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Dynamic Pattern of Estradiol Binding to Uterine Receptors of the Rat

INHIBITION AND STIMULATION BY UNSATURATED FATTY ACIDS*

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The binding of estradiol to uterine cytosoluble receptors from 24-day-old rats was reduced or potentiated by unsaturated fatty acids (NEFAs), depending on the concentrations of estradiol and unsaturated NEFAs. At estradiol concentrations of up to 1.5×10^{-8} M, unsaturated NEFAs inhibited estradiol binding to the 8 S cytosol receptor. This inhibition was dose-dependent (10–70%, $p < 0.001$) and a function of NEFA unsaturation. Scatchard analysis indicated that unsaturated NEFAs caused a large decrease in receptor affinity for estradiol. Polyunsaturated NEFAs had no apparent effect on estradiol binding at estradiol concentrations of $2–4 \times 10^{-8}$ M. At high estradiol concentrations (above 4×10^{-8} M), estradiol binding was increased 130–250% ($p < 0.01$) by polyunsaturated NEFAs. This increased binding was particularly associated with proteins sedimenting at 12.5 S and the 8 S binding was, in fact, reduced. Metabolic studies showed that the reduced binding in the presence of unsaturated fatty acids was correlated with a decrease in reversibly bound estradiol at low estradiol concentrations. The increase in estradiol binding at high estradiol concentrations is the result of a reduction in reversibly bound estradiol and an increase in nonorganic solvent-extractable (water-soluble) estradiol. The amounts of these water-soluble estradiol derivatives depended on both estradiol and unsaturated NEFA concentrations. 70% of the water-soluble estradiol derivatives were trichloroacetic acid-precipitable, suggesting a covalent protein-steroid link. Thus, changes in the hydrophobic fatty acid environment of the uterine cytosol estrogen receptor could modify estrogen-receptor function by altering binding site conformation and/or by inducing changes in estradiol metabolism.

There is now considerable evidence that nutritional status can influence the production, excretion, and metabolism of estrogens, hormones which play a major role in the pathogenesis of human breast and endometrial cancers (1, 2). Most epidemiological data suggest that there is a relationship between a high fat diet and the incidence of these cancers (3–9), and the results of animal experiments also indicate that dietary fat may be involved in the promotion of breast tumorigenesis (10, 11).

Recent studies have shown that both the transport in the plasma and the intracellular activity of steroid hormones may be modulated by nonesterified fatty acids (NEFAs).[1] Unsaturated fatty acids strongly inhibit the binding of steroids to several plasma proteins, such as murine α-fetoprotein (12–15), human sex steroid-binding protein (16), corticosteroid-binding globulin (17), while saturated fatty acids potentiate such binding. These changes in steroid binding capacities may account for the apparent connection observed between high free circulating levels of hormone, obesity, and cancer (18–20).

Other studies suggest that NEFAs may influence cell growth and multiplication by modifying membrane fluidity, with resulting changes in enzymatic and receptor activities (21, 22). Thus, 17 β OH dehydrogenase, the key enzyme in estrogen metabolism, is inhibited by unsaturated fatty acids (23). The binding of estradiol to human uterine, breast, and melanoma tissues has also been reported to be dramatically potentiated by unsaturated fatty acids. This potentiation seems to be due to the irreversible binding of estradiol to uterine proteins in the presence of unsaturated fatty acids (24, 25). Finally, studies on rats, using an estradiol concentration of 10^{-8} M, indicate that unsaturated fatty acids inhibit the reversible binding of estradiol to uterine receptors. The inhibition was found to be dependent on both the concentration and the degree of unsaturation of the fatty acid used (14, 26, 27). However, the above studies were carried out with a single concentration of estradiol and there is no indication of the way in which estrogen concentration and the ratio between estradiol and fatty acids may influence such binding.

This present report contains the results of a detailed study of the influence of saturated and unsaturated fatty acids on the binding of estradiol to the uterine cytosoluble receptors of the 24-day-old rat. Attention is focused on the role of estradiol concentration and the estradiol/NEFA ratio in the modulation of estradiol binding to uterine proteins. The data indicate that, depending on the estradiol/NEFA ratio, there may be an apparent decrease in the reversible binding of estradiol to uterine protein and/or a dramatic increase in irreversible binding of estradiol to uterine protein.

MATERIALS AND METHODS

Reagents

6,7-^{3}H-Estradiol-17β (41 Ci/mmol), purchased from Amersham International Ltd., was purified by chromatography on celite using an isooctane/ethyl acetate/methanol/water system (17:3:7:3). The radioactive estradiol eluting between 5 and 6 retention volumes was used in experiments.

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[1] The abbreviations used are: NEFAs, non-esterified fatty acids; DES, diethylstilbestrol; DCC, dextran-coated charcoal.

