Influence of androgens and age on androgen receptor and 5α-reductase II transcription

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Abstract

Objective: The regulation of the androgen receptor (AR) and 5α-reductase II (5RII) gene in genital skin fibroblasts is of particular interest in understanding androgen-dependent embryonic formation of external male genitalia.

Design: Human genital skin fibroblasts from pre- and postpubertal male individuals (aged 5 months to 51 years) were incubated with testosterone and dihydrotestosterone under various conditions to study the regulation of AR and 5RII transcript concentrations dependent on androgen concentration and donor age.

Methods: A competitive reverse transcribed PCR (RT-PCR) protocol was designed to achieve simultaneous relative quantification (semi-quantification) of AR and 5RII mRNAs in standardized whole RNA samples from each donor.

Results: Concentrations of AR and 5RII mRNAs are not influenced by androgens in genital skin fibroblasts. Moreover, comparison of AR transcript concentrations in genital skin fibroblast cell lines revealed weak variations independent of donor age, while 5RII transcription exhibited clear individual differences with a declining tendency towards higher ages.

Conclusions: The transcription of AR and 5RII is not directly regulated by testosterone or dihydrotestosterone in pre- or postpubertal human genital skin fibroblasts. However, donor age seems to play a role in gradual depression of 5RII transcription.

Introduction

The androgen receptor (AR) and 5α-reductase II (5RII) play a crucial role in the development of the male reproductive tract. In normal male individuals, the AR first recognises and binds the androgens testosterone (T) or dihydrotestosterone (DHT) within the cytoplasm of androgen target cells. Then it dimerizes and migrates into the nucleus where it binds with cofactors to the promoter of androgen sensitive target genes initiating the appropriate cell response (1–3). During urogenital ontogenesis, T–AR complexes induce the virilization of the Wolffian ducts. In fibroblasts of the sinus urogenitalis and genital folds, T is first reduced to DHT by the enzyme 5RII, then development of external genitalia is initiated by the AR–DHT complex (4).

In various cell types androgens seem to excite an influence on AR and 5RII expression in different and sometimes even contradicting manners. This occurs particularly at the transcription level. In rat ventral prostate, 5RII expression is enhanced by DHT (5), while AR transcription in this and other tissues (epididymis, vas deferens, kidney and brain) is up-regulated in castrated rats, probably due to T depletion (6). In smooth muscle cells from rat penis (7) and in hamster harderian gland cells (8), androgens enhance AR transcription. In the human prostate cancer cell line LNCaP androgens repress AR transcription, but enhance AR protein stability (9, 10). As genital skin fibroblasts (GSFs) are crucial for androgen-dependent development of male external genitalia, normal expression of AR and 5RII is of considerable importance. Therefore we focused our study on the effect of T and DHT on AR and 5RII transcription in cultured GSFs from pre- and postpubertal individuals; additionally, age-dependent individual differences of AR or 5RII mRNA concentrations in GSFs from several normal males were investigated.

Materials and methods

Cell culture

Foreskins from 10 normal prepubertal boys and two adult fertile men of varying ages were obtained at
circumcision, dissected mechanically, and incubated in medium (DMEM–F12, 5% CO₂, 10% charcoal-stripped fetal calf serum, antibiotics) at 37°C to culture genital skin fibroblasts (GSFs). Cells of the second passage were stored in liquid nitrogen for later experiments. After thawing, GSFs were first grown to approximately 80% confluence and then pre-incubated in medium without androgens for 48 h. Cells were afterwards incubated in medium containing varying T or DHT concentrations for ascending periods (Table 1). Finally, GSFs were tailed at their 5'-end with a T7 RNA polymerase recognition sequence. Products were in vitro transcribed with T7 RNA polymerase (Promega, Heidelberg, Germany) and digested twice with DNase (Gibco-BRL, Ludwigshafen-Leopoldshausen, Germany) according to the manufacturer’s instructions. RNA competitors were quantified and finally diluted to obtain working stock aliquots for further usage in semi-quantitative competitive RT-PCRs.

