A Partial Characterization of a Sertoli Cell-Secreted Protein Stimulating Leydig Cell Testosterone Production

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Abstract. To examine whether immature rat Sertoli cells in culture secrete a factor(s) which stimulates testosterone production by mature mouse Leydig cells, Sertoli cell-enriched cultures were prepared from 3-week-old male rats with trypsin and collagenase.

Sertoli cells were plated at an initial density of 3-5×10⁵ cells/35 mm well and cultured in 3 ml serum free media supplemented with insulin (10 μg/ml). Sertoli cell culture medium (SCCM) collected every 3rd day was added to Leydig cells (10⁶ cells in 1 ml of MEM with 2% steroid-free FCS) prepared from 10-week-old mice by mechanical separation and incubated for 3 h at 34°C. Secreted testosterone was determined by RIA. SCCM 15 times concentrated by Amicon YM10 membrane demonstrated a dose-dependent stimulation of testosterone production, whereas there was no effect on testosterone secretion when Leydig cells were maximally stimulated by LH. Leydig cell stimulating activity was retained by both a dialysis membrane with a pore size of 24 Å and an ultrafiltration membrane with a molecular weight cut-off of 10 kDa. However, activity was reduced by heating at 60°C for 30 min and almost lost after incubation with 0.1% trypsin for 1 h at 37°C. This activity was not retained by means of a Con A-Agarose column and was demonstrated only in break-through fractions. HPLC gel filtration of a 15 times concentrated SCCM preparation on a TSK gel G3000SW revealed Leydig cell-stimulating activity at approximately 13 kDa. In conclusion, immature rat Sertoli cells in culture secrete a factor(s) which stimulates testosterone production from adult mouse Leydig cells. This factor(s) is a heat-sensitive protein with a molecular size around 13 kDa.

Key words: Sertoli cell, Leydig cell, Interaction, Testosterone production.

LH STIMULATES testosterone production by Leydig cells and spermatogenesis in the testes progresses under the control of both a high concentration of testosterone and FSH stimulation. If Sertoli cells, which are a major component of seminiferous tubules and supporting a series of spermatogenic cells, requiring a sufficient amount of androgen, produce a factor or factors which stimulate(s) testosterone production from interstitial cells, a quite significant intratesticular paracrine control mechanism exists.

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Accumulated evidence suggests the existence of a factor or factors which stimulates testosterone production by normal mature or immature Leydig cell in testicular interstitial fluid [1-3], in Sertoli cell culture medium [4-10], and in culture medium of seminiferous tubules [11, 12]. But this factor has not yet been purified or identified and some biochemical characteristics of this factor from different species and sources are not yet agreed upon by different investigators. Recently, Melsert et al. [13, 14] reported that albumin fractions of both rat testicular fluid, and rat, bovine and human sera were effective in stimulating LH-stimulated pregnenolone production by immature rat Leydig cells. And Papadopoulos [15] purified a human Sertoli cell-secreted protein with
a molecular size of 80 kDa that stimulated progesterone production from a mouse tumor Leydig cell line.

In the present study, we demonstrate the existence of a factor which stimulates testosterone production by mature mouse Leydig cells in a spent medium of immature rat Sertoli cell cultures and explain some new biochemical characteristics of this factor including fractionation of the activity using an HPLC system.

**Materials and Methods**

**Animals and chemicals**

Three-week-old Wistar rats and 10-week old ICR mice were purchased from Clea Japan Inc. (Tokyo, Japan). Amicon ultrafiltration cell and Diaflo ultrafiltration membranes (YM 10) were purchased from Amicon Corporation (Danvers, MA) and W. R. Grace & Co. (Danvers, MA). Seamless cellulose tubing (pore size 24 Å) was purchased from Union Carbide Co. 1, 2-3H(N)-Testosterone (1480.0 GBq/mmol) was purchased from DuPont/NEN Research Products. Foetal calf serum was purchased from Sera-Lab Ltd. (Sussex, England). Luteinizing hormone (LH), insulin, trypsin, soybean trypsin inhibitor (STI), retinol, T₃, DNase, gentamycin, HEPES and collagenase were purchased from Sigma (St. Louis, MO). Dulbecco’s Modified Eagle’s Medium (MEM), Ham’s F-12 Nutrient Mixture and Hank’s Balanced Salt Solution (HBSS) were purchased from Gibco (Grand Island, NY). Other chemicals and reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Primary Sertoli cell cultures**

