Effects of age, photoperiod and follicle-stimulating hormone on lactate production by cultured Sertoli cells from prepubertal Siberian hamsters (*Phodopus sungorus*)

S. C. Newton¹*, M. J. Welsh² and A. Bartke¹

¹Southern Illinois University, School of Medicine, Department of Physiology, Carbondale, IL 62901-6512, USA; and ²Department of Anatomy and Cell Biology, University of Michigan, School of Medicine, Ann Arbor, MI 48109, USA

Summary. To define a functional difference in Sertoli cells of animals exposed to different photoperiodic conditions, we isolated Sertoli cells from the testes of juvenile Siberian hamsters and cultured them in serum-free medium. In all age groups studied, Sertoli cells isolated from hamsters with delayed and normal puberty responded to follicle-stimulating hormone (FSH) with an increase in lactate production. The increase in lactate production induced by 1000 ng FSH ml⁻¹ was significantly greater in Sertoli cells isolated from hamsters with delayed puberty than in those with normal puberty. These results suggest that Sertoli cells of Siberian hamsters exposed to short photoperiod *in vivo* may respond to increases in plasma FSH concentrations associated with photostimulation or spontaneous sexual maturation by an increase in secretory activity that may be critical for the initiation of spermatogenesis.

Keywords: Sertoli cells; Siberian hamster; photoperiodism; follicle-stimulating hormone; lactate

Introduction

The Siberian hamster (*Phodopus sungorus*) is a seasonally breeding rodent, which breeds in the spring and summer in its natural habitat and in photoperiods of 14 h under laboratory conditions (Hoffmann, 1973). A short photoperiod (< 10 h light day⁻¹) causes a delay of several months in sexual maturation (Hoffmann, 1978). Such a delay in puberty resembles the situation described in some other rodent species (Horton, 1984), but differs from the effects of ambient photoperiod on reproductive function in the golden hamster (Darrow *et al.*, 1980). We studied the effects of short and long photoperiods on testicular development in Siberian hamsters to identify functional changes in the Sertoli cells that are related to the onset of spermatogenesis. This experimental model allows separation of the changes related to chronological age from those related to pubertal development.

Sertoli cells are important mediators of the effects of hormones, toxicants, temperature and other factors on spermatogenesis (Steinberger, 1971). Of the numerous products of the Sertoli cell, lactic acid has been proposed as a possible mediator of the effects of follicle-stimulating hormone (FSH) on spermatogenesis (Robinson & Fritz, 1981; Mita & Hall, 1982; Mita *et al.*, 1982). In rats, lactate was reported to be the preferred energy substrate for developing germ cells *in vitro* (Jutte *et al.*, 1981) and was shown to increase oxygen consumption (Jutte *et al.*, 1981), incorporation of [³H]uridine (Jutte *et al.*, 1982) and [³H]leucine (Jutte *et al.*, 1983) and ATP production (Grootegeod *et al.*, 1984) in germ-cell cultures. The key role of FSH in the control of pubertal development in

*Present address: University of South Carolina, School of Medicine, Department of Cell Biology and Neurosciences, Columbia, SC 29208, USA.

Siberian hamsters is supported by the changes in plasma FSH concentrations during maturation (Yellon & Goldman, 1984) and by the demonstration that suppressing the normal increase in plasma FSH in photostimulated hamsters by injections of porcine follicular fluid delayed testicular growth (Milette *et al.*, 1988). Follicle-stimulating hormone alone or in combination with luteinizing hormone (LH) causes an increase in testicular weight, restoration of seminiferous tubule diameter and complete restoration of spermatogenesis in testicularly regressed adult Siberian hamsters (Niklowitz & Hoffmann, 1989).

