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THE EFFECTS OF VARIOUS SERINE PROTEASE INHIBITORS ON ESTROGEN RECEPTOR STEROID BINDING

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Summary—Two serine protease inhibitors, phenylmethanesulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP), were utilized to investigate the possible involvement of serine hydroxyl groups on 17β-estradiol binding to the rat estrogen receptor (ER). Single point saturation analysis and Scatchard analysis demonstrated that both 5 mM PMSF and 5 mM DFP were able to inhibit steroid binding to the ER after incubation at 37°C, but neither were able to inhibit steroid binding of the nonactivated ER (0–4°C). The reducing agent dithiothreitol (DTT) was used to differentiate between the interaction of PMSF with serine groups or with sulfhydryl groups of the receptor. When incubated in the presence of 5 mM PMSF, various concentrations of DTT up to 25 mM were not able to overcome the inhibition of this agent, indicating that there was no interaction of PMSF with sulfhydryl groups. Thus, these findings indicate that serine hydroxyl groups are involved in steroid binding of the rat ER.

INTRODUCTION

Serine protease inhibitors are used to prevent degradation of cellular proteins following disruption and homogenization of tissues or whole cells [1]. Two of the best known protease inhibitors are diisopropylfluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF). These and several other compounds have been observed to stabilize or inhibit various proteins, including the glucocorticoid receptors (GR). Ganesan *et al.* [2] reported that PMSF significantly inhibited prostatic estrogen receptor (ER) steroid binding, and that dithiothreitol (DTT) enhanced the inhibition of estradiol binding.

Jansen *et al.* [3] reported that DFP inhibits the serine proteases and the esterases trypsin, acetyl trypsin, chymotrypsin and α-chymotrypsin [3, 4]. Serine residues have a high nucleophilicity within the active sites of a number of hydrolytic enzymes. Other nucleophiles in proteins include threonine, aspartic acid, glutamic acid, cysteine, lysine, histidine, arginine, methionine, tryptophan and tyrosine [5, 6].

In addition to phosphorylation of serine (the most commonly known reaction), it was also found that DFP phosphorylates tyrosyl residues without inhibiting protease activity [7–9].

Sulfonyl fluorides are generally more reactive and more specific than sulfonyl chlorides. For the sulfonyl halides, inhibition is accomplished by sulfonylation of an active site serine hydroxyl group. PMSF reacts specifically and exclusively with the active site serine hydroxyl group. PMSF reacts specifically and exclusively with the active site serine of α-chymotrypsin even when used in excess [10].

There have been various reports of stabilization or inhibition of receptors for various ligands by DFP, PMSF and various other protease inhibitors. Puca *et al.* [11] reported that DFP inhibited transformation of calf uterus ER when present during a 60-min 20°C incubation, but not when added after the incubation. This group concluded that calf uterus ER under transforming conditions acquires or exposes a serine binding site, that ER transformation is due to the effect of serine protease and that the receptor is endowed with this activity.

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Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropylfluorophosphate; DTT, dithiothreitol; ER, estrogen receptor; NGF, nerve growth factor; DCC, dextran-coated charcoal; DES, diethylstilbestrol; GR, glucocorticoid receptor.

Nerve growth factor (NGF) is a hormone-like polypeptide required for growth and development of the sensory and sympathetic nervous systems; its receptors are within the cell membrane. Incubation of heterogeneous chicken dorsal root ganglia cells or homogeneous clonal rat pheochromocytoma cell line (PC-12) with 1–2 mM PMSF inhibits binding of NGF, and is indicative of a reactive serine group in NGF receptor [1].

Various protease inhibitors have been shown to interact directly with the GR and inhibit steroid binding. Baker *et al.* [12] were one of the first to demonstrate this inhibition of glucocorticoid binding by the protease inhibitors PMSF, and the chloromethyl ketones of phenylalanine (TPCK) and lysine (TLCK). These protease inhibitors significantly inhibited rat hepatoma cell GR binding despite the presence of 7 mM 2-mercaptoethanol. In contrast to these results, Mayer *et al.* [13] showed that a reducing agent (5 mM DTT) could overcome the effects of TLCK and TPCK, thus suggesting the presence of thiol groups at or near the ligand binding site of the GR.

