CHARACTERISTICS OF GLUCOCORTICOID-BINDING SITES OF RAT LIVER: DIFFERENT EFFECTS OF ADRENALECTOMY ON THE BINDING

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Abstract—Hydrocortisone (HC) in rat liver cytoplasmic fraction was bound to three different binding sites with high, medium and low affinity. Dissociation constants (Kd) were approx. 2.1, 22 and 208 nM; and the densities of these binding sites were about 40, 50 and 10% of total number of binding sites, respectively. The binding site for dexamethasone (DM) of the cytoplasmic fraction was the medium affinity one among these three components. The maximum number of binding sites (Bmax) of HC and DM was significantly increased by adrenalectomy. The Bmax of HC was about twice as great as that of DM in normal and adrenalectomized rat liver. DM inhibited 3H-HC binding in a dose-dependent manner but inhibition did not exceed 50% in either normal or adrenalectomized rats. Following adrenalectomy, the Bmax of the medium affinity-site for HC was significantly increased, while the high affinity component disappeared. By adding DM to the cytoplasmic fraction of adrenalectomized rat liver in vitro and in vivo, the Bmax of the medium affinity-site was significantly decreased, and a high affinity component of HC was revealed with a significant increase in the number of binding sites. These results indicate that the binding site for DM is one component of the HC binding site; and following adrenalectomy, the number of each type of binding site for glucocorticoids increases differently from the others.

It has been reported by numerous workers that there are some binding sites for glucocorticoids in the cytoplasmic fraction of various tissues (1-11). They subdivided the binding sites into three or four types of protein by sucrose density gradient centrifugation, ion exchange or gel chromatography and demonstrated the specificity of each binding site for glucocorticoids.

On the other hand, an increase in the number of glucocorticoid binding sites in rat liver has been observed following adrenalectomy (12-16), but little is known about the effect of adrenalectomy on each glucocorticoid binding site. To determine the density and affinity of glucocorticoid binding sites, to which ligands bind with different affinity, we used the kinetic analysis method reported for use in the determination of adrenergic \( \beta_1 \) and \( \beta_2 \) receptors of the heart, lung and brain (17, 18). In this study, we have demonstrated some binding sites for glucocorticoids and showed the different effects of adrenalectomy on these binding sites by means of kinetic analysis.

Materials and Methods

Male albino rats of the Wistar strain weighing 150–200 g were used throughout the experiments. Adrenalectomized rats were maintained on food ad libitum and drinking water supplemented with 0.9% NaCl. After
decapitation, the liver was perfused via the vena cava inferior with 20 ml of ice-cold 0.9% NaCl. The liver was isolated and homogenized with 6 vol. of ice-cold 10 mM Tris-HCl (pH 7.5) buffer (containing 1 mM EDTA and 2 mM dithiothreitol). Following centrifugation at 100,000 x g for 60 min, lipoprotein was removed from the resultant supernatant by aspiration. The remaining supernatant was used for the glucocorticoid binding assay as a cytoplasmic fraction of rat liver. Adrenalectomized rats were used 2 days after operation unless otherwise noted.