In adult Syrian hamsters (*Mesocricetus auratus*), the number of testicular FSH receptors and the responsiveness of Sertoli cells to FSH *in vitro* are influenced by photoperiod exposure *in vivo*. Thus, exposure to short photoperiods results in an increased concentration, but a reduced total number of testicular FSH receptors (Klemcke *et al.*, 1987; Sinha-Hikim *et al.*, 1989). Furthermore, Sertoli cells isolated from testicularly regressed Syrian hamsters respond to FSH with a greater increase in cAMP synthesis than do cells isolated from testicularly active animals (Heindel *et al.*, 1981; Berkowitz & Heindel, 1984; Heindel, 1988). In the present study, Sertoli cells were isolated from Siberian hamsters aged 12–15, 18–21, 24–27 or 30–33 days, born and raised in a long or a short photoperiod, and were maintained in culture. These cultures were used to examine the effects of age and photoperiod on the ability of Sertoli cells to produce lactate and to respond to FSH *in vitro*.

Materials and Methods

Animals

Siberian hamsters were obtained from a breeding colony originating from animals kindly provided by B. Goldman (University of Connecticut, Storrs, CT, USA) and maintained at Southern Illinois University in Carbondale, USA. Tekland laboratory chow and water were provided *ad libitum*. Short (S) photoperiods provided 6 h of light day⁻¹ and long (L) photoperiods provided 16 h of light day⁻¹. All reagents were obtained from Sigma Chemical Company, St Louis, MO, USA, unless otherwise indicated.

Isolation and culture of Sertoli cells

Enriched populations of Sertoli cells were prepared by a modification of the procedure of Welsh & Wiebe (1975), as described by Ireland & Welsh (1987). The testes were aseptically removed, decapsulated and placed in a solution of phosphate-buffered saline lacking calcium and magnesium (solution A) and subjected to sequential digestions with collagenase (2–4 mg per 20 ml solution) and pancreatin (2 mg enzyme per 20 ml solution). In animals exposed to short photoperiods, the tubules were washed with the aid of low-speed (60 g) centrifugation in 15 ml sterile polystyrene centrifuge tubes (Baxter Scientific products, McGraw Park, IL, USA) because the tubules were small. The resulting cell clusters were resuspended in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium, supplemented with growth factors (final concentrations in media were 10 µg insulin ml⁻¹, 5 µg transferrin ml⁻¹, and 0.25 ng epidermal growth factor ml⁻¹) and antibiotics (final concentrations in media were 2 U penicillin ml⁻¹, 2 µg streptomycin ml⁻¹, 1 µg gentamicin ml⁻¹ and 5.0 ng amphotericin B ml⁻¹; Mather *et al.*, 1983; Newton *et al.*, 1989). This was similar to the 3F medium of Rich *et al.* (1983) and Wright *et al.* (1989), which was considered a serum-free basal medium. Insulin in high concentrations increased the secretion of androgen-binding protein and lactate (Rich *et al.*, 1983; Oonk *et al.*, 1985), while the effects of transferrin and epidermal growth factors were less defined (Rich *et al.*, 1983). Cells were cultured on 35 mm diameter culture dishes (Falcon plastics), at 34°C in an atmosphere of 95% air and 5% carbon dioxide. Approximately two plates per animal were obtained from S animals and from 12–15-day-old L animals and three plates per animal from the older L hamsters. The media were changed daily, each dish containing 2 ml of medium. The cells were allowed 4 days to attain confluency and the experiments were performed on the fifth day after isolation.

Assays

Experiments ran for 8 h, because this was determined to be the optimal duration for measurements of lactate production by isolated rat Sertoli cells (Oonk *et al.*, 1985). After that, the media were collected for lactate determinations (Gutmann & Wahlefeld, 1974). An aliquot of each of the samples was de-proteinized with perchloric acid (1 mol l⁻¹). This acid solution was centrifuged and the supernatant was assayed for lactate using lactate dehydrogenase (Sigma catalog number 826-6). In this procedure, the amount of reduced nicotinamide adenosine dinucleotide (NADH), measured as absorbance at 340 nm formed from NAD (Sigma N7004), provides a measure of the original lactate

concentration in the sample. Monolayers of Sertoli cells were incubated for, at most, 24 h at 34°C with 1 ml of NaOH solution (0.3 mol l^{-1}), which caused the cells to lyse so that DNA and protein could be determined (Giles & Myers, 1965; Bradford, 1976; Patterson, 1979).

