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Prolactin and the dietary protein/carbohydrate ratio regulate the expression of SNAT2 amino acid transporter in the mammary gland during lactation

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ABSTRACT

The sodium coupled neutral amino acid transporter 2 (SNAT2/SAT2/ATA2) is expressed in the mammary gland (MG) and plays an important role in the uptake of alanine and glutamine which are the most abundant amino acids transported into this tissue during lactation. Thus, the aim of this study was to assess the amount and localization of SNAT2 before delivery and during lactation in rat MG, and to evaluate whether prolactin and the dietary protein/carbohydrate ratio might influence SNAT2 expression in the MG, liver and adipose tissue during lactation. Our results showed that SNAT2 protein abundance in the MG increased during lactation and this increase was maintained along this period, while 24 h after weaning it tended to decrease. To study the effect of prolactin on SNAT2 expression, we incubated MG explants or T47D cells transfected with the SNAT2 promoter with prolactin, and we observed in both studies an increase in the SNAT2 expression or promoter activity. Consumption of a high-protein/low carbohydrate diet increased prolactin concentration, with a concomitant increase in SNAT2 expression not only in the MG during lactation, but also in the liver and adipose tissue. There was a correlation between SNAT2 expression and serum prolactin levels depending on the amount of dietary protein/carbohydrate ratio consumed. These findings suggest that prolactin actively supports lactation providing amino acids to the gland through SNAT2 for the synthesis of milk proteins.

1. Introduction

Lactation is a physiological process necessary to provide all nutrients to the newborns. This process is fundamental for all mammals, and it requires large amounts of building block substrates for the synthesis of proteins, triglycerides and lactose among others for the milk synthesis. Thus, circulating amino acids from the mother are actively taken up for protein synthesis. It has been evidenced that during the lactation period the most abundant amino acids transported into the mammary gland are alanine and glutamine [1] and are particularly important in regulating the amino acid intracellular pool. Specifically, glutamine is an efflux substrate for other amino acid transporters like the amino acid heteroexchanger system L (LAT1), facilitating the uptake of branched-chain amino acids, particularly leucine that activates the TOR pathway involved in protein synthesis [2,3].

These amino acids, alanine and glutamine, as well as other small neutral amino acids are mainly transported across membranes through System A which is comprised by three subtypes known as SNATs (sodium-coupled neutral amino acid transporters) 1, 2 and 4 [4]. However, SNAT2, which represents the classical characteristics of System A, is widely expressed in mammalian cells and is Na^+-dependent [5–7]. Additionally, SNAT2 is regulated by environmental conditions, proliferative stimuli, developmental changes, and hormonal signals [8].

Several studies showed that the mammary glands possess characteristics of System A activity [9,10], determined by using the non-metabolizable analog MeAIB. In addition, SNAT2 in the mammary gland shows a unique characteristic of this transport system observed in other cell types called adaptive regulation [11].

Abbreviations: BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CRE, cAMP response element; DAB, diaminobenzidine; ECL, enhanced chemiluminescence; HPRT1, hypoxanthine phosphoribosyl transferase 1; HPR, horseradish peroxidase; JaK2, janus kinase 2; MAPK, mitogen-activated protein kinase; MeAIB, methylaminoisobutyric acid; MG, mammary gland; MMLV, Moloney murine leukemia virus; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PVDF, polyvinylidene difluoride; RIA, radioimmunoassay; RIPA, radio-immunoprecipitation assay; RT-PCR, real time polymerase chain reaction; SNAT2, sodium-coupled neutral amino acid transporter 2; STAT5, signal transducer and activator of transcription 5; TBS, Tris-buffered saline

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Several hormones play an important role in the regulation of SNAT2 gene expression in the mammary gland. There is evidence during gestation of an increase in the SNAT2 mRNA abundance in this tissue that is associated with concomitant changes in serum estradiol levels [12]. Recently, it has been demonstrated that upregulation of SNAT2 gene expression during gestation is mediated by the estrogen receptor α bound to the coactivator glyceraldehyde 3-phosphate dehydrogenase that specifically binds to an estrogen response element found in the SNAT2 gene promoter [13]. Interestingly, we have evidence that after gestation, there is a second increase of SNAT2 mRNA that reaches its maximal in the peak of lactation in the mammary gland [12]. There is preliminary evidence that suggests that prolactin could be responsible for the maintenance and regulation of this amino acid transporter during lactation, however this has not been established.

Prolactin is a single polypeptide with a molecular weight of 23 kDa that is produced in the pituitary gland [14]. This hormone is secreted with a pulsatile pattern during this physiological stage [15]. Prolactin has two receptor isoforms, a long- and a short-isoform [16]. Interestingly, the mammary gland during lactation mainly expresses the long-isoform which activates several signaling pathways, particularly those involving JAK2/STAT5, PI3K/PKB and MAPK [14]. It is known that prolactin is involved in the activation of the expression of several genes that include the β-casein gene to synthesize one of the most abundant proteins in milk *via* the STAT5 transcription factor [17]. Therefore, in order to sustain an elevated rate of milk protein synthesis, the supply of large amounts of amino acids is necessary. There is evidence that the expression of several amino acid transporters is up-regulated during lactation, including SNAT2 [18–20].

