

THE INFLUENCE OF HEPARIN ON LH- AND DIBUTYRYL-cAMP-STIMULATED TESTOSTERONE PRODUCTION IN VITRO BY ADULT RAT TESTES

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1. Introduction

In addition to the anti-coagulant and anti-lipemic effects of heparin [1,2], this sulfated mucopolysaccharide has been shown to inhibit certain gonadal enzymes involved in steroid hormone biosynthesis. A relatively specific inhibition of testicular Δ^4 -5 α -reductase as well as a comparatively weaker inhibition of C₁₇—C₂₀ lyase and 17 β -hydroxysteroid dehydrogenase activities, was reported [3] when immature rat testes were incubated in vitro in the presence of heparin. Heparin inhibition of LH-sensitive adenylate cyclase has been demonstrated [4] in ovarian membrane fractions prepared from PMSG-treated rats. Since interaction of LH with specific ovarian and testicular membrane receptors and subsequent stimulation of membrane-associated adenylate cyclase represents an early and apparently obligatory event in LH-stimulated gonadal steroid hormone biosynthesis [5–7], the demonstration that heparin inhibits ovarian LH-sensitive adenylate cyclase [4] suggests that heparin may inhibit gonadal LH-stimulated hormone production. In an effort to test this postulate, we studied the effects of heparin on LH-stimulated testosterone production in vitro by adult rat testes. Also examined were the possible effects of heparin on testosterone production in vitro by rat testes incubated

in the presence of (Bu)₂-cAMP. This analogue of cAMP has been shown to fully mimic the effects of cAMP generated in the testes in response to LH stimulation [6–8].

2. Materials and methods

Adult male Sprague-Dawley rats (75 days) were sacrificed by decapitation and testes were rapidly removed, decapsulated and weighed. Approximately one-half testis from each animal were transferred to 25 ml Erlenmeyer incubation flasks containing 1.0 ml Krebs Ringer bicarbonate glucose (KRBG) buffer, pH 7.4 (supplemented with 1.0 mg glucose/ml) and either 0, 50, 100, 1000 or 5000 USP units of sodium heparin (specific activity, 168 USP units/mg; from porcine intestinal mucosa, grade I, Sigma Chemical Co., St Louis). In addition to varying concentrations of heparin, each incubate, depending on the experiment, contained either 10 ng bovine LH (NIH-LH-B10, biological potency 1.06 \times NIH-LH-S1 and < 0.05 NIH-FSH-S1/mg) or final conc. 5 mM (Bu)₂-cAMP (sodium salt, Grade II, Sigma Chemical Co.). Incubations were conducted for 4 h at 33°C in a Dubnoff metabolic incubator (144 osc./min) under a 95% O₂:5% CO₂ atmosphere.

At the conclusion of the 4 h incubation period, 0.1 ml aliquots of incubation media were transferred to conical glass extraction tubes containing 0.5 ml absolute methyl alcohol and stored at –6°C until extraction of steroids with freshly distilled diethyl ether (4 \times 3 vol.) and estimation of testosterone content in duplicate samples by a previously described

Abbreviations: LH, luteinizing hormone; (Bu)₂-cAMP, dibutyladenosine 3':5'-cyclic monophosphoric acid; cAMP, adenosine 3':5'-cyclic monophosphoric acid; PMSG, pregnant mare's serum gonadotropin

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radioimmunoassay method [9,10]. Sensitivity of the testosterone assay method was 8 pg and all samples were analyzed in a single assay procedure in order to avoid inter-assay variation. The intra-assay coefficient of variation for the testosterone radioimmunoassay procedure was 6.9%. Statistical analysis of data was accomplished using the *t*-test for independent means, and $P < 0.05$ between controls and the appropriate experimental groups was considered to be statistically significant.

3. Results and discussion

The *in vitro* incubation of decapsulated rat testes followed by assessment of testosterone released into the incubation medium, a system originally described [8], has been used extensively as a model for the study of factors which regulate testicular androgen production [10–13]. The values shown in table 1 for testosterone released into the incubation media by rat testes (expressed as ng testosterone/total testis/4 h) in the absence of heparin and in the presence of 10 ng LH or 5 mM (Bu)₂-cAMP are in good agreement with previous studies assessing the effects of these compounds on rat testicular androgen production *in vitro* [8,11]. When LH or (Bu)₂-cAMP concentrations in incubates were increased above 10 ng or 5 mM, respectively, no further stimulation of testicular testosterone production was observed (data not shown) indicating that testicular androgen production

was maximally stimulated by the LH or (Bu)₂-cAMP concentrations used in the present study.

