FEBS Letters 579 (2005) 4654–4658 FEBS 29831

Suppression of hypothalamic deiodinase type II activity blunts TRH mRNA decline during fasting

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Received 11 July 2005; revised 15 July 2005; accepted 17 July 2005

Available online 2 August 2005

Edited by Barry Halliwell

Abstract Fasting is characterized by disrupted thyroid feedback, with suppressed levels of thyroid hormones and paraventricular thyrotropin releasing hormone (TRH). We found that third ventricle administration of the deiodinase inhibitor, iopanoic acid, dose-dependently reduced deiodinase type II (DII) activity selectively in the hypothalamus. This suppression of DII by iopanoic acid during fasting prevented elevated DII activity and blunted the decline in hypothalamic TRH mRNA levels. Because fasting-induced elevation in hypothalamic DII activity is paralleled by increased hypothalamic T3 concentration, our study suggests that T3 formation by DII in the hypothalamus is the cause of disrupted thyroid feedback during fasting.

Keywords: Hypothalamus; Deiodinase type II; Fasting; Enzymatic activity; Iopanoic acid

1. Introduction

Hypothyroidism leads to a change in the activity of iodothyronine deiodinases, enzymes that are necessary for activation and inactivation of circulating thyroid hormones. The regulation of these enzymes occurs in a tissue-specific manner [1]. Deiodinase type II (DII), which converts T4 to the active thyroid hormone, T3, is present in the central nervous system [2,3], pituitary [4], brown adipose tissue [5], and placenta [6] and it shows increased activity when plasma T4 declines. It has been suggested that the major role of DII is to maintain T3 homeostasis producing adequate intracellular levels of T3 in order to ensure all T3-dependent cellular functions in the tissue [7,8].

Hypothyroidism due to failure of the thyroid gland induces a rise in hypothalamic TRH levels [9], which, in turn, triggers release of thyroid stimulating hormone (TSH) from the anterior pituitary. This classic negative feedback of the thyroid axis is paradoxically reversed during fasting whereby suppressed circulating T4 levels coincide with suppressed production and release of TRH in the hypothalamic paraventricular nucleus and median eminence [10]. The central mechanism that underlies the emergence of this apparent paradox in thyroid feedback is not known.

We have previously shown [11,12] that an increase in the enzymatic activity and mRNA levels of arcuate nucleus DII occurs during short term fasting. T4 replacement in fasted animals did not reverse the increase in DII activity and mRNA levels in the hypothalamus. Instead, DII activity during fasting appears to be controlled by the inverse shift of circulating leptin and corticosterone concentrations [12]. Thus, it is conceivable that the increased DII activity during fasting underlies elevated local T3 production (in spite of the systemic hypothyroidism), which, in turn, could trigger suppressed TRH production and release. This study was undertaken to test this hypothesis.

2. Materials and methods

2.1. Experiment 1

Ten Sprague–Dawley male rats (200–250 g BW; Taconic Farms, Inc.) were used. Each animal was implanted with one cannula into the third ventricle (Bregma −0.8 mm) connected to a micro-osmotic pump (Alzet Corp., Palo Alto, CA; 1.0 μl/h for 3 days). Animals were divided into two experimental groups: a group ($n = 5$) infused with 0.9% saline and fed ad libitum, and a second group ($n = 5$) infused with 0.9% saline and fasted for 48 h. Animals were infused for the duration of the experiment. Rats were sacrificed and the hypothalamus was collected and immediately frozen and stored at −80 °C until the activity measurement was performed. The enzymatic activity was carried out as previously described [12].

2.2. Experiment 2

Sixteen Sprague–Dawley male rats (200–250 g BW; Taconic Farms, Inc.) were used. Each animal was implanted with one cannula into the third ventricle (Bregma −0.8 mm) connected to a micro-osmotic pump (Alzet Corp., Palo Alto, CA; 1.0 μl/h for 3 days). Animals were divided into four experimental groups: a control group ($n = 4$) infused with 0.9% saline, a second group ($n = 4$) infused with 10^{-7} M iopanoic acid (IOP; TCI America) diluted in 0.9% saline, a third group ($n = 4$) infused with 10^{-5} M IOP diluted in 0.9% saline, and a fourth group ($n = 4$) infused with 10^{-3} M IOP diluted in 0.9% saline. Animals were infused for the duration of the experiment. Twenty-four hours after implantation, all animals were food-deprived for 48 h. Animals were then sacrificed and the hypothalamus, hippocampus, cerebellum, and pituitary were collected and immediately frozen and stored at −80 °C until the activity measurement was performed. The enzymatic activity was carried out as previously described [12].

