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CONCANAVALIN A-INDUCED GLUCOCORTICOID RESISTANCE IN RAT THYMUS CELLS: DECREASED CYTOPLASMIC AND NUCLEAR RECEPTOR BINDING OF DEXAMETHASONE

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SUMMARY

Concanavalin A (Con A) rapidly stimulates glucose uptake in isolated rat thymocytes, whereas cortisol has been shown to reduce the uptake of glucose. If Con A is added to thymocytes before or together with cortisol, no decrease in glucose uptake effect is observed. But if cortisol is added as little as 10 min before Con A (in the absence of glucose) the typical glucocorticoid response is seen superimposed on the Con A effect. The Con A-induced glucocorticoid antagonism may be accounted for in part by alteration in thymocyte glucocorticoid receptor metabolism. Con A exposure within 30 min lowers saturable dexamethasone binding by 40%. At concentrations as low as 20 μg/ml Con A significantly reduces dexamethasone binding to thymocytes in both cytoplasmic and nuclear components, but does not alter the temperature-sensitive cytoplasmic-to-nuclear translocation of dexamethasone receptor complexes. When Con A exposure follows dexamethasone, only a reduction in the concentration of cytoplasmic binding sites occurs. At all concentrations tested, Con A did not affect binding of dexamethasone to isolated cytoplasmic glucocorticoid receptors. Con A reduces the number of thymocyte glucocortocoid receptors without altering the affinity of the residual steroid binding proteins.

INTRODUCTION

Exposure of rat thymocytes to glucocorticoids triggers a series of events which culminate in cell death. Cell death is preceded by a rapid (15–25 min) inhibition of glucose uptake with subsequent depression of RNA and protein synthesis (for review see [1]).

Actinomycin D- and cycloheximide-sensitive steps [2, 3] appear to mediate the decrease in glucose transport, and in turn depend on prior or simultaneous interaction of the steroid with a cytoplasmic receptor and subsequent translocation to the nucleus of the hormone–receptor complex.

In contrast to glucocorticoids, plant lectins such as Con A have been shown to stimulate RNA synthesis [4] and glucose metabolism in lymphoid cells [5, 6]. Thymocytes respond within 5 min to Con A with a dramatic increase in glucose uptake. This process is not dependent on RNA or protein synthesis [7]. Con A stimulation of glucose uptake resembles the increased facilitated diffusion of hexose that has been observed with phytohemagglutinin acting on peripheral lymphocytes [8]. Our earlier work indicated that thymocytes pre-exposed to maximal stimulatory concentrations of Con A are incapable of responding to glucocorticoids with a typical inhibition of glucose uptake. The cortisol-induced decrease in glucose uptake is present, however, when Con A stimulation follows cortisol exposure for 30 min in the absence of glucose [7]. In an attempt to understand the underlying mechanisms of the steroid-resistant state observed in Con A-stimulated thymocytes we have explored the possibility that Con A alters glucocorticoid receptor levels.

EXPERIMENTAL

Unlabeled cortisol and dexamethasone were purchased from Calbiochem, dissolved in buffer, and used at 10^{-6} M or at the concentration indicated in the figure legends. [^{3}H]-dexamethasone, 22.6 Ci/mmol, was purchased from New England Nuclear. Solutions of Con A (Pharmacia) were prepared fresh daily in Krebs–Ringer bicarbonate buffer with or without glucose at the concentrations indicated. Other chemicals used in this study were reagent grade and obtained from Fisher Scientific.

Thymus cell preparation. Thymus cell suspensions from male Sprague–Dawley rats adrenalectomized 6–8 days prior to use were prepared in Krebs–Ringer bicarbonate buffer equilibrated with 95% oxygen: 5% carbon dioxide [9]. Cells were used at a cytocrit of 0.1–0.3 ml of packed cells per ml of cell suspension measured by the microhematocrit method.

Determination of glucose uptake. Glucose uptake was measured by disappearance of glucose from the medium in a 1-h incubation at 37° using inverted microcentrifuge tubes [9, 10]. Results are given as micromoles of glucose used per ml of packed cells.