Simultaneous reverse transcription (RT) of AR/5RII transcripts and competitors was achieved by specific antisense priming. Whole RNA (1 μg) from each GSF sample was mixed with both AR- and 5RII-RNA competitors in variable concentrations (Figs 1 and 2) in one reaction tube. Twenty picomoles of each AR and 5RII antisense primer and DEPC-H₂O were added to a final volume of 10 μl. Mixtures were incubated at 70°C for 5 min. Then, 1x first-strand buffer, 1 mM dNTPs, 10 mM DTT and 10U RNase inhibitor (Boehringer-Mannheim, Mannheim, Germany) were added to a final volume of 20 μl. Reverse transcription was performed with 40U Superscript II RT (Gibco BRL) at 37°C for 1 h. For AR, an exon-spanning fragment of the target transcript stretching from nucleotide position 1653 (according to Lubahn et al. (15)) in exon 1 to position 2845 in exon 4 was amplified from 1 μl cDNA samples. PCR solutions contained 20 pmol sense primer AR Is (5'-TGG ATG GAT AGC TAC TCC GG-3') and antisense primer AR Iia (5'-ACT ACA CCT GGC TCA ATG AGC CAG AGT CAT CCC TGC TTC A-3') as previously described (12). The amplification procedure was stopped after 21 cycles, i.e. within the exponential phase of the PCR, as determined by preliminary experiments (not shown). Samples were analyzed for equivalent amplification product concentration on 2% agarose gels (13).

**Construction of artificial standards (competitors) and semi-quantitative competitive RT-PCR**

Standardized cDNA synthesis and PCR for all investigated RNA samples was performed with the addition of artificial standards (competitors). Competitors share primer complementary sequences with the target amplicons of AR and 5RII transcripts to allow similar amplification behaviour and direct competition of target and competitor for the primers during the PCR step. This represents the basis for the quantification experiments described here. Competitors were constructed as described by Celi et al. (14). Briefly, GSF whole RNA was first reverse transcribed by poly-dT priming and then submitted to PCRs using the following composite primers: antisense primer hARE3a–AR Iia Comp (5'-ACT ACA CCT GGC TCA ATG AGC CAG AGT CAT CCC TGC TTC A-3') and sense primer T7–AR IsComp (5'-TAA TAC GAC TCA CTA TAG GGA GTG GAT GGA TAG CTA CTC CGG -3') for the construction of the AR competitor; antisense primer 5RII E2a–4a Comp (5'-GTG GCC AGG GCA TAG CCG ATC TGT GTA CCA CCC ATC AGG G-3') and sense primer T7–5RII E1 SIIs Comp (5'-TAA TAC GAC TCA CTA TAG GGA GAG CTG CCT TCC TTC GCG GTG-3') for the construction of the 5RII competitor. With these primers, PCR products were generated which are 155 bp (AR) and 176 bp (5RII) shorter than the respective target amplicons. By their sense composite primers, shortened PCR products were tailed at their 5'-end with a T7 RNA polymerase recognition sequence. Products were in vitro transcribed with T7 RNA polymerase (Promega, Heidelberg, Germany) and digested twice with DNase (Gibco-BRL, Ludwigshafen-Leopoldshausen, Germany) according to the manufacturer’s instructions. RNA competitors were quantified and finally diluted to obtain working stock aliquots for further usage in semi-quantitative competitive RT-PCRs.

**RNA analysis**

Whole RNA from GSFs was isolated using RNeasy columns according to the manufacturer’s instructions (Quiagen, Hilden, Germany). RNA was quantified photometrically (absorbency at 260 nm measured in a DNA/RNA calculator from Pharmacia, Freiburg, Germany). To determine the quality and integrity of RNA, 5 μl aliquots were electrophoresed on formaldehyde-denaturing 1% agarose gels (11). Standardization of whole RNA from different GSF cultures was performed by RT-PCRs for ubiquitous ribosomal protein L7 transcription with modified primers L7neus (5'-GAA ACC ATG GAG GGT GTA GA-3') and L7neua (5'-GAA AAA TAA TCA TGG TAG AC-3') as previously described (12). The amplification procedure was stopped after 21 cycles, i.e. within the exponential phase of the PCR, as determined by preliminary experiments (not shown). Samples were analyzed for equivalent amplification product concentration on 2% agarose gels (13).
In vitro and finally were repurified by agarose gels and QuiagQuick columns. DNA polymerase (Boehringer-Mannheim). Products columns (Quiagen) and blunt ended (‘polished’) with T4 ends. The products were electrophoresed on 2% agarose -SSC/0.1% SDS for 2

Signals of hybridized probes were developed following hybridization and washing procedures was performed at 42°C.