We define an undetectable level of DII enzymatic activity as a sample in which the amount of iodine released did not differ from the blank control (where the homogenate was omitted).

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.07.035

2.3. Experiment 3

Twenty Sprague–Dawley male rats (200–250 g BW; Taconic Farms, Inc.) were used for this experiment implanted with a cannula into the third ventricle (Bregma −0.8 mm) as described above. Animals were divided into two experimental groups: a control group ($n = 10$) was infused with 0.9% saline while the second group ($n = 10$) was infused with 10^{-7} M IOP (TCI America) diluted in 0.9% saline (Alzet micro-osmotic pump 1 μl/h, 3 days). All the animals were infused for the duration of the experiment. Twenty-four hours after the implantation of the pump, the animals were further divided in the following groups: $n = 5$ infused with 0.9% saline and fed ad libitum; $n = 5$ infused with 0.9% saline and fasted for 48 h; $n = 5$ infused with 10^{-7} M IOP and fed ad libitum; $n = 5$ infused with 10^{-7} M IOP and 48 h fasted. The animals were then perfused and processed for semi-quantitative in situ hybridization histochemistry.

An 826 bp fragment of complementary DNA (cDNA) of TRH was amplified based on the RT-PCR reaction, using specific oligonucleotide primers derived from the coding region of the rat TRH sequence [13]. Total RNA was extracted from the hypothalamus by guanidium thiocyanate–phenol–chloroform method using TriZol reagent (Life Tecnologies, Grand Island, NY) and transcribed using the first-strand cDNA Synthesis Kit (Pharmacia Biotech, Piscataway, NJ). PCR reaction was carried out using the following protocol: 3 μg cDNA template, 0.5 μM primers, 1.25 mM $MgCl_2$, 80 μM dNTP and 2 U *Taq* DNA polymerase. The resulting fragment, purified from agarose gel using QIA quick Gel Extraction Kit (QIAGEN Inc.), was digested with *Eco*RI and *Bam*HI, inserted in pBluescript vector (Stratagene, La Jolla, CA). Linearized DNA was transcribed using SP6 polymerase (antisense cRNA probe) and T7 polymerase (sense cRNA probe; Riboprobe Combination System SP6/T7, Promega Corporation, Madison, WI) and labeled with ^{35}S-UTP (Amersham; 10 mCi/ml). The hybridization was carried out as previously described [11].

The density of the hybridization product was assessed in the different experimental groups. In order to digitally analyze, quantitate and compare the amount of TRH mRNA, an Image-1/AT image processor (Universal Imaging Corporation, West Chester, PA) using an Olympus IMT-2 inverted microscope with dark field optics (Olympus Corporation, Lake Success, New York) and a Hamamatsu CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) was employed. Six sections per animal were selected from the same area to assess the intensity of the hybridization product. The total surface covered by the hybridization product was assessed within a test region measuring 2×10^5 mm^2 that contains the paraventricular nucleus. The threshold for measurement was assessed for each slide by determining the background labeling in the nearby ventromedial nucleus.

2.4. Experiment 4

Twenty male Sprague–Dawley rats (200–250 g BW; Taconic Farm, Inc.) were used in this study. Each animal was implanted with one cannula into the third ventricle (Bregma −0.8 mm) connected to a micro-osmotic pump (Alzet Corp., Palo Alto, CA; 1.0 μl/h for 3 days). Animals were divided into four experimental groups: group 1 ($n = 4$) and group 2 ($n = 4$) infused with 0.9% saline, group 3 ($n = 4$) and group 4 infused with 10^{-7} M IOP (TCI America) diluted in 0.9% saline. Rats were infused for the duration of the experiment. Twenty-four hours after implantation, groups 2 and 4 were food-deprived for 48 h. All animals were then sacrificed and the hypothalamus was collected and immediately frozen and stored at −80 °C.