Other protease inhibitors have been tested on the GR. Baker and Fanestil [14] observed that 2–3 mM diethylpyrocarbonate, a histidine-selective reagent, inhibited estrogen binding of rat α-fetoprotein and progestin binding in chick oviduct cytosol [15]. Inhibition of these systems was overcome by hydroxylamine. This research suggested the presence of histidine near the steroid binding site.

The simplest interpretation of these observations is that binding of hormone to GR probably involves sulfhydryl groups and histidine [6]. Furthermore, protease inhibitors found to act directly with steroid receptors might prove useful in purifying the receptor (e.g. inhibitor-linked affinity columns), or characterizing specific chemical groups on the receptor.

The purpose of this study was to investigate the effect of the protease inhibitors, DFP and PMSF, on ER steroid binding. Our results reveal that DFP and PMSF are able to inhibit steroid binding after incubation at 37°C for 15 min, but neither is able to inhibit steroid binding of the nonactivated ER (18 h incubation at 0–4°C), indicating the involvement of serine residues in hormone binding.

EXPERIMENTAL

Chemicals

[^{3}H]17β-estradiol (95 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals were supplied by Sigma Chemical Co. (St Louis, MO).

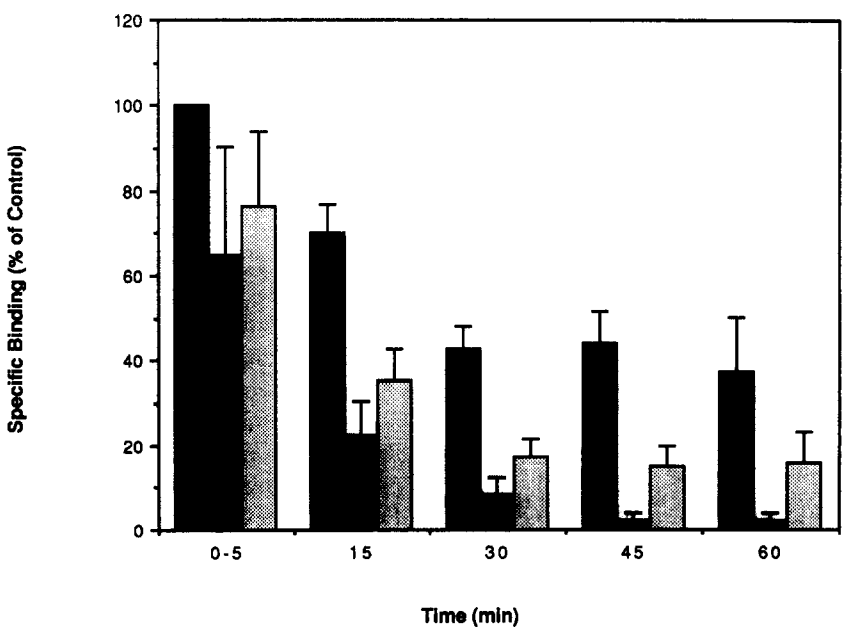

Fig. 1. Single-point assay of the effect of time on estradiol binding to rat uterus ER in the presence or absence of PMSF or DFP. Cytosol was incubated at 37°C for 0–60 min with 5 nM [^{3}H]estradiol ± 200-fold excess of DES in the absence (■) or presence of 5 mM PMSF (▨) or 5 mM DFP (▩). After the appropriate incubation time, 0.5% DCC was added to remove free steroid, and specific binding was determined. Results represent the mean and SE of 3 trials.

Buffers

Potassium phosphate (17 mM K_2HPO_4) dissolved in double-distilled water was adjusted to pH 7.4 at room temperature. Stock solutions of DTT and PMSF were prepared in 17 mM phosphate buffer, while DFP was prepared in ethanol.

Tissue handling and storage

Uteri from mature female Sprague–Dawley rats weighing approx. 300–350 g were purchased from Charles River Labs (Wilmington, MA), housed in individual containers, and provided food and water, *ad libitum*. After dissection, specimens were placed in plastic vials, frozen in liquid nitrogen, and stored at −70°C until use.

Preparation of cytosol

All procedures were performed at 0–4°C. Four volumes of buffer were added to pulverized tissue and homogenized with a Polytron PT-10 probe (Brinkman Instrument Co. Inc., Westbury, NY) with two 5-s bursts at the full setting with 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.* [16] using bovine serum alumin as the protein standard.

