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Transferrin Secretion in Response to Different Modes of FSH Stimulation and Cycloheximide in Superfused Sertoli Cell Cultures

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The influence of different modes of FSH stimulation and cycloheximide on transferrin secretion by rat Sertoli cells was investigated using a superfusion culture system. Sertoli cells from 18-day-old rats were cultured in serum-free medium on Matrigel-covered slides first in static conditions for 19 hours, and then superfused at a flow rate of 2.5 ml/hour. After an equilibration period of 48 hours to establish the basal rate of transferrin secretion, the cultures were exposed to various modes of FSH stimulation. Sertoli cells stimulated intermittently (20 min/2 hours) up to 22 hours responded to each consecutive FSH pulse with a rapid increase of transferrin secretion followed by a decline toward basal values. Continuous 22-hour exposure to FSH elicited an immediate increase followed by irregular fluctuations and a transient decline towards the baseline. With either mode of FSH stimulation, there was a secondary prolonged increase in transferrin secretion. Although cultures stimulated intermittently or continuously during the entire experimental period (22 hours) secreted similar cumulative amounts of transferrin (10.8 ± 0.5 μg and 11.1 ± 0.8 μg, respectively), there was a direct correlation between the secreted amount of transferrin and the duration of FSH exposure up to 8 hours. Addition of cycloheximide decreased both basal and FSH-stimulated transferrin secretion. However, even when cycloheximide was added 1 hour before FSH, an early secretory peak in response to FSH was still observed. It was concluded that, regardless of the mode of FSH stimulation, transferrin secretion shows a biphasic response in which an initial increase within minutes of FSH exposure is followed by a return to basal levels, and then a delayed and prolonged secondary increase. Secondly, cumulative amounts of transferrin can vary with the mode of FSH stimulation; finally, Sertoli cells appear to secrete mainly newly synthesized transferrin, but a small storage pool may also exist for immediate release by FSH independently of new protein synthesis.

Key words: Sertoli cells, superfusion culture, transferrin secretion, effect of FSH, effect of cycloheximide.

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Sertoli cells play a crucial role in spermatogenesis by providing a unique microenvironment believed to be essential for germ cell differentiation (Steinberger, 1981; Sharpe, 1983; Steinberger et al, 1984). This is accomplished by selective transport of substances from the circulation (Waites and Gladwell, 1982) as well as secretory activities (Mather et al, 1983; Shabanowitz and Kierszenbaum, 1986). Among various proteins secreted by the Sertoli cells, transferrin is a

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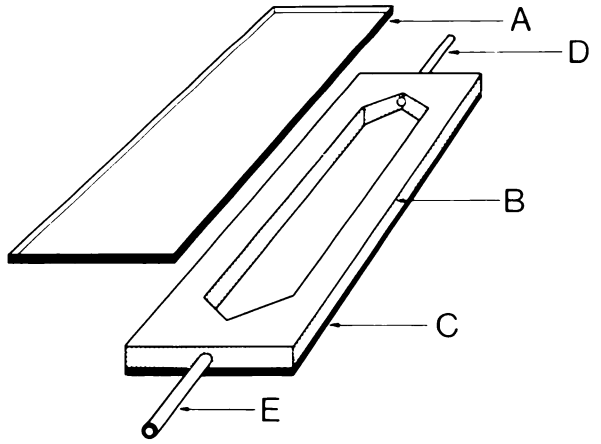

Fig. 1. Diagram of the superfusion culture chamber (not in actual proportions). **A.** Removable top glass plate. **B.** Gasket. **C.** Bottom glass plate. **D.** Inlet. **E.** Outlet. Sertoli cells were plated on the bottom glass plate covered with Matrigel.

major product (Skinner and Griswold, 1980). Its actions in the testis (Holmes et al, 1983; Dighe et al, 1984; Huggenvik et al, 1984; Morales and Clermont, 1986) and the regulation of its synthesis and secretion (Skinner and Griswold, 1982; Huggenvik et al, 1987), including vectorial secretion (Janecki and Steinberger, 1986; 1987a, 1987b; Hadley et al, 1987), have been intensively investigated during the last few years using various culture techniques. However, the culture systems commonly used were stationary and did not allow the study of the dynamics of Sertoli cell secretion under various experimental conditions, or in response to different modes of hormonal stimulation *in vitro*.