Triiodothyronine (T3) was extracted from the hypothalamus by adding methanol 95% containing PTU 10^{-4} M. Tissues were homogenized and centrifuged at 13 000 rpm and the pellets re-suspended twice using methanol solution. The supernatants were evaporated to dryness and resuspended in GAB buffer (0.2 M glycine/0.13 M acetate with 0.02% BSA). T3 was determined by radioimmunoassay system (RIA). Samples and standard curve were incubated at 4 °C with polyclonal antibody against T3 (Fitzgerald Industries International, Concord, MA) in RIA buffer GAB. Three days later, 10 000 cpm of radiolabeled [^{125}I] T3 (Specific activity 2200 Ci/mmol; Perkin–Elmer Life Sciences, Boston MA) was added to each tube. After two days of incubation, a rabbit gamma globulin (Jackson ImmunoResearch Laboratories) diluted in 0.1 M EDTA and 16% polyethylene glycol with goat antirabbit IgG (Antibodies Incorporated, Davis CA) in GA buffer (0.2 M glycine/0.13 M acetate) was added to precipitate the antibody–T3 complex. After centrifugation the precipitates were counted in a γ-counter. T3 is expressed in picogram per milligram of weight tissue.

2.5. Statistical analyses

Means were compared between experimental groups using one-way analysis of variance (ANOVA) with mean comparisons by the Student–Newman–Keuls method. A level of confidence of $P < 0.05$ was used to determine significant differences.

3. Results

3.1. Experiment 1

As previously reported by our group [11,12], fasting in saline infused animals (96.33 ± 1.69 fmol/h/mg protein) increased DII enzymatic activity compared to ad libitum fed rats (79.75 ± 1.55 fmol/h/mg protein; Fig. 1).

3.2. Experiment 2

The intracerebroventricular infusion of IOP in fasted rats showed that IOP at a concentration of 10^{-7} M can selectively inhibit the activity of DII in the hypothalamus without interfering with its activity in other brain regions or in the pituitary (Table 1). In the hypothalamus, IOP infusion inhibits DII activity at all three concentrations used (undetectable levels), while in the pituitary, as well as in the cerebellum, a partial inhibition (about 45% and 58%, respectively) occurs at a concentration of 10^{-5} M, and a total inhibition occurs at 10^{-3} M (Table 1). In the hippocampus, only the 10^{-3} M concentration induces a complete inhibition of DII activity, while all of the other concentrations had no effect on the enzyme function (Table 1).

3.3. Experiment 3

As previously shown in fasted compared to fed rats, in situ hybridization for TRH mRNA in the paraventricular nucleus of the hypothalamus of saline infused rats showed that fasting induces a decrease in TRH mRNA levels (OD = 1801 ± 177) compared to fed control animals (OD = 2850 ± 50; $P < 0.05$; Fig. 2). When the animals were treated with IOP, fasting blunted the decrease in TRH mRNA levels (OD = 2293 ± 178) compared to that of IOP-treated fed animals (OD = 2577 ± 123; $P > 0.05$; Fig. 2). In fed groups, IOP treatment slightly but not significantly decreased TRH mRNA levels compared to the saline-treated rats ($P > 0.05$). On the other hand, in IOP-treated fasted rats the levels of TRH mRNA was statistically higher than the levels of the saline-treated fasted group ($P < 0.05$).

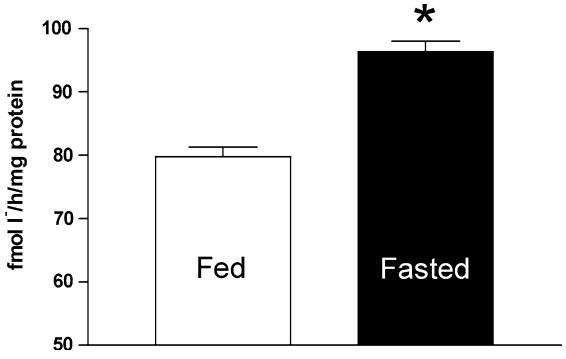

Fig. 1. Graph showing hypothalamic DII activity in ICV saline-treated rats that were either fed ad libitum ($n = 5$) or fasted for 48 h ($n = 5$). Results are expressed as means ± S.E.M. *$P < 0.001$.

