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Mammary 5'deiodinase (5'D) during the breeding cycle of the rat: indirect evidence that 5'D type I is specific to the alveolar epithelium

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The present study analyses the activity of 5'deiodinases type I and II in mammary gland during the breeding and estrous cycle of the rat, and includes indirect evidence that 5'D-I is present only in the alveolar epithelium. Data show that the mammary gland exhibits 5'D-II activity throughout the developmental period and its activity varies along the allometric growth of the gland. 5'D-I is detected during the differentiation stages of the alveolar epithelium (puberty, late pregnancy) and its activity rises significantly (10-fold) 24 h after delivery. Data also show that 5'D-I activity is not present in the fat pads of the gland. These findings suggest that during its differentiation and functional stages, the mammary gland requires an elevated and compartmentalized production of T3.

Keywords: 5'deiodinase activity; mammary 5'D; gestation; lactation; thyronines; breeding cycle; estrous cycle

Introduction

Thyroid hormones play a crucial role in the development and differentiation of various tissues, besides participating in the regulation of the energetic expenditure of the organism. These effects are mainly mediated by the interaction of T3 with its nuclear receptors. Local production of this thyronine is accomplished by specific enzymes named 5'deiodinases (5'D). These deiodinating enzymes are currently identified by operational definitions, as none of the catalytic activities has been purified. Type I 5'D (5'D-I) is normally found in organs of high hormonal turnover rate i.e. liver, kidney and thyroid gland and is characterized by relatively high K_m and V_{max} values, which suggests that this enzyme has an elevated catalytic efficiency. Type II is amply distributed and displays moderate catalytic efficiency because its K_m and V_{max} values are one order of magnitude lower than type I (Larsen, 1991).

Thyronines are essential for growth and differentiation of the mammary gland (Vonderhaar & Greco, 1979), they regulate the synthesis of alfa lactalbumin and therefore of lactose synthetase activity (Ziska *et al.*, 1988), and are part of the galactopoietic hormonal complex (Tucker, 1981). Furthermore, we have previously reported that the lactating mammary gland contains substantial 5'D-I activity, which depends on the suckling stimulus, and that enzyme activity is directly related to the number of pups (Valverde-R & Aceves, 1989; Aceves & Valverde-R, 1992). These findings lead us to propose that the high energetic expenditure needed for milk production demands an elevated local conversion from T4 and T3. The present study was designed to distinguish if the different periods of growth and development of mammary tissue are accompanied by specific deiodinating activities, and to determine the tissue localization of 5'D-I during lactation. Results show that 5'D-I is only detected within the mammary gland either when the gland alveolar epithelium is differentiating (puberty or late pregnancy) or when it is functional (lactation). These data support the notion that the high energy expenditure associated to differentiation and or lactation of the mammary gland requires an elevated T3 local supply.

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Results

Figure 1 shows mammary 5'D type I and type II activity during the different stages of the rat breeding cycle. Independently of the physiological state, the mammary gland always contains 5'D-II. During puberty (5th week) and at 14 and 20 days of pregnancy, 5'D-I activity is also detected. This enzymatic type rises significantly (10-fold) 24 h after delivery and continues to increase until it reaches a maximum value 15 days postpartum. On weaning, a continuous and significant descent in 5'D-1 activity is observed. Eight days after weaning (30 days postpartum) the only activity detected is type II.

Figure 2 shows 5'D-II activity during the estrous cycle. The only activity detected corresponds to this isotype. The lowest activity was observed in diestrus (1 and 2), it rises in proestrus and continues to increase until it reaches a maximum value in estrus.

Histological examination of de-epithelialized subcutaneous tissue obtained from the region where the fourth mammary gland is normally located, showed the presence of normal looking adipose tissue (Figure 3A). A careful search for remanent epithelial components was unsuccessful. In contrast, the contralateral intact fourth mammary gland showed the characteristic lactating hyperplasia (Figure 3B).

Figure 4 shows the selective effect of prepuberal de-epithelialization on mammary 5'D activity during lactation. The de-epithelialized glands (treated) exhibit a significant increase in 5'D-II but 5'D-I activity disappears. In contrast, the contralateral glands (intact) show similar 5'D-I activity to untreated animals (control).