Recently, we described a new superfusion culture system that was developed and validated in our laboratory for investigating the dynamics of Sertoli cell secretions (Janecki et al, 1987). In this system, rat Sertoli cells maintain morphologic and functional integrity for up to 12 days of culture and form highly polarized monolayers that restrict the passage of macromolecules, such as inulin. We have also previously demonstrated (Jakubowiak et al, 1987a, 1987b) that pulsatile stimulation with FSH has a dual effect on the pattern of transferrin secretion in that a peak of transferrin secretion in response to each consecutive pulse is followed by a delayed and more prolonged secondary rise. When the cells are exposed continuously to FSH, transferrin secretion is also biphasic, but more irregular. In the present article we describe the results of further studies concerning the patterns of transferrin secretion following different modes of exposure to FSH and cycloheximide (an inhibitor of protein synthesis) in superfused rat Sertoli cell cultures.

Materials and Methods

Collagenase (type II), DNAse (type I), hyaluronidase (S-I), steroid hormones (progesterone, hydrocortisone), BSA (fraction V), EDTA, N-pTosyl-l-lysine chloromethyl ketone, vitamin E, and cycloheximide were supplied by Sigma (St. Louis, MO). Culture media, Hanks Balanced Salt Solution (HBSS), Ca^{2+} and Mg^{2+}-free HBSS and fetal bovine serum were obtained from Flow Laboratories (Rockville, MD). Epidermal growth factor, insulin, and Matrigel were purchased from Collaborative Research, Inc. (Waltham, MA). Trypsin (1:250) and antibiotics were supplied by Gibco (Grand Island, NY), vitamin A (Aquasol A) by USV (Tuckahoe, NY), HEPES buffer by Research Organics, Inc. (Cleveland, OH), and [^{3}H]inulin (260 mCi/g) by ICN Radiochemicals (Irvine, CA). Rat transferrin and rabbit antirat transferrin antibody were provided by Cappel Laboratories (Cochranville, PA). FSH (NIH oFSH-S16) was a gift from the NIADDK National Hormone and Pituitary Program. Silastic^R sheeting (0.06-inch thick), silicone rubber (732 RTV), and silastic tubing (0.03-inch ID) used to construct the superfusion chambers were from Dow Corning (Midland, MI).

Isolation and Culture of Sertoli Cells

Sertoli cells were isolated from 18-day-old Sprague-Dawley rats (Harlen, Houston, TX) as previously described (Janecki and Steinberger, 1987a). Briefly, each detunicated testis was dispersed with a plastic syringe in Ca^{2+} and Mg^{2+}-free HBSS containing 0.5 mM EDTA and incubated (30 to 40 minutes, 34 C, with shaking) in HBSS (pH 7.4) containing 0.2% trypsin, 20 μg/ml DNAse and 20 mM HEPES. Trypsin action was stopped by 10% FBS in HBSS, the tissue fragments were washed with HBSS + 0.2% BSA, and then incubated for 30 to 50 minutes (34 C; with shaking) in HBSS (pH 7.4) containing 0.2% collagenase, 0.15% hyaluronidase, 20 mM N-pTosyl-L-lysine chloromethyl ketone, 15 μg/ml DNAse, 0.1% BSA, and 20 mM HEPES. The tubule fragments depleted of most myoid cells were then sedimented by gravity, washed in HBSS + 0.2% BSA, and dispersed mechanically using 5-ml Pasteur pipets with a 0.5-mm mouth opening. The resulting cell aggregates (3 to 10 cells) were spun at 50 × g for 4 minutes, suspended in DMEM-F12 medium (Dulbecco's Modified Eagle's Medium: Ham F-12 [1:1] supplemented with insulin [2 μg/ml], EGF [10 ng/ml], vitamins A and E [200 ng/ml] progesterone and hydrocortisone [10^{-8} M each], sodium bicarbonate [1.2 g/l], HEPES [15 mM], penicillin [200,000 U/l], streptomycin [0.2 g/l] and fungizone [2.5 mg/l] pH 7.3) and plated (2 to 3 × 10^6 cells/cm^2) on the bottom of superfusion culture chambers that had been covered with Matrigel (diluted 1:1 with DMEM-F12). The superfusion chambers (described in detail by Janecki et al, 1987) were modified for the current experiments as shown in Fig. 1. The top (A) and bottom (C) of the chamber were formed by 1 × 3-inch glass slides. The sides (gaskets B) were made from Silastic sheeting and sealed to

the bottom slide with silicone rubber. The inlets (D) and outlets (E) were formed by Silastic tubing sealed to the gaskets with silicone rubber. The top slide (A) was removed for cell plating and during stationary culture phase (19 hours at 34 C in humidified atmosphere of 97% air and 3% CO_2) preceding the superfusion.

