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SHORT COMMUNICATION

β-Diethylaminoethyl-2,2-diphenylpentanoate (SKF 525-A)-Mediated Translocation of Uterine Estrogen Receptor from the Cytosolic to the Nuclear Compartment in Isolated Rat Uteri

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SUMMARY

The current study examines whether β-diethylaminoethyl-2,2-diphenylpentanoate (SKF 525-A), a recognized inhibitor of microsomal monooxygenase activity, interacts with the uterine estrogen receptor in a manner similar to that of classical estrogens. Incubation of SKF 525-A with isolated uteri from immature rats diminished the levels of cytosolic estrogen receptor and increased the amount of nuclear estrogen receptor. Similar results were obtained with uteri from ovariectomized or ovariectomized and adrenalectomized rats, indicating that the action of SKF 525-A did not depend on the availability of ovarian or adrenal hormones. Additionally, experiments with uterine cytosol from immature rats, employing analysis by Scatchard and Lineweaver-Burk plots, demonstrated that SKF 525-A (0.05 mM) competitively inhibited [^{3}H]estradiol binding to the estrogen receptor, suggesting that both compounds bind to the same site on the receptor. The K_i for SKF 525-A was determined to be 100 μM, indicating that SKF 525-A has a relatively low affinity for the receptor. Further confirmation of this finding was obtained by assessing the relative inhibition of [^{3}H]estradiol binding to uterine cytosol estrogen receptor by SKF 525-A versus that of unlabeled estradiol. The affinity of SKF 525-A for the estrogen receptor appears to be about 0.001% that of estradiol. These studies demonstrate that, like estradiol, SKF 525-A interacts with the estrogen receptor. Additionally, it was concluded that the "estrogenic" (uterotropic) activity observed *in vivo* [Calhoun *et al. Proc. Soc. Exp. Biol. Med.* **136**:47 to 50 (1971)] with SKF 525-A, is mediated through the uterine estrogen receptor.

During the development of a method for assessing whether a compound that is estrogenic *in vivo* is estrogenic per se or is a proestrogen which is metabolically activated, we devised a method involving the simultaneous incubation of a potential proestrogen with rat liver microsomes and immature rat uteri (1). In this method the microsomal monooxygenase activates the proestrogen, and the uterine estrogen receptor serves as a target monitor. When we attempted to use SKF 525-A,[1] a well-known inhibitor of hepatic microsomal monooxygenase activity (2), to block proestrogen metabolism, we observed that SKF 525-A (Fig. 1) per se, in the absence of proestrogen, caused translocation of uterine cytosolic estrogen receptor into the nuclear compartment.

A subsequent review of the literature revealed that SKF 525-A administration increased uterine wet weight (a response considered indicative of estrogenic activity) in both immature and ovariectomized mature rats (3, 4). Multiple administration of SKF 525-A to ovariectomized animals caused an increase in uterine wet and dry weight (3). We reasoned that our serendipitous observation offered an explanation at the molecular level for the uterotropic action of SKF 525-A and therefore initiated the current study.

Immature female Sprague Dawley CD rats (19–22 days old) were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). Animals were used intact or subjected to ovariectomy or adrenalectomy and ovariectomy under ether anesthesia and used 7 days after surgery.

[2,4,6,7-^{3}H]Estradiol (85–110 Ci/mmole) was obtained from Amersham Corporation (Arlington Heights, Ill.) and had a radiochemical purity of more than 98%. SKF 525-A·HCl was a gift from Smith Kline & French Laboratories (Philadelphia, Pa.). Metyrapone [2-methyl-1,2-di-(3-pyridyl)-1-propanone], also a gift, was from Ciba Pharmaceutical Company (Summit, N. J.). Insta-Gel was from Packard Instrument Company (Downers Grove,

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[1] The abbreviations used are: SKF 525-A, β-diethylaminoethyl-2,2-diphenylpentanoate; DCC, dextran-coated charcoal.

