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Changes in messenger RNA abundance of amino acid transporters in rat mammary gland during pregnancy, lactation, and weaning

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Abstract

During lactation, the mammary gland increases the needs for nutrients to fulfill the milk production requirements. Among these nutrients, amino acids play an important role for the synthesis of milk proteins. Amino acids are supplied to the mammary gland through amino acid transporters, although some are synthesized in situ. The purpose of this study was to establish the pattern of changes in messenger RNA abundance of the amino acid transporters ASC, system L, EAAC1, GLAST, CAT-1, and Tau in the mammary gland of the rat during different stages of pregnancy and lactation. Rats were fed during pregnancy and lactation a 20% casein diet. Food intake increased significantly during the lactation period. Amino acid transporter ASC expression increased during the first days of pregnancy about 2-fold, and it was increased in a lesser extent again during the peak of lactation. The expression of system L (LAT-1) and CAT-1 transporters was increased only during the lactation period. On the other hand, the expression of the transporters for anionic amino acids EAAC1 and GLAST was low during both stages. Finally, taurine transporter expression decreased during pregnancy; and it was significantly lower during lactation. These results showed that amino acid transporters were not expressed similarly in the mammary gland during pregnancy and lactation, indicating that the expression of these transporters did not respond only to the metabolic needs of the gland but depended on the dietary protein supply and possibly the specific hormonal changes that occur during pregnancy and lactation.

1. Introduction

Pregnancy and lactation greatly increase the nutrient needs. These are met by an increase in food intake and by mobilization of body reserves, which accumulate during pregnancy and are used during late pregnancy for fetal growth and during lactation to sustain an active milk synthesis. Thus, utilization of carbohydrates and lipids by maternal tissues is minimized in favor of the fetus, placenta, and mammary gland [1,2].

During lactation, uptake of amino acids from blood into the mammary gland is enhanced [3-12] to support the high requirement of amino acids needed to sustain an active synthesis of milk proteins such as caseins [11,13-16]. Furthermore, oxidation of certain amino acids, mainly branched-chain amino acids (BCAAs), increases during the peak of lactation [17,18]. Some enzymes involved in BCAA catabolism are induced in response to lactation in the mammary gland [17], shuttling more BCAA, especially leucine, to increase in situ the novo fatty acid synthesis [12]. In addition, β-amino acids such as taurine, which is present in maternal milk, are obtained from maternal sources. Therefore, amino acid transport into the lactating mammary gland can control the rate of substrates entry for the synthesis of different milk components.

Amino acid uptake by the mammary gland is carried out by different transporters located in the plasma membrane of the mammary epithelial cells. Despite changes in metabolic needs between pregnancy and lactation, and the different pattern of hormones produced in the different stages of pregnancy and lactation, there are few studies that show the changes in the expression of the amino acid transporters along these 2 stages in the mammary gland. We have demonstrated that activity and expression of the sodium-coupled neutral amino acid transporter 2 (SNAT2) in rat mammary tissue increase progressively during pregnancy until day 18 and decrease rapidly near the end of pregnancy [19]. During lactation, SNAT2 messenger

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RNA (mRNA) increases again around days 12 to 16, coinciding with the peak of milk production [20]. The activity and expression of several amino acid transporters, including the glutamate transporter [21-24], transporters for neutral amino acids with branched or aromatic side chains [25], β-amino acids [26,27], and cationic amino acids [28,29], have been described in the lactating mammary gland of various species. Among the expressed glutamate transporters GLAST, GLT-1, and EAAC-1, the GLAST transporter has been shown to be present in the mammary gland; and its expression is modified by the diet [30]. Transport of neutral amino acids by a Na^+-independent mechanism involves transporters LAT1 and LAT2, which are expressed in the mammary gland; however, the main contribution of the transport of these amino acids in the gland is via transporter LAT2 [25]. Cationic amino acids are transported in the mammary gland via transporter CAT-1 and CAT-2; however, it has been demonstrated that CAT-1 resembles strongly the properties of system y+ [31]. There is no information about the expression of β-amino acid transporters in the mammary gland. Data for the ASC system have been controversial; Neville et al [32] did not find evidence of ASC system activity in mouse mammary tissue, whereas Verma and Kansal [33] demonstrated the presence of this system in the same tissue. It has been demonstrated that there are 2 isoforms of system ASC: ASCT1 and ASCT2. However, ASCT1 is mainly found in the brain [34], whereas ASCT2 has been found in several tissues [35] and possibly represents most of the ASC system activity in the mammary gland. However, it is not known whether the expression of the amino acid transporters in the mammary gland is up-regulated during lactation, which is the stage of high demand of milk production, or during pregnancy to prepare the gland for lactation and if the pattern of expression is the same for the different amino acid transporters.

