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ENHANCEMENT OF AFFINITY TO RECEPTORS IN THE ESTERIFIED GLUCOCORTICOID, HYDROCORTISONE 17-BUTYRATE 21-PROPIONATE (HBP), IN THE RAT LIVER

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Abstract—To investigate the affinity of glucocorticoid (GC) to its receptor, the binding of [^{3}H]hydrocortisone 17-butyrate 21-propionate ([^{3}H]HBP) and [^{3}H]dexamethasone ([^{3}H]DEX) in rat liver was analyzed kinetically. Scatchard analysis of [^{3}H]hydrocortisone ([^{3}H]HC) binding yielded a curvilinear plot with upward concavity with high and low affinities. The dissociation constant (K_d) value of high affinity was 1.9 nM and of low affinity 68.7 nM. A Scatchard plot of [^{3}H]HBP binding showed a straight line with high affinity. The K_d value was 9.8 nM. The K_d values for the low affinity site of HC were in good agreement with the K_i values obtained from displacement experiments of [^{3}H]DEX and [^{3}H]HBP binding. The K_i values of HC for [^{3}H]DEX and [^{3}H]HBP were 51.9 and 42.3 nM respectively. The association rate constant for HBP to the GC receptor was 2.9 times lower than that for HC. The dissociation rate constant for HBP was 6.1 to 8.3 times lower than that for HC. The K_d values for [^{3}H]HBP (9.5 nM) and [^{3}H]HC (30.0 nM) obtained from the above two rate constants were approximately the same as the K_i and K_d values (in the case of HC, the value of the low affinity site). These results suggest that esterification of the hydroxyl group(s) in the side chain of GC by butyrate and propionate increased the affinity to the GC receptor, and that a decrease in the dissociation rate from the receptor caused the increase in the affinity to the GC receptor.

Many chemical modifications have been made to increase the topical anti-inflammatory potency of natural glucocorticoids (GCs) for dermatological therapy. The important changes are halogenation at C-6 and/or C-9 [1, 2], methylation or hydroxylation at C-16 [3, 4], and introduction of a 1,2 double bond [5]. Esterification at the C-17 and/or the C-21 hydroxyl group(s) has also been suggested to greatly increase the lipophilicity of its parent compound [6, 7]. On the other hand, a good correlation has been observed between the relative physiological and pharmacological potencies of the GCs and their affinities for the receptor of the cytoplasm in tissue *in vitro* and *ex vivo* [8–11].

In this study, we examined the binding characteristics of a newly synthesized anti-inflammatory GC, hydrocortisone 17-butyrate 21-propionate (HBP) (Fig. 1), which has weak systemic effects and potent topical effects [12], in comparison to those of its parent compound, hydrocortisone (HC).

MATERIALS AND METHODS

Male Wistar rats, each weighing between 150 and 200 g, were used in all experiments. The animals were fasted for 18 hr but allowed to drink water *ad lib*. The hepatic supernatant fraction was prepared as previously reported [13]. 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 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 2 mM dithiothreitol. The homogenate was centrifuged at 100,000 *g* for 60 min. After removal of the lipoprotein from the resultant supernatant by aspiration, the remaining supernatant was used for the GC binding assay as the cytoplasmic fraction.

Assay of GC binding. The binding of GC was assayed as described previously [13, 14]. [^{3}H]GC was incubated at 0° with 300 μl of 10 mM Tris–HCl (pH 7.4) containing the cytoplasmic fraction, EDTA and dithiothreitol. Unless otherwise noted, the reaction mixture was incubated for 2 hr. The reaction was terminated by the addition of 500 μl activated

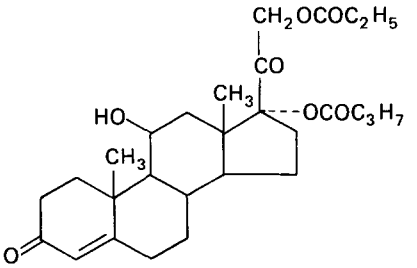

Fig. 1. Chemical structure of hydrocortisone 17-butyrate 21-propionate (HBP).