Sertoli cell-enriched cultures were prepared as described previously [16]. Decapsulated testes were incubated with 0.2% (wt/vol) trypsin and 0.001% (wt/vol) DNase in HBSS for 15 min at 25°C to separate tubules. The tubules were washed, minced with scissors and incubated further with 0.2% (wt/vol) collagenase in MEM/F-12 for 30 min at 32°C. After filtration through 100 µ mesh, single cell and small cell clamps were plated at an initial density of 3–5 x 10⁶ cells / 35 mm well in 3 ml MEM/F-12 supplemented with insulin (10 µg/ml), retinol (1 x 10⁻⁷ M), vitamin E (100 ng/ml), T₃ (1 x 10⁻¹⁰ M), and selenium (2 x 10⁻⁸), and incubated at 37°C in a humidified atmosphere of 97% air and 3% CO₂. The Sertoli cell-conditioned medium (SCCM) was changed 1 day after plating and on the 4th, 7th and 10th days, the medium was changed, pooled and stored at −80°C until analyzed for LH-like activity. The concentration of testosterone in a random sample of pooled media was less than 0.4 ng/ml.

**Bioassay method for LH-like activity**

Leydig cells were collected from 10-week-old ICR mice by mechanical separation [17]. In brief, testes were decapsulated, cut into small pieces and the seminiferous tubules were teased apart with fine scarpels. Then MEM containing 2% FCS was added to a final concentration of 6–8 testes/30 ml and gently stirred with a magnetic stirrer for 10 min at room temperature. The medium was filtered through a fine mesh and the filtrate was centrifuged at 200 x g for 10 min. The supernatant was discarded and the cells were resuspended in fresh MEM 2% FCS. Leydig cells (10⁶ cells) were incubated with samples with or without LH at 34°C for 3 h in 1 ml of MEM 2% FCS. The incubation medium was spun down at 800 x g, and testosterone concentrations in the supernatant were determined by RIA without chromatography with the antibody to testosterone-11-succinate-BSA. The intraassay coefficient of variation was 6.3%. All samples collected from one bioassay were always analyzed in the same RIA.

**Chromatography**

1) Con A-agarose affinity chromatography

SCCM was concentrated 15 times with an amicon YM-10 membrane, dialysed against DPBS pH 7.4, and applied to a Con A-agarose lectin affinity column (2.5 ml) packed and equilibrated at 4°C with DPBS pH 7.4. After the sample was loaded on the column, the flow was stopped for 1 h to allow interaction of the glycoproteins with the immobilized Con A, after which the column was washed with 20 ml DPBS. Then DPBS containing 0.2 M α-methyl-D-glucoside was applied to elute Con A-bound glycoproteins.
2) Gel filtration high pressure liquid chromatography

The solvent of fifteen times-concentrated SCCM was replaced by DPBS with amicon ultrafiltration apparatus. A portion of this solution (1 ml; 400 μg protein) was loaded on a TSK3000 gel filtration HPLC column (Toyo Soda, Japan) preequilibrated with DPBS pH 7.4 as a solvent at a flow rate of 0.5 ml/min with a Pharmacia HPLC system (Pharmacia LKB, Sweden) equipped with an LKB 2249 LC gradient pump, LKB 2510 Uvicord SD UV monitor, LKB 2154–001 sample injector and a Shimazu Chromatopak C-R6A data recorder.

Other methods

The protein concentrations were determined by the method of Lowry et al. [18] using BSA as the standard. Comparisons between individual response values within a single assay were made by unpaired t-test.

Results

In vitro bioassay of LH-like activity with adult mouse Leydig cells

A standard curve of LH activity for testosterone production by adult mouse Leydig cells during a 3 h incubation at 34°C is shown in Fig. 1. A dose-dependent relationship between the LH concentration and testosterone production was demonstrated. LH was saturated at a concentration of about 500 mIU/ml under these experimental conditions.