Incubation of thymus cells for assessment of steroid binding. Aliquots (250 μl) of cell suspension were placed in 1 dram screw-cap glass vials with or without

Con A and incubated at 37° for the time periods indicated in the figure legends. Subsequently steroid was added in a 10 μl vol. to a final concentration of 10–20 nM [^{3}H]-dexamethasone or 10–20 nM [^{3}H] dexamethasone plus 1–2 μM unlabeled dexamethasone. The cells were then incubated for an additional 30 min at 37°. Twenty min after addition of dexamethasone, samples were taken to determine the total (cell plus supernatant) and free (supernatant) concentration of [^{3}H] steroid in each vial. Within each experiment the concentrations of free steroid generally differed by $<10\%$, and were normalized to equivalent free steroid concentration ($\simeq 1 \times 10^{-8}$ M) to allow valid comparison among groups. At the end of the incubation the cells were cooled to 3° for determination of cytoplasmic and nuclear receptor binding.

Assessement of cytoplasmic and nuclear dexamethasone receptor binding. [^{3}H]-dexamethasone binding in cytoplasmic cell fractions was determined by breaking the incubated cells by 6-fold dilution in 1.5 mM aqueous $MgCl_2$ containing Dextran-coated charcoal [11], and measuring the radioactivity in the supernatant. Saturable receptor binding of dexamethasone was considered to be the difference between [^{3}H]-dexamethasone binding alone and [^{3}H]-dexamethasone plus unlabelled dexamethasone. Binding values are shown as c.p.m. [^{3}H]-dexamethasone bound/ml of packed cells. Con A treatment did not influence the nonsaturable binding of dexamethasone in either cytoplasmic or nuclear fractions or the cytocrit of the cell suspension. The data is shown as [^{3}H]-dexamethasone bound/ml of packed cells as calculated from a measure of the cytocrit in the final incubation. [^{3}H]-dexamethasone binding in nuclear fractions was determined by counting the radioactivity in the pellets of 500-fold dilutions of incubated cells in 1.5 mM $MgCl_2$ [11].

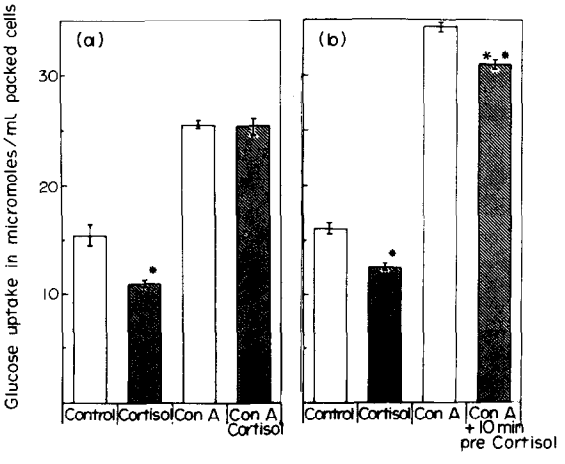

Fig. 1. Aliquots of a thymocyte suspension (40 μl) were incubated with Con A (250 μg/ml) or cortisol (1 μM) alone or combined for 1 h at 37° as described in Materials and Methods. Values are the means ± S.E. of 4–6 individual determinations from a representative experiment. *Differs significantly from control ($P < 0.05$). **Differs significantly from Con A at ($P < 0.05$). (A) Cells were exposed to Con A and cortisol simultaneously. (B) Thymocytes were exposed to cortisol or buffer for 10 min in the absence of glucose, prior to the addition of Con A.

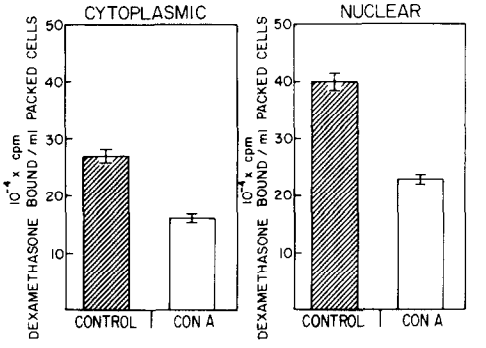

Fig. 2. Equilibrium binding of dexamethasone to Con A-stimulated thymocytes. Cell suspensions of thymocytes were incubated with or without Con A (250 μg/ml) for 30 min at 37°. Subsequently 10 nM [^{3}H]-dexamethasone was added to the incubation vials, or 10 nM [^{3}H]-dexamethasone plus 1 μM unlabeled dexamethasone. The incubation was continued for an additional 30 min, after which the cells were assayed in 4 replicates for cytoplasmic and nuclear dexamethasone-receptor binding.

