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17β-Estradiol affects proliferation and apoptosis of rat prostatic smooth muscle cells by modulating cell cycle transition and related proteins

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Abstract

Abundant evidence indicates that estrogens have an important role in the pathology of benign prostatic hyperplasia (BPH). To investigate the effect of 17β-estradiol (E_2) on the proliferation and apoptosis of prostatic smooth muscle cells (PSMCs), rat PSMCs were obtained and exposed to gradient concentrations (0.1−100 nmol/l) of E_2 over varying amounts of time. The progression of cell cycle, cellular apoptosis, cyclin D1, Bcl-2 and Bax proteins were detected. The data show that the effect of E_2 on rat PSMCs is bilateral: it promotes cell proliferation by enhancing the expression of cyclin D1, which accelerates G_1 to S phase transition; on the other hand, it induces apoptosis of the cells by up-regulating the expression of Bax. We thus suggest that an increase in estrogen may exert a launching effect in the pathology of BPH.

Keywords: Estradiol; Prostate; Smooth muscle cell; Proliferation; Apoptosis; Cell cycle

1. Introduction

Benign prostatic hyperplasia (BPH) is the most common benign neoplasm in older men (Isaacs, 1990). It is well accepted that the disease is associated with aging and the presence of functional testes (Rotkin, 1983). The prostate is an androgen-dependent organ and it is the mesenchyme, rather than the epithelium, that is the major target for androgen (Cunha et al., 1987). However, the action of androgen alone cannot explain the development of BPH (Coffey and Pienta, 1987). For instance, it is still unclear why the prostate does not develop into hyperplasia in young males who have high levels of serum androgen yet does develop into hyperplasia in older males with low androgen levels and who are prone to suffer from this disturbance. One of the prevalent hypotheses is that estrogen may have an important role in the pathology of BPH (Farnsworth, 1999; Grayhack, 1965).

McNeal (1990) suggests that BPH is primarily a stromal disease that originates in the periurethral transition region of the prostate. Within BPH it has been shown that the stroma contains about three times more estrone and estradiol than the epithelium (Kozák et al., 1982). In castrated dogs, a glandular form of BPH was induced by a combination of treatment of 17β-estradiol together with androstanediol, whereas androgen alone failed to produce this effect (Juniewicz et al., 1989). Rhodes et al. (2000) found that estradiol caused a dose-dependent stimulation of prostate growth. Estradiol can stimulate *in vitro*-cultured stromal cells of the prostate to proliferate (Collins et al., 1994). Moreover, the estrogen receptor has been located in the prostate stroma (Konishi et al., 1993; Royuela et al., 2001). In short, accumulated evidence points to a role of estrogen in BPH.

Smooth muscle cells are the major cellular components of human BPH tissue (Shapiro et al., 1992). In the guinea pigs, smooth muscle is the predominant component of the prostatic stroma (Tilley et al., 1985). The proliferation of smooth muscle cell results in the dynamic obstruction of the bladder outlet. Some evidence indicates that estrogen may directly stimulate the prostatic smooth muscle cell (PSMC) rather than the fibroblast (Levine et al., 1992).

Abbreviations: BPH, benign prostatic hyperplasia; PSMC, prostatic smooth muscle cell; E_2, 17β-estradiol.

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However, little information has been collected on the mechanism of how the estrogen affects the PSMC *in vitro*. The purpose of this paper is to investigate the effect of 17β-estradiol (E_2) on the proliferation and apoptosis of PSMC via cell cycle analysis and related protein detection.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats, weighing 233 ± 28 g and bred in Wuhan University's experimental animal center were used for these studies and maintained in a controlled environment with free access to food and water. The animal use protocol is approved by the Institutional Animal Care and Use Committee of Wuhan University.

2.2. Materials

Phenol red-free RPMI 1640 medium, charcoal/dextran treated fetal bovine serum (FBS) and standard FBS were obtained from HyClone Laboratories Inc. (UT, USA). E_2 (E4389) and soybean trypsin inhibitor were purchased from Sigma–Aldrich (MO, USA). Mouse or rabbit monoclonal antibodies to cyclin D1 (sc-8396), Bax (sc-526) and Bcl-2 (sc-783) were obtained from Santa Cruz Biotechnology (CA, USA). The α-smooth muscle actin (α-SMA) and desmin antibodies were purchased from Bioss Co. (Beijing, China) and Boster Co. (Wuhan, China), respectively. An ELISA Cell Death Detection kit was obtained from the Roche Diagnostics Corporation (Basel, Switzerland).

