

LOSS OF GLUCOCORTICOID REGULATION OF PLASMINOGEN ACTIVATOR
ACTIVITY IN ANUCLEATE RAT HEPATOMA CELLS

P.A. Barouski and T.D. Gelehrter

Department of Human Genetics and Internal Medicine
University of Michigan Medical School, Ann Arbor, MI 48109

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Summary: Glucocorticoids decrease the plasminogen activator activity of rat hepatoma cells through production of an inhibitor. We have examined the dexamethasone regulation of plasminogen activator in anucleate rat hepatoma cells to investigate the role of the nucleus in the steroid regulation of this membrane-associated phenomenon. Dexamethasone did not affect either the intra- or extra-cellular plasminogen activator activity of the anucleate cells, and did not induce production of an inhibitor of plasminogen activator. Therefore, glucocorticoid regulation of plasminogen activator activity requires the presence of an intact nucleus.

Glucocorticoids alter several membrane-associated properties in hepatoma tissue culture (HTC)¹ cells, an established line of rat hepatoma cells (1). Dexamethasone, a synthetic glucocorticoid, decreases both the intra- and extra-cellular activity of plasminogen activator (2,3), a serine protease which catalyzes the conversion of the serum protein, plasminogen, to the active fibrinolysin, plasmin. Seifert and Gelehrter (4) have previously reported that the mechanism of dexamethasone-induced inhibition of plasminogen activator (PA) activity appears to be the production of an inhibitor of PA.

Steroid hormones are thought to induce cellular changes through transcriptional activation of specific genes after translocation of cytosol hormone-receptor complexes into the nucleus and site-specific binding of the complexes to the chromatin (5). However, steroid hormones or hormone-receptor complexes might also mediate cellular changes by directly affecting cytoplasmic or membrane functions. To investigate these alternative mechanisms for steroid hormone action, we examined the glucocorticoid

1. The abbreviations used are: HTC, hepatoma tissue culture; PA, plasminogen activator.

regulation of PA activity in anucleate HTC cells. Dexamethasone did not cause a decrease in either the intra- or extra-cellular PA activity, or induce the production of a PA inhibitor in the anucleate cells. Therefore, glucocorticoid regulation of PA activity in HTC cells requires the presence of an intact nucleus.

METHODS

Cell culture: HTC cells were grown in spinner culture without antibiotics in Eagle's minimal essential medium for suspension culture (Gibco autoclavable powder) supplemented with 50 mM Tricine, 0.5 g/l NaHCO₃, 2 mM glutamine, 5% calf serum, and 5% fetal bovine serum (4).

Enucleation: HTC cells were enucleated in suspension by centrifugation through a discontinuous Ficoll density gradient in the presence of 10 µg/ml cytochalasin B according to a modification of the procedure described by Wigler and Weinstein (6,7). Control cells were centrifuged through a Ficoll gradient in the absence of cytochalasin B, or incubated without centrifugation in 15% Ficoll containing cytochalasin B. Identical results were observed with either treatment of control cells. Efficiency of enucleation was routinely >92% as determined in aceto-orcein stained preparations or by measuring the relative rates of [³H] thymidine incorporation in cytoplasts and whole cells (7).

Glucocorticoid treatment: Cells and cytoplasts were plated at densities of 2×10^6 cells and 6×10^6 cytoplasts per 35 mm tissue culture dish in serum-containing medium plus 0.02% CaCl₂. After four hours incubation at 37°C, cells and cytoplasts were washed and incubated in serum-free medium containing 0.1% bovine serum albumin, 50 µg/ml neomycin, and 0.01% ethanol with or without 0.1 µM dexamethasone.

Plasminogen Activator Assay: Conditioned medium was collected from the cells and cytoplasts after 0, 2, 4, 6, and 16 hours incubation, and detached cells removed by centrifugation. Cell extracts were prepared as previously described (4); protein concentration was determined by the method of Lowry et al. (8).

PA activity was measured in a fibrin plate assay (4) using 2 µg human plasminogen in 16 mm diameter wells of [¹²⁵I] fibrin-coated plates (9); solubilized radioactivity was measured after 6 or 6.5 hours incubation at 37°C. Total radioactivity was the amount solubilized by 50 µg trypsin. The background activity, usually 3 to 6% of the total radioactivity, was the amount solubilized by an assay mixture of plasminogen with either 0.2% triton or unconditioned medium. The PA activity of the sample was corrected for background activity, and expressed as a percentage of the total radioactivity. The cell extracts and the conditioned medium samples have no plasminogen-independent fibrinolytic activity. Dexamethasone added directly to the assay has no effect on fibrinolytic activity.

