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Estrogen Receptor Stereochemistry: Ligand Binding Orientation and Influence on Biological Activity

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SUMMARY

Racemic (Rac) 4′- and 5-deoxyindenestrol A (4′-DIA and 5-DIA), monohydroxyl analogs of the diethylstilbestrol (DES) oxidative metabolite indenestrol A (IA), were synthesized, and their enantiomers were resolved and isolated. Each compound was then tested for estrogen receptor (ER) binding affinity, uterotropic activity, and nuclear ER levels, to further define the stereochemical preference of the ER and to structually evaluate the function of each IA hydroxyl group for binding and biological activity. Competitive binding to cytosolic ER determined the relative binding affinity of racemic mixtures of 4′- and 5-DIA as 1.3 and 3.7, respectively, compared with that of DES, 286. The ER exhibited a binding preference for the *S*-enantiomer of both compounds, with relative binding affinities of 4′-DIA-*R*, 0.2; 4′-DIA-*S*, 1.8; 5-DIA-*R*, 0.9; and 5-DIA *S*, 5.6. 4′-DIA-Rac produced 3 times the *in vivo* stimulation of 5-DIA-Rac in the uterotropic bioassay (with mouse uterine doubling doses of 302.4 and 800 μg/kg, respectively). Nuclear ER levels measured 1 hr after *in vivo* treatment with either 160 μg/kg 4′-DIA or 80 μg/kg 5-DIA showed a maximum binding level of 2 (4′-DIA) and 1.5 (5-DIA) times saline control, with these doses producing levels nearly equal to that caused by a 10 μg/kg dose of IA. Metabolic studies were carried out by treating mice with [^{3}H]4′- and [^{3}H]5-DIA-Rac, to determine the differential binding affinity and biological stimulation of 4′-DIA and 5-DIA. The *in vivo* metabolism of the [^{3}H]DIA compounds showed formation of [^{3}H]IA-Rac in urine extracts, as analyzed by chiral high performance liquid chromatography. Furthermore, *in vitro* incubation of unlabeled 4′- and 5-DIA-Rac with mouse liver microsomes showed stereospecific metabolism, with IA-*S* primarily formed from 4′-DIA-Rac and IA-*R* from 5-DIA-Rac. Metabolism of 4′-DIA-Rac to the more active IA *S*-enantiomer and of 5-DIA-Rac to the less active IA *R*-enantiomer contributes to the different biological activities, because the ER exhibits a chiral preference for these compounds. The higher binding affinity of 5-DIA indicates that the phenyl ring hydroxyl group is required for high affinity binding; however, both hydroxyl groups are needed for subsequent biological activity. These data further suggest that the ER demonstrates stereochemical ligand binding and that IA binds in an orientation relative to 17β-estradiol in which the IA phenyl ring corresponds to the estradiol A-ring.

Estrogen hormone action and biological responses in a number of cell or animal models are believed to be mediated in part by binding interactions with an intracellular receptor protein. It is now clear that the receptor functions as a ligand-induced transcription factor (1). Recent evidence (2) indicates that ER binding to specific DNA sequences appears not to be ligand dependent, although the increased transcriptional activity does require ligand binding (3) for stimulation of hormonal responsiveness.

For the past several years, we have investigated the role of the ligand structure in mediating ER hormonal activity in the rodent uterus (4, 5). A series of stilbestrol estrogens have been studied and compared structurally with steroidal estrogen, as a means of segregating the binding activity from the biological response.

Certain derivatives of the symmetrical DES molecule are useful tools for defining the preferred orientation of ligands in the binding site, relative to the position of E_2. It was proposed from studies with E_2 that the 3-hydroxyl group affects binding affinity, whereas the 17β-hydroxyl elicits the biological stimulation (6). However, the binding orientation of certain stilbestrol derivatives, unsymmetrical, high affinity, synthetic estrogens, is unknown (7). Determining which of the two phenolic hydroxyl groups is responsible for binding affinity and which for biological activity would provide additional information about the position of these compounds within the binding site of the receptor protein, relative to the binding orientation of E_2.

