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DIFFERENTIAL SENSITIVITY OF ISOLATED RAT THYMOCYTES TO GLUCOCORTICOIDS FOLLOWING CONCANAVALIN A STIMULATION

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SUMMARY

Concanavalin A (Con A) rapidly stimulates leucine incorporation into trichloroacetic acid (TCA) precipitable, but not TCA soluble material in isolated rat thymocytes. This stimulation of leucine incorporation by Con A is dose dependent, time dependent, and is inhibited by the addition of cycloheximide. In non mitogen stimulated thymocytes, cortisol (10^{-6} M) inhibits leucine incorporation into protein by 30%, whereas in Con A stimulated cells, cortisol inhibits leucine incorporation by 50%. The cortisol effect in Con A stimulated cells is present when cortisol and Con A are added simultaneously, is diminished when cortisol is added at intervals after Con A and is dependent on the concentration of cortisol administered. The inhibitory effect of cortisol on leucine incorporation is dose and time dependent in both control and Con A treated cells. The magnitude of the cortisol effect does not differ in control and Con A stimulated thymocytes for the first 3 h of steroid exposure, after which time a significant increase in the glucocorticoid effect occurs only in the Con A stimulated cells. This increased response to cortisol occurs at a time which is coincident with the mean maximal response of these cells to Con A alone. Both progesterone and cortexolone (10^{-6} M) inhibit leucine incorporation in control cells only slightly. The magnitude of these effects does not change by stimulation of the thymocytes with Con A. Under the conditions of these experiments, the rate of accumulation and the levels of nuclear glucocorticoid receptors are the same in both control and Con A stimulated thymocytes. These data indicate that thymocyte responsiveness to glucocorticoid is increased following exposure to concanavalin A. This enhanced response to glucocorticoid probably occurs subsequent to nuclear accumulation of steroid receptor complexes.

INTRODUCTION

Thymus cells respond to physiological concentrations of glucocorticoids both *in vitro* and *in vivo*. Some of these catabolic effects include inhibition of glucose transport, inhibition of precursor incorporation into DNA, RNA and protein, increased nuclear fragility and ultimately cell death [1]. All of these responses appear to be dependent on both actinomycin D and cycloheximide sensitive steps which are subsequent to hormone receptor interaction and nuclear localization of the glucocorticoid receptor complex.

In addition to being a target cell for glucocorticoids, thymocytes and lymphoid cells respond to and have specific receptors for the plant lectin concanavalin A [2–7]. Cellular responses of lymphocytes to Con A which have been described include stimulation of glucose transport and stimulation of precursor incorporation into RNA, DNA and protein [3–7].

Because of the widely opposing actions of Con A and glucocorticoids on thymocytes, we and others [6, 7] have sought to clarify to what extent these molecules antagonize each other's action in whole cells. In previous studies, [6, 7] we have shown that stimulation of thymocytes with high concentrations of concanavalin A (100–200 μg/ml) stimulates glucose uptake, reduces the magnitude of the glucocorticoid induced inhibition of glucose uptake and significantly decreases the number of intracellular glucocorticoid receptor complexes. In the present investigation, we demonstrate that unlike our previous studies, which employed high Con A concentrations, stimulation of thymocytes with low mitogen concentrations (3–12 μg/ml) can effectively and specifically increase the magnitude of the inhibitory action of glucocorticoids on leucine incorporation into protein. This amplification probably occurs at a step subsequent to nuclear steroid receptor accumulation.

Abbreviations used: F, cortisol (11β, 17α, 21 trihydroxy-4-pregnene-3, 20-dione) cortisone (17α, 21-dihydroxy-4-pregnene-3, 11, 20 trione) corticosterone (11β, 21-dihydroxy-4-pregnene-3, 20 dione) cortexolone (17α, 21-dihydroxy-4-pregnene-3, 20-dione) progesterone (4-pregnene-3, 20-dione). MEM-L, minimum essential medium without leucine. Con A, concanavalin A.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (Canadian Breeding Farms and Laboratories) weighing 100 gms were used throughout. Bilateral adrenalectomy was performed under ether anesthesia approx. 1 week before the experiment, and surgical ablations were confirmed at autopsy by examination of the peritoneal cavity.