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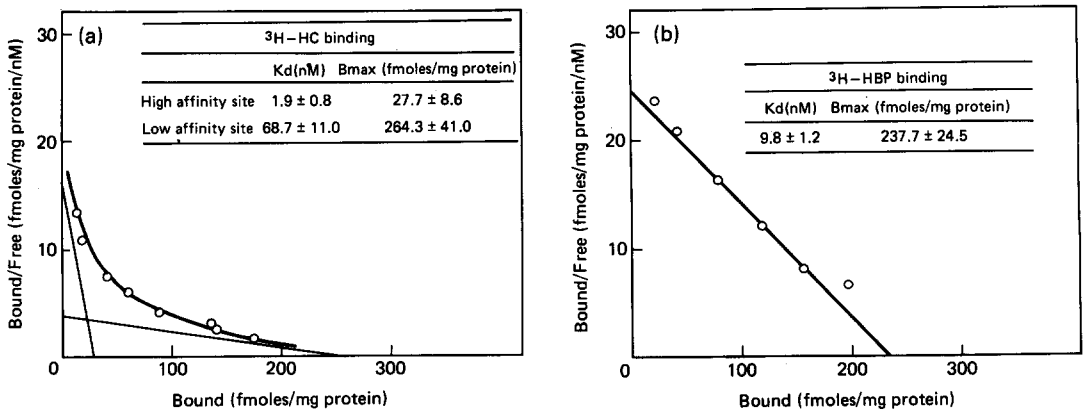

Fig. 2. Scatchard plot analyses of specific binding of [^{3}H]HC and [^{3}H]HBP to cytoplasmic fractions of the rat liver. Concentrations of [^{3}H]HC and [^{3}H]HBP used were 1.0 to 100.0 nM respectively. Each value represents the mean obtained from three separate experiments with [^{3}H]HC (a) and [^{3}H]HBP (b).

charcoal mixture [1% charcoal and 0.1% dextran (mol. wt 60,000–90,000)/10 mM Tris–HCl, pH 7.4] and subsequent mixing for 15 sec. The mixture was centrifuged at 3000 rpm for 10 min at 4°, and then 0.2 ml of the supernatant fraction 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 38–44%. The radioactivity found in the presence of 3 μM non-labeled GC was considered to be non-specific binding of [^{3}H]GC, and this value was subtracted from each experimental value to obtain the amount of specifically bound [^{3}H]GC. Non-labeled HC, HBP and dexamethasone (DEX) were used to determine the specific binding of [^{3}H]HC, [^{3}H]HBP and [^{3}H]DEX respectively.

[^{3}H]DEX and [^{3}H]HBP (both 5 nM) were used for the determination of the affinity of various GCs by measuring the displacement of specific binding of [^{3}H]DEX and [^{3}H]HBP. To measure the association constant, several different concentrations of [^{3}H]-HBP (0.37 to 49.1 nM) and [^{3}H]HC (4.9 to 17.3 nM) were used as the ligand.

The dissociation constant (K_d) and B_{max} values for the binding of [^{3}H]HC and [^{3}H]HBP in hepatic cytoplasmic fractions were determined using Scatchard [15] analysis. Concentrations of [^{3}H]HC and [^{3}H]HBP between 1.0 and 100.0 nM were used for the Scatchard analysis. Kinetic analysis of non-linear Scatchard plots was carried out on a computer system [16] based on the method of Feldman [17] using a PC-8001 (NEC) personal computer.

Protein concentration was determined by the method of Lowry *et al.* [18] using bovine serum albumin as the standard.

Substances used. HC, DEX, betamethasone (BM), and betamethasone 17-valerate (BV) were obtained from Roussel UCLAF, Sigma, Japan Siber Hegner, and SIRS, respectively. [1,2-^{3}H]HC (sp. act. 50.7 Ci/mmole), [1,2-^{3}H]HBP (sp. act. 59 Ci/mmole), and [1,2,4-^{3}H]DEX (sp. act. 46 Ci/mmole) were obtained from New England Nuclear and RCC Amersham, respectively. HB, HBP, clobetasol, and clobetasol 17-propionate were synthesized in our laboratory. The PCS scintillator was obtained from Amersham/Searle.

RESULTS

Scatchard plot analyses of [^{3}H]HBP and [^{3}H]HC binding. [^{3}H]HC bound to the cytosol with high and low affinities (Fig. 2a). On the other hand, [^{3}H]HBP bound to the cytoplasmic fraction with high affinity (Fig. 2b). Ninety percent of the [^{3}H]HC bound had low affinity binding sites and had the same B_{max} value as HBP.