Northern blots

For Northern blotting, 4 µg whole RNA per lane were loaded on RNA gels and electrophoresed. Gels were equilibrated and blotted with 20× SSC onto positively charged nylon membranes using a vacuum blotter from Hoefer (Pharamacia). Digoxigenin-labelled AR and 5RII RNA probes were generated by RT-PCR without competitors using the protocols described above, in which the antisense primers ARIIa and 5RIIE 4a were tailed by a T7 RNA polymerase recognition sequence at their 5' ends. The products were electrophoresed on 2% agarose gels, isolated from the gels, purified by QuiaQuick columns (Quiagen) and blunt ended (‘polished’) with T4 DNA polymerase (Boehringer-Mannheim). Products were repurified by agarose gels and QuiaQuick columns and finally in vitro transcribed with T7 RNA polymerase (Promega) using digoxigenin labelled rNTPs according to the respective manufacturer’s instructions. Northern blot filters were initially pre-hybridized for 1–2 h and then hybridized overnight with Dig-easy-Hyb (Boehringer-Mannheim) containing AR or 5RII RNA probes at 68°C. Blots were washed twice for 5–10 min with 2× SSC/0.1% SDS at room temperature; stringent washings were performed at 68°C with 0.1× SSC/0.1% SDS for 2×15 min. Chemoluminescence signals of hybridized probes were developed following the ‘Digoxigenin Users Guide for Northern-blots’ (Boehringer-Mannheim). Gels were stripped and reprobed with β-actin DNA probe as described above; however, hybridization and washing procedures was performed at 42°C.

Results

Evaluation of accuracy of semi-quantitative competitive RT-PCR

RT-PCRs on AR- and 5RII transcripts were performed on declining amounts of LNCaP cells (AR positive) and hVHF 9–2 (5RII positive) with constant competitor concentrations (0.5 amol AR or 0.02 amol 5RII RNA competitors respectively). As shown in Fig. 1, concentration measurements on AR and 5RII mRNA are proportional to the quantities of whole RNA samples and thus to initial target amounts. At least 4-fold differences in target concentrations can be represented by our method using only one competitor concentration. Three sets of each eight semi-quantitative competitive RT-PCRs were performed to evaluate the methodical variability at three stages of the method: RNA isolation, cDNA synthesis and PCR. One set was performed on eight separate whole RNA samples from cell cultures obtained from a single individual, a second set on a pool of whole RNA and a third set of PCRs on a cDNA pool comprising competitors. A(t/s) values of each RT-PCR set was analyzed for standard deviations from their mean value, which was defined as 100%. For AR RT-PCRs, the highest standard deviation could be demonstrated for the RNA pool set (± 10.2%), the lowest for the cDNA-pool set (± 5%). The standard deviation of ± 9.5% for the set on separated RNA samples reflects the results from the subsequent cDNA synthesis (RNA pool set). For 5RII semi-quantitative competitive RT-PCRs, the standard deviation pattern is similar, but higher at all stages (± 14.8% (set on separate RNA samples), ± 14% (RNA pool set), ± 11% (cDNA pool set)). These results indicate that cDNA synthesis is the most critical step for semi-quantifications by RT-PCRs. However, maximum standard deviations of as little as 10.2% for AR and 14.8% for 5RII semi-quantitative competitive RT-PCRs were considered to be low enough to allow proper semi-quantifications. Evaluation of simultaneous competitive RT-PCRs of both AR and 5RII demonstrated the same results as separate competitive RT-PCRs of each transcript.

