Photoperiod-dependent regulation of inhibin in Siberian hamsters:

I. Ovarian inhibin production and secretion

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Abstract

Follicle-stimulating hormone (FSH) stimulates ovarian follicle development and the production of protein hormones including inhibin A and inhibin B. The inhibins are dimeric proteins (α - β_A or α - β_B) secreted by growing follicles that suppress FSH in a classical endocrine negative feedback loop. Siberian hamsters, *Phodopus sungorus*, exhibit seasonal variation in FSH levels. Given the role of inhibin in FSH regulation, we hypothesized that ovarian inhibin expression differs between animals reared in long (16 h light:8 h darkness) and short (6 h light:18 h darkness)

photoperiods. To examine inhibin expression in animals housed under long or short photoperiods, hamster inhibin α -, β_A -, and β_B -subunits were cloned and used to detect and localize inhibin subunit mRNA in developing follicles. Ovarian inhibin α -subunit mRNA levels were significantly higher in long day-exposed (LD) than in short day-exposed (SD) hamsters. In addition, dimeric inhibin, as well as inhibin α -, β_A -, and β_B -subunit protein levels were higher in the LD than in the SD hamster ovaries.

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Introduction

The inhibins are dimeric protein hormones produced by developing ovarian follicles that in many species inhibit the secretion of follicle-stimulating hormone (FSH) from pituitary gonadotropes (Woodruff *et al.* 1988). Inhibin A and inhibin B share a common α -subunit that assembles with one of two highly related β subunits (β_A or β_B) to form the active hormones. All three subunits are stimulated by FSH and are initially synthesized as pre-prohormones. The biologically active form of the ligands is a dimer of the C-terminal regions of the subunits linked by a single intermolecular disulfide bond (Hsueh *et al.* 1987, Rivier *et al.* 1987). Inhibin A and inhibin B are thought to inhibit FSH release similarly; however, the pattern of secretion for each ligand differs during the reproductive cycle. The synthesis and secretion of the inhibins are best characterized during the rat estrous cycle (Woodruff *et al.* 1996, Bernard & Woodruff 2001). At the onset of a new cycle, inhibin A and inhibin B levels increase in the follicle population that has been recruited by the so-called 'secondary' FSH surge. FSH levels fall in response to rising inhibins. Estradiol is not produced in measurable levels in this early phase of the cycle, suggesting that the inhibition of FSH is reliant largely on ovarian inhibin (Woodruff *et al.* 1996, Bernard & Woodruff 2001). Inhibin B rises much more quickly and reaches maximal levels during the early

follicular phase (metestrus, diestrus). Inhibin A rises more slowly and closely parallels the rise in estradiol secretion from the Graafian follicles. All three inhibin subunits produced in the Graafian follicles are inhibited by the preovulatory surges of the gonadotropins which also induce follicle degradation prior to ovulation. The half-lives of the subunit mRNAs and the ligands are short. Thus, as a consequence of FSH and luteinizing hormone (LH) action, inhibin A and inhibin B levels fall (Woodruff *et al.* 1993a,b, Halvorson *et al.* 1994, Weiss *et al.* 1995, Winters *et al.* 1996). The pituitary is thereby relieved from inhibin repression and elevated FSH levels persist, leading to recruitment of a new cohort of follicles into the next cycle.

The β -subunits can also self-assemble into dimeric proteins, which are antagonists to inhibin action. Activin A (β_A - β_A), activin B (β_B - β_B) and activin AB (β_A - β_B) have all been purified from follicular fluid (Ling *et al.* 1986a,b, Schmelzer *et al.* 1990, Robertson *et al.* 1992, Yokoyama *et al.* 1995). Activin A has been detected in the serum of animals at the time of the preovulatory and secondary FSH surges in rats, but at no other time does activin appear to circulate at detectable levels (Besecke *et al.* 1997). Thus, the differential production of inhibins or activins must be exquisitely controlled at the cellular level to ensure the secretion of the appropriate ligand at specific times during the cycle. Relatively little is known regarding the mechanism(s) underlying dimer assembly, processing, or

Table 1 Inhibin oligonucleotides used for RT-PCR amplification of hamster inhibin subunit cDNAs. The oligonucleotide sequences and the location and source of these sequences are listed

	Sequences	Sequence no.	GenBank accession no.
Oligo			
α 5'	TGTCCTGGCCAAAGTGARGGC	345–365*	NM012590
α 3'	CAGGAAAGGAGTGGTCTCAGG	888–903	NM012590
β A5'	CTCATGGAGCAGACCTCGGAG	508–528	NM017128
β A3'	GTGAGGATGGTCTTCAGACTG	1024–1044	NM017128
β B5'	TATGTCCTGGAGAAGGGCAGC	422–1220	XM046786
β B3'	GCGATGTCTGCTATCGCCCA	1200–1220	XM046786

*The α -subunit forward primer was modified at nucleotide 18 after unsuccessful amplification and sequence comparison of multiple species α -subunit cDNA sequences.

secretion; thus, it is necessary to identify animal models that will permit a more detailed analysis of each of these processes. Towards that end, we have investigated the regulation of inhibin and activin in the Siberian hamster (*Phodopus sungorus*).