Superfusion

Following the stationary culture phase, the top slide (A) was pressed against the gasket (B) and held firm by binder clips. The cultures were then superfused for 48 hours with DMEM-F12 medium at a flow rate of 2.5 ml/hour to equilibrate the system and to establish the basal rate of transferrin secretion. Subsequently, the cultures superfused at the same rate were stimulated with FSH, either continuously or intermittently, according to a chosen mode using a microprocessor-controlled programmable timer. The actual concentrations of FSH in the chambers were monitored by adding trace amounts of [^{125}I]FSH or [^{3}H]inulin to the same superfusion medium and counting the radioactivity in the effluent samples. In some experiments cycloheximide (25 μg/ml) was added either together with, 1 hour prior to, or 1 hour after the addition of FSH. This concentration inhibited the incorporation of [^{3}H]leucine into TCA-precipitable material by >95% (not shown). Unstimulated control cultures were handled in a similar manner except that FSH was omitted. The effluent media were collected at the indicated time intervals and stored at −20 C until assay. Details of the superfusion culture system have been described previously (Janecki et al, 1987).

Transferrin Radioimmunoassay. The transferrin concentration in the effluent media was measured by radioimmunoassay (RIA) according to the method described by Skinner and Griswold (1982). The minimum detectable dose was 13.7 ng/ml, and the intraassay and interassay coefficients of variation were 5.8% and 9.9%, respectively.

Calculations and Statistical Evaluation of Data. Each experiment was performed 2 or more times yielding generally similar results. However, since the secretory profiles of transferrin from replicate chambers varied slightly within and between experiments, it was not practical to pool the data. Therefore, results from representative experiments were expressed as the percent of mean basal values determined for each culture chamber prior to FSH exposure. To compare the effects of various treatments on the cumulative amounts of secreted transferrin, the results were expressed as percent of values from nonstimulated control cultures. The data were analyzed statistically using Student's two-tailed *t* test.

Results

Sertoli Cell Culture

The Sertoli cells attached rapidly to Matrigel-coated slides and formed a dense confluent monolayer during the 19-hour stationary culture phase. The number of residual germ cells decreased after 24 hours of superfusion to <5% of total cell population, and at the end of the experiment over 95% of Sertoli cells were viable, as evidenced by trypan blue exclusion. Superfused Sertoli cell monolayers on Matrigel-coated slides were morphologically similar to those cultured on Millipore filters in two-compartment chambers (Janecki et al, 1987).

Transferrin Secretion in Response to Different Modes of FSH stimulation

In basal culture (without FSH), the Sertoli cells maintained a relatively constant rate of transferrin secretion (Fig. 2A). Continuous exposure to FSH led to an initial sharp increase of transferrin secretion, ranging in different cultures from 130% to 200% of basal levels, which, despite the continuing presence of FSH, returned after 2 to 4 hours to near baseline values (Fig. 2B). After 6 to 10 hours of FSH exposure, a secondary prominent increase occurred and was maintained throughout the remaining period of the experiment. Sertoli cells exposed to FSH intermittently (20 minutes/2 hours) responded to each consecutive pulse of FSH with an immediate increase of transferrin secretion followed within 20 to 40 minutes by a return to basal values (Fig. 2C). As in continuously stimulated cultures, a secondary prolonged increase of transferrin secretion also occurred, causing the values between FSH pulses to increase progressively above baseline. The cumulative amounts of transferrin secreted by 10^7 cells during the 22-hour period in response to either continuous or pulsatile FSH stimulation did not differ significantly from each other, but were significantly higher than in control, nonstimulated cultures. (Fig. 2, inset).