Unlabeled Compounds—Estradiol and diethylstilbestrol were supplied by Roussel Uclaf Research Centre (Romainville, France). The following saturated and unsaturated fatty acids were purchased from Sigma: tetradecanoic acid (C14:0, myristic acid), hexadecanoic acid (C16:0, palmitic acid), *cis*-7-hexadecanoic acid (C16:1, palmitoleic acid), *cis*-9-octadecanoic acid (C18:1, oleic acid), 9,12-octadecadienoic acid (C18:2, linoleic acid), 6, 9, 12-octadecatrienoic acid (C18:3, linolenic acid), 5,8,11,14-eicosatetraenoic acid (C20:4, arachidonic acid), 4,7,10,13,16,19-docosahexaenoic acid (C22:6) and 7,10,13,17-docosatetraenoic acid (C22:4). NEFA purity was checked by thin layer chromatography (benzene/methanol/acetic acid, 96:4:1, v/v). Stock solutions of NEFAs (1 mg/ml) were prepared, using hexane for unsaturated fatty acids and chloroform for saturated fatty acids.

Tissue Preparation

Female CD rats (24-days old, Charles River) were lightly anesthetized. The uteri were rapidly removed, any fat trimmed off, rinsed, and stored frozen in liquid nitrogen until processed. Uteri were suspended in TE buffer (50 mM Tris-HCl, 1.5 mM EDTA, 20 mM molybdate, pH 7.4) and homogenized in an all-glass homogenizer (Kontes, Vineland, NJ). The homogenate was centrifuged at 105,000 × *g* for 1 h, and the resulting supernatant, referred to as "cytosol," was used in the binding experiments. All manipulations were carried out at 4 °C.

Binding Studies

Incubations—Uterine cytosol was diluted with TE buffer to give a final concentration of 2 mg of protein/ml. Aliquots (0.5 ml) of this solution were incubated overnight at 4 °C with a range of labeled estradiol concentrations (0.1–7.5 × 10^{-8} M), either alone, or in the presence of varying concentrations of NEFAs (0.1–6 × 10^{-4} M). NEFAs and estradiol were dried under nitrogen and cytosol immediately added. Nonspecific binding was determined using a 200-fold excess of unlabeled diethylstilbestrol (DES) and was about 5% in the absence of fatty acids or in the presence of saturated fatty acids, 12% in the presence of 3 × 10^{-4} M unsaturated fatty acids, 20% in the presence of 6 × 10^{-4} M unsaturated fatty acids. The fatty acid solubilities were checked and found to be 80% (saturated fatty acids) and 95% (unsaturated fatty acids) in the presence of 2 mg/ml cytosoluble proteins over the whole range of fatty acid concentrations used (0.1–6 × 10^{-4} M).

Dextran-coated Charcoal (DCC) Method

Bound and free hormone fractions were separated by incubation with a suspension of DCC (0.5% charcoal, 0.05% dextran, v/v) for 2 h at 4 °C, and centrifugation at 3000 rpm for 10 min. Aliquots of supernatant were counted in 4 ml of PCSII (Amersham International) scintillant in a Kontron S4000 scintillation counter. The system was checked to ensure that all free hormone was extracted by DCC treatment, even at the highest NEFA concentration.

Density Gradient Centrifugation

Cytosol protein was incubated overnight at 4 °C with 0.4 × 10^{-8} M, 2.5 × 10^{-8} M and 4 × 10^{-8} M tritiated estradiol, either alone or in the presence of various non-radioactive fatty acids. Nonspecific binding was estimated by carrying out parallel centrifugations in the presence of a 200-fold excess of unlabeled DES. Unbound estradiol was removed by DCC treatment, the cytosol mixtures were layered onto 5–20% sucrose gradients and centrifuged at 60,000 rpm for 77 min in a Kontron TV865 rotor. Fractions were collected from the top of the gradient using an autoDensiFlow IIc (Haakebuchler) fraction collector. 0.15 ml of each fraction was mixed with scintillation fluid and the radioactivity counted. Density gradients were calibrated with horseradish peroxidase (3.6 S) and Aspergillus niger glucose oxidase (7.9 S) markers according to the method of Martin and Ames (28).

Estrogen Metabolite Analysis

Bound and free steroids were separated with DCC and the aqueous bound fractions extracted with organic solvent (cyclohexane/ethylacetate, 1:1, v/v) as previously described (25). Aqueous and organic phases were separated by freezing at −20 °C.

Organic Phase—The solution was evaporated to dryness and the organic solvent-soluble metabolites were separated by thin layer chromatography in benzene/methanol/acetic acid (96:4:1). Estrone, estradiol, and C22:6 were used as standards. Bands of gel (1-cm wide) were scraped off and their radioactivity measured in 5 ml of PCS II.

