Cold Suppression of Follicle-Stimulating Hormone Activity on Proliferation and Survival of Newt Spermatogonia

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In newts elevated titers of plasma prolactin (PRL), induced by low temperature, cause apoptosis in the penultimate mitotic stage of spermatogonia, and this cell death is suppressed by antiserum against newt PRL, but only during the initial 3 days of exposure (Yazawa et al., 1999). Thus, factors other than PRL must be involved in spermatogonial death. Follicle-stimulating hormone (FSH) may be a plausible candidate. Accordingly, the current study examined the activity of FSH on the proliferation and survival of spermatogonia at low temperatures in vivo and in vitro. Porcine FSH (pFSH) administration in vivo inhibited spermatogonial death induced at 12°C, but failed to do so at 8°C. Also pFSH promoted in vitro the proliferation of spermatogonia at 12°C, but not at 8°C. Furthermore, dibutyryl cyclic AMP stimulated in vitro DNA synthesis of secondary spermatogonia at 12°C, but not at 8°C. These different responses to temperatures were not caused by different levels of mRNA for the receptor of follicle-stimulating hormone, the number of FSH binding sites, or FSH binding affinity to its receptors in the testicular cells. Thus, the results indicate that a temperature-sensitive period exists during the postreceptor process and is responsible for the lack of response of newt testis to FSH at 8°C.

INTRODUCTION

Temperature plays a dominant role in controlling endocrine functions in poikilothermic vertebrates, especially during the reproductive process. For example, newt spermatocytogenesis ceases from late autumn to early spring, and it is believed that this is caused by spermatogonial cell death (Mazzi et al., 1967; Yazawa et al., 1999, 2000, 2001). It has been demonstrated experimentally in newts that low environmental temperature induced elevated titers of plasma prolactin (PRL) followed by apoptosis of spermatogonia during their penultimate mitotic stage; furthermore, this spermatogonial death was suppressed for 3 days by injections of anti-newt PRL antiserum to newts (Yazawa et al., 1999). The fact that spermatogonia died after 3 days, even though they were exposed to anti-newt PRL, suggests that factors other than PRL are involved in this cell death. Follicle-stimulating hormone (FSH) may be a likely candidate as one of these factors, because mammalian FSH (1) maintains spermatogonial viability and stimulates their proliferation (Abé and Ji, 1994; Ji and Abé, 1994); (2) is indispensable for completion of the last spermatogonial mitosis (Yazawa et al., in preparation); and (3) inhibits PRL-induced spermatogonial cell death both in vivo and in vitro (Mazzi and Vellano, 1968; Yazawa et al., 2000).
is possible that FSH activity decreases at low temperature and this reduced activity could be responsible for the spermatogonial death beginning 3 days after exposure to anti-newt PRL. In fact, Tanaka et al. (1981) reported that acidic gonadotropin (FSH) of the male newt pituitary disappears during the winter months, and therefore, plasma levels of FSH would be expected to decline during the same period. Also, the binding affinity of FSH to newt testis is reduced at high temperature (Kubokawa and Ishii, 1984a,b). These findings led previous authors to hypothesize that temperature controls the quantity of gonadotropins binding to receptors and as such ultimately controls gonadal functions. However, Licht et al. (1990) reported that suppression of testosterone secretion by cold temperature in LH-stimulated turtle testes is attributable to postreceptor processes. Thus, the step at which low temperature acts in the gonadotropin pathway in the testes of poikilothermic vertebrates is still not resolved.

The current report focused on several questions regarding the function of FSH and its receptor in newt testes in an attempt to identify the step at which cold temperature acts in the gonadotropin pathway. At low environmental temperature (1) can FSH suppress apoptosis? (2) can FSH promote spermatogonial proliferation? (3) is the production of FSH receptor (FSH-R) mRNA and PRL receptor (PRL-R) mRNA affected? and (4) do the number of FSH binding sites and FSH binding affinity to its receptors in the testicular cells decrease? Whether dibutyryl cyclic AMP promotes spermatogonial proliferation at low temperatures was also examined. The results of these studies indicate that low temperature affects a step in the postreceptor pathway.

