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Differential and Interactive Effects of Ligand-Bound Progesterone Receptor A and B Isoforms on Tyrosine Hydroxylase Promoter Activity

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Abstract

The classical progesterone receptors (PRs) are expressed in some hypothalamic dopaminergic and brainstem noradrenergic neurons. Progesterone influences prolactin and luteinising hormone release from the anterior pituitary gland, in part by regulating the activity of these catecholaminergic neurons. The aim of this study was to determine the effects of PRs on tyrosine hydroxylase (TH) promoter activity. When CAD, SK-N-SH and CV-1 cells were transfected with TH promoter constructs and PR-A or PR-B expression vectors, progesterone treatment caused three- to six-fold increases in TH-9.0kb promoter activity in PR-B expressing cells, but a modest increase or no change in PR-A expressing cells. Using CAD cells, deletional analysis mapped the site of PR action to the –1403 to –1304 bp region of the TH promoter. Mutational analysis of putative regulatory sequences in this region indicated multiple DNA elements are required for complete PR-B transactivation. Electrophoretic mobility shift assays were unable to demonstrate direct PR-B binding to TH promoter DNA sequences. However, chromatin immunoprecipitation (ChIP) analysis indicated PR-B was recruited to the TH promoter. Two different PR-B DNA binding domain mutants had opposite effects on PR-B mediated TH promoter activation. A GS to AA mutation located in the p-box of the first zinc finger of PR-B inhibited progesterone transactivation of the TH promoter, whereas a C to A mutation in the zinc finger increased transactivation. PR-A was able to inhibit PR-B transactivation in a dose-dependent manner, although the degree of PR-A inhibition was dependent on the TH promoter deletion construct. These data indicate that ligand-bound PR-B is recruited to DNA elements in the TH promoter and acts as a transcriptional activator of the TH gene and that changes in the ratio of PR-A to PR-B may affect the ability of progesterone to increase TH expression.

Keywords

catecholamine; tyrosine hydroxylase; progesterone receptor; gene expression; transcriptional regulation; steroid hormones

Introduction

Progesterone influences selective dopamine and norepinephrine neuronal groups involved in reproduction. Most notably, the tuberoinfundibular dopaminergic neurons in the arcuate nucleus of the hypothalamus act to regulate prolactin secretion from the anterior pituitary gland. (1, 2). These neurons express oestradiol-inducible progesterone receptor (PR) and

progesterone can have both stimulatory and inhibitory actions on their activity, resulting in altered prolactin secretion (3–8). Progesterone treatment increases tyrosine hydroxylase (TH) mRNA levels in the arcuate nucleus of oestradiol-treated ovariectomized female rats and the progesterone receptor antagonist RU486 blocks this stimulatory effect of progesterone (7). These data suggest a stimulatory action on TH gene expression through the classical nuclear PR. The inhibitory effect of progesterone is observed on the afternoon of pro-oestrus, with the initial response of decreased TH phosphorylation state in the median eminence followed by decreased TH mRNA levels in the arcuate nucleus (8–10). The stimulatory effect of progesterone on TH content is not limited to the hypothalamic neurons, as progesterone increases TH content in the olfactory bulb (11). Some brainstem noradrenergic neurons project to the hypothalamus/ preoptic area and are associated with increased luteinising hormone release (12–15). Noradrenergic neurons, particularly those located within the nucleus tractus solitarius (A2 neurons), express oestradiol-inducible PR (16). Progesterone can modulate norepinephrine turnover in hypothalamic areas (17, 18), although it is unknown if these changes are associated with TH gene expression in brainstem noradrenergic groups.

TH catalyzes the initial and rate-limiting step in catecholamine biosynthesis and is highly regulated by short-term direct control of enzyme activity and medium- to long-term changes in gene expression (19, 20). The complexity of transcriptional control is substantiated by the multiple DNA regulatory elements in the 5' region upstream of the transcription start site (19). Some steroid hormones, including glucocorticoids, oestrogens, androgens, and retinoic acid, acting through their respective receptors, influence TH promoter activity (21–24), although little is known about progesterone action on the TH promoter.

Progesterone can act through the classical nuclear receptors or the more recently identified membrane receptors and membrane components (25–29). The classical nuclear receptors are composed of PR-A and PR-B isoforms. These two isoforms are expressed from a single gene through two distinct promoters (30). The PR-A isoform lacks the activation function-3 domain and can inhibit PR-B transactivation of specific promoters (31–33). The ratio of PR-A and PR-B will often determine progesterone-mediated actions (33, 34). The aims of this study were to 1) determine the independent effects of PR-A and PR-B on TH promoter activity, 2) map the site(s) of PR action on the TH promoter, 3) evaluate PR-B binding to DNA elements and 4) determine the interactive effects of PR-A and PR-B on TH promoter activity.

Materials and Methods

Cell line maintenance

SK-N-SH and CV-1 (ATCC, Manassas, VA, USA) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cath.a differentiated CAD cells were described previously (35) and maintained in DMEM:F-12 (1:1) media supplemented with 8% FBS and penicillin/streptomycin. All cells were maintained in 37°C humidified incubator at 5% CO₂.

PR and TH promoter constructs

Rat PR-B was cloned by PCR from rat ovary and rat pheochromocytoma PC-12 cell-line cDNA. The rat PR-B cDNA showed 100% homology to Genbank HM037361.1 sequence and was ligated into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). Rat PR-A was generated by utilizing a BamHI restriction site located between the PR-B and PR-A translation start sites. The PR-DNA binding domain (DBD) mutants GS to AA and C to A mutations were generated by PCR. These mutations occur at the underlined amino acids of

the highly conserved GSCKV (amino acids 578–582 of rat PR-B) sequence located within the PR-DBD and have been described elsewhere (34, 36). Rat TH promoter luciferase constructs TH-2.4, TH-5.6, and TH-9.0 were generated by restriction digests of the TH-9.0 choline acetyl transferase (CAT) construct (37), and ligated into PGL3 basic vector (Promega, Madison, WI). TH-1.8, TH-1.6, TH-1.4, TH-1.35, TH-1.3, TH-1.25, TH-1.20, TH-0.773, and TH-0.272 constructs were generated by PCR amplification from rat genomic DNA or the TH-2.4 construct. All TH promoter constructs span to the +27 position of the TH gene and were subcloned into PGL3 basic. The 2X progesterone response element (2xPRE) luciferase construct described previously (38) was obtained from Addgene (Cambridge, MA). Sequence identities for all constructs were verified by DNA sequencing on a Beckman Coulter CEQ 8000.

