Plasminogen Activator Inhibitor Type 1 Biosynthesis and mRNA Level Are Increased by Dexamethasone in Human Fibrosarcoma Cells

PETER A. ANDREASEN,^{1,2*} CHARLES PYKE,¹ ANDREA RICCIO,³ PETER KRISTENSEN,¹ LARS S. NIELSEN,¹ LEIF R. LUND,¹ FRANCESCO BLASI,^{3,4} AND KELD DANØ¹

Finsen Laboratory, Rigshospitalet, DK-2100 Copenhagen Ø.,¹ Institute of Biochemistry C, University of Copenhagen, DK-2200 Copenhagen N.,² and Institute of Microbiology, University of Copenhagen, DK-1353 Copenhagen K.,⁴ Denmark, and International Institute of Genetics and Biophysics, Consiglio Nazionale delle Ricerche, 80123 Naples, Italy³

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Dexamethasone increases type 1 plasminogen activator inhibitor (PAI-1) activity released from the human fibrosarcoma cell line HT-1080. We demonstrated that dexamethasone caused about 10-fold increases in the intracellular and extracellular levels of PAI-1 protein, as measured by an enzyme-linked immunosorbent assay, in the rate of PAI-1 biosynthesis, and in the PAI-1 mRNA level. The effects on PAI-1 biosynthesis and mRNA level were detectable within 4 h and were maximal 16 to 24 h after the addition of dexamethasone. Cycloheximide did not inhibit the dexamethasone-induced increases in the capacity of the cells to synthesize PAI-1 and in the PAI-1 mRNA level.

Plasmin-mediated proteolysis has been implicated in a variety of physiological processes, including thrombolysis and breakdown of extracellular matrix proteins during tissue destruction under normal and pathological conditions (e.g., in cancer). Plasmin formation from the abundant inactive proenzyme plasminogen is catalyzed by either urokinase-type plasminogen activator (u-PA) or tissue-type PA (for reviews, see references 7, 10, and 28). Regulation of PA enzyme activity can for instance be exerted by specific and fast-acting inhibitors; such inhibitors have been identified in a variety of tissues, body fluids, and cell cultures. At least two types of inhibitors may be distinguished by immunochemical criteria, type 1 PA inhibitor (PAI-1) and type 2 PAI (PAI-2); both react with u-PA, as well as with tissue-type PA (2, 4, 9, 12, 14, 21, 25, 31). Recent structural studies of the protein and cDNA have shown that both PAI-1 and PAI-2 belong to the serpin (serine protease inhibitors) group and that PAI-1 is an argserpin with an arginine residue at the reactive center (3, 15, 16, 22, 24).

Dexamethasone increases PAI-1 inhibitory activity in the medium of cultured rat HTC hepatoma cells (14) and human HT-1080 fibrosarcoma cells and UCT/gli-1 glioblastoma cells (1). We report here that this glucocorticoid effect in HT-1080 cells depends on an increase in intracellular and extracellular levels of PAI-1 protein and an increased rate of biosynthesis of the protein, which in turn can be traced back to an increase in the cellular level of PAI-1 mRNA.

Increase of PAI-1 protein by dexamethasone treatment: immunological evidence. HT-1080 cells were grown to confluency in Dulbecco modified Eagle minimum essential medium supplemented with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (4.29 mg/ml), β -mercaptoethanol (0.00039% [vol/vol]), hypoxanthine (2.72 mg/ml), thymidine (0.76 mg/ml), and 10% fetal calf serum. Confluent cultures were washed with 0.01 M sodium phosphate (pH 7.4)–0.15 M NaCl–1 mM CaCl₂–0.5 mM MgCl₂ (PBS) and maintained in medium without serum for 4 days. Parallel serum-deprived

cultures were then incubated with 20 ml of medium without serum and without or with dexamethasone for various periods of time up to 48 h.

PAI-1 in cell extracts and conditioned culture media was quantitated by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (19a), except that the second layer of polyclonal rabbit antibodies (2) was affinity purified (18) by using a column with immobilized PAI-1 which had been purified by immunosorbent chromatography on a column with monoclonal anti-PAI-1 immunoglobulin G (IgG) (21). The intracellular PAI-1 level, as well as its extracellular accumulation in the culture medium, was increased 5- to 10-fold by incubation with dexamethasone for 48 h (Fig. 1). The effect on the intracellular PAI-1 level was apparent within 4 h of exposure to dexamethasone and leveled off after 10 to 20 h, whereas the extracellular PAI-1 level was significantly increased after 16 h and continued to increase during the 48 h of incubation.

