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Differential Actions of Gonadotropin-Releasing Hormone and Human Chorionic Gonadotropin on Interstitial Fluid Volume and Immunoglobulin G Concentrations in Adult Rat Testis

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ABSTRACT: Gonadotropin-releasing hormone (GnRH) agonists regulate testicular interstitial fluid (tIF) volume, most probably via specific receptors on Leydig cells. The aim of this study was to confirm the interaction between GnRH and Leydig cells in regulation of testicular fluid, and to examine the effects on serum proteins in testis. Unilateral intratesticular injection of a GnRH agonist (100 ng/testis) caused a 50% reduction in tIF volume within 2 hours. Destruction of Leydig cells by treatment with ethane dimethane sulfonate also caused a similar decline in tIF volume; however, GnRH agonist treatment had no additional influence on this response in Leydig cell-depleted testes. GnRH agonist treatment had no effect on serum protein permeability in testis as indicated by maintenance of the tIF/serum immunoglobulin G (IgG) concentration gradient. Injection of human chorionic gonadotropin (hCG, 100 IU) had no effect on tIF volume at 2 hours, but increased the permeability of the testicular vasculature to serum IgG. At 20 hours after hCG injection, tIF volume was increased twofold, while the testicular permeability barrier to IgG appeared to have been restored. These data indicate that the acute inhibitory action of GnRH on vascular fluid permeability is dependent upon Leydig cells, confirming that these cells are the primary site of GnRH action on testicular vasculature. The data also indicate that supraphysiological doses of hCG cause a rapid increase in testicular permeability to serum proteins, which occurs prior to the well-characterized stimulation of tIF volume. These data provide further evidence that the concentration of serum proteins in tIF and the volume of tIF are both under regulatory control involving Leydig cells, but are independently regulated.

Key words: Vasculature, permeability, inflammation, testosterone, regulation.

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Local regulation of the formation and content of testicular interstitial fluid (tIF) is of considerable importance to normal testis function (Bergh and Damber, 1993; Desjardins, 1993). Numerous studies have established that tIF formation is maintained by androgen-dependent regulation of testicular blood pressure and flow characteristics, mediated via the seminiferous epithelium (Damber et al, 1987a; Maddocks and Sharpe, 1989; Collin et al, 1993). Accordingly, ablation of Leydig cells in adult rats by treatment with ethane dimethane sulfonate (EDS) causes a decrease in tIF volume that can be prevented by testosterone replacement, whereas direct disruption of the germ cells by various means results in a progressive increase in tIF volume (Sharpe, 1983; Sharpe et al, 1991).

Hyperstimulation of Leydig cell with luteinizing hormone (LH) or human chorionic gonadotropin (hCG) in adult rats causes a transient decrease in testicular blood flow, which is immediately followed by increased testicular blood flow and pressure, and opening of the vascular endothelial cell junctions, leading to an increase in tIF volume 16–24 hours after treatment (Sharpe, 1979; Setchell and Sharpe, 1981; Widmark et al, 1986a; van Vliet et al, 1988). Treatment with EDS eliminates these responses, confirming the Leydig cell as the site of action, although androgens are not involved (Setchell and Rommerts, 1985; Veijola and Rajaniemi, 1985; Sowerbutts et al, 1986). In fact, later vascular changes in response to hCG treatment are associated with an increase in polymorphonuclear cells within the testis (Bergh et al, 1986; Widmark et al, 1987), suggesting that this is primarily an inflammatory response, most likely mediated by the proinflammatory cytokine, interleukin-1, secreted by Leydig cells (Veijola and Rajaniemi, 1986; Bergh and Söder, 1990; Lin et al, 1993; Bergh et al, 1996; Collin and Bergh, 1996).