Earlier, we studied (4, 6) one such synthetic estrogen, designated IA (Fig. 1), an *in vivo* metabolite of DES oxidative metabolism that has weak biological activity but high ER binding affinity. The phenolic ring of IA was shown to have a high structural correlation (close overlay fit) to the A-ring of E_2. Because the E_2 A-ring hydroxyl group is associated with

ABBREVIATIONS: ER, estrogen receptor; HPLC, high performance liquid chromatography; HAP, hydroxylapatite; IA, indenestrol A; DIA, deoxyindenestrol A; E_2, 17β-estradiol; DES, diethylstilbestrol; Rac, racemic.

binding activity, it was relevant to determine whether the IA phenolic ring hydroxyl has the same function. It was also of interest to evaluate the role of the IA indanyl ring hydroxyl in causing biological activity. Previously, we showed that the IA enantiomers bind to the ER with different affinities, indicating that the ER ligand binding site demonstrates a stereochemical preference (4). The exact location of the IA stereochemical group, in relation to an ER binding domain stereochemical recognition site, may have a further effect on mechanisms of ER action, including nuclear receptor occupancy and biological activity (4).

For this study, two derivatives of IA, namely 4′-DIA and 5-DIA, were synthesized, and the pure individual enantiomers of the two DIAs were separated and isolated. Each compound possesses only one of the two hydroxyl groups present on the IA molecule. These compounds were then used in competitive binding assays and uterine bioassays for determination of the function of each hydroxyl group in ligand binding and resulting biological activity.

Experimental Procedures

Materials. IA, [1-ethyl-2-(4-hydroxyphenyl)-3-methyl-5-hydroxyindene, and 4′- and 5-DIA were prepared by Chemsyn Science Laboratories (Lenexa, KS). [2,4,6,7-^{3}H]E_2 (110 Ci/mmol), with >98% radiochemical purity, and Liquifluor were obtained from Du Pont-New England Nuclear (Boston, MA). Unlabeled *E*-DES, β-glucuronidase, and NADPH were obtained from Sigma (St. Louis, MO). HPLC-grade solvents were obtained from Fisher (Fairlawn, NJ). All other chemicals were reagent grade.

Unlabeled 4′- and 5-DIA were labeled by the catalytic tritiation labeling method by Du Pont-New England Nuclear. After removal of the labile tritium with ethanol, the labeled compounds were further purified by silica gel column chromatography by eluting the 7- × 200-mm column with a solvent mixture of 25% ethyl acetate in hexane. The compounds were further purified by C-18 reverse phase HPLC. The purified products had a specific radioactivity of ~300 mCi/mmol, with >98% radiochemical purity.

HPLC. The 4′- and 5-DIA racemic mixtures were separated and purified into individual enantiomers according to earlier procedures (8). HPLC was performed on an IBM Instruments (Danbury, CT) LC/9533 ternary gradient liquid chromatography system equipped with a variable-wavelength UV detector, set at 280 nm, and a Bakerbond Chiralpak OP(+) column (4.6 × 250 mm; J.T. Baker Co., Phillipsburg, NJ) packed with optically active helical polymer of triphenylmethyl methacrylate-coated silica gel. Radioactivity from the HPLC column was measured with a Radiomatic (Tampa, FL) Flo-One/Beta radiodetector. For preparative HPLC, a solvent mixture of methanol/water (80:20, v/v) was used at a flow rate of 0.5 ml/min. A flow splitter (8:2 ratio) was used to collect fractions.

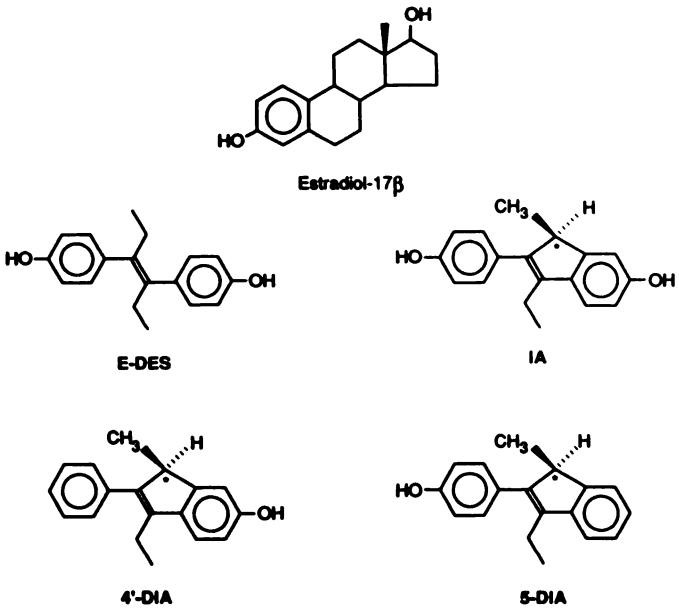

Fig. 1. Structures of E_2, DES, and IA derivatives.