Aqueous Phase—Water-soluble metabolites were incubated overnight with Helix pomatia digestive juice at pH 5.3 and the steroid fraction was extracted with ether. The radioactivity in the ether extracts was measured. The remaining aqueous solution was made 10% in trichloroacetic acid and the protein left to precipitate overnight at 4 °C. The precipitate was collected by centrifugation, washed 3 times with 10% trichloroacetic acid, and dissolved in 0.5 ml of 1 N NaOH. Aliquots of both the precipitate and the aqueous supernatant were counted for radioactivity. Bound radioactivity corresponding to estradiol and its metabolites is expressed as fmole estradiol equivalents per milligrams of protein.

Fatty Acid Analysis

Fatty acids were extracted from the cytosol with cyclohexane/ethyl acetate (1:1) and quantified by gas chromatography as previously described (29).

Estrone and Estradiol Assay

Estrone and estradiol were assayed by radioimmunoassay using specific anti-estrone (6-thyroglobulin serum) and anti-17-β-estradiol (6-BSA serum) antibodies purchased from Miles Yeda (Israel).

Total Protein Assay

Proteins were measured by the method of Lowry *et al.* (30).

Statistical Analyses

Data were analyzed using Student's *t* test.

RESULTS

Differential Effect of NEFAs on the Binding of Estradiol to Uterine Cytosol Proteins

Endogenous Estrogen and NEFA Concentrations in Uterine Cytosol—The endogenous NEFA concentration of the uterine cytosol of 24-day-old rats, determined by gas chromatography, was 83 ± 20 × 10–^{6} mol/g soluble protein, *i.e.* 1.6 × 10^{-4} M (M = mole/liter of cytosol); 45% were saturated fatty acids (C14:0, C16:0, C18:0) 31% were unsaturated fatty acids (C16:1, C18:1), and 23% were polyunsaturated (C18:2, C18:3, C20:4, C22:6). The estradiol concentration, determined by radioimmunoassay, was 0.5 × 10^{-9} M. Thus, the ratio of free fatty acids to estrogen was 3 × 10^{5}. On the basis of these data, all binding studies were performed with the addition of exogenous NEFAs and estradiol which produced NEFA/estradiol ratios between 0.5 × 10^{4} and 1 × 10^{5}.

Dose-dependent Effect of NEFA on Estradiol Binding—The effect of increasing concentrations of saturated and unsaturated fatty acids on the binding of estradiol to cytosol protein was examined at two estradiol concentrations, 0.7 × 10^{-8} M (Fig. 1) and 5.6 × 10^{-8} M (Fig. 2). Estradiol binding to uterine estrogen receptor in the presence of different classes of fatty acids was measured and compared to that of estradiol binding in the absence of fatty acid (100%). All data shown are for specific binding (*i.e*, after subtraction of nonspecific binding).

Fig. 1 shows that, for estradiol = 0.7 × 10^{-8} M, the binding of estradiol was unaffected by saturated fatty acid (C18:0). The effect of unsaturated fatty acids on estradiol binding was dose-dependent: there was a small, but reproducible, increase in estradiol binding at very low unsaturated fatty acid concentrations (0.5 × 10^{-4} M); between 0.5 × 10^{-4} M and 3 × 10^{-4} M fatty acid, there was a dose-dependent inhibition of estradiol binding; above 3 × 10^{-4} M, this dose-dependence was no longer observed. Binding was reduced to 50% of control values in the presence of 1.2 × 10^{-4} M polyunsaturated NEFAs (C20:4 and C22:6) and in the presence of 1.8 × 10^{-4} M monoenoic fatty acids (C16:1, C18:1). Estradiol binding to cytosol was reduced by 80% at NEFA (oleic and docosahexaenoic acids) concentrations above 3 × 10^{-4} M, but by only