Inhibitor Assay: Samples of cell extract and conditioned medium were also examined for the ability to inhibit urokinase, a plasminogen activator purified from human urine (10). Cell extracts or conditioned medium were incubated in a fibrin-coated well with 1.0 or 1.5×10^{-3} Ploug units of urokinase (10) for thirty minutes at ambient temperature; the PA assay was then initiated by the addition of plasminogen. PA activity of the sample incubated without urokinase was also measured.

The amount of inhibition in cell extracts or conditioned medium from cells incubated with dexamethasone was calculated by this formula:

$$\text{amount of inhibition} = \left\{ 1 - \frac{\left[\text{PA activity (dexamethasone sample)} - \text{PA activity (urokinase)} \right] - \left[\text{PA activity (dexamethasone sample)} \right]}{\left[\text{PA activity (control sample)} - \text{PA activity (urokinase)} \right] - \left[\text{PA activity (control sample)} \right]} \right\} \times 100\%$$

This equation corrects for any endogenous PA activity of the sample and measures the inhibitory effect specific to dexamethasone treatment. The comparison of inhibition in control and dexamethasone samples eliminates the potential complications introduced by the presence in either the cell extract or the conditioned medium of inhibitors which are not induced by dexamethasone (11).

Materials: Fibrinogen (77% clottable) was purchased from Calbiochem-Behring and purified as described by Strickland and Beers (9); fibrinogen, (98.3% clottable) also obtained from Calbiochem-Behring, was used without further purification. [¹²⁵I] fibrinogen (specific activity 100-150 μCi/ml) was purchased from Abbott; [³H] thymidine (1 mCi/ml) and [¹⁴C] amino acid mixture (100 μCi/ml) were New England Nuclear products. Plasminogen was purified from outdated human plasma by lysine-Sepharose chromatography (12). Dexamethasone was a gift from Dr. Walter Gall of Merck and Company. Ficoll (Type 400) and cytochalasin B were purchased from Sigma; urokinase B grade, was obtained from Calbiochem-Behring. All other compounds were of reagent grade.

RESULTS AND DISCUSSION

Anucleate rat hepatoma cells retain several membrane-related functions. HTC cytoplasts still adhere to tissue culture dishes, and ≥ 98% exclude trypan blue, even after sixteen hours incubation in serum-free medium. PA activity (13) and active transport of α-aminoisobutyric acid (7) are maintained at levels comparable to, or higher than, those in intact cells. Similar to L-cell cytoplasts (14), anucleate HTC cells remain capable of locomotion, redistributing from areas of high density to areas of lower density on tissue culture plates.

Nevertheless, HTC cytoplasts are not responsive to glucocorticoid regulation of PA activity. In intact HTC cells, dexamethasone dramatically decreases both intra- and extra-cellular PA activity (Figure 1). Intracellular PA activity (upper panel) was decreased to less than 50% of control activity after two hours of incubation with dexamethasone, and was not detectable after six hours of incubation. Similarly, extracellular PA activity (lower panel) of cells treated with dexamethasone was reduced relative to that of control cells. In the anucleate HTC cells, however,

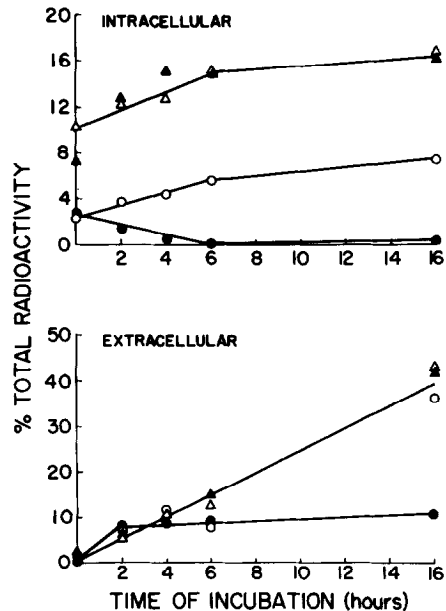


Figure 1: Time course of dexamethasone effects on intra- and extra-cellular PA activity. HTC cells (circles) and cytoplasts (triangles) were incubated for 0-16 hours in the presence (closed symbols) or absence (open symbols) of 0.1 μM dexamethasone. Triton X-100 extracts (4 μg protein) of cells or cytoplasts (upper panel) and medium (100 μl) conditioned by cells or cytoplasts (lower panel) were assayed for PA activity on [^{125}I]-fibrin plates as described in Methods. Each point represents the average of duplicate assays from a single culture. Total radioactivity solubilized by trypsin was 130,000 cpm/well.

dexamethasone had no effect on either the intra- or extra-cellular PA activity, even after 16 hours of treatment. The PA activity of the cytoplasts was two to three times greater than that of intact cells when expressed per mg of protein; but, the nucleated cells generally contained twice as much protein per cell (7).

