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Competitive Inhibition of [^{3}H]Dexamethasone Binding to Mammary Glucocorticoid Receptor by Leupeptin (42482)

LUKE C. C. HSIEH, CHI SU, AND FRANCIS S. MARKLAND, JR.

Department of Biochemistry, and Comprehensive Cancer Center, University of Southern California, School of Medicine, Los Angeles, California 90033

Abstract. The inhibitory effect of leupeptin on [^{3}H]dexamethasone binding to the glucocorticoid receptor from lactating goat mammary cytosol has been studied. Leupeptin (10 m*M*) caused a significant (about 35%) inhibition of [^{3}H]dexamethasone binding to glucocorticoid receptor. Binding inhibition is further increased following filtration of unlabeled cytosolic receptor through a Bio-Gel A 0.5-m column. Binding inhibition was partially reversed by monothioglycerol at 10 m*M* concentration. A double reciprocal plot revealed that leupeptin appears to be a competitive inhibitor of [^{3}H]dexamethasone binding to the glucocorticoid receptor. Low salt sucrose density gradient centrifugation revealed that the leupeptin-treated sample formed a slightly larger (approximately 9 S) receptor complex (leupeptin-free complex sediments at 8 S).

Leupeptin, a microbial proteinase inhibitor (1), has been used at 0.5–1 m*M* (2) and 20–50 m*M* (3–5) concentrations to prevent proteolysis in steroid hormone receptor preparations. Interestingly, it is active in purified systems as a sulfhydryl proteinase inhibitor at much lower (μM) concentrations (1). Baker *et al.* (6) and more recently Hubbard *et al.* (7) reported that some serine and cysteine protease inhibitors significantly inhibited glucocorticoid binding to hepatoma and rat liver cytosolic receptors. The possibility of using these protease inhibitors to probe the steroid–receptor interaction was suggested. While studying methods to prevent proteolysis of the mammary glucocorticoid receptor during purification, we observed that lower than normal binding was obtained when leupeptin was added prior to incubation with [^{3}H]dexamethasone. Agarwal and Philippe (8) had previously reported inhibition of [^{3}H]-cortisol binding by leupeptin in rat liver cytosol. In the present report, we examine the effect of leupeptin on [^{3}H]dexamethasone binding to goat mammary glucocorticoid receptor.

Materials and Methods. *Chemicals.* Leupeptin was purchased from Chemicon (El Segundo, CA). [1,2,4,6,7-^{3}H]Dexamethasone (78 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Catalase was purchased from Mannheim Boehringer (Indianapolis, IN). Cytochrome *c* (type VI), hemoglobin (type I), γ globulin, monothioglycerol, and unlabeled dexamethasone were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Preparation of cytosol. Fresh, perfused goat mammary tissue, cleaned of adhering connective tissue and fat, was homogenized in 2 vol of 10 m*M* Tris–HCl buffer containing 10% glycerol, pH 7.4, at 2°C with a Polytron PT10 homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 100,000*g* for 60 min at 2°C. The supernatant (cytosol) was carefully removed without the upper fatty layer. Cytosol was stored in liquid nitrogen until use.

Partial purification of unlabeled glucocorticoid receptor. Cytosolic receptor (6 ml) prepared in 10 m*M* Tris–HCl buffer, pH 7.4, containing 10% glycerol was filtered through a Bio-Gel A 0.5-m column (bed vol 50 ml; fractionation range 10–500 $\times$ 10^3 Da) equilibrated with the 10 m*M* Tris–HCl buffer, pH 7.4, containing 10% glycerol and 10 m*M* sodium molybdate, at a flow rate of 80 ml/hr. Receptor fractions (2 ml) were identified by postlabeling with 12.5 n*M* [^{3}H]dexamethasone with or without 1.25 μM unlabeled dexamethasone at 25°C for 1 hr. Fractions containing receptor were pooled and used for leupeptin inhibition studies.

Leupeptin inhibition of [^{3}H]dexamethasone binding to glucocorticoid receptor. Cytosol (or the partially purified receptor) was incubated with 20 n*M* [^{3}H]dexamethasone $\pm$ 2 μM un-

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labeled dexamethasone for 4 hr at 2°C either in the presence or absence of leupeptin or monothioglycerol or in combination with both. The specific dexamethasone binding is determined by hydroxylapatite method (9). To an aliquot (500 μl) of a receptor-containing ethanol extract of hydroxylapatite, 5 ml of Scinti-Verse-II was added. Radioactivity was determined using a Beckman LS-3150T scintillation counter with a tritium counting efficiency of 35%.