Siberian hamsters exhibit seasonal variation in FSH levels. These changes can be recapitulated in laboratory animal breeding colonies by altering daily light exposure (Schlatt *et al.* 1993). Because follicle development can be inhibited by movement of the animals from long to short photoperiods (Lerchl & Schlatt 1993, Schlatt *et al.* 1993), we predicted that the regulation of inhibin subunit assembly could be readily examined in this animal model. Moreover, given the role of inhibin in FSH regulation, we hypothesized that ovarian inhibin production would differ between long day-exposed (LD) and short day-exposed (SD) animals. Significantly, ovarian inhibin synthesis does differ between LD and SD animals, with more overall inhibin production in ovaries collected from LD animals. In a companion paper (Kenny *et al.* 2002), we demonstrate that FSH is not the only stimulus necessary to stimulate inhibin, suggesting that the production and assembly of inhibin A and inhibin B relies on additional cues that have not yet been identified.

Materials and Methods

Animals and experimental procedures

Female Siberian hamsters (21–32 g, 53–60 days of age) were bred at Northwestern University and were individually housed in access-controlled rooms isolated from males. Food and water were available *ad libitum* and light cycles of 16 h light:8 h darkness (LD) or 6 h light:18 h darkness (SD) were provided. The ovaries were collected from animals after asphyxiation in a CO₂ chamber. The tissue was stored at –80 °C until further processed. Blood obtained by cardiac puncture was allowed to clot on ice for

2 h and serum was separated and stored at –80 °C in assay-specific aliquots. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals

Cloning of hamster inhibin α -, β A- and β B-subunits: cDNA library screening, RT-PCR, and 5'-RACE

Siberian hamster ovarian total RNA was extracted and was reverse transcribed into cDNA using Maloney murine leukemia virus reverse transcriptase in the presence of random hexamer oligonucleotides and deoxynucleotide triphosphates (dNTPs) (Promega Corp., Madison, WI, USA). PCR was performed on the RT reaction using standard techniques and inhibin α -, β A- and β B-subunit-specific primers which were initially generated from rat inhibin subunit sequences with the exception of the α -subunit forward primer, which was modified after unsuccessful amplification and sequence comparison of multiple species α -subunit cDNA sequences. All primers used to amplify the inhibin subunits from Siberian hamster ovarian RNA are listed in Table 1. PCR products were cloned into pCRScript vector (Stratagene, La Jolla, CA, USA), and sequenced with T7 and T3 primers using BigDye Terminator Cycle Sequencing (ABI Prism, Foster City, CA, USA). The hamster α -subunit cDNA sequence was 88%, 87% and 84% identical to orthologous sequences in mouse, rat and human respectively. The amplified hamster α -subunit cDNA was 559 bp in length and corresponds to signal peptide sequence, nucleotides 345 to 903 of the rat α -subunit sequence (GenBank accession number NM012590). The hamster β -subunits cDNAs were sequenced and β A was 95%, 95% and 92% and β B was 92%, 91% and 89% identical to the mouse, rat and human orthologs respectively. The hamster β A-subunit was 563 bp in length and corresponds to signal peptide sequence, base-pairs 508 to 1044 of the rat β A-subunit sequence (GenBank accession number NM017128). The

amplified hamster β_B -subunit was 800 bp in length and corresponds to signal peptide sequence and mature peptide sequence, nucleotides 422 to 1220 of human β_B -subunit sequence (GenBank accession number XM046786).

The inhibin α -subunit fragment generated above was random prime labeled and used to screen a Siberian hamster ovarian cDNA library (ZAP-cDNA synthesis kit, Stratagene) using standard techniques. The *in vivo* excised clones were sequenced with T7 and T3 as described above. All sequences were aligned using Sequencher (version 3.1.1, Gene Codes Corp., Ann Arbor, MI, USA) on a Macintosh G3 computer (Apple, Cupertino, CA, USA). The 5' end of the cloned Siberian hamster inhibin α -subunit was 242 bp downstream of the putative start site of translation when compared with the rat inhibin α -subunit sequence (GenBank accession number NM012590). Therefore, to obtain additional 5'-sequence we used RLM-RACE following the manufacturer's instructions (Ambion Inc., Austin, TX, USA). The open-reading frame (ORF) of the hamster α -subunit nucleotide sequence was 88%, 89% and 81% identical to mouse, rat and human respectively.

RNA isolation and Northern blot analysis

Total RNA was extracted from ovarian tissue collected from female Siberian hamsters housed in short or long photoperiods using TRIZOL Reagent (Life Technologies Inc., Rockville, MD, USA). Concentration of the isolated RNA was determined by absorption at 260 nm on a spectrophotometer and the integrity of the preparation examined by agarose gel electrophoresis. For RNA blot analysis, 20 μ g total RNA were electrophoresed per lane on a 1.0% agarose, 1.1 M formaldehyde and $1 \times (3\text{-[N-Morpholino]propane sulfonic acid})$ (MOPS) using standard procedures. RNA was transferred to Hybond N nylon membranes (Amersham) by capillary action overnight with $20 \times$ SSC. Radiolabeled cDNA probes were synthesized from plasmid DNA templates using deoxycytidine 5'-triphosphate [α - 32 P] 3000 Ci (111 TBq)/mmol (NEN Life Sciences Products, Boston, MA, USA) and Ready-to-Go beads (-dCTP) (Amersham). The membranes were hybridized to α , β_A and β_B probes in hybridization buffer (50% formamide, $5 \times$ SSC, 20 mM NaH_2PO_4 (pH 6.8), 1% SDS, 5% dextran sulfate, and 100 μ g/ml herring sperm DNA). After hybridization, the blots were washed sequentially in $2 \times$ SSPE+0.5% SDS at 20 °C, $2 \times$ SSPE+0.5% SDS at 65 °C and $0.2 \times$ SSPE+0.1% SDS at 65 °C. First, the blots were exposed to a Phosphor screen (Molecular Dynamics, Piscataway, NJ, USA) for 4 h and analyzed using STORM (Molecular Dynamics) and Image Quant (Molecular Dynamics). The optical densities for α -, β_A - and β_B -subunits were normalized by dividing the optical density for RPL19 in the same lane. Secondly, the blots were exposed to X-OMAT film (Eastman Kodak) for 2 to 6 days.

