Benign prostatic hyperplasia (BPH) epithelial cell line BPH-1 induces aromatase expression in prostatic stromal cells via prostaglandin E2

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Abstract

Estradiol (E2) level in stroma of benign prostatic hyperplasia (BPH) increases with age, and this increase was associated with an elevated expression of aromatase in prostatic stromal cells (PrSCs). Here, we showed that conditioned medium (CM) of BPH-1 (a benign hyperplastic prostatic epithelial cell line), but not of prostate cancer cell lines (LNCaP, DU-145, and PC-3), stimulates aromatase expression in PrSCs. Cyclooxygenase-2 (COX-2) mRNA level in BPH-1, as well as prostaglandin E2

(PGE2) concentration in BPH-1 CM, was significantly higher than that of prostate cancer cell lines. CM of BPH-1 treated with NS-398 (a specific inhibitor of COX-2) failed to stimulate aromatase expression in PrSCs. And PGE2 can stimulate aromatase expression in PrSCs. Our data suggested that BPH-1 induced aromatase expression in PrSCs through the production of PGE2 in a paracrine mechanism.

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Introduction

Steroid hormones play a significant role in growth and function of the normal prostate as well as the development of benign prostate hyperplasia (BPH). Although serum estrogen levels are low in healthy men (Isidori *et al.* 2000), intra-prostatic estradiol (E2) levels (both absolute levels and those relative to testosterone) increase in men with age, accompanied by an increase in the prostate volume (Rubens *et al.* 1974, Seppelt 1978, Shibata *et al.* 2000). The increased expression of aromatase disrupts the balance of estrogen/androgen in the prostate. Therefore, the estrogen-dominant status in men after middle age has been implicated in the induction and progression of BPH (Shibata *et al.* 2000).

Estrogens are synthesized from androgens using the enzyme aromatase, a member of the cytochrome P450 family, encoded by the *cyp19* gene (Fisher *et al.* 1998). Many studies have reported aromatase expression in the prostate by either RT-PCR or enzymatic activity using biochemical assays (Kaburagi *et al.* 1987, Stone *et al.* 1987, Matzkin & Soloway 1992, Tsugaya *et al.* 1996, Negri-Cesi *et al.* 1999). The level of E2 in stroma of BPH increases with age (Krieg *et al.* 1993), and this increase was associated with an elevated expression of aromatase in prostatic stromal cells (PrSCs), especially those around hyperplastic glands in BPH patients (Hiramatsu *et al.* 1997). We hypothesize that, in the prostate of elderly men, aromatase expression in PrSCs is

induced by adjacent epithelial cells through secretion of a paracrine factor, leading to the increased production of estrogen. Herein, we report that this paracrine factor is prostaglandin E2 (PGE2).

Materials and Methods

Cell lines

The benign prostate hyperplastic epithelial cell line BPH-1 was provided by Dr Helmut Klocker (Innsbruck Medical University, Austria). Prostate cancer epithelial cell lines, LNCaP, DU-145, and PC-3, were purchased from American Type Culture Collection (Rockville, MD, USA).

Collection of conditioned medium (CM)

BPH-1, LNCaP, DU-145, and PC-3 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics. Upon reaching 30–40% confluence, the cultures were changed to DMEM/F12 serum-free medium and were continuously cultured for 48 h. The CM was normalized to the same cell number for different cell lines. Then media were collected, centrifuged at 300 *g* for 3 min, and stored at –20 °C until used.

Analyses of active factor in CM that induced expression of aromatase

CM was boiled for 10 min and cooled down. The CM and DMEM/F12 medium were extracted with chloroform thrice. Aqueous phase was collected, filtered, and stored in -20 °C; organic phase was freeze-dried, redissolved in DMEM/F12 medium, filtered, and then stored in -20 °C.