Animal and tissue preparation. Ovariectomized female CD-1 (ICR) BR mice, from Charles River Breeding Laboratories (Wilmington, MA), were kept in a controlled environment with 14 hr of light and 10 hr of darkness. Food and water were given *ad libitum*. After sacrifice of the animals by cervical dislocation at 8 to 10 weeks of age, the uteri were removed and frozen on dry ice. The frozen uteri were then placed in ice-cold TEGM buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, 3 mM $MgCl_2$, pH 7.6) and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) for 15 sec, at setting 6.5, at a ratio of 50:1 (mg of tissue weight/ml of buffer). The homogenate was filtered through 100–125-μm-mesh Nitex filtering media and then centrifuged at 1000 × *g* for 10 min to pellet the nuclei. The supernatant was decanted and centrifuged at 45,000 rpm for 50 min. The nuclear pellet was washed twice with TEGM buffer. After the final wash, TEGM buffer was added to the pellet at a volume equal to the original homogenate volume. The nuclear suspension was then used for nuclear binding assays. The 105,000 × *g* supernatant was used for cytosol receptor binding assays.

Binding assays. The exchange assay to measure the amount of ER in mouse uterine nuclear and cytosol preparations was performed in duplicate assay tubes according to the method of Anderson *et al.* (9), with modifications (10), incorporating 60% HAP as the assay reagent (11). The competitive binding assay was carried out according to our previous analysis (12). Aliquots of 100 μl of cytosol were incubated with 5 nM [^{3}H]E_2 and increasing concentrations of unlabeled competitor (0.5 nM to 5 μM). The mixtures were incubated at 4° for 18 hr, and then 250 μl of 60% HAP in TEGM buffer were added to each tube. Tubes were then centrifuged at 1000 × *g* for 10 min, and the resulting HAP pellet was washed twice with 3 ml of TEGM buffer and then suspended in toluene-based Liquifluor (Du Pont-New England Nuclear). The radioactivity was measured in a Beckman CS 9800 scintillation counter. The C_{50} values were determined using Ligand Competition Analysis software by EMF Software (Baltimore, MD) and represent the molar excess of competitor required to produce a 50% decrease in specific ER binding.

Bioassay. The uterine bioassay was performed as described previously (13). The compounds were dissolved in corn oil, and 100 μl were injected subcutaneously into a group of three mice on 3 successive days. A control group of mice were injected with corn oil. The mice were sacrificed by cervical dislocation on the fourth day. A ratio of uterine weight (mg) per body weight (g) was calculated and then plotted versus dose, to determine the dose of compound required to obtain a doubling of the uterine weight ratio (doubling dose) calculated for control mice.

Measurements of DNA. The amount of DNA was determined by the method of Labarca and Paigen (14), with modifications (15). A 100-μl aliquot of nuclear suspension was mixed with 2.7 ml of 2.2 M NaCl, 100 μl of TEGM, and 100 μl of a Hoechst 33258 (30 μg/ml of H_2O) solution. Fluorescence was measured on a Perkin-Elmer 650-40 fluorescence spectrophotometer (excitation, 356 nm; emission, 458 nm), and DNA concentrations of samples were determined by comparison with a standard curve, with calf thymus DNA used as the standard.

***In vitro* and *in vivo* metabolic assays.** Homogenization and all subcellular fractionation procedures were carried out at 4°. Mouse liver was homogenized with 0.1 M phosphate buffer, using a glass-Teflon homogenizer. Microsomal pellets were obtained from 15,000 × *g* supernatant by centrifugation at 105,000 × *g* for 1 hr. The pellets were washed two times with the same buffer and resuspended in 0.1 M phosphate buffer for incubation. *In vitro* metabolic assays of DIA

compounds were performed with mouse liver microsomes. DIA racemic mixtures and individual enantiomers were incubated for 1 hr at 37° with mouse liver microsomes in the presence of 4 mM of NADPH, in a total incubation volume of 2 ml of 0.1 M phosphate buffer at pH 7.4. The incubation was terminated by extracting the products with three 2-ml aliquots of ethyl acetate. The products were analyzed by chiral HPLC as previously reported (8).

