A disrupted cholecystokinin A receptor gene induces diabetes in obese rats synergistically with ODB1 gene

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Division of1Chemotherapy and 2Gastroenterology, National Kyushu Cancer Center, Fukuoka 815; 3Department of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, Tokyo 173; 4Third Department of Internal Medicine, Asahikawa Medical College, Asahikawa Hokkaido 078; and 5Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima 771–01, Japan

Takiguchi, Soichi, Yutaka Takata, Nobuhiko Takahashi, Kazuhiro Kataoka, Tsukasa Hirashima, Kazuya Kawano, Kyoko Miyasaka, Akihiro Funakoshi, and Akira Kono. A disrupted cholecystokinin A receptor gene induces diabetes in obese rats synergistically with ODB1 gene. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E265–E270, 1998.—Otsuka Long-Evans Tokushima fatty (OLETF) rats develop hyperglycemia, hyperinsulinemia, and mild obesity, which are characteristic of human non-insulin-dependent diabetes mellitus. We have shown that two recessive genes, ODB1 mapped on the X chromosome and ODB2 mapped on chromosome 14, are involved in the induction of the diabetes in OLETF rats. Recently we found that OLETF rats are the naturally occurring cholecystokinin type A receptor (CCKAR) gene knockout rats. In this study, we focused on the genotype of CCKAR gene and the ODB1 gene in regulation of glucose homeostasis in the F2 cross of the OLETF rats. Relatively high plasma glucose levels were observed in the F2 offspring with the homozygously disrupted CCKAR gene. A synergistic effect for increasing plasma glucose levels in F2 rats between disrupted CCKAR gene and the ODB1 gene was shown. The CCKAR gene was found to map very close to ODB2 by a linkage analysis using microsatellite markers. These results suggest that CCKAR gene maintains normoglycemia in rats.

Otsuka Long-Evans Tokushima fatty rats; non-insulin-dependent diabetes mellitus model; ODB2 gene


MATERIALS AND METHODS

Animals. OLETF rats of the 29th generation were supplied from Tokushima Research Institute. F344 and BN rats were purchased from Charles River Japan. The animal facilities were free of specific pathogens. Temperature (23°C), humidity (55%), and lighting (0700–1900) at the facilities were controlled. All rats were freely given a standard diet (CRF-1;
Oriental Yeast (Tokyo, Japan) and tap water. Segregation studies were performed between OLETF and F344 and/or BN rats (10, 11). A diagram of the studies is shown in Fig. 1.

DNA from segregation studies. As described by Sambrook et al. (18), the nuclei were separated from rat liver tissues obtained from the offspring of the segregation studies, and the DNA was purified by the standard phenol-chloroform method. In the studies, F1 rats were obtained by mating female (f) OLETF rats with male (m) F344 or BN rats. F2 rats were produced by brother-sister matings of the F1 rats. Hyperglycemia was mostly observed in males because of hormone dependence, although the genetic background was the same for both sexes (13). Therefore, all male rat progenies were killed and analyzed at 30 wk of age. Small samples of the livers were subjected to DNA analysis. The DNA samples from the backcross study, OLETF (m) × F1(f) [OLETF(f) × BN(m)] (11), was also analyzed.

Southern blot analysis. Genomic DNA (10 µg) was digested with a restriction enzyme (BanH I), separated by 0.7% agarose gel electrophoresis, and blotted onto Hybond-N nylon membranes (Amersham). The blots were hybridized with a 32P-labeled, random-primed rat CCKAR cDNA probe (a gift from Dr. S. A. Wank, National Institutes of Health, Bethesda, MD) containing the full coding sequence (27). The blots were washed once for 20 min in 2× saline-sodium citrate (SSC); 1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0/0.1% sodium dodecyl sulfate at room temperature and then twice for 20 min, each time in 0.1× SSC/0.1% sodium dodecyl sulfate at 62°C. Autoradiography was performed using X-ray film (X-OMAT, Kodak).

Oral glucose tolerance test. An oral glucose tolerance test (OGTT) was performed for 30-wk-old rats being fasted for 16 h before the test. Glucose (2 g/kg) solution was given per os, and plasma levels of glucose were measured before and 30, 60, 90, and 120 min after its administration (10, 11). Rats were classified into three types on the basis of the results by the OGTT: 1) diabetic, with a peak level of plasma glucose >300 mg/dl and a level of plasma glucose at 120 min >200 mg/dl, and only rats showing both conditions by OGTT were classified as CCKAR(-/-), CCKAR(+/-), or CCKAR(+/+). Then each group was further classified by the genotypes of D14Mit4, a microsatellite marker class 1, and carboxypeptidase B1, a gene on chromosome X, obtained previously (12) as OLETF-type PRPS1I1, (-/-), (-/+), (+/+), OLETF-type PRPS1I1, (-/-), (-/+), (+/+), and F344-type PRPS1I1, (-/-), (-/+), (+/+). The blood glucose level was compared between two groups and another.

