INHIBITION OF THE BINDING OF PROGESTERONE RECEPTOR TO NUCLEI: EFFECTS OF o-PHENANTHROLINE AND RIFAMYCIN AF/013

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Summary: When preparations of chick oviduct progesterone receptor, labeled with [^{3}H]progesterone, are suitably incubated with o-phenanthroline or rifamycin AF/013, the ability of the [^{3}H]progesterone-receptor to bind to purified oviduct nuclei is almost completely abolished, although the steroid-receptor complex itself remains essentially intact. m-Phenanthroline and rifampicin do not cause significant inhibition of nuclear-binding ability. These findings are discussed in relation to known effects of the same compounds on nucleic acid polymerases. The effectiveness of o-phenanthroline suggests that the receptor may be a metalloprotein in which the metal ion participates in the attachment of receptor to nuclear binding sites.

The compounds o-phenanthroline, a metal chelator, and rifamycin AF/013, a lipophilic derivitive of the rifamycin class of antibiotics, both inhibit the activity of RNA and DNA polymerases (1-6). Evidence suggests that both inhibitors interfere with the binding of polymerases to DNA. Such interference has been demonstrated in the action of AF/013 against RNA polymerases (4,6,7) and is implied in the action of o-phenanthroline against terminal deoxynucleotidyl transferase (3) and various DNA polymerases (2), in which case chelation of enzyme-bound zinc ion appears to occur (2).

We have examined the effects of these two compounds on the *in vitro* binding of progesterone receptor to nuclei, with the following considerations in mind: 1) Both DNA and chromosomal proteins have been implicated as binding sites for steroid receptors within the nucleus (8,9). If o-phenanthroline and AF/013 should happen to interact somewhat generally with DNA-binding proteins, they might inhibit steroid receptor binding to DNA and, consequently, steroid receptor binding to nuclei. 2) A major site of steroid hormone action is on RNA synthesis (8). A finding that steroid receptors interact with inhibitors of polymerases would strengthen the possibility that receptor molecules may

themselves have or be associated with some type of polymerase activity.

3) Since inhibitors such as AF/013 have been used to block initiation of RNA polymerase in investigations of steroid effects on RNA synthesis (10), a direct interaction of receptors with such inhibitors could affect the interpretation of results in such studies.

MATERIALS AND METHODS

Materials. Progesterone [1,2-^{3}H(N)], 48 Ci/mmole, was obtained from New England Nuclear; o-phenanthroline, A.C.S. reagent grade, from Fisher Scientific; m-phenanthroline from the G. Frederick Smith Chemical Co., Columbus, Ohio, (recrystallized before use); and rifampicin from Schwarz/Mann. Rifamycin AF/013 was provided by Gruppo LePetit, Milan, Italy. The phenanthrolines and rifamycins were dissolved in dimethylsulfoxide (DMSO) and then diluted to 10% DMSO by the rapid addition of H_2O.

Preparation of progesterone receptor. Oviducts were removed from White Leghorn chicks that had been injected for 2 to 6 weeks with diethylstilbestrol, 5 mg/day in sesame oil, starting at approximately one week of age. The tissue was rinsed and then homogenized in two volumes of 0.04 M Tris, 0.012 M thioglycerol buffer, pH 7.5 (Tris-TG), using a "Tissumizer" (Tekmar model SDT). The temperature was kept at 0-4°. After centrifuging the homogenate for 10 min at 12,000 g, the supernatant was centrifuged at 149,000 g for 1 hr. The high-speed supernatant, from which the lipid layer was removed, represented the cytosol. The protein concentration was adjusted to 20 mg/ml, and [^{3}H]progesterone (2 x 10^{-8}M) was added to the cytosol at least 1 hr prior to use. For some experiments, the "labeled" cytosol was treated with $(NH_4)_2SO_4$ to 35% saturation to precipitate the [^{3}H]progesterone-receptor. In such instances, the receptor, pelleted by centrifugation for 20 min at 12,000 g, was redissolved in the same volume of Tris-TG as the original cytosol.

