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EVIDENCE FOR INVOLVEMENT OF TYROSINE IN ESTRADIOL BINDING BY RAT UTERUS ESTROGEN RECEPTOR

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Summary—The possibility of tyrosine involvement in steroid binding by rat uterus estrogen receptor (rER) was investigated. Chemical modification of rER with reagents such as tetranitromethane (TNM) and *N*-acetylimidazole (NAcI) inhibited estradiol binding. Steroid binding was inhibited to a greater extent at pH 8 than at pH 6, indicating the participation of tyrosine (TNM has increasing affinity for cysteine over tyrosine at pH 6). Inhibition patterns remained similar for incubations at 0–4°C or 37°C. NAcI inhibited rER steroid binding at 37°C, but not at 0–4°C. Hydroxylamine incubated in the presence of rER and NAcI appeared to reverse this inhibition. Thus, these findings indicate that the phenyl ring and possibly the phenolic hydroxyl of tyrosine are involved in steroid binding of the receptor.

INTRODUCTION

The primary amino acid sequence for the estrogen receptor has been elucidated for several species [1–4], however, its mechanism of action remain unclear.

Previous work in our laboratory [5] shows that phenylmethylsulfonyl fluoride (PMSF) inhibits estrogen receptor (ER) binding. Subsequent work (manuscript in preparation) shows that diisopropylfluorophosphate (DFP) and PMSF inhibit binding of ER at 37°C, but not at 0–4°C. These studies indicate involvement of a nucleophilic group (e.g. serine, tyrosine, cysteine, amino residues) in steroid binding by estrogen receptor. Chemical modification of the estrogen receptor with various protein modifying agents was the method chosen to explore this possibility.

Common acetylation reagents for the modification of hydroxyl groups of proteins include acetic anhydride and *N*-acetylimidazole (NAcI) [6]. Acetic anhydride also reacts with amino and sulfhydryl groups. NAcI reacts with these groups as well, but reacts with amino groups to a much lesser extent than does acetic anhydride, and is less likely to induce undesired structural modifications [7–10].

NAcI is most stable in pH 7.5 [6]. In most instances, all reactive tyrosines can be acetylated with a 60-fold molar excess of NAcI. Once formed, the *O*-acyl tyrosyl residue may be readily hydrolyzed to regenerate the original tyrosyl group. Since hydrolysis is done under mild conditions, it is possible to assess the role of tyrosine in protein function [6, 8, 10]. The acetylated protein is incubated with hydroxylamine at pH 7.5. Time required for hydrolysis is a function of the hydroxylamine concentration [10]. This reversibility of tyrosine acetylation is analogous to the reversibility of sulfhydryl modification with mercurials and dithiothreitol (DTT). In this way, the ability of hydroxylamine to overcome any inhibition of estradiol binding caused by NAcI would be evidence for the involvement of tyrosine in the binding of estradiol to ER.

Tetranitromethane (TNM) is a mild reagent for the specific nitration of tyrosyl residues of proteins at pH 8.0. The product is 3-nitrotyrosine. Oxidation of cysteine is differentiated by carrying out this reaction at pH 6 whereby

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Abbreviations: rER, rat estrogen receptor; NAcI, *N*-acetylimidazole; TNM, tetranitromethane; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylfluoro phosphate; $^*E_2$, [^{3}H]17β-estradiol; NH_2OH, hydroxylamine; DCC, dextran-coated charcoal; DES, diethylstilbestrol; HAP, hydroxylapatite.

tyrosine is not altered [5, 10–14]. There are also reports that TNM oxidized tryptophan groups and staphylococcus nuclease; however, if the reagent concentrations were low enough, the reaction occurred only with tyrosine residues and slightly with sulfhydryl residues [11, 16].

NAcI and TNM have been used to study tyrosine involvement in some proteins [17, 18]. The purpose of this study was to investigate the functional relationship between various domains of the estrogen receptor using two specific chemical modification agents, TNM and NAcI. Our results indicate that the phenyl ring and possibly the phenolic hydroxyl of tyrosine are involved in steroid binding of the receptor.

