Differential UDP-galactose-4′-epimerase (GALE) enzymatic activity and mRNA expression in the rat mammary gland during lactation

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Abstract We have investigated the UDP-galactose-4′-epimerase (GALE) enzymatic activity and mRNA expression in the rat mammary gland during lactation. We report a dramatic increase in the GALE enzymatic activity correlated with an increase in the mRNA transcript expression. These results indicate a transcriptional regulation of the enzyme during lactation in the rat mammary gland. Our data are of double interest for further investigation: first, the mammary gland provides a suitable model for the characterisation of the transcriptional regulation elements of GALE which are still unknown in mammals; second, GALE expression could help to compensate UDP-galactose deficiency in classic galactosaemia.

Key words: UDP-galactose-4′-epimerase; Enzymatic activity; mRNA expression; Galactose metabolism; Galactosemia; Mammary gland

1. Introduction

The mammary gland has been well studied because of its particular function to induce the secretion of lactose to produce milk. The biochemistry [1] and endocrinology of lactogenesis are well-documented [2]. Lactogenesis becomes important in the parturient mammary gland, where a complex of hormones act on this quiescent tissue to initiate the secretion of milk [3]. Lactose synthesis is a temporal, tissue- and sex-specific metabolic process. Lactose, which is a disaccharide consisting of glucose and galactose, represents the major free carbohydrate of milk [4,5] and is not found elsewhere in the body.

The enzymes involved in the metabolism of lactose precursors UDP-galactose and glucose [6] and in galactose metabolism [7] have been very well documented. Although most of these enzymes do not significantly vary their enzymatic activity around the period of lactation, a few studies have reported an increase of the enzymatic UDP-galactose-4′-epimerase (GALE) activity in the mammary gland of rabbits and rats [8]. The rate of increase of the GALE activity, however, remains uncertain since the data reported vary between a two-fold [9] and a 300-fold increase [10] in rats during lactation.

GALE has a crucial role in galactose metabolism where it is thought to maintain the UDP-galactose/UDP-glucose ratio [11], essential for the incorporation of galactose in cells as a source of energy. At present there is no accurate biological model allowing the study of mammalian GALE expression. We therefore proposed to investigate the rat mammary gland as a tissue model for studying GALE expression. Our data show a differential expression of the GALE mRNA transcript and enzymatic activity in the mammary gland at different physiological stages.

2. Materials and methods

2.1. Animals

Two-month-old virgin female rats of the Wistar strain were used. Rats were kept in a light- (lights on 07:00–19:00 h) and temperature-(22–24°C) controlled room, with free access to pelleted food and water.

The virgin females were caged with fertile males on the night of prooestrus. The presence of spermatozoa was checked in the vaginal smear the following morning (designated day 0 of pregnancy). Rats on days 7 and 19 of gestation were used. These stages correspond to the beginning (BG) and the end of gestation (EG) respectively. The day of parturition was considered to be day 0 of lactation. Litters of lactating rats were adjusted to 10 pups post partum. Lactating rats from days 7, 12 and 19 were used. These stages corresponding to the beginning (BL), the middle (ML) and the end of lactation (EL) respectively. After 21 days of lactation, pups were removed from the rats which were taken 7 days after weaning (W).

Rats were killed by decapitation at different physiological stages: virgin (V), during gestation (BG and EG), during lactation (BL, ML, and EL) and after weaning (W). Cortex, muscle (quadriceps), abdominal and inguinal mammary glands were removed. Tissues used for RNA extraction were homogenised with an ultra-Turax homogeniser and frozen at −80°C in acid guanidinium thiocyanate solution until analysis. Tissues used for enzymatic activity assays were frozen at −80°C immediately after removal.

2.2. GALE activity assay

Mammary glands and cortex from rats were solubilised by sonication in 300 μl distilled water. Samples were centrifuged at 2000 rpm for 2 min and supernatants were collected. For each stage at least eight animals were killed. Endogenous GALE activity was measured and calculated as described by Shin et al. [12,13].

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

GALE and β-actin mRNA levels were assessed using semiquantitative RT-PCR assays. For each stage at least eight animals were killed. The different tissues were treated independently for RNA extraction and RT-PCR.

After homogenisation in acid guanidinium thiocyanate solution, total RNAs were extracted using a phenol/chloroform solution and quantitated by optical density at 260 nm wavelength. The quality of isolated RNA was verified by agarose gel electrophoresis with ethidium bromide staining.