In vivo metabolic assays were carried out with a group of five ovariectomized mice. [^{3}H]4′- or [^{3}H]5-DIA-Rac (2.5 μCi) was injected intraperitoneally. Mice were housed in a metabolic cage for 24 hr and given food and water *ad libitum*. Urine was collected and treated with β-glucuronidase for 6 hr at 37°, and the product was extracted with three 10-ml aliquots of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate, and the solvent was evaporated under vacuum. The products were fractionated with silica gel column chromatography by elution with 25% ethyl acetate in hexane, and the fractions were analyzed by chiral HPLC.

Results

The structures of *E*-DES, IA, and the two DIA molecules are shown in Fig. 1. 4′-DIA has a single hydroxyl group on the indene moiety of the indenestrol structure, whereas 5-DIA has the hydroxyl group on the phenyl ring. Both IA and DIA compounds have the same stereogenic center at the C3-position and exist as a mixture of enantiomers. Individual DIA enantiomers were separated (Fig. 2) using a HPLC Chiralpak column, as previously described for the separation of IA enantiomers (8). Absolute configurations for the IA enantiomers were deduced by X-ray crystal structure analysis (4); the first eluting peak of the DIA enantiomers was assumed to have an *R*-configuration and the second peak from HPLC the *S*-configuration, as previously established for IA.

To determine the effect of the hydroxyl groups of IA on the ER binding mode and biological responses, the binding affinities and biological activities were evaluated (Table 1). Competitive binding experiments with mouse uterine cytosol showed that 5-DIA-Rac (Fig. 3B) bound the ER with a 3 times higher binding affinity (27.0 nM) than 4′-DIA-Rac (75.6 nM) (Fig. 3A). We previously reported (4) that the enantiomers of IA are bound by the ER with different affinities; the IA *R*-enantiomer has a 10–20 times lower binding affinity than the IA *S*-enantiomer. Enantiomer 4′-DIA-*S* (64.4 nM) has a higher binding

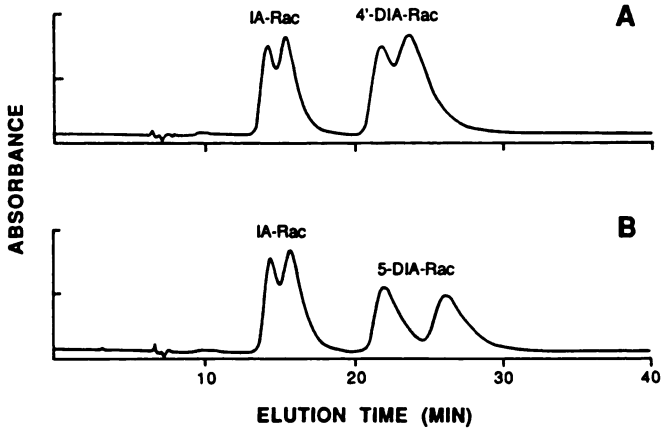

Fig. 2. Chiral HPLC of DIA. A Bakerbond Chiralpak OP(+) column, which was packed with optically active polymer of triphenylmethyl methacrylate-coated silica gel, was used to separate and isolate the optically active individual 4′- and 5-DIA enantiomers. The column was eluted with 20% water/methanol, at a flow rate of 0.5 ml/min. Individual enantiomers were purified from repeated runs and have >98% purity.

TABLE 1

Relative binding affinities and biological activities

Relative binding index was determined by the competitive binding assay and represents the mean of three experiments. Binding affinity for E_2 was set to 100. The uterine doubling dose determined by the uterine bioassay represents the dose required to obtain a uterine wet weight/body weight ratio 2 times that of control. Results are the mean of two experiments.

Compound	Relative binding index	Uterine doubling dose
		μg/kg
E_2	100[a]	5.0 ± 1.0
DES	286[a]	5.0 ± 1.5
IA-Rac	143[a]	126.2 ± 13.5
IA-*S*	286[a]	
IA-*R*	23[a]	
4′-DIA-Rac	1.3 ± 0.30	302.4 ± 13.5
4′-DIA-*S*	1.8 ± 0.56	
4′-DIA-*R*	0.2 ± 0.10	
5-DIA-Rac	3.7 ± 0.13	800.0 ± 7.5
5-DIA-*S*	5.6 ± 0.12	
5-DIA-*R*	0.9 ± 0.30	

[a] Previously reported values from Ref. 4.

