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Luteinizing Hormone Secretion by Male Rat Pituitary Cells Perifused *In Vitro*: Effect of Experimental Left Varicocele and Orchiectomy

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It has been shown previously that experimental left varicocele in the rat, results in a bilateral decrease in intratesticular testosterone. In the present work, pituitary responsiveness to GnRH as a possible mediator of this effect has been examined. Unilateral varicoceles were created in adult rats. A second group of animals underwent a sham operation and a third underwent bilateral orchiectomy. Thirty days after surgery, rats from all three groups were sacrificed and their pituitaries were removed. Dispersed pituitary cells were perifused in Bio-Gel columns with varying concentrations of GnRH. The concentration of LH in the collected eluent was determined by radioimmunoassay. The mean, overall GnRH-stimulated LH immunoreactive secretion rate (ng/min/10^7 cells) by pituitary cells from rats with varicocele (0.062 ± 0.11) was no different from the overall release from the sham-operated controls (0.051 ± 0.007). The dose-response curves for GnRH-stimulated release of LH by dispersed pituitary cells in the two groups also were not different. The overall GnRH-stimulated LH release by cells from the orchiectomized rats (0.171 ± 0.032) was significantly greater than release by cells from the sham-operated and varicocele rats, and the concentration-response curve from the orchiectomy group was significantly elevated over those of the other two groups. These results indicate that GnRH-stimulated immunoreactive LH release is not altered in rats with experimental left varicocele and, thus, is not the source of an endocrinopathy that leads to decreased intratesticular testosterone concentrations in these animals.

Key Indexing Terms: Varicocele; Pituitary Cells; Perifusion; LH.

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The clinical relationship of varicocele to infertility remains controversial. The existence of an association between the two is supported by the finding of a varicocele in approximately 30% of men evaluated in infertility clinics, whereas the incidence in the general population is only around 15% (Saypol, 1981), and by the fact that approximately two-thirds of patients show improvement in semen quality after varicocelectomy (see Pryor and Howards, 1987 for

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review). Nevertheless, the accepted incidence of infertility in males in the general public is only 5% (Lipshultz and Howards, 1983), a figure lower than the proportion of men with varicocele; thus it is clear that only a subpopulation of patients with varicocele are actually infertile.

The pathophysiology of varicocele is also incompletely understood. This makes it difficult to determine the mechanism by which a varicocele can induce infertility in some males but not in others, or whether varicocele is simply an attendant finding in some males with infertility who are infertile for other reasons. Since precise, invasive studies of biologic mechanisms cannot be performed in humans, our laboratory has used the experimental varicocele model in rats to study this lesion further.

Saypol et al (1981) demonstrated a bilateral elevation of intratesticular temperature and testicular blood flow after the establishment of left unilateral varicocele in dogs and rats. Repair of the experimental varicocele by high ligation of the dilated left spermatic vein reversed the bilateral physiologic changes in blood flow and temperature (Green et al, 1984; Hurt et al, 1986) and sperm count and motility (Hurt et al, 1986) to normal. Further work with this rat model has demonstrated that the physiologic effects of elevated blood flow and increased intratesticular temperature are not dependent on a primary injury to the left testis (Hurt et al, 1987) and that the varicocele does not induce hypoxia in the testicular circulation (Turner et al, 1988). It has recently been shown, however, that a bilateral decrease in intratesticular testosterone (T) occurs in the rat after the establishment of unilateral left varicocele (Rajfer et al, 1987). The etiology of this decreased intratesticular T is not clear, but two general possibilities are (1) an alteration in pituitary signaling to the Leydig cells through LH secretion, or (2) alteration of Leydig cell responsiveness to LH secreted normally by the pituitary. Both of these possibilities must be examined to eliminate or accept either possibility by itself. The present study was undertaken to determine if rat pituitary responsiveness to GnRH is altered by experimental varicocele.

Materials and Methods

Animals

Mature, male, Sprague-Dawley rats (400–700 g) were obtained from institutional vivarium sources. The rats were acclimated to a 12 h dark:12 h light photoperiod for 1 wk prior to initial surgery and maintained on that photoperiod thereafter. The animals were fed lab chow and water *ad libitum*.

