Journal of Andrology, Vol. 26, No. 5, September/October 2005

Protein C Inhibitor Expression by Adult Rat Sertoli Cells: Effects of Testosterone Withdrawal and Replacement

MATTHEW D. ANWAY,* MATTHEW D. SHOW,† AND BARRY R. ZIRKIN†

*From the *Center for Reproductive Biology, Washington State University, Pullman, Washington; and the †Division of Reproductive Biology, Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland.*

ABSTRACT: Protein C inhibitor (PCI), a member of the plasma serine protease inhibitor family, has been reported to be abundantly expressed in the seminal vesicles and testes. In this study, we examine the localization and regulation of the *PCI* gene and protein expression in testes and freshly isolated Sertoli cells from control rats, rats treated with luteinizing hormone–suppressive testosterone/estradiol (TE)-containing Silastic capsules for 7, 14, 28, and 56 days, and rats treated with TE for 56 days, followed by high levels of testosterone for 7 or 14 days. The administration of the TE capsules for 56 days resulted in reduced testicular testosterone, from approximately 100 ng/mL in the controls to approximately 10 ng/mL, accompanied by a 73% reduction in testicular weight. PCI mRNA levels in freshly isolated Sertoli cells were reduced by 30% and 54% following TE treatment for 28 and 56 days, respectively. When rats that had received TE capsules for 56 days were provided replacement testosterone, there was a 40% increase in PCI mRNA levels within 7 days in the absence of any change in testicular weight, and PCI mRNA levels returned to control values by 14 days. The decrease in PCI mRNA levels in TE-treated rats was paralleled by a decrease in PCI protein levels in whole testis lysates and in seminiferous tubule fluid (STF). Protease activity was significantly increased in STF following 56 days of TE treatment. Taken together, these results indicate that 1) PCI in the testis is expressed by Sertoli cells; 2) the testicular expression of PCI is responsive to intratesticular testosterone levels; and 3) protease activity within the seminiferous epithelium is elevated when intratesticular concentration is decreased, perhaps as a consequence of decreased PCI.

Key words: Estradiol, androgen, testis, protease inhibitor.
J Androl 2005;26:578–585

Protein C inhibitor (PCI) is a member of the plasma serine protease inhibitor (serpin) family of proteins. In humans, PCI is the inhibitor of activated serine protease protein C, the major protease of the serum anticoagulant protein C pathway (Suzuki et al, 1983) as well as several other serine proteases, including thrombin (Rezaie et al, 1995), urokinase (Espana et al, 1993) and kallikreins (Espana et al, 1995), acrosin (Hermans et al, 1994), and prostate-specific antigen (Christensson and Lilja, 1994).

PCI has been shown to be expressed in several human and rodent tissues, including the seminal vesicles, prostate, epididymis gland, and testis (Laurell et al, 1992; Wakita et al, 1998). Mice lacking a functional PCI protein are infertile (Uhrin et al, 2000). Disrupted Sertoli cell–tight junctions and apoptotic germ cells at all stages of the cycle of the seminiferous epithelium characterize the testes of these mice, suggesting that PCI expression has a vital function in spermatogenesis (Uhrin et al, 2000). PCI (−/−) mice have been reported to have a twofold increase in urokinase-like proteolytic activity in testis extracts when compared to control mice, an effect that was reversed with purified recombinant human PCI (Uhrin et al, 2000). On the basis of these observations, Uhrin et al (2000) ascribed the infertility observed in the PCI (−/−) mice to disorganization of germ cell development within the seminiferous epithelium resulting from increased proteolytic activity.

It is not yet known whether the expression of the *PCI* gene is sensitive to androgen levels. On the one hand, Wakita et al (1998) reported that *PCI* gene expression in the rat seminal vesicle depends on the presence of testosterone in blood serum. This conclusion was based on the observation that within 48 hours following castration, PCI mRNA levels, relatively high in untreated rats, became undectectable, and this effect was reversed upon the replacement of testosterone. Consistent with this, the administration of estradiol to rats, which decreases serum testosterone levels, resulted in a time-dependent decrease in PCI mRNA levels in the seminal vesicles (Wakita et al, 1998). However, this treatment had no effect on PCI mRNA levels in the testis (Wakita et al, 1998), suggesting that testicular *PCI* gene expression is not sensitive to androgen levels. Intratesticular testosterone levels were not

Supported by NIH grant HD44258 (B.R.Z.).
Correspondence to: Dr Matthew D. Anway, Center for Reproductive Biology, Washington State University, Pullman, WA 99164-4231 (e-mail: manway@mail.wsu.edu).
Received for publication December 29, 2004; accepted for publication April 9, 2005.
DOI: 10.2164/jandrol.05001

measured, however, and, as pointed out by Wakita et al (1998), the administration of estradiol for only 7 days may not have been adequate to reduce intratesticular testosterone levels. Therefore, it is not known whether PCI within the testis is responsive to intratesticular testosterone concentration or, alternatively, whether PCI in the testis responds differently to reduce testosterone than does PCI within other components of the reproductive tract.

We recently published a method for isolating adult Sertoli cells rapidly and with high purity (Anway et al, 2003). In the current study, we used this method to conduct an ex vivo study designed to assess the effect of reduced and repleted intratesticular testosterone on the expression of PCI mRNA by freshly isolated rat Sertoli cells. Our intent was to test the hypothesis that the expressions of PCI mRNA and protein in the testis are responsive to intratesticular androgen concentration.

Materials and Methods

Animals

Male Sprague-Dawley rats aged 8–12 weeks were purchased from Charles River (Kingston, Mass). All rats were housed in a vivarium under a 14h:10h light-dark cycle and provided water and rat chow ad libitum.

