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·*Original Article*·

Upregulation of macrophage migration inhibitory factor and calgizzarin by androgen in TM4 mouse Sertoli cells

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Abstract

Aim: To identify proteins induced by androgen in Sertoli cells during spermatogenesis. **Methods:** We analyzed protein profiles in TM4 Sertoli cells treated with dihydrotestosterone (DHT) using surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) . **Results:** We found increases in the expression of a 5.0-kDa protein at 15 min, an 11.3-kDa protein at 24 h and 4.3 kDa, 5.7 kDa, 5.8 kDa, 9.95 kDa and 9.98 kDa proteins at 48 h after the treatment. In contrast, the expression of 6.3 kDa and 8.6 kDa proteins decreased at 30 min, and 4.9 kDa, 5.0 kDa, 12.4 kDa and 19.8 kDa proteins at 48 h after the treatment. The 11.3-kDa protein was identified as macrophage migration inhibitory factor (MIF) known to having various functions. The 9.98-kDa protein was identified as calgizzarin related to calcium channels. The timing of their expression suggests that MIF and calgizzarin are involved in late regulation of spermatogenesis in Sertoli cells by androgen. **Conclusion:** MIF and calgizzarin are two important androgen-responsive proteins produced by Sertoli cells and they might play a role in regulating spermatogenesis. *(Asian J Androl 2006 Sep; 8: 549–554)*

Keywords: androgen; Sertoli cell; spermatogenesis; surface enhanced laser desorption ionization time-of-flight mass spectrometry; macrophage migration inhibitory factor; calgizzarin

1 Introduction

Androgen is the crucial hormone responsible for the initiation and maintenance of spermatogenesis [1]. Testosterone exists in testis in a much higher concentration than in sera. Testosterone alone is able to restore spermatogenesis under experimental conditions where follicle stimulating hormone (FSH) is virtually absent, such as in hypogonadal mice genetically deficient in gonadotropin-releasing hormone [2]. Testosterone is secreted from Leydig cells under the control of luteinizing hormone (LH) and affects the expression of target genes in Sertoli cells in testes. Because Sertoli cells support the development of germ cells through various factors secreted by themselves, androgen indirectly affects sper-

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matogenesis [3].

The information about target genes of androgen in Sertoli cells is still obscure. It has been reported that androgen induces expression of Pem and Myc in Sertoli cells [4, 5], but whether these proteins are involved in spermatogenesis is unclear. Sertoli cells secrete several factors inducing NOS believed to affect spermatogenesis [6, 7], but all factors related to spermatogenesis are still not identified. It is important to identify relevant proteins to clarify the role of Sertoli cells in spermatogenesis.

In the present study, we analyzed the effects of androgens on protein expression during spermatogenesis in Sertoli cells using SELDI-TOF mass spectrometry [8, 9]. We used dihydrotesto sterone (DHT) instead of testosterone in the present study because the activity of DHT was the same as that of testosterone. Several proteins whose experssion in Sertoli cells were affected by stimulation with androgen were identified. Two of them were identified as macrophage migration inhibitory factor (MIF) and calgizzarin. The possible involvement of MIF and calgizzarin in spermatogenesis is discussed.

2 Materials and methods

2.1 Isolation of cell extract from mouse TM4 Sertoli cell line

Mouse TM4 Sertoli cell line was obtained from American Type Culture Collection [10] and maintained in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 2.5% fetal calf serum and 5% horse serum. For the treatment with DHT, cells were cultured without fetal calf serum and horse serum for 24 h, and then with DHT (10^{-8} mol/L) for different periods of time (15 min, 30 min, 1 h, 24 h, 48 h, 54 h and 72 h). Cells were harvested, washed with phosphate-buffered saline (PBS), and sonicated in lysis buffer (9 mol/L urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate and 1 mmol/L dithiothreitol). The cell extract was stored at –80ºC until use. The protein concentration was measured with a BCA kit with a HiTrap Q FF column (Japan Bio Rad Laboratories, Tokyo, Japan). The TM4 Sertoli cell line cultured without DHT was used as the control in the present study.

2.2 Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS)

2.2.1 SAX2 and CM10 ProteinChip array protocol

The cell extract was adjusted to 2 mg protein/mL with the lysis buffer and diluted to 200 μg/mL with binding buffer. An SAX2 or CM10 ProteinChip array, 8-spot (Ciphergen Biosystems, CA, USA) was placed in a bioprocessor (Ciphergen, CA, USA) and washed with 250 μL of the binding buffer three times. The binding buffer used for SAX2 is as follows: 0.1% Triton X-100, 50 mmol/L TrisHCl (pH 8.5), 50 mmol/L sodium acetate (pH 4.5), and that for CM10 is as follows: 0.1% Triton X-100, 50 mmol/L sodium acetate (pH 4.5 or pH 5.5), 50 mmol/L sodium phosphate pH 6.5 or pH 7.5. Four hundred microliters of the sample was applied to the array. The array was placed on a shaker and gently agitated for 30 min. The sample was removed and the array was washed with 250 μL of the same binding buffer for 5 min three times, followed by a brief MillQ water wash twice. The array was removed from the bioprocessor, a 0.5-μL aliquot of saturated sinapinic acid (dissolved in 50% acetonitrile containing 0.5% trifluoroacetic acid) was added to each spot twice and the array was air dried.

