INTRODUCTION

Growth hormone releasing hormone and somatostatin (SRIF) are the main hormones produced in hypothalamus which regulate growth hormone secretion (1). Recently it was found that, despite these two hormones, another peptide, ghrelin, exists that has the same function. Ghrelin and its receptors are produced in almost all tissues including the central nervous system. Besides influence on growth hormone secretion, ghrelin also regulates food intake and energy metabolism centrally as well as peripherally. In our study, active ghrelin and growth hormone levels in serum were measured. We also investigated gene expression of proghrelin, growth hormone releasing hormone (GHRH) and growth hormone receptor (GH-R) in the hypothalamus and the active form of ghrelin receptor (GHSR-1a) in hypothalamus and pituitary. Expression of growth hormone and growth hormone releasing hormone receptor (GHRHR) in the pituitary were also measured. The results of our study indicate that active ghrelin and growth hormone levels in serum increased during pregnancy. Expression of ghrelin in hypothalamus and its receptor also increased in hypothalamus and pituitary during pregnancy. We also observed that growth hormone gene expression rose in pituitary, while its receptor mRNA level in hypothalamus decreased. Additionally, growth hormone expression in placenta decreased during pregnancy. Moreover, GHRH in hypothalamus and its receptor in pituitary showed reduced levels during pregnancy. Our results may indicate that ghrelin is a important factor influencing growth hormone release during pregnancy.

Key words: growth hormone, ghrelin, growth factors, growth factors receptors, hypothalamus, pregnancy, placenta
MATERIAL AND METHODS

Animals

In this study adult female Wistar rats were used. Presence of sperm in the vaginal smear was defined as a first day of pregnancy. After fertilization, rats were placed in separate plastic cages (Techniplast). Animals were kept under conditions described earlier (16). Rats were sacrificed by decapitation in fourth (group B), thirteenth (group C) and eighteenth day (group D) of pregnancy and 24 hours after delivery (group E). As controls (group A) rats in the diestrus phase of the estrous cycle were used. The days of pregnancy were chosen according to the suggestion of Witschi (17). The Local Ethics Committee for Experiments on Animals approved the experiment protocol.

Radioimmunoassay

Serum was collected immediately after decapitation and frozen at -80°C. Active ghrelin and growth hormone serum concentration were measured using specific RIA kit (Linco Res., Inc., St. Charles, MO, USA) according to RIA protocol. Intra- and interassay variations for growth hormone were 6.3% and 12.4%, whilst for active ghrelin were 5.5% and 10.7 respectively.

RNA isolation and DNase digestion

Hypothalamus, placenta and pituitary were isolated, cut and frozen at -80°C in 2 ml tube (Eppendorf Biopur; Eppendorf AG, Hamburg, Germany). After addition of Trizol LS Reagent (Invitrogen Corp., Carlsbad, CA, USA), tissues were homogenized by use of Omni tip homogenizing kit (Omni International, Marietta, USA). RNA isolation and DNase digestion was isolated according to the Trizol manufacturer's protocol. Before isolation, tissues were washed four times in Molecular Grade Water (Eppendorf AG, Hamburg, Germany). After addition of Trizol LS Reagent (Invitrogen Corp., Carlsbad, CA, USA), tissues were homogenized by use of Omni tip homogenizing kit (Omni International, Marietta, USA). RNA was isolated according to the Trizol manufacturer’s protocol with some modifications. After RNA isolation, DNase digestion using RQ1 RNase-Free DNase (Promega Corp., Madison, WI, USA) was set up.

