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Increased testicular Sertoli cell population induced by an estrogen receptor antagonist

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ABSTRACT

Sertoli cell proliferation is prolonged in neonatal boars treated with the aromatase inhibitor letrozole, but porcine testicular aromatase synthesizes a potent, non-aromatizable androgen, 1-hydroxytestosterone, as well as estradiol. Therefore, experiments were conducted to determine whether the Sertoli cell proliferative response to letrozole is due to a loss of estrogen or a loss of androgen signaling. Littermate boars were treated with letrozole, the estrogen receptor blocker ICI 182,780, or vehicle, from 1 week of age and testes collected at 6.5 weeks. Sertoli cell number was increased 30% by letrozole or ICI 182,780 compared with vehicle. Neither treatment affected testosterone, gonadotropins or prolactin. We conclude that Sertoli cell proliferation in neonatal boars is restricted by the local activation of estrogen receptors. The response to letrozole is apparently not mediated by the novel capacity of the porcine gonadal aromatase for 1-hydroxytestosterone but by estradiol synthesis; therefore, aromatase inhibition may have similar effects on Sertoli cell proliferation in other species.

1. Introduction

Suppressing aromatase activity in pigs using an enzyme inhibitor, letrozole, for a period as short as from 1 to 12 weeks of age leads to a 25% increase in numbers of Sertoli cells in the postpubertal testis with a commensurate increase in sperm production (At-Taras et al., 2006a; Berger et al., 2008). The increased number of Sertoli cells, which can be detected as early as 6.5 weeks of age (Berger et al., 2012), likely results from extending the window of proliferation rather than decreasing the rate of apoptosis (Kao et al., 2012), but beyond this aspect, the mechanism remains unclear. Considerable interest remains in the mechanisms that regulate the proliferation of Sertoli cells since the number of Sertoli cells determines the capacity for sperm production in livestock. Deficient Sertoli cell proliferation and dysfunction are generally accepted as contributors to testicular dysgenesis syndrome. Therefore, understanding the mechanism of the testicular response to aromatase inhibition in the neonatal pig may lead to a better understanding of how Sertoli cell proliferation can be maintained in patients suffering from testicular dysgenesis and manipulated to increase spermatogenesis in other species.

The aromatase enzyme converts androgens to estrogens in all vertebrates (Conley and Hinshelwood, 2001) and is thought to be encoded by a single gene, *CYP19A* (Corbin et al., 1995), in all mammalian taxa except Suids (Conley et al., 2009). The pig has three CYP19 genes (Conley and Hinshelwood, 2001; Graddy et al., 2000), one expressed in the gonads (Conley et al., 1996; Corbin et al., 1995) and hypothalamus (Corbin et al., 2009), a second in the placenta (Corbin et al., 1995), and the third in the pre-implantation blastocyst (Choi et al., 1996). The gonadal/hypothalamic form of porcine aromatase is unique in synthesizing an unusual metabolite from testosterone, 1-hydroxytestosterone (1-OHT), a non-aromatizable androgen, in addition to synthesizing estradiol (Corbin et al., 2009, 2004). As expected, the porcine testis secretes 1-OHT as well as estradiol, and the synthesis of both is suppressed by an aromatase inhibitor. Therefore, while the effects of aromatase inhibition might be attributed to loss of estrogen synthesis alone, in the pig it is accompanied by loss of 1-OHT, this potent, non-aromatizable androgen product. This alternative leads to the important question of whether the increased proliferation of Sertoli cells following aromatase inhibition is due to reduced estrogenic or androgenic stimulation. If increased Sertoli cell numbers are mediated via reduced endogenous estrogens, inhibition of aromatase activity as a strategy to increase testicular sperm production might be adapted to cattle and other food animal species. If, on the other hand, reduced aromatase activity mediates increased Sertoli cell proliferation through reduced 1-OHT concentrations, the effects of neonatal aromatase inhibition on testis development would be expected to be specific to the pig.