Intratesticular Testosterone

To experimentally reduce intratesticular testosterone, rats were administered subdermal 2.5 cm of testosterone (T)/0.1 cm of estradiol (E)-filled polydimethylsiloxane (Silastic, Dow Corning, Midland, Md) capsules, or empty capsules as controls, according to methods previously described (Stratton et al, 1973; Ewing et al, 1977). To study the effects of reduced intratesticular testosterone on PCI expression, rats were implanted with TE capsules for 7, 14, 28, and 56 days. To study the effects of testosterone repletion, rats that had received TE treatment for 56 days were implanted with 3- × 8-cm (24 cm total) capsules for 5–7 (T7) or 14–15 (T14) days. Some rats received 3- × 8-cm testosterone-filled capsules for 56 days (TM). This dose was shown to maintain quantitative spermatogenesis, although at lower than normal intratesticular testosterone concentration (Zirkin et al, 1989). All protocols were approved by the Johns Hopkins University School of Public Health Animal Care and Use Committee.

Sertoli Cell Isolation

Sertoli cells were isolated following methods previously described (Anway et al, 2003) but omitting the 10-minute trypsin digestion. Briefly, 2 decapsulated testes were incubated in 0.5 mg/mL of collagenase in Hanks balanced salt solution (HBSS, pH 7.4) at 34°C, with shaking for 15 minutes to eliminate the interstitial cells. After 3 washes, the seminiferous tubules were incubated in a mixture of enzymes (0.1% collagenase, 0.2% hyaluronidase, 0.04% DNase I, and 0.03% trypsin inhibitor in HBSS, pH 7.4) at 34°C, with shaking for 40 minutes. The Sertoli cells were pelleted by centrifugation, and the resulting supernatant was collected for peritubular myoid cell cultures (see below). The Sertoli cell pellet was washed in HBSS and repelleted by centrifugation. This step was repeated a total of 3 times. The Sertoli cells then were resuspended in HBSS, subjected to hypotonic shock in a dilute HBSS solution (1:3.5 HBSS/water), collected by centrifugation, resuspended in HBSS, and filtered through 53-μM nylon mesh. The cells were washed and resuspended in Ham media and Dulbecco modified Eagle medium mixed 1:1 (F12/DMEM).

Cell viability, assessed by trypan blue exclusion, consistently was greater than 98%. Sertoli cells were identified by their distinct morphology (Griswold et al, 1988). The total number of isolated Sertoli cells per testis was approximately 4–7 million, as estimated by hemacytometer counts. Isolated Sertoli cells were stained for vimentin protein, and purity was assessed by determining the percentage of stained cells, as previously described (Anway et al, 2003). Purity was approximately 75%–80% for all Sertoli cell preparations. The contaminants were peritubular myoid cells (approximately 10%) and germ cells (10%–15%).

Peritubular Myoid Cell Isolation

Peritubular myoid cells, contained in the supernatants after pelleting Sertoli cells, were collected by centrifugation (1000 × *g* for 2 minutes). The myoid cells were plated on 100-mm plates in DMEM plus 5% fetal calf serum and allowed to proliferate for 3 days. The purity of the peritubular myoid cells was estimated to be greater than 95% by staining for smooth muscle b-actin, as previously described (Anway et al, 2003).

Germ Cell Isolation

Pachytene spermatocytes, round spermatids, and elongating spermatids were isolated from the testes of 12-week-old rats by unit gravity sedimentation (Staput) according to methods previously described (Aguilar-Mahecha et al, 2001). Purity was determined by cell morphology under phase-contrast microscopy. The purities of pachytene spermatocytes, round spermatids, and elongating spermatids were estimated to be 90%, 90%, and 85%, respectively.

Leydig Cell Isolation

Leydig cells were isolated as previously described (Klinefelter et al, 1987). Briefly, the testicular artery was cannulated and perfused with collagenase (1 mg/mL) in dissociation buffer (199 medium with 2.2 mg/mL HEPES, 1.0 mg/mL bovine serum albumin, 25 mg/L trypsin inhibitor, and 0.7 mg/mL sodium bicarbonate, pH 7.4) to clear blood from the testes. Testes were decapsulated and digested in collagenase (0.25 mg/mL) at 34°C, with shaking for 15 minutes. The dissociated cells were subjected to centrifugal elutriation and Percoll gradient centrifugation purification, as previously described (Klinefelter et al, 1987). The final purity of the Leydig cells obtained this way, determined by staining the cells for 3β-hydroxysteroid dehydrogenase (3β-HSD), consistently was 95%.

RNA Isolation and Northern Blot Analyses

Total RNA was purified from rat testis, cultured peritubular myoid cells, freshly isolated Leydig cells, Sertoli cells, pachytene

spermatocytes, round spermatids, and elongating spermatids by the Trizol method (Invitrogen Corporation, Carlsbad, Calif). The cDNA clone for PCI was generated for Northern blot analyses by reverse transcriptase-polymerase chain reaction (RT-PCR). First, total RNA was isolated from the testis of a 4-month-old rat. Testis total RNA (2 μg) was reverse transcribed in a 20-μL reaction at 46°C for 60 minutes, using 0.2 U of Superscript II (Invitrogen) and 50 ng of oligo-dT primer in single-strength first-strand synthesis buffer, according to manufacturer's specifications. PCR was performed in a reaction volume of 50:1 containing 0.5:1 of cDNA, single-strength buffer, 20 μM deoxy nucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 300 nM antisense primer, 300 nM sense primer, and 0.5 U of AmpliTaqR DNA Polymerase (Perkin Elmer, Boston, Mass). The PCR conditions were 30 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 2 minutes. PCR products were cloned into p-GemT Easy Vector (Promega, Madison, Wis) according to manufacturer's specifications and sequenced to verify the product. PCI cDNA was generated using sense primer 5′-GGACTCCTTCTGAAACTCA-3′ and antisense primer 5′-AACTGCAGTCAGCCAGGTT-3′ corresponding to base pairs 1272–1942 of the cDNA GenBank accession number NM_022957.