Primary culture of PrSCs

Human PrSCs were derived from fresh surgical prostate specimens of BPH patients with informed consent and cultured as described previously by us (Zhang *et al.* 1997). After four to five passages, the cells were harvested and resuspended at a concentration of 4×10⁴ cells/ml. An aliquot of 4 ml cell suspension was seeded in a 25 cm² flask. After the cells were attached, they were changed to DMEM/F12 serum-free medium and cultured continuously for 24 h. Change of culture medium was performed by replacing 1/3 volume of the medium with the CM from different cell lines. For RNA extraction, cells were harvested after 24 h. For protein extraction, whole cell lysate was prepared after 48 h.

RNA extraction, RT-PCR, and real-time RT-PCR

Total RNA was isolated from the cells using Trizol reagent (Invitrogen) and reverse transcribed. RT-PCR was carried out on a DNA-Engine Thermocycler (Bio-Rad). Real-time quantitative PCR was carried out on a MJ Research DNA Engine Opticon Continuous Fluorescence Detection System (Opticon Monitor II, MJ Research Inc., Waltham MA, USA) using SYBR Green I-based method. The relative gene

expression was determined using the comparative C_T method and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The primers used are listed in Table 1.

Western blot analysis

Protein was extracted with RIPA buffer and was quantified using Bradford method. Equal amount of protein (35 µg) was loaded to each well. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 10% non-immune serum for 1 h, and then incubated with primary antibody (Aromatase, mouse monoclonal antibody, 1:200, Serotec, MCA2077T) at 4 °C overnight. After washing three times with TBST (Tris-buffered saline with 0.1% Tween-20) buffer, the membrane was incubated with the secondary antibody (goat-anti-mouse horseradish peroxidase (HRP)-conjugated 1:3000, Bio-Rad, 1706516) at room temperature for 1 h, developed by enhanced chemiluminescence and visualized with Hyperfilm-ECL (Amersham, Piscataway, NJ, USA).

ELISA

The concentration of PGE2 in CM from different prostate epithelial cells was determined with ELISA-Kit (Minneapolis, MN, USA, R&D, DE2100) following manufacturer's protocol.

Statistical analysis

Data are expressed as mean±s.d. Significance was assessed using Student's paired *t*-test. **P*<0.05 was considered as significant.

Table 1 Primers used in RT-PCR

Gene	Primer sequences (5'–3')	Product length (bp)	Annealing temperature (°C)
GAPDH	Forward GGGGAGCCAAAGGGTCATCATCT	457	58
	Reverse GACGCCTGCTTCACCACTTCTTG		
Aromatase	Forward GAATATTGGAAGGATGCACAGACT	342	58
	Reverse GGGTAAAGATCATTCCAGCATGT		
COX-2	Forward ATAAGTGCGATTGTACCCG	247	52
	Reverse TAGCCATAGTCAGCATTGTAAGT		
EP1	Forward CGCTATGAGCTGCAGTACCC	508	55
	Reverse CCAGGATCTGGTTCCAGGAG		
EP2	Forward CAACCTCATCCGCATGCAC	419	55
	Reverse CTCAAAGGTCAGCCTG		
EP3	Forward ACCCGCCTCAACCACTCTACACA	410	63
	Reverse ATGGCGCTGGCGATGAACAAC		
EP4	Forward CCTCCTGAGAAAGACAGTGCT	366	54
	Reverse AAGACACTCTCTGAGTCCT		

Results

The effect of CM from different prostatic epithelial cell lines on the expression of aromatase in PrSCs

To examine the effect of CM from different prostatic epithelial cell lines on the expression of aromatase, we first treated PrSCs with CMs from different cell lines and then we performed real-time RT-PCR to detect mRNA expression of aromatase. In BPH-1 CM-treated PrSCs, mRNA expression of aromatase increased significantly. For comparison, CMs from DU-145, LNCaP, and PC-3 have little effect on the mRNA expression of aromatase (Fig. 1A). To further examine the effect of different CMs on expression of aromatase, we performed western blot analysis to determine the expression of aromatase at the protein level. In cells treated with BPH-1 CM, the protein expression of aromatase is significantly higher than those treated with CMs from other cell lines. This finding is consistent with the result obtained from real-time RT-PCR (Fig. 1B).

