Cellular and Biochemical Actions of Adrenal Glucocorticoid Hormones on Rat Thymic Lymphocytes

by Donald A. Young,* Bruce P. Voris,* and Mary L. Nicholson*

The molecular, biochemical, and cellular effects of adrenal glucocorticoid hormones on thymic lymphocytes are reviewed, with emphasis on their relationship to the growth suppressive and lethal actions that occur in lymphoid tissues when glucocorticoids are administered to the whole animal. The data support the hypothesis that the hormonal inhibition of growth and development is a consequence of its ability to suppress cellular energy production, causing the cells to behave as though they were in a more stringent environment. Slight changes in ratios of adenine and guanine nucleotides appear to account for the realignment of metabolic priorities that occur, with processes related to growth and development curtailed in favor of those more essential to immediate cell survival.

The lethal glucocorticoid actions appear to be the result of the operation of separate mechanisms (unrelated to energy metabolism) that lead to lethal attack at the level of the nuclear membrane. Resistance to the lethal effects appears to occur via the selection (in the case of cancer cells where the animal or patient is undergoing chemotherapy with glucocorticoids) or the normal development (in the case of immunologically noncommitted thymocytes progressing to immunologically committed ones) of cells with harder membranes. This progression is associated with a change in a few cellular proteins. One such protein appears identical in both kinds of cells, offering itself as a candidate for an intracellular mechanism conferring resistance.

Evidence is also presented for the appearance of hormone-induced proteins that could be metabolic regulators that mediate the individual cellular and biochemical actions of glucocorticoids.

It is proposed that toxins could alter cellular metabolism through mechanisms similar to those utilized by steroid hormones, or possibly alter the sensitivity of cells to steroids, or vice versa.

Most physicians are aware of two somewhat different effects of adrenal glucocorticoid hormones on thymus cells; these can be seen not only in animals but also in patients. We have come to regard them as representing two distinctly different phenomena, that probably subserve different biological functions. First, in the tiny amounts that circulate normally from day to day, glucocorticoids chronically suppress the growth and development of thymus cells. This suppression may be appreci-

*E. Henry Keutmann Laboratories, Departments of Medicine (DAY, MLN) and of Radiation Biology and Biophysics (DAY, BPV), University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, New York 14642.

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increased amounts of steroids that are secreted by the adrenals during periods of stress.

It seems obvious that there must be important biological implications to both kinds of hormone actions mentioned above; yet at this point one can only speculate as to how they are useful to the animal. We have supposed that the suppression of growth and development may serve to prolong the life of the immunologically noncommitted lymphocytes; and that upon commitment these cells are released from this suppression and are then free to grow and divide. It also seems possible to us that the killing of the immunologically noncommitted lymphocytes during a period of stress (that often involves tissue damage due to infection or trauma) may serve to prevent autoreactivity to those normally intracellular substances that might enter the circulation as the result of cellular disruption. Thus, hormone-induced cell killing would serve to prevent the development of autoimmune disease.

While more information is needed before we fully understand the biological utility of these powerful hormone effects on lymphoid cells, the effects themselves have proved to be quite useful to biologists investigating the mechanistic details of how steroid hormones influence the behavior of their target cells. Thymus cells, when used as a model system for understanding hormone actions, offer at least two distinct advantages over other steroid-hormone target-cell systems; they remain responsive to physiological levels of steroids when the cells are incubated outside the animal, and they elicit a number of metabolic hormone effects that evolve more rapidly than almost all other steroid hormone effects seen in other target cells (1-3). During the past decade or so a major effort in our laboratory has been aimed at sorting out the various metabolic and other cellular hormone effects produced by adding glucocorticoids to thymus cells incubated in vitro, and at understanding these effects in the context of the growth inhibitory and lethal actions as produced by glucocorticoids in vivo. The remainder of this discussion will be devoted to a summary of some of our findings and conclusions.