RESULTS

The results in Fig. 1A confirm previous observations that cortisol treatment of thymocytes for 1 h significantly (28%) reduces the uptake of glucose [13], while Con A exposure markedly stimulates glucose uptake [7]. Simultaneous exposure of thymocytes to Con A and cortisol (4th bar) results in a stimulatory effect on glucose uptake which is equivalent in magnitude to the Con A response alone. Thus, thymocytes exposed to mitogen no longer show inhibition of glucose uptake when exposed to cortisol. The results in Fig. 1B demonstrate that the ability of thymocytes to respond to cortisol in the presence of Con A is strictly dependent on the order of addition of steroid and mitogen. Thymocytes exposed to cortisol in the absence of glucose for as little as 10 min at 37° prior to Con A show a typical glucocorticoid inhibition of glucose uptake even in the face of a maximal Con A stimulation of glucose uptake. These data suggest that the antagonism by Con A of the cortisol effect shown in Fig. 1A is due to a block by Con A of one of the steps in cortisol action taking place within the first 10 min of exposure of cells to cortisol.

The data in Fig. 2 demonstrates that when Con A-stimulated thymocytes are unresponsive to cortisol (with regard to glucose uptake), both cytoplasmic and nuclear receptor binding of [^{3}H]-dexamethasone at 37° is decreased. Thus, Con A treatment for 30 min at 37° reduces cytoplasmic binding by 42% and nuclear binding by 45%. As expected, at 37° nuclear binding exceeds cytoplasmic binding. Con A treatment clearly does not block formation of the nuclear complex. Similar results are obtained under conditions where glucose is omitted from the buffer.

As seen in Fig. 3, the effect of Con A on glucocorticoid receptor binding is rapid and the magnitude of

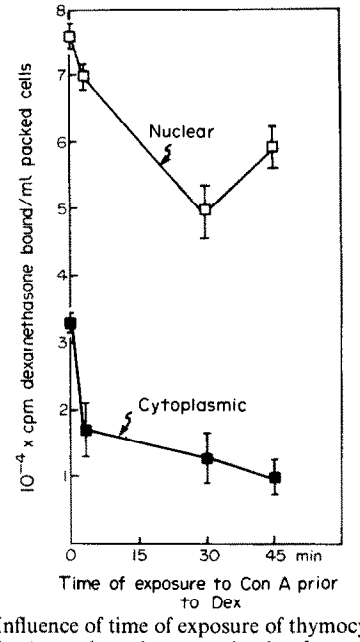

Fig. 3. Influence of time of exposure of thymocytes to Concanavalin A on the subsequent levels of cytoplasmic and nuclear glucocorticoid receptors. Thymocytes were exposed to Con A (250 μg/ml) at 37° for the times indicated. [^{3}H]-dexamethasone or [^{3}H]-dexamethasone plus unlabeled dexamethasone were then added and the incubations continued for 30 min, after which binding of [^{3}H]-dexamethasone was assessed. All cell suspensions were maintained at 37° for a total of 75 min. Each group is shown as the mean ± S.E. of 4 replicate determinations from a typical experiment.

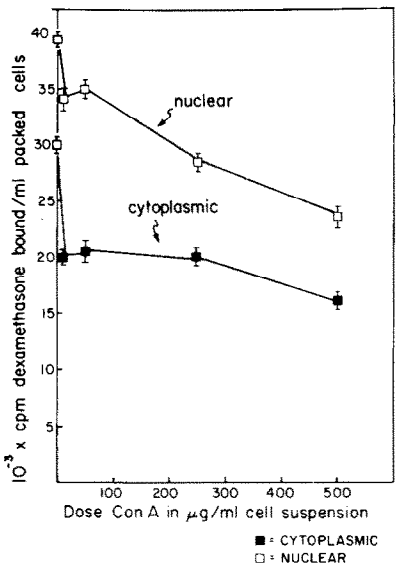

Fig. 5. The effect of Con A concentration on the levels of cytoplasmic and nuclear dexamethasone receptor complex in isolated thymocytes. Thymus cells were incubated with various concentrations of Con A for 45 min at 37°C prior to the addition of 10 nM [^{3}H]-dexamethasone or 10 nM [^{3}H]-dexamethasone plus 1 μM unlabelled dexamethasone. Following an additional 30-min incubation at 37° the levels of cytoplasmic and nuclear steroid binding proteins were measured. Each point represents the mean ± S.E. of four individual determinations of receptor levels.

the response dependent on the time of exposure. Initially the relative effect is considerably greater on cytoplasmic than on nuclear binding, but the difference diminishes with time. It should be noted that the conditions in Fig. 1 correspond to the 30-min time period in this figure.

