Regulation of regional expression in rat brain PC2 by thyroid hormone/characterization of novel negative thyroid hormone response elements in the PC2 promoter

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Shen, Xiaoxiong, Qiao-Ling Li, Gregory A. Brent, and Theodore C. Friedman. Regulation of regional expression in rat brain PC2 by thyroid hormone/characterization of novel negative thyroid hormone response elements in the PC2 promoter. Am J Physiol Endocrinol Metab 288: E236–E245, 2005; doi:10.1152/ajpendo.00144.2004.—The tissue-specific endoproteolytic processing of prohormone precursors within the secretory pathway. We previously showed that changes in thyroid status altered pituitary PC2 mRNA and that this regulation was due to triiodothyronine-dependent interaction of the thyroid hormone receptor (TR) with negative thyroid hormone response elements (nTREs) contained in a large proximal region of the human PC2 promoter. In the current study, we examined the in vivo regulation of brain PC2 mRNA by thyroid status and found that 6-n-propyl-2-thiouracil-induced hypothyroidism stimulated, whereas thyroxine-induced hyperthyroidism suppressed, PC2 mRNA levels in the rat hypothalamus and cerebral cortex. To address the mechanism of T3 regulation of the PC2 gene, we used human PC2 (hPC2) promoter constructs transiently transfected into GH3 cells and found that triiodothyronine negatively and 9-cis-retinoic acid positively regulated hPC2 promoter activity. EMSAs, using purified TRα1 and retinoid X receptor-β (RXRβ) proteins demonstrated that TRα bound the distal putative nTRE-containing oligonucleotide in the PC2 promoter, and RXR bound to both nTRE-containing oligonucleotides. EMSAs with oligonucleotides containing deletion mutations of the nTREs demonstrated that the binding to TR and RXR separately is reduced, but specific binding to TR and RXR together persists even with deletion of each putative nTRE. We conclude that there are two novel TRE-like sequences in the hPC2 promoter and that these regions act in concert in a unique manner to facilitate the effects of thyroid hormone and 9-cis-retinoic acid on PC2.

posttranslational processing; hypothyroidism; prohormone convertase; pituitary; regulation

A VARIETY OF REGULATORY PEPTIDES AND PROTEINS are generated from inactive precursors by endoproteolytic processing. The majority of prohormones are cleaved at paired basic residues to generate bioactive hormones in a cell-specific manner by prohormone convertases (PCs), members of the mammalian family of the subtilisin-like endoproteases (47, 54). Seven members of the PC family have been cloned: both PC1 and PC2 are found exclusively in neural and endocrine cells equipped with a regulatory secretory pathway (10, 18, 49, 50), PC4 is found in the testis (37), and the other PCs are ubiquitously distributed (46, 48). PC1 and PC2 process a variety of prohormones and prohormone precursors, including proopiomelanocortin (POMC), prosomatostatin, provasopressin, prorenotropin, pro-thyrotropin-releasing hormone (pro-TRH), and pro-opiomelanocortin peptide (4, 9, 14, 15, 17, 57).

The important role of PC1 and PC2 in hormonal biosynthesis has been elucidated by the studies of PC1- and PC2-null mice and in a patient with defective PC1. PC1-null mice are short because of impaired pro-growth hormone-releasing hormone processing and are hyperglycemic because of impaired proinsulin processing (60, 61). PC2-null mice have absent proglucagon processing leading to hypoglycemia and also have impaired proinsulin processing (16). POMC, proenkephalin, and prodynorphin processing are blunted (1, 26). A patient lacking PC1 had severe childhood onset obesity, postprandial hypoglycemia, infertility, and low levels of ACTH and cortisol with elevated levels of POMC (23, 40). The findings that the knockout mice have processing profiles that are expected from the known biochemistry of PC1 and PC2 support the belief that these two enzymes are necessary and sufficient for the biosynthesis of most hormones/neuropeptides.

