Changes of agouti-related protein in hypothalamus, placenta, and serum during pregnancy in the rat

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Abstract

Agouti-related protein (AGRP) is a homolog of the agouti protein and acts as an antagonist of peptides derived from proopiomelanocortin through melanocortin receptors. This peptide is produced mainly in the hypothalamus, particularly during negative energy balance and influences increased food intake. In the hypothalamus, this peptide is co-expressed in arcuate nuclei with neuropeptide Y, another important peptide that regulates energy metabolisms. In our study, we analyzed changes in the *Agrp* mRNA level in the hypothalamus as well as mRNA and protein levels in placenta during different stages of rat pregnancy. We also

investigated the AGRP level in the blood serum. In this study, we found the AGRP level in serum increased, while its gene expression in the hypothalamus increased only up to the 13th day of pregnancy, and decreased on the 18th day. This study demonstrates that AGRP is expressed during late pregnancy in placenta. Moreover, we found that AGRP expression is higher on the 18th than on the 13th day of pregnancy. Our results indicate that AGRP may play an important role during pregnancy in the mother's and, possibly, also in the fetus's energy balance.

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Background

Peptides derived from proopiomelanocortin prohormone (POMC), after posttranslational modifications, particularly α melanocyte-stimulating hormone (α MSH), regulate appetite in the central nervous system by decreasing food intake (Kim *et al.* 2002, Pritchard *et al.* 2002). In 1997, a protein with a high homology to agouti protein was identified and called agouti-related protein (AGRP). This protein is an antagonist to melanocortin receptors, mainly MC3R and MC4R (Ollmann *et al.* 1997, Shutter *et al.* 1997, Pritchard *et al.* 2004). This type of receptor is the most common isoform in the brain and is responsible for appetite regulation (Yeo *et al.* 1998). This may indicate that AGRP is essential in the regulation of energy balance.

It was found that AGRP expression is associated with changes in the leptin level, one of the main hormones which regulates food intake via the hypothalamus. It was further observed that AGRP is produced in neurons that exhibit leptin receptor expression. Mizuno *et al.* (1999) reported that *Agrp* mRNA production was inhibited by leptin injection into the hypothalamus. They also found that the AGRP level was fivefold higher in ob/ob mice. Moreover, AGRP may also influence augmentation of the POMC expression (Schwartz *et al.* 1997, Korner *et al.* 1999, Cowley *et al.* 2001). However, in the ob/ob mouse model, changes in the AGRP expression are more significant than the POMC expression

(Ollmann *et al.* 1997, Thornton *et al.* 1997). In addition, Hoggard *et al.* (2004) also demonstrated that α MSH reduced leptin secretion from adipocytes, but this action of MSH was inhibited by the addition of AGRP. On the other hand, there were no changes of the leptin expression level upon AGRP stimulation in the absence of α MSH. These data suggest that there is a feedback loop between leptin and α MSH, and that AGRP may exert a significant effect on the regulation of this mechanism, and hence, on the nutritional status.

Moreover, AGRP is co-expressed in Arcuate nuclei with neuropeptide Y (NPY), another orexigenic peptide (Hahn *et al.* 1998, Chen *et al.* 1999). This indicates that changes in energy expenditure regulated by other hormones similar to leptin and insulin may also be associated with changes in NPY expression (Schwartz *et al.* 1996, Ollmann *et al.* 1997, Shutter *et al.* 1997, Korner *et al.* 2001, van den Top *et al.* 2004). Moreover, both insulin and leptin exert similar effects and inhibit the expression of AGRP and NPY. It was further shown that NPY also stimulates food intake by inhibiting POMC secretion (Cowley *et al.* 1999). These are important data indicating the crucial role of AGRP in the regulation of energy balance.

