Characteristics of Binding of a New Anti-Inflammatory Glucocorticoid, Hydrocortisone 17-Butyrate 21-Propionate (HBP), to Glucocorticoid Receptors of Rat liver

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Abstract—Characteristics of the specific binding of hydrocortisone 17-butyrate 21-propionate (HBP) to the cytoplasmic fraction from rat liver were investigated. The inhibition constant (K_i) of HBP for the site of 3H-dexamethasone (3H-DM) binding was approximately equal to the value of DM and significantly smaller than that of hydrocortisone (HC). The maximum number of binding sites (B_max) and dissociation constant (K_d) for 3H-HBP were also approximately equal to those for 3H-DM. The Scatchard and Hofstee plots analyses of 3H-HC binding indicated that the HC binding sites consisted of three components with different affinity, while that of 3H-DM had only one site with an intermediate affinity. HBP and hydrocortisone 17-butyrate (HB) bound to other binding sites of HC in addition to the site for DM. The IC50 value for synthetic glucocorticoids determined from the inhibition curves of 3H-HC binding in the first phase agreed with the values determined by the displacement study of 3H-DM binding. Furthermore, the first phase of HBP in the inhibition curve of 3H-HC binding disappeared from the curve, and only the second phase remained following the addition of DM. These results indicate that the esterification of C-17 and C-21 OH increases the affinity of the binding site for synthetic glucocorticoid and attenuates the affinity for the other binding sites of HC.

It is well known that glucocorticoids have the ability to prevent or suppress the development of local heat, redness, swelling and tenderness by which inflammation is recognized (1). Hydrocortisone (HC) and other anti-inflammatory glucocorticoids are not selectively concentrated in inflamed tissues and sometimes induce adverse effects (1). On the other hand, numerous workers have reported that the anti-inflammatory actions of glucocorticoids are mediated by the formation of a glucocorticoid-receptor complex, which is transferred into nuclei and induces an anti-inflammatory protein named lipomodulin or macrocortin (2-5). Recently, we have synthesized a new anti-inflammatory glucocorticoid, hydrocortisone 17-butyrate 21-propionate (HBP), which possesses weak systemic effects and potent topical effects (6). The present investigation was designed to examine the pharmacological characteristics of the binding site for HBP in comparison with those for other natural and synthetic glucocorticoids.

Materials and Methods
Male Wistar rats weighing 150 to 200 g were used in all experiments. Adrenalectomized rats were maintained ad libitum on food and drinking water supplemented with 0.9% NaCl solution. The hepatic supernatant fraction was prepared as we reported previously (7). In brief, livers were perfused via the portal vein with 20 ml of ice-cold
0.9% NaCl solution following decapitation. The livers were isolated immediately and homogenized with 6 vol. of ice-cold Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 2 mM dithiothreitol. The homogenate was centrifuged at 100,000 x g for 60 min. After removal of the lipoprotein from the resultant supernatant by aspiration, the remaining supernatant was used for the glucocorticoid binding assay as the cytoplasmic fraction.

**Assay of glucocorticoid binding:** Assays of the binding of glucocorticoid were carried out as described previously (7). 3H-Glucocorticoids were incubated at 0°C for 2 hr with 300 ul of 10 mM Tris-HCl (pH 7.4) containing the cytoplasmic fraction, EDTA and dithiothreitol. The reaction was terminated by the addition of 500 ul activated charcoal mixture (1% charcoal and 0.1% dextran (MW 60,000–90,000)/10 mM Tris-HCl (pH 7.4)) and subsequent mixing for 15 sec. The mixture was centrifuged at 3,000 rpm for 10 min at 4°C, and then 0.2 ml of the supernatant was transferred into a counting vial containing PCS-xylene (1:1) scintillation counting fluid (commercially available xylene-surfactant based liquid scintillation cocktail).

