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Effect of epristeride on expression of TGF-β receptor in rat prostatic epithelial cells *in vitro*

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KEY WORDS prostatic hyperplasia; epristeride; rats; transforming growth factor beta receptors; reverse transcriptase polymerase chain reaction; Western blotting

ABSTRACT

AIM: To assess the effect of epristeride on the expression of transforming growth factor β type Ⅱ receptor (TβR Ⅱ) in rat prostatic epithelial cells *in vitro*. **METHODS**: RT-PCR and Western blot were used to quantitatively detect the mRNA and protein expressions of TβR Ⅱ in rat prostatic epithelial cells treated or untreated with epristeride. Immunocytochemical staining method was used to qualitatively analyze the expression of TβR Ⅱ protein. **RESULTS**: After treatment with epristeride 180 or 360 nmol/L, TβR Ⅱ mRNA expression levels were 0.56 ± 0.08 and 0.59 ± 0.07, respectively, which were significantly up-regulated compared with control cells (0.38 ± 0.04, $P < 0.05$); expression level of TβR Ⅱ protein were 3163 ± 920 and 6769 ± 1941, respectively, which were also markedly up-regulated compared with control cells (536 ± 240, $P < 0.05$). Immunostaining showed weak positive reaction in control cells, while strong staining of TβR Ⅱ was found in epristeride-treated cells. **CONCLUSION**: Epristeride may up-regulate the expression of TβR Ⅱ to induce apoptosis of prostatic epithelial cells.

INTRODUCTION

Development of benign prostatic hyperplasia (BPH) appears to be dependent on the conversion of testosterone to dihydrotestosterone (DHT), which is enzymatically mediated by a steroid 5α-reductase[1]. Epristeride (SK and F105657) is a novel uncompetitive 5α-reductase inhibitor, which can reduce the prostate growth by inhibiting the conversion of testosterone to dihydrotestosterone[2]. Although epristeride is shown to decrease prostate size and intraprostatic DHT level[3], its molecular mechanisms underlying growth inhibition induced by epristeride are incompletely described.

Our previous study demonstrated that epristeride induced prostatic atrophy histologically in experimental models and reduced the number of prostatic epithelial cells via an apoptotic mechanism[3]. The transforming growth factor beta (TGF-β) family is composed of a series of isoforms, which regulates very distinct cellular functions, including proliferation and differentiation[4]. TGF-β has been detected in normal and abnormal human prostate[5]. TGF-β1 seems to be a potent inhibitor of prostatic epithelial cell proliferation and has been shown to mediate apoptosis of epithelial cells *in vitro*[6]. Signaling by TGF-β is dependent on binding to cell surface receptors, three of which designated as TβR Ⅰ, Ⅱ, and Ⅲ have been cloned. In a proposed model, TGF-β binds to the type Ⅱ receptor, and then the type Ⅰ receptor is recruited and phosphorylated by the type Ⅱ receptor. It has also been demonstrated that castration-induced involution in the rat prostate was accompanied by an increase in TGF-β expression, and administration of TGF-β1 can induce programmed cell death in the rat prostate[7]. These results have established an important role of TGF-β in programmed cell death in the rat prostate.

To investigate whether epristeride-induced atrophic changes in BPH were mediated by TGF-β, A semi-quantitative RT-PCR was established to assess the expression of TβR Ⅱ mRNA in cultured primary prostatic epithelial cells. Protein level was detected quantitatively by Western blot and immunocytochemistry.

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MATERIALS AND METHODS

Drug and reagents Epristeride was provided by Yangzhou Pharmaceutical Factory, China. All primary antibodies used in the experiment were purchased from Santa Cruz Biotech Inc (California, CA, USA).

Rat ventral prostatic epithelial cell culture Primary culture of rat prostatic epithelial cells was carried out as described previously[8]. Cells were cultured in 2 mL of a medium consisting of RPMI-1640 (GIBCO) supplemented with 10 % bovine calf serum, glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, epidermal growth factor (EGF) 10 μg/L, cholera toxin 10 μg/L, and transferrin 5 mg/L. Cultures were incubated in a humidified atmosphere of 5 % carbon dioxide and 95 % air at 37 ℃. On the 12th day, cells were harvested and the following tests were carried out.

