Liver 5'-deiodinase activity is modified in rats under restricted feeding schedules: evidence for post-translational regulation

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Abstract

Restricted feeding schedules (RFSs) produce a behavioral activation known as anticipatory activity, which is a manifestation of a food-entrained oscillator (FEO). The liver could be playing a role in the physiology of FEO. Here we demonstrate that the activity of liver seleno-enzyme deiodinase type 1 (D1), which transforms thyroxine into triiodothyronine (T3), decreases before food access and increases after food presentation in RFSs. These changes in D1 activity were not due to variations in D1 mRNA. In contrast, a 24 h fast promoted a decrease in both D1 activity and mRNA content. The adjustment in

hepatic D1 activity was accompanied by a similar modification in T3-dependent malic enzyme, suggesting that the local generation of T3 has physiological implications in the liver. These results support the notion that the physiological state of rats under RFSs is unique and distinct from rats fed freely or fasted for 24 h. Data also suggest a possible role of hepatic D1 enzyme in coordinating the homeorhetic state of the liver when this organ participates in FEO expression.

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Introduction

Experimental animals, restricted to only 2 or 3 h of food access for several weeks (restricted feeding schedule (RFS)), anticipate food availability with an increment in locomotor activity and water drinking behavior (Mistlberger 1994, Stephan 2002). This behavioral arousal, known as anticipatory activity (AA), reflects an endogenous timing system independent of the circadian clock located in the suprachiasmatic nuclei (SCN). The presence of a food-entrained oscillator (FEO) was evidenced from the middle 1980s (Stephan 2002). Recently, circadian fluctuations of clock genes in peripheral tissues including the liver were reported (Yamazaki *et al.* 2000). These circadian rhythms of clock genes in peripheral tissues uncouple from the SCN by RFSs (Damiola *et al.* 2000). Although the expression of timing mechanisms has been identified, little is known about the nature of the metabolic adaptations of the liver during RFSs. We have reported the entrainment of both metabolites of energetic significance and hormones such as insulin, glucagon and corticosterone in food-restricted rats (Escobar *et al.* 1998, Díaz-Muñoz *et al.* 2000). We also demonstrated significant changes in crucial parameters of liver metabolism such as

the energetic charge ($E_c = \frac{1}{2} \text{ATP} + \text{ADP} / \text{ATP} + \text{ADP} + \text{AMP}$) and the cytoplasmic and mitochondrial redox states (NAD^+/NADH ratio), which were coincident with the onset of the AA (Díaz-Muñoz *et al.* 2000). It has been reported that dexamethasone resets clock gene rhythmicity in the liver and other peripheral tissues but not in the SCN, and induces rhythmic fluctuations in clock genes when applied to fibroblasts maintained in culture (Balsalobre *et al.* 2000). Also, daily injections of adrenaline induced expression of mPer2 and mBmal1 in the liver (Terazono *et al.* 2003) as well as behavioral activation similar to AA (Mendoza *et al.* 2003). Altogether, these findings strongly suggest that endocrine influences on the hepatic metabolic state could be an important component of the mechanisms underlying FEO physiology. Among the most influential hormones for hepatic metabolic status are thyroid hormones (THs). It has been estimated that approximately 18% of the hepatic genes are regulated by THs and their effects include multiple cellular pathways, such as carbohydrate and fatty metabolism, cell proliferation, and signal transduction, among others (Feng *et al.* 2000, Flores-Morales *et al.* 2002). Thyroxine (T4) is the major thyronine secreted by the thyroid gland and functions as a pro-hormone since its deiodination product,

triiodothyronine (T3), is the active hormone. The family of enzymes that converts T4 into T3 is known as 5'-deiodinases, and two different tissue-specific isoforms have been characterized in vertebrates: type 1 (D1) and type 2 (D2) (Hesch *et al.* 1975). D1 is a rapid enzyme producing T3 for local and systemic demands. In liver this enzyme is highly sensitive to fasting regimens (mainly carbohydrates) showing a reduction in the T4 to T3 conversion within the first 24 h, and associated with a systemic decrease in T3 concentration after 48 h (Gavin *et al.* 1988). Moreover, it has been documented that the decrease in D1 activity during fasting is accompanied by a similar decline in D1 mRNA levels, suggesting the existence of pretranslational mechanisms (O'Mara *et al.* 1993). In addition, we have previously reported the encoding of D1 by two messengers that differ in the length of their 3'-untranslated region (3'UTR), and also that the expression of the large form increases with hyperthyroidism in liver and with β -adrenergic stimulation in heart and mammary gland (Navarro *et al.* 1997, Aceves & Rojas-Huidobro 2001). Functional studies analyzing the participation of these different messengers have not been undertaken during fasting or catabolic situations. Hence, the present study was designed to gain further insight into the possible role of the liver as an FEO component, and to test the hypothesis that RFSs induce a unique homeorhetic state that is different from the condition prevalent in rats with free food access or fasted for 24 h. To accomplish this objective, we determined the levels of circulating THs, hepatic D1 activity and mRNA content, as well as the presence in this organ of an enzymatic indicator of thyroidal status, such as the malic enzyme, in RFS, freely fed, and 24 h fasted rats.