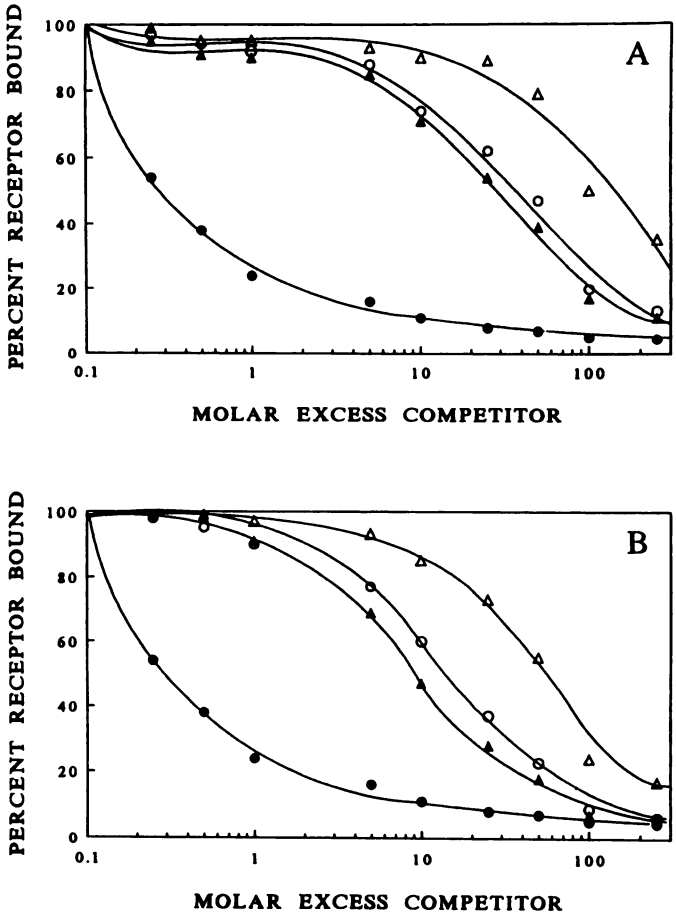

Fig. 3. Competitive binding analysis of 4′- and 5-DIA. A 200-μl aliquot of mouse uterine cytosol was incubated with 5 nM [^{3}H]E_2 and 0.5 nM to 5 μM unlabeled 4′-DIA (A) or 5-DIA (B) compounds. After incubation for 18 hr at 4°, the receptor was precipitated by addition of 60% HAP. The precipitate was washed with buffer, and the radioactivity was counted as described in Experimental Procedures. △, *R*-enantiomer; ○, Rac; ▲, *S*-enantiomer; ●, DES.

affinity than 4′-DIA-*R* (556.0 nM), whereas 5-DIA-*S* (17.7 nM) has a higher binding affinity than 5-DIA-*R* (117.0 nM). Competitive binding studies clearly indicated that the ER maintains a stereochemical binding preference for the *S*-enantiomers of DIA. These data agree with previous observations with the IA compounds (4).

Nuclear ER levels were measured 1 hr after intraperitoneal injection of 4′-DIA and 5-DIA (Fig. 4). Treatment with 80, 120, and 160 μg/kg doses of 4′-DIA caused maximal occupancy in the 120–160 μg/kg range at 1 hr. The 160 μg/kg dose gave a binding level 2 times the saline control level and about half the 472 fmol/100 μg of DNA level produced by a 20 μg/kg dose of DES. A 20 μg/kg dose of IA-Rac occupied 242 fmol of ER/100 μg of DNA at 1 hr. Dose-response experiments with 5-DIA (data not presented) indicated maximum nuclear binding at an 80 μg/kg dose, but values only reached 1.5 times those found in control animals.

Uterotropic bioassays were performed to determine the biological activity of the DIA compounds (Table 1). 4′-DIA produced a biological response approximately 3 times that stimulated by 5-DIA, based on uterine doubling doses of 302.4 and 800.0 μg/kg, respectively. Both of the DIA compounds have weaker biological activity than IA-Rac (126.2 μg/kg).

