HORMONAL REGULATION OF PROSTATE SPECIFIC ANTIGEN AND HUMAN GLANDULAR KALLIKREIN IN MALES AND FEMALES IN VIVO: EFFECTS OF ANDROGENS AND ANTIANDROGENS ON PLASMA AND URINARY PSA AND HK2 LEVELS

by

Christina V. Obiezu

A thesis submitted in conformity with the requirements for the Degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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CHRISTINA V. OBIEZU

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ABSTRACT

The PSA and hK2 genes are known to be regulated by androgens and progestins in vitro. To determine if this also occurs in vivo, plasma and urinary PSA and hK2 were immunoassayed in 32 female-to-male transsexuals before, at 4 and at 12 months of testosterone treatment, as well as in 3 groups of male-to-female transsexuals undergoing 3 types of androgen-suppressing therapy. Due to testosterone, serum and urinary PSA as well as urinary hK2 were upregulated dramatically (>90%) by the 4th month; serum hK2 remained undetectable. Conversely, the antiandrogen cyproterone acetate (CA), alone or in combination with estradiol, suppressed plasma and urinary PSA and hK2 (>90%) either at 4 or 12 months of therapy. All changes were statistically significant (P<0.001) at 4 months compared to baseline. The demonstrated in-vivo upregulation of PSA and hK2 by testosterone, and their in-vivo downregulation by CA may have clinical applications in hyperandrogenic females and prostate cancer patients.
ACKNOWLEDGEMENTS

First and foremost, I would like to express my warmest gratitude to my supervisor, Dr. Eleftherios P. Diamandis, for his guidance and support during the course of this study; his tremendous insight and enthusiasm provided the foundation for the success of this work. My gratitude also extends to our invaluable collaborators, Dr. Erik J. Giltay and Louis G. Gooren; without their meticulous collection and preparation of the clinical samples, this study would not have been possible. Special thanks to the members of my committee, Dr. Alex Romaschin and Dr. Khosrow Adeli, for their mentorship and helpful advice. Dr. David Howarth and Dr. Andreas Scorilas provided their expertise for histochemical and statistical analyses. Many thanks to my fellow graduate students in our laboratory, from whose friendship and suggestions I have benefited a great deal. I wish to acknowledge Dr. Marc Mittelman for my initiation into the field of medical research. My warmest thanks to my father, my brother and all my friends who provided me with a lot of support during my graduate studies.

I dedicate this work to the memory of my late Mother who has taught me to strive to reach my goals and my full potential.
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1. The Human Kallikreins</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Prostate Specific Antigen (PSA) Protein</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1 PSA in extraprostatic Tissues and Fluids</td>
<td>4</td>
</tr>
<tr>
<td>1.1.2 Serum PSA and Seminal Plasma PSA</td>
<td>4</td>
</tr>
<tr>
<td>1.1.3 Urinary PSA</td>
<td>6</td>
</tr>
<tr>
<td>1.2 Human Glandular Kallikrein (hK2) Protein</td>
<td>7</td>
</tr>
<tr>
<td>1.2.1 hK2 in Seminal Plasma, Serum and Urine</td>
<td>9</td>
</tr>
<tr>
<td>2. The Androgen System: Function and Dysfunction</td>
<td>10</td>
</tr>
<tr>
<td>2.1 Androgens</td>
<td>10</td>
</tr>
<tr>
<td>2.1.1 Androgens in Males</td>
<td>10</td>
</tr>
<tr>
<td>2.1.2 Androgens in Females</td>
<td>14</td>
</tr>
<tr>
<td>2.1.3 Androgenic Dysfunction in Females</td>
<td>15</td>
</tr>
<tr>
<td>2.2 The Androgen Receptor: Structure and Function</td>
<td>16</td>
</tr>
<tr>
<td>2.3 Antiandrogens</td>
<td>17</td>
</tr>
<tr>
<td>3. Regulation of PSA and hK2 Gene Expression</td>
<td>19</td>
</tr>
<tr>
<td>3.1 PSA and hK2 Gene Regulation by the AR</td>
<td>19</td>
</tr>
<tr>
<td>3.1.1 Regulation by the Androgen-AR complex</td>
<td>19</td>
</tr>
<tr>
<td>3.1.2 Regulation via Non-Androgenic Activation of the AR/Bypass of AR Activation</td>
<td>22</td>
</tr>
<tr>
<td>3.2 Androgen, AR or other steroid receptor independent regulation</td>
<td>24</td>
</tr>
<tr>
<td>4. PSA and hK2 as Markers of Disease and Androgen Irregularity</td>
<td>25</td>
</tr>
<tr>
<td>4.1 PSA and hK2 in Prostatic Diseases</td>
<td>25</td>
</tr>
<tr>
<td>4.2 PSA and hK2 in Breast Tissue Tumorigenicity</td>
<td>27</td>
</tr>
<tr>
<td>4.3 PSA in Females with Elevated Androgens</td>
<td>28</td>
</tr>
</tbody>
</table>
II. RATIONALE