In contrast to testicular blood flow and tIF formation, relatively little is known about the regulation of the uptake of serum proteins by the testis. In a previous study (Hedger and Hettiarachchi, 1994), we observed that destruction of the seminiferous epithelium by experimental cryptorchidism increased tIF volume without affecting the concentration gradient of immunoglobulin G (IgG) be-

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tween serum and tIF, which is an indicator of changes in vascular protein permeability (Garlick and Renkin, 1970; Bell et al, 1983). This observation was consistent with separate regulation of tIF volume and serum protein permeability in the testis, which is mediated by specific transendothelial transport (Banks and Kastin, 1992; Holash et al, 1993; Turner and Rhoades, 1995). However, there is evidence that permeability to proteins in the testis is under hormonal control. Testicular permeability to the serum proteins, IgG and albumin, in developing rats increases substantially during the pubertal period (Setchell et al, 1988; Pöllänen and Setchell, 1989; Pöllänen et al, 1995), and suppression of gonadotropins by subcutaneous implants reduced permeability to IgG in normal adult rats (Pöllänen et al, 1995). Because depletion of germ cells by cryptorchidism had no effect on testicular IgG concentrations (Hedger and Hetiarachchi, 1994), a direct role for Leydig cells is implicated. However, the use of the EDS model to study the role of Leydig cells in regulating vascular permeability to serum proteins was obscured by the nonspecific effect of the carrier solvent, dimethyl sulfoxide (DMSO), on vascular permeability throughout the body (Balin et al, 1987; Hedger and Hettiarachchi, 1994). The use of local agents that act specifically upon Leydig cells may provide an alternative approach to this question.

Locally produced gonadotropin-releasing hormone (GnRH), or a GnRH-related peptide, has been implicated in local regulation of steroidogenesis by Leydig cells, and in modulation of testicular vasculature (Sharpe et al, 1981; Sharpe et al, 1983a; Damber et al, 1984; Hedger et al, 1985b; Widmark et al, 1986b; Damber et al, 1987b). Local administration of low doses of a potent GnRH agonist causes a transient reduction in rat tIF volume (Sharpe et al, 1983a,b) and an increase in testicular vascular resistance (Widmark et al, 1986b). However, intratesticular injection of higher doses of GnRH agonist, which elicit a concomitant release of endogenous LH, causes an increase in tIF volume and a decrease in vascular resistance at later time points, similar to the effect of exogenous LH/hCG (Sharpe, 1979; Sharpe et al, 1983a,b; Widmark et al, 1986a,b). An inflammation-like response also has been observed in hypophysectomized rats treated with GnRH agonist (Damber et al, 1984, 1987b).

These data indicate that GnRH exerts both an acute inhibitory effect on testicular blood-flow and interstitial fluid formation, and a longer-term inflammation-like effect similar to that induced by LH/hCG. The presence of GnRH receptors on Leydig cells and the effects of GnRH agonists on testicular steroidogenesis have led to the assumption that this cell type is the primary site of the vascular action of GnRH in the testis (Bourne et al, 1980). However, it is possible that other testicular cells also possess these receptors (Hedger et al, 1985a), and a direct effect of GnRH on the capillary endothelial cells, or mediation via other testicular cells, cannot yet be excluded. The following experiments were designed to investigate the direct physiological interaction between GnRH and Leydig cells in regulation of both tIF volume and protein transport.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (80–100 days old) were obtained from the Monash Central Animal House and housed at Monash Medical Centre for the duration of the experimental procedure under conditions of controlled day length (12 hours light: 12 hours dark) with unlimited access to food and water. Experimental procedures were approved by the Monash University Standing Committee on Ethics in Animal Experimentation and conformed to the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation/Australian Agricultural Council Code of Practice for the Care and Use of Animals for Experimental Purposes.

Experimental Details

Experiment 1: Effect of Intratesticular GnRH Injection on tIF Volume and Serum and IF [IgG]—Animals were randomly assigned (n = 5 rats/group) to 1 of 3 experimental groups and received 1) no treatment (control group), 2) a single injection (IP) of 25% DMSO (Sigma Chemical Co, St Louis, Mo) in water (200 μL/100 g body weight), or 3) a single injection (IP) of EDS (supplied by Dr Michael Fuller, Department of Chemistry, Monash University) to a final dose of 7.5 mg/100 g body weight dissolved in DMSO:water. Seven days later, 1 testis received an intertubular injection (20 μL, subcapsular) of 0.9% saline containing 1 mg/mL gelatin (Sigma) and 5 mg/mL bovine serum albumin/protein (Sigma; saline-carrier solution). The contralateral testis received a similar injection of the potent GnRH agonist, D-Ala^6, des-GlyNH_2^{10} GnRH-ethylamide (100 ng/20 μL; Sigma) in the carrier solution. After 2 hours, animals were ether-anesthetized and a sample of blood (1–2 mL) was collected by cardiac puncture. The blood was allowed to clot (4 hours, 20°C) and the serum fraction collected. The testes were removed, and tIF was collected from a 2-mm incision in the lower pole of the testis, as previously described (Sharpe, 1979). All samples were stored at −20°C until assay.