Hypothalamic AGRP and NPY also mediate the action of ghrelin, another peptide regulating food intake, which is produced in the stomach and in the hypothalamus (Kojima *et al.* 1999, Kamegai *et al.* 2001, Seoane *et al.* 2003, Chen *et al.* 2004). Results published by Chen *et al.* (2004) demonstrated

that *Agrp*^{−/−}, *Npy*^{−/−} double knockout mice treated with ghrelin had a 4-h food intake attenuation. However, in a mouse model with one gene knocked out, either *Agrp* or *Npy*, there was no attenuation of food intake. These results indicated that AGRP may play an important role in appetite regulation. On the other hand, Tang-Christensen *et al.* (2004) found that NPY has a short-term effect on food intake, whereas the effect mediated by AGRP is prolonged. Moreover, mice with knockout of MC3R and MC4R genes did not show increased food intake after ghrelin stimulation, which may additionally imply the importance of AGRP in food intake regulation (Shaw *et al.* 2005).

All these data suggest the importance of AGRP in the regulation of energy status in relation to numerous peptides. In our study, we presented changes in the expression of *Agrp* in the hypothalamus, and its protein level in serum during hypermetabolic state, which is observed during pregnancy. Moreover, we present here that AGRP is also expressed in placenta during late pregnancy, which not only may influence energy homeostasis in the mother, but also may affect fetus development.

Material and Methods

Animals

In this experiment, female Wistar rats were used. Vaginal smear analysis was executed every day at the same time of day. The presence of sperm was defined as the first day of pregnancy. After fertilization, pregnant rats were kept under conditions described previously (Szczepankiewicz *et al.* 2006). Experimental rats were analyzed on day 4 (before implantation and placenta development, group B), day 13 (embryonic period, group C), day 18 (fetal period, group D) and one day after parturition (group E), as suggested by Witschi (1962). Rats in the diestrus phase of estrous cycle were used as controls (group A). In each group, ten animals were used. The Local Ethics Committee for Experiments on Animals approved the experiment protocol.

RIA

Serum was collected immediately after decapitation and frozen at −80 °C. AGRP was measured using a specific RIA kit (Phoenix Peptide, Karlsruhe, Germany) according to the manufacturer’s protocol. Intra- and inter-assays were 4.3 and 9.2% respectively.

Table 1 PCR primers and number of probes

Gene	Primer sequences	Roche probes number
<i>Agrp</i> (AF_206017)	Forward 5' CAGCAGACCGAGCAGAAGATGT 3' Reverse 5' GCACAGGTCGCAGCAAGGT 3'	#29
<i>Hprt</i> (NM_012583)	Forward 5' CAGTCAACGGGGACATAAAAG 3' Reverse 5' ATTTTGGGGCTGTACTGCTTGA 3'	#22

Immunocytochemistry

Placenta was cut out after decapitation from the mother on the 13th and 18th days of pregnancy. Placental tissue was fixed in the Bouin’s fluid and incubated for 72 h. Then it was embedded in paraffin and sectioned at 6 µm. After deparaffinization of sections, blocking of endogenous peroxidase activity was carried out by incubation in 1% H₂O₂ for 30 min at room temperature. In the next step, the sections were washed in PBS. After washing, the sections were preincubated for 30 min with 5% normal goat serum (DAKO, Glostrup, Denmark). A total of 1% of BSA (Sigma–Aldrich) was added to the goat serum. After that, the sections were incubated with a goat anti-rat AGRP (Phoenix Peptide), or in its solvent as a negative control for 1 h and then they were washed in PBS. Sections were incubated with peroxidase and secondary antibody (DAKO LSAB 2 System HRP). Peroxidase activity was detected using the DAB technique (DAKO liquid DAB substrate – chromogen system) and nuclei were counterstained with hematoxylin. A negative control without the primary antibody step was prepared. Observation was done with use of Zeiss LSM 510 Meta and analysis of figures was performed with use of LSM 510 vs 3.2 SP1 software.