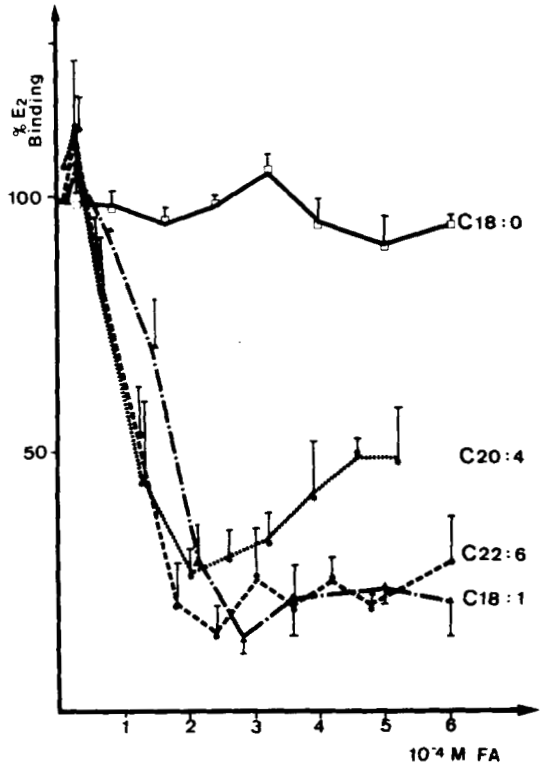

FIG. 1. **Effect of increasing NEFA concentrations on estradiol (E_2) binding to uterine cytosol in the presence of 0.7 × 10^{-8} M estradiol.** 0.5 ml of cytosol (1 mg of protein) was incubated overnight at 4 °C in the presence of 0.7 × 10^{-8} M [^{3}H]estradiol alone or together with increasing fatty acid concentration (*FA*) (0.1–6 × 10^{-4} M). Free estradiol was removed with DCC; specific binding was calculated after subtraction of nonspecific binding. The results are plotted as % of control (estradiol binding in absence of NEFAs) *versus* NEFA concentrations. Means ± S.D. of five to six experiments for each fatty acid.

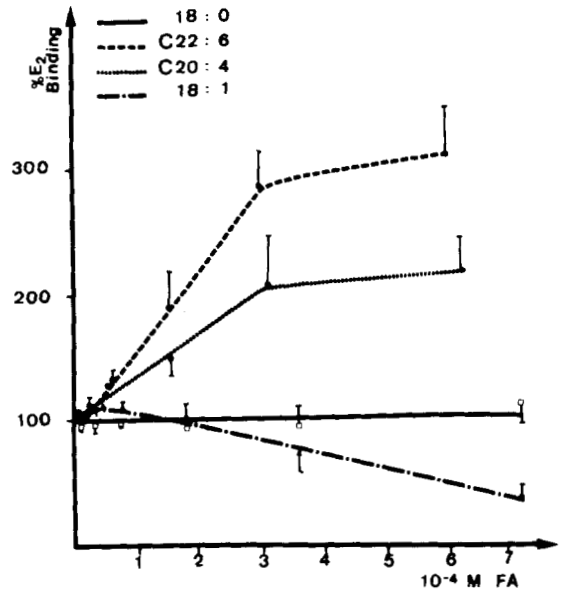

FIG. 2. **Effect of increasing NEFA concentration on estradiol (E_2) binding to uterine cytosol in the presence of 5.6 × 10^{-8} M.** 0.5 ml of cytosol (1 mg of protein) was incubated overnight at 4 °C in the presence of 5.6 × 10^{-8} M [^{3}H]estradiol alone or together with increasing fatty acid concentrations (*FA*) (0.1–6 × 10^{-4} M). Free estradiol was removed with DCC. Specific binding was calculated after subtraction of nonspecific binding. The results are plotted as % of control (estradiol binding in absence of NEFAs) *versus* NEFA concentrations. Means ± S.D. of five to six experiments for each fatty acid.

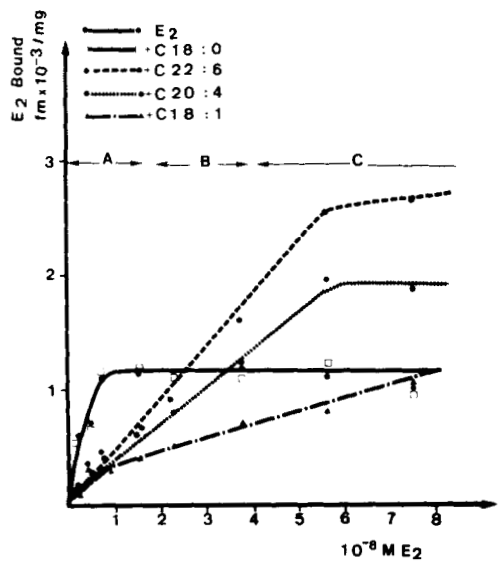

FIG. 3. **Differential effect of NEFAs on the specific estradiol (E_2) binding to uterine cytosol proteins as a function of estradiol concentration.** One-half milliliter of uterine cytosol (1 mg of protein) was incubated overnight at 4 °C with increasing [^{3}H]estradiol concentrations (0.25–8 × 10^{-8} M) in the absence or presence of 3 × 10^{-4} M of C22:6, C20:4, C18:1, C18:0. Specific estradiol binding was determinated after subtraction of nonspecific binding (measured in presence of an excess 200-fold of unlabeled DES); free hormone was removed by the DCC method. The results are expressed as fmoles of [^{3}H]estradiol bound per milligram of cytosol proteins. Data are from a single experiment representative of a total of three experiments for each fatty acid.

50% in the presence of the same concentration of arachidonic acid.

Fig. 2 shows that, at high estradiol concentration (5.6 × 10^{-8} M), the pattern of estradiol binding to cytosol proteins in the presence of increasing NEFA concentrations was quite different. Saturated fatty acids had no effect at the concentrations tested. All the unsaturated fatty acids caused a slight increase in binding at very low fatty acid concentrations (0.5 × 10^{-4} M). Above this concentration, monounsaturated fatty acids (C18:1) was inhibitory, while increasing concentrations of polyunsaturated fatty acid, up to 3 × 10^{-4} M, increased estradiol binding to cytosol. The greatest increase was seen with C22:6 (290%). Above 3 × 10^{-4} M, the polyunsaturated fatty acid-induced increase in binding remained essentially constant.