Therefore, the objective of the present work was to determine whether the rat mammary gland expresses the amino acid transporters CAT-1, GLAST, EAAC-1, ASCT-2, rB16a (taurine), and LAT-1 and to determine changes in mRNA abundance during pregnancy, lactation, and weaning periods.

Table 1
Composition of the diet used in this study

Ingredients	g/kg diet
Vitamin-free casein[a]	200
Dextrose[a]	344.2
Starch[a]	344.2
Corn oil	50
Inorganic nutrient mix[b]	50
Vitamin mix[c]	10
Choline citrate	1.65

[a] Teklad test diets.
[b] Rogers-Harper, Teklad test diets.
[c] Vitamin mix, Teklad 40060 (milligrams per kilogram diet): *p*-aminobenzoic acid, 110; ascorbic acid, 991; biotin, 0.4; vitamin B12, 30; calcium pantothenate, 66; choline dihydrogen citrate, 3497; folic acid, 2; inositol, 110; menadione, 50; niacin, 99; pyridoxine HCl, 22; riboflavin, 22; thiamin HCl, 22; vitamin A palmitate, 40; cholecalciferol, 4; and vitamin E acetate, 242.

2. Materials and methods

2.1. Animals

A total of 45 female Wistar rats with a weight of 200 to 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-hour light/dark cycle and allowed free access to water and diet (Table 1). The diet was provided in dry form and contained (grams per kilogram diet) 690 of cornstarch and sucrose (1:1 ratio), 200 of vitamin-free casein, 50 corn oil, 50 mineral mix, and 10 vitamin mix (Teklad, Madison, WI). The same diet was administered throughout pregnancy, lactation, and weaning.

Gestational age was determined by vaginal smear to detect spermatozoa. Mammary gland tissue was obtained as described below from virgin (n = 3 rats), pregnant (1, 5, 10, 14, 16, and 18 days) (n = 3 rats per day), lactating (1, 5, 10, 12, 14, 16, and 18 days) (n = 3 rats per day), and postweaning rats (24 hours) (n = 3 rats). After delivery, litters were adjusted to 8 pups per dam. Pups were weighed after birth every 5 days. Rats were separated from their pups at day 21 postpartum. Nonpregnant, nonlactating rats 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.

2.2. Preparation of mammary tissue

Rats were anesthetized with CO_2 and then killed by decapitation. The mammary gland was immediately removed and frozen in liquid nitrogen. The tissue was conserved at −80°C until use.

2.3. RNA preparation

Total RNA was extracted following the method of Chomczynski and Sacchi [36]. Mammary tissue was homogenized in guanidinium buffer containing 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate (pH 7.0), 0.1 mol/L 2-mercaptoethanol, and 0.5% *N*-lauryl sarcosine with a polytron (PT2000; Kinematica, Lucerne, Switzerland) at the lowest setting. The homogenate was centrifuged at 12 000*g* for 15 minutes at 18°C, and the resulting supernatant was layered onto a cesium chloride solution containing 5.7 mol/L CsCl and 25 mmol/L sodium acetate (pH 5.2). The cesium chloride gradient was formed by centrifugation at 113 000*g* for 18 hours at 18°C to yield total RNA. The RNA was precipitated with 100% ethanol

