Effect of suckling and adrenergic stimulation on peripheral deiodination in lactating rats: differential expression of type 1 deiodinase mRNA forms

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Abstract

Previous works led us to propose that peripheral iodothyronine deiodination is mainly regulated by the reciprocal interaction between the thyroid and the sympathetic nervous system (SNS). In this study, we analyzed the role suckling exerts, through SNS activation, upon deiodination of thyronines in liver, heart, brown adipose tissue and mammary gland during lactation. Our results showed that resuckling causes a concurrent stimulatory response on deiodinase type 1 (D1) in heart and mammary gland, but

not in liver and brown adipose tissue. The stimulatory response was mimicked by norepinephrine and by the β -adrenergic agonist isoproterenol, through the over-expression of the large form of D1 mRNA. These results suggested that, during lactation, peripheral thyronine deiodination is co-ordinated by the SNS, and suckling is a major modulatory influence.

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Introduction

In vertebrates, thyroxine (T₄), the major hormone produced by the thyroid gland, must be converted by 5'-deiodination (5'D) into tri-iodothyronine (T₃) to exert its control upon several functions; i.e. growth and development, thermogenesis and energetic expenditure (Kohrle 1999). This biotransformation, which takes place in target tissues, is catalyzed by two selenoenzymes called deiodinases type 1 and type 2 (D1 and D2 respectively). D1 is sensitive to inhibition by propylthiouracil (PTU), has a ping-pong catalytic mechanism and is expressed in organs such as liver, kidney, thyroid, heart, pituitary and lactating mammary gland (MG). D2 has a sequential catalytic mechanism, is highly resistant to PTU, and is present in brain, skin, placenta and brown adipose tissue (BAT) (Larsen *et al.* 1981, Leonard & Kohrle 1996). Extensive clinical and experimental data indicate that the reciprocal functional relationship between the thyroid and the sympathetic nervous system (SNS) constitutes a critical part of the homeorhetic mechanisms aimed at preserving the organism's metabolic fitness (Aceves *et al.* 1994, Silva 1996). Lactation is an excellent example of these homeorhetic adjustments and, in terms of peripheral thyronine deiodination, we have shown that (1) despite the hyperphagia present during lactation, the organ-specific adjust-

ments in peripheral deiodination resemble those observed in fasted or hypothyroid animals (Valverde-R & Aceves 1989, Aceves *et al.* 1994); (2) these adjustments are not modified by overfeeding or by thyroid hormone administration (Aceves *et al.* 1994); (3) this 'pseudohypothyroidism' is exacerbated when the lactational demand (litter size) is increased (Valverde-R & Aceves 1989); (4) D1 enzyme is encoded by two messengers that differ in the length of their 3'-untranslated region (3'UTR), and the expression of the large form is directly correlated to thyroid status (Navarro *et al.* 1997); (5) MG D1, which is encoded by the short messenger, is positively regulated by suckling through the efferent sympathetic mammary innervation (Aceves *et al.* 1999a); and (6) this local MG D1 regulation involves segmental mechanisms that, depending on the size of the litter, may allow a differential, gland by gland adjustment of energetic expenditure (Aceves *et al.* 1999b). These findings add further support to our proposal that, during lactation, deiodination represents a functional intersection of the dyad thyroid-SNS to maintain the elevated energy supply needed by the lactating mammary gland (Aceves *et al.* 1999b). Thus, the present study was designed to gain further insight into the background of this thyroid-SNS interaction, and to test the hypothesis that suckling, through SNS activation, modulates the homeorhetic adjustments that characterize lactation.

Materials and Methods

Reagents

Non-radioactive thyronines were obtained from Henning Co. (Berlin, Germany). Radiolabeled reverse T3 (rT3; specific activity 1174 $\mu\text{Ci}/\mu\text{g}$) and T4 (specific activity 1174 $\mu\text{Ci}/\mu\text{g}$) were purchased from New England Nuclear (Boston, MA, USA). Dithiothreitol (DTT) and oxytocin (OT) were obtained from Calbiochem (La Jolla, CA, USA), and norepinephrine (NE) and isoproterenol (ISO) from Sigma Chemical (St Louis, MO, USA). Oligonucleotides were synthesized by GIBCO-BRL (Gaithersburg, MD, USA). All other reagents were of the highest purity commercially available.

