Single base substitution in growth hormone receptor gene influences the receptor density in bovine liver

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Abstract

OBJECTIVES: Nucleotide sequence polymorphisms in the coding gene regions may influence the biological properties of proteins encoded by a gene. The A/T substitution in exon 8 of the growth hormone receptor (*GHR*) gene results in changed amino acid sequence 279 (Phe/Tyr) in the transmembrane domain of the receptor protein and therefore could influence its functional parameters. We searched for the relationship between the A/T nucleotide polymorphism in the *GHR* gene the receptor binding capacity and dissociation constant.

METHODS: Nucleotide sequence variations in the exon 8 (coding for the transmembrane domain of the receptor) of the bovine *GHR* gene and in fragments of adjacent introns were analysed using PCR-SSCP and sequencing techniques. GH receptor binding capacity (B_{max}) and dissociation constant (K_d) for GHR were determined by the Scatchard analysis in livers of ten bulls carrying the AA or AT *GHR* genotypes.

RESULTS: Two single nucleotide polymorphisms (SNPs) were identified – the C/T transition in intron 8 at position 863+32 and the A/T transversion in exon 8 at position 836, the latter resulting in Phe/Tyr amino acid substitution in the receptor protein. The results showed significant differences in the GHR binding capacity between these genotypes. B_{max} was significantly greater ($p \leq 0.01$) in bulls carrying TT genotype of GHR in comparison to those with the AT genotype. No significant differences in the dissociation constants (K_d) were found.

CONCLUSION: Our results demonstrated that single base substitution in the transmembrane domain encoding region of GH receptor gene may influence the physiological properties of the receptor.

ORIGINAL ARTICLE

Abbreviations

GH	- growth hormone
bGH	- bovine growth hormone
GHR	- growth hormone receptor
Phe	- Phenylalanine
Tyr	- Tyrosine
SNP	- single nucleotide polymorphism
RFLP	- restriction fragment length polymorphism
QTL	- quantitative trait locus
PCR	- polymerase chain reaction
PAS	- Polish Academy of Sciences
BW	- Black-and-White (cattle)
HF	- Holstein-Friesian (cattle)
EDTA	- ethylene diamine tetraacetic acid
SSCP	- single strand conformation polymorphism
TBE	- tris - boric acid - EDTA (buffer)
DNA	- deoxyribonucleic acid
RRA	- radioreceptor assay
JAK2	- Janus kinase 2
STAT5	- signal transducer and activator of transcription 5

INTRODUCTION

Growth hormone is the most important regulator of growth and metabolism in mammals, stimulating anabolic processes such as cell proliferation, skeletal growth and protein synthesis. Therefore, there is great research interest in finding the specific relationship between the growth hormone gene and physiological properties. This hormone affects target cells' function depending on the GH receptor (GHR) which mediates the biological actions of this hormone by transducing the GH signal across the cell membrane and inducing transcription of many genes [1, 2]. Therefore, the genes encoding GH and GHR are viewed as promising candidates for the research directed towards obtaining the desired characteristics of animal organism [3].

GHR is a member of the cytokine/hematopoietin superfamily of receptors and contains three functional domains: intracellular (signal-transducing), transmembrane, and extracellular (ligand-binding). A single gene of GHR is located on chromosome 20 in cattle [4]. Several genetic polymorphisms have been reported for the bovine *GHR* gene. It has been shown that milk yield and composition, growth performance and other characteristics are correlated with the polymorphism of the *GHR* gene [5,6,7]. Moreover, an association was found between RFLP-AluI, -AccI and -StuI in the 5' region of the gene and the ligand-binding activity of GH receptor [8].

Blott *et al.* [9] have identified a single nucleotide polymorphism – the A/T transversion in exon 8 at position 836 in the *GHR* gene. The nucleotide substitution changes the amino acid sequence at position 279 (Phe/Tyr) in the GHR protein. This mutation in the *GHR* gene has been shown to be a strong quantitative trait locus (QTL) for milk yield and milk protein synthesis and fat content.

The objective of this study was to search for polymorphisms in the bovine *GHR* gene and to examine effects of the its possible polymorphism on GH binding activity in bovine liver.

MATERIAL AND METHODS

The study was performed on Black-and-White (BW) bulls with more than 80% of Holstein-Friesian (HF) blood. From birth to slaughter (at 348 days of age), all animals were housed at the local station of the Institute of Genetics and Animal Breeding PAS. Twice a day they were fed with diets, formulated according to age, and under a standardized feeding regimen (corn silage, concentrates and hay).