III. HYPOTHESIS

IV. OBJECTIVES

V. MATERIALS AND METHODS
1. Drug Treatment
2. Blood and Urine Sample Collection
3. PSA Immunoassay
4. hK2 Immunoassay
5. Immunoassays for other Serum and Urine Parameters
6. Statistical Analysis of Immunoassay Results
7. Immunohistochemical Localization of PSA, hK2 and AR

VI. RESULTS
1. Results of Samples from Female-to-Male Transsexuals
   1.1 Immunoassay Results
      1.1.1 PSA in Plasma and Urine
      1.1.2 hK2 in Plasma and Urine
   1.2 Correlation of PSA and hK2 with Other Tested Parameters
   1.3 Immunohistochemical Staining Results
2. Results of Samples from Male-to-Female Transsexuals
   2.1 Immunoassay Results for Patients under
      Ethinyl Estradiol + CA Treatment
      2.1.1 Plasma PSA and hK2
      2.1.2 Urinary PSA and hK2
   2.2 Immunoassay Results for Patients under CA Treatment
      with or without Transdermal Estradiol
      2.2.1 Plasma and Urinary PSA
      2.2.2 Plasma and Urinary hK2
   2.3 Correlation of PSA and hK2 with Other Tested Parameters
VII. DISCUSSION

1. Androgen Induced PSA and hK2 Upregulation *In Vivo* 59

2. Antiandrogenic Suppression of PSA and hK2 *In Vivo* 63

3. Conclusions 65

VIII. REFERENCES 66
## LIST OF TABLES

| Table VI-1.2a | Mean testosterone, LH and FSH values in serum at baseline, 4 and 12 months of testosterone treatment | 45 |
| Table VI-1.2b | Spearman correlation values, for parameters measured in serum 4 months post treatment, with plasma, urinary PSA as well as with urinary hK2 | 46 |
| Table VI-1.2c | Spearman correlation values, for parameters measured in serum 12 months post treatment, with plasma, urinary PSA and with urinary hK2 | 47 |
LIST OF FIGURES

Figure II-2.1  Structures of some Androgens and Antiandrogens  11

Figure II-2.1.1a  Androgen Production and Action from the Gonads  12

Figure II-2.1.1b  Androgen Production from the Adrenal Cortex  13

Figure III-3.1  Schematic Representation of the ARE Organization in the hKLK2 and hKLKL3 Promoters  21

Figure VI-1.1.1a  Mean Plasma PSA Concentrations at 0, 4 and 12 Months of Testosterone Treatment  42

Figure VI-1.1.1b  Mean Urinary PSA Concentrations at 0, 4 and 12 Months of Testosterone Treatment  42

Figure VI-1.1.2  Mean Urinary hK2 Concentrations at 0, 4 and 12 Months of Testosterone Treatment  44

Figure VI-1.3a  Histochemical Staining for PSA in Breast Tissue  49

Figure VI-1.3b  Histochemical Staining for hK2 in Breast Tissue  50

Figure VI-1.3c  Histochemical Staining for the AR in Breast Tissue  51

Figure VI-2.1.1a  Median Plasma PSA under Ethinyl Estradiol + Cyproterone Acetate Treatment  53

