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Differential Effects of GnRH and Androgens on *Cres* mRNA and Protein in Male Mouse Anterior Pituitary Gonadotropes

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ABSTRACT: The *Cres* gene defines a new subgroup in the family 2 cystatins of cysteine protease inhibitors. However, unlike typical cystatins, CRES does not inhibit cysteine proteases but rather inhibits the serine protease prohormone convertase 2, an enzyme with roles in proprotein processing in the neuroendocrine system. *Cres* is expressed in the gonadotropes and colocalizes with LHβ, suggesting a role in the regulation of gonadotrope secretion. Our present studies were carried out to examine the regulation of *Cres* mRNA and protein expression by GnRH and steroid hormones, thus providing clues regarding its role in gonadotropes. Castration profoundly reduced *Cres* mRNA, while replacement with estradiol (E_2), testosterone (T), or dihydrotestosterone (DHT) further decreased *Cres*, suggesting negative regulation by GnRH or steroid hormones. The administration of Antide, a GnRH antagonist, resulted in a 3-fold increase in *Cres* mRNA, supporting a negative regulation by GnRH. Because all hormonal manipulations in vivo resulted in alterations in steroid hormones, organ culture was used to assess the effects of GnRH independent of steroids. Mouse pituitaries cultured in the absence of GnRH or steroids showed high *Cres* mRNA levels, while DHT or E_2 resulted in decreases of 25% and 68%, respectively. However, an 85% decrease in *Cres* mRNA occurred following the administration of GnRH, demonstrating that GnRH, and to a lesser degree E_2, negatively regulate *Cres* mRNA in gonadotropes. Examination of CRES protein by immunohistochemistry showed that levels were profoundly reduced following castration, while DHT and in part T, but not E_2, restored CRES levels. Castrated mice treated with Antide showed little effect. However, castrated mice treated with Antide + DHT showed a dramatic recovery of CRES, suggesting that androgens act directly at the level of the gonadotrope to regulate CRES protein. Together, our studies suggest that *Cres* mRNA and protein are low at peak gonadotrope secretory activity, possibly as a means to allow proprotein processing events to occur that are integral to gonadotrope function.

Key words: Steroid hormones, cystatin, gonadotrope, anterior pituitary gland.

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CRES (cystatin-related epididymal spermatogenic) defines a new subgroup in the family 2 cystatins of cysteine protease inhibitors by virtue of its reproductive-specific expression and its lack of consensus sites necessary for the inhibition of cysteine proteases, suggesting distinct biochemical activities (Cornwall and Hsia, 2003). Indeed, in contrast to the prototypical family 2 cystatin, cystatin C, CRES does not inhibit the cysteine proteases papain or cathepsin B, but rather inhibits the serine protease prohormone convertase 2 (PC2), a subtilisin/Kex2p-like endoprotease involved in prohormone processing in the neuroendocrine system (Cornwall et al, 2003).

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The prohormone convertases are a family of calcium-dependent proteases that function within the secretory pathway and cleave proproteins at mono- or dibasic sites to generate biologically active proteins (Bergeron et al, 2000). Substrates for convertases include the precursors of neural and peptide hormones, cell surface receptors, growth factors, and cell adhesion molecules. Of the 7 prohormone convertase family members, PC1, PC2, and PC4 are primarily found within the neuroendocrine and reproductive systems, respectively, while other family members including furin, PC5/6, PACE4, and PC7/8 exhibit broader patterns of expression. Although convertases may cleave the same substrate in vitro, in vivo the substrate specificity and efficiency of these proteases are thought to be governed not only by the levels of expression of a particular convertase but also by the levels of endogenous inhibitors and by the presence of cell-specific chaperones (Berman et al, 1999). Thus, one possible role for CRES may be to regulate important proprotein processing events in the reproductive and neuroendocrine systems.

CRES is expressed in the proximal caput epididymal epithelium (Cornwall et al, 1992), round spermatids in the testis (Cornwall and Hann, 1995), anterior pituitary

gonadotropes (Sutton et al, 1999), and corpora lutea in the ovary (Hsia and Cornwall, 2003). Within gonadotropes, CRES is packaged into secretory granules of the regulated secretory pathway (Sutton et al, 1999), which contain luteinizing hormone (LH) as well as known prohormone convertase substrates, including secretogranin, chromogranin, prodynorphin, and pituitary adenylate cyclase activating peptide (PACAP) (Koves et al, 1998; Laslop et al, 1998; Berman et al, 1999). Prohormone convertase mRNAs that are expressed in the gonadotropes include PC1, PC2, furin, PC6, and PACE4 (Dong and Day, 2002). Several of the secretory granule proteins that are released with LH and CRES are thought to be involved in the autocrine/paracrine feedback regulation of gonadotropes (Sion et al, 1988; Dragatsis et al, 1995; Tsujii and Winters, 1995). The presence of CRES exclusively within gonadotropes and the fact that it inhibits PC2 but not PC1 (Cornwall et al, 2003) suggest that CRES could be a mechanism to regulate the specificity of hormone processing enzymes within these cells and ultimately feedback activity. The need to tightly control protease activities within neuroendocrine cells is demonstrated by the phenotype of mice null for 7B2, a chaperone and inhibitor of PC2, which consists of multiple endocrine defects resulting from incorrect prohormone processing (Westphal et al, 1999).

Previous experiments from our laboratory demonstrated that within male mouse gonadotropes, intracellular levels of CRES protein varied in concert with LH following castration and testosterone replacement, supporting the idea that CRES might play an integral role in regulating gonadotrope secretion (Sutton et al, 1999). The secretion of gonadotropins from the anterior pituitary gland is a tightly controlled process regulated by the complex interactions of the hypothalamic-pituitary-gonadal (HPG) axis. The pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus regulates the synthesis and secretion of the gonadotropin LH as well as various autocrine/paracrine factors from the gonadotrope cell. In the male, LH regulates the synthesis and secretion of testosterone from testicular Leydig cells, which in turn can elicit negative feedback either directly at the level of the pituitary gland gonadotropes or indirectly by regulating GnRH release from the hypothalamus. The experiments presented herein were designed to examine in the male mouse the regulation of *Cres* mRNA and protein steady state levels by components of the HPG axis to identify the hormonal conditions in which *Cres* is up- or down-regulated, thus providing clues regarding its physiological role in gonadotropes. The levels of LHβ mRNA and protein were also determined and served as indicators of the hormonal state of the animal following the various treatments, while serum LH was examined as a measure of gonadotrope secretory activity.

Materials and Methods

Experimental Animals

Mature intact and castrate ICR and CD-1 male mice were purchased from Harlan Sprague Dawley, Inc (Indianapolis, Ind) and Charles River Laboratories (Wilmington, Mass), respectively. Animals were housed separately under a constant 12:12 light:dark cycle with mouse chow and water ad libitum. At the time animals were euthanized, blood was collected by cardiac puncture for RIA determination of serum T, DHT, E_2, and LH levels. Seminal vesicles were collected and weighed as a measure of androgen replacement, and pituitary glands were processed for immunohistochemistry as described below or flash frozen in liquid N_2 and stored at $-80°C$ until RNA isolation. LH RIAs were performed by Matthew Hardy, PhD, The Population Council, New York, NY, and T and E_2 RIAs were performed by Sam Prien, PhD, Department of Obstetrics and Gynecology, Texas Tech University Health Sciences Center, Lubbock, Tex. The DHT RIA was performed using the Active DHT RIA kit (Diagnostic Systems Laboratories, Inc., Webster, Tex) according to the manufacturer's protocol. All animal studies were conducted in accordance with the NIH guidelines for the Care and Use of Experimental Animals.

