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Agouti-related peptide and melanocortin-4 receptor mRNAs expressions in arcuate nucleus during the pregnancy and lactation of rats

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ABSTRACT

Pregnancy is associated with a range of physiological adjustments to adapt the body to the demands of the growth of the fetus and subsequent lactation. It has been observed that agouti-related peptide (AGRP) and melanocortin-4 receptor (MC4R) are involved in energy homeostasis. A randomized controlled experimental study was planned to investigate the expression of AGRP and MC4R mRNAs in the stages of pregnancy and lactation in rat arcuate nucleus (ARC) of the hypothalamus. Thirty-two adult female rats were randomly divided into six groups. Pregnant rats were assigned into three groups (n = 6) of 7, 14, and 21 days of pregnancy. Two more groups were also assigned of non-suckling rats (n = 5), immediately separated from their pups after parturition, and suckling rats (n = 5), allowed to suckle five pups until day 8 (increasing milk). The sixth group consisted of four ovariectomized rats, which were assigned two weeks after surgery and served as control. Using real-time PCR, the relative expressions (compared to controls) of MC4R and AGRP mRNAs in ARC were calculated in the pregnant, suckling and non-suckling rats. Expression of AGRP mRNAs in pregnant rats on days 14 and 21 was higher than that observed in suckling and non-suckling rats ($P<0.05$). Expression of MC4R mRNAs in pregnant rats on days 14 and 21 was lower than that observed in suckling rats, and was higher on

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day 7 than that observed in both suckling and non-suckling rats ($P<0.05$). In conclusion, expression of AGRP in pregnancy and MC4R in lactation in ARC of rats controls energy homeostasis.

Key words: agouti-related peptide, melanocortin-4 receptor, arcuate nucleus, pregnancy, lactation, rat

Introduction

Pregnancy is associated with a range of physiological alterations to enable the pregnant female to cope with the demands of supporting growth of fetuses, parturition, and forthcoming lactation (RUSSELL et al., 2001). These include significant changes in appetite and food intake. In rats, for example, increased food intake begins to occur during early pregnancy, even before implantation has occurred (LADYMAN, 2008). Therefore, the hormonal alterations occurring in pregnancy induce adaptive changes in the body weight homeostasis of mothers, to prepare them for the energy requirements of the development of fetuses and impending lactation (GRATTAN, 2002). In lactating rats, food consumption increases as much as 2-3 times when compared with that of non-lactating non-pregnant rats (FELL et al., 1963).

Agouti-related peptide (AGRP) is a part of the melanocortin system that is involved in the control of body weight homeostasis, regulation of fat metabolism, and food intake (MORTON and SCHWARTZ, 2001). Three melanocortins, α, β, and γ melanocyte-stimulating hormones (MSH), are produced from their precursor protein, pro-opiomelanocortin (POMC). POMC produces α-MSH as the ligand for the centrally located melanocortin 3 and 4 receptor subtypes (MC3R and MC4R, respectively). Activation of these receptors has a significant effect on food intake and body mass (POGGIOLI et al., 1986). On the other hand, the naturally occurring MC3R and MC4R antagonist, AGRP, is expressed in the hypothalamus arcuate nucleus (ARC), and its central administration stimulates food intake (HAGAN et al., 2000).

Ingestion behavior, energy balance, and the use of substrate are controlled by melanocortin and leptin signaling, but only defects of leptin signaling cause infertility and hypothalamic hypogonadism (ISRAEL et al., 2012). Although gonadotropin releasing hormone (GnRH) neurons do not express leptin receptors, leptin influences GnRH neuron activity through regulation of immediate downstream mediators, including the neuropeptide Y (NPY) and/or α-MSH and AGRP, the agonist and antagonist of melanocortin, respectively (ISRAEL et al., 2012). Neurons of AGRP/NPY and POMC in the ARC of hypothalamus project onto GnRH neurons of the medial preoptic area (NILSSON et al., 2005). Moreover, both NPY and AGRP inhibit luteinizing hormone (LH) pulsatile release (VULLIEMOZ et al., 2005), and the overexpression of these neuropeptides maybe one of the multiple mechanisms responsible for infertility associated with leptin signaling deficiency.