Cell transfection and luciferase assay

Cells were plated at a density of 75,000 cells/ well on 24-well plates, and were transfected with 500 ng PR-B or PR-A, 500 ng TH promoter and 25 ng CMV-Renilla luciferase (to normalise transfection efficiency) by the calcium phosphate method. Deviations from these transfected amounts are indicated in figure legends or results section. Eighteen hours later, media was replaced with serum free media containing 1% ITS universal culture supplement premix (BD Biosciences, Bedford, MA) for CAD cells or media containing 10% charcoal-stripped FBS for CV-1 and SK-N-SH cells. Four hours later, cells were treated with 0.1% ethanol (vehicle) or 200 nM progesterone for 20 h. Luciferase assays were performed with the Promega Dual-Luciferase Reporter Assay System following manufacturer's protocol using a Bio-Tek Clarity dual injector luminometer (Bio-Tek Inc., Winooski, VT).

Western blotting

Transfected CAD cells were collected and lysed in buffer containing 150 mM sodium chloride, 50 mM Tris (pH=7.5), 1.0% Triton X-100, 1.0 mM EDTA, 1 mM sodium fluoride, aprotinin (10 µg/ml), leupeptin (10 µg/ml) and pepstatin A (10 µg/ml). Fifteen micrograms of lysates were separated on 7.5% SDS-PAGE gels and subjected to western blot analysis as described previously (10), with minor modifications and using anti-PR (1:2000, Dako, Carpinteria, CA) and anti-tubulin (1:2500, Millipore, Billerica, MA) antibodies.

Immunofluorescence

CAD cells (400,000 cells/ 35 mm dish) were transfected with 2.5 µg PR-A or PR-B. Cells were treated 200 nM progesterone for 20 h. Cells were fixed in 4.0% paraformaldehyde, permeabilised in 100% methanol and incubated with rabbit anti-PR (Dako) followed by Alexafluor 596-conjugated goat anti-rabbit IgG (Invitrogen). DNA was stained with Hoescht Dye 33258 (1 µg/ml). Cells were visualised with an Olympus BW50 fluorescence microscope using a 60× water objective.

Protein isolation and electrophoretic mobility shift assay (EMSA)

Whole cell extracts were prepared by transfecting CAD cells (1×10^6 cells/ 100 mm plate) with 10 µg of pcDNA3 or PR-B constructs for 18 h. Twenty-four hours after transfection, cells were treated with 200nM progesterone for 2 h. Cells were then collected and incubated in lysis buffer for 30 min on ice. Lysates were cleared by centrifugation and protein concentration was determined. ³²P-labeled dsDNA probes were generated by PCR and the sequences are as follows:

PRE control (39):

5'-TGATCTTTGAGAACAACTGTTCTTAAACGAGGATCA-3'

PRE in 1353-1304:

5'-
GGAGCGGGATCTTTGAGAACAAACTGTTCTTAAAACGAGGATCGTCCGAG-3
,

TH 1403-1354:

5'-
AGCAAGGCAGTGATGTGGCAGGCAGTTCCTGTTGTGAAAGAGCTGGGAAG-
3'

TH 1378-1329:

5'-
TTCCTGTTGTGAAAGAGCTGGGAAGGGAGCGGGCTGGGCCTGGTACGTAC-3
,

TH 1353-1304:

5'-
GGAGCGGGCTGGGCCTGGTACGTACAGCAGGCCATTTCTGAGGGTCCGAG-3
,

Cold probes were generated by PCR and concentrations were determined with a spectrophotometer. Whole cell extracts were incubated in EMSA buffer (50 mM potassium chloride, 10mM Tris (pH=7.5), 1 mM EDTA, 5% glycerol, 0.05% IGEPAL CA-630, 1 mM dithiothreitol and 1µg of dA:dT) with radiolabeled dsDNA probes for 20 min at room temperature. Reactions were then subjected to electrophoresis on 4% nondenaturing acrylamide gels and visualized by a Phosphorimager.

Chromatin immunoprecipitation (ChIP) assays

CAD cells (1×10^6 cells/ 100 mm plate) were transfected with 5 µg of FLAG-PR-B and 2 µg of TH-1600 constructs for 18 h. Media was then replaced with serum-free media containing 1% ITS supplement premix. Twenty-four hours later, cells were treated with 200 nM progesterone for 0, 1 and 2 h followed by *in vivo* crosslinking with formaldehyde (Sigma-Aldrich, St. Louis, MO). ChIP assays were performed using the EZ-ChIP chromatin immunoprecipitation kit (Millipore) following the manufacturer's protocol with either 2 µg of mouse anti-FLAG antibodies (Sigma-Aldrich) or mouse non-immune IgGs (Santa Cruz Biotechnologies, Santa Cruz, CA). Eluted ChIP samples were subjected to 25 cycles of PCR. The primers for PCR were 5'-CAGAGCAAGGCAGTGATGTGG-3' and 5'-CTCGGACCCTCAGAAATGGC-3' amplifying -1406 to -1304 of the TH promoter. PCR products were separated on 10% PAGE gels and stained with ethidium bromide. ChIP samples were also analyzed by real-time PCR on a Cepheid SmartCycler (Sunnyvale, CA) using IQ Sybr Green Supermix (Bio-Rad, Hercules, CA). Fold enrichments were determined for the anti-FLAG ChIP samples relative to the corresponding IgG ChIP samples after input Ct subtraction.

Statistical analysis

Fold activation luciferase assay experimental data are expressed as mean $\pm$ SE and are representative data of at least two independent experiments performed in triplicate. Data were analysed by one-way or two-way ANOVA followed by Tukey's multiple comparison post hoc test or when indicated in the figure legend by Student's t-tests. Percent activation luciferase assay experimental data are expressed as mean $\pm$ SE of at least three independent experiments and were analyzed by one-way ANOVA followed by Tukey's multiple comparison post hoc test. ChIP real-time PCR data is expressed as the mean $\pm$ SE of three independent experiments and was analyzed by one-way ANOVA followed by Tukey's

multiple comparison post hoc test. P values less than 0.05 were considered significantly different.