Preincubation and nuclear-binding assay. Conditions for determining the binding of progesterone receptor to nuclei have been described previously by Buller et al. (11). In our procedure, each experiment consisted of two main parts: preincubation of the [^{3}H]progesterone-receptor, followed by a nuclear-binding assay. In the preincubation, 0.4 ml of cytosol or $(NH_4)_2SO_4$-precipitated receptor, labeled with [^{3}H]progesterone, was incubated at 23° or 0° with 0.15 ml 42% glycerol and 0.1 ml additive. The additive was either H_2O, 10% DMSO, or the phenanthrolines or rifamycins in 10% DMSO. For the nuclear-binding assay, the preincubation solutions were all cooled to 0° and further incubated for 1 hr with 0.2 ml Tris-TG and 0.15 ml purified oviduct nuclei containing 50-100 µg DNA. The nuclei had been prepared and stored according to Method III of Spelsberg et al. (12) (omitting the use of Triton X-100), and they were suspended in a buffer of 0.01 M Tris, 25% glycerol and 0.002 M $MgCl_2$, pH 7.5. At the end of the nuclear incubation, 3.5 ml wash (0.01 M Tris, 10% glycerol, 0.001 M $MgCl_2$ and 0.1% Triton X-100 buffer, pH 7.5) was added to each tube and the nuclei sedimented at 1,000 g for 5 min. Supernatants were aspirated off, the nuclei resuspended in 3.5 ml wash and again recovered by centrifugation. Each nuclear pellet was then suspended in 0.5 ml H_2O and transferred to a scintillation vial. Ten ml of scintillation fluid, consisting of Beckman Biosolv BBS-3, Spectrofluor PPO-POPOP and toluene, 2:1:16 (v/v/v) were added. Radioactivity was determined with 40% efficiency. To measure nuclear binding, experimental points were determined from duplicate or triplicate incubations, and additional replicate incubations were carried out to assess the recovery of DNA. DNA was determined by the method of Burton (13).

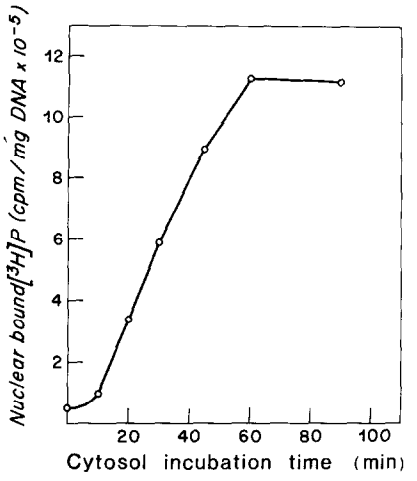

Fig. 1. Formation of activated [^{3}H]progesterone-receptor in cytosol incubated at 23°, showing time-dependence of the process. Preincubation of the cytosol at 23° and assay for nuclear binding at 0° were carried out as described in Materials and Methods.

RESULTS

When chick oviduct cytosol containing [^{3}H]progesterone is heated at 23°, the [^{3}H]progesterone-receptor complex is converted into the form (the "activated" form) that will bind to nuclei when the cytosol is subsequently incubated with oviduct nuclei at 0°. This process is illustrated in Fig. 1 (see also ref. 11). When o-phenanthroline and rifamycin AF/013 were preincubated with samples of cytosol at 23°, the characteristic nuclear binding of the [^{3}H]progesterone-receptor was found to be markedly inhibited. The concentration dependence of this inhibition is shown in Fig. 2.

