Glucocorticoid responsiveness of the porcine GnRH receptor (GnRHR) gene

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Abstract

The binding of GnRH to its receptor results in the synthesis and secretion of the gonadotropins as well as stimulation of the gene encoding its own receptor. Thus, the interaction between GnRH and GnRHR represents a central point for regulation of reproductive function. Glucocorticoids can alter reproduction by reducing GnRH responsiveness of gonadotropes within the anterior pituitary gland, potentially via transcriptional regulation of the GnRHR gene. Consistent with this, transcription of the murine GnRHR gene is stimulated by glucocorticoids. To determine the effect of glucocorticoids on porcine GnRHR gene expression, we isolated approximately 5000 bp of 5' flanking sequence for the porcine GnRHR gene and produced reporter constructs containing the GnRHR promoter fused to the cDNA encoding luciferase (-5000LUC). The gonadotrope-derived αT3-1 cell line was transiently transfected with -5000LUC for 12 h and treated with increasing concentrations of the glucocorticoid agonist, dexamethasone (1, 10, 100 and 1,000 nM) for an additional 12 h prior to harvest. A dose-dependent increase in luciferase activity was observed with maximal induction noted at 100 nM dexamethasone (2-fold over untreated controls; $P < 0.05$). The dexamethasone induction of the -5000 promoter was blocked by the glucocorticoid antagonist, mifepristone (100 pM). To determine the location of the glucocorticoid response element(s) within the GnRHR promoter, we performed transient transfection assays with luciferase reporter constructs containing progressively less 5' flanking region for the porcine GnRHR gene. Dexamethasone-stimulated luciferase activity was maintained following reduction of the full length GnRHR promoter to 320 bp upstream of the translational start site. However, further deletion to 270 bp of proximal promoter eliminated glucocorticoid responsiveness, suggesting the presence of a glucocorticoid response element(s) within this region. Sequence analysis of this region has revealed a number of putative elements including a consensus activator protein-1 element and binding sites for cyclic AMP response element-binding protein, GATA-1 and glucocorticoid receptor. Electrophoretic mobility shift assays (EMSA) using ^{32}P-labeled oligomers spanning this region revealed increased protein binding to the -307/-287 bp oligonucleotide in nuclear extracts from αT3-1 cells treated with 100 nM dexamethasone compared to vehicle. In summary, glucocorticoid responsiveness of the porcine GnRHR gene is conferred by an element(s) located between 287 and 307 bp of proximal promoter.

Introduction

The interaction between GnRH and its receptor is a critical point for regulation of reproductive function in mammals. GnRH is secreted from the hypothalamus and travels through the hypothalamic-hypophyseal portal system to the anterior pituitary gland where it binds to its cognate receptor on gonadotrope cells. The binding of GnRH to its receptor results in the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH), as well as up-regulation of GnRHR itself (1).

In addition to regulation of glucose, protein and fat metabolism, glucocorticoids also exhibit anti-inflammatory and immunosuppressive actions. Recent investigation into stress-related reproductive disorders implicated glucocorticoids in the physiological regulation of GnRH. Consistent with this, high cortisol levels in women have been associated with reduced amounts of circulating LH (2). In contrast to inhibition of GnRH production, glucocorticoids also have been shown to increase GnRHR mRNA levels (3). Further, activity of the murine GnRHR gene promoter is up-regulated by the glucocorticoid agonist, dexamethasone (4). An AP-1 element located between 255 and 331 bp upstream of the transcriptional start site has been implicated as the glucocorticoid response element. Activation of the murine GnRHR promoter by dexamethasone is dependent upon binding of the transcription factor, c-Jun, to the AP-1 element. In this study, we wanted to examine the effect of glucocorticoids on transcriptional regulation of the porcine GnRHR gene.

Materials and Methods

- Vectors were constructed containing portions of the full-length porcine GnRHR promoter fused to the cDNA encoding luciferase.
- Transient transfections were performed in gonadotrope-derived αT3-1 cells using a liposome-mediated protocol (Fugene6, Roche Molecular). The reporter vector (0.9 µg) was cotransfected with a control vector, pSV-β-galactosidase (0.1 µg).
- Following 12 h of transfection, cells were treated with dexamethasone, vehicle (70% ethanol), or the appropriate inhibitor for an additional 12 h prior to harvest.
- Three transfections were completed in triplicate using DNA from three different plasmid preparations.
- Luciferase (Promega) and β-galactosidase (Tropix) assays were performed.
- Luciferase values were divided by β-galactosidase activity to normalize for transfection efficiency and means were expressed as fold changes over untreated control.
- Means for adjusted luciferase activity were compared using either Dunnett's two-tailed t-test or Tukey's studentized range test.
- EMSAs were performed using four ^{32}P-labeled oligonucleotides spanning the -320/-270 bp region of GnRHR promoter and crude nuclear extracts obtained from αT3-1 cells treated with 100 nM dexamethasone or vehicle.

Objectives

1. Establish glucocorticoid responsiveness of the porcine GnRHR promoter in αT3-1 cells.
2. Isolate the putative glucocorticoid response element(s) within the porcine GnRHR gene promoter.
3. Examine the molecular mechanisms underlying glucocorticoid stimulation of GnRHR gene expression.

