Studies on Androgen Receptor mRNA expression in Pancreas, Hypothalamus and Ovary of Androgen Sterilized Rats

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Objective To investigate the androgen receptor (AR) mRNA expression in pancreas, hypothalamus and ovary of androgen sterilized rats (ASR)

Methods ASR model was established by subcutaneous injection of testosterone propionate to SD female rats at the age of 9 days. Around the age of 106 days (proestrus), all rats were killed, serum $\Delta$4-andronestedione ($\Delta$4-A), total testosterone (TT), free testosterone (FT), insulin (Ins) and C-peptide (C-P) were measured by radioimmunoassay (RIA). Total RNA in pancreas, hypothalamus and ovary were extracted and the amount of AR mRNA was quantitatedly analyzed by RT-PCR with single base mutant template as inner standard.

Results Serum concentrations of $\Delta$4-A, TT, FT, Ins and C-P in ASR model rats were significantly higher than those in control group ($P<0.05$, $P<0.01$). The expression of AR mRNA in pancreas, hypothalamus and ovary increased significantly ($P<0.05$, $P<0.01$) of model rats as compared with control group.

Conclusion The elevated serum androgen levels in ASR model could enhance the expression of AR mRNA levels in pancreas, hypothalamus and ovary, which further induce hyperinsulinemia and anovulation.

Key words: androgen sterilized rat; androgen receptor; androgen; insulin

As a precursor for estrogen synthesis by the ovary follicular cell, androgen is one of the most important hormones in female human’s physiologic life. Excessive amount of androgen secretion may cause acne, hirsutism, oligomenorrhea, amenorrhea and even affect

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conception. Moreover, hyperandrogen is related to glucide, lipid metabolism and obesity. To explore the pathological mechanism of how hyperandrogenism affect reproduction and endocrine of female, androgen sterilized rats (ASR), an animal infertility model induced by high androgen, was used in this study. Total RNA in pancreas, hypothalamus, and ovary were extracted and the amount of AR mRNA was quantitatively analyzed by RT-PCR with single base mutant template as inner standard.

Materials & Methods

ASR model establishment and grouping

Neonatal female Sprague-Dawley (SD) rats were provided by Experimental Animal Center, Fudan University. All rats were housed in clean grade, with 12 h light/dark cycles and weaned at 21 d of life, fed with standard rat granule forage. ASR model was duplicated according to the methods proposed by Yu et al. [2]: rats of 9 d old in the experimental group received a subcutaneous injection of 1.25 mg testosterone propionate (equivalent to 0.05 mL, in neural tea oil, Shanghai Ninth Pharmaceutical Factory) at the nape of the neck. And then their vaginal smears were examined daily for 11 consecutive days from 70 d of age. The rats with persistent vaginal cornification without cyclic estrus change were used as ASR model. Normal control rats of 9 d old received a subcutaneous injection of 0.05 mL neutral tea oil at the nape of the neck. The vaginal smears showed cyclic estrus change at 70 d of age.

Specimen preparation

Around 106 d of age, ASR model and normal control rats were anesthetized with 4% chloral hydrate (400 mg/kg weight) intraperitoneal injection and killed on the afternoon of proestrus cycle. The pancreas, hypothalamus and ovaries were quickly removed and homogenized; RNA was extracted with one-step method[3]. The serum was separated after centrifugation and stored for hormonal assay.

Serum hormone assay

Serum △4-androstenedione (△4-A), total testosterone (TT), free testosterone (FT), insulin (Ins) and C-peptide (C-P) were measured by radioimmunoassay (RIA). The inter- and intra-assay coefficients of variation were less than 5% and 10% respectively. The RIA kits were purchased from America DPC Company.

Quantitative PCR of AR mRNA

AR mRNA was quantitatively analyzed by PCR with single base mutant template as inner standard[4]. EcoRI cleave site is lacked in the target gene of AR cDNA to be amplified (281-1 196 bp). A single base changed mutated template was made by changing the target gene 886 bp from G to T to add an EcoRI cleave site GAAT ↓ TC (GA ↓ ATTC). The target gene and mutant template DNA were amplified in the same PCR reaction. After digestion by EcoRI, the target AR gene (no cleavage, 916 bp) could be quantitative analyzed
based on the amount of mutant template DNA fragment (two DNA fragments, 605 bp and 311 bp after EcoRI cleavage).