2.3. PSMC culture

PSMCs were enzyme-dispersed using a modified method originally described by Ricciardelli et al. (1989). Briefly, aseptically dissected ventral prostate was placed in a cold D-Hank's balanced salt solution. After removing the connective tissue, the prostate was cut into small pieces (about 1–3 mm^3) and incubated in 2 g/l collagenase II (Invitrogen, Carlsbad, CA, USA) with 0.5 g/l soybean trypsin inhibitor. After digestion, the tissues were transferred into a centrifuge tube containing 3 ml medium with 100 ml/l FBS and centrifuged at 70×*g* for 5 min. The cell pellet was re-suspended and plated at a density of 1×10^4/ml into a 50 ml culture flask. The preferential adhesion technique was used to reduce contaminating fibroblasts at this stage. Because of the known estrogenic effects of phenol red, the cells were cultured in phenol red-free RPMI 1640 containing 100 ml/l FBS, 1×10^5 units/l penicillin, 100 ng/l streptomycin and 4 mmol/l L-glutamine at 37 °C in 5% CO_2. The α-SMA and desmin antibodies were used to identify the cells by immunocytochemistry. *Passages of 3–4* were used for this study.

2.4. Analysis of PSMC growth

The effect of E_2 on PSMC growth was determined through cell counting. The cells were plated into 6-well plastic plates at a seeding density of 3×10^5 cells/well, and cultured in medium with or without 10 nmol/l E_2, respectively, in the presence of 100 ml/l charcoal/dextran treated FBS. On the indicated days, triplicate wells were trypsinized and re-suspended in 10 ml of isotonic saline solution. Duplicate samples from each well were counted with a haemocytometer under a phase-contrast microscope. To determine whether the effect of E_2 on PSMC growth is dose-dependent, the cells were cultured in medium with gradient concentrations of E_2 (0–100 nmol/l) for 3 days in the presence of 100 ml/l charcoal/dextran treated FBS.

2.5. Analysis of cell cycle progression

To investigate the effect of E_2 on the cell cycle progression of the PSMC, the cells were seeded into 6-well plastic plates at 3×10^5 cells/well and incubated for 2 days in the medium with 100 ml/l standard FBS. After 24 h of serum deprivation to synchronize their cell cycles, the PSMCs were re-stimulated with 100 ml/l charcoal/dextran FBS and gradient concentrations of E_2 (0–100 nmol/l) for a further 3 days. The cells were then harvested by trypsinization and fixed in 700 ml/l cold ethanol at 4 °C for 10 h. The cells were washed twice with ice-cold PBS buffer, and incubated with RNase (100 mg/l) and DNA intercalating dye propidium iodide (50 mg/l) for 30 min in a 37 °C aqueous bath before analysis. The cell cycle phases were analyzed using an FC500 flow cytometer and CXP software (Beckman Coulter, Mountain View, CA, USA). A minimum of 1×10^4 events were analyzed. Triplicate samples were assessed for each group and each assay was repeated twice. The proliferative index (PI) was calculated with the formula: $\mathrm{PI}\ (\%) = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$.

2.6. Detections of apoptosis

The apoptotic rate represented by the percentage of sub-G_1 peak in flow cytometry histogram with propidium iodide stain was used to estimate the number of apoptotic cells. To retrieve the discrepancy of the above assay in discriminating the apoptotic cell and corpuscle fragment, the Cell Death Detection ELISA Plus kit was used to measure histone-bound DNA fragments (nucleosome) in an ELISA format. The cells were treated for 3 days with different concentrations (0–100 nmol/l) of E_2. Three samples from each group were prepared according to the protocol provided by the manufacturer and analyzed on a microplate spectrophotometer at 405 nm. Data were expressed as means of absorbance from triplicate experiments performed in each sample.

2.7. Protein assay for cyclin D1

To investigate the latent mechanisms of the cell cycle promoting effect of E_2, a regulator for the G_1 checkpoint, cyclin D1, was analyzed through flow cytometry. The PSMCs treated with or without 10 nmol/l of E_2 for 1–5 days were fixed in suspension in 37 g/l paraformaldehyde, washed with PBS and treated with 2 g/l Triton-X100 and 50 g/l block serum

for 15 min on ice. After washing, the cells were incubated with the primary antibody to cyclin D1 for 45 min on ice, followed by staining with the corresponding FITC-conjugated second antibody for 45 min. Then the washed samples were placed in tubes and read on the FC500 flow cytometer. The control cells were incubated in the absence of the primary antibody. The relative expression levels of the tested proteins were expressed by FITC fluorescence intensity.