For all assays, 1 μg of each RNA sample was reverse transcribed in a final volume of 20 μl containing 1.25 μM of random hexamers, 10 mM of each deoxy (d)-ATP/dCTP/dGTP/dTTP (dNTP; Promega, Madison, WI), 1.25 U/μl of rRNasin Ribonuclease Inhibitor (Promega), reverse transcriptase buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine and 50 mM DTT) and 10 U/μl of AMV reverse transcriptase (Promega). Additional reactions were carried out omitting the reverse transcriptase to detect amplification from contaminating genomic DNA. An additional tube per assay did not contain RNA (blank) to detect contamination of solutions and pipettors.

1 μg of cDNA of each sample was used for PCR. Sense and anti-

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sense primers were designed to amplify across introns. This strategy was used in order to detect by sizing any unspliced RNA and contaminating genomic DNA. Each PCR reaction was assessed in a final volume of 50 μl containing 0.05 mM of dNTP, 0.4 μM of primers, *Taq* DNA polymerase buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C), 15 mM $MgCl_2$ and 1% Triton X-100) and 2.5 U of *Taq* DNA polymerase. To quantify reaction products of PCR, 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) was added to the reaction mix. Reactions were overlaid with 100 μl of light mineral oil and amplified for cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C followed by a final extension of 5 min at 72°C. For each assay, PCR fragments were separated on 10% non-denaturing acrylamide gels for 90 min at 150 V. PCR products were detected by ethidium bromide staining and the quantity of ^{32}P incorporated in the PCR products was determined by exposing the gel to a phosphorimager screen. The same procedure was applied to measure mRNA variations isolated from rat skeletal muscle and cortex of the same animals. The linear range for each PCR amplification was determined by removing aliquots after 25, 28, 30, 35 and 40 PCR cycles. Linearity was also determined for different amounts of cDNA templates.

Rat GALE primer sequence was designed on the basis of the rat GALE sequence [14]. The following primers were used: 5′-CAA TTC CAT TCG TGG AGA AG-3′ and 5′-AGG CAG GTA CGG GTT TCC CA-3′, with an expected product size of 320 bp. Rat β-actin primer sequence was chosen from the rat β-actin gene sequence [15] as: 5′-GGT CAC CCA CAC TGT GCC CAT CTA-3′ and 5′-GAC CGT CAG GCA GCT CAT AGC TCT-3′, with an expected product size of 263 bp.

2.4. Statistical analysis

Variations in GALE activity and GALE mRNA level at the different physiological stages tested were statistically analysed by a one-way analysis of variance (ANOVA).

3. Results

3.1. GALE activity assay

GALE activity was quantitated using a radiometric assay and was expressed in nmol/min/mg proteins. The activity values were calculated from the UDP-glucose standard curve (Fig. 1A) where the UDP-glucose concentration was found to be linear up to a concentration of 0.15 mM.

Enzymatic activity was measured at different stages during lactation in the rat mammary gland and in the cortex. At least eight assays were performed in triplicate. GALE activity was found to vary during the lactation in the mammary gland (Fig. 1B). In the virgin mammary gland GALE activity was around 1 nmol/min/mg protein. This activity, however, showed a more than five-fold increase during the middle of lactation (ML) with a peak at nearly 5 nmol/min/mg protein. This increase was shown to fall to less than 1 nmol/min/mg protein at the end of lactation (EL), to reach a basal level of activity. An analysis of variance showed significant increase of GALE activity in the beginning (BL) ($F(2,44) = 43.081$; $P = 0.0049$) and middle (ML) ($P < 0.001$) of lactation in comparison with the basal activity in the virgin (V) and weaning (W) stages.

No significant variation was detected in the cortex during the various stages of lactation (data not shown).

3.2. Expression of GALE mRNA

We examined the mRNA expression of GALE in rat mammary glands, cortex and skeletal muscle by RT-PCR analysis in virgin and weaning rats and during gestation and lactation. In a preliminary study GALE transcripts were detected by RT-PCR analysis performed on total RNA from different tissue samples using specific primers combined with the incorporation of ^{32}P-labelled dCTP to quantitate relative amounts of transcripts. In order to accurately determine relative amounts of mRNA, we analysed the levels of mRNA production of both β-actin and GALE during the time course of the PCR. Amplification of β-actin and GALE mRNA from control mammary gland were positive functions of the number of PCR cycles carried out, as shown in Fig. 2A. The slope of the curve increased between 25 and 30 cycles and stabilised at 35 cycles. Thus, the number of 30 cycles was considered to be the optimum for the determination of relative amounts of mRNA.