Chemicals. [3,4,5-^{3}H(N)]-L-leucine 115.0 Ci/mmol and [1-^{14}C]-L-leucine 56.9 mCi/mmol (New England Nuclear) were diluted to a concentration of 1 μCi/ml in Minimum Essential Medium (Eagle) without L-leucine (MEM-L). Concanavalin A (Pharmacia) stock solution was prepared fresh daily in Krebs-Ringer-bicarbonate buffer containing glucose (1 mg/ml). Cortisol, dexamethasone, cortexolone, corticosterone and cortisone (Steraloids) were dissolved in absolute ethanol to prepare stock solutions at $\simeq 2.0 \times 10^{-4}$ M. Aliquots were evaporated in glass tubes and brought to volume with MEM-L prior to the addition of the cell suspension. Other chemicals used in this study were reagent grade and obtained from Fisher Scientific Company or Sigma.

Tissue preparation. Animals were decapitated at the time of the experiment and the thymus cell suspensions were prepared in Krebs–Ringer-bicarbonate buffer with glucose, and equilibrated with 95% O_2–5% CO_2 as described previously [8]. Cell suspensions were prepared in MEM-L at concentrations of 3–6 $\times$ 10^7/ml. Measurement of all cell numbers was made with a hemocytometer.

Measurement of leucine incorporation into TCA precipitable material. Forty μl aliquots of a thymocyte cell suspension prepared in MEM-L were added to an equal volume of MEM-L containing mitogens and/or glucocorticoids in a 96 well microtiter plate. The plate was covered and incubated for 5 h at 37°C in a 5% CO_2–95% air atmosphere. A 20 μl aliquot of [^{14}C]- or [^{3}H]-L-leucine prepared in MEM-L at a concentration of 1 μCi/ml was added to each well and the incubation continued for an additional 30 min. The microtiter plate was placed on ice for 10–15 min and 20 μl of cold 50% TCA was added.

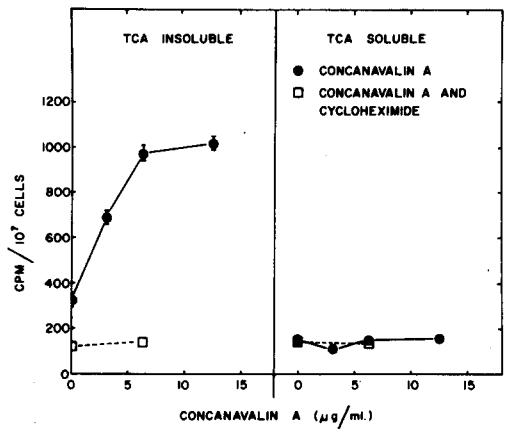

Fig. 1. The effect of concanavalin A and cycloheximide alone and in combination on leucine incorporation in isolated rat thymocytes. Cells were incubated with concanavalin A (●) and/or cycloheximide (□) (1 $\times$ 10^{-5} M) in MEM-L for 5 h. A pulse of [^{14}C]-L-leucine was then added and leucine incorporation into trichloroacetic acid precipitable and trichloroacetic acid soluble material was determined as described in Materials and Methods. Each point represents the mean $\pm$ one standard error of four separate experimental points from a single thymus cell pool.

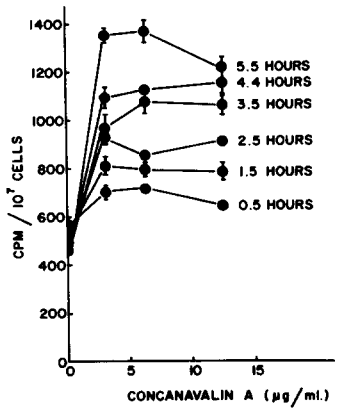

Fig. 2. Influence of time of exposure of thymocytes to concanavalin A on leucine incorporation. Concanavalin A at the concentrations indicated was added to thymocytes at 37°C at 0, 1, 2, 3, 4, and 5 h after the start of their incubation. Groups of cells were then pulsed with [^{14}C]-L-leucine for 30 min and leucine incorporation into TCA insoluble material was measured as described in Materials and Methods. Values are the mean $\pm$ one standard error of four individual determinations from a representative experiment.

