Enhanced ACTH release precedes the onset of diabetes in the NOD mice?

Yanina Revsin, Melly S. Oitzl and E. Ronald de Kloet
Abstract

Defects in the hypothalamus-pituitary-adrenal (HPA) axis regulation may play a role in further exacerbation of the autoimmune response against the pancreas beta-cells preceding the onset of diabetes. In a model of autoimmune type 1 diabetes, the nonobese diabetic (NOD) mouse, were reported: 1) increased levels of circulating glucocorticoids, suggesting activation of the HPA axis and 2) disturbances in brain areas regulating the HPA system, such as the hippocampus. Therefore, in the present study we hypothesized that altered HPA axis regulation in NOD mice may signal the onset and progression of the disease. Hence, we examined molecular markers of the hippocampus and the HPA axis in diabetic and non-diabetic littermates. The results revealed that a group of non-diabetic mice presents high ACTH release and low corticosterone levels suggesting adrenal hyporesponsiveness. This finding raises therefore the question whether it is possible that the profound ACTH activation precedes the onset of type 1 diabetes mellitus in a group of non-diabetic NOD mice. During full-blown diabetes glucocorticoids are elevated and ACTH is significantly lower as compared to non-diabetic littermates, suggesting a switch of hypo- to hyperresponsiveness of the adrenals to ACTH. Moreover, negative feedback regulation seems compromised because of downregulation of the glucocorticoids receptor in hippocampus and hypothalamus.
IN THE NONOBSE DIABETIC (NOD) mouse, a model of autoimmune type 1 diabetes (T1D), autoimmunity against beta cells is evident as early as 4 weeks of age, when infiltration of mononuclear cells into pancreatic islets (insulitis) is observed. The frequency of insulitis reaches 70% to 90% by 9 weeks of age, and by 20 weeks of age, almost 100% of mice of both sexes develop insulitis. Despite the development of insulitis in almost all NOD mice, only some of them develop overt diabetes: 80% to 90% of females and 10% to 50% of males. In this T1D model, brain alterations were described, such as: 1) increased number of glial fibrillary acidic protein reactive astrocytes in the hippocampus during the early post-weaning period (4 weeks of age, not yet diabetic animals), which was aggravated in diabetic mice (Saravia et al, 2002), 2) decreased cell proliferation after diabetes onset (Beauquis et al, 2007) and, 3) increased expression of hypothalamic arginine-vasopressin (AVP) and oxytocin proteins and mRNAs in diabetes (Saravia et al, 2001). Moreover, diabetic NOD mice also present increase levels of circulating glucocorticoids (Fitzpatrick et al, 1992. Amrani et al, 1994) suggesting activation of the hypothalamus pituitary adrenal (HPA) axis.

Animals at risk to develop endocrine/organ-specific autoimmune diseases show various pre-autoimmune aberrancies that need to interact abnormally before autoimmune disease can fully develop. In this abnormal interaction additional aberrancies in other regulatory systems may play a role in a further exacerbation of the self-directed immune response, such as defects in the HPA axis system (Lam-Tse et al, 2002).

This concept and the findings in pre-diabetic and diabetic states suggest that the NOD mouse is an excellent model to study the sequential changes in HPA axis activity. Therefore, in the present study the hypothesis was tested that altered HPA axis regulation may signals the onset and progression of the disease. Hence, we examined molecular markers of the hippocampal-HPA axis in NOD diabetic mice and NOD non-diabetic littermates. C-peptide concentrations were measured as an indicator for beta-cell activity, as well cytokines levels to monitor the autoimmune condition. The data suggest that in the pre-diabetic state a subgroup of animals can be identified which exhibit elevated ACTH and C-peptide levels.

Research Design and Methods

Animals

A NOD mouse colony was bred at the animal facility of the LACDR Leiden from adult NOD mice from Prof. Dr. Drexhage’s laboratory (Department of Immunology, Erasmus University, Rotterdam, The Netherlands). Newborn pups were culled (4 males and 4 females per dam) and group housed (4 mice per cage). After weaning the rats were kept undisturbed under constant humidity (55±5 %) and temperature (23±2 °C) conditions with 12-12 light-dark hours cycle (lights on at 8 am). Food and water was provided ad libitum. The animal experiments were performed in accordance with the European...
Communities Council Directive 86/609/EEC and with approval from the animal care committee of the Faculty of Medicine, Leiden University.

**Treatment**

From week 12 of age glycemia levels were daily measured to assess diabetes (Accu-Chek Compact, Roche, Germany). Animals with blood glucose levels higher than 11mM were classified as overtly diabetic. At 25 days after diabetes onset male mice were decapitated between 9 am and 11 am. At the same time, randomly chosen non-diabetic littermates were sacrificed as control animals. After decapitation the brain was quickly removed, frozen in isopentane and stored at -80 °C until processing for later use in *in situ* hybridization. Trunk blood was collected for corticosterone, ACTH, C-peptide and cytokines (IL-1α, IL-6 and TNFα) measurements by radioimmunoassay (RIA) and Bio-Plex system respectively.