Surgical Treatments

Rats were randomly assigned to one of three surgical groups, 16 rats per group.

Varicocele: rats were anesthetized with inspired Halothane and unilateral left varicoceles were induced as previously described (Saypol et al, 1981; Green et al, 1984; Hurt et al, 1986; Turner et al, 1987).

Sham operation: After induction of Halothane anesthesia, the animals were exposed to the same surgery and passage of ligatures around the left renal vein as occurred in the varicocele group. In this case, however, the sutures and 0.85-mm guide probe were removed without any ligation having occurred and the incisions were closed.

Bilateral Orchiectomy: After induction of Halothane anesthesia, animals were bilaterally orchiectomized through vertical incisions over the left and right hemiscrotum. Incisions were closed in two layers using 4-0 chromic on the tunica dartos and 4-0 silk on the skin.

LH Secretion by Dispersed Pituitary Cells In Vitro

Thirty days after the surgical treatments described above, the animals were weighed, anesthetized with ether, and rapidly decapitated. Trunk blood was collected and allowed to clot at 4 C for 4 h, after which the serum was collected by centrifugation and stored at − 20 C until measurement of LH by radioimmunoassay (RIA). The animals that had been treated to induce a unilateral left varicocele were laparotomized and checked for the presence of the left spermatic varicosity. Each testis was collected and weighed. The anterior pituitary was placed in Earle's balanced salt solution (EBSS, Gibco, Grand Island, NY) and diced into approximately 0.5-mm fragments. These were incubated for 20 min in 10 ml EBSS with 0.2% (w/v) Trypsin and 0.15 mg DNAase (dioxyribonuclease I, Sigma Chemical Co., St. Louis, MO) at 37 C. The fragments were then washed with calcium- and magnesium-free EBSS and the cells were dispersed by gentle trituration with a 1.0-ml pipette (Pipetman, Rainin Instrument Co., Inc., Woburn, MA). Stranded DNA was removed and an aliquot of cells was taken for determination of the number of viable cells by the Trypan blue exclusion test using a standard hemocytometer. Cells were gently mixed with Bio-Gel P2 (200–400 mesh Bio-Rad Laboratories, Richmond, CA) that had been preswollen overnight. The pituitary cell-Bio-Gel mixture was packed into 2.0 ml plastic syringes (Sabre International Products, Ltd., Reading, Berkshire, England) that served as the perifusion chambers. Cells were maintained at 37 C by submersion of the chambers in a water bath and perifusion was performed with medium 199 (M199, Gibco, containing 0.25% BSA, 10 μg/ml penicillin, 2.5 μg/ml streptomycin, 187.5 ng/ml amphotericin B, and 5 μg/ml gentamicin) at a mean flow rate of approximately 0.43 ml/min.

Cells were allowed to equilibrate to perifusion conditions for 4 h, at which time GnRH was administered