Blood samples of 10 ml were taken from the jugular vein of each animal to test tubes containing K_2EDTA (1.6 mg/ml blood).

Samples of liver tissues were collected from the middle part of the small lobe within 15 min after slaughter, frozen in liquid nitrogen and stored at –70 °C until radioreceptor assay (RRA).

All procedures involving animals were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and were approved by the Local Ethics Commission (permission No. 67/2001).

We searched for a polymorphism in the *GHR* gene exon 8 and in fragments of the adjacent introns. PCR-SSCP analyses were performed using primers GHRex8-F and GHRex8-R of the sequence given by Blott *et al.* [9]. A DNA fragment of 342 bp, containing whole exon 8 (91 bp), 130 bp of intron 7, and 121 bp of intron 8, was amplified. Polymerase chain reactions were performed using a PCR-mix with: primers at 5.0 pmol/ml, 1 U *Taq* polymerase (Polgen, Łódź, Poland), 1 µl *Taq* polymerase buffer, four dNTPs, each at a final concentration of 0.2 mM, *ca.* 100 ng of genomic DNA, and H_2O up to 10 µl. The PCR reaction cycle consisted of an initial denaturation period at 95 °C for 120 s, 36 cycles of 10 s denaturation at 95 °C, 45 s annealing at 58 °C, and 120 s elongation at 72 °C, followed by a 10 min final extension at 72 °C. The yield and specificity of PCR products were evaluated by electrophoresis in 2% agarose gel (Gibco, BRL, England) with ethidium bromide.

For SSCP analysis, 8% polyacrylamide gels in TBE buffer (90 mM Tris-boric acid, 2 mM EDTA, pH 8.0) were pre-run for 2 h at 120 V/50 mA/5 W using a Hoefer SE 600 electrophoresis apparatus (Amersham Biosciences, Buckinghamshire, UK). A thermostatically controlled water circulator was used to maintain the gel at a constant temperature of 8 °C. Samples of PCR product of 10 µl were mixed with 10 µl of denaturation buffer (75% formamide, 0.25% bromphenol blue, 0.5 M EDTA), denatured for 5 min at 95 °C, rapidly chilled on ice, and then loaded onto the gel. The electrophoresis was run at 80 V/40 mA/5 W for approx. 26 h. After electrophoresis gels were stained using the Silver Staining System (Kucharczyk, Poland).

PCR products with different SSCP patterns were purified with the QIAquick® PCR Purification Kit (QIAGEN) and DNA samples were sequenced using an ABI377 sequencer (Applied Biosystems, Foster City, Ca, USA) and analysed with the Sequence Analyser 2.01 program.

Frozen liver samples (2 g) were homogenized in 20 ml of buffer A (25 mM Tris, 150 mM NaCl and 5 mM $MgCl_2$, pH 7.4) three times for 5 s at 10 000 rpm, with 40 sec intervals using an Ultra-Turrax T 125 homogenizer (Janke and Kunkel IKA, Staufen, Germany). The homogenates were first centrifuged at 4 °C for 10 min at 150 g, then 15 ml of supernatant was collected and centrifuged at 4 °C for 35 min at 8 000 g. The obtained pellet was resuspended in 7.5 ml of buffer A and used for RRA as well as for protein concentration determination [10].

Recombinant bovine GH (Monsanto, St. Louis, USA) was used both as unlabeled standard and ^{125}I-labeled ligand, iodinated with the chloramine T method (specific activity 1 600 Ci/mM). The incubation was performed in triplicates by adding to plastic tubes: 0.3 ml of buffer A with 0.1% BSA (Sigma, St. Louis, USA), 0.1 ml of membrane preparation, and 0.1 ml of ^{125}I GH. (60×10^3, 120×10^3, 180×10^3, 240×10^3 dpm, respectively). Incubations were done during 16 h at room temperature in a total volume of 0.5 ml. Nonspecific binding was estimated in the presence of 1 000-fold excess of unlabelled bGH. At the end of incubation, 5×1 ml of buffer A with 1% BSA were added to each tube and the mixture was then applied to GFA filters (Whatman, Clinton, NJ, USA), presoaked with 2% BSA in buffer A. Bounded ligands were separated from free ligands, was by vacuum filtration. The retained activity was measured in a Cobra II γ-counter (Packard Instrument Company Inc, Meriden, CT, USA) with a counting efficiency of 80% for ^{125}I. Receptor binding capacity – B_{max} (fmol/mg protein) and dissociation constant ($K_d\times10^{-11}$ mol/l) for GHR were determined by the Scatchard analysis [11]. The assays were performed separately with liver tissues derived from five bulls of each genotype. Three independent Scatchard analyses were performed for each sample.

