Vol. 143, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1987 Pages 329-336

Dexamethasone Coordinately Inhibits Plasminogen Activator Gene Expression and Enzyme Activity in Porcine Kidney Cells

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Received January 16, 1987

Abstract. The peptide hormone, calcitonin, induces urokinase-type plasminogen activator (uPA) enzyme activity in cultured LLC-PK_1 pig kidney cells. This induction occurs as a consequence of transcriptional activation of the uPA gene. Treatment with the synthetic glucocorticoid hormone, dexamethasone, was found to inhibit calcitonin induction of uPA enzyme activity by as much as 80%. The inhibitory effect of dexamethasone was attributed to at least two mechanisms: induction of an inhibitor of uPA enzyme activity, and reduction in uPA mRNA levels. Study on reduction of uPA mRNA levels showed that dexamethasone significantly reduced the transcription rate of the calcitonin--induced uPA gene, without affecting the half-life of uPA mRNA. Although dexamethasone has been reported to induce inhibitors of plasminogen activator enzyme activity and to inhibit transcription of various genes, the system described here appears novel in that both actions are coordinated.

Plasminogen activators are believed to play important roles in many aspects of cellular regulation, such as fibrinolysis (1), tissue remodelling (2), inflammation (3), tumor metastasis (4), and angiogenesis (5). Therefore, understanding how plasminogen activator genes and proteins are regulated will increase the knowledge of the physiological significance of plasminogen activators (see ref in 6 and 7).

An established line of pig kidney cells, LLC-PK_1, responds to the peptide hormone, calcitonin, by producing and secreting urokinase-type plasminogen activator (uPA). Calcitonin induction of uPA enzyme activity, which is mediated by cAMP (8,9), is due mainly to the transcriptional activation of the uPA gene (10,11). The uPA gene is a direct, primary target of calcitonin induction, because induction of uPA mRNA is not diminished in the absence of protein synthesis (10,12).

0006-291X/87 $1.50

Treatment with the anti-inflammatory synthetic glucocorticoid hormone, dexamethasone, inhibits plasminogen activator enzyme activity in several cells(13-19). In some cells, dexamethasone treatment increases plasminogen activator activity (20,21). Dexamethasone was recently reported to suppress uPA mRNA levels in human fibrosarcoma cells and in synovial fibroblasts (19).

Here we describe the coordinate actions in which dexamethasone inhibits calcitonin-induced uPA gene expression and uPA enzyme activity.

Materials and Methods

Materials

Synthetic salmon calcitonin was a gift of Dr. S. Guttman (Sandoz AG, Basel, Switzerland). Actinomycin D and cycloheximide were obtained from Sigma, and dexamethasone was from Fluka. Human plasminogen was prepared according to Deutsch and Mertz (22). Human urokinase was obtained from Leo Pharmaceutical Products (Bellerup, Denmark). BA 85 nitrocellulose membrane filters were from Schleicher and Schuell. [α^{32}P]dCTP and ^{125}I were obtained from New England Nuclear, and [^{3}H]uridine was from Amersham.

Cell culture

LLC-PK$_1$ cells (23) were cultured as described (10). In most experiments about 2×10^5 cells were plated on 35 mm dishes. Two days later, the cells were washed with phosphate-buffered saline containing calcium and magnesium [PBS(+)], and supplied with serum-free medium and with additional reagents as described in figure legends.

Determination of uPA enzyme activity

uPA enzyme activity in samples of conditioned medium or of cell lysates was measured as the plasminogen-dependent rate of fibrinolysis with the use of ^{125}I-fibrin coated multiwell plates (24). Samples were diluted 100-fold with 0.1M Tris·HCl, pH 8.1, and 10 μl of diluted samples were added to 250 μl of the same buffer containing 8 μg/ml human plasminogen. The assay mixtures were incubated at 37°C for 1 h and the amounts of solubilized ^{125}I-fibrin peptides were measured by γ-counting. Results were expressed in Ploug units, as determined by reference to a standard preparation of urokinase.

uPA cDNA probe and genomic DNA

Plasmid pYN15, a cDNA probe used to detect uPA mRNA in Northern hybridizations was described (25). Plasmid pYNg1, porcine uPA genomic DNA used for nuclear transcription experiment, consists of 5 kb of the 5′ flanking region, 6 kb of the transcribed region, and 500 bp of the 3′ flanking region, which were cut out from the uPA genomic clone λYN4 (25) and inserted into Eco RI and NarI (nearest to Tth111I site) sites of pBR322.

RNA preparation and Northern blot hybridization

RNA was prepared from LLC-PK$_1$ cells cultured in 35 mm dishes ($1-2\times10^6$ cells per dish) as described (10), and dissolved and stored in water. Northern blot hybridizations were performed as described (10).

Nuclear transcription

Preparation of nuclei, transcription, and filter hybridization were done according to McKnight and Palmiter (26), except that [^{3}H]UTP was used as a radioactive precursor instead of [α^{32}P]UTP, and that a genomic uPA DNA, pYNg1, was immobilized to nitrocellulose filters to hybridize the specific uPA transcripts instead of uPA cDNA.