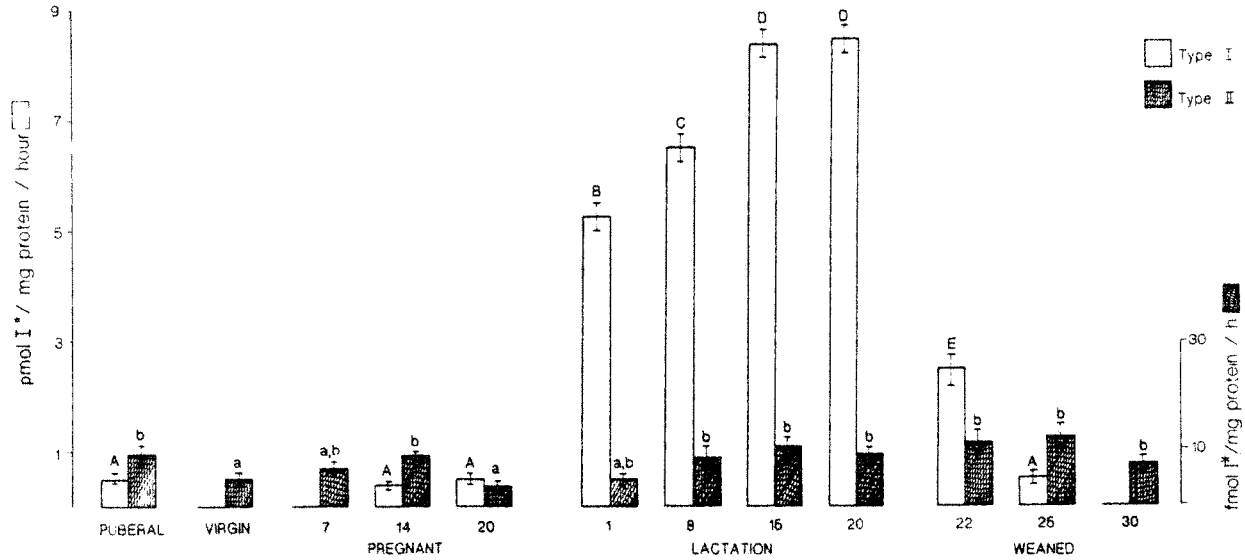

Figure 1 Mammary 5'D activity during the breeding cycle. Puberal (5th week), virgins (8th week), pregnant (days 7, 15 and 20), primiparous lactating mothers (ppartum days 1, 8, 15 and 20) with litter adjusted to 10 pups/each. In the weaned group (ppartum days 22, 25 and 30), litters were removed at the 21st ppartum day. The distinction between enzymatic types was performed in parallel assays with and without 10 mM PTU. The values for 5'D-I were obtained by subtracting type II activity (PTU-insensitive) from total activity. The type II values were assessed using: 8 nM T4, 20 mM DTT and 10 mM PTU. Capital and lower case letters refer to comparisons between type I or type II activity respectively. Means with different letters are significantly different ($P<0.05$); $n=6$

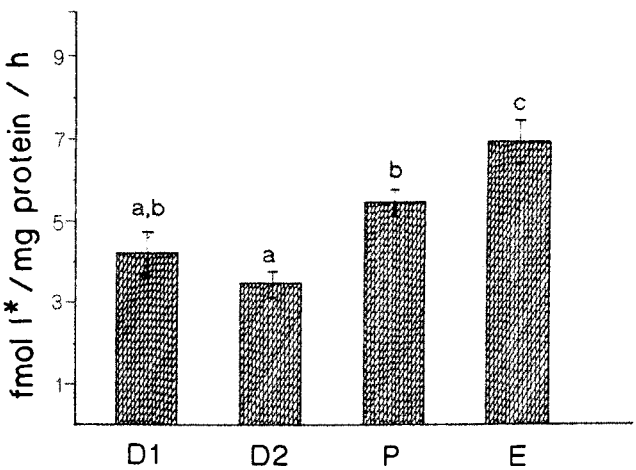

Figure 2 Mammary 5'D-II activity during the estrous cycle. Results correspond to virgin rats (8th week). D1: early diestrus; D2: late diestrus; P: proestrus; E: estrus. Means with different letters are significantly different ($P<0.05$); $n=4$