The statistics used to evaluate the precision of the estimates of the apparent equilibrium dissociation constant (K_d) and maximum number of binding sites (B_{max}) were standard error (S.E.) and 95% confidence interval, respectively. The slope of the plot was tested for difference against zero, and the data obtained were not interpreted unless the slope was different from zero with 95% confidence. The data were processed by analysis of variance using the GLM procedure of the SAS program (1989). The models contained fixed effects of GHR genotype. Body weight at slaughter was treated as covariate. Since all bulls were slaughtered at the same age, its effect was not considered in the analysis. All data were expressed as least square means with standard errors.

The *GHR* allele frequencies were calculated by allele counting according to the Hardy-Weinberg equilibrium [12].

RESULTS

DNA samples were analysed for nucleotide sequence polymorphism using the SSCP techniques. Within the analysed group of bulls, five SSCP patterns were detected (Figure 1). The individual patterns were highly reproducible. Different SSCP variants of the 342 bp PCR DNA fragments were sequenced. Two SNPs (single nucleotide polymorphisms) were identified: the A/T transversion in exon 8 at position 836, and the C/T transition in intron 8 at position 863+32. The former polymorphism (in exon 8) was previously reported by Blott *et al.* [9] and the latter was the novel polymorphism not previously reported.

Since the substitution A/T at position 836 of the *GHR* gene results in changed amino acid sequence at position 279 (Phe/Tyr) in the receptor protein and could influence the functional parameters of the receptor, we were able to determine the ligand-binding activity of different *GHR* genetic variants.

One hundred and thirty-six animals were genotyped by SSCP for the A/T transversion in exon 8. DNA sequencing in ten randomly chosen DNA samples, repre-

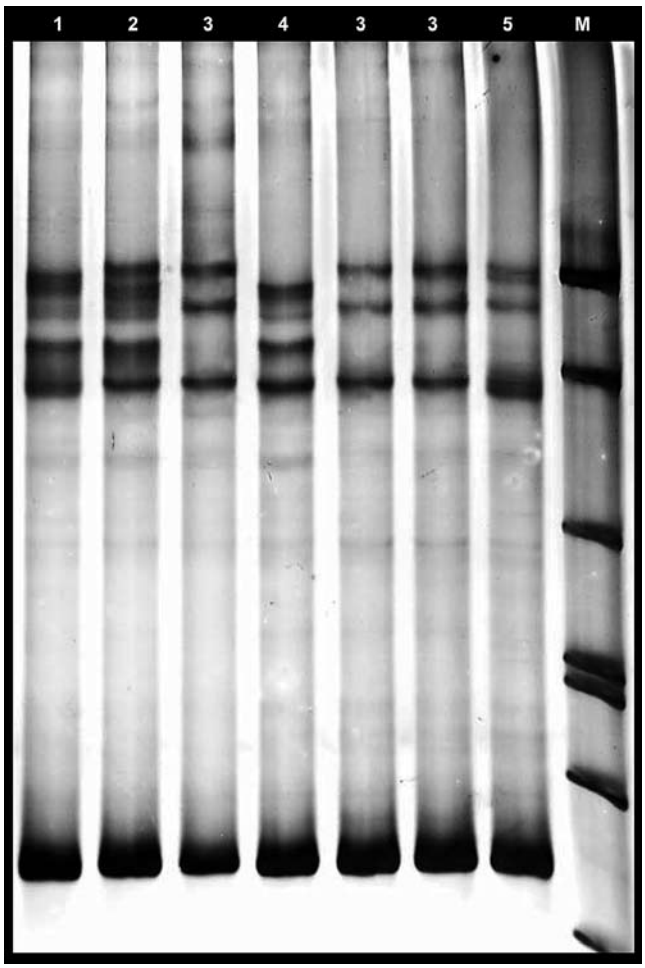

Figure 1. Polyacrylamide gel electrophoresis of the 342 bp gene fragment showing the SSCP polymorphism within exon 8/ intron 8 of the bovine GHR gene. Different SSCP patterns were observed. Pattern "1" corresponded to AT at position 836 and CT at position 863+32, pattern „2" - AT at position 836 and TT at position 863+32, pattern „3" - TT at position 836 and TT at position 863+32, pattern „4" - AA at position 836 and TT at position 863+32, and pattern „5" - TT at position 836 and CT at position 863+32.

senting different SSCP patterns, confirmed the presence of T or A nucleotide. Seventy-two animals had the TT genotype, 63 – the AT genotype, and only one bull carried the homozygous genotype AA.