**Glucocorticoid Suppression of Growth and Development of Lymphoid Cells**

Figure 1 illustrates the order of appearance and relative magnitudes of several well-known metabolic and cellular changes that are seen after the addition of physiological levels of glucocorticoids (less than 10^-6M) to surviving thymus cells (1-3). The most prominent among these hormone effects is a large inhibition of glucose transport, that reaches 25-30% by about 1/2 hr after hormone addition (4). This effect precedes by about 1 hr the evolution of some other inhibitory effects on transport, measured by the ability of cells to accumulate both natural and nonmetabolizable (α-aminoisobutyric acid) amino acids (AIB in Fig. 1), or by the inability to take up nucleosides such as uridine (5). By about 2 hr there is also a slight hormone-induced decline in steady-state levels of ATP (6) that appears to be the consequence of an inhibition of mitochondrial ATP production (7). At this time one can also measure declines in overall rates of macromolecular “synthesis,” as measured by inhibition of incorporation of radioactive precursors into either RNA or proteins (5-8). In the case of uridine incorporation into RNA, the early hormonal inhibition (during the first 2 hr) seems to reflect mostly an inhibition of uridine transport (2). On the other hand in the case of the incorporation of amino acids into proteins the decline in labeling here reflects a decrease in protein synthesis per se (2, 6).
thymus cells may be the consequence of the limitation of glucose uptake (13). In retrospect (see below) this idea no longer seems tenable (7). At present the only cellular process that seems to be directly influenced by the inhibition of glucose uptake is a concomitant reduction of fatty acid synthesis (14).

More recently, however, our attention has been directed to the second hormone action on carbohydrate metabolism, the inhibition of carbohydrate-supported mitochondrial ATP production (6, 7). This action does seem to have a major influence on other cellular processes, particularly those related to cellular growth and development. We have found that several of the biochemical phenomena associated with growth, most notably the rapid uptake of nucleosides (2, 5) and overall rates of protein biosynthesis (6, 7, 10) exhibit very large declines as a response to the small steady-state hormone-induced changes in adenine nucleotides, that in turn are a consequence of the hormone-induced limitation of ATP production (6, 7, 10). Our studies on mechanisms have largely concentrated on those linking the changes in nucleotides to changes in protein synthesis. Here initiation seems extremely sensitive (9, 10). We suspect that the important parameter (that is linked to the hormone-induced rise in AMP and ADP) is a related hormone-induced rise in either GMP or GDP that limits the initiation reaction possibly through the mechanisms proposed by Walton and Gill (15).

As a general working hypothesis that would encompass these observations we have supposed that most cells have regulatory mechanisms that link energy-utilizing reactions to the cells' own energy status. Since ATP turnover tends to be very rapid (half-life of only a few minutes) (16), even a slight decline in ATP production without an appropriate decline in ATP utilization would soon exhaust cellular ATP supplies (7). Our observations suggest that those processes related to growth and development are curtailed in order to conserve ATP for other processes (such as ion transport) that are essential to immediate cell survival. What seems to be happening is that glucocorticoids seem to "fool" the thymus cells into reacting as though they are in a more stringent environment. As a result normal adaptive mechanisms that are present in most other cell types are brought into play; these selectively reduce the growth and development of the cells (3).

While space here does not permit the development of data to support these ideas, the data in Figure 3 are included to illustrate the relationship between the changes in the balance of adenine nucleotides (here plotted as the adenylate energy

Figure 2. Schematic presentation of the molecular metabolic events involved in the action of glucocorticoid hormones on thymic lymphocytes. Hormone receptor complexes when they reach specific sites along the chromatin, here labeled 1, 2, 3, and 4, initiate the transcription of four separate mRNAs that in turn code for four separate "induced proteins." Each of these is presumed to initiate a separate primary hormone action, here shown as a decrease in glucose transport, a decrease in mitochondrial ATP production, a decrease in AIB accumulation and an increase in nuclear fragility. The question mark and dotted line reflect our uncertainty as to whether a single mRNA and protein could conceivably be responsible for both the inhibition of AIB accumulation and the increase in nuclear fragility. Our data support the idea that the slight hormone-induced decrease in the adenylate energy charge (here shown as a decrease in ATP and an increase in ADP and AMP) is responsible for both the hormone-induced suppression of nucleoside uptake and the suppression of overall rates of protein synthesis. It seems likely that suppression of protein synthesis may actually be mediated through changes in guanine nucleotides that mimic those seen in adenine nucleotides.

which appears to be the result of a deficit at the level of the peptide initiation reaction (3, 9, 10).

