Glucocorticoid-mediated induction of glucocortin: A rapid primary response common to major target tissues

(gene expression/thymus cells/cycloheximide-insensitive induction/*in vitro* translation/giant two-dimensional gel electrophoresis)

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ABSTRACT Glucocorticoids are known to rapidly induce four proteins in rat thymus cells in time to account for the earliest known metabolic hormone effects. We report here an additional protein, which we refer to as “glucocortin” ($M_r = 17,000$, $pI = 4.7$). It is of special interest since it is the only protein rapidly induced in all of the glucocorticoid target cells we have examined. We have characterized the kinetics of glucocortin mRNA induction in isolated thymus cells. Giant two-dimensional gel electrophoretic analysis of *in vitro* translation products reveals a 2-fold increase in the level of translatable mRNA within 15 min of dexamethasone addition, with maximal stimulation (≈ 7 -fold) by 45 min. Cycloheximide does not reduce the hormone-mediated increase in glucocortin mRNA, suggesting that the induction represents a primary response to glucocorticoids. This protein is induced by dexamethasone in a dose-dependent manner, with maximal induction at 10^{-6} M and partial inductions at concentrations as low as 10^{-10} M. It is strongly induced by cortisol at 10^{-6} M, but it is not induced by estradiol or testosterone or by thyroid hormone, even at concentrations as high as 10^{-6} M. Deoxycorticosterone has no effect at 10^{-8} M but does generate a half-maximal effect at 10^{-6} M, a finding consistent with its status as a partial glucocorticoid agonist. In summary, glucocortin appears to be a primary glucocorticoid-induced protein that represents the most rapid induction so far detected, and it appears to be the only one that may be common to all glucocorticoid target cells.

Previous research has provided evidence that the earliest effects of glucocorticoids in surviving normal rat thymus cells—i.e., the inhibition of glucose transport that begins after 20 min (1) and the inhibitions of α -aminoisobutyric acid uptake (2) and ATP production (3–5) that begin after 1 hr—evolve separately (6) and are dependent upon the synthesis of new mRNA (7, 8) and protein (9). The development and application of ultra-high-resolution giant two-dimensional (2-D) gel electrophoresis, which allows one to monitor the relative rates of synthesis of as many as 5000 proteins simultaneously (10–17), has revealed several early protein inductions in thymus cells (13) that appear in time to mediate these initial metabolic hormone effects. More recently, we have sought (and found) a small number of rapidly evolving protein inductions in several other major glucocorticoid target cells, including fat, liver, and fibroblasts, with the aim of discovering one or more inductions common to these tissues (12, 14–17).

We report here a protein not observed previously in thymus cells, which we refer to as “glucocortin.” It is the only early induction common to all target cells so far examined. We describe the time course of the mRNA induction and its specificity for glucocorticoids and establish

that glucocortin represents a primary steroid hormone response.

MATERIALS AND METHODS

Materials. Rats were obtained from Charles River Breeding Laboratories. Dexamethasone, hydrocortisone, 11-deoxycorticosterone, 17β -estradiol, testosterone, 3,3',5-triiodothyronine, and diethyl pyrocarbonate were obtained from Sigma. Dexamethasone for animal injections was purchased from Elkins-Sinn (Cherry Hill, NJ). Collagenase was purchased from Millipore; guanidine thiocyanate and hydrochloride were from Fluka; oligo(dT)-cellulose (type T-2) was from Collaborative Research (Waltham, MA); and L-[35 S]methionine (>1000 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear (translation grade). Reticulocyte lysates were obtained from Promega Biotec (Madison, WI), and ampholytes were from LKB. 14 C-labeled molecular weight standards were obtained from Bio-Rad.

Animals. Male Sprague–Dawley rats (100–125 g), adrenalectomized and maintained on 0.9% NaCl, were used within 6–12 days to obtain thymus, fat, and liver tissue.