Animals

Virgin (250 g) and primiparous (300 g) Sprague–Dawley rats were housed in individual cages in a room with a 14 h light:10 h darkness cycle. On postpartum day 1, the size of the litter was adjusted to ten pups per mother and all the experimental procedures were conducted on postpartum day 10 ± 1 . All animals were fed Purina Lab Chow and tap water *ad libitum*. Procedures regarding care, administration of treatment and euthanasia of animals were reviewed and approved by the supporting Direccion general de asuntos del personal academico (DGAPA)/UNAM Committee. All animals were killed by decapitation and two samples of liver, heart, BAT and MG of each individual were dissected. One sample of each tissue was immediately frozen on liquid nitrogen and the other sample was homogenized in a guanidine thiocyanate solution.

Enzymatic assay

Deiodinase activity was determined by a modification of the radiolabeled iodide release method as previously standardized for each tissue (Aceves *et al.* 1994). All tissues were homogenized in a solution containing 10 mM HEPES (pH 7.0), 0.32 M sucrose, 1.0 mM EDTA and 20 mM DTT, and centrifuged at 2800 g for 30 min at 4 °C. Assay conditions for tissues containing D1 activity (liver, heart and MG) were 0.2–200 μg protein, 2 nM ^{125}I -rT3, 0.5 μM non-radiolabeled rT3 and 5 mM DTT. For BAT (which expresses D2 enzyme) the assay was carried out with 200 μg protein, 2 nM ^{125}I -rT4 and 20 mM DTT. After 3 h of incubation, released acid-soluble radioiodide was isolated by chromatography in Dowex 50W-X2 columns (Bio-Rad, Richmond, CA, USA). Proteins were measured by the Bradford method (Bio-Rad protein assay; Bio-Rad). Results are expressed as fmol or pmol radioiodide released/mg protein/h.

RT-PCR

D1 or D2 mRNAs were identified by using a previously standardized semi-quantitative PCR procedure in which

an amplicon of the structural protein cyclophilin (Cyc) was simultaneously amplified (Aceves *et al.* 1999a). Briefly, the reverse transcription (RT) reaction was primed with oligo(dT) and subscripted with 5 μg total RNA. The PCR reaction was carried out using 5 μl RT mixture and the following primers: for D1, M2s and M6as, for D2, D22s and D26as and for Cyc, Cyc1s and Cyc7as (see Fig. 1). Amplification was carried out for 32 cycles. Each cycle consisted of melting at 94 °C $\times$ 45 s, annealing at 52 °C $\times$ 45 s, and an extension at 72 °C $\times$ 1 min. As a control, a reaction mixture containing an RNA sample with the appropriate oligonucleotide primers, but without the RT, was included in every experiment. The sizes of the resultant PCR fragments were 251 bp for D1, 805 bp for D2 and 521 bp for Cyc, and were resolved on a 2% agarose gel and visualized using ethidium bromide. The sizes of the bands were confirmed by a restriction digested pUC plasmid (1 kb DNA ladder; GIBCO-BRL). After a Polaroid picture had been taken, the pictures were digitized using a Hewlett Packard Scanner Jet 11CX, and the signals were analyzed by using an editing version of the NIH-image program. Values obtained were normalized according to the Cyc mRNA levels detected in each sample.