The tissue distribution of PC1 and PC2 differs in that PC1 is present in higher amounts in the anterior lobe, whereas higher levels of PC2 are found in the intermediate lobe (3, 18, 50). PC1 is present in higher amounts in pancreatic β-cells, whereas PC2 is more abundant in α-cells (38). Both PC1 and PC2 are widely distributed in the brain, with higher levels of PC2 in most regions (44). Rat intermediate lobe PC1, PC2, and POMC mRNA levels increased with treatment with the dopamine antagonist haloperidol and decreased with the dopamine agonist bromocriptine (3, 4, 18, 50). This regulation was at the level of transcription and involved the intracellular cAMP pathway (24). Regulation of processing enzymes by thyroid status has been briefly studied in rat pituitary. Rats made hypothyroid by thyroidectomy or 6-n-propyl-2-thiouracil (PTU) treatment had an increase in anterior pituitary PC1, PC2 (10), and peptidylglycine α-amidating monooxygenase (PAM; a processing enzyme involved in amidation) mRNA levels (41) and a decrease in paired basic amino acid converting enzyme 4 (PACE4; a more ubiquitously distributed PC) mRNA levels (25). Rats made hyperthyroid by daily injection of thyroxine (T4) showed decreased anterior pituitary PC1, PC2, and PAM mRNA levels and increased PACE4 mRNA levels (10, 25).

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The regulation of many thyroid-responsive genes such as TRH, thyroid-stimulating hormone (TSH), growth hormone, and α-myosin heavy chain by thyroid hormone is via nuclear thyroid hormone receptors (TRs) at the transcriptional level (5, 6, 8, 29, 36). Triiodothyronine (T₃) mediates transcriptional regulation through interactions in the promoter region of target genes bearing consensus DNA sequences, referred to as thyroid hormone response element (TRE) (27). Heterodimerization between TR and retinoid X receptor (RXR) usually augments the ligand-dependent stimulation or repression (27). However, RXR-independent mechanisms may also be involved in thyroid hormone regulation, such as in the TRE in the human type 1 deiodinase promoter (56).

We have previously shown that T₃ negatively regulates both PC1 and PC2 in the rat anterior pituitary and in rat somatotrope cells (GH3 cells) (30, 31). We also localized regions on both human promoters that contain putative negative TREs (nTREs) (30, 31). In the current paper, we first studied the in vivo regulation of PC2 mRNA by thyroid status in different neuropeptide-rich rat brain regions using in situ hybridization and real-time PCR. To address the mechanism of T₃ regulation of the PC2 gene at the promoter level, we used GH3 cells, a rat somatotrope cell line expressing endogenous TRs (21) and PC2 promoter. Electrophoretic mobility shift assays (EMSAs) were performed with the use of oligonucleotides containing two putative nTREs in the hPC2 promoter to further localize putative nTREs.

MATERIALS AND METHODS

Animals and treatments. Adult male Sprague-Dawley rats (250–270 g) were housed in a room with controlled light, temperature, and humidity. Three groups of six animals each received treatment as follows: in the control group, implantation of a placebo pellet with normal chow; in the hyperthyroid group, implantation of a thyroid hormone (T₄; 1-thyroxine) pellet (15 mg) (Innovative Research of America, Sarasota, FL) with normal chow; in the hypothyroid group, added to rat serum was 80–100%. Serum TSH assays were performed without knowledge of the treatment status of the rat.

The rat serum TSH levels were determined with the use of a highly sensitive double antibody method developed by A. F. Parlow [Director of the National Hormone & Pituitary Program, Harbor-University of California Los Angeles (UCLA) Medical Center, Torrance, CA], similar to that described for measuring mouse TSH (45). The assay used highly purified rat TSH as the iodinated ligand, a guinea pig anti-rat TSH at a final tube dilution of 1:500,000 as the primary antibody, and a partially purified extract of rat pituitary containing TSH as the reference preparation. Cross-reactivity of either highly purified rat FSH or rat LH in this mouse TSH RIA was <1%. Displacement curves obtained by testing sera of hypothyroid rats in graded dilutions did not depart significantly from parallelism with displacement curves for the reference preparation. The sensitivity of the assay is such that TSH levels in hyperthyroid and euthyroid animals are distinguishable. Recovery of exogenous rat TSH activity added to rat serum was 80–100%. Serum TSH assays were performed according to the basis of published cDNA sequences (4, 58). The sequences of the primers were as follows: 5'-agatccggaaagctc-3' (PC2 sense), 5'-ccgtggaacgaatttgc-3' (PC2 antisense), 5'-gtccacaeacgtctt-3' (GAPDH sense), and 5'-ggagcatggaagatgctt-3' (GAPDH antisense). Quantitative measurement of PC2 and GAPDH cDNA using total RNA was performed by the QuantiTect SYBR Green RT-PCR system according to the manufacturer’s instruction (Qiagen). The real-time cycler conditions were as follows: reverse transcription at 50°C for 30 min, and, after denaturation at 95°C for 15 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturation at 95°C for 15 s and annealing and extension at 58°C for 1 min. The accumulating DNA products were monitored by the iCycler iQ optical system (Bio-Rad, Hercules, CA), with the data being stored continuously during the reaction. Product purity was confirmed by dissociation curve analysis and agarose gel electrophoresis in the presence of ethidium bromide. The calculations of the initial mRNA copy numbers in each sample were made according to the cycle threshold method (33).