Cell Preparation and Labeling. Thymus cell suspensions from 6–30 animals, prepared as described (5), were incubated in Krebs–Ringer bicarbonate buffer (KRB buffer) (pH 7.4 in equilibrium with 95% O_2 /5% CO_2) and gassed every 15 min. For experiments analyzing the effects of glucocorticoids and other hormones on the synthesis of glucocortin, 100- μ l aliquots of cell suspensions (5–10% packed cell volume) were incubated at 37°C in 3-ml plastic stoppered test tubes in the presence or absence of hormone at the concentrations indicated. After 15 min of incubation with hormone, thymus cells were labeled with [35 S]methionine at a final concentration of 1.0 mCi/ml for an additional 45 min. Cells were then diluted 1:10 with KRB buffer, pelleted, washed, and lysed in lysis buffer as described (11), in preparation for electrophoresis. When RNA was to be isolated, experiments were performed with larger volumes (10 ml) of thymus cell suspensions (10–20% packed cell volume) incubated in 50-ml stoppered plastic Erlenmeyer flasks.

Fat cell suspensions were prepared from the epididymal fat pads of rats and mice (Swiss Webster, nonadrenalectomized) by collagenase digestion of the surrounding connective tissue (18) and incubated (10% packed cell volume) with shaking in Krebs–Ringer phosphate buffer (KRP buffer) (19) at 37°C. Fat cells were treated with dexamethasone (10^{-6} M) for 2 hr in the presence of [35 S]methionine at a concentration of 167 μ Ci/ml. Incubates were then diluted 1:5 with KRP buffer and fat cells were centrifuged up through a 1-ml layer of silicone oil. Cells were then lysed in lysis buffer in preparation for electrophoresis.

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Abbreviation: 2-D, two-dimensional.

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Steroid hormone solutions were prepared as 2.2×10^{-5} M aqueous solutions (except for estradiol, which is less soluble in water, and was made to 1.4×10^{-5} M) assuming the following molar extinction coefficients: dexamethasone, 1.5×10^4 liters/mol per cm at 250 nm; cortisol, 1.63×10^4 at 230 nm; deoxycorticosterone, 1.72×10^4 at 240 nm; testosterone, 1.68×10^4 at 240 nm; estradiol, 1.68×10^4 at 240 nm. Triiodothyronine was made at a concentration of 2.2×10^{-5} M in 0.01 M NaOH.

RNA Isolation and *in Vitro* Translation. RNA was isolated from thymus cells and liver by using the guanidine thiocyanate extraction procedure (20), with the following modifications for thymus cells. At the end of an incubation, cells were pelleted and lysed hypotonically in 10 mM MgCl_2 /0.5% Nonidet P-40/0.1% diethyl pyrocarbonate with brief, but vigorous, mixing on a Vortex. Particulate material was removed with a 2-min centrifugation at $1000 \times g$. The supernatant was then added to 2 vol of 6 M guanidine thiocyanate/25 mM sodium citrate, pH 7.0/0.5% *N*-lauroylsarcosine/0.1 M 2-mercaptoethanol, and RNA was precipitated and further purified by successive extractions with guanidine hydrochloride followed by ethanol precipitation (20). Poly(A)⁺ RNA was obtained by using standard oligo(dT)-cellulose selection techniques (21), except that batch isolations were performed (either in small test tubes or Eppendorf centrifuge tubes) instead of column chromatography. Typically, 100 mg of oligo(dT) was used to obtain poly(A)⁺ RNA from 2–4 mg of total RNA, with a yield of 1–2%. Purified RNA was stored in 70% ethanol or lyophilized in small (1–10 μg) aliquots.