Hormonal Treatments

Castration and Steroid Maintenance—Orchiectomies were by the scrotal route under ketamine/xylazine anesthesia. To examine variation in pituitary *Cres* and LHβ mRNA levels between individual mice, the pituitary glands from 7 intact mice and 3 mice bilaterally castrated for 7 days were removed for isolation of RNA and reverse transcription polymerase chain reaction (RT-PCR) analysis. In castration and hormonal maintenance studies, hormonal maintenance was begun at the time of castration and included daily subcutaneous injections of vehicle (sham and castrate), 25 μg testosterone propionate (Naik et al 1984; Fallest et al, 1995), 25 μg 5α-androstan-17β-ol-3-one (DHT) (Fallest et al, 1995), or 300 ng 17β-estradiol (E_2) (Fallest et al, 1995) in 100 μL sesame oil (Sigma Chemical Co, St. Louis, Mo) for 1 week. Animals (8–10 mice/group) were euthanized on the day following the final injections; 6–7 pituitary glands were pooled for preparation of RNA and subsequent RT-PCR analysis and 2–3 pituitary glands processed for immunohistochemistry. The castration and hormone replacement experiments were repeated with an additional 8–10 mice/treatment group, and both sets of data are shown.

Antide Studies—Antide, a GnRH antagonist (Bachem California, Torrance, Calif), was solubilized in water (1 mg/mL) and then diluted to 0.6 mg/mL in a final solution of 0.9% NaCl/20% propylene glycol and stored at $-80°C$ until use. Animals were castrated and given subcutaneous injections of the appropriate combination of vehicle(s) and hormone treatment(s): oil + saline/propylene glycol (castrate); oil +

Antide (60 μg) (Fallest et al, 1995); or DHT (25 μg) + Antide. Animals were euthanized on the day following the final injections. The pituitary glands from 6–7 mice in each treatment group (8–10 mice/group) were pooled for preparation of RNA and RT-PCR analysis, while 2–3 pituitary glands were processed for immunohistochemistry. To examine the effect of Antide on *Cres* mRNA levels over time, intact mice were given 1 injection of Antide (60 μg) and killed 4, 12, and 24 hours later. The pituitary glands from mice in each group (6–7 mice/group) were pooled for preparation of RNA and RT-PCR analysis. Both Antide experiments were repeated with an additional 6–10 mice/treatment group, and both sets of data are shown.

Organ Culture—To examine the effect of steroid hormones on *Cres* expression in vitro, pituitary glands from intact male mice were hemisected and placed directly into a 24-well plate (3–6 pituitary sections/well) containing 0.5 mL DMEM with high glucose (Invitrogen, Carlsbad, Calif) and 0.05% ethanol (vehicle), 10 nM E_2 (Ravindra and Aronstam, 1992; Muyan et al, 1993; Shupnik, 1996), or 10 nM DHT (Muyan and Baldwin, 1992; Muyan et al, 1993) and cultured for 6 hours in the absence of serum. To examine the effect of GnRH treatment on *Cres* expression in vitro, hemisected pituitary glands were placed into a 24-well plate (3–6 sections/well) containing 0.5 mL DMEM with high glucose and treated with 10 nM GnRH in PBS (Sigma) in 5-minute pulses every 45 minutes (Weiss et al, 1990; Cassina et al, 1997) with a pulse at the beginning of culture and the glands harvested immediately after 9 pulses (6 hours), a protocol that has been shown to stimulate transcription of LHβ (Shupnik, 1996). For all incubations, the 24-well tissue culture plates were placed into a large plastic dish that was then placed in a 37°C shaking water bath to facilitate gas exchange with room air by agitation. To control for the possible effect of multiple media changes in the GnRH studies, pituitary glands in all treatment groups were given fresh changes of the appropriate media coincident with GnRH pulses. At the end of the 6-hour culture period, pituitary glands from each treatment group were placed directly into Trizol reagent for RNA preparation. Also, 1 group of pituitary glands was placed directly into Trizol reagent after removal from the animal to serve as an in vivo control group. The organ culture experiments were repeated on 3 separate occasions using 3–6 pituitary sections/treatment.

Semiquantitative RT-PCR

Because of the small amount of total RNA extracted from an individual mouse pituitary gland and the necessity for multiple replicate RT-PCR reactions of each primer pair, including no–reverse-transcription (RT) controls, for most experiments pituitary glands were pooled from 6–7 mice in each treatment group and total RNA isolated using Trizol reagent (Invitrogen) following the manufacturer's protocol. All animal experiments were repeated. The RNA was quantitated by A_{260}/A_{280} and visualized by gel electrophoresis in 1% agarose gel containing borate buffer (pH 8.2) and 0.66 M formaldehyde. For RT-PCR, 2.5 μg total RNA was incubated in a RT reaction buffer containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 0.5 mM deoxynucleotide triphosphates, 20 U RNasin (RNase inhibitor, Promega, Madison, Wis), and 2.5 μM oligo-dT (Promega) in a final volume of 25 μL for 30 minutes at 37°C in the presence of 2.5 U RNase-free DNase I (Roche, Indianapolis, Ind). After heat inactivation of DNase I at 75°C for 5 minutes (Huang et al, 1996), an aliquot was reserved for PCR amplification as a no-RT control to confirm the absence of contaminating DNA. Then 50 U MuLV reverse transcriptase (Perkin-Elmer Biosystems, Foster City, Calif) was added to the remainder, and reverse transcription was carried out at 42°C for 30 minutes, 99°C for 5 minutes, and 5°C for 5 minutes.

Three μL of each RT and no-RT reaction was amplified by PCR in separate reactions using primers recognizing *Cres*, LHβ, and GAPDH cDNAs. LHβ was amplified as a biological control in each experiment, and GAPDH was amplified as a constitutive control to measure the relative efficiency of each RT reaction. The identity of PCR products generated with each primer pair was confirmed by sequence analysis. PCR master mixes containing 10 mM Tris (pH 8.3), 50 mM KCl, 0.5 μM each of forward and reverse primers, 0.25 μCi [α-^{32}P] dCTP, and 1.25 U Taq DNA polymerase (Sigma) were prepared so that RNA samples from each treatment group within a particular experiment were amplified from a single master mix. $MgCl_2$ and dNTP concentrations, as well as cycle number, were optimized for each set of primers. Specifically, to determine that amplification by PCR was within the exponential phase, identical RT-PCR reactions containing pooled pituitary gland RNA were amplified for increasing cycle numbers in the presence of [α-^{32}P] dCTP for each primer pair and analyzed by agarose electrophoresis. Amplification with each primer set produced cycle-number dependent increases in the amount of PCR product (data not shown). For each primer pair, a cycle number in the middle of the exponential phase was chosen so that differences could be detected in either direction.

Cres PCR reactions were carried out in 2.5 mM $MgCl_2$ and 0.25 mM dNTPs for 40 cycles. LHβ reactions consisted of 2 mM $MgCl_2$ and 0.3 mM dNTPs for 30 cycles, and GAPDH reactions were amplified using 2 mM $MgCl_2$ and 0.2 mM dNTPs for 26 cycles. The cycling parameters consisted of 45 seconds at 95°C for denaturation, 25 seconds annealing at T_a for each primer set, and 1 minute at 72°C for extension, after which the reactions were incubated at 72°C for 7 minutes using a minicycler (MJ Research, Inc, Watertown, Mass). RT-PCR products were then analyzed by electrophoresis in 1.5% agarose/1 × Tris/acetate/EDTA gels, which were dried under vacuum onto filter paper for 2 hours at 50°C. The dried gel was then exposed to a Phosphorimager (Molecular Dynamics, Sunnyvale, Calif), and integrated optical densities (IOD) were generated based on the amount of radioactivity using ImageQuant (Molecular Dynamics). The integrated optical densities for *Cres* and LHβ were normalized to that of GAPDH.