2.8. Protein assays for Bcl-2 and Bax

To investigate the mechanisms underlying E_2-induced PSMC apoptosis, two apoptosis-related proteins, Bax and Bcl-2, were examined. The cells treated as above were collected and lysed in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). Following centrifugation at 12,000×*g* for 5 min at 4 °C, the supernatants were collected and stored at −70 °C until use. Equal amounts of protein extracts (20 mg/lane) were subjected to SDS-PAGE on a 10% separating gel and electrophoretically transferred onto PVDF membrane. After being blocked with 50 g/l skim milk powder and 1 g/l Tween-20 in TBS buffer for 1 h, the membranes were then incubated with either anti-Bax or anti-Bcl-2 antibody for 10 h at 4 °C, followed by the corresponding horseradish peroxidase-conjugated second antibody for another 1 h. Anti-β-actin antibody was used as an internal standard for protein concentration and integrity. The reaction was visualized by DAB staining. Quantitative analysis for all the pixels in each band was carried out with GeneTools software (Syngene, Cambridge, UK). The relative expression levels of the proteins were expressed as ratio of Bax or Bcl-2 raw volumes (integrated intensity of all the pixels in each band) divided by the corresponding β-actin value.

2.9. Statistical analysis

Data were expressed as mean ± standard deviation (SD). SPSS 13.0 software (SPSS Inc., IL, USA) was used in the process. Comparison of data in more than two groups was analyzed by one-way ANOVA with a post hoc SNK test. The independent samples *t* test was used for a comparison of data between two groups. The difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. The effect of E_2 on PSMC growth

As shown in Fig. 1, the average numbers of the control and 10 nmol/l E_2-treated cells were very similar on Day 1. On Days 2–4, the average cell numbers in the E_2-treated group were significantly higher than those in control cultures. By Day 5, the two groups became confluent. The result indicates that E_2 had a transient growth-promoting effect on the cells. Fig. 2 shows that the effect of E_2 on the PSMC was dose-dependent at the concentrations from 0.1 nmol/l to 10 nmol/l.

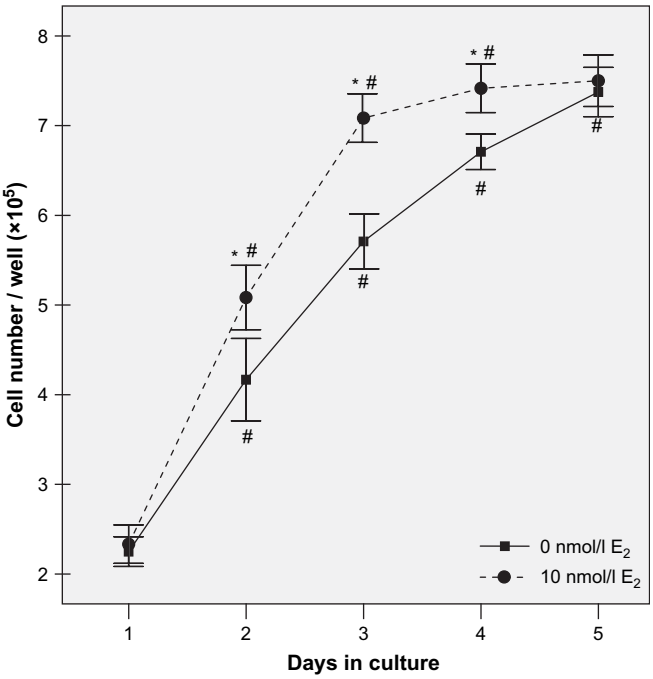

Fig. 1. Cell count for growth of PSMCs treated with or without 10 nmol/l of E_2 on different days. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with the previous adjacent group.

However, when the E_2 concentrations were higher than 10 nmol/l, the effect was decreased.