Sucrose-density gradient centrifugation. Cytosol was incubated with 20 n*M* [^{3}H]dexamethasone with or without 40 m*M* leupeptin and aliquots were analyzed by sucrose density centrifugation. The dextran-coated charcoal (0.5% activated charcoal, 0.05% Dextran T-70)-treated (10) sample (200 μl) was layered on 5-ml linear 5 to 20% (w/v) sucrose gradients prepared in 10 m*M* Tris–HCl buffer, pH 7.4, containing 10% glycerol. Catalase (11.3 S), γ globulin (7.1 S), hemoglobin (4.4 S), and cytochrome *c* (2.0 S) were employed as external marker proteins. The gradients were centrifuged in a Beckman swinging-bucket SW60-Ti rotor for 16 hr at 54,000 rpm at 2°C and were fractionated from the bottom using a Beckman fraction recovery system (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA). Radioactivity in the gradient fractions was measured and the position of marker proteins was determined spectrophotometrically at 280 nm.

Protein determination. Protein concentrations of cytosol and receptor fractions were determined by the Coomassie dye binding method (11).

Results. Table I demonstrates that leupeptin at 10 m*M* concentration caused greater than 35% inhibition of [^{3}H]dexamethasone binding to cytosolic glucocorticoid receptor. The binding inhibitory effect of 10 m*M* leupeptin is further increased after removal of low molecular weight contaminants, including endogenous reducing substances, by gel filtration of the crude cytosolic glucocorticoid receptor on a Bio-Gel A 0.5-m column. This procedure should also separate receptor containing fractions from low molecular weight proteases, which migrate in the molecular weight range of 20,000–30,000. At least some of these proteases would probably interact directly with leupeptin. Addition of monothioglycerol (10 m*M*) to the partially purified (gel-filtered) receptor preparation was partially effective in preventing binding inhibition by leupeptin as

TABLE I. EFFECT OF LEUPEPTIN AND MONOTHIOGLYCEROL ON [^{3}H]DEXAMETHASONE BINDING TO GLUCOCORTICOID RECEPTOR[a]

	Concentration of leupeptin (m*M*)	Specific glucocorticoid binding[b] (cpm/mg protein)	Binding inhibition (%)
Cytosol			
A. Control		7300 ± 50	—
+ leupeptin	10	4660 ± 90	36
B. Monothioglycerol (10 m*M*)		7930 ± 170	—
+ leupeptin	10	5330 ± 50	33
Gel-filtered cytosol			
C. Control		16420 ± 110	—
+ leupeptin	10	2840 ± 90	83
D. Monothioglycerol (10 m*M*)		16780 ± 70	—
+ leupeptin	10	5970 ± 180	64

[a] In Experiment A, cytosol was prepared as described under Materials and Methods, and was incubated with 20 n*M* [^{3}H]dexamethasone ± 2 μ*M* radioinert dexamethasone for 4 hr at 2°C either with or without leupeptin. Specifically bound [^{3}H]dexamethasone was measured by the hydroxylapatite method (9). In Experiment B, cytosol was prepared as in Experiment A: aliquots of stock monothioglycerol were added to the final concentration indicated. In Experiment C, a separate preparation of cytosolic receptor was obtained as in A and partially purified by filtering unliganded receptor preparation through a Bio-Gel A 0.5-m column. Void volume fractions, which contained receptor, were pooled and used. The higher specific binding of the partially purified receptor is due to the removal of some non-dexamethasone binding proteins during gel filtration. In Experiment D, receptor was prepared as in C and aliquots of stock monothioglycerol were added to the final concentration indicated.

[b] Results are the means ± SD of three experiments.

shown in Table I. However, with crude cytosol, the effect of monothioglycerol was not significant.