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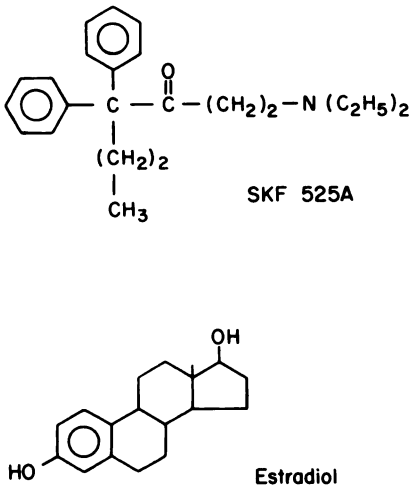

FIG. 1. *SKF 525-A and 17β-estradiol*

Ill.). All other materials were from previously described sources (5). Incubation of isolated rat uteri was by the method of Ruh *et al.* (6). Cytosolic and nuclear estrogen receptor was determined by an exchange with [^{3}H]estradiol using the methodology of Anderson *et al.* (7) and Clark and Peck (8). All modifications were previously described (5). Saturation analysis was carried out by a procedure utilizing DCC as described by Clark and Peck (8) with subsequent analysis by Scatchard plot (9). Calculation of K_i values was as previously described (5). The relative inhibitory potency of SKF 525-A versus unlabeled estradiol toward [^{3}H]estradiol binding to uterine cytosol was determined as described in Fig. 3. The DCC used throughout this investigation contained 10 mM Tris-HCl, 1% charcoal, and 0.05% dextran at pH 8. Radioactivity was determined in a Packard Model 460CD scintillation spectrometer employing the channel's ratio method of quench correction.

As indicated by the significant increase in nuclear receptor, the incubation of immature rat uteri in the presence of 0.05–1.0 mM SKF 525-A or 20 nM estradiol resulted in the translocation of estrogen receptor into the nuclear compartment (Table 1, Experiments 1–5). The higher concentrations of SKF 525-A (0.5–1.0 mM) and 20 nM estradiol also caused a decrease in the amount of cytosolic estrogen receptor. However, in the case of SKF 525-A, the decrease was not always significant (Table 1, Experiments 3 and 4). Even the lowest concentration of SKF 525-A (0.05 mM) caused a significant increase in nuclear receptor concentration (Table 1, Experiment 4), whereas the higher levels of SKF 525-A (0.5–1.0 mM) did not always cause a significant decrease in the concentration of cytosolic receptor (Table 1, Experiment 3 versus Experiment 5). For this reason, elevation of nuclear estrogen receptor concentration is a more sensitive mon-

TABLE 1

Effect of incubation of uteri from immature rats with SKF 525-A or metyrapone on distribution of uterine cytosolic (R_C) and nuclear (R_N) estrogen receptors

For each determination (n = 1), uteri from three rats were incubated in Krebs-Ringer solution at 37° for 1 hr under an atmosphere of O_2. Prior to incubation the uteri were subjected to vigorous bubbling of O_2 through the solution for 2 min. After incubation, the uteri were washed with buffer (10 mM Tris-HCl, 1.5 mM EDTA·2Na, pH 7.4). Methodology for cytosolic and nuclear estrogen receptor determination is cited the text.

Experiment	Compound added[a]	[^{3}H]Estradiol bound[b]		
		R_C	R_N	$R_C + R_N$
			fmoles/uterus	
1	Control (4)	1034 ± 60	94 ± 13	1128 ± 50
	SKF 525-A, 0.5 mM (4)	306 ± 41[c]	317 ± 16[c]	623 ± 49[c]
2	Control (7)	629 ± 31	63 ± 9	693 ± 37
	SKF 525-A, 0.5 mM (3)	179 ± 37[c]	294 ± 21[c]	473 ± 52[d]
	Metyrapone, 0.5 mM (3)	692 ± 39	81 ± 10	774 ± 48
	Estradiol, 20 nM (2)	150 ± 5[c]	498 ± 8[c]	648 ± 14
3	Control (3)	784 ± 112	70 ± 5	854 ± 107
	SKF 525-A, 0.1 mM (3)	953 ± 83	109 ± 6[d]	1062 ± 87
	SKF 525-A, 0.3 mM (3)	911 ± 212	234 ± 28[e]	1145 ± 194
	SKF 525-A, 0.5 mM (3)	630 ± 24	301 ± 20[c]	931 ± 43
	SKF 525-A, 1.0 mM (2)	534 ± 2	422 ± 24[c]	956 ± 23
	Estradiol, 20 nM (1)	208	866	1074
4	Control (7)	1322 ± 94	77 ± 8	1399 ± 86
	SKF 525-A, 0.05 mM (4)	1436 ± 137	105 ± 9[f]	1541 ± 144
	SKF 525-A, 0.10 mM (4)	1096 ± 93	113 ± 7[d]	1209 ± 887
	SKF 525-A, 0.50 mM (2)	1059 ± 62	298 ± 28[c]	1357 ± 35
5	Control (7)	1142 ± 52	90 ± 12	1232 ± 45
	SKF 525-A, 0.5 mM (5)	668 ± 70[c]	403 ± 22[c]	1071 ± 71
	SKF 525-A, 1.0 mM (5)	493 ± 40[c]	495 ± 19[c]	988 ± 50[f]

[a] Number of incubations (n value) in parentheses.