Figure 5 shows the Eadie-Hofstee plots for 5'D activity in lactating mammary glands. Plots in Figure 5A correspond to assays with and without 10 mM PTU, in which high rT3 (0.01 to 1.0 μM) and low (5 mM) DTT concentrations were used. K_m and V_{max} for each enzymatic type were: total activity = 0.44 μM and 9.4 pmol/mg protein/h and type I = 0.57 μM and 9.73 pmol/mg protein/h, respectively. Figure 5B shows the results from assays with 10 mM PTU, 2 to 32 nM rT3 or T4, and 20 mM DTT. K_m and V_{max} values were: for rT3 8.9 nM and 7.34 fmol/mg protein/h and for T4 9.0 nM and 18.41 fmol/mg protein/h respectively. These results allowed the calculation of the catalytic efficiency rate (V_{max}/K_m) for both types of 5'D activity. thus, for 5'D-I and using rT3 (9.73 pmol/mg protein/hour/0.57 μM), the rate is 17.0. In the case of 5'D-II and using rT3 (0.00734 pmol/mg protein/hour/0.0089 μM) the rate is 0.82; whereas using T4 (0.0184 pmol/mg protein/hour/0.0090 μM) the rate is 2.0.

Table 1 summarises the kinetic constants of the de-epithelialized glands. The analysis show that this tissue only contains PTU-insensitive activity whose catalytic efficiency (V_{max}/K_m) rises practically three fold when using T4 at 20 mM DTT. These data suggest that T4 is the preferential substrate.

Discussion

In previous work, we showed that the lactating mammary gland displays deiodinating activity type I (Aceves & Valverde-R, 1989). The kinetic constants there reported for total mammary 5'D were K_m 0.416 μM and V_{max} 1.4 nMol/mg protein/h. Present results confirm that mammary 5'D has a high rT3-affinity (K_m 0.44 μM), however, now we report significantly lower values for V_{max} and specific activity. This discrepancy is the result of procedural errors in our previously published work (1989). In those experiments we used the Hartree method to measure protein concentrations unaware that HEPES buffer interfered with the method. Thus our protein contents may have been underestimated by up to 50% (Peterson, 1979). Secondly, while calculating the specific enzymatic activity, we made a calculation error which resulted in the overestimation of V_{max} and specific activity by as much as 135-fold. The present work measures protein by the Bradford method and calculates kinetic parameters by linear regression of Eadie-Hofstee plot data. We also show that the mam-

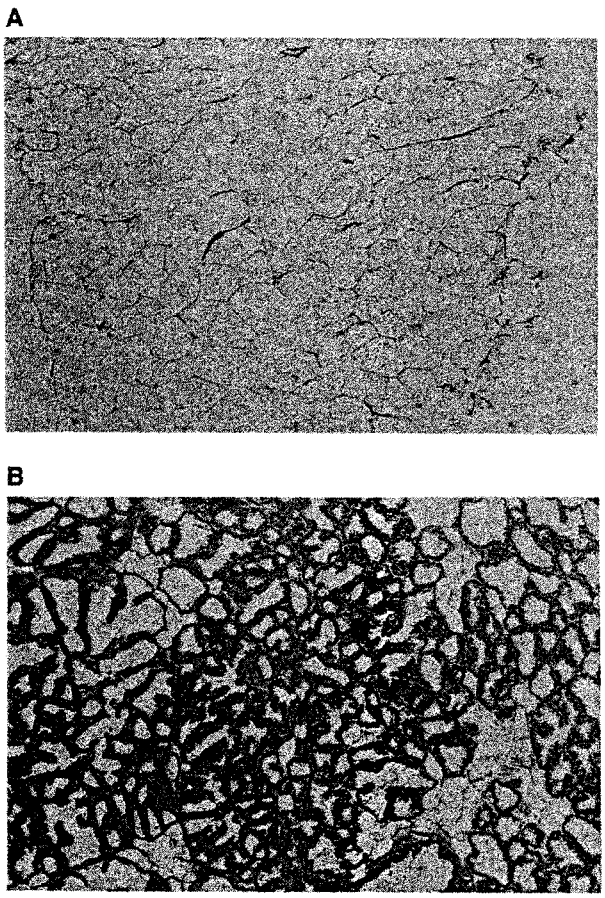

Figure 3 Photomicrographs showing the effects of alcohol treatment (**A**) and microscopical appearace of the contralateral intact lactating mammary gland (**B**). Tissues were fixed in 10% formaldehyde, included in paraffin and sections were stained with H&E, Magnification, 1000 ×