**Glucocorticoid binding assay:** The binding assay was carried out as Giannopoulos reported previously (19). Three hundred μl of cytoplasmic fraction was incubated with tritiated glucocorticoid at 0°C for 2 hr. The reaction was terminated by adding 500 μl activated charcoal solution (1% charcoal and 0.1% dextran [MW 60,000–90,000]/10 mM Tris-HCl buffer as used for the preparation of the cytoplasmic fraction) and was then mixed for 15 sec. The mixture was centrifuged at 3,000 rpm for 10 min. From the supernatant, 0.2 ml was transferred to a counting vial containing PCS-xylene (1:1) scintillation counting fluid. The radioactivity was measured with a Packard model 3255 liquid scintillation spectrometer at a counting efficiency of 38–44%. The radioactivity found in the presence of 10 μM nonlabeled glucocorticoid was considered to be nonspecifically bound tritiated glucocorticoid and was subtracted from each experimental value to obtain the amount of specifically bound tritiated glucocorticoid, as the nonlabeled glucocorticoids hydrocortisone (HC) and dexamethasone (DM) were used for the determination of specific 3H-HC and 3H-DM binding, respectively. Five nM 3H-HC and 3H-DM were used for the measurement of the affinity of various steroids. Specific binding of 3H-HC and 3H-DM was found to be 90–95% of the total binding. These specific binding values increased linearly with the protein concentration up to 3 mg per assay and reached equilibrium within 100–120 min under these experimental conditions. The dissociation constant (Kd) and maximum number of binding sites (Bmax) for 3H-HC and 3H-DM of the cytoplasmic fraction were determined by Scatchard analysis (20). Densities and apparent dissociation of binding sites for HC and DM were calculated using Hofstee plots obtained from the displacement of 5 nM tritiated HC and DM binding by nonlabeled HC and DM according to the method of Barnett et al. (17) and Minneman et al. (18). The final Kd and the inhibition constant (Ki) were determined by means of the equation proposed by Cheng and Prusoff (21). The binding assays were always carried out in triplicate. The protein (prot) concentration was determined by the method of Lowry et al. (22) using bovine serum albumin as a standard. Each value represents the mean±S.E.M.

**Drugs and materials:** HC, DM and Bethamethasone 17 valerate (BV) were obtained from Roussel UCLFA, Sigma and SIRS, respectively. 1,2,4-3H-DM (SA 46 Ci/nmol) and 1,2-3H-HC (SA 50.7 Ci/nmol) were obtained from RCC Amersham and New England Nuclear, respectively. PCS scintillator was obtained from Amersham/Searle.

**Results**

1. **Effect of adrenalectomy on 3H-DM and 3H-HC binding to cytoplasmic fraction:** As shown in Fig. 1, Scatchard plots of 3H-DM binding in normal rat consisted of a single straight line. Values for Kd and Bmax were 12.1 nM and 440 fmoles/mg prot, respectively. The Bmax was significantly increased by adrenalectomy (Fig. 1). On the other hand, Scatchard analysis of 3H-HC binding in normal rat revealed the presence of two different HC affinity binding sites: the
Fig. 1. Scatchard analysis of $^3$H-DM binding to cytoplasmic fractions obtained from normal and Adx rat livers. Between 1 to 60 nM $^3$H-DM were used. Each point represents the mean obtained from 4 to 5 separate experiments.

Fig. 2. Scatchard analysis of $^3$H-HC binding to cytoplasmic fractions obtained from normal and Adx rat livers. Concentrations from 1 to 60 nM $^3$H-HC were used. Each point represents the mean obtained from 4 to 6 separate experiments.
high affinity site had a $K_d$ of 2.9 nM, while that of the low affinity site was 32.5 nM. The $B_{\text{max}}$ of the high and low affinity sites ($B_{\text{max}1}$ and $B_{\text{max}2}$) were 89 and 541 fmoles/mg prot, respectively (Fig. 2). Following adrenalectomy, the maximum number of binding sites of the low affinity ($B_{\text{max}2}$) by Scatchard analysis was significantly increased with a slight decrease in the $K_d$ value, whereas the high affinity component of $3^\text{H}$-HC binding sites completely disappeared from Scatchard plots (Fig. 2). In both normal and adrenalectomized rat, the $B_{\text{max}}$ of HC was larger than that of DM. Fifty to 70% of the HC binding sites were DM binding sites (Figs. 1, 2). In Scatchard analysis, no large $K_d$ value for a low affinity site for HC (208.0 nM in normal and 227.0 nM in adrenalectomized rat) obtained from Hofstee plot analysis was observed.