Table 2
Primers used to measure the expression of the amino acid transporters in the mammary gland during pregnancy and lactation

Gene	Upper primer	Lower primer	Annealing temperature (°C)	GenBank accession no.
ASCT2	5′ TTC TGG GCC TGG TCG TGT 3′	5′ TGG TGG CAT CAT TGA AGG AGT 3′	56.5	AJ132846
CAT1	5′ GTG CCC TTC CTT CTT GTC TCT 3′	5′ CTG GGG CAC CTT CTG AAA 3′	55.4	U70476
EAAC1	5′ CAT GCC GAT CGG TAT TTT GTT 3′	5′ ATT GCA AGC CCA CTC AGG A 3′	53.5	U39555
Glut/Asp	5′ TGC CCT TTC TTC TGA CTC TCA 3′	5′ CAT TGG GGA GCC TCA CTT T 3′	54.6	S59158
LAT1	5′ TGG CCG TGA AAG AAA CCT 3′	5′ CAG TCC CCA AAG TCA GAA AGA 3′	55	AB015432
Taurine	5′ TTC CAT CCT GGG CTT CAT 3′	5′ ATG GTC ACA GCT TTT GGG TAG 3′	54.6	M96601
β-Actin	5′ GGT CGT ACC ACT GGC ATT GTG 3′	5′ GGA GCG CGT AAC CCT CAT AGA 3′	56.8	NM031144

and 3 mol/L sodium acetate (pH 5.2), washed twice in 75% ethanol, and resuspended in sterile deionized water. The RNA was quantified in a spectrophotometer (Beckman DU 640, Palo Alto, CA) by optical density at 260 nm and stored at −80°C until use.

2.4. Relative gene expression by quantitative real-time polymerase chain reaction

The first-strand complementary DNA was synthesized from 100 ng of total RNA with the Multiscribe master Mix (PE Applied Biosystems, Foster City, CA). Amino acid transporter mRNA abundance was quantified by polymerase chain reaction (PCR) using Power SYBR Green Master Mix (PE Applied Biosystems). The procedure was optimized regarding primer concentrations and denature/extension temperatures. Different primer concentrations were probed, and the optimum found at 0.2 μmol/L was used for all the genes tested. The optimized temperatures used for each gene were as follows: ASCT-2, 56.5°C; CAT-1, 55°C; EAAC-1, 53.5°C; LAT-1 and GLAST, 55°C. The reaction was carried out in a 25-μL final reaction volume containing 0.2-μmol/L concentration of each forward and reverse primer, 3 μL of 100 ng complementary DNA (template), 9 μL distilled water, and 12.5 μL of kit-supplied SYBR PCR master mix (including SYBR Green I dye, Ampli Taq Gold DNA polymerase, dNTPs, passive reference, and optimized buffer components). Positive and negative reference samples were tested along with the unknown samples in each run. Two different pairs of primers were separately used: 1 pair to amplify the gene of interest and the other pair for the endogenous control gene β-actin. Because mammary gland during gestation and lactation has changes in cell differentiation, we studied first changes in mRNA abundance of different housekeeping genes including glyceraldehyde-3-phosphate dehydrogenase, 18S, and β-actin. The less variable gene was β-actin, and its variability was about ±10.2% (0.91-1.12) along pregnancy and lactation with respect to the beginning of gestation; but it did not reach significant difference according to 1-way analysis of variance. Thus, it was selected as the reference gene. The forward and reverse primers for each amino acid transporter and β-actin genes were designed with the Oligo 5.0 software (National Biosciences, Inc, Plymouth, MN) and are described in Table 2. The ABI PRISM 7000 system was used for the reaction and detection (Applied Biosystems). A disassociation curve was generated in each run to verify the specificity of PCR products.

2.5. Statistical analysis

Values are expressed as mean ± standard error of the mean. Results were evaluated using 1-way analysis of variance for fixed effects followed by Fisher protected least significant difference test (SPSS 11.0 for Macintosh; SPSS, Chicago, IL). Differences were considered significant at P less than .05.