**In situ hybridization**

Determination of mRNA levels of MR, GR, AVP and CRH were measured on coronal brain cryosections (14 μm) containing hippocampus (distance from bregma -1.7 to -2.06 mm) and PVN (distance from bregma: -0.7 to -1.06 mm) (Paxinos and Franklin, 2001). Two or three sections from each mouse were mounted on poly-l-lysine (Sigma) coated slides and stored at −80 °C. *In situ* hybridization procedure follows the previously described by Revsin *et al* 2008. The mean of 4-6 measurements of each riboprobe were calculated for each animal.

**Radio immuno assay (RIA)**

Trunk blood was collected individually in labeled potassium-EDTA coated tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany). Blood samples were kept on ice and later centrifuged for 15 minutes at 3000 rpm at 4 °C. Plasma was transferred to clean tubes and stored frozen at -20 °C until the determination of corticosterone, ACTH by MP Biomedical RIA kit (ICN, Biomedicals Inc., CA). C-peptide concentrations were determined with a RIA kit following the manufacturer’s instructions (Linco Research St. Charles, Missouri, USA).

**Cytokine determination**

Cytokines were determined at the Luminex Core Facility (University Medical Center, Department of Pediatrics, Utrecht, The Netherlands) as previously described (de Jager *et al*, 2005). The Bio-Plex system employing the Luminex multi-analyte profiling technology (xMAP), allows individual and multiplex analysis of up to a hundred different mediators in a singe well containing a sample volume of 10 μl.
Data analysis and statistics

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Software (version 4). For corticosterone and ACTH plasma levels, 28 non-diabetic and 13 diabetic mice were used. For C-peptide and cytokines concentrations and in situ hybridizations 6 mice per group were randomly chosen from the pool of non-diabetic and diabetic mice; the mRNAs expression values were assessed by optical density (o.d.) of the signal on autoradiographic film. Statistical analysis was performed by one way ANOVA plus Bonferroni post test. Statistical differences were considered significant when p<0.05.

Results

Corticosterone, ACTH, C-peptide and pro-inflammatory cytokine concentrations.

In diabetic mice (glycemia: 21.72±3.61 mM, p<0.05 vs non-diabetic), basal corticosterone levels increased significantly compared to non-diabetic littermates (Figure 1A). While the corticosterone levels were elevated, basal plasma ACTH in the same animals was significantly decreased after diabetes onset as compared to non-diabetic mice (glycemia: 8.64 ±0.68 mM) (Figure 1B). However, a subgroup of non-diabetic animals (non-diabetic group 2) exhibits profoundly elevated ACTH levels. Based on this observation of different ACTH concentrations between the non-diabetic mice, 2 subgroups were identified: non-diabetic group 1 = non-diabetic mice with non altered ACTH (100.9± 25.17 pg/ml), and non-diabetic group 2 = non-diabetic mice with high ACTH (1934± 203.7 pg/ml) (>1000 pg/ml, p<0.001 vs non-diabetic group 1). These two subgroups were euglycemic and did not differ in body weight (data not shown).

C-peptide determination was performed to assess beta-cell function. Figure 1D shows that non-diabetic mice with highly elevated ACTH levels (non-diabetic group 2) also had significantly elevated C-peptide concentrations compared to non-diabetic group 1 and diabetic littermates. The C-peptide concentrations of non-diabetic group 1 mice were similar to non-fasting levels from control c57Bl/6 mice (0.5 to 1 nM, data not shown), indicating that the non-diabetic group 2 animals produce elevated C-peptide secretion. Additionally, C-peptide levels of diabetic mice are significantly decreased as compared to non-diabetic mice (group 1 and group 2).

Pro-inflammatory cytokines IL-1 alpha, IL-6 and TNF-alpha did not vary among the groups (data not shown).

HPA axis disturbances

In situ hybridization revealed no differences in the mRNAs expression of MR in the hippocampus and CRH in the PVN between diabetic and non-diabetic mice (data...
not shown). However, GR mRNA in the hippocampus (CA1 and DG) and PVN was significantly decreased (Figure 2A and 2B), while AVP mRNA was significantly elevated in diabetic mice as compared to non-diabetic (Figure 2C).

The two groups of non-diabetic mice, which differed in ACTH and C-peptide levels, did not exhibit differences in expression of any of the above-mentioned mRNAs.