The secretory patterns of transferrin in response to different modes of FSH stimulation are shown in Fig. 3. A biphasic response was consistently observed regardless of the duration of FSH exposure. Cultures exposed to FSH during the initial 0.5 to 8 hours (Fig. 3 A, B, C) showed a slight decline in transferrin secretion during the latter half of the 22-hour experiment. This decline was not observed in cultures exposed to FSH either continuously (Fig. 3D) or for 2 × 4 hours (Fig. 3E). Moreover, the total amount secreted during the entire 22-hour experimental period differed with the mode of FSH exposure. As shown in Fig. 4, cultures stimulated with FSH for 0.5 and 4 hours secreted significantly less transferrin than those stimulated for 8 hours, 2 × 4 hours, or 22 hours. During the initial 4 hours of the experiment, duration of FSH exposure correlated well ($r = 0.97$) with the amount of transferrin secreted (Fig. 5).

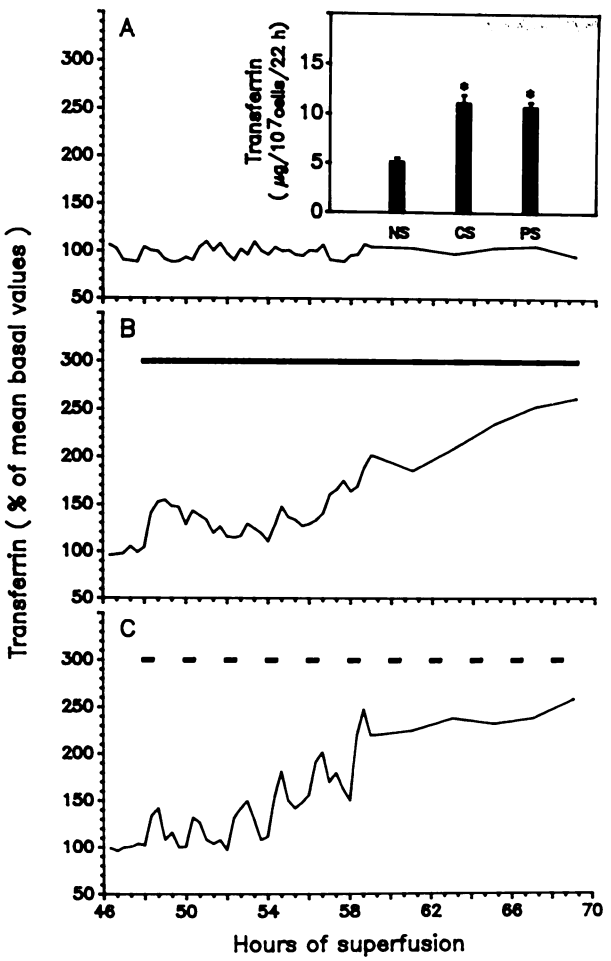

Fig. 2. Effect of continuous and pulsatile FSH stimulation on transferrin secretion by superfused Sertoli cells (representative experiment). After 48 hours of superfusion with FSH-free medium, Sertoli cell monolayers were superfused without or with FSH (0.1 μg/ml). **A.** Nonstimulated control. **B.** Continuous FSH stimulation. **C.** Pulsatile FSH stimulation (20 minutes/2 hours). The heavy horizontal lines in this and all subsequent figures indicate the time of FSH exposure. The effluent media were collected every 20 minutes for up to 59 hours, and then every 2 hours. Transferrin secretion in this and subsequent figures is expressed as percent of mean basal values determined as described in Materials and Methods. Inset: Total amount of transferrin secreted by 10^7 cells during the 22-hour experimental period (mean ± SD from three culture chambers). NS, nonstimulated control; CS, continuous stimulation; PS, pulsatile stimulation. *Significantly different from nonstimulated control ($P < 0.01$).

Influence of Cycloheximide on Basal and FSH-stimulated Transferrin Secretion

Addition of cycloheximide (25 μg/ml) to FSH-nonstimulated cultures caused an immediate decrease of basal transferrin secretion, which, after 4.5 hours,