The radioactivity was measured in a Packard model 3255 liquid scintillation counting spectrometer at a counting efficiency of 38 to 44%. Each assay was performed in duplicate or triplicate. The radioactivity found in the presence of 3 μM non-labeled glucocorticoid was considered to be non-specific binding of 3H-glucocorticoid, and this value was subtracted from each experimental value to obtain the amount of specifically bound 3H-glucocorticoid. Non-labeled HC and dexamethasone (DM) were used to determine the specific bindings of 3H-HC and 3H-DM, respectively. To determine the specific binding of 3H-HBP, 3 μM DM was used. Five nM 3H-HC and 3H-DM were used for the measurement of the affinities of various steroids determined by the displacement of specific bindings of 3H-HC and 3H-DM. The specific bindings of 3H-HC and 3H-DM were calculated to be approximately 95% of the total binding. These specific bindings increased linearly with the protein concentration added up to 3 mg/assay and reached an equilibrium within 120 min under these experimental conditions. The dissociation constant (Kd) and Bmax values for the bindings of 3H-HC, 3H-DM and 3H-HBP in hepatic cytoplasmic fractions were determined using Scatchard (8) analysis. Densities and apparent dissociation constants for the bindings of HC and DM were calculated using Hofstee plots obtained from the displacement of the bindings of 5 nM 3H-HC and 3H-DM by non-labeled HC and DM according to the method of Kuriyama et al. (9), except the calculations by computer. K, values were determined by the previously reported equation (10). The Hill coefficients for the bindings of 3H-HC, 3H-DM and 3H-HBP were determined by Hill plots.

Protein concentration was determined by the method of Lowry et al. (11) using bovine serum albumin as the standard.

**Substances used:** Aldosterone, progesterone and 17-β estradiol were obtained from Sigma. HC, DM, betamethasone (BM), betamethasone 17-valerate (BV) were obtained from Roussel UCLAF, Sigma, Japan Siber Hegner and SIRS, respectively. (1, 2, 4-3H) DM (S.A. 46 Ci/mmol) and (1, 2-3H) HC (S.A. 50.7 Ci/mmol) were obtained from RCC Amersham and New England Nuclear, respectively. HB, HBP, Clobetasol-17 propionate and (1, 2-3H) HBP (S.A. 109 mCi/mmol) were synthesized in our laboratory. PCS scintillator was obtained from Amersham/Searle.

**Results**

**Specificity of 3H-DM binding to cytoplasmic fraction:** The specific binding of 3H-DM was significantly inhibited by various steroids in a concentration-dependent manner. The IC50 values for DM, aldosterone, progesterone and β-estradiol were found to be 7.5 x 10^-9, 4.3 x 10^-8, 1.0 x 10^-6 and 2.0 x 10^-5 M, respectively (data not shown). These results clearly indicate that the specific binding of 3H-DM reflects the association of DM with glucocorticoid binding sites in the hepatic cytoplasmic fraction.

**Inhibition of specific binding of 3H-DM to cytoplasmic fraction by various glucocorticoids:** In the case of normal rats, the
specific binding of 3H-DM was significantly inhibited by HC, HBP, DM and other glucocorticoids, and the inhibition curves with these glucocorticoids were parallel (Fig. 1). The IC50 values for the various glucocorticoids were determined from the inhibition curve presented in Table 1. The affinity of HB and HBP for 3H-DM binding sites was similar, but significantly higher than that of HC. The affinity of BV was also significantly higher than that of BM. By the esterification of the C-17 OH of HC and BM, the affinities of the steroids for the DM binding site were significantly increased (Table 1). Similar results were obtained from the experiments using adrenalectomized (Adx) rats (Table 1).