Labeling of cRNA probes and in situ hybridization. For in situ hybridization, a construct containing bases 227–708 of rat PC2 (4) in pBluescript II SK-vector (Strategene, La Jolla, CA), a generous gift from Dr. Richard Mains (Univ. of Connecticut), was used. Antisense and sense RNA probes were generated by transcription with T7 and T3 RNA polymerase, respectively, with a DIG DNA Labeling Kit (Roche Molecular Biochemicals, Indianapolis, IN). In situ hybridization was performed as previously described (52) with slight modifications. Briefly, brain tissue was embedded in paraffin, and microtome sections of 6 µm were obtained. After the paraffin was removed by treatment with xylene, sections were digested with proteinase K, postfixed with 4% paraformaldehyde, washed in phosphate buffer, and treated with 0.2 M HCl. The sections were hybridized overnight at 42°C and then treated with ribonuclease A for 30 min. In situ...
hybridization signals were detected immunohistochemically with alkaline phosphatase-conjugated anti-digoxigenin (DIG) antibody according to the manufacturer’s instructions (DIG Nucleic Acid Detection Kit, Roche). Quantitation of the signal was performed using ImagePro Plus software (Media Cybernetics, Silver Spring, MD).

EMSAs. EMSAs were performed as described previously (31) with double-stranded oligonucleotide probe corresponding to the hPC2 promoter regions containing consensus TRE half-sites previously identified as conferring negative regulation by thyroid hormone (31). These oligonucleotide probes correspond to the hPC1 promoter sequence, from +43 to +76 bp (wild type 1; WT1) and from +117 to +137 bp (wild type 2; WT2). Mutations in the nTREs were generated with the mutated DNA containing a restriction site as follows: gtcgac (Sal I) replaced nucleotides +60 to +65 of TRE1 (TRE Mut1), and ggatcc (Bam HI) replaced nucleotides +118 to +123 of TRE2 (TRE Mut2) (see Fig. 4A). All of the oligonucleotide probes were radiolabeled with [γ-32P]ATP (6,000 Ci/mmol; ICN, Costa Mesa, CA) and RXRB (0.5 μg; Biomol, Plymouth Meeting, PA) proteins in reaction buffer [25 mM HEPES, 0.5 mM EDTA, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg of poly(dI-dC)] for 10 min on ice, and, subsequently, the incubation was continued with radiolabeled DNA probes for 30 min. For antibody supershift experiments, specific antibodies were added to mixtures 10 min after the addition of the radiolabeled DNA probes. Protein-DNA complexes were analyzed on 6% polyacrylamide gels that were run using 25 mM Tris, 190 mM glycine, and 1 mM EDTA buffer and were visualized by autoradiography.

Statistical analyses. One-way ANOVA with Dunnett’s post hoc t-tests was used. Significance was at P < 0.05.

RESULTS

Changes in PC2 mRNA expression in rat hypothalamus, hippocampus, and cortex by thyroid status. Our laboratory (31) has recently shown that PTU-induced hypothyroidism stimulated and thyroid hormone (T3)-induced hyperthyroidism suppressed PC2 mRNA levels in the rat anterior pituitary. In the current study, we extended these experiments to examine the thyroid hormone regulation of PC2 mRNA levels in several neuropeptide-rich brain regions. Rats were made hypothyroid and hyperthyroid by a 3-wk procedure slightly modified from our prior protocol (30); hypothyroidism was induced with low-iodine-PTU chow instead of drinking water containing PTU, and hyperthyroidism was induced by the implantation of sustained-release T4 pellets instead of injection of T3. The hypothyroidism of PTU-treated rats was confirmed by demonstrating increased plasma TSH levels in the PTU-treated rats (18.9 ± 2.6 mU/l, mean ± SE) vs. control rats (4.7 ± 2.7 mU/l, P < 0.0001). The hyperthyroidism of T4-treated rats was confirmed by demonstrating decreased plasma TSH levels (0.57 ± 0.04 mU/l, P < 0.005, compared with controls). There was no overlap in TSH levels between the euthyroid and hypothyroid animals or between the euthyroid and hyperthyroid animals.