There is no follicular development during pregnancy compared to the changes during the estrous cycle in the rat (GREENWALD, 1966). During pregnancy, it has been documented that serum LH levels tend to decrease, becoming lowest at mid-pregnancy and then recovering at the end of gestation (MORISHIGE et al., 1973). During the first 11 days of pregnancy in rats, serum LH concentrations were higher than those observed between days 13-19, and a progressive increase was subsequently seen beginning on day 20 and continuing to term, which was not contiguous with the postpartum ovulation inducing surge of LH (MORISHIGE et al., 1973). Furthermore, follicular maturation and ovulation are inhibited during lactation/suckling in various mammals (BUTLER, 2000). Inhibition of the estrous cycle in lactating rats mostly results from inhibition of LH and GnRH secretion (FOX et al., 1990).

The present study was conducted to evaluate the mRNAs expression of AGRP and MC4R in ARC of rats as pathways for infertility during the pregnancy and lactation, and to demonstrate the relationshiP between nutrition and fertility.

Materials and methods

Thirty-two adult (3-4 months old) female Sprague-Dawley rats (*Rattus norvegicus*), weighing 200 ± 20 g, were used in the present randomized controlled experimental study. The rats were housed in the Center of Comparative and Experimental Medicine of Shiraz University of Medical Sciences, Shiraz, Iran, under controlled temperature (22˚C) and lighting (12:12 light to dark ratio; light on at 7:30 AM) conditions. All experimental procedures on the rats were carried out between 12.00-2.00 PM and based on the recommendations of the Animal Care Committee of the Shiraz University of Medical Sciences.

For grouping based on days of pregnancy, 18 rats were randomly selected and their pregnancy confirmed using the vaginal smear method (Sabet Sarvestani et al., 2014). The stage of their estrous cycle was determined by microscope observation of the vaginal smears. The female rats in proestrous or estrous stages were transferred to cages with mature male rats in a 3:1 ratio and left overnight. The female rats were examined for the presence of a vaginal plug the next morning and separated from the males. On days 4 and 5 post-coitus, vaginal smears were evaluated once again and their cellular characteristics determined under a light microscope. The presence of diestrous or metestrous cells in vaginal smears on days 4-5 post-coitus was taken as a positive indication for pregnancy. The pregnant rats were subsequently randomly assigned to three equal groups of 7, 14, and 21 days of pregnancy (n = 6).

Ten pregnant rats were selected randomly and were assigned as suckling and non-suckling groups. Immediately after parturition, the non-suckling rats (n = 5) were separated from their pups. The suckling rats (n = 5) were allowed to suckle their five

pups for eight days (the increasing phase of lactation). Four ovariectomized rats, selected randomly, were used as the control group. The control group rats were anesthetized by an intra-peritoneal injection of xylazine (7 mg/kg, Alfazyne, Woerden, Netherlands) and ketamine (100 mg/kg, Woerden, Netherlands) and were ovariectomized via incision of the ventral midline. Further procedures were carried out after ensuring a 2-week recovery period.

The pregnant, suckling, non-suckling and ovariectomized rats were decapitated and the brains were removed immediately. The pregnancy of 18 rats was re-confirmed by observation of pregnant uteri. The diencephalon was dissected out by an anterior coronal section, anterior to the optic chiasm, and at the mammillary bodies' posterior border a posterior coronal cut was made. To separate ARC from AVPV, a third coronal cut was made through the middle of the optic tract, just rostral to the infundibulum (SALEHI et al., 2013b). The ARC specimens were stored in liquid nitrogen until further analysis.

RNA extraction, DNase treatment, cDNA synthesis and relative real-time PCR procedures were performed as described elsewhere (SALEHI et al., 2013a). Primers were designed with Allele ID 7 software for the reference gene, AGRP (NM_033650.1) and MC4R (NM_013099.2). For data normalization, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rat gene (M32599) was used as the reference gene (Table 1).

Table 1. Sequences of real-time PCR primers used to evaluate relative expression of AGRP and MC4R genes in the rat

Amplicon length (bp)	Primer sequence	Primer
181	5` TGGGTGTCATAAGCCTGTTGG 3`	MC4R-F
	5` GCGTCCGTGTCCGTACTG 3`	MC4R-R
189	5` TGAAGAAGACAGCAGCAGACC 3`	AGRP-F
	5` TGAAGAAGCGGCAGTAGCAC 3`	AGRP-R
112	5` AAGAAGGTGGTGAAGCAGGCATC 3`	GAPDH-F
	5` CGAAGGTGGAAGAGTGGGAGTTG 3`	GAPDH-R

Relative expressions of AGRP and MC4R based on the threshold cycle (CT) method were calculated for quantitative real-time PCR data. Using Line-gene K software (LARIONOV et al., 2005), CT for each sample was calculated. Fold expression of the target mRNAs over reference values was calculated by the equation $2^{-\Delta\Delta CT}$ (LIVAK and SCHMITTGEN, 2001), where ΔCT is determined by subtracting the internal control (corresponding GAPDH CT value) from the specific CT of the AGRP or MC4R. $\Delta\Delta CT$ was obtained by subtracting the ΔCT of each experimental sample from that of the control ovariectomized rats.