The primary objective of this study was to evaluate whether reduced aromatase activity exerted its effect through a reduction in endogenous estradiol acting through classical nuclear estrogen

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receptors or an alternate mechanism. To test the hypothesis that the effects of reduced aromatase activity were mediated via the classical nuclear estrogen receptors, ICI 182,780, which acts as an antagonist for both ESR1 and ESR2 and appears to act as an agonist at the membrane GPER (Fitts et al., 2011), was used in these trials, which also compared response to letrozole and vehicle controls. At the same time, we extended our previous observations on circulating gonadotropins following letrozole administration to earlier timepoints to evaluate early, transient effects of reduced aromatase activity. We also evaluated the effects of ICI 182,780 on postnatal circulating gonadotropins and steroids and testicular steroid levels and evaluated the effects of treatments on prolactin, a reported stimulant of FSH binding and proliferation in cultured porcine Sertoli cells (Guillaumot et al., 1996; Scarabelli et al., 2003).

2. Materials and methods

2.1. Animals and treatments

All pigs were of Sygen ancestry and derived from stock provided by PIC USA (Franklin, KY) and semen provided by Genus plc (Franklin, KY). Littermate boars were randomly allocated to receive vehicle (canola oil), letrozole, or ICI 182,780 (Tocris USA, Ellisville, MO, USA). The littermate receiving the vehicle treatment was given the canola oil vehicle orally once per week (1–5 weeks of age) and received surgical implants of osmotic pumps delivering a sterile 1:1 mixture of phosphate buffered saline (PBS) and dimethylsulfoxide, the osmotic pump vehicle (Schaub and Wood, 2009). The letrozole-treated littermate received 0.1 mg letrozole suspended in canola oil vehicle/kg body weight weekly beginning at 1 week of age with the last treatment at 5 weeks of age and surgical implants of osmotic pumps containing the osmotic pump vehicle. The ICI 182,780-treated littermate received the canola oil vehicle orally and an osmotic pump containing the ICI 182,780 in a sterile 1:1 mixture of PBS and dimethylsulfoxide. Data were collected from five replicates comprising littermates from five different litters.

Osmotic pumps were inserted under general anesthesia induced with 0.3 mg Telazol® (Fort Dodge Animal Health, Overland Park, KS, USA)/kg body weight. An incision was made behind the right ear and a cavity created using blunt dissection to receive the osmotic pump, previously equilibrated with PBS; the osmotic pump was inserted along with 1/2 cc of penicillin/streptomycin. The incision was closed with three or four sutures (Prolene™, Ethicon, Inc., Somerville, NJ, USA). The initial osmotic pump was a 2ML4 (Durect Corporation, Cupertino, CA) designed to deliver compound for 4 weeks and inserted at 1 week of age. This pump was replaced at 5 weeks of age with a 2ML2 osmotic pump inserted behind the left ear. The concentration of ICI 182,780 was calculated to deliver a minimum of 125 μg/kg body weight at removal since this dose effectively inhibited uterotropic effects of estrogens in postnatal pigs (Tarleton et al., 1999). Piglet weight was monitored to ensure this dosage was met.

Blood samples were collected weekly (weeks 2–5) and prior to euthanasia by jugular venipuncture , stored on ice, centrifuged for at least 10 min at 1300×g, and the plasma aliquotted and stored at −20 °C until analyzed. Following euthanasia (86 mg pentobarbital sodium/kg body weight, Vortech Pharmaceuticals, Dearborn, MI, USA) at 6.5 weeks of age, testes were removed and weighed, and a cross-section corresponding to the equator of the testis was fixed overnight in 4% paraformaldehyde in PBS. Samples were rinsed in PBS, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. An additional testis sample was immediately frozen on dry ice and stored at −80 °C until prepared for analysis of aromatase activity.

