Inhibition of cultured rat prostatic epithelial cell growth by epristeride in vitro

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KEY WORDS epristeride; prostate; epithelium; cultured cells; epidermal growth factor receptors; IGF type I receptor; reverse polymerase chain reaction; flow cytometry

ABSTRACT

AIM: To study the molecular mechanism of rat prostate atrophy induced by epristeride. METHODS: MTT test was used to determine the effect of epristeride on the growth of prostatic epithelial cell induced by exogenous epithelial growth factor (EGF) or insulin-like growth factor-I (IGF-1). RT-PCR and flow cytometry were then used to quantitatively detect the mRNA and protein expressions of EGFR and IGF-I R of the epithelial cells treated or untreated with epristeride. RESULTS: Epristeride attenuated growth of epithelial cells induced by exogenous EGF, IGF-1. Epristeride 360 nmol/L inhibited EGFR and IGF-1 R expression at mRNA level, while epristeride 180 nmol/L had no marked effect on EGFR and IGF-1 R mRNA expression. Both epristeride 180 nmol/L and 360 nmol/L could down regulate EGFR and IGF-1 R protein levels. CONCLUSION: The molecular mechanisms of prostatic epithelial cell atrophy induced by epristeride might be associated with alternation in the expression of growth factor receptors such as EGF and IGF-1.

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 5a-reductase. DHT has a greater affinity than testosterone for the androgen receptor and is thought to actively modulate prostate growth. Epristeride is a potent and specific inhibitor of type II 5a-reductase and has proved to be effective in reducing prostate size and the circulating and intraprostatic levels of DHT. But its molecular mechanism against BPH is not very clear.

The prostate gland requires androgens for proliferation and maintenance of its function. BPH development has been associated with aging and hormonal control, but age-related changes in androgen secretion alone do not explain the hyperplastic development of the gland. Efforts to identify pivotal growth factors and studies on their effects have been prompted by the observation that prostatic cells in culture need substances than androgens for proliferation. In addition to hormones, a whole battery of other regulators is involved in the fine-tuning of prostate growth and differentiation. It has been proved that the proliferation of epithelial cells in the prostate is influenced by factors such as insulin-like growth factor (IGF) and epidermal growth factor (EGF), and their expression undergoes changes in proliferative prostatic disease. IGF-I is one of most important positive growth factors. Several studies have indicated that IGF are mitogenic in prostate tumor cells and normal prostate cells. The prostate stromal cells secrete IGF-I and the epithelial cells respond to IGF through the interaction of these growth factors with the type I IGF receptor. In vivo and in vitro studies have detected specific receptors for IGF-I on human prostatic epithelial cells in culture. It has been demonstrated that BPH, a proliferative disorder, is associated with abnormal expression of several of the gene products of IGF axis molecules by the prostatic cells. IGF-II mRNA expression in cultured stromal cells from BPH patients is markedly elevated. The principal IGF produced in the rat prostate is IGF-I whereas
in humans, the predominant species is IGF-1 (8).

EGF is mitogenic polypeptide that consists of 53 amino acids, and act on cells by binding the EGF receptor (EGFR) (9). High concentrations of EGF were determined in rat prostate (10). EGFR expression was observed in rat epithelial cells (11). EGF is androgen-regulated, androgen withdrawal by castration in mice is followed by a reduction in prostatic EGF levels which, however, can be restored by administration of testosterone. Several lines of evidence support a role for EGF in the pathophysiology of the prostate gland.

This study was designed to assess the effect of episteride on the expression of growth factors such as epidermal growth factor receptor (EGFR) and insulin-like growth factor I receptor (IGF-I R) in rat prostatic epithelial cells in vitro to provide further evidence of the mechanism of action of episteride against BPH.

MATERIALS AND METHODS

Drug and reagent Episteride, a white powder, purity ≥ 99%, insoluble in water, provided by Yangzhou Pharmaceutical Factory, China, was dissolved in dimethylsulfoxide (Me2SO). All primary antibodies used in the experiment were purchased from Santa Cruz Biotechnology, Inc, California, DA. TRIzol and RPMI-1640 medium were the products of GIBCO.

Rat ventral prostatic epithelial cell culture Primary culture of rat prostatic epithelial cells was carried out as described previously (12,13). Cells were cultured in 2 mL of a medium consisting of RPMI-1640 supplemented with 10% bovine calf serum, 2 mM tritiated thymidine, 100 IU/mL penicillin, 100 mg/L streptomycin, 10 μg/mL epidermal growth factor (EGF), 10 μg/L cholera toxin, 5 mg/mL transferrin. Cultures were incubated in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C. On the 12th day, cells were harvested and the following tests were carried out.

