Heterogeneity of class I INS VNTR allele association with insulin secretion in obese children

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Le Fur, Sophie, Cédric Auffray, Franck Letourneur, Corinne Cruaud, Catherine Le Stunff, and Pierre Bougnères. Heterogeneity of class I INS VNTR allele association with insulin secretion in obese children. Physiol Genomics 25: 480–484, 2006. First published March 28, 2006; doi:10.1152/physiolgenomics.00311.2005.—On the basis of the near-complete linkage disequilibrium of the insulin variable number of tandem repeats (INS VNTR) allele with the neighboring −23Hph1 A/T single-nucleotide polymorphism, previous studies have documented the association of class I ("short") and class III ("long") INS VNTR alleles with metabolic parameters, including circulating insulin levels. Using a new method to sequence class I alleles, we revisited this association in 346 obese children. Class I alleles are made of several types of repeats, whose repartition determines subclasses IC and ID. Fasting insulin was found to be higher in obese children with ID/ID genotypes (135 ± 12 pmol/l, n = 64) than with ID/IC or IC/IC genotypes (91 ± 5 pmol/l, n = 97, P = 0.0005). In response to oral glucose, peak insulin levels and insulin-to-glucose area under the curve ratios were higher in ID/ID (872 ± 122 pmol/l and 109 ± 15, respectively) than in ID/IC or IC/IC patients (586 ± 42 pmol/l and 76 ± 5, P = 0.02 and P = 0.04, respectively). Fasting and postglucose insulin levels were comparable in carriers of IC and of class III alleles. Our results support that the molecular structure of the VNTR allele, not only its overall length, is associated with variations of insulin secretion. ID/ID homozygosity appears responsible for the increased insulin levels previously attributed to the whole class I VNTR group. It will be important to test the ramifications of this observation for class I association with Type 1 (susceptibility) and Type 2 diabetes (protection).

in a variable number of tandem repeats; genetic association; heterogeneity

IN HUMANS, a variable number of tandem repeat (VNTR) allele is located 596 bp upstream of the insulin gene (1). The extensive polymorphism of the insulin VNTR (INS VNTR) allele is generated by variations in both repeat number and sequence. Approximately 70% of European INS VNTR alleles are of class I (26–63 repeats) and 30% of class III (138–209 repeats). The polymorphism of INS VNTR varies across human populations (26), and allele ontology based on sequence comparisons has indicated that class I and class III diverged independently from ancestral African VNTR alleles (25).

In Europeans, the entire INS VNTR class I allele is in almost-complete linkage disequilibrium with the neighboring −23Hph1 “A” allele and class III with the “T” allele (4), allowing this single-nucleotide polymorphism (SNP) to be used as a surrogate marker for the VNTR polymorphism in association studies. The −23Hph1 “A” allele was found to be associated with increased levels of insulin gene transcription (4, 14, 27) and circulating insulin levels (9, 10, 16). Different degrees of association have been reported between the INS VNTR class I/III genotype and insulin-related traits or diseases. Associations were found between the class III allele and Type 2 diabetes in most (4, 13, 20) but not all (12) studies. Associations of the INS VNTR allele with fetal growth (2, 11) or poly cystic ovaries (22, 28) are still debated.

In fact, class I and class III are composed of a number of different alleles. Because of the sequencing difficulties due to the G-rich VNTR sequence, a minisatellite variant-repeat PCR method had to be developed to determine INS VNTR sequences precisely (24). Two class I sublineages have been characterized in Caucasians (24) according to the presence (ID) or absence (IC) of an F repeat within the last four repeats at the 3’ end of the INS VNTR allele, of a 5’ CA motif, and of a central FAC block. We questioned whether this molecular variation could affect the reported association of class I alleles with insulin secretion (9, 10, 16). We performed this study in obese juveniles because their insulin secretion varies over a large individual phenotypic range, a situation favorable to the detection of genotypic effects.

MATERIALS AND METHODS

Patients. Three hundred forty-six obese children were recruited from families of Western Europe, with careful assessment of family history, the four grandparents’ birthplaces, and patronymic names. We enrolled for the study only patients with all grandparents born in continental Europe and having names of non-African origin [to avoid inclusion of African VNTR alleles (25)]. Inclusion criteria were a body mass index (BMI) exceeding the 85th percentile before 6 yr of age and a monotonous weight curve since birth (16). A subgroup of 88 super-obese patients was defined on the basis of a BMI exceeding the 99.6th percentile (16).