Cell viability was determined by trypan blue exclusion, and >95% of the cultured cells were viable. Occasionally, to check for possible contamination with Leydig cells, Sertoli cell cultures were stained histochemically for 3 β -hydroxysteroid dehydrogenase as described by Welsh & Wiebe (1975) and were virtually negative for the presence of this enzyme. Possible contamination of Sertoli cell monolayers by peritubular (myoid) cells was evaluated by use of alkaline phosphatase histochemistry according to the procedure of Chapin *et al.* (1987). According to a report by Palombi & Di Carlo (1988), alkaline phosphatase histochemistry selectively detects desmin-containing contractile cells in cultures of tubular and peritubular cells (see also Anthony & Skinner, 1989). Sertoli cell monolayers isolated in this study were >95% free of contamination with peritubular myoid cells, possibly because of the isolation technique and the use of serum-free media. It is generally accepted that peritubular cells do not contain FSH receptors (Orth & Christensen, 1977) and therefore do not respond to FSH stimulation. However, paracrine regulation cannot be ruled out and therefore peritubular (myoid) cell contamination was kept to a minimum. Contamination with spermatogenic cells was generally <5% after five changes of medium. Cultures from L animals generally contained more spermatogenic cells than those from S animals.

The effects of FSH on lactate production were examined using 125, 250, 500, 1000, 2000 and 5000 ng ovine FSH (oFSH-S17, kindly provided by NIH) ml^{-1} of culture medium. The data were expressed as percentage of a mean basal lactate value determined within each experiment to correct for the effects of cell density on Sertoli cell function and intra-isolation variability.

Statistical analysis

Data were analysed by ANOVA, appropriate to the design, and a Student Neuman–Keuls test at the 95% confidence limit was used to determine which treatment group means were significantly different. Student's *t* tests were performed when only two groups were compared. Pearson's correlation coefficient and probability were determined to identify responses that were dose related. The statistics were analysed with the aid of the Crisp statistical package for personal computers (Crunch Software Corp., San Francisco, CA, USA).

Results

Sertoli cells isolated from hamsters exposed to long or short photoperiods responded to oFSH with a characteristic shape change (Fig. 1) and by a significant increase in the accumulation of lactate in the incubation medium. Under basal conditions, Sertoli cells from S hamsters produced significantly more lactate than those from L animals (101.1 ± 11.9 versus $39.97 \pm 4.59 \mu\text{g} \mu\text{g}^{-1}$ DNA in 8 h; $P < 0.001$). The response of Sertoli cells derived from S 18–21-day-old hamsters to oFSH was dose dependent over the whole range of oFSH concentrations used in this study (125–5000 mg ml^{-1}) with a correlation of 0.70 and a slope of $0.025 \mu\text{g lactate} \mu\text{g}^{-1}$ DNA as a percentage of basal ng^{-1} oFSH ($P < 0.00001$). The median effective dose of oFSH for lactate production was 500 ng ml^{-1} (Fig. 2b).

Lactate production by Sertoli cells isolated from L exposed hamsters of the same age was dose dependent between 125 and 1000 ng ml^{-1} with a correlation of 0.63 and a slope of $0.039 \mu\text{g lactate} \mu\text{g}^{-1}$ DNA as a percentage of basal ng^{-1} oFSH ($P < 0.00001$) (Fig. 2b). In the presence of higher doses of oFSH, lactate production from L Sertoli cells was not significantly different from basal values ($P > 0.05$). On the basis of these results, doses of 500 and 1000 ng FSH ml^{-1} were selected for the study of the effects of photoperiod exposure *in vivo* on lactate production *in vitro* from Sertoli cells in other age groups.

Lactate production by Sertoli cells

From hamsters 12–15 days old. Lactate production from L Sertoli cells was significantly greater than basal concentrations in the presence of 500 or 1000 ng oFSH ml^{-1} ($P < 0.05$). Lactate values measured in the presence of 1000 ng oFSH were significantly greater than in the presence of 500 ng ml^{-1} ($P < 0.05$). Lactate production in response to oFSH was dose related, with a correlation coefficient of 0.75 and a slope of $0.073 \mu\text{g lactate} \mu\text{g}^{-1}$ DNA as a percentage of basal ng^{-1} oFSH.