Influence of androgens and age on AR and 5RII transcription

To analyze the influence of androgens on AR and 5RII transcription, we incubated the prepubertal cell line hVHF 26–2 in medium containing ascending T concentrations for 12 h. Then, whole RNA was isolated and three semi-quantitative competitive RT-PCRs for AR and 5RII transcripts per incubation step were applied. As shown in Fig. 2A on the PAA gels from a representative experiment, relations between target and standard are similar for AR and 5RII at all incubation steps. As demonstrated by semi-kinetic RT-PCRs, similar L7 transcript amounts indicate equal whole RNA quantity and quality in these samples. When mean A(t/s) values were depicted as a function of T concentration, no characteristic difference was found indicating the absence of an influence of T on AR or 5RII mRNA levels. This result was supported by Northern blots (Fig. 2B). In whole RNA from hVHF 26–2 cells exposed to 0, 10 and 100 nM T, we detected two bands at 10.5 and 4.9 kb responding to the AR probe. The stronger 10.5 kb band represents physiologically active AR mRNA; the weak signal at 4.9 kb is most likely caused by an AR transcript degradation product (20). Exposure of the blot to the 5RII probe unveiled signals at 2.4 kb. After subtraction of background effects, bands specific for both transcripts displayed similar optical densities.
(OD) in the 0 nM, 10 nM and the 100 nM T sample (10.5 kb band (AR mRNA) = OD 0.84, 0.78 and 0.809 respectively; 2.4 kb band (5RII mRNA) = OD 1.26, 1.39 and 1.26 respectively). Our findings could be reproduced by experiments based on prolonged T exposure of prepubertal (hGHF 26–2) and postpubertal (hGHF 33–2) GSFs for up to 120 h (Table 1). In an attempt to analyze the effect of the more potent androgen DHT on AR and 5RII transcription, we exposed the prepubertal GSF cell line hGHF 9–2 to 100 nM DHT for increasing periods of time. As shown in Fig. 3, no incubation time-dependent correlation of transcript levels could be

Figure 1 PAA gels with semi-quantitative competitive RT-PCRs on AR (left gel) and 5RII (right gel) and graphical analysis. Declining amounts of whole RNA (1, 0.75, 0.5, 0.25 and 0 μg) from LNCaP cells (AR) and prepubertal GSF cell line hGHF 26–2 (5RII) were each mixed with 0.5 amol AR and 0.02 amol 5RII RNA competitor respectively and submitted to semi-quantitative competitive RT-PCRs. Densities from target and standard (competitor) product bands on PAA gels were quantified and compared, resulting in a value which represents a relative equivalent of transcript concentrations (A(t/s)). A(t/s) values are shown below the gels as a function of initial amount of whole RNA per sample. t: target band; s: standard band; M: molecular size markers with DNA fragments of 527, 404 and 307 bp (left gel) or 404 and 307 bp (right gel) length.
found, indicating that AR and 5RII mRNA concentrations are not dependent on the presence of DHT.

To test whether the results outlined above could be confirmed by experiments made on other GSF cell lines, we incubated GSF lines from pre- and postpubertal normal male individuals of between 5 months and 51 years old for 48 h in medium either without T or with 100 nM T. Transcript levels in GSF grown without T were set to 100%. Mean AR mRNA levels in GSF grown with T were between 111.9 and 88.7%, mean 5RII transcript levels between 123.9% and 74.7% of their control without T. Also, no characteristic effect between

