Corticotropin-Releasing Factor-Binding Protein Concentrations in Plasma of Patients with Hypothalamic-Pituitary-Adrenal Disorders

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Abstract. Immunoreactive corticotropin-releasing factor-binding protein (CRF-BP) concentrations in human plasma were determined by means of radioimmunoassay for human CRF-BP. CRF-BP antiserum to the C-terminal fragment of human CRF-BP (298-322) was produced, and CRF-BP (298-322) was used as the tracer and the standard. Large amounts of human CRF did not affect measurement of plasma CRF-BP with this radioimmunoassay. The basal plasma CRF-BP concentration in normal subjects was 4.19 ± 0.57 nmol/L (mean ± SD). The CRF-BP concentration was low in patients with Cushing's syndrome, except those with preclinical Cushing's syndrome, and high in patients with Addison's disease, hypopituitarism and isolated ACTH deficiency. After surgery, the plasma CRF-BP concentration in patients with Cushing's syndrome rose, peaked, and then decreased to the control level. In patients with Addison's disease, the high plasma CRF-BP concentration decreased to the control level after hydrocortisone replacement, the same as plasma ACTH concentration. These findings suggest that the immunoreactive CRF-BP concentration in human plasma was decreased by glucocorticoids, at least under chronic conditions.

Keywords: CRF-binding protein, Cushing's syndrome, Adrenal insufficiency, RIA

Received: January 6, 1994
Accepted: June 30, 1994
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CORTICOTROPIN-releasing factor (CRF) is the most potent stimulator of the synthesis and release of ACTH [1]. CRF is present both in peripheral plasma and in hypophysial portal blood [2]. Plasma CRF greatly increases in the third trimester of pregnancy [3], but plasma ACTH remains within the normal range at this time [4]. Most plasma CRF is bound to CRF-binding protein (CRF-BP). This explains why the plasma ACTH of pregnant women remains within the normal range.

CRF-BP specifically binds CRF in human plasma, and this bound form of CRF is biologically inactive [5-7].

CRF-BP is present in human plasma, but not in human cerebrospinal fluid [7], or in the plasma of the rat, sheep or rabbit [7]. CRF-BP has an approximate molecular weight of 38,000 and is a glycoprotein [8] possessing an intramolecular disulfide bond [7]. In our previous studies, CRF-BP binding to CRF was decreased by glucocorticoids [9], and umbilical cord plasma was found to decrease just before delivery [10].

Recently, CRF-BP has been purified from normal human plasma [11] and CRF-BP gene cloned and sequenced [12]. CRF-BP is composed of 322 amino acids (MW = 37,000) [12], and the CRF-BP gene is expressed in rat and human brain as well as in human liver and placenta [13].

We previously tried to develop a CRF-BP RIA with the C-terminal fragment of human CRF-BP.
and have reported our preliminary data [14], but the cross-reactivity of the CRF-BP antiserum with CRF-BP was less than 20%. In the present study, we developed another CRF-BP antiserum and human CRF-BP RIA, and determined plasma levels in patients with hypothalamic-pituitary-adrenal disorders.

**Materials and Methods**

1. **Subjects**

The healthy subjects were between 27 and 60 years old (13 men and 10 women). The subjects with hypothalamic-pituitary-adrenal disorders consisted of patients with Cushing’s disease (n=9), Cushing’s syndrome due to adrenal adenoma (n=8), ectopic ACTH syndrome due to bronchial carcinoma (n=1), preclinical Cushing’s syndrome due to adrenal adenoma (n=4), Addison’s disease (n=7), hypopituitarism (n=4), and isolated ACTH deficiency (n=1). In patients with Addison’s disease or hypopituitarism, with the exception of untreated patients, steroid administration was discontinued for 36 h before the study. We also assessed changes in the plasma CRF-BP concentration in patients with Cushing’s disease (n=3) and in Cushing’s syndrome due to adrenal adenoma (n=1) before and after surgery, and in patients with untreated Addison’s disease (n=1) and in isolated ACTH deficiency (n=1) after starting glucocorticoid replacement. After an overnight fast, venous blood was withdrawn into EDTA tubes and centrifuged at 4°C. The plasma was stored at -20°C until assayed.

2. **CRF-BP RIA**

1) Peptide and antiserum

Synthetic C-terminal 25-residues of human CRF-BP (298–322) (Mitani Sangyo, Co., LTD. Tokyo, Japan) were conjugated to thyroglobulin (Sigma, St. Louis, Mo.) with carbodiimide, as previously described [2]. Japanese white rabbits were immunized with the conjugate in Freund’s adjuvant.