In situ hybridization

Fresh frozen ovaries were sectioned (20 μ m) on a Leica CM3050 cryostat (Deerfield, IL, USA). Alternate sections were mounted on six microscope slides. Slides were labeled for hybridization to α , β_A and β_B sense and α , β_A and β_B anti-sense probes. Cross-sections of the ovary were taken with respect to the attached oviduct to maintain a standard orientation between samples. Sections were fixed in 4% paraformaldehyde, treated with 0.2% (v/v) acetic anhydride in 0.1 M triethanolamine for 10 min, dehydrated through an ethanol series (50%, 70%, 95% and 100%), and stored in a vacuum dessicator until further processed (<18 h). cRNA probes (sense and anti-sense riboprobes) were synthesized from linearized plasmid DNA templates using T7 or T3 RNA polymerase and easy tides uridine 5'-triphosphate [α - 32 P] 3000 Ci (111 TBq)/mmol (NEN Life Sciences). A coverslip was placed on the slide after addition of the hybridization solution (50% formamide, 0.3 M NaCl, 0.01 M Tris, 1 mM EDTA, $1 \times$ Denhardt's, 20% dextran sulfate, 500 μ g/ml tRNA, 500 μ g polyA RNA, and 1×10^8 c.p.m./ml 32 P-labeled probe) and hybridized for 18 h at 42 °C in heavy mineral oil, as previously described (Woodruff *et al.* 1987, 1989). Oil and coverslips were removed, and the sections were treated with 20 μ g/ml RNase-A for 1 h at 37 °C. After RNase treatment, the slides were washed sequentially in $2 \times$, $1 \times$ and $0.5 \times$ SSC for 2 h at 42 °C. Slides were exposed to Biomax film (Eastman Kodak) for 6 h and subsequently prepared for autoradiography using Kodak NTB-2 emulsion. Slides were exposed to the emulsion for 2 weeks and developed using D-19 developer and fixer (Eastman Kodak). Processed tissues were stained with hematoxylin and silver grains were visualized using a Nikon Eclipse E600 (Nikon Instruments Inc., Melville, NY, USA). Digital images were captured using Metamorph (v4.6; Universal Imaging Corp, Downingtown, PA, USA). Three or four animals were analyzed for each treatment.

Hormone assays

Serum FSH levels were determined in duplicate by RIA with NIH-FSH RP-2 (NIDDK, Rockville, MD, USA) as standard, and anti-rat (r) FSH-I-8 as trace (Northwestern University RIA Core). The limit of detection in the assay was 2 ng/ml with inter- and intra-assay variations of 5.22% and 4.15% respectively. Serum LH levels were determined in duplicate by RIA with NIH-LH RP-3 (NIDDK) as standard and anti-rLH-S-11 as trace (Northwestern University RIA Core). The limit of detection in the assay was 0.2 ng/ml with inter- and intra-assay variations of 11.5% and 9.6% respectively. Inhibin A, inhibin B and activin A assay kits were purchased from Serotec (Oxford, Oxon, UK), and the characteristics of these dimer-specific assays have been described previously

(Groome & Lawrence 1991, Groome *et al.* 1994, 1996, Knight *et al.* 1996, Muttukrishna *et al.* 1996). Serum and ovarian inhibin A, inhibin B and activin A levels were determined in duplicate. Each ovarian sample was obtained by homogenizing one ovary in 0.5 ml 0.85% (w/v) NaCl as described (Herath *et al.* 2001). The homogenized samples were centrifuged at $15\,000 \times g$ for 20 min at 4 °C. Inhibin A and inhibin B assays were performed on freshly homogenized tissue. The inhibin assays were validated within the laboratory using recombinant human (rh) inhibin A, rh-inhibin B and Siberian hamster serum. The inhibin A standard was the WHO standard (91/624). The inhibin B standard was from National Institute for Biological Standards and Control (NIBSC). No cross-reactivity between heterologous ligands occurred using purified recombinant ligands for any of the assays. Rh-ligands were spiked at three dilutions into serum from ovariectomized female Siberian hamsters. Inhibin A recovery was 75, 82 and 80% for high, medium and low standards and inhibin B recovery was 90, 87 and 86% for high, medium and low standards. Native Siberian hamster ligands diluted linearly and in parallel to the standard curve (data not shown). The assays were sensitive (7.8 pg/ml inhibin A; 15.6 pg/ml inhibin B) and precise. The intra-assay variation was 5.71% and 5.69% for inhibin A and inhibin B assays respectively. The interassay variation was 23.2% and 24.1% for inhibin A and inhibin B assays respectively. The activin A standard was bovine follicular fluid and the limit of detection of this assay was 5 ng/ml.