MTT Test In order to evaluate the effect of episteride on growth of primary cultured prostatic epithelial cells induced by exogenous IGF-1, cells were seeded on a flat-bottom 96-well plate (Falcon, Lincoln Park, NJ) at a concentration of 1 x 10^4 cells per well in RPMI 1640 containing 10% FCS. Twenty four hours later, cultures were down-shifted to serum-free medium, to which recombiant IGF-1 (0-25 μg/L) was added alone or in combination with various concentrations of episteride (180 mM/L, 360 mM/L). After 72 h of culture, cell proliferation was assessed by addition of 20 μL of vital dye (MTT, 5 g/L) to the culture. The blue dye taken up by the cells after 4 h incubation was dissolved in Me2SO (100 μL/well), and its optical density at 490 nm was read by an automated microplate reader (Bio-Tech, Winooski, VT). Results of a preliminary study with the MTT assay showed that absorbance was directly proportional to the number of cells. The cell viability (percentage of growth) was calculated for each well: % Viability = A_{x00 treated cells}/A_{x00 control cells} × 100%.

Effect of episteride on growth of rat prostatic epithelial cells induced exogenous EGF was carried out as described above. Exogenous EGF concentration was 0, 0.2, 1, 5, and 25 μg/L, respectively. Episteride concentration was 180 μmol/L and 360 μmol/L respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) Cultured prostatic cells were plated at 1 x 10^6 cells in 25 cm^2 culture flasks containing serum-free RPMI-1640 for 24 h. Then the cells were treated with two different concentrations of episteride (180 μmol/L and 360 μmol/L) for 72 h, and untreated cells were used as control. Cells were washed and further incubated for 48 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 x g for 5 min. The supernatants were discarded and cells were washed twice with PBS. RNA from the various fresh cell pellets was immediately extracted with phenol and guanidinium thiocyanate (Tri-reagent, GIBCO). Synthesis of cDNA was carried out with reverse transcriptase (M-MLV, GIBCO) from 1 μg of total RNA. Reverse-transcribed single-stranded cDNA 2 μL was subjected to PCR in 50 μL of 1 x buffer [10 mMol/L Tris-HCl (pH 8.3), 50 mMol/L KCl, 1.5 mMol/L MgCle, 50 μmol/L dNTPs, 2.5 U of Thermus aquaticus polymerase (Sigma), and 0.2 μmol/L of specific primers for indexes to be detected and β-actin]. All of the primer sequences used in the present study, PCR product sizes and PCR conditions are listed in Tab 1. Oligonucleotides specific to EGF receptor, IGF-I receptor mRNA were designed from known genomic and cDNA information (14,15).

Experiments were performed using known positive control for EGF receptor (rat liver), IGF-I receptor (Embryo 20-d rat kidney) to determine the optimal number of cycles of PCR to which cDNA products should be co-amplified, and β-actin was amplified as an internal standard for quantitation under the same PCR conditions. PCR products were separated by electrophoresis on 3%
Tab 1. Nucleotide sequences of oligonucleotide primers used for RT-PCR and related information.

<table>
<thead>
<tr>
<th>Oligonucleotide primers (5'-3')</th>
<th>Sizes of PCR products (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin (internal standard)</td>
<td>211 same as co-amplified</td>
<td>EGFR or IGF-1 R</td>
</tr>
<tr>
<td>F: CTCCTATGGCAACACAGTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R: GTACTCTGTCCGCTGAGCTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF receptor</td>
<td>457 94°C 1 min, 56°C</td>
<td></td>
</tr>
<tr>
<td>F: GTAGCAGCTGGAACATCAGTGG</td>
<td>1 min, 72°C 5 min,</td>
<td></td>
</tr>
<tr>
<td>R: CCAGAGACATAGATTA</td>
<td>28 cycles</td>
<td></td>
</tr>
<tr>
<td>IGF-1 receptor</td>
<td>546 94°C 1 min, 58°C</td>
<td></td>
</tr>
<tr>
<td>F: ATTACCCGATGCTCATCTCTC</td>
<td>1 min, 72°C 5 min,</td>
<td></td>
</tr>
<tr>
<td>R:AACGCACTGTAGTACCTCT</td>
<td>30 cycles</td>
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agarose gels. Relative ethidium bromide-stained band intensities were assessed by densitometry (Molecular Dynamics, ImageQuant Software, Sunnyvale CA).

Flow cytometry analysis The EGFR and IGF-1 R protein levels were measured by flow cytometry (16). Rat ventral prostatic epithelial cells 1 x 10^6 were examined by flow cytometry for each sample. Cells were exposed to various concentrations of epiristeride (180 nmol/L and 360 nmol/L) for 72 h, and each group included three samples. The primary antibodies used were rabbit primary antibody against EGFR (diluted to 1:100) and rabbit primary antibody against IGF-1 R (diluted to 1:150), and the secondary antibody used was a FITC-conjugated antibody goat anti-rabbit IgG (DAKO, Denmark) (1:50). Negative control included substitution of the first antibody with normal rabbit serum or omission of the first and/or the second antibodies. The antigen density was measured by a FACStar flow cytometry (Becton Dickinson, Mountain View, CA) and the percentages of EGFR, IGF-1 R positive cells were recorded respectively. The experiment was performed at least three times.