Figure 1 indicates that leupeptin appears to be a competitive inhibitor of [^{3}H]dexamethasone binding to the glucocorticoid receptor. After incubation of goat mammary cytosol with [^{3}H]dexamethasone (20 n*M*) and leupeptin (40 m*M*) for 4 hr at 2°C, low salt sucrose density gradient centrifugation reveals that the glucocorticoid receptor sediments at approximately 9 S (average of two determinations) (Fig. 2). This is similar to the sedimentation profile of molybdate-treated cytosolic receptor under similar conditions (9–10 S, data not shown). Leupeptin-free complexes sedimented at 8 S in low salt–sucrose density gradient as shown in Fig. 2. The inclusion of monothioglycerol (40 m*M*) did not prevent receptor from forming a 9-S aggregate. When leupeptin (40 m*M*) was added after incubating cytosol with [^{3}H]dexamethasone (20 n*M*) for 3 hr (with a total incubation time of 4 hr) at

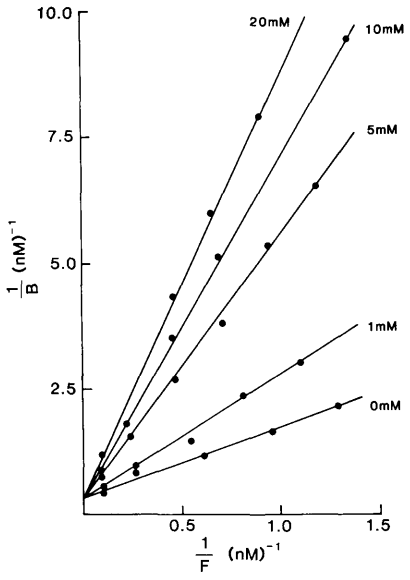

FIG. 1. Competitive inhibition of [^{3}H]dexamethasone bindng to the glucocorticoid receptor by leupeptin. Binding of [^{3}H]dexamethasone to goat mammary tissue cytosol was determined in the absence and presence of a constant concentration of leupeptin as described under Materials and Methods. B, specifically bound [^{3}H]dexamethasone; F, free [^{3}H]dexamethasone determined by subtracting total bound [^{3}H]dexamethasone from total concentration of [^{3}H]dexamethasone. The concentrations of leupeptin are indicated adjacent to the appropriate lines.

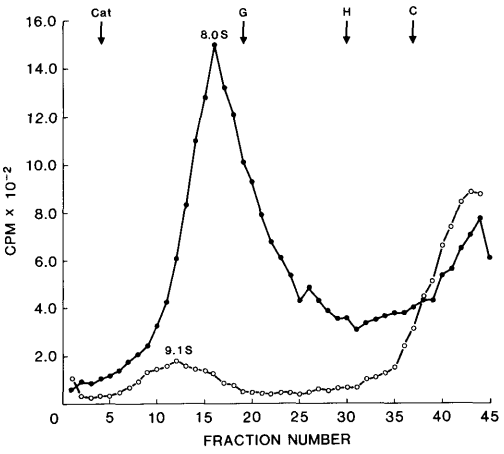

FIG. 2. Sucrose density gradient profiles of goat mammary tissue cytosol [^{3}H]dexamethasone–receptor complexes in the absence (●) and presence (○) of 40 mM leupeptin. Cytosol and 5–20% sucrose gradients were prepared in 10 mM Tris–HCl buffer, pH 7.4, containing 10% glycerol. Centrifugation was for 16 h in a SW60-Ti rotor at 54,000 rpm and 2°C. The radioactive peak centered at fraction 45 is free [^{3}H]dexamethasone which apparently dissociated from the dextran-coated charcoal-treated samples in both leupeptin-containing and leupeptin-free cytosol during gradient centrifugation. Low binding activity in the presence of leupeptin is due to inhibition of [^{3}H]dexamethasone binding to the receptor. Abbreviation for marker proteins are Cat, catalase; G, γ globulin; H, hemoglobin; C, cytochrome *c*. Marker proteins were detected spectrophotometrically as described under Materials and Methods.

2°C, the leupeptin-treated cytosolic glucocorticoid receptor sedimented at about 8 S with much greater bound radioactivity recovered in a low salt–sucrose density gradient than observed under conditions where leupeptin was added to receptor samples prior to [^{3}H]-dexamethasone addition (data not shown).