The ER has a higher binding affinity for 5-DIA-Rac than for 4′-DIA-Rac (5), but 5-DIA has a 2.6-fold weaker biological activity than 4′-DIA, as determined by the uterine bioassay (Table 1). To determine whether the weaker biological activity of 5-DIA was a result of different metabolism of the two deoxy compounds, a series of *in vivo* and *in vitro* metabolic experiments were carried out. Experiments showed that IA could be metabolically formed from DIA. HPLC analysis of the urinary extracts from mice treated with [^{3}H]4′-DIA-Rac and [^{3}H]5-DIA-Rac showed the formation of [^{3}H]IA compound (Fig. 5). When unlabeled 4′- or 5-DIA-Rac was incubated with mouse liver microsomes in the presence of NADPH at 37°, IA was formed (Figs. 6 and 7). As shown in Fig. 6A, the IA *S*-enantiomer is the predominant metabolite when 4′-DIA-Rac is incubated with the microsomes, whereas IA *R*-enantiomer is the major metabolite from 5-DIA-Rac (Fig. 6B). Mass spectral analysis showed that the converted IA has the same molecular weight and ion as the standard IA (data not presented) (16). To determine whether *R*- and *S*-enantiomers of DIA are metabolized to *R*- or *S*-enantiomers of IA, pure *R*-enantiomers of 4′- (Fig. 7A) and 5-DIA (Fig. 7B) were incubated with mouse liver microsomes. No cross-conversion of individual enantiomers was observed. The stereochemistry was maintained, because 4′-DIA-*R* was metabolized to IA-*R* and 5-DIA-*R* was converted into the IA *R*-enantiomer.

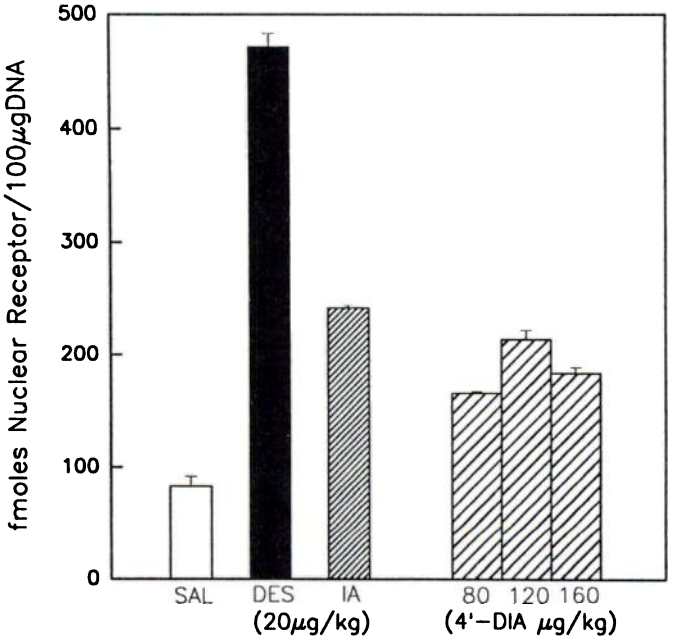

Fig. 4. Nuclear ER levels after treatment with DES, IA-Rac, or 4′-DIA-Rac. Groups of three animals were injected intraperitoneally with varying doses (80, 120, or 160 μg/kg of body weight) of 4′-DIA-Rac, 20 μg/kg DES, or 20 μg/kg IA-Rac and were killed 1 hr later. Nuclear ER were assayed by the exchange assay, as described in Experimental Procedures. Results are expressed as means ± ranges of values from duplicate samples and are representative of three separate experiments. *SAL*, saline control.

Discussion

In this study, we demonstrated the binding mode of mouse uterine ER with DIA, as well as the resulting biological re-