In addition to these metabolic inhibitions one can also see changes at the level of the whole cell, albeit evolving more slowly. By 1-2 hr there is an increase in nuclear fragility (see below). By about 6 hr there is a swelling of nuclei and clumping of chromatin (11). After about 12 hr there are more clear-cut signs of cell destruction, with increases in calcium uptake (12), release of DNA, inability to exclude certain dyes and finally lysis of a large number of the cells (3).

Figure 1 was included here to give a one sense of the order of appearance of the effects and of their relative magnitudes. Figure 2 illustrates our working hypothesis about the interrelationships between these several hormone actions. Since the effect on glucose is so large and appears so rapidly, at one time it had been proposed (first by Ingl and later by Munch, who studied this action in detail) that many if not all of the other hormone actions in

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charge) and rates of protein synthesis. One can appreciate that a slight decline in the energy charge is associated with a very large fall in overall rates of protein biosynthesis brought about here by a variety of means. In all those instances studied, the glucocorticoid-induced suppression in overall rates of protein biosynthesis can be accounted for in terms of such small hormone-induced reductions in energy charge (2, 3, 6, 7, 9, 10). While the effect on glucose transport is not the cause of the decrease in energy production it may be essential to prevent the cells from overriding the shift in adenine nucleotides (that occurs as a response to suppression in mitochondrial function) via a compensatory increase in glycolysis (7). More recent data obtained from fat cells seems to support the applicability of these mechanisms to understanding glucocorticoid actions in other kinds of cells as well (17).

**Glucocorticoid-Induced Cell Killing**

We will now leave these metabolic hormone effects and consider briefly the lethal actions of glucocorticoids. It has been supposed by many that these were merely an extension of the metabolic suppressive effects. This idea no longer seems very likely to us since thymus cells survive well under minimal incubation conditions where overall rates of transport and macromolecular synthesis are reduced below levels ordinarily seen with glucocorticoids, and since we have found that a total blockade of protein synthesis actually seems to prolong the life of the cells in the presence of glucocorticoids, protecting them from the lethal actions (18). The structural changes that accompany cell lysis have been described; in about 6 hr one sees (with the electron microscope) a loss of the normally feathery pattern of chromatin. Instead it seems to coalesce around the periphery of the nucleus (11).

In order to study mechanisms we began (in 1967) to search for some early (less than 6 hr) measure of hormone-induced structural changes at the level of the nucleus. We eventually found that within 1-2 hr there are substantial hormone-induced increases in what we called “nuclear fragility” (18). Normally one can lyse thymus cells in distilled water (with slight amounts of magnesium chloride added to stabilize the DNA) and collect their nuclei, most of which survive. If one incubates cells in simple salt solutions, there tends to be a slow deterioration with small increases in the numbers of nuclei breaking as the cells are lysed. More importantly we found that quite early after the addition of glucocorticoid hormones there is a substantial increase in the number of nuclei that lyse (when the whole cells are broken) providing evidence for some kind of hormone-induced structural change. This increase in “nuclear fragility” is entirely specific for the hormone. Moreover, the effect is blocked by inhibitors of the binding of the steroids to their specific receptors, and also by inhibitors of protein synthesis. Some typical data are presented in Figure 4.

There have been several possibilities that could account for the hormone-induced changes seen in chromatin. These include removal of histones, or changes in their acetylation or phosphorylation. Wylie and associates have found clear-cut evidence
FIGURE 4. Time course of the development of the effect of cortisol increasing nuclear fragility in the presence and absence of added glucose: (●) the amount of DNA recovered in supernatant fractions of cells incubated with glucose and with cortisol; (○) with glucose and without cortisol; (□) without glucose and with cortisol; (●) without glucose or cortisol. Cell suspensions (0.5 ml) were incubated with or without cortisol (10^{-6}M) and with or without glucose (1 mg/ml) for 240 min. Aliquots of cell suspensions were lysed at the times indicated in the figure. Data presented are the means of determinations from five flasks ± 1 S.E. The difference between cortisol and controls is significant at the p < 0.06 level by 60 min, at the p < 0.01 level by 2 hr, and at the p < 0.001 level by 4 hr, both in the presence and in the absence of added glucose. The difference between glucose and no glucose, either in the presence or absence of cortisol, does not become significant until 4 hr, when the difference is significant at p < 0.01. The insert, included for comparison, shows the time of onset and course of development of effects of cortisol on glucose transport (measured as levels of intracellular glucose-6-phosphate) and on rates of incorporation of radiolabelled valine into protein (18). Data from Giddings and Young (18).