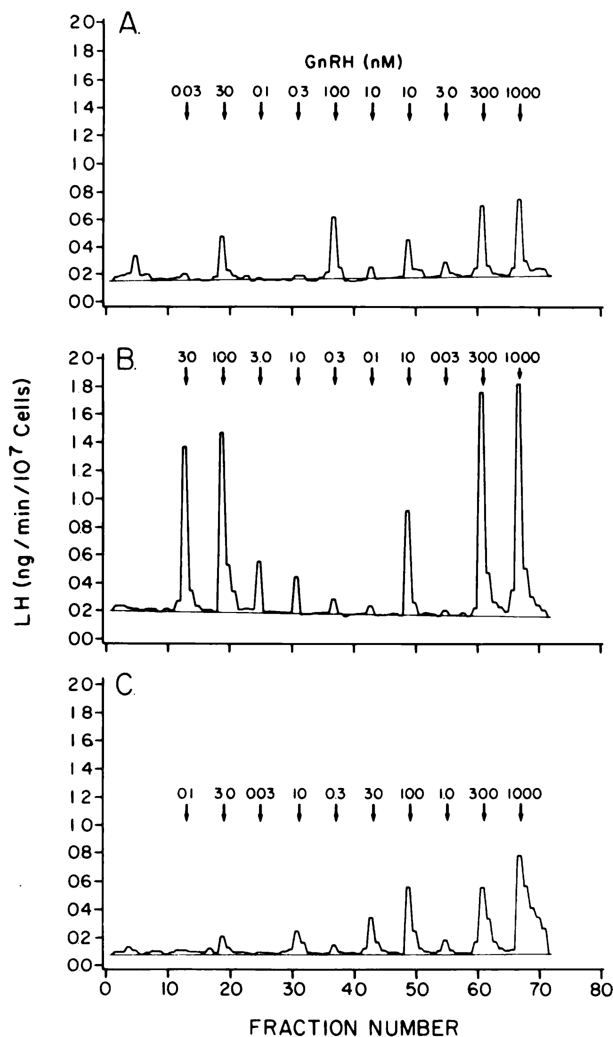

Fig. 1. Examples of LH secretion data from individual columns containing dispersed rat pituitary cells that were equilibrated for 4 h prior to perifusion *in vitro* with 10 random GnRH pulses over a 3-h time period. Each column contained dispersed pituitary cells from four male rat pituitaries suspended in 2.0 ml Bio-Gel, maintained at 37 C, and subjected to a 0.4-ml/min perifusion rate. The data shown are representative of that obtained from dispersed pituitary cells from: A-sham operated animals; B—bilaterally orchiectomized animals; and C—animals with experimental, left varicocele.

as 2.5-min pulses at 30-min intervals. The concentrations were 0.03, 0.1, 0.3, 1.0, 3.0, 10, 100, 300, and 1,000 nM. Except for the 300 and 1,000 nM concentrations, which was administered last to avoid depleting the cells of releasable LH, these pulses were applied in random order in four repetitions of three parallel columns. Individual columns contained pituitary cells (four pituitaries/column) from sham, varicocele, or orchiectomy animals. Eluents were collected as 5-min fractions and stored at − 20 C until measurement of LH by RIA.

Radioimmunoassay

Levels of LH in serum and eluent fractions were measured by RIA using reagents kindly supplied by Dr. A.F. Parlow and the National Pituitary Hormone Program of NIADDK. Anti-rat LH serum (S-7) and rLH reference preparation (RP-2) were used. The assay standards were assayed in triplicate and all samples were run on a single assay. The intraassay coefficient of variation was 5.3% at 0.2 ng/tube.

Data and Statistical Analysis

Expression of the LH data from the perifusion experiments was dependant on the numbers of pituitary cells and the flow rate of each column. The RIA-determined LH concentration (ng/ml) was converted to a secretory rate for each fraction (ng/min/10^7 cells) by dividing it by the number of cells (in millions) and multiplying by 10 X the flow rate (ml/min). The overall secretory response to GnRH was expressed as the sum of the secretory rates above baseline during the 30-min period immediately following the administration of the appropriate GnRH concentration. This was accomplished by calculating the slope of the baseline from the nadir between GnRH-stimulated surges and the first pre-infusion and the last post-infusion LH value. The baseline value for each fraction was calculated using the sample number and the slope. The amount of LH secreted above baseline was calculated by subtracting the individual baseline value from the total value of LH for that fraction. The resulting values were summed over the 30-min period following the initiation of the GnRH pulse, and the sum was divided by the number of fractions (six 5-min fractions/30-min period) to produce an overall value for the response expressed as secretory rate in ng/min/10^7 cells. The sequence of GnRH concentrations were then arranged in ascending order, and the means ± SE of these data were calculated for each experiment.

Concentration-response curves were calculated for each group using least-square regression analysis for the log of the concentration expressed in nM. Differences among concentration-response relationships determined for the experimental groups were assessed using a general linear model with the least-square means obtained from the model being compared with the corresponding t-statistics. Differences among the overall secretory means were determined using analysis of variance followed by the Duncan's Multiple Range test.