**Mutant template preparation**

The mutant template was prepared by PCR. The sequences of the primer are as follows:

Primer 1: 5′-CGG GTT CTC CAG CTT GA T GCG-3’
Primer 2: 5′-CTC TGG CCG AAT TCA AAG GTC T-3’
Primer 3: 5′-CAC AGG CTA CCT GGC CCT GG-3’
Primer 4: 5′-AGA CCT TTG AA T TCG GCC AGA G-3’

PCR reaction was carried out with Taq DNA polymerase and consisted of mixture of the following reactions: 8 pmol primer 1 and primer 2, 0.5 µL AR cDNA, 2 u Taq, 0.5 µL dNTP. The following PCR protocol was used to make mutant template 281-899 bases: denaturized at 94°C for 3 min, then at 94°C for 1 min, 62°C for 1 min, 72°C for 2 min for 32 cycles of amplification. The reaction was completed with a single cycle at 72°C for 10 min to complete the extension. The mutant template 877-1 196 base was made by the same protocol with primer 3, primer 4 and 0.5 µL AR cDNA. The single base mutant template 916 bp was made by PCR amplification with primer 1, primer 3 and 0.5 µL of each PCR product mentioned above. After being purified, the mutant template was quantitatively analyzed and diluted at different concentrations to amplify synchronous with AR cDNA of normal rats. And 10^{-20} mol/L mutant template was close to the AR cDNA concentration in normal rats, so it is the suitable concentration for quantitative PCR.

**Quantitative PCR**

The PCR amplification was performed with primer 1, primer 3, 2 µL AR cDNA after reverse transcription of 1 µg total rat RNA and 2 µL mutant template (10^{-20} mol/L). After being cleaved with EcoRI enzyme, the amplified samples were analyzed by electrophoresis on a 2% agarose gel. Three bands under ultraviolet lamp were seen (Figure 1). The density ratio between AR cDNA target gene’s PCR product and mutant template amplification product represented expression level of AR mRNA by measuring the bands density with automatic density image analysis instruments.

Lane 1-6: digested PCR products from 2 µL mutant template (10^{-30}mol/L) added into 2 µL AR cDNA of reverse transcription from 1 µg total RNA of rats
Lane 7: 100 bp DNA ladder marker
Lane 8: digested PCR products from 2 µL mutant template (10^{-30}mol/L) (without AR cDNA)

Figure 1 Electrophoretic result of digested quantitative PCR products with single base mutant template as inner standard
Table 1  Comparison of serum hormone between ASR model and control group (x ± s)

<table>
<thead>
<tr>
<th>Item</th>
<th>n</th>
<th>Control</th>
<th>ASR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>△4-A (µg/L)</td>
<td>12</td>
<td>0.087 ± 0.066</td>
<td>1.178 ± 0.378</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TT (ng/L)</td>
<td>12</td>
<td>1.210 ± 0.360</td>
<td>5.200 ± 1.590</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FT (ng/L)</td>
<td>12</td>
<td>0.057 ± 0.058</td>
<td>0.369 ± 0.045</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ins (µU/L)</td>
<td>12</td>
<td>4.184 ± 0.388</td>
<td>4.737 ± 0.669</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>C-P (µg/L)</td>
<td>12</td>
<td>0.083 ± 0.009</td>
<td>0.121 ± 0.008</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

AR mRNA level

AR mRNA level was significantly elevated in pancreas, hypothalamus, and ovary of ASR model compared with that in the control group (Table 2).