Total RNA (10 μg) from isolated germ and Sertoli cell preparations was fractionated in a 1% agarose/formaldehyde gel, transferred overnight to a nylon membrane (HybondTM-N; Amersham Pharmacia, Piscataway, NJ), and ultraviolet (UV) cross-linked (UV Stratagene 1800, Stratagene Inc, La Jolla, Calif). PCI, clusterin (Collard and Griswold, 1987), protamine-2 (Anway et al, 2002), full-length transferrin (which detects both Sertoli cell transferrin) and germ cell hemiferrin mRNA (Huggenvik et al, 1987), and ribosomal protein S2 (ChoB; used as a control for RNA loading) (Mukherjee et al, 1996) cDNAs were radiolabeled with (α-^{32}P) deoxy adenine triphosphate (dATP), using the Rad Prime DNA Labeling Kit (Invitrogen). Northern blots were hybridized overnight at 65°C with labeled cDNA probes in ExpressHyb hybridization solution (Clontech, Palo Alto, Calif). Following hybridization, blots were washed in 2× saline sodium citrate (SSC) with 1.0% sodium dodecyl sulfate (SDS) for 30 minutes at 65°C; 1× SSC with 0.5% SDS for 30 minutes at 65°C; and 0.1% SSC with 0.1% SDS for 30 minutes at 65°C. After blots were washed, they were placed in a phosphor screen cassette for 8–12 hours. The signals were detected using a Typhoon 9200 and quantified using ImageQuant software (Amersham Pharmacia). Northern blots were normalized to the mRNA levels of clusterin, with the control ratios given a numerical value of 1.0 and treatment groups represented as a percentage of the control. Normalization was to clusterin mRNA, because previous studies have shown that clusterin mRNA levels do not change following androgen withdrawal (Roberts et al, 1992; Turner et al, 2001).

Radioimmunossays

Interstitial fluid (IF) was collected according to a previously described method (Turner et al, 1984). In brief, an 18-gauge needle was used to puncture 3 holes in the tunica at one end of a testis. The testis was then placed in a tube and centrifuged at 400 × *g* to separate the IF from the tissue (Turner et al, 1984). All samples were stored at −80°C until assayed for testosterone. IF testosterone concentrations were determined in duplicate for each sample as previously described (Turner et al, 1984). Testosterone was assayed by radioimmunoassay with a testosterone antibody from ICN (Costa Mesa, Calif) and ^{3}H-T from NEN (Boston, Mass). The sensitivity of the assay was 10 pg per tube.

Western Blot Analyses

For some studies, testes or isolated cells were homogenized in protein suspension buffer (100 mM NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 0.1% Triton X-100, and 0.5% protease inhibitor cocktail [P8340; Sigma]). For others, seminiferous tubule fluid (STF) was obtained from rat testes by a method previously described (Turner et al, 1984). Briefly, an incision was made in the tunica albuginea of the distal pole of the testis, and the testis was centrifuged at 54 × *g* at 0°C for 15 minutes to collect IF, as above. The testis was then decapsulated, and the seminiferous tubules were rinsed to remove residual IF. The seminiferous tubules were extruded through the hub of a syringe and then centrifuged at 6000 × *g* at 0°C for 15 minutes to collect the supernatant above the collapsed seminiferous tubules, which was composed of STF. The STF was placed in a new 1.5-mL tube, and protein concentration was measured. For all samples, the protein concentrations, determined by the BCA method (Pierce, Rockford, Ill), were similar, ranging from 0.47 to 0.62 mg/μL.

Protein samples were sonicated for 45 seconds and then centrifuged at 13 000 × *g* for 5 minutes at room temperature to eliminate insoluble material. The supernatant was added to an equal volume of 2× loading buffer (100 mM Tris, pH 6.8, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and stored at −20°C until analyzed. Prior to analyses, samples were reduced with 0.1% beta-mercaptoethanol, boiled for 2 minutes, and loaded on a 10% SDS-polyacrylamide gel as previously described (Laemmli, 1970). Protein was transferred to Protran Nitrocellulose (Schleicher & Schuell, Keene, NH) with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, Calif), according to manufacturer's specifications.

PCI protein was detected on Western blots using anti-PCI antibody (Uhrin et al, 2000). Membranes were blocked for 1 hour with blocking solution (5% nonfat dry milk in 1× TBSS (25 mM Tris, 137 mM NaCl, and 3 mM KCl) and 0.1% Tween 20 at room temperature, followed by anti-PCI antibody (1:1000) overnight in blocking solution at room temperature. After 3 washes in 1× TBSS plus 0.1% Tween 20, membranes were incubated with secondary anti-rabbit horseradish peroxidase (HRP)-linked immunoglobulin G (IgG; 1:3000, NA 931; Amersham Pharmacia) for 1 hour in 1× TBSS at room temperature. The membranes were washed 3 times in 1× TBSS plus 0.1% Tween 20 and then 3 times in 1× TBSS (standard wash following all secondary antibody incubations). All signals were detected using a SuperSignal WestPico Chemiluminescent kit (Pierce) according to manufacturer's specifications. Membranes were stripped using Restore Western Blot Stripping Solution (Pierce) according to manufacturer's specifications. Membranes with total testis protein were blocked and probed with anti-beta actin antibody (1:1000, A5441; Sigma) for 3 hours in blocking solution, followed by anti-mouse HRP-linked IgG (1:3000, NA 931; Amersham Pharmacia) for 1 hour at room temperature. A