2. Effect of adrenalectomy on inhibition of specific $3^\text{H}$-HC binding by HC: Following adrenalectomy, specific $3^\text{H}$-HC binding was significantly increased with a slight decrease in the concentration of HC at which $3^\text{H}$-HC is displaced by 50% (IC50) (Fig. 3). The HC inhibition curve for $3^\text{H}$-HC binding in normal rat did not have the theoretical sigmoid shape, but the curve for adrenalectomized rat became sigmoidal in shape (Fig. 3). Hofstee plot analysis of the inhibition of $3^\text{H}$-HC binding by HC in normal rat cytoplasmic fraction revealed the presence of three types of binding sites: the high affinity site had a $K_d$ of 2.1 nM, while those of the medium and low sites were 22.3 and 208.0 nM, respectively (Fig. 4). The densities of these binding sites were 33, 59 and 7% of the total number of binding sites, respectively. Following adrenalectomy the high affinity component observed in normal rat disappeared from the Hofstee plot, and the density of the medium affinity component was significantly increased with a slight increase in affinity (Fig. 4). The $K_d$ of medium and low affinity sites were 15.8 and 227 nM, respectively. The densities of these medium and low affinity sites were 95 and 5% of the total number of binding sites, respectively.

These results clearly indicate that $K_d$ values for HC for high and low affinity components obtained by Scatchard analysis correspond to the high and medium affinity components obtained by Hofstee plot analysis.
3. Effect of adrenalectomy on specific \(^3\)H-DM binding and inhibitory effect of DM and HC: HC and DM inhibited \(^3\)H-DM binding significantly in a dose-dependent manner, and inhibition curves of sigmoid shape were observed in both normal and adrenalectomized (Fig. 5). Adrenalectomy significantly increased the specific binding of 5 nM \(^3\)H-DM with a slight decrease in the IC50 of HC and DM for \(^3\)H-DM binding (Fig. 5). Hofstee plot analysis revealed the presence of one type of binding site in normal and adrenalectomized rat liver (data not shown). The K_i of the DM binding site was slightly decreased by adrenalectomy (8.4 → 4.7 nM).

4. Inhibitory effect of HC and DM on specific \(^3\)H-HC binding to cytoplasmic fraction from normal and adrenalectomized rat liver: As mentioned above, at 10^-6 M, HC and DM inhibited \(^3\)H-DM binding completely. Concerning the \(^3\)H-HC binding, HC inhibited the \(^3\)H-HC binding completely at 3 X 10^-8 M, whereas DM could not inhibit the \(^3\)H-HC binding completely. The inhibitory effect of DM on \(^3\)H-HC binding reached a plateau at 30 nM with 50% inhibition (Fig. 6). Nor did BV inhibit \(^3\)H-HC binding more than 50% (data not shown). The inhibition constants (K_i) of DM for HC binding in normal and adrenalectomized rats were 6 and 4 nM, respectively (Fig. 6). These values were
Fig. 6. Inhibitory effect of HC and DM on specific $^3$H-HC binding to cytoplasmic fraction from normal rat liver. Each point represents the mean obtained from 3 and 6 separate experiments.

approx. equal to those obtained for the displacement of $^3$H-DM binding by DM (Fig. 5).

These results clearly indicate that the DM binding site is one component of the HC binding site and the density of the DM sites is approx. 50% of the total number of HC binding sites.

5. Effect of DM on adrenalectomy increased HC binding in vitro and in vivo: To determine whether the low affinity component of HC obtained by Scatchard analysis (medium affinity component of Hofstee plot analysis) is a DM binding component or not, the effect of DM addition was investigated in vitro and in vivo. As shown in Fig. 7, at one or two days after adrenalectomy, a significant increase in $B_{max}$ and the disappearance of the high affinity component of $^3$H-HC were observed; however, DM (1 mg/kg, i.p., every 6 hr for 1 day or 2 hr before isolation) administration to the adrenalectomized rat significantly decreased the $B_{max}$ of the low affinity site to below the normal rat level and revealed the presence of a high affinity component with a significant increase in $B_{max}$ (normal: 89 fmoles versus adrenalectomized: 110 and 310 fmoles/mg prot) (Fig. 7A and B.)

