CLINICAL STUDY

Sequencing analysis of the ghrelin receptor (growth hormone secretagogue receptor type 1a) gene

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Abstract

Objectives and design: Ghrelin is a novel 28 amino acid peptide which is reported to have several endocrine and non-endocrine actions. It possesses strong growth hormone (GH)-releasing activity, which is mediated via the GH secretagogue receptor type 1a (GHS-R1a). We hypothesised that there might be functional variant variations in the GHS-R1a gene affecting phenotypes linked to the GH/insulin-like growth factor-I (IGF-I)-axis.

Methods: To test our hypothesis we chose patients from our OPERA (Oulu Project Elucidating Risk of Atherosclerosis) study with low (n = 96) and high (n = 96) IGF-I levels, sequenced their GHS-R1a gene exons and performed association studies.

Results: We found five single-nucleotide polymorphisms (SNPs) which did not change the amino acid sequence. We were unable to detect associations between the SNPs and the IGF-I plasma concentrations, but instead we showed that SNP 171C>T was associated with the values of the area under the insulin curve (AUCIN) in an oral glucose tolerance test and with IGF-binding protein-1 (IGFBP-1) concentrations (P<0.05). SNP 477G>A was associated with the low density lipoprotein and very low density lipoprotein cholesterol plasma levels and AUCIN values (P<0.05).

Conclusions: This study was the first genomic screening of the GHS-R1a gene in a population. It suggests that genetic variations in the GHS-R1a gene are not the main regulators of IGF-I levels. However, the variants may be associated with IGFBP-1 concentrations and insulin metabolism.

European Journal of Endocrinology 150 457–463

Introduction

Ghrelin is a novel 28 amino acid peptide hormone which was originally extracted from rat stomach (1) and which has subsequently been shown to be expressed at lower levels in the hypothalamus and pituitary and also in several peripheral tissues (2). Ghrelin at pharmacological doses strongly stimulates growth hormone (GH) release in rodents and humans (1, 3) acting synergistically with growth hormone-releasing hormone (GHRH) (4). Ghrelin is also adipogenic in rodents (5) and has orexigenic potency both in animals (6, 7). However, ghrelin is downregulated in human obesity (8). Ghrelin has been proposed to act as a link between the regulation of energy balance and GH secretion (9).

Ghrelin is an endogenous ligand for the type 1a growth hormone secretagogue receptor (GHS-R1a). GHS-R1a binds acylated ghrelin and other growth hormone secretagogues and mediates their stimulating activity on GH secretion (1, 10). This receptor is a member of the G-protein-coupled receptor superfamily and it was first detected from the hypothalamus, pituitary and arcuate nucleus, where it is concentrated (10). It is also expressed at lower levels in the thyroid gland, pancreas, spleen, myocardium and adrenal glands (2), which supports the notion that, in addition to stimulating GH release, ghrelin also participates in other biological functions.

The biological activities of GH are mediated through insulin-like growth factor-I (IGF-I) and GH is characterised as the major driving force of IGF-I synthesis. IGF-I is produced mainly in the liver and released into the bloodstream, where it circulates as a free molecule or is bound to the IGF-binding protein (IGFBP) (11). Six different IGFBPs have been described (12), of which IGFBP-1 seems to affect IGF-I bioactivity effectively by regulating the amount of free IGF-I in the circulation. Decreased portal levels of insulin lead to elevated levels of IGFBP-1 which, in turn, reduces free IGF-I and diminishes the negative feedback on GH release in somatotrophs (13). The effects of ghrelin on the GH/IGF-I axis are not completely clear. Obesity is characterised by decreased ghrelin levels (8) and GH deficiency, while IGF-I levels are within the normal range or even elevated (14). Decreased IGF-I levels...
have been associated with type 2 diabetes (15), diabetes-related complications and heart diseases (16, 17). Anorectic patients have elevated ghrelin (18) and IGFBP-1 levels but subnormal free IGF-I levels, while their GH levels are within the normal range (19). There is evidence that the role of ghrelin in physiology may be sexually dimorphic; decreased expression levels of the ghrelin receptor GHS-R1a gene in transgenic mice result in decreased GH and IGF-I levels more often in female than in male mice (20). This also suggests that a connection between GHS-R1a and IGF-I levels might exist. However, sequential variations in the GHS-R1a gene have not been described previously in a population-based study. We therefore screened the region of the GHS-R1a gene encoding the entire protein product to find sequence variations that might have an effect on protein structure and function and might be related to different phenotypes in the GH/IGF-I axis, e.g. IGF-I plasma levels.