Effect of NEFAs on Estradiol Binding as a Function of Estradiol Concentration—The inflexion point, the concentration of estradiol at which the effects of fatty acids on estradiol binding changed from inhibitory to stimulatory, was studied by examining estradiol binding in the presence of fixed concentrations of various fatty acids (Fig. 3). At estradiol concentrations equal to or below those required to saturate estradiol-binding sites (1.5 × 10^{-8} M), polyunsaturated fatty acid (C22:6, C20:4) inhibited the specific binding of estradiol by 10–70% ($p < 0.001$) (*position A*). At concentrations of estradiol (2–4 × 10^{-8} M, Fig. 3, *position B*), polyunsaturated fatty acids had no apparent effect on estradiol binding, while at higher estradiol concentrations (above 4 × 10^{-8} M, Fig. 3, *position C*), the binding of estradiol to cytosol protein was greatly increased (130–250%) and reached a plateau in the presence of polyunsaturated fatty acids ($p < 0.01$). Estradiol

binding was lower than control values at all estradiol concentrations in the presence of the monounsaturated fatty acid, oleic acid (C18:1). However, the difference between estradiol-cytosol binding in the presence and absence of oleic acid was progressively reduced with increasing estradiol concentrations. The Scatchard plot (Fig. 4) indicates that the number of binding sites was not significantly affected, while the apparent affinity constant of estradiol binding to cytosol was dramatically reduced from control values in the presence of fatty acid.

Fig. 5, *A–C*, shows the results of sucrose (5–20%) density gradient analysis of estradiol binding to uterine protein in the presence of constant fatty acid concentration. Three strategic estradiol concentrations were chosen: *A*, 0.4×10^{-8} M (where estradiol binding was decreased by unsaturated fatty acid); *B*, 2.25×10^{-8} M (where estradiol binding was apparently unaffected by unsaturated fatty acid); *C*, 5.4×10^{-8} M (where estradiol binding was potentiated by unsaturated fatty acid).

Estradiol was bound principally to a protein fraction sedimenting at 8 S at all three estradiol concentrations tested (0.4×10^{-8} M, 2.25×10^{-8} M, and 5.6×10^{-8} M) in the absence of fatty acids (Fig. 5). This binding was abolished when C22:6 was added to the lowest estradiol concentration (Fig. 5*A*). The 8 S peak was reduced at the intermediate estradiol concentration in the presence of C22:6, and a second binding peak was observed at 12.5 S (Fig. 5*B*). At the highest estradiol concentration tested, addition of C22:6 did not decrease binding to the 8 S receptor but mainly enhanced binding to the 12.5 S region (Fig. 5*C*).

Analysis of the Estradiol Metabolites Bound to Cytosol Proteins in the Presence of Unsaturated Fatty Acids

The binding of estradiol to target tissue receptors was generally reversible and almost all (90%) of the bound steroid

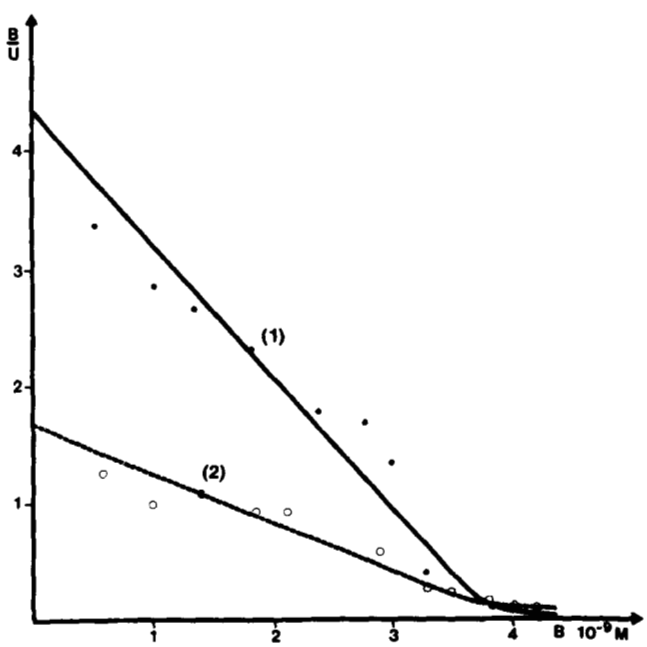

FIG. 4. **Scatchard (48) plots for the interaction of estradiol 17-β in absence** (*curve 1*) or in presence (*curve 2*) of docosahexaenoic acid (0.6×10^{-4} M). Experimental conditions were as in Fig. 1. Estradiol assay contained 1 mg of uterine cytosol proteins and 0.08 $\times 10^{-8}$ M to 7.6×10^{-8} M of estradiol. Each point is the mean value of duplicate assay. B represents bound estradiol; *U* represents unbound estradiol. Graphic Rosenthal correction (not shown) (49) was used to calculate the estradiol associations constant and number of binding sites, $K_1 = 1.1 \times 10^9$ M^{-1}; $n_1M_1 = 3 \times 10^{-9}$ M without NEFA. $K_2 = 0.3 \times 10^9$ M^{-1}; $n_2M_2 = 2.8 \times 10^{-9}$ M with NEFA.