For each animal experiment, 4–6 RT-PCR replicates were performed on each RNA preparation on separate occasions. The absolute values of the IODs of corresponding PCR reactions in different RT-PCR replicates varied considerably due to differences in the age of the radioisotope, the exposure

time on the Phosphoimager, and radioisotope incorporation efficiency. Therefore, for each RT-PCR replicate, we calculated a modified Z score for each PCR reaction by dividing the IOD from each PCR reaction by the average of all IODs for that primer pair, ie, for n treatments, $Z_n = y_n / (\Sigma y_n)/n$. The validity of this transformation was verified by ensuring that the standard deviations of transformed values within a PCR replicate were not statistically different (one-way analysis of variance [ANOVA]) between different replicates. The Z value of each *Cres* and LHβ PCR reaction, which reflected incorporated radioactivity relative to that of simultaneously performed PCR reactions, was normalized to the corresponding GAPDH PCR reaction. This assay does not measure *Cres* mRNA levels relative to LHβ or GAPDH mRNA levels, but rather compares *Cres* mRNA levels between pituitary gland RNA samples analyzed simultaneously by RT-PCR.

Oligonucleotide Primer Pairs

PCR primers (Invitrogen) were designed from the known sequences for mouse *Cres*, LHβ, and GAPDH cDNAs using PrimerSelect from Lasergene Suite (DNA Star, Madison, Wisc) and are as follows: *Cres* sense: 5′ CAAGGAAAGTGAGGACAAATATGTC 3′ and antisense: 5′ GTGACAGACTTGAACCACAGGTT 3′, T_a (annealing) = 64°C; LHβ sense: 5′ AAATGGGGTGGGGTACAGCGAGACG 3′ and antisense: 5′ TTGGGAAGGAGGGAGGGAGGGATGAT 3′; T_a = 64°C; GAPDH sense:5′ AAGGTCGGAGTCAACGGATT 3′ and antisense 5′ TTGATGACAAGCTTCCCGTT 3′, T_a = 55°C.

Indirect Immunofluorescence Analysis

Following cardiac puncture, animals were perfused with 4 mL room temperature PBS followed by 4 mL of cold 4% parafomaldehyde in PBS. Pituitary glands were removed and further fixed in 4% paraformaldehyde in PBS pH 7.4 for 1 hour at 4°C, then washed successively at 4°C for 30 minutes each in PBS, 0.9% NaCl, 0.45% NaCl/50% ethanol, and 70% ethanol, and stored in 70% ethanol overnight. The glands were then dehydrated by incubation in 95% ethanol for 30 minutes at 4°C followed by 100% ethanol for 30 minutes and two 1-hour incubations in 100% ethanol at 4°C. Tissues were washed in xylenes for 40 minutes followed by 1 hour at room temperature and then embedded in paraffin. Four-micron sections were cut and mounted onto glass slides by the Texas Tech University Health Sciences Center Electron Microscopy Center. Sections were deparaffinized by incubating at room temperature twice (for 10 minutes each) in xylenes, once (for 3 minutes) in 100% ethanol, and once (for 3 minutes) in 95% ethanol. The slides were air-dried, and the sections were circled with a Pap pen (Fisher Scientific, Pittsburgh, Pa) to allow small incubation volumes. After a 20-minute incubation in PBS for rehydration, the sections were covered with 100% normal goat serum and incubated in a humidified chamber at 37°C for 90 minutes to block nonspecific binding sites. The sections were then rinsed with 5 drops 5% goat serum/PBS and incubated in a humidified chamber at 37°C for 2 hours with a mixture of guinea pig anti-rat LHβ (1:3000) and either rabbit pre-immune, rabbit anti-mouse CRES antiserum, or rabbit anti-mouse CRES antiserum preincubated with recombinant CRES protein (1:400 for each). The guinea pig anti-rat LHβ antiserum was developed by A.F. Parlow and was a gift from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases. The specificity of the anti-LHβ antiserum has been demonstrated previously (Deschepper et al, 1985). After incubation with primary antibodies, the sections were washed 3 × 5 minutes at room temperature in PBS and incubated for 1 hour at 37°C in a dark humidified chamber with a mixture of 1:50 fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig antiserum and 1:50 Texas Red-conjugated goat anti-rabbit antiserum (both from Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa). The sections were washed in the dark 3 × 5 minutes at room temperature with PBS and 1 × 5 minutes in PBS, pH = 8.5 and then inverted onto cover slips with ~10 μL mounting medium (92 mM Tris [pH = 8.5], 18.5% dimethylsulfoxide, 23% methanol, 0.092 mg/mL Mowiol 4–88 [Fisher Scientific]) to which 1 mg/mL p-phenyline diamine (Sigma) was added immediately before use. After curing overnight in the dark, the sections were examined using an Olympus Corp BX-60 microscope equipped for epifluorescence and photographed both with a wide yellow filter for Texas Red and a narrow band filter for FITC. Multiple sections from 2–3 individual pituitary glands from mice in each treatment group of each animal experiment were examined by immunofluorescence analysis, and representative sections are shown.

Statistical Analysis

RT-PCR data were statistically evaluated by ANOVA, with differences between groups determined by Sheffé's S method. The use of ANOVA for analysis of transformed data was validated using Hartley's test for homogeneity of population variances. *P* values of less than .05 were considered significant. Values are means ± SEM.

Results

Effect of Castration and Steroid Hormone Replacement on Cres mRNA Levels

In our previous experiments we observed a decrease in intracellular CRES protein in anterior pituitary gonadotropes following castration, suggesting a regulation by steroid hormones (Sutton et al, 1999). To determine if *Cres* mRNA responded similarly, we examined the steady-state levels of *Cres* mRNA in individual pituitary glands from mice following 7 days of castration as compared to intact male mice. As shown in Figure 1, intact mice exhibited large interanimal variation in the levels of *Cres* mRNA, while castration consistently resulted in reduced levels of *Cres* mRNA. The decrease in *Cres* mRNA levels following castration could be due to the loss of steroid hormone effects on the pituitary gonadotropes or to the increase in GnRH output from

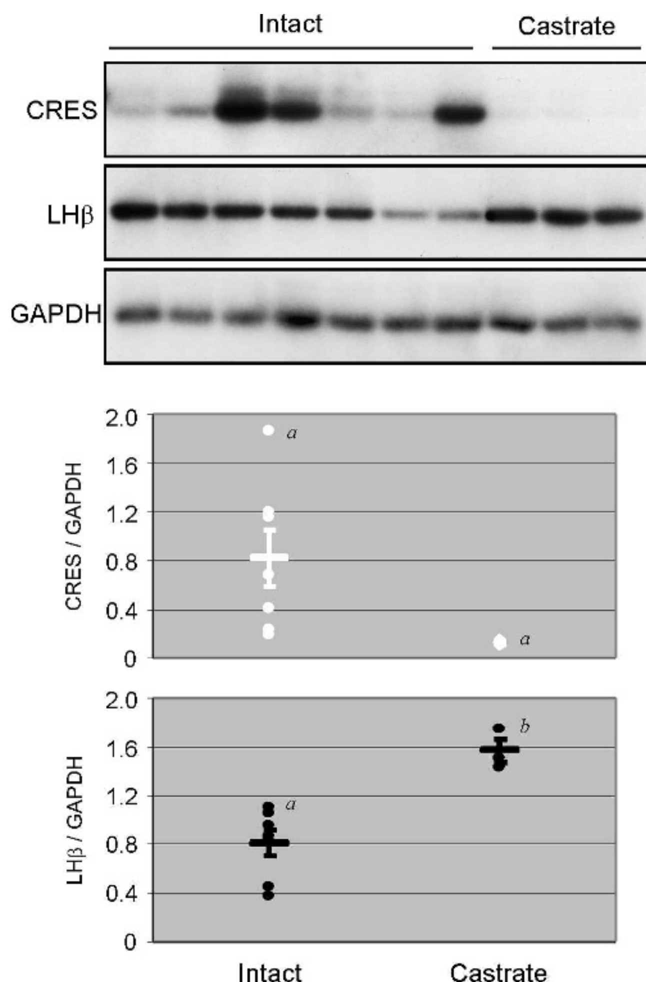


Figure 1. Examination of *Cres* and LHβ mRNAs in individual male pituitary glands and the effect of castration. Individual pituitary glands from intact (n = 7 mice) and 7-day castrated mice (n = 3 mice) were examined for CRES and LHβ mRNAs by semiquantitative RT-PCR. The top panel shows a representative RT-PCR experiment. The bottom panels show the mean ± SEM of normalized CRES and LHβ mRNAs in intact and castrated pituitary glands. Groups with different letters (*a*,*b*) are statistically significant at $P < .05$.