HT-1080 cells contain very low levels of PAI-1-activator complexes compared with the total PAI-1 concentration (1). This is expected based on the fact that u-PA is present in HT-1080 conditioned medium in the proenzyme form (P. A. Andreasen, unpublished results), which does not react with PAI-1 (2), and that HT-1080 cells produce very low levels of tissue-type PA (1). Therefore, the presence of PAI-1 together with the activators in the samples did not interfere with the determination of PAI-1 in the ELISA.

Immunofluorescence staining with monoclonal anti-PAI-1 IgG (19a) showed a much stronger immunoreactivity in dexamethasone-treated cells than in control cells. In both instances, the staining was mainly located perinuclearly and had a granular appearance (results not shown).

Dexamethasone increased the rate of biosynthesis of PAI-1. To study the rate of biosynthesis of PAI-1, HT-1080 cells were washed three times with PBS, incubated for 30 min at 37°C with 10 ml of PBS to exhaust cellular methionine stores, scraped from the dishes, and pelleted by centrifugation. The cells originating from one dish were suspended in 0.5 ml of medium without methionine and incubated with 15

* Corresponding author.

μCi of [^{35}S]methionine for 15 min at 37°C . The cells were washed and then either extracted directly or first chased by incubation in complete medium at 37°C for various times, followed by repelleting and washing. Incorporation of radioactivity into total protein in cell extracts was linearly related to time within the pulse-labeling period; the accumulation of labeled proteins in the incubation medium during the chase period had a lag of 15 to 30 min, proceeded linearly with time for 1 to 2 h, and leveled off to reach a plateau within 4 h (results not shown).

For isolation of labeled PAI-1, cell extract or incubation medium was diluted fivefold with 0.1 M Tris (pH 8.1)–0.01% Tween 20–0.1 M NaCl and passed twice over 1-ml Sepharose 4B columns with polyclonal rabbit anti-IgG PAI-1; the columns had been equilibrated with the same buffer. The columns were then washed with 10 column volumes of 0.1 M Tris (pH 8.1)–0.01% Tween 20–1 M NaCl. Bound PAI-1 was eluted with 0.1 M CH_3COOH (pH 2.7)–1 M NaCl–0.01% Tween 20, dialyzed against 0.03% sodium dodecyl sulfate (SDS), lyophilized, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 6 to 16% gradient gels (11, 17) followed by autoradiography. A single labeled band, comigrating with PAI-1, was obtained. The yield of PAI-1 in this purification procedure was higher than 60%, as revealed by ELISA. No inhibitor was retained on columns with preimmune rabbit IgG. The labeled band comigrating with PAI-1 in SDS-polyacrylamide gels could be removed by passage of the preparation through a column with monoclonal anti-PAI-1 IgG from hybridoma clone 1 (21) but not by passage through a column with a monoclonal antibody of irrelevant specificity (antitrinitrophenyl IgG [30]) (results not shown). Therefore, this labeled band represented homoge-

TABLE 1. [^{35}S]methionine labeling of PAI-1 in extracts and conditioned medium of control cells and cells cultured in the presence of dexamethasone^a

Fraction	Dexamethasone concn (M)	Chase period (h)	PAI-1 radioactivity (cpm/ 10^7 cells)
Extract	0	0	436
		4	102
	10^{-6}	0	3,805
		4	270
Medium	0	0	0
		4	374
	10^{-6}	0	0
		4	4,022

^a HT-1080 cells were kept under serum-free culture conditions for 48 h in the absence or presence of dexamethasone. The cells were then pulse-labeled with [^{35}S]methionine for 15 min and chased for 4 h. Cell extracts and incubation media were analyzed for [^{35}S]methionine incorporation into PAI-1 by immunoaffinity chromatography followed by SDS-PAGE and liquid scintillation counting of PAI-1-containing slices of the individual gel lanes. The number of cells was calculated from the amount of DNA (8) in the pellets remaining after preparation of cell extracts, assuming a content of 6 pg of DNA per cell.

neous PAI-1 (see Fig. 5A). This immunoaffinity chromatography with polyclonal antibodies was found to give a more precise quantitation of labeled PAI-1 in cell extracts than did immunoprecipitation or immunoaffinity chromatography with monoclonal antibodies. Incorporation of radioactivity into PAI-1 was linear with time in the pulse-labeling period in both control and dexamethasone-treated cells, showing that the incorporation in this period was proportional to the rate of PAI-1 biosynthesis. The results shown in Table 1 indicate that a 48-h pretreatment with dexamethasone induced an approximately 10-fold increase in PAI-1 biosynthesis.