Procedures. Plasma insulin levels were measured in duplicate as previously reported (16). In brief, after children had fasted overnight for 12 h after 3 days of standardized diet at the Obesity unit, we measured plasma insulin levels under unstressed conditions (intravenous microcatheter placed in a peripheral vein 24 h before sampling). Duplicate insulin measurements on the following days in 100 children showed a 3–7% intrasubject coefficient of variation, as previously reported (16). We checked that all children had been gaining weight the month before the study to ensure that sampled insulin values truly reflected the natural history of β-cell function. The oral glucose tolerance test (OGTT) consisted of ingestion of 1.75 g/kg glucose (75 g maximum) in 200 ml of lemon-flavored water at 10°C and venous blood sampling at 0, 30, 60, 90, and 120 min. The plasma insulin level was measured in duplicate with a standard radioassay. Insulin and
Table 1. Clinical and biological characteristics in obese children stratified by the INS VNTR class I and class III genotypes and class I allele structures

<table>
<thead>
<tr>
<th>Class I Allele Structures</th>
<th>I/I</th>
<th>ID/ID</th>
<th>IC/ID</th>
<th>IC/IC</th>
<th>I/III</th>
<th>III/III</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>161</td>
<td>64</td>
<td>77</td>
<td>20</td>
<td>158</td>
<td>27</td>
</tr>
<tr>
<td>Male/Female</td>
<td>67/94</td>
<td>24/40</td>
<td>38/39</td>
<td>5/15</td>
<td>68/90</td>
<td>11/16</td>
</tr>
<tr>
<td>Age, yr</td>
<td>11.6</td>
<td>11.7</td>
<td>11.6</td>
<td>11.3</td>
<td>11.8</td>
<td>11.9</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>29.8</td>
<td>30.9</td>
<td>29.1</td>
<td>29.0</td>
<td>29.5</td>
<td>29.5</td>
</tr>
<tr>
<td>Fasting glucose, mM</td>
<td>4.55</td>
<td>4.61</td>
<td>4.32</td>
<td>4.43</td>
<td>4.43</td>
<td>4.74</td>
</tr>
<tr>
<td>Fasting insulin, pmol/l</td>
<td>109</td>
<td>135</td>
<td>98</td>
<td>98.5</td>
<td>98.4</td>
<td>88.9</td>
</tr>
<tr>
<td>Peak insulin, pmol/l</td>
<td>711</td>
<td>872</td>
<td>591</td>
<td>563</td>
<td>630</td>
<td>477</td>
</tr>
<tr>
<td>AUCinsulin/AUCglucose</td>
<td>91</td>
<td>109</td>
<td>77</td>
<td>71</td>
<td>80</td>
<td>63</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 346 children. INS VNTR, insulin variable number of tandem repeats; AUC, area under the curve. *P = 0.09 vs. IC/IC, 0.02 vs. IC/ID, 0.04 vs. [IC/IC and IC/ID], and 0.01 vs. [I/III and III/III]. †P = 0.09 vs. IC/IC, 0.01 vs. IC/ID, 0.02 vs. [IC/IC and IC/ID], and 0.02 vs. [I/III and III/III]. ‡P = 0.02 vs. IC/IC, 0.008 vs. IC/ID, 0.005 vs. [IC/IC and IC/ID], and 0.001 vs. [I/III and III/III].

Table 2. Clinical and biological characteristics in the subgroup of superobese children

<table>
<thead>
<tr>
<th>Class I Allele Structures</th>
<th>I/I</th>
<th>ID/ID</th>
<th>IC/ID</th>
<th>IC/IC</th>
<th>I/III</th>
<th>III/III</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>20</td>
<td>20</td>
<td>5</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>Male/Female</td>
<td>25/20</td>
<td>13/7</td>
<td>11/9</td>
<td>1/4</td>
<td>1/44</td>
<td>3/3</td>
</tr>
<tr>
<td>Age, yr</td>
<td>13.8</td>
<td>13.5</td>
<td>14.3</td>
<td>12.9</td>
<td>14.5</td>
<td>12.2</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>36.9</td>
<td>38.2</td>
<td>36.2</td>
<td>35.0</td>
<td>37.0</td>
<td>34.8</td>
</tr>
<tr>
<td>Fasting glucose, mM</td>
<td>4.56</td>
<td>4.58</td>
<td>4.51</td>
<td>4.65</td>
<td>4.53</td>
<td>4.85</td>
</tr>
<tr>
<td>Fasting insulin, pmol/l</td>
<td>170</td>
<td>223</td>
<td>119</td>
<td>164</td>
<td>121</td>
<td>132</td>
</tr>
<tr>
<td>Peak insulin, pmol/l</td>
<td>1,060</td>
<td>1,465</td>
<td>650</td>
<td>685</td>
<td>722</td>
<td>505</td>
</tr>
<tr>
<td>AUCinsulin/AUCglucose</td>
<td>136</td>
<td>178</td>
<td>89</td>
<td>104</td>
<td>87</td>
<td>71</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 88 children. *P = 0.05 vs. [IC/IC and IC/ID] and 0.03 vs. [I/III and III/III]. †P = 0.009 vs. [IC/IC and IC/ID] and 0.02 vs. [I/III and III/III]. ‡P = 0.009 vs. [IC/IC and IC/ID] and 0.001 vs. [I/III and III/III].