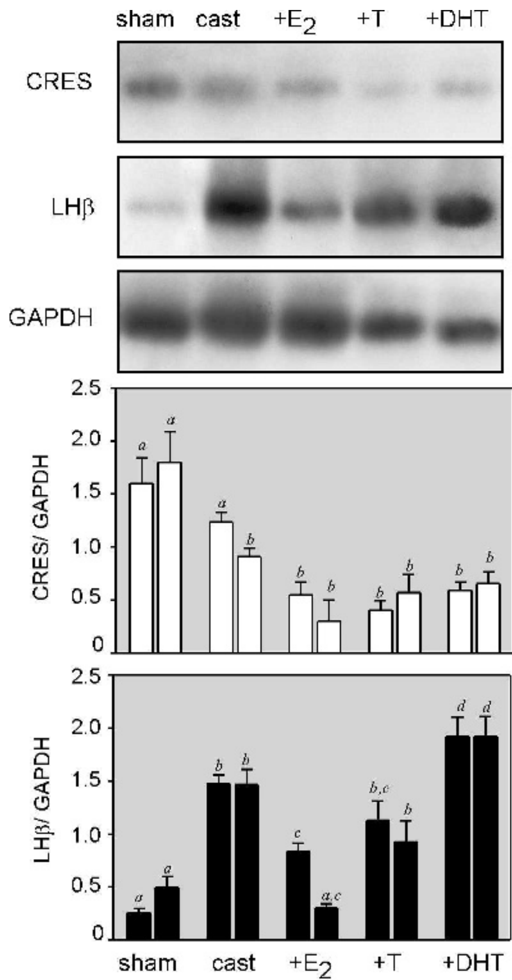
the hypothalamus as a result of the loss of negative feedback from gonadal steroid hormones. LHβ mRNA levels in individual pituitary glands from intact mice showed less variation between animals than observed for *Cres* mRNA (Figure 1). Furthermore, in contrast to *Cres*, LHβ mRNA was consistently increased 2-fold following 7 days of castration, an established response to the increase in GnRH following the loss of negative feedback from steroid hormones.

To determine if the administration of steroid hormones would reverse the effects of castration on *Cres* expression, castrated mice were treated with testosterone (T), estradiol (E_2), or dihydrotestosterone (DHT), a nonaromatizable androgen, to eliminate the possibility that aromatization of testosterone to estradiol is involved in the testosterone-mediated negative feedback on gonadotropin levels. For these and subsequent experiments, pituitary glands were pooled from 6–7 mice in each treatment group to allow for sufficient RT-PCR replicates to minimize experimental variability. Animal experiments were repeated to control for biological variability, and both sets of data are presented. Relative to castrate levels, the combination of castration and hormone maintenance with E_2, TP, or DHT further decreased *Cres* mRNA levels by an average of 60%, 54%, and 42% respectively (Figure 2), suggesting that steroid hormones may negatively regulate *Cres* mRNA. Alternatively, *Cres* mRNA could be affected by changes in GnRH as a result of negative feedback from the exogenous steroid hormones.

LHβ mRNA increased following castration but was reduced relative to castrate levels with E_2 and TP treatment, reflecting the positive regulation of LHβ by GnRH and subsequent down-regulation of expression due to decreased GnRH as a result of negative feedback from exogenous steroid hormones (Figure 2). Surprisingly, DHT treatment did not reduce LHβ mRNA like TP but rather consistently resulted in an increase in LHβ mRNA over that in castrated animals. The unexpected stimulatory effect of DHT on LHβ mRNA in mice has been reported by other investigators, who also observed small increases in serum LH and LHβ mRNA in castrated mice treated with DHT (Lindzey et al, 1998). While the explanation for this observation is not known, pharmacological effects of DHT or method of hormone replacement have been proposed.

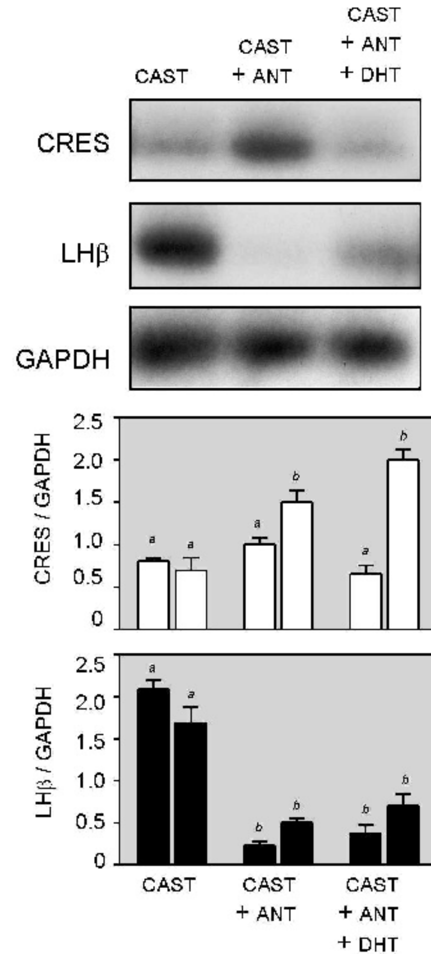
Effect of GnRH Withdrawal and Androgen Treatment on Cres mRNA Levels in Male Pituitary Glands

Because the previous studies did not delineate between steroid hormones and GnRH as potential regulators of *Cres* expression, the next set of experiments was designed to assess the effect of GnRH withdrawal as well as the direct effect of androgen treatment independent of GnRH on *Cres* mRNA levels. Male mice were castrated and treated for 1 week with Antide, a GnRH antagonist, or Antide + DHT. Antide treatment increased *Cres* mRNA approximately 2- to 3-fold above that in castrated animals (Figure 3). Additional treatment with DHT resulted in a variable response between the 2 animal experiments, with a decrease in *Cres* mRNA relative to castrated + Antide animals observed in the first experiment and an increase in *Cres* mRNA relative to castrated + Antide in the second experiment. The differential response of *Cres* mRNA to DHT between the 2 groups of mice may reflect differences in the efficiency of the DHT re-



	sham	cast	$+E_2$	+T	+DHT
T	213	<20	<20	100	<20
	693	<20	<20	250	<20
DHT	65	<20	<20	37	110
	80	<20	NA	50	158
LH	2.8	7.9	3.8	11.0	8.0
	5.7	9.4	12.3	6.6	7.4

Figure 2. Effects of castration and steroid hormone treatment on Cres and LHβ mRNAs in the male pituitary gland. Male mice were sham operated (sham) or castrated (cast) and given daily injections of sesame oil vehicle (sham and cast), estradiol ($+E_2$, 300 ng), testosterone propionate (+T, 25 μg), or dihydrotestosterone (+DHT, 25 μg) for 7 days. Pituitary glands were pooled from each group (n = 6–7 mice/group), and total RNA was analyzed by semiquantitative RT-PCR to measure the relative levels of *Cres*, LHβ, and GAPDH mRNAs. The animal experiment was repeated, and both sets of data are presented. The top panel shows a representative RT-PCR experiment from experiment 1, and the bottom 2 panels show the combined results of normalized *Cres* and LHβ IODs from all RT-PCR measurements (n = 6 replicates) (mean ± SEM) from each animal experiment. The first bar in each pair represents experiment 1, while the second bar represents experiment 2. Groups with different letters are statistically different at $P < .05$ and reflect comparisons between treatments within each animal experiment. Serum testosterone (T) (ng/dL), DHT (pg/mL), and LH (ng/mL) for each animal experiment are presented in the table. For each animal experiment RIAs were performed on serum pooled from mice in each treatment group. ND, not determined.



