Characterization and regulation of the gene expression of amino acid transport system A (SNAT2) in rat mammary gland

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MILK PRODUCED BY THE MAMMARY GLAND during lactation is fundamental for the development and growth of the newborn, providing nutrients in the quantity and quality required to maintain metabolic functions. Thus, to fulfill this role, the lactating mammary gland requires, among other nutrients, large amount of amino acids to sustain different functions, such as synthesis of milk proteins as energy substrates (36) and as synthesis of fatty acids (39). Several studies (2, 39) in different species where arteriovenous differences in amino acids across the mammary gland are measured indicate that all amino acids are captured by the gland, with alanine and glutamine the most actively transported. These amino acids are preferentially transported by system A in most tissues (21), and this activity could play a key role in the mammary gland as well as in other tissues (3).

Several studies showed that mammary glands possess characteristics of system A activity (20, 27), as determined by using the nonmetabolizable analog α-(methylamino)isobutyric acid (MeAIB) (35), such as Na⁺ dependence, pH sensitivity, preference for the uptake of short-chain neutral amino acids like alanine and serine, as well as the amide amino acids glutamine and asparagine, and relatively lower affinity for threonine and the branched-chain amino acids (10, 15, 21). In addition, system A in mammary gland presents a unique characteristic of this transport system observed in other cell types called adaptive regulation (35). Adaptive regulation occurs when cells are incubated in a medium lacking extracellular amino acid substrates, and as a result there is an increase in system A transport activity. This activity is repressed with actinomycin D or when cells are transferred to an amino acid-rich medium (14).

In recent years, a gene family of sodium-coupled neutral amino acid transporters (SNAT; SCL38 gene family) coding for proteins that possess the classically described system A transport activities, in terms of their functional properties and patterns of regulation, has been cloned (18). Several isoforms have been described and designated SNAT1 (38), SNAT2 or SAT2 (33, 40), and SNAT4 (34). SNAT2 is widely expressed in different cell types (4), and its mRNA concentration increases in cultured cells during adaptive regulation or with the addition of cAMP (8). A recent study (22) has demonstrated that the increase of SNAT2 expression in response to amino acid deprivation is associated with the presence of an amino acid response element located in intron 1 of the gene in several species.

Because of the importance of system A activity in the mammary gland during lactation, as well as its role in the control of other amino acid transport systems, it is important to understand the regulation of expression of the SNAT2 isoform in the mammary gland. The results of the present study show that SNAT2 expression in rat mammary gland explants is controlled at multiple levels by a variety of mechanisms that involve PKA and PKC, as well as estradiol. Presumably, all these signals tightly control the expression of SNAT2, depending on the physiological status of the gland during pregnancy and lactation.

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MATERIALS AND METHODS

Materials. Nylon membrane filter Hybond N+, deoxycytidine 5'-[α-32P]triphosphate (3,000 Ci/mmol), and the rediprime DNA labeling system were from Amersham, UK.

Animals. Female Wistar rats with a weight of 200–250 g were obtained from the animal research facility at the Instituto Nacional de Ciencias Médicas y Nutrición. Animals were housed in individual stainless steel cages at 18°C with a 12:12-h light-dark cycle and 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 (35) from virgin, pregnant, lactating, and postweaning rats. After normal pregnancy and delivery, the litter size was adjusted to 8 pups/dam. Rats at the end of lactation (21 days postpartum) were separated from their pups. Non-pregnant, nonlactating animals were used as control rats and are referred to as “virgin rats.” This study was approved by the Animal Care Committee of the Instituto Nacional de Ciencias Médicas y Nutrición, México, in accordance with international guidelines for the use of animals in research.

Preparation of mammary tissue explants. Mammary tissue explants were prepared as described previously (35). Briefly, rats were anesthetized and then killed by decapitation. The mammary gland was isolated and then killed by decapitation. The mammary gland was freed from the connective tissue, the mammary tissue was diced into 2- to 3-mm explants. The tissue explants were rinsed repeatedly with buffer at 37°C prior to assay.