Materials and Methods

Reagents

Thyronines were obtained from Henning Co. (Berlin, Germany). Radiolabeled reverse T3 (rT3) (1174 $\mu\text{Ci}/\mu\text{g}$), T3 (1150 $\mu\text{Ci}/\mu\text{g}$) and T4 (1150 $\mu\text{Ci}/\mu\text{g}$) were purchased from New England Nuclear (Boston, MA, USA). Dithiothreitol (DTT) was obtained from Calbiochem (La Jolla, CA, USA) and T3 and T4 antibodies 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 and housing

Adult male Wistar rats weighing 180–220 g were maintained in a 12 h light:12 h darkness cycle (lights on at 0800 h) and constant temperature ($22 \pm 1^\circ\text{C}$). Animals were kept in groups of five with free access to water. All

experimental procedures were conducted according to the institutional guide for care and use of animals under biomedical experimentation (Universidad Nacional Autónoma de México).

Experimental design

Rats were sorted at random into the following experimental groups (Davidson & Stephan 1999): (i) control rats fed freely; (ii) rats with a daily RFS from 1200 to 1400 h for 3 weeks; and (iii) rats with a 24 h fast. To obtain serum and liver samples, rats from groups (i) and (ii) were randomly killed at 0800 h (before AA), 1100 h (during AA), and 1400 h (after food access). Rats fasted for 24 h were killed between 1100 and 1400 h.

Biochemical procedures

Determination of serum T4 and T3 Serum levels of T3 and T4 were measured by homologous RIA methods previously standardized by Valverde & Aceves (1989) with intra- and interassay coefficients of variation of 9 and 12.8% for T3 and 12 and 15% for T4 respectively.

Liver and blood sampling Rats were killed by decapitation and samples of blood and hepatic tissue were removed immediately. For determination of malic enzyme activity, one sample (~ 0.3 g) was homogenized in a 3 ml volume containing 0.25 M sucrose, 1 mM EDTA, pH 7.4 supplemented with a cocktail of peptidase inhibitors, and subjected to differential centrifugation to obtain the cytosolic fraction (Aguilar-Delfin *et al.* 1996). A second sample (~ 1 g) to be used for the determination of D1 enzyme activity and quantification of mRNA was dissected and immediately frozen in dry ice.

Determination of D1 activity Liver samples were prepared by homogenization in ice-cold buffer (10 mM Hepes, pH 7.0, with 0.32 M sucrose and 1.0 mM EDTA) and stored at -70°C before use. D1 activity was determined by a modification of the radiolabeled iodide release method (Valverde & Aceves 1989). Assay conditions were as follows: 2 μg protein, 2 nM [^{125}I]rT3, 0.5 μM non-radiolabeled rT3, and 5 mM DTT. Incubation time was 1 h at 37°C . The released acid-soluble radioiodide was isolated by chromatography in Dowex 50W-X2 columns. Proteins were measured by the Bradford method (Bio-Rad protein assay; Bio-Rad, Richmond, CA, USA). Results are expressed as nmol released iodide per milligram of protein per hour (nmol I/mg protein per h).

Determination of mRNA for D1 Since the two messengers that encode the D1 enzyme only differ in the last 465 nucleotides of the 3'UTR, the analysis of their expression was carried out at two different times. Total D1 mRNA (including both short and long messengers) was

identified by using a previously standardized semiquantitative PCR procedure in which an amplicon of the structural protein cyclophilin (Cyc) was co-amplified (Aceves & Rojas-Huidobro 2001). To verify the presence of the long form of D1 mRNA a PCR amplification was carried out for 40 cycles with oligonucleotides whose sequences are only present in the 3'UTR of the long mRNA form. The sizes of the resultant PCR fragments were 250 bp for total D1, 136 bp for long mRNA D1 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 was taken, the pictures were digitized using a Hewlett Packard Scanner Jet 11CX, and the signals were analyzed by an edited version of the NIH-image program. Values obtained were normalized according to the Cyc mRNA levels detected in each sample. Three different RNA samples for each group were analyzed.