Results

Liganded PR-B transactivates the TH-9.0 promoter

The first experiment investigated the effects of progesterone actions through PR-A and PR-B on the TH promoter. CAD (mouse neuronal TH expressing), CV-1 (monkey kidney), and SK-N-SH (human neuroblastoma-derived TH expressing) cells were transfected with pcDNA3 (control vector), PR-A or PR-B, together with either a rat TH-9.0 luciferase promoter construct or a 2xPRE luciferase construct (positive control). Cells were treated with vehicle (0.1% ethanol) or 200 nM P4 followed by luciferase assays. Progesterone treatment of pcDNA3-transfected cells did not increase TH-9.0 (Fig. 1A, 1B and 1C) or 2xPRE (not shown) promoter activity in the three cell lines. For PR-A transfected cells, there was a small increase (less than 2 fold) for both TH-9.0 and 2xPRE promoter activities in CAD and CV-1 cells after progesterone treatment (Fig. 1A and 1B). Progesterone-treated PR-B-transfected cells showed 6-, 4-, and, 3-fold increases in TH-9.0 activity and 5-, 6-, and 10-fold increases in 2xPRE activity in CAD, CV-1, and SK-N-SH cells, respectively (Fig. 1A, 1B and 1C). Immunofluorescence experiments and western blot analysis were also performed on PR-A or PR-B transfected CAD cells to confirm PR localization and appropriate molecular weight. Both PR-A and PR-B overexpression resulted in a strong nuclear PR fluorescence staining (Fig. 1D). Strong bands of the expected molecular size were observed for PR-A (~90 kDa) and PR-B (~117 kDa) with and without progesterone treatment (Fig. 1E). It is notable a triplet with higher apparent molecular size bands was observed in the PR-A transfected cells only after progesterone treatment. A minor band of around the PR-A molecular weight was observed in the PR-B (no progesterone treatment) lane.

Mapping the location of PR-B action on the TH promoter

To determine the regions of the TH promoter necessary for PR-B actions, a number of TH promoter deletion constructs were generated and tested in CAD cells (Fig. 2). Progesterone treatment of the TH promoter deletion constructs TH-9.0, TH-2.4, TH-1.8, TH-1.6 and TH-1.4 exhibited between 6- to 10-fold increases in promoter activities. Deletion of 50 bp from the TH-1.4 construct (TH-1.35) decreased progesterone-mediated activation to approximately 3-fold. An additional 50 bp deletion to TH-1.3 further decreased progesterone mediated increase to less than 1.5-fold. Further deletions (TH-1.25, TH-1.2, TH-0.773, and TH-0.272) eliminated or reduced activation to less than 1.5 fold. The slight increase in the progesterone-mediated pGL3 vector (pGL3 basic) luciferase activity has been reported elsewhere (34). These data indicate PR-B responsiveness falls between -1403 and -1304 bp within the TH promoter.

Influence of specific DNA elements on PR-B transactivation of the TH promoter

The DNA sequence of the TH promoter between -1403 and -1304 bp (Fig. 3A) was analyzed using Transcription Element Search Software and Searching Transcription Factor Binding Sites databases to determine potential hormone response elements (HRE) or other potential progesterone modifying regulatory elements, including P300 and specificity protein 1 (SP1) (40). These potential regulatory elements were mutated to the indicated sequences (Figure 3A) in the TH-1.4 construct and tested in luciferase assays. CAD cells were transfected with wild type (wt) or mutant TH-1.4 constructs and with PR-B expression vector. Cells were treated with vehicle or progesterone and fold activation was determined for each mutant. These values were then compared to the wt fold activation that was set to 100% (Fig. 3B). Mutation of a potential HRE (HREm) caused a 35% decrease in

progesterone activation. Mutation of a putative p300 element caused a 20% reduction in activation. The p300 element is 6 bp upstream of the HRE site such that this site may also be utilized as the other half-site of the HRE. Two different mutations were generated for the potential SP1 element (SP1m1 and SP1m2). The SP1m1 TH-1.4 construct showed no change in activation compared to the wt TH-1.4, but the SP1m2 showed a 40% reduction in activation. The SP1m2 had an additional 3' adenosine mutation beyond the SP1m1. When both the HREm and SP1 mutants were combined (HREm/SP1m1 and HREm/SP1m2), the HREm/SP1m1 had the same reduction in activation as the HREm alone but the HREm/SP1m2 showed more than 60% reduction in progesterone-mediated activation.

Determination of direct DNA binding by PR-B to the TH promoter

EMSA experiments were performed to determine if PR-B would directly bind DNA from the -1403 to -1304 bp region of the TH promoter. CAD cells were transfected with pcDNA3 or PR-B and then treated with progesterone for 2 h. Whole cell extracts were obtained and used for EMSA experiments. In a first experiment, a control PRE probe showed a unique shifted band with PR-B whole cell extract, whereas TH promoter probes spanning -1403 to -1354 bp, -1378 to -1329 bp, and -1353 to -1304 bp of the TH promoter did not show shifted bands (data not shown). These data indicated that PR-B was able to bind a consensus PRE, but not sequence in the TH promoter. Since weak binding might not have been detected, cold probes of 50 bp length that span from -1403 to -1354 bp, -1378 to -1329 bp, and -1353 to -1304 bp of the TH promoter were used to determine if molar excesses of TH sequences could compete for PR-B-PRE control DNA complexes. A control cold PRE probe, which had a 35 bp PRE containing sequence replacing the inner 35 bp of the -1353 to -1304 bp probe, was also used as a positive control. When no competitors were present, the radiolabeled PRE probe showed a unique shifted band in the PR-B extract lane (PRE-PR-B complex) but not the pcDNA3 extract lane (Fig. 4A). Cold probes containing the indicated TH promoter sequences at 200, 400, and 1000 fold molar excess were unable to completely compete for PRE-PR-B complexes, but each TH sequence appeared to cause a slight decrease in the PR-B-PRE radiolabeled complex compared to the no competitor lane. However, there were no major differences between the 200, 400, and 1000 fold molar excess experiments. The control PRE contained within the -1353 to -1304 bp TH promoter sequence was able to completely compete off the radiolabeled PRE probe from PR-B at all molar excesses.

ChIP assays were performed to evaluate the recruitment of PR to the TH promoter. CAD cells were transfected with FLAG-PR-B and the TH-1600 promoter construct. Cells were treated with progesterone for 0, 1 or 2 h followed by ChIP analysis using anti-FLAG antibodies or mouse non-immune IgGs (control). PCR amplification from FLAG-PR-B immunoprecipitants showed increased TH promoter -1406 to -1304 bp band intensity at 1 and 2 h after progesterone treatment as compared to the untreated (0 hour) cells (Fig. 4B). The non-immune mouse IgG immunoprecipitants showed little or no TH promoter DNA. Real time PCR was used to determine fold enrichments in TH promoter DNA from anti-FLAG precipitants compared to IgG precipitants (Fig. 4C). The 1 and 2 hour progesterone treated cells showed 17.0 and 12.9 fold enrichments compared to the 1.8 fold enrichment for the 0 hour progesterone treated cells.