Reverse transcription polymerase chain reaction (RT-PCR) Cultured prostatic cells were plated at a desity of 1×10^5 cells in 25-cm^2 culture flasks containing serum-free RPMI-1640 for 24 h. Then cells were treated with two different concentrations of epristeride (180 and 360 nmol/L) for 72 h, untreated cells were used as control. Cells were washed and further incubated for 24 h in drug- and serum-free culture medium. All the treated and control groups were harvested with trypsin/edetic acid (0.05 %/0.01 μmol · L^{-1}), centrifuged at 3500 × *g* for 5 min. The supernatants were discarded and cells were washed twice with PBS (in mmol/L: Na_2HPO_4 8.1, NaH_2PO_4 1.9, and NaCl 0.145; pH 7.4). Total RNA from the various fresh cell pellets was immediately extracted by TRIzol (GIBCO). PCR primers were as following: TβR Ⅱ, 5′-GGTCATTATCCAAGTGACGG-3′ (sense), 5′-CAGCTTGGCCTTGTAGACCT-3′ (antisense), amplicon 303 bp; β-actin, 5′-CCTCTATGCCAACACAGTGC-3′ (sense), 5′-GTACTCCTGCTTGCTGATCC-3′ (antisense), amplicon 211 bp. Oligonucleotide primers specific to TβR Ⅱ mRNA were designed from known genomic and cDNA information[9]. Synthesis of cDNA was carried out with reverse transcriptase (M-MLV, GIBCO) from 1 μg of total RNA. Reverse-transcribed single strand cDNA 2 μL was subjected to 25 cycles of PCR in 50 μL of buffer [Tris-Cl 10 mmol/L (pH 8.3), KCl 50 mmol/L, $MgCl_2$ 1.5 mmol/L, dNTPs 50 μmol/L, Thermus aquaticus polymerase 2.5 U (Sigma), and 0.2 μmol/L of specific primers for TβR Ⅱ and β-actin].

Experiment was performed using known positive control for TβR Ⅱ (rat kidney) to determine the optimal number of cycles of PCR to which cDNA products should be co-amplified, β-actin was amplified as an internal standard on all of the samples simultaneously for quantitation. Each cycle consisted of denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 5 min. PCR products were separated by electrophoresis on 3 % agarose gel. Relative ethidium bromide-stained band intensities were assessed by densitometry (Molecular Dynamics, ImageQuant Software, Sunyvale, CA). Each RT-PCR was repeated thrice using different preparation of RNA.

Western blot Total protein was obtained from cultured prostate epithelial cells. Rat prostate epithelial cells 1×10^5 were cultured in the presence or absence of epristeride (180 or 360 nmol/L) in serum-free medium for 72 h, then harvested and lysed with 100 μL ice-cold lysis buffer (Tris-Cl 20 mmol/L, pH 7.4, NaCl 50 mmol/L, edetic acid 5 mmol/L, NaF 50 mmol/L, sodium pyrophosphate 30 mmol/L, $NaVO_4$ 100 μmol/L, PMSF 1 mmol/L, aprotinin 10 mg/L, leupeptin 10 mg/L, and 1 % of Triton X-100) at 4 ℃ for 30 min, and ultracentrifuged at 100 000 × *g*, 4 ℃ for 45 min. The protein in the supernatant was mixed with sample buffer [Tris-Cl 50 mmol/L, pH 6.8, DTT 10 mmol/L, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol], fractionated by 7.5 % SDS-polyacrylamide gel, and electrotransferred onto a nitrocellulose membrane. The membrane was probed consecutively with anti-TβR Ⅱ rabbit polyclonal antibody (dilution 1 : 500) and horseradish peroxidase-conjugated anti-rabbit IgG. The blots were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). The densities of the immunoblots were quantitated with an image analyzer.

Immunocytochemical assay of TβR Ⅱ protein expression After a 72 h treatment with epristeride, cells (1.0×10^4/well) were collected and smeared on the slides precoated with 0.01 % poly-*L*-lysine (Sigma). The TβR Ⅱ immunoreactivity was detected by the ABC (avidin-biotin-peroxidase complex) method. The primary antibody used was anti-TβR Ⅱ rabbit polyclonal antibody (Santa Cruz Biotech Inc) (1:150) and secondary antibody used was a biotinylated-antibody goat anti-rabbit IgG (Huamei Biotech, China) (1 : 200). The experiment was performed at least three times and representative photos were taken by Olympus System (Japan).

Statistical analysis Statistical analysis was per-

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formed with t-test. $P < 0.05$ was considered significant.

RESULTS

Effect of epristeride on expression of TβR Ⅱ mRNA of prostatic epithelial cell by RT-PCR To evaluate expression of TβR Ⅱ mRNA in prostatic epithelial cell treated with epristeride, RT-PCR was performed on extracted mRNA aliquots from serum-free cultured cells. Fig 1 showed the result of analysis by agarose gel electrophoresis of RT-PCR products from treated and untreated samples. Band intensity of specific product obtained by scanning densitometry was divided by the intensity of 211-bp β-actin band. In the epithelial cells treated with epristeride 180 or 360 nmol/L, TβR Ⅱ mRNA expression showed an increasing trend in a dose-dependent manner and TβR Ⅱ mRNA levels were 0.56 ± 0.03 and 0.59 ± 0.07, respectively, which were up-regulated significantly compared with that of untreated cells (0.38 ± 0.01, $P < 0.05$).