Protein extraction and Western blot analysis

Protein was extracted from whole ovaries by grinding the tissue on dry ice with a mortar and pestle. The tissue was collected in protein extraction buffer (10 mM Tris, 0.5 M NaCl, 1 mM MgCl₂, 0.1% Triton X-100, and protease inhibitors (Complete mini protease inhibitor cocktail tablets, Roche Laboratories, Indianapolis, IN, USA). Protein samples were electrophoresed under reducing or nonreducing conditions in NuPage Bis-Tris 4–12% gradient gels (Novex, Invitrogen Corp., Carlsbad, CA, USA). Samples were incubated in NuPage LPS sample buffer at 70 °C for 10 min before loading. The gels were run in MES-SDS Running Buffer and NuPage Reducing Agent using the NuPage mini gel apparatus. Proteins were transferred to Protran nitrocellulose (Schleicher & Schwell, Keene, NH, USA). The recombinant human inhibin standards used were rh-inhibin A and rh-inhibin B (R&D Systems Inc., Minneapolis, MN, USA), and the recombinant human activin standards used were rh-activin A and rh-activin B produced in our laboratory by Magdalena Suszko. The blots were blocked in 10% milk, 1 × Tris-buffered saline (TBS) and 0.1% Tween 20 for 1 h at room temperature. The blots were incubated with 0.15 µg/ml primary antibodies in 5% milk, 1 × TBS, 0.1%

Tween for 1 h. The antibodies used (rabbit anti α - (305–214-ET, amino acids 1–26 GY), β_A - (305–24-D, amino acids 81–113) or β_B (305–25-D, amino acids 80–112)-subunit polyclonal antibodies (Shaha *et al.* 1989, Jin *et al.* 2001)) were kindly provided by Wylie Vale (Salk Institute, La Jolla, CA, USA). The blots were washed three times for 15 min in 1 × TBS and 0.1% Tween and incubated with a 1:10 000 dilution of horseradish peroxidase conjugated donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) in 5% milk, 1 × TBS and 0.1% Tween for 30 min. The blots were washed three times for 15 min in 1 × TBS and 0.1% Tween. The immunoblots were analyzed by ECL development (Amersham Pharmacia Biotech, Inc.). The immunoblots were exposed to x-ray film for 1 to 120 min.

Statistical analyses

Values are reported as the means $\pm$ S.E. Correlations between ligands were determined using simple linear regression analyses. In all cases, $P < 0.05$ was considered significant. Paired comparisons were made using paired *t*-test.

Results

RT-PCR amplification, cloning, and sequencing of the Siberian hamster inhibin α -subunit cDNAs

The full-length Siberian hamster inhibin α -subunit cDNA was cloned by a combination of library screening, RT-PCR, and 5'-RACE (GenBank accession number AF432351). The cDNA was determined to contain an open reading frame (ORF) of 1098 bp, flanked by 61 bp and 210 bp of 5'- and 3'-untranslated regions (UTRs). The ORF is predicted to encode a protein of 366 amino acids, identical in length to the rat and mouse α -subunits (Fig. 1; Woodruff *et al.* 1987, Albano *et al.* 1993). There is an in-frame stop codon 6 bp upstream of the first ATG in 5'-UTR and a consensus polyadenylation signal 22 bp upstream of the poly A tail in the 3'-UTR. The sequence encoding the mature region of the hamster α -subunit nucleotide sequence was 91%, 90% and 82% identical to the mature subunit of the mouse, rat and human respectively. The precursor predicted amino acid sequence was 92%, 92% and 82% identical to the mouse, rat and human predicted precursor sequences respectively. The Siberian hamster protein sequence encoding the mature region of the inhibin α -subunit conservation is fairly high, sharing 96%, 96%, and 84% identity to mouse, rat, and human respectively (Mason *et al.* 1986, Woodruff *et al.* 1987, Albano *et al.* 1993). A dibasic Arg–Arg proteolytic cleavage site at amino acids 232 and 233 is preserved in the hamster protein, suggesting that the mature inhibin α -subunit will include the α C-terminal 133 amino acids.

Figure 1 Amino acid sequence alignment for mouse (GenBank accession no. X69618), rat (GenBank accession no. NP036722) and Siberian hamster (GenBank accession no. AF432351) inhibin α -subunit. Conserved amino acids are darkly shaded and conserved cysteine residues are lightly shaded. The start of the mature region of the α -subunit is indicated by an arrow and the mature α -subunit is 133 amino acids in length. The N-glycosylation sites (stars) are at amino acids 147 and 302, as previously described in rat and mouse (Mason *et al.* 1996).

In addition, seven cysteines and a single predicted glycosylation site are conserved between rat, mouse, and Siberian hamster mature proteins. A notable difference between human and rodent mature (α C) α -subunit is that human α C may be glycosylated at one or two N-linked sites (Mason *et al.* 1996).

Partial precursor and mature regions of the inhibin β_A - and β_B -subunits were amplified by RT-PCR and ligated into plasmids. The nucleotide sequences for the regions amplified for the β_A -subunit and the β_B -subunit cDNAs were 95% and 92% identical to rat and mouse β_A - and β_B -subunit sequences respectively.