Effects of PR-B DBD mutations on TH promoter transactivation

Given that EMSA experiments above did not clearly demonstrate direct progesterone-TH promoter DNA interactions and ChIP experiments showed recruitment to the TH promoter, two previously described DNA binding domain (DBD) mutants for PR-B (34, 36) were generated and used in transcription assays. These mutants were tested on TH-1.4 (Fig. 5A), TH-9.0 (Fig. 5B), and the 2xPRE (Fig. 5C) luciferase constructs. The GS to AA (GS/AA)

mutation decreased fold activation to 2.2-, or 1.6- compared to 7- and 6-fold activation for wtPR-B on TH-1.4 and TH-9.0 promoter constructs, respectively. The GS/AA mutation completely eliminated fold activation for the 2xPRE promoter construct. Surprisingly, the C to A mutation (C/A) showed increased fold activation to 9.5- and 11.5- compared to 7- and 6-fold activation for wtPR-B on TH-1.4 and TH-9.0 promoter constructs, respectively. In contrast to the TH promoter, C/A decreased fold activation of the 2xPRE luciferase control to 2.5-fold compared to 6-fold for wtPR-B. PR-B was also co-expressed with the GS/AA or the C/A mutants and progesterone-mediated promoter activation were determined for the TH-1.4 promoter. The co-expression of wtPR-B and GS/AA reduced activation to 4-fold compared to the 7-fold increase for PR-B alone and 2.5-fold for GS/AA alone. Co-expression of the C to A mutation with wtPR-B had fold activation equal to that of PR-B alone and lower than the C/A mutant expressed alone. Western blots performed on lysates from similarly transfected cells verified overexpression of the various wt or mutant PRs (Fig. 5D).

PR-A inhibition of PR-B transactivation of TH promoter deletion constructs

PR-A can often inhibit PR-B mediated promoter transactivation and the ratio of PR-A:PR-B can determine progesterone actions. We sought to determine the effects of changing PR-A:PR-B ratios on TH-9.0 promoter activity. Cells were transfected with 500ng of PR-B alone, 500ng of PR-A alone, or 500ng PR-B with 50ng, 250ng, 500ng, 1000ng, or 2000ng PR-A. As the ratio of PR-A to PR-B was increased, there was a decrease in the progesterone-mediated TH-9.0 promoter activity compared to progesterone-treated cells transfected with PR-B alone (Fig. 6A). We also examined the effects of increasing amounts of PR-B on a fixed amount of PR-A (Fig. 6B). Cells were transfected with 500ng of PR-B alone, 500ng of PR-A alone, or 500ng PR-A with increasing amounts of PR-B (50ng, 250ng, 500ng, 1000ng, or 2000ng PR-B). As levels of PR-B increased there was a corresponding increase in progesterone-mediated TH promoter transactivation.

We next determined whether PR-A was able to inhibit PR-B actions on other TH deletion promoter constructs. Cells were transfected with PR-B alone or a 1:1 ratio of PR-A:PR-B with the indicated TH promoter deletion or 2xPRE luciferase constructs (Fig. 6C). The fold activation of the PR-A:PR-B transfected cells was compared to the fold activation of PR-B alone for each deletion construct with the PR-B alone activation set to 100%. The co-expressed PR-A:PR-B activation for TH-9.0, TH-5.6, TH-2.4 and TH-1.4 was reduced to 54.7%, 62.0%, 68.3% and 82.5% of PR-B activation alone, respectively, whereas PR-A:PR-B activation for 2xPRE was reduced to 36.8% of PR-B alone activation. Significant changes were observed between the TH-9.0, TH-2.4, TH-1.4, and 2xPRE. A representative western blot is also shown to illustrate PR-B alone or 1:1 PR-A: PR-B over-expression in CAD cells (Fig. 6D).

Discussion

The findings from this study indicate that rat PR-A and PR-B differentially influenced TH promoter activity, with progesterone-bound PR-B causing marked activation. Deletional analysis mapped the region of PR-B activation domain to 100 bp between -1403 bp and -1304 bp of the TH promoter. Mutation of potential PR related sequences within the PR activation region indicated specific DNA sequence elements are necessary for PR activity, although a well-defined PRE was not identified. Although EMSA assays indicated no direct binding of ligand-bound PR-B to TH promoter elements, ChIP analysis revealed that progesterone treatment resulted in recruitment of PR-B to the region. PR-A was able to inhibit PR-B transactivation activities, but the extent of inhibition was determined by not only the ratio of PR-A:PR-B but also the TH promoter deletion construct used.

Progesterone, acting through PR-B, but not PR-A, was able to activate the TH promoter, even though PR-A and PR-B are identical except for the N-terminal 164 amino acid extension of PR-B. These data suggest that the N-terminal activation function-3 domain of the PR-B isoform is essential for transactivation activities on TH promoter. PR-B mediated the strongest activation of the 2xPRE promoter as well. Similar differences between the PR isoforms have been observed for other genes as well (34, 41). When PR-B was over-expressed, progesterone treatment effectively increased TH promoter activity in both TH expressing (CAD and SK-N-SH) and non-TH expressing (CV-1) cells, suggesting PR-B actions are not dependent on cell type. These data contrast with nuclear receptor related 1 protein (NURR1) and retinoic acid receptor actions on the TH promoter, which do show cell type specificity (24, 42). After progesterone treatment, both PR-A and PR-B predominantly localized in the cell nucleus of CAD cells, supporting their roles as nuclear transcription factors. It is notable that a triplet for PR-A was observed by Western blot analysis only after progesterone treatment, whereas a single band was observed without progesterone treatment. Others have also observed similar mobility changes (43–44) for human PR and one possibility is that multiple hormone-induced post-translational modifications may occur for the rat PR-A isoforms in CAD cells.

The progesterone responsive region in the TH promoter is directly adjacent to the regions of activation defined for both retinoic acid and androgen receptors. The action of androgens, through the androgen receptor, maps to –1562 to –1328 bp, whereas retinoic acid, through retinoic acid receptor, maps to the –1500 to –1487 bp region upstream of the transcription initiation site (23, 24). In the current study, progesterone action, through PR-B, mapped to –1403 and –1304 bp, slightly downstream of the androgen and retinoic acid responsive regions. However, the close proximity of these diverse steroid responsive regions, suggest that this region of promoter may have additional, yet unidentified, features supportive of transactivation activities of steroid hormones. In contrast to progesterone, the related ovarian steroid hormone, oestradiol, through oestrogen receptor β , causes a decline in TH promoter activity, whereas oestrogen receptor α increases TH promoter activity through a CREB response element around –45 bp upstream from the transcription initiation site and through a membrane-initiated signalling pathway (22, 45). Glucocorticoids, through the glucocorticoid receptor, increase TH promoter activity and the site action mapped to a region –2400bp upstream of the transcription initiation site (21). These data would suggest that in spite of similarities between the consensus palandromic glucocorticoid and progesterone response elements (46), these similar steroid hormones employ distinct and separate TH promoter elements to elicit gene activation.

