Actinomycin D Prevents Nuclear Processing of Estrogen Receptor*

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SUMMARY

This communication describes a novel effect of ac- tinomycin 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, or inhibitors of translation or of other cell functions, 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 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% non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 6 μg/ml of insulin (Sigma), 5% calf serum (Gibco), 25 μg/ml of gentamicin (Scher- ing), 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 ng hydrocortisone and 1 μg/ml of ovine pro-

² K. B. Horwitz, and W. L. McGuire, manuscripts submitted for publication.
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/ml of serum). This procedure removed more than 96% of a trace amount of estradiol in the charcoal was removed by centrifugation and sterilized serum was stored at -70°C until use. Flasks were fluid-changed with fresh medium every 48 h. For experimental media, 10 μM estradiol (Sigma) prepared in ethanol was 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 5064, Cancer Chemotherapy National Service Center, NCI) 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 EDTA in Ca++, Mg++-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 thioglycollate, 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 10 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 mostly 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 cytosolic and 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 A 280/290 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 (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 Rn to and binding to Rn and the initial accumulation of total receptor in the nucleus are not inhibitable. However, all subsequent processing stops (Fig. 1, arrow). The slight downward slopes of the dashed lines show that the effect on ER processing occurs within 15 min of ACD addition, so that entry of ACD into nuclei must be quite rapid.

To rule out the possibility that high ER levels in the presence of ACD are somehow an artifact of salt extraction or the protamine sulfate assay, we measured [3H]estradiol in nuclei purified after [3H]estradiol treatment of whole cells. As seen in Fig. 2, the apparent ACD effect on 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 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 untreated. 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 receptors (▲), total nuclear receptors (Ο, Δ). Each point represents triplicate determinations from four pooled T-75 flasks.](http://www.jbc.org/)

RESULTS AND DISCUSSION

Unstrept MCF-7 cells have unfulfilled 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 (17). Beginning 30 min after estradiol addition and continuing for 5 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 inhibitable. However, all subsequent processing stops (Fig. 1, arrow). The slight downward slopes of the dashed lines show that the effect on ER processing occurs within 15 min of ACD addition, so that entry of ACD into nuclei must be quite rapid.

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Actinomycin Stops Nuclear Estrogen Receptor Processing

The bar heights represent total cell receptors (Rc) during the early recovery phase after estradiol withdrawal while high concentrations (5 μg/ml) result in enzyme superinduction. The low AcD dose would be sufficient to saturate the high affinity DNA binding sites (7), which suggests that the high AcD requirement for tyrosine aminotransferase superinduction or maintenance of elevated nuclear ER is somehow involved with binding to the second lower affinity sites (7).

In preliminary experiments, we have tested several other inhibitors of cell function for their effect on ER binding, translocation, and processing during estradiol treatment. At 1 μM, all other intercalators and inhibitors tested were ineffective; the list includes other inhibitors of transcription (dactinomycin, adriamycin, distamycin A, α-amanitin), inhibitors of replication (chloroquine, nalidixic acid, mitomycin C, novobiocin, ethidium bromide), a translation inhibitor (cyclomexime), and inhibitors of other cell processes (colchicine, cytochalasin B). The sole exception was chromomycin A3. This compound behaves identically to AcD, and like AcD is the only inhibitor which shows specificity for G-C base-pairs (1).

The failure of other intercalators and translation inhibitors to prevent ER processing suggests indirectly that the AcD effect is not due to inhibition of RNA and protein synthesis. However, because of the inhibitors' complex actions, the possibility cannot be ruled out that actinomycin is regulating the nuclear exit of receptors distinct from its effect on chromatin, or that it alters the proteins' turnover rate at some extranuclear site. Blocking of transcription is the classic and best studied effect of this antibiotic, which suggests that inhibition of ER processing may occur because AcD directly blocks ER access to DNA. In that case, AcD may distinguish between two Ran binding sites in nuclei. Newly translocated receptor binds to a site on chromatin or DNA insensitive to inhibition. AcD or chromomycin A3 stops subsequent processing by preventing Ran insertion at a second base-specific region on DNA. The existence of two receptor binding sites in nuclei, one for chromatin, another for DNA, have been postulated in the chick oviduct for progesterone receptor (21). Palmer et al. (22) have also proposed a two-step nuclear receptor translocation mechanism involving a rate-limiting movement of steroid receptors from initial nonproductive chromatin binding sites to productive sites. Alternatively, a model involving only one binding site requires that nuclear ER binding is immediately to DNA; AcD may then mechanically prevent ER release from this site. As so little is understood about the mechanism of actinomycin action, none of these various explanations can be ruled out by our data.

The effect of AcD on nuclear ER suggests that another explanation for the AcD-induced superinduction of proteins seen after treatment with steroids (13, 14, 23) is by an alteration in nuclear steroid-receptor turnover rate; although this question must be examined in each experimental system. Finally, it is possible that the association of anti-estrogens with ER results in changes analogous to those induced by actinomycin.

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K B Horwitz and W L McGuire


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