RNA preparation. Mammary tissue was homogenized in guanidinium buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, and 0.5% N-laurylsarcosine with a polytron (PT2000; Kinematica, Lucerne, Switzerland) at the lowest setting. The homogenate was centrifuged at 12,000 g for 15 min at 18°C, and the resulting supernatant was layered onto a CsCl solution containing 5.7 M CsCl and 25 mM sodium acetate, pH 5.2. The CsCl gradient was formed by centrifugation at 113,000 g for 18 h at 18°C to yield total RNA. The RNA was precipitated with 100% ethanol and 3 mM sodium acetate, pH 5.2, washed twice in 75% ethanol, and resuspended in formamide. The RNA was quantified by optical density at 260 nm and stored at −80°C until use.

Probe preparation. The probe was prepared by RT-PCR using poly(A)+ mRNA isolated from mammary gland. Poly(A)+ RNA was purified from mammary gland explants by guanidine thiocyanate extraction and ultracentrifugation through a cesium chloride cushion (5) followed by a single round of oligo(dT) cellulose chromatography. The sense primer was 5'–CTACTCTATAACCCCAAGAGA-3', which corresponds to nucleotide positions 475–495 in SNAT2 cDNA, and the antisense primer was 5'–ACGATAGGCTCCACGAGA-3', which corresponded to nucleotide positions 1824–1842 in SNAT2 cDNA (GenBank accession no. NM181090). 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, and extension for 1.30 min at 72°C for 34 cycles; final extension for 7 min at 72°C. The reaction (25 μl) was separated by electrophoresis on a 1.5% agarose-Trits-acetate-EDTA gel containing ethidium bromide and viewed under UV light. Afterward, manual sequencing of the product was performed to establish its molecular identity.

Northern blot analysis. For Northern analysis, 15 μg of RNA were loaded into each lane and were electrophoresed in a 1.0% agarose gel containing 37% formaldehyde. The RNA was then blotted onto nylon membranes (Hybond N+) and cross-linked with a UV crosslinker (Amersham). cDNA probe was labeled with Redivue [α-32P]dCTP (110 TBq/mmol) using the Rediprime DNA labeling kit. Membranes were prehybridized with rapid-hyb buffer (Amersham) at 65°C for 1 h and then hybridized with the cDNA probe for 2.5 h at 65°C. Membranes were washed once with 2 SSC-0.1% SDS (1 SSC = 0.15 M sodium chloride, 15 M sodium citrate) at room temperature for 20 min and then twice for 15 min with 0.1 SSC-0.1% SDS at 65°C. Digitalization of the images and quantitation of radioactivity (cpm) of the bands were done by using the Instant Imager (Packard Instrument, Meriden, CT). Membranes were also exposed to Extascam film (Kodak, Guadalajara, Mexico) at −70°C with an intensifying screen.

Preparation of tissue for in situ RT-PCR. The alcohol-fixed mammary gland sections (4 μm) were embedded in paraffin and used for system A mRNA expression determined by in situ RT-PCR. Mammary gland sections were mounted on silane cover slides, deparaffinized for 18 h at 60°C, and sequentially immersed in xylene (30 min at 37°C), absolute ethanol, 50% ethanol, and water. Cells were rendered permeable by incubation for 10 min at room temperature in 0.02% HCl, followed by a 90-s incubation with 0.01% Triton X-100 and then a 30-min incubation at 37°C with 1 μg/ml protease K (GIBCO-BRL, Gaithersburg, MD). DNA was removed by incubation for 15 min at 37°C with DNase, 3 U/tissue section (GIBCO-BRL). Reverse transcription was performed at 37°C for 1 h, incubating the tissue in 50 μl of reverse transcriptase buffer (1X, GIBCO-BRL), 0.1 M dithiothreitol, 2 mM dNTP mix (GIBCO-BRL), 0.1 μg/μl of oligo (dT), and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). The sections were sealed using the Assembly tool (PerkinElmer, Branchburg, NJ). The RNA was then removed by incubation with 3 U RNase and fixed again by immersion with 4% paraformaldehyde in Sorenson buffer for 3 min at 4°C, and PCR was performed, incubating the tissue sections with 50 μl of 1X reaction buffer (GIBCO-BRL), 2 U of Taq polymerase, 15 mM MgCl2, and dNTPs labeled with digoxigenin (Boehringer Mannheim, Mannheim, Germany). The slides were sealed again and placed in the thermocycler (PerkinElmer). After an initial denaturation at 95°C for 5 min, 35 cycles were performed using an annealing temperature of 56.2°C for 1 min and an extension temperature of 72°C for 1.5 min. PCR products were detected with monoclonal mouse antidigoxigenin antibodies coupled to alkaline phosphatase and tetrazolium nitroblue (Boehringer Mannheim) and counterstained using nuclear fast red. Expression of actin was used as a positive control, and the negative control consisted of performing the whole procedure without the system A primers.

