Actinomycin D Prevents Nuclear Processing of Estrogen Receptor*

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Kathryn B. Horwitz and William L. McGuire‡
From the Department of Medicine, The University of Texas Health Science Center, San Antonio, Texas 78284

SUMMARY

This communication describes a novel effect of actinomycin D (AcD) in inhibiting the nuclear processing or turnover of estrogen receptors in the human breast cancer cell line, MCF-7. In the absence of AcD, estradiol treatment results in rapid (5 min) hormone binding and translocation of unfilled cytoplasmic receptors (Rc) and binding of unfilled nuclear receptors (Rn). Thereafter, filled nuclear receptors (RnE) progressively deplete and, by 3 to 5 h, 70% are lost or processed. We now show that 1 to 2 μM AcD or chromomycin A3, both of which intercalate at G-C base-pairs on DNA, selectively and completely block RnE processing. In contrast, estrogen binding, translocation of receptor complex, and RnE accumulation in the nucleus are completely insensitive to AcD inhibition. At 1 to 2 μM, all other intercalators and inhibitors tested, including other inhibitors of transcription and replication, fail to prevent binding, translocation, or the nuclear processing step. AcD inhibition of RnE processing is dependent on dose; at lower doses (100 nM decreasing to 1 nM), progressively greater RnE depletion occurs. AcD completely prevents RnE depletion if given together with or within 30 min after estradiol; at any time between 30 min and 5 h after estradiol, the processing of RnE is stopped instantly by addition of AcD.

Because of the complexity of actinomycin action, several mechanisms can be proposed to explain its effect on nuclear ER levels.

Actinomycin D has been a powerful tool in investigations into the biochemistry of nucleic acids and their involvement in replication and transcription (1). The antibiotic intercalates into double-stranded DNA with its chromophore between successive G-C base-pairs; two pentapeptides lie in the minor groove of the double helix (2). Two binding sites, distinguished by different affinities for DNA, have been described; both are partially blocked by the presence of chromosomal proteins (3).

The nature of the inhibitory action of AcD* is complex and partially concentration-dependent. AcD suppresses the synthesis of all cellular RNA fractions by preferentially blocking chain elongation catalyzed by DNA directed RNA polymerase. However, at low concentrations, there is differential inhibition of various RNA classes with ribosomal RNA being most sensitive (1). DNA synthesis in intact cells or by isolated DNA polymerases is also sensitive to AcD but requires the presence of much higher inhibitor concentrations (4). Actinomycin may also have direct effects on protein synthesis by inhibiting the transport of nuclear mRNA into cytoplasm (5) or by altering the rate of mRNA translation (6, 7).

In cells which are actively synthesizing specific proteins under steroid hormone stimulation, steroid removal results in rapid deinduction. However, if AcD is present under such conditions, not only is deinduction prevented, but the protein may in fact accumulate further. The mechanism for this "superinduction" of inducible proteins remains controversial; it may involve stabilization of specific mRNA or inhibition of protein turnover rate (8, 9) or selective enhancement of mRNA translation (10, 11). The list of proteins superinduced by AcD is large (12). The effect of AcD on the induction of tyrosine aminotransferase by dexamethasone in hepatoma cells has been most thoroughly studied (8, 9); enhancement by AcD of estrogen-inducible proteins has also been reported in several experimental systems (11, 13, 14).

We have been studying the mechanisms involved in estrogen and anti-estrogen action as these are mediated by the estrogen receptor in MCF 7 human breast cancer cells. In particular, we have described a complex response system in which ER binding, translocation, and subsequent turnover or nuclear processing appear to mediate induction of progesterone receptor (15). During processing, progressive depletion of approximately 70% of estradiol filled nuclear receptors occurs before a new steady state level of RnE is established. This may represent de facto receptor loss from the cell or, alternatively, a loss of ligand binding capacity. With nonsteroidal anti-estrogens, the nuclear processing step may be defective.2 We have now used a variety of inhibitors of various cell functions to study the mechanisms involved in RnE turnover or processing, and find that only AcD and chromomycin A3 (which like AcD intercalates at G-C base-pairs) selectively and completely block RnE processing. This novel effect of actinomycin on nuclear ER depletion may serve as the basis for models of nuclear ER action and may provide alternative explanations for the biological effects seen in studies using actinomycin together with steroids.