Interestingly, It has been demonstrated that incubation of mammary gland explants with prolactin increases the uptake of amino acids transported *via* System A [21]. However, there are no studies that have established whether prolactin in fact increases the expression of SNAT2 in the mammary gland.

Moreover, it has been demonstrated that the circulating levels of prolactin are dependent on the amount of dietary protein consumed. The lower the amount of protein, the lower the concentration of serum prolactin [22]. As a result there is a decrease in milk production that is reflected in the growth pattern of the pups. It is important to study whether the changes in the dietary protein/carbohydrate ratio may affect the expression of SNAT2 not only in the mammary gland but also in other tissues such as the adipose tissue and liver. It has been demonstrated that the prolactin receptor is present in these tissues among others [23–27]. However, there is no knowledge whether during lactation the SNAT2 gene is regulated in a similar fashion in these tissues compared with the mammary gland.

Therefore, the aim of the present work was to demonstrate if there are changes in SNAT2 protein abundance before delivery and during lactation, to study if prolactin was able to increase the expression and promoter activity of SNAT2 in mammary gland explants and T47D cells and finally, to establish whether the dietary protein/carbohydrate ratio may influence serum prolactin levels and SNAT2 gene expression in the mammary gland, liver and adipose tissue.

2. Materials and methods

The experimental design and procedures of this study were reviewed and approved by the Animal Care Committee of the Instituto Nacional de Ciencias Médicas y Nutrición, México, in accordance with the international guidelines for the use of animals in research.

2.1. Animals

This study was divided into two independent studies in order to achieve the objectives described.

2.1.1. Study 1

This study was designed to determine the change in SNAT2 gene expression during late gestation and lactation. Female Wistar rats weighing 200–250 g were obtained from the animal research facility at the Instituto Nacional de Ciencias Médicas y Nutrición. The animals were housed in individual stainless steel cages at 21 °C with a 12:12 h light–dark cycle. The animals were allowed free access to water and chow diet. Gestational age was determined by vaginal smear to detect spermatozoa. Mammary gland explants were obtained as previously reported [11] from pregnant rats at day 20, lactating rats at days 5, 12, and 18, or weaning (rats 21 days postpartum separated from their pups for 24 h). After normal pregnancy and delivery, the litter size was adjusted to 8 pups/dam. Five rats per group were used.

2.1.2. Study 2

This study was designed to determine if dietary protein modifies SNAT2 protein abundance during late gestation and lactation. The rat strain, the maintenance thereof, and the gestational age determination were the same as study 1. The animals were allowed free access to water and to a low-protein/high-carbohydrate (10/73%), normal-protein/normal-carbohydrate (20/63%) or high-protein/low-carbohydrate (30/53%) diets, according to the American Institute of Nutrition (AIN93) lab rodent diet recommendations [28]. The adipose tissue, liver and mammary gland were obtained from pregnant rats on day 20, or rats that had been lactating for 5 and 12 days. After delivery, the litter size was adjusted to 8 pups/dam. Five rats per group were used. The food intake of the dams was recorded daily, and the dams and pups were weighed every other day.

2.2. Quantitative real-time PCR

Total RNA was extracted from the mammary gland by the guanidinium thiocyanate–cesium chloride method as previously described [12]. The RNA concentration was measured using a NanoDrop spectrophotometer 1000 (ND-1000; Thermo Scientific, Wilmington, De, USA). RNA integrity was corroborated by visualizing the 28S and 18S ribosomal subunits in a 1% (w/v) agarose gel and quality was assessed with the 260/280 nm ratio absorbance of 2.0. RNA (3 μg) was reverse-transcribed to cDNA by the use of Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). For the real-time PCR analyses, 300 ng of cDNA was used in a final reaction volume of 10 μl per reaction. Predesigned TaqMan assay (Applied Biosystems, Foster City, CA, USA) probes for sodium-coupled neutral amino acid transporter SNAT2 (Rn00710421_m1) and HPRT1 (hypoxanthine phosphoribosyl transferase 1) (Rn01527840_m1) were used. RT-PCR was performed using the following PCR amplification conditions: denaturation for 5 min at 95 °C, annealing for 1 min at 56.2 °C, extension for 1.30 min at 72 °C for 34 cycles; and final extension for 7 min at 72 °C. The amplification and detection of specific products was performed with the ABI PRISM 7000 (Applied Biosystems). The mRNA level of the SNAT2 was normalized to the HPRT1 gene. HPRT1 was used as a housekeeping gene since cyclophilin or β-actin showed great variation among samples. The relative amount of mRNA was calculated using the comparative CT method (User Bulletin no. 2; PE Applied Biosystems).

2.3. Western blot

Proteins were extracted from the mammary gland, liver and adipose tissue using RIPA (radio-immunoprecipitation assay) lysis buffer containing the following: 50 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 1% NP40, 0.25% Na-deoxycholate, and 1 mmol/l PMSF. We added a 1× Roche mini complete protease mixture. The protein concentration was measured in duplicate using the Lowry method. Before being loaded, the samples were prepared by mixing 40 μg of protein with Laemmli buffer in a 1:1 ratio and heated at 80 °C for 5 min.