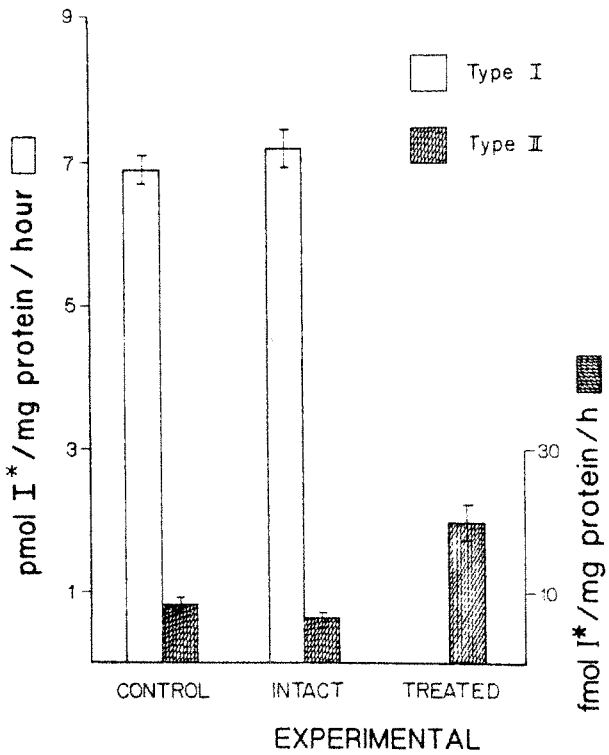

Figure 4 Cellular localization of mammary 5'D-I during lactation. In the experimental group, the epithelial gland components of prepuberal rats (4 weeks old) were cleared from the left mammary line under general anesthaesia by infusing through the nipples pure ethyl alcohol (see Material and methods). Glands from the right mammary line were left intact. The control group corresponds to untreated lactating rats. ($n = 10$)

mary gland displays type II deiodinating activity and we include its kinetic characterization in both intact and de-epithelialized mammary glands.

The finding that 5'D-I is also present during puberty and gestation confirms the importance of thyroid hormones during mammogenesis and strongly suggests that this enzymatic type is specific to the alveolar epithelium. Indeed, the presence of 5'D-I activity only during puberty and its absence in adult virgin animals is consistent with studies showing that thyroid hormones are indispensable for the development and commitment of primordia i.e. alveolar epithelium buds, which only occur during the first four estral cycles (Schmidt, 1970; Vonderhaar & Greco, 1979). Equally, the presence of 5'D-I during the second half of gestation is consonant with the differentiation and growth of the alveolar epithelium (Schmidt, 1970). The notion that 5'D-I is specific to the alveolar epithelium is further supported by the concomitant disappearance of 5'D-I and the well-known secondary alveolar involution on weaning (Oka & Yokorama, 1965), as well as the absence of this enzymatic type in de-epithelialized glands.

Regarding isotype 5'D-II our results show that it is present throughout the breeding cycle and that its activity varies along the allometric growth of the gland. In fact, it increases during puberty, estrus and gestation, stages in which the adipose, ductal and myoepithelial components of the gland are developed and/or grow. Fat pads are indispensable for optimal growth and differentiation of the alveolar epithelium (Imagawa *et al.*, 1990). Present results show that fat pads, which are the only tissue preserved after de-epithelialization, display a significant increase in V_{max} and 5'D-II activity per mg of protein and thus suggest that this mammary component may well be the only one containing this type of activity. This finding contrasts with previous reports which show that rat epididymal fat pads contain 5'D-I (Leonard *et al.*, 1982) and that no type of deiodinating activity is detected in nonlactating human breast adipose tissue (Rao *et al.*, 1985). Further investigations are needed to explain the discrepancy (e.g. tissue type, physiological state, species, etc).