To rule out the possibility that o-phenanthroline and AF/013 were inhibiting heat-induced receptor activation rather than the binding capability of the activated receptor, further studies were carried out using receptor that had been precipitated with $(NH_4)_2SO_4$. This receptor preparation was in the activated form (11) and could not be further activated by heat (data not shown). Both compounds still inhibited nuclear-binding activity when they were preincubated with this maximally activated receptor preparation (Table I). The effectiveness of o-phenanthroline required an elevated temperature (23°) and

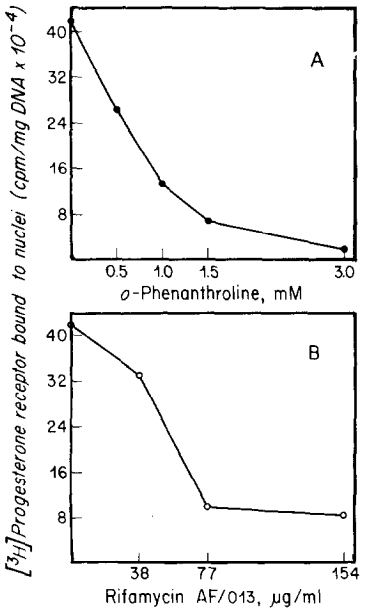

Fig. 2. Inhibition of the nuclear-binding activity of [^{3}H]progesterone receptor in oviduct cytosol when o-phenanthroline or rifamycin AF/013 are preincubated with the cytosol for 45 min at 23°. Preincubation and nuclear-binding assay were as described in Materials and Methods, except that the aliquots of cytosol contained 15 mg protein/ml. Inhibitor concentrations are expressed in units commonly employed by others (1-7).

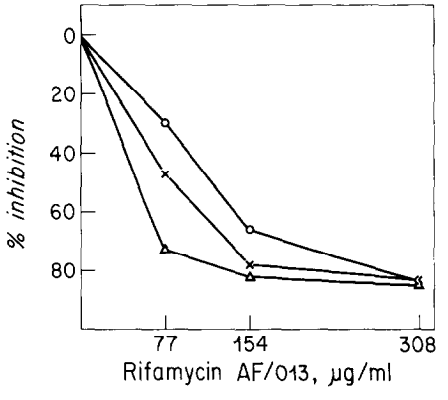

Fig. 3. Rifamycin AF/013 inhibition of [^{3}H]progesterone-receptor binding to nuclei: effect of varying the receptor concentration. Aliquots of [^{3}H]progesterone-receptor, prepared by $(NH_4)_2SO_4$ precipitation and redissolved in Tris-TG buffer, were preincubated with varying concentrations of inhibitor in a final volume of 0.65 ml for 20 min at 0°. 0.1 ml receptor, △——△ ; 0.2 ml receptor, X——X ; 0.4 ml receptor, ○——○ . In the absence of inhibitor, nuclear binding of the receptor complex was directly proportional to the amount of receptor complex in the incubation.

TABLE I. EFFECT OF PHENANTHROLINES AND RIFAMYCINS ON THE NUCLEAR BINDING OF [^{3}H]PROGESTERONE-RECEPTOR PREPARED BY AMMONIUM SULFATE PRECIPITATION*

Additive	Preincubation Temperature (°C)	Nuclear-Bound [^{3}H]P Receptor (% of control) Preincubation time (min) 0**	15	30	60
o-Phenanthroline (3 mM)	0	101	101	100	99
	23	101	50	24	16
Rifamycin AF/013 (154 µg/ml)	0	28	28	27	21
	23	28	13	10	10
m-Phenanthroline (3 mM)	23			100	90
Rifampicin (308 µg/ml)	23			100	100

*Preincubations and nuclear-binding assays were carried out as described in Materials and Methods. Control samples were incubated with 10% DMSO at 0° and 23° for all times shown. The quantity of steroid-receptor complex remaining after preincubation was measured by charcoal assay (14) and correction was made for some breakdown of receptor complex that occurred with AF/013 and m-phenanthroline at 23°.

**Addition was made to the receptor at 0° immediately prior to addition of nuclei.

increased with the time of preincubation, whereas AF/013 produced substantial inhibition (72%) at 0° even when added immediately prior to the start of the nuclear-binding assay.

It is apparent from the preincubation requirement that o-phenanthroline produces its inhibitory effect on the receptor preparation and not on the nuclei. Although the same conclusion for AF/013 is not as apparent, additional studies have shown (Fig. 3) that inhibition due to AF/013 is inversely dependent on the receptor concentration, indicating that AF/013 also acts, directly or indirectly, on the receptor molecule.