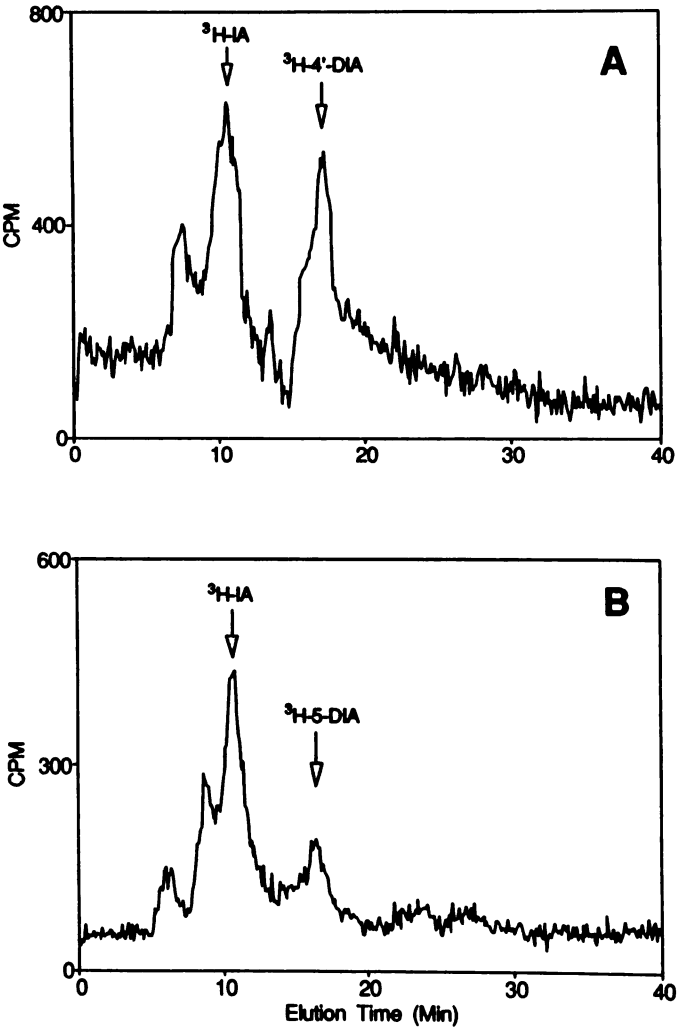

Fig. 5. Formation of [^{3}H]IA from *in vivo* metabolism of [^{3}H]4′-DIA and [^{3}H]5-DIA. Five ovariectomized mice/group were injected intraperitoneally with 2.5 μCi of [^{3}H]4′-DIA-Rac (A) or [^{3}H]5′-DIA-Rac (B). Urine collected during 24 hr was treated with β-glucuronidase for 6 hr at 37° and then extracted three times with ethyl acetate. After drying, evaporation of the solvent, and fractionation by silica gel column chromatography, the samples were analyzed by chiral HPLC.

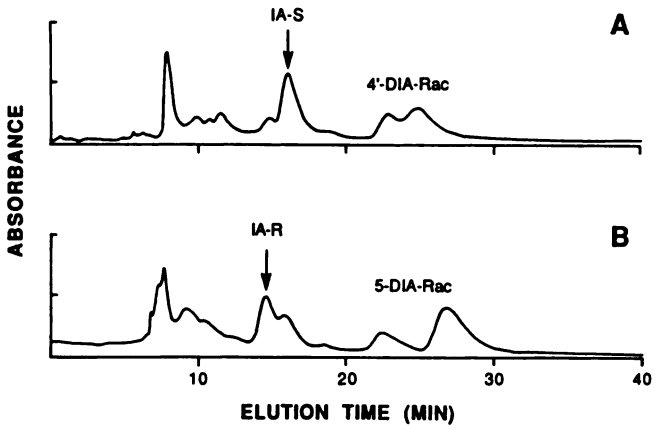

Fig. 6. Formation of IA from *in vitro* metabolism of 4′- and 5-DIA-Rac. Unlabeled 4′-DIA-Rac (A) or 5-DIA-Rac (B) was incubated with mouse liver microsomes in the presence of 4 mM NADPH. After incubation for 1 hr at 37°, the mixture was extracted with ethyl acetate and the extract was fractionated with silica gel column chromatography. Samples were analyzed by chiral HPLC.