EXPERIMENTAL

Chemicals

[^{3}H]17β-estradiol (95 Ci/mmol) was purchased from New England Nuclear (Boston, Mass). All other chemicals were from Sigma Chemical Co. (St Louis, Mo.), except hydroxylamine-HCl (Baker-Adamson), and tetranitromethane and *N*-acetylimidazole (generous gifts of Robert F. Steiner, University of Maryland, Baltimore).

Buffers

For TNM studies, potassium phosphate buffer (17 mM K_2HPO_4) was dissolved in doubled distilled water and adjusted at room temperature to pH 6 or 8. For NAcI studies, 50 mM HEPES buffer (pH 7.5) was used. Stock solutions of DTT and the water-soluble inhibitors were prepared in phosphate buffer.

Preparation of cytosol

All procedures were carried out at 0–4°C, unless otherwise indicated. Four volumes of phosphate buffer was added to the pulverized frozen tissue and homogenized with a Polytron PT-10 probe (Brinkman Instrument Co. Inc., Westbury, N.Y.) with two 5-s bursts at the full setting and a 60-s cooling period in between bursts. The homogenate was then centrifuged at 105,000 *g* for 30 min. The supernatant was decanted and used as the cytosol. The protein concentration was determined by the method of Lowry *et al.* [19] using bovine serum albumin as the protein standard.

Steroid binding assays

Cytosol was diluted with buffer to a protein concentration of 1.2–7.2 mg/ml and incubated with [^{3}H]17β-estradiol ($^*E_2$) (0.5–5.0 nM) for 16–20 h at 0–4°C or 5–60 min at 37°C. The total incubation volume was 0.25 ml. Parallel incubations were carried out in the presence of a 200-fold excess of diethylstilbestrol (DES) to determine nonspecific binding. Free steroid was removed by incubating with 0.25 ml of a dextran-coated charcoal (DCC) suspension (0.5% norit A, 0.005% dextran in buffer, pH 7.4) for 10 min at 0–4°C, regardless of incubation temperature. DCC was pelleted by centrifugation at 5000 *g* for 10 min. Aliquots of the supernatant containing protein-bound steroid were counted in a liquid scintillation counter. In studies with TNM, 0.25 ml of a 60% hydroxyapatite (HAP) suspension was added to the tubes containing the reaction mixture instead of DCC to terminate the reaction. The tubes were then allowed to incubate on ice for 15 min before being centrifuged at 5000 *g* for 5 min. The supernatant was decanted and the resulting HAP pellet was washed three times with 1 ml of phosphate buffer. The washed pellet was then resuspended in 0.5 ml 95% ethanol and incubated at room temperature for 30 min. The HAP was centrifuged once again and aliquots of the supernatant were counted.

Measurement of radioactivity

Samples (100 μl aliquots) were mixed with 4 ml of scintillation cocktail fluid (Packard Instrument Co., Downer Grove, Ill.) and measured in a Packard Model 2425 Automatic Tricarb Liquid Scintillation Spectrometer (30–40% efficiency) or a Beckman LS5801 (Irvine, Calif.; 60% efficiency).

Statistical analysis

Statistical analysis of data was done using ANOVA (analysis of variance). Tukey's test was then used for further analysis of control means versus individual treatment means [20].

RESULTS

Effect of tetranitromethane modification on rat ER estradiol binding

Tetranitromethane (1 mM) inhibited rat ER steroid binding in a pH dependent manner. When cytosol was incubated in the presence of TNM, the binding of 5 nM [^{3}H]estradiol to the rat estrogen receptor was not inhibited by 1 mM TNM at pH 6 for 4 h at 0–4°C (Fig. 1A) or at 37°C for 15 min (Fig. 1B). Thus, TNM modifi-