RESULTS

To determine the mechanism(s) of regulation of the SNAT2 gene expression, we studied whether adaptive regulation was accompanied with a concomitant increase of SNAT2 mRNA in mammary gland explants. With this purpose, explants were incubated in an amino acid-free medium for different periods of time ≤300 min. As shown in Fig. 1, SNAT2 mRNA concentration increased steadily during the first 250 min of amino acid deprivation, reaching a 20-fold induction. When incubation of mammary gland explants in amino acid-free medium was prolonged ≤300 min, SNAT2 mRNA concentration began to decay. Interestingly, when mammary gland explants were incubated with an equimolar mixture of the amino acids used by the amino acid transport system A (1 mM each alanine, glycine, and serine), the induction of SNAT2 was suppressed (Fig. 1). However, when explants were incubated with a mixture of large neutral and cationic amino acids (1 mM each phenylalanine, valine, and lysine), a rapid induction of SNAT2 was observed at 50 min, and it was less prolonged compared with explants incubated in an amino acid-free medium (Fig. 1). These results clearly indicated that SNAT2 gene expression in mammary gland explants was susceptible to the presence or absence of small neutral amino acids in the
medium. To identify whether the expression of SNAT2 was dependent on RNA synthesis, lactating mammary gland explants were preincubated for 300 min in the presence of 50 mM actinomycin D. As can be seen in Fig. 1, there was no induction of SNAT2 mRNA, indicating that abundance of SNAT2 mRNA was indeed dependent on RNA synthesis during adaptive regulation.

To study the rate of SNAT2 mRNA decay, explants were preincubated for 200 min in an amino acid-free medium and were then switched to a medium containing a mixture of small neutral (alanine (Ala), glycine (Gly), and serine (Ser)) or large neutral and cationic amino acids (phenylalanine (Phe), valine (Val), and lysine (Lys)) or in an amino acid-free medium containing actinomycin D. A rapid decay of SNAT2 mRNA was observed, and the estimated half-life for the decay was 67 min, indicating that SNAT2 mRNA has a rapid turnover rate. These results indicate that the presence of the amino acid substrates for system A in the medium rapidly repressed the expression of SNAT2 mRNA (Fig. 2).

It has been demonstrated (32) in various tissues that system A activity is upregulated by several hormones. Glucagon and dibutyryl-cAMP significantly stimulate system A activity and SNAT2 gene expression in liver cells (15). However, there is evidence that rat mammary gland lacks glucagon receptor (37). We then studied whether SNAT2 mRNA expression in mammary gland explants was responsive to distinct signaling pathways, one coupled to phospholipase C, generating diacylglycerol and inositol 1,4,5-triphosphate, and the other coupled to adenylate cyclase, increasing cAMP levels. To assess whether any of these signaling pathways were involved in regulating system A gene expression in mammary gland, explants were incubated in medium containing alanine, glycine, and serine at 1 mM each, in the presence of 10 μM forskolin or 100 nM PMA. The results showed a strong induction with either compound, indicating that signaling can occur via PKA or PKC (Fig. 3).
To determine whether stimulation of SNAT2 gene expression during adaptive regulation was mediated via PKA or PKC, mammary gland explants were incubated with the specific inhibitors of PKA or PKC, H89 or GF109203X, respectively, and the inhibitors for adenylate cyclase or phospholipase C, MDL-12330 HCl or ET-18-CH3, respectively, in an amino acid free-medium for 200 min (Fig. 4). As expected, explants incubated in the amino acid free-medium showed an increase in SNAT2 mRNA expression ~5-fold; however, the presence of any of the inhibitors prevented the induction of the SNAT2 gene, indicating that inhibition of both signaling pathways reduced SNAT2 gene expression. As expected, incubation of mammary gland explants in an amino acid-free medium in the presence of forskolin or PMA increased SNAT2 mRNA concentration compared with explants incubated with a medium containing amino acids (Fig. 4).