Inhibition of specific binding of [^{3}H]HBP and [^{3}H]DEX to cytoplasmic fractions by various GCs. [^{3}H]HBP binding was dose-dependently inhibited by HBP, DEX, HC and other GCs. The inhibition curves of these GCs were parallel. The K_i values were determined from the inhibition curves. The K_i values and the order of potency of GCs obtained from the inhibition of [^{3}H]HBP binding were in good agreement with those obtained from the experiments of the inhibitions of [^{3}H]DEX binding (Table 1). Esterification of the C-17 and/or the C-21 hydroxyl group(s) of HC, BM or clobetasol significantly increased the affinity to the receptor. The K_i values of HC for [^{3}H]DEX and [^{3}H]HBP binding sites were in good agreement with the K_d value for the low affinity site of [^{3}H]HC. The coefficient of correlation was 0.980, and a significant correlation was observed ($P < 0.01$) between the K_i values obtained from the displacement of [^{3}H]DEX and [^{3}H]HBP bindings.

These results suggested that the binding site of HBP is the same as that of DEX and the low affinity site of HC. Furthermore, the esterification of GC by certain fatty acids such as butyrate, propionate and valerate significantly increased the affinity to the receptor site.

Association and dissociation rates for [^{3}H]HBP and [^{3}H]HC to the binding sites. To estimate the association rate to the binding site with HC, more than 4 nM [^{3}H]HC was used as the ligand, since the

Table 1. Inhibition of [^{3}H]DEX and [^{3}H]HBP binding to cytoplasmic fractions of the rat liver by various esterified and non-esterified glucocorticoids

Glucocorticoids	[^{3}H]DEX		[^{3}H]HBP		K_i (DEX) / K_i (HBP)
	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)	K_i (nM)	
Hydrocortisone (HC)	66.4 ± 3.2*	51.9 ± 2.5*	54.1 ± 5.1	42.3 ± 4.0	1.22
HC 17-butyrate (HB)	11.0 ± 1.3*	8.6 ± 1.5*	7.4 ± 5.3	5.8 ± 4.1	1.48
HC 17-butyrate 21-propionate (HBP)	8.7 ± 2.6*	6.8 ± 2.0*	9.2 ± 2.1	7.2 ± 1.6	1.05
Betamethasone (BM)	6.3 ± 1.7*	4.9 ± 1.3*	5.9 ± 0.8	4.6 ± 0.6	1.06
Betamethasone 17-valerate (BV)	3.4 ± 0.5*	2.6 ± 0.4*	3.9 ± 1.1	3.0 ± 0.9	0.86
Clobetasol	157.0 ± 20.0	117.2 ± 15.6	155.5 ± 35.7	121.5 ± 27.9	0.97
Clobetasol 17-propionate	4.4 ± 0.8	3.4 ± 1.0	6.3 ± 0.7	4.9 ± 0.5	0.69
Dexamethasone (DEX)	7.5 ± 1.6*	5.9 ± 1.3*	10.6 ± 1.2	8.2 ± 0.9	0.72

Each value represents the mean ± S.E.M. obtained from five to nine separate experiments.
K_i values were obtained from the equation $K_i = IC_{50}/1 + \text{ligand concentration}/K_d$ [19].
* Values are quoted from our previous data [20].

low affinity site for HC represented the character of the GC receptor.

The binding of [^{3}H]HBP was slower than that of [^{3}H]HC (Fig. 3, a and b). [^{3}H]HC binding and [^{3}H]-HBP binding reached a steady state in 60 and 120 min respectively. The rate constants of association (k_1) and dissociation (k_2) were obtained from the equation: $k_{ob} = k_1[L] + k_2$ (k_{ob}, observed apparent rate constant; L, several concentrations of ligands) using several concentrations of [^{3}H]HC and [^{3}H]HBP. The association rate for [^{3}H]HC was approximately three times higher than that for [^{3}H]HBP (Fig. 3, a and b, and Table 2). The dissociation rate was also determined by another method. The k_2 values were obtained by adding an excess of unlabeled HC and HBP during the equilibrated state of the bindings (Fig. 4, a and b). The dissociation rate for HBP was 1/6 to 1/8 times that for HC (Fig. 4, a and b, and Table 2).

The K_d (k_2/k_1) values computed from the above two methods were 12.8 and 9.5 nM for HBP and 26.7 and 30.0 nM for HC respectively (Table 2).