Fig. 1. Phase-contrast micrograph of Siberian hamster Sertoli cells in culture under (a) basal conditions and (b) the influence of stimulation by follicle-stimulating hormone ($1 \mu\text{g oFSH-S17 ml}^{-1}$ for 30 min). Note the difference in the shape of the cells; $\times 160$.

In Sertoli cells, doses of 500 and $1000 \text{ ng oFSH ml}^{-1}$ could also stimulate lactate production above basal rates, lactate production by Sertoli cells treated with $1000 \text{ ng oFSH ml}^{-1}$ being significantly greater than the corresponding value for cells treated with $500 \text{ ng oFSH ml}^{-1}$ ($P < 0.01$). These responses were dose related, with a correlation coefficient of 0.91 ($P < 0.00001$) and a slope of $0.096 \mu\text{g lactate } \mu\text{g}^{-1} \text{ DNA as a percentage of basal ng}^{-1} \text{ oFSH}$. Lactate production by S Sertoli cells treated with $1000 \text{ ng oFSH ml}^{-1}$ was significantly greater than in identically treated L Sertoli cells (t test; $P < 0.05$; Fig. 2a).

From hamsters 24–27 days old. Both the doses of oFSH significantly stimulated lactate production by L Sertoli cells ($P < 0.01$). Lactate production in the presence of $1000 \text{ ng oFSH ml}^{-1}$ was not significantly different from the values measured at $500 \text{ ng oFSH ml}^{-1}$ ($P > 0.05$). Nevertheless, the effect of oFSH on lactate production from L Sertoli cells was dose related, with a correlation coefficient of 0.62 ($P < 0.00001$) and slope of $0.054 \mu\text{g lactate } \mu\text{g}^{-1} \text{ DNA as a percentage of basal ng}^{-1} \text{ oFSH}$. Both 500 and $1000 \text{ ng oFSH ml}^{-1}$ also stimulated lactate production from S Sertoli cells ($P < 0.01$). Values obtained with $1000 \text{ ng oFSH ml}^{-1}$ were significantly greater than in the presence of $500 \text{ ng FSH ml}^{-1}$ ($P < 0.05$). The effect of FSH on lactate production by S Sertoli cells was dose related, with a correlation coefficient of 0.85 and slope of $0.087 \mu\text{g lactate } \mu\text{g}^{-1} \text{ DNA as a}$

Fig. 2. Effects of various doses of follicle-stimulating hormone (FSH) on lactate secretion from Sertoli cells isolated from Siberian hamsters reared in long (▨) or short (□) photoperiods (a) 12–15 (b) 18–21 (c) 24–27 or (d) 30–33 days old at the time of culture. Lactate secretion (mean $\pm$ SEM) is expressed as $\mu\text{g lactate } \mu\text{g}^{-1} \text{ DNA}$ as a percentage of basal, after incubation for 8 h. Different letters indicate significant differences ($P < 0.05$) between treatment groups. Numbers under the columns indicate number of plates per treatment group.

percentage of basal ng^{-1} oFSH. Lactate production by S Sertoli cells treated with $1000 \text{ ng oFSH ml}^{-1}$ was significantly greater than by identically treated L Sertoli cells (t test; $P < 0.03$; Fig. 2c).

From hamsters 30–33 days old. Lactate production from L Sertoli cells was greater than basal levels with either of the doses of oFSH. Values obtained from L Sertoli cells treated with $1000 \text{ ng oFSH ml}^{-1}$ were not significantly different from values measured at $500 \text{ ng oFSH ml}^{-1}$ ($P > 0.05$), but the results were dose related, with a correlation coefficient of 0.62 and a slope of $0.037 \mu\text{g lactate } \mu\text{g}^{-1} \text{ DNA}$ as a percentage of basal ng^{-1} FSH. The production of lactate by S Sertoli cells was also greater than basal with either 500 or $1000 \text{ ng oFSH ml}^{-1}$, the effect of the higher dose being significantly greater ($P < 0.05$). Lactate production by S Sertoli cells was dose related, with a correlation coefficient of 0.66 ($P < 0.0001$) and a slope of $0.074 \mu\text{g lactate } \mu\text{g}^{-1} \text{ DNA}$ as a percentage of basal ng^{-1} oFSH. Lactate production by S Sertoli cells treated with $1000 \text{ ng oFSH ml}^{-1}$ was significantly greater than by identically treated L Sertoli cells (t test; $P < 0.03$; Fig. 2d).