Steroid binding assays

Cytosol was diluted with buffer to a protein concentration of 2–3 mg/ml and incubated with [^{3}H]17β-estradiol (0.5–5 mM) for 16–20 h at 0–4°C or 5–60 min at 37°C in the absence or presence of PMSF, DFP and DTT. The total incubation volume was 0.25 ml. Parallel incubations were carried out in the presence of a 200-fold excess of DES to determine nonspecific binding. Specific binding was determined as the difference between total and nonspecific binding. Free steroid was removed by incubating 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 (100 μl) of the supernatant containing protein-bound steroid were then taken for measurement of radioactivity. Data were analyzed by single-point assays and by Scatchard analysis [17].

Measurement of radioactivity

Aliquots were mixed with 4 ml of scintillation cocktail fluid (Packard Instrument Co., Downer Grove, IL) and measured in a Packard Model 2425 automatic tricarb liquid scintillation spectrometer. Efficiencies were between 30 and 40%.

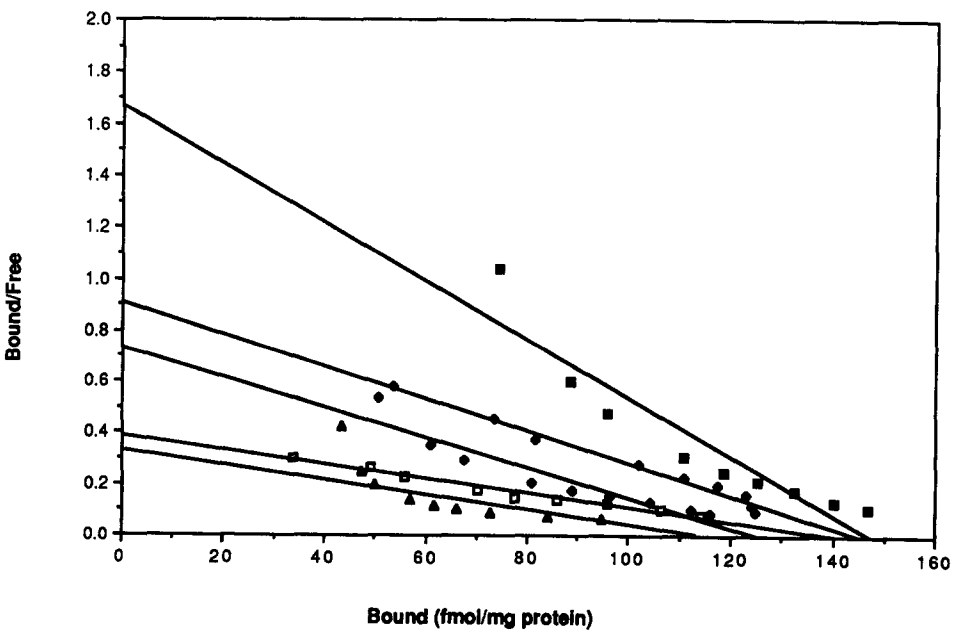

Fig. 2. Scatchard analysis of ER in the presence of 0–5 mM PMSF at 37°C for 15 min. Rat uterus cytosol was incubated with 0.5–5 nM [^{3}H]estradiol ± a 200-fold excess of DES in the absence (■) or presence of 0.1 (◆), 1.0 (◇), 2.5 (□) or 5.0 (△) mM PMSF. After 15 min of incubation, 0.5% DCC was added to remove free steroid, and specific binding was determined.

Data analysis

Data were analyzed using analysis of variance (ANOVA) and Tukey's test [18], a *post hoc* test, to compare experimental means with control means.

RESULTS

Specific binding vs incubation time at 37°C

Single-point assays were performed to determine the effect of time and protease inhibitor concentration on rat uterus ER estradiol binding. Rat uterus cytosol was incubated at 37°C with 5 nM [^{3}H]17β-estradiol in the presence or absence of a 200-fold excess of diethylstilbestrol (DES), 5 mM PMSF and 5 mM DFP (Fig. 1). After 15 min, 70% of estradiol binding was retained in the absence of inhibitor. In the presence of either PMSF or DFP, however, specific estradiol binding decreased to 22 and 35% of control, respectively. Based on these results, assays at 37°C were incubated for 5–15 min.