2.2. Analyses

Sertoli cell numbers were determined following GATA-4 labeling of thick sections (25 μm thick) from the paraffin-embedded testis using 17 μm-thick counting frames randomly selected by the CAST Grid software (Computer Assisted Stereological Toolbox, Olympus) as previously described (At-Taras et al., 2006a). Number per testis are a product of the average density per frame in a minimum of 30 counting frames per testis, the volume per counting frame, and the weight of the testis providing the tissue. The total number is not corrected for tissue shrinkage during embedding; this shrinkage was equivalent across samples. Estradiol, testosterone, estrogen conjugates, LH, and FSH were measured in plasma exactly as previously described (Berger et al., 2012). Plasma was stored at −20 °C until assayed and measured in duplicate by radioimmunoassay as previously validated in the pig (At-Taras et al., 2006b). All samples from a litter were measured in the same assay. A sheep anti-estradiol 17ß-6-BSA antibody (Niswender #224; G. Niswender, Colorado State University, Fort Collins, CO.), tritiated estradiol (NET-317, Perkin Elmer Life Sciences, Boston, MA) and estradiol standards (E950; Steraloids, Wilton, NH) were used in the estradiol assays. A sheep anti-testosterone antibody (Niswender #S250), 3H-testosterone (NET370, Perkin Elmer Life Sciences) and testosterone standards (A6940, Steraloids) were used in the testosterone assay. A rabbit anti-estrone-3-glucuronide (Munro R-583, 1:12 000 dilution; courtesy of C.J. Munro, Clinical Endocrinology Laboratory, University of California, Davis, CA) and tritiated estrone sulfate (estrone sulfate [6,7-3H(N)], NET203; Perkin–Elmer Life Sciences) were used in the estrogen conjugate radioimmunoassay. Testicular tissue steroid levels were determined as previously described (Berger et al., 2012) following homogenization of frozen tissue in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 5 mM β-mercaptoethanol, and 0.5 mM AEBSF (Sigma) and concentrations expressed per mg protein determined by Coomassie dye (Coomassie Protein Assay Kit, Thermo Fisher Scientific Inc., Rockford, IL, USA). A mouse monoclonal anti-bovine LH (518B7, Roser) and porcine LH (EX275A, Papkoff) standards and a rabbit anti-porcine FSH (R285; courtesy of H. Papkoff, University of California, Davis, CA) and iodinated porcine FSH (EX274B, Papkoff) standards were used in the LH and FSH assays, respectively. Sensitivities for the estradiol, testosterone, estrogen conjugates, LH, and FSH radioimmunoassays were 10 pg/ml, 0.1 ng/ml, 0.1 ng/ml, 0.25 ng/ml, and 0.5–3 ng/ml respectively and extraction efficiencies for estradiol and testosterone averaged 82% and 83%. The mean intra-assay CV's for these five radioimmunoassays were 14%, 7.6%, 8.7%, 8.7 and 11.3%, and the interassay CV's were 9.6%, 11.2%, 13%, 7.8% and 12%. Prolactin concentrations in plasma were measured by radioimmunoassay as previously described (Horigan et al., 2009) with the following modifications; rabbit anti-PRL antiserum (National Hormone and Peptide Program) was used at a dilution of 1:10,000 dilution, and samples were incubated with ^{125}I-pPRL (20,000 cpm) for 48 h at 4 °C. Sensitivity of the assay was 0.25 ng/ml, and the intra- and interassay CV's were 2.43% and 17.6%, respectively. Microsomes were prepared from testicular tissue and aromatase activity determined by the tritiated water assay (Conley et al., 2002; Corbin et al., 2003).

2.3. Statistical analysis

The Proc Mixed procedure of SAS Statistical Programs (SAS Institute, Cary, NC) was used to analyze these data. Litter effects were removed to meet normality constraints and the residuals of Sertoli cell numbers and testes weight were analyzed for treatment effects using Proc Mixed. Similarly, testicular aromatase activity and body weight were analyzed using Proc Mixed with litter as a random effect. Estradiol, estrogen conjugate, LH, and FSH values