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 some time 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 thymolytic 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

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 $(2 \times 10^7 \text{ cells/ml})$ 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 NaH₂⁸³PO₄, the cells were first incubated in minimal essential medium for sus-

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 or prior to 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.

Abbreviation: TCA, trichloroacetic acid.

 TABLE 1. Inhibitory action of cortisol in vitro on hexose and rubidium ion transport and on orthophosphate accumulation by rat thymocutes

Precursor and time of pulse labeling after cortisol addition	Radioactivity (dpm/mg cell protein) Control Cortisol		% Inhibition due to cortisol
[¹⁴ C]Glucose, 1–1.5 hr	2070	1450	$30 \pm 5(4)$
[¹⁴ C]2-Deoxyglucose, 1–1.5 hr	1710	1350	21 ± 1 (12)
[¹⁴ C]2-Deoxyglucose, 3-3.5 hr	1690	1310	$22 \pm 3 (5)$
[14C]3-O-Methylglucose, 1-1.5 hr	2430	1910	$21 \pm 5(4)$
⁸⁶ RbCl, 1–2 hr	3050	2770	$9 \pm 1 (3)$
⁸⁶ RbCl, 3–4 hr	2450	1830	25 ± 2 (4)
NaH232PO4, 1-2 hr	15,640	14,150	$10 \pm 2(3)$
NaH ₂ ³² PO ₄ , 1–2 hr; 1.0 mM		•	
iodoacetate added at 0.4 hr	11,580	8,950	$23 \pm 6 (3)$
NaH2 ³³ PO4, 3–4 hr	61,900	47,600	23 ± 2 (12)
NaH ₂ ³² PO ₄ , 3–4 hr; 1.0 mM			
iodoacetate added at 2.5 hr	10,650	6,320	44 ± 4 (3)

Cortisol, 1 μ M. Values for radioactivity incorporated are for the amounts in the TCA-soluble fraction of the cells. Values for % inhibition are means \pm SE (number of experiments are in parentheses). Each experiment included six or more separately labeled aliquots of cells for each condition studied.

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 ⁸⁶RbCl, or in a phosphate-free medium in experiments with NaH₂-³³PO₄. 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-ml conical centrifuge tubes containing either 0.005-0.015 µCi (about 1 Ci/g) of ⁸⁶RbCl (Abbott Laboratories), 0.075-0.50 µCi (0.2-2.0 Ci/g) of NaH₂³²PO₄, 0.025-0.150 µCi (0.2-2.0 Ci/g) of NaH233PO4, 0.34 µCi of [U-14C]glucose (16 Ci/mol) or 0.10 µCi of [1-14C]2-deoxyglucose (10.6 Ci/mol) (New England Nuclear Corp.) or 0.5 μ Ci 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 \times a for 5 min, and the cell pellets were washed in cold incubation medium (3 \times 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 ⁸H and ¹⁴C have been reported (8). Efficiencies for ⁸⁶Rb, ⁸³P, and ⁸²P 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). Ionexchange 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 μ M), estradiol (1 μ M), and 17 α -ethyl-19-nortestosterone (1 μ M) 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 ⁸⁶RbCl appears to be a valid index of the Na⁺-K⁺ transport system in thymocytes. The percentage inhibition of ⁸⁶RbCl 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 μ M 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 NaH₂³³PO₄ from the medium (Table 1). Incubation of cells with 10 μ M progesterone did not influence phosphate accumulation (data not shown). In other studies the inhibitory effect of cortisol was also observed when labeling with NaH₂³³PO₄ was carried out in the presence of unlabeled orthophosphate over a concentration range of 10⁻⁷ M to 10⁻² M. The data for phosphate uptake in Table 1 are for the TCA-soluble fraction 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 NaH₂³³PO₄ 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 NaH₂³³PO₄ 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 hot 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 μ M 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 (II- --II) incorporation into the TCA-insoluble fraction, $[{}^{14}C]\alpha$ -aminoisobutyrate (AIB) (\Box -- \Box) incorporation into the TCA-soluble fraction, and [6-3H] uridine incorporation into the TCA-soluble $(\bullet - - - \bullet)$ and TCA-insoluble $(O_{-} - O)$ fractions were as described previously (8). NaH₂³²PO₄ or $NaH_{2}^{33}PO_{4}$ incorporation in the presence $(\bullet - - \bullet)$ and absence (\blacktriangle) of iodoacetate, and the incorporation of ⁸⁶RbCl (O---O) and hexose (average for the three hexoses studied) (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

