Plasma Adiponectin Levels are Increased Despite Insulin Resistance in Corticotropin-releasing Hormone Transgenic Mice, an Animal Model of Cushing Syndrome


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Abstract. Adiponectin (AdN), an adipokine derived from the adipose tissue, has an insulin-sensitizing effect, and plasma AdN is shown to be decreased in obesity and/or insulin resistant state. To clarify whether changes in AdN are also responsible for the development of glucocorticoid-induced insulin resistance, we examined AdN concentration in plasma and AdN expression in the adipose tissue, using corticotropin-releasing hormone (CRH) transgenic mouse (CRH-Tg), an animal model of Cushing syndrome. We found, unexpectedly, that plasma AdN levels in CRH-Tg were significantly higher than those in wild-type littermates (wild-type: 19.7±2.5, CRH-Tg: 32.4±3.1µg/mL, p<0.01). On the other hand, AdN mRNA and protein levels were significantly decreased in the adipose tissue of CRH-Tg. Bilateral adrenalectomy in CRH-Tg eliminated both their Cushing’s phenotype and their increase in plasma AdN levels (wild-type/sham: 9.4±0.5, CRH-Tg/sham: 15.7±2.0, CRH-Tg/ADX: 8.5±0.4µg/mL). These results strongly suggest that AdN is not a major factor responsible for the development of insulin resistance in Cushing syndrome. Our data also suggest that glucocorticoid increases plasma AdN levels but decreases AdN expression in adipocytes, the latter being explained possibly by the decrease in AdN metabolism in the Cushing state.

Key words: Adiponectin, Glucocorticoid, Insulin resistance, Cushing syndrome, Obesity

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dehydrogenase) is overexpressed [8, 9]. Since visceral adipose tissue is considered to be the major source of adipokines, the increase in visceral fat mass with the resultant increase in plasma adipokine levels may at least partly be responsible for the occurrence of insulin resistance in Cushing syndrome with glucocorticoid excess. Indeed, glucocorticoid is shown to stimulate the release of resistin from mouse adipocytes [10], and serum resistin levels are elevated in Cushing syndrome [11], indicating that resistin may be involved in the glucocorticoid-induced insulin resistance.

Adiponectin (AdN), also known as Acrp30, GBP 28, apM1 or AdipoQ identified by four independent groups [12-15] is recognized as one of the adipokines. AdN gene encodes a secreted protein expressed exclusively in both white and brown adipose tissues. AdN protein belongs to the complement 1q family, which is shown to form a characteristic homomultimer in the blood. Unlike other adipokines, AdN is shown to improve insulin resistance, and indeed its serum concentration is decreased in obese and insulin-resistant states [16, 17]. Clinical studies also show the existence of an inverse relationship between visceral fat mass and serum AdN levels [18, 19] and lower AdN levels are associated with a higher incidence of diabetes [20, 21]. The role of AdN in the pathogenesis of insulin resistance in Cushing syndrome, however, has not been completely examined. Previous in vitro studies have shown that glucocorticoid exerts an inhibitory effect on AdN production and secretion [22, 23]. In contrast, in patients with Cushing disease or with glucocorticoid administration, serum AdN levels were not altered [24, 25].

Thus, in this study, we tried to address the question of whether AdN is involved in the development of glucocorticoid-induced insulin resistance in vivo. Using the corticotropin-releasing hormone (CRH) transgenic mice (CRH-Tg), an animal model of Cushing syndrome, we examined both circulating AdN levels and AdN mRNA/protein levels in the adipose tissue. Unexpectedly, we found an increase and decrease in AdN in plasma and AdN mRNA/protein levels in the adipose tissue, respectively, and the increase in plasma AdN levels were abolished following adrenalectomy. The present results suggest that AdN is not responsible for the glucocorticoid-induced insulin resistance and that glucocorticoid increases circulating AdN concentrations.