Table 2  Comparison of AR mRNA levels in pancreas, hypothalamus and ovary between ASR models and control group (x ± s)

<table>
<thead>
<tr>
<th>AR mRNA levels</th>
<th>n</th>
<th>Control</th>
<th>ASR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>12</td>
<td>0.815 ± 0.182</td>
<td>2.244 ± 0.364</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>12</td>
<td>3.709 ± 0.457</td>
<td>4.396 ± 0.326</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ovary</td>
<td>12</td>
<td>18.298 ± 3.829</td>
<td>41.313 ± 4.490</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Discussion

Serum TT, FT and their precursor △4-A were enhanced significantly, and the vaginal smears showed persistent vaginal cornification without estrus cycle in ASR model. For the above reasons, ASR could be regarded as an satisfied anovulation model caused by hyperandrogenism for research.

In female individuals, testosterone and its metabolic products are important steroid hormones. Testosterone could combine with androgen receptor and be transferred to nucleus to promote a cell reaction. Androgen receptors are widely distributed in the female body. Hyperandrogenism cause a series of pathologic changes through androgen receptor in female. In this study, the amount of AR mRNA was quantitatively analyzed by RT-PCR with single
base mutant template as inner standard. This quantitative PCR method eliminate different renaturation speed caused by different primer sequences and length, and avoid error as well in the quantitative PCR caused by different amplified length’s effects on amplified efficiency. The quantitative effect is better than the PCR method using GAPDH or β-actin as inner standard.

Like polycystic ovary syndrome with hyperandrogenism and hyperinsulinism, the pancreas secretion in ASR model is excessive, the serum insulin and C-peptide were also significantly higher than that in the control group. Zhang et al.[5] found that glucose tolerance in ASR model declined. Many scholars reported that hyperinsulinemia and insulin resistance could induce hyperandrogenism[6], but there were few study on hyperandrogenism’s impact on insulin’s synthesis and secretion. Hyperandrogenism and hyperinsulinemia are also found in ASR model. RT-PCR demonstrated that androgen receptors were distributed in pancreas. The level of AR mRNA elevated significantly in ASR model, which suggests that hyperandrogenism up-regulate the level of AR mRNA in pancrea, enhance the number and density of androgen receptor, and stimulate the synthesis and excretion of insulin from pancreatic islet β cells while insulin, could stimulate ovary to synthesize more androgen. Thus endocrine-metabolism in the ASR model’s went into vicious circle, which resulted in pathologic conditions forming anovulation, obesity, or abnormal glucose tolerance, etc.

Like in peripheral tissue, androgen’s role and activity in central nervous system are mediated through the androgen receptor[7]. Androgen receptors in hypothalamus are not only closely related to sexual differentiation, but also responsible for modulating some neurotransmitter and neuro-modulator release. Previous studies suggest that there are ARs on β-endorphin neuron in the arcuate nucleus of hypothalamus, and the hypothalamic proopiomelanocortin (POMC) mRNA’s synthesis increased[8], while the content of follicular stimulating hormone (FSH) and luteinizing hormone (LH) decreased[9]. In this study, hypothalamic AR mRNA increased significantly in ASR model, and the high-expression of androgen passed the blood-cerebral barrier, which up-regulated the synthesis of AR in the neurons of hypothalamus, and influenced the synthesis and release of some kinds of neurotransmitters and neuro-modulators; however, it inhibited the synthesis and secretion of gonadotropin, and finally contributed to the hypogonadotropin status in ASR model.

Like hypothalamus and pancreas, the expression of ovary AR mRNA was also enhanced significantly in ASR model, which rendered the ovary hypersensitive to androgen and strengthened androgen’s inhibition effect on ovary. Hyperandrogenism, together with hypogonadotropin and hyperinsulinemia in the model, caused anovulation of the ovary.

Recently, the increasing incidence of PCOS, characterized by its hyperandrogenism and insulin resistance, has drawn more and more attention in the field of reproductive endocrine. In this study the ASR model exhibited many proven characteristics, such as hyperandrogenism, hyperinsulinemia and anovulation. This ASR model could be widely used in the study on
pathologic changes of hyperandrogenism in women’s neuro-endocrine-metabolism network and bring about a bright future in exploring the pharmacology aspect of anti-androgenic drugs.

Reference


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