In short, the present work corrects the kinetic constants previously described by us for mammary 5'D activity; it demonstrates that independently of the physiological status, the mammary gland displays 5'D-II activity, and suggests that the alveolar epithelium is the only mammary component which contains 5'D-I activity. The relative importance of 5'D-I activity can be inferred by the fact that during lactation the catalytic efficiency of mammary deiodination increases 8.5 times. These data support and complete our previous suggestion that during its differentiation and functional stages, the mammary gland requires an elevated and compartimentalized production of T3.

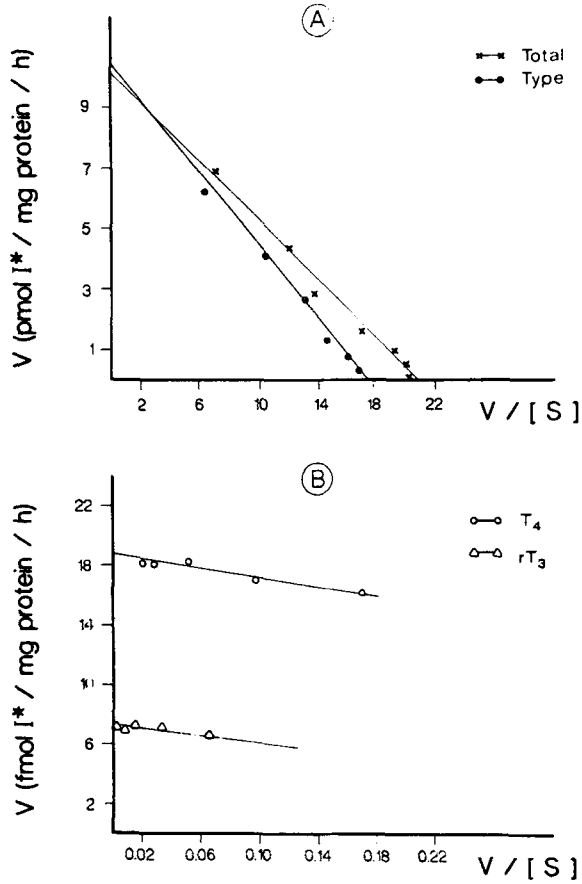

Figure 5 Eadie-Hofstee plots from lactating mammary 5'D. Results are the mean values of triplicate observations on pooled lactating mammary glands from three rats. (A) shows assays with 0.01 to 1.0 μM rT_3 and 5 mM DTT. Type I activity (PTU-sensitive) was calculated as the difference between total activity and type II (PTU-insensitive). (B) shows assays with 10 mM PTU, using 2 to 32 nM rT3 or T4 and 20 mM DTT (further details in text)

Table 1 Kinetic parameters for mammary 5'D-II activity

Enzymatic type	*Substrate ([ ]range)*	*DTT mM*	*PTU mM*	K_m *nM*	V_{max} *fmol/mg prot/h*	V_{max}/K_m *rate*
Total	rT_3 (2-32 nM)	5	–	9.0	7.30	0.81
II	rT_3 (2-32 nM)	5	10	9.2	7.13	0.77
II	rT_3 (2-32 nM)	20	–	9.1	17.39	1.89
II	T_4 (2-32 nM)	20	–	9.7	51.38	5.29

Results are the mean of triplicate observations on pooled de-epithelialized mammary gland homogenates from 10 rats. The kinetic parameters were calculated by linear regression of Eadie-Hofstee plot data

Materials and methods

Reagents

Nonradioactive thryonines were obtained from Henning Co. (Berlin, Germany). Radiolabeled rT_3 (sa: 1174 μCi/μg) and T_4 (1200 μCi/μg) were purchased from New England Nuclear (Boston, MA), and were purified by high voltage paper electrophoresis (Sakurada *et al.*, 1978) before use. Propylthiouracil (PTU) was obtained from US Biochemical Co. (Cleveland, OH) and the dithiothreitol (DTT) from Calbiochem (La Jolla, CA). All other reagents were of the highest purity commercially available.