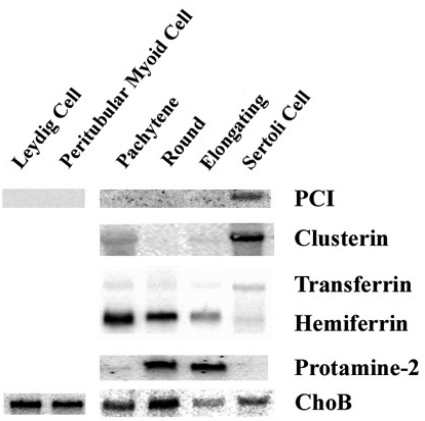

Figure 1. Northern blots of protein C inhibitor (PCI) in RNA purified from cultured peritubular myoid cells and freshly isolated Leydig and Sertoli cells, pachytene spermatocytes, round spermatids, and elongating spermatids from 120-day-old rats. Each lane was loaded with approximately 10 μg of total RNA. Membranes were probed with cDNAs for the Sertoli cell–expressed genes *PCI,* clusterin, and tranferrin and for germ cell–expressed genes hemiferrin and protamine-2. Ribosomal protein S2 (ChoB) cDNA was used as an RNA loading control.

duplicate set of membranes with STF protein was generated and probed with anti-clusterin antibody (1:1000) overnight, followed by anti-rabbit HRP-linked IgG for 1 hour. All films were digitized and intensities quantified by MacBAS software version 2.2 (Fuji Photo Film Co, Edison, NJ). Intensities were normalized to actin expression; control ratios were given a numerical value of 1.0, and treatment groups were represented as a percentage of the control.

Proteolytic Activity

Protease activity was measured in the STF from control rats and from rats administered TE for 56 days with the QuantiCleave Protease Assay kit (Pierce) using 100 μL of 1× phosphate-buffered solution (PBS) instead of 100 μL of the succinylated casein protein. STF was collected and diluted 1:5 with 1× PBS, and 10 μL was used in each reaction at 37°C for 0–30 minutes. Following incubation, trinitrobenezenesulfonic acid (TNBSA) was added and allowed to react for 20 minutes at room temperature. Protease activity was determined by colorimetric analysis, with absorbancies measured at 450 nm.

Statistical Analysis

Data are expressed as the mean ± SEM of 3–5 experiments. Sample differences were analyzed by 1-way analysis of variance (ANOVA). If ANOVA showed $P < .05$, group mean differences were determined by the Scheffe multiple range test. Means were considered significantly different at $P < .05$.

Results

Sertoli Cell PCI Gene Expression

To identify the cell type(s) in the testis that expressed PCI mRNA, we analyzed RNA from freshly isolated pachytene spermatocytes, round spermatids, elongating spermatids, Sertoli cells, Leydig cells, and cultured peritubular myoid cells by Northern blot analysis. As seen in Figure 1, PCI mRNA was detected in Sertoli cells but not in pachytene spermatocytes, round spermatids, or elongating spermatids. Known Sertoli cell–expressed genes transferrin and clusterin were expressed in the isolated Sertoli cells and were absent from the round and elongating spermatid samples. The faint signal for clusterin in the pachytene spermatocyte sample probably was due to Sertoli cell contamination. As with the isolated germ cells, PCI mRNA was not detected in freshly isolated Leydig cells or in cultured peritubular myoid cells (Figure 1). Ribosomal S2 (ChoB) mRNA was detected in all samples. These results indicate that PCI is expressed predominantly, if not exclusively, by the Sertoli cells within the testis.

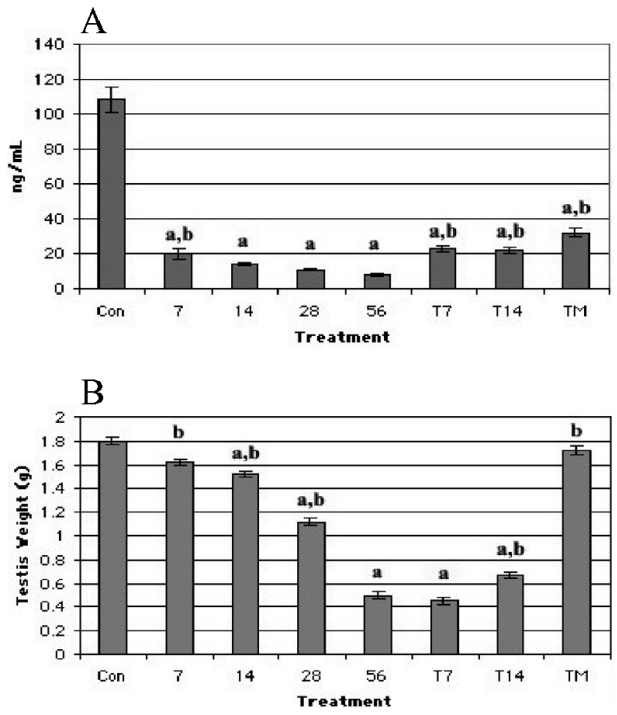

Figure 2. Effects of exogenous treatment with testosterone capsules on interstitial fluid (IF) testosterone concentrations **(A)** and testis weights **(B)** in control (Con) rats, in rats following testosterone/estradiol (TE) treatment for 7, 14, 28, or 56 days, and in rats administered TE for 56 days and then administered high levels (24 cm of Silastic implants) of testosterone for 5–7 (T7), 14–15 (T14), or 56 (TM) days. Each time point represents the mean ± SEM (n = 4) derived from 3 independent treatment groups. a—statistically different at $P < .05$ from control value; b—statistically different from TE for 56 days.