Materials and Methods

Animals

We used CRH transgenic overexpression mouse (CRH-Tg), an animal model of Cushing syndrome, which was characterized previously [26], and also their wild-type littermates (WT). All the experiments were carried out using male mice. All mice were maintained under controlled conditions of light (lights on, 0600-1800 h) at 24°C in a humidity-controlled room and allowed free access to standard chow and drinking water. All procedures were approved by the animal committee of the Kochi Medical School.

Evaluation of metabolic parameters in CRH-Tg mice

For the intraperitoneal (ip) glucose tolerance test, CRH-Tg and WT littermates (12 weeks old) were fasted overnight and then injected ip with glucose [2g/kg body weight (BW)]. Tail blood samples were serially obtained before and at 30, 60, 120 min after injection, and blood glucose levels were determined by the enzymic method (FreeStyle: Nipro Inc., Japan). For the insulin tolerance test, another set of CRH-tg and WT mice (10 weeks old) were fasted overnight and then injected ip with insulin (0.7 unit/kg BW). Blood glucose levels were determined as described above.

For the determination of blood hormone levels, CRH-Tg mice and their WT littermates (6 or 14 weeks old, n=6-8 in each group) were decapitated between 0900-1200 h. Trunk blood was collected for measuring plasma corticosterone and insulin levels using commercially available RIA/ELISA kits (corticosterone RIA kit, MP Biomedicals, OH, USA, and insulin ELISA kit, Morinaga Inc., Japan). For the determination of plasma AdN, two different ELISA kits were used (CircuLex Inc., Japan and Linco Research, MO, USA). Additionally, an ELISA kit for specific high molecular weight (HMW) form of AdN was used to analyze the molecular isoform of plasma AdN (Wako Chemicals, Japan).

In both groups of mice, the thymus, adrenal glands, subcutaneous fat (inguinal region), brown fat (scapular region), epididymal fat, and peri-renal fat were obtained for weighing. In addition, epididymal fat was frozen in liquid nitrogen, and kept at –80°C until RNA or protein extraction.
INCREASED ADIPOНЕCTIN IN CRH-Tg MICE

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Table 1. Body/tissue weights and basal plasma parameters in CRH-Tg and control mice

<table>
<thead>
<tr>
<th></th>
<th>6 weeks old</th>
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<th>14 weeks old</th>
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<tbody>
<tr>
<td></td>
<td>WT (n = 7)</td>
<td>CRH-Tg (n = 6)</td>
<td>WT (n = 8)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>20.8±0.5</td>
<td>16.6±0.5*</td>
<td>29.0±0.6</td>
</tr>
<tr>
<td>Sc fat/BW (%)</td>
<td>15.2±1.4</td>
<td>15.1±1.4</td>
<td>13.6±0.8</td>
</tr>
<tr>
<td>Br fat/BW (%)</td>
<td>5.9±0.6</td>
<td>10.6±1.1*</td>
<td>5.5±0.5</td>
</tr>
<tr>
<td>Ed fat/BW (%)</td>
<td>10.7±0.6</td>
<td>9.8±1.0</td>
<td>14.6±1.3</td>
</tr>
<tr>
<td>Pr fat/BW (%)</td>
<td>3.2±0.2</td>
<td>2.7±0.2</td>
<td>5.2±0.6</td>
</tr>
<tr>
<td>Thymus/BW (%)</td>
<td>2.3±0.1</td>
<td>0.7±0.1*</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Adrenal/BW (%)</td>
<td>0.2±0.0</td>
<td>0.3±0.0*</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>117.7±5.5</td>
<td>85.7±4.6*</td>
<td>78.8±5.0</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>2.3±0.4</td>
<td>4.4±0.8*</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>Plasma cort (ng/mL)</td>
<td>14.5±4.7</td>
<td>99.5±33.2*</td>
<td>13.5±2.7</td>
</tr>
</tbody>
</table>

*P<0.05 vs. wild-type littersmates at the same age.

Adrenalectomy (ADX)

CRH-Tg and WT littersmates (12 weeks old) were divided into 3 groups, i.e., (1) WT with sham operation, (2) CRH-Tg with sham operation, and (3) CRH-Tg with ADX, (n=8-12 in each group). ADX or sham operation was carried out under pentobarbital anesthesia. Two weeks after operation, all mice were decapitated between 0900-1200 h, and trunk blood and tissues were obtained as described above.