Effect of adrenalectomy on binding of 3H-DM and 3H-HBP to cytoplasmic fraction: As shown in Fig. 2, Scatchard plots of the binding of 3H-DM consisted of a single straight line. As reported previously (7, 12), adrenalectomy increased the Bmax of 3H-DM binding without altering the affinity of the binding site for DM. Kd and Bmax values of DM obtained from normal and Adx rats were 12.1±2.1 nM, 440±52 fmoles/mg prot. and 10.6±4.2 nM, 841±63 fmoles/mg prot., respectively (Fig. 2). In normal and Adx rats, HBP showed almost equal values to those of DM in Kd and Bmax. Kd and Bmax values of HBP obtained from normal and Adx rats were 10.1±4.2 nM, 345±80 fmoles/mg prot. and 10.2±3.2 nM, 886±37 fmoles/mg prot., respectively (Fig. 2). It was also found that the Kd values for the bindings of 3H-DM and 3H-HBP obtained from Scatchard plot analysis coincided with the Kd values of DM and HBP determined by the displacement of specific 3H-DM binding. Similar results were obtained in Hill plot analysis of the bindings of 3H-DM and 3H-HBP. The Hill plot also showed that the coefficient of the bindings of 3H-DM and 3H-HBP in normal and adrenalectomized rats were approximately 1.0 (data not shown). These results indicate that cooperativity is not involved in the bindings of DM and HBP.

![Fig. 1. Inhibition of specific binding of 3H-DM to cytoplasmic fraction from normal rat liver by various glucocorticoids. Each inhibition curve was obtained from 3 to 5 separate experiments. 3H-DM: 3H-dexamethasone, HC: hydrocortisone, BV: betamethasone 17-valerate, DM: dexamethasone, HBP: hydrocortisone 17-butyrate 21-propionate.](image)

| Glucocorticoid                  | Normal rat IC50 (nM) | Normal rat Kd (nM) | Normal rat Bmax (fmoles/mg prot.) | Adx IC50 (nM) | Adx Kd (nM) | Adx Bmax (fmoles/mg prot.) |
|--------------------------------|----------------------|--------------------|-----------------------------------|---------------|-------------|-----------------------------|
| Hydrocortisone (HC)            | 66.4±3.2             | 51.9±2.5           | 38.1±10.0                         | 23.0±6.1      |             |                             |
| Hydrocortisone 17-butyrate (HB)| 11.0±1.3*            | 8.6±0.9***         | 8.6±1.5***                        | 8.0±1.0*      |             |                             |
| Hydrocortisone 17-butyrate 21-propionate (HBP) | 8.7±2.8***           | 6.8±2.0***         | 7.9±3.2***                        | 5.5±2.2**     |             |                             |
| Betamethasone 17-valerate      | 3.4±0.5*             | 2.6±0.4*           | 3.3±0.7                           | 2.3±0.5       |             |                             |
| Dexamethasone                  | 7.5±1.6              | 5.9±1.3            | 6.1±2.6                           | 4.2±1.6       |             |                             |
| Betamethasone                  | 6.3±1.7              | 4.9±1.3            | N.T.                              | N.T.          |             | N.T.                        |
| Clofetabosil 17-propionate     | 4.4±0.8              | 3.4±0.6            | N.T.                              | N.T.          |             | N.T.                        |

Each value represents the mean±S.E.M. obtained from 3 to 5 separate experiments. N.T.: not tested. Control values of normal and Adx rats were 112.5±7.7 and 264.9±10 fmoles/mg prot., respectively. *P<0.05, compared with IC50 and Kd values of betamethasone, respectively. ***P<0.001, compared with IC50 and Kd values of HC, respectively.
and they suggest that the binding site for DM may be identical with that for HBP.

**Fig. 2.** Scatchard plots analysis of the binding of $^3$H-DM and $^3$H-HBP to cytoplasmic fraction obtained from normal and adrenalectomized (Adx) rat livers. Concentrations of $^3$H-DM and $^3$H-HBP used were 1 to 60 and 5.5 to 220 nM, respectively. The data represents the mean±S.E.M. obtained from 3 to 6 separate experiments.