Determination of malic enzyme activity Malic enzyme activity (L-malate-NADP+ oxidoreductase (decarboxylating), EC 1.1.1.40) was determined in liver cytosolic fractions according to the method reported by Wise & Ball (1964). The conversion of malate to pyruvate and CO₂ was coupled to the reduction of NADP+ and recorded at 340 nm.

Statistical analysis

Data from the control, the 24 h fast and RFS groups are reported as means $\pm$ S.E.M. Differences between groups and time points were tested with a two-way ANOVA (feeding schedule $\times$ time of day), followed by a Tukey's multiple-comparison post-hoc test, with α set at $P < 0.05$. Statistical analysis was performed with the statistical package Statistica (Version 4.5, 1993).

Results

Serum THs profile

Serum levels of T3 and T4 did not show any changes in control and experimental groups (Fig. 1). A 24 h fast did not alter the circulating T3 or T4 concentrations. As a consequence of the invariability in T3 and T4 serum levels, the ratio of both thyronines did not change in any experimental condition.

D1 enzyme

Liver D1 activity in rats fed freely was similar at the three times studied. Twenty-four hour fasted animals showed a slight but significant decrease (23%) in comparison with control animals. D1 activity in the RFS group was even

Figure 1 TH serum levels (T4 and T3) of rats exposed during 3 weeks to an RFS (food presentation from 1100 to 1400 h) and their respective freely fed controls. Food presentation from 1200 to 1400 h. Experimental groups were killed before (0800 h), during food AA (1100 h) and 2 h after the meal (1400 h). The control group with a 24 h fast was killed at 0800 h. Values represent means $\pm$ S.E.M., $n=5$. No significant differences were detected.

lower at 0800 and 1100 h – before feeding – than animals fed freely or fasted, but at 1400 h – after food presentation – showed a dramatic increase reaching a value even higher than in control freely fed rats (Fig. 2, upper panel). To explore whether the changes observed in D1 activity for all experimental groups were due to an increase in transcriptional activity, the amounts of total and long D1 mRNAs were quantified. Figure 2 middle panel shows staining of gels with the corresponding amplicon for total D1 and visualized using ethidium bromide. The long D1 was undetected in all the samples even with a second round of amplification, indicating that total D1 mRNA corresponded only to the short messenger form. Data showed that only the group of fasting animals exhibited a significant decrease in the messenger. In contrast, all rats in the RFS group presented a similar D1 mRNA signal with no apparent changes at any of the times of sample processing. Figure 2 lower panel shows D1 mRNA signals analyzed by densitometry and normalized to the structural protein Cyc.

Malic enzyme activity

To test whether the changes in D1 activity found in the RFS group had some impact in hepatic T3-dependent metabolic responses, we measured the activity of the malic enzyme. The Fig. 3A shows that malic enzyme activity in animals freely fed remained constant during the three

Figure 2 Comparison of D1 activity and mRNA amounts for rats after a 24 h fast and 3 weeks with an RFS as well as their respective freely fed controls. Upper panel, D1 activity, $n=5$. Middle panel, ethidium bromide-stained gel showing RT-PCR products for Cyc (510 bp) and total D1 (250 bp) after 32 cycles of amplification. RT-, RNA sample and the appropriate oligonucleotides primers, but no reverse transcriptase; H₂O, water with all PCR reagents. Lower panel, quantification by densitometry of a Polaroid negative from an ethidium bromide-stained gel. The experiments were repeated three times with independent RNA samples and the values were normalized with Cyc RNA amplicon (D1/Cyc). Values represent means $\pm$ S.E.M. Different superscripts indicate significant differences between groups ($P<0.05$).

sampled time points, but presented a significant decrease in rats with a 24 h fast. The RFS group at 0800 and 1100 h – before feeding – showed a similar decrease in malic enzyme activity to that obtained in the 24 h fast group. However, the RFS group at 1400 h – after feeding – presented a significant increase in this enzyme activity, even above the values observed in rats fed freely. Figure 3B depicts a significant correlation between D1 and malic enzyme activities taking into consideration all the experimental groups in our protocol.