Results

Body and Testis Weights

No differences were observed in the mean body weights of the sham (551 ± 14 g), varicocele (534 ± 13 g), or orchiectomy (560 ± 13 g) animals. Likewise, there were no differences in the mean testis weights at 30 days after surgery in the sham (1.81 ± 0.03 g) or varicocele (1.79 ± 0.03 g) rats.

Serum LH Concentrations

Serum LH concentrations were not significantly different in the sham (0.49 ± 0.057 ng/ml) or varicocele (0.561 ± 0.078 ng/ml) animals. Serum LH concentrations in the orchiectomy animals (8.724 ± 0.803 ng/ml) were significantly elevated ($p < 0.0001$) relative to the other two groups.

GnRH-Stimulated LH Release

Dispersed pituitary cells in all individual columns responded to random GnRH pulses in a manner consistent with the concentration of the GnRH pulse (Fig. 1). Regression analysis of data from all columns revealed that GnRH-stimulated LH release (ng/min/10^7 cells) occurred in a concentration-dependent manner in tissues obtained from sham ($r^2 = 0.78$; $p = 0.0001$), varicocele ($r^2 = 0.61$; $p = 0.0001$), and orchiectomy ($r^2 = 0.7$; $p = 0.0001$) rats (Fig. 2). The concentration-response relationship generated in cells from sham rats was no different ($p = 0.419$) from that obtained in cells from varicocele rats. However, the concentration-response curves calculated for both the sham and varicocele pituitary cells were significantly different ($p = 0.0001$) from that of the orchiectomy rats. Overall, GnRH-stimulated LH release by cells from varicocele rats (0.062 ± 0.011 ng/min/10^7 cells) was not different ($p = 0.712$) from overall release by cells from sham rats (0.051 ± 0.007 ng/min/10^7 cells). In contrast, overall GnRH-stimulated LH release by cells from orchiectomy rats (0.171 ± 0.032 ng/min/10^7 cells) was greater than release by cells from the other two groups (sham: $p = 0.0023$ and varicocele: $p = 0.0040$).

Discussion

The present experiment was stimulated by our earlier report that male rats with unilateral, experimental varicocele exhibited a bilateral decrease in intratesticular T concentrations without evidence of alterations in peripheral androgen concentrations (Rajfer et al, 1987). Intratesticular activities of two steroidogenic enzymes, 17,20-lyase and 17-hydroxlyase, were also significantly reduced bilaterally by unilateral experimental varicocele. Other detected changes in testicular physiology due to experimental varicocele, eg blood flow and temperature (Saypol et al, 1981; Hurt et al, 1986), are specific to the testis. While it is possible that changes in steroidogenic function can be due exclusively to direct effects of varicocele on the testis, this is not necessarily the case. For example, Ando et al (1984, 1985) also found evidence for decreased T, 17,20-lyase and 17-hydroxlase in human males with varicocele. In addition they reported that pituitaries of men with varicocele exhibited an increased responsiveness to a single dose of GnRH (Ando et al, 1985). These results indicated an alteration in pituitary function in these men with varicocele and decreased T synthesis.

Whether or not human males with varicocele actually experience alterations in T synthesis is controversial (see Hudson, 1988 for review). Ando et al (1984, 1985), for example, found that men with varicocele had decreased serum T concentrations whether or not their sperm counts were normal, whereas Hudson et al (1985, 1986) found no decrease in serum T concentrations in similarly categorized varicocele patients. Resolution of the question of the effects of varicocele on T synthesis in the human

