Establishment of a Novel Model for Studying the Effects of Extracts of Chinese Herb Medicine on Human Type II 5α-Reductase in Vitro

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Human steroid 5α-reductase type II (hSRD5A2) and dihydrotestosterone (DHT) play important roles in benign prostatic hyperplasia (BPH). The aim of our study was to establish a novel model to investigate the inhibitory effects of extracts and compounds of Chinese herb medicine on hSRD5A2. The gene, hSRD5A2, was artificially synthesized and cloned into pcDNA3.1 (+) vector, which was transfected into CHO cells by liposome. Transfected cells were screened through G418 and MTX. The expressed protein of hSRD5A2 by cells was purified and detected by western blotting. A minin reactive system comprising hSRD5A2 and testosterone (T) as substrate together with NADPH as hydrogen donor was established for screening inhibitors of hSRD5A2. The reaction system was optimized in the concentrations of T, NADPH, and hSRD5A2 and reaction temperature, time, and activity of hSRD5A2 were determined by the production of DHT. Furthermore, we screened some extracts and compounds of Chinese herb medicine using this model. The concentrations of T, NADPH, and hSRD5A2 were 0.02 μM, 0.8 mM, and 0.05 U/μl, respectively, in the model; maximum activity of hSRD5A2 was achieved at 37°C and 60 min reaction, and mangiferin had significant inhibitory effect on the activity of hSRD5A2. The model in this study is convenient and reliable for screening and evaluation of inhibitors of hSRD5A2; mangiferin may be a potential medicine for the treatment of BPH.

Key words—human steroid 5α-reductase type II purification; screening model; inhibitor; mangiferin

INTRODUCTION

Benign prostatic hyperplasia (BPH) is one of the most common ailments in elderly men; among those aged 50-60 and 80-90 years; 40% and 90%, respectively, have been diagnosed with BPH. The principal prostatic androgen is dihydrotestosterone (DHT), which is formed by the steroid enzyme, hSRD5A2, from its substrate testosterone (T). In several androgen target tissues including the prostate T is converted to DHT, which is the most potent natural androgen. This process amplifies the androgenic response, perhaps because of the higher affinity of the androgen receptor (AR) for DHT than for T. Both hSRD5A2 and DHT perform critical roles physiologically and pathologically. The plasma level of DHT has been reported elevated in patients with BPH.

Androgens mediate their activities by binding to AR, which belongs to the nuclear receptor superfamily and functions as a transcription factor. Upon binding ligand, AR undergoes a conformational change, translocates to the nucleus, and binds to the specific DNA sequence of target genes. These result in positive and/or negative regulation of gene expression. Physiologically, the two most important androgens are T and 5α-DHT. Although T and DHT can bind to the same AR, there is a clear difference between these two androgens in terms of binding affinity potential. T has a half lower binding affinity than DHT to the AR, and the dissociation rate of T from the receptor is five-fold faster than that of DHT. For that reason, DHT is regarded as a stronger male hormone than T, and believed as a major player in BPH.

Metabolically, DHT is made from T by the action of hSRD5A. This enzyme also catalyzes NADPH-dependent reduction of the Δ4 double bond of several other steroid substrates. So far, two distinct forms of hSRD5A isoenzymes, called type I and type II, have been characterized based on their different pH optimum. Although the structure of the type I and type II SRD5A genes is similar, the protein sequence homology is relatively low (about 47%). SRD5A1 is predominantly expressed in the liver, adrenal gland, and non-genital skin. In contrast, SRD5A2 is ex-
pressed mainly in androgen-dependent tissues such as the prostate, epididymis, seminal vesicles, and hair follicles. SRD5A2 is the predominant form in prostate and plays a more critical role in the pathogenesis of BPH.

**MATERIALS AND METHODS**

**Construction of Recombinant pcDNA3.1 (+) Expressing hSRD5A2/His-tag** The gene sequence of hSRD5A2 was obtained according to GENE BANK sequence (NM_000348). To increase expression of hSRD5A2, one signal peptide and regulation sequences were added before the N-terminal of hSRD5A2 in proper order. In addition, mammalian code preference and spatial structure of RNA of hSRD5A2/His-tag were optimized. To purify conveniently, histidine tag, enterokinase (EK) recognition site were also added before the N-terminal of hSRD5A2. HSRD5A2 sequence was artificially synthesized and cloned into pcDNA3.1 (+) between sites of BamHI and XhoI. The objective gene and digested plasmid were detected by electrophoresis. (GenScript Limited Company, China), and transformed into E. coli DH5α. The cells were spread on LB-agarose plate with 100 μg/ml ampicillin. The plate was incubated upside down at 37 °C overnight. The recombinant pcDNA3.1 (+) plasmids were extracted from positive E. coli DH5α and double digested with BamHI and XhoI. The objective gene and digested plasmid were detected by electrophoresis. The plasmids were sent for DNA sequencing by GenScript.