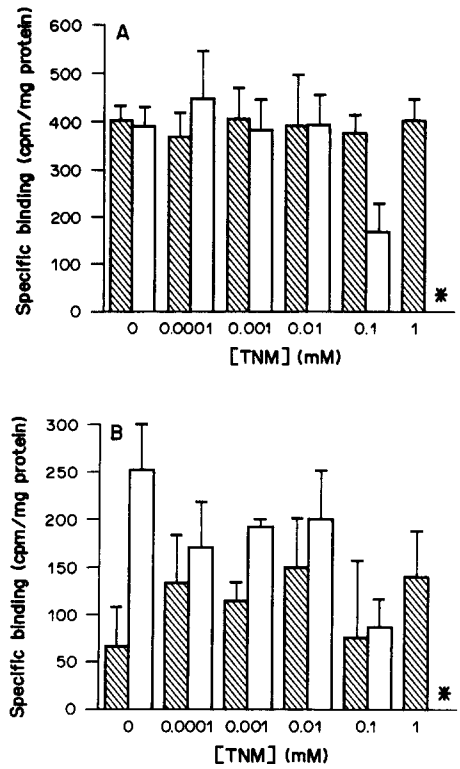

Fig. 1. Titration of ER with TNM at pH 6 and pH 8. Rat uterus cytosol was incubated at pH 6 (▨) or pH 8 (□) with 5 nM *E_2 ± a 200-fold excess of DES in the absence or presence of 100 nM, 0.001 mM, 0.01 mM, 0.1 mM or 1 mM TNM for 4 h at 0–4°C (Panel A) or 15 min at 37°C (Panel B). Specific binding was assessed by the HAP method. (*$P < 0.05$.)

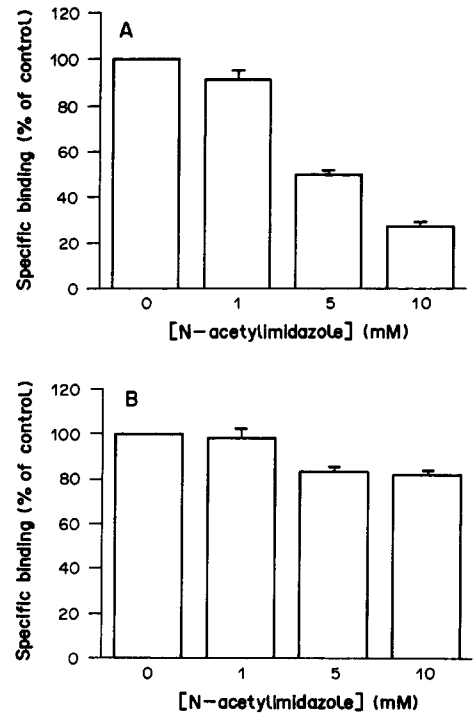

Fig. 2. Titration of ER with NAcI. Rat uterus cytosol was incubated with 5 nM *E_2 ± a 200-fold excess of DES in the absence or presence of 1, 5, or 10 mM NAcI for 15 min at 37°C (Panel A) or 16 h at 0–4°C (Panel B). HEPES buffer (50 mM) at pH 7.5 was used. Specific binding was determined by the DCC method.

cation at pH 6 showed no inhibition of steroid binding. In addition, the combination of acidic pH and elevated temperature results in the degradation of the receptor, as indicated by consistently low control values. Modification of cytosolic ER with 1 mM TNM at pH 8, however, showed significant inhibition of estradiol binding at both 0–4°C and 37°C. These data suggest that high concentrations of TNM (1 mM) are necessary to modify tyrosine residues to the extent that they inhibit steroid binding.

Effect of N-acetylimidazole on ER steroid binding

The inhibition of steroid binding by TNM modification implicated tyrosine involvement in the mechanism of action of ER. NAcI was used to further investigate the interaction of ER tyrosine with steroid. Rat ER was incubated with various concentrations of NAcI at 0–4°C for 16 h and 37°C for 15 min. The data illustrated in Fig. 2A demonstrated the concentration-dependent manner in which NAcI effected steroid binding at 37°C. Estradiol binding was inhibited 50% in the presence of 5 mM NAcI and 74% in the presence of 10 mM NAcI. At lower temperatures (0–4°C), NAcI did not inhibit estradiol binding regardless of concentrations (Fig. 2B).

Hydroxylamine reversal of NAcI modification

The possibility that tyrosine is intimately involved in steroid binding was further explored by hydroxylamine reversal of NAcI modification. Hydroxylamine is known to hydrolyze acetylated moieties. If NAcI modified ER by tyrosyl modification, hydroxylamine should hydrolyze the acetyl group of tyrosine specifically and the steroid binding by ER would be normal.