Results and Discussion

Dexamethasone Inhibition of Calcitonin-induced uPA Enzyme Activity

The effect of dexamethasone on calcitonin-induced uPA enzyme activity is shown in Fig. 1. Various doses of dexamethasone were added to the culture medium of LLC-PK_1 cells at the same time as 10 ng/ml calcitonin. The medium was collected 24 h later and the uPA enzyme activity measured. The inhibitory effects of dexamethasone on calcitonin-induced uPA enzyme activity were observed at concentrations as low as 10^{-9} M, and the greatest inhibition, about 80%, was noted at 10^{-7} to 10^{-6} M. To explain these results, we considered two possibilities: 1) dexamethasone induced the activity of a uPA inhibitor, and 2) dexamethasone caused a decrease in uPA production.

Dexamethasone Activation or Induction of an Inhibitor of uPA Enzyme Activity

We sought to demonstrate more directly that dexamethasone treatment induced the activity of an inhibitor of uPA. Conditioned medium was

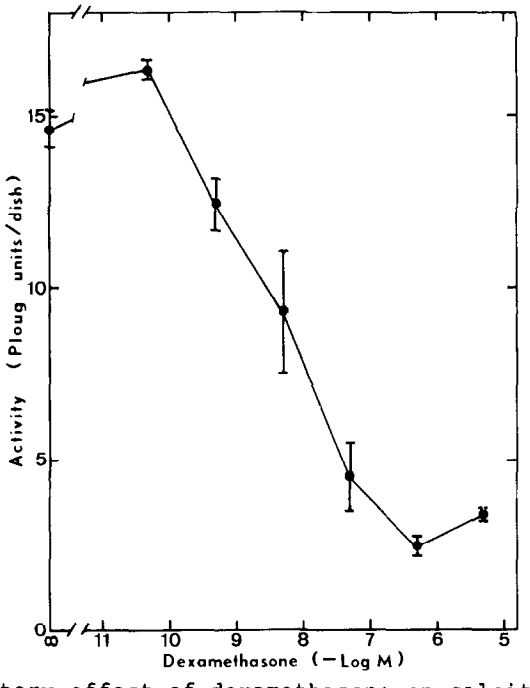

Figure 1. Inhibitory effect of dexamethasone on calcitonin-induced uPA enzyme activity. Cells were washed once with PBS(+), and then provided with 2 ml of serum-free medium containing 10 ng/ml calcitonin and various concentrations of dexamethasone. Twenty-four hours later, the incubation medium was removed and assayed for uPA enzyme activity.

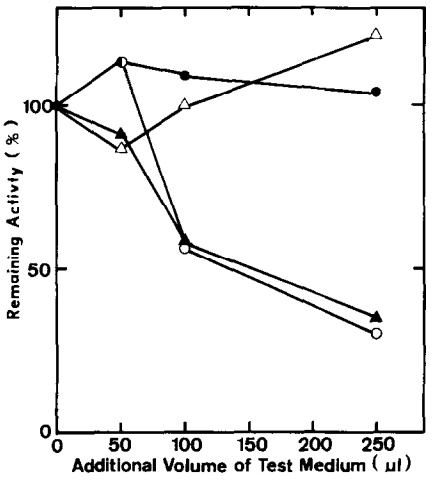

Figure 2. Inhibitory effect of medium conditioned by dexamethasone-treated cells on uPA enzyme activity. 2×10^6 cells were plated on 100 mm dishes and provided with serum-containing medium. Two days later, cells were washed with PBS(+), and then provided with serum-free medium without (●) or with 10^{-7} M dexamethasone (○, ▲, △). 24 h later, portions of the dexamethasone-conditioned medium were untreated (○); dialyzed overnight against PBS (without calcium and magnesium) (▲); or heat treated (95°C, 5 min) (△). Various volumes of these media were added to 50 μl aliquots of medium collected from cells in which uPA had been induced by treatment with 10 ng/ml calcitonin of 24 h. PBS(+) was added as necessary to make a final volume of 300 μl. The mixtures were incubated at 37°C for 30 min, and then uPA enzyme activity was assayed. The 100% value was 22.5 Ploug units/ml.

obtained from cells cultured with or without dexamethasone for 24 h. Then the ability of dexamethasone-conditioned medium to inhibit uPA activity in medium taken from calcitonin-induced cells was tested. The results of this mixing experiment showed that dexamethasone conditioned medium inhibited uPA enzyme activity in medium from calcitonin-induced cells (Fig. 2). As increasing volumes (50-250 μl)of dexamethashhone-conditioned medium were added to fixed volumes (50 μl) of calcitonin-conditioned medium, the extent of the inhibition of uPA enzyme activity increased. This suggested a stoichiometric effect. The inhibitory activity of the dexamethasone-conditioned medium was unaffected by overnight dialysis against PBS(-), but was abolished by heating at 95°C for 5 min. This suggested that the inhibitory activity in the dexamethasone conditioned medium has characteristics of a protein. Direct addition of 10^{-6} M dexamethasone to the assay had no effect.