	cast	+Antide	+Antide+DHT
T	<20	<20	<20
	<20	<20	<20
DHT	<20	<20	2357
	<20	31	113
LH	12.8	4.1	4.5
	7.5	0.7	1.0

Figure 3. Effects of Antide and DHT treatment on Cres and LHβ mRNAs in the pituitary glands of castrated male mice. Male mice were castrated (cast) and given daily injections of vehicle, Antide (60 μg, cast + ant), or Antide (60 μg) + DHT (25 μg) (cast + ant + DHT) for 7 days. Pituitary glands were pooled, and total RNA from each group (n = 6 mice/group) was analyzed by semiquantitative RT-PCR to measure the relative levels of Cres, LHβ, and GAPDH mRNAs. The animal experiment was repeated, and both sets of data are shown. The top panel shows a representative RT-PCR experiment from animal experiment 1 and the bottom panels show the normalized Cres and LHβ IODs from all RT-PCR measurements (n = 6 replicates) (mean ± SEM) from each animal experiment. The first bar of each pair represents experiment 1, while the second bar represents experiment 2. Groups with different letters are statistically different at $P < .05$ and reflect comparisons between treatments within each animal experiment. Serum testosterone (T) (ng/dL), DHT (pg/mL), and LH (ng/mL) for each animal experiment are presented in the table. RIAs were performed on serum pooled from mice in each treatment group.

placement. Indeed, this is indicated by the large difference between the 2 experiments in the serum DHT levels in the DHT-replaced mice. In the first experiment the mean serum DHT levels were 2357 pg/mL, while in the second experiment the mean DHT levels were 113 pg/mL (Figure 3). Thus those mice exposed to higher levels of circulating DHT exhibited decreased levels of *Cres* mRNA. Together, these results suggest that both GnRH and androgens negatively regulate *Cres* mRNA in the pituitary gland. We hypothesized that one possible reason for the modest effects of Antide on *Cres* mRNA in castrated mice was that the pituitary glands were harvested 16 hours after the last Antide injection, when the effects of Antide could have been waning.

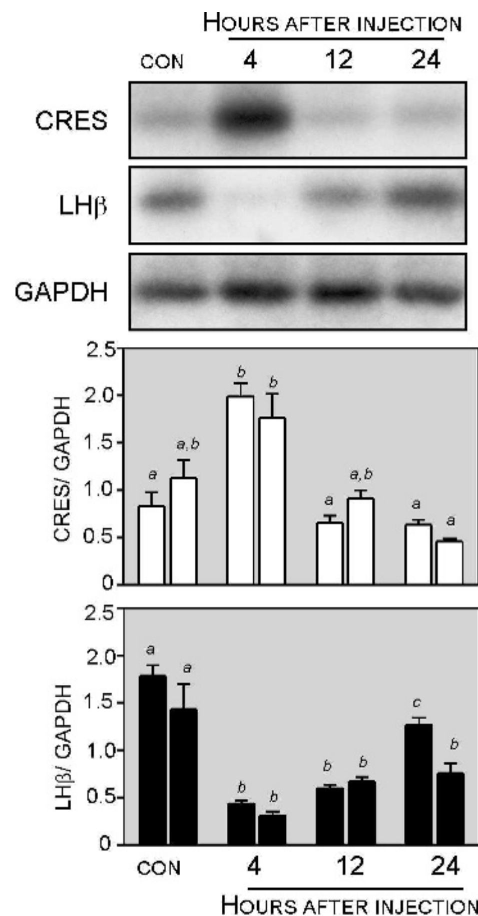
In contrast to *Cres*, Antide treatment dramatically reduced LHβ mRNA to an average 9% and 12% of castrate levels in the absence and presence of DHT, respectively (Figure 3).

Effects of Short-Term GnRH Withdrawal on Cres mRNA Levels in Male Pituitary Glands

To further examine the acute effects of GnRH withdrawal on *Cres* mRNA, intact animals were given a single injection of vehicle or Antide and pituitary glands were harvested 4, 12, or 24 hours later. Figure 4 shows that *Cres* mRNA levels were consistently increased approximately 3-fold following 4 hours of Antide treatment but returned to control levels after 12 hours, demonstrating a negative regulation of *Cres* mRNA by GnRH and illustrating the transient nature of the Antide treatment.

Compared to control animals, LHβ mRNA was reduced by 75% following 4 hours of Antide treatment but began to recover to control, intact levels over the time course, exhibiting average mRNA levels 39% and 63% of control at 12 and 24 hours, respectively. These observations were consistent with the measured serum testosterone levels in these animals, which were reduced to nondetectable levels 4 and 12 hours after Antide treatment and which recovered to approximately one-half of control values by 24 hours (Figure 4).

These studies indicate that the recovery of *Cres* mRNA to control levels after Antide treatment occurs more rapidly than that of LHβ mRNA or serum testosterone, both of which remained considerably reduced at 24 hours. Since even the short-term treatment of mice with Antide disrupted both GnRH and androgens, this experiment also did not rule out androgens as potential regulators of *Cres* mRNA. However, the return of *Cres* mRNA to control levels prior to the recovery of serum testosterone argues against androgens mediating the Antide-induced increase in *Cres* mRNA. Although the in vivo studies together supported GnRH as a negative regulator of *Cres* mRNA, they did not allow us to make a clear distinction between the effects of steroid hormones and



	Control	4hr Antide	12 hr Antide	24 hr Antide
T	487 315	<20 <20	<20 <20	205 134
LH	6.0 2.1	0.5 0.4	0.4 0.5	3.14 0.8

Figure 4. Effect of acute Antide treatment on *Cres* and LHβ mRNA levels in the pituitary glands from intact male mice. Intact male mice were treated with a single injection of vehicle (con) or Antide, and pituitary glands were harvested after 4, 12, and 24 hours. Pituitary glands were pooled from each group, and total RNA (control, n = 18 mice; Antide-treated, n = 6 mice/group) was analyzed by RT-PCR to measure the relative levels of *Cres*, LHβ, and GAPDH mRNAs. Animal experiments were repeated, and both sets of data are shown. The top panel shows a representative RT-PCR experiment from experiment 1, and the bottom panels show the normalized *Cres* and LHβ IODs from all RT-PCR measurements (n = 6 replicates) (mean ± SEM) from each animal experiment. The first bar of each pair represents experiment 1, while the second bar represents experiment 2. Groups with different letters are statistically different at $P <$ .05 and reflect comparisons between treatments within each animal experiment. Serum testosterone (T) (ng/dL) and LH (ng/mL) for each animal experiment are presented in the table. RIAs were performed on serum pooled from mice in each treatment group.

GnRH on *Cres* mRNA levels. Thus we utilized an in vitro pituitary culture system to measure the direct effects of steroid hormones and GnRH on *Cres* mRNA levels.

Effects of Steroid Hormones and GnRH on Cres mRNA Levels in Pituitary Gland Organ Cultures

To measure the direct effects of steroid hormones on *Cres* mRNA, pituitary glands were cultured in media containing ethanol (vehicle), DHT (10 nM), or E_2 (10 nM) for 6 hours. Alternatively, pituitary glands were exposed to GnRH for 5 minutes every 45 minutes over the 6-hour culture period in an attempt to mimic endogenous GnRH pulses. The data presented represent the means of 3 replicate experiments. Figure 5 shows that, relative to control pituitary glands cultured in the presence of vehicle alone (control media [con]), DHT treatment resulted in a minor decrease (25%) in *Cres* mRNA while E_2 and GnRH treatments significantly reduced *Cres* mRNA by 68% and 85%, respectively. These results confirmed our in vivo studies showing a negative regulation of *Cres* mRNA by GnRH and also demonstrated a direct action of E_2 on the pituitary gland to affect *Cres* mRNA levels. *Cres* mRNA levels were also dramatically different between pituitary glands cultured in con and those taken directly from the animal and not cultured (nc). While it is difficult to directly compare between in vitro and in vivo models, *Cres* mRNA was increased 2-fold in control cultures compared to that in the animal, which may reflect the loss of negative regulation by GnRH.