EMSA experiments were unable to clearly demonstrate direct DNA binding of PR-B to relevant TH promoter DNA sequences. However, ChIP analysis indicated PR-B is recruited to the TH promoter in a ligand-dependent manner. Combined these data imply that PR-B may act indirectly on the TH promoter through other factors that tether PR-B to specific DNA sequences in the –1403 to –1304 bp region. Indeed, the lack of direct binding to sequences from this region indicates a different type of DNA-protein interaction than that of the described for classical PRE binding by a PR dimer. Mutation of the potential TH HRE decreased progesterone-mediated activation, but did not eliminate it. The TH HRE mutated in this study is identical to the one described for effects of the androgen receptor on the TH promoter. In that case, mutation of the site was also unable to completely eliminate androgen-mediated transactivation of the TH promoter (23). Recent studies demonstrate human PR can influence specific promoters through interactions with other transcription factors including activator protein 1 and SP1 and not through direct DNA-PR interactions (40, 47–49). Mutation of a SP1 site in the –1403 to –1304bp region of the TH promoter had varied results on progesterone-mediated activation. Both SP1 mutations used in this study should have eliminated the putative SP1 site, however one mutant (SP1m1) had no influence

on activation but a second mutation (SP1m2) decreased activation. The SP1m2 mutation carried an additional 3' mutation, indicating a site adjacent to the putative SP1 and most likely not the SP1 site may be involved in progesterone responsiveness. Combined mutations (HRE/SP1m2) further decreased activation, suggesting that multiple DNA elements are necessary for complete progesterone responsiveness on the TH promoter. More work is needed to identify other factors involved in PR-B actions on the TH promoter and whether SP1 is able to bind to this region.

The functional domains of PR are similar to other steroid hormone receptors with a c-terminal ligand binding domain, multiple activation function domains and a centrally located DBD containing two 'zinc finger' structures responsible for recognition of specific DNA sequence (50). Interestingly in the present study, two DBD mutants for PR-B exhibited different actions on the TH promoter, even though both mutations were adjacent in the first zinc finger. The GS to AA mutation, located in part of the p-box of the first zinc finger, was able to inhibit or eliminate progesterone actions on the TH-9.0, TH-1.4, and 2xPRE constructs. When the GS to AA mutant was co-expressed with wt PR-B, it suppressed wt PR-B ability to increase TH-1.4 promoter activity. This might be due to heterodimer formation between mutant and wt forms of PR-B as has been described elsewhere (34) or binding competition between the mutant and wt forms of PR-B for DNA or protein interaction sites. Surprisingly, the zinc finger DBD C to A mutation had the opposite effect of the GS to AA mutation and increased progesterone transactivation on both the TH-1.4 and TH-9.0 promoter constructs, even though this mutation decreased PR-B actions on the 2xPRE control. The C580 (C587 in human PR-B) residue is one of the four highly conserved cysteine molecules, which coordinates binding of the zinc atom in the first zinc finger and mutation of this residue eliminates binding to PRE sequences (36). Similar to the GS to AA mutation, co-expression of the C to A mutation with wt PR-B reversed the effect of the mutant receptor, albeit resulting in the increased fold activation being decreased to PR-B alone values. Without having a clearly defined PRE binding sequence in this region of the TH promoter, it is possible that the C to A mutation might enhance protein-protein interactions that tether PR-B to this region of the TH promoter, whereas the GS to AA mutation may negatively affect these interactions. Overall, these data indicate that PR-B does not activate the TH promoter using the classical steroid hormone model of receptor homodimers binding to a PRE, but involves more intricate interactions. As more progesterone responsive genes are being analyzed, the complexity of PR actions is becoming apparent.

PR-A alone caused little or no transactivation of the different TH promoter constructs, but had the ability to oppose PR-B action, suggesting that the PR isoforms have remarkably different roles in regulating TH gene expression. Although human PR-A can activate some promoters, the opposing effect of the PR isoforms, in which PR-A inhibits PR-B-induced transactivation, has been observed for other promoters as well (33, 34, 41). The physiological consequence of the PR-A and PR-B interactions may be that the ratio of progesterone receptor isoforms impact progesterone's ability to enhance TH transcription in vivo and facilitates the timing for progesterone's action. Recent studies indicate higher levels of PR-B mRNA expression in the hypothalamus on the afternoon of pro-oestrus (51), suggesting a potential change in the ratio of PR-B and PR-A at specific times during the rat estrous cycle. Examination of the effect of PR-A inhibition on different TH promoter deletion constructs showed that the longer TH-9.0 promoter was more sensitive to PR-A inhibition than the TH-1.4 promoter construct. These data suggest upstream PR-A responsive elements or other regulatory sites that act independent of the progesterone-mediated actions that reside between -1403 to -1304 bp of the TH promoter. More studies are needed to examine the mechanisms of how PR-A is able to inhibit PR-B actions on the various promoter deletion constructs.

The tuberoinfundibular dopaminergic neurons are perhaps the best characterized catecholaminergic neuronal population with respect to regulation by oestradiol and progesterone (7, 9, 52, 53). The current study sheds new light on how progesterone, acting on different PR isoforms, may effect TH gene transcription and will likely provide important information in understanding the mechanism(s) for the changes in TH mRNA levels observed *in vivo*. Progesterone treatment for seven days causes an increase in TH mRNA in ovariectomized oestradiol-treated rats, which is prevented by concomitant treatment with RU486 (7). The current data suggest that PR-B likely mediates progesterone's stimulatory action on TH mRNA levels by interaction with a specific regulatory region of the TH gene. The stimulatory progesterone effect was observed with oestradiol, but not with vehicle treatment (7). Indeed, oestradiol preferentially upregulates PR-B as compared to PR-A expression in the hypothalamus (54) and the increased PR-B to PR-A ratio may contribute to the progesterone stimulatory effect. Although sparsely explored, a stimulatory effect for progesterone during late pregnancy is suggested by the ability of RU486 treatment to decrease TH protein levels in the medial basal hypothalamus (55). In contrast to its stimulatory action, progesterone can also have an inhibitory effect on the tuberoinfundibular dopaminergic neurons with a decrease in TH activity, TH phosphorylation levels and TH mRNA levels in tuberoinfundibular dopamine neurons observed on the evening of pro-oestrus, but RU486 treatment on pro-oestrus had no effect on the progesterone induced suppression of TH mRNA levels (6, 8–10). Combined these studies indicate progesterone has multiple effects on TH *in vivo*. Additional studies are needed to determine spatiotemporal mechanisms of PR-mediated regulation of tuberoinfundibular dopaminergic neuronal activities looking at both influences on TH transcription rates and TH activity through potential changes in the ratio of PR-A to PR-B or through membrane progesterin or membrane component progesterone receptors (29, 51).

