NOTE

Hypothalamic-Pituitary-Adrenal Axis in WBN/Kob Rats with Non-Insulin Dependent Diabetes Mellitus

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Abstract. In an attempt to define the hypothalamic-pituitary-adrenal (HPA) axis in non-insulin dependent diabetic rats (WBN/Kob), we measured plasma corticotropin releasing factor (CRF), as well as arginine vasopressin (AVP), ACTH, and corticosterone (B). CRF concentrations in the median eminence (ME), the remainder of the hypothalamus (rHY) and the neurointermediate lobe of the pituitary (NIL). We also measured Iodine-125-labeled ovine CRF ([125I]oCRF) binding in the brain and peripheral tissues. Body and thymus weight in WBN/Kob rats were significantly lower than in control Wistar rats, but adrenal weight was higher in WBN/Kob rats. Plasma ACTH levels were significantly higher in WBN/Kob rats than in the control rats. However, plasma CRF, AVP and B levels in WBN/Kob rats were not different from those in the control rats. The CRF concentration was significantly decreased in the ME of the diabetic rats, compared with control rats, but CRF concentrations in the rHY and NIL were unchanged in the two groups. A significant reduction in [125I]oCRF binding in the anterior pituitary was demonstrated in WBN/Kob rats, but no significant difference between the diabetic and control rats in CRF binding was observed in the frontal cortex, spleen or adrenal gland. These findings suggest that the HPA axis is chronically stimulated in the non-insulin dependent diabetic rats.

Key words: Hypothalamic-pituitary-adrenal (HPA) axis, Corticotropin releasing factor (CRF), ACTH, Corticosterone, Diabetes mellitus

THE FUNCTION of the hypothalamic-pituitary-adrenal (HPA) axis in diabetes mellitus (DM) is still controversial. Small but significant elevations in plasma ACTH and corticosterone (B) levels have been observed in streptozotocin (STZ)-induced diabetic rats [1]. In insulin treated alloxan diabetic rats, used as an “acute diabetes” model, the adrenocortical sensitivity to ACTH was reportedly increased and the characteristics of the feed back element were altered [2]. Diabetic animals have been found to have a marked increase in endorphin equivalents, in the neurointermediate lobe of the pituitary (NIL), with no change being observed in β endorphin-like immunoreactivity or ACTH in the anterior pituitary and plasma [3, 4]. In order to further evaluate HPA function in diabetes mellitus, we utilized Wistar Bonn/Kobori (WBN/Kob) rats, a diabetic strain of rats with endoexocrine pancreatic insufficiency. The characteristic features of these diabetic rats are gradual onset of symptoms with polyuria, polydipsia, hyperglycemia without ketonuria, insulin deficiency, high sensitivity to exogenous insulin, and multifocal fibrosis with exocrine pancreatic insufficiency [5]. In the present study, we measured corticotropin releasing factor (CRF), arginine vasopressin (AVP), ACTH and B in plasma, and the concentration of...
CRF in the median eminence (ME) of the hypothalamus, the remainder of the hypothalamus (rHY) and NIL, as well as Iodine-125-labeled ovine CRF ([125I]oCRF) binding in the brain and peripheral tissues.

**Materials and Methods**

**Animals**

All studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Male WBN/Kob rats (approximately 56 weeks old) or age-matched male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) were used. All the animals were housed in a light (12 h on/off) and temperature-controlled room, with food and water available ad libitum.

**Materials**

Iodine-125-labeled ovine CRF ([125I]oCRF) (specific activity, approximately ~2200 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). Unlabeled rat/human CRF (r/hCRF) was purchased from Peptide Laboratories (Osaka, Japan).

**Tissue preparation**

The animals were sacrificed by decapitation at 1000 h and the frontal cortex, the median eminence (ME) of the hypothalamus, the remainder of the hypothalamus (rHY), the anterior and the neurointermediate lobe (NIL) of the pituitary, thymus, spleen and adrenal gland were dissected and frozen in liquid nitrogen for all of the assays. Trunk blood was collected in glass tubes containing 10 mg disodium EDTA. Whole blood was centrifuged for 10 min at 3000 rpm to separate plasma. All tissues and plasma were stored at −30 °C until assay.