Experiment 2: Effect of Low Dose of GnRH—Rats (n = 5 rats/group) were given an intratesticular injection of 1 ng/testis of GnRH agonist and tIF volume was measured 2 hours later, as in experiment 1.

Experiment 3: Effect of hCG Injection—Animals (n = 5 rats/group) were injected (100 μL, sc) with 0.9% saline alone (control), or with saline containing 100 IU hCG (Pregnyl, Organon, Cambridge, United Kingdom). Two hours later, the controls and half the hCG-injected rats were killed, and 20 hours later the remaining hCG-injected rats were killed. Serum and tIF were collected, as in experiment 1.

Measurement of IgG

Total IgG levels in tIF and serum were measured by enzyme-linked immunosorbent assay, as previously described (Hedger and Hettiarachchi 1994).

Measurement of Testosterone

Testicular IF was assayed without extraction for testosterone content by ^{125}I-labeled testosterone radioimmunoassay (RIA; Kerr et al, 1985). Serum testosterone levels were measured by ^{3}H-testosterone RIA after hexane-chloroform (4:1) extraction (Risbridger et al, 1981).

Statistics

Comparisons between the saline-injected and GnRH agonist-injected testes were performed by paired *t* test. Comparisons between different treatment groups or time points were made by 1-way or 2-way ANOVA as appropriate, in conjunction with Student-Newman-Keuls multiple range test. All statistical analyses were performed using Sigmastat version 1.0 Software (Jandel Corp, San Rafael, Calif).

Results

Role of Leydig Cells in Mediating the Effect of a GnRH Agonist on tIF Volume and Permeability to IgG

There was a 50% decline in tIF volume in testes of EDS-treated Leydig cell-deficient rats compared with untreated normal or DMSO-treated controls (Figure 1A). Unilateral testicular injection of GnRH agonist (100 ng/testis) reduced tIF volume by 35% to 40% compared with the contralateral saline-injected testis 2 hours later in both untreated normal and DMSO-treated controls, but it had no additional effect on tIF volume in the EDS-treated rats.

The IF/serum [IgG] ratio increased in both the DMSO-treated and EDS-treated rats, compared with untreated normal rats, but GnRH agonist treatment had no effect on the IF/serum [IgG] ratio in any experimental group (Figure 1B). As observed previously (Hedger and Hettiarachchi, 1994), serum [IgG] levels were significantly reduced ($P < .05$) in the serum of rats treated with DMSO alone (2.08 ± 0.16 mg/mL; mean ± SEM; n = 5) compared with untreated controls (3.52 ± 0.52 mg/mL) or EDS-treated rats (3.20 ± 0.19 mg/mL). These extratesticular effects of DMSO and EDS on IgG levels did not affect the results of the study.

There was no significant difference in tIF testosterone concentrations between saline-injected and GnRH agonist-injected testes in any experimental group; however, tIF testosterone levels were significantly elevated in the GnRH agonist-injected normal (1654 ± 301 ng/mL; mean ± SEM; n = 5) and DMSO-treated control rats (1840 ± 180 ng/mL), compared with samples collected from rats that had not received an intratesticular injection of GnRH agonist (400 ± 101 ng/mL; n = 5). Testosterone levels