These results indicated that SNAT2 expression is regulated by different signal transduction pathways in lactating mammary gland; however, it has not been established whether the SNAT2 gene is expressed differentially during pregnancy and lactation. Thus experiments were designed to determine whether the expression of SNAT2 mRNA changed during pregnancy or lactation. Mammary gland explants from rats at different stages of pregnancy, lactation, and weaning were studied. Northern blot analysis revealed a single band of 4.5 kb corresponding to SNAT2 mRNA in explants of mammary gland derived from pregnant and lactating rats. Furthermore, SNAT2 mRNA concentration increased progressively during pregnancy until day 18 and decreased rapidly near the end of pregnancy. During lactation the pattern of expression of SNAT2 mRNA showed small fluctuation without considerable changes in the first days of lactation. However, there was an increase of SNAT2 mRNA concentration around days 12–16 that coincided with peak milk production (Fig. 5). After postweaning, SNAT2 mRNA decreased rapidly. This finding was strengthened by in situ RT-PCR in mammary gland explants, indicating that lactocytes increased SNAT2 mRNA concentration predominantly during pregnancy (Fig. 6). To understand whether SNAT2 expression was induced by hormonal changes occurring during pregnancy, we performed studies in lactating mammary gland explants incubated in the presence of progesterone, 17β-estradiol, or both. The addition of progesterone to explants incubated in a medium containing alanine, glycine,
and serine at 1 mM each to repress adaptive regulation did not change the expression of SNAT2 mRNA. However, the incubation of explants in the same medium in the presence of 17β-H9252-estradiol markedly increased the concentration of SNAT2 mRNA. The effect of 17β-H9252-estradiol was time dependent, and after 3 h of incubation SNAT2 mRNA increased ~6-fold (Fig. 7). The induction of SNAT2 with 17β-estradiol was repressed by the addition of the specific inhibitor for estrogen receptor-α, ICI-182780 M (Fig. 8). These results indicate an additional level of regulation of SNAT2 gene expression involving hormones present during pregnancy.

DISCUSSION

The results of this study demonstrated that expression of SNAT2 in the mammary gland is regulated by different effectors. As expected, incubation of mammary gland explants in an amino acid-free medium showed adaptive regulation. These results are in agreement with previous reports (35) that show that the activity of system A, measured as uptake of MeAIB into mammary gland explants, increases if tissue is incubated in an amino acid-free medium. The increase in SNAT2 mRNA during adaptive regulation in the mammary gland explants follow kinetics similar to those observed in HepG2 cells or fibroblasts (1, 4, 17). Expression of SNAT2 mRNA occurs rapidly in HepG2 cells after the explants in the amino acid-free medium are incubated (1). The expression was sensitive to the presence of amino acids, mainly the small neutral amino acids, since the mixture of alanine, glycine, and serine prevented the elevation of SNAT2 mRNA concentration, but this reduction was not observed with a mixture of large neutral and cationic amino acids. Adaptive regulation of SNAT2 gene is controlled at the transcriptional level, and a study by Palić et al. (22) determined that a specific amino acid response element is located in the intron 1 and is responsible for regulating SNAT2 expression under conditions of amino acid deprivation.

The estimated half-life of SNAT2 mRNA was ~67 min, indicating the rapid degradation rate of SNAT2 mRNA. This short half-life is in agreement with others estimated by measuring the activity of system A after treatment with glucagon that was between 1.4 and 1.5 h for both in vivo and in vitro studies (6). This rapid turnover allows for tight regulation of utilization of amino acids such as alanine and glutamine, the amino acids more actively transported in the mammary gland (2, 39). Nonetheless, the physiological relevance of adaptive regulation in the mammary gland is not clear, since there is
Fig. 6. In situ RT-PCR of SNAT2 mRNA expression in virgin (×200), pregnant (×100), lactating (×100), and postweaning (×100) rat mammary gland explants. SNAT2 mRNA was amplified after reverse transcription, with the primers indicated in MATERIALS AND METHODS.

Fig. 7. A and C: effect of 17β-estradiol or progesterone, respectively, in rat mammary gland explants incubated in a medium containing a mixture of small neutral amino acids (Ala, Gly, and Ser, 1 mM each). B and D: quantitative analysis of SNAT2 mRNA. Values are means ± SE; n = 3. Different letters indicate a significant difference among groups (P < 0.05).
SNAT2 is activated in the same fashion via PKA or PKC. Either pathway was similar, indicating that transcription of the SNAT2 gene is activated by adenylate cyclase in the expression of SNAT2 mRNA. Our results also show that the induction with dibutyril cAMP is mediated, in part, by an increase in system A activity. We (35) previously demonstrated that system A activity is induced by glucagon in the mammary gland. The present study clearly demonstrates that the increase of system A activity is not depleted (11).