Intratesticular Testosterone and Testis Weight

The administration of luteinizing hormone–suppressive TE capsules to rats resulted in decreases in IF testosterone (Figure 2A) and testis weight (Figure 2B) over time after the treatment. IF testosterone levels decreased significantly from 100 ± 7.2 ng/mL in the control rats to 20 ±

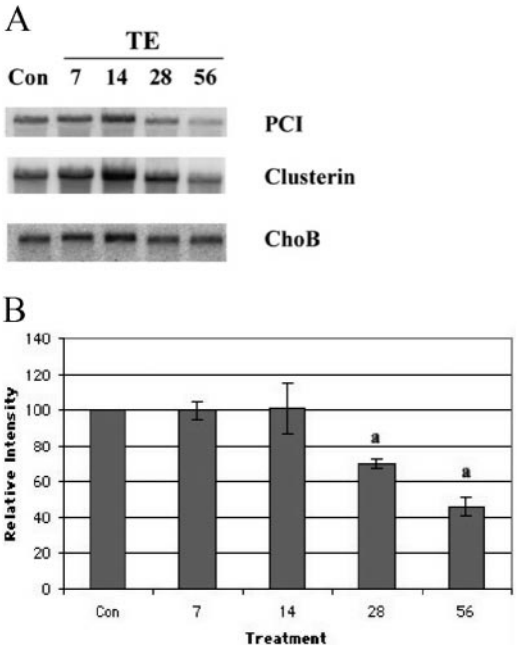

Figure 3. **(A)** Northern blots of protein C inhibitor (PCI), clusterin, and ribosomal protein S2 (ChoB) expression in Sertoli cells isolated from control (Con) rats and from rats following testosterone/estradiol (TE) treatment for 7, 14, 28, or 56 days. Each lane is loaded with approximately 10 μg of total RNA purified from freshly isolated Sertoli cells. **(B)** Quantification of Sertoli cell PCI mRNA levels following TE treatment of rats for 7, 14, 28, and 56 days, normalized to clusterin mRNA. Each time point represents the mean ± SEM (n = 3) from 3 independent Sertoli cell isolations. a—statistically different from control value.

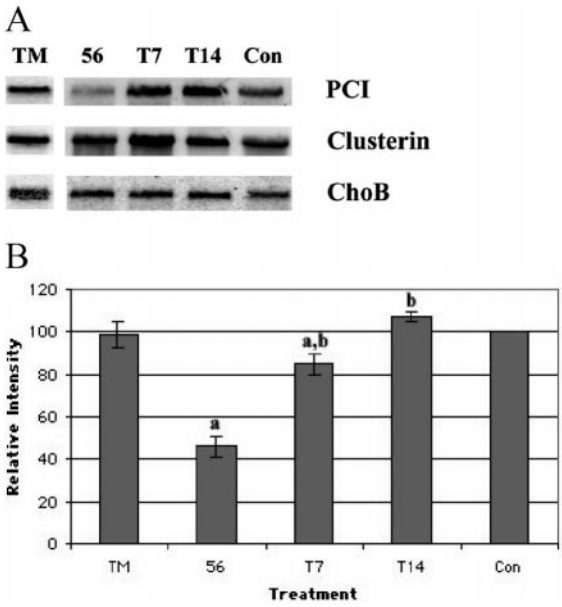

Figure 4. **(A)** Northern blots of protein C inhibitor (PCI) expression in Sertoli cells isolated from control (Con) rats following testosterone/estradiol (TE) treatment for 56 days (56) and from rats administered TE for 56 days and then administered high levels (24 cm of Silastic implants) of testosterone for 5–7 (T7), 14–15 (T14), or 56 (TM) days. Rats were administered empty 24-cm capsules for 56 days as a control. Each lane was loaded with approximately 10 μg of total RNA purified from freshly isolated Sertoli cells and was probed with PCI, clusterin, and ribosomal protein S2 (ChoB) cDNA. **(B)** Quantification of PCI mRNA levels following testosterone repletion, normalized to clusterin mRNA. Each time point represents the mean ± SEM (n = 3) from 3 independent Sertoli cell isolations. a—significantly different from the control value; b—statistically different from the TE/56-day value.

3.0 ng/mL following 7 days of TE treatment and then decreased further, to 8 ± 1.1 ng/mL, by 56 days of TE treatment (Figure 2A). In rats administered 24 cm of testosterone-filled Silastic implants (testosterone maintenance dose, TM), a dose shown to maintain spermatogenesis (Zirkin et al, 1989), the IF testosterone level was reduced, when compared to controls, to 30 ± 2.2 ng/mL, by 56 days of treatment (Figure 2A). The administration of the TM dose to rats that previously had received TE capsules for 56 days increased the IF testosterone levels to 24 ± 1.4 ng/mL and 25 ± 1.6 ng/mL at 7 (T7) and 14 (T14) days thereafter, respectively. Reduced intratesticular testosterone concentrations correlated with decreased testis weights (Figure 2B); testis weights decreased from 1.80 ± 0.03 g in the controls to 0.50 ± 0.03 g by 56 days of treatment with TE. Testis weight was maintained at normal levels in the TM group, indicating that spermatogenesis was maintained. Providing testosterone to rats after 56 days of TE did not alter testis weight by 7 days. After 14 days of testosterone repletion, however, testis weights increased to 0.68 ± 0.03 g, due to an increase in germ cell numbers when compared to the TE group (Figure 2B), as determined by morphological analyses of fixed tissues.

Analysis of PCI mRNA Following Testosterone Withdrawal and Repletion

PCI gene expression was analyzed by Northern blots (Figure 3A) of total RNA from Sertoli cells freshly isolated from control rats and from rats administered TE capsules for 7, 14, 28, or 56 days. Quantification of the blots (Figure 3B) showed that PCI mRNA levels, when normalized to clusterin mRNA levels, were unchanged from control levels in Sertoli cells isolated 7 or 14 days after TE treatment but that they were decreased significantly, by 30% and 54%, following 28 and 56 days of TE treatment, respectively. Clusterin mRNA levels were not significantly changed following the TE treatment (data not shown), as reported previously (Roberts et al, 1992; Turner et al, 2001).