**Tissue extraction**

The ME and NIL were homogenized in 2 ml of 0.1 N HCl. Duplicate 200 µl aliquots of each tissue homogenate were dried for protein measurement. The rest of the homogenate was centrifuged for 10 min at 10,000 × g at 4 °C, and the supernatant was lyophilized. The dried extracts were stored at −30 °C until assay.

The rHY and adrenal gland were homogenized in a 2 ml solution composed of 80% acetone and 20% 0.5 N HCl. Duplicate 20 µl aliquots of the homogenate were dried for protein measurement. The remainder of the homogenate was centrifuged for 10 min at 10,000 × g at 4 °C. The supernatant was transferred to another glass tube and 2 ml of petroleum ether was added. The mixture was centrifuged for 5 min at 3,000 × g. The lower layer was transferred to another tube and dried under N2 gas at 40 °C. The dried extracts were stored at −30 °C until assay. Tissue protein was measured with a protein assay kit (Bio-Rad Laboratories, CA, USA).

**Plasma glucose concentration and plasma insulin levels**

The plasma glucose concentration was measured by Antsense (Daikin, Osaka, Japan). The plasma insulin level was measured with a Ab Bead Insulin Radioimmunoassay Kit (Eiken, Tokyo, Japan).

**Plasma AVP, ACTH and B assay**

Plasma AVP, ACTH and B levels were measured with commercially available kits (Mitsubishi Petroleum Chemicals, Tokyo, Japan and ICN Biomedicals Inc., Costa Mesa, CA, respectively).

**CRF radioimmunoassay**

Tissue and plasma CRF concentrations were assayed as previously described [6]. The recovery rates of CRF at 10 and 50 pg/ml in the plasma CRF radioimmunoassay were 55.0 ± 7.0% and 54.4 ± 9.5%, respectively. The intra-assay variation of plasma CRF at 3.1, 8.1, 11.8 and 16.3 pg/ml was 24.2, 9.4, 14.3, and 14.1%, respectively.

**[125I]oCRF binding assay**

Tissues were disrupted in ice-cold buffer (PBS, 10 mM MgCl2, 2 mM EGTA, pH 7.2) with a tissue homogenizer (NITI-ON, Funabashi, Japan) at setting 5 for 15 sec. The homogenate was centrifuged at 40,000 × g for 12 min at 4 °C and washed by resuspending in the same buffer and recentrifug-
ing. After the wash, the tissues were resuspended in the same buffer with a homogenizer to a final protein concentration of 0.2-0.8 mg/ml.

One hundred microliters of the membrane suspension were added to a 1.5-ml polypropylene microtube containing 100 µl [125I]oCRF (final concentration range, 100-150 pM) and 100 µl incubation buffer (tissue preparation buffer with 0.1% BSA, 10⁻⁴ M bacitracin and 100 kallikrein inhibitor units/ml aprotinin) with or without unlabeled r/h CRF. Nonspecific binding was determined in the presence of 300 nM r/h CRF. The reaction was allowed to proceed for 2 h at room temperature, since these conditions were found to be at equilibrium, i.e. at the plateau of the association kinetics curve. The tissue was separated from the incubation medium by centrifugation in a TOMY microfuge (Tokyo, Japan) for 10 min at 12,000 x g at room temperature. The resulting pellet was washed with 1 ml Dulbecco’s PBS (Gibco, Grand Island, NY) containing 0.01% Triton X-100, pH 7.4. The contents were recentrifuged for 10 min at 12,000 x g. The supernatant was aspirated, and the radioactivity of the pellet was measured in a γ-counter (Alloka, Tokyo, Japan) at 80 % efficiency.

Statistical analysis

Data are presented as the mean ± SEM. Statistical evaluations were made with the Mann Whitney-U test. A level of P<0.05 was accepted as statistically significant.

Results

Body weight, thymus weight and adrenal weight in WBN/Kob and control Wistar rats are shown in Table 1. Body and thymus weight in WBN/Kob rats were significantly lower than in control Wistar rats, but adrenal weight was higher in WBN/Kob rats.