RNA isolation and reverse transcription

Total RNA from the placenta and hypothalamus was isolated using Trizol LS Reagent (Invitrogen Corp.) according to the manufacturer’s protocol. After RNA isolation, DNase digestion was performed using RQ1 RNase-Free DNase (Promega Corp). The quantity and quality of RNA were analyzed using an Eppendorf spectrophotometer (Eppendorf AG, Hamburg, Germany). A total of 2 µg RNA were used to perform the reverse transcription (RT)–PCR analysis. RT was executed using an ImProm-II RT System (Promega Corp). Negative controls without RT that has been used in further analysis were also prepared.

Real-time PCR

Primers for the *Agrp* and internal standard hypoxanthine phosphoribosyl transferase (*Hprt*) were designed using Primer 3 software accessible in the internet to allow the amplification of regions that span introns. Sequence divisions on exon–intron boundaries were analyzed using the ‘Blat’ option from the internet page UCSC Genome Browser Home

(www.genome.ucsc.edu) for the rat. For the designed primers, we adjusted universal Tag Man probes (Roche Diagnostic) employing the Universal Probe Finder available on the Roche Internet Homepage. The probe numbers that we used were # 29 and # 22 for the *Agrp* (AF_206017) and *Hprt* (NM_012583) respectively (Table 1). The internal standard was selected from five genes (data not shown) after Best Keeper analysis (Pfaffl *et al.* 2004). Real-time expression was prepared using a LightCycler 2.0 instrument (Roche Diagnostic) and a LightCycler TaqMan Master kit (Roche Diagnostic). After initial optimization, the product was recovered from capillaries and resolved on 2% agarose gel at the presence of DNA molecular standard (Promega Corp). Afterwards the products were photographed and analyzed using Gel Logic 100 Imaging System and Kodak 1D analysis software version 3.6 (Estman Kodak Company) to verify the correct product size. Quantification was made using the relative standard curve method.

Sequencing of *Agrp*

In addition, real-time PCR with SYBR Green using LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostic) was performed. Melting curve analysis was also done. The product was recovered from capillaries and resolved in agarose. The PCR products for *Agrp* 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 *Agrp* products.

Western blotting

Total protein was isolated simultaneously with total RNA from placenta according to the manufacturer's protocol. Protein quantity was analyzed using Bradford reagent (Sigma-Aldrich Inc.) and measured on a spectrophotometer (Eppendorf AG). Equal amounts of proteins (8 µg) were used in the analysis. Samples were heated with XT Sample Buffer and XT Reducing Agent (Bio-Rad) at 95 °C for 5 min and loaded onto a 12% Criterion XT Precast Gel (Bio-Rad). Protein electrophoresis was performed using Bio-Rad Criterion apparatus in electron transfer XT Mops Running Buffer (Bio-Rad). The protein molecular mass standards Cruz Marker MW Standards (Millipore) were used. We performed wet electrotransfer of proteins from the gel to PVDF membranes (Millipore) for 1 h at 50 V at 4 °C using Trans-Blot Cell (Bio-Rad). The transfer efficiency was verified by incubating part of the gel with repeated sample in a Coomassie Brilliant Blue (Sigma-Aldrich Inc.) solution. Nonspecific protein binding was reduced during overnight incubations in blocking buffer (2% BSA in TBST). The membranes were incubated with specific goat antibodies, anti-rat AGRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in a TBST buffer, immunoreactive complex

Figure 1 Changes of AGRP concentration in blood serum of the pregnant rats. Statistically significant differences are marked with letters corresponding to the groups (small letters – $p \leq 0.05$, capital letters – $P \leq 0.01$). $P = 0.05$ is not statistically significant.

was detected with secondary donkey anti-goat IgGs conjugated to HRP and Immobilon Western Chemiluminescent HRP Substrate (Millipore). Western blots were visualized and analyzed using Versa Doc apparatus and quantity one 1-D analysis software (Bio-Rad). Negative controls with blocking peptides (Santa Cruz Biotechnology) against AGRP were also performed.

Statistical analysis

Statistical analysis for the AGRP level in serum and its expression in the hypothalamus was performed using ANOVA, followed by the multiple range Duncan's test with 99 and 95% confidence intervals. For the *Agrp* expression in placenta, the *t*-test was used. Results were shown as means $\pm$ S.E.M.