Similar results were obtained by adding DM (0.1 $\mu$M) to the cytoplasmic fraction from adrenalectomized rat liver in vitro (Fig. 7B).

These results indicate that the low affinity component of HC binding sites in the Scatchard analysis is the binding component of DM, and the significant increase in the high affinity component following adrenalectomy is masked by the more significantly increased low affinity component (DM binding component).
Discussion

Numerous workers have subdivided the binding sites of glucocorticoids and suggested the role of each binding site (1–11). They explained the biological potency of natural and synthetic glucocorticoids by the affinity for type II protein or GR₁ moiety. These studies involved chromatographic analysis of the binding protein, while subdivision of the binding sites using kinetic analysis was hardly reported at all.

In the present study, we demonstrated the presence of three binding sites, their specificity for glucocorticoid and the different effects of adrenalectomy on these binding sites by kinetic analysis. HC, a natural glucocorticoid, bound to three types of binding sites with different affinity, i.e., high, medium and low. The following results clearly indicate that the medium affinity component for HC is the only component which synthetic glucocorticoid can bind, and its density is approximately 50% of the total binding sites of HC. 1) The $K_d$ value of the medium affinity component of HC from Scatchard analysis is equal to the value of the inhibition constant ($K_i$) of HC from the displacement of $^3$H-DM binding. 2) DM binds to 50% of the binding sites of HC. 3) The $B_{max}$ value of DM is 50–70% of the HC.

In addition, the inhibition activity of $^3$H-DM...
binding to the cytoplasmic fraction of rat liver is roughly correlated with the antiinflammatory action of glucocorticoid, as reported earlier (23). In view of these results, it seems reasonable to assume that the medium affinity binding site corresponds to the type II protein of Litwack et al. (6) and/or the GR1 moiety of Agrawal (10).

Most attention has been focused on the study of the binding site mentioned above. For this reason, the presence and the role of a binding site to which natural glucocorticoid binds with a higher affinity than to the DM binding site has not been reported. Following adrenalectomy, the Bmax value of the medium affinity component was significantly increased, and the high affinity component disappeared from the Scatchard plot. However, this high affinity component could be found by decreasing the number of binding sites of medium affinity by adding DM. These results clearly indicate that the Bmax increase in the high affinity component was masked by the increase in the medium affinity component, and the Bmax increase of the former is smaller than that of the latter in adrenalectomized rat liver.

Although the physiological role of the high affinity binding site for HC remains to be elucidated, the results obtained in this study suggested that the number of high affinity sites is regulated differently from that of the DM binding site when the concentration of glucocorticoid in cytoplasm is changed.

Adrenalectomy was found to induce an increase in the glucocorticoid and mineralocorticoid receptor level for the decrease in the corticoid level (12–16, 24). Feldman (13) and Giannopoulos (16) interpreted that the increase, at least in part, is owing to the ability to measure the previously occupied receptor by glucocorticoid. Recently, using an exchange assay method for glucocorticoid receptor in rat liver, Rosner and Polimeni (15) made it clear that the increase of the receptor by adrenalectomy is not due to the decrease of the previously occupied receptor, but due to the loss of the ability of glucocorticoid to suppress the receptor level. Claire et al. (24) suggested that this increase in glucocorticoid receptor level in adrenalectomized rat liver is indirect evidence for down regulation of the receptor number.

Although details of this mechanism are not yet clear, it is felt that the number of corticoid binding sites is regulated by the corticoid itself. More recently, the involvement of the cycloheximide-sensitive step in decreasing the number of DM binding sites in the AT-20 cell was investigated (25). We could not demonstrate any inhibition of an increase in the number of binding sites following the administration of cycloheximide to adrenalectomized rat liver because the death of the animal occurred following cycloheximide injection as reported in the results of mineralocorticoid receptor experiments (24).

It is generally believed that the steroid receptor complex undergoes an energy dependent activation, enters the nucleus, binds to the DNA, and subsequently controls some gene expressions (26, 27). Whether the role of the high affinity binding site for HC is involved in the above steps or not is under our investigation.

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