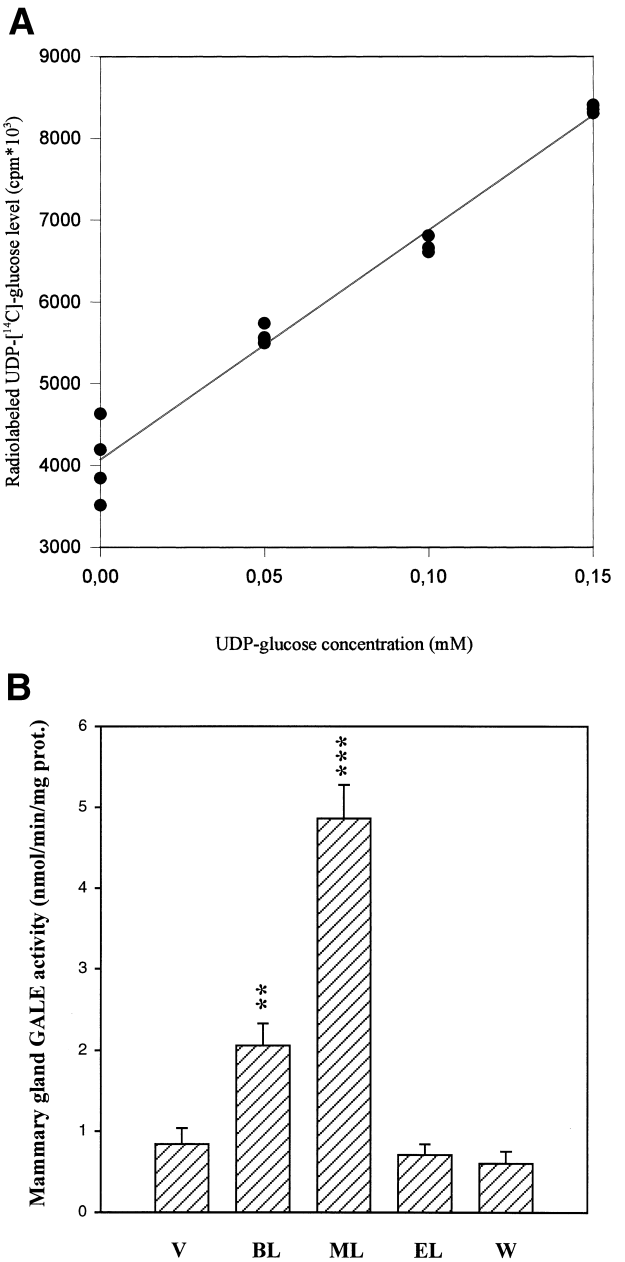

Fig. 1. A: UDP-glucose standard curve. Levels of radiolabeled UDP-[^{14}C]glucose in relation to the initial UDP-glucose concentration. Each point represents one assay. B: Effect of the lactation period on the GALE mammary gland activity. GALE activity was measured in virgin (V), lactating (beginning (BL), middle (ML) and end (EL) of lactation) and in weaning (W) rats. GALE activity is expressed in nmol/min/mg prot. Each bar chart represents the mean of triplicates of at least eight values of activity ± S.E.M.