Leydig cell stimulating activity in rat SCCM

Fifteen-times concentrated rat SCCM stimulated testosterone production by adult mouse Leydig cells in a dose-dependent fashion from 25 μl (10 μg protein) up to 100 μl (40 μg protein), but the activity came to a plateau at a dose of 200 μl (Fig. 2). SCCM failed to show a similar dose-response relationship when incubated with the maximally stimulating dose (10 IU/ml) of LH (Fig. 3).

Some characteristics of Leydig cell stimulating factor

Leydig cell stimulating activity was retained by both a dialysis membrane with a pore size of 24 Å and an ultrafiltration membrane with a mol wt cut-off of 10 kDa. However, activity was reduced by heating for 30 min at 60°C and lost after incubation with 0.1% trypsin for 1 h at 37°C (Fig. 4).

This activity was not retained by a Con A-Agarose column and was demonstrated only in
The effects of increasing amounts of 15 times-concentrated immature rat SCCM on the LH-stimulated secretion of testosterone by mature mouse Leydig cells. Leydig cells (1×10^6) were incubated with appropriate amounts of 15 times-concentrated SCCM and a maximally stimulating dose of LH (10 IU) at 34°C for 3 h in 1 ml of medium. The results are the mean ± SEM of quadruplicate incubations for each treatment.

Fig. 4. Effects of various treatments on Leydig cell-stimulating activity of SCCM. Leydig cells (1×10^6) were incubated with ten-times concentrated SCCM (400 µl) at 34°C for 3 h in 1 ml of medium. The results are the mean ± SEM of triplicate or quadruplicate incubations for different SCCM preparations (*, P<0.05, ** P<0.01 vs. control).

Fig. 5. Elution profile of SCCM on Con A-Agarose. SCCM was concentrated 15 times and dialyzed against DPBS (pH 7.4). An aliquot (1300 µl; 500 µg protein) was chromatographed at 4°C, and 600 µl fractions were collected at a flow rate of 10 ml/h. An aliquot (500 µl) of each fraction was incubated with mouse Leydig cells (1×10^6) at 34°C for 3 h in 1 ml of medium, and the effect on testosterone production was determined in duplicate by RIA. α-MG, 0.2 M α-methyl-D-glucoside.

Fig. 6. HPLC gel filtration chromatography of SCCM. An aliquot (1 ml; 400 µg protein) of 15 times-concentrated SCCM was chromatographed at room temperature, and 500 µl fractions were collected at a flow rate of 500 µl/min. An aliquot (400 µl) of each fraction was incubated with Leydig cells (1×10^6) at 34°C for 3 h in 1 ml of medium, and the effect on testosterone production was determined in duplicate by RIA.

HPLC gel filtration of 15 times concentrated SCCM preparation on TSKgel G3000SW revealed activity at about 13 kDa (Fig. 6).
Discussion

We detected and partially characterized a factor which stimulated testosterone production by mature mouse Leydig cells in spent medium from cultured immature rat Sertoli cells. Using an HPLC gel filtration column, we demonstrated that the approximate molecular weight of this factor was about 13 kDa. Previous investigators reported the molecular size of this paracrine factor(s), but their results were fundamentally based on ultrafiltration methods with some filter papers having different pore sizes. Sharpe et al. [1] and Janecki et al. [8] reported factors with molecular weights of more than 10 kDa and more than 1 kDa in the interstitial fluid from adult rat and immature rat SCCM, respectively. Verhoeven and Cailleau [5,12] showed a factor with a molecular weight between 10 kDa and 30 kDa in immature rat SCCM or in spent media from human testicular tubules, Papadopoulos et al. [9] reported that a factor in SCCM from adult rats has a molecular weight of between 10 kDa and 50 kDa. He [15] also identified and purified another protein with a molecular size of 80 kDa which stimulated progesterone production by mouse tumor Leydig cells in culture medium of human Leydig cells. But it was not clearly shown whether this protein stimulated testosterone production by normal human or rat Leydig cells or not. Mersert et al. [13,14] reported that albumin fractions purified from rat testicular fluid and rat, bovine, and human sera had stimulatory effects on pregnenolone production by isolated immature rat Leydig cells. But Hedger et al. [3] showed that bovine albumin had no effect on testosterone production by adult rat Leydig cells. They [3] reported a factor with a molecular size of more than 30 kDa in the interstitial fluid of adult rats which stimulated testosterone production by adult rat Leydig cells. Other researchers also investigated the molecular size of this paracrine factor with dialysis membranes. Sharpe et al. [1] presented a nondialysable factor (MW > 10 kDa) in adult rat interstitial fluid. On the other hand, Syed et al. [11] showed a dialysable factor (MW < 10 kDa) in the spent media of the adult rat seminiferous tubule incubations. In this study, repeated freezing and thawing made it difficult to fractionate this factor by gel filtration, suggesting that this protein is quite unstable or easily aggregates with other macro-