Table 1
DII activity measurements (expressed in fmoles I^-/h/mg protein) in the pituitary, hypothalamus, hippocampus and cerebellum of third ventricle implanted rats ($n = 4$ for each group) with saline, 10^{-7}, 10^{-5} and 10^{-3} M iopanoic acid (IOP)

	Saline	10^{-7} M IOP	10^{-5} M IOP	10^{-3} M IOP
Pituitary	1014 ± 39.37	1077.5 ± 13.44	453.67 ± 30.36[a,b]	ND[a,b,c]
Hypothalamus	96.33 ± 1.69	ND[a]	ND[a]	ND[a]
Hippocampus	68.0 ± 3.0	72.67 ± 1.15	80.33 ± 6.75	ND[a,b,c]
Cerebellum	53.67 ± 5.25	50.33 ± 3.18	31.0 ± 5.77[a,b]	ND[a,b,c]

Results are expressed as means ± S.E.M.
ND, not detectable.
[a] $P < 0.05$ compared to the saline control.
[b] $P < 0.05$ compared to the 10^{-7} M IOP.
[c] $P < 0.05$ compared to the 10^{-5} M IOP.

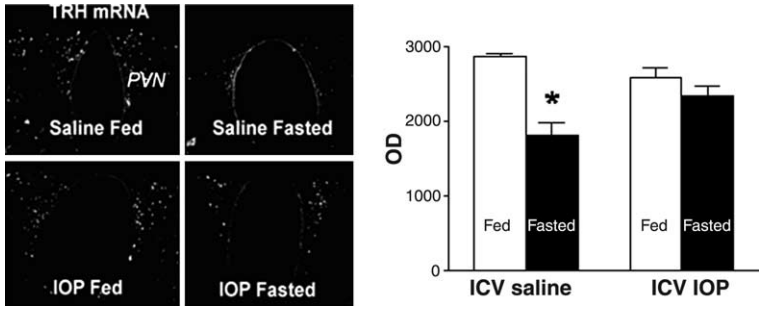

Fig. 2. In situ hybridization for TRH mRNA in the PVN revealed that while fasting suppressed TRH mRNA levels in the saline-treated animals ($P < 0.05$), ICV IOP (dissolved in saline) injections blunted the fasting-induced decline in TRH mRNA levels of the PVN. Results are expressed as means ± S.E.M. *$P < 0.05$ compared to saline-treated fed, IOP-treated fed and fasted rats. $P > 0.05$ between fed saline and fed IOP-treated rats. OD, arbitrary optical density.

3.4. Experiment 4

In the present as well as previous studies, we showed [11,12] that during food deprivation DII activity levels are increased in the hypothalamus. To assess whether this elevation in activity also induces increased tissue levels of T3, we performed T3 measurements in the hypothalamic tissue of fasted and fed animals. Moreover, to determine whether the effect of IOP in blunting TRH mRNA decline during fasting is due to a change in the tissue T3, we assessed hypothalamic T3 levels in IOP infused rats that were either fasted or fed ad libitum. Hypothalamic T3 levels were significantly higher in saline-treated fasted rats (2.32 ± 0.13 pg/mg wet tissue) compared to the saline-treated ad libitum fed animals (1.71 ± 0.03 pg/mg wet tissue; Fig. 3). On the other hand, in IOP-infused rats, food deprivation did not affect hypothalamic T3 levels (1.81 ± 0.03 pg/mg wet tissue) and was not significantly different from the levels of the fed animals (1.95 ± 0.04 pg/mg wet tissue). No significant differences ($P > 0.05$) in T3 levels were found between the fed saline-treated, fed IOP-treated and fasted IOP-treated rats.

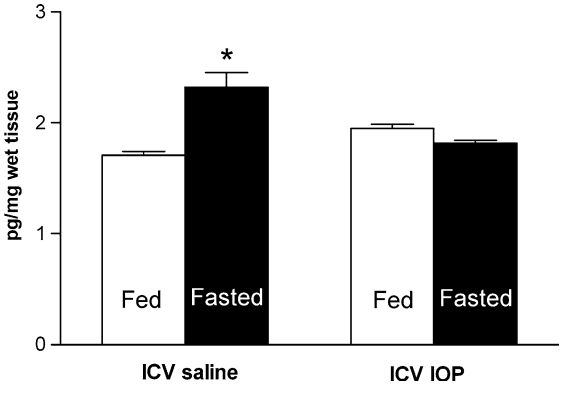

Fig. 3. Graph showing hypothalamic T3 levels in fed and fasted rats after ICV injection of saline or IOP ($n = 4$ for each group). Results are expressed as means ± S.E.M. *$P < 0.05$ compared to saline-treated fed, IOP-treated fed and fasted rats. $P > 0.05$ between fed saline, fed IOP and fasted IOP-treated rats.