2) Iodination

CRF-BP (298–322) was labelled by the lode-gen method [15].

3) RIA procedure

Human CRF-BP (298–322) was used as the standard. Standards (0.05 ml) with heat-inactivated horse serum or a plasma sample (0.05 ml) were incubated with antiserum (final dilution, 1:10,000) and 125I-CRF-BP (298–322) in a total of 0.5 ml of RIA buffer (25 mM Tris-saline pH 7.5, containing 0.1% bovine serum albumin, 5 mM MgCl2 and 0.04% sodium azide) for 48 h at 4°C. B/F separation was performed by the polyethylene glycol method. All samples were assayed in duplicate.

3. **Gel filtration**

The plasma samples (300 µl) were applied to a 1 X 50 cm Sephacryl S-200 (Pharmacia, Tokyo, Japan) column equilibrated with 0.1 mol/L phosphate buffer containing 1 g/L gelatin, pH 7.4 at 4°C. Immunoreactive CRF-BP in each fraction was determined by CRF-BP RIA.

4. **ACTH RIA**

Plasma ACTH levels were determined with a Mitsubishiyuka ACTH immunoradiometric assay kit (Tokyo, Japan), as previously reported [9].

5. **Statistical analysis**

The CRF-BP concentrations were statistically evaluated by analysis of variance with Duncan’s multiple range test. P<0.05 was considered significant.

**Result**

1. **Characterization of RIA**

Assay sensitivity was 2 fmol/tube, and IC50 was 60 fmol/tube (Fig. 1). The intra-assay and inter-assay coefficients of variation were 5.7% and 11.8%, respectively. There was no cross-reactivity with human CRF (hCRF). Adding 10 ng hCRF to the samples did not affect the measurement of plasma CRF-BP concentrations in this RIA. The dilution curves of plasma CRF-BP paralleled that of the standard. Gel filtration of plasma revealed that immunoreactive CRF-BP eluted in the position of the peak fraction that had binding activity to labelled hCRF. The elution position of CRF-BP immunoreactivity was just before that of B-lactoglobulin (MW 35,000).
2. Basal CRF-BP concentration in plasma (Fig. 2)

The basal plasma CRF-BP concentration in normal subjects was $4.19 \pm 0.57$ nmol/L (mean ± SD). The CRF-BP concentration was low in patients with Cushing’s syndrome due to adrenal adenoma ($3.29 \pm 1.00$ nmol/L), patients with Cushing’s disease ($3.39 \pm 0.81$ nmol/L), and a patient with ectopic ACTH syndrome, but not in patients with preclinical Cushing’s syndrome. CRF-BP levels were high in patients with Addison’s disease (5.78 ± 1.10 nmol/L), hypopituitarism (6.02 ± 1.76 nmol/L) and the patient with isolated ACTH deficiency. However, in two patients with primary and secondary adrenal insufficiency, respectively, who were extremely emaciated, the plasma CRF-BP concentration was within the normal range.

3. Correlation between CRF-BP concentration and cortisol in plasma

There was a significant negative correlation between the plasma CRF-BP concentration and plasma cortisol in patients with hypothalamic-pituitary-adrenal disorders ($Y = -8.101X + 982$, $r = -0.8043$, $P < 0.01$).

4. Postoperative changes in plasma CRF-BP in patients with Cushing’s syndrome (Fig. 3)

The low plasma CRF-BP in 4 patients with Cushing’s syndrome increased after surgery. In patient 2 (Cushing’s disease) the plasma ACTH remained undetectable, and steroid replacement was continued for 10 months after the transsphenoidal surgery. In patient 3 (Cushing’s disease), the pituitary adenoma tissue was left in the sella because of invasion of the cavernous sinus, and plasma ACTH and cortisol increased again 4 months after transsphenoidal surgery. Patient 1 (Cushing’s disease) and patient 4 (Cushing’s syndrome due to adrenal adenoma) were in remission after surgery.

5. Changes in plasma CRF-BP and ACTH concentrations in patients with adrenal insufficiency after glucocorticoid replacement (Fig. 4)

In one patient with Addison’s disease, plasma ACTH and CRF-BP did not change during the first 8 months because the medication was taken only occasionally. After 8 months, the patient started taking the medication (hydrocortisone) exactly as prescribed, and the plasma ACTH and CRF-BP levels decreased to normal. In the patient with isolated ACTH deficiency, the high plasma CRF-BP decreased to normal one month after the start of glucocorticoid replacement.