In addition to adaptive regulation, several studies have demonstrated that system A activity is induced by glucagon in different cells and tissues (15). Recent evidence (8) demonstrated that cAMP also induces the expression of SNAT2 in cultured cells, and these changes have been associated with an increase in system A activity. We (35) previously demonstrated that the activity of this transport system is also increased by dibutyryl cAMP in the mammary gland. The present study clearly demonstrates that the increase of system A transport activity by cAMP is mediated, in part, by an increase in the expression of SNAT2 mRNA. Our results also show that the expression of this gene is activated by adenylate cyclase and phospholipase C-dependent pathways (7). As a result of the activation via both pathways, it is not clear whether SNAT2 gene expression is preferentially activated via PKA or PKC. Surprisingly, our data show that SNAT2 expression is upregulated by both signal transduction pathways. The induction with either pathway was similar, indicating that transcription of SNAT2 is activated in the same fashion via PKA or PKC. There is evidence that, although dams consume a low- or an adequate-protein diet, the intracellular pool during the peak of lactation of amino acids, including the amino acid substrates for system A, is not depleted (11).

A protein is increased and the new synthesized transporter located in intracellular vesicles and cycled to the membrane to increase uptake of most amino acids, including the amino acid substrates for system A. The increase in rodent mammary gland and then fall rapidly after parturition (23), and perhaps this explains, in part, the increase in expression of SNAT2 during pregnancy, although there are other factors that influence the expression of this gene as described below.

Studies measuring amino acid arteriovenous concentration differences across the mammary gland (26, 28) show that uptake of most amino acids, including the amino acids transported by system A, occurs very actively during lactation. However, the pattern of expression of SNAT2 in the mammary gland during pregnancy and lactation has not been determined previously. Our data show that upregulation of SNAT gene expression occurs mainly during pregnancy and during the peak of lactation. These results suggest that SNAT2 gene expression is probably regulated during pregnancy by hormonal changes that occur in this condition. Our data show that SNAT2 gene expression is induced by β-estradiol but not by progesterone. This is in agreement with previous studies (29, 30) that show an increase in the activity of systems L and A in breast cancer cell lines incubated with β-estradiol. Induction of system A by estrogens in breast cancer cell lines only occurred in those lines expressing estrogen receptor-α. During pregnancy, it has been shown (24, 25) that expression of estrogen receptor-α decreases, but estrogen receptor-β remains high; however, there is no experimental evidence that associates expression of SNAT2 with the presence of this receptor. More research is needed to understand this interaction. In addition, due to the rapid induction of SNAT2 by β-estradiol in the mammary gland explants, there is the possibility that this effect could be mediated by a nongenomic action of the estrogen receptor (13, 19). It has been demonstrated (19) that estrogen receptor-α is also present in the plasma membrane and that binding of β-estradiol activates cellular signaling systems that involve either Go and Gq proteins. Furthermore, it has been shown (12, 31) that β-estradiol is able to be activated by a nongenomic mechanism phospholipase C at the membrane and stimulates PKC pathway in osteoblasts (16) and also activates PKA and PKC in neurons altering synaptic transmissions in the cells. Further studies are required to understand whether activation of SNAT2 by β-estradiol occurs through a genomic or nongenomic mechanism.

It is not clear why expression of SNAT2 increases during pregnancy and decreases during the first part of lactation. Amino acids are needed for lactocyte formation during pregnancy but also for synthesis of milk lipids and proteins during lactation. This change in expression could also be associated with the possibility that the mammary gland is preparing the milk-producing cells for lactation by synthesizing amino acid transporters during pregnancy for lactation. It has been shown (9) that system A protein in skeletal muscle is located in vesicles that can recycle to the plasma membrane to increase activity in response to insulin. Perhaps the synthesis of system A protein is increased and the new synthesized transporter located in intracellular vesicles and cycled to the membrane after receiving a hormonal signal that occurs during lactation. We are conducting studies to detect whether in fact this event takes place.

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