Normality of the data on the relative expression of AGRP and MC4R mRNAs was evaluated by the Kolmogorov-Smirnov test using SPSS version 11.5 (SPSS Inc, Chicago, Illinois). The relative expressions of AGRP and MC4R mRNAs were not normally distributed. Therefore, data was log-transformed to obtain a normal distribution that was used in one-way ANOVA analyses. Expressions of AGRP and MC4R mRNAs were compared between groups using one-way ANOVA and LSD post hoc test. The correlation coefficient of expression of AGRP and MC4R mRNA was evaluated using the Pearson correlation test. We considered $P<0.05$ as significant.

Results

Mean expression of AGRP mRNAs in the ARC of pregnant rats on days 14 and 21 was significantly higher as compared to suckling and non-suckling rats ($P<0.05$, Fig. 1). Relative expression of AGRP mRNAs in the ARC of pregnant rats on days 7, 14 and 21 were 3.4, 4.7 and 4.2 times more than suckling and 1.7, 2.3 and 2.1 times more than non-suckling rats, respectively.

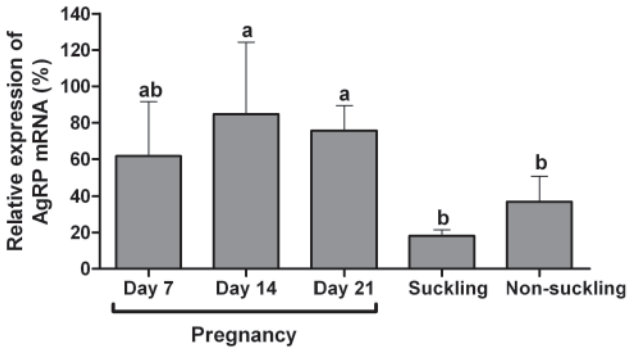

Fig. 1. Mean (± standard error) of the relative expression of agouti-related peptide (AGRP) mRNAs in the arcuate nucleus during pregnancy and suckling in rats. Different letters indicate significant difference ($P<0.05$).

Conversely, mean expression of MC4R mRNAs in the ARC of pregnant rats on day 7 was lower than that observed in suckling and non-suckling rats ($P<0.05$, Fig. 2). The mean expression of MC4R mRNAs in the ARC of pregnant rats on days 14 and 21 was also lower compared to suckling rats ($P<0.05$). The relative expression of MC4R mRNAs in the ARC of pregnant rats on days 7, 14 and 21 were 6.4, 5.6 and 5.2 times less than suckling and 4.6, 4 and 3.7 times less than non-suckling rats, respectively. A negative non-significant correlation was observed between expression of AGRP and MC4R mRNAs in the ARC of pregnant and suckling rats ($r = -0.24$, $P = 0.3$, Fig. 3).

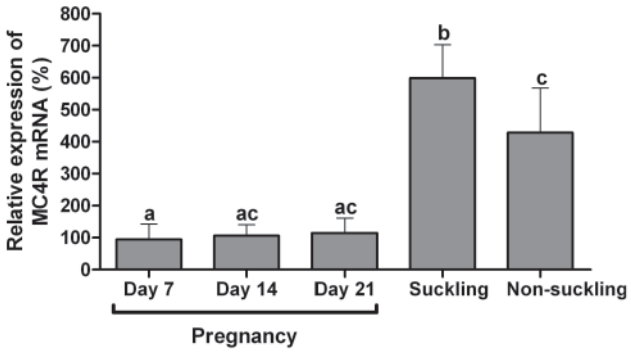

Fig. 2. Mean (± standard error) of the relative expression of melanocortin-4 receptor (MC4R) mRNAs in the arcuate nucleus during pregnancy and suckling in rats. Different letters indicate significant difference ($P<0.05$)