In contrast to *Cres* mRNA, LHβ mRNA was unaffected by any of the hormone treatments relative to con, suggesting that the regimen of GnRH pulses used in our studies was not optimal for the up-regulation of LHβ mRNA (Figure 5). Previously, this regimen has been reported to up-regulate the transcription of rat LHβ (Shupnik, 1990), while in other studies this regimen of GnRH pulses was not successful, suggesting either that there are species differences in LHβ responsiveness in organ culture or that other variables may be involved (Weiss et al, 1990; Winters et al, 1992; Chedrese et al, 1994). A profound decrease (67%) in LHβ mRNA was observed in cultured pituitary glands (con) compared to nc pituitary gland, which may reflect the loss of GnRH.

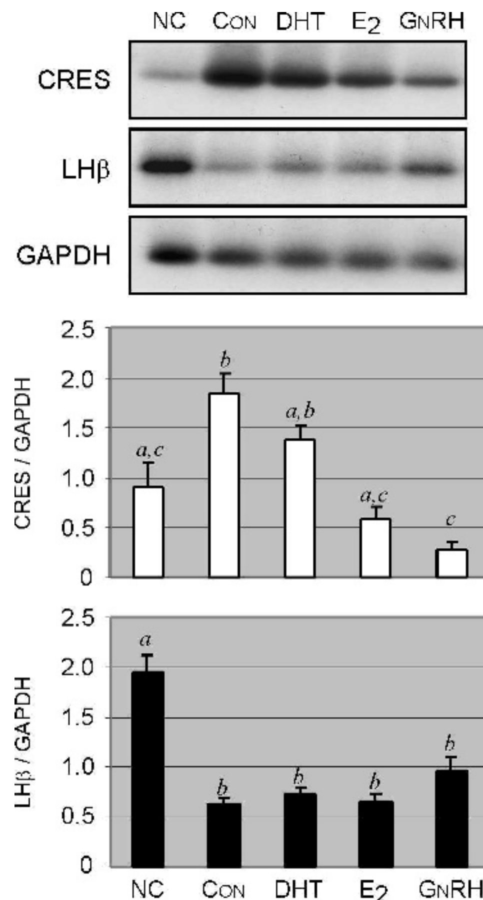


Figure 5. Effects of DHT, E_2, and pulsatile GnRH on *Cres* and LHβ mRNA levels in pituitary gland organ cultures. RNA was isolated from noncultured (NC) male pituitary glands and hemisected pituitary glands cultured in media treated with vehicle (con), DHT (10 nM), or E_2 (10 nM) for 6 hours or pulsatile GnRH (10 nM, 5-minute pulse every 45 minutes) for 6 hours (n = 3–5 hemisections/treatment). Total RNA from each group of pituitary sections was analyzed by RT-PCR to measure the relative levels of *Cres*, LHβ, and GAPDH mRNAs. The top panel shows a representative RT-PCR experiment, and the bottom panels show the average of normalized *Cres* and LHβ IODs from 3 separate experiments, each of which consisted of 6 RT-PCR replicates (mean ± SEM). Groups with different letters were statistically different at $P < .05$.

Effects of Castration and Steroid Replacement on CRES Protein in Anterior Pituitary Gonadotropes

Our previous experiments demonstrated an increase in the intracellular levels of both CRES and LHβ proteins following testosterone treatment in castrated mice (Sutton et al, 1999). To assess whether this effect is a result of the androgenic activities of testosterone or its aromatization to E_2, pituitary glands from castrated animals given hormone replacement were examined for CRES protein by indirect double-label immunofluorescence. As shown in Figure 6, consistent with our previous studies, CRES protein levels in gonadotropes were undetectable following castration, while TP administration increased the amount of intracellular CRES protein over that present in castrate mice. Immunofluorescence analyses with the control preimmune serum showed no staining (data not shown) (Sutton et al, 1999). In contrast to TP administration, E_2 treatment resulted in only low levels of background

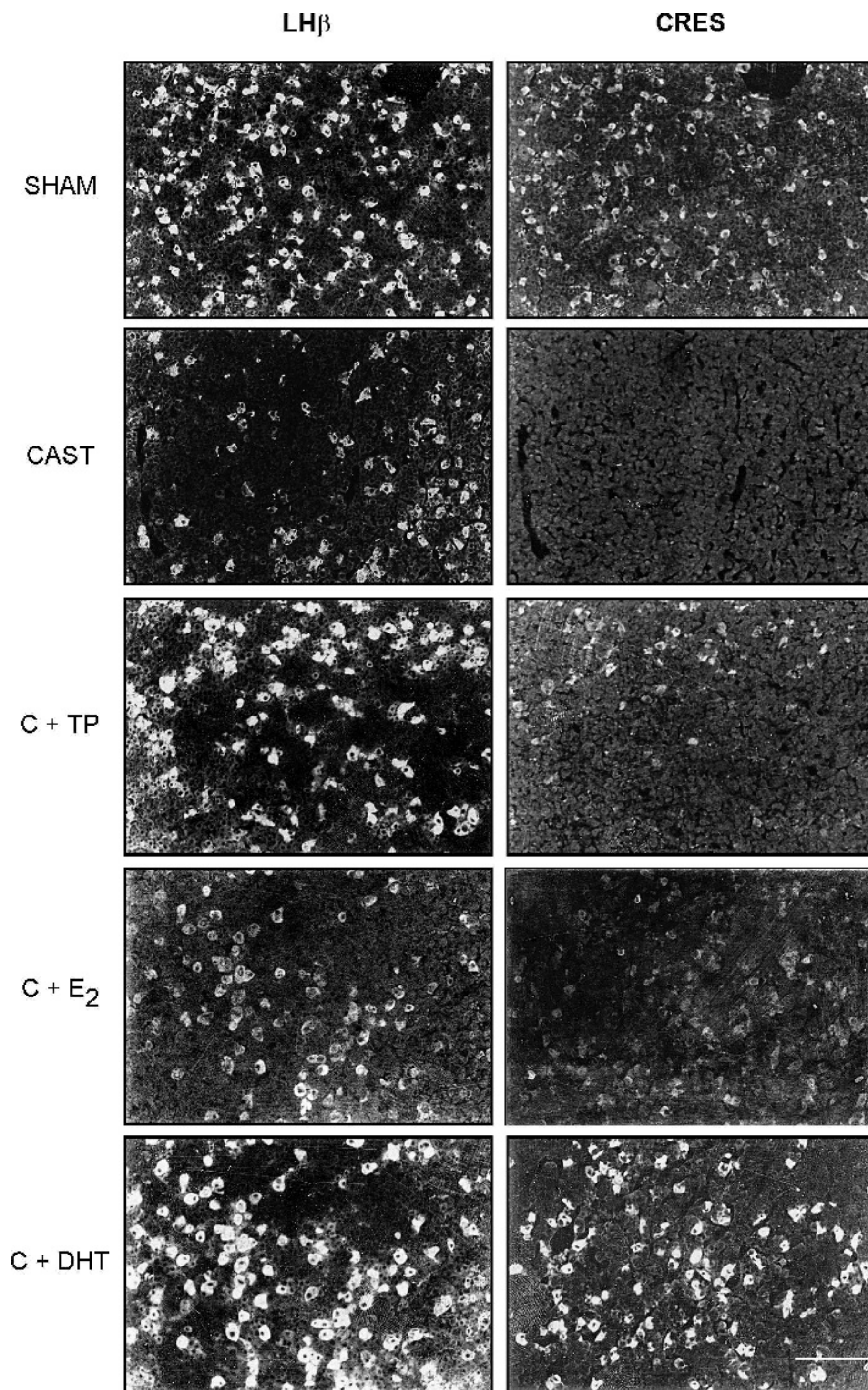


Figure 6. Effects of castration and steroid hormone maintenance on CRES and LHβ protein levels in male pituitary glands. Pituitary gland tissue sections from sham operated (sham) and castrated (cast) male mice given daily injections of vehicle, testosterone (25 μg), estrogen (E_2) (300 ng), or dihydrotestosterone (DHT) (25 μg), as described in Figure 2, were analyzed by double-label immunofluorescence for CRES and LHβ proteins as described in "Materials and Methods." The sections were photographed with filters to detect Texas Red (CRES) or FITC (LHβ) at 40× magnification. Representative photographs from each treatment group with a final print magnification of 70× are shown. Bar = 142 μm.