**Fig. 3.** Hofstee plot analysis for inhibition of $^3$H-HC binding by HC in cytoplasmic fraction of normal rat liver. Insert: Inhibition of specific $^3$H-HC binding by HC. Each value represents the mean obtained from 6 separate experiments.
corticoids: As shown in Fig. 3, the specific binding of $^3$H-HC was completely inhibited by HC at $3 \times 10^{-5}$ M, but this inhibition curve did not exhibit a theoretical sigmoid. These results suggest that this curve may consist of several components. In fact, the Hofstee plot analysis for the inhibition of $^3$H-HC binding by HC showed that HC bound to the cytoplasmic fraction with three different types of affinity; i.e., high, intermediate and low. The $K_d$ values of these binding sites obtained from Hofstee plot analysis were found to be 2.1, 22.3 and 203.0 nM, while the densities of these sites were approximately 35, 60 and 5%, respectively. Inhibitions of the specific binding of $^3$H-HC by DM and BV were observed with the concentrations up to 30 nM, and this reached a plateau at 50% inhibition. Further inhibition was not observed, however, by increasing the concentration of these drugs (Fig. 4). On the other hand, HB and HBP inhibited the binding of $^3$H-HC more than 50%, but did not inhibit the binding completely (Fig. 5). The inhibition of $^3$H-HC binding by HB and HBP was best explained by two distinct affinity states of the binding site for each competitor as previously observed in other receptors (13, 14). The IC50 values for DM, BM, BV, HB and HBP on the binding of $^3$H-HC were almost equal to the values obtained from the displacement of $^3$H-DM binding (Table 1). Densities in the 1st and the 2nd phases were about 45% of the total specific binding sites of HC (Table 2). Affinities of HB and HBP for the component of the 2nd phase were significantly lower, and IC50 values were found to be greater than 500 nM.

**Characteristics of HBP binding sites:**
Scatchard analysis of the binding of $^3$H-HC

![Fig. 4. Inhibition of specific binding of $^3$H-HC to cytoplasmic fraction from normal rat liver by DM and BV. Each inhibition curve was obtained from 4 separate experiments.](image)

![Fig. 5. Inhibition of specific binding of $^3$H-HC to cytoplasmic fraction from normal rat liver by HB and HBP. Each inhibition curve was obtained from 4 separate experiments.](image)

| Glucocorticoids | 1st Phase | 2nd Phase |
|-----------------|-----------|-----------|
|                 | Density (%) | IC50 (nM) | Density (%) | IC50 (nM) |
| Hydrocortisone 17-butynoate (HBP) | 41.3±10.3 | 5.2±2.6 | 38.8± 6.6 | 590.0±220.0 |
| Hydrocortisone 17-butynoate (HB) | 45.3± 9.8 | 6.7±1.5 | 46.0±10.1 | 750.0±220.0 |
| Dexamethasone | 44.6± 8.1 | 3.6±2.2 | 0 | — |
| Betamethasone | 50.5± 0.5 | 1.6±0.9 | 0 | — |
| Betamethasone 17-valerate | 51.6± 8.3 | 1.2±0.2 | 0 | — |

Each value represents the mean±S.E.M. obtained from 4 to 5 separate experiments.
to the cytoplasmic fraction from normal rat liver revealed the presence of two types of binding sites. The \( K_d \) of the high affinity site was 2.5 nM, while that of low affinity site was 33.2 nM (Fig. 6). The \( B_{\text{max}} \) values for high and low affinity sites were 61 and 262 (±323–61) fmoles/mg prot., respectively. It was also found that the \( K_d \) values of high and low affinity binding sites for \(^3\text{H}-\text{HC} \) determined by Scatchard analysis were similar to the \( K_d \) values for the binding sites with high and intermediate affinities obtained from Hofstee plot analysis for the inhibition of \(^3\text{H}-\text{HC} \) binding by HC. In the presence of 0.1 \( \mu \text{M} \) DM and HBP, the \( B_{\text{max}} \) values for the binding of \(^3\text{H}-\text{HC} \) was significantly decreased, especially in the low affinity site (Fig. 7). Similarly, it was found that the 1st phase disappeared from the competing binding curve for HBP in the presence of 0.1 \( \mu \text{M} \) DM, and only the 2nd phase was observed (data not shown). These results clearly indicate that the high affinity binding site for HBP corresponds to the DM binding sites as well as to the binding site for HC with an intermediate affinity.