A dexamethasone-induced inhibitor of urokinase was detected in cell extracts and conditioned medium from whole cells, after 2 and 4 hours incubation, respectively. In contrast, dexamethasone did not induce this inhibitor in the anucleate cells, even after 16 hours incubation (Figure 2). Similarly, dexamethasone did not induce tyrosine aminotransferase (15), or regulate amino acid transport (7) in HTC cytoplasts.

The complete failure of anucleate cells to respond to dexamethasone regulation of PA activity was not due to a lack of glucocorticoid receptors.

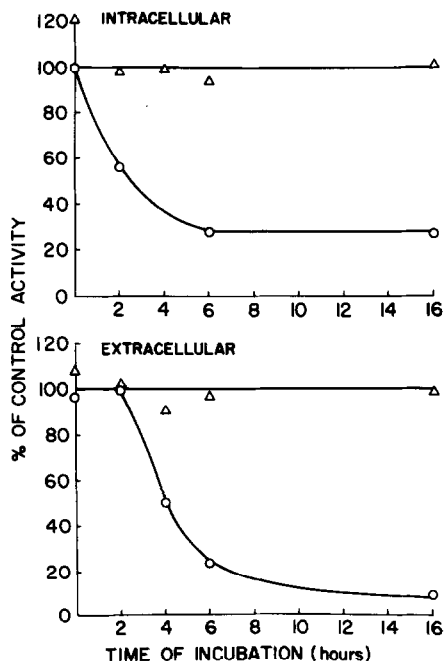


Figure 2: Time course of dexamethasone induction of urokinase inhibitory activity. HTC cells (circles) and cytoplasts (triangles) were incubated for 0-16 hours in the presence or absence of $0.1 \mu\text{M}$ dexamethasone. Triton X-100 extracts of cells or cytoplasts ($4 \mu\text{g}$ protein) or conditioned medium ($100 \mu\text{l}$) were incubated for 30 minutes with or without 1×10^{-3} or 1.5×10^{-3} Ploug units of urokinase, respectively, and then assayed for 6.5 hours on $[^{125}\text{I}]$ -fibrin plates. Dexamethasone-induced inhibition of urokinase was calculated as described in Methods. Percentage of control activity remaining ($100 - \% \text{ inhibition}$) is plotted against time of incubation. Total radioactivity was $130,000 \text{ cpm/well}$.

McDonald and Gelehrter (7) have demonstrated saturable, specific glucocorticoid binding in anucleated HTC cells, although the extent of specific binding was reduced relative to intact cells. Nor was it the result of the binding was reduced relative to intact cells. Nor was it the result of the cytochalasin B treatment. Incubation of the intact cells with cytochalasin B prior to dexamethasone addition had no effect on the levels of intra- or extra-cellular PA activity, or on the production and release of inhibitor into the medium (data not shown). Finally, the decreased rate of protein synthesis observed in cytoplasts (6,7) also can not explain the lack of glucocorticoid responsiveness; in intact cells whose protein synthesis was blocked to a comparable level by cycloheximide, dexamethasone still decreased PA activity and induced inhibitor production, albeit to a reduced extent (Figure 3).

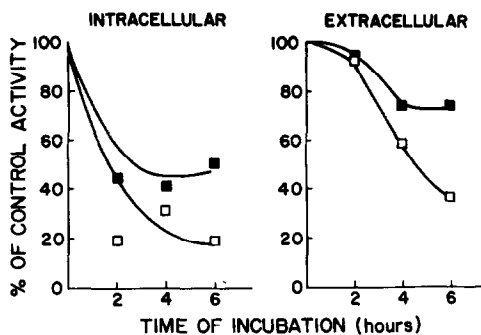


Figure 3: Effect of cycloheximide on the time course of dexamethasone induction of urokinase inhibitory activity. Control cells (open squares) or cells treated with $0.25 \mu\text{M}$ cycloheximide for four hours prior to, and then during dexamethasone induction (closed squares) were incubated for 0-6 hours in the presence and absence of $0.1 \mu\text{M}$ dexamethasone. Cycloheximide treatment reduced protein synthesis $56 \pm 3\%$ (mean \pm standard deviation, $n=8$). Triton X-100 extracts of cells ($9 \mu\text{g}$ protein) and conditioned medium ($100 \mu\text{l}$) were incubated for 30 minutes with or without 1×10^{-3} Ploug units of urokinase, then assayed for 6 hours on [^{125}I]-fibrin plates. Dexamethasone-induced inhibition of urokinase was calculated as previously described. Total radioactivity was 58,000 cpm/well.

We conclude that the steroidal regulation of PA activity requires the presence of an intact nucleus; regulation of this membrane-associated phenomenon does not appear to be mediated by direct interaction of the hormone or hormone-receptor complex with either the membrane or other cytoplasmic elements. Glucocorticoids probably regulate PA activity by transcriptional activation of the gene(s) for an inhibitor of plasminogen activator.

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