MATERIALS AND METHODS

Cell Culture—MCF-7 cells, a gift of Dr. Herbert Soule (Michigan Cancer Foundation) were plated at 2 × 10⁶ cells in Falcon plastic flasks (75 cm²), and grown in 5% CO₂ in air at 37°C. Growth medium consisted of autoclavable Eagle’s minimal essential medium containing Earle’s salts (MEM medium, Gibco) supplemented with 1% nonessential amino acids (Gibco), 2 mM L-glutamine (Gibco), 6 ng/ml of insulin (Sigma), 5% calf serum (Gibco), 25 μg/ml of gentamicin (Schering), and 0.1% NaHCO₃.

Experimental Media—Two days after plating, growth medium was replaced by control harvest medium consisting of growth medium supplemented with 10 μM hydrocortisone and 1 μg/ml of ovine pro-

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1 The abbreviations used are: AcD, actinomycin D; ER, estrogen receptor; Rc, unfilled cytoplasmic estrogen receptor; Rn, unfilled estrogen receptor; RnE, filled nuclear estrogen receptor; MEM medium, Eagle’s minimal essential medium; HTC, rat hepatoma cells in culture; POPOP, 1,4 bis(2-(5-phenyloxazolyl) benzene.

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Lactin, (NHI-P-S-12, NCI). The medium also contained 5% calf serum stripped of endogenous hormones by a 30-min incubation at 45°C with a dextran-coated charcoal pellet (0.25% Norit A, 0.025% dextran in 0.01 M Tris-HCl, pH 8.0 at 4°C, 2 ml/m of serum). This procedure removed more than 98% of a tritiated estradiol (Sigma) preparation in ethanol added to control harvest medium (0.6 ml of ethanol per 500 ml of medium) to obtain the final concentration (10 nM) used. AcD (NSC 30564, Cancer Chemotherapy National Service Center) prepared in ethanol at 1000-fold excess was added to estradiol-containing media to obtain the final concentrations described in the text.

Cell Harvest—Confluent cells were removed by a 10-min incubation at 37°C with 1 ml of EDTA in Ca2+ (Ca+++-free Hank's balanced salt solution, washed once with Hank's solution at 4°C, and once in phosphate buffer (5 mM sodium phosphate, pH 7.4 at 4°C, 10 mM thiglyceral, and 10% glycerol).

Cytosol and Nuclear Extracts—Cells were resuspended in phosphate buffer (1 ml of buffer/ml of packed cells) and homogenized in a Teflon-glass Dounce homogenizer (Kontes) using the B pestle, until they were more than 90% disrupted (about 30 strokes) as seen by phase microscopy.

The homogenate was centrifuged at 800 x g for 15 min and the supernatant (crude cytosol) was saved. The nuclear pellet was washed twice with 1 ml of phosphate buffer and the supernatants were added to the crude cytosol. This was centrifuged at 105,000 x g for 30 min in a Beckman 50 Ti rotor, and the supernatant (cytosol) was used immediately. DNA content in the cytosol pellet which contains mainly microsomes and mitochondria was less than 2% of the total DNA.

The nuclear pellet was resuspended in 1 to 3 ml of Tris buffer (10 mM Tris-HCl, pH 8.5 at 4°C, 1.5 mM EDTA, 10% glycerol), containing 0.6 M KCl, and incubated for 1 h during which time the pellet was resuspended every 15 min.

Solubilized proteins from the nuclear extract were then obtained by centrifugation at 105,000 x g for 30 min. DNA used for calculation of the number of nuclear sites was determined from the high speed nuclear pellet by the diphenylamine method of Burton (16) using calf thymus DNA as the standard.

Protamine Sulfate Exchange Assay—Concentrated cytosol protein content was measured by A260/280 nm (17), then adjusted to a concentration of 2 mg/ml with phosphate buffer, pH 7.4. The nuclear extract was diluted at least 8-fold to reduce the salt concentration, a step essential for precipitation by protamine (18).