3. Results

3.1. Weight gain and food intake during pregnancy and lactation

At the beginning of the study, dams weighed in average 284 ± 5.8 g. Dams were fed a 20% casein diet during pregnancy and gained approximately 112 ± 13.5 g (Fig. 1A), with an average daily food intake of 20 ± 4.8 g (Fig. 1B). After pregnancy, the dams lost 58 ± 13.8 g and maintained an average weight of 317 ± 12.3 g during lactation (Fig. 1A). However, food intake during lactation increased from 26 ± 4.4 g/d at the beginning of lactation to about 100 ± 1.7 g/d at the end of lactation (Fig. 1B). The change in food intake is associated with an increase in the pups' weight gain; and after 21 days of lactation, pups gained approximately 39.3 ± 1.9 g (Fig. 1C).

3.2. The Na^+-dependent amino acid transporter ASCT2 is expressed in the mammary gland predominantly during pregnancy

Transporter ASCT2 mRNA abundance in the mammary gland increased 3.2-fold ($P < .0001$) at day 10 of pregnancy when compared with the virgin rats. After day 10 of pregnancy, mRNA abundance decreased ($P < .0001$). There was a 2-fold increase in ASCT2 mRNA abundance from day 5 to 10 of lactation ($P < .04$), followed by a decrease thereafter ($P < .003$) until the first day of weaning (Fig. 2A).

3.3. The Na^+-independent transport of neutral amino acids by L system is preferentially expressed in the mammary gland during lactation

Transporter LAT-1 mRNA abundance increased ($P < .05$) from day 5 to 10 of lactation, reaching a significant 20-fold increase at day 10 ($P < .002$), when compared with the virgin rat mammary gland. LAT-1 mRNA expression was the highest on lactation days 14 and 16 ($P < .0001$). There was a sharp rise in mRNA abundance on day 1 postweaning ($P < .0001$). During pregnancy, there was no difference in LAT-1 mRNA expression when compared with the virgin rats (Fig. 2B).

3.4. The Na^+-independent cationic amino acid transporter CAT-1 is preferentially expressed in the rat mammary gland during lactation

Abundance of CAT-1 mRNA increased during lactation compared with pregnancy ($P < .0015$). The maximum level of expression was attained at day 14, where a 12-fold increase ($P < .0001$) was observed, when compared with the first day of lactation. During pregnancy, there were no differences in mRNA abundance compared with virgin rats (Fig. 2C).

3.5. The Na^+-dependent glutamate transporters EAAC1 and GLAST are expressed only at low levels in the rat mammary gland during pregnancy, lactation, and postweaning

There was low mRNA abundance of EAAC1 and GLAST transporters during lactation (Figs. 3A, B). For EAAC1, mRNA abundance was lower in pregnancy when compared with the virgin rats; and its expression remained similar across pregnancy, lactation, and postweaning. On the other hand, the GLAST transporter mRNA abundance dropped gradually from day 1 to the middle of pregnancy ($P < .0001$) and remained similar across lactation and postweaning (Fig. 3A, B).

3.6. The Na^+-dependent transport of β-amino acids is preferentially expressed during pregnancy in the rat mammary gland

Taurine transporter mRNA abundance peaked at day 5 of pregnancy ($P < .0001$), followed by a consistent decrease through pregnancy. This level of expression was maintained until day 18, to decrease again at the first day of lactation ($P < .006$) to levels not significantly different from the virgin rats (Fig. 3C).