Figure 2 Analysis of inhibin α -, β_A - and β_B -subunit mRNA levels in ovaries of long day-exposed (LD) and short day-exposed (SD) hamsters. RNA (20 μ g) was electrophoresed and transferred to Hybond N nylon membrane. The blot was hybridized to α -, β_A - and β_B -specific probes, exposed to a Phosphor screen and analyzed as described in Materials and Methods. The relative α -subunit mRNA intensity level was significantly higher in the ovaries of LD than of SD hamsters (* $P < 0.001$). There was no significant difference in β_A - or β_B -subunit mRNA levels in ovaries of LD or SD hamsters. RPL19 was used as a loading control. These results are representative of the results of three independent experiments ($n = 12$ per photoperiodic condition).

Abundance of inhibin subunits in ovaries collected from hamsters reared in long and short photoperiods

Female hamsters were weaned at 20 days of age and immediately housed in an LD or SD photoperiod for 4–5 weeks. Relative levels of inhibin α -, β_A - and β_B -subunit mRNAs were measured by RNA blot analysis (Fig. 2). Inhibin α -subunit mRNA levels were significantly higher in LD than in SD animals. No significant difference was detected in the abundance of the β_A - or β_B -subunit mRNA levels when total ovarian RNA was compared between LD and SD animals. The abundance of the inhibin α -subunit was greater than both β_A - and β_B -subunits at all times.

Inhibin subunit mRNA compartmentalization in long and short photoperiod hamster ovaries

The total abundance of the inhibin α -subunit mRNA differed between ovaries collected from LD and SD hamsters. To study the pattern of inhibin subunit expression as it relates to follicle dynamics, hamsters were

weaned and immediately housed in an LD or an SD photoperiod for 4–5 weeks. The animals were not cyclic, as determined by the lack of vaginal opening in all animals studied. The ovaries contralateral to those used for determination of the overall abundance of the subunit mRNAs were prepared for *in situ* hybridization using subunit-specific riboprobes as previously described (Woodruff *et al.* 1987). Follicles of all size classes up to 520 μ m were detected in the ovaries from LD animals (Fig. 3A–F), while few follicles reached 400 μ m in the SD animals (Fig. 3G–L). On average, fewer follicles were positive for the inhibin α -subunit in the SD ovaries when compared with the LD follicles (compare Fig. 3A with 3G). In addition, the overall level of inhibin α -subunit expression was lower in SD, size-matched follicles (compare Fig. 3D with 3J). Thus few follicles expressed the inhibin α -subunit in SD ovaries, and the level of inhibin α -subunit expression was lower than in LD ovaries. No corpora lutea were detected in any animal regardless of housing conditions.

Both β_A - and β_B -subunits were detected in a subset of follicles positive for the α -subunit. In general, the expression of β -subunits overlapped with each other and with the α -subunit. In some follicles, the β_B -subunit was preferentially expressed in the antral granulosa cells while the β_A -subunit was found throughout the mural and antral granulosa cell layers (see arrows, Fig. 3E and F).

Regulation of inhibin subunit synthesis and processing in ovaries from long and short photoperiod Siberian hamsters

Serum was collected from LD and SD hamsters and FSH, LH, inhibin A, inhibin B, and total activin A were measured (Table 2). The serum levels of inhibin A and inhibin B were low to undetectable (<7.8 pg/ml) in both LD and SD animals using currently available assays. Sporadic, low levels of inhibin A were detected in both LD and SD animals, but not consistently. The inhibin A and inhibin B assays were validated for hamster serum using pregnant mare serum gonadotropin-induced serum samples (see the companion paper Kenny *et al.* 2002). The levels of activin A were also undetectable (<5 ng/ml) in both LD and SD hamster serum. FSH levels were significantly higher in LD hamsters than in SD hamsters. There was no statistically significant photoperiodic difference in the serum levels of LH.

Because the component subunit mRNAs were detected in the ovary yet no circulating inhibin or activin could be measured, we examined the abundance of dimeric inhibin or the three subunits by protein blot analysis. In the ovary, two dominant precursor species of dimeric inhibin were detected. A high molecular weight form (approximately 80–90 kDa) was detected (Fig. 4A, black arrowhead). In ovarian homogenates from LD females, two precursor forms were detected between 80 and 90 kDa, likely

Figure 3 *In situ* hybridization of inhibin α -, β_A - and β_B -subunit mRNA in ovaries of LD and SD hamsters. cRNA probes for the α -, β_A - and β_B -subunits were produced and hybridized to ovarian tissue sections as described in Materials and Methods. (A–C) α -, β_A - and β_B -subunit mRNA expression in cross sections of the entire ovary of LD hamsters were analyzed on x-ray film. Both β_A - and β_B -subunits were detected in a subset of follicles positive for the α -subunit (arrows; A–C). Some follicles expressed only the α -subunit (arrow heads; A). A scale bar is located in panel C for panels A–C. (D–F) α -, β_A - and β_B -subunit mRNA expression is shown in antral (left follicle) and Graafian (right follicle) follicles in adjacent ovarian sections of LD hamsters. The localization of β_A - and β_B -subunit expression in mural (left arrow; E and F) and cumulus (right arrow; E and F) granulosa cells is illustrated. A scale bar is located in panel F for panels D–F. (G–I) α -, β_A - and β_B -subunit expression in cross sections of the entire ovary of SD hamsters were analyzed on x-ray film. Both β_A - and β_B -subunits were detected in a subset of follicles positive for the α -subunit (arrows; G–I). Some follicles expressed only the α -subunit (arrow heads; G). A scale bar is located in panel I for panels G–I. (J–L) α -, β_A - and β_B -subunit mRNA expression are shown in the largest antral follicles found in the ovaries of SD hamsters.