Real-time PCR

Total RNA (2 µg) was used to perform RT-PCR analysis. Reverse transcription was executed using Verte Kit (Novazym Poland) with RNase inhibitor (Promega Corp., Madison, WI, USA). For all genes RT- (without reverse transcriptase) control was prepared. The cDNA was amplified using real-time PCR with designed primers for proghrelin, GHRH, GH and its receptors and internal standard with hypoxanthine phosphoribosyl transferase (HPRT) (Table 1). PCR primers were designed using accessible in internet Primer 3 software to allow amplification of regions that span intron. Sequences division on exon - intron regions were analyzed using “Blat” option from internet page UCSC Genome Browser Home for the rat. Internal standard was selected from five genes (data not shown) after Best Keeper analysis (18). Real-time expression was prepared by using Light Cycle 2.0 instrument (Roche Diagnostic Penzberg Germany) and LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostic Penzberg Germany). Analysis was made using LightCycler Software Version 4.5 based on the relative quantification method with efficiency correction (standard curve method). Results were presented as a ratio of studied gene expression to HPRT.

Immunocytochemistry

Placenta was collected after decapitation from the females on the 13th and 18th day of pregnancy. Placental tissue was fixed in the Bouin’s solution and incubated for 72 hours. After were dehydrated, embedded in paraffin and sectioned at 6 µm. For immunohistochemistry specimens were then deparaffinized and incubated for 15 min with 1% H2O2 in order to block endogenous peroxidase activity. After BPS washing, the sections were preincubated for 30 min with mix of 5% NGS (normal goat serum, DAKO, Glostrup, Denmark) and 1% of BSA (bovine serum albumin, Sigma-Aldrich). After that, sections were incubated with a polyclonal goat anti-rat GH (L-20) 1:200 (sc–10364, Santa Cruz Biotechnology, Inc. USA) (19). Negative controls were either performed by omitting primary antibody, or pre-incubating primary antibody with commercially available peptide (sc–10364 P, Santa Cruz Biotechnology, Inc. USA). Sections then were incubated with biotinylated secondary antibody for 30 min and finally with peroxidase also for 30 min (DAKO LSAB 2 System HRP). Peroxidase activity was detected using the DAB chromogen system (DAKO Liquid DAB Substrate). Sections were additionally counterstained with hematoxylin. Analysis were performed with LSM 510 Meta confocal microscopy (Carl Zeiss) operated by LSM 510 v. 3.2 SPI software.

Table 1. PCR primers, annealing temperature and size of PCR product.

<table>
<thead>
<tr>
<th>Gene/ accession number</th>
<th>Sequences</th>
<th>Annealing temp.</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proghrelin AB029433</td>
<td>forward 5’ AAAGCCACAGACAGAAAGGAAT 3’ reverse 5’ GAGGGGAGCATTTGAACCTGATT 3’</td>
<td>62°C</td>
<td>141 bp</td>
</tr>
<tr>
<td>GHSR-1a AB001982</td>
<td>forward 5’ GTCTCATCAGGAGAAGCTA 3’ reverse 5’ CCAGCAGGAGGAAACAA 3’</td>
<td>60°C</td>
<td>128 bp</td>
</tr>
<tr>
<td>GHRH NM 031577</td>
<td>forward 5’ GGTTGTTTCTTCTGCTCTC 3’ reverse 5’ TCCTTTGCTCTTTCTGTCC 3’</td>
<td>61°C</td>
<td>197 bp</td>
</tr>
<tr>
<td>GHRH-R NM 012850</td>
<td>forward 5’ CGGITTCTTCTAACACCA 3’ reverse 5’ AGCAATGAGCCGCAACA 3’</td>
<td>60°C</td>
<td>157 bp</td>
</tr>
<tr>
<td>GH NM 001034848</td>
<td>forward 5’ CAGATCCAGCAGGTGGCCTT 3’ reverse 5’ TGAATGGATGAGCAGCAACG 3’</td>
<td>60°C</td>
<td>120 bp</td>
</tr>
<tr>
<td>GH-R NM 017094</td>
<td>forward 5’ CCTTCTATTGGAATCCTAC 3’ reverse 5’ CCCTCAAAACATCGGACT 3’</td>
<td>62°C</td>
<td>201 bp</td>
</tr>
<tr>
<td>HPRT NM 012583</td>
<td>forward 5’ AGTCAGCCAGAGGACATAAAAG 3’ reverse 5’ ATTTTGGGCTGTACTGCTGA 3’</td>
<td>61°C</td>
<td>146 bp</td>
</tr>
</tbody>
</table>
Sequencing of GH

In addition, real-time PCR reaction with SYBR- Green using LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostic Penzberg Germany) was performed. Melting curve analysis was also added. The product was recovered from capillaries and resolved in agarose. The PCR products for GH were cut out from gel and purified using DNA Gel Extraction Kit (Millipore Billerica, MA USA). Afterwards the product was sequenced by IBB PAN (Warsaw, Poland) using ABI Prism BigDye Terminator Cycle Sequencing Kit to confirm the presence of GH products.