PCI mRNA levels were analyzed in freshly isolated Sertoli cells following TE treatment for 56 days and then after repletion of intratesticular testosterone with 24 cm of testosterone-containing capsules for 7 or 14 days (Figure 4A and B). PCI mRNA level was significantly reduced from controls following TE treatment for 56 days, but repletion of intratesticular testosterone levels for 7 days increased PCI mRNA levels by 40% when compared

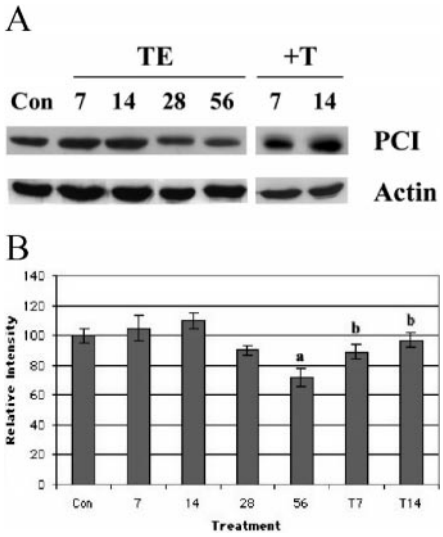

Figure 5. **(A)** Western blots of protein C inhibitor (PCI) and actin in total testis protein from control (Con) rats, rats administered testosterone/estradiol (TE) for 7, 14, 28, and 56 days, and rats administered TE treatment for 56 days (56), followed by the administration of high levels of testosterone for 5–7 (T7) and 14–15 (T14) days. Each lane was loaded with approximately 20 μg of protein. **(B)** Quantification of PCI protein in the testes of rats administered TE for 7, 14, 28, and 56 days and in rats administered TE for 56 days, followed by high levels of testosterone for 5–7 (T7) or 14–15 (T14) days, normalized to actin levels. Each time point represents the mean ± SEM (n = 3) from 3 independent treatment groups. a—statistically different from the control value; b—statistically different from the TE 56-day value.

to the level in the TE 56-day samples (Figure 4B). The repletion of intratesticular testosterone for 7 days did not increase the testis weight when compared to the TE 56-day testes (Figure 2B), which suggests that the increase in Sertoli cell *PCI* gene expression seen at 7 days after testosterone repletion resulted from increased androgen levels in the testis and not from altered numbers of germ cells. Repletion of intratesticular testosterone level for 14 days restored PCI mRNA to control levels, completely reversing the decrease in the mRNA level following the 56-day TE treatment (Figure 4B). The repletion for 14 days resulted in increased testis weights when compared to the 56-day TE levels, although not to control values. The reduction of IF testosterone from control levels after providing control rats with the TM dose for 56 days (Figure 2A) did not alter PCI mRNA levels when compared to the untreated controls (Figure 4B), suggesting that the very high IF testosterone concentration of the controls is not required for maximal *PCI* gene expression.

Analysis of PCI Protein Expression Following Testosterone Withdrawal and Repletion

PCI protein expression was analyzed by Western blots of total protein from the testes of control rats and rats administered TE capsules for 7, 14, 28, or 56 days (Figure 5). With the knowledge that PCI is secreted by Sertoli

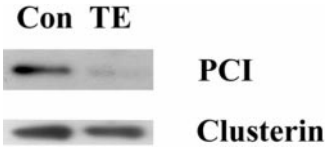

Figure 6. Western blots of seminiferous tubule fluid (STF) protein C inhibitor (PCI) and clusterin protein from control (Con) rats and following TE treatment for 56 days (TE). Each lane was loaded with approximately 10 μL of diluted STF protein sample.

cells, we also examined the PCI protein content of the STF collected from control rat testes and the testes of rats that received TE treatment for 56 days (Figure 6). As was true of mRNA levels, PCI protein levels in the testes did not change significantly following TE treatment for 7, 14, or 28 days, but they did decrease significantly, by 27%, following TE treatment for 56 days (Figure 5). This effect was reversed by 7 and 14 days of testosterone replacement (Figure 5). The level of PCI also decreased in the STF following 56 days of TE treatment (Figure 6). Clusterin protein levels were similar between the controls and after 56 days of TE treatment. It should be noted that the total STF protein concentration was similar between control and TE-treated rats, suggesting that testosterone withdrawal reduced both PCI protein expression and its secretion into the seminiferous epithelium.

STF Proteolytic Activity

Proteolytic activity was measured in the STF from control rats and rats treated with TE capsules for 56 days following the incubation of the STF for 0–30 minutes (Figure 7). The proteolytic activity was significantly increased in the STF from the TE-treated rat testes. Normalizing each

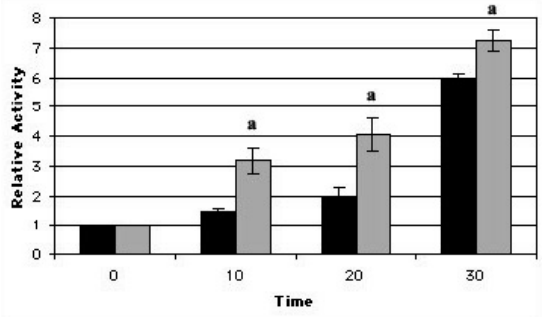

Figure 7. Protease activity within the seminiferous tubule fluid (STF) of control rats (black bars) and rats administered testosterone/estradiol (TE) for 56 days (gray bars). Each STF sample was diluted 1:5 with phosphate-buffered saline (PBS) and incubated for 0–30 minutes as described in ''Materials and Methods.'' The 10-, 20-, and 30-minute time points were normalized to the 0-time point, which was given the numerical value of 1.0. Represented is trinitrobenezenesulfonic acid (TNBSA)-labeled protein as measured by the absorbancy at 450 nm in comparison to the 0-time point. Each time point represents the mean SEM (n = 3) from 3 independent treatment groups. a—statistically different from the control value.

sample to the 0-time point, the STF from the TE-treated rat testes had 2.1-fold, 2.0-fold, and 1.2-fold increases in proteolytic activity following 10-, 20-, and 30-minute incubations, respectively.