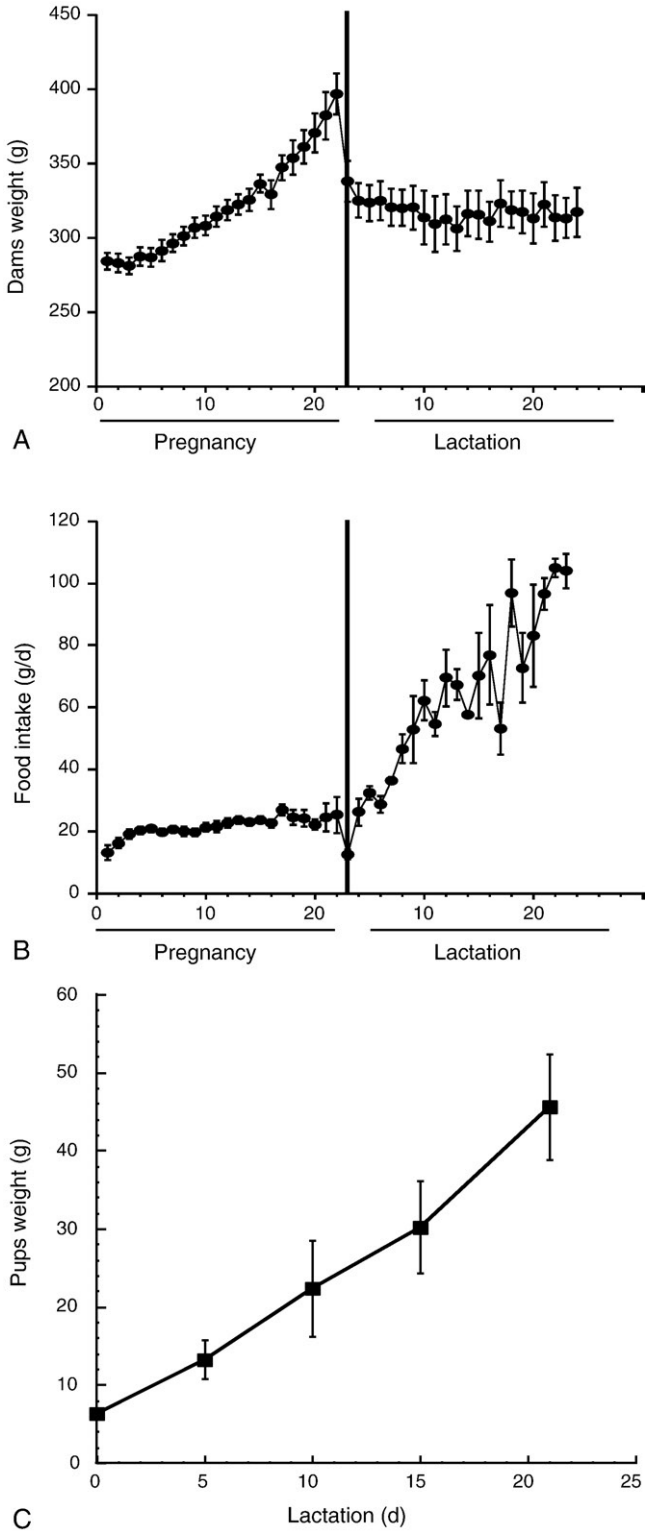

Fig. 1. Weight gain (A) and food intake (B) during pregnancy and lactation of dams consuming a 20% casein diet. Weight gain (C) of pups during lactation.

4. Discussion

The lactating mammary gland has an increased requirement of amino acids to synthesize milk proteins. As a consequence, there is also an increase in the uptake of amino acids by the gland as has been described in several species by measuring plasma amino acid arteriovenous differences across the mammary gland [37-40]. However, it has not been shown whether the elevated needs of amino acids for the mammary gland coincide with an elevated expression of the