Subjects and methods

Subjects

The subjects were chosen from our OPERA (Oulu Project Elucidating Risk of Atherosclerosis) study, which is an epidemiological case-control study addressing the risk factors and disease end-points of atherosclerotic cardiovascular diseases. The series was collected during 1991–1993 at the Department of Internal Medicine of the University of Oulu. This study has been described in more detail elsewhere (21, 22). The subjects for this study were selected from the hypertensive cohort, which consisted of 600 age-stratified Finnish subjects (300 men and 300 women). Fasting blood samples were collected, and the oral glucose tolerance tests (OGTTs) were performed (with 75 g glucose) at the Department of Internal Medicine of the University of Oulu. Intima-media thicknesses (IMTs) were measured from the common carotid arteries by ultrasonography. The routine clinical laboratory tests were carried out in the Central Laboratory of Oulu University Hospital. Subsequently, several parameters have been measured using fasting plasma samples and the genomic DNA extracted from them. Measurements of the total IGF-I and IGFBP-1 concentrations were carried out using commercial kits (DSL-10-2800 ACTIVE Non Extraction IGF-I ELISA, Diagnostic Systems Laboratories, Inc., Webster, TX, USA and IGFBP-1 IOMA test, Oy Medix Biochemica Ab, Kauniainen, Finland). Ghrelin concentrations were assessed (23) by radioimmunomassay (RIA) using a commercial kit (Peptide Radioimmuno Assay Kit, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). Fasting insulin levels were measured by two-site immunoenzymometric assay (AIA-PACK IRI, Tosoh Corp., Tokyo, Japan), and fasting glucose levels were determined by the glucose dehydrogenase method (Diagnostica, Merck, Darmstadt, Germany).

Quantitative insulin sensitivity check indexes (QUICKI) were calculated as described elsewhere (24). The measurements of plasma triglyceride concentrations and very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels have been described in detail elsewhere (25). All the subjects volunteered for the study, which was approved by the Ethical Committee of the University of Oulu. The study was conducted according to the principles of the Declaration of Helsinki 1975, as revised in 2000.

Since we were also interested in studying the association of sequential variations with some diabetes-related parameters, we chose subjects from the hypertensive group of the OPERA study, which includes more diabetics than the control group. Another reason for choosing the study subjects from the hypertensive cohort was that our earlier results suggested that the effect of low ghrelin on blood pressure levels was stronger in a hypertensive state. When the hypertensive and non-hypertensive study cohorts were analysed separately, the association between ghrelin and blood pressure levels remained only in the hypertensive cohort (23). Therefore, ghrelin and its receptor as well as their genetic variation might play a specific role in hypertension.

Subjects with high \( n = 96 \) and low \( n = 96 \) total IGF-I plasma concentrations were chosen. The number of patients with type 2 diabetes was 11 in the group with high IGF-I levels and 16 in the group with low levels \( (P = 0.299) \). Both groups were sex-stratified, consisting of 48 men and 48 women. Physiological age-dependent reduction in IGF-I levels was taken into account by stratifying both of the study groups for age: the average age (mean) in the group with high IGF-I levels was 51.1 years and that in the group with low levels was 51.7 years. In the group with high IGF-I levels the mean concentration of IGF-I was 136.5 ng/ml (95% confidence interval \( CI = 127.4–145.5 \)) and that of IGFBP-1 was 2.9 ng/ml (95% \( CI = 2.4–3.4 \)). The mean IGF-I concentration in the group of low IGF-I levels was 30.2 ng/ml (95% \( CI = 27.0–33.4 \)), and the mean IGFBP-1 concentration was 3.9 ng/ml (95% \( CI = 2.5–5.3 \)). The difference in the IGFBP-1 levels between the groups with high and low IGF-I levels was not statistically significant \( (P = 0.517) \).

Amplification of genomic DNA by PCR and sequencing of amplified DNA

Two overlapping fragments covering the first exon and one fragment covering the second exon of the GHS-R1a gene were amplified from genomic DNA by PCR using DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) or AmpliTaq Gold (PE Applied Biosystems, Foster City, CA, USA) enzymes. The primers, (Sigma-Genosys Ltd., The Woodlands, TX,
USA), fragment sizes, annealing temperatures and the number of cycles for each fragment are shown in Table 1. The PCR products were purified with calf intestinal phosphatase, (CIP; Finnzymes) and Exonuclease I (New England BioLabs Inc., Beverly, MA, USA) and sequenced in both directions using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Bucks, UK). Detection of sequences was carried out with an ABI 377 sequencer (PE Applied Biosystems).