To safeguard against population stratification in children carrying the ID/ID genotype versus those carrying other INS VNTR genotypes, we included only individuals of European origin. In addition, the cohort was subjected to “genomic control” (23). In brief, a set of 400 markers covering the human genome at 10-cM average resolution (ABI Prism Linkage Mapping Sets version 2.5, Applied Biosystems) were genotyped. These markers were selected based on chromosomal location and heterozygosity. χ²-Tests have been realized to detect potential associations between each marker and our phenotypic traits; they were not significant in all instances (P > 0.05). Therefore, we concluded that there was no evidence for population stratification in the cohort.

Statistical analysis. Plasma insulin concentrations in the fasting state, peak insulin levels, and AUCinsulin had skewed distribution and were log transformed for analysis. ANOVA and Student’s t-test were then used for comparisons of the genotypic groups in the whole obese cohort (Table 1). The Mann-Whitney test and Kruskal-Wallis analysis by rank were applied instead to compare genotypic groups of smaller size in the subgroup of superobese children (Table 2). We used χ²-tests to compare proportions of repeats between IC and ID alleles (Fig. 1) and categorical parameters.

Data were evaluated using Statview 5.0 (SAS Institute). Values are reported as means ± SE.

RESULTS

We sequenced 480 class I INS VNTR alleles from 346 patients, divided into 175 IC alleles and 305 ID alleles. The distribution of these alleles according to their number of repeats is shown in Fig. 1. Class I alleles distributed in three main length subclasses around distinct peaks (31, 40, and 42...
repeats), as reported in nonobese Caucasian subjects (3). Allele ID42.4 accounted for 21% of class I alleles. Variant repeat distribution at the INS VNTR allele differed substantially between IC and ID (Fig. 1). ID alleles had a comparable proportion of A, C, J, and S repeats but fewer B and more F repeats than IC alleles ($P < 0.03$).

Table 1 shows the main clinical and biological characteristics of our cohort according to INS VNTR genotypes. IC/IC and IC/ID genotypes were analyzed separately and then pooled for analysis because values of studied parameters were strictly similar in these two genotypic groups. The mean fasting plasma insulin level was higher in ID/ID homozygotes (135 pmol/l) than in IC hetero- or homozygotes (91 ± 5 pmol/l, $P = 0.0005$) or in class III hetero- or homozygotes (96 ± 4 pmol/l, $P = 0.001$). Comparable differences between ID/ID and other class I and class III genotypes were observed for the insulin peak level and AUC insulin/AUC glucose after the OGTT (Table 1). In superobese children, the fasting and peak insulin levels (OGTT) and AUC insulin reached twofold higher values in ID/ID patients than in other genotypes (Table 2).

The fasting insulin level and AUC insulin/AUC glucose correlated with BMI in all INS VNTR genotypic groups, as expected (10, 16) (Fig. 2). ID/ID homozygotes, however, showed a much stronger correlation and steeper regression slopes than other genotypes (Fig. 2). This was also true for the relationship between AUC insulin/AUC glucose and BMI, as described by the equation $y = 10.5x - 224$ ($r = 0.68$) in ID/ID homozygotes, $y = 3.5x - 24$ ($r = 0.45$) in other class I/I genotypes, and $y = 1.8x + 22$ ($r = 0.23$) in carriers of a class III allele.

We found no correlation between fasting insulin levels or AUC insulin/AUC glucose and numbers of B or F repeats or with the number of AAAA quadruplets included in the structure of class I alleles.