Figure VI-2.1.1b  Median Plasma hK2 under Ethinyl Estradiol + Cyproterone Acetate Treatment  53
Figure VI-2.1.2a Median Urinary PSA under Ethinyl Estradiol + Cyproterone Acetate Regime 55
Figure VI-2.1.2b Median Urinary hK2 under Ethinyl Estradiol + Cyproterone Acetate Regime 55
Figure VI-2.2.1a Median PSA in Plasma under CA and Transdermal Estradiol + CA Treatment 56
Figure VI-2.2.1b Median PSA in Urine under CA and Transdermal Estradiol + CA Treatment 56
Figure VI-2.2.2a Median hK2 in Plasma under CA and Transdermal Estradiol + CA Treatment 57
Figure VI-2.2.2b Median hK2 in Urine under CA and Transdermal Estradiol + CA Treatment 57
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>3α-AG</td>
<td>3-alpha androstenedione glucuronide</td>
</tr>
<tr>
<td>A2M</td>
<td>alpha-2-macroglobulin</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ACT</td>
<td>alpha-1 antichymotrypsin</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropicin</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>androgen response element</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BPH</td>
<td>benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CA</td>
<td>cyproterone acetate</td>
</tr>
<tr>
<td>CAH</td>
<td>congenital adrenal hyperplasia</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DFP</td>
<td>diflunisal phosphate</td>
</tr>
<tr>
<td>DHT</td>
<td>5-alpha dihydrotestosterone</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRE</td>
<td>digital rectal examination</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
</tr>
<tr>
<td>hK1</td>
<td>human tissue kallikrein</td>
</tr>
<tr>
<td>hK2</td>
<td>human glandular kallikrein</td>
</tr>
<tr>
<td>HSCCE</td>
<td>human stratum corneum chymotryptic enzyme</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KGF</td>
<td>keratinocyte growth factor</td>
</tr>
<tr>
<td>KLK-L</td>
<td>kallikrein-like</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LNCaP</td>
<td>lymph node metastasized prostate cancer</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>mililitre</td>
</tr>
<tr>
<td>mM</td>
<td>milimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NES-1</td>
<td>normal epithelial cell specific gene 1</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>PC</td>
<td>prostate cancer</td>
</tr>
<tr>
<td>PCI</td>
<td>protein C inhibitor</td>
</tr>
<tr>
<td>PCOS</td>
<td>polycystic ovary syndrome</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate specific antigen</td>
</tr>
<tr>
<td>SA-ALP</td>
<td>streptavidin-alkaline phosphatase conjugate</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone binding globulin</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>Tb$^{3+}$</td>
<td>terbium ion</td>
</tr>
<tr>
<td>TGF-α</td>
<td>transforming growth factor alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TLSP</td>
<td>trypsin-like serine protease</td>
</tr>
<tr>
<td>TSH</td>
<td>thyrotropin</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

1. THE HUMAN KALLIKREINS

The human kallikreins, comprising a subgroup of serine protease enzymes, play a wide variety of roles ranging from the cleavage of vasoactive peptides that regulate blood pressure to the degradation of extracellular matrix proteins for cell mobilization. In each case, they perform their enzymatic function with a high degree of specificity (Evans et al., 1988). The first discovered member of this group of enzymes, tissue kallikrein (hK1) (Frey et al., 1932; McDonald et al., 1988) is responsible for the cleavage of the low-molecular weight kininogen, thus releasing the vasoactive peptide Lys-bradikinin which regulates blood pressure (Bhrola et al., 1992). Because its enzymatic function is similar to that demonstrated by other rat and mouse kallikreins, this activity soon became the requirement for any protein to be classified as a kallikrein.

Another protein named prostate specific antigen (PSA), was first discovered in, and purified from the male prostate (Wang et al., 1977, 1979, 1981). By virtue of its similarity to hK1 and due to its ability to produce small amounts of kinin-like molecules in seminal fluid (Fichtner et al., 1996), this protein also came to be regarded as a kallikrein. However, it soon became apparent that PSA does not possess exactly the same enzymatic characteristics as hK1 since it has a chymotrypsin-like enzymatic activity as opposed to the trypsin-like activity of hK1 (Deperthes et al., 1997). Hence, the cleavage of kinins does not appear to be the main function of PSA.
For some time, hK1, PSA and the subsequently discovered human glandular kallikrein (hK2) were thought to be the only members of the human kallikrein family which then was primarily defined by their kinin-producing enzymatic activity (Schachter et al., 1980). A revolution to the term 'kallikrein' and the accompanying concept occurred with the discovery of the kallikrein multigene family in mice, consisting of many genes sharing a high degree of sequence similarity, with all genes colocalizing to murine chromosome 7 (Evans et al., 1985; Van Leeunen et al., 1986). Following this, it became evident that in terms of the defining characteristics of kallikreins, the emphasis should rest on their genetic sequence similarity and localization, rather than on the enzymatic activity of the protein.

With the discovery of human stratum corneum chymotryptic enzyme (HSCCE) (Lundstrom & Egelrud, 1991), normal epithelial cell-specific gene 1 (NES-1) (Lin et al., 1996), followed recently by their localization to chromosome 19q13.2-13.4 (Luo et al., 1998; Yousef et al., 1999), it became imperative that the more broader, functional definition be adopted for human kallikreins. Under this notion, the human kallikrein family grew considerably with the discovery of additional, kallikrein-like proteins like neuropsin (Suzuki et al., 1995; Shimizu et al., 1998; Yoshida et al., 1998a), zyme/proteaseM/neurosin (Ainsawicz et al., 1996; Little et al., 1997; Yamashiro et al., 1997) and trypsin-like serine protease (TLSP) (Yoshida et al., 1998b). A recent breakthrough in this field came with the discovery of 6 additional kallikrein-like genes: Prostase/KLKL1 (Nelson et al., 1999; Yousef et al., 1999), KLKL2, KLKL3, KLKL4, KLKL5 (Yousef et al.,
1999). Based on the sequence similarity and colocalization of these genes to the human chromosome 19q13.3-13.4, the multigenic nature of human kallikreins has been proven beyond a doubt.