3.2. The effect of E_2 on PSMC cell cycle progression

Cell cycle analysis (Table 1 and Fig. 3) shows that at the concentrations from 0.1 nmol/l to 10 nmol/l, the rates of the PSMC at the G_0/G_1 phase were significantly decreased, while those at the *S* and G_2/M phase increased in a concentration-dependent manner, which resulted in a significant increase

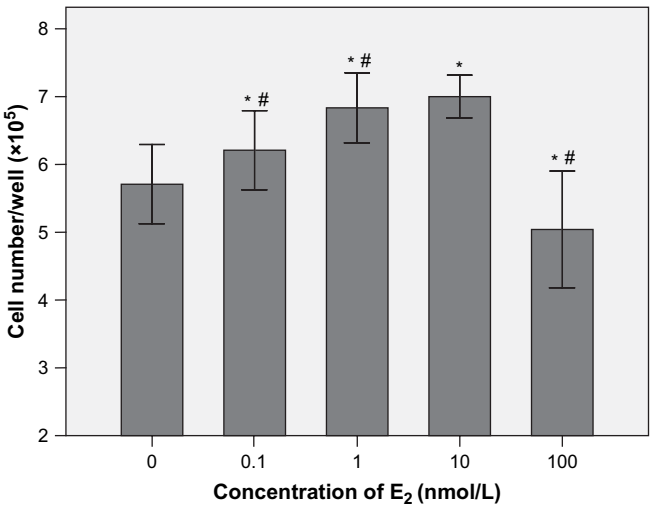

Fig. 2. Cell count for growth of PSMCs treated with different concentrations of E_2 for 72 h. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with the previous adjacent group.

Table 1
Cell cycle progression of PSMCs treated with different concentrations of E_2 for 3 days (%, mean ± SD)

Concentrations of E_2 (nmol/l)	G_0/G_1	S	G_2/M	PI
0	74.98 ± 5.19	12.39 ± 2.64	12.63 ± 3.74	25.02 ± 5.19
0.1	68.85 ± 2.31*,#	13.50 ± 2.26	17.66 ± 2.05	31.15 ± 2.31*,#
1	43.63 ± 5.98*,#	18.50 ± 4.98*,#	37.87 ± 9.67*,#	56.36 ± 5.98*,#
10	41.50 ± 4.00*	21.16 ± 4.83*	37.34 ± 7.35*	58.50 ± 4.00*
100	80.09 ± 2.37*,#	7.98 ± 1.92*,#	11.92 ± 1.15,#	19.91 ± 2.37*,#

*$P < 0.05$, compared with the control group; #$P < 0.05$, compared with the previous adjacent group.