**MATERIALS AND METHODS**

**Animals**

Adult male newts, Cynops pyrrhogaster, were purchased from a dealer (Hamamatsu Seibutsu Kyozai Ltd., Hamamatsu, Japan), maintained at 22°C under 12L:12D illumination, and fed frozen Tubifex.

**Treatment of Newts with FSH**

Forty-two adult male newts, previously maintained at 22°C for a month, were divided into three groups: one group was maintained at 22°C, one was transferred to 12°C, and the third group to 8°C. Half of the newts in each group were injected with porcine FSH (pFSH; 0.5 unit/newt; Sigma) every other day over a 7-day period, while the animals in the other half were each administered an equal volume of physiological saline (OR-2) (Wallace et al., 1973). Twenty-four h after the last injection, the testes were excised and fixed in Bouin’s solution.

**Histology**

The testes were dehydrated in a series of graded ethanol, embedded in paraffin, and sectioned serially at 5 µm thickness. The sections were stained with hematoxylin–eosin. To estimate the extent of spermatogonial degeneration, 10 sections each separated by 24 consecutive sections from the middle of the testis were examined with the light microscope. The incidence of degenerated spermatogonia was expressed as the percentage of animals containing degenerated spermatogonia, and the frequency was expressed as the percentage of sections containing degenerated spermatogonia/animal (Yazawa et al., 1999).

**Organ Culture of Testicular Fragments**

Newt testes were cut into small pieces (1–2 mm in diameter) and three pieces were placed on each nucleopore filter (Coaster Corp., Cambridge, MA) contained in a 35-mm plastic dish (Falcon, Lincoln Park, NJ; No. 1008). The testicular fragments were cultured at various temperatures in humidified air for 1 week in basal medium containing pFSH (5 µg/ml) or N6, 2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate [dibutyryl cyclic AMP (dbcAMP); 1 mM; Sigma]. The basal culture medium consisted of Leibovitz-15 supplemented with 10 mM Hepes adjusted to pH 7.4 with 1 N NaOH.

**Assay for Spermatogonial Proliferation**

Proliferating spermatogonia were determined by immunohistochemical detection of 5-bromo-2-de-
oxyuridine (BrdU) incorporated into replicating DNA with a kit according to the manufacturer’s instructions (Amersham Int. plc, UK). Testicular fragments cultured at various temperatures for a week in control medium or in medium supplemented with FSH or dbcAMP were labeled for 6 h with BrdU and then prepared for histological sections. The proliferative activity of spermatogonia was expressed as the percentage of cysts incorporating BrdU in 3 sections, each separated by intervals of 24 consecutive sections, all obtained from the middle of the fragments. The average ±SE was obtained for three experiments.

Statistics

The incidence of degenerated spermatogonia was submitted to χ² analysis. Other results were analyzed by the Tukey multiple comparison test. A probability level of <0.05 indicated a statistically significant difference.

RT–PCR and Southern Blot Analysis

Total RNA was prepared from testis fragments by the guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). One microgram of total RNA from testes, which had been treated with DNase I (Gibco-BRL, Tokyo, Japan), was reverse-transcribed using oligo(dT) primer with the Thermoscript RT–PCR system (Gibco BRL). PCR was carried out with KOD DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). The following primers were used for genes, newt FSH-R, PRL-R, and EF-1α: FSH-R F, 5’-CGGGATCCGGAAGAAATACAGAAT-3’; FSH-R R, 5’-ATCCCTCGTGACTTGCGTGCTGCT-3’; PRL-R F, 5’-CAGAGGGAAAAAACACTTACCTC-3’; PRL-R R, 5’-GATGTGGAGGCTCATAGATTA-3’; EF-1α F, 5’-CCTGCAGGACGTCTACAA-3’; EF-1α R, 5’-CTTCAGTGGTACGTACCTGG-3’. The annealing temperatures were 63°C (FSH-R) and 55°C (PRL-R and EF-1α), and 28 cycles (FSH-R), 35 cycles (PRL-R), and 22 cycles (EF-1α) were performed. These PCR cycles were in a linear range of amplification for each primer pair. The amplified products were separated on agarose gels, transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech), and detected by Southern hybridization. Labeling of cDNA probe, hybridization, and detection of signals were carried out using the AlkPhos direct system (Amersham Pharmacia Biotech).