Discussion

In the present study, we developed an hCRF-BP RIA with the C-terminal 25 amino acids of CRF-
BP, as the standard and tracer, and C-terminal antiserum to this fragment. The plasma dilution curve and the gel filtration analysis results indicate that this RIA cross-reacts with the entire CRF-BP molecule. Moreover, the plasma CRF-BP concentration in normal subjects determined by RIA with the entire CRF-BP [16] was quite similar to that determined by our RIA. CRF-BP (298–322) and CRF-BP (1–322) therefore seem to cross-react on an equimolar basis in our RIA. The measurement of CRF-BP was unaffected by the addition of large amounts of hCRF to the RIA. This suggests that the C-terminal side of the CRF-BP molecule does not play an important role in the binding of CRF to CRF-BP. In our previous reports [9], CRF-BP binding was increased in patients with Addison’s disease and reduced in patients with Cushing’s syndrome. There was a good negative correlation

Fig. 3. Plasma CRF-BP levels in patients with Cushing’s syndrome before and after the surgery. The shaded area indicates the normal range. Patients 1–3, Cushing’s disease; Patient 4, Cushing’s syndrome due to adrenal adenoma; Ope, operation.

Fig. 4. Plasma CRF-BP (——) and ACTH (------) concentrations in a patient with Addison’s disease (left) and a patient with isolated ACTH deficiency (right) before and after glucocorticoid replacement. The shaded area indicates the normal range.
between the plasma cortisol concentration and CRF-BP binding in patients with Cushing's syndrome before and after surgery. In a Scatchard analysis, the number of binding sites was low in patients with Cushing's syndrome. This change in the plasma CRF-BP concentration is similar to that during the postoperative recovery of CRF in patients with Cushing's syndrome [19]. In the present study, the plasma CRF-BP concentration was high in patients with primary and secondary adrenal insufficiency and low in patients with Cushing's syndrome of adrenal and pituitary origin. This finding is in agreement with our previous data [9] and strongly suggests that glucocorticoid reduces plasma CRF-BP, at least under chronic conditions. In patients with Cushing's syndrome, the low plasma CRF-BP increased and peaked from 1 to 3 months after surgery. Thereafter, it returned to the control concentration. In our previous report [9], CRF-BP binding activity recovered 6–12 months after surgery in patients with Cushing's syndrome. We also reported that CRF-BP was a glycoprotein and oligosaccharide chains were important for CRF-BP binding [8]. Therefore, one of the reasons to explain the different recovery of the immunoreactive CRF-BP concentration and CRF-BP binding activity is that glycosylation of CRF-BP may be immature in the early stage of recovery in these patients. In patients with primary and secondary adrenal insufficiency, the high plasma CRF-BP decreased to the control level from 2 to 4 months after starting glucocorticoid replacement. This finding has not been reported yet. In these patients, who were extremely emaciated, the plasma CRF-BP concentration was not high. This suggests that nutrition also affects the plasma CRF-BP concentration. Plasma CRF-BP seems to be of hepatic origin in men and in nonpregnant women, based on the presence of CRF-BP mRNA in the liver [12]. When there is emaciation, the synthesis of CRF-BP in the liver may be decreased, as has been shown in the case of corticosteroid-binding globulin [17] and thyroglobulin [18]. Any direct effect of ACTH on the plasma CRF-BP concentration is unlikely, because high plasma CRF-BP was found in Addison's disease and in isolated ACTH deficiency. Recently, it was reported that plasma CRF-BP is decreased by acute injection of hCRF, but not by ovine CRF. This decrease in plasma CRF-BP was thought to be due to the dimerization of CRF-BP after binding to hCRF [20]. The physiological role of CRF-BP is still unknown, except for the inactivation of hCRF. In our previous study [21], the effect of CRF-BP on the clearance of CRF from plasma was minimal. CRF-BP mRNA was detected in the brain as well as in the liver [13]. Placental CRF-BP may therefore play a role in maintaining pregnancy or delivery, and brain CRF-BP may also play a role in the regulation of CRF activity as an endogenous specific antagonist to hCRF.

In summary, the mechanisms regulating the synthesis and release of CRF-BP are still unknown. However, the CRF-BP concentration in human plasma was reduced by glucocorticoids, at least under chronic conditions.

References