After 20 min, the plate was then mixed gently on ice for 10–15 min and centrifuged in a Servall RC-3 (HL-8 rotor) at 4°C for 10 min at 1500 *g*. The TCA supernatant was carefully aspirated, discarded and the procedure repeated twice using 10% TCA. After the final aspiration step, 100 μl of 2N NaOH was added to each well to dissolve the precipitate. A 50 μl aliquot of the supernatant was recovered and counted in a PCS/xylene 2:1 scintillant in a Beckman LS-150 liquid scintillation spectrometer having a 40% efficiency for ^{3}H and 60% efficiency for ^{14}C.

Measurement of leucine incorporation into TCA soluble material. After the pulse of radiolabeled leucine, the cells were centrifuged as described above and the supernatant was aspirated and discarded. The cells were then washed with 100 μl of cold phosphate buffered saline, recentrifuged and the supernatant aspirated and discarded. This procedure was repeated for a second time after which 100 μl of cold 10% TCA was added to each well and allowed to sit on ice for 10–15 min. The microtiter plate was then centrifuged as described and a 50 μl aliquot of the supernatant sampled for determination of radioactivity.

RESULTS

Figure 1 shows the influence of exposure of thymocytes to several concanavalin A concentrations on leucine incorporation into both TCA soluble and insoluble material. Concanavalin A stimulates leucine incorporation into TCA precipitable material without altering the intracellular level of TCA soluble material. At the 5 h incubation period used in these experiments, maximal response of the thymocytes to Con A occurs at concentrations betwen 6 and 12 μg/ml.

This response is generally a 2–3 fold stimulation over basal leucine incorporation in non-mitogen stimulated thymocytes. Slightly higher concentrations of Con A, up to 25 μg/ml do not lead to any further stimulation of leucine incorporation and occasionally slightly suppress the maximal response. Figure 1 also shows that the stimulatory effect of Con A on leucine incorporation into protein is blocked when thymocytes are simultaneously exposed to Con A and cycloheximide. Cycloheximide by itself significantly lowers leucine incorporation into TCA insoluble material. Cycloheximide has no effect on intracellular TCA soluble leucine in either control or Con A stimulated cells. The experiments performed in Fig. 1 were conducted at a leucine concentration of $\simeq 2 \times 10^{-5}$ M. These are conditions which probably do not flood the intracellular leucine pool and therefore the observed effects on leucine incorporation may be due either to a stimulation of protein synthesis or a decrease in protein degradation. At the present time, we have no information on which process is being altered by Con A.

Figure 2 shows results of experiments designed to assess the rate at which Con A stimulates leucine incorporation into TCA insoluble material in isolated thymocytes. In this experiment and in all others, we have investigated kinetic parameters of Con A and glucocorticoid effects on thymocytes. All of the cells have been maintained *in vitro* for 5.5 h at 37°C. Thus, for the 0.5 h time point shown in this graph, cells were incubated for 5 h after which Con A was added just prior to the pulse of [^{14}C]-leucine. The data in Fig. 2 indicate that the effect of Con A on leucine incorporation is a rapid one, with significant stimulation observed within 30 min of exposure of cells to Con A. The magnitude of the stimulatory effect of Con A also appears to be time dependent. In general, 6–12 μg/ml ($\simeq 2 \times 10^{-6}$ M) Con A is required to achieve maximal stimulation, however, at mitogen concentrations below this (3 μg/ml) the response tends to be more variable.