**DISCUSSION**

Class I VNTR alleles are associated with increased transcriptional activity of the insulin gene in the adult (3, 5) and fetal (27) pancreas as well as of reporter genes in transfected rodent cell lines (18, 21). In our cohort, the class I VNTR alleles encompassed more than 104 different alleles characterized by their length (27–47 repeat units) and repeat sequence or distribution. Class I alleles can be subclassified into two main lineages: ID and IC. The ID subclass can be distinguished from the IC subclass by the presence of a 5' CA motif and a central FAC block as well as differences in repeat number, sequence, and distribution. Allele ontogeny indicates that IC and ID alleles, which are rare in Africans, seem to have followed distinct pathways during human evolution (25).

We have previously reported a strong association between class I INS VNTR alleles taken as a group and elevated insulin secretion (10, 16). These results were consistent with the
widely accepted hypothesis that the number of repeats, or length, of the INS VNTR structure is a major component of its functional effects. The present data indicate, instead, that ID/ID homozygosity carries most of the effect previously attributed to the I/I genotype in obese juveniles. This observation adds credit to the hypothesis that it is the VNTR allele per se, not the neighboring Hph1 SNP or other SNPs, that is involved in the association with insulin-related traits.

The recessive effect of the ID subclass is difficult to understand. Recessivity would require some kind of a threshold effect or a physical interaction between the two alleles, as suggested in a previous study (6) of VNTR association with autoimmune diabetes.

Almost all ID alleles are longer than IC alleles (Fig. 1). This length difference may result in distinct secondary structures of the DNA strand. The sequence of the INS VNTR allele is particularly G rich and tends to form unusual DNA structures through the formation of G quartets and complementary C quartets (7, 8). MAZi is a zinc finger transcription factor able to modulate insulin transcription through the recognition of inter- and intramolecular G quartets formed by the INS VNTR allele (17). Because long ID alleles could form more G quartets than IC alleles, they have a higher probability to bind MAZi and activate insulin gene transcription. Class III alleles, however, which are much longer than ID alleles, are associated with lower insulin values and activation of insulin gene transcription (18) than ID alleles. These observations do not support the view that functional differences between ID and IC or class III alleles are based on the length of the VNTR allele and number of G quartets.

We next examined whether differences in repeat type and distribution appeared to account for the effects of ID and IC alleles on insulin secretion. Kennedy et al. (14) found that single-nucleotide differences in the VNTR repeat sequence can affect insulin gene transcription and correlate with the ability to form unusual DNA structures at inter- and intramolecular levels. It was postulated that VNTR variants differ in their ability to stimulate transcription as a function of the binding of inter- and intramolecular quartets to the transcription factor MAZi. Different VNTR repeats binds MAZi with different affinity. A repeats binds MAZi with the highest affinity. A functional role for A repeats, however, does not explain the differences observed between ID and IC alleles because these alleles have a similar frequency and copy number of A repeats (Fig. 1), whereas class III alleles have more A repeats but a lower capacity to activate insulin gene transcription. Other repeats or multirepeat motifs did not provide likely explanations to our observation. F repeats are the only repeats known to contain potential methylation sites (24). ID alleles contain more F repeats than IC alleles (Fig. 1), but this does not relate directly with insulin levels, because class III alleles contain a similar percentage of F repeats as ID alleles (24). The same reasoning holds for the presence of a 5’ CA motif and a central FAC block in ID and class III alleles, in contrast with IC alleles. We found no association of increased insulin levels with AAAA quadruplets, which are reported to have the greatest transcriptional activity (14). Therefore, our attempt to find a simple model of repeat sequence, number, or composition within ID or IC alleles with insulin levels remained unsuccessful. Moreover, understanding the different effect of ID and IC genotypes upon insulin levels is not restricted to understanding differences between these alleles but should also integrate the necessity for ID/ID homozygosity.

In conclusion, our data, indicating that among class I VNTR alleles, ID/ID homozygotes are associated with increased insulin levels compared with other genotypes, may have a medical relevance for the obesity-to-diabetes transition. With the risk of Type 2 diabetes in obese juveniles being proportional to their degree of obesity, it is important that in super-obese European children, ID/ID homozygosity is associated with insulin levels that are twofold higher than in other genotypes. This may indicate a higher capacity of ID/ID patients to compensate for the insulin resistance associated with massive obesity. It is also important in this respect to note that ID alleles are not present in people of African ancestry.

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