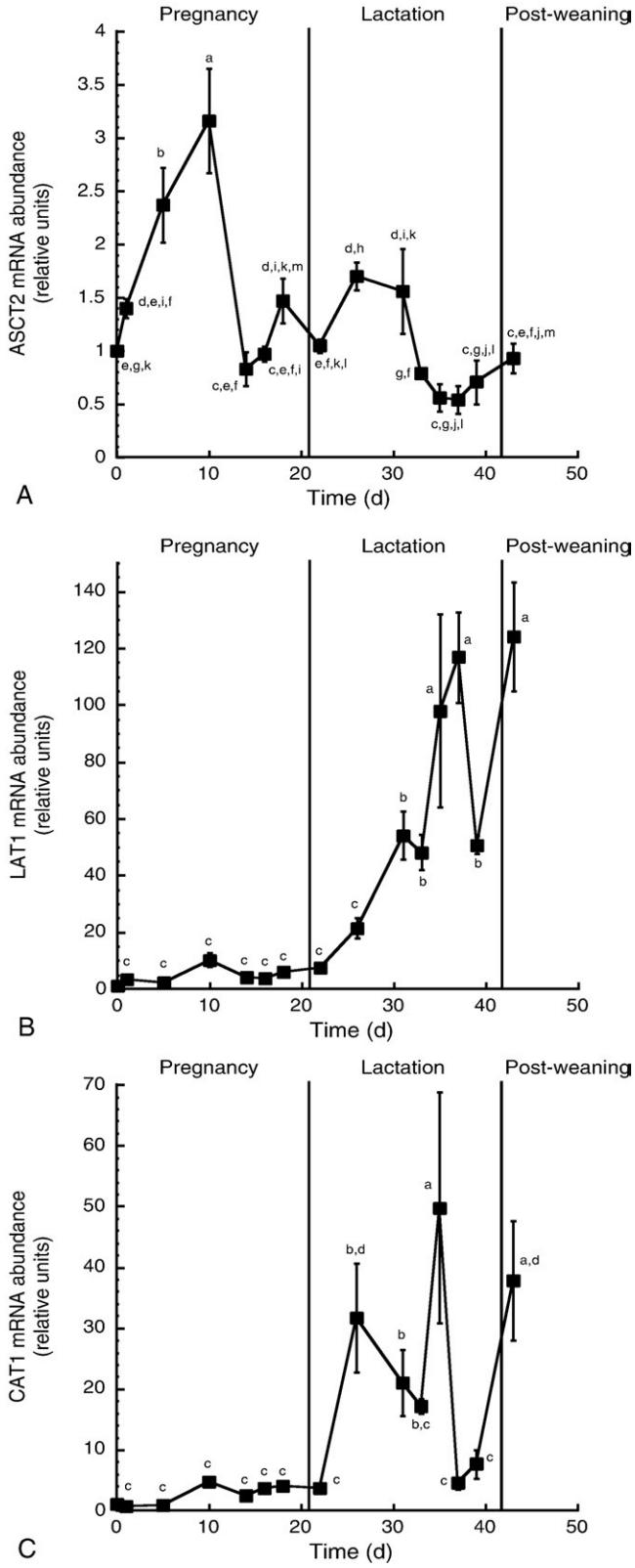

Fig. 2. Expression of amino acid transporters ASC (A), L system LAT-1 (B), and CAT-1 (C) in the mammary gland of rats during pregnancy and lactation. Messenger RNA concentration was determined by quantitative reverse transcriptase PCR as described in "Materials and methods." n = 3 dams per each point. Values with different letter superscripts are significantly different among groups; a > b > c > d > e ($P < .05$).