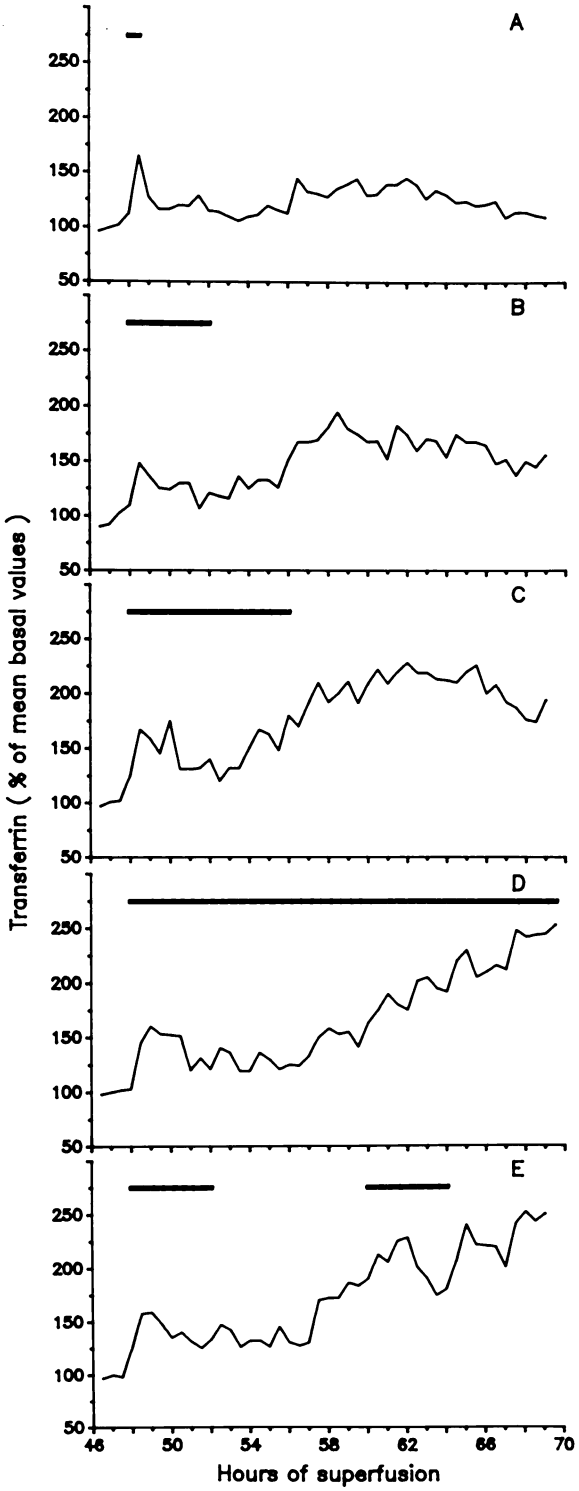

Fig. 3. Influence of varying modes of FSH stimulation on the profile of transferrin secretion. During the 22-hour period, Sertoli cell monolayers were exposed to FSH (0.2 μg/ml) for either 0.5 hour (A), 4.0 hours (B), 8.0 hours (C), 22 hours (D), or 2 × 4.0 hours (E). Effluent media were collected every 30 minutes.

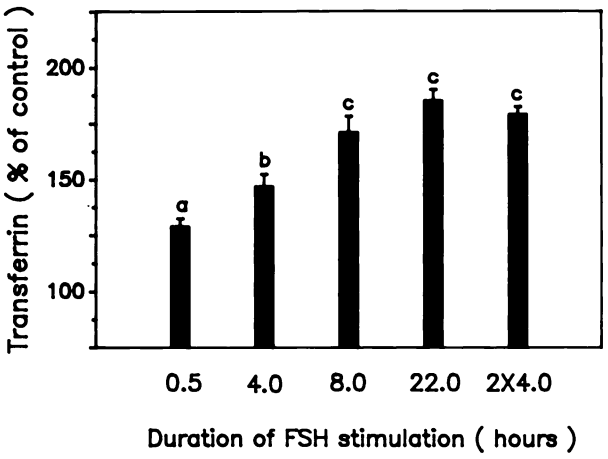

Fig. 4. Effect of varying modes of FSH stimulation on cumulative transferrin secretion during the 22-hour experimental period. The cultures were treated as described in the Fig. 3 legend. Data are mean ± SD from nine chambers in three separate experiments. [a]Different from nonstimulated control ($P < 0.01$); [b]Different from 0.5-hour stimulation ($P < 0.01$); [c]Different from 0.5-hour and 4-hour stimulation ($P < 0.01$).