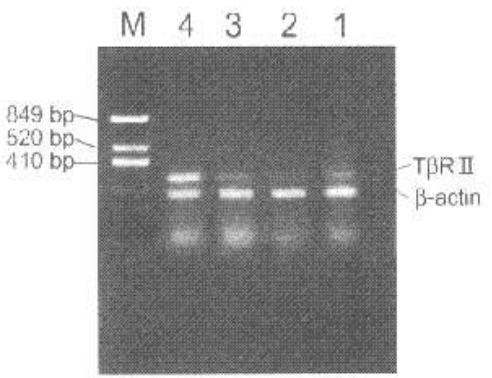

Fig 1. Analysis of TβR Ⅱ mRNA levels by RT-PCR. Co-amplification of TβR Ⅱ (303 bp) with β-actin (211 bp). RT-PCR products were analyzed on ethidium bromide-stained agarose gel. Bands were examined by scanning densitometry. The results were obtained from a representative experiment performed in triplicate. Lane M: Marker; Lane 1: Positive control (rat kidney); Lane 2: Control; Lane 3: Epristeride 180 nmol/L; Lane 4: Epristeride 360 nmol/L.

Immunodetection of TβR Ⅱ in untreated and epristeride-treated cells The expression of TβR Ⅱ at the protein level was analyzed by Western blot. The expression intensities of specific immunoreactive bands for TβR Ⅱ (70 kD) were quantified by densitometric scanning. In the epithelial cell treated with epristeride (180 or 360 nmol/L), TβR Ⅱ protein expression levels were 3163 ± 920 and 6769 ± 1941, which were significantly up-regulated compared with that of control (536 ± 240, $P < 0.05$, Fig 2).

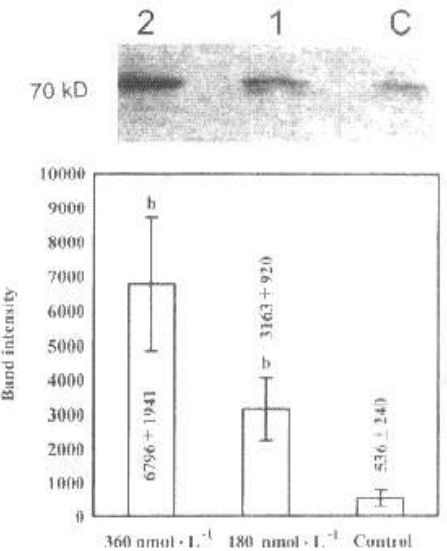

Fig 2. TβR Ⅱ protein levels analyzed by Western blot. Band intensities were examined by scanning densitometry. The results were obtained from a representative experiment performed in triplicate. Lane C: Control (epristeride-untreated group); Lane 1: Epristeride 180 nmol/L; Lane 2: Epristeride 360 nmol/L. $n = 3$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* control.

Immunocytochemical staining for TβR Ⅱ protein Using immunocytochemical staining method, strong positive reaction of TβR Ⅱ can be seen in the epithelial cell treated with epristeride, while in the control cells, TβR Ⅱ immunostaing was weak (Fig 3).

DISCUSSION

Although the mechanisms leading to the development of human BPH are still largely unknown, there are several findings that suggest the involvement of growth factor in its pathogenesis. Our data demonstrated that inhibition of epristeride on growth of prostatic epithelial cells *in vitro* was associated with up-regulation of TβR Ⅱ mRNA expression. The data provided an additional molecular mechanism by which epristeride affected TGF-β physiology in the prostate gland.

Epristeride is a novel 5α-reductase inhibitor, its effect of decreasing prostatic weight and intraprostatic DHT level