Figure 3 shows the influence of exposure of thymocytes to cortisol and Con A alone and in combination on leucine incorporation into TCA insoluble material. Exposure of non Con A stimulated thymocytes to cortisol at 10^{-8}, 10^{-7}, and 10^{-6} M results in inhibition of leucine incorporation by 7, 18 and 24% respectively. When cells are exposed to Con A concentrations greater than 3 μg/ml simultaneously with cortisol, the magnitude of the inhibitory effect of the glucocorticoid on leucine incorporation is potentiated. For example, at a Con A concentration of 6.25 μg/ml, cortisol at 10^{-8}, 10^{-7}, and 10^{-6} M inhibits leucine incorporation into protein by 18, 41, and 55% respectively. Thus under conditions of simultaneous exposure of cells to concanavalin A and cortisol we have more than doubled the inhibitory glucocorticoid effect on leucine incorporation. It is worthy to note here that the enhanced response to cortisol in Con A stimulated cells can be viewed both in terms of

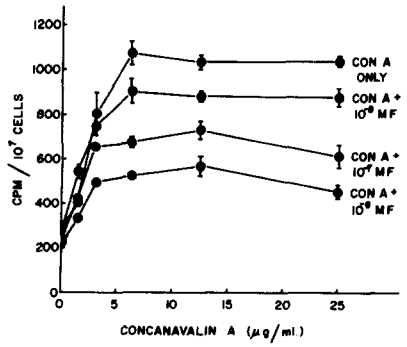

Fig. 3. The effect of cortisol concentration on leucine incorporation in concanavalin A stimulated thymocytes. Cells were incubated for 5 h at 37°C with cortisol and concanavalin A alone or in combination at the concentrations shown. A pulse of [^{14}C]-L-leucine was then added, the incubation continued for 30 min and leucine incorporation into TCA insoluble material was measured as described. Values are the mean ± one standard error from four individual determinations from a representative experiment.

absolute magnitude and % inhibition in relation to the appropriate control. We believe that viewing our data as % inhibition is more meaningful since this procedure allows comparison of the cortisol effect in Con A and control cells wherein the relative rates of leucine incorporation differ markedly. This altered response to glucocorticoid in concanavalin A stimulated cells still appears to be a function of the dose of cortisol administered.

The data in Fig. 4 show that the enhanced inhibitory effect of cortisol on leucine incorporation is highly specific for glucocorticoids. For example, in control cells, cortisol, corticosterone, cortexolone, progesterone and cortisone at 10^{-6} M inhibited leu-

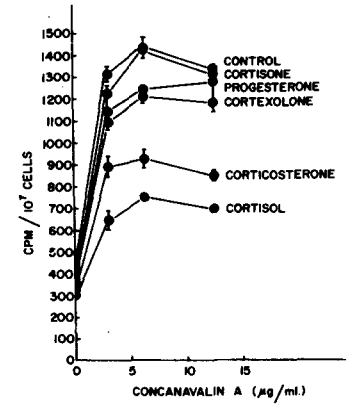

Fig. 4. The influence of glucocorticoids of different biological potency on leucine incorporation in concanavalin A stimulated thymocytes. Cortisol, progesterone, cortexolone, cortisone, and corticosterone, each at 10^{-6} M were incubated with thymocytes stimulated with concanavalin A for 5 h at 37°C. Following this incubation, a pulse of [^{14}C]-L-leucine was added for 30 min and leucine incorporation into TCA precipitable material was measured as described. Each point represents the mean ± one standard error from four separate determinations from a single thymus cell pool.