for chromatin degradation in some lymphocytes as early as 3 hr (19). While it seems possible that changes in nuclear fragility we have observed could be secondary to events occurring within the nucleus (for example, by increasing the osmolarity within the nuclear envelope) it also seems possible that a more specific glucocorticoid attack at the level of nuclear membrane might initiate the subsequent nuclear events.

At this point we do not have direct evidence favoring either one of these alternatives. Nevertheless there is quite compelling evidence that membrane properties and responsiveness to glucocorticoids are somehow intertwined. Some of this comes from studies aimed at understanding mechanisms for resistance to glucocorticoid killing seen in those cancer cells that have been selected by prolonged glucocorticoid treatment (20). Here we used mouse P1798 lymphosarcoma cells, a tumor line derived from thymus cells. As in normal thymus cells glucocorticoid killing begins with a measurable increase in nuclear fragility, at about 1-2 hr, long before other structural changes appear (6 hr). However, the most striking finding is that cells that are resistant to killing appear to have harder nuclear membranes that can be detected in the absence of hormones. On the basis of such studies we have proposed that emergence of resistance, at least in some lines of cancer cells, may occur via the selection of cells with harder nuclear membranes; the glucocorticoids are still able to initiate those intracellular processes that lead to their destructive effects, but the membranes are sufficiently tough to withstand these glucocorticoid-induced changes (20). Data in Figure 5, explained in the legend, illustrate some of these results. It is particularly interesting to note that when cells that are resistant to glucocorticoid killing in the animal deteriorate sufficiently (at 6 hr) to exhibit the same degree of basal nuclear fragility as do the sensitive cells (at 3 hr) they then are equally susceptible to a further glucocorticoid-induced increase in nuclear fragility.

Some more recent studies have suggested that such changes in membrane properties may not be limited to cancer cells (22, 23). As normal thymus cells become immunologically committed they become resistant to glucocorticoid killing. One can select those normal cells that are resistant to glucocorticoids by obtaining survivors after treatment of the animal for two days to kill the sensitive cells (24). Figure 6 shows large differences in the tendency of normally resistant thymus cells to lyse upon hypotonic shock as compared to normally sensitive thymus cells. It seems likely that this increased resistance to hypotonic shock, now at the level of the whole cell, also reflects changes in membrane properties. Taken altogether these studies suggest that cancer cells develop resistance to glucocorticoid-induced cell killing by the selection of cells that are expressing the same properties that are involved in the development of resistance that occurs normally as immunologically noncommitted thymus cells become committed.

Other recent studies seem to provide molecular confirmation of this latter idea. Utilizing O’Farrells’ two-dimensional methods for the separation of individual cellular proteins on SDS-acrylamide gels (25) we have found that in both normal thymus cells and in mouse lymphosarcoma cells there are changes in the synthesis of a small subset of cellular proteins that accompany the emergence of resistance to the lethal actions of glucocorticoids (22, 23, 26). The most interesting finding however is that the most prominent of these proteins that appear as normal
thymus cells become resistant (#110 in Fig. 7) seems to be identical in size and isoelectric point with the protein whose presence is associated with complete resistance to glucocorticoid killing in the lymphosarcoma cells (#116 in Fig. 7); when these proteins are mixed they coelectrophorese (26). We therefore seem to have a candidate for a protein whose presence in cells confers resistance to glucocorticoid killing. It would not be surprising to find that this protein is somehow responsible for changes in cellular membranes.