Discussion

Cultures of Sertoli cells isolated from immature Siberian hamsters were viable and virtually free of other testicular cell types. When treated with FSH, the cultured cells exhibited a clear morphological response (shape change); in rat Sertoli cells such a change accompanies an increase in intracellular cyclic AMP (Kierszenbaum *et al.*, 1985).

Lactate production by Sertoli cells from L and S hamsters 18–21 days old were stimulated with ovine FSH (125–1000 ng ml⁻¹) in a dose-related manner. Although lactate may not be the sole regulator of spermatogenesis, it is an indicator of Sertoli cell function. Radioimmunoassay using commercially available rat transferrin (Cappel Laboratories, West Chester, PA, USA) failed to detect Siberian hamster transferrin in serum and Sertoli cell medium (S. C. Newton & A. Bartke, unpublished data). Results presented here resemble those obtained by Jutte *et al.* (1983) in cultures of rat Sertoli cells. The high dose of FSH needed to stimulate secretion of lactate may be due to species differences in responsiveness to FSH. High doses of FSH (2000 and 5000 ng ml⁻¹) stimulated lactate production in S Sertoli cells, but not in L Sertoli cells, indicating that Sertoli cells isolated from L hamsters may be more susceptible to desensitization at higher doses of FSH. In another seasonal breeder, the adult golden (Syrian) hamster, injections with human chorionic gonadotrophin (hCG) produced a dose-dependent decrease in the number of testicular LH receptors in gonadally active animals (Amador *et al.*, 1985). In contrast, testes from testicularly regressed golden hamsters showed an increase in testicular LH and hCG receptors after injections of hCG (with the exception of a high dose, 0.9 i.u. hCG g⁻¹ body weight), thus resembling the responses measured in immature animals (Amador *et al.*, 1985). Testicularly regressed golden hamsters could also respond to a second challenge of hCG, 24 h after a priming dose, with increased serum testosterone concentrations (Amador *et al.*, 1985).

Regulation of FSH receptors by FSH has been demonstrated in several species (Orth & Christensen, 1977; Thanki & Steinberger, 1978; Tsutsui & Ishii, 1978; Salhanick & Wiebe, 1980; Klemcke *et al.*, 1987). No data are available on the effects of photoperiod or puberty on testicular FSH binding in Siberian hamsters. However, it is possible that the increased sensitivity of Sertoli cells from S hamsters to FSH reflects either upregulation of FSH receptors in response to suppression of endogenous FSH release (Yellon & Goldman, 1984) or the ability of FSH to increase, rather than deplete, the number of FSH-binding sites in S animals.

Berkowitz & Heindel (1987) reported that the inhibin-like activity of conditioned media of golden hamster Sertoli cells was greatest from cells isolated from S hamsters. Moreover, the pituitaries from S hamsters were more sensitive to the action of inhibin. The result is a decrease in FSH release from the anterior pituitary and possible upregulation of the FSH receptors on Sertoli cells.