With the use of quantitative real-time RT-PCR analysis, the data in Fig. 1 show that, in hyperthyroid rats, hypothalamic PC2 mRNA levels were downregulated by 50% by T4 treatment compared with control euthyroid animals (P < 0.05). Also, in the cortex, PC2 mRNA levels were suppressed to ~60% (P < 0.05) of those of control animals. However, no significant change was detected in the mRNA levels of PC2 in hippocampus between T4-treated rats and control rats. On the other hand, in hypothyroid rats, a significant increase in PC2 mRNA levels in hypothalamus (2-fold stimulation) and cerebral cortex (2-fold stimulation) compared with control euthyroid animals (P < 0.005) was observed, whereas no significant differences were detected in the mRNA levels of PC2 in hippocampus between PTU-treated rats and control rats. These results suggest that, for PC2 expression, the hypothalamic nuclei and cerebral cortex are responsive to thyroid status.

Localization of PC2 mRNA expression in the rat hypothalamus, cortex, and hippocampus. Thyroid hormones are essential for control of a variety of metabolic and developmental processes in particular brain regions (2). To determine the localization of PC2 mRNA in neuropeptide-rich brain regions by thyroid status, we used in situ hybridization with a nonradioactive cRNA probe to examine PC2 expression in more detail. Using a PC2 sense probe, we detected no signal in the hypothalamus, cortex, and hippocampus (data not shown). However, we found that PC2 mRNA was ubiquitously distributed and abundant in neuropeptide-rich brain regions using a PC2 antisense probe (Fig. 2A). PC2 was highly expressed in fields CA1, CA2, and CA3 of the hippocampus, with lower expression in the dentate gyrus, but its expression was not altered by thyroid status (data not shown). PC2 mRNA was abundant in the cerebral cortex, with highest concentrations observed in the retrosplenial agranular cortex. The in situ hybridization pattern in rat cortex was similar in all three...
treatment groups; however, the PTU-treated rats had higher PC2 expression than the control rats, and the T4-treated rats had lower PC2 expression than the control rats. Additionally, PC2 mRNA expression in the hypothalamus was found in periventricular, paraventricular, medial preoptic, and suprachiasmatic nuclei, and the lateral hypothalamic area, with the highest expression in the basal hypothalamus. A similar PC2 mRNA expression pattern was observed in all three groups in the hypothalamus, but the expression in PTU-treated rats was higher and in T4-treated rats was lower. Overall, PC2 expression in the different brain regions was more intense than for PC1 (51), with the exception of lower PC2 staining in the dentate gyrus. Quantitation of mRNA levels in the three brain regions is depicted in Fig. 2.

T3 and 9-cis-RA regulate hPC2 promoter activity preferentially through nTRE2. We previously studied a series of deletion transient transfection assays in GH3 cells in the presence or absence of T3. The minimal construct, −44 to +137 bp, exhibited both basal and T3-regulated luciferase activity (31). EMSAs with purified TRα1 and RXRβ protein showed that regions from +51 to +71 bp and from +118 to +137 bp of this promoter bind to TRα1 as both a monomer and a homodimer and with TRα1/RXRβ as a heterodimer. We have extended our previous mapping of the two nTREs (now termed nTRE1 and nTRE2) by performing mutational analysis. Our experience with the negative thyroid hormone regulation of the hPC1 promoter, in which mutation of the central nucleotides of the nTRE half-site led to reduced but still significant inhibition by T3 (30), led us to design mutants of the hPC2 promoter in which all six nucleotides of each nTRE half-site were replaced by a restriction site (this also facilitated screening of the mutants) (Fig. 3A). In the current series of experiments, we tested the regulation by T3, 9-cis-RA, or all-trans-RA of the hPC2 promoter construct from −226 to +137 bp with either the nTRE1 or the nTRE2 intact or mutated (Fig. 3, B–D). In confirmation of the prior results from our laboratory (31), T3 reduced PC2 promoter activity in the wild-type construct by ∼50% (P < 0.05) (Fig. 3B). Mutation of nTRE1 (PC2 Mut1) alone only partially (∼25%) reduced the T3 inhibition of hPC2 promoter luciferase activity (P < 0.05, compared with no T3) (Fig. 3B). However, mutation of the nTRE2 region (PC2 Mut2) abolished the T3 inhibition [P = not significant (NS), com-