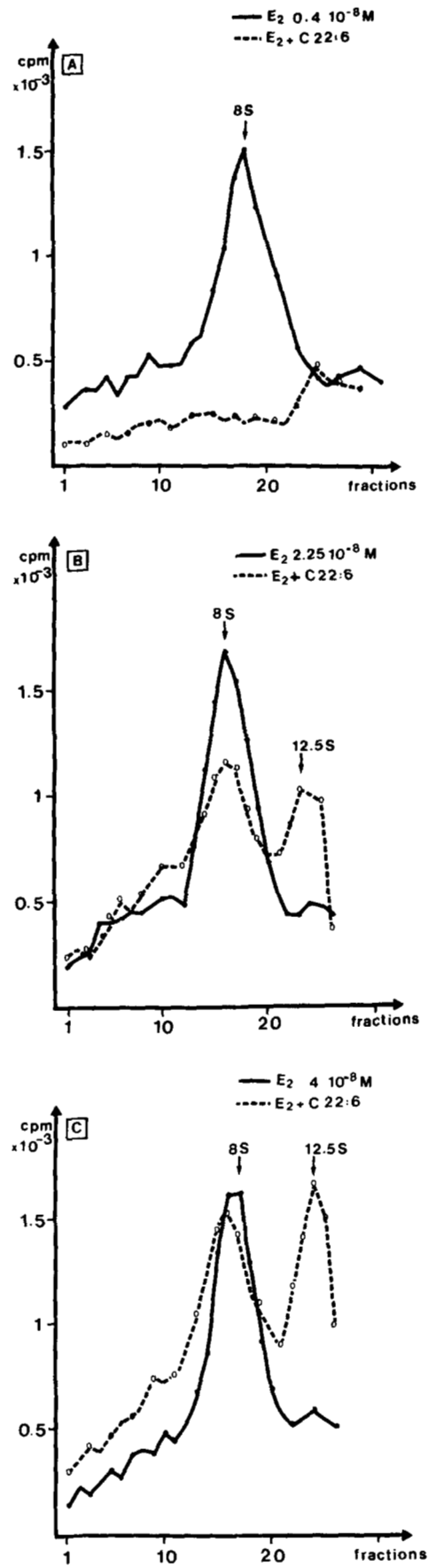

FIG. 5. **Density gradient analysis of the effect of the fatty acid, docosahexaenoic acid (C22:6) on estradiol (E_2) binding**

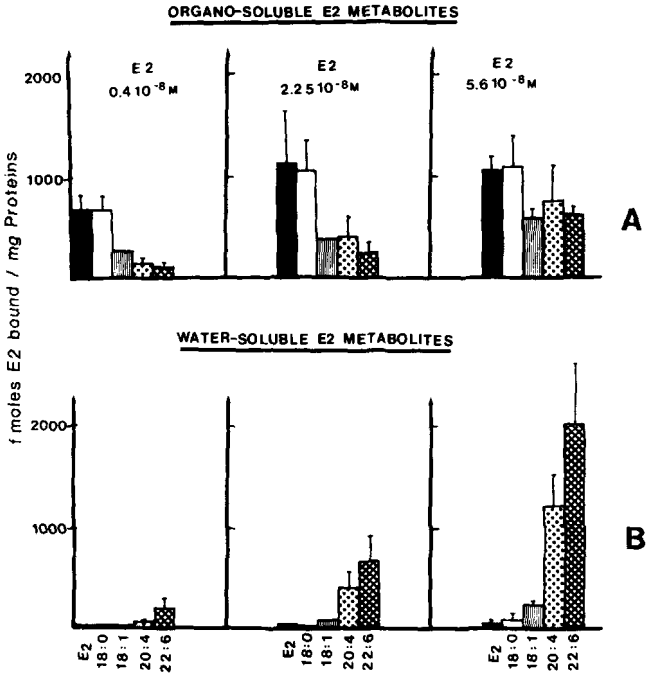

FIG. 6. ***A* and *B*, analysis of estradiol (E_2) metabolites bound to uterine cytosol proteins.** The experimental conditions of incubation were the same as described in Fig. 3. Separation or organic solvent-soluble and water-soluble fractions was outlined under "Materials and Methods." The results are expressed as fmoles of estradiol equivalents of [^{3}H]estradiol per milligram of protein. Means ± S.D. of triplicate experiments for each cytosol preparation are shown.

could be extracted with organic solvent (Fig. 6*A*), although traces of radioactive estradiol metabolites (<10%) remained in the aqueous phase (Fig. 6*B*) at all the estradiol concentrations tested.