2.2.2 ProteinChip reader and bioinformatics

The SAX2 or CM10 array was transferred into the ProteinChip Reader, model PBS IIc (Ciphergen, CA, USA), which generates nanosecond laser pulses from an ultraviolet-emitting pulsed nitrogen laser (377 nm). The mass spectra of proteins/peptides were generated using an average of 150 laser shots at a laser intensity of 185–200 arbitrary units. An external calibration was performed using low molecular weight marker (MWM) (Somatostatin [1 637.9], bovine insulin B-chain [3 495.94], human recombinant insulin [5 807.65] and hirudin BKHV [7 033.61]), or median MWM (equine cardiac cytochrome C [12 360.2], equine cardiac myoglobin [16 951.5] and rabbit GAPDH [35 688]), or high-MWM (equine cardiac myoglobin [16 951.5] and rabbit GAPDH [35 688], bovine serum albumin [66 433] and *Escherichia coli* beta-galactosidase [116 351]). Data interpretation was augmented using ProteinChip software (version 3.1.1, Ciphergen, CA, USA). The protein expression patterns were analyzed using Biomarker Wizard (Ciphergen, CA, USA), which generates consistent peak sets across multiple spectra and allows for automatic comparison. The TagIdent tool from the ExPAsy molecular biology server (http://www.expasy.ch/tools/tagident.html) was used to create a list of candidate proteins that roughly matched the characteristics of interesting protein peaks observed. This tool searches within the SWISS-PROT and TrEMBL protein databases for proteins that match the requested molecular mass and isoelectric point (pI).

2.3 Enrichment of proteins of interest for identification

To enrich the proteins of interest, the cell extract prepared from 5×10^7 cells was diluted 10-fold with the binding buffer. The sample was fractionated with increasing concentrations of NaCl (0–1 000 mmol/L), with the purification progress being monitored using NP20 (Ciphergen Biosystems, CA, USA) array. The column fraction eluted with 200 mmol/L NaCl was dialyzed against MillQ water and freeze-dried. The sample was dissolved in MillQ water and loaded onto a HiTrap Q FF column (Amersham Biosciences Corp., Piscataway, NJ, USA). The sample was fractionated with decreasing concentrations of ammonium sulfate, with the fractionation process being monitored using SAX2 arrays. The flow-through fraction of the HiTrap Q FF column was run on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for further analysis.

2.4 SDS-PAGE and N-terminal amino acid sequence analysis (Edman degraduation)

The sample (20 μL) was mixed with NuPAGE LDS sample buffer (8 μL), NuPAGE Reducing Buffer (3.2 μL) and deionized buffer (0.8 μL) (NP 0060: Invitrogen Corp., Carlshad, CA, USA), heated at 70 ºC for 10 min and electrophored, 20 μL per lane, in 12% NuPAGE Novex Bis-Tris gel (NP 0342BOX: Invitrogen). A 20-kDa protein separated in the gel was transferred to PVDF membrane electrophoretically, and the target band was excised and analyzed by the Edman degradation method using Applied Biosystems 476a Protein Sequencer (Applied Biosystem, Japan). The amino acid sequence obtained was compared with that in the SWISS-PROT database using the FASTA sequence alignment program (http://fasta.bioch.virgina.edu).

3 Results

3.1 SELDI profiling of the TM4 cell extract

We analyzed protein profiles of TM4 cells treated with DHT using SAX2 or CM10 array and compared them with those of the control cells. When the intensity of protein was more than twice or less than half compared to the control, the difference was estimated to be