Figure 2 Changes in hypothalamic expression of *Agrp* in the course of pregnancy. Statistically significant differences are marked with letters corresponding to the groups (small letters – $p \leq 0.05$, capital letters – $P \leq 0.01$). $P = 0.05$ is not statistically significant.

Figure 3 (A) Immunocytochemistry localization of AGRP in 18th day of pregnancy in placenta. (B) Control reaction.

Results

In our study, we found that AGRP level in the serum increased significantly on the 13th and 18th days of pregnancy, and there was a slight increase on the 4th day in comparison with controls and a decrease after delivery, but its level was still higher than in control rats (Fig. 1).

The expression of *Agrp* in the hypothalamus increased substantially in the course of pregnancy. After 18 days, we found extenuation of mRNA level for *Agrp* (Fig. 2).

Figure 4 Western blotting of placental AGRP in 13th and 18th days of pregnancy. Lanes 1–3 present 18th day of pregnancy; lanes 4–6 present 13th day of pregnancy.

Figure 5 Changes in placenta expression of *Agrp* in the course of pregnancy. Statistically significant differences are marked with letters corresponding to the groups (small letters – $p \leq 0.05$, capital letters – $P \leq 0.01$). $P = 0.05$ is not statistically significant.

We also found, using real-time PCR, immunocytochemistry (Fig. 3), and western blotting (Fig. 4) methods that AGRP was expressed in placenta on the 18th day of pregnancy. Moreover, the use of the real-time PCR allowed us to observe that the expression of *Agrp* was considerably higher on the 18th than on the 13th day of pregnancy (Fig. 5).

Discussion

In this study, we found that the *Agrp* expression significantly increased up to the 13th day of pregnancy and decreased on the 18th day. However, the *Agrp* mRNA level was still considerably higher than in control rats. Rocha *et al.* (2003) presented similar results showing that *Agrp* expression increased substantially in the hypothalamus during pregnancy. Furthermore, they found that another peptide, NPY, which is co-produced with AGRP by the same neurons and also regulates food intake, was not altered during pregnancy. Moreover, it was found that the injection of ghrelin agonist GH-releasing peptide 2 into the hypothalamus led to fat mass gain by AGRP in NPY-deficient animals (Tschöp *et al.* 2002). These and previous data (Korner *et al.* 2001) may indicate that NPY and AGRP exert similar effects, but may be regulated differently. Also Ciofi *et al.* (1991) found that NPY was not changed during pregnancy. It may suggest that the AGRP may play a key role in the regulation of feeding during pregnancy.

It was found that *Agrp* expression in the hypothalamus was inhibited by leptin (Mizuno *et al.* 1999). During pregnancy, leptin level increased in the serum; however, mRNA level for the leptin receptor, both short and long forms, decreased (Amico *et al.* 1998, Garcia *et al.* 2000, Szczepankiewicz *et al.* 2006). These data may partly explain the increase of mRNA level for *Agrp* in the hypothalamus. On the other hand, we

found that *Agrp* expression significantly decreased on the 18th day of pregnancy, although mRNA level was still higher than in control rats, in comparison with the rats on the 4th day of pregnancy.

Mechanisms responsible for the increase of food intake and retainment of positive energy balance during pregnancy are particularly important. Therefore, the decline in the expression of anorexigenic POMC peptides during pregnancy is obvious (Rocha *et al.* 2003). Rocha *et al.* (2003) also found significant changes in the melanocortin receptor 4 gene expression. However, the expression of this receptor was higher in pregnant than in control rats. This suggests that AGRP, as an antagonist of MC4R (Ollmann *et al.* 1997, Shutter *et al.* 1997), may be involved in the attenuation of POMC-derived peptides function during pregnancy. Furthermore, AGRP may act as an inverse agonist of MC4R (Haskell-Luevano & Monck 2001, Breit *et al.* 2006) and contribute to food intake via this receptor. However, the competition between AGRP and MSH in melanocortin receptor binding has not yet been well known and may be associated with other factors such as syndecan-3 that acts as a co-receptor for MC4R in AGRP binding (Reizes *et al.* 2001). On the other hand, it was found that AGRP (83–132) acts as a competitive antagonist for human MC4R (Pritchard *et al.* 2004), suggesting a need for further exploration of these mechanisms.