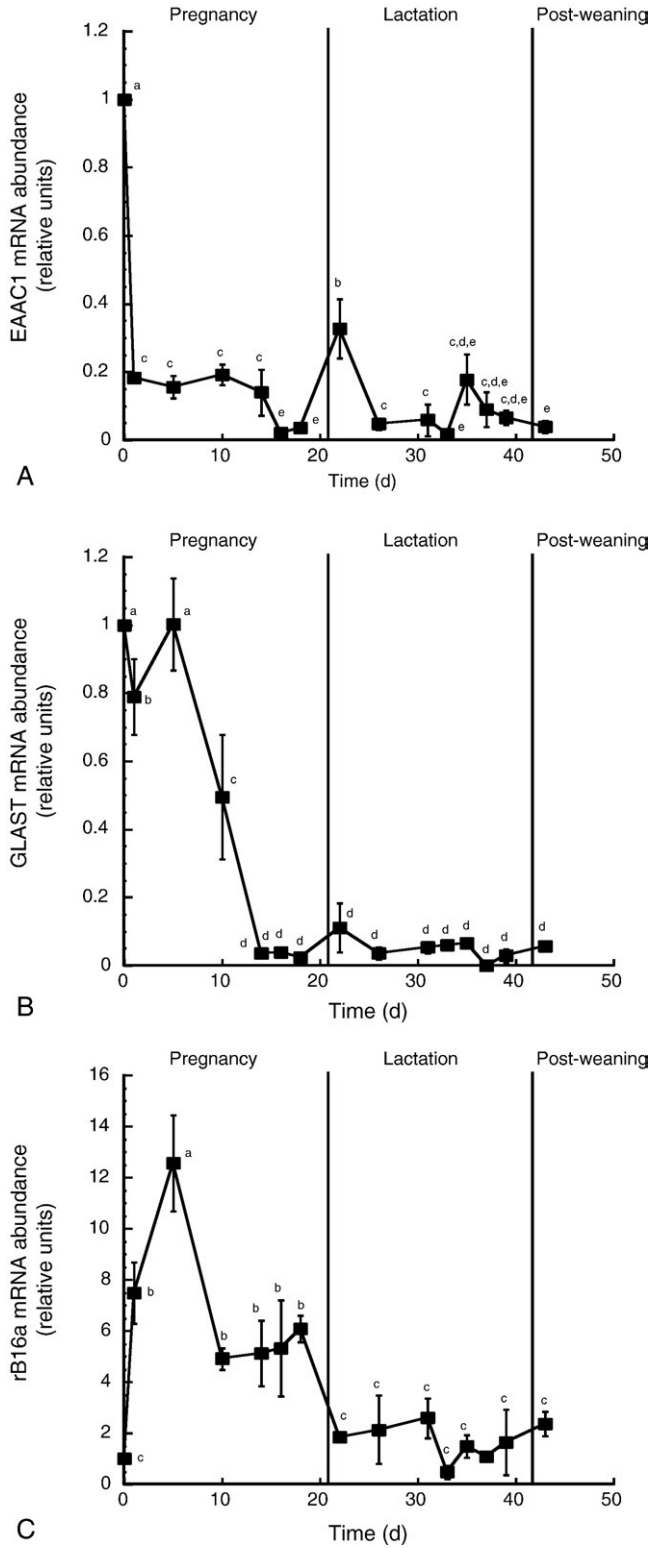

Fig. 3. Expression of amino acid transporters EAAC1 (A), GLAST (glutamate transporter) (B), and rB16a (taurine transporter) (C) in the mammary gland of rats during pregnancy and lactation. Messenger RNA concentration was determined by quantitative reverse transcriptase PCR as described in "Materials and methods." n = 3 dams per each point. Values with different letter superscripts are significantly different among groups; a > b> … l > m ($P < .05$).

amino acid transporters to fulfill the amino acid requirements. Our study clearly showed that the expression of the amino acid transporters in terms of abundance of specific mRNA changed during specific stages of pregnancy or lactation, indicating that the regulation of the expression of the genes for each transporter is controlled by different factors, presumably involving specific hormones and the amount of diet consumed.

The presence of the ASC system in the mouse mammary gland has been suggested [41], along with specificity for methionine transport. Our study confirms the presence of ASCT2 transporter in the rat mammary gland and also shows that it is highly expressed during pregnancy, but is also present during lactation. A very similar pattern of expression was observed previously for transport system A (SNAT2) [20]. During lactation, the most actively transported amino acids into the mammary gland are alanine and glutamine, which are substrates of system ASC [42] and A, respectively [11]. It has been recently shown that alanine concentration increases in the mammary gland during lactation, possibly to increase adenosine triphosphate concentration [43], as a result of an increase in the activity of the amino acid degrading enzyme alanine aminotransferase in the mammary gland [44]. Thus, the increase in alanine in the mammary gland is associated with an increased uptake of this amino acid via system ASC and A. We have recently shown in mammary gland explants that SNAT2 mRNA abundance is activated by estradiol and also by prolactin, indicating that both hormones are stimulators of the SNAT2 gene [20]. There are no studies that show that ASC system is also responsive to these hormones, but its pattern of expression indicates that these hormones might be involved in its regulation.