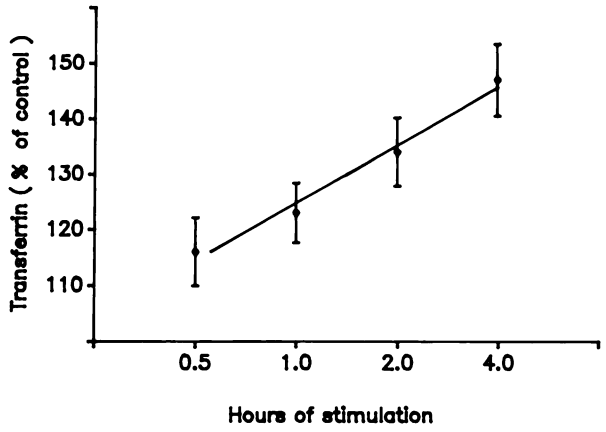

Fig. 5. Correlation between the duration of FSH exposure and the amount of transferrin secreted during the initial 4-hour experimental period. The data are mean ± SD from three separate experiments ($r = 0.97$).

was near the lower limit of detection (Fig. 6A). However, when cycloheximide was added either together with FSH, 1 hour before or 1 hour after the exposure to FSH, an immediate peak of transferrin secretion preceded its decline (Fig. 6B, C, D). Therefore, even in the presence of protein synthesis inhibitor, Sertoli cells responded to FSH with increased transferrin secretion. Delaying addition of cycloheximide relative to FSH exposure resulted in a greater amount of secreted transferrin during the 4.5 hours of FSH exposure. Transferrin levels began returning to baseline about 4 hours after cycloheximide withdrawal (Fig. 7).

Discussion

Using superfused Sertoli cell cultures, we investigated the pattern of transferrin secretion in response to different modes of FSH stimulation during a 22-hour period. The major findings were 1) FSH exerted a biphasic effect on transferrin secretion: an immediate rapid increase followed by a decline and then a delayed and more prolonged secondary response; 2) the cumulative amounts of transferrin secreted during the entire 22-hour experimental period in response to pulsatile and continuous FSH exposure were similar, although the amount secreted during the early response (4 hours) was directly related to the duration of a single FSH exposure ranging from 0.5 to 4.0 hours; and 3) both FSH-stimulated and nonstimulated Sertoli cells appear to secrete mainly

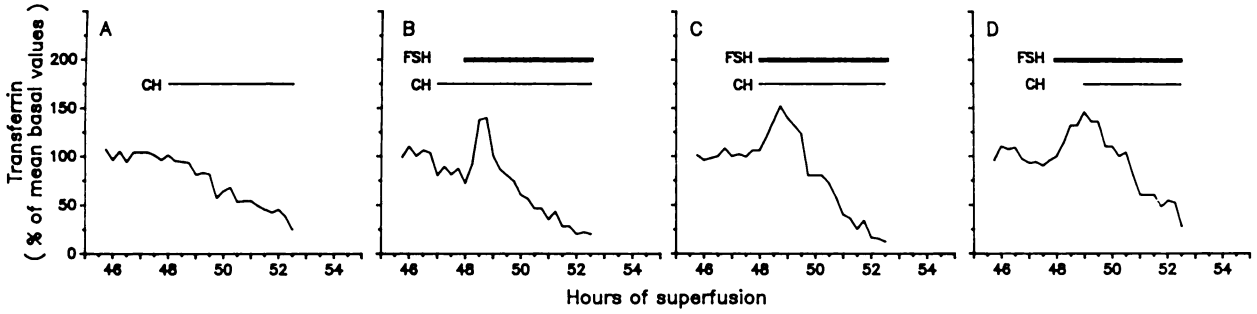

Fig. 6. Influence of cycloheximide (CH) on transferrin secretion in nonstimulated (A) and FSH-stimulated (B, C, D) cultures. After an initial 48 hours of superfusion with medium alone, cycloheximide (25 μg/ml) was added to the cultures either together (C), 1 hour before (B), or 1 hour after (D) exposure to FSH (0.2 μg/ml). The effluent media were collected every 15 minutes.

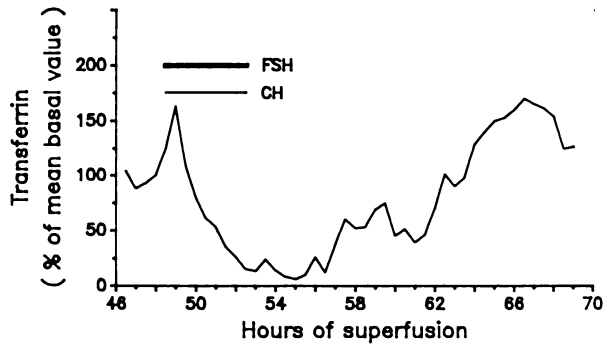

Fig. 7. Recovery of transferrin secretion after inhibition of protein synthesis by cycloheximide. Sertoli cell monolayers were exposed for 4 hours simultaneously to cycloheximide (25 μg/ml) and FSH (0.2 μg/ml) and then superfused with medium alone. The effluent media were collected every 30 minutes.

newly synthesized transferrin that was blocked by the addition of cycloheximide; however, some intracellular storage of transferrin may also exist that can be released in response to FSH independently of new protein synthesis.