[b] Statistical analysis (experimental versus control) by Student's t-test. In Experiment 3, estradiol was not tested. Values are means ± standard error. Except where otherwise indicated, $p > 0.05$.

[c] $p \leq 0.001$.

[d] $p \leq 0.01$.

[e] $p \leq 0.005$.

[f] $p \leq 0.05$.

TABLE 2

Effect of incubation of uteri from ovariectomized and adrenalectomized rats on distribution of uterine cytosolic (R_C) and nuclear (R_N) estrogen receptor

Uteri were incubated as described in Table 1.

Experiment and surgical treatment	Compound added[a]	[^{3}H]-Estradiol bound[b]		
		R_C	R_N	$R_C + R_N$
			fmoles/uterus	
1. Ovariectomy	Control (7)	797 ± 41	76 ± 19	873 ± 33
	Estradiol, 20 nM (2)	407 ± 27[c]	426 ± 47[c]	837 ± 16[d]
	SKF 525-A, 0.5 mM (5)	522 ± 43[c]	252 ± 17[c]	774 ± 31[d]
2. Ovariectomy and adrenalectomy	Control (4)	861 ± 36	48 ± 6	909 ± 38
	Estradiol, 20 nM (1)	212	474	686
	SKF 525-A, 0.5 mM (3)	360 pm 41[c]	352 ± 10[c]	712 ± 50[e]
3. Ovariectomy and adrenalectomy	Control (4)	1019 ± 25	102 ± 6	1121 ± 26
	Estradiol, 20 nM (1)	392	444	836
	SKF 525-A, 0.5 mM (4)	630 ± 41[c]	350 ± 6[c]	980 ± 40[f]

[a] Number of incubations (n value) in parentheses.
[b] Statistical analysis (experimental versus control) by Student's t-test. Values are means ± standard error.
[c] $p \leq 0.001$.
[d] Not significant ($p > 0.05$).
[e] $p \leq 0.05$.
[f] $p \leq 0.025$.

itor of translocation than is a decrease in cytosolic receptor. Interestingly, incubation of uteri with equal molar amounts (with respect to SKF 525-A) of metyrapone, also an inhibitor of monooxygenase activity (2), failed to bring about translocation of the receptor into the nuclear compartment (Table 1, Experiment 2). Occasionally, the presence of SKF 525-A caused a significant decrease in the amount of total uterine estrogen receptor (cytosolic

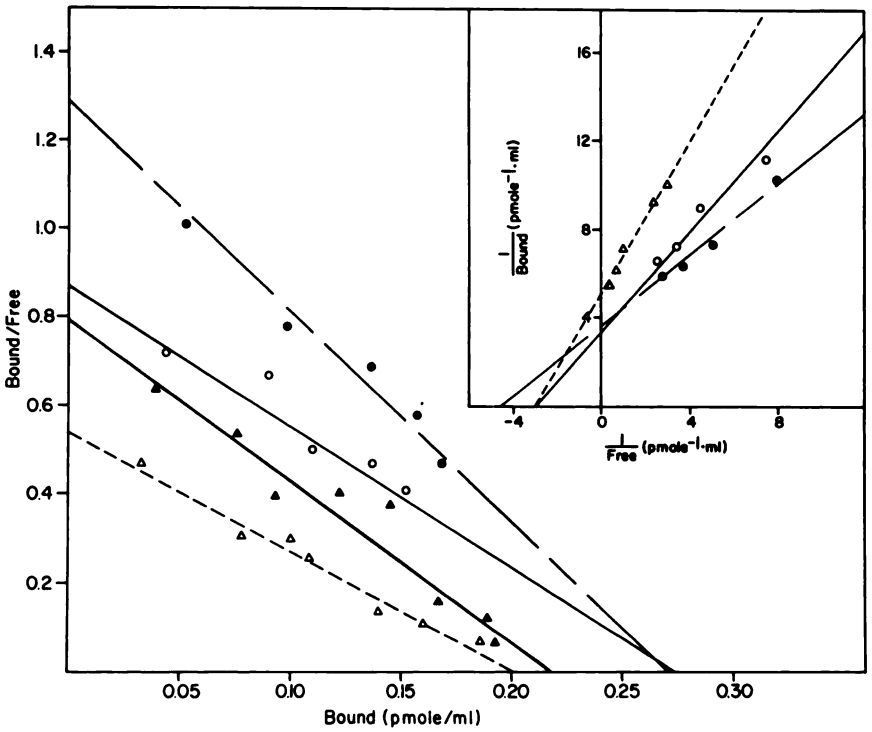

FIG. 2. *Scatchard plot analysis of inhibition by SKF 525-A of [^{3}H]estradiol binding to rat uterine cytosol*