The development of diabetic characteristics in WBN/Kob rats was investigated by measuring plasma glucose and insulin levels. Plasma glucose levels were significantly increased and plasma insulin levels were significantly decreased in WBN/Kob rats compared with control rats (Table 1).

Plasma CRF levels in WBN/Kob rats were not significantly different from those in control rats. Plasma ACTH levels were significantly increased in WBN/Kob rats compared with control Wistar rats, and B levels were very similar in the two groups. Plasma AVP levels of diabetic rats tended to be higher in WBN/Kob than in control rats, but there was no statistically significant difference between the two (Fig. 1).

The CRF concentration in the ME was significantly decreased in WBN/Kob rats compared with control rats. However, the concentrations of CRF in the rHY and NIL in the WBN/Kob and control rats were not statistically different (Fig. 2).

To evaluate further HPA responses in WBN/Kob rats, we measured [125I]oCRF binding in the brain and peripheral tissues. [125I]oCRF binding in the pituitary was dramatically decreased in WBN/Kob rats compared with control rats, but there was no difference in [125I]oCRF binding in the frontal cortex, spleen or adrenal gland (Fig. 3).

Discussion

In the present study, plasma ACTH levels in WBN/Kob rats were higher than in control rats. The mechanisms involved in the increased ACTH levels in the diabetic rats are unknown. We have also observed higher ACTH levels in other genetic diabetic rats, Otsuka Long Evans Tokushima Fatty

### Table 1. Body weight, adrenal weight, thymus weight, and plasma glucose and immunoreactive insulin levels in WBN/Kob and control Wistar rats

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Adrenal weight (mg)</th>
<th>Thymus weight (mg)</th>
<th>Plasma glucose (mg/dl)</th>
<th>Immunoreactive insulin (µU/ml)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>440.6 ± 10.1</td>
<td>9.48 ± 0.36</td>
<td>62.3 ± 4.7</td>
<td>147.8 ± 2.3</td>
<td>77.9 ± 11.3</td>
</tr>
<tr>
<td>WBN/Kob</td>
<td>355.0 ± 7.5**</td>
<td>11.3 ± 0.42*</td>
<td>15.1 ± 2.4**</td>
<td>678.7 ± 30.8**</td>
<td>8.69 ± 0.7**</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM (n=6). * and ** denote significant changes at * P<0.01 and ** P<0.001, respectively from control Wistar rats.
(OLETF), compared with control animals (unpublished data). These data are in agreement with the fact that Streptozotocin treated rats showed elevated plasma ACTH levels compared with non-treated rats [1]. Since elevated ACTH levels have been observed in the different diabetic animals, it is likely that the alterations may be a consequence of increased glucose levels, and/or a chronic stressed condition, but the possible strain specificity of the HPA axis can not be excluded.

To further evaluate ACTH secretion in WBN/Kob rats, we measured CRF concentrations in the brain. The CRF concentration was significantly decreased in the ME in WBN/Kob rats as compared with control Wistar rats, but not in the rHY or NIL. We previously reported that after water immersion-restraint stress, the CRF concentration was significantly decreased in the ME, but not in the rHY or NIL [7]. Although the reduction in the CRF concentration most likely has been observed following acute stress, it has been reported that not only acute but also chronic stress reduces CRF-like immunoreactivity in the arcuate nucleus/median eminence (ME) [8]. Hypersecretion of CRF into the portal vessels in the chronic stressed condition may therefore be responsible for the decrease in the CRF concentrations in the ME. [125I]oCRF binding was significantly decreased in the pituitary in WBN/Kob rats compared with control Wistar rats. Aguilera et al. reported that, after chronic CRF administration, the CRF receptor concentration was decreased in anterior pituitary membranes without any change in binding affinity [9–11], suggesting CRF receptors were reduced.
in the pituitary. Since the measurement of CRF binding in the present study was obtained with a ligand concentration in excess of the Kd value, it is likely that the decrease in [125I]oCRF binding in WBN/Kob rats represents a decrease in receptor density. Taking together the decrease in the CRF concentration in the ME and in [125I]oCRF binding in the anterior pituitary, it is likely that CRF secretion from the ME to the hypophysial portal vein is increased, and this hypersecretion may directly stimulate ACTH secretion from the anterior pituitary in WBN/Kob rats. In contrast to the marked reduction in CRF binding in the pituitary, CRF receptor densities in the frontal cortex, spleen and adrenal gland were unchanged in WBN/Kob and control rats. These data are consistent with a previous observation that the CRF receptor content was reduced after immobilization stress in the pituitary but not in the brain areas [9]. The differential regulation of CRF receptors between the pituitary and other tissues may be related to differences in the nature of hormone-receptor interactions in each tissue [9].