Quantitative analysis of AdN mRNA

Total RNA was extracted from the adipose tissue using Trizol reagent (Invitrogen, Carlsbad, CA). RNA samples obtained were treated with DNase, and first strand DNA was synthesized with reverse transcriptase (QuantiTect RT kit: Qiagen, Germany). Each sample was then applied for quantitative real-time PCR analysis of AdN mRNA using TaqMan probes (ABI PRISM 7000: Applied Biosystems, CA). Primer sets for the mouse GAPDH (Applied Biosystems) were used as an internal control.

Western blotting

Tissue protein was extracted from the adipose tissue using T-PER Protein Extract Reagents (Pierce, IL). After heat treatment (at 95 °C for 5 min), the extracts were applied for electrophoresis, and then incubated with anti-mouse AdN antibody (CircuLex, Japan), and with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA). Finally, the bands were detected by chemiluminescence using ECL plus the Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK), and optical densities of bands were measured by the densitometer.

Statistical analysis

All data were expressed as the mean ± SEM. Statistical difference between the control and experimental group was determined by ANOVA followed by Fisher’s PLSD test, and P values below 0.05 were considered significant.

Results

CRH-Tg shows overt glucocorticoid excess

In CRH-Tg, plasma corticosterone levels were markedly elevated compared with those in WT littermates at the ages of both 6 and 14 weeks (Table 1). Plasma insulin levels and body fat weights in CRH-Tg were also significantly increased at the age of 14 weeks. Increased adipocyte size rather than its number was reported in adipose-specific 11β HSD-1 overexpression mice [8], suggesting that the former mechanism due to glucocorticoid excess is responsible for the increased fat weight in CRH-Tg. In addition, CRH-Tg showed atrophic thymus and hypertrophic adrenal glands compared with WT littermates, reflecting the actual existence of ACTH-induced hyperadrenocorticism.
In vitro [22, 23]. In contrast, no difference between WT and CRH-Tg was observed in adN mRNA levels in subcutaneous and brown adipose tissue (data not shown). The protein levels of adN in the epididymal adipose tissue, determined by Western blotting, were also decreased in CRH-Tg (Figs. 2, 3), suggesting that local adN production is decreased in cRH-tg, an animal model of Cushing syndrome. Unexpectedly, however, we found that plasma adN concentrations were significantly higher in CRH-Tg at both 6 and 14 weeks, despite the increase in their fat weight (Fig. 4 and Table 1). We also observed that plasma concentration of high molecular weight (HMW) form of plasma adN in cRt-tg were tended to be higher than those in WT, although it did not reach statistical significance (HMW AdN: WT 2.6±0.3, CRH-Tg 3.3±0.2µg/mL, p=0.09).

CRH-Tg showed glucose intolerance with insulin resistance

Glucose tolerance test revealed that CRH-Tg had impaired glucose metabolism (Fig. 1A), probably due to the insulin resistance caused by glucocorticoid excess. This was confirmed by impaired hypoglycemic response to insulin injection in CRH-Tg (Fig. 1B).

Tissue AdN expression was decreased whereas plasma AdN was increased in CRH-Tg

Adipose AdN mRNA levels in epididymal adipose tissue in CRH-Tg were significantly decreased compared with those in WT littermates (Fig. 2A), in agreement with the previous studies showing that glucocorticoid decreased adN gene expression in adipocytes in vitro [22, 23].
Discussion

In this study, we tried to clarify whether AdN is involved in the pathogenesis of glucocorticoid-induced insulin resistance frequently observed in Cushing syndrome or in patients under pharmacological doses of glucocorticoid administration. For this purpose, we used CRH-Tg, an animal model of Cushing syndrome, the metabolic characteristics of which are quite similar to those in patients with glucocorticoid excess. Indeed, the obtained data in this study clearly showed that CRH-Tg had marked hypercortisolemia, increased adipose tissues, hyperinsulinemia with insu-