Antibiotic present throughout the incubation*	Precursor and time of pulse labeling after cortisol addition†	Radioactivity incorporated (dpm/mg cell protein)			Antibiotic
		Control	Antibiotic	Cortisol	+ cortisol
Cycloheximide	[¹⁴ C]2-Deoxyglucose, 1–1.5 hr	1780	1740	1370	1710
Cycloheximide	[¹⁴ C]Glucose, 1–1.5 hr	1140	1370	923	1310
Cycloheximide	[¹⁴ C]3-O-Methylglucose, 1–1.5 hr	1990	2920	1670	2770
Cycloheximide	⁸⁶ RbCl, 3–4 hr	1920	1880	1550	1950
Cycloheximide	NaH ₂ ³³ PO ₄ , 3–4 hr	24,500	24,000	19,700	23,700
Puromycin	[¹⁴ C]2-Deoxyglucose, 1–1.5 hr	1680	1790	1310	1820
Actinomycin D	^{[14} C]2-Deoxyglucose, 1–1.5 hr	1850	1780	1410	1780
Actinomycin D	NaH ₂ ³³ PO ₄ , 3–4 hr	25,400	26,100	19,800	26,600

* Cycloheximide (25 μ M), puromycin (25 μ M), or actinomycin D (4.0 μ g/ml) was added to control cell suspensions and to cell suspensions with 1 μ M 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.



FIG. 2. Reversal or prevention of cortisol action by inhibitors of RNA and protein synthesis. Suspensions of thymocytes were incubated with and without 1 μ M cortisol. At the indicated times after addition of cortisol or ethanol (control), 4 μ g/ml actinomycin D, 25 μ M cycloheximide, or a combination of both antibiotics was added to portions of the cell suspensions. After further incubation, aliquots of the suspensions were labeled (at the indicated times after ethanol or cortisol addition) with NaH₂³²PO₄, [¹⁴C]glucose, or [¹⁴C]3-O-methylglucose. Other details as in Table 2.

Fig. 1. Also shown, for comparison, is the more rapid onset of inhibition of uridine incorporation that occurs when exposure to cortisol is in vivo (7), with only the labeling carried out in vitro. It is likely that the less rapid effect of cortisol in vitro may be due at least in part to the enriched medium used for incubation, since more pronounced early effects of cortisol in vitro on incorporation of orthophosphate into ATP are obtained in the presence of iodoacetate. In any case, under appropriate conditions of measurement, significant inhibitory effects of cortisol in vitro on all parameters illustrated in Fig. 1 are evident with pulse labeling of 60-90 min after cortisol addition. When studied under identical conditions (without iodoacetate present), inhibition by cortisol of hexose transport is evident but very small with labeling from 10 to 30 min (Fig. 1). However, inhibition of hexose uptake by cortisol increases to a maximum value of 20-30% by about 60 min, whereas the other inhibitory effects of cortisol continue to progress in magnitude over a considerable period of time. If the inhibition by cortisol of incorporation of orthophosphate into ATP represented an effect on a compartment of ATP involved primarily in transport, this would in turn produce inhibition of transport of hexose as well as of other molecules. An alternative possibility is that the action of cortisol on transport is not dependent on ATP synthesis. Also, thymocytes represent a heterogeneous population of cells, and it is not yet known whether these diverse inhibitory processes all occur in the same cell population or in all thymocytes.

Role of RNA and protein synthesis in the action of cortisol

The influence of antibiotic inhibitors of RNA and protein synthesis on hexose and rubidium ion transport and on orthophosphate incorporation by thymocytes incubated with and without cortisol is summarized in Table 2. Actinomycin D, puromycin, and cycloheximide did not inhibit transport or phosphorylation in the absence of cortisol over the time period studied. However, when antibiotic was present throughout the 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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