Virtually all bound estradiol was organic-solvent extractable in the presence of saturated fatty acids, at all estradiol concentrations tested (Fig. 6, *A* and *B*).

Organic solvent-extractable estradiol accounted for less than the total estradiol bound whenever unsaturated fatty acids were present in the mixture. At an estradiol concentration of 0.4×10^{-8} M, the decrease in estradiol binding in the presence of unsaturated fatty acid was due, essentially, to a decrease in organic solvent-extractable material; a small increase in water-soluble estradiol metabolite, especially with C22:6, was observed. At the intermediate estradiol concentration tested (2.5×10^{-8} M), where the unsaturated fatty acids had no apparent effect on estradiol binding as determined by DCC treatment, there was a decrease in organic solvent-extractable estradiol and a significant increase in water-soluble estradiol metabolites. In the extreme case, at an estradiol concentration of 5.6×10^{-8} M in the presence of C22:6, the organic solvent-extractable estradiol accounted for less than 25% of the total estradiol bound. The increases in estradiol binding induced by unsaturated fatty acid were essentially due to a dramatic increase in water-soluble estradiol derivatives.

to uterine cytosol in presence of (*A*) [^{3}H]estradiol 0.4×10^{-8} M, (*B*) [^{3}H]estradiol 2.25×10^{-8} M, and (*C*) [^{3}H]estradiol 4×10^{-8} M. An 0.5-ml aliquot of cytosol (1 mg of protein) was incubated overnight at 4 °C with each [^{3}H]estradiol concentration, either alone or in the presence of 3×10^{-4} M of C22:6. The reacted cytosol was then layered onto a 5–20% sucrose gradient and centrifuged at 60,000 rpm for 1 h and 17 min in a (Kontron) TV 865 rotor. Fractions were collected and their radioactivity counted. The data shown are net counts per minute after subtraction of nonspecific binding values.

Thin layer chromatography analysis of the organic solvent extracts from experiments performed at 2.25×10^{-8} M and 5.6×10^{-8} M estradiol showed essentially unmetabolized estradiol (95%) in the absence of fatty acids. 80% of unmetabolized estradiol was found in the presence of fatty acids; 8% of the estradiol metabolites were more polar than estradiol and 12% were less polar than estradiol, *i.e* with polarities close to those of estrone and estradiol fatty acid esters.

Helix pomatia digestive juice hydrolysis of the water-soluble estradiol derivatives showed that sulfate and glucuronide conjugates accounted for only 10% of the aqueous phase. Most (70%) of the water-soluble estradiol derivatives were trichloroacetic acid-precipitable.

Analysis of the estradiol metabolites bound to 8 and 12.5 S cytosol species after density gradient fractionation of a high concentration estradiol incubate (4.1×10^{-8} M) is presented in Table 1. The data, expressed on the basis of the protein concentrations in the 8 S region (fractions 12–19, Fig. 5*C*) and 12.5 S region (fractions 20–24, Fig. 5*C*), show that almost all (90%) of the estradiol bound to 8 and 12.5 S proteins could be extracted with organic solvent in the absence of fatty acid. The amount of organic solvent-extractable estradiol metabolites was decreased in the 8 S region and slightly increased at 12.5 S in the presence of C22:6; the water-soluble estradiol sedimenting at 8 S increased 10-fold, while that sedimenting at 12.5 S increased 100-fold in the presence of fatty acid. The majority of these water-soluble estradiol metabolites from the 8 and 12.5 S regions could be precipitated along with the protein by the addition of trichloroacetic acid. 65% of the radioactivity in the 8 S region and 85% of that in the 12.5 S region were trichloroacetic acid-precipitable.

DISCUSSION

These results demonstrate that unsaturated fatty acids can mediate alterations in estradiol binding to uterine cytosol. The estradiol-cytosol interaction was apparently unaffected by the addition of saturated fatty acids, but estradiol binding was either reduced or potentiated by unsaturated fatty acid, depending on the concentrations of estradiol and unsaturated NEFAs.

At a low estradiol concentration, the unsaturated fatty acids appeared to inhibit the binding of estradiol to the 8 S cytosol receptor in a dose-dependent fashion. This effect was also a function of the degree of fatty acid unsaturation (14, 26, 27). Polyunsaturated fatty acids, such as C20:4 and C22:6, appeared to have the greatest effect on the binding to 8 S receptor. Scatchard analysis indicated that these fatty acids decreased the receptor affinity for estradiol. Such an effect would be consistent with the capacity of fatty acids to interact with receptor hydrophobic sites (31), producing a conformational change in the receptor and resulting in a lower affinity for estradiol.