The AGRP expressed in the hypothalamus may be associated with the regulation of brain energy homeostasis during pregnancy. We found that the AGRP serum level increased significantly during pregnancy. However, it was found that AGRP is frequently present as a trimer and crosses the blood–brain barrier very slowly (Kastin *et al.* 2000). It suggests that the peripheral AGRP is probably not responsible for a direct regulation of different peptides secretion in the brain. On the other hand, the *Agrp* expression in the hypothalamus decreased on the 18th day of pregnancy, while the AGRP level in the serum was significantly lower only after delivery. These data may suggest that another tissue may produce AGRP during pregnancy, and be responsible for its serum level. In addition, the hypothalamic AGRP may act particularly in a paracrine and autocrine manner. These data are in part consistent with the results obtained by Caminos *et al.* (2008), as they found that the *Agrp* expression in hypothalamus was the lowest on the 16th day of pregnancy in both groups of rats: those that were food restricted and those with access to food *ad libitum*.

In our study, we found that AGRP was expressed in placenta in mRNA as well as protein levels. Moreover, we found that the *Agrp* expression increased and was higher on the 18th day than on the 13th day of pregnancy. Similar results were obtained by Caminos *et al.* (2008) who also found that placental *Agrp* expression was significantly higher, but this increase was observed later, on the 21st day of pregnancy, and only in rats with access to food *ad libitum*, whereas in the group of food-restricted rats, *Agrp* expression in placenta increased significantly in all studied stages of pregnancy. Their

results may indicate that increase of AGRP expression influences regulation of food intake, particularly during nutritional deficiency, being an important peptide responsible for maintenance of energy balance. However, results obtained by Beloosesky *et al.* (2006) indicated that *Agrp* expression decreased in rat placenta up to the 16th day of pregnancy, and was not observed on the 18th day. The reason for this inconsistency is unclear; however, their results have not been confirmed so far.

Our results imply that the placenta may be a source of AGRP in mother serum. Moreover, the AGRP serum level did not decrease before delivery, indicating that the placenta is a very important source of AGRP. However, AGRP was also found to be produced in other tissues such as the adrenal gland (Bicknell *et al.* 2000).

On the other hand, Nilsson *et al.* (2005) found that in the newborn mouse, the AGRP system in arcuate nuclei was not fully developed, and this process was not finished before the third postnatal week. They also suggested that the same action takes place in newborn rats. These results indicated that placental AGRP may also regulate fetal development. Although there are no data about AGRP crossing through placenta, this mechanism is possible.

In conclusion, we found that the AGRP expression in the hypothalamus and placenta, and its serum level increased during pregnancy. Moreover, the placenta may be the source of AGRP in the course of pregnancy. These data may indicate that this peptide acts as a key factor during pregnancy, for both mother energy expenditure and fetal development. As we have described previously, AGRP changes correlate positively with food intake increase during pregnancy (highest on the 18th day of pregnancy), but this increase was not significant (Szczepankiewicz *et al.* 2006). However, there are only few published data about changes of AGRP, MSH, and its receptors during pregnancy. Therefore, more experiments are required to confirm our findings.

Declaration of interest

There are no potential conflicts of interests.

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Authors' contribution

D S, participated in the design of the study, tissue collections, protein isolation, and vaginal smear analysis, performed real-time PCR analysis, and drafted the manuscript. E P O, vaginal smear analysis, tissue collection, and RIA analysis. P K, western blot analysis and statistical analysis. M S, vaginal smear analysis, RNA isolation, and reverse transcription. K A,

immunocytochemistry. T W, tissue collection and RIA analysis. M S, tissue collections. K W N, participated in the design of the study and coordinated the study.

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