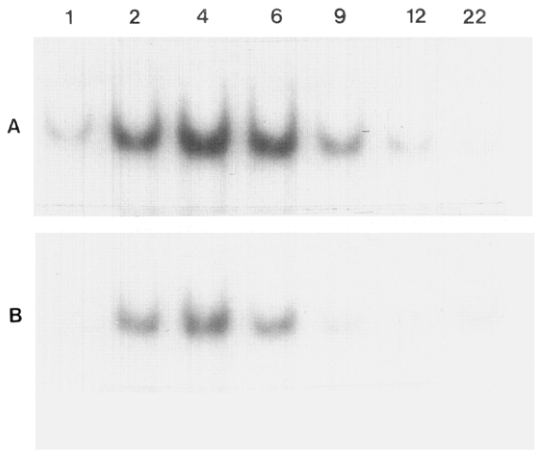

Figure 3. Time course of dexamethasone inhibition of the accumulation of calcitonin-induced uPA mRNA. Cells were washed once with PBS(+), and then provided with 2 ml of serum-free medium containing (a) 10 ng/ml calcitonin, or (b) 10 ng/ml calcitonin and 10^{-7} M dexamethasone. At the times (h) indicated, RNA was prepared and 10 μg subjected to Northern blot hybridization analysis.

Two inhibitors specific for plasminogen activator have been isolated and characterized, from placenta (27) and from endothelial cells (28,29). We do not know whether the dexamethasone-induced inhibitor of uPA enzyme activity in LLC-PK_1 cells is related to either of the two inhibitors.

Dexamethasone Inhibition of uPA mRNA Accumulation

The degree of inhibition noted in the mixing experiment (Fig. 2) was not high enough to explain 80% inhibition noted in Fig. 1. When as many as five volumes of dexamethasone-conditioned medium were added to one volume of calcitonin-conditioned medium, as much as 30% uPA enzyme activity remained in the calcitonin-conditioned medium (Fig. 2). Accordingly, we considered the possibility that dexamethasone also acted through another mechanism of inhibition, namely the inhibition of de novo synthesis of uPA.

The time course of the effect of dexamethasone on calcitonin-induced uPA mRNA accumulation is shown in Fig. 3. The results with cells treated only with calcitonin, showing a peak at 4 h, were similar to those reported previously (10). When cells were treated with both calcitonin and dexamethasone, uPA mRNA levels were always less than without dexamethasone treatment. We considered two possibilities

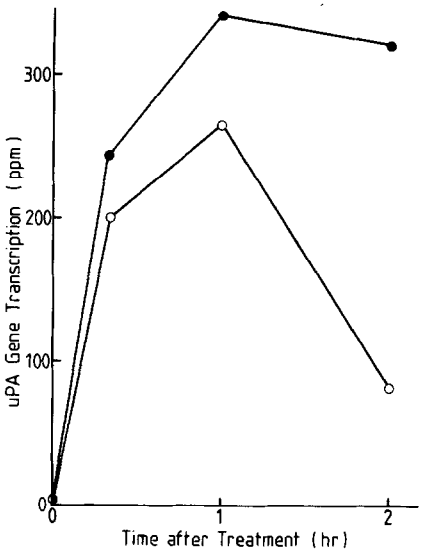

Figure 4. Decreased uPA gene transcription in nuclei from calcitonin-induced cells treated with dexamethasone. 1×10^7 cells were plated on 150 mm dishes and provided with serum-containing medium. Two days later, cells were washed with PBS(+), and then provided with serum-free medium with 1 ng/ml calcitonin without (●) or with (○) 10^{-6} M dexamethasone. At the times indicated, nuclear fractions were prepared, transcription was continued for 90 min in the presence of [^{3}H]UTP, and the extent of uPA gene transcription was measured by filter hybridization. The experiment was done twice, with similar results.

for these results: dexamethasone caused a decrease in transcription of the uPA gene, or caused a decrease in the stability of uPA mRNA.

The results of nuclear transcription experiments (Fig. 4) showed that dexamethasone inhibited uPA gene transcription induced by calcitonin. Cells were treated with calcitonin in the presence or absence of dexamethasone. Various times later, nuclei were isolated and uPA gene transcription initiated in intact cells were continued in the isolated nuclei. uPA gene transcription in nuclei from dexamethasone-treated cells was less than that in untreated cells, and declined more quickly.

In contrast, dexamethasone appeared to have no effect on the stability of uPA mRNA. The half-life of uPA mRNA was about 1.5 h, regardless of the presence or absence of dexamethasone (data not shown).

The stimulatory effects of glucocorticoid hormones on gene transcription have been well documented (see ref in 30). More recently,

inhibitory effects have been noted. These include inhibition of transcription of the β-globin gene (31), the α-fetoprotein gene(32, 33), the proopiomelanocortin gene (34-36), and the bovine prolactin promoter transfected into rat pituitary tumor cells (37). It will be of interest to determine which regions of the porcine uPA gene (25) are responsible for the inhibitory actions of dexamethasone on calcitonin-induced uPA gene transcription.

Acknowledgements

We thank Drs. D. Monard and Ch. Moroni for critical reading of the manuscript.

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