**Figure 2** Influence of increasing T concentrations after 12 h incubation on AR and 5RII mRNA levels in prepubertal GSF (hVHF 26–2). (A) whole RNA samples (1 μg) from cells grown in 0, 5, 10, 50 and 100 nM T were submitted to semi-quantitative competitive RT-PCRs for AR and 5RII transcripts with 0.2 amol AR and 0.02 amol 5RII competitor simultaneously. Experiments were performed in triplicate. Top panel: 2% agarose gel with products from semi-kinetic RT-PCRs indicating similar L7 transcript amounts. Bottom panel: one representative PAA gel per transcript is demonstrated. A(t/s) values for AR and 5RII transcript levels in control cells grown without T were set to 100% and compared with the three experiments for each T concentration. In the graphs, A(t/s)-values for transcripts in T-exposed cells are expressed as a percentage of their control for each T concentration chosen. Average values of the three measurements are connected by a line for graphical reasons, demonstrating no significant influence of T on AR and 5RII transcription. t: target band; s: standard band; M: molecular size markers with DNA fragments of 527, 404 and 307 bp (left gel) or 404 and 307 bp (right gel) length. (B) Northern blots with specific signals of similar density at 10.5 kb (AR mRNA) and 2.4 kb (5RII mRNA) in lanes with whole RNA from GSF incubated with 0, 10 and 100 nM T. Actin signaling indicates equal loading.
transcript concentrations and the presence or absence of T in different GSF cell lines was found. In contrast to AR transcription, 5RII mRNA levels in GSFs grown without T displayed marked individual differences which are additionally dependent on donor ages. Higher transcript levels were found in early infancy with maximum values in GSFs from the donors aged 2 years 7 months and 4 years 3 months. Then, A(t/s) values decline reaching the lowest values at donor ages of 42 and 51 years (Fig. 4). These observations were supported by semi-quantitative competitive RT-PCRs for 5RII on RNA from GSF cell lines hVHF 2±2 (11 months old) and hVHF 33±2 (42 years old) with competitor concentrations decreasing from 0.1 to 0.005 amol/mg RNA (data not shown). For hVHF 2±2 the target ampliﬁcate emerged on PAA gels despite competition by a high competitor concentration (0.1 amol); for hVHF 33±2 the target product only appeared reproducible at 0.005 amol competitor per μg RNA.

Discussion

Simultaneous, competitive RT-PCRs allow accurate semi-quantification of transcripts

Combined reverse transcription and competitive PCR is a reliable method for speciﬁc gene transcript quantiﬁcation. In contrast to Northern blots and ribonuclease protection assays (11, 21), semi-quantitative competitive RT-PCR is especially less labor and often also less material intensive and comprises only two substantial steps and thus only two error sources: cDNA synthesis and PCR. PCR is most critical for proper quantification, as exponential ampliﬁcation is followed by stages of linear and/or stagnant ampliﬁcation (plateau phase) at higher cycles. This problem is encompassed by artiﬁcial standards (competitors). During the exponential phase and plateau, these standards behave similarly to the targets due to target identical primer recognition sequences (22, 13). This was documented by moderate technical variations found at the PCR level (±5% for the AR and 11% for the 5RII transcript). As published by Price et al. (23) and veriﬁed by us (Fig. 1), relative transcript level differences can be distinguished reliably over at least a 4-fold range by adding just one constant competitor concentration to the samples. Synthesis of cDNA, however, is also subject to variations which may be critical for accurate quantiﬁcation: reverse transcription with poly-dT primers has been reported to reach only 40–50% efﬁcacy (24, 25). This step is normally standardized by low cycle-number PCRs on reverse transcribed house-keeping gene transcripts (13). We ourselves made kinetic RT-PCRs on house-keeping gene L7 transcripts to assure similar whole RNA sampling.
(Fig. 2). However, manual kinetic RT-PCR is imprecise, as differences lower than 2-fold (26) cannot be distinguished reliably. Additionally, possible RT efficiency variances which are specific to different transcripts cannot be outlined by kinetic RT-PCRs on housekeeping gene transcripts. Therefore this step was standardized by the addition of RNA competitors before cDNA synthesis, as reported by Henvel et al. (27). In fact, we could demonstrate that in-tube RT efficacy variances are normalized to tolerable values. However, quantification had to be repeated to provide reliable results. If two transcripts were examined, whole RNA amounts and labour-intensity increased considerably. A preservation of both advantages of our method, fully standardized RT-PCR by RNA competitors and reduced material and time consumption, was made possible by specific-primed RT of AR and 5RII transcripts with RNA competitors in one tube followed by separate PCRs. We achieved reliable semi-quantification of two transcripts per μg RNA. Theoretically, multiple transcripts can be quantified with little expenditure by semi-quantitative competitive RT-PCRs.