4. Discussion

The results of this study suggest that activation of hypothalamic DII during fasting contributes to elevated local T3 production, which, in turn, could trigger suppression of TRH mRNA levels in the paraventricular nucleus. Although IOP treatment did not reduce hypothalamic T3 levels in fed animals, it did prevent T3 elevation induced by fasting. We found that in fed animals treated with IOP hypothalamic T3 levels were slightly elevated compared to the saline-treated fed animals and this could be the reason for the slight suppression of TRH levels in the IOP-treated fed group. While it may appear to be a paradox, we suggest that the elevated hypothalamic T3 levels in IOP-treated fed group could be due to increased transport of circulating T3 to the hypothalamus (rather than local formation). However, fasting-induced DII increase was blocked by IOP hence the diminished suppression of TRH.

To block DII activity, we employed IOP. IOP has been previously shown to be a potent competitive inhibitor of DII in vivo as well as in vitro [14–17].

In our study, we observed that IOP had a differential effect in all brain areas examined. We propose that this differential effect is the consequence of the accessibility of IOP. Because the cannulae in the third ventricle were positioned in the

anterior portion of the hypothalamus (Bregma −0.8), it is reasonable to hypothesize that the strongest effect of IOP at the lower dose (10^{-7} M) was found in the hypothalamus, compared to the hippocampus, cerebellum and pituitary gland.

In the central nervous system, the majority of the active form of thyroid hormone, T3, derives from the intracellular 5′-monodeiodination of T4 by type II 5′-monodeiodinase (DII) [18,19]. DII activity measurements in rat brain have shown the highest enzymatic activity in the arcuate nucleus/median eminence (ARC/ME) fragments of the hypothalamus, while a minimal activity has been found in the paraventricular nucleus (PVN), where TRH-containing cells are located [2]. The appearance of DII mRNA in the ependymal zone and the ME [3], together with our previous observation of glial fibrillary acidic protein (GFAP) in cells expressing DII mRNA [20], an earlier report [21] on the expression of DII in glial cells in neonatal rat brain and our recent finding of DII immunoreactivity in the hypothalamus [22] strongly indicate that DII producing cells are astrocytes and tanycytes. These glial cells provide an extensive network of cellular processes in the ARC [23,24] and suggest a paracrine action on PVN-projective ARC neurons via the production of thyroid hormones.

We have recently shown [12] that during food deprivation, the increased DII activity is due to an inverse shift in circulating levels of corticosterone and leptin. Now we showed that increased DII activity during fasting triggers elevated local T3 levels and suppression of DII activity by IOP prevents this elevation. Furthermore, IOP infusion prevents fasting-triggered decrease in TRH mRNA levels. Thus, we propose that this previously unsuspected existence of hypothalamic hyperthyroidism in the face of systemic hypothyroidism during fasting may be responsible for the decline of TRH mRNA levels and for the reversal of negative feedback of the thyroid axis.

The arcuate nucleus has been found to contain an abundant population of thyroid receptor-producing neuronal nuclei [25], as well as populations producing various regulatory peptides and neurotransmitters such as neuropeptide Y, opioid peptides, growth hormone releasing hormone and dopamine, all of which are known to influence the production and release of TRH [26–28]. We have demonstrated the existence of a monosynaptic pathway between the arcuate nucleus that contains DII-producing glial cells, and the paraventricular TRH neurons that project to the median eminence with direct access to fenestrated capillaries [20]. In addition, we [29] and others [30] have shown that the arcuate nucleus NPY/AgRP neurons provide a massive inhibitory input on TRH cell bodies and proximal dendrites via symmetric synapses. Another study [31] also reported that TRH cells are symmetrically contacted by nerve terminals containing AgRP, which is co-produced in the NPY arcuate neurons. In the present study we showed that activation of DII induces alterations in arcuate nucleus T3 levels and that may be responsible for the decrease in TRH mRNA levels during fasting. Thus we hypothesize that the increased in T3 levels during food deprivation could affect and altered neuropeptide expression in leptin-responsive arcuate neurons that strongly project to paraventricular TRH neurons. Studies are underway to delineate this signaling modality in the hypothalamic regulation of the thyroid axis.

Acknowledgments: This work was supported by NIH Grant DK 061619 (to S.D.). We thank Jayodita Sanghvi for her technical assistance.

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