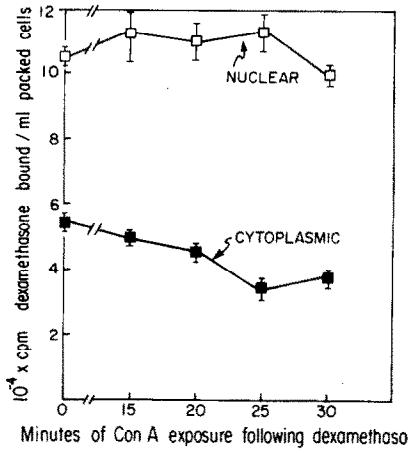

Fig. 4. Time course of Con A-induced decrease in glucocorticoid receptor binding following exposure of cells to [^{3}H]-dexamethasone. Thymocytes were exposed to 10 nM [^{3}H]-dexamethasone or 10 nM [^{3}H]-dexamethasone plus 1 μm unlabeled dexamethasone for 30 min at 37°. Subsequently Con A (250 μg/ml) was added for the times indicated, after which levels of cytoplasmic and nuclear dexamethasone binding were measured. The data are shown as the mean ± S.E. of four replicate determinations for both types of steroid receptor complex from single incubations.

The data in Fig. 4 demonstrate that when thymocytes are exposed to Con A following [^{3}H]-dexamethasone, a reduction occurs only in the level of cytoplasmic binding, without change in nuclear binding. The magnitude of the Con A response is again time-dependent. These results demonstrate that Con A decreases cytoplasmic glucocorticoid binding even in the presence of steroid.

The effect of Con A on dexamethasone binding appears to be dose-related (Fig. 5). Preexposure of thymocytes to Con A concentrations as low as 20 μg/ml reduces the level of both cytoplasmic and nuclear binding. As shown in Fig. 6, Con A at the concentrations studied does not impair direct binding of [^{3}H]-dexamethasone to the isolated cytoplasmic receptor at 3°. Since Con A does reduce cytoplasmic binding in whole cells at 3° (data not shown), these results show that the effect is probably not due to direct interaction of Con A with the receptor.

The Scatchard plots in Fig. 7, showing the concentration dependence of cytoplasmic binding of [^{3}H]-dexamethasone with untreated cells and cells treated with Con A, indicate that Con A reduces the number of dexamethasone binding sites without altering significantly the association constants of the residual binding sites. Control treated cells have an apparent association constant for dexamethasone of $4.2 \times 10^7\ M^{-1}$, with approximately 54×10^{-12} moles bound per ml of packed cells. Con A treated

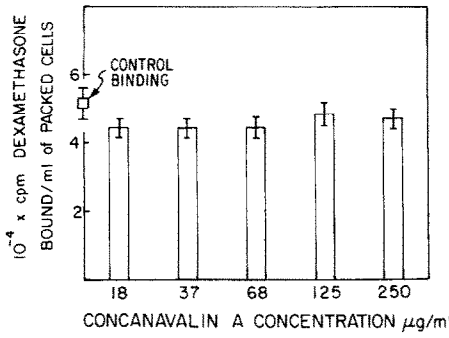

Fig. 6. Influence of Con A on the binding of dexamethasone to glucocorticoid receptors in thymus cell cytosol. Unexposed thymus cell suspensions ($V = 0.2$–0.3) were diluted 1 to 6 in 1.5 mM $MgCl_2$ for 15 min at 3°. Broken cell membranes and nuclei were sedimented by centrifugation at 3000 *g* in a Sorvall GLC centrifuge. The supernatant was then used for the assessment of dexamethasone binding. Quadruplicate 50 μl samples were incubated with 10 nM [^{3}H]-dexamethasone with or without 1 μM dexamethasone, and 1×10^{-8} M [^{3}H]-dexamethasone plus the concentrations of Con A shown. The incubation time was 90 min at a temperature of 3°. 100 μl aliquots of dextran coated charcoal were then added to each sample, vortexed and allowed to incubate for 15 min at 3°. Subsequently all samples were centrifuged at 10,000 *g* in a Beckman microfuge to pellet the charcoal and nonreceptor bound steroid. 75 μl aliquots of the supernatant were placed in scintillation vials for determination of radioactivity. The values shown are the mean ± S.E. of three individual measurements. The data is expressed per ml of packed cells as calculated from the original cytocrit.