Modifications in the conformation of estrogen receptors by non-steroid molecules have been reported. The conformation of estrogen receptors may be modified by such molecules as alkyphenol, tetracaine, sulfated polysaccharides, and fatty acids (31–33). Recent studies indicate that unsaturated fatty acids had an inhibitory effect, similar to that observed in the present study, on brain glucocorticoid and progestin receptors (34).

In addition, the interaction of fatty acid with the receptor could influence the activation of the receptor by estrogens (35), and, consequently, its binding to chromatin. However, further studies are required to establish this point.

The effect of unsaturated fatty acids was quite different when examined in the presence of high concentrations of

TABLE I

Analysis of estradiol metabolites specifically bound to 8 S and 12.5 S regions

0.5 ml of cytosol (1 mg of protein) was incubated overnight at 4 °C with 4×10^{-8} M estradiol alone or in presence of 3×10^{-4} M C22:6. The reacted cytosol was then layered onto 5–20% sucrose gradient and centrifuged at 60,000 rpm for 1 h and 17 min in a TV 865 rotor. Separation of solvent-soluble and water-soluble fractions is described under "Material and Methods." The results are expressed as femtomol eq of [^{3}H]estradiol per mg of protein determined for each peak. Means of two determinations.

Cytosol proteins		Specific binding activity	Organosoluble estradiol metabolites	Water-soluble estradiol metabolites
		fmol/mg protein	*fmol/mg protein*	*fmol/mg protein*
8 S	Estradiol	7,256	7,095	160
	Estradiol + C22:6	5,737	4,229	1,525
12.5 S	Estradiol	4,712	4,645	66
	Estradiol + C22:6	11,055	5,366	5,092

estradiol. There was a dramatic increase in the estradiol binding activity in the presence of polyunsaturated fatty acids. The density gradient analyses indicate that this increased binding was to a larger, 12.5 S species, and that the binding to the 8 S receptor was, in fact, reduced. This dynamic pattern of estradiol binding suggests that, for high estradiol concentrations, unsaturated fatty acid may induce conformational changes in the receptor leading to an association of estradiol receptor with non-receptor components. However, stimulation of a multienzyme system in the rat uterus by unsaturated fatty acids cannot be excluded. This system may be associated with, or independent of, the receptor and could cause a metabolic change in estradiol leading to tight binding of the metabolized steroid to cytosol protein.

The interaction between uterine receptor and estradiol was, for the most part, reversible, as indicated by the fact that 90% of the estradiol bound could be extracted with organic solvent in the absence of unsaturated fatty acids. There was a radical change in the nature of estradiol binding at high estradiol concentrations (2.5×10^{-8} M) in the presence of unsaturated fatty acids. There was a reduction in the amount of reversibly bound estradiol and an increase in the amount of estradiol which was not organic solvent-extractable. The quantity of this water-soluble estradiol form was a function of both estradiol and unsaturated fatty acid concentrations. These results emphasize the importance of the ratio between estradiol and NEFAs in the immediate receptor environment of the cytosol in the determination of estrogen receptor function.

The fact that these water-soluble estradiol derivatives were 70–80% trichloroacetic acid-precipitable indicates that they may be composed of estradiol covalently linked to protein to form "estroproteins." The remaining 30% of nonprecipitable water-soluble estradiol could be in the form of estradiol metabolites bound to small peptides such as glutathione (36, 37). Similar increases in water-soluble estrogen metabolites in the presence of unsaturated fatty acids has been reported for the cytosols of human uterus, breast, and melanoma (25), rat liver microsomes (36, 37), and Syrian hamster embryo fibroblasts (38).

The biochemical pathway responsible for the formation of the water-soluble estrogen adducts is, as yet, unknown. The present data suggest that the unsaturated fatty acids influence estrogen metabolism via an action on an oxidative system such as the prostaglandin synthetase, lipoxygenase system, or the eosinophil peroxidase activity (25, 39–41). It is also possible that estrogen, acting in conjunction with unsaturated fatty acid, could control the functional activity of the receptor via the phosphokinase system, as suggested by some authors (42–44). A receptor having type II low affinity binding sites (45, 46) may also be involved, since the water-soluble estradiol derivatives are preferentially produced at high estradiol concentrations.

The question remains as to just how general is the irreversible interaction of estradiol with cytosol proteins which has now been shown to occur both *in vitro*, in this study (25), and *in vivo* in homogenates of breast cancer tissue (47).

However, whatever the mechanism of NEFA action, the present finding emphasize the importance of NEFAs of dietary or membrane origin in estrogen activity and metabolism, and indicate a new aspect of the way in which non-esterified fatty acids may influence the biological response of a target tissue to estrogen.

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