Statistical analysis Statistical analysis was performed with t-test.

RESULTS

Effect of exogenous IGF-1 on prostatic epithelial cell treated or untreated with epiristeride In order to examine the effect of epiristeride on the growth of prostatic epithelial cell, IGF-1 was added to each well at final concentrations of 0, 0.2, 1, 5, and 25 µg/L. There was no stimulating effect on prostatic epithelial cells at concentration of 0.2 µg/L of IGF-1. Prostatic epithelial cells untreated with epiristeride proliferated to IGF-1 at concentration 1, 5, 25 µg/L in a concentration-dependent manner, while, epiristeride inhibited the growth induced by exogenous IGF-1, the inhibition percentages of epiristeride (both 180 nmol/L, 360 nmol/L) at IGF-1. 25 µg/L were 26.4 %, 43.9 %, respectively (Fig 1a).

![Graph](image)

**Fig 1. Effects of epiristeride on growth of cultured rat prostatic epithelial cells in vitro stimulated by exogenous IGF-1 (1a), EGF (1b). Results are expressed as percentage of IGF-1 or EGF-untreated control. n = 3. x ± s. a P < 0.05, b P < 0.01 vs control cells untreated with epiristeride at the same concentration of IGF-1 or EGF, respectively. c P < 0.05, d P < 0.01 vs control cells untreated with IGF-1 or EGF.**

Effect of epiristeride on growth of cultured rat prostatic epithelial cells induced by exogenous EGF was also assessed by MTT test. Different concentrations of EGF were added alone or in combination with epiristeride (180 nmol/L and 360 nmol/L). Prostatic epithelial cells untreated with epiristeride responded to EGF at concentrations of 0.2, 1, 5, and 25 µg/L in a concentration-dependent manner; however, there were no significant dif-
ferences between control cells and episteride treated cells at the same EGF concentration, except those treated with episteride 360 nmol/L at EGF 25 μg/L, in which the inhibition percentage was 33.5% (Fig 1b).

Effect of episteride on expression of EGF receptor, IGF- I receptor mRNA of prostatic epithelial cell by RT-PCR  To evaluate expression of EGF receptor and IGF- I receptor mRNA in prostatic epithelial cells treated with episteride, RT-PCR was performed on extracted mRNA aliquots from serum-free cultured cells. Fig 2 shows the result of 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. Different receptors/β-actin values were given in Table 2. In the epithelial cells treated with episteride (180 nmol/L and 360 nmol/L), the levels of IGF- I receptor mRNA were 0.51 ± 0.12, 0.39 ± 0.07, respectively. The mRNA level of cells treated with episteride 360 nmol/L was decreased as compared with that of control (0.67 ± 0.13, P < 0.05). The mRNA levels of EGF receptor in the epithelial cells treated with episteride, 100 nmol/L and 360 nmol/L were 0.47 ± 0.03 and 0.38 ± 0.06, respectively. Only the mRNA level of cells treated with episteride 360 nmol/L was down-regulated compared with the control (0.55 ± 0.06, P < 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>EGF</th>
<th>IGF- I R</th>
</tr>
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<tbody>
<tr>
<td>Positive control</td>
<td>0.69 ± 0.12</td>
<td>0.74 ± 0.68</td>
</tr>
<tr>
<td>Control</td>
<td>0.53 ± 0.06</td>
<td>0.67 ± 0.13</td>
</tr>
<tr>
<td>Episteride 100 nmol/L</td>
<td>0.47 ± 0.03</td>
<td>0.51 ± 0.12</td>
</tr>
<tr>
<td>Episteride 360 nmol/L</td>
<td>0.33 ± 0.06</td>
<td>0.39 ± 0.07</td>
</tr>
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</table>

**Fig 2.** Analysis of EGF, IGF- I R mRNA levels by RT-PCR. Co-amplification of EGF (457 bp) or IGF- I R (546 bp) with β-actin (211 bp). RT-PCR products were analyzed on ethidium bromide-stained agarose gels. Bands were examined by scanning densitometry. EGF/β-actin, IGF- I R/β-actin values for each individual case was given in Tab 2. M marker; 1, positive control (rat liver) for EGF; 2, control group; 3, episteride 180 nmol/L; 4, episteride 360 nmol/L; 5, positive control (embryo 20-d rat kidney) for IGF- I R; 6, control group; 7, episteride 180 nmol/L; 8, episteride 360 nmol/L.