3'UTR amplification

The length of 3'UTR D1 mRNA was assessed by using a previously standardized 3'-rapid amplification cDNA ends (RACE) procedure (Aceves *et al.* 1999a) and 5 μg total RNA from liver, heart and MG. The RT reaction was primed with oligo(dT) containing a terminal specific sequence, AP (see Fig. 1). Amplification was carried out for twenty cycles consisting of melting at 94 °C $\times$ 45 s, annealing at 50 °C $\times$ 45 s, and an extension at 72 °C $\times$ 1 min, with J3s and UAP oligos (see Fig. 1) and a second tandem of 40 cycles of melting at 94 °C $\times$ 45 s, annealing at 52 °C $\times$ 45 s, and extension at 72 °C $\times$ 1 min with the same oligos. The resultant PCR fragments were 956 and 506 bp for large and short mRNA forms respectively. To verify the presence of the long form of D1 mRNA in each tissue a PCR amplification was carried out with oligos whose sequence is only present in the 3'-terminal region of the long mRNA form (As and Bas), with a resultant fragment of 136 pb (see Fig. 1). Amplified fragments were electrophoresed on 2% agarose gel and visualized using ethidium bromide.

Statistical analysis

Data are expressed as the means $\pm$ s.d. Differences between experimental groups were analyzed using a one-way ANOVA and Tukey's HSD test. Differences with a $P < 0.05$ were considered statistically significant.

All experiments had parallel controls, which consisted of intact virgin rats and mothers with continuous suckling.

B

primer	size	location	sequence	purpose
D22s	21-mer	545-565	GAG TGC ACA GGA GAC TGA CTG	PCR
D26as	21-mer	135-1330	CTT CTC CAG CCA ACT TCG G AC	PCR
Cyc1s	21-mer	7- 27	AGA CGC CGC TGT CTC TTT TCG	PCR
Cyc4as	23-mer	527-507	CCA CAC AGT CGG AGA TGG TGA TC	PCR

Figure 1 (A) Schematic representation of two mRNAs of D1 enzyme. Arrows represent the position and direction of the different oligonucleotides used. Exact position, length and sequence are illustrated. (B) The characteristics of oligonucleotides used by D2 and Cyc mRNA amplification. RT, reverse transcriptase.

Whenever the glands were not resuckled by pups, accumulated milk was removed by administration of OT (30 mU; i.p.) 1 min before they were killed. This procedure ensures that accumulated milk will not act as a dilution factor when tissue proteins are quantified.

Results

Figure 2 shows the changes exhibited by 5'D activity in all groups. Enzymatic values differed between virgin and lactating rats in all tissues analyzed. Lactation was accompanied by significantly higher 5'D activity values in heart and MG, and lower activity in liver and BAT. After

12 h of non-suckling, the mothers exhibited a significant ($\approx 50\%$) decrease in heart and MG D1 activity, whereas in liver and BAT the enzymatic activity remained unchanged. Within the initial 12-h non-suckling interval, resuckling (4 h) could restore the enzymatic activities to control values in heart and MG. Adrenergic stimulation exerted a differential effect on 5'D activities. In virgin animals, NE and ISO administration increased heart D1 and BAT D2 activities. In lactating animals, both NE and ISO elicited a significant 5'D stimulatory response in heart and MG, but not in BAT. In contrast, in neither virgin nor lactating animals were these adrenergic agents able to evoke a detectable response in liver D1 activity. As depicted in Fig. 3 these changes in enzyme activity were

Figure 2 Comparison of 5' deiodinase (D) activity in different tissues from virgin and lactating rats. Control animals included intact virgin animals and lactating mothers in which suckling was continuous (SC), the rest of the lactating rats were separated from their pups for 12 h. One group was killed after 12 h of non-suckling (12 hNS), a second group was resuckled by their pups for 15 min and killed 4 h later (resuckled group; RS), the last two groups were treated with 40 µg/100 g body weight norepinephrine (NE) and 100 µg/100 g body weight isoproterenol (ISO) respectively, and killed 4 h later. Virgin animals received the same doses of both substances. Data are expressed as the means $\pm$ S.D. ($n=6$). Means with different letters are significantly different ($P<0.05$).

parallel with those exhibited by D1 and D2 mRNAs, thus suggesting that these treatments exert their action at transcriptional level.