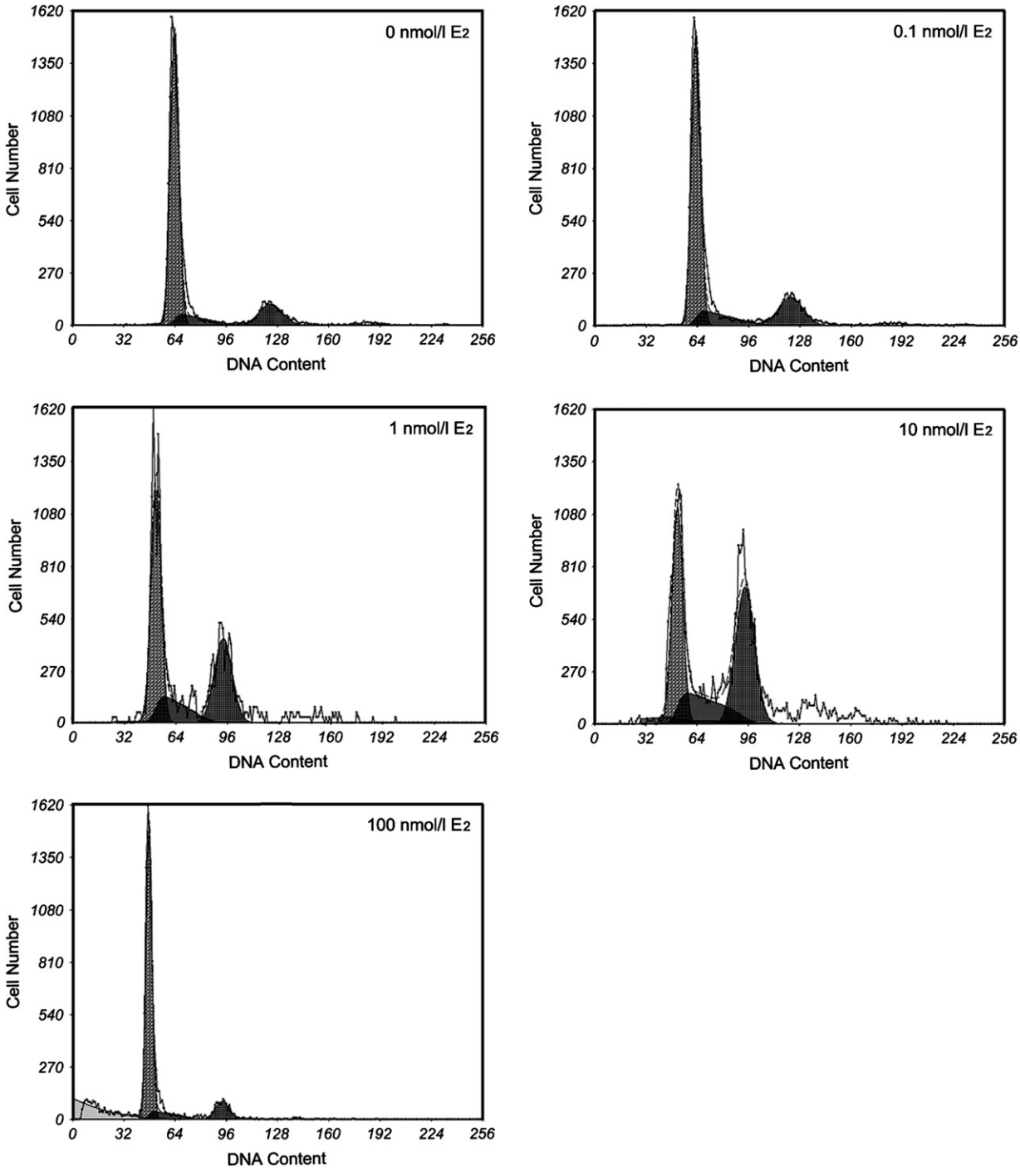

Fig. 3. Representative histograms of flow cytometric analysis for cell cycle distribution of synthetic PSMCs treated with different concentrations of E_2 for 3 days. The cells were labelled with propidium iodide. Samples were analyzed under the excitation light of 488 nm and detected at 610 nm.

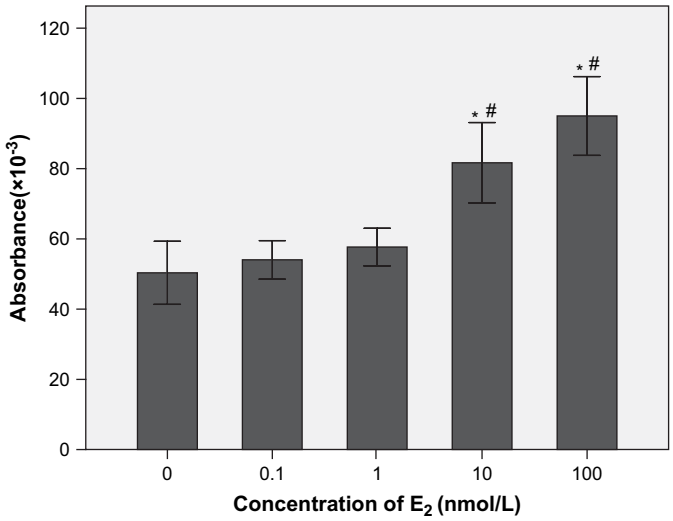

Fig. 4. ELISA analysis for nucleosomes in PSMCs treated with different concentrations of E_2 for 3 days. The relative levels of nucleosomes are expressed as average values of absorbance at 405 nm. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the previous adjacent group.

of proliferative index. It indicates that E_2 stimulates the growth of PSMCs by accelerating their cell cycle progression from the G_1 to the S and G_2 phases. When the concentration of E_2 reached 100 nmol/l, self-inhibition of the hormone's action was also observed. The sub-G_1 population appeared and increased *along with accruement* in concentrations of E_2 (Fig. 3).