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References

1. Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. *Physiol Rev.* 2000; 80:1523–1631. [PubMed: 11015620]
2. Ben-Jonathan N, Hnasko R. Dopamine as a prolactin (PRL) inhibitor. *Endocr Rev.* 2001; 22:724–763. [PubMed: 11739329]
3. Fox SR, Harlan RE, Shivers BD, Pfaff DW. Chemical characterization of neuroendocrine targets for progesterone in the female rat brain and pituitary. *Neuroendocrinology.* 1990; 51:276–283. [PubMed: 1970131]
4. Lonstein JS, Blaustein JD. Immunocytochemical investigation of nuclear progesterin receptor expression within dopaminergic neurones of the female rat brain. *J Neuroendocrinol.* 2004; 16:534–543. [PubMed: 15189328]
5. Steyn FJ, Anderson GM, Grattan DR. Expression of ovarian steroid hormone receptors in tuberoinfundibular dopaminergic neurones during pregnancy and lactation. *J Neuroendocrinol.* 2007; 19:788–793. [PubMed: 17850461]
6. Arbogast LA, Ben-Jonathan N. The preovulatory prolactin surge is prolonged by a progesterone-dependent dopaminergic mechanism. *Endocrinology.* 1990; 126:246–252. [PubMed: 1967162]
7. Arbogast LA, Voogt JL. Progesterone reverses the estradiol-induced decrease in tyrosine hydroxylase mRNA levels in the arcuate nucleus. *Neuroendocrinology.* 1993; 58:501–510. [PubMed: 7906870]

8. Arbogast LA, Voogt JL. Progesterone suppresses tyrosine hydroxylase messenger ribonucleic acid levels in the arcuate nucleus on proestrus. *Endocrinology*. 1994; 135:343–350. [PubMed: 7912184]
9. Liu B, Arbogast LA. Phosphorylation state of tyrosine hydroxylase in the stalk-median eminence is decreased by progesterone in cycling female rats. *Endocrinology*. 2008; 149:1462–1469. [PubMed: 18096660]
10. Liu B, Arbogast LA. Progesterone decreases tyrosine hydroxylase phosphorylation state and increases protein phosphatase 2A activity in the stalk-median eminence on proestrous afternoon. *J Endocrinol*. 2010; 204:209–219. [PubMed: 19945993]
11. Guerra-Araiza C, Miranda-Martinez A, Neri-Gomez T, Camacho-Arroyo I. Sex steroids effects on the content of GAD, TH, GABA(A), and glutamate receptors in the olfactory bulb of the male rat. *Neurochem Res*. 2008; 33:1568–1573. [PubMed: 18363096]
12. Barraclough CA, Wise PM. The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. *Endocr Rev*. 1982; 3:91–119. [PubMed: 6120832]
13. Mohankumar PS, Thyagarajan S, Quadri SK. Correlations of catecholamine release in the medial preoptic area with proestrous surges of luteinizing hormone and prolactin: effects of aging. *Endocrinology*. 1994; 135:119–126. [PubMed: 8013343]
14. Legan SJ, Callahan WH. Suppression of tonic luteinizing hormone secretion and norepinephrine release near the GnRH neurons by estradiol in ovariectomized rats. *Neuroendocrinology*. 1999; 70:237–245. [PubMed: 10529618]
15. Szawka RE, Franci CR, Anselmo-Franci JA. Noradrenaline release in the medial preoptic area during the rat oestrous cycle: temporal relationship with plasma secretory surges of prolactin and luteinising hormone. *J Neuroendocrinol*. 2007; 19:374–382. [PubMed: 17425612]
16. Haywood SA, Simonian SX, van der Beek EM, Bicknell RJ, Herbison AE. Fluctuating estrogen and progesterone receptor expression in brainstem norepinephrine neurons through the rat estrous cycle. *Endocrinology*. 1999; 140:3255–3263. [PubMed: 10385422]
17. Wise PM, Rance N, Barraclough CA. Effects of estradiol and progesterone on catecholamine turnover rates in discrete hypothalamic regions in ovariectomized rats. *Endocrinology*. 1981; 108:2186–2193. [PubMed: 6785074]
18. Rance N, Wise PM, Barraclough CA. Negative feedback effects of progesterone correlated with changes in hypothalamic norepinephrine and dopamine turnover rates, median eminence luteinizing hormone-releasing hormone, and peripheral plasma gonadotropins. *Endocrinology*. 1981; 108:2194–2199. [PubMed: 6785075]
19. Kumer SC, Vrana KE. Intricate regulation of tyrosine hydroxylase activity and gene expression. *J Neurochem*. 1996; 67:443–462. [PubMed: 8764568]
20. Dunkley PR, Bobrovskaya L, Graham ME, von Nagy-Felsobuki EI, Dickson PW. Tyrosine hydroxylase phosphorylation: regulation and consequences. *J Neurochem*. 2004; 91:1025–1043. [PubMed: 15569247]
21. Hagerty T, Morgan WW, Elango N, Strong R. Identification of a glucocorticoid-responsive element in the promoter region of the mouse tyrosine hydroxylase gene. *J Neurochem*. 2001; 76:825–834. [PubMed: 11158254]
22. Maharjan S, Serova L, Sabban EL. Transcriptional regulation of tyrosine hydroxylase by estrogen: opposite effects with estrogen receptors alpha and beta and interactions with cyclic AMP. *J Neurochem*. 2005; 93:1502–1514. [PubMed: 15935066]
23. Jeong H, Kim MS, Kwon J, Kim KS, Seol W. Regulation of the transcriptional activity of the tyrosine hydroxylase gene by androgen receptor. *Neurosci Lett*. 2006; 396:57–61. [PubMed: 16356647]
24. Jeong H, Kim MS, Kim SW, Kim KS, Seol W. Regulation of tyrosine hydroxylase gene expression by retinoic acid receptor. *J Neurochem*. 2006; 98:386–394. [PubMed: 16805833]
25. Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci U S A*. 2003; 100:2231–2236. [PubMed: 12574519]
26. Cai Z, Stocco C. Expression and regulation of progestin membrane receptors in the rat corpus luteum. *Endocrinology*. 2005; 146:5522–5532. [PubMed: 16123161]