The dynamics and molecular mechanisms of transferrin or other Sertoli cell secretions are poorly understood (Tindall et al, 1985). Huggenvik et al (1987) reported an increase of transferrin mRNA in static cultures of rat Sertoli cells 2 hours after treatment with a combination of FSH, testosterone, insulin, and retinol. The increase continued for the duration of the experiment (approximately 50 hours). Possibly, the secondary increase of transferrin secretion observed in FSH-treated cultures in our studies may have been due to increased transferrin mRNA. However, since only the combined effect of these factors has been tested by the above authors, comparison with our results is difficult.

The significance of different modes of hormonal stimulation on secretory responses has been reported for various cell types, including pituitary, ovarian, and testicular cells (Smith and Vale, 1981; Peluso et al, 1984; Verhoeven et al, 1986). Our results showed that pulsatile and continuous FSH stimulation during a prolonged (22-hour) period results in similar total amounts of transferrin, suggesting that pulsatile FSH delivery may not be critical in long-term regulation of testicular transferrin. However, the importance of pulsatile FSH stimulation in short-term Sertoli cell responses cannot be excluded, particularly since episodic circulating FSH levels have been reported for males of several mammalian species (review by Desjardins, 1981).

A relatively brief FSH exposure results in maximal transferrin secretion, since cultures stimulated for either 8 or 22 hours secreted similar cumulative amounts of transferrin during the 22-hour period. It has been reported that a 4-hour stimulation by FSH during a 24-hour period elicits maximal Sertoli cell response (Verhoeven et al, 1980). In our experiments, however, FSH stimulation lasting under 8 hours yielded less total transferrin during the 22-hour period. Also, the rate of transferrin secretion in cultures exposed to FSH for 8 hours or less declined during the later part of the experimental period. Interestingly, this delayed decrease did not occur with 2 × 4 hours of FSH stimulation (Fig. 3E).

Several groups of investigators employing static cultures reported desensitization of Sertoli cell responsiveness, as shown by decreased FSH binding, adenylyl cyclase activity, and cAMP levels after exposure to FSH (Verhoeven et al, 1980; Francis et al, 1981; Le Gac et al, 1985). Interestingly, in the study by Francis et al, aromatization of testosterone following FSH exposure actually increased when cAMP levels were declining. In our study, we cannot exclude some degree of Sertoli cell desensitization, although the rate of transferrin secretion was increased even when FSH was present continuously for 22 hours. Experiments correlating transferrin secretion with cAMP levels in response to different FSH treatments should provide additional information concerning the dynamics of Sertoli cell responses to FSH.

It is not clear at present how secretory proteins are processed within the Sertoli cells, namely, whether they are secreted immediately after being synthesized (constitutive secretion) or are stored and then released in response to stimulation (regulated secretion). Lack of morphologic evidence for the presence of secretory vesicles in Sertoli cells (Fawcett, 1975) suggests absence of stored secretory proteins. On the other hand, a storage pool for ABP in Sertoli cells has been suggested (Kierszenbaum et al, 1980; review by Tindall et al, 1985). Data from our experiments indicate that Sertoli cells may possess both mechanisms of transferrin secretion. The decrease in the rate of transferrin secretion after inhibition of protein synthesis suggests that a substantial amount of transferrin is secreted immediately after being synthesized. However, the rapid increase of transferrin response to FSH when protein synthesis was blocked by the addition of cycloheximide suggests some storage of transferrin available for immediate release upon FSH stimulation. A similar distribution between the two secretory pathways has been report-

ed for the parathyroid gland (Chu et al, 1983). Whether the secretion of transferrin (and possibly other Sertoli cell proteins) involves both theoretically possible secretory pathways (Kelly, 1985) remains to be determined. It should be pointed out also that other explanations of our data, e.g., a more rapid recycling of the transferrin receptors in response to FSH, are possible.

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