Uterine cytosol (20,000 g) was prepared from immature rat uteri, using one-half uterus per milliliter of buffer (10 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, pH 7.4). To 0.25-ml aliquots of cytosol, SKF 525-A at various concentrations was added in 25 μl of buffer. Subsequently, 15 min later, samples were incubated at 30° for 30 min in the presence of increasing quantities of [^{3}H]estradiol (0.08–2.22 pmoles added in 25 μl of buffer). Final incubation volume was 0.35 ml. Separation of bound from free [^{3}H]estradiol was achieved with DCC (methodology cited in text). The X and Y intercepts were determined by linear regression analysis (correlation coefficients 0.95–0.99). Cytosolic protein concentration, 0.87 mg/ml. *Inset*, Lineweaver-Burk plot of same data points. One point was omitted to save space but was included in the analysis to determine the regression line. The regression line for 0.1 mM SKF 525-A was omitted from the Lineweaver-Burk plot. ●, Control, no SKF 525-A; ○, SKF 525-A, 0.05 mM; ▲, SKF 525-A, 0.10 mM; △, SKF 525-A, 0.30 mM.

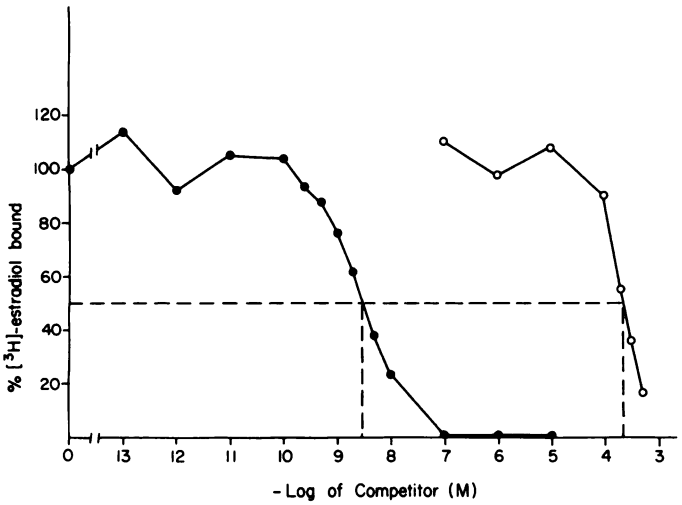

FIG. 3. *Relative potency of SKF 525-A versus unlabeled estradiol with respect to inhibiting the binding of [^{3}H]estradiol to rat uterine cytosol*

Uterine cytosol (20,000 g) was stripped of potential endogenous compounds by treatment with a DCC pellet (equivalent to 2 ml of DCC suspension per uterus) for 20 min at 0°, with vortexing every 10 min. DCC was removed by centrifugation (800 × *g* for 10 min). Cytosol equivalent to one-eighth of a uterus and 0.3 pmole of [^{3}H]estradiol (final volume 0.3 ml) were incubated for 30 min at 37° in the presence of various concentrations of competitor (●, estradiol; ○, SKF 525-A) which was added in 25 μl of 1:1 dimethylformamide/buffer (see legend to Fig. 2 for composition of buffer). Unbound [^{3}H]estradiol was removed by DCC and centrifugation (800 × g for 10 min). Supernatant equal to 0.5 ml was counted in 5 ml of Insta-Gel. Values were corrected for nonspecific binding of [^{3}H]estradiol, with 30 pmoles of diethylstilbestrol. All data points were determined in duplicate.

plus nuclear) when compared with parallel control incubation. However, the loss of total receptor was not readily reproducible (Tables 1 and 2).

In a subsequent series of experiments (Table 2), SKF 525-A (0.5 mM) was incubated in the presence of uteri prepared from ovariectomized or adrenalectomized and ovariectomized animals. In each case, SKF 525-A caused the translocation of estrogen receptor into the nuclear compartment. Receptor translocation was also observed when 20 nM estradiol was used. The translocation of estrogen receptor by SKF 525-A does not appear to depend on endogenous estradiol in the uterus because this effect is observed in uteri from ovariectomized or adrenalectomized and ovariectomized rats (Table 2).