Characterization of the active component in BPH-1 CM that induces the expression of aromatase

To characterize the active factor in BPH-1 CM that induced the expression of aromatase, the BPH-1 CM was processed with different methods (boiling or extracting with organic reagent). Our results showed that the boiled BPH-1 CM retained the ability to induce aromatase expression. Following the extraction of the BPH-1 CM with chloroform, we separated the organic phase from the aqueous phase. The organic phase fraction was still able to induce the expression of aromatase, while aqueous phase fraction had little effect on aromatase expression. As a control, the organic phase fraction extracted from serum-free culture medium had no effect on aromatase expression (Fig. 1C).

The expression of cyclooxygenase-2 (COX-2) in different prostatic epithelial cell lines

The above results suggested that the active component that induced aromatase expression might be a non-protein substance. Recent studies showed that prostaglandin receptor activated by PGE2 could bind the PII promoter and then increase the aromatase transcription; therefore, we tested the expression of COX-2, a key enzyme in PGE2 syntheses, in different prostatic epithelial cell lines. In addition, we measured directly the concentration of PGE2 in different CMs. Our results showed that the expression of COX-2 was significantly higher in BPH-1 than in other cell lines (Fig. 2A). The concentration of PGE2 in BPH-1 CM was significantly higher than that of CMs from other cell lines (Fig. 2B).

Figure 1 (A) Real-time RT-PCR analysis of aromatase mRNA expression in PrSCs treated with CM from different prostatic epithelial cell lines. $n=4$, $*P<0.05$ versus control. (B) Representative western blotting results of aromatase in PrSCs treated with CM from different prostatic epithelial cell lines. (C) Real-time RT-PCR analysis of aromatase mRNA expression in PrSCs treated with different components of BPH-1 CM. 1. Control; 2. BPH-1 CM; 3. Organic compound extracted with chloroform from BPH-1 CM; 4. Aqueous phase after extraction with chloroform from BPH-1 CM; 5. Organic compound extracted with chloroform from serum-free DMEM/F12; 6. Boiled BPH-1 CM.

Figure 2 (A) Real-time RT-PCR analysis of COX-2 mRNA expression in different prostatic epithelial cell lines. (B) ELISA of PGE2 concentration (pg/ml) in CM of different prostatic epithelial cell lines. Error bars: S.D., $n=3$.

Figure 3 ELISA of PGE2 in CM of BPH-1 treated with NS-398 (10^{-6} mol/l). $n=3$, * $P<0.05$ versus control.

Figure 4 (A) Real-time RT-PCR analysis of aromatase mRNA expression in PrSCs treated with BPH-1 CM with or without NS-398 (10^{-6} mol/l) and PGE2 (10^{-7} mol/l). $n=4$, * $P<0.05$ versus control, ** $P<0.05$ versus BPH-1 CM. (B) Representative western blotting results of aromatase expression in PrSCs treated with BPH-1 CM with or without NS-398 (10^{-6} mol/l) and PGE2 (10^{-7} mol/l). (C) RT-PCR analysis of prostaglandin E receptors subtype EP1, EP2, EP3, and EP4 mRNA expression in PrSCs. Lanes 1–4: subtypes of EP1–4; M: DNA marker.

BPH-1 induced aromatase expression of PrSCs through the production of PGE2

To further confirm that the active component responsible for the induction of aromatase expression is indeed PGE2, we added NS-398 (Cayman, Cat No. 70590), a COX-2 specific inhibitor, into the culture medium of BPH-1 cells. The PGE2 concentration in NS-398-treated CM was significantly lower than non-treated BPH-1 CM (Fig. 3). We next examined the effect of NS-398 on aromatase expression at both the mRNA level (Fig. 4A) and the protein level (Fig. 4B). PGE2

(0.1 μ mol/l) induced the expression of aromatase (fifth sample in Fig. 4A and B). Treatment of NS-398 to PrSCs alone had no direct effect on aromatase expression (fourth sample in Fig. 4A and B). Treatment of BPH-1 CM was able to induce the expression of aromatase (second sample in Fig. 4A and B). However, BPH-1 CM derived from cells cultured with NS-398 failed to induce aromatase expression (third samples in Fig. 4A and B), indicating that the active component secreted by BPH-1 to induce aromatase expression is PGE2.