Discussion

Based on the hypothesis that FEO function involves a metabolic and/or neural communication between periph-

Figure 3 (A) Malic enzyme activity of rats exposed during 3 weeks to an RFS (food presentation from 1100 to 1400 h) and their respective freely fed controls. Experimental groups were killed before (0800 h), during the food AA (1100 h) and 2 h after the meal (1400 h). The control group with a 24 h fast was killed at 0800 h. Values represent means $\pm$ S.E.M., $n=5$. Different superscripts indicate significant differences between groups ($P<0.05$). (B) Correlation between D1 and malic enzyme activities. The correlation was analyzed by plotting the corresponding activity values for each enzyme: D1 (Fig. 2, upper panel) and malic enzyme (A). r =correlation coefficient, $P<0.005$.

eral organs and selective brain areas (Stephan 2002), our group has shown that liver presents adaptations in two of the principal parameters that regulate intermediate metabolism, namely energy charge and redox state in food-restricted rats (Díaz-Muñoz *et al.* 2000). In the present study, we examined the effect of food restriction and a 24 h fast on thyroid economy, one of the most influential endocrine factor in the hepatic metabolic status (Feng *et al.* 2000, Flores-Morales *et al.* 2002). Our findings corroborate that liver is capable of distinguishing both regimens and that subsequent metabolic responses include a differential deiodinative pattern. Neither of these regimens modified thyronine circulating levels, however, the RFS exerted a dramatic change in liver D1 activity between

fast/food periods. These results are in agreement with previous reports showing that the AAs secondary to RFSs exhibit specific regulatory parameters of liver metabolism (Díaz-Muñoz *et al.* 2000) and support the notion that the physiological state of rats under an RFS is unique and distinct from rats fed freely or fasted for 24 h (Persons *et al.* 1993). Our results confirm previous data on fasting regimens showing that although changes in systemic thyroid status are evident until 48 or 72 h after fasting, liver D1 activity and mRNA content start to decrease within 24 h or less (Reimers *et al.* 1986, O'Mara *et al.* 1993, Kmiec *et al.* 1996). The clear dissociation between constant D1 mRNA content and the significant changes in D1 activity during an RFS suggest the occurrence of translational or post-translational mechanisms. Moreover the fact that our assay *in vitro* was carried out with optimal DTT saturation discounts the possibility that changes in D1 activity were due to a limitation of thiol cofactor availability and indicates that these adjustments involve other modifications, i.e. changes in mRNA transcription velocity, protein stability, or installation of some covalent mechanism such as phosphorylation or methylation (Mori *et al.* 1996, Roger *et al.* 1997). Moreover, the lack of signal in the long D1 mRNA amplification for all the samples is in agreement with previous work where we demonstrated that the large mRNA form was expressed only in metabolic overdemand situations like chronic hyperthyroidism, lactation or adrenergic stimulation (Aceves & Huidobro 2001), and it suggests that in normal or fasting regimens D1 enzyme is encoded by the short messenger. The low D1 activity measured before food presentation (0800 and 1100 h) was coincident with a significant increase in corticosterone serum levels (Díaz-Muñoz *et al.* 2000), whereas the enhancement in D1 activity after feeding (1400 h) was concurrent with a peak in serum insulin (Díaz-Muñoz *et al.* 2000). These correlations could be part of a possible mechanism to account for the changes in D1 activity in RFS rats, since Davies *et al.* (1996) and Sato *et al.* (1983) reported that hepatic D1 activity is decreased by glucocorticoid administration and stimulated by insulin treatment respectively. The demonstration in this study that liver D1 adjustments could be entrained with RFSs strongly supports the notion that considers the liver a peripheral integrator of nutrient availability (Escobar *et al.* 1998), and suggests that local T3 production could be operated as a gatekeeper toward anabolic or catabolic processes. Malic enzyme is a lipogenic enzyme that provides most of the NADPH necessary for *de novo* synthesis of long-chain fatty acids (Goodridge 1989) and T3 is one of their primary regulators by increasing the level of its mRNA (Goodridge & Adelman 1976). Thus, the concurrent pattern of D1 and malic enzyme activities observed during RFSs suggests the occurrence of a 'hypothyroidal state' in the liver at the times previous to food presentation, whereas a 'hyperthyroidal state' seems to arise as a consequence of food intake. The hepatic

'hypothyroidal state' is coincident with our previous reports showing a decrease in glycogen, enhancement in ketone body production, oxidized redox state in both cytosol and mitochondria, and an increase in ATP and adenine nucleotide levels. In contrast, the 'hyperthyroidal state' is accompanied by deposition of glycogen, and ketone body formation, shift of the cytoplasmic and mitochondrial redox states toward a reduced state, and a decrease in ATP levels (Díaz-Muñoz *et al.* 2000). In summary, our data suggest a possible role of hepatic D1 enzyme coordinating a liver homeorhetic state when this organ participates in FEO expression, and emphasize an important issue to be elucidated, that of the mechanism by which an RFS is able to coordinate the response among peripheral organs and selected brain areas to elicit behavioral and physiological FEO expression.

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