Fig. 2. A: in situ localization of PC1 mRNA in rat hippocampus, cortex, and hypothalamus of control, T4-treated, and PTU-treated rats, using nonradioactive digoxigenin (DIG)-labeled antisense cRNA probes. PC1 mRNA levels in the hippocampus were not altered by thyroid status. Pe, periventricular hypothalamic nucleus; Pa, paraventricular hypothalamic nucleus; MPO, medial preoptic area; 3V, 3rd ventricle; f, fornix; LPO, lateral preoptic area; CA1, -2, and -3: field CA of hippocampus; RS, retrosplenial granular cortex; M, motor cortex; S1, primary somatosensory cortex. Scale bars = 100 μm. B: quantitation with ImagePro Plus software of PC2 mRNA of cortex, hypothalamus, and hippocampus of euthyroid, hypothyroid, and hyperthyroid animals. *P < 0.05 and **P < 0.01 vs. control.
pared with no T₃. As expected, mutation of both nTRE1 and nTRE2 (dMut) also showed no regulation by T₃ treatment (Fig. 3B). Mutation of the nTRE2 region (PC2 Mut2) and both elements (dMut) (both P < 0.05, compared with wild type) but not nTRE1 (PC2 Mut1) showed diminished activity in the absence of T₃ (Fig. 3B), indicating that nTRE2 is needed for activity in the absence of ligand. It is also worth noting that all hPC2 promoter constructs had >100 times the luciferase activity of the promoterless control construct, indicating that all hPC2 mutants exhibited substantial promoter activity. These results indicate that nTRE2 is primarily responsible for both the basal activity and the negative regulation by T₃ on the hPC2 promoter.

In contrast to T₃ treatment, 9-cis-RA treatment resulted in significant stimulation of the wild-type hPC2 promoter (P < 0.05), similar to the previous results of our laboratory (31). However, in constructs with mutations of either nTRE1 (PC2 Mut1) or nTRE2 (PC2 Mut2) alone or together (PC2 dMut), 9-cis-RA did not stimulate promoter activity compared with no 9-cis-RA (P = NS). These data indicate that the nTRE1 and nTRE2 regions are both important cis-acting DNA elements in the hPC2 promoter that facilitate transcriptional regulation of PC2 by 9-cis-RA.

On the other hand, when all-trans-RA (10⁻⁷ M) was added to wild-type or mutant hPC2 promoter-transfected GH3 cells, no stimulatory effects were noted (Fig. 3D). These results indicate that the retinoic acid receptor (RAR) does not interact with this region of the PC1 promoter.

EMSAs with wild-type and mutant oligonucleotides with TRα1 and RXRβ proteins. To further determine whether TR/RXR binding sites of the hPC2 promoter can uniquely interact with TRα1 and RXRβ protein and whether T₃ can cause a change in protein binding to these regions, we performed EMSA experiments using radiolabeled oligonucleotide probes corresponding to two regions of the hPC2 promoter that each contain a nTRE half-site (Fig. 4A). Corresponding mutant probes contained the six oligonucleotides of the nTRE half-site replaced with a restriction site. In all experiments, the amount of probe added to each lane was normalized by the number of counts. As indicated in Fig. 4B, when TRα1 was added to the reactions with the Wt1 probe, a TRα1 homodimer and a higher weight multimer were detected (lane 2). The addition of RXRβ protein to the Wt1 probe led to an intense RXR homodimer band (lane 3). The addition of RXRβ protein to TRα1 and the Wt1 probe led to a strong RXR homodimer and a TR plus RXR heterodimer complex (lane 4). In contrast to the Wt1 probe, the Mut1 had decreased TR homodimer binding (lane 5), decreased RXR homodimer binding (lane 6), and a modest decrease in the TR plus RXR heterodimer complex (lane 7). Interestingly, we observed that Wt2 had almost no binding with TRα1 and lower binding to RXRβ (lanes 8 and 9). When RXRβ and TRα1 were added to the Wt2 probe, a clear dimer