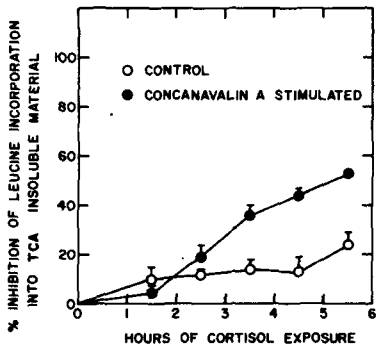

Fig. 5. Development of the cortisol inhibition of leucine incorporation in concanavalin A and control thymocytes. Cortisol (10^{-6} M) was added to unstimulated thymocytes or cells which were stimulated with concanavalin A (12.5 μg/ml) at time 0. The duration of exposure to cortisol is indicated in the figure. A pulse of [^{3}H]-L-leucine was added during the last 30 min of incubation. Leucine incorporation into TCA precipitable material was measured as described. The data are expressed as the % inhibition of the appropriate control. Values shown are the mean ± one standard error of at least four determinations.

cine incorporation by 29, 24, 17, 11, and 0% respectively. As the data in Fig. 4 illustrate, only the response of the thymocytes to the biologically potent glucocorticoids (cortisol, corticosterone) was enhanced when these cells were stimulated with concanavalin A. These data suggest that the enhanced response of Con A stimulated thymocytes to glucocorticoids is a steroid dependent process which presumably is mediated via receptor interaction.

The data in Fig. 5 show that the enhanced glucocorticoid inhibition of leucine incorporation into protein in Con A stimulated lymphocytes occurs at a time when our cells become maximally responsive to the mitogen itself i.e. 3–4 h (Fig. 2). Within the first 2 h of exposure to cortisol (10^{-6} M) both concanavalin A stimulated thymocytes and unstimulated controls respond similarly. After this period, however, the magnitude of the inhibitory effect of cortisol on leucine incorporation into protein is enhanced in the concanavalin A stimulated cells. Five and one half hours after cortisol treatment, leucine incorporation is inhibited by 24% in unstimulated cells and by 53% in the concanavalin A stimulated cells. Thus, the onset of the inhibitory response to cortisol is similar in both groups of cells after which a significant increase in the glucocorticoid response occurs in the mitogen stimulated cells. It should be noted that in this experiment, [^{3}H]-leucine rather than [^{14}C]-leucine was used. This lower effective leucine concentration has no effect on the magnitude of the Con A response.

The studies in the next figure were designed to test the possibility that the enhanced glucocorticoid response, which we have observed in concanavalin A stimulated cells (Figs 2–5), may be accounted for by an alteration in the level or in the rate of accumulation of nuclear glucocorticoid receptor complexes. Previous studies in our laboratory [6, 7] have indicated that stimulation of thymocytes with high concentrations of Con A (>20 μg/ml) can result in a decrease in nuclear glucocorticoid receptors under equilibrium conditions. The data in Fig. 6 show the results of a typical kinetic study in which the time course of nuclear [^{3}H]-dexamethasone-receptor binding was measured in both control and Con A stimulated cells (6.25 μg/ml). These results demonstrate that within experimental error no significant variation occurs in either the rate or the extent to which nuclei from either group of cells accumulate glucocorticoid receptors. The reduction in total nuclear glucocorticoid receptor binding which occurs after 90 min of incubation cannot be completely accounted for by the small number of thymocytes which die after this time (10–20%) and therefore may be related to the "nuclear processing" phenomenon recently described for estradiol-receptors in MCF-7 tumor cells [9].

DISCUSSION

The data in this report provide us with several pieces of information which are not currently known about the interactions of glucocorticoids and mitogens at the cellular level in thymocytes. We have shown that the magnitude of the inhibitory effect of glucocorticoids on thymocytes can be enhanced when these cells are exposed to low concentrations of Con A. Thymocytes which have been stimulated with Con A still respond to steroids according to classical definitions outlined for steroid mediated processes.