Scatchard analysis and protease inhibitors in estradiol binding

Scatchard analysis were conducted for ER in the absence or presence of 0.1–5 mM PMSF (Fig. 2) and 4.6 μM–5 mM DFP (Fig. 3) at 37°C for 15 min. The results indicated that increasing concentrations of both PMSF and DFP increased the K_d but did not alter the B_{max}. Similar incubations in the presence of PMSF and DFP for 18 h at 0–4°C had no effect on estradiol binding to the receptor (data not shown).

The effect of PMSF and DTT on estradiol binding

To investigate any possible involvement of sulfhydryl groups on PMSF-induced inhibition, rat uterus cytosol was incubated with 5 nM [^{3}H]17β-estradiol in the absence or presence of a 200-fold excess of DES, 1 or 5 mM PMSF and 1, 5, 10 or 25 mM of the reducing agent DTT for 15 min at 37°C. Addition of DTT to incubation mixtures containing PMSF failed to reverse the observed PMSF-induced inhibition of ER steroid binding (Fig. 4), indicating that sulfhydryl groups are not involved in the PMSF-induced inhibition.

DISCUSSION

All serine proteases are inhibited by DFP and most are inhibited by PMSF. However, PMSF will also inhibit some cysteine proteases [19]. The effect of protease inhibitors on estradiol binding seen in these studies agrees with those

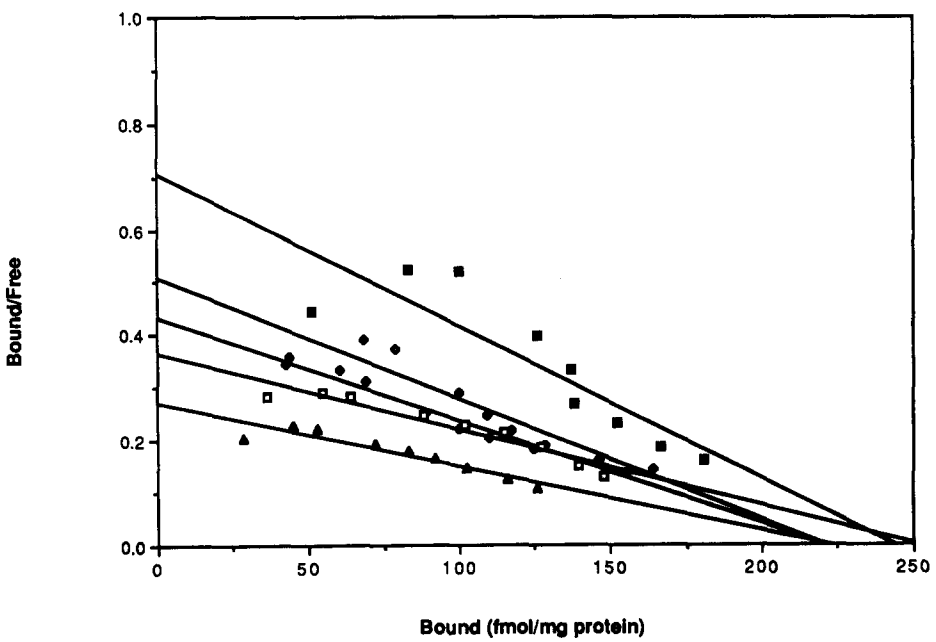

Fig. 3. Scatchard analysis of ER in the presence of 0–5 mM DFP at 37°C for 15 min. Rat uterus cytosol was incubated with 0.5–5 nM [^{3}H]estradiol ± a 200-fold excess of DES in the absence (■) or presence of 4.6 μM (◆), 1.0 (◇), 2.5 (□) or 5.0 (△) mM DFP. After 15 min of incubation, 0.5% DCC was added to remove free steroid, and specific binding was determined.