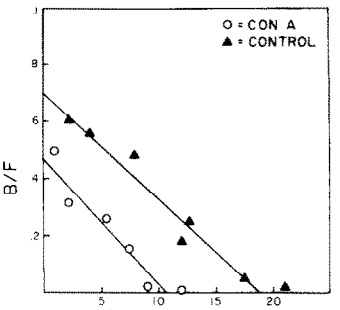

Fig. 7. Scatchard plot of cytoplasmic dexamethasone binding to cytoplasmic receptor of Con A-treated and untreated thymus cell suspensions. Thymocytes ($V = 0.28$) were incubated with or without Con A (250 μg/ml) for 30 min at 37°. Subsequently 250 μl aliquots of cells were incubated for 30 min at 37° with [^{3}H]-dexamethasone at concentrations from 1 μM to 0.6 pM, with continued exposure to Con A, after which cytoplasmic binding was measured. B = [^{3}H]-dexamethasone bound per ml cell suspension. F = free [^{3}H]-dexamethasone per ml of cell suspension.

cells have an apparent association constant for dexamethasone of $6.0 \times 10^7\ M^{-1}$ with approximately 29×10^{-12} mol bound per ml packed cells which represents a 43% reduction from control values. Analogous results are found when thymocytes are exposed to Con A in the absence of steroid, disrupted by hypotonic shock and binding of dexamethasone studied with cytosol fractions.

DISCUSSION

The results of this and previous investigations [7] indicate that thymocytes perturbed by the mitogen Con A may be incapable of responding to cortisol with a typical inhibition of glucose uptake, when stimulation by Con A precedes or accompanies exposure to cortisol. In the earlier study [7] this loss of glucocorticoid sensitivity was not seen when mitogenic stimulation followed 30 min of steroid treatment.

The data presented in Fig. 1 confirm and extend these results [8]. Following a steroid exposure of only 10 min, thymocytes were capable of responding with a typical inhibition of glucose uptake. Our new finding suggests that the antagonistic action of Con A on cortisol-induced inhibition of glucose uptake must take place at some time within the first 10 min of cortisol action on thymocytes. Thus receptor binding both to cytoplasmic fractions, nuclear association of the steroid–receptor complexes and early RNA induction, are likely candidates for Con A modification.

As for possible mechanisms of this early mitogen action, it is known that phytohemagglutinin rapidly (15 min) increases both cyclic GMP levels[14] and RNA polymerase I [15] in peripheral lymphocytes. Furthermore, cyclic GMP has been shown to mimic the action of PHA on RNA polymerase I [16]. On the basis of these studies we have investigated the action of cyclic nucleotides on the cortisol-induced inhibition of glucose uptake response. Preliminary work indicates that cyclic GMP derivatives but not cyclic AMP derivatives can mimic the action of Con A in blocking the cortisol effect on glucose uptake [17]. Similarly, cyclic GMP derivatives reduce the levels of glucocorticoid binding, though not to the extent observed with Con A in the present study. Therefore, although the reduction in level of glucocorticoid receptors seen in the present study provides a simple explanation for the loss of at least 40% of the glucocorticoid sensitivity (if one assumes that nuclear steroid receptor complex is in a one-to-one relationship with biological response), it does not explain the entire loss of cortisol sensitivity at 1 h or the action of cyclic GMP derivatives. Ongoing studies on the nature of the biologically active nuclear acceptor sites should provide insight into this problem.

With regard to the mechanism by which Con A treatment reduces both cytoplasmic and nuclear levels of glucocorticoid receptor, it is possible that this effect may result from lowered levels of ATP. Receptor-binding of glucocorticoids in thymus cells is known to depend on ATP levels [18–20], and recent evidence [21] suggests that Con A activates lymphocyte ATPase and thereby reduces intracellular ATP.

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DISCUSSION

Toft. Does the receptor bind Con A?

Cidlowski. There is no significant competition between con A and dexamethasone for the isolated receptor.