Cell-free translation of poly(A)⁺ RNA was performed by using a nuclease-treated rabbit reticulocyte lysate. The suggested translation protocol was followed (Promega Biotec), except that just prior to beginning the incubation RNA was pretreated with 3.5 mM CH_3HgOH for 5 min at 22°C (22). After translation (60 min, 30°C), samples were diluted with 0.5 vol of water and centrifuged in a Beckman Airfuge at $100,000 \times g$ for 10 min at 2°C. Duplicate 1- μl aliquots of the supernatants were used to determine cpm incorporated into trichloroacetic acid-precipitable protein. Samples were then frozen, lyophilized, and dissolved in lysis buffer (11) for subsequent analysis by giant 2-D gel electrophoresis.

Giant Gel Electrophoresis. Procedures for separation of proteins on giant gels have been described in detail (11). Briefly, proteins were focused under reducing conditions in the first dimension with 2% ampholytes (1.6% pH 5–8, 0.4% pH 3.5–10) and separated in the second dimension on concave exponential 10–16% polyacrylamide gradients in the presence of sodium dodecyl sulfate. The pH gradients were determined from first-dimension gel slices equilibrated in double-distilled, degassed water, and molecular weights were determined from ^{14}C -labeled marker proteins. Autoradiography of dried gels was performed as described (11).

Quantitation of Glucocorticoid Induction. Quantitation of changes in the relative rates of synthesis of individual proteins separated by 2-D gel electrophoresis was performed as described (23). Briefly, the maximal optical density of a protein spot on an autoradiogram is measured with a computerized pinhole densitometer that subtracts adjacent background. The corrected maximal reading is converted to cpm/ mm^2 by using a calibration curve of known radioactive standards, thus taking into account any nonlinearity in the film response. Comparison of this number obtained with and without hormone treatment for the protein of interest provides a measure of the magnitude of the change (induction ratio). In all cases, quantitation is performed on gels (equal cpm applied) exposed for equal periods of time. In addition, gels are further normalized by quantitating at least 10 proteins in the same region as the protein of interest that do not change with hormone treatment. It should be noted that in certain

cases the induction ratio may represent an underestimate of the absolute induction, since the photocell scanner assumes a fixed area for each spot, when, in fact, an increase in maximal density is sometimes accompanied by an increase in area.

RESULTS

Investigators in this laboratory have been examining glucocorticoid target tissues by using ultra-high-resolution giant 2-D gel electrophoresis to search for very rapid hormone-induced changes in the synthesis of individual cellular proteins that could serve to identify the primary genetic sites of hormone action as well as the proteins that act as mediators of the initial hormone effects. Extensive comparisons of different target tissues based on several hundred giant 2-D gel separations have revealed a handful of rapid inductions in each tissue (12–17), with only occasional overlaps (12). However, it has become evident that there is one induction, not previously singled out, that is the only one thus far identified as being common to each of these tissues. In this report we refer to this protein ($M_r = 17,000$; $pI = 4.7$) as glucocortin, describe the rapidity and cycloheximide insensitivity of its mRNA induction in thymus cells, and show that this response is specific for glucocorticoids.

The results in Fig. 1 show that the dexamethasone-mediated induction of glucocortin can be equally well detected by separating whole-cell proteins pulse-labeled with [^{35}S]methionine or by separating the products of *in vitro* translation of recovered poly(A)⁺ RNA on giant 2-D gels. The parallel in the results obtained with these two methods demonstrates that the induction occurs at a pretranslational level. The identical 2-D gel migration characteristics of this protein, whether synthesized in whole cells or *in vitro* (same molecular weight and pI values and location with respect to neighboring marker proteins), also indicate that little, if any, specific cellular processing of the primary translation product occurs. For the other proteins in Fig. 1 as well as for glucocortin, the close parallel between the relative rates of synthesis *in vivo* and *in vitro* suggests that the level of mRNA is the limiting factor in the expression of these gene products.

In our giant 2-D gel separations, glucocortin migrates very close to a more-heavily labeled neighboring protein (slightly lower pI and molecular weight; see Fig. 1), raising the possibility that these two proteins are related. However, three lines of evidence make this seem unlikely. (i) Only glucocortin changes with hormone treatment; (ii) glucocortin does not label with [^{35}S]cysteine, whereas the adjacent protein remains heavily labeled; and (iii) partial proteolytic digests of these two proteins show no similar peptides (unpublished data). Thus, despite similar 2-D gel migration properties, these two proteins appear to be distinct gene products.