representing additional glycosylation, while only one form was detected in the ovarian lysates from SD females. A second precursor (two bands approximately 36–41 kDa) was detected in ovarian lysates from LD and SD females (Fig. 4A, black arrows). Mature dimeric inhibin was not detected in ovarian lysates from LD or SD females (Fig. 4A, gray arrows). When samples were reduced to break the disulfide bonds, the mature inhibin α -subunit and the

precursor $\alpha N\alpha C$ subunit were detected in LD ovaries, while no fully processed mature α -subunit could be detected in the ovaries from females housed in a short photoperiod (Fig. 4B). Similarly, mature β_A - and β_B -subunits were detected in ovaries of LD females but not of SD females (Fig. 4C and D).

Ovarian inhibin levels were also measured in LD and SD Siberian hamsters (Fig. 5). The levels of inhibin A

Table 2 Gonadotropin, inhibin A, inhibin B and activin A serum levels and body weights of LD- and SD-exposed hamsters. The circulating levels of FSH, LH, inhibin A, inhibin B and activin, and the body weights are reported as means ± s.e. The range of values for each measurement is listed in parentheses. LTS refers to values less than the standards used in each assay or lower than the limit of detection of the assay (<7.8 pg/ml inhibin A; <15.6 pg/ml inhibin B; <5 ng/ml activin A). These results are representative of three independent experiments with more than six animals per treatment

	Long photoperiod (LD)	Short photoperiod (SD)
FSH (ng/ml)	8.1 ± 0.7 (4.3–13.0)*	4.3 ± 0.6 (1.0–7.1)
LH (ng/ml)	0.6 ± 0.2 (0.2–1.7)	0.8 ± 0.3 (0.2–3.2)
Inhibin A (pg/ml)	LTS (LTS–82.6)	LTS (LTS–60.8)
Inhibin B (pg/ml)	LTS (LTS–47.3)	LTS (LTS–40.8)
Activin A (ng/ml)	LTS (LTS)	LTS (LTS)
Body weight (g)	34.2 ± 3.4 (30–44)	23.6 ± 2.3 (20–29)

**P*<0.05, LD compared with SD animals.

and inhibin B were higher in the LD than SD hamsters. In the LD hamsters, ovarian inhibin A levels ranged from 6.85 to 38.39 pg/mg ovary with a mean value of 22.93 ± 2.63 pg/mg ovary, and ovarian inhibin B levels ranged from 11.05 to 50.00 pg/mg ovary with a mean value of 27.59 ± 3.89 pg/mg ovary. In the SD hamsters, inhibin A levels were less than the standard range and inhibin B levels ranged from less than the standard range to 5.81 pg/mg ovary with a mean value of 3.43 ± 0.43 pg/mg ovary.

Discussion

The regulation of reproductive function relies on carefully orchestrated feed-forward and feedback signals produced by the brain, pituitary and gonads. In many species, environmental cues are also used to time reproductive effort such that offspring are born at those times of year most conducive to their survival. For example, the Siberian hamster (*Phodopus sungorus*) uses seasonal changes in day length to time changes in gonadal size and activity, body weight, and pelage color (Hoffmann 1973, Lerchl & Schlatt 1993, Ebling 1994). These changes can be recapitulated in laboratory animal breeding colonies by altering daily light exposure. Thus, the Siberian hamster can be used to study the impact of photoperiod on the endogenous hormones that communicate between the brain, pituitary, and testis or ovary. One of the most important endocrine hormones emanating from the ovary to control pituitary function is inhibin. Thus, the intent of this study was to examine the role of inhibin in the regulation of photoperiod-dependent seasonal changes in reproductive function in the Siberian hamster.

Inhibin regulates normal reproductive function in a number of species by inhibiting FSH secretion and it was our prediction that inhibin would also be important to the regulation of reproductive function in the female Siberian

hamster. Inhibin subunit expression in the ovaries has been characterized in several other rodent species (Meunier *et al.* 1988, 1989, Woodruff *et al.* 1988, Jih *et al.* 1993, McAsey *et al.* 1995, O'Shaughnessy & Gray 1995, Otsuka *et al.* 1997, Oshima *et al.* 1999). The hamster inhibin subunits were cloned and used to examine changes in the abundance of each of the subunits in LD or SD animals. Each of the hamster inhibin subunits are highly conserved at the protein level and all of the hallmark glycosylation sites, cysteine motifs and cleavage sites are present in the hamster, just as they are in other rodent species (Woodruff *et al.* 1987, Albano *et al.* 1993). In addition, we estimate that α -subunit mRNA levels are 10- to 20-fold higher in abundance in the hamster ovary than are the β -subunit mRNAs. Interestingly, α -subunit mRNA levels are less than half as great in SD as in LD ovaries. This can be accounted for by the fewer number of follicles expressing the α -subunit, the smaller overall size of the follicle pool, and the lower intensity of inhibin α -subunit expression in the smaller follicles of the SD ovaries. β -Subunit expression is restricted to a smaller number of follicles compared with the α -subunit in both photoperiods and subtle differences in cellular distribution within the granulosa cell compartment of the follicle is noted. Specifically, the β_B -subunit is detected in the antral granulosa cell layer of some of the follicles, while no morphological difference could be detected in these follicles when compared with follicles with homogeneous expression of the β_B -subunit.