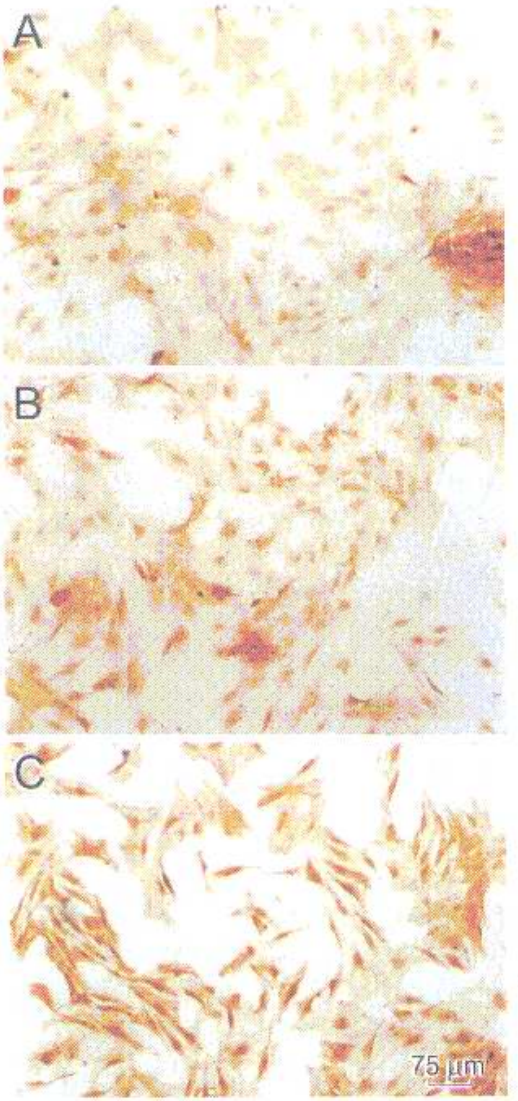

Fig 3. Immunocytochemical detection of TβRⅡ expression in prostatic epithelial cells. (A) untreated; (B) treated with episteride 180 nmol/L; (C) episteride 360 nmol/L. ×133.

have been reported[3]. However, molecular mechanisms associated with its growth-inhibitory effect are not clear. Concentrations of episteride used in this experiment were determined according to our preliminary experiment results, which showed that these two concentrations were equal to the intraprostatic concentration of episteride when SD rats were treated with episteride by gavage at minimal effective doses 3 and 6 mg/kg.

Apoptosis has been proposed as a mechanism to explain involution in androgen-deprived prostates. In rats, castration triggers apoptosis in epithelial and stromal cells throughout the ventral lobe[10,11]. The TGF-β signaling system plays an established role in apoptosis in different transformed and non-transformed epithelial cells[12] and also in the stroma[13]. TGF-β is a potent inhibitor of prostatic epithelial cells and it induces epithelial apoptosis[14,15]. TGF-β receptors are predominantly expressed in prostatic epithelial cells. Undoubtedly, the TGF-β system is regulated by androgens. Castration increases the levels of TGF-β mRNA, which return to normal after androgen replacement. TGF-β receptors are also up-regulated by castration. The findings that the TβRⅡ is under negative androgen control is also consistent with the proposed role of TGF-β as a growth inhibiting factor in normal prostate[7]. It had been also reported that TβRⅠ and TβRⅡ were up-regulated and localized in the glands undergoing involution after treated with finasteride. The up-regulation of these receptors could be one of the earliest changes observed in the responsive secretory cell as a result of inhibitory signal[16]. Our observation of a marked increase both in TβRⅡ mRNA level and in protein level in epithelial cells treated with episteride 360 nmol/L is a further confirmation of a mediation of episteride action by the TGF-β pathway.

In summary, our data demonstrated that episteride could up-regulate TβRⅡ mRNA and protein expression and episteride-induced apoptosis might be correlated with up-regulation of TβRⅡ. These results provide further understanding of the mechanism of episteride against BPH.

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爱普列特对体外大鼠前列腺上皮细胞 TGF-β 受体表达的影响

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关键词 前列腺增生；爱普列特；大鼠；转化生长因子-β 受体；逆转录聚合酶链反应；蛋白质印迹

目的：研究爱普列特对体外培养前列腺上皮细胞转化生长因子β受体Ⅱ (TβRⅡ)表达的影响. **方法**：逆转录 PCR 法及 Western blot 定量检测体外培养前列腺上皮细胞 TβRⅡ mRNA 及蛋白表达，免疫组织化学染色定性分析爱普列特对前列腺上皮细胞 TβRⅡ表达的影响. **结果**：TβRⅡ mRNA 表达水平分别 0.56 ± 0.08 和 0.59 ± 0.07，与对照组细胞(0.38 ± 0.04，$P < 0.05$)相比明显上调；TβRⅡ蛋白的表达水平分别为 3163 ± 920 和 6769 ± 1941，与对照组细胞(536 ± 240，$P < 0.05$)相比明显上调；免疫组化结果表明未经爱普列特处理的上皮细胞 TβRⅡ表达为弱阳性，经爱普列特处理后的上皮细胞 TβRⅡ表达明显增强. **结论**：爱普列特诱导前列腺上皮细胞凋亡可能与上调 TβRⅡ表达有关.

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