Molecular Mechanisms: Hormone-Induced Proteins Initiate the Biological Effects of Glucocorticoids

Figure 8 schematically summarizes the early molecular steps that are probably involved in the initiation of the biological effects of glucocorticoids (1, 21, 27, 28). Steroids seem to penetrate cells readily. Combination with intracellular receptors leads to a transformed hormone receptor complex that has a high, but nonspecific affinity for chromatin and is therefore found within the nucleus. However, when the hormone receptor complex reaches (perhaps by sliding along the chromatin) certain specific sites, the interaction presumably leads to increased transcription of specific mRNAs (hormone-induced mRNAs). When, after migration to the cytoplasm, these mRNAs are translated by ribosomes, there is a change in the amounts of "hormone-induced" regulatory proteins within the cell. These proteins in turn find their way to their sites of action.

Much indirect evidence that we have obtained (early studies in collaboration with Munck's laboratory) tends to support the probable involvement of
FIGURE 7. In both P1798 lymphosarcoma cells and normal rat thymus cells resistance to glucocorticoid hormone-induced cell killing is associated with the increased synthesis of similar proteins (22). Thymus cells were selected for resistance by treating rats with 10 mg/kg/day dexamethasone (suspended in tricaprylin) for 3 days, followed by 2 days untreated. Suspensions of both thymus (1% packed cell volume) and tumor cells (5×10⁷ cells/ml) were labelled in KRB or RPMI-1640 (respectively) with ³⁵S-methionine (100μCi/100 ml cell suspension, 500-1200 Ci/m mole) for 1 hr at 37°C. Cells were washed in their respective incubation media and resuspended in cell lysis buffer. Samples applied to the gels contained 500,000 cpm and were focused for 4 hr at 400 V. The figures are enlarged segments of the autoradiograms from nonequilibrium gels of glucocorticoid-sensitive and -resistant P1798 lymphosarcoma cells (left) and sensitive and resistant normal thymus cells (right). The pH increases from left to right. This section of the gel spans a molecular weight range from 60,000 daltons (top) to 25,000 daltons (bottom). Circled proteins are invariant peaks used here for orientation. Arrows upward indicate consistent increases in the density of the protein spot.

such hormone-induced mRNAs and proteins in the initiation of even the most rapidly evolving steroid hormone actions, i.e., the inhibition of glucose uptake. Inhibitions of receptor binding (29), RNA synthesis (29), addition of poly(A) to mRNA (30), or translation of the message (31), all block the emergence of the hormone action on glucose transport. This is also the case for those other, more slowly evolving, hormone actions that we have studied (3). Nevertheless, it must be admitted that hormone-induced messages and proteins that appear in time to account for the action on glucose uptake have not previously been detected. However, the recent development by Voris of very high resolution two-dimensional separations of proteins on giant acrylamide gels (32) has allowed us to detect a number of candidates (for these hormone-induced regulatory proteins) that change substantially in amounts within the time framework sufficient to account for all the metabolic and cellular hormone actions mentioned. For example, there is a large increase in protein 1 in Figure 9 within 30 min after the addition of glucocorticoids to thymus cells. It is conceivable this protein is an inhibitor of glucose...
transport. Of course these results do not establish with certainty the idea of hormone-induced proteins initiating the metabolic hormone effects; however, they do go a long way in this direction providing concrete candidates for the molecular changes that were predicted (29). Some of our current research is aimed at further subcellular localization of the six or more proteins that have been detected. It is conceivable that the inhibition of glucose transport could be the result of the insertion of one of these into cell membranes, the decrease in mitochondrial ATP production could be the result of a protein interacting with mitochondria, and the lethal action could be the result of a protein interacting with the nuclear membrane (as shown in Fig. 8).

**Relevance to Toxicology**

Finally we would like to suggest a few implications of these ideas as they might relate to toxicology. We have proposed that the slight changes in ATP production brought about by glucocorticoids in thymus cells are sufficient to trigger adaptive mechanisms that slow down cellular growth and development. On this basis it would seem that any toxic products that interfere even slightly with ATP production in these or other cell systems might also importantly influence biosynthetic processes. We have also suggested that resistance to glucocorticoid killing may occur via the selection of cells with hardier membranes, those better able to withstand lethal glucocorticoid attack. It seems reasonable to assume that should toxic substances interfere with cellular membrane functions the resistance of toxins might occur via the selection of cells with altered cellular membrane properties. It is also possible that toxins might alter membrane properties in such a way as to make target cells more or less susceptible to glucocorticoids, or vice versa.

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