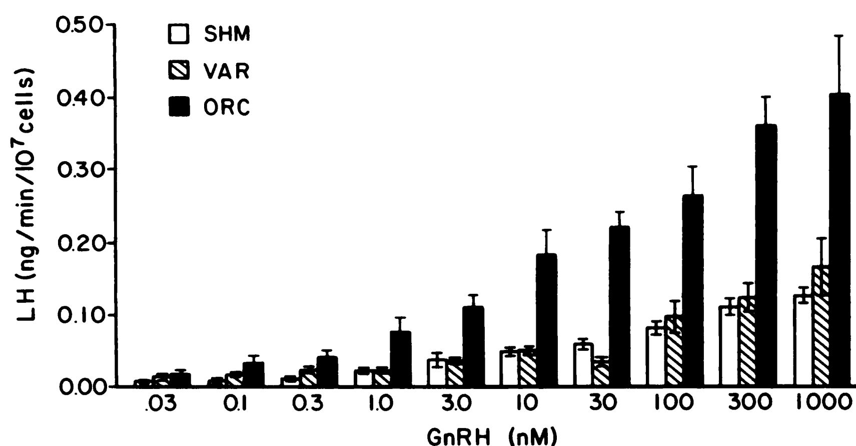

Fig. 2. Mean ± SE. LH responses of perifused pituitary cells to GnRH from sham (open bars), varicocele (hatched bars), and bilaterally orchiectomized rats (closed bars). Data are expressed as the mean secretory rate over the entire 30-min period after administration of the appropriate concentration of GnRH.

testis has been difficult since only sera or seminal plasma are generally available for study in humans. It has not been possible to examine human intratesticular T concentrations as it has been in laboratory species.

Interestingly, Hudson et al (1985, 1986) found that a subset of men with varicocele, irrespective of their seminal plasma T concentrations, also exhibited an increased pituitary responsiveness to GnRH; therefore, despite some differences in the specifics of their data, Ando et al (1984, 1985) and Hudson et al (1985, 1986) both postulated the presence of an endocrinopathy in at least some men with varicocele. Our finding of reduced intratesticular T in the experimental varicocele model (Rajfer et al, 1987) was consistent with this hypothesis; we therefore wanted to determine whether or not pituitary sensitivity and responsitivity to GnRH was altered by the presence of experimental varicocele.

Our decision to use the method of continuous perifusion of dispersed anterior pituitary cells to address this issue was based primarily on two factors. First, we (Borges et al, 1982) and others (Smith and Vale, 1981; Loughlin et al, 1984) have previously demonstrated concentration-response relationships between GnRH and LH release by perifused cells. Secondly, and of considerable importance, the response of acutely dispersed cells to GnRH in a perifusion system has been shown to reflect the gonadal hormone environment of the donor animal at the time of sacrifice, whereas our studies of monolayer preparations of pituitary cells have failed to demonstrate these effects (Evans et al, 1983). Thus, we reasoned that acutely dispersed cells in a perifusion system would offer the best opportunity to document the effects of varicocele on gonadotrophic function.

Our results demonstrating a concentration-response relationship between GnRH and immunoreactive LH secreted by pituitary cells from male rats are consistent with previous studies (Borges et al, 1982; Loughlin et al, 1984). In addition, our observations of enhanced GnRH-stimulated LH release by cells from rats after bilateral orchiectomy support those of others using a rat pituitary organ culture technique (Badger et al, 1978) and unpublished findings in our own laboratory using the present technique.

Under the same experimental conditions in which cells from control and orchiectomized male rats responded appropriately to GnRH, the response characteristics of pituitary cells from rats with experimental varicocele were indistinguishable from those exhibited by cells from the control animals. This suggest that gonadotrophic function is probably not affected in rats bearing experimental varicoceles.

The differences in results in the rat and those described by Ando et al (1984, 1985) and Hudson et al (1985, 1986) in men may relate at least in part to experimental design. Clearly, the definition of full concentration-response relationships is a much more powerful approach than use of a single dose GnRH challenge, as has been employed in the human studies. Without question, single dose/concentration administration of GnRH carries with it the possibility that an arbitrary difference in LH release will be detected (eg, the response to the 30-nM concentration shown in Fig. 2). Thus, our data in rats with experimental varicoceles point out the need for detailed studies in the human, ideally with monitoring of both endogenously driven LH pulses and LH release in response to a full range of exogenously administered GnRH doses.

Finally, it is acknowledged that the current study examined the response of pituitary cells in secreting immunoreactive LH, leaving open the possibility that experimental varicoceles may have induced an undetected change in the secretion of bioactive LH, or a change in the pulsatile pattern in which LH is secreted *in vivo*. These latter possibilities remain to be tested, as does the hypothesis that Leydig cell responsiveness to LH, rather than anterior pituitary gland responsiveness to GnRH, is altered by the varicocele condition.

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