake of rubidium ion in this system was found to be competitive with that of potassium ion and was abolished by the presence of ouabain (data not presented). Thus, as has been found for other tissues, the uptake of ³²RbCl appears to be a valid index of the Na⁺-K⁺ transport system in thymocytes. The percentage inhibition of ³²RbCl uptake produced by cortisol was not altered by the presence of 1.0 mM KCl. In studies not presented, incubation of thymocytes for 3 hr with 1 µM progesterone or deoxycorticosterone had no influence on the rate of rubidium uptake.

Exposure of thymocytes to cortisol in vitro also resulted in a decreased rate of accumulation of NaH₂³²PO₄ from the medium (Table 1). Incubation of cells with 10 µM progesterone did not influence phosphate accumulation (data not shown). In other studies the inhibitory effect of cortisol was also observed when labeling with NaH₂³²PO₄ was carried out in the presence of unlabeled orthophosphate over a concentration range of 10⁻⁷ M to 10⁻³ M. The data for phosphate uptake in Table 1 are for the TCA-soluble fraction.

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**Table 1. Inhibitory action of cortisol in vitro on hexose and rubidium ion transport and on orthophosphate accumulation by rat thymocytes**

<table>
<thead>
<tr>
<th>Precursor and time of pulse labeling after cortisol addition</th>
<th>Radioactivity (dpm/mg cell protein)</th>
<th>% Inhibition due to cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>[¹⁴C]Glucose, 1-1.5 hr</td>
<td>2070</td>
<td>30 ± 5 (4)</td>
</tr>
<tr>
<td>[¹⁴C]2-Deoxyglucose, 1-1.5 hr</td>
<td>1710</td>
<td>21 ± 1 (12)</td>
</tr>
<tr>
<td>[¹⁴C]2-Deoxyglucose, 3-3.5 hr</td>
<td>1690</td>
<td>22 ± 3 (5)</td>
</tr>
<tr>
<td>[¹⁴C]3-O-Methylglucose, 1-1.5 hr</td>
<td>2430</td>
<td>21 ± 5 (4)</td>
</tr>
<tr>
<td>³²RbCl, 1-2 hr</td>
<td>3050</td>
<td>9 ± 1 (3)</td>
</tr>
<tr>
<td>³²RbCl, 3-4 hr</td>
<td>2450</td>
<td>25 ± 2 (4)</td>
</tr>
<tr>
<td>NaH₂³²PO₄, 1-2 hr</td>
<td>15,640</td>
<td>20 ± 2(3)</td>
</tr>
<tr>
<td>NaH₂³²PO₄, 1-2 hr; 1.0 mM iodoacetate added at 0.4 hr</td>
<td>11,580</td>
<td>23 ± 6 (3)</td>
</tr>
<tr>
<td>NaH₂³²PO₄, 3-4 hr</td>
<td>61,900</td>
<td>23 ± 12 (12)</td>
</tr>
<tr>
<td>NaH₂³²PO₄, 3-4 hr; 1.0 mM iodoacetate added at 2.5 hr</td>
<td>10,650</td>
<td>44 ± 4 (3)</td>
</tr>
</tbody>
</table>

Cortisol, 1 µM. Values for radioactivity incorporated are for the amounts in the TCA-soluble fraction of the cells. Values for % inhibition are means ± SE (number of experiments in parentheses). Each experiment included six or more separately labeled aliquots of cells for each condition studied.
only. Exposure of the cells to cortisol also led to a decrease in the radioactivity recovered in the TCA-insoluble fraction (about 15% of the total radioactivity) equal to and reflecting the decrease in the TCA-soluble fraction.

Essentially all of the NaH$_4$PO$_4$ present in the TCA-soluble fraction of the thymocytes was in a charcoal-adsorbable form as early as 5 min after addition of label. A significant amount of intracellular radioactivity was recovered directly as orthophosphate in a butanol-extractable form only after 60 min of incubation with label. Additional data obtained with the use of thin-layer chromatography (data not presented) revealed that most of the TCA-soluble radioactivity was present as nucleoside triphosphate. Evidence has been presented previously for the existence of carrier-mediated transport of inorganic phosphate in Ehrlich mouse ascites tumor cells (23) and in human red blood cells (24). However, our studies with NaH$_4$PO$_4$ as precursor, in contrast to those with 3-O-methylglucose and rubidium ion, do not permit distinction between transport and phosphorylation. The data presented suggest a probable close temporal relationship between phosphate transport and ATP synthesis.

Enhancement of the action of cortisol by iodoacetate

The magnitude of the inhibitory influence of cortisol on incorporation of orthophosphate into ATP was markedly enhanced when the pulse labeling was carried out in the presence of iodoacetate (Table 1). Similar enhancement was obtained with 2,4-dinitrophenol and other inhibitors of oxidative phosphorylation (unpublished results). These observations indicate that inhibition of phosphorylation by cortisol is not due to an effect on hexose uptake or glycolysis, but involves inhibition of oxidative phosphorylation at a site distinct from that which is affected by 2,4-dinitrophenol (9). Interference with cellular ATP synthesis by iodoacetate or 2,4-dinitrophenol would then result in the cortisol-dependent step becoming more prominent and (or) evident at an earlier time during incubation of cells with steroid.