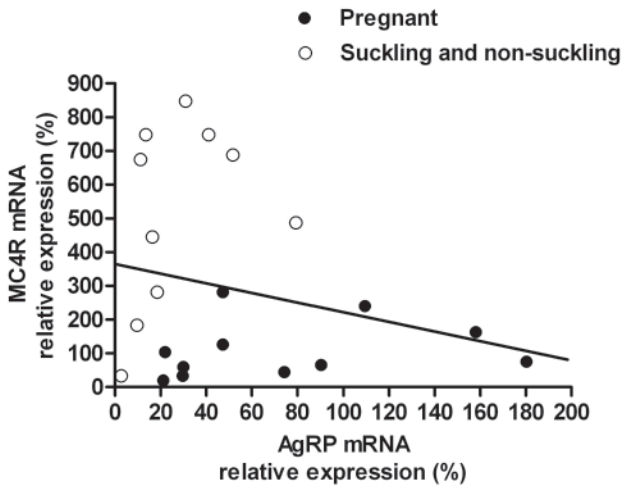

Fig. 3. Scatter plot of the relative expression of agouti-related peptide (AGRP) and melanocortin-4 receptor (MC4R) mRNAs in the arcuate nucleus of pregnant, suckling and non-suckling rats

Discussion

The mean expression of AGRP mRNAs in the ARC of pregnant rats was greater than that in suckling and non-suckling rats. Consistent with our findings, elevated levels of AGRP, but not POMC, MC4R or NPY, in Wistar rats have been recorded that during pregnancy, suggesting that AGRP could play a role in the hyperphagia that occurs during pregnancy (ROCHA et al., 2003). The ARC contains the orexigenic NPY and AGRP neurons, and the POMC neurons, from which the satiety factor α-MSH is derived (ELIAS et al., 1999). NPY and AGRP neurons are responsive to leptin and are involved in integration of the leptin signals with the neural circuits involved in energy homeostasis (ELIAS et al., 1999). However leptin stimulates POMC neurons and inhibits the NPY and AGRP neurons (ELIAS et al., 1999). Leptin normally negatively regulates NPY and AGRP mRNA expression levels (ROCHA et al., 2003). In the ARC, NPY neurons express progesterone receptors (DUFOURNY and SKINNER, 2002). However, during pregnancy the expression of AGRP, which is co-localized with NPY and presumably with the progesterone receptor, appears to be enhanced (ROCHA et al., 2003). Therefore, in the rat, not only is pregnancy a state of leptin resistance, but also a state of α-MSH resistance. It is tempting to speculate that this inability to respond to α-MSH is due to the increase in AGRP, an endogenous antagonist of MC3R and MC4R. During pregnancy, an increase in mRNA expression of AGRP has been reported in the hypothalamus (ROCHA et al., 2003) and high levels of AGRP prevent the α-MSH mediated anorectic activation of these receptors (OLLMANN et al., 1997). Leptin resistance is part of the metabolic adaptation of the maternal body and is required for a successful pregnancy. Moreover, administration of exogenous AGRP or NPY inhibits pulsatile LH release and reproductive function (VULLIEMOZ et al., 2005). Reductions in hypothalamic AGRP and NPY mRNA expression were also described in studies of MC4R knockout mice (IRANI et al., 2005). The possibly cooperative role of AGRP and gonadotropin inhibitory hormone in the ARC of the hypothalamus has been implicated in ovarian function regulation in ewes (JAFARZADEH SHIRAZI et al., 2011b).

The levels of NPY, which plays an important role in mediating food intake, are significantly increased during lactation in some hypothalamic areas, including the ARC (CHEN et al., 1999). A different study recorded the expression of AGRP during diestrus in rats, the stage of the cycle when estrogen levels are basal and similar to lactation (ZANDI et al., 2014), to be higher than that observed in suckling rats in the present study. AGRP expression during diestrus in ewes was less than at other stages of the estrous cycle (JAFARZADEH SHIRAZI et al., 2011a). In contrast to our findings, during lactation, AGRP expression was significantly increased in a subset of the AGRP neurons in the ARC, in comparison with the diestrous phase (CHEN et al., 1999). These findings suggest that AGRP may be involved in the increase in food intake during pregnancy.

Mean expression of MC4R mRNAs in the ARC of pregnant rats during the present study showed a post-partum decrease. Recent studies have indicated that melanocortin receptor agonists can stimulate ARC kisspeptin expression in ewes, thus providing an additional mechanism by which melanocortin signaling can impact reproductive outcome (BACKHOLER et al., 2009). The KiSS-1 mRNA expression in ARC was the highest in the first week of pregnancy, and decreased 4-fold in the third week of pregnancy in rats (SABET SARVESTANI et al., 2014). The present study demonstrated that expression levels of MCR4 decreased during pregnancy, when levels of prolactin are low. It has been shown that dopamine, which is an inhibitor of prolactin, has an inhibitory effect on MSH secretion (HALBACH and DERMIETZEL, 2006). Probably, axons from dopamine neurons project to both MSH and prolactin neurons, to inhibit LH during gestation. Unlike non-pregnant rats, pregnant rats showed no response to exogenous α-MSH, indicating that at least part of the inability of leptin to cause a reduction in food intake is due to the decreased α-MSH ability to suppress appetite (LADYMAN, 2008). During pregnancy, the obvious alterations of the expression of POMC are apparent, decreasing mid-pregnancy and increasing during late pregnancy. However, the expression of POMC is only indirectly related to expression of α-MSH (DOUGLAS et al., 2002).