Conclusions have often been drawn regarding inhibin secretion based on inhibin expression. In this study, we measured inhibin A and B serum levels in order to investigate the correlation between subunit expression and circulating inhibin levels. Surprisingly, little or no inhibin was detected in serum samples collected from animals housed under long or short photoperiods. It is possible that inhibin is secreted but at a level that is too low to detect using the current inhibin ELISAs or that the current inhibin ELISAs are insufficiently sensitive to detect

Figure 4 Western blot analysis of inhibin and inhibin α -, β_A - and β_B -subunits in ovarian lysates from LD and SD hamsters. Proteins were extracted from individual ovaries and 40 μ g protein were loaded in each well, as described in Materials and Methods. (A) Immunoblot analysis of ovarian extracts under nonreducing conditions using the α -subunit polyclonal antibody. Inhibin was detected as two dominant precursor proteins in ovaries of LD and SD hamsters (black arrowhead and black arrows). The standard, rh-inhibin A, was detected as a mature dimer ($\alpha C/\beta$) and precursor proteins. (B) Immunoblot analysis of ovarian extracts under reducing conditions using the α -subunit polyclonal antibody. Two mature forms (αC , asterisks) and precursor forms ($\alpha Na\alpha C$) of the α -subunit were detected. The standard, rh-inhibin A, was detected in its nonglycosylated and diglycosylated mature α -subunit form (αC). (C) Immunoblot analysis of ovarian extracts under reducing conditions using the β_A -subunit polyclonal antibody. The mature β_A -subunit (β_A) was detected in the ovary of LD hamsters. The standard, rh-activin A conditioned media, was detected as mature and precursor proteins ($pro\beta_A$) of the inhibin β_A -subunit (gray star). (D) Immunoblot analysis of ovarian extracts under reducing conditions using the β_B -subunit polyclonal antibody. The mature β_B -subunit (β_B) and precursor proteins ($pro\beta_B$) were only detected in the ovaries of LD hamsters. The standard, rh-activin B conditioned media, was detected as mature and precursor proteins of the inhibin β_B -subunit (gray star).

inhibin in the hamster. The current inhibin ELISAs use monoclonal antibodies against amino acids 1–32 of the human αC region of the α -subunit (Groome *et al.* 1990). A comparison of the 1–32 amino acid sequence of the human and Siberian hamster mature α -subunit reveals that there are four different amino acids which may lead to a reduced immunoactivity in the inhibin A and inhibin B ELISAs. The current inhibin ELISAs do not detect bovine or mouse inhibin A and inhibin B. A comparison of the structure of the N-terminal region of the mature α -subunit between hamster (GenBank accession no. AF432351) and

human (GenBank accession no. BC006391) revealed differences at amino acids 4, 7, 12 and 28, while a comparison between bovine (GenBank accession no. A25732) and human (GenBank accession no. BC006391) and between mouse (GenBank accession no. X69618) and human (GenBank accession no. BC006391) both revealed differences at amino acids 4–6, 12 and 28. In a companion paper, we demonstrate that inhibin A and inhibin B can be detected in the serum in response to exogenous gonadotropins, so we have confirmed that the ELISAs used can detect inhibin A and B in Siberian hamsters (Kenny *et al.*

Figure 5 Measurement of ovarian inhibin A and inhibin B content by ELISA. LTS refers to values less than standards used in each assay or lower than the limit of detection of the assay (<7.8 pg/ml inhibin A; <15.6 pg/ml inhibin B). There was a significant difference in levels of inhibin A and inhibin B between the LD and SD animal groups (* P <0.05). These results represent three independent experiments (n =9).

2002). We did detect inhibin A and inhibin B at low levels in a few animals housed under long and short photoperiods (Table 2), suggesting that some inhibin is being secreted sporadically but not in sufficient quantity to be detected in the assay. When ovarian homogenates were measured in the assay, dimeric inhibin A and inhibin B were detected in the LD but no inhibin A and significantly lower levels of inhibin B were detected in the SD samples. By Western blot analysis, little mature inhibin was detected in the SD samples. We hypothesize that the low circulating levels of inhibin A and inhibin B likely reflect a low rate of mature inhibin synthesis and secretion, but the current inhibin ELISAs may not be sufficiently sensitive to Siberian hamster inhibin to draw this conclusion. The development of a Siberian hamster-specific assay using recombinant hamster inhibins as the standards would resolve this issue.

FSH is known to regulate inhibin synthesis and secretion (Woodruff *et al.* 1987, 1996). Serum FSH levels did differ between the two populations of animals with no associated difference in LH levels. Thus, while the animals were clearly responsive to the different photoperiods, the levels of FSH in the LD animals appear to have been insufficient to promote cyclicity. It is possible that additional environmental cues are necessary to promote full reproductive function in this species (Goldman 1991, Ouarour *et al.* 1991, Puchalski & Lynch 1991, Badura & Goldman 1992, Debeljuk & Lasaga 1999, Drazen *et al.* 2001, Horton & Yellon 2001, Pak *et al.* 2001). The LD animals used were 7 weeks old and, based on previous studies, were thought to be sexually mature (Lerchl & Schlatt 1993, Schlatt *et al.* 1993). However, no vaginal opening was detected, thus contributing to our conclusion that the animals were not cycling. The animals in this study were individually housed in an environment free of male pheromones. Thus, cyclicity was solely dependent on light and endogenous hormones.