Table 1 summarizes some typical data obtained on the induction of glucocortin in the major glucocorticoid target cells, thymus cells, fat cells, liver cells, and fibroblasts. For these comparisons the induction was examined within the first 2 or 3 hr of glucocorticoid treatment. It is also worth noting that the magnitude of the induction is larger than the other early glucocorticoid-induced proteins found in these tissues (data not shown). A variety of other cells not shown in Table 1 in which the induction of glucocortin has been observed includes primary isolates of mouse B lymphocytes, mouse mammary cells (c127), cultured human lung tumor cells (A549), and AKR-2B cells (unpublished data).

Since the most rapidly evolving biological effect of glucocorticoids, the inhibition of glucose uptake, may be measurable within 20 min of hormone addition (1) and has appeared to be dependent upon the earlier synthesis of new mRNA and protein (7–9), we were interested in determining

FIG. 1. Close-up regions of giant 2-D gel separations of proteins synthesized in whole cells and *in vitro* showing the glucocorticoid-mediated induction of glucocorticin. Isolated thymus cells were incubated with (+ Dex) or without (– Dex) 10^{-6} M dexamethasone for 1 hr. [35 S]Methionine was present from 15 to 60 min (whole cells), or at 60 min cells were lysed and poly(A) $^{+}$ RNA was isolated and subsequently translated (*in vitro* translation) with a reticulocyte lysate (in the presence of [35 S]methionine). Proteins were separated on giant 2-D gels and detected autoradiographically. Arrows indicate the position of glucocorticin. Each of the four gel regions shown here represents 3% of the total area of a giant 2-D gel separation. The presence of a few proteins in the – Dex gel regions not seen in the + Dex gels is due to differences in how the photographs were cropped. Equal amounts of trichloroacetic acid-precipitable cpm exposed for equal periods of time are shown (– Dex, + Dex). For whole cells, this represents 5×10^6 cpm for 5 days, and for *in vitro* translation, this represents 0.76×10^6 cpm for 23 days. The pH range of these gel regions and the position of 14 C-labeled molecular weight standards ($\times 10^{-3}$) are indicated.

Table 1. Induction of glucocorticin in several major glucocorticoid target tissues

Tissue or cell type	Magnitude of induction	Time, hr	Assay
Thymus cells	5.3	1–2	Whole cells
	5.6	2	Translation
Fat cells			
Rat	7.0	0–2	Whole cells
Mouse	2.3	0–2	Whole cells
Liver	6.8	3	Translation
Fibroblasts (mouse)			
3T3	9.0	0–3	Whole cells (16)
L1	5.5	0–3	Whole cells (17)

For whole-cell labeling studies, isolated suspensions of thymus and fat cells were incubated with dexamethasone (10^{-6} M) from zero time and labeled with [35 S]methionine for the times indicated. For *in vitro* translation studies, poly(A) $^{+}$ RNA was isolated from thymus cells that had been incubated with or without dexamethasone for 2 hr, whole liver tissue from animals injected with dexamethasone (2 mg/kg), or carrier solution, 3 hr prior to sacrifice. [35 S]Methionine-labeled cellular proteins and *in vitro* translation products were analyzed on giant 2-D gels, and the magnitude of induction of glucocorticin was quantitated. Each induction ratio is the average from at least two experiments, except mouse fat cells and 3T3 fibroblasts, which are from a single representative experiment. The data from fibroblasts have been published elsewhere (references in parentheses) and are included here for completeness.

whether an increase in glucocorticin mRNA could occur rapidly enough to account for this effect. As is shown in Fig. 2, the level of translatable mRNA is increased in thymus cells as early as 15 min after dexamethasone addition and peaks at 45 min. The occurrence of this peak, which we find reproducibly, followed by a leveling off at 1–2 hr, suggests the possibility of a counter-regulatory mechanism limiting the magnitude of the response. In other experiments (data not shown) we have found that the induction at 4–6 hr remains at about the level seen at 2 hr. Six other glucocorticoid-induced mRNAs have been identified in thymus cells (unpublished data), but none appears as rapidly as glucocorticin.