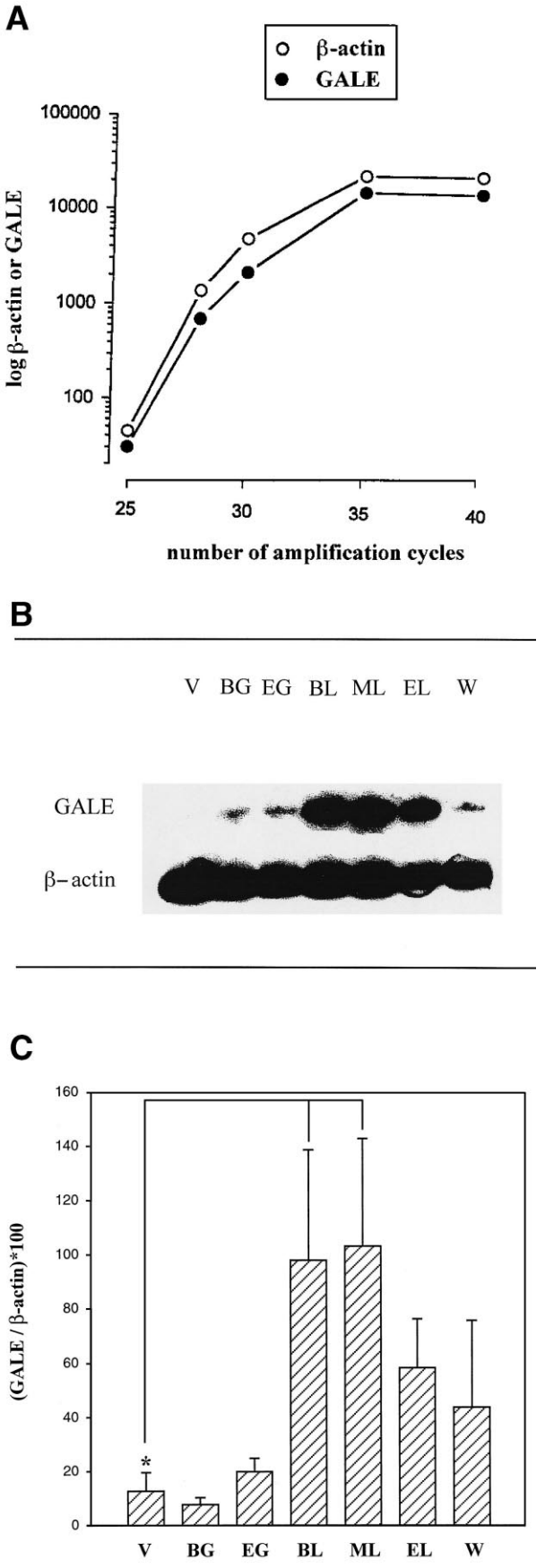

Fig. 2. A: Effect of the number of PCR cycles on the mammary gland β-actin and GALE amplification. Amplification is expressed in log units. B: Representative phosphorimage of mammary gland GALE and β-actin RT-PCR products. GALE and β-actin amplification products were tested in virgin (V), gestating (beginning (BG) and end (EG) of gestation), lactating (beginning (BL), middle (ML) and end (EL) of lactation) and weaning (W) rats. GALE, the upper band, is 320 bp, and β-actin, the lower band, is 263 bp. C: Effect of the gestation and lactation period on mRNA GALE expression in the mammary gland. Relative amounts of GALE amplification product are expressed as the ratio of GALE to β-actin. Each bar chart represents the mean of at least eight experiments ± S.E.M., for the physiological stages as follows: virgin (V), gestating (beginning (BG) and end (EG) of gestation), lactating (beginning (BL), middle (ML) and end (EL) of lactation) and weaning (W).
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Expression of GALE was normalised against β-actin in the different tissues obtained by independently performed RNA extraction (Fig. 2B). GALE mRNA expression was increased about six times during lactation with a peak at the beginning and the middle of lactation compared to a basal level in virgin, gestating and weaning animals (Fig. 2c). The increase of GALE mRNA expression during lactation was statistically significant versus basic expression ($F(6,49) = 3.167$; $P < 0.05$) (Fig. 2C). Skeletal muscle and cortex taken from the same animals showed no significant variations in GALE mRNA expression during these physiological stages (data not shown).

4. Discussion

The present report is the first comparative analysis of GALE expression of both enzymatic activity and mRNA transcript in the rat mammary gland during lactation. We tested the GALE specific activity at three different stages of lactation in comparison to virgin and weaning stages when there is no stimulation of the mammary gland. We report a general increase of GALE activity in the rat mammary gland with particularly a more than five-fold increase of specific activity in the middle of lactation. On weaning the activity dropped rapidly to the unstimulated level. These results are in accordance with the remarkable increase of GALE activity during lactation shown by Shatton et al. [10] and thus confirm a particular regulation of GALE in this tissue during the different physiological stages tested.

As the increase in GALE activity of lactating mammary glands was found to be due to a structural change of the existing enzyme that leads to a more active form [16], we attempted to determine whether it is a case of transcriptional or post-translational regulation of GALE enzyme in the rat mammary glands during lactation. We therefore tested mRNA expression of GALE in rat mammary glands during lactation. Interestingly, we found this variation not only in the enzymatic activity but also in the mRNA transcript which increased about six-fold during the beginning and the middle of lactation and then dropped in the end.

These variations in GALE activity and mRNA are in accordance with the lactose level in mammary glands which increases, at the end of gestation to reach a peak of concentration on day 15 of lactation [17]. Moreover, GALE is specifically expressed during lactose production in the mammary gland since no increase of GALE mRNA expression was found in muscle and cortex as expected.

Our results are the first evidence of a differential expression

of GALE mRNA during lactation in the rat mammary gland. The significant increase of GALE activity indicates the capability of the mammary gland to respond to a functional requirement by an increase of mRNA production. The physiological variation supports the idea of a possible transcriptional regulation of the enzyme during lactation. In this way our work opens a new field for studying the regulation of GALE expression in the mammary gland providing an accurate model of expression. Indeed, although the human GALE cDNA [18] has been sequenced no studies have been reported concerning the molecular regulation of this enzyme in mammals.

Furthermore, GALE plays a key role in galactose metabolism. Mutations of the gene encoding this enzyme are responsible for a minor form of galactosaemia [19]. Moreover, the knowledge of the regulation of the *gale* gene may open the way to develop a new strategy to induce the production of UDP-galactose which has been shown to be deficient in patients with 'classical' galactosaemia [20]. The analysis of such regulatory elements may be possible by using the differential expression of GALE in the mammary gland during lactation.

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