Time course of induction of the inhibitory effects of cortisol

The progression of various inhibitory effects of cortisol added in vitro on transport, phosphorylation, and related processes in thymocytes with time of incubation is summarized in

![Fig. 1. Time course of induction of inhibitory effects by 1 μM cortisol present in vitro in thymic cell suspensions. Cell suspensions were prepared and incubated with and without cortisol as described in the text and Table 1. The abscissa indicates the time interval from cortisol addition to midpoint of pulse labeling. [14C]Glycine (— — —) incorporation into the TCA-insoluble fraction, [14C]α-aminobutyrate (AB) (□ □) incorporation into the TCA-soluble fraction, and [6-3H]uridine incorporation into the TCA-soluble (□ — □) and TCA-insoluble (O — O) fractions were as described previously (8). NaH$_4$PO$_4$ or NaH$_4$HPO$_4$ incorporation in the presence (O — O) and absence (△ △ △) of iodoacetate, and the incorporation of 86RbCl (L — L — L) and hexose (average for the three hexoses studied) (Δ — Δ — Δ), were as described in Table 1. For comparison, the inhibition of [6-3H]uridine into the TCA-insoluble fraction measured in vitro with exposure to cortisol for various times in vivo (O — O) (7) is also shown.

Table 2. Cortisol does not inhibit transport and phosphorylation by rat thymocytes in the presence of some antibiotics that do not inhibit these processes in the absence of steroid

<table>
<thead>
<tr>
<th>Antibiotic present throughout the incubation*</th>
<th>Precursor and time of pulse labeling after cortisol addition†</th>
<th>Radioactivity incorporated (dpm/mg cell protein)</th>
<th>Antibiotic + cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide</td>
<td>[14C]2-Deoxyglucose, 1–1.5 hr</td>
<td>1780</td>
<td>1740 1370 1710</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>[14C]Glucose, 1–1.5 hr</td>
<td>1140</td>
<td>1370 923</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>[14C]3-O-Methylglucose, 1–1.5 hr</td>
<td>1990</td>
<td>2920 1670 2770</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>86RbCl, 3–4 hr</td>
<td>1920</td>
<td>1880 1550 1950</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>NaH$_4$PO$_4$, 3–4 hr</td>
<td>24,500</td>
<td>24,000 19,700 23,700</td>
</tr>
<tr>
<td>Puromycin</td>
<td>[14C]2-Deoxyglucose, 1–1.5 hr</td>
<td>1680</td>
<td>1790 1310 1820</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>[14C]2-Deoxyglucose, 1–1.5 hr</td>
<td>1850</td>
<td>1780 1410 1780</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>NaH$_4$PO$_4$, 3–4 hr</td>
<td>25,400</td>
<td>26,100 19,800 26,600</td>
</tr>
</tbody>
</table>

* Cycloheximide (25 μM), puromycin (25 μM), or actinomycin D (4.0 μg/ml) was added to control cell suspensions and to cell suspensions with 1 μM cortisol also present.
† At least two separate experiments were carried out for each parameter studied. Other conditions for incubation and labeling were as described in the text and Table 1.
incubation with cortisol, the usual inhibitory effects on the steroid were not evident. Thus, in the presence of cortisol, inhibition of RNA or protein synthesis appeared to enhance transport and phosphorylation, i.e., to “reverse” the inhibition by steroid. The data are interpreted as indicating that cortisol induces the synthesis of inhibitory protein or proteins not present in the cell in the absence of steroid, and that this effect involves new RNA synthesis, perhaps new mRNA which codes for the new protein. It is of interest that Stevens et al. (25) reported evidence for early stimulation of synthesis of cytoplasmic protein in vivo in mouse lymphoid cells after a single injection of cortisol acetate.

Further evidence for the above concept of cortisol action is summarized in Fig. 2. When actinomycin D is added to the thymocyte incubation system after the addition of cortisol (in contrast to addition of actinomycin D before or at the time of cortisol addition as in Table 2), the effects of cortisol are no longer prevented or reversed by this antibiotic during the subsequent 1–2 hr. In contrast, addition of cycloheximide at a time subsequent to cortisol results in prevention or reversal of the effects of the steroid. In fact, the inhibition of hexose transport, already fully expressed at 60 min after cortisol, is essentially reversed by the presence of cycloheximide from 60 to 90 min after steroid addition. These results indicate that the inhibitory protein (or proteins) induced by cortisol have a much more rapid rate of turnover than does most of the cell protein (which averages about 10% per hr (10)). Furthermore, the mRNA template for induced protein appears to be more stable than the induced protein, since actinomycin D did not reverse steroid effects over a time period during which cycloheximide did achieve this reversal. It has been shown previously that a very brief exposure to cycloheximide before labeling does not reverse cortisol action (10).

It has been reported that rat liver hepatoma cells growing in tissue culture showed an increase in synthesis of tyrosine aminotransferase in the presence of dexamethasone, a synthetic glucocorticoid, and that subsequent addition of actinomycin D resulted in a “superinduction” of the enzyme (26, 27). In contrast, in the present study addition of this antibiotic subsequent to cortisol did not enhance the steroid effect.

The data reported in the present communication, supplemented by our previous studies as well as those of other laboratories, suggest the following tentative working hypothesis in explanation of the primary action of cortisol on lymphoid cells. Exposure of these cells to a thymolytic steroid leads to synthesis of new mRNAs, which code for one or more proteins with a rapid rate of turnover that is greater than that of the mRNA. The newly synthesized protein or proteins exert an inhibitory influence on transport processes and on ATP synthesis, as well as possibly on phosphorylation reactions coupled with ATP synthesis. The loci of action of the inhibitory protein or proteins appear to be at the plasma membrane, reflected in transport phenomena, and at one of the loci involved in oxidative phosphorylation.

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