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Biosynthesis of proTRH-derived peptides in prohormone convertase 1 and 2 knockout mice

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ABSTRACT

Prohormone convertases (PCs) 1 and 2 are the primary endoproteases involved in the post-translational processing of proThyrotropin Releasing Hormone (proTRH) to give rise to TRH and other proposed biologically active non-TRH peptides. Previous evidence suggests that PC1 is responsible for most proTRH cleavage events. Here, we used the PC1 and PC2 knockout (KO) mouse models to examine the effects of PC1 or PC2 loss on proTRH processing. The PC1KO mouse presented a decrease in five proTRH-derived peptides, whereas the PC2KO mouse showed only lesser reduction in three TRH (Gln-His-Pro), TRH-Gly (Gln-His-Pro-Gly), and the short forms preproTRH$_{178–184}$ (pFQ$_7$) and preproTRH$_{186–199}$ (pSE$_{14}$) of pFE$_{22}$ (preproTRH$_{178–199}$). Also, PC1KO and not PC2KO showed a decrease in pEH$_{24}$ indicating that PC1 is more important in generating this peptide in the mouse, which differs from previous studies using rat proTRH. Furthermore, downstream effects on thyroid hormone levels were evident in PC1KO mice, but not PC2KO mice suggesting that PC1 plays the more critical role in producing bioactive hypophysiotropic TRH. Yet loss of PC1 did not abolish TRH entirely indicating a complementary action for both enzymes in the normal processing of proTRH. We also show that PC2 alone is responsible for catalyzing the conversion of pFE$_{22}$ to pFQ$_7$ and pSE$_{14}$, all peptides implicated in regulation of suckling-induced prolactin release. Collectively, results characterize the specific roles of PC1 and PC2 in proTRH processing *in vivo*.

1. Introduction

Most neuropeptide hormones are initially synthesized as large inactive precursors. These precursors are posttranslationally processed to form smaller bioactive products that are stored in secretory vesicles and released when the cell receives specific stimuli [2,31]. Rat PreproTRH, one of the best-studied precursors, is a 29 kDa protein composed of 255 amino acids [processing reviewed by 15, 16, 25]. The N-terminal 25 amino acid signal peptide is first cleaved upon entrance to the endoplasmic reticulum to create proTRH. ProTRH contains 5 copies of the TRH progenitor sequence Gln-His-Pro-Gly flanked by paired basic amino acids (Lys-Arg or Arg-Arg), four nonTRH peptides occurring between the TRH progenitors, an N-terminal flanking peptide, and a C-terminal flanking peptide [21,32]. The N-terminal flanking peptide (preproTRH$_{25–50}$-R-R-preproTRH$_{53–74}$) is further cleaved at the C-terminal side of an arginine pair site to produce preproTRH$_{25–50}$ and preproTRH$_{53–74}$. The final result is a total of seven proTRH-derived peptides [13,21]. TRH is a critical regulator of thyroid hormone. Thyroid hormone, the peripheral end product of the hypothalamic (TRH)-pituitary (TSH)-thyroid (T3/4) (HPT) axis, is a key stimulator of energy expenditure, largely by increasing basal metabolic rate in cells through the action of T3 on the uncoupling protein-3 (UCP3) [*e.g.* 12,28]. This regulation takes place through release of hypophysiotropic TRH from neurons situated in the medial and periventricular parvocellular subdivisions of the paraventricular (PVN) nucleus of the hypothalamus through their axons to the portal vessels in the median eminence (ME) [27]. TRH released into circulation stimulates TSH secretion from the pituitary that in turn stimulates the synthesis and release of thyroid hormone the active thyroid hormone being triiodothyronine (T3). Our early findings on the processing of proTRH show that endoproteolytic cleavages to generate biologically active TRH occurs at paired basic residues by the action of primarily PC1 (also referred to as PC3 [33]) and secondarily by PC2 [7,18,19,30]. Following cleavages catalyzed by PC2, carboxypeptidase E (CPE) removes the basic residue(s) [10]. Gln-His-Pro-Gly is then amidated by the action of

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peptidylglycine alpha-amidating monooxygenase (PAM), which uses the C-terminal Gly as the amide donor, and the Gln residue undergoes cyclization to a pGlu residue to yield TRH [6,25].

Although the preponderance of our understanding of proTRH processing has come from research conducted in the rat, the mouse offers a unique advantage in genetic knockout technology. Mouse preproTRH shares about 88% homology with the rat preproTRH and has 256 amino acids as well as 5 copies of the TRH progenitor sequence [29]. However, little is known about the impact that loss of PC1 and/or PC2 has on proTRH processing or the thyroid axis. Therefore, the present study examined the biosynthesis and processing of hypophysiotropic proTRH in PC1 and PC2 KO mouse models compared with wild-type mice. We have analyzed the proTRH-derived peptide content within the paraventricular hypothalamic region by HPLC fractionations followed by specific radioimmunoassays (RIAs) against TRH, TRH-Gly and end products of proTRH processing from the N- and C-terminal side of the prohormone.

2. Materials and methods

2.1. Mice

PC1KO mice were developed by the deletion of exon 1 and several putative upstream transcriptional control elements (CRE, ICS, GHF-1, AP-1, and Sp1) from the PC1 (PCSK1) gene [8] as originally described in [39]. PC2KO mice was generated from a strain of mice lacking active SPC2 by introducing the neomycin resistance gene (Neor) into the third exon of the mSPC2 [9]. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Institute of The Howard Hughes Medical Institute, University of Chicago.