The results shown in Fig. 2 indicate that cycloheximide, even at concentrations shown to block >97% of protein synthesis in thymus cells (9), does not prevent the dexamethasone-mediated increase in translatable mRNA for glucocorticin. This indicates that the mRNA induction in thymus cells is a primary response to glucocorticoids. Some more slowly evolving steroid hormone-mediated mRNA inductions may conceivably represent secondary responses in that they require ongoing protein synthesis (24–32), possibly reflecting the need for an intermediary hormone-induced gene product or perhaps a rapidly turning-over protein.

To determine the specificity of this response for glucocorticoids we tested several different steroid hormones and thyroid hormone for their ability to induce glucocorticin in

FIG. 2. Time course and cycloheximide insensitivity of the induction of translatable mRNA for glucocorticoid. Thymus cells (6×10^9 for each condition) were incubated in the presence of dexamethasone (10^{-6} M) (●), cycloheximide (2.5×10^{-5} M) (○), or dexamethasone and cycloheximide (◐) for the times indicated. Cycloheximide was added at -15 min to ensure a significant inhibition of protein synthesis at time zero (9) when dexamethasone was added. At the end of each incubation poly(A)⁺ RNA was isolated and translated, and translation products (equal cpm) were analyzed by giant 2-D gel electrophoresis. The relative induction of translatable mRNA for glucocorticoid was quantitated. Each point represents the average of three translations from two experiments. Control (without dexamethasone) levels for all comparisons represent the average of three time points (15, 60, and 120 min). No significant change in control levels occurred during this time period.

isolated thymus cells. The results are shown in Fig. 3. Neither estradiol, testosterone, nor triiodothyronine at concentrations of 10^{-6} M increases the synthesis of glucocorticoid, whereas the glucocorticoids dexamethasone and hydrocortisone (10^{-6} M) result in a 5- to 6-fold induction. Deoxycorticosterone produces about a half-maximal effect at the high concentration of 10^{-6} M; however, at 10^{-8} M no induction is seen. Dexamethasone is so potent that a significant induction is seen at 1 hr, even at concentrations as low as 10^{-10} M. To our knowledge this makes the induction of glucocorticoid

mRNA the most sensitive response to glucocorticoids so far reported and one that occurs within the same time frame as the induction of mouse mammary tumor virus RNA transcription (33).

DISCUSSION

We report here the very rapid glucocorticoid-mediated induction of a previously unreported protein and its mRNA (glucocorticoid; $M_r = 17,000$, $pI = 4.7$) that represents a specific response common to all target tissues we have examined (for examples, see Table 1). Our results indicate that this induction occurs at a pretranslational level. At this point we cannot distinguish between increased synthesis of the mRNA, which could be due to an activation of transcription or enhanced processing or transport, and decreased degradation. However, unless the hormone-receptor complex has a direct effect at a site other than the DNA, the cycloheximide insensitivity and rapidity of this response would argue strongly for increased transcription.

These results are also of interest since most other steroid hormone-mediated induction responses reported to date appear to be tissue-specific (12-17, 30, 34). A notable exception is the more slowly evolving induction of metallothionein ($M_r = 6600$) by glucocorticoids, which has been shown to occur in many undifferentiated mouse cell lines (35) as well as HeLa cells (36) and rat hepatocytes (37), but not in muscle cells or primary thymocytes from either mice (35) or rats (38). Another exception might be the induction of a M_r 41,000-44,000 protein reported to occur after prolonged exposure of several target tissues to glucocorticoids (39). However, *in vivo* this response required chronic (5 days) administration of hormone and in tissue culture it required at least 4 hr of hormone treatment to obtain a maximal 2-fold increase, which would suggest that the induction may not be a primary response.