**Discussion**

The results obtained in this study clearly indicate that the affinity of glucocorticoids for the binding site of DM is significantly increased by the esterification of the C-17 OH of glucocorticoids with butyrate or valerate. Furthermore, a significant decrease or loss of the affinity for binding sites of HC, to which DM does not bind, has been found in C-17 OH esterified HC.

It has been reported that BM and BV possess 25 and 360 times higher anti-inflammatory potency as compared with that of HC, respectively (1, 15). Williams et al. (16) also reported that BV was the most potent anti-inflammatory agent when applied...
topically due to its ability to soak quickly into the skin.

On the other hand, Wieland et al. (17) reported that the esterification of the C-21 OH of glucocorticoid prolonged the anti-inflammatory action of glucocorticoid. These results coupled with the present findings strongly suggest that potentiation of the anti-inflammatory action of C-17 and C-21 OH esterified HC may be due to the increase of the affinity of this synthetic glucocorticoid to the DM binding site in addition to the ability of soaking into the skin.

Agarwal (18) subdivided the specific binding site for glucocorticoids into three populations, i.e., GR₁, GR₂ and GR₃, and demonstrated that natural glucocorticoids have specific affinities for GR₁ and GR₂, especially for GR₂, while synthetic glucocorticoids bind to GR₁ and GR₃, especially for GR₃. Litwack et al. (19) also found the binding protein which has specific affinity for DM or HC. Beato and Feigelson (20) also reported the presence of three binding proteins for glucocorticoid, i.e. A, B and G protein, and concluded that the G protein, to which DM bound with high affinity, was the true glucocorticoid binding site which is involved in physiological and pharmacolgical actions of glucocorticoids.

In this study, we have also observed that HC binds to three different types of binding sites: high, intermediate and low in its affinity. The density of these binding sites was found to be approximately 35, 60 and 5% of the total binding sites, respectively. Synthetic glucocorticoids (DM, BM, BV) only bound to the binding site with intermediate affinity. On the other hand, HB and HBP bound to the intermediate affinity site for HC as well as to another binding site for HC. The latter binding site is considered to be the high affinity site for HC. It is also noteworthy that the affinity of HB and HBP to the intermediate affinity site was 6 times higher than that of HC, whereas that to the high affinity site was 1/250 of HC. These results strongly suggest that the intermediate affinity site for HC may be the most important component interconnecting the effect of glucocorticoids, and it may be identical to the GR₁, GR₃ or G protein reported by Agarwal (18) and Beato and Feigelson (20).

It is well known that the effects of corticosteroids are numerous, and the sites of action of these hormones are widely distributed (1). The ⁹H-DM binding protein is also known to be distributed over numerous tissues and organs (21). Recently, we have found that HBP possesses more potent anti-inflammatory activity than HB and BV, when applied on ear edema and dermatitis induced by croton oil in rats (6). Furthermore, we have observed a more potent protective effect of HBP on the edema induced by carrageenin injection and the granuloma induced by cotton pellet implantation (6) as compared with those of HB and BV. Considering these data together with the results obtained in this study, they suggest that a high affinity of HBP to the binding sites for glucocorticoid and high ability of HBP to soak into the skin, which are induced by esterification of C-17 and C-21 OH of HC with butyrate and propionate, respectively, may be involved in the potent topical anti-inflammatory action of HBP.

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