The K_d values for HBP in the above experiments were in good agreement with the K_d value derived from saturation studies of Scatchard analysis, but those for HC were a little smaller than the K_d values from Scatchard analysis or K_i values obtained in the displacement experiments. However, the K_d values for HBP were 1/3 to 1/8 times that for HC.

DISCUSSION

Most physiological effects of GCs result from their specific binding to an intracellular receptor protein [21–23]. The relative potencies of GCs in various systems correlate with their affinities to the receptor [8–11]. Bodor *et al.* [24] reported the enhancement of GC activity in esterified compounds using the

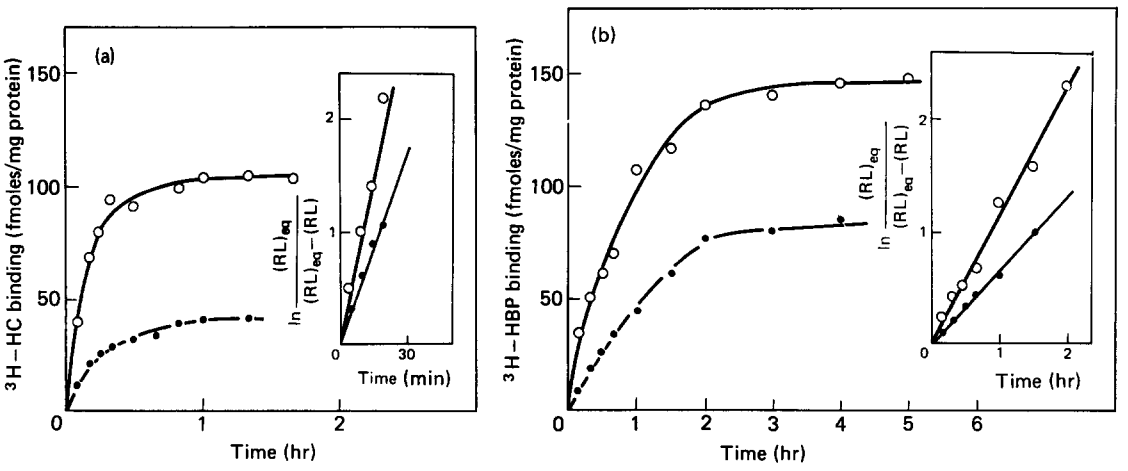

Fig. 3. Time course of associations of specific [^{3}H]HC and [^{3}H]HBP binding. [^{3}H]HC (4.9 and 17.3 nM) (a) and [^{3}H]HBP (4.3 and 17.3 nM) (b) were used as ligands. (RL) and (RL_{eq}) show the concentration of bound radioligand and of bound ligand at equilibrium respectively. [^{3}H]HC and [^{3}H]HBP were incubated with cytoplasmic fractions of the rat liver for the indicated times at 0°, and specific binding was measured.

Table 2. Association and dissociation rate constants of HBP and HC in the rat liver cytoplasmic fraction

	Association experiments			Dissociation experiments	
	k_1 (nM^{-1}, min^{-1})	k_2 (min^{-1})	K_d (k_2/k_1, nM)	k_2 (min^{-1})	K_d (k_2/k_1^*, nM)
HBP	0.040 ± 0.003	0.514 ± 0.014	12.8 ± 1.1	0.425 ± 0.072	9.5
HC	0.117 ± 0.005	3.146 ± 0.201	26.7 ± 0.7	3.510 ± 0.764	30.0

Each value represents the mean ± S.E.M. obtained from three separate experiments.
* The k_1 values obtained from the association experiments were used.

vasoconstrictive activity test, which is often used to evaluate GC activity. GC esters such as 17-propionate, 17-isopropionate, 17-butyrate, 17-valerate and 17- and 21-dipropionate of betamethasone, 17- and 21-dipropionate of beclomethasone, and 17-butyrate of HC are more potent than those of each parent glucocorticoid, betamethasone, beclomethasone and HC [24]. Similar results were obtained for many other GCs by esterification of the C-17 and/or the C-21 hydroxyl group(s) [24].