**Establishment of Stable Transfected CHO Cell Line** CHO cells (dhfr-) were incubated with DMEM (Invitrogen) culture medium containing HT supplement at 37 °C in a humidified, 5% CO2 incubator. CHO cells were transfected by Lipofectamine2000 according to the manufacturer’s instructions. After the transfected cells were incubated for 48 h, the cells were screened with the medium DMEM +10% FBS and the medium DMEM +10% FBS +1 ×HT +800 μg/ml G418 successively. Thereafter, HT-free DMEM culture medium was changed to medium containing 800 μg/ml G418, 10 μM MTX, and 10% FBS. After 6 days of growth in culture medium, hSRD5A2/His-tag expression in cell supernatant was detected by EIA. To induce high level of secretion, the concentration of MTX was increased gradually (from the starting concentration of 10⁻⁶ M) by amplifying the copies of hSRD5A2/His-tag gene. After 2-week culture, the cells were digested by 0.1% trypsin, with a single CHO cell distributed into one well of the plate. After 4-week culture, cells treated with the same method as above were distributed into bottom of large culture bottle. After 4-week culture and screening, a large-scale volume of cell supernatant was obtained. The cell supernatant was collected and purified by centrifugation at 8000 rpm and 4°C for 10 min.

**RT-PCR** RNA was extracted with chloroform/isoamyl alcohol and precipitated with 70% ethanol. The resulting RNA was quantified spectrophotometrically then 0.5–1 g/l RNA used to synthesize cDNA. The cDNA was amplified using specific primers as follows: forward, 5'-GAATCCCATCATCACCATCATCACGAATTCT-3'; reverse, 5'-AAATATAAATGGATTATTATCTCTCTTCT-3'; 35 PCR cycles yielded a hSRD5A2 fragment (828 bp) using an automated thermocycler (PTC100, MJ Research, Hercules, CA, USA) with cycles of 98°C for 30 s, 55°C for 45 s, and 72°C for 1 min. The cDNA from RT-PCR reaction was separated on 1% agarose gel and visualized by 1% ethidium bromide staining with a UV source.

**Western Blotting Analysis and Purification of HSRD5A2** Cell culture containing hSRD5A2/His-tag was collected and cell supernatant harvested by centrifugation. Thereafter, the supernatant containing hSRD5A2, with a N-terminal histidine tag, was loaded on Ni Sepharose 6 Fast Flow (GE Healthcare Sweden) column with a flow rate of 0.4 ml/min, ten bed volumes of wash buffer at a flow rate of 1 ml/min and one bed volume of elution buffer in order. After the liquid eluted out, it was dialyzed against 1×PBS, pH7.4 for 48 h, 10 μl of which was used for western blotting analysis. About 0.5 ml of the eluted fraction of the purified protein was obtained from the method above, which concentration was estimated by the BCA protein assay reagent kit (Pierce, Rockford, IL, USA). The sample of 10 μl protein was then separated by 10% SDS-PAGE with a Bio-Rad mini-protein cell (Hercules, CA, USA) then transferred to nitrocellulose membrane (Amer sham, Arlington Heights, IL, USA). After incubation with blocking solution (5% non-fat milk; Sigma, USA), the membrane was incubated with the mAb of anti-histidine tag (Genescript) at 1:10,000 dilution at 37°C for 1 h. The membrane was washed and incubated with the secondary antibody of goat anti-mouse pAb-HRP (Boster, China) at 1:10,000 dilution at 37°C for
2 h. After adding the luminescence substrate, the labeled band was visualized by exposing the membrane with X-ray film. They were then detected using an ECL detection system (Amersham, Pharmacia Biotech, Uppsala, Sweden). The relative intensity of the band on the film was analyzed by relative protein analytic software.