molecules like inhibin, which is also secreted by Sertoli cell in the male, and which readily aggregates. Further purification is needed to determine the exact molecular size of this factor.

Inhibin and activin (homodimer of β subunit of inhibin) have been reported to have stimulatory and suppressive effects on Leydig cell steroidogenesis, respectively [19]. But Grootenhuis et al. [20] showed that highly purified 32 kDa inhibin had no effect on Leydig cell steroidogenesis. Hedger et al. [3] also reported that not only inhibin but also bovine albumin, ovalbumin, epidermal growth factor, insulin-like growth factor-1, and transforming growth factor-β were inactive. In our study, the Leydig cell-stimulating factor was demonstrated to be different from inhibin, because this factor did not bind with a Con-A agarose affinity column and it was a heat labile protein, in contrast to inhibin, which is a heat resistant glycoprotein [16].

It has been demonstrated in several studies that this factor is heat sensitive. But Benahmed et al. [6] used a Sephadex G 100 gel filtration column and reported heat stable factors in immature pig SCCM with molecular sizes of 10–15 kDa and 20–30 kDa. Syed et al. [11] showed a heat stable, trypsin resistant low molecular weight substance in the spent media of the seminiferous tubule incubations from cryptorchid rats. This discrepancy was presumably due to the different species and different experimental conditions.

In the present study, SCCM protein failed to show the activity stimulating Leydig cell testosterone production when incubated with a maximally stimulating dose of LH (10 IU/ml). Although these results are consistent with previous reports using a culture medium of Sertoli cells or testicular tubules as sources of this factor(s) [8,11,12], Sharpe et al. [1] and Hedger et al. [3] have described how interstitial fluid from mature rats continued to stimulate testosterone production by rat Leydig cells even in the presence of a maximum dose of LH or hCG. The explanation for this dissimilarity, namely whether these biologically active factors from different sources have different action mechanisms or whether the results are due to the difference in the biological activity of the factors, remains to be established.

Although Verhoeven et al. [4] and Risbridger et al. [2] reported that this factor stimulated an early step in the steroidogenic pathway, the actual action
The mechanism of this factor remains to be elucidated. Risbridger et al. [2] showed that this factor activated the cholesterol side-chain cleavage enzyme which was rate-limiting in the biosynthesis of testosterone and was one of the action sites of LH. They also showed that this factor further stimulated testosterone production in the presence of a maximum stimulated dose of LH, suggesting that this factor’s action mechanism is different from that of LH. Papadopoulos et al. [9] demonstrated that this factor in spent medium from rat seminiferous tubule cultures did not require protein synthesis for testosterone formation whereas LH did, suggesting a different action mechanism of this factor and LH. Perrard-Sapori et al. [21] showed that coculture of Leydig and Sertoli cells produced an increase in both hCG receptor and hCG-induced testosterone production.

In conclusion, our results further support the theory of the existence of a factor which stimulates Leydig cell testosterone production in spent media from immature rat Sertoli cell cultures. Although this factor has not yet been identified, we found, with an HPLC system, that the apparent molecular size of this factor is approximately 13 kDa, and demonstrated some new biochemical characteristics.

References