Table I shows that the compounds m-phenanthroline, which does not chelate metals or inhibit polymerase activity (1,2) and rifampicin, which is not active against eukaryotic RNA polymerases (4), do not appreciably inhibit the

binding of [^{3}H]progesterone-receptor to nuclei.

The stability of the steroid-receptor complex to o-phenanthroline and AF/013 was examined by means of glycerol gradient centrifugation. Maximally inhibited samples of cytosolic receptor were used (Fig. 4). Although the inhibitors produced a slight loss of steroid from the receptor and may have caused some increase in aggregation, the greater part of the receptor complex in both instances sedimented as the 4S peak characteristic of the heated cytosolic progesterone-receptor complex. With both inhibitors, the amount of receptor complex in the 4S peak represented at least 75% of the control. When amounts of steroid-receptor complex remaining in preincubated cytosol were determined by charcoal assay (14), no decrease was detected in samples containing o-phenanthroline (3 mM), compared to controls, and an approximately 10% decrease was

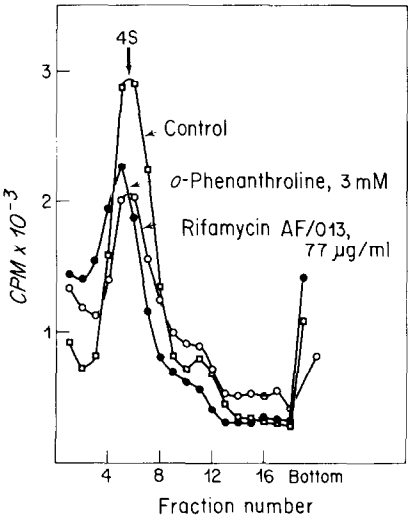

Fig. 4. Sedimentation patterns of cytosolic [^{3}H]progesterone-receptor heated at 23°, with or without inhibitors, and then centrifuged in low-salt glycerol gradients. Samples of preincubated cytosol from the experiment of Fig. 2 were cooled to 0° and diluted 1:2 with Tris-TG buffer to lower the glycerol concentration. Aliquots (0.2 ml) were layered on linear 7-28% glycerol gradients (4.7 ml) prepared with a Beckman Gradient Former from 1% and 40% glycerol solutions in 0.04 M Tris, 0.012 M thioglycerol, 0.01 M KCl buffer, pH 7.5. Gradients were centrifuged for 16 hrs at 149,000 g and 2°C in a Beckman SW 50.1 rotor. [^{14}C]ovalbumin was used as a sedimentation standard (15). Fractionation and tritium analysis have been described previously (14).

found in samples incubated with rifamycin AF/013 (154 μg/ml) (data not shown). Thus, the extent of the observed inhibition of nuclear binding cannot be accounted for by destruction of the progesterone-receptor complex.

DISCUSSION

The present studies show that two polymerase inhibitors, o-phenanthroline and rifamycin AF/013, which interfere in the binding of polymerases to DNA (2-4,6,7) also inhibit the binding of a steroid receptor to nuclei. Two related substances which do not interfere with the binding of polymerases to DNA, i.e., m-phenanthroline and rifampicin* (1,2,4), do not inhibit nuclear binding of the steroid receptor. Although not proven, the observed inhibition appears to involve a direct effect of the inhibitor on the receptor molecule, and suggests that steroid receptors have certain properties in common with polymerases. The question now remains whether other types of DNA-binding proteins are also susceptible to the action of the inhibitors, or whether the results reported here imply a unique relationship between steroid receptors and polymerases.

The finding that o-phenanthroline inhibits receptor binding to nuclei enhances the possibility raised by Shyamala (16), that steroid receptors are metalloproteins. Since most polymerases are zinc-containing enzymes (1), it would be most informative to examine purified preparations of steroid receptors for this metal.

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*Although rifampicin inhibits the initiation reaction of bacterial RNA polymerase, it does not appear to block the binding of that enzyme to DNA (4). Since this is the case, the question of whether rifampicin interacts with the steroid receptor is left open.

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