Dialyzed liquid containing 50 μg hSRD5A2/his-tag was digested with 1 μl of EK with 2 U/μl in the tube at room temperature for 16 h. The mixture was loaded on Ni Sepharose 6 Fast Flow (GE Healthcare, Sweden) column to eliminate his-tag and hSRD5A2/his-tag, which was not digested by EK, the elution liquid was also dialyzed against 50 mM Tris-HCl pH 8.0. Thereafter, the dialyzed liquid was loaded on DEAE-Sepharose Fast Flow weak anion exchanger to obtain hSRD5A2 according to the manufacturer’s instructions. The elution liquid was buffer of 50 mM Tris-HCl pH 8.0 containing 200 mM NaCl.

Establishing Model for Screening of HSRD5A2 Inhibitors In 96-well plate, the experiment and control wells were all in triplicate. The hSRD5A2, T, and NADPH were placed together in the reaction wells; after reaction at 37°C for 60 min, 200 μl of reaction liquid was transferred to another 96-well plate to end the reaction. The concentrations of DHT were assessed by EIA according to the manufacturer’s instructions (Adlitteram). The activity of hSRD5A2 was determined by production of DHT. The hSRD5A2, T as substrate, NADPH as hydrogen donor, time, and temperature were optimized in a minin reactive system so as to establish an in vitro model for screening inhibitors of hSRD5A2. Finasteride was selected as positive control inhibitor of hSRD5A2, with ethanol as negative control.

Extracts of Chinese Herb Medicine Mangiferin (content, 98.5%) was purchased from the Plant Diversity Research Center of China; some extracts were afforded by Phytochemistry Department of Anhui University of Traditional Chinese Medicine (China). The Chinese herb medicine was crushed and extracted with ethanol. The ethanol extracts were concentrated in a vacuum evaporator (Shanghai Zhenji Lab. Instrument Limited Co. China) and resulting residues weighed and dissolved to 1% solution in 50% ethanol.

Statistical Analysis Data are mean values of at least three different experiments and expressed as mean ± S.D. Differences between the groups were compared by one-way ANOVA. p < 0.05 was considered statistically significant.

RESULTS

Molecular Cloning of HSRD5A2/his-tag Gene

The 828-bp DNA fragment included a signal peptide fragment, an insert regulation fragment, histidine tag, EK site, and hSRD5A2 gene fragment. The selected sequence was chemically synthesized and cloned into the eukaryon expressive vector pcDNA3.1 (+). The target fragment was confirmed by DNA sequencing as reported in NCBI. The recombinant plasmid was double digested with BamHI and XhoI to give a DNA fragment of expected size. Enzymatic hydrolysis verified that hSRD5A2/his-tag gene was correctly cloned into the vector (Fig. 1A).

Identification of HSRD5A2/his-tag Total RNA was isolated from CHO cell lines and the cDNA of hSRD5A2/his-tag was obtained by RT-PCR assay. The PCR product was analyzed by 1% agarose gel and a band about 828 bp was detected, which was identical to the theoretical value as we expected. After cloning into PMD18-T simple vector, it was confirmed by DNA sequencing. The result showed that the coding sequence of hSRD5A2/his-tag was successfully obtained. RT-PCR analysis revealed that only the hSRD5A2/his-tag transfected CHO produced a 828-bp fragment. Neither vacant CHO nor vacant plasmid control produced any bands when RT-PCR was performed by using the specific hSRD5A2/his-tag primers. The product of RT-PCR was separated on a 1% agarose gel electrophoresis and visualized with 1% ethidium bromide staining with a UV source (Fig. 1B).

HSRD5A2/his-tag Detected by Western Blotting after Purification After harvesting the supernatant from cell culture bottle, affinity chromatography was performed with Ni Sepharose 6 Fast Flow for the purification of hSRD5A2 containing a histidine-tag. Eluted fraction of 0.5 ml was obtained. The western blotting analysis showed that hSRD5A2 carrying a his-tag was on the position of about 30 kDa, the control protein without his-tag was not seen on lane 2 (Fig. 1C).