2.2. Tissue collection and extraction

Mice were euthanized using an intraperitoneal injection of sodium pentobarbital (80 mg/kg) followed by decapitation. The PVN was rapidly microdissected and placed in 0.15 ml of 2 M acetic acid containing a protease inhibitor cocktail (P8340; Sigma–Aldrich St. Louis, MO). Samples were then boiled at 100 °C for 10 min, homogenized, sonicated, and centrifuged at 16,000 × *g* for 30 min at 4 °C to retrieve the supernatant for subsequent HPLC/RIA analyses.

2.3. Antibodies for proTRH-derived peptides

The antibodies used in the current study were anti-TRH, anti-TRH-Gly, anti-pFE$_{22}$, anti-pEH$_{24}$ (preproTRH$_{83–106}$) and anti-pLE$_{26}$ (preproTRH$_{25–50}$) against rat. Epitopes for the antibodies used in the present studies were generated against specific portions of the rat proTRH. Each antibody was developed in house and each antibody has been used successfully in previous studies to determine proTRH processing [*e.g.* 30]. The specific sequences and the percent homology of each sequence compared with the corresponding proTRH sequence in mouse are described in Table 1. A comparison of the full-length preproTRH in rat and mouse is depicted in Fig. 1. Also, Fig. 2 illustrates the proposed model of rat proTRH processing using *in vitro* and primary neuronal cultures [16].

2.4. RIAs

PVN extracts were subjected to HPLC fractionation followed by RIA to measure TRH, TRH-Gly, pFE$_{22}$, pEH$_{24}$ and pLE$_{26}$ as previously described [18,21,30]. Six animals per group were used for analyses of all proTRH-derived peptides. In addition to RIAs to measure proTRH-derived peptides, an RIA for the active thyroid hormone T3 was performed on serum taken from PC1KO, PC2KO, and their respective wild-type control mice. The T3 RIA was conducted using a kit from MP Biomedicals (06B-256447; Solon, OH) according to manufacturers guidelines. Four to six animals per group were used for T3 analysis. All RIAs for were performed using the same sample volume and in duplicate or triplicate.

Table 1
Epitope/amino acid sequence recognized by the antibodies used to detect proTRH-derived peptides and the percent homology to the corresponding sequence in mouse. Numbers refer to the amino acids of the proTRH sequence (see Fig. 1). As TRH and TRH-Gly are repeated within the sequence, specific amino acids used to generate each antibody are listed. The TRH antibody was generated against the cyclized form of the peptide. The antibodies listed have been used previously to measure specific proTRH-derived peptides in the rat [1].

Antibody	Epitope/amino acid sequence	Percent homology to mouse sequence
Anti-pEH24	83–106	78
Anti-pFE22	178–199	86
Anti-pLE26	25–50	88
Anti-TRH-Gly	QHPG	100
Anti-TRH	QHP	100

3. Results

3.1. PC1 is critical for the maturation of proTRH and production of T3

In our early studies, we showed that PC1 is the primary processing enzyme involved in the maturation of proTRH [30]. However, we did not know whether this was also true *in vivo*. Therefore, we tested the production of proTRH-derived peptides in wild type compared to PC1KO mice and determined whether the production of T3 was affected. Results depicted in Fig. 3 follow the proposed model for proTRH-derived peptides generated by PC1 cleavage (Fig. 2). There was a 79.6 and 68.3% reduction in the PC1KO mouse for TRH and TRH-Gly, respectively. Despite this dramatic reduction, the production of mature TRH was not completely blocked in the PC1KO mouse, which can be partially compensated by PC2 as demonstrated in our early studies [30]. Calculation of the percent change in each peptide (in pmol) between wild type and PC1KO mice revealed that all peptides analyzed decreased by more that 60% with the greatest decrease in pFE$_{22}$ both the short (86.6%) and extended (82.2%) forms (see Fig. 2). Previous studies suggest that proTRH is rapidly processed because the ratio of TRH to other proTRH-derived peptides is typically between 2:1 and 3:1 rather than the theoretical 5:1 [18,19]. Here, we show this pattern is also true for both the wild type and PC1KO mouse models. For example, the molar ratio of TRH:pLE$_{26}$ in wild type mice was 2.38 and in PCIKO mice the molar ratio of TRH:pLE$_{26}$ was 2.64. There was, however, an ~35.9% reduction in the molar ratio of TRH:TRH-Gly in PC1KO (1.34) compared to wild type (2.09) mice suggesting that the amidation of TRH-Gly was affected in the PC1KO mice. We also wanted to explore whether changes in PVN proTRH processing in the absence of PC1 would coincide with a change in circulating thyroid hormone concentrations. We found that the PC1KO mouse had significantly lower serum T3 compared to its wild type control (Fig. 5). Altogether, these data validate the importance of PC1 in proTRH processing.

3.2. PC2 contributes to proTRH processing and converts pFE22 to pFQ7 and pSE14

Having determined the role of PC1 in proTRH processing using the PC1KO mouse; we then used the same approach to determine the role of PC2 in proTRH processing using the PC2KO mouse. Loss of PC2 resulted in a decrease in both TRH (44%) and TRH-Gly (44%; Fig. 4), yet not to the same extent as was observed in the PC1KO