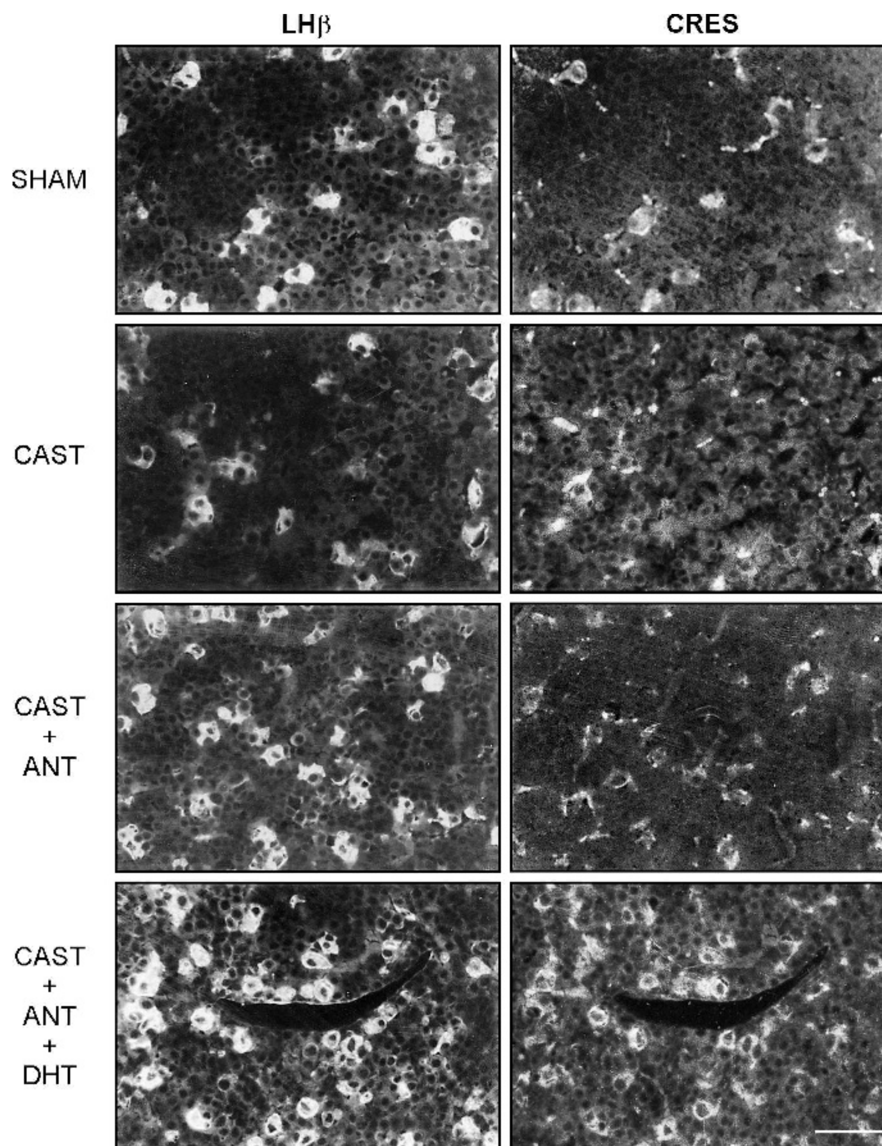


Figure 7. Effects of castration, Antide, and DHT treatment on CRES and LHβ protein levels in male mouse pituitary glands. Animals were treated as described in Figure 3, and pituitary gland sections were analyzed by double-label immunofluorescence for CRES and LHβ proteins as described in "Materials and Methods." Sections were photographed at 40× magnification with a final print magnification of 140× . Bar = 71 μm.

staining that were also detected with preimmune serum (data not shown). DHT maintenance, however, dramatically increased the amount of CRES protein in the gonadotropes to levels similar to, if not higher, than those of sham operated animals, suggesting that androgens, rather than estrogens affect CRES protein in gonadotropes.

Consistent with numerous previous reports, intracellular LHβ protein levels were decreased following castration, an effect reversed with TP maintenance (Figure 6). The increase in LHβ was due to the androgenic effects of testosterone, as E_2 treatment had little effect and DHT profoundly increased LHβ relative to castrate levels. Interestingly, CRES and LHβ were similarly altered in response to each hormonal treatment, suggesting that the proteins may be regulated similarly.

Effects of GnRH Withdrawal and Androgen Treatment on CRES Protein in Anterior Pituitary Gonadotropes

We next wanted to determine if the increase we observed in CRES protein levels following DHT treatment in castrate animals was a direct effect of DHT upon the pituitary gland or an indirect effect mediated by the hypothalamus via alterations in GnRH pulses. Thus, we examined CRES protein levels in pituitary glands from

castrated animals treated with or without Antide and DHT, as described in Figure 3. CRES protein decreased following 1 week castration (Figure 7) but appeared unchanged when castrated animals were treated with Antide. However, DHT, when given concurrently with Antide, caused a potent increase in intracellular CRES protein, an effect independent of GnRH. Thus, androgens act directly on the pituitary gland to affect intracellular levels of CRES protein.

LHβ protein levels responded similarly to CRES in that a dramatic decrease in intracellular protein was observed following castration, while the administration of Antide resulted in a very modest increase (Figure 7). The administration of DHT to Antide-treated mice, however, resulted in a profound increase in LHβ protein, indicating that, like CRES, androgens directly affect LHβ protein in the gonadotrope cells.

Discussion

GnRH Negatively Regulates Cres mRNA Levels in the Pituitary Gland

Taken together, the in vivo and in vitro studies strongly support GnRH as a negative regulator of *Cres* mRNA. Furthermore, these studies support GnRH as the primary regulator of *Cres* mRNA, with more minor effects attributed to steroid hormones. Firstly, both chronic and acute treatment of mice with Antide, a GnRH antagonist, resulted in a profound up-regulation of *Cres* mRNA levels. Correspondingly, when GnRH was added in a pulsatile manner to pituitary glands cultured in vitro, a significant decrease in *Cres* mRNA was observed relative to that of control pituitary glands cultured in the presence of vehicle alone. We have also observed that the levels of *Cres* mRNA in the pituitary gland vary considerably between individual intact mice (Figure 1). This variability is consistent with a primary regulation by pulsatile GnRH rather than more temporally stable steroid hormones. Indeed, GnRH pulses in mice varies a great deal both within and between individual male mice, with some mice having no GnRH pulses within a given 9-hour period and others having as many as 6 (Coquelin and Desjardins, 1982). The mechanism(s) by which GnRH pulses can suppress *Cres* expression remains to be determined. However, intracellular effects of GnRH in gonadotropes are mediated by its Gq/11α coupled receptor, which activates several signal transduction pathways, including the IP3 pathway, which mobilizes intracellular Ca^{++} stores and PKC and several PKC-stimulated MAPK pathways (Gharib et al, 1990; Pawson and McNeilly, 2005). Activation of specific signaling pathways could result in either reduced *Cres* transcription or increased *Cres* mRNA turnover. Indeed, both transcriptional and posttranscriptional effects of GnRH on gonadotropin mRNA levels have been described (Chedrese et al, 1994; Pawson and McNeilly, 2005).