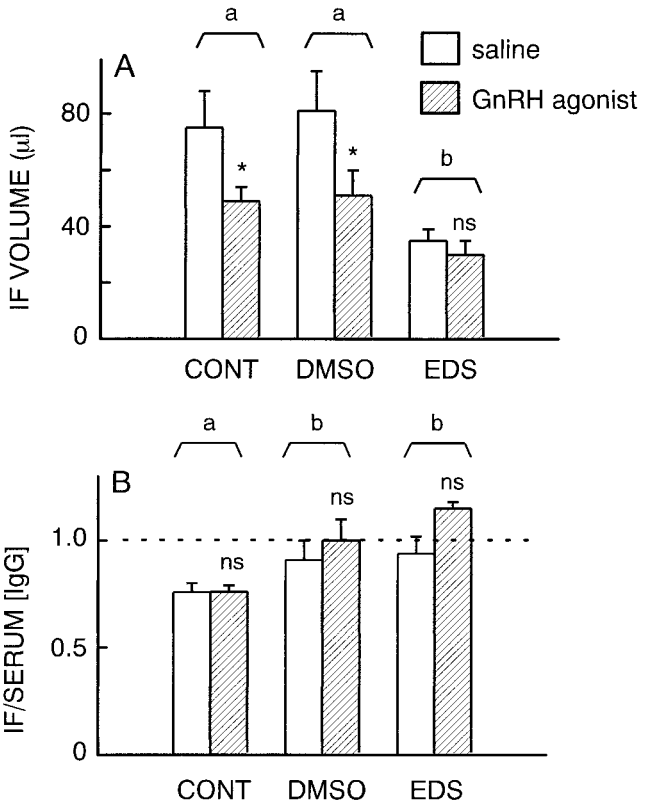

Figure 1. Effect of unilateral intratesticular GnRH agonist injection on **(A)** IF volume and **(B)** the IF-to-serum [IgG] ratio in untreated control (CONT), DMSO-treated, and EDS-treated adult rats. Values are mean ± SEM (n = 5 rats/group). Comparisons are between the saline-injected and GnRH agonist-injected testis. *$P < .05$; ns indicates not significant ($P > 0.05$) by paired *t* test. Treatment groups with different letter superscripts are significantly different ($P < .05$).

were considerably lower in tIF from EDS-treated rats (4 ± 2 ng/mL).

Evaluation of the Influence of Pituitary LH

Because the elevated testosterone values indicated that the GnRH agonist had stimulated pituitary function, leading to a release of LH and stimulation of Leydig cells, the effect of a lower dose of GnRH agonist, and increasing LH/hCG were also examined over a similar time period. Unilateral treatment with 1 ng GnRH agonist per testis significantly reduced tIF volume ($P < .05$) by a similar magnitude to that observed with the higher GnRH agonist dose (65 ± 9 µL cf 42 ± 4 µL in the saline-injected testis; mean ± SEM; n = 5), but serum testosterone levels remained within the normal range (8.8 ± 1.2 ng/mL cf 9.4 ± 2.7 ng/mL in control animals).

In contrast to the effect of the GnRH agonist, hCG treatment had no significant effect on tIF volume 2 hours later, although by 20 hours, IF volume had increased by more than twofold (Figure 2A). Serum IgG concentrations were not significantly affected by hCG treatment at either time point (data not shown). Although hCG did have a

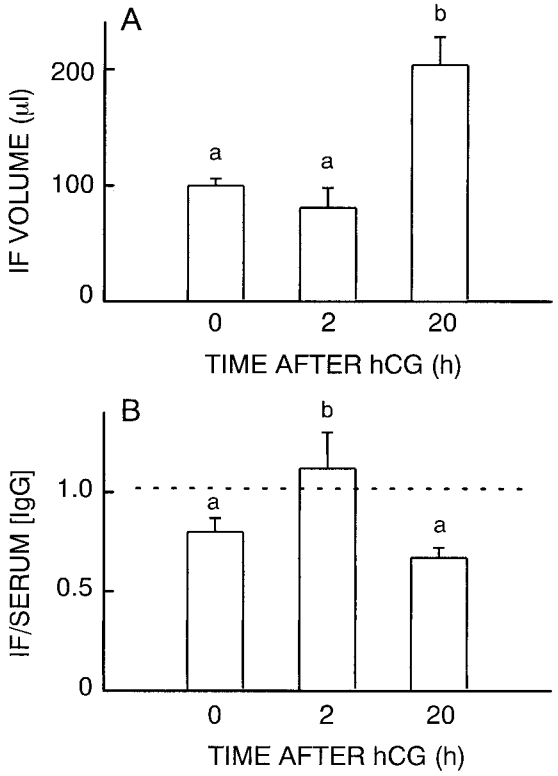

Figure 2. Effect of subcutaneous hCG-injection on **(A)** IF volume and **(B)** the IF-to-serum [IgG] ratio in adult rats. Values are mean ± SEM (n = 5 rats/group). Comparisons are with control saline-injected rats (CONT): time-points with different letter superscripts are significantly different ($P < .05$).

transient stimulatory effect on the IF/serum [IgG] ratio at 2 hours after injection, there was considerable variation in the response among different rats (Figure 2B).