As shown in table 1, incubation of testes with either 50 or 100 USP units of heparin did not significantly ($P > 0.1$) alter LH-stimulated testosterone production when compared to LH-stimulated testes incubated in the absence of heparin. However, the presence of 500, 1000 or 5000 USP units of heparin in incubates resulted in significant reductions of LH-stimulated testosterone production to 61.5%, 61.0% and 19.8%, respectively, of the corresponding hormone production by LH-stimulated testes incubated without heparin. In contrast to these findings, neither 50, 100, 500 or 1000 USP units of heparin/incubate significantly altered testosterone production in (Bu)₂-cAMP-stimulated testes (although 500 units heparin/incubate in (Bu)₂-cAMP-stimulated testes evoked an apparent 20% reduction in testosterone production, this value was not significantly different, $P > 0.1$, from androgen production in testes incubated in the presence of (Bu)₂-cAMP and absence of heparin. Moreover, the higher concentration of 1000 units heparin/incubate did not produce a discernible reduction in testosterone production in (Bu)₂-cAMP-stimulated testes — see table 1). A concentration of 5000 USP units heparin in incubates did, however, significantly reduce (Bu)₂-cAMP-stimulated hormone production to ~38% of the corresponding non-heparin-treated control values.

To our knowledge, the present work represents the first evidence that heparin is capable of reducing the capacity of testes to produce testosterone in response to LH. Also, the finding that lower concentrations of heparin appeared to be required to inhibit LH-stimulated testosterone production (e.g., 500–1000 USP units heparin/incubate) than was required to inhibit (Bu)₂-cAMP-stimulated testosterone production (5000 USP units/incubate) suggests possible multiple loci of heparin inhibition of testicular steroidogenesis. In this context, it is tempting to speculate that lower concentrations of heparin (500–1000 USP units/incubate) may inhibit testicular LH-sensitive adenylate cyclase with a resultant decrease in intracellular cAMP levels in response to hormone treatment, since (Bu)₂-cAMP was capable of overcoming heparin inhibition of testosterone production at these concentrations. This interpretation would be consistent with the observation that heparin does appear to inhibit

Table 1
The influence of heparin on LH- and (Bu)₂-cAMP-stimulated testosterone production *in vitro* by adult rat testes^a

Heparin (USP units/incubate)	LH-stimulated	(Bu) ₂ -cAMP-stimulated
0	858 ± 61	758 ± 74
50	802 ± 81	855 ± 131
100	682 ± 109	718 ± 79
500	528 ± 77 ^c	598 ± 86
1000	531 ± 99 ^b	741 ± 66
5000	170 ± 29 ^d	291 ± 46 ^d

^a Values obtained for testosterone are the mean ± SEM of determinations performed on 6 testes/experiment

^b $P < 0.02$; ^c $P < 0.01$; ^d $P < 0.001$, compared with appropriate control (no heparin) values

ovarian LH-sensitive adenylate cyclase [4]. On the other hand, the highest concentration of heparin used in incubates in the present study (5000 USP units) inhibited both LH- and (Bu)₂-cAMP-stimulated androgen production, a result which may indicate that inhibition of other testicular steroidogenic enzymes such as C₁₇—C₂₀ lyase and/or 17 β -hydroxysteroid dehydrogenase [3] was achieved at this higher concentration of heparin.

The specific mechanism(s) involved in heparin inhibition of testicular LH-stimulated testosterone production are not known. However, heparin and other naturally-occurring sulfated glycosaminoglycans are negatively-charged polymers and it has been suggested that by adsorbing to opposite charges on exposed surface membranes of the ovary, heparin may interfere with LH binding sites or restrict lateral mobility of the components of membrane-associated adenylate cyclase and thus inhibit generation of intracellular cAMP [4]. Partial support for this concept is derived from the findings [14] which show that polyanions such as dextran sulfate and poly(L-aspartate) inhibit thyroid-stimulating hormone (TSH) stimulation of bovine thyroid membrane adenylate cyclase activity. It seems unlikely, however, that charge effects of polyanions alone are totally responsible for inhibition of ovarian LH-sensitive adenylate cyclase since, in contrast to heparin, polyanions such as chondroitin-4-sulfate, chondroitin-6-sulfate or hyaluronic acid were reported to be ineffective in inhibiting ovarian LH-sensitive adenylate cyclase [4]. Also, because LH is a glycoprotein hormone and a significant biochemical property of heparin is its ability to electrostatically interact with a variety of proteins [15], the possibility cannot be excluded that heparin may interact with this hormone through charge effects and render it conformationally inactive for binding to gonadal membrane LH receptors.

The present findings that heparin inhibits testicular responsiveness to LH in vitro and the observation that sulfated mucopolysaccharides may be involved in regulation of the ovulatory process in rodents [4,16] indicate the need for further study of the possible effects of these compounds on gonadal function. Studies are currently in progress to investigate the possible effects of a variety of glycosaminoglycans on testicular adenylate cyclase activity and testosterone production in response to LH stimulation.

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