Natural androgens do not influence AR and 5RII transcription in GSFs

Our observations indicate that testosterone and dihydrotestosterone, independently of cell line, incubation time or androgen concentration, do not control AR or 5RII transcription in cultured GSFs (Figs 2 and 3, Table 1). Our results are supported by previous studies (28–30), which point to an androgen-independent regulation of 5α-reductase activity in skin from external genitalia. In contrast, these former studies indicate that 5α-reductase in cells from the pubic skin appears to be under the control of androgens. Seemingly, this is the result of predominant 5α-reductase type I expression in this cell type (31); this 5α-reductase iso-enzyme seems to underlie androgen-dependent expression control mechanisms. For AR, our results are in accordance with the data from Wolf et al. (10), who did not observe any effect of 96 h of androgen incubation on AR transcript levels in GSFs. However, Nirdé et al. (32) reported a DHT-driven AR transcription regulation observable at short periods of incubation (0.5–2 h). This contrast may be explained by different experimental designs. Firstly, Nirdé et al. (32) incubated their cells in 1 nM DHT; this is a 5-fold lower concentration than the lowest T concentration used by us (5 nM). However, as we demonstrated 5RII transcription in GSFs, T can be expected to be partly reduced to DHT by 5RII translation products. Thus, GSFs should display at least residual or retarded AR transcription regulation in response to T. We could not detect such event, the same holds true for incubation with 100 nM DHT (Fig. 3). Second, Nirdé et al. (32) applied AR transcript quantification on GSFs from newborn boys. Our detailed experiments were performed on GSFs from infants of approximately 4 years of age. It could be possible that AR transcription regulation is controlled by androgens only during a restricted phase ending during early infancy. However, AR transcript levels in GSFs from 5-month-old individuals did not differ significantly from those in GSFs from older individuals (Fig. 4), making pronounced changes of AR transcription regulation mechanisms improbable. Additionally, these GSFs also did not display any specific reaction to 100 nM T administration after 48 h (data not shown). The results of Nirdé et al. (32) show wide differences of AR transcript level reactions to DHT incubation between four different GSF cultures. Whether these variations and the differences dependent on DHT incubation times are influenced by the technical approach they took remains to be answered. Several deficiencies are
apparent in the method used by Nirdé et al. (32) for the application of reverse transcribed competitive PCRs, as they did not monitor transcript-specific differences in cDNA efficiency and DNA competitor was added after the RT step. They also measured optical densities of products from RT competitive PCRs on agarose gels, which may present with a high background and additionally do not provide optimal separation of similarly lengthened DNA fragments. These problems are eliminated in the method described here as we used RNA competitors and PAA gels for densitometric evaluations of product bands. Additionally, we analyzed 12 different GSF cultures, in contrast to four cultures tested by Nirdé et al. (32).

Levels of 5RII mRNA showed marked individual variations (Fig. 4). Seemingly, they are predominantly correlated with the donor age. After higher transcription rates in early infancy, 5RII mRNA amounts decrease considerably to the lowest values in GSFs from a 12-year-old boy and a fertile man. These results are in accordance with those of Pinsky et al. (33), who reported a 40-fold variation of 5α-reductase activity between different foreskin GSF strains. Although previous indications for high 5α-reductase levels in external genitalia of prepubertal individuals exist (28–30), we could not find direct reports which support or contradict a possible age dependence of 5RII transcript levels in GSFs. Hodgins (34) suspected that high 5α-reductase activity in embryonic external genitalia may serve to protect these tissues from the potentially antiandrogenic action of progesterone. Correspondingly, the low 5RII mRNA levels observed by us in GSFs from pubertal and adult donors could reflect the decreased necessity for such protection at older ages. The extremely low 5RII mRNA levels in adult GSFs may also be explained by their insignificance for further maturation of external genitalia. The finding that the highest levels occurred in GSFs from individuals of approximately 2 to 4 years old, however, does not fit with these explanations, although it could be explained by individual differences alone.

In contrast, AR transcription is similar in all GSF cultures tested independent of age. For maturation of male external genitalia in embryonic stages, infancy and puberty, constant and reliable AR levels may be critical in GSFs, as they play an important role in the organization of these tissues. The need for such constancy may persist in adulthood to guarantee instant response of genital tissues to androgens. If this is the case, AR mRNA levels should be insensitive to androgen-driven regulation during maturation and adulthood, as this would lead to instabilities in AR expression depending on hormonal variations. Similarly, the last consideration could also explain the absence of androgen-driven autoregulation of 5RII transcription.

In conclusion, impairment of AR or 5RII transcription may well be an important mechanism in virilization disorders. Whether this holds true in respective patients still remains to be investigated.

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