Discussion. In view of the potent proteinase inhibitory properties of leupeptin (1), we have employed this agent in an attempt to prevent proteolysis during receptor purification. However, it was observed that leupeptin caused an apparent inhibition of [^{3}H]dexamethasone binding to goat mammary cytosolic glucocorticoid receptor. Several other proteinase inhibitors, including tosyl-phenylalanine chloromethyl ketone (TPCK), tosyllysine chloromethyl ketone (TLCK), and L-trans-epoxysuccinylleucylagmatine (E-64), were also reported to be inhibitors of steroid

binding to several cytosolic steroid hormone receptors (6, 7, 12, 13). By comparison of the inhibitory concentrations of leupeptin (10–20 mM) to those of other proteinase inhibitors which interfere with glucocorticoid binding, such as TPCK (2 mM), TLCK (2 mM), and E-64 (36 μM), leupeptin is a relatively weak glucocorticoid binding inhibitor. This is consistent with the relatively high [^{3}H]dexamethasone binding inhibition constant ($K_i = 1.7 \pm 0.1 \times 10^{-3}$ M, $n = 2$) for leupeptin, calculated from the double reciprocal plot (Fig. 1). The apparently higher K_i reported for inhibition of [^{3}H]dexamethasone binding by leupeptin than for inhibition of thiol proteases suggests that the mechanism of binding inhibition is not via the protease inhibition pathway. However, the K_i determined (Fig. 1) may represent a higher value than the true K_i since crude cytosol was used in the determination. It is likely that a number of other (nonglucocorticoid) receptors and/or other molecules present in the cytosol might react with leupeptin and effectively reduce its concentration as demonstrated in Table IA (compare Experiments A and C). Thus, the value we report for K_i is an upper limit. The lack of complete reversal by monothioglycerol of leupeptin inhibition of [^{3}H]dexamethasone binding to the glucocorticoid receptor (Table I) is similar to the finding of Hubbard *et al.* (7), who showed that in the presence of 5 mM dithiothreitol, the inhibitory effect of E-64 was not altered.

The apparent competitive nature of leupeptin inhibition of [^{3}H]dexamethasone binding could suggest that leupeptin and [^{3}H]dexamethasone share a common binding site on the glucocorticoid receptor. Leupeptin is a mixture of two major components, leupeptin AC-LL (acetyl-L-leucyl-L-leucyl-D,L-argininal) and leupeptin Pr-LL (propionyl-L-leucyl-L-leucyl-D,L-argininal), and their analogs containing isoleucine or valine in place of one or two leucine residues (1). It is known that the aldehyde group in leupeptin is essential for an effective inhibitory effect on several proteinases (14, 15). The importance of thiol groups to the steroid–receptor interaction has already been noted (16–21), and it was postulated that cysteine in the glucocorticoid receptor binding site is involved in both the binding of glucocorticoid to the receptor and the covalent binding of dexamethasone mesylate (22, 23). Thus the leupeptin aldehyde group might react with the thiol group of cysteine in the receptor binding site and form a reversible hemithioketal which prevents [^{3}H]-dexamethasone from binding to the receptor. The increase in [^{3}H]dexamethasone binding stimulated by monothioglycerol in the presence of leupeptin might indicate that monothioglycerol reacted with leupeptin and decreased the effective concentration of leupeptin available to compete with [^{3}H]dexamethasone for glucocorticoid receptor. On the other hand, the possibility of other nucleophiles, for example amino groups, present in the vicinity of the binding site which might also react with the aldehyde group on leupeptin, is not ruled out.

The formation of glucocorticoid receptor complexes (or aggregates) of similar size to those obtained with molybdate was reported in cytosols treated with leupeptin (3–5, 24) and with tosyl-lysyl chloromethyl ketone (25). The results obtained from sucrose density gradient centrifugation suggest that leupeptin may modulate the structure of the unliganded glucocorticoid receptor complex. The mechanism by which leupeptin alters the receptor complex is unknown at the present time. However, as suggested by Sherman *et al.* (4), leupeptin may stabilize an intact native receptor form. The slower sedimenting form (8 S) observed in the absence of leupeptin may result from proteolysis or partial disaggregation.

The biochemical mechanism of steroid- and temperature-mediated glucocorticoid–receptor complex activation (transformation) is still not completely understood. There is recent evidence that activation of the glucocorticoid receptor (26) and estradiol receptor (27) may be caused by a proteolytic mechanism. In fact, Puca *et al.* (27) have reported the interesting observation that the estrogen receptor may have inherent proteolytic activity that is responsible for its own activation. With all steroid hormone receptors, a steroid is required to trigger the subsequent activation processes (e.g., binding to nuclei or DNA) as a consequence of its (steroid hormone) binding to receptor. The suppression of steroid–hormone receptor activation by protease inhibitors (25, 28) suggests that a possible correlation may exist between the competitive inhibition by the protease inhibitors (e.g., leupeptin) of steroid binding to the receptors and the ability of receptor to be activated.

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