Discussion

PCI expression is abundant in the male reproductive tracts of rodents and humans (Laurell et al, 1992; Wakita et al, 1998). In both species, its biological function(s) is unclear. Previous immunocytochemical analyses indicated that PCI protein is localized to Leydig cells, developing germ cells, and spermatozoon heads in the testes of sexually mature rodents and humans (Hermans et al, 1994; Zheng et al, 1996; Uhrin et al, 2000; Odet et al, 2004). We show that PCI mRNA is expressed predominantly in Sertoli cells of the rat testis and that germ cells, peritubular myoid cells, and Leydig cells express little or no PCI mRNA, at least as detectable by Northern blot analysis. Our results are supported by recent microarray analyses of isolated Sertoli, germ, and myoid cells, which similarly demonstrated PCI mRNA expression by Sertoli cells of the mouse testis (Shima et al, 2004). Taken together, these observations suggest that the PCI protein is secreted bidirectionally from the Sertoli cell.

Our results show a 50% reduction in PCI mRNA levels associated with a twofold increase in protease activity within the seminiferous tubules, suggesting that protease activity is inversely related to PCI expression levels in the testis. Although the function(s) of PCI in the male reproductive tract is (are) unknown, its functions in other systems suggest that it is involved in regulating protease activity within the testis (Uhrin et al, 2000). PCI is likely to interact with proteases secreted from Sertoli cells, such as urokinase and/or tissue plasminogen activators (Espana et al, 1993); germ cells, such as acrosin (Hermans et al, 1994; Zheng et al, 1996); and/or from Leydig cells, such as protein C (He et al, 1995; Odet et al, 2004). For example, urokinase and kallikrein-like protease activities were shown to be increased by 3.1- and 1.8-fold, respectively, in the testes of the mice lacking PCI protein, an effect that was inhibited by the addition of recombinant PCI (Uhrin et al, 2000). Uhrin et al (2000) demonstrated that male mice lacking a functional PCI protein were infertile and that this infertility was associated with a 2.2-fold increase in proteolytic activity when compared to control mice. PCI also has been suggested to play a role in sperm motility and fertilization; lack of PCI, or nonfunctional PCI protein, was shown to result in decreased sperm motility in ejaculated semen as well as inhibition of sperm–egg binding in vitro (Moore et al, 1993; Zheng et al, 1996; Elisen et al, 1998).

The present study supports the contention that PCI is regulated, at least in part, by testosterone. Thus, PCI mRNA was reduced significantly from control levels in the Sertoli cells in response to reduced intratesticular testosterone, suggesting that PCI mRNA is sensitive to intratesticular testosterone levels, responds to the loss of germ cells resulting from reduced intratesticular testosterone, or both. Previous studies have shown that changes in the germ cell population with which Sertoli cells are associated can alter Sertoli cell gene expression (Chung et al, 1998; Zabludoff et al, 2001). However, the observation that PCI mRNA levels in TE-treated rats increased in response to 7 days of exogenously administered testosterone supports the contention that PCI mRNA responds to intratesticular testosterone levels, because germ cells would not have been restored in 7 days. This observation does not rule out the possibility that PCI mRNA also responds to changes in germ cell content or number, however.

The method used to reduce intratesticular testosterone in the present study, a combination of testosterone and estradiol, requires discussion, because estrogen receptors are expressed in the testis, making it conceivable that estrogen might, itself, directly affect PCI expression. Although we cannot completely rule this out, it seems unlikely for the following reasons. Neither the human nor the rodent PCI promoters have estrogen response elements, but both contain multiple androgen receptor response elements (unpublished data). Additionally, the administration of estradiol to rats for 8 days had no effect on the testis expression of PCI, and although the seminal vesicle PCI mRNA levels did decrease, this occurred only after serum testosterone levels decreased (Wakita et al, 1998). The conclusion that PCI mRNA responds to testosterone is consistent with reports of decreased *PCI* gene expression in the seminal vesicles following castration and of increased expression following the administration of testosterone to castrated rats (Wakita et al, 1998; Suzuki and Hayashi, 2000). Additionally, microarray studies have shown that testosterone supplementation administered to hypogonadal mice resulted in an almost twofold increase in the expression of PCI by 24 hours (Sadate-Ngatchou et al, 2004).

In summary, we have shown that Sertoli cell expression of PCI decreases following reduced levels of intratesticular testosterone and that it can be restored by the administration of testosterone alone to rats in which intratesticular testosterone was reduced experimentally. Although the function of PCI within the testis remains uncertain, the association of reduced PCI with increased proteolytic activity suggests that PCI is involved in the protease/protease inhibitor balance within the seminiferous epithelium.

Acknowledgments

We thank Michael Griswold at Washington State University, Pullman, Wash, for the clusterin antibody and Margarethe Geiger at the University of Vienna, Vienna, Austria, for the PCI antibody.

References

Aguilar-Mahecha A, Hales BF, Robaire B. Expression of stress response genes in germ cells during spermatogenesis. *Biol Reprod.* 2001;65: 119–127.

Anway MD, Folmer J, Wright WW, Zirkin BR. Isolation of sertoli cells from adult rat testes: an approach to ex vivo studies of sertoli cell function. *Biol Reprod.* 2003;68:996–1002.

Anway MD, Ravindranath N, Dym M, Griswold MD. Identification of a murine testis complementary DNA encoding a homolog to human a-kinase anchoring protein-associated sperm protein. *Biol Reprod.* 2002; 66:1755–1761.

Christensson A, Lilja H. Complex formation between protein C inhibitor and prostate-specific antigen in vitro and in human semen. *Eur J Biochem.* 1994;220:45–53.

Chung SS, Zhu LJ, Mo MY, Silvestrini B, Lee WM, Cheng CY. Evidence for cross-talk between Sertoli and germ cells using selected cathepsins as markers. *J Androl.* 1998;19:686–703.

Collard MW, Griswold MD. Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. *Biochemistry.* 1987; 26:3297–3303.