In addition to alanine, the anionic amino acids glutamate and aspartate are abundant in the lactating mammary gland. It is not clear whether the concentration of these amino acids increases as a consequence of their transport or because they are actively synthesized in situ. Several studies have demonstrated the activity of glutamate transporters in the lactating mammary gland of rats and mice [21-24]. In contrast, although our results showed that the transporters for anionic amino acids GLAST and EAAC1 are present in the mammary tissue, their mRNA concentration did not change during pregnancy, lactation, or postweaning periods. Despite glutamate and aspartate being abundant free amino acids in the mammary gland and also in casein proteins [9,15], we suggest that the transport of these amino acids may not be a limiting factor controlling their intracellular concentration and that, perhaps, the endogenous synthesis is more important. For instance, it has been shown that glutamate is synthesized in the mammary gland by transamination of the BCAAs with the mitochondrial isoform of the BCAA aminotransferase and that the expression of this enzyme increases during lactation, indicating increased synthesis of glutamate during this stage [17].

Most of the large neutral amino acids are indispensable amino acids that are obtained from the diet. To sustain protein synthesis in the mammary gland, these amino acids are transported by the L system, which consists of LAT1, LAT2, and 4F2hc proteins [45]. Our results showed an important increase in LAT1 expression during lactation, but not during pregnancy. This increase paralleled the increase in food and protein intake observed during lactation. These results are in accordance with those of Shennan et al [25], who described the presence of LAT-1 in mammary tissue from lactating rats. In addition, a recent study also showed that LAT-1 expression is higher in lactation than in pregnancy in mammary gland from mice [43].

Cationic amino acids are actively transported into the lactating mammary gland by Na^+-independent transport systems [46-49], but the cationic amino acid transporters have been poorly described in the mammary gland [28,50]. In this study, CAT-1 mRNA abundance increased during lactation when compared with the virgin rats. This pattern parallels LAT1 expression in the mammary gland, and this may explain in part the interaction between cationic and large neutral amino acids in the mammary gland because large neutral amino acids such as leucine stimulate the efflux of lysine [48,49].

Taurine is one of the most abundant amino acids in milk, and a high intracellular taurine level has been showed in the mammary gland [26]. Sturman et al [27] demonstrated that radiolabeled taurine injected intraperitoneally into lactating rats is transported into milk. Our results showed that taurine transporter mRNA was highly abundant during pregnancy in the rat mammary gland, but was not different in lactation from that observed in the virgin rats. These results are in agreement with those observed in microarray analysis that showed a reduction in the expression of this transporter in mice mammary gland during lactation in comparison with pregnancy [43]. The expression of the rate-limiting enzyme required for taurine synthesis, cysteine sulfinate decarboxylase (EC 4.1.1.29), is highly induced during pregnancy and lactation [51,52], suggesting that taurine might be synthesized de novo in the mammary gland and not only transported from the blood.

This study showed the differential mRNA abundance of several amino acid transporters across stages of pregnancy and lactation in the rat mammary gland. Our results showed that the mRNA abundance of the transporters studied did not follow the same pattern along pregnancy and lactation. Transporters for anionic amino acids decreased rapidly their expression from the beginning of pregnancy, and no change was observed during lactation. Taurine and ASCT2 transporters expression is strongly induced during pregnancy, with modest changes during lactation. CAT1 and LAT1 expression did not occur during pregnancy, but were upregulated during lactation. Results from this and other studies demonstrate that mRNA concentration of the amino acid transporters studied changes differentially during pregnancy and lactation [20,28]. Therefore, expression of

each transport gene must be regulated in a different manner. Apparently, some transporters are not responding to the metabolic and nutritional needs of the mammary gland cells. More studies are needed to understand the physiologic reason for this differential pattern of expression of the amino acid transporters in the mammary gland during pregnancy and lactation.

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