Triplicate aliquots of diluted cytosol (200 µl) or diluted nuclear extracts (500 µl) were precipitated with 250 µl of 1 mg/ml of protamine (Sigma) prepared in ethanol and added to control harvest medium (0.6 ml of ethanol per 500 ml of medium) to obtain the final concentration of 250 µg/ml used. AcD (1 µM) was added together with estradiol and incubations were continued to 5 h (triangles). One set (arrow) received estradiol plus AcD continuously for 5 h. Control cells shown at zero time were not treated with AcD. In parallel flasks, cells were treated with estradiol alone for 5 min to 2 h, then AcD (1 µM) was added together with estradiol and incubations were continued to 5 h (triangles). One set (arrow) received estradiol plus AcD continuously for 5 h. Control cells shown at zero time were not treated. At the end of incubation, cells were harvested, washed, homogenized, and assayed for cytosolic estrogen receptors by the single saturation dose protamine method. Unoccupied cytosol estrogen receptors (4°C, A, •) were determined by incubating nuclear pellets for 15 min at 4°C with vigorous vortexing every 2 to 3 min. Nuclei appeared free of cytoplasmic debris as seen by phase contrast microscopy. Nuclei were pelleted by an 800 x g, 5-min centrifugation, and the supernatant plus supernatants from two 2-ml washes in sucrose/phosphate buffer were pooled. One hundred-microliter aliquots were counted in 5 ml of toluene/PPO/POPOP at a counting efficiency for 3H of 42%. Washed nuclear pellets were extracted twice in 1 ml of ethanol. One hundred-microliter aliquots of the extracts were counted in 5 ml of toluene/PPO/POPOP at a counting efficiency for 3H of 42%.

RESULTS AND DISCUSSION

Unstressed MCF-7 cells have unfilled estrogen receptors in both cytoplasm (Rc) and nuclei (Rn) (19). In 5 min, estradiol binds Rc and translocates it to the nucleus; estradiol also binds Rn directly (18). Beginning 30 min after estradiol addition and continuing for 3 to 5 h, a progressive depletion of RnE occurs without reappearance of unfilled sites, until a new steady state level of RnE is achieved at about 30% of the initial maximum value (Fig. 1). If AcD is added at any time (5 min to 2 h) during estradiol treatment and incubations are then continued to 5 h, nuclear ER are immediately fixed at the levels they had reached before addition of the inhibitor. If estradiol and AcD are added together at the start of treatment, estrogen binding and translocation of Rc and binding to Rn and the initial accumulation of total receptor in the nucleus are not inhibited. However, all subsequent processing of nuclear ER processing is not changed.

The inhibitory effects of AcD on ER processing are only achieved when it is present in high concentrations (Fig. 3). This study shows that within 10 min of estradiol addition all

![Fig. 1. Cytoplasmic and nuclear ER levels in cells treated continuously for 5 min to 5 h at 37°C with 10 nM estradiol (circles). In parallel flasks, cells were treated with estradiol alone for 5 min to 2 h, then AcD (1 µM) was added together with estradiol and incubations were continued to 5 h (triangles). One set (arrow) received estradiol plus AcD continuously for 5 h. Control cells shown at zero time were not treated. At the end of incubation, cells were harvested, washed, homogenized, and assayed for cytosolic estrogen receptors by the single saturation dose protamine method. Unoccupied cytosol estrogen receptors (4°C, A, •), total nuclear receptors (30°C, O, △). Each point represents triplicate determinations from four pooled T-75 flasks.](image-url)
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FIG. 2. Effect of AcD on nuclear ER measured directly by uptake of [3H]estradiol. Cells grown in four T-75 flasks were incubated 5 min to 5 h with [3H]estradiol (3.5 μM) alone or together with a 100-fold excess of diethylstilbestrol. Parallel flasks contained [3H]estradiol plus 1 μM AcD. The cells were harvested, cooled, washed with ice cold MEM and phosphate/sucrose buffer, then incubated 15 min at 4°C and repeated vortexing with 0.5% Triton X-100 in phosphate/sucrose buffer. Nuclei were then pelleted and washed. Triplicate aliquots of ethanol-extracted nuclei and the combined postnuclear supernatants and washes were counted directly for radioactivity. Nonspecific binding was less than 5% and has been subtracted.

FIG. 3. Effect of AcD dose on extent of nuclear ER processing. Cells were treated for 10 min or 5 h with estradiol (10 nm) alone or together with 0.05 μM (0.06 μg/ml) to 2 μM (2.5 μg/ml) AcD. At the end of incubation, cells were harvested, washed, homogenized, and assayed for cytoplasmic and nuclear ER by the single saturating dose protamine method. Unoccupied cytoplasmic (4°C, black), unoccupied nuclear receptors (4°C, open), occupied nuclear receptors (30-4°C, hatched/ stippled). The bar heights represent total cell receptors (Rc + Rn + RnE) determined in triplicate from four pooled T-75 &eske.

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