Cell membrane proteins were extracted from livers of ten randomly chosen bulls carrying different *GHR* genotypes – TT or AT, and then Scatchard analysis was performed. The AA genotype was not considered in the binding analysis because only one bull carrying this variant was detected. Our results revealed significant differences in the receptor binding capacity (B_{max}) between animals of the two *GHR* genotypes. Bulls carrying genotype TT (with Phe at position 279 of GHR) expressed a significantly greater ($p \leq 0.01$) B_{max} as compared to the B_{max} determined for GHR from bulls carrying the AT genotype (Figure 2). No significant differences between the two genotypes examined were found in their receptor affinity (K_d) (Figure 3), but the tendency was for lower K_d value in animals with the TT genotype of *GHR*.

DISCUSSION

Two SNPs were identified using the PCR-SSCP analysis and sequencing of the *GHR* gene fragment comprising exon 8 and fragments of flanking introns. The C/T transition in intron 8 has not been reported previously, but the A/T transversion at position 836 was described by Blott *et al*. [9] as the mutation F279Y. Initially, a strong QTL for milk traits was found in the segment of bovine chromosome 20, and then the causative mutation A→T in the *GHR* gene was identified by sequencing. The effect of this mutation is the amino acid substitution Phe→Tyr within the highly conservative transmembrane domain of GH receptor. This mutation significantly influenced milk production traits in cattle, namely the protein and fat content in milk and the milk yield [9].

We adopted the PCR-SSCP method to identify this mutation. Due to the clearly different SSCP patterns we could easily distinguish between the genotypes AA, AT, and TT. In the studied cohort of 136 BW bulls the frequency of genotypes TT, AT, AA was 0.529, 0.456, and 0.007, respectively; only one bull carried the homozygous AA genotype. The allele frequency was: T - 0.76 and A - 0.24.

We assumed that the amino acid substitution within the transmembrane domain of the protein could influence the GH receptor functions. Therefore, the effect of this mutation on the ligand-binding activity of the receptor was examined. The functional parameters (B_{max} and K_d) of liver GH receptors were studied using the Scatchard analysis. The results showed a significant effect of the *GHR* genotype on the maximum number of binding sites of the receptor (B_{max}). This parameter appeared higher in TT homozygotes as compared to AT heterozygotes (AA homozygotes were not analyzed because only one bull carrying this genotype was found).

Previously, we studied the GH binding parameters to the GH receptor in the livers of dairy and beef bulls [8]. We showed significant differences in the GHR B_{max} and K_d between the dairy and beef breeds. The B_{max} was greater ($p \leq 0.05$) in Polish BW bulls as compared to beef breeds, while K_d was the lowest in the dairy breed ($p \leq 0.01$). Moreover, the relationship was examined be-

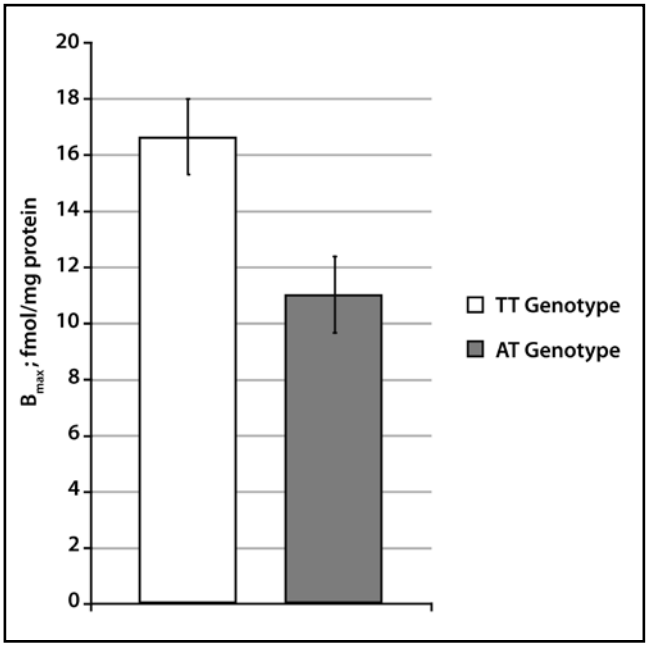

Figure 2. GH receptor capacity (B_{max}; fmol/mg protein) in the bovine liver of two *GHR* genotypes - TT homozygotes and AT heterozygotes. Mean values ± SE for 5 determinations, values are significantly different ($p \leq 0.01$).