In the present study, no difference was found between the plasma CRF levels in diabetic rats and controls. The sources of plasma CRF are not only the hypothalamic paraventricular nucleus but peripheral tissues, i.e., pancreas, adrenal gland and intestine [12, 13]. These results suggest that hypothalamic CRF contributes to a very limited part of plasma CRF. The small or moderate change in CRF secretion in the hypophysial portal vein may not be sufficient to affect peripheral plasma CRF concentrations.

Plasma AVP levels in diabetic rats tended to be higher than in control rats, but the difference was not statistically significant. Although plasma osmolality could not be measured in this study, it has been reported that plasma AVP levels were elevated in diabetic animals [14], with the speculation that hyperglycemia and/or hyperosmolality stimulate AVP secretion. Both AVP and CRF are likely to play important roles in the responsiveness of the HPA axis during stress [15-17]. While AVP is a weak ACTH stimulator by itself, it markedly potentiates the effects of CRF [18, 19]. Furthermore, it is well known that AVP increases CRF and ACTH secretion in both in vivo and in vitro experiments [20, 21], suggesting that central AVP may modify the hypersecretion of CRF and ACTH in diabetic rats.

In contrast to plasma ACTH, plasma B levels in the two groups were not significantly different. An alteration in adrenal function may explain the discrepancy. Thymus weights were lower in diabetic rats than in the controls, suggesting that the plasma steroid levels might have been increased during maturation. In Streptozotocin-induced diabetes (STZ) rats, adrenal weight was increased and thymus weight was decreased compared with the
controls [1, 22]. Both plasma and urine B levels in STZ rats were significantly higher than control [22]. These differences could be attributed to the use of different animals, as the WBN/Kob rats that we used in this study have genetic diabetes. In addition, we utilized aged rats (56 weeks old) as WBN/Kob rats need rather long periods to establish the overt diabetic state, suggesting that aging factors may influence the adrenal function. The high plasma glucose levels, more than 600 mg/dl, and dramatic decrease in immunoreactive insulin (IRI) in WBN/Kob rats may change steroid synthesis and/or secretion in the adrenal glands. In addition we have found no change in the difference between plasma corticosterone concentrations in OLETF rats and controls. B concentrations in the adrenal gland in OLETF rats were not different from control rats (unpublished data). Additional studies will be required to determine the precise mechanisms controlling corticosterone concentrations in diabetic rats.

In 1964, Lentle et al. reported that patients with complicated diabetes have higher levels of plasma cortisol throughout the day than do healthy persons or patients with uncomplicated diabetes [23]. Some investigators have reported that the increase in plasma ACTH and cortisol in non-insulin dependent diabetes mellitus correlated positively with the duration of diabetes [24], the poor control state [25] and the degree of retinopathy [23, 26]. Recently, Tsigos et al. reported that diabetic neuropathy is associated with increased activity of the hypothalamic-pituitary-adrenal axis [27]. We have previously reported that in the non-insulin dependent diabetic patients, plasma cortisol and ACTH levels were both higher than in control patients [28], suggesting that the pituitary-adrenal axis in diabetes mellitus patients may be stimulated.

In summary, we utilized WBN/Kob rats that have genetic non-insulin-dependent diabetes as a diabetic animal model. CRF concentrations in ME and CRF binding in the pituitary were significantly decreased in WBN/Kob compared with control Wistar rats. Plasma ACTH was increased in diabetic rats. These findings suggest that the HPA axis is chronically stimulated in the non-insulin dependent diabetic rats. In order to confirm this conclusion, we should have conducted the same experiments on insulin-treated WBN/Kob rats.

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