Elisen MG, van Kooij RJ, Nolte MA, Marquart JA, Lock TM, Bouma BN, Meijers JC. Protein C inhibitor may modulate human sperm–oocyte interactions. *Biol Reprod.* 1998;58:670–677.

Espana F, Estelles A, Fernandez PJ, Gilabert J, Sanchez-Cuenca J, Griffin JH. Evidence for the regulation of urokinase and tissue type plasminogen activators by the serpin, protein C inhibitor, in semen and blood plasma. *Thromb Haemost.* 1993;70:989–994.

Espana F, Fink E, Sanchez-Cuenca J, Gilabert J, Estelles A, Witzgall K. Complexes of tissue kallikrein with protein C inhibitor in human semen and urine. *Eur J Biochem.* 1995;234:641–649.

Ewing LL, Desjardins C, Irby DC, Robaire B. Synergistic interaction of testosterone and oestradiol inhibits spermatogenesis in rats. *Nature.* 1977;269:409–411.

Griswold MD, Morales C, Sylvester SR. Molecular biology of the Sertoli cell. *Oxf Rev Reprod Biol.* 1988;10:124–161.

He X, Shen L, Bjartell A, Malm J, Lilja H, Dahlback B. The gene encoding vitamin K-dependent anticoagulant protein C is expressed in human male reproductive tissues. *J Histochem Cytochem.* 1995;43: 563–570.

Hermans JM, Jones R, Stone SR. Rapid inhibition of the sperm protease acrosin by protein C inhibitor. *Biochemistry.* 1994;33:5440–5444.

Huggenvik JI, Idzerda RL, Haywood L, Lee DC, McKnight GS, Griswold MD. Transferrin messenger ribonucleic acid: molecular cloning and hormonal regulation in rat Sertoli cells. *Endocrinology.* 1987;120: 332–340.

Klinefelter GR, Hall PF, Ewing LL. Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. *Biol Reprod.* 1987;36:769–783.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680–685.

Laurell M, Christensson A, Abrahamsson PA, Stenflo J, Lilja H. Protein C inhibitor in human body fluids. Seminal plasma is rich in inhibitor antigen deriving from cells throughout the male reproductive system. *J Clin Invest.* 1992;89:1094–1101.

Moore A, Penfold LM, Johnson JL, Latchman DS, Moore HD. Human sperm–egg binding is inhibited by peptides corresponding to core region of an acrosomal serine protease inhibitor. *Mol Reprod Dev.* 1993; 34:280–291.

Mukherjee A, Park-Sarge OK, Mayo KE. Gonadotropins induce rapid phosphorylation of the 3′,5′-cyclic adenosine monophosphate response element binding protein in ovarian granulosa cells. *Endocrinology.* 1996;137:3234–3245.

Odet F, Guyot R, Leduque P, Magueresse-Battistoni B. Evidence for similar expression of protein C inhibitor and the urokinase-type plasminogen activator system during mouse testis development. *Endocrinology.* 2004;145:1481–1489.

Rezaie AR, Cooper ST, Church FC, Esmon CT. Protein C inhibitor is a potent inhibitor of the thrombin–thrombomodulin complex. *J Biol Chem.* 1995;270:25336–25339.

Roberts KP, Santulli R, Seiden J, Zirkin BR. The effect of testosterone withdrawal and subsequent germ cell depletion on transferrin and sulfated glycoprotein-2 messenger ribonucleic acid levels in the adult rat testis. *Biol Reprod.* 1992;47:92–96.

Sadate-Ngatchou PI, Pouchnik DJ, Griswold MD. Identification of testosterone-regulated genes in testes of hypogonadal mice using oligonucleotide microarray. *Mol Endocrinol.* 2004;18:422–433.

Shima JE, McLean DJ, McCarrey JR, Griswold MD. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. *Biol Reprod.* 2004;71:319–330.

Stratton LG, Ewing LL, Desjardins C. Efficacy of testosterone-filled polydimethylsiloxane implants in maintaining plasma testosterone in rabbits. *J Reprod Fertil.* 1973;35:235–244.

Suzuki K, Hayashi T. *cis*-elements required for expression of human protein C inhibitor gene in HepG2 cells and its androgen-dependent expression in rat reproductive organs. *Semin Thromb Hemost.* 2000;26: 75–83.

Suzuki K, Nishioka J, Hashimoto S. Protein C inhibitor. Purification from human plasma and characterization. *J Biol Chem.* 1983;258:163–168.

Turner KJ, Morley M, MacPherson S, Millar MR, Wilson JA, Sharpe RM, Saunders PT. Modulation of gene expression by androgen and oestrogens in the testis and prostate of the adult rat following androgen withdrawal. *Mol Cell Endocrinol.* 2001;178:73–87.

Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B, Gunsalus GL. On the androgen microenvironment of maturing spermatozoa. *Endocrinology.* 1984;115:1925–1932.

Uhrin P, Dewerchin M, Hilpert M, Chrenek P, Schofer C, Zechmeister-Machhart M, Kronke G, Vales A, Carmeliet P, Binder BR, Geiger M. Disruption of the protein C inhibitor gene results in impaired spermatogenesis and male infertility. *J Clin Invest.* 2000;106:1531–1539.

Wakita T, Hayashi T, Yuasa H, Nishioka J, Kawamura J, Suzuki K. Molecular cloning, tissue distribution and androgen regulation of rat protein C inhibitor. *FEBS Lett.* 1998;429:263–268.

Zabludoff SD, Charron M, DeCerbo JN, Simukova N, Wright WW. Male germ cells regulate transcription of the cathepsin l gene by rat Sertoli cells. *Endocrinology.* 2001;142:2318–2327.

Zheng X, Zechmeister-Machhart M, Uhrin P, Hufnagl P, Geiger M, Binder BR. Effect of protein C inhibitor (PCI) on in vitro fertilization. *Immunopharmacology.* 1996;33:140–142.

Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. *Endocrinology.* 1989;124: 3043–3049.