The PCR-RACE method was employed to analyze the 3'UTR D1 mRNA from liver, heart and MG. Figure 4 shows that two fragments were amplified from heart and liver cDNAs, indicating the presence of two D1 mRNA

forms in these tissues. In previous work, we have amplified and sequenced these fragments and showed that the longer form uses the second polyadenylation site in the 3'UTR region of the mRNA (Navarro *et al.* 1997). A more intense signal was evident for the short fragment than the larger one, suggesting that the short mRNA is more abundant. Nevertheless, it is difficult to interpret the

Figure 3 Comparison of mRNAs for 5′deiodinases in different tissues from virgin (V) and lactating (L) rats. Ethidium bromide-stained gel showing RT-PCR products for D1 (221 bp) and cyclophilin (Cyc) (520 bp) after 40 cycles of amplification. A, ladder; CS, control animals with continuous suckling; 12 hNS, mothers whose pups were removed for 12 h; RS, mothers whose pups were resuckled after 12 h of non-suckling; NE, animals treated with NE; ISO animals treated with ISO; RT–, RNA sample and the appropriate oligonucleotide primers, but without reverse transcriptase; H₂O, water with all the PCR reagents. The experiments were repeated twice with independent RNA samples.

relative quantities of two isoforms considering that a co-amplification took place, and the replication efficiency is dependent on the fragment size (Stolovitzky & Cacchi 1996). In the case of MG, only the short fragment was evident in lactating animals. When we corroborated the presence of the long mRNA form using PCR amplification for the last portion of the 3′UTR, fragments of the expected size were obtained in liver and heart from virgin

and lactating animals in all experimental conditions. The quantity of the large fragment was increased in virgin animals treated with adrenergic agonists (NE and ISO), whereas in lactating rats this increase was exhibited in continuous suckling, resuckling and adrenergic stimulation (NE and ISO). In MG, this fragment was amplified in cDNAs from resuckled and adrenergic-stimulated lactating animals.

Figure 4 PCR-RACE amplification of 3'-untranslated region (3'UTR) of D1 mRNAs from virgin (V) and lactating (L) rats (ethidium bromide-stained gel). Oligo(dT) with specific sequence denoted AP (see Fig. 1 and Materials and Methods) was used for RT reactions. In the upper panel, PCR was carried out with complementary sequence oligo(dT), UAP, J3s, and 1 µl (liver) or 5 µl (heart and mammary gland (MG)) aliquots of the RT reaction. In the lower panel, PCR was carried out with oligonucleotides As and Bas, and 1 µl (liver) or 5 µl (heart and MG) aliquots of the RT reaction. A, ladder; CS, control animals with continuous suckling; 12 hNS, mothers whose pups were removed for 12 h; RS, mothers whose pups were resucked after 12 h; NE, animals treated with NE; ISO, animals treated with ISO; RT -, RNA sample and the appropriate oligonucleotide primers, but without reverse transcriptase; H₂O, water with all the PCR reagents. The experiments were repeated twice with independent RNA samples.

Discussion

The present results confirm and extend our previous analysis of the thyroid–SNS interaction during lactation.

Besides corroborating that lactating rats exhibit an organ-specific thyroid hormone–deiodinative rearrangement that resembles hypothyroidism (Valverde-R & Aceves 1989, Aceves *et al.* 1994), the present data show for the first time

that suckling concurrently stimulates heart and MG D1. This simultaneous activation is mediated by β -adrenergic receptors and suggests that this SNS–D1 interaction is part of a physiological mechanism co-ordinating the metabolic demands imposed by milk production. This notion agrees with previous observations showing that (1) the characteristic homeorhetic adjustments of lactation are modulated by the lactational intensity (litter size, number and frequency of suckling, etc.) (Prentice & Prentice 1988, Valverde-R & Aceves 1989); (2) mammary D1 is stimulated *in vivo* by the NE released in the sympathetic nerve endings (Aceves *et al.* 1999b); and (3) in the heart, a classic noradrenergic target tissue, lactation increases the NE turnover rate (Bauman & Currie 1980).