FSH Binding Experiments

The preparation of membranes for the receptor studies was performed by the method of Kubokawa and Ishii (1980). Membrane preparations (100 μg) in 25 mM Tris–HCl (pH 7.2) containing 10 mM MgSO₄ and 0.1% BSA derived from testes of newts that had been kept at 8, 12, or 22°C for a week were incubated at 8° or 12°C for 36 h or at 22°C for 18 h, respectively, with 30,000 cpmp of ¹²⁵I-labeled human FSH (NEN, Boston, MA), and various concentrations of unlabeled human FSH (Calbiochem, La Jolla, CA) in a total volume of 400 μl. The reaction was stopped by the addition of 1 ml of ice-cold 25 mM Tris–HCl (pH 7.2) containing 10 mM MgSO₄ and 0.1% BSA. Bound hormone was separated from unbound hormone by centrifugation at 10,000g for 10 min. The pellet was washed once with chilled buffer. The radioactivity of the pellet was counted using an automatic gamma counter (1480 Wizard, Wallac OY, Turku, Finland). Nonspecific binding (<500 cpmp) was determined by adding excess unlabeled hFSH (1000 ng per tube) to the reaction mixture. Specific binding was defined as the difference between total and nonspecific binding. The Scatchard plot was calculated by using duplicated data in the competitive experiment.

RESULTS

FSH Suppression of Spermatogonial Degeneration at Low Temperatures

The newt testis is composed of a number of lobules within which germ cells and surrounding Sertoli cells form clusters called cysts. Lobules formed at the cephalic region gradually take more caudal positions as they mature so that a spermatogenic wave is produced extending longitudinally throughout the testis (Callard et al., 1978; Tanaka and Iwasawa, 1979). Therefore, any spermatogenic stage from spermatogonia to the most advanced stage for the season could be observed when longitudinal sections of the newt testis are made.
Degeneration of spermatogonia at the mitotic penultimate stage occurred in all animals exposed for 1 week to the low temperatures of 12° and 8°C (Fig. 1B), whereas no degeneration was found in animals kept at 22°C (Fig. 1A) (Yazawa et al., 1999). Both the incidence and frequency of degenerated spermatogonia were elevated to approximately 100% at the low temperatures, whereas those animals maintained at 22°C showed no spermatogonial degeneration (0%) (Fig. 2). The administration of FSH to animals maintained at 12°C suppressed spermatogonial cell death. The incidence of cell death (29%) was not significantly different from that of the initial control or the group maintained at 22°C. The frequency of cell death with FSH injection at 12°C was remarkably lower than that in the saline-injected group, but significantly higher than that in the animals in the initial control group or those at 22°C. On the other hand, FSH did not inhibit spermatogonial cell death in animals maintained at 8°C.

FSH Stimulation of Spermatogonial Proliferation at Low Temperatures in Vitro

To examine the effect of FSH on the stimulation of spermatogonial proliferation at low temperatures, testicular fragments were cultured in medium containing BrdU and the percentage of spermatogonia that incorporated BrdU was determined in histological sections (Fig. 3). The proliferative activity of secondary spermatogonia was about equal in FSH-treated fragments cultured at 12° and 22°C and in both cases significantly higher than that in controls. In FSH-treated fragments maintained at 8°C there was no stimulation of spermatogonial proliferation.