27. Li X, O'Malley BW. Unfolding the action of progesterone receptors. *J Biol Chem.* 2003; 278:39261–39264. [PubMed: 12893816]
28. Cahill MA. Progesterone receptor membrane component 1: an integrative review. *J Steroid Biochem Mol Biol.* 2007; 105:16–36. [PubMed: 17583495]
29. Thomas P. Characteristics of membrane progesterin receptor alpha (mPRalpha) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progesterin actions. *Front Neuroendocrinol.* 2008; 29:292–312. [PubMed: 18343488]
30. Kraus WL, Montano MM, Katzenellenbogen BS. Cloning of the rat progesterone receptor gene 5'-region and identification of two functionally distinct promoters. *Mol Endocrinol.* 1993; 7:1603–1616. [PubMed: 8145766]
31. Sartorius CA, Melville MY, Hovland AR, Tung L, Takimoto GS, Horwitz KB. A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. *Mol Endocrinol.* 1994; 8:1347–1360. [PubMed: 7854352]
32. Tung L, Abdel-Hafiz H, Shen T, Harvell DM, Nitao LK, Richer JK, Sartorius CA, Takimoto GS, Horwitz KB. Progesterone receptors (PR)-B and -A regulate transcription by different mechanisms: AF-3 exerts regulatory control over coactivator binding to PR-B. *Mol Endocrinol.* 2006; 20:2656–2670. [PubMed: 16762974]
33. Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP. Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol Endocrinol.* 1993; 7:1244–1255. [PubMed: 8264658]
34. Brayman MJ, Julian J, Mulac-Jericevic B, Conneely OM, Edwards DP, Carson DD. Progesterone receptor isoforms A and B differentially regulate MUC1 expression in uterine epithelial cells. *Mol Endocrinol.* 2006; 20:2278–2291. [PubMed: 16740655]
35. Qi Y, Wang JK, McMillian M, Chikaraishi DM. Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation. *J Neurosci.* 1997; 17:1217–1225. [PubMed: 9006967]
36. Takimoto GS, Tasset DM, Eppert AC, Horwitz KB. Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages: analysis with zinc finger mutants and the progesterone antagonist ZK98299. *Proc Natl Acad Sci U S A.* 1992; 89:3050–3054. [PubMed: 1557412]
37. Tinti C, Yang C, Seo H, Conti B, Kim C, Joh TH, Kim KS. Structure/function relationship of the cAMP response element in tyrosine hydroxylase gene transcription. *J Biol Chem.* 1997; 272:19158–19164. [PubMed: 9235905]
38. Giangrande PH, Kimbrel EA, Edwards DP, McDonnell DP. The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. *Mol Cell Biol.* 2000; 20:3102–3115. [PubMed: 10757795]
39. Roemer SC, Donham DC, Sherman L, Pon VH, Edwards DP, Churchill ME. Structure of the progesterone receptor-deoxyribonucleic acid complex: novel interactions required for binding to half-site response elements. *Mol Endocrinol.* 2006; 20:3042–3052. [PubMed: 16931575]
40. Owen GI, Richer JK, Tung L, Takimoto G, Horwitz KB. Progesterone regulates transcription of the p21(WAF1) cyclin- dependent kinase inhibitor gene through Sp1 and CBP/p300. *J Biol Chem.* 1998; 273:10696–10701. [PubMed: 9553133]
41. Wang H, Lee EW, Zhou L, Leung PC, Ross DD, Unadkat JD, Mao Q. Progesterone receptor (PR) isoforms PRA and PRB differentially regulate expression of the breast cancer resistance protein in human placental choriocarcinoma BeWo cells. *Mol Pharmacol.* 2008; 73:845–854. [PubMed: 18042733]
42. Kim KS, Kim CH, Hwang DY, Seo H, Chung S, Hong SJ, Lim JKT, Anderson T, Isacson O. Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. *J Neurochem.* 2003; 85:622–634. [PubMed: 12694388]
43. Hurd C, Nag K, Khattree N, Alban P, Dinda S, Moudgil VK. Agonist and antagonist-induced qualitative and quantitative alterations of progesterone receptor from breast cancer cells. *Mol Cell Biochem.* 1999; 199:49–56. [PubMed: 10544951]

44. Abdel-Hafiz H, Dudevoir ML, Horwitz KB. Mechanisms underlying the control of progesterone receptor transcriptional activity by SUMOylation. *J Biol Chem*. 2009; 284:9099–108. [PubMed: 19211567]
45. Maharjan S, Serova LI, Sabban EL. Membrane-initiated estradiol signaling increases tyrosine hydroxylase promoter activity with ER alpha in PC12 cells. *J. Neurochem*. 2010; 112:42–55. (2010). [PubMed: 19818101]
46. Nelson CC, Hendy SC, Shukin RJ, Cheng H, Bruchovsky N, Koop BF, Rennie PS. Determinants of DNA sequence specificity of the androgen, progesterone, and glucocorticoid receptors: evidence for differential steroid receptor response elements. *Mol Endocrinol*. 1999; 13:2090–2107. [PubMed: 10598584]
47. Tseng L, Tang M, Wang Z, Mazella J. Progesterone receptor (hPR) upregulates the fibronectin promoter activity in human decidual fibroblasts. *DNA Cell Biol*. 2003; 22:633–640. [PubMed: 14611684]
48. Faivre EJ, Daniel AR, Hillard CJ, Lange CA. Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. *Mol Endocrinol*. 2008; 22:823–837. [PubMed: 18202149]
49. Daniel AR, Knutson TP, Lange CA. Signaling inputs to progesterone receptor gene regulation and promoter selectivity. *Mol Cell Endocrinol*. 2009; 308:47–52. [PubMed: 19549591]
50. Bain DL, Heneghan AF, Connaghan-Jones KD, Miura MT. Nuclear receptor structure: implications for function. *Annu Rev Physiol*. 2007; 69:201–220. [PubMed: 17137423]
51. Liu B, Arbogast LA. Gene expression profiles of intracellular and membrane progesterone receptor isoforms in the mediobasal hypothalamus during pro-oestrus. *J Neuroendocrinol*. 2009; 21:993–1000. [PubMed: 19807848]
52. Pasqualini C, Levie V, Guibert B, Faucon-Biguier N, Kerdellue B. Inhibitory actions of acute estradiol treatment on the activity and quantity of tyrosine hydroxylase in the median eminence of ovariectomized rats. *J Neuroendocrinol*. 1991; 3:575–580. [PubMed: 19215508]
53. Blum M, McEwen BS, Roberts JL. Transcriptional analysis of tyrosine hydroxylase gene expression in the tuberoinfundibular dopaminergic neurons of the rat arcuate nucleus after estrogen treatment. *J Biol Chem*. 1987; 262:817–821. [PubMed: 2879835]
54. Scott RE, Wu-Peng XS, Pfaff DW. Regulation and expression of progesterone receptor mRNA isoforms A and B in the male and female rat hypothalamus and pituitary following oestrogen treatment. *J Neuroendocrinol*. 2002; 14:175–183. [PubMed: 11999716]
55. Soaje M, Valdez S, Bregonzio C, Penissi A, Deis RP. Dopaminergic mechanisms involved in prolactin release after mifepristone and naloxone treatment during late pregnancy in the rat. *Neuroendocrinology*. 2006; 84:58–67. [PubMed: 17090971]

Figure 1.