Table 2. Effects of ADX on body/tissue weights and plasma parameters in CRH-Tg and control mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CRH-Tg/sham (n=12)</th>
<th>CRH-Tg/sham (n=8)</th>
<th>CRH-Tg/ADX (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>26.5±0.5</td>
<td>25.7±1.1</td>
<td>29.5±1.0**</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>88.7±5.9</td>
<td>120.8±20.3</td>
<td>65.5±8.7*</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>1.5±0.1</td>
<td>7.3±1.2*</td>
<td>0.7±0.2*</td>
</tr>
<tr>
<td>Plasma cort (ng/mL)</td>
<td>40.4±3.1</td>
<td>150.2±16.8*</td>
<td>60.3±6.8*</td>
</tr>
<tr>
<td>Sc fat/BW (‰)</td>
<td>12.8±0.5</td>
<td>26.0±0.6*</td>
<td>8.9±1.9*</td>
</tr>
<tr>
<td>Br fat/BW (‰)</td>
<td>8.0±0.4</td>
<td>20.5±2.4*</td>
<td>7.6±0.9*</td>
</tr>
<tr>
<td>ED fat/BW (‰)</td>
<td>13.9±1.0</td>
<td>29.3±3.7*</td>
<td>16.6±1.5*</td>
</tr>
<tr>
<td>PR fat/BW (‰)</td>
<td>3.4±0.4</td>
<td>5.5±1.9*</td>
<td>2.0±0.4*</td>
</tr>
<tr>
<td>Thymus/BW (‰)</td>
<td>3.1±0.2</td>
<td>0.7±0.1*</td>
<td>4.2±0.8*</td>
</tr>
<tr>
<td>Adrenal/BW (‰)</td>
<td>0.4±0.0</td>
<td>0.6±0.1*</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
| *P<0.05 vs. wild-type/sham group, **P<0.05 vs. CRH-Tg/sham group.


Effect of ADX in CRH-Tg

Plasma corticosterone levels were significantly lower in CRH-Tg/ADX compared with those in CRT-Tg/sham (Table 2). Hypertrophy and atrophy in the adipose tissues and thymus, respectively, as well as other metabolic parameters, were completely reversed in CRH-Tg/ADX, confirming the elimination of the glucocorticoid excess. Interestingly, ADX also abolished the elevation of circulating AdN observed in CRH-Tg (Fig. 5). These results strongly suggest that the glucocorticoid excess in CRH-Tg is responsible for the increase in plasma AdN levels.
obesity with elevated adN levels. We also found that plasma AdN levels in CRH-Tg were significantly higher than those in WT littermates. ADX abolished the increase in circulating AdN levels of CRH-Tg, suggesting that glucocorticoid excess itself, but not CRH and/or ACTH, is responsible for the change in AdN. Our in vivo data strongly suggest that AdN is not a major factor for the development of insulin resistance in Cushing syndrome. Rather, glucocorticoid excess could cause increase in circulating AdN levels but not tissue AdN expression.

AdN production is known to be decreased in adipose tissue in obese patients, in contrast to the increase of other adipokines like TNFα, PAI-1, and resistin [3, 18]. It has also been shown that there is a strong association with increased adiposity and reduced AdN expression, in agreement with the results obtained in CRH-Tg. This may be caused by an alteration in the differentiation state of adipocytes in obesity, or by an obesity-associated hyperinsulinemia because insulin inhibits AdN production in vitro [22]. Indeed, our results showed an inverse relationship between plasma insulin and AdN expression in the adipose tissue. Several cell culture studies have previously suggested that glucocorticoid inhibits AdN production in adipocytes [22, 23]. Our data obtained in vivo support these in vitro data, showing that AdN expression is decreased at both mRNA and protein levels in adipose tissue during chronic glucocorticoid excess. IL-6 and TNFα, two other representative adipokines, are known to be increased in simple obesity, and they are also shown to have a potent inhibitory effect on AdN production [27], although this has not been determined in this study. Regardless of the molecular mechanism(s), it is generally accepted that reduced AdN production with resultant decrease in plasma AdN levels is at least partly responsible for the insulin resistance in obese patients, because AdN is shown to improve insulin sensitivity both in vitro and in vivo [3, 17].