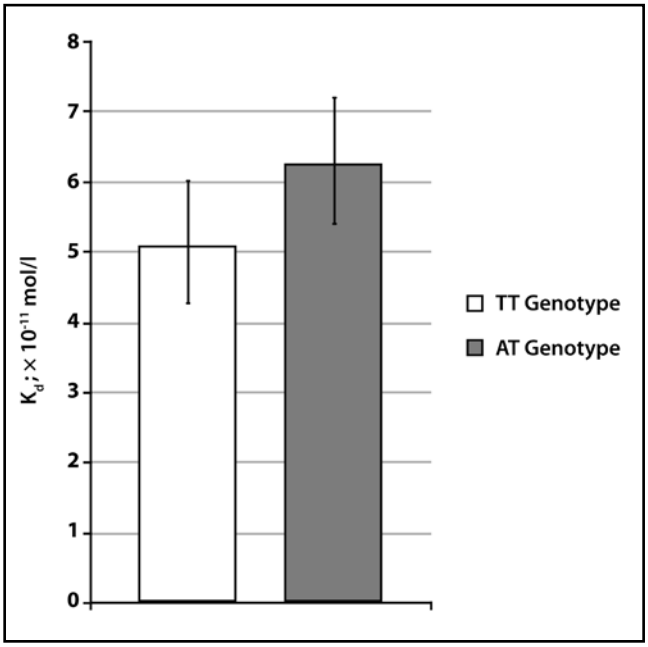

Figure 3. GH receptor binding affinity (K_d; × 10^{-11} mol/l) in the bovine liver of two *GHR* genotypes - TT homozygotes and AT heterozygotes. Values are means ± SE for 5 determinations, values show no significant difference.

tween the GHR ligand-binding activity and the nucleotide sequence polymorphism (RFLP) at AluI, AccI and StuI sites in the 5' flanking region of the bovine *GHR* gene. Significant differences were found in the K_d value between the (+/+) and (+/–) GHR genotypes in BW bulls ($P \leq 0.05$), and all heterozygotes examined had greater K_d than did homozygotes (+/+). A significant difference was also observed between heterozygotes and homozygotes (–/–) in the AluI polymorphism ($p \leq 0.05$).

In the present study we found differences in the maximum binding capacity B_{max} of GHR in livers of bulls differing in *GHR* genotypes in the transmembrane domain ($p \leq 0.01$). Greater GHR capacity to bind GH might result in an increased potential to respond to the hormonal stimulus and affect the functioning of cells regulated by this hormone; e.g. it might result in higher expression levels of genes regulated by GH in TT genotype animals. A direct role for the GH receptor in mediating the effect of GH on milk production and secretion has been reported in bovine mammary tissue [13] and bovine GH was shown to stimulate *in vivo* expression of milk protein genes in pregnant rabbits [14].

The relationships observed in the present work correspond with the results of an analysis of the association between the GHR genotype and the dairy traits of cattle. Animals with the TT genotype, with a greater B_{max} value, and possibly expressing more IGF1 in liver, may be also superior in the breeding value for the milk yield and composition. Thus, these results might provide some suggestions about the possible mechanisms that underlie the effect of a particular *GHR* genotype on cattle physiology, growth regulation mechanism and productive traits. However, the T/A polymorphism in exon 8 of the GHR gene was shown not to modify the JAK2-STAT5 signaling pathway from the growth hormone receptor in cells transfected with cDNA expression plasmids for the 2 versions of GHR and cotransfected with a STAT5/luciferase reporter gene construct [Zhou and Jiang, 2006]. Thus, if this polymorphism has a causative effect on milk production, this effect is unlikely to be mediated by the JAK2-STAT5 pathway, the currently known major signaling pathway from the growth hormone receptor.

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