Expression of FSH-R and PRL-R mRNAs at Low Temperatures

The differences, detected above in FSH suppression of spermatogonial degeneration and FSH stimulation of spermatogonial proliferation at different temperatures, could stem from the synthesis of different amounts of mRNA of FSH-R and/or PRL-R depending on temperatures. Therefore, the expression levels of the mRNAs in testis enriched in spermatogonia (containing a few primary spermatocytes) were examined from newts that had been maintained for a week at 8°, 12°, or 22°C (Fig. 4). The amount of FSH-R
mRNA expressed was similar at all temperatures examined. On the other hand, the mRNA levels of PRL-R were significantly higher at 8°C than at 12°C or 22°C.

**Number of FSH Binding Sites and FSH Binding Affinity at Low Temperature**

Next, whether the number of FSH binding sites and FSH binding affinity to its receptors in the testicular cells vary at different temperatures was investigated. Cell membranes were prepared from testes of newts that had been maintained at different temperatures for a week. The binding of \(^{125}\)I-labeled human FSH to the receptors in the membrane preparations was examined by a series of competition experiments at the same temperature at which newts had been kept. Equilibrium parameters of the FSH-R was estimated by Scatchard plots (Fig. 5). The equilibrium constants of association for specific binding of hFSH were \(3.34 \times 10^8\) M\(^{-1}\) at 8°C, \(3.15 \times 10^8\) M\(^{-1}\) at 12°C, and \(2.99 \times 10^8\) M\(^{-1}\) at 22°C. On the other hand, the capacities were 880 fmol/mg of protein at 8°C, 896 fmol/mg of protein at 12°C, and 1004 fmol/mg of protein at 22°C. These results indicated that equilibrium constants and capacities were almost the same at different temperatures, suggesting that the number of FSH binding sites and FSH binding affinity to its receptors in the testicular cells were not changed at low temperature.

**Dibutylryl Cyclic AMP Stimulation of Spermatogonial Proliferation in Vitro at Low Temperatures**

Having determined that the expression of FSH-R mRNA and binding sites for FSH as well as the FSH binding capacity to membrane fractions are independent of the temperatures tested, whether the postreceptor process is temperature dependent was then examined. In these experiments, the in vitro effect of dbcAMP on the proliferation of secondary spermatogonia was assessed (Fig. 6). One millimolar dbcAMP stimulated spermatogonial proliferation equally at both 12°C and 22°C, but did not overcome the low...
proliferation of spermatogonia at 8°C. This result indicates that the temperature-dependent step is located within the postreceptor process.

DISCUSSION

The current in vivo and in vitro studies demonstrated that: (1) spermatogonial cell death induced at low temperature was significantly suppressed by FSH at 12°C and 22°C but not at 8°C; (2) the proliferative activity of secondary spermatogonia was promoted by FSH at both 12°C and 22°C and to a similar degree, but barely at 8°C; (3) expression levels of the mRNA for FSH-R, the number of FSH binding sites, and FSH binding affinity to its receptors in the testicular cells were unaffected by the various temperatures; and (4) dbcAMP promoted spermatogonial proliferation at 12°C and 22°C but not at 8°C. These results are consistent with previous findings that gonadotropin functions are temperature dependent in various poikilothermic vertebrates (Licht and Pearson, 1969; Tsui and Licht, 1974; Licht, 1975; Licht et al., 1989, 1990; Jalali et al., 1976; Pierantoni et al., 1987), including newts (Tanaka and Takikawa, 1984). In nature, such temperature sensitivity of gonadotropins contributes to the seasonal regulation of the reproductive cycle in poikilothermic vertebrates. Our studies and others have attempted to locate the point in the gonadotropin pathway at which the temperature sensitivity exists. FSH interacts with FSH-R, a G-protein-coupled transmembrane receptor expressed on Sertoli cells (Heckert and Griswold, 1992), and thus there are several points at which the action of FSH could be regulated: the levels of circulating FSH in the blood could vary; the quantity of mRNA for the FSH-R in the testis could change, and/or FSH binding affinity or the number of FSH binding sites could differ.