Testes from adult L Siberian hamsters exhibit full spermatogenesis, with paired testes of approximately 400 mg, while S animals produce no advanced germ cells, with paired testes of approximately 15 mg (Hoffmann, 1978). The presence or absence of germ cells has been reported to influence the function of Sertoli cells, including a decrease in RNA synthesis in Sertoli cells (Rivarola *et al.*, 1986), decreased secretion of androgen-binding protein (ABP) in response to FSH or dbcAMP when germ cells are added to Sertoli cells *in vitro* (Le Magueresse & Jegou, 1988; Le Magueresse *et al.*, 1988), appearance of a 48 kDa glycoprotein in rat Sertoli cell cultures in the absence of germ cells (Galdeiri *et al.*, 1984). Some proteins that may be important in spermatogenesis, such as plasminogen activator, are secreted at a particular stage of spermatogenesis (Fritz & Karmally, 1983; Vihko *et al.*, 1984; Shabanowitz *et al.*, 1986; Wright & Luzzaraga, 1986), suggesting that germ cells affect Sertoli cell function. Germ-cell-dependent changes in phosphorylation of Sertoli cell proteins have been demonstrated (Ireland & Welsh, 1987) and, on this basis, a signal transduction mechanism by which germ cells may affect Sertoli cell function has been suggested to be the phosphatidylinositol pathway (Ireland & Welsh, 1987; M. J. Welsh & M. E. Ireland, unpublished). Testes from prepubertal animals (S) have a lower complement of spermatogenic cells *in vivo* and therefore are not subjected to the possible paracrine regulation of Sertoli cell function by spermatogenic cells compared with testes from L animals. This is also apparent after cell isolation, since L Sertoli cells have more contaminating spermatogenic cells. It is possible that L Sertoli cells have the receptors for these paracrine regulators, while S Sertoli cells have less-developed receptors.

Sertoli cells isolated from hypophysectomized or cryptorchid adult golden hamsters that had undergone testicular regression could respond to FSH with an accumulation of cAMP similar to that in Sertoli cells isolated from immature hamsters or adult hamsters exposed to short

photoperiod (Heindel, 1990). This response was specific for FSH in that cholera toxin, forskolin or isoproterenol caused a response similar to that in L adults. Cryptorchidism causes a decrease in testicular weight (regression) due to damage to advanced germ cells (Aumuller, 1980). Hypophysectomy causes regression by removing the source of gonadotrophins. In either case, a lack of advanced germ cells is observed, and responsiveness of cultured Sertoli cells to the actions of FSH is increased. This strongly suggests an action of germ cells, possibly paracrine, on Sertoli cell responsiveness to FSH (Heindel, 1990). Indeed, pretreatment of Sertoli cells with germ cells has been observed to reduce, by about 50%, the subsequent response to FSH as measured by cAMP production from ATP by Sertoli cell membrane fractions (M. J. Welsh & M. E. Ireland, unpublished).

The stimulatory effect of FSH on lactate production by Sertoli cells isolated from animals of all age groups studied was dose related. However, at all ages examined, the correlation coefficient and the slope were numerically greater in S Sertoli cell cultures than in L Sertoli cell cultures. In Sertoli cells from L animals, 18–21 days old or older, production of lactate in response to 500 or 1000 ng oFSH ml⁻¹ was not significantly different. This implies a shift in the dose–response curve with a plateau beginning near 500 ng FSH ml⁻¹. In other words, lactate production in response to 1000 ng ml⁻¹ in L Sertoli cells appears to decrease with age of the hamsters, as was reported for cultured Sertoli cells in rats (Rich *et al.*, 1983) and golden hamsters (Berkowitz & Heindel, 1987). Lactate production from S Sertoli cells treated with 1000 ng oFSH ml⁻¹ was greater than from identically treated L cells in all age groups examined.

The responsiveness of Sertoli cells to FSH varies with the age of the animal. In rats, response to both basal adenylate cyclase and FSH stimulation of adenylate cyclase declines between the ages of 12 and 60 days (Van Sickle *et al.*, 1981). The binding of FSH to Sertoli cells increases up to 16 days of age and then declines to adult levels (Thanki & Steinberger, 1978; Salhanick & Wiebe, 1980). This age-related loss of Sertoli cell responsiveness to FSH was observed in the present study in L Siberian hamsters, but not in S animals. It can be concluded that Sertoli cells from immature Siberian hamsters are well prepared to respond to FSH when FSH is increased during the process of photostimulation or spontaneous sexual development. Our results demonstrate that Sertoli cells from L hamsters undergo a gradual decrease in responsiveness to FSH with age, while those from S animals maintain a prepubertal condition with a greater response to FSH than is observed in age-matched animals raised in long photoperiods. Whether these differing responses are due to desensitization of the FSH response or to the residual influence of advanced germ cells on Sertoli cell function remains to be determined.

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