Progesterone treatment increased TH promoter activity through the PR-B isoform in CAD, CV-1, and SK-N-SH cells. A) CAD cells were cotransfected with TH-9.0 promoter luciferase construct or a 2xPRE luciferase control construct, with pcDNA3, PR-A or PR-B and with CMV-Renilla luciferase (transfection efficiency) for 18 h. Cells were treated with vehicle or 200 nM progesterone for 20 h followed by dual luciferase assays. Similar transfections were carried out in CV-1 (B) and SK-N-SH (C) cells for both TH-9.0 and 2xPRE luciferase constructs. Experiments were carried out at least twice in triplicate and one representative experiment is shown. Each value represents the mean $\pm$ SEM relative to vehicle-treated, pcDNA3-transfected cells, for which fold activation was set to 1. * $p < 0.05$ versus vehicle-treated. D) Immunofluorescence was used to detect PR expression (red) in PR-A or PR-B transfected CAD cells treated with progesterone for 20 h. DNA (blue) was stained with Hoescht Dye. E) Western blots to detect PR-A or PR-B expression and tubulin were performed on lysates from pcDNA3, PR-A, or PR-B transfected CAD cells treated with vehicle (–) or progesterone (+) for 20 h.

Figure 2.

Mapping the location of PR-B actions on the rat TH promoter through TH promoter deletion constructs. CAD cells were cotransfected with the indicated TH promoter deletion luciferase constructs, PR-B and CMV-Renilla luciferase (transfection efficiency) for 18 h. Cells were treated with vehicle or 200 nM progesterone for 20 h followed by dual luciferase assays. Experiments were carried out independently at least twice in triplicate and one representative experiment is shown. Each value represents the mean $\pm$ SEM relative to values obtained from vehicle treated cells for each deletion construct, for which fold activation was set to 1. Student's t-tests were used to compare vehicle to progesterone treatment for each deletion construct. * $p < 0.05$ versus vehicle-treated.

Figure 3.

Specific DNA sequences within the -1403 to -1304 bp region of the TH promoter influence PR-B mediated transactivation. A) DNA sequence of the rat TH promoter spanning -1403 to -1304 was mutated at the potential DNA elements to the indicated sequences. B) CAD cells were cotransfected with wtTH-1.4 luciferase construct or the indicated TH-1.4 mutated promoter luciferase constructs, PR-B, and CMV-Renilla luciferase (transfection efficiency) for 18 h. Cells were treated with vehicle or 200 nM progesterone for 20 h followed by dual luciferase assays. Each value represents the mean $\pm$ SEM relative to wtTH-1.4, which was set to 100% for each independent experiment. Experiments were performed 3–5 times. * $p < 0.05$ versus TH-1.4. # $p < 0.05$ versus HREm.

Figure 4.

PR-B is recruited to the TH promoter, but does not appear to directly bind specific DNA sequences within -1403 to -1304 bp of the TH promoter. A) Competition EMSAs were performed using a radiolabeled dsDNA PRE probe incubated with pcDNA3 or PR-B whole cell extracts (WCE) with the indicated cold probes at 200, 400, or 1000 molar excess and analyzed by EMSA on 4% gels. Arrows indicate PR-B-DNA complexes. The entire gel is shown for the 200× experiment and PR-B-DNA shifted complex is shown for the 400× and 1000× experiments (boxed region). The other major shifted bands are most likely due to other DNA binding proteins in the whole cell extracts. The cold PRE in 1353-1304 probe was able to completely compete off the radiolabeled PRE at all molar excesses used. B and C) ChIP analysis of FLAG-PR-B interaction with the TH promoter. CAD cells transfected with FLAG-PR-B and TH-1600 were treated with progesterone for 0, 1 or 2 h followed by ChIP. B) PCR amplified products were separated on acrylamide gels and stained with ethidium bromide. C) Quantitative PCR was performed on ChIP products to determine fold enrichment of anti- FLAG ChIP over IgG ChIP for each time point relative to 1.0% input ct values. Data represents mean fold enrichment ± SEM of the 3 individual ChIP assays for each time point. *p<0.05 versus 0 hour treatment.

Figure 5.

Effects of two different PR-B DNA binding domain mutants on PR-B transactivation of TH-1.4, TH-9.0, and 2xPRE luciferase promoter constructs. CAD cells were cotransfected with A) TH-1.4, B) TH-9.0 or C) 2xPRE constructs, with wtPR-B, GS/AA mutated PR-B, or C/A mutated PR-B and CMV-Renilla (transfection efficiency) for 18 h. Cells were treated with vehicle or 200 nM progesterone for 20 h followed by dual luciferase assays. Experiments were carried out independently at least twice in triplicate and one representative experiment is shown. Each value represents the mean $\pm$ SEM relative to values obtained from vehicle-treated wtPR-B transfected cells, for which fold activation was set to 1. * $p < 0.05$ versus vehicle-treated groups. # $p < 0.05$ versus wildtype PR-B progesterone-treated groups. D) Lysates from CAD cells transfected with pcDNA3, PR-A, PR-B, PR-B (GS/AA), or PR-B (C/A) were analyzed by western blot to evaluate expression of PR-A, PR-B and α -tubulin.

Figure 6.

Influence of PR-A on PR-B mediated transactivation of the TH promoter. A, B) CAD cells were cotransfected with TH-9.0 luciferase constructs with PR-B, PR-A or the indicated μ g ratios of A) PR-A to PR-B or B) PR-B to PR-A and CMV-Renilla (transfection efficiency) for 18 h. C) CAD cells were transfected with 0.5 μ g PR-B or 0.5 μ g of PR-A and PR-B each with the indicated TH promoter deletion constructs and CMV-Renilla for 18 h. Cells were treated with vehicle or 200 nM progesterone for 20 h followed by dual luciferase assays. Fold activations were determined compared to vehicle-treated cells. A, B) Each value represents the mean $\pm$ SEM relative to values obtained from progesterone-treated PR-B transfected cells for which activation was set to 100%. * $p < 0.05$ versus PR-B alone. # $p < 0.05$ versus PR-A alone. C) Fold activation from progesterone treatment was determined for both the PR-B alone and the 0.5:0.5 μ g ratio of PR-A:PR-B transfected cells. The PR-B alone activation (dashed line) was set to 100% for each of the deletion constructs and the percentage of luciferase activity was determined for the PR-A:PR-B transfected cells. Each value represents the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$ versus TH-9.0. D)

Western blot analysis of lysates from CAD cells transfected with 0.5 µg PR-B alone or 0.5:0.5 µg PR-A:PR-B to evaluate expression of PR-A, PR-B and tubulin.