Our finding that MCR4 levels of expression significantly increase with lactation is in line with previous observations about the important role of the positive effects of MSH on thyrotropin-releasing hormones (TRH) (RIIS and MADSEN, 1985). The thyroid hormones, triiodothyronine (T3) and thyroxin (T4) increase in lactation in the goat (RIIS and MADSEN, 1985) and the cow (KAFI et al., 2012). So, it is likely that MSH neurons stimulate TRH neurons and, in turn, cause milk production. In addition, a small increase in plasma levels of the growth hormone (GH) occurred during lactation in the rat (SAR and MEITES, 1969). Moreover, immunoneutralization of rat GH can reduce milk secretion (MADON et al., 1986). This, alongside other studies that showed that growth-hormone-releasing hormone (GHRH) co-localizes with MSH (GERACIOTI et al., 2009), is consistent with the present study, which revealed that expression levels of MC4R increased during lactation. A previous study demonstrated a significant increase in the expression of estradiol receptors (ERs) during lactation in the rat (VANOYE-CARLO et al., 2009) that is consistent with our findings, because co-localization of ERα in neurons containing POMC has been demonstrated (DEWING et al., 2007). POMC neurons in the ARC project onto GnRH-immunoreactive cells in the medial preoptic area (LERANTH et al., 1988), in which activation of MC4R stimulates LH secretion (WATANOBE et al., 1999). These findings suggest that AGRP may be involved in the increase in food intake during lactation.

Conclusions

A negative non-significant correlation was observed between expression of AGRP and MC4R mRNAs in the ARC of pregnant and suckling rats. Consistent with our findings, a negative non-significant correlation between AGRP and MC4R mRNAs during the estrous cycle was previously observed in rats (ZANDI et al., 2014). Therefore, expression of AGRP mRNA during pregnancy and expression of MC4R mRNA during lactation in ARC of rats simultaneously control nutrition and fertility in these stages.

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SAŽETAK

Gravidnost je povezana s nizom fizioloških prilagodbi kojima tijelo odgovara zahtjevima povezanima s rastom fetusa i kasnije laktacije. Uočeno je da su agutiju srodan peptid (AGRP) i melanokortin-4 receptor (MC4R) uključeni u energetsku homeostazu. Randomiziranim kontroliranim pokusom planirano je u arkuatnoj jezgri (ARC) hipotalamusa štakorica istražiti ekspresiju mRNA AGRP i mRNA MC4R tijekom gravidnosti i laktacije. Trideset dvije odrasle štakorice nasumično su bile podijeljene u šest skupina. Gravidne su štakorice podijeljene u tri skupine (n = 6), s obzirom na 7., 14. i 21. dan gravidnosti. Još dvije skupine (n = 5) činile su nedojne štakorice koje su odvojene od svoje mladunčadi odmah nakon poroñaja te dojne štakorice kojima je dozvoljeno dojenje 5 mladunaca do osmog dana rastuće laktacije. Četiri ovarijektomizirane štakorice, dva tjedna nakon operacije, dodijeljene su u 6. kontrolnu skupinu. Koristeći PCR u stvarnom vremenu, relativna ekspresija (usporedba s kontrolama) mRNA MC4R i mRNA AGRP u ARC izračunata je za gravidne, nedojne i dojne štakorice. Ekspresija mRNA AGRP kod gravidnih štakorica 14. i 21. dan bila je veća od one opažene kod dojnih i nedojnih štakorica ($P<0,05$). Ekspresija MC4R mRNA u gravidnih štakorica 14. i 21. dan bila je manja u odnosu na dojne štakorica i veća 7. dan u odnosu na skupine dojnih i nedojnih štakorica ($P<0,05$). Zaključno, ekspresija AGRP u ARC tijekom gravidnosti i MC4R u ARC tijekom laktacije kontrolira energetsku homeostazu štakorica.

Ključne riječi: agutiju srodan peptid, melanokortin-4 receptor, arkuatna jezgra, gravidnost, laktacija, štakor