Although it is well documented that in several organs D2 is regulated by the SNS (Silva 1996, Kohrle 1996, 1999), in the case of D1 the evidence is scarce and contradictory. The possibility of an important effect of catecholamines on extrathyroidal conversion of T4 to T3 was suggested by clinical studies where β -receptor antagonists decreased the plasma concentration of T3 in thyrotoxic or hypothyroid patients maintained on a fixed dose of T4 (Murchison *et al.* 1979, Sulkin *et al.* 1984). Subsequently, *in vitro* studies in liver and kidney demonstrated that D1 inhibitory effects of propranolol were related to its lipid solubility and not to its β -adrenergic blocking activity (Heyma *et al.* 1978, Sulkin *et al.* 1984). Moreover, when kidney preparations were treated with NE and β -agonists, the expected stimulatory D1 response did not appear (Heyma *et al.* 1980). Besides confirming the lack of β -adrenergic influence upon liver D1, our findings demonstrate that β -adrenergic mechanisms do stimulate, in a concerted manner, D1 enzyme in heart and lactating MG. This finding indicates that catecholamines need to be added to the ample variety of regulatory influences known to affect D1 activity in an organ-specific manner; i.e. thyroid status, carbohydrates, selenium, thyrotropin, angiotensin II, cAMP, etc. (Kohrle 1996, Yonemoto *et al.* 1999, Bates *et al.* 2000).

As we have mentioned before, rat liver contains two different transcripts of D1 mRNA, and we have shown that the expression of the large form is dependent on thyroid status (Navarro *et al.* 1997). We now demonstrate that these two mRNA forms are present in all D1-containing tissues analyzed herein, and that the larger form is stimulated by resuckling and β -adrenergic stimulation in heart, but not in liver. Our data also indicate that these stimuli induce the *de novo* expression of this longer messenger in lactating MG. The physiological role of this differential mRNA expression has not been elucidated; however, studies on other transcripts have demonstrated the involvement of the 3'UTR in the control of translation efficiency and/or mRNA stability (Whale & Keller 1992). Thus, DeSavage *et al.* (1992) have shown that amyloid protein, which is expressed by two different transcripts containing two distinct polyadenylated signals, is prefer-

entially translated with the large mRNA form. Our results strongly suggest that the large D1 mRNA is preferentially expressed in response to a metabolic overdemand that requires an elevated and rapid supply of local T3 production, i.e. hyperthyroidism, supraphysiological administration of β -adrenergic agonist or vigorous resuckling after 12 h of non-suckling. Current studies in our laboratory are exploring the possible differences in translation efficiency and/or stability of these messenger forms, as well as the role of thyroid–SNS interaction on its regulation.

During lactation, adrenergic stimulatory deiodinase response was preserved in predominantly β -adrenergic receptor-containing tissues (heart and MG) and disappeared in tissues (BAT) that contain a mixed population of α - and β_3 -adrenergic receptors. These findings coincide with previous reports showing that in a catabolic situation, such as lactation (Giralt *et al.* 1987, Aceves *et al.* 1994) or fasting (Silva & Larsen 1986), the sensibility of D2 BAT to NE is impaired. The exact mechanism implicated in this differential regulation is unknown; however, several studies suggest that the hormonal and nutritional 'milieu' characteristic of lactation (hypothyroidism, hypoglycemia, hypoinsulinemia, elevated concentrations of growth hormone, etc.) could modulate the adrenergic outflow and response in different target tissues (Silva & Larsen 1986, Aceves *et al.* 1994, Larsen & Berry 1995, Hernandez & Obregon 2000). Thus, whereas the increased sympathetic tone triggered by suckling may be responsible for maintaining the elevated heart and MG D1 activity observed in the present study, the impaired D2 BAT adrenergic response could be secondary to the accompanying hypothyroidism and the increased growth hormone levels that characterize lactation.

In summary, present results add further support to the notion that, during lactation, peripheral thyronine deiodination represents a functional intersection of the dyad thyroid–SNS. This reciprocal interaction in which suckling plays a major regulatory role subserves the organ-specific maintenance of the elevated energy supply in lactating mammary gland.

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