Heterologous mammalian FSH was used in the current study, because newt FSH is not available. Kubokawa and Ishii (1984b) reported that thermal dependence of the FSH binding to FSH-R in the newt testis is not influenced by animal source of the hor-

FIG. 5. Scatchard plots of specific binding of hFSH to a membrane preparation of newt testis. These plots were constructed from data of competition experiments. The binding reactions for membrane preparation derived from testes of newts kept at 8°, 12°, and 22°C were carried out at 8°, 12°, and 22°C, respectively.

FIG. 6. Effects of dibutylryl cAMP on proliferative activity in secondary spermatogonia at various temperatures in testicular organ culture. Percentages of BrdU-labeled spermatogonia in each group are shown. Each point is the mean of triplicate determinations with standard errors. Values with the same letters do not differ from each other significantly at the 5% level.
mone. Licht et al. (1975, 1990) also demonstrated in reptilian testis using homologous gonadotropins that thermal dependence of the response to exogenous gonadotropin is predominantly a property of the gonadal tissue (receptor) rather than the hormones. So it seems reasonable that the present results reflect the properties of newt FSH receptor.

First, with respect to changing amounts of FSH in the circulation, there is indirect evidence that reduced levels of plasma FSH can lead to spermatogonial death. We found that exogenous FSH exerted antagonistic effects on PRL-induced spermatogonial apoptosis (Yazawa et al., 2000). In nature, the acidic gonadotropin (FSH) of the newt pituitary disappears during winter months (Tanaka et al., 1981), and the plasma levels could decline at this period. Certainly, the present results showing that exogenous FSH prevented spermatogonial cell death at 12°C support this point. Also, in the Djungarian hamster, a seasonal breeding mammal, the plasma FSH level drops rapidly after the animals are exposed to a short photoperiod (Tsutsui et al., 1988; Furuta et al., 1994), and following this treatment apoptosis of the testicular cells and involution of the testis occur. However, administration of exogenous FSH promotes regrowth of the testes and completes spermatogenesis even under photoinhibited conditions (Niklowitz et al., 1989).

Thus, FSH is a primary regulator of apoptosis during spermatogenesis in these seasonally breeding animals. However, precisely how different plasma levels of FSH function in newts at low temperature remains to be investigated.

Second, it was found that the expression levels of mRNA for FSH-R, the number of FSH binding sites, and FSH binding affinity to its receptors in the testicular cells were independent of the temperatures tested, and thus there was no regulation of FSH at the receptor level (Figs. 4 and 5). However, others have reported that temperature affects the rate, affinity, and extent of FSH binding in the gonads of poikilothermic vertebrates (Licht and Midgley, 1976; Kubokawa and Ishii, 1980, 1984a,b) and hypothesized that temperature adaptations at the level of gonadotropin binding to receptors might control gonadal function (Kubokawa and Ishii, 1984a,b). The newt FSH-R for rat FSH have the optimum affinity between 15°C and 20°C and also have similar affinities between 5°C and 30°C which are higher than affinities at 35°C and 40°C. Nevertheless, our current experiments found that the regulation of FSH is controlled in the postreceptor pathway. This conclusion is supported by the current studies with dbcAMP. Cyclic AMP is a second messenger of intracellular FSH-R signaling that mimics FSH action, e.g., promotion of spermatogonial proliferation (Nishimune et al., 1981; Haneji et al., 1986). In our experiments, dbcAMP failed to correct the decline in proliferative activity of secondary spermatagonia induced at 8°C, but did so at higher temperatures. In the turtle testis, Licht et al. (1990) also found that suppression of gonadotropin action at low temperature (5°C) is affected by postreceptor processes rather than by receptor binding per se. This finding, together with the present results, indicates that FSH exerts its function in newt testes by a postreceptor pathway.

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