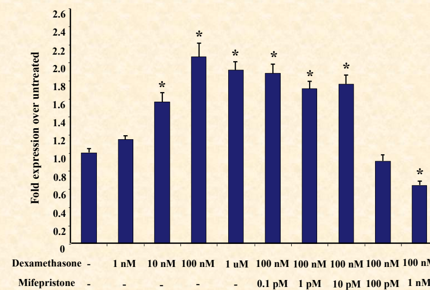

Figure 1. Luciferase activity of αT3-1 cells containing porcine GnRHR promoter constructs following treatment with increasing concentrations of the glucocorticoid agonist, dexamethasone, or 100 nM dexamethasone in combination with increasing amounts of the glucocorticoid antagonist, mifepristone. Optimal promoter activity occurred after treatment with 100 nM dexamethasone and activity was reduced to basal levels by 100 pM mifepristone. Bars with asterisks are different from controls ($P < 0.05$).

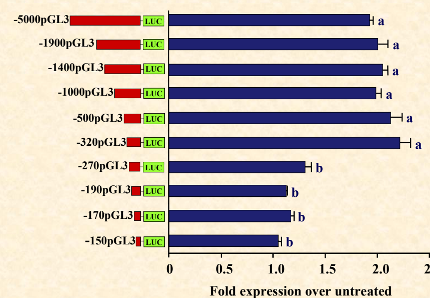

Figure 2. Transient transfection of αT3-1 cells with luciferase vectors containing sequential 5' deletions of the full-length porcine promoter. Reduction of proximal promoter from 320 to 270 bp inhibited glucocorticoid-stimulated luciferase activity by 50%, indicating the presence of a glucocorticoid response element(s). Bars with different superscripts differ ($P < 0.05$).

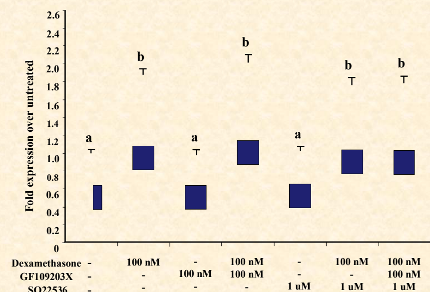

Figure 3. Dexamethasone responsiveness of the porcine GnRHR gene promoter following treatment with PKC (GF109203X) and PKA (SQ22536) inhibitors. Neither inhibitor decreased dexamethasone-stimulated promoter activity, suggesting involvement of other signaling pathways. Bars with different superscripts differ ($P < 0.05$).

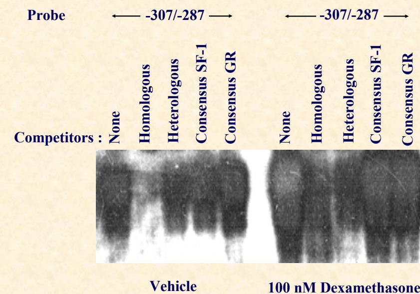

Figure 4. EMSA using a radiolabeled probe corresponding to the -307/-287 bp region of the porcine GnRHR promoter. Increased binding was detected for nuclear extracts from αT3-1 cells treated with 100 nM dexamethasone versus those treated with vehicle (70% ethanol).

Results

- Transient transfection of reporter constructs containing the full-length porcine GnRHR promoter into gonadotrope-derived αT3-1 cells resulted in a dose-dependent increase in luciferase activity following treatment with increasing concentrations of the glucocorticoid agonist, dexamethasone. Optimal promoter activity occurred after treatment with 100 nM dexamethasone (Fig. 1).
- Specificity of this response was confirmed by treatment with increasing amounts of the glucocorticoid antagonist, mifepristone. Promoter activity was reduced to basal levels following treatment with 100 pM mifepristone (Fig. 1).
- Sequential 5' deletions of -5000pGL3 demonstrated that approximately 320 bp of porcine GnRHR promoter are required for glucocorticoid responsiveness (Fig. 2).
- Protein kinase A and C inhibitors did not reduce glucocorticoid-stimulated promoter activity, indicating that other signaling pathways are involved (Fig. 3).
- Sequence analysis of the -320/-270 bp region within the porcine GnRHR gene promoter revealed an SF-1 and two putative GR binding sites as well as AP-1 and GATA-1 elements.
- EMSA with probes spanning the 50 bp region between 270 and 320 bp of 5' flanking region indicated that one of the probes, representing -307/-287 bp of proximal promoter, revealed increased binding to nuclear extracts from αT3-1 cells treated with dexamethasone compared to vehicle (Fig. 4).

Conclusions

Our results indicated that glucocorticoid responsiveness of the porcine GnRHR gene promoter is conferred by element(s) located within 287 to 307 bp upstream of the translational start site. Consistent with this, EMSA analysis indicated enhanced binding at this site with nuclear extracts from glucocorticoid-treated αT3-1 cells compared to those treated with vehicle. Finally, signaling pathways other than protein kinase A and C are stimulated following treatment of αT3-1 cells with dexamethasone.

Future Aims

- Perform transient transfections with vectors containing block replacement mutations of the glucocorticoid response element(s) within the porcine GnRHR promoter.
- Determine factors binding to the glucocorticoid response element(s) within the porcine GnRHR promoter using EMSAs.
- Further examine the signaling cascades activated following glucocorticoid stimulation of αT3-1 cells.

References

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Acknowledgments

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