**Association studies and statistical analysis**

Statistical analysis was performed using the SPSS statistical package (versions 9.0 and 11.5; SPSS Inc., Chicago, IL, USA). The frequencies of the observed single-nucleotide polymorphisms (SNPs) were compared in the groups with low and high IGF-I plasma levels by a chi-square test. The allele frequencies were equally distributed between the groups (data not shown). However, SNP 171C > T was associated with the IGFBP-1 and area under the insulin curve (AUCIN) values, showing statistical significance in ANOVA ($P = 0.009$ and $P = 0.020$ respectively; Table 3). When adjustments for age, BMI and sex were carried out, significance was maintained (ANCOVA, $P = 0.038$ and $P = 0.038$ respectively; Table 3). The wild-type C/C homozygotes had the highest AUCIN values and the lowest IGFBP-1 plasma levels compared with the heterozygotes or variant homozygotes. The differences were statistically significant between the wild-type homozygotes and heterozygotes in pair-wise comparisons (AUCIN: $P = 0.011$ and $P = 0.032$ after Bonferroni correction; IGFBP-1: $P = 0.012$ and $P = 0.037$ after Bonferroni correction; Table 3).

**SNP 477G > A** was associated with the AUCIN values. There was a significant difference in the AUCIN values between the 477G > A genotypes (ANOVA, $P = 0.007$, Table 3). Significance was maintained

**Table 1 Primers and annealing temperatures used in PCR amplification. Sequencing was performed using the same primers.**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing temperature</th>
<th>Number of cycles</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’ ctctcagcgtctgac 3’</td>
<td>5’ ggaaaggctgtgccagtg 3’</td>
<td>56 °C</td>
<td>35</td>
<td>569</td>
</tr>
<tr>
<td>2</td>
<td>5’ cagtgagctgctcctg 3’</td>
<td>5’ ggaaaggctgtgccagtg 3’</td>
<td>56 °C</td>
<td>30</td>
<td>491</td>
</tr>
<tr>
<td>3</td>
<td>5’ ctctcagcgtctgac 3’</td>
<td>5’ ctctcagcgtctgac 3’</td>
<td>56 °C</td>
<td>30</td>
<td>430</td>
</tr>
</tbody>
</table>

Fragment 1 was amplified using AmpliTaq Gold DNA polymerase (PE Applied Biosystems) and the other fragments were amplified using DyNAzyme (Finnzymes).

**Extracellular space**

**Cytosol**

Figure 1 Genomic screening for sequence variations in the type 1a growth hormone secretagogue receptor (GHS-R1a) gene revealed five single-nucleotide polymorphisms (SNPs). The SNPs were found in the codons of amino acids, which are indicated by arrows in this presentation of the predicted structure of the receptor. The structure of the receptor was illustrated by adapting the information from the original paper (26).

**Association studies**

The frequencies of the discovered SNPs were compared between the groups with high and low IGF-I levels by a chi-square test. The allele frequencies were equally distributed between the groups (data not shown). However, SNP 171C > T was associated with the IGFBP-1 and area under the insulin curve (AUCIN) values, showing statistical significance in ANOVA ($P = 0.009$ and $P = 0.020$ respectively; Table 3). When adjustments for age, BMI and sex were carried out, significance was maintained (ANCOVA, $P = 0.038$ and $P = 0.038$ respectively; Table 3). The wild-type C/C homozygotes had the highest AUCIN values and the lowest IGFBP-1 plasma levels compared with the heterozygotes or variant homozygotes. The differences were statistically significant between the wild-type homozygotes and heterozygotes in pair-wise comparisons (AUCIN: $P = 0.011$ and $P = 0.032$ after Bonferroni correction; IGFBP-1: $P = 0.012$ and $P = 0.037$ after Bonferroni correction; Table 3).