3.3. The effect of E_2 on apoptosis

FCM analysis on the sub-G_1 rate reveals that the absorbance of PSMC in the 10 nmol/l and 100 nmol/l groups was 2.12 ± 0.41 and 4.59 ± 0.96 on Day 3, respectively. Both of them presented significant differences to the control group (1.09 ± 0.33), $P < 0.01$. The other two groups (0.1 nmol/l and 1 nmol/l) did not demonstrate significant differences compared to the control. Results indicated that the apoptotic rates rose significantly in cells of the 10 nmol/l and 100 nmol/l groups. This result was compatible with the ELISA data (as shown in Fig. 4). A time course study through FCM analysis demonstrated that at the same concentration (10 nmol/l), E_2-induced apoptosis was time-dependent (Table 2).

3.4. The effect of E_2 on cyclin D1 protein expressions

Administration of 10 nmol/l of E_2 to the PSMC cultures induced a significant increase in the expression levels of cyclin D1 in comparison with the control cells in all culture durations (Table 3). However, cyclin D1 showed a tendency to decrease on Day 5.

Table 2
Apoptosis rate of PSMC treated with different concentrations of E_2 on different days determined by flow cytometry (%, mean ± SD)

Concentrations of E_2 (nmol/l)	1 day	3 days	5 days
0	0.86 ± 0.40	1.09 ± 0.33	1.18 ± 0.32
10	1.22 ± 0.20	$2.12 \pm 0.41^{*}$	$4.33 \pm 1.71^{*,\#}$

$^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with the group of previous adjacent checkpoint at the same concentration.

Table 3
Expressions of cyclin D1 by PSMCs treated with different concentrations of E_2 on different days (fluorescence channel, mean ± SD)

Concentrations of E_2 (nmol/l)	1 day	3 days	5 days
0	2.59 ± 0.46	2.61 ± 0.35	2.52 ± 0.30
10	$3.96 \pm 0.50^{*}$	$5.59 \pm 0.53^{*,\#}$	$3.68 \pm 0.43^{*,\#}$

$^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the group of previous adjacent checkpoint at the same concentration.

3.5. The effects of E_2 on expressions of Bax and Bcl-2

Western blotting analysis showed that after the exposure of the PSMC to 10 nmol/l E_2, the levels of Bcl-2 showed no obvious changes in comparison with the control cells (Table 4 and Fig. 5), but the expression of Bax was significantly increased, leading to a corresponding increment in the ratio of Bax/Bcl-2. Moreover, Bax protein was augmented in a time-dependent manner.

4. Discussion

The widely accepted hypothesis that BPH is caused by androgens and aging remains imperfect, as some contradictions are difficult to clarify. The important role of estrogen in etiology of BPH has attracted much attention in the recent decades. Krieg et al. (1993) attributed the decline in epithelial dihydrotestosterone levels with age to a concurrent fall in 5α-reductase in the epithelial cells. Tissue testosterone levels were all low and unaffected by donor age. However, prostatic stromal estradiol and estrone levels of BPH patients increased very significantly with age (Farnsworth, 1999). Estrogens can stimulate the growth of stromal cells derived from hyperplastic prostate (Collins et al., 1994). Ricciardelli et al. (1994) put forward that smooth muscle cells were the target of estrogen by studying the effect of E_2 on guinea-pig smooth muscle prostate cells *in vitro*. As far as the mechanism is concerned, Ricciardelli suggested that E_2 stimulates proliferation of guinea-pig prostate smooth muscle cells *in vitro* by an estrogen

Table 4
Expression of Bax and Bcl-2 by PSMC treated with or without 10 nmol/l of E_2 in different culture durations (integrated intensity ratio, mean ± SD)

Group (time and concentration of E_2)		Bax	Bcl-2	Bax/Bcl-2
1 day	0 nmol/l	0.95 ± 0.09	0.26 ± 0.04	3.74 ± 0.67
	10 nmol/l	0.99 ± 0.08	0.29 ± 0.06	3.46 ± 0.70
3 days	0 nmol/l	0.93 ± 0.13	0.32 ± 0.12	3.31 ± 1.32
	10 nmol/l	$1.15 \pm 0.08^{*,\#}$	0.24 ± 0.04	$4.87 \pm 0.62^{*,\#}$
5 days	0 nmol/l	0.99 ± 0.11	0.30 ± 0.06	3.48 ± 0.87
	10 nmol/l	$1.27 \pm 0.07^{*,\#}$	0.27 ± 0.03	$4.76 \pm 0.68^{*}$

$^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the group of the previous adjacent checkpoint at the same concentration.