Our studies show that glucocorticoid is not induced by sex steroids, such as estradiol and testosterone, or by thyroid hormone, even at concentrations as high as 10^{-6} M. Other results from this laboratory show that stress-related gene induction agents such as cadmium and heat shock (23, 38) as well as polypeptide hormones such as insulin (unpublished data) and several growth factors (16) do not induce this protein. However, it is interesting that in some experiments

FIG. 3. Specificity of the glucocorticoid induction for glucocorticoids. Isolated thymus cells were incubated for 1 hr in the presence of various hormones (at the concentrations indicated) with [³⁵S]methionine labeling between 15 and 60 min. Proteins were separated on giant 2-D gels and autoradiographed, and the relative rate of synthesis of glucocorticoid was quantitated. Data are expressed as a percentage of the maximal induction seen with 10^{-6} M dexamethasone in this experiment, which is 6.3-fold. E, estradiol; T, testosterone; TH, thyroid hormone; DOC, deoxycorticosterone; HC, cortisol; and Dex, dexamethasone. Each bar represents the average from three independent incubations.

growth factors seem to reduce the magnitude of the glucocorticoid-mediated induction (see ref. 16; protein p18c in table 3). Whether this growth factor effect represents a decrease in the hormone-induced mRNA level, possibly related to the counter-regulatory mechanism proposed above or perhaps a specific inhibition of translation, is unknown. The response seen with deoxycorticosterone at 10^{-6} M, but not at 10^{-8} M, is consistent with other studies showing biological effects similar to glucocorticoids at such high concentrations (40–43) and with observations that deoxycorticosterone is more effective than other steroids at displacing glucocorticoids from their receptor (44).

It should be noted that the identification of early (<2 hr) glucocorticoid-induced proteins in a number of different target tissues has required the ultra-high-resolving power of "giant" 2-D gel protein separatory techniques (11–13). Even so, until recently the quality of our giant 2-D gel separations for low molecular weight acidic proteins ($M_r < 20,000$, $pI < 5.0$) was not adequate to allow reproducible resolution of glucocorticoid from neighboring proteins. As a result, in an earlier report describing glucocorticoid-induced proteins in rat thymus cells (13) this induction was not identified. Thus, it can be appreciated that relatively low-abundance proteins such as glucocorticoid (<0.03% of total [35 S]methionine incorporation during maximal induction) that migrate, even in giant gels, in very close proximity to proteins synthesized in much greater amounts (see Fig. 1) would probably be obscured on smaller 2-D gel separations.

Many steroid hormone-mediated gene inductions are dependent upon ongoing protein synthesis and thus are considered "secondary" responses (24–32). The requirement for protein synthesis has led to the idea that mediator protein(s) (24), either hormone-induced or perhaps rapidly turning-over, are necessary for such inductions. A notable example of such a secondary response is the induction of α_1 -acid glycoprotein (α_1 -AGP) mRNA in liver (31, 32). Vannice *et al.* have suggested that a glucocorticoid-inducible RNA-processing factor allows production of stable α_1 -AGP mRNA transcripts (32). The discovery of rapid, primary glucocorticoid-induced proteins like glucocorticoid demonstrates that such mediator proteins may exist. Another possible role for glucocorticoid, suggested by its time course of induction and ubiquity, might be to inhibit glucose uptake. However, its induction in liver tissue, where glucose transport is not inhibited by glucocorticoids, would argue against this. The ability of such low concentrations of dexamethasone (10^{-10} M) to induce glucocorticoid suggests that it may be involved in the permissive actions of glucocorticoids. Further studies aimed at elucidating the role that this inducible protein plays in mediating the biological effects of glucocorticoids are necessary.

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