Nevertheless, we unexpectedly found that circulating AdN concentration was not decreased but rather increased in CRH-Tg compared with WT littermates. Since previous studies using genetically or diet-induced mice manifesting obesity almost consistently showed insulin resistance with decreased circulating AdN levels [28, 29], our CRH-Tg with increased adiposity is an intriguing report of an animal model of obesity with elevated AdN levels. Our data also suggest that the pathophysiological role of AdN in CRH-Tg is different from that in previously reported animal models with obesity. AdN improves insulin sensitivity by increasing energy expenditure and fatty acid oxidation through activation of AMP-activated protein kinase (AMPK), and by increasing the expression of peroxisome proliferator activated receptor α (PPARα) target genes [16, 17]. Thus, it is possible that glucocorticoid excess induces AdN resistance in CRH-Tg [30]. Further studies are needed to clarify this issue.

Clinically, the effect of glucocorticoid excess on blood levels of AdN is controversial. In patients with Cushing syndrome, serum AdN levels were reported not to be different from those in control, and were not changed significantly following the surgical removal [11, 31]. However, there are many factors affecting the plasma level of AdN, including body (fat) weight, age, gender, nutritional state, drugs being administered, and genetic variation in AdN gene [SNP276] [17], and such factors might have influenced the results, especially in small-scale clinical studies. In other studies, the administration of dexamethasone or hydrocortisone did not influence the serum AdN levels [25, 32], although Lewandowski et al. have suggested the possibility that longer administration of glucocorticoid might alter the serum levels of AdN. Recently, Uchida et al. have reported that high doses of glucocorticoid significantly increased serum AdN concentrations in patients with IgA nephropathy [33]. Since CRH-Tg is an animal model with genetically identical background and with unequivocally increased plasma glucocorticoid, we assume that chronic glucocorticoid

![Fig. 5. Effect of ADX on plasma AdN levels. Plasma AdN levels were determined by ELISA in mice of each group at 12 weeks old. Open, closed, and hatched bars represent WT/sham, CRH-Tg/sham, and CRH-Tg/ADX, respectively. *P<0.05 vs. WT/sham. #P<0.05 vs. CRH-Tg/sham.](image-url)
excess has a positive effect on circulating AdN levels, although further large scale clinical studies are needed to confirm the conclusion in humans.

The mechanism responsible for the paradoxical changes of AdN expression in chronic glucocorticoid excess, i.e., decreased AdN expression in the adipose tissue but increased circulating AdN is not clear in this study. The most plausible possibility is that glucocorticoid somehow inhibited the degradation of plasma AdN. Although the degradation pathway of AdN is not clarified, the extended half-life of plasma AdN is a possible mechanism, which might be a new therapeutic approach for improving insulin sensitivity. In addition, AdN is known to show a variety of molecular forms, such as trimer, hexamer, or even dodecamers and 18mers, and most insulin-sensitizing effects of AdN have linked to the high molecular weight (HMW) form [16, 17]. The distribution of AdN isoform is controlled at the level of secretion from adipocytes. Molecular chaperones in the endoplasmic reticulum (ER), including ER proteins of 44 kDa (ERp44) and ER oxidoreductase 1-L alpha (Ero1-Lα), play an important role in the secretion of adiponectin [34, 35]. Although our data showed that HMW form of plasma AdN were also tended to be increased in CRH-tg, it is possible that quantitative difference in the molecular forms of AdN is responsible for the paradoxical change in plasma AdN in CRH-Tg. Besides the mechanism(s), considering the generally accepted insulin-sensitizing nature of AdN, our data strongly suggest that the insulin resistance due to glucocorticoid excess is not caused by the decrease in plasma AdN levels, i.e., AdN is not involved in the pathogenesis of insulin resistance in Cushing syndrome.

References