Effects of Concentrations of T, NADPH, HSRD5A2 on Activity of HSRD5A2 The hSRD5A2 reaction system comprised T, NADPH, hSRD5A2, and potassium phosphate buffer solution (pH 7.0), finasteride as positive control medicine. To confirm the optimal
Fig. 1. The Coding Sequence of hSRD5A2 in Plasmid and Its Expressed Product Detected by Double Restriction Enzyme Digestion, RT-PCR, and Western Blotting, Respectively

A. Digestion of pcDNA3.1(+) and pcDNA3.1(+/−)/hSRD5A2/his-tag by BamHI and XhoI. Lane 1. DNA marker (2000, 1000, 750, 500, 250, 100 bp). Lane 2. pcDNA3.1(+) digested with enzyme of BamHI and XhoI (negative control). Lane 3. pcDNA3.1(+/−)/hSRD5A2/his-tag double digested with BamHI and XhoI (828 bp product was observed). B. RT-PCR product of coding sequence of hSRD5A2/his-tag. Lane 1. DNA marker. Lane 2 and Lane 3. RT-PCR product of coding sequence of hSRD5A2/his-tag; 828-bp product was observed. C. HSRD5A2/his-tag detected by western blotting. Lane M. Protein marker (14, 30, 40, 50, and 60 kDa). Lane 1. Protein with histidine tag as positive control. Lane 2. Protein without histidine tag. Lane 3. HSRD5A2 with histidine tag; approximate molecular weight of 30 kDa corresponding to the hSRD5A2 with histidine tag was observed.

Fig. 2. Effects of Different Concentrations of T, NADPH, and hSRD5A2 on Activity of hSRD5A2

A. Concentrations of DHT had linear correlation with T from 0 to 0.02 μM (n = 6). B. Concentrations of DHT had linear correlation with NADPH from 0.05 to 0.8 mM (n = 6). C. Concentrations of DHT had linear relationship with hSRD5A2 from 0 to 0.05 U/μl (n = 6). Concentration of DHT showed maximum level at concentration of hSRD5A2 in 0.05 U/μl (we regarded the 0.00082 g of hSRD5A2 as 1 U). All results are expressed as mean ± S.D.; n represents no. of samples.
Effects of Time and Temperature on Activity of hSRD5A2

It was necessary to confirm the relationship between reaction time, temperature, and activity of hSRD5A2. To confirm the optimal reaction temperature in the reaction system, the concentrations of T, hSRD5A2, and NADPH and time were fixed. The concentration of DHT reached peak after reaction at 37°C for 2 h, however, even if the temperature continued to increase, the concentration of DHT was below maximum level (Fig. 3A). On the other hand, the concentration of DHT reached peak after reaction at 37°C for 60 min. Although the reaction time was prolonged, the concentration of DHT did not change (Fig. 3B). Therefore 37°C and 60 min were confirmed to be the optimal temperature and time in the reaction system.

Inhibitory Effects of Extracts of Chinese Herb Medicine on hSRD5A2 Activity

Based on the above, we fixed the concentrations of hSRD5A2, NADPH, and T, time, and temperature, which were same as the above. When the finasteride concentration was varied from 0.005 to 0.16 μM, the concentration of DHT had linear relationship with finasteride. We screened about twenty aqueous and ethanolic extracts of Chinese herb medicines including jasminoidin, alkaloid of sophora flavescens, curcumae, mangiferin, berberine, and cinnamic acid and found that all except mangiferin had no or very weak inhibitory activity on hSRD5A2 (data not shown). However, mangiferin had significant inhibitory effect on hSRD5A2. Although the inhibitory activity of mangiferin was superior to that of neomangiferin, both compounds were weaker than finasteride (Fig. 4).

DISCUSSION

Many investigators have targeted the hSRD5A2 enzyme so as to develop new therapeutics for BPH. To screen inhibitors for hSRD5A2, several experimental systems have been adopted. For instance, in vitro enzymatic assay using prostate tissues of human or experimental animals is preferentially used,13,14 as well as establishing a stable cell line expressing hSRD5A2 as an inhibitory screening model,14 in which is contained SRD5A1, SRD5A2, or endogenous NADPH.4,16,17 It is hard to obtain adequate amount of human specimens, and there is only 60% homology between human and rodent SRD5A2, making it difficult for direct extrapolation, not separating hSRD5A2 from other interference factors. To overcome these limitations, in our study, we established a novel model for screening inhibitors for hSRD5A2. The described method for the determination of hSRD5A2 activity depends on the sum of the formed products from T, using the absolute calibration curve method. In addition, in the first time, highly purified hSRD5A2 was synthesized by biotechnology. The method does not require any radiolabeled compound; therefore it is convenient and reliable for enzyme experiments of hSRD5A2 activity or the development of its inhibitors.18