**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 factors in its pathogenesis. Our data demonstrated that inhibition by episteride on the growth of prostatic epithelial cells in vitro was associated with down-regulation of these growth factors expressions. The data provided an additional molecular mechanism by which episteride affected growth factor physiology in the prostate gland.

Episteride is a novel 5α-reductase inhibitor, its effect on decreasing the prostatic weight and intraprostatic DHT levels has been reported [21]. However, molecular mechanisms associated with its growth-inhibitory effects are not clear. We found through the MTT test that episteride could attenuate the growth-stimulating effect of exogenous IGF- I on rat ventral prostate epithelial cell, and episteride seemed to be more effective in inhibiting the growth of epithelial cells at high concentrations (5, 25 μg/L) of IGF- I , though it could not inhibit the growth of epithelial cells induced by DHT [21]. These results demonstrated that besides inhibition of 5α-reductase, episteride was in part associated with inhibition of IGF- I pathway in vitro. Concentrations of episteride used
in this experiment were determined according to our preliminary experimental results, which showed that these concentrations were equal to the intraprostatic concentration of episteride when SD rats were treated with episteride orally at a minimum effective doses of 3 mg/kg and 6 mg/kg (unpublished data). Our finding demonstrated that episteride was a multiple mechanism drug acting in BPH. RT-PCR and flowcytometry showed that episteride could decrease the expression of IGF-I receptor both in mRNA and protein levels in prostatic epithelial cells. In the human prostate increasing evidence suggests that epithelial cells are the predominant target cells for IGF-I action. In fact, in vivo IGF-I receptor is localized in the epithelial cells\(^8\), and in vitro human prostatic epithelial cell proliferation is stimulated by exogenous IGF-I\(^7\). Our data confirmed such an IGF regulation. It was found that the antiproliferative effects of finasteride could be attributed to the suppression of IGF-I autocrine/paracrine loops, which in turn reduced the ventral prostate weight\(^10\). Finasteride, similar to episteride, is also a selective and specific II isotype 5α-reductase inhibitor, which can decrease the intraprostatic DHT level and at the same time it can also increase the T level in the prostate. The dosage of finasteride used clinically would have to increase with increase of testosterone levels in the prostate, whereas episteride did not\(^12\). Our findings regarding involvement of episteride in inhibition of IGF-I receptor demonstrates negative regulation of episteride on the growth of prostatic epithelial cells.

Effect of episteride on growth of epithelial cells stimulated by exogenous EGF was similar to that of IGF,
but far less than that induced by IGF-I, though the stimulating effect of EGF was more potent than IGF-I. The possible reason may be that androgen might be inducing 5α-reductase activity via IGF-I. Increased signal transduction through EGFR can lead to abnormal cell growth; over-expression of EGFR in vitro has oncogenic potential, and its immunohistochemical over-expression has been reported in BPH and correlated with epithelial cell number. We have found that epistride could down regulate EGF receptor mRNA expression, which may also be another mechanism of acting of epistride against BPH.

In summary, our data demonstrated that epistride inhibited EGF receptor and IGF-I receptor mRNA expression and decreased its protein levels. These results provide further understanding of the mechanism of action of epistride in the treatment of BPH.

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爱普利特抑制体外培养的大鼠前列腺上皮细胞生长

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关键词 爱普利特；前列腺；上皮；培养的细胞；表皮生长因子受体；IGF Ⅰ型受体；逆转录聚合酶链反应；流式细胞术

目的：研究爱普利特对体外培养前列腺上皮细胞表皮生长因子受体和胰岛素样生长因子-Ⅰ受体表达的影响，探讨其抗前列腺增生的分子机制。

方法：MTT 法检测爱普利特对外源性表皮生长因子（epidermal growth factor，EGF），胰岛素样生长因子（insulin-like growth factor I，IGF-Ⅰ）诱导的大鼠前列腺上皮细胞增殖的作用，逆转录 PCR 及流式细胞术定量检测体外培养前列腺上皮细胞 EGFR 和 IGF-ⅠR mRNA 及蛋白表达。

结果：爱普利特 180 nmol/L 和 360 nmol/L 可以明显抑制 IGF-Ⅰ5、25 μg/L 诱导的细胞增殖；爱普利特 360 nmol/L 可以抑制 EGF 25 μg/L 诱导的细胞增殖；爱普利特 360 nmol/L 可以明显下调 EGFR、IGF-ⅠR mRNA 的表达，而爱普利特 180 nmol/L 则没有明显作用；爱普利特 180 nmol/L 和 360 nmol/L 均可以明显下调 EGFR 和 IGF-ⅠR 蛋白的表达。

结论：爱普利特抗前列腺增生的分子机制与纠正增生时异常表达的生长因子有关。