Classification of F2 and backcross rats by genotypes of CCKAR and ODB2. The CCKAR genotypes of all F2 rats (Fig. 1A) were determined, and the rats were classified as CCKAR(-/-), CCKAR(+/-), or CCKAR(+/+). Then each group was further classified by the genotypes of D14Mit4, a microsatellite marker of chromosome 14, obtained previously (13) as OLETF-type D14Mit4, (-/-), (-/+), (+/+), and OLETF-type D14Mit4, (-/-)BN, (-/+), (+/+). The blood glucose level was compared between two groups and another.

Statistical analysis. Values are expressed as means ± SE. Determination of plasma glucose levels was performed by the multiple analysis of variance with repeated measure and analysis of variance followed by Fisher’s protected least-significant difference test, respectively. Segregation of various genotypes was examined by the χ² test. Incidence of diabetic syndrome in each genotype was analyzed by Fisher’s exact test. Statistical significance was assumed with a P value < 0.05.

RESULTS

Identification of CCKAR genotypes. A PCR method was designed for the rapid identification of the CCKAR genotypes [the homozygously disrupted allele (-/-), the normal allele (+/+), and the heterozygously disrupted allele (+/-)] of progeny rats in the segregation study (see MATERIALS AND METHODS). The normal CCKAR allele was detected in F344 rats and the interrupted CCKAR allele (-/-) in OLETF rats. When the F1 progenies, obtained by mating female OLETF and male F344 rats, were analyzed, both the 165-bp and 453-bp DNA fragments were amplified in the PCR mixture (Fig. 2B). The results showed that all the F1 progenies had both a disrupted and normal CCKAR gene. Southern blotting results supported this (Fig. 2C). Expression of the CCKAR gene in the pancreas was almost the same in the F1 rats as in F344 rats, irrespective of age or sex, as shown by Northern blot analysis (unpublished results).
also analyzed by PCR, and 43 had a homozygous disrupted CCKAR gene (−/−), 80 had a heterozygous CCKAR gene (+/−), and 37 had a homozygously normal CCKAR gene (+/+). The frequency of F2 rats with different CCKAR genotypes was roughly distributed according to Mendel’s law. The relationship between the CCKAR genotypes and the OGTT results was analyzed for all F2 rats, and plasma glucose in rats with the CCKAR gene (−/−) was found to be at a relatively higher level than in rats of the (+/−) or the (+/+), genotype on average (Fig. 3). The difference of plasma glucose levels at 30, 60, and 90 min was significant among the CCKAR genotypes (−/−) vs. (+/−) or (+/+)]. Rats with a heterozygously disrupted CCKAR (+/−) gene seemed to have similar plasma glucose levels to rats with the normal gene (+/+) (Fig. 3).

When the male F2 [OLETF(f) × F344(m)] rats (Fig. 1A) were analyzed according to CCKAR genotypes and PRPSII, the highest incidence rates (35%) of DM (20%) and IGT (15%) were found in the (−/−) F2 genotype of F2 rats; each genotype of the F2 rats was analyzed in relation to their OGTT results (s^2 = 15.97, P = 0.0069; Table 1). When the incidence of diabetic syndrome in each genotype was analyzed by Fisher’s exact tests, (−/−) vs. (+/+) F344, (+/−) F344 vs. (+/+) F344, and (−/−) F344 vs. (+/+) F344 were P = 0.005, P = 0.230, and P = 0.15, respectively. Similar results were obtained by the analysis of the F2 offspring of OLETF and BN rats (data not shown).

Backcross male offspring obtained by mating male OLETF and female F1 [OLETF(f) × BN(m)] rats were analyzed for their plasma glucose level at OGTT. The difference in plasma glucose levels at 30, 60, and 90 min was significant among the two groups of disrupted CCKAR genotypes OLETF F2 rats [OLETF(f) × BN(m)]
Table 1. Incidence of diabetes mellitus in various genotypes of F2 from crosses of OLETF(m) and F344(f) rats

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DM</th>
<th>IGT</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-/-) OL</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>(+/-) OL</td>
<td>43</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td>(+/-) F344</td>
<td>23</td>
<td>1</td>
<td>4.3</td>
</tr>
<tr>
<td>(+/-) F344</td>
<td>37</td>
<td>1</td>
<td>2.7</td>
</tr>
<tr>
<td>(+/-) F344</td>
<td>19</td>
<td>0</td>
<td>&lt;5.2</td>
</tr>
<tr>
<td>Totals</td>
<td>167</td>
<td>7</td>
<td>4.4</td>
</tr>
</tbody>
</table>

DM, diabetes mellitus; IGT, impaired glucose tolerance; (-/-), homozygously disrupted CCKAR gene; (+/-), heterozygously disrupted CCKAR gene; (+/+), wildtype CCKAR gene; OL, OLETF type PRPSII; (Fisher 344, F344), F344 type PRPSII; m, male; f, female.