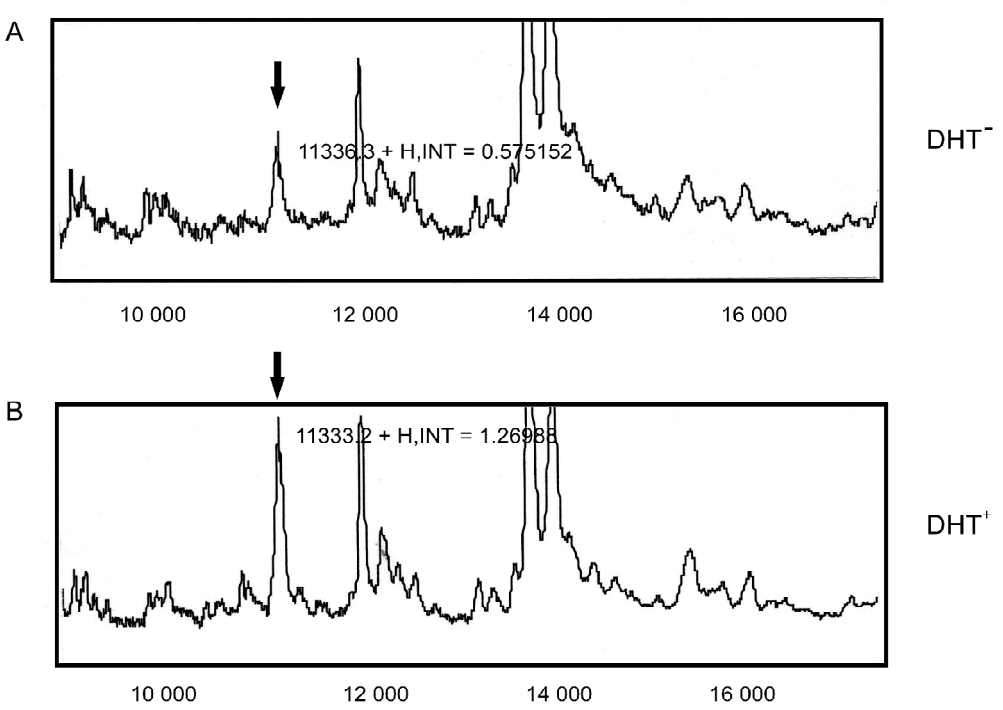

Figure 1. Protein profiles of TM4 Sertoli cells cultured without (A) or with (B) dihydrotestosterone (DHT), analyzed on CM10 ProteinChip assay. The protein profiles from 10 000–16 000 m/z are shown. The arrows show a protein that increased in amount following treatment with DHT.

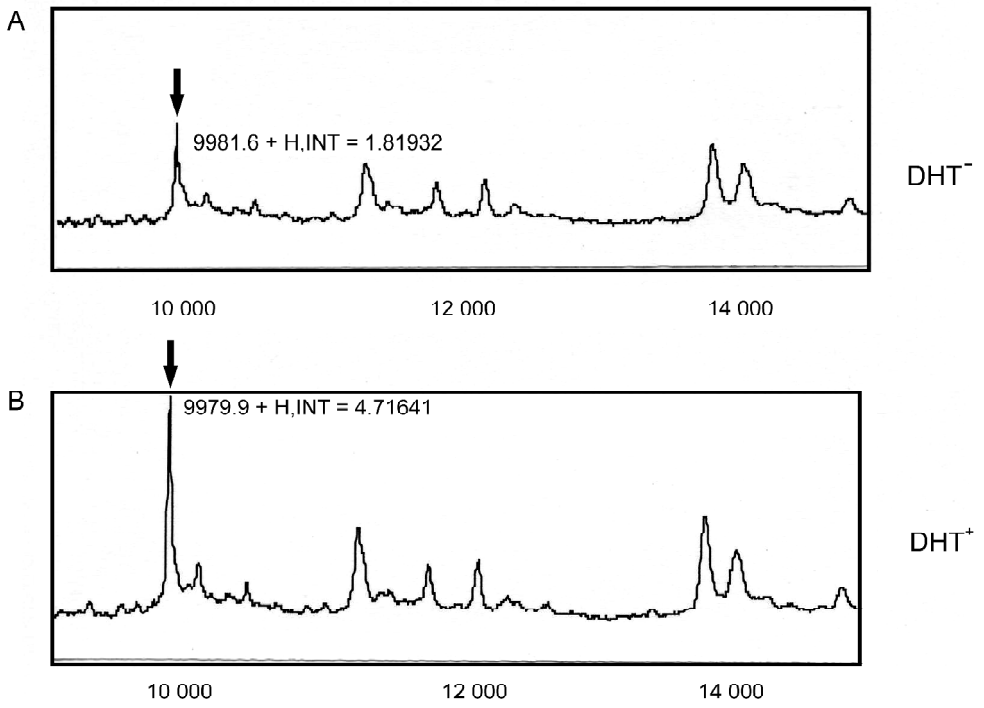

Figure 2. Protein profiles of TM4 Sertoli cells cultured without (A) or with (B) dihydrotestosterone (DHT), analyzed on SAX2 ProteinChip assay. The protein profiles from 10 000–14 000 m/z are shown. The arrows show a protein that increased in amount following treatment with DHT.