SNP 477G > A was associated with the AUCIN values. There was a significant difference in the AUCIN values between the 477G > A genotypes (ANOVA, $P = 0.007$, Table 3). Significance was maintained
Table 2 Genotype and allele frequencies for the sequence variations observed in the study.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype frequency (n)</th>
<th>Allele frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60C &gt; T</td>
<td>C/C 82, C/T 247, T/T 91</td>
<td>C/T 92, T/T 0.8</td>
</tr>
<tr>
<td>171C &gt; T</td>
<td>C/C 189, C/T 20, T/T 81</td>
<td>C/T 92, T/T 0.8</td>
</tr>
<tr>
<td>447C &gt; G</td>
<td>C/C 189, C/G 20, G/G 81</td>
<td>C/G 92, G/G 0.8</td>
</tr>
<tr>
<td>477G &gt; A</td>
<td>C/C 81, C/A 85, A/A 26</td>
<td>C/A 92, A/A 0.8</td>
</tr>
<tr>
<td>531C &gt; A</td>
<td>C/C 188, C/A 4, A/A 4</td>
<td>C/A 92, A/A 0.8</td>
</tr>
</tbody>
</table>

The nomenclature of the SNPs shows the bases taking part in nucleotide substitutions and the numbers stand for the nucleotides at which the substitutions have taken place; number one corresponds to the first nucleotide of the first codon. The GenBank access number for the SNPs are shown in the column entitled dbSNP rs#.

Discussion

The GH/IGF-I axis has an important role in normal growth and metabolism. A GH pulse in the circulation has been thought to be the major driving force for IGF-I synthesis and secretion. Some association studies between ghrelin, an endogenous ligand for the GH secretagogue receptor, and IGF-I levels exist. In a recent study, serum IGF-I levels were not altered by ghrelin administration (27). Fasting, which is characterised by increased ghrelin secretion, has been shown to lead to a decrease in IGF-I levels (28).
Considering the role of ghrelin in hyposomatotrophism of human obesity, no causative relationship could be demonstrated in a recent study (29). This and other earlier observations provide evidence that the effects of ghrelin on GH secretion and energy homeostasis may be distinct. Our results suggest that the five ghrelin receptor polymorphisms found in this study have no evident association with IGF-1 levels. However, two SNPs were found to be associated with glucose metabolism-related parameters: AUCIN values and IGFBP-1 plasma levels.

Insulin is a powerful downregulator of IGFBP-1 synthesis, and IGFBP-1 levels have been shown to be elevated in type 1 diabetes (30). However, the situation in type 2 diabetes and insulin resistance may be different. The increased AUCIN values observed in the 171C/C type 2 diabetes and insulin resistance may be different. Actually, in our population, SNPs 60C>T and 447C>G have too low an allelic frequency (allelic frequency <1%) to fulfil the criteria for polymorphism.

It must be mentioned that, in theory, there is always a risk for false positive signals when sequencing PCR products. We increased the reliability of the method by sequencing the exons in both directions. Also, the detected SNPs were not located close to the primers. Therefore, our genotyping results can be regarded as being quite reliable. Another thing that must be remembered is that the sample size for each genotype is not very large in any of our five SNPs. If we had analysed a larger number of subjects, the differences would probably have been clearer. However, our results provide useful information for the genes and pathways that should be studied further.

It is important to point out that the role of endogenous ghrelin in the regulation of GH secretion in a physiological situation is unknown, and that the ghrelin/GHS-R1a interaction at the molecular level has not yet been fully clarified. GHS-R1a has been demonstrated to be a highly conserved gene throughout evolution (35), and no mutations with an effect on the protein structure have been described. It is likely that this receptor has an essential or even vital function in organisms, and our result of no amino acid-changing mutations in 192 patients is therefore not very surprising.

In conclusion, our study was the first to describe a genomic screening of the GHS-R1a gene in a population. We found five SNPs, but no amino acid-changing mutations in the protein-coding area. The SNPs were not associated with IGF-1 levels. Instead, SNP 171C>T was associated with IGFBP-1 levels and AUCIN values and SNP 477G>A was associated with AUCIN values and VLDL and HDL levels. The data suggest that genetic variations in the protein coding area of the ghrelin receptor GHS-R1a gene are not the main regulators of IGF-1 levels. However, the variants may be associated with the regulation of insulin metabolism and IGFBP-1 levels.

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Acknowledgements

This study was supported by the Research Council for Health of the Academy of Finland and the Finnish Foundation for Cardiovascular Research. Sakari Kakko, MD, PhD, and laboratory technicians Heidi Häikö and Marja-Leena Kytökkangas at the Department of Internal Medicine at the University of Oulu are gratefully thanked for providing assistance with some of the experiments.

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Received 15 September 2003

Accepted 15 December 2003