**Discussion**

Our findings identify a subgroup of non-diabetic NOD mice showing elevated ACTH concentrations without the concomitant increase in corticosterone. Although the prediabetic phenotype seems to be characterized by adrenal hyposresponsiveness in view of high basal ACTH vs typical basal corticosterone levels, the opposite is observed in long-term diabetes. During full blown type 1 diabetes basal corticosterone level is elevated and basal plasma ACTH level is significantly lower as compared to non-
diabetic littermates, a condition suggesting hyperresponsiveness of the adrenals to ACTH. Moreover, downregulation of the glucocorticoid receptor in the hippocampus and PVN of diabetic animals suggests that the capacity of corticosterone to suppress the HPA axis is diminished. Such an impaired negative feedback would further promote hypercorticism.

The finding raises the question whether the activation of ACTH release precedes full-blown diabetes in the NOD mouse model of autoimmune type 1 diabetes. We showed in the present study, that enhanced ACTH release occurs concomitantly with increased C-peptide release (non-diabetic group 2), indicative of increased beta-cell activity. Mild transiently elevated insulin levels have been reported very early during the pre-diabetic period in NOD mice, developing between 2 and 4 weeks of age and persisting until 8 weeks of age (Amrani et al., 1998; Homo-Delarche, 1997; Orban et al., 2001). Moreover, transient hyperinsulinemia has also been observed in another model of spontaneous T1D, the BioBreeding (BB) rats, a few days before the onset of T1D, and perfused inflamed, but not uninflamed, islets exhibit beta-cell hyperactivity (Nakhooda et al., 1978; Teruya et al., 1993). In view of these and our results, it seems likely that the group of mice
with elevated C-peptide might develop T1D. Therefore, ACTH release may precede the cascade of endocrine events triggered by the destruction of insulin-producing cells. However, definite proof for these sequences of events requires longitudinal studies as well as the demonstration of more severe insulitis in these animals (non-diabetic group 2).

It is also noteworthy that in this subgroup in the face of ACTH hypersecretion, corticosterone levels remain unaffected, indicating decreased sensitivity of the adrenals to ACTH and/or impaired adrenal function. Such a relatively reduced adrenal function would facilitate the progression of autoimmunity, but how would such hyporresponsiveness develop? Lymphocytic infiltration of the adrenal gland was previously described in NOD mice. However, no signs of immune destruction of the adrenal neither in pre-diabetic nor in diabetic NOD mice were detected (Breidert et al, 1998). Moreover, lymphocytic infiltration of the adrenal glands was not accompanied by changes in corticosterone levels (Beales et al, 2002). From these studies, we can discard the idea that the lack of a corticosterone response is due to adrenal immune destruction in the subgroup of pre-diabetic NOD mice with high ACTH concentration. Although our results on plasma cytokine concentrations did not provide any difference between groups at the time of decapitation, we can not exclude changes in the adrenal cytokine balance to explain the lack of increased corticosterone concentrations in view of the increase in ACTH levels. Apparently, the adrenal glands were hyposensitive to ACTH and additional analysis of ACTH receptor expression levels, as well as the concentration of the various cytokines in the adrenal glands might help to elucidate this phenomenon.

In full-blown diabetes, we found, as previously described by Fitzpatrick et al in 1992, corticosterone hypersecretion indicating HPA axis activation. However, ACTH levels are significantly decreased in NOD diabetic mice, suggesting hyperresponsiveness of adrenals to ACTH. These data are in parallel with the ones described by our laboratory in the pharmacological model of T1D, the STZ-diabetic mice (Revsin et al, 2008). In the later model, we described a possible underlying mechanism for this observation: 11-days diabetic mice show upregulation of adrenocortical ACTH receptors (melanocortin 2 and 5) and increased corticosterone release from adrenocortical cell cultures challenged to ACTH.

In conclusion, the present data indicate that the profound ACTH activation is present in a group of non-diabetic NOD mice without increased corticosterone levels. It is conceivable that this state of adrenal hyporesponsiveness facilitates autoimmunity to the beta-cells; hence, increased ACTH release may precedes the onset of type 1 diabetes mellitus which in its full development switch to hyperresponsiveness of the adrenals and hypercorticism.

Acknowledgments

We thank Dr. M. Frolich for the insulin measurement and Dr. G. Rijkers for the cytokines
measurements. This work was supported by grants from The Netherlands Organization for Scientific Research (NWO-WOTRO) Grant 88-252 and the Royal Netherlands Academy for Arts and Sciences.

References

Amrani, A, Chaouloff, F, Mormede, P, Dardenne, M, Homo-Delarche, F. Glucose, insulin, and open field responses to immobilization in nonobese diabetic (NOD) mice. Physiol Behav. 56:241-246, 1994


HPA axis activation in NOD mice