To characterize the receptor subtype, we performed an RT-PCR analysis for the four subtypes of prostaglandin E receptors in PrSCs. The results showed that EP2 and EP3 subtypes were expressed at high level, which may be involved in PGE2 regulation of aromatase expression in PrSCs (Fig. 4C).

Discussion

Aromatase is the critical enzyme that converts testosterone into E2, and its expression was prominently high in PrSCs within hyperplastic glands in BPH patients (Hiramatsu *et al.* 1997). Herein, we provided evidence to indicate that prostatic epithelial cells induced PrSCs to express aromatase through the secretion of PGE2.

We first compared the effects of CMs from different prostatic epithelial cell lines on the expression of aromatase in PrSCs. Only CMs from BPH-1, a prostatic hyperplastic epithelial cell line, can induce aromatase expression, while the CMs from prostatic cancer epithelial cell lines (LNCaP, PC-3, and DU-145) had little effect on aromatase expression. Our observations indicated that prostatic epithelial cells could induce the expression of aromatase, resulting in the increase of estrogen concentration in prostatic stroma.

The expression of aromatase is highly tissue specific. The complex expression and regulation of aromatase is achieved through the use of multiple exon 1 that encodes the 5' untranslated region (Sebastian & Bulun 2001). Therefore, the regulation of aromatase expression is very complicated, many growth factors, chemokines, and steroid hormones can regulate expression of aromatase (Simpson *et al.* 1997). Initially, we attempted to identify the active component that induced aromatase expression by boiling BPH-1 CM or extracting BPH-1 CM with chloroform. Following boiling, most proteins were denatured, whereas BPH-1 CM could still induce aromatase expression, indicating that the factor in BPH-1 CM regulating aromatase expression is a material other than proteins. Organic phase but not aqueous phase from BPH-1 CM chloroform extraction can induce aromatase expression, while organic phase extracted from serum-free culture medium had no effect on aromatase expression, which further suggested that active component in BPH-1 CM was a material other than protein, probably acyclic/alicyclic or steroid molecules.

Recent studies reported that PGE2 up-regulated aromatase expression in breast cancer (Zhao *et al.* 1996), endometriosis and

uterine leiomyomata (Bulun *et al.* 2005), and adrenocortical carcinoma cell lines (Watanabe *et al.* 2006). COX-2 is a key enzyme in PGE2 biosyntheses. We examined COX-2 expression in different epithelial cell lines by real-time RT-PCR. Our results showed that the expression of COX-2 in BPH-1 was significantly higher than prostatic cancer cell lines, and BPH-1 cells secreted more PGE2 than prostatic cancer cell lines. NS-398 is a COX-2 specific inhibitor. At certain range of concentrations, it inhibits COX-2 activity but has no effect on COX-1. Therefore, we added NS-398 into our BPH-1 culture medium to inhibit PGE2 syntheses, which abolished the ability of BPH-1 CM to induce aromatase expression. Separately, the expression of aromatase in PrSCs increased dramatically by stimulating cells directly with PGE2, indicating that the factor secreted by BPH-1 to induce aromatase expression in PrSCs is indeed PGE2.

In conclusion, the results of the present study suggested that prostatic epithelial cells induced aromatase expression in PrSCs through the production of PGE2 in a paracrine mechanism. We assume that in the prostate of elderly men, epithelial cells induced aromatase expression of PrSCs by the production of PGE2 in a paracrine manner. The high levels of aromatase expression led to an increase in estrogen, which may be involved in the pathogenesis of BPH.