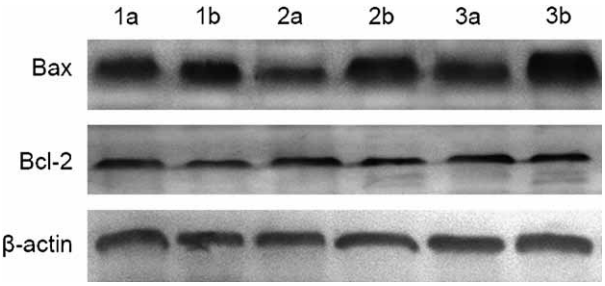

Fig. 5. Representative graphs of Western blotting analysis for Bax and Bcl-2 proteins from PSMC samples treated without (A) or with (B) 10 nmol/l of E_2 in different culture durations. Lane 1, 2 and 3 show that the cells were treated for 1 day, 3 days and 5 days, respectively. The equal loading of the samples was confirmed by β-actin as an internal control.

receptor-dependent mechanism. Hong et al. (2004) found that estrogen could stimulate the growth of prostatic stromal cells and increase smooth muscle cell markers, which may be achieved through a pathway involving TGF-beta 1. However, there have been opposite findings. García-Flórez et al. (2005) found that administering E_2 to castrated rats decreased the absolute volume of the PSMC in the rat ventral prostate. Levine et al. (1992) found estrogens did not stimulate the proliferation of their stromal cell cultures.

The present study shows that the effect of E_2 on subcultured PSMC is bilateral: it promotes cell proliferation by enhancing the expression of cyclin D1, which accelerates G_1 to S phase transition. On the other hand, it also induces apoptosis of the cells by up-regulating the expression of Bax at high concentrations. These results agree with the study of Scarano et al. (2005), who showed that hypertrophy of smooth muscle cells was observed in the estradiol-treated guinea pigs through histological and histochemical procedures.

It is well established that cyclin D1 is one of the key regulators that drives a cell from G_1 to S phase (Donnellan and Chetty, 1998). Estrogens, which activate cyclin D1 gene expression with estrogen receptor-α, inhibit expression with estrogen receptor-β (Liu et al., 2002). Our results reveal the modulating role of cyclin D1. However, we could not reveal the change of estrogen receptor subtype from this study.

In our experiment, E_2 treatment did not affect the expression of Bcl-2, but resulted in an up-regulation of Bax, leading to an increased ratio of Bax/Bcl-2, which is accepted as a crucial factor in triggering apoptosis. When the hormone reached a critical concentration, Bax-induced apoptosis overwhelmed the proliferation-promoting effect of E_2 and the growth of PSMCs demonstrated a self-inhibitory character. This observation may be related to the fact that complex interactions of hormones were inhibited after adding activated charcoal and dextran to the serum to the culture; thus the use of E_2 alone manifested a common character of hormones: i.e., low concentrations of a hormone can stimulate a tissue, while high concentrations have the opposite effect. vom Saal et al. (1997) found that when fetal mice were exposed to estradiol or diethylstilbestrol, prostate weight first increased then decreased with every dose, resulting in an inverted-U dose-response relationship. Our results support their result *in vivo*, although the curve was not obvious. Arguably a much greater range of doses of estradiol was required to show the inverted-U dose-response relationship.

Furthermore, we found that when we increased the culture time in a fixed concentration (10 nmol/l) of E_2, the proliferative index did not increase infinitely and the growth of cells slowed down after 5 days. Similarly, the increased expression of Bax might be responsible for the phenomenon.

We thus suggest that the relative increase of estrogen in older males may exert a launching effect in the pathology of PSMC proliferation which results in a stromal-predominant BPH, with the assistance of other factors, such as androgen, prolactin and the interaction between the stroma and the epithelium, amongst other things. This might provide an alternative interpretation for the etiology of BPH.

In accounting for the growth-promoting effect of E_2 on PSMCs, the present study supports the view that combined estrogen and androgen-deprivation therapies may provide a more appropriate alternative to surgical treatment of BPH, especially in cases where there is extensive stromal cell hyperplasia (Ricciardelli et al., 1994). Aromatase inhibitors have been proved effective in decreasing the estrogen level and have been used for many years in experimental BPH therapy which aims to decrease the estrogen levels (Ito et al., 2000). This experiment supports the availability of this therapy in theory.

Despite the anatomical differences between the humans and rats, our experiment provides an appropriate foundation for the further study of human prostatic cells.

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