Animals

The study was conducted on Wistar female rats. Animals were housed in an automatically controlled environment (21 ± 1°C; 12 h light-dark cycle) with *ad libitum* Purina Chow and water. All animals were killed by decapitation, and the mammary glands of each individual were dissected and immediately frozen in acetone-dry ice. Procedures regarding care, administration of treatment and euthanasia of animals were reviewed and approved by the supporting DGAPA/UNAM Committee.

Enzymatic assay

Individual mammary glands were homogenized (1:10 w/vol) in ice-cold buffer (10 mM HEPES, pH 7.0, with 0.32 M sucrose, 1.0 mM EDTA, and 10 mM DTT), and centrifuged (2500 *g*) during 10 min at 4°C. 5'D activity was determined by a modification of the release of radiolabeled iodide method as previously described (Aceves & Valverde-R, 1989). All assays contained 200 μg protein, substrate and cofactor concentrations were optimal for each experimental procedure (see below). Parallel control runs for each assay consisted of tubes without homogenate. Incubation time was 3 h at 37°C. Released acid-soluble radioiodide was isolated by chromatography on Dowex 50W-X2 columns. For all enzymatic assays the minimum activity was at least twice the background released ^{125}I from control tubes, which was in all cases less than 3%. Reaction conditions were chosen so that less than 30% of substrate was consumed during the assay. Specific activity was calculated by subtracting the background activity (control tubes) and correcting to 80% because of losses due to handling, according to Pazos-Moura *et al.*, 1991. Proteins were measured by the Bradford method (Bio-rad protein assay, BIO-RAD, Richmond, CA). Results are expressed as picomoles of radioiodide released per milligram protein/hour.

Experimental procedures

Breeding cycle 5'D activity was assessed ($n = 6$) under the following physiological conditions: puberal (5th week), virgins (8th week), pregnant (days 5, 14 and 20), lactating mothers (ppartum days 1, 8, 15 and 20) with litter adjusted to 10 pups/each, or weaned (ppartum days 22, 25 and 30). In this latter group litters were removed on the 21st ppartum day. Determination of 5'D type I at different stages was carried out in parallel assays with and without 10 mM PTU, containing: 2 nM [^{125}I]rT3, 0.5 μM rT3 and 5 mM DTT. Type I activity (PTU-sensitive) was calculated as the difference between total activity and type II (PTU-insensitive). Type II was assessed using: 8 nM T4, 20 mM DTT and 10 mM PTU.

Estrous cycle This study was conducted in virgin rats (8th week; $n = 4$). Enzyme activity was assessed using: 8 nM T4, 20 mM DTT and 10 mM PTU.

Cellular localization of 5'D-I This study was conducted in lactating rats ($n = 10$) in which the left mammary line was de-epithelialized (Zhang *et al.*, 1991). Prepuberal rats (4 weeks old) were anesthetized (sodium pentobarbital 3 mg/100 g bw), and the epithelial mammary gland components were cleared from the left mammary line (treated) by infusing through the nipples 50 μl of pure ethyl alcohol (three times at intervals of 5 days each). Glands from the right mammary line were not infused (intact). An additional group of females that were not subjected to mammary de-epithelialization were maintained as controls (control) At delivery, litters were adjusted to six pups. Mothers were sacrificed at day 15 of lactation. Histological analysis of mammary glands was performed by fixation on 10% formaldehyde, paraffin embedding, and Hematoxylin & Eosin (H&E) staining. Charac-

terization of 5'D type I and II activities were also carried out in parallel assays as described above (see Breeding cycle).

Kinetic parameters of 5'D Enzymatic characterization was assessed on lactating, virgin and de-epithelialized mammary glands. 5'D-I and II activities were run at intervals of high (0.01 to 1.0 μM) and low (2 to 32 nM) rT_3 concentrations, 5 or 20 mM DTT and, with and without 10 mM PTU. 5'D-II activity was also analysed with T4 (2 a 32 nM) and 20 mM DTT. The kinetic parameters were calculated by linear regression of Eadie-Hofstee plot data ($y = b + mx$; in which $b = V_{max}$ and $m = K_m$) (Segel, 1975).

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