During the pulse-labeling period, no labeled PAI-1 was detected in the medium. However, during the chase period, there was a time-dependent release of labeled PAI-1 from the cells into the medium, the recovery in medium being complete after 4 h in both control and dexamethasone-treated cultures (Table 1). Thus, dexamethasone did not have any detectable effect on PAI-1 secretion, in contrast to findings reported for at least one other protein (6).

The rate of PAI-1 synthesis began to increase 4 h after the addition of dexamethasone and reached a new steady-state after about 16 h (Fig. 2). This time course for induction followed quite closely the time course for the increase in intracellular PAI-1 (Fig. 1). Withdrawal of dexamethasone from the medium resulted in a return of the rate of PAI-1 synthesis to control values after 24 h. A half-maximal effect on the rate of PAI-1 synthesis was reached at a dexamethasone concentration of 5×10^{-9} M, and a maximal effect was reached at 10^{-7} M (results not shown).

Dexamethasone increased the PAI-1 mRNA level. Total RNA and poly(A)⁺ RNA were analyzed by hybridizing Northern blot (RNA blot) and dot blot filters (19a, 20) with a plasmid, pPAI1-A1, carrying a full-length cDNA for PAI-1 (3). Northern blot analysis (Fig. 3A and B) showed that both total and poly(A)⁺ RNA from control cells and cells treated with dexamethasone for 48 h contained two RNA species (3.4 and 2.4 kilobases) which hybridized with PAI-1 cDNA. The relative abundance of the two species was the same in the control and dexamethasone-treated cells. The presence of two mRNA species has been reported previously for various cell types, including HT-1080 (3, 15, 22, 24). Se-

FIG. 1. Time course of dexamethasone-induced increase in PAI-1 protein in HT-1080 cells. Dexamethasone (final concentration, 10^{-6} M) was added to serum-free cultures of HT-1080 cells at the indicated times before harvest. Cell extracts (○) and conditioned media (●) were analyzed for PAI-1 by ELISA. The bars indicate standard deviations. The number of cells was calculated from the amount of DNA (8) in the pellets remaining after preparation of cell extracts, assuming a content of 6 pg of DNA per cell.

FIG. 2. Time course of dexamethasone-induced increase in PAI-1 biosynthesis in HT-1080 cells. Dexamethasone (final concentration, 10^{-6} M) was added to serum-free cultures of HT-1080 cells at the indicated times before analysis of the cells for the capacity to synthesize PAI-1. The cells were pulse-labeled with [35 S]methionine for 15 min. Radioactively labeled PAI-1 was isolated from cell extracts by immunoaffinity chromatography and subjected to SDS-PAGE and autoradiography. The relative amounts of label incorporated into PAI-1 were determined by spectrophotometric scanning of autoradiographic films.

quence determination for the corresponding cDNAs has shown that these two RNA species are identical in their 5' flanking region and in the coding region but differ in the 3' noncoding region, possibly due to different polyadenylation sites (3, 15, 22). Quantitative dot blots analyzed by spectro-

FIG. 4. Time course of induction of PAI-1 mRNA by dexamethasone in HT-1080 cells. The dot blots shown in Fig. 3C and D were analyzed quantitatively by spectrophotometric scanning of the autoradiographic films. The relative amounts of PAI-1 mRNA were calculated from the slopes of the lines relating the areas and intensities of the dots to the amounts of RNA in the dots, normalized against the corresponding relative amounts of β -actin mRNA.

photometric scanings of autoradiographic films (Fig. 3C and 4) showed that dexamethasone increased the level of PAI-1 mRNA about 10-fold after 48 h and that the time course of the increase followed quite closely the time courses for the increases in the intracellular PAI-1 level and PAI-1 biosynthesis. As a control, hybridization to a human β -actin probe (27) was identical in control and dexamethasone-treated cells (Fig. 3D).