Recently, with some new GCs for topical use, esterification of the side chain hydroxyl group(s) of GC has been suggested to potentiate the pharmacological activity of the agent [25–28]. The anti-inflammatory activities of the C-17- and C-21-hydroxyl-group-esterified HC (HBP) were more potent than HC or HC-17 propionate [12]. The potentiation induced by esterification was reported to be caused by the change in physicochemical properties such as lipophilicity and solubility [6, 7]. The permeability of the skin to HBP is also increased by esterification with butyrate and propionate [29]. In this study, the affinities of HB, HBP, BV and clobetasol 17-propionate to the receptor were higher

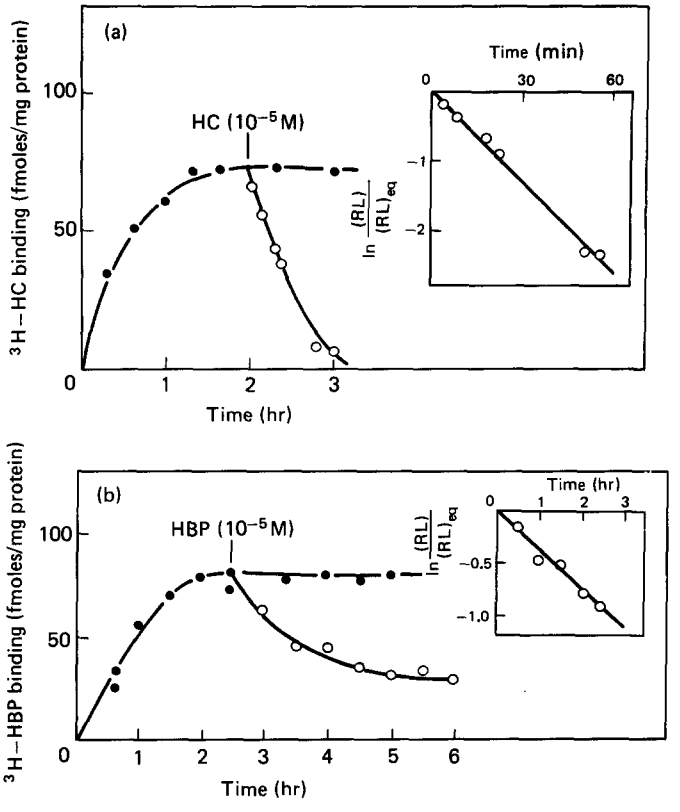

Fig. 4. Time course of dissociations of specific [^{3}H]HC and [^{3}H]HBP bindings. [^{3}H]-HC (14.0 nM) (a) and [^{3}H]HBP (5.6 nM) (b) were used as ligands. [^{3}H]HC and [^{3}H]HBP were incubated with the cytoplasmic fraction of the rat liver for the indicated times at 0° and specific binding was measured. A large excess of HC (10^{-5} M) or HBP (10^{-5} M) was added at 2 or 2.5 hr after the incubation respectively.

than those of each parent compound, HC, BM and clobetasol. In the case of HBP, the increase in the affinity to the receptor was due to the decrease in the dissociation reaction at the receptor site. Concerning the binding sites of HC and HBP, we previously demonstrated that synthetic GCs such as DEX, betamethasone and betamethasone 17-valerate (BV) and esterified HC such as HBP and hydrocortisone 17-butyrate (HB) do not inhibit [^{3}H]HC binding completely even though the concentrations of GCs increased to 3 μM in rat cytoplasmic fraction [20]. On the other hand, in this study, [^{3}H]DEX and [^{3}H]HBP bindings were inhibited completely by HC. The K_i values for [^{3}H]DEX and [^{3}H]HBP were almost the same, and these values were in good agreement with the K_d values of the low affinity site of HC in Scatchard analysis. These data suggest that the low affinity site of HC is the GC receptor, and that HC has another specific binding site of high affinity different from the GC receptor.

Concerning the high affinity site of HC, Beato *et al.* [30] found a binding protein in the cytoplasmic fraction of the rat liver, designated A-binder, which is clearly not transcortine and has high affinity for HC but not for synthetic GC such as DEX. Thus, the high affinity site of HC in this study might be the A-binder reported by Beato *et al.* [30]. The present data demonstrate that the respective esterifications of the C-17 and C-21 hydroxyl groups of HC with butyrate and propionate resulted in a loss of high affinity for the non-GC receptor but increased the affinity for the GC receptor. Furthermore, these data suggest that the potentiation of anti-inflammatory potency of GC by esterification of the hydroxyl group of the parent GC is due not only to the increase in the lipophilicity but also to the increase in the affinity to the GC receptor, mainly due to the decrease in the dissociatin of GC from the GC receptor.

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