DISCUSSION

In the mating study F344 and/or BN rats were used as partners to the OLETF rats because both strains are normoglycemic and are thought to have no abnormal genes increasing the plasma glucose level. We suspected that the disrupted CCKAR gene is segregated in a group of F2 progenies in the study. The PCR method was useful for analysis of the types of CCKAR gene in the F1 and F2 progenies. The distribution of the disrupted CCKAR gene was elucidated in F1 progenies, with both males and females heterozygous (+/+) for the CCKAR gene (Fig. 2, B and C). This result indicates that the CCKAR gene is located on an autosomal chromosome in rats, consistent with the result of our fluorescence in situ hybridization analysis (24). When we classified the CCKAR genotypes of 160 F2 male rats and analyzed the relationship with their OGTT values at 30 wk of age, we found that 43 F2 rats with the CCKAR(+/-) genotype had significantly high blood glucose levels. However, approximately one-half the F2 rats with the disrupted CCKAR(−−) gene were normoglycemic, and two F2 of 37 with the wild type gene (+/+) had relatively high plasma glucose levels. Therefore, factor(s) other than disrupted CCKAR gene act(s) to elevate blood glucose levels in F2 rats, and these factors likely act together. The lack of CCKAR may partly account for the rise in blood glucose levels of some F2 rats. Then, the plasma glucose levels were compared in backcross offspring with CCKAR(−−) genes and (+/−) F2 rats of OLETF and BN rats, and a higher plasma glucose level was recognized in the former rats than in the latter (Fig. 4). These results

Table 2. Rat progenies analyzed by genotypes of CCKAR and D14Mit4

<table>
<thead>
<tr>
<th>CCKAR Genotype</th>
<th>D14Mit4-Genotype</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Analyses of F2 rats</td>
<td></td>
</tr>
<tr>
<td>BN/BN</td>
<td>34</td>
</tr>
<tr>
<td>BN/OL</td>
<td>1</td>
</tr>
<tr>
<td>OL/OL</td>
<td>0</td>
</tr>
<tr>
<td>Analyses of backcross progenies</td>
<td></td>
</tr>
<tr>
<td>BN/OL</td>
<td>39</td>
</tr>
<tr>
<td>OL/OL</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are with different genotypes. Oral glucose tolerance test data were obtained from the F2 rats and the backcross progenies.
suggested that in addition to the disrupted CCKAR gene, other factor(s) act to elevate the plasma glucose level, that is, hyperglycemia in OLETF rats is a polygenic event. When the male F2 [OLETF(f) × F344(m)] rats were analyzed according to CCKAR genotype and PRPSII (a microsatellite marker closely linked with ODB1 gene), the highest incidence of DM and IGT was found in the (−/−) IOL genotype, as analyzed in relation to OGTT results (Table 1). According to the analysis with χ² tests, the disrupted CCKAR and OLETF type ODB1 seem to act synergistically to elevate the plasma glucose level in F2 rats. Similar results were obtained on the analysis of the F2 offspring of OLETF and BN rats (data not shown). These findings suggested that the CCKAR and the ODB1 genes act together to induce hyperglycemia in F2 rats. Recently, ODB2 was suggested as a gene responsible for hyperglycemia in OLETF rats and mapped to chromosome 14 (11), on which CCKAR gene is also located (24). The CCKAR gene and D14Mit4, the microsatellite marker of ODB2, which CCKAR gene is also located (24). The CCKAR OLETF rats and mapped to chromosome 14 (11), on which CCKAR gene is also located (24). The CCKAR and ODB1 genes act together to induce hyperglycemia in F2 rats. Recently, ODB2 was suggested as a gene responsible for hyperglycemia in OLETF rats and mapped to chromosome 14 (11), on which CCKAR gene is also located (24). The CCKAR gene and D14Mit4, the microsatellite marker of ODB2, were found to be very closely linked in the F2 rats and in the backcross offspring. These analytic results suggested that the CCKAR and ODB2 genes are the same gene or very closely located on chromosome 14.

At present, disrupted CCKAR gene is the only gene that has been documented and analyzed in correlation with hyperglycemia in OLETF rats. The disruption of the CCKAR gene was recessive as for the hyperglycemic phenotype, which is consistent with the Northern blotting results obtained from rats with heterozygously disrupted CCKAR gene (+/−), which produces an average amount of CCKAR mRNA in the pancreas (unpublished result).

The genetic background of the NIDDM of OLETF rats is very complicated (8, 10, 11, 13). According to the results of this study, a null mutation in the CCKAR gene seems to elevate the plasma glucose level in OLETF rats in correlation with other factors. The mechanism behind this is likely to be the lack of the “incretin” effect of CCK (6), rather than a reduction in the effectiveness of insulin in the muscle, caused by obesity. In the F2 rats with homozygously disrupted CCKAR gene, the insulin response after the oral ingestion of glucose might be reduced. In this respect, the insulin levels in the F2 rats needed to be determined, but unfortunately it was not measured in the segregation study because it was difficult to gather enough blood to follow the time course.

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