Because FSH levels differed between LD and SD animals yet serum inhibin levels were not adequately detected in the current assay, we examined inhibin synthesis and assembly in ovarian homogenates by protein blot analysis. The advantage of this method is that mature and precursor forms of the proteins can be detected to allow a more complete understanding of the synthesis and assembly of inhibin before it is secreted. Few studies have investigated the synthesis of inhibin subunits by the normal ovary, and there is a dearth of understanding regarding the regulation of inhibin translation and post-translational modification in any animal model (Findlay *et al.* 2001, Pangas & Woodruff 2001). Much of our knowledge regarding inhibin synthesis is derived from the products of recombinant cell lines (Pangas & Woodruff 2001). Thus, this analysis represents the first comprehensive investigation of naturally occurring inhibin subunit production. Inhibin proteins are produced by the hamster ovaries and the predominant forms detected in the LD and SD animals are precursors, the mature β -subunit (β) disulfide linked to the precursor α -subunits, α N α C or pro- α C. The abundance of these precursor proteins is higher in LD than in SD animals. No mature inhibins (α C/ β) are detected in the LD or SD ovarian lysates. When the protein is reduced, the pro- α C/ β band is lost because the disulfide bonds joining the pro-region of the α -subunit to the mature domain of the α -subunit and the bond linking the mature α -subunit to the mature β -subunit are broken. As a result, the higher molecular weight dimer is reduced and the mature α -subunit (18 kDa) and mature β -subunits (14 kDa and 13 kDa for β_A - and β_B -respectively) are detected. The antibody used in this analysis recognizes amino acids 1–32 of the mature α -subunit, thus, a band representing the precursor protein is not detected under reducing conditions. By contrast, little mature α -subunit is detected in the SD animals, even under reducing conditions. This observation is consistent with the differences observed in α -subunit mRNA levels.

Similarly, β_A - and β_B -subunits are both detected in ovarian lysates of LD females but not of SD females. This result is somewhat surprising given the equivalent β_A and β_B mRNA levels detected in the ovaries collected from animals under the two photoperiods. While a direct comparison between the abundance of the inhibin α -subunit and the two β -subunits cannot be carried out due to differences in antibodies, it is likely that the α -subunit is in sufficient abundance relative to the β -subunits to produce inhibin in excess of activin. Consistent with this argument, although not definitive, is our inability to detect activin in serum samples collected from these animals.

Although LD animals were not cycling (based on a lack of vaginal opening), follicles were developing through the large antral follicle stage and inhibin subunits were detected in the granulosa cells of these developing follicles. No evidence of previous ovulation was detected, based on

the absence of corpora lutea. In addition, serum FSH but not LH was significantly higher in LD compared with SD animals. Likely as a consequence of these FSH levels, inhibin α -subunit expression was higher within given follicles and was expressed in a larger number of follicles in LD animals. Higher levels of inhibin precursors were detected in ovarian homogenates of long photoperiod than short photoperiod animals. This final finding was not anticipated. Little is known regarding the processing of inhibin by any cell including the recombinant inhibin cell lines. The dibasic arginines are important for cleavage, but it is not known what protease is responsible for this regulation. These data suggest that some processing has occurred but the disulfide bond linking the precursor domain to the mature domain of the inhibin α -subunit is intact. Thus, a processing enzyme such as a disulfide isomerase may be regulated and contribute to inhibin production in heretofore unappreciated ways. The identification of the proteases and isomerases necessary for inhibin processing will be key to our understanding of the regulation of the normal reproductive cycle. In addition, we detected little inhibin secretion in the animals studied. This may reflect limited secretion of precursor inhibin subunits into the systemic circulation of LD and SD animals or that the current inhibin ELISAs are not sensitive enough to measure circulating inhibin in this animal model.

In conclusion, both serum FSH and ovarian inhibin A and inhibin B levels are higher in LD than in SD animals. Serum inhibin levels do not differ conclusively between the photoperiods. These data suggest that inhibin does not significantly contribute to the seasonal differences in FSH (at least not in non-cycling LD females). In contrast, there is significant inhibin A and inhibin B production within the ovaries and the amount produced differs between photoperiods. α -Subunit mRNA is higher in LD than in SD animals, as is α -subunit protein forms. β -Subunit mRNAs do not differ between the photoperiods but subunit protein synthesis appears to be higher in the LD than in the SD animals. These studies raise significant new questions regarding our understanding of the transcriptional, translational, post-translational modification, and secretion of inhibin by the ovary. The advantage of the Siberian hamster system is that subtle changes in follicular status can apparently be investigated with minimal changes in circulating gonadotropins. Based on these studies, the response of the inhibin system to high levels of gonadotropins was investigated in order to evaluate whether local or non-gonadotropin factors are involved in each of these steps in inhibin secretion (see the companion paper Kenny *et al.* 2002). The results of these studies are significant in that they change our understanding of the link between transcription and the subsequent processes needed to release a bioactive inhibin molecule. Future studies will focus on whether these steps in inhibin biosynthesis are regulated in other animal models such as the rat and the human.

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