In the hormone replacement study the decrease in *Cres* mRNA following androgen treatment to castrated mice suggested there might also be a direct effect of androgens on *Cres* mRNA. However, several lines of evidence suggest that this response may also reflect the influence of GnRH rather than testosterone. Testosterone replacement was expected to lower GnRH and return *Cres* mRNA to intact levels. However, in both animal experiments the TP-replaced mice had lower serum T (100 ng/dL, 250 ng/dL) and higher serum LH (11, 6.6 ng/mL) than the sham controls (213 ng/dL, 693 ng/dL) (2.8, 5.7 ng/mL), suggesting that GnRH remained elevated, which could account for the persistently reduced levels of *Cres* mRNA in the TP-replaced mice. In addition, androgen replacement has been shown to prevent the down-regulaton of GnRH receptor that normally occurs in response to castration-induced increases in GnRH pulse frequency (Naik et al, 1984). Thus similar levels of GnRH may produce more pronounced effects, ie, lower *Cres* mRNA levels in androgen replaced mice relative to castrated mice. Finally, the surprising stimulation of DHT on LHβ mRNA suggests that DHT does not reduce GnRH pulse frequency. Consequently, the low levels of *Cres* mRNA in DHT-replaced mice could reflect increased effects of GnRH resulting from increased GnRH receptor levels. This would be consistent with the increased LHβ mRNA in the same mice and agrees with other studies (Lindzey et al, 1998). Lastly, pituitary glands cultured in vitro in the presence of androgens showed only a small decrease in *Cres* mRNA levels that was not significantly different from control cultures. Taken together, these experiments support GnRH rather than androgens as the primary regulator of *Cres* mRNA in the male pituitary gland.

Estrogen Negatively Regulates Cres mRNA in the Pituitary Gland

Previous studies have demonstrated that E_2 can feed back to the hypothalamus and/or pituitary gland to suppress the postcastration decrease in hypothalamic GnRH content as well as the increase in serum gonadotropins (Gharib et al, 1990; Lindzey et al, 1998). Thus, E_2-treated castrated mice would be expected to demonstrate an increase in pituitary *Cres* mRNA relative to castrate levels resulting from decreased GnRH input to the pituitary gland. The opposite effect on *Cres* mRNA following E_2 treatment

of castrated mice suggests that E_2 may elicit direct effects on *Cres* mRNA in the pituitary gland. Consistent with this, E_2 acted directly upon the pituitary gland in our organ culture studies to significantly reduce, although not as profoundly as GnRH, *Cres* mRNA relative to control cultures. Interestingly, 3 EREs are superimposed over consensus sites for C/EPB known to be important in *Cres* transcription (Hsia and Cornwall, 2001), suggesting that the inhibitory activity of E_2 on *Cres* mRNA could be due to interference with C/EPBβ binding.

Divergent Expression of Cres and LHβ mRNAs

In addition to providing evidence that GnRH, and to a lesser degree estrogen, negatively regulates *Cres* mRNA in the male mouse pituitary gland, our in vivo studies comparing *Cres* mRNA with LHβ mRNA after various hormonal manipulations also provided clues with regard to CRES function in the gonadotropes. Specifically, *Cres* mRNA appeared to be regulated oppositely to that of LHβ mRNA. When serum testosterone levels were profoundly reduced, as in the castrate state, the expected and observed response in the pituitary gland was the up-regulation of LHβ mRNA and ultimately increased secretion of LH, a response designed to stimulate testosterone production from the gonad. These same hormonal conditions profoundly decreased *Cres* mRNA levels, suggesting that low levels of CRES are required during high levels of gonadotrope activity.

Androgens Increase Intracellular Levels of Both CRES and LHβ Proteins in the Anterior Pituitary Gland

In contrast to the divergent regulation of *Cres* and LHβ mRNAs, intracellular CRES and LHβ protein levels appear to be regulated similarly by steroid hormones. This likely is due to the presence of CRES and LH proteins within the same population of secretory granules, thus allowing the secretion of both proteins to be regulated similarly. Both CRES and LHβ protein levels were greatly reduced following castration. This was particularly apparent when we compared the signal intensity *and* the size of the gonadotropes, which together provide the most accurate reflection of the qualitative differences in CRES or LHβ proteins, since the distribution of gonadotropes within a pituitary gland is not homogeneous. The dramatic effects of TP and DHT and the lack of E_2 effects on CRES and LHβ proteins demonstrate that the effects of testosterone on CRES and LHβ are mediated by the androgen receptor rather than by its aromatization to E_2. Furthermore, the increase in protein levels in animals receiving Antide and DHT demonstrates that the increase is due to the direct action of androgens on the pituitary gland rather than to indirect effects mediated by GnRH from the hypothalamus. Androgens have been demonstrated to reduce LH release from rat gonadotropes by modulating components of the Ca^{++} signaling pathway (Tobin et al, 1997) and by reducing the synthesis and glycosylation of LH protein (Muyan and Baldwin, 1992). However, in this experiment the profound increase in LHβ protein observed with concurrent Antide and DHT treatment is not likely due solely to a decrease in secretion, since the serum LH levels were similar between Antide only and Antide + DHT treated groups. In addition, DHT has been shown to increase serum LH in castrated mice (Lindzey et al, 1998). This suggests that DHT may act directly on the pituitary gland to increase intracellular levels of both CRES and LHβ protein, likely via posttranscriptional mechanisms.

Difference in Cres mRNA and Protein Levels

In several experiments, we observed alterations in *Cres* mRNA that did not result in a corresponding change in CRES protein. For example, castrated animals treated with Antide for 1 week exhibited an increase in *Cres* mRNA relative to that in castrated animals, yet a corresponding increase in intracellular CRES protein was not observed. A similar lack of change in protein was also noted in animals treated with a single Antide injection (data not shown). Serum LH did not increase in the Antide-treated animals, indicating that increased gonadotrope secretory activity was not the reason for the apparent lack of an increase in intracellular CRES protein. Together, these studies further support that posttranscriptional mechanisms may regulate CRES protein levels. Also in support, CRES protein was greatly increased in castrated mice after DHT treatment despite a decrease in *Cres* mRNA. The disparate responses of mRNA and protein in this experiment could be the result of a posttranscriptional increase in CRES protein synthesis, a decrease in secretion of CRES following androgen treatment, or the accumulation of CRES protein due to an increase in its stability. Finally, these studies cannot exclude the possibility that CRES may be androgen-regulated at the level of translation, so that androgens cause an increase in the rate of CRES protein production despite lower mRNA levels.

Functional Significance of CRES in Gonadotropes

The regulation of *Cres* mRNA and protein at multiple cellular levels by components of the HPG axis suggests that CRES plays a role in gonadotrope function. Taken together, the data presented here suggest that *Cres* mRNA and protein levels were low at times of peak gonadotrope secretory activity such as are seen in castrated animals. Conversely, *Cres* mRNA levels were

generally higher in those groups of intact animals with higher average serum testosterone, suggesting that increased CRES levels are favored at times when negative feedback mechanisms maintain lower overall gonadotrope activity. We propose that in gonadotropes CRES regulates the activity of a proprotein processing protease whose activity is desirable at times of high gonadotrope synthetic and secretory activity. Particularly intriguing is the possibility that CRES inhibits PC2, a prohormone convertase responsible for processing peptides such as opioids, granins, and PACAP, which are involved in the local regulation of gonadotropin release via autocrine and paracrine feedback mechanisms. Since many neuroendocrine peptides are not sorted and secreted correctly unless they are correctly processed (Garcia et al, 2005), the failure of these peptides to mature due to CRES-mediated protease inhibition would preclude their release from gonadotropes. However, at times of high secretory activity, such as the castrated state, low CRES protein levels would allow increased release of regulatory feedback peptides along with the increased release of LH. In this way, CRES would contribute to the myriad of subtle influences known to be involved in integrating the diverse functions of the HPG axis.

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