A cell-free system was employed to demonstrate the interaction between SKF 525-A and uterine estrogen receptor. Incubation of uterine cytosol with various nonsaturating concentrations of [^{3}H]estradiol in the presence of increasing concentrations of SKF 525-A revealed two distinct types of inhibition of [^{3}H]estradiol binding to cytosolic estrogen receptor (Fig. 2). The effects of a low concentration (0.05 mM) of SKF 525-A on the binding characteristics of [^{3}H]estradiol are depicted graphically by Scatchard and Lineweaver-Burk plots. In the Scatchard plot, the lines representing no SKF 525-A and 0.05 mM SKF 525-A intersect the ordinate at different points and have a common convergence on the abscissa, indicating that 0.05 mM SKF 525-A diminished the ratio of bound to free [^{3}H]estradiol without altering the number of [^{3}H]estradiol binding sites of receptor protein(s). The diminished ratio of bound to free [^{3}H]estradiol was reflected in a change in the K_D for [^{3}H]estradiol, which increased from 2.10×10^{-10} M (no SKF 525-A) to 3.15×10^{-10} M (0.05 mM SKF 525-A). The values for the binding sites were 0.31 and 0.32 pmole of [^{3}H]estradiol bound per milligram of cytosolic protein for control (no SKF 525-A) and 0.05 mM SKF 525-A, respectively. In the Lineweaver-Burk plot (Fig. 2, *inset*), the lines representing no SKF 525-A and 0.05 mM SKF 525-A have a common convergence on the ordinate and intersect the abscissa at different points, indicating that 0.05 mM SKF 525-A caused a change in the K_D value while the number of binding sites remained constant. From the above data, it was concluded that 0.05 mM SKF 525-A inhibited the binding of [^{3}H]estradiol in a competitive manner. The K_i value (assuming competitive inhibition) for SKF 525-A was calculated to be 100 μM, suggesting that SKF 525-A is a weak inhibitor of estradiol binding and has a low affinity for the cytosolic estrogen receptor. Additionally, we determined that 2.2×10^{-4} M SKF 525-A and 3.1×10^{-9} M unlabeled estradiol inhibited by 50% the binding of [^{3}H]estradiol to cytosolic estrogen receptor (Fig. 3). This indicates that the relative binding affinity of SKF 525-A is about 0.001% that of estradiol. In another experiment (data not shown), similar values were obtained. Also, in that experiment concentrations of 10^{-8} and 10^{-9} M SKF 525-A did not affect [^{3}H]estradiol binding to cytosol. Binding characteristics, similar to those described above, have been reported for other xenobiotic estrogens, such as *o,p'*-DDT and Kepone (5, 10, 11). The line on the Scatchard plot (Fig. 2) representing 0.3 mM SKF 525-A demonstrated, when compared with the line representing no SKF 525-A, a diminished ratio of bound to free [^{3}H]estradiol ($K_D = 3.7 \times 10^{-10}$ M) and a decrease in the number of binding sites (0.23 pmole of [^{3}H]estradiol bound per milligram of cytosolic protein). A similar effect (Fig. 2) was observed for the intermediate concentration of 0.1 mM SKF 525-A ($K_D = 2.8 \times 10^{-10}$ M and 0.25 pmole of [^{3}H]estradiol bound per milligram of protein). On the basis of these data and the Lineweaver-Burk plot it was concluded that 0.1–0.3 mM SKF 525-A inhibition of [^{3}H]estradiol binding was of the noncompetitive "mixed" type.

We concluded that the observed competitive inhibition reflects the ligand (SKF 525-A)-receptor interaction responsible for the translocation *in vitro* of estrogen receptor demonstrated in this study. However, the significance, if any, of the *noncompetitive* inhibition can only be conjectured. It is tempting to speculate that, at the higher concentrations, SKF 525-A interacts with the receptor at a site other than the one normally occupied by an estrogen and changes the receptor architecture, which disrupts the normal binding process between receptor and ligand, and that this change is reflected in the occasional loss of estrogen receptor observed in the receptor translocation experiments (Tables 1 and 2). In fact, Mueller and Kim (12) and Mueller (13) have reported the displacement of [^{3}H]estradiol bound to rabbit uterine receptor by certain xenobiotics, presumably by binding to an allosteric site. However, the relationship

between this phenomenon and our observation remains to be established. Clearly, additional studies are necessary to clarify the situation.

In summary, we conclude that SKF 525-A interacts with the rat uterine estrogen receptor, causing its translocation into the nuclear compartment, and that this event is responsible for the estrogenic activity of SKF 525-A observed *in vivo*. Given the structural diversity of compounds with estrogenic characteristics that have been identified in recent years (14), the emergence of SKF 525-A as a representative of another structural class of estrogens or antiestrogens is not a total surprise.

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