Statistical analysis

Statistical analysis for peptides level changes in serum during pregnancy as well as the changes of genes expression for studied peptides and their receptors was performed using ANOVA, followed by the Multiple Range Duncan’s test with 99% and 95% confidence intervals. For GH expression changes in placenta t-test was used. Results were shown as means±S.E.M.

RESULTS

Serum level changes

Our results indicate that both active ghrelin level and growth hormone level significantly increased in serum during 13th and 18th day of pregnancy and decreased after delivery (Fig. 1). Ten rats were used in each group.

Ghrelin and its receptor expression changes

Ghrelin gene expression in the hypothalamus (Fig. 2) rose during pregnancy and these changes were significant. However, ghrelin expression decreased significantly after delivery. Gene expression for the active form of ghrelin receptor (GHSR-1a) was significantly enhanced in hypothalamus (Fig. 3) and pituitary (Fig. 4) during pregnancy. However, in both tissue ghrelin receptor mRNA levels fall after delivery. Eight rats were used in each group.

Growth hormone and its receptor expression changes

Growth hormone expression in the pituitary (Fig. 5) rose during pregnancy and these changes were significant. After delivery the growth hormone gene expression decreased, but this change was not substantial. We also found that, during pregnancy, GH expression significantly decrease in placenta tissue (Fig. 6). This was additionally confirmed by immunocytochemistry as we observed presence of growth hormone in placenta on 13th day of pregnancy. However, we did not find it in placenta on 18th day of pregnancy (Fig. 7).

Moreover, we also detected that expression of the growth hormone receptor significantly decreased during pregnancy in the hypothalamus (Fig. 8).

Growth hormone releasing hormone (GHRH) and its receptor expression changes

Using real-time PCR, we observed significant decrease in GHRH mRNA level in the hypothalamus on the 13th day of pregnancy, but from the 18th day, GHRH expression was rising and was still lower in comparison to control and rats in their 4th day of pregnancy (Fig. 9). Pituitary GHRH receptor mRNA concentration increased in the 13th day of pregnancy in comparison to the control rats and to the 4th day of pregnancy.
Fig. 4. Ghrelin receptor isoform 1a mRNA level in the pituitary during pregnancy in the rat (n=8). Statistically significant differences are marked with letters corresponding to the groups (small letters - P≤0.05, capital letters - P≤0.01).

Fig. 5. Growth hormone expression in the pituitary (n=8). Statistically significant differences are marked with letters corresponding to the groups (small letters - P≤0.05, capital letters - P≤0.01).

Fig. 6. Growth hormone expression in the placenta (n=8). Statistically significant differences are marked with letters corresponding to the groups (small letters - P≤0.05, capital letters - P≤0.01).

Fig. 7. Immunocytochemistry localization of growth hormone on 13th day of pregnancy in placenta (A). Arrows indicate localization of growth hormone in placenta. B – no signal for growth hormone on 18th day of pregnancy in placenta. C–negative control, 100 µm=scale bars.

but mRNA levels significantly decreased in the 18th day of pregnancy and increased again after delivery (Fig. 9). Eight rats were used in each group.