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Fig. 3. Effect of triiodothyronine (T₃), 9-cis-retinoic acid (9-cis-RA), and all-trans-retinoic acid (all-trans-RA) on wild-type (Wt) or deleted mutant (Mut) hPC2 promoter (hPC2) constructs. TRE, thyroid hormone response element. A: schematic representations of hPC2 luciferase constructs (−226 to +137). Shown are wild type (PC2 Wt), deletion mutant TRE1 (PC2 Mut1), deletion mutant TRE2 (PC2 Mut2), and double mutant TRE1 and TRE2 (PC2 dMut). B: GH3 cells transfected with Wt or Mut hPC2 promoter luciferase construct and treated with or without T₃ (10⁻⁶ M) for 24 h. Data from triplicate measurements are expressed as means ± SE. *P < 0.05 vs. without T₃. **P < 0.05 vs. without T₃. C: GH3 cells transfected with Wt or Mut hPC2 promoter luciferase construct and treated with or without 9-cis-RA (10⁻⁷ M) for 24 h. Data from triplicate measurements are expressed as means ± SE. *P < 0.05 vs. without 9-cis-RA. D: GH3 cells transfected with Wt or Mut hPC2 promoter luciferase construct and treated with or without all-trans-RA (10⁻⁷ M) for 24 h. Data from triplicate measurements are expressed as means ± SE.
was detected corresponding to the RXR homodimer and TR plus RXR heterodimer (lane 10). Mut2 did not appear to bind to TR/H9251 (lane 11) and had much lower binding to RXR/H9252 (lane 12) and TR/H9251 plus RXR/H9252 (lane 13).

To confirm the specificity of the interactions between the RXRβ protein and the hPC2 promoter probe, we tested the effect of 9-cis-RA and T3 using EMSAs. As shown in Fig. 4C, 9-cis-RA increased the binding of RXRβ alone to the hPC2 probe (compare lane 3 with lane 1). Similarly, 9-cis-RA increased the binding of RXRβ plus TR to the hPC2 probe (compare lane 4 with lane 2). In contrast, T3 did not affect the binding of RXRβ to the hPC2 probe (compare lane 5 with lane 1). These results confirm the specificity of the binding between RXRβ and the hPC2 promoter and agree nicely with our studies showing that 9-cis-RA increases PC2 promoter activity (Fig. 3C).

To test whether the binding of the TRα1/RXRβ proteins to the hPC2 promoter probe was specific, we examined the effects of T3 or TRα1 and RXRβ antibodies on the protein-probe complex (Fig. 4D). As can be seen in lanes 1 and 2, both TRα1 (small arrow) and RXRβ (large arrow) form homodimer bands with the Wt1 probe, with stronger binding with RXRβ compared with TRα1. When TRα1 and RXRβ are added to Wt1, the band complex formed (indicated by the larger arrow) most likely represents binding of an RXRβ homodimer and a TRα1 plus RXRβ heterodimer to the probe (lane 3). The addition of T3 to the Wt1 probe in the presence of TRα1 plus RXRβ caused a dramatic reduction in this complex (lane 4). The addition of anti-TRα1 antibody reduced the TR homodimer band but had no effect on the complex (lane 5), whereas the addition of anti-RXRβ antibody reduced the complex band of TRα1 plus RXRβ and led to a higher weight complex (super-
We wanted to investigate the in vivo regulation of PC2 mRNA by thyroid status in various regions of the rat brain where proneuropeptide processing would occur to complement an earlier study of PC1 regulation by thyroid status (51). In the present study, we found that basal brain expression of PC2 was of a similar pattern as that reported by Schafer et al. (44). We then demonstrated that PTU-induced hypothyroidism stimulated and T₄-induced hyperthyroidism suppressed PC2 mRNA expression in the rat hypothalamus and cerebral cortex, as measured by real-time PCR and localized by in situ hybridization. We have identified brain regions in which PC2 expression is influenced by thyroid status and other areas, such as the hippocampus, in which PC2 expression is not influenced by thyroid status. The mechanism underlying these findings may be differential expression of TR coactivators and corepressors in these regions. The consequence of brain region PC2 sensitivity or resistance to thyroid status is not known. Increased PC2 activity in the hypothalamus, as a result of hypothyroidism, may function to increase the conversion of pro-TRH to active TRH, stimulating TSH production in the pituitary.

We also studied the role of RXR in PC2 expression. It is known that thyroid hormone and RXR can act in concert to mediate T₃ and RXR regulation. This was confirmed in the present study by demonstrating that basal brain expression of PC2 in RXR⁺/⁻ mice was greater than in Wt mice. This indicates that both TR and RXR act in concert to mediate PC2 expression. The mechanism underlying these findings may be differential expression of TR coactivators and corepressors in these regions. The consequence of brain region PC2 sensitivity or resistance to thyroid status is not known. Increased PC2 activity in the hypothalamus, as a result of hypothyroidism, may function to increase the conversion of pro-TRH to active TRH, stimulating TSH production in the pituitary.