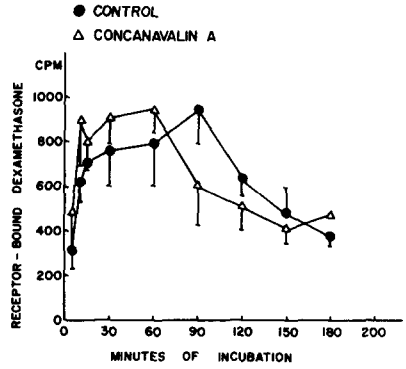

Fig. 6. The time course of nuclear dexamethasone receptor binding in control and concanavalin A stimulated thymocytes. Suspensions of thymocytes were exposed to 6.25 μg/ml Con A or MEM-L alone for 5 h. After this time, the cell suspensions from both groups were divided and half of each incubated with 2×10^{-8} M [^{3}H]-dexamethasone without or with 2×10^{-6} M unlabeled dexamethasone for the time periods indicated. The data shown represent the difference between cells which were incubated with [^{3}H]-dexamethasone alone and cells incubated with [^{3}H]-dexamethasone plus unlabeled dexamethasone. Values shown are the mean ± one standard error from four separate determinations. The methods used for measurement of nuclear receptor binding were the same as previously described [6, 7].

Thus, we have shown that thymocytes, stimulated with Con A, respond to glucocorticoids in a dose-dependent, time dependent manner, with a high degree of steroid specificity. In addition, our studies on nuclear glucocorticoid receptor binding in both Con A stimulated and control thymocytes suggest that nuclear receptor steroid interactions are unchanged at least with regard to the rate of nuclear accumulation of steroid–receptor complexes or the number of complexes associated with nuclei at a one-half saturating concentration of steroid. We therefore believe that these data show that Con A enhances steroid hormone action via mechanisms which probably are subsequent to nuclear receptor binding.

Earlier work in our laboratory [6, 7] provided us with information, which would appear to be different from that we now observe. In fact, however, these studies are not in opposition and complement each other. In those studies the concentrations of Con A which were required to maximally stimulate glucose uptake in thymocytes [6, 7] were $\simeq 100\,\mu$g/ml. Under these conditions, significant reductions in nuclear dexamethasone receptors occurred and the inhibitory action of cortisol on glucose uptake was absent. Although stimulatory for glucose uptake, these concentrations of Con A ($\simeq 100\,\mu$g/ml) only marginally stimulate leucine incorporation into protein (Cidlowski, unpublished observation). Biphasic dose response curves, wherein low mitogen concentrations cause an effect and high concentrations do not, are not unusual for the actions of mitogens on lymphoid cells [10]. It also becomes evident that the dose response curves for Con A stimulation of glucose uptake [6] and stimulation of leucine incorporation (Fig. 1) differ. Only 1/10 of the concentration of Con A required to stimulate glucose uptake is needed to maximally stimulate leucine incorporation. This difference may be related to the length of time of exposure of the cells to mitogen. In our former studies on glucose uptake [6, 7] cells were only exposed to mitogen for 1 h, whereas a 5.5 h exposure is used in the current investigation.

The question of mitogen/glucocorticoid antagonism has been looked at in another model system, the human peripheral lymphocyte [11, 12]. As we report here for the thymocyte model, these studies on lymphocytes demonstrated that glucocorticoids are capable of blocking or inhibiting precursor incorporation into macromolecules in mitogen stimulated cells. Direct comparison of their results [11, 12] with ours, however, is difficult because the human peripheral lymphocyte model represents a mixed cell population in which changes in cell cycle stages [12] may occur during experimentation. No changes in cell number or thymidine incorporation into DNA occur under the conditions of our experiments. Ono *et al.* [11], however, have looked at glucocorticoid responsiveness in Con A stimulated lymphocytes during times in which alterations in cell number probably were not occurring. They observed that the inhibition of uridine incorporation into RNA caused by cortisol (6×10^{-5} M) was greater in phytohemagglutinin stimulated cells than control cells.

In summary, we have provided evidence that the magnitude of the inhibitory action of cortisol on leucine incorporation into protein in thymocytes can be enhanced via stimulation of the cells with low concentrations of concanavalin A. The enhanced response to glucocorticoids occurs at a time period when thymocytes are responding maximally to Con A. These studies clearly demonstrate that the response of cells to glucocorticoids is markedly dependent on cellular homeostasis and provide us with a model to further investigate the mechanisms underlying the glucocorticoid hyperresponsiveness observed in mitogen stimulated thymocytes.

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