DISCUSSION

Our results demonstrate that active ghrelin in serum increased during pregnancy and decreased after delivery. Gualillo et al. (20) found that ghrelin levels in serum did not change significantly during pregnancy in the rat. On the other hand, Shibata et al. (21) observed that ghrelin levels declined substantially by the 15th day of pregnancy, but on 20th day increased to levels comparable with non-pregnant rats. However, both studies only measured total ghrelin levels in serum, without
accounting for whether it is active or not. They also found that ghrelin level and ghrelin gene expression in the stomach did not change during normal pregnancy. Similar results regarding ghrelin expression in rat stomach were also found in the previous studies (22). This may indicate that stomach ghrelin is not responsible for ghrelin distribution during pregnancy. Similarly to rat studies, during human pregnancy the ghrelin levels increase at first, with the highest level in week 18, with subsequent fall afterwards and another increase after delivery (23). Recently, it has been reported that active acyl ghrelin decreased slightly during late pregnancy, but this change was not significant. Conversely, studies have shown that inactive des acyl ghrelin increased significantly during late pregnancy (15). Interestingly, placenta ghrelin mRNA level rose during pregnancy, but did not influence ghrelin level in serum (21). Gualilillo et al. (22) found that ghrelin expression was undetectable until the 12th day of pregnancy, but then suddenly increased on the 16th day of pregnancy and later declined. All presented data may indicate that ghrelin sources responsible for ghrelin serum level changes during pregnancy may not be of stomach and placental origin, but also other tissues such as the hypothalamus and pituitary, where ghrelin is also expressed, may be involved (2). Additionally, ghrelin is also expressed in the ovary. Moreover, it was found that ghrelin influences ovary cells proliferation, estradiol production and has an antiapoptotic action on these cells (24). These data suggest that ghrelin is also responsible for regulation of estrous cycle and fertility.

Shibata et al. (21) found, with use of Northern blot analysis, that ghrelin expression in hypothalamus is lower on the 15th day of pregnancy, but generally there was similar level of expression during pregnancy in comparison to non-pregnant rats. Furthermore, they also found augmentation of ghrelin mRNA in pituitary, but it was not significant. However, in our study, we observed substantial increase of ghrelin expression in the hypothalamus, while in the pituitary mRNA level was stable in the course of pregnancy (data not shown). Probably, these dissimilarities may result from use of different methods (Northern blot vs. real-time PCR). Moreover, we found that GHSR-1a mRNA level also increased in both hypothalamus and pituitary. Our results may suggest that increasing ghrelin level in serum may be connected with higher ghrelin mRNA level in the hypothalamus and that ghrelin acts by GHSR-1a in the hypothalamus and the pituitary.

Ghrelin and its receptor in the hypothalamus are mainly expressed in arcuate nuclei (3, 25), the region that is responsible for control of food intake. Ghrelin action on regulating food intake is strongly mediated by neuropeptide Y (NPY) and Agouti related protein (AgRP) which are also produced in hypothalamic arcuate nuclei (26-28). Moreover, ghrelin increases expression of NPY and AgRP in the hypothalamus (29). Because both NPY and AgRP expression increases during pregnancy (30, 31) our results may indicate that increase of hypothalamic and circulating active ghrelin causes an increase of NPY and AgRP expression and, in result, participate in the control of energy expenditure. Furthermore, the ghrelin receptor is expressed in other hypothalamic nuclei e.g. paraventricular, ventromedial lateroanterior, supraoptic nuclei (32), additionally supporting ghrelin action by the hypothalamus and may explain the increase of ghrelin and its receptors’ mRNA level during pregnancy. This indicates that ghrelin plays important role during this metabolic state.

Apart from energy homeostasis, ghrelin is also essential in GH release but this role is not well understood. In the human, the main source of GH during pregnancy is the placenta (PGH-placental GH), while in the pituitary GH levels decline (23). On the other hand, we did not find any information about placental GH isoform in the rat. In our study we found that GH level in serum and GH expression in the pituitary increased whereas in the placenta decreased during pregnancy. Our result is comparable to the results obtained by Escalada et al. (33) and Shibata et al. (21). Escalada et al. (33) who also found that the main source of GH in serum is associated with higher GH mRNA level in the rat pituitary. They also found that somatostatin expression declines during late pregnancy which may be connected with maintaining the high levels of GH. On the contrary, according to the most recent data, ghrelin does not affect growth hormone level during pregnancy (34). Moreover, in the same study it was found that placenta does not produce ‘pituitary’ GH during pregnancy. Additionally, it was recently found, that cortistatin-14 (CST-14) may regulate GH secretion from pituitary not only by specific receptor for CST, but also by somatostatin and ghrelin receptor. Moreover, this action is different from somatostatin action (35). These data prove that numerous peptides influence GH secretion.