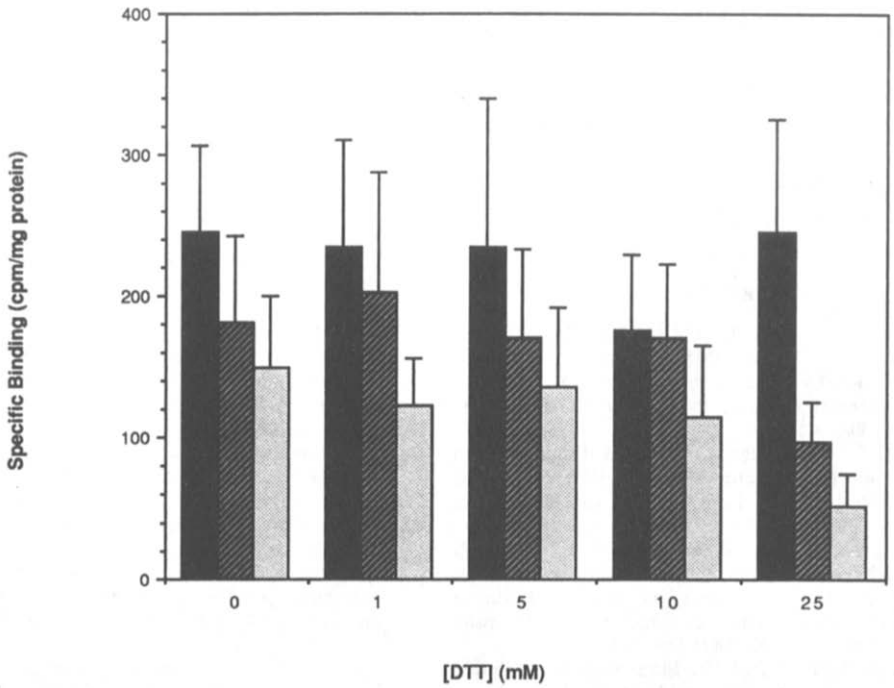

Fig. 4. Effect of PMSF and DTT on estradiol binding at 37°C for 15 min. Rat uterus cytosol was incubated with 5 nM [^{3}H]estradiol ± 200-fold excess of DES in the absence (■) or presence of 1 mM (▨) or 5 mM (⊡) PMSF and various concentrations of DTT (1, 5, 10 and 25 mM). After the appropriate incubation time, 0.5% DCC was added to remove free steroid and specific binding was determined. Results represent the mean and SE of 3 trials.

of Ganesan *et al.* [2] for PMSF, and Lukola and Punnonen [20] for ER inhibition by PMSF and Puca *et al.* [11] for ER inhibition by DFP.

The effect of DFP and PMSF on estradiol binding was studied by single-point saturation binding analysis. The results presented in Fig. 1 suggest that the incubation of the receptor at 37°C exposes the site for PMSF and DFP interaction which in turn effects the binding of the hormone to its receptor. The effects of PMSF and DFP on estradiol binding to the ER have also been shown with Scatchard analysis (Figs 2 and 3). Increasing concentrations of both PMSF and DFP increase the K_d of the ER. Any combination of the 17 serine residues that are located within the rat uterus ER steroid binding domain may participate in covalent binding. In addition, 16 of these serines are identical in the rat and human ER [21, 22], while the 17th serine contains a conservative substitution in the human ER (threonine).

Puca *et al.* [11] showed that DFP inhibited transformation of calf uterus ER when incubated for 60 min at 20°C, and that ER acquires or exposes a serine binding site for aprotinin. They believed that ER transformation was due to serine protease activity with which the ER was endowed. The results presented here are consistent with those observations and the prediction by Baker *et al.* [12] that some of the structure that characterizes the active site of serine proteases would be present in steroid hormone receptors and would be proximal to areas of hormone–receptor interaction. Serine 195 of the serine protease chymotrypsin forms a transient covalent linkage with the enzyme substrate complex to catalyze hydrolysis of a peptide bond [23]. Indeed, a charge relay system similar to the one found in chymotrypsin and elastase that activates a serine at the active site when substrate binds might also be responsible for proteolysis during transformation of the ER.

Ganesan *et al.* [2] showed that PMSF significantly inhibited prostate ER. Inhibition of 17β-estradiol to ER by PMSF was enhanced in the presence of 1 mM DTT, indicating the direct involvement of sulfhydryl groups in the interaction of PMSF with the ER. Alternatively, reduction of some sulfhydryl groups on the ER may expose a serine hydroxyl group which interacts with PMSF [2]. However, the results presented in Fig. 4 are not consistent with the hypothesis that sulfhydryl groups directly interact with PMSF since DTT did not have any effect in reversing the PMSF-induced inhibition.

Based on single-point saturation analysis and Scatchard analysis of chemically modified ER with the reagents PMSF and DFP, we conclude that serine groups are involved in the binding of steroid to the rat ER. The details of this interaction require the modification of specific serine residues via site directed mutagenesis.

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