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References

- Bulun SE, Imir G, Utsunomiya H, Thung S, Gurates B, Tamura M & Lin Z 2005 Aromatase in endometriosis and uterine leiomyomata. *Journal of Steroid Biochemistry and Molecular Biology* **95** 57–62.
- Fisher CR, Graves KH, Parlow AF & Simpson ER 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. *PNAS* **95** 6965–6970.
- Hiramatsu M, Maehara I, Ozaki M, Harada N, Orikasa S & Sasano H 1997 Aromatase in hyperplasia and carcinoma of the human prostate. *Prostate* **31** 118–124.
- Isidori AM, Strollo F, More M, Caprio M, Aversa A, Moretti C, Frajese G, Riondino G & Fabbri A 2000 Leptin and aging: correlation with endocrine changes in male and female healthy adult populations of different body weights. *Journal of Clinical Endocrinology and Metabolism* **85** 1954–1962.
- Kaburagi Y, Marino MB, Kirdani RY, Greco JP, Karr JP & Sandberg AA 1987 The possibility of aromatization of androgen in human prostate. *Journal of Steroid Biochemistry* **26** 739–742.
- Krieg M, Nass R & Tunn S 1993 Effect of aging on endogenous level of 5 alpha-dihydrotestosterone, testosterone, estradiol, and estrone in epithelium and stroma of normal and hyperplastic human prostate. *Journal of Clinical Endocrinology and Metabolism* **77** 375–381.

- Matzkin H & Soloway MS 1992 Immunohistochemical evidence of the existence and localization of aromatase in human prostatic tissues. *Prostate* **21** 309–314.
- Negri-Cesi P, Colciago A, Poletti A & Motta M 1999 5alpha-reductase isozymes and aromatase are differentially expressed and active in the androgen-independent human prostate cancer cell lines DU145 and PC3. *Prostate* **41** 224–232.
- Rubens R, Dhont M & Vermeulen A 1974 Further studies on Leydig cell function in old age. *Journal of Clinical Endocrinology and Metabolism* **39** 40–45.
- Sebastian S & Bulun SE 2001 A highly complex organization of the regulatory region of the human *CYP19* (aromatase) gene revealed by the Human Genome Project. *Journal of Clinical Endocrinology and Metabolism* **86** 4600–4602.
- Seppelt U 1978 Correlation among prostate stroma, plasma estrogen levels, and urinary estrogen excretion in patients with benign prostatic hypertrophy. *Journal of Clinical Endocrinology and Metabolism* **47** 1230–1235.
- Shibata Y, Ito K, Suzuki K, Nakano K, Fukabori Y, Suzuki R, Kawabe Y, Honma S & Yamanaka H 2000 Changes in the endocrine environment of the human prostate transition zone with aging: simultaneous quantitative analysis of prostatic sex steroids and comparison with human prostatic histological composition. *Prostate* **42** 45–55.
- Simpson ER, Michael MD, Agarwal VR, Hinshelwood MM, Bulun SE & Zhao Y 1997 Cytochromes P450 11: expression of the *CYP19* (aromatase) gene: an unusual case of alternative promoter usage. *FASEB Journal* **11** 29–36.
- Stone NN, Laudone VP, Fair WR & Fishman J 1987 Aromatization of androstenedione to estrogen by benign prostatic hyperplasia, prostate cancer and expressed prostatic secretions. *Urological Research* **15** 165–167.
- Tsugaya M, Harada N, Tozawa K, Yamada Y, Hayashi Y, Tanaka S & Maruyama K 1996 Aromatase mRNA levels in benign prostatic hyperplasia and prostate cancer. *International Journal of Urology* **3** 292–296.
- Watanabe M, Noda M & Nakajin S 2006 Effect of epidermal growth factor and prostaglandin on the expression of aromatase (*CYP19*) in human adrenocortical carcinoma cell line NCI-H295R cells. *Journal of Endocrinology* **188** 59–68.
- Zhang J, Hess MW, Thurnher M, Hobisch A, Radmayr C, Cronauer MV, Hittmair A, Culig Z, Bartsch G & Klocker H 1997 Human prostatic smooth muscle cells in culture: estradiol enhances expression of smooth muscle cell-specific markers. *Prostate* **30** 117–129.
- Zhao Y, Agarwal VR, Mendelson CR & Simpson ER 1996 Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the *CYP19* (aromatase) gene. *Endocrinology* **137** 5739–5742.

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