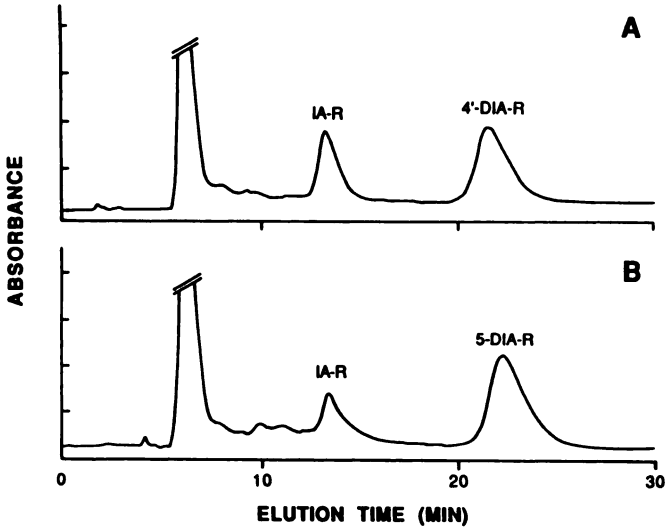

Fig. 7. *In vitro* formation of IA *R*-enantiomer from 4′- and 5-DIA *R*-enantiomers. Unlabeled 4′-DIA-*R* (A) or 5-DIA-*R* (B) was incubated with mouse liver microsomes in the presence of 4 mM NADPH. Samples were analyzed by chiral HPLC.

sponses. Previous studies (5) showed that the ER exhibits stereochemical recognition at the hormone binding site and that the mode of binding is different among the indenestrol derivatives. The fact that 5-DIA has a higher binding affinity for ER than does 4′-DIA (Table 1) indicates that the phenyl ring hydroxyl group of IA is the primary contact region with ER and the indanyl ring is the secondary contact. This finding is consistent with the report by Anstead *et al.* (17) for ER binding activity and binding orientation of hydroxylated 2,3-diarylindenes.

It has been reported (18) that the phenolic hydroxyl group of a steroid is more important for receptor binding than the 17β-hydroxyl group. We previously proposed (4) that IA binds in an orientation relative to E_2 in which the phenolic hydroxyl group of IA corresponds to the hydroxyl group on the E_2 A-ring. In this orientation, the two sets of hydroxyl groups match closely, and the indanyl ring of IA and the D-ring of the steroid show close correlation, with their respective hydroxyl groups deviating only slightly from overlap. The higher binding affinity and lower biological activity of 5-DIA, in comparison with 4′-DIA, demonstrates that the phenolic hydroxyl group confers high affinity binding, whereas the comparison of biological activities of IA and the DIA compounds suggests that the ER needs both hydroxyl groups for a ligand-protein conformation that results in subsequent biological activity. The binding affinity of DIA-Rac is mostly due to the active *S*-enantiomer. This is in agreement with data found for IA, in which a 10–20-fold binding difference exists between the individual IA enantiomers, with the IA *S*-enantiomer having the higher affinity (4).

Although 4′-DIA produced the same level of nuclear receptor occupancy with lower doses than 5-DIA, the levels were quite low, compared with the nuclear receptor occupancy obtained by IA. The biological activity correlates with the amount of nuclear occupancy produced, because nuclear binding is required for biological activity. However, this does not occur with the derivatives of IA (4). Although 4′-DIA produced half the levels of nuclear ER, compared with 5-DIA, it exhibited higher biological activity. Neither compound produced as much biological activity as IA, indicating the requirements of both hydroxyl groups for hormone stimulation.

The differential binding affinity and biological stimulation of 4′-DIA and 5-DIA are explained by the metabolism of these compounds. As shown in Fig. 5, *in vivo* metabolism of both [^{3}H]4′- and [^{3}H]5-DIA resulted in the formation of [^{3}H]IA. Further, as measured *in vitro*, 4′-DIA-Rac is metabolized predominantly to the active IA *S*-enantiomer, and 5-DIA-Rac is metabolized predominantly to the less active IA *R*-enantiomer. The higher biological activity of 4′-DIA-Rac, compared with 5-DIA-Rac, is probably due to stereochemical metabolism of the different DIA compounds to either active or inactive enantiomers of IA. The 5-DIA causes higher nuclear ER levels at one half the dose of 4′-DIA at 1 hr. This occurs probably because the initial higher affinity binding of 5-DIA, which is likely due to the activity of the phenolic hydroxyl group, is not overcome pharmacokinetically by conversion of 4′-DIA to the active IA *S*-enantiomer in only 1 hr. Further, the binding levels and bioactivity of 4′-DIA are not equal to the values seen for pure IA, because as expected not all of the 4′-DIA is metabolized to IA. The increased binding affinity due to the metabolism is not seen in competitive binding assays with mouse uterine cytosol, because the microsomal enzyme system needed for conversion is not present in the cytosol preparations.

This study confirms our earlier finding (4) that ER displays stereochemical ligand preference. The ligand orientation for the stilbene estrogen structure within the binding domain appears to be consistent with the phenolic ring hydroxyl as the initial contact point.

Acknowledgments

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