Discussion

These data provide the first confirmation that the acute inhibitory action of GnRH on testicular capillary fluid permeability is mediated through Leydig cells. In contrast to tIF formation, IgG levels in tIF were unaffected by acute GnRH treatment whether or not Leydig cells were present, and irrespective of the changes in tIF volume due to EDS treatment. Over the same time course, hCG had no apparent effect on fluid volume, but caused a rapid increase in testicular IgG concentration, which returned to normal when tIF volume was increased 18 hours later. These studies provide further support for the concept that Leydig cells are centrally involved in regulation of tIF in testis (Setchell and Rommerts, 1985; Damber et al, 1987a; Collin et al, 1993; Collin and Bergh, 1996; Turner et al, 1996).

It is logical to assume that there would be a relationship between tIF formation and the concentration of molecules in tIF, and that changes in tIF volume and fluid permeability may play an important role in regulating the local concentration of these components, such as locally produced testosterone (Sharpe, 1984). However, a simple relationship between tIF volume and the concentration of the serum-derived protein, IgG, does not appear to exist. Very large increases and decreases in tIF volume have been observed between treatment groups, with little or no change in testicular IgG concentrations, in both this study and our previous study (Hedger and Hettiarachchi, 1994). These data support our previous observation that the volume of testicular fluid and the concentrations of serum-borne proteins in the testis are not directly related, presumably because they involve separate transport mechanisms that are themselves under separate regulation by local and pituitary factors (Hedger and Hettiarachchi, 1994).

Dynamic regulation of testicular fluid volume involves changes in the interendothelial cell junctions and blood flow characteristics (Sharpe, 1979; Setchell and Sharpe, 1981; Bergh et al, 1986; Widmark et al, 1986a; Bergh and Damber, 1988). Access of serum proteins to testis, on the other hand, is regulated by facilitated diffusion and active transport mechanisms, as in other microvessels (Garlick and Renkin, 1970; Milici et al, 1987; Ghinea and Milgrom, 1995). Whereas testicular microvessels are unfenestrated and relatively poorly permeable to many marker substances (Kormano, 1967, 1968), permeability to many serum-derived proteins such as albumin and IgG actually appears to be higher than in most other vascular beds (Sharpe, 1979; Setchell et al, 1988; Pöllänen and Setchell, 1989; Hedger and Hattiarachchi, 1994). The permeability of different proteins, however, varies quite widely, and because relative permeability is not proportional to the size of the protein, this appears to be due to differences in specific transcapillary transport mechanisms (Pöllänen and Setchell, 1989; Banks and Kastin, 1992; Turner and Rhoades, 1995). The specificity and regulation of these transcapillary transport mechanisms in testis is poorly understood. In the present study, hCG treatment caused a rapid increase in permeability to IgG, which occurred much earlier than the increase in tIF volume that follows hCG treatment. However, while this observation implicates Leydig cells in control of protein permeability in testicular endothelium in addition to controlling vascular flow responses, there is insufficient information to establish whether similar mechanisms are involved.

Finally, while GnRH and its mRNA have been detected in normal testis (Hedger et al, 1985b; Bahk et al, 1995; Botté et al, 1998) and GnRH bioactivity has been found in Sertoli cell culture medium (Sharpe et al, 1981; Nagendranth et al, 1983), in fact the origin and physiological significance of this peptide in testis remains to be clari-

fied. The confirmation that lymphocytes produce GnRH (Emanuele et al, 1990; Azad et al, 1991) and the observation that lymphocytes freely circulate through the testis (Wang et al, 1994) suggests the possibility of GnRH-mediated lymphocyte–Leydig cell communication. This communication may be particularly important during testicular inflammation, which is characterized by both an increase in intratesticular leukocytes and a reduction in IF volume, similar to that observed after GnRH injection (O'Bryan et al, 2000). The possible role of GnRH in this response deserves further investigation.

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