It is known that DNA binding is needed for thyroid hormone negative regulation by thyroid hormone, a recent study demonstrated that PC2 is one negatively regulated by T₃ in the cortex. Positive TREs generally are composed of paired hexameric half-sites [(A/G)GGT(C/G)A] (27, 39). In addition, immediate flanking sequences of hexameric half-sites may also modulate TR-DNA interactions (56). The configuration of promoters negatively regulated by thyroid hormone through nTREs is largely unknown. Although both DNA binding-dependent and -independent mechanisms have been proposed for negative regulation by thyroid hormone, a recent study demonstrated that DNA binding is needed for thyroid hormone negative regulation.

**Figure 5.** Model of the binding of negative (n)TRE1 and nTRE2 on the PC2 promoter to RXR and TR and subsequent effects on PC2 promoter activity. Size of font for TR and RXR indicates relative strength of binding. Downward arrows, inhibiting promoter activity. Upward arrows, stimulating promoter activity. Number of arrows indicates magnitude of effect on promoter activity.
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regulation of genes involved in the hypothalamic-pituitary-thyroid axis (53). Most nTREs identified so far, such as those in mouse and human TRH (28, 43), rat sodium, potassium ATPase (55), TSH β-subunit, and glycoprotein α-subunit (7), exhibit variable half-site sequences that may or may not contain consensus TRE sequences.

We previously identified two potential nTRE half-sites on the PC2 promoter (31). We hypothesized that the negative transcription mediated by T3 on this region likely involves one or both of these two putative nTRE half-sites and in this paper demonstrated that the +60 to +65 region (nTRE1) appears to bind more strongly to both RXR and TR than the +128 to +133 region (nTRE2).

Recent studies have demonstrated that RXR heterodimerization can augment T3-mediated gene regulation to increase TR DNA interactions (11, 19). However, the magnitude of the augmentation varies significantly, especially when different cell lines were used. Both 9-cis-RA and T3 stimulated rat growth hormone promoter, and the combination of T3 and 9-cis-RA exerted additive effects on this promoter in GH3 cells (31). In our study, using the hPC2 promoter transiently transfected into GH3 cells, we found that T3 negatively regulated hPC2 promoter activity, whereas 9-cis-RA stimulated the activity, indicating that binding to RXR abrogates rather than augments the effects of T3 on TR. All-trans-RA had no effect on promoter activity, indicating that RAR does not interact with this region of the PC1 promoter. The nTRE2 region was more important for mediating basal expression (in the absence of T3) and the negative effects of T3 on PC2 promoter activity than nTRE1, although both of these regions were equally important for mediating the positive effects of 9-cis-RA on PC2 promoter activity. We interpret the difference between stronger binding of nTRE1 to TR and RXR, but the more pronounced role of nTRE2 in mediating the negative effects of T3 on the PC2 promoter activity, as indicating that activators mediating signaling from the nTRE2 site binding to TR and/or repressors mediating signaling from the nTRE1 site render the nTRE2 region more important for promoter activity. Similarly, although RXR binds more strongly to nTRE1 than nTRE2, both sites contribute approximately equal amounts to the effect of 9-cis-RA on PC2 promoter activity. The effect of nTRE2 binding to TR and signaling the negative effect of T3 on the PC2 promoter activity likely reflects the physiological relevance of thyroid status, while 9-cis-RA is likely to be a modulator and not a primary regulator. Although the interactions between TR and RXR and these two response elements are likely to be complex, we propose a model integrating the divergent effects on binding and promoter activity in Fig. 5.

In summary, this study demonstrates abundant expression of PC2 mRNA in rat brain and shows that thyroid status regulates PC2 expression in the hypothalamus and cortex. These results suggest that this prohormone convertase is involved in neuropeptide precursor processing not only in pituitary but also in neuropeptide-rich brain regions. We also demonstrated that the nTRE1 appears more important for binding to TR, yet nTRE2 appears more important for mediating the negative effects of T3 on PC2 promoter activity. We speculate that alterations of PC2 activity by thyroid hormone may mediate some of the pathophysiological consequences of hypo- or hyperthyroidism.

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