FIG. 3. Northern blot and dot blot analysis of the effect of dexamethasone on the level of PAI-1 mRNA in HT-1080 cells. Total RNA and poly(A)⁺ RNA was prepared from serum-free cultures incubated with 10^{-6} M dexamethasone for the indicated times before harvest. For Northern blot analysis (A and B), 2 μ g of poly(A)⁺ RNA (panel A) or 10 μ g of total RNA (panel B) was electrophoresed in agarose gels under denaturing conditions and blotted onto a nitrocellulose filter. Dot blots (C and D) were prepared by applying twofold serial dilutions of denatured total RNA, starting from 3.2 μ g, onto nitrocellulose. The filters were hybridized to 32 P-labeled cDNA probes for PAI-1 (panels A to C) or β -actin (panel D) and analyzed by autoradiography. The positions of rRNA in the Northern blots are indicated to the left of panel A, and the sizes of the two PAI-1 mRNA bands, as determined from their electrophoretic mobilities, are indicated to the right of panel B. Kb, Kilobases.

FIG. 5. Effect of dexamethasone on PAI-1 biosynthetic capacity and PAI-1 mRNA levels in the presence of cycloheximide. HT-1080 cells were incubated for 16 h without additions (lanes 1, 1', and 1'') or with 10^{-6} M dexamethasone (lanes 2, 2', and 2''), $10 \mu\text{g}$ of cycloheximide per ml (lanes 3, 3', and 3''), or 10^{-6} M dexamethasone plus $10 \mu\text{g}$ of cycloheximide per ml (lanes 4, 4', and 4''). After incubation, the cells were removed for analyses of PAI-1 biosynthetic capacity and PAI-1 mRNA levels. (A) After a 30-min wash with PBS to remove cycloheximide, the cells were pulse-labeled for 15 min with [^{35}S]methionine. Labeled PAI-1 was purified from cell extracts by immunoaffinity chromatography and analyzed by SDS-PAGE and autoradiography. The positions of M_r markers (rabbit phosphorylase *b* [97,000], bovine serum albumin [67,000], ovalbumin [43,000], carbonic anhydrase [30,000], soybean trypsin inhibitor [20,100], and β -lactalbumin [14,400]) are indicated on the left, and the position of PAI-1 is indicated on the right. Separate control experiments (results not shown) demonstrated that the presence of $10 \mu\text{g}$ of cycloheximide per ml during the pulse-labeling abolished the incorporation of label into all proteins. (B) Dot blots with poly(A)⁺ RNA were prepared by applying twofold serial dilutions of denatured RNA, starting from 100 ng, onto nitrocellulose. The filters were hybridized to ^{32}P -labeled cDNA probes for PAI-1 (lanes 1' to 4') and β -actin (lanes 1'' to 4'').

Dexamethasone effect on PAI-1 biosynthesis and the PAI-1 mRNA level in the presence of cycloheximide. During a 16-h incubation, cycloheximide ($10 \mu\text{g}/\text{ml}$) alone had no detectable effect on either the capacity of HT-1080 cells to synthesize PAI-1 or the cellular PAI-1 mRNA level, and it did not prevent the effects of dexamethasone on PAI-1 biosynthesis and the mRNA level (Fig. 5).

The effects of glucocorticoids on mRNA levels have in most instances been accounted for by an increased transcriptional rate (for a review, see reference 29), but glucocorticoids have also been reported to affect the cellular levels of specific mRNAs posttranscriptionally (13, 32). Further studies are needed to elucidate the mechanism of the dexamethasone-induced increase in cellular PAI-1 mRNA levels in HT-1080 cells. Since the PAI-1 mRNA increase was not affected by the presence of cycloheximide, it may have resulted from a direct effect of the glucocorticoid-receptor complex on the PAI-1 gene, without the intervening induction of other regulatory proteins.

Since fibronectin and other components of the extracellular matrix are substrates for plasmin, plasminogen activation may regulate the breakdown of the extracellular matrix (for a review, see reference 10). PAI-1 may protect against such breakdown, since PAI-1 is deposited pericellularly by HT-1080 cells together with components of the extracellular

matrix, including fibronectin (26). Besides increasing PAI-1, dexamethasone also suppresses the amount of u-PA released from HT-1080 cells (1), and there have been similar findings with a variety of u-PA-producing cell lines (10). Furthermore, glucocorticoids increase the production of fibronectin in HT-1080 and other cells (5, 19, 23). All these changes tend to enhance the amount of extracellular matrix proteins, and this enhancement may be an important biological effect of glucocorticoids.

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