In these series of experiments, rat uterus cytosol was incubated with 5 nM *E_2 ± a 200-fold excess of DES alone, or simultaneously with increasing concentrations of hydroxylamine or hydroxylamine (0–20 mM) and NAcI (0 or 10 mM) for 15 min at 37°C. Hydroxylamine alone in the incubation mixture has no effect between 1 and 10 mM. Inhibition of steroid binding caused by NAcI appears to be reversed by hydroxylamine concentrations between 1 and 20 mM (Fig. 3).

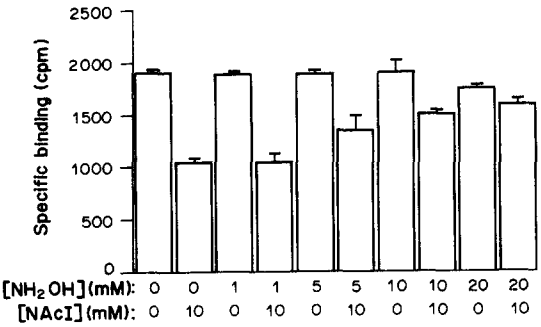

Fig. 3. Titration of rat ER with hydroxylamine in the absence or presence of 10 mM NAcl at 37°C for 15 min. Rat uterus cytosol was incubated with 5 nM $^*E_2 \pm$ a 200-fold excess of DES in the absence or presence of 0–20 mM hydroxylamine and/or 10 mM NAcl for 15 min at 37°C. Specific binding was determined by the DCC method.

DISCUSSION

Tyrosine involvement in steroid binding

The main product of nitration of proteins with TNM is the 3-nitrotyrosine derivative [6]. Oxidation of cysteine is differentiated by carrying the reaction out at pH 6 where tyrosine remains unaltered [6, 11–15].

At both temperatures, 1 mM TNM did not inhibit estradiol binding at pH 6 and completely abolished estradiol binding at pH 8 (Fig. 1) indicating the participation of the phenyl moiety of the tyrosine group. Lower concentrations of TNM were ineffective inhibitors of estradiol binding at pH 6 or 8.

NAcl was used to further assess the possibility that phenolic hydroxyl of tyrosine is involved in steroid binding. Concentrations of 1, 5 and 10 mM NAcl had little effect on estradiol binding at 0–4°C over 16 h, but the same concentrations inhibited estradiol binding in a concentration-dependent manner at 37°C for 15 min (Fig. 2).

Hydroxylamine was used to reverse the NAcl-induced modification by hydrolysis of the acetyl group bound to tyrosine [6, 8, 10]. Incubation of ER with various concentrations of hydroxylamine in the absence and presence of 10 mM NAcl for 15 min at 37°C indeed showed a trend of hydroxylamine to reverse inhibition of estradiol binding by NAcl (Fig. 3). Thus, hydroxylamine reversal indicated the involvement of tyrosyl hydroxyl moieties in estradiol binding.

Our present findings that tyrosine is involved in steroid binding is consistent with those of Kuhn and Raymoure [18] and Inano and Tamaoki [17]. Kuhn and Raymoure were unable to detect inhibition of steroid binding by progesterone binding globulin in the presence of NAcl. On the other hand Inano and Tamaoki [17] used TNM to inhibit steroid binding by estradiol 17β-dehydrogenase from human placenta. However, they did not use NAcl, nor were they using rat uterus.

On the basis of inhibition of this protein by TNM, they concluded that the phenyl ring and not the hydroxyl moiety is involved in binding. The difference between our results and those of Kuhn and Raymoure [18] might best be explained by a difference in microenvironment of the steroid binding sites, and the fact that we are dealing with the cellular steroid binding protein while they used a blood steroid binder.

Based on single point saturation analysis of chemically modified estrogen receptor with the reagents tetranitromethane and *N*-acetylimidazole at different temperatures, we conclude that tyrosine groups are involved in the binding of steroid to rat estrogen receptor. The details of this interaction require the modification of specific tyrosine residues via site directed mutagenesis.

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