A 1 40
MPMFIVNTNV PRASVPEAFL SELTAGLAGA TAKPAGYIAV
80
HVVPDALMTF SATNDPCALC SLHSIAKIAG AGNRNVSKLL
115
CGLLSDRLHI SPDRVYINYV DMNAANVGWN GSTFA

B 1 40
MPTETERCIE SLIAVFQKYS GKDGNNTGLS KTEFLSFMNT
80
ELAAFTKNGK DQGVLDRMMK KLDLNCDGGL DFGEFLNLIG
98
GLAIACHDSF IGTSAKRI

Figure 3. (A): N-terminal amino acids sequence of macrophage migration inhibitory factor (MIF). The underline indicates the sequence of the 11.3-kDa protein determined by Edman degradation method. (B): N-terminal amino acids sequence of calgizzarin. The underline indicates the sequence of the 9.98-kDa protein determined by Edman degradation method.

significant.

Seven species of proteins were found to be upregulated in TM4 cells treated with DHT: a 5-kDa protein at 15 min, an 11.3-kDa protein at 24 h and five others of 4.3 kDa, 5.7 kDa, 5.8 kDa, 9.95 kDa and 9.98 kDa at 48 h. We also observed that six protein species were downregulated, 6.3 kDa and 8.6 kDa proteins at 30 min, and four others of 4.9 kDa, 5.0 kDa, 12.4 kDa and 19.8 kDa at 48 h after DHT stimulation.

3.2 Purification and sequence determination

To identify the 11.3-kDa protein separated on CM10 array pH 8.5 (Figure 1), we purified it by SDS-PAGE and subjected it to Edman degradation analysis. The results showed that it was MIF (Figure 3A) [11]. To identify the 9.98-kDa protein separated on SAX2 array (pH 8.5) (Figure 2), we purified it by SDS-PAGE and subjected it to Edman degradation analysis. The results showed that it was calgizzarin (Figure 3B) [12]. The other proteins could not be identified because of their small molecular weight and it was difficult to purify them.

4 Discussion

This is the first report on the analysis of protein profiles in Sertoli cells treated with DHT using SELDI-TOF mass spectrometry. In the present study, we used the TM4 Sertoli cell lines instead of primary culture of Sertoli cells because the results might be more stable and reliable than those using primary culture cells. We identified several proteins in TM4 Sertoli cells that increased or decreased in amount after treatment with DHT. So far, the expression of Myc and Pem has been reported to increase in Sertoli cells treated with androgen [4, 5]. Our results show that androgen affects several proteins, with different response times.

We showed that DHT treatment caused increased expression of seven proteins and decreased expression of six proteins in TM4 Sertoli cell lines. One of the proteins that increased, the 11.3-kDa protein, was identified as MIF [11]. Although MIF was first reported to be an inhibitory factor secreted by T lymphocytes [13], now, MIF is a pluripotent mediator of the physiological and pathophysiological regulation in diverse tissues [14, 15]. As the conventional membrane receptor for MIF has not been found, the action mechanism of MIF is still unclear. Because the inhibitory effect of MIF on the migration of macrophage is Ca^{2+}-dependent, the removal of calcium from the external medium inhibits the ability of MIF to modulate macrophage migration. In testes, MIF is secreted by Leydig cells and accumulated into the interstitial fluid [16]. MIF increases calcium influx in peritubular myoid cells [17]. MIF plays an important role in calcium homeostasis in Leydig cell-seminiferous tubule interaction. When Leydig cells are disturbed by Leydig cell-specific toxin ethane dimethane sulfate, the production of MIF switches to Sertoli cells [18]. In the present study, we first identify that the production of MIF depends on DHT in Sertoli cells. Although the function of MIF in Sertoli cells is unclear, our result shows that MIF is possibly related to calcium-dependent signal transduction in spermatogenesis because the Sertoli cells support the function of germ cells in seminiferous tubules.

We also identify a 9.98-kDa protein as calgizzarin. Calgizzarin is a target protein regulated by Ca^{2+}-bound S100B. Intracellular Ca^{2+} is involved in regulating various biochemical events in excitable cells, such as contraction, secretion and mitogenesis. These events are mediated by a family of Ca^{2+}-binding proteins. S100B is one protein of the family. S100B usually binds the target proteins, including calgizzarin, and shows the various functions *in vivo*. The S100B play important roles in neurodegeneration and cell cycle regulation [19, 20]. Therefore, calgizzarin seems to plays an important role in calcium-dependent signal transduction or cell cycle regulation in Sertoli cells or germ cells.

In the present study, we demonstrate that androgen affects the expression of several protein species, including MIF and calgizzarin, in Sertoli cells in mice. The identification of the other proteins affected and their function in Sertoli cells during spermatogenesis remain to be established. In primates, including humans, the androgenic effect in Sertoli cells should also be investigated using SELDI-TOF mass spectrometry.

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