The IC50 value of finasteride (51 nM) was measured by the model established by us (from Fig. 4), which indicated that our model is more sensitive than those previously reported (237 nM).18 The Lineweaver-Burk plot, which was constructed by polling 1/v versus 1/[s], showed good linearity. Km and Vmax
values obtained from hSRD5A2 are 0.0545 ± 0.002 µM and 120 ± 1.4 pmol/min/U hSRD5A2, respectively (mean ± S.D.; n = 6). The obtained K_m value is lower than the reported (0.78 ± 0.30 µM), while the obtained V_max value is higher than the reported (15 ± 4.7 pmol/min/mg protein), suggesting that our method is more sensitive.

Natural extracts are widely used to treat BPH in the orient, and are becoming more popular in western countries. Although it has not yet been incorporated into the mainstream of medical care because of limited scientific evidence and lack of mechanistic understanding, alternative medicine using natural extracts is becoming an increasingly attractive approach worldwide. As a preliminary step towards the development of hSRD5A2 inhibitors, we tested the inhibitory effects of some natural extracts of Chinese medicine.

In China, the medicine of Zi-Shen Pill (ZSP) was originally reported in a manuscript by Li Gao (1279–1368 AD; Yuan Dynasty of China). It consisted of three kinds of medicinal plants Anemarrhena asphodeloides Bge (Liliaceae, rhizome), Phellodendron amurense Rupr (Rutaceae, bark), and Cinnamomum cassia Presl (Camphoraceae, bark) with a ratio of 10:10:1 in weight. All plants grow in China, Korea, and Japan and have traditionally been used to treat BPH for a long time. Treatment with extract of ZSP decreases prostate mass via inhibiting the growth of prostatic cells. Moreover, administration of extract of ZSP reduces serum DHT level but not serum testosterone, implying that extract of ZSP possesses hSRD5A2-inhibitory activity. It was reported that there are two testosterone hSRD5A2-inhibitory active constituents, named cis-hinokiresinol and 2,6,4’-triidroxy-4-methoxybenzophenone, existing in A. asphodeloides Bge. Mangiferin exists in A. asphodeloides Bge (by content, 0.5 – 1.95%) in China and Japan and has inhibitory effect on hSRD5A2, which has not been reported until now.

In our study, we determined that mangiferin has inhibitory effect on hSRD5A2. It has also been reported that mangiferin has anti-oxidative activities, can inhibit expression of iNOS and TNF-α, and enhance TGF-β expression. Mangiferin, a C-glucosylxanthone (1,3,6,7-tetrahydroxyxanthone-C2-beta-D-glucoside), has inhibitory activity on DPP-IV, PTP1B, and α-glucosidase with IC_50 values 7.30 µg/ml, 50 µM, and 5.234 µg/ml, respectively. However, the IC_50 value of mangiferin (370 µg/ml) measured in our study compared with the reported inhibitor (11.85 µM). From the molecular structure of mangiferin, a C-glucosylxanthone (1,3,6,7-tetrahydroxyxanthone-C2-beta-D-glucoside), we speculate that the inhibitory effect of mangiferin on hSRD5A2 is due to direct inhibition of hSRD5A2 itself rather than its non-specific anti-oxidant potential. Inhibition activity of mangiferin may derive from the precipitation activity of the protein. Mangiferin possibly binds to complexes of hSRD5A2 and substrate, and this
binding inhibits conversion of T to DHT. Five constituents of mangiferin such as 5-hydroxymethyl-2-furaldehyde, neomangiferin, mangiferin, isomangiferin, and reregalo side B were identified. There is also a possibility that other components in mangiferin can competitively or noncompetitively inhibit hSRD5A2. The finding of active component and its molecular action mechanism will be an interesting further study.

The results indicate that mangiferin (400 μg/ml) has nearly the same inhibitory effect on activity of hSRD5A2 compared with finasteride (0.05 μM). However, when finasteride was used for 6 months in patients with BPH, atrophy of epithelium can last for 30 months. Finasteride causes adverse effects such as gynecomastia, impaired muscle growth, and severe myopathy. If mangiferin has less adverse effects and is active in vivo, it may be an important candidate treatment for BPH. Further research is needed to explore the therapeutic usefulness of mangiferin.

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**REFERENCES**