Intracerebroventricular injection of ghrelin stimulate GH secretion in humans and rats and this action is dose dependent (5, 36-38). On the other hand, Tolle et al. (39) found that
correlation between ghrelin and GH secretion does not exist and ghrelin influences only food intake and sleeping cycles. Moreover, immunoneutralization of ghrelin did not change GH secretion, while GHRH neutralization attenuated GH release (40). However, ghrelin acts by GHSR-1a on hypothalamus neurons producing GHRH (41). Additionally, GHRH influence on the increase of ghrelin mRNA and peptide levels in the pituitary leads to GH secretion (42). It was also found that co-administration of ghrelin and GHRH is necessary for maximum GH release in comparison to ghrelin or GHRH actions alone (43). Kamegai et al. (42) also found that GHSR inhibitor significantly reduced GH release mediated by GHRH. It may indicate that ghrelin/GHRH system is responsible for GH release. However, Seoane and colleagues (37) also observed, that in low doses (3 nmol/kg body mass) ghrelin and GHRH influence GH secretion in the same manner, while in higher doses (12 nmol/kg body mass) ghrelin increases GH release more significantly than GHRH. This suggests that high doses of ghrelin may be more important to GH release than GHRH. In our study, we found that ghrelin expression increased in the hypothalamus and its receptor expression increased in both the hypothalamus and the pituitary during pregnancy, whereas GHRH mRNA level decreased from the 4th to the 13th day of pregnancy, increasing on the 18th day. Alternatively, Escalada et al. (33) found that GHRH mRNA level did not change during pregnancy. We also found that expression of the receptor for GHRH increased up to the 13th day and subsequently fell significantly downwards and then increased again after delivery. These results may indicate that increase of GHRH-R till the 13th day of pregnancy is in response to GHRH decrease at that time. Moreover, our results show that there is no connection between ghrelin, ghrelin receptor and GHRH during the first half of pregnancy and that ghrelin is probably very important for GH release in this period. On the other hand, increase of GHRH mRNA level and decrease of its receptor expression during second half of pregnancy indicates that GHRH may not influence GH secretion directly, but indirectly, by maintaining a high mRNA level of ghrelin and GHSR-1a. After delivery, both GHRH and ghrelin affect on GH secretion.

In our study, we also found that GH receptor gene expression substantially decreases during pregnancy in the pituitary. Because high levels of GH may attenuate GHSR mRNA level (44), this mechanism may be protective. High level of GH, NPY and somatostatin also inhibit GHRH release (41). However, our results and the data mentioned above indicate that other mechanisms may exist that are responsible for GHRH changes during pregnancy.

In conclusion, the present study has shown that active ghrelin and growth hormone level increase during pregnancy. These changes might be important not only for maternal metabolism, but also for the fetus. Our results also suggested that ghrelin may be important for GH release during pregnancy from the pituitary. Furthermore, we found that during the first half of pregnancy, a relationship between GHRH ghrelin and the active isoform of its receptor probably does not exist. However, from the 13th day of pregnancy there may be an association between these two entities. Furthermore, GHRH by this mechanism, but not only by its own receptor, may indirectly regulate growth hormone secretion. In turn, growth hormone resistance associated with decreased receptor expression in pituitary may protect against high levels of GH and assure the maintenance level of this peptide. We also found that placenta is a source of GH, but the pituitary is probably more responsible for GH increase during pregnancy. However, one should take into account that changes in mRNA expression may not correspond to the same changes on protein level. Therefore, part of our results considering only mRNA expression changes needs further confirmation.

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Conflict of interests: None declared.

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