1.1 The Prostate Specific Antigen (PSA) Protein

Prostate specific antigen is a 33 kDa glycoprotein, first discovered to be produced by the ductal epithelial cells of the male prostate. (Wang et al., 1981; Sinha et al., 1987). After translation into a 261 aa-long prepropeptide, post-translational formation of five intramolecular disulfide bonds, and variable glycosylation events at Asp$^{45}$, PSA is secreted, upon cleavage of the 7 aa-long signal peptide at its N-terminus, as a propeptide into the seminal fluid (Lunwall & Lilja, 1987). Following the additional cleavage of seventeen N-terminal amino acids, PSA attains its mature, enzymatically active form (Takayama et al., 1997). The main function of this chymotrypsin-like enzyme in seminal fluid is to aid sperm motility by cleaving seminogelin I and II proteins of the seminal coagulum (Lilja, 1985). The presence of the catalytic triad (His$^{41}$, Asp$^{96}$, Ser$^{189}$ in PSA), known to be conserved in kallikreins, clearly identifies this protein as a serine protease (Rittenhouse et al., 1998). In prostatic tissues, PSA exists in its uncomplexed form (free PSA), 70% of which is proteolytically active (Christensson et al., 1990).
1.1.1  **PSA in Extraprostatic Tissues and Fluids**

With the advent of the development of highly sensitive immunoassays for PSA, it became evident that PSA production is not exclusive to the male prostate; indeed, PSA was demonstrated to be produced in both female and male extraprostatic tissues. Of these, most notable are cancerous, hyperplastic and normal breast tissue, the breast milk of lactating women, nipple aspirate fluid, amniotic fluid and various malignancies of the pituitary, lung and ovary (see Table 1.1.1 for detailed reference). With the exception of nipple aspirate fluid, where PSA concentrations may approach as much as 3000 ng/mL (Foretova et al., 1996; Sauter et al., 1996), PSA levels are at least $10^5$ – $10^6$ fold lower in extraprostatic tissues and fluids than in seminal plasma (Rittenhouse et al., 1998).

1.1.2  **Serum PSA and Seminal Plasma PSA**

Two main forms of PSA exist in serum: free PSA and PSA complexed with various serine protease inhibitors (serpins). In serum, a significant portion of PSA is complexed to $\alpha$1-antichymotrypsin (ACT) and to a lesser extent to $\alpha$-2 macroglobulin (A2M), with the PSA-ACT complexes accounting for more than 80% of total serum PSA (Pizzo et al., 1988). These complexes are removed from the circulation by the liver via liver serpin receptors (Pizzo et al., 1988) while the PSA-A2M leave the circulation through the interaction with A2M receptors found on reticuloendothelial cells (Travis et al., 1983).
Table I-1.1.1: Extraprostatic sources of PSA

<table>
<thead>
<tr>
<th>Tissue / biological fluid</th>
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<tr>
<td>Normal, hyperplastic and cancerous breast tissue</td>
<td>Yu et.al., 1994</td>
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<td>Yu et.al., 1995</td>
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<tr>
<td></td>
<td>Yu et.al., 1996</td>
</tr>
<tr>
<td>Nipple aspirate fluid</td>
<td>Foretova et.al., 1996</td>
</tr>
<tr>
<td></td>
<td>Sauter et.al., 1996</td>
</tr>
<tr>
<td>Breast milk of lactating women</td>
<td>Yu et.al., 1995b</td>
</tr>
<tr>
<td>Breast cyst fluid</td>
<td>Manello et.al., 1996</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>Yu &amp; Diamandis, 1995c</td>
</tr>
<tr>
<td></td>
<td>Melegos et.al., 1996</td>
</tr>
<tr>
<td>Cancerous ovarian tissue</td>
<td>Yu et.al., 1995d</td>
</tr>
<tr>
<td>Cancerous lung tissue</td>
<td>Levesque et.al., 1995</td>
</tr>
</tbody>
</table>
In males, the main source of PSA is the prostate gland. Total serum PSA levels are normally below 2 ng/mL with around 0.2-0.3 ng/mL for young men below the age of 30. Higher levels than 4 ng/mL indicate that the tight containment of PSA in prostatic tissues has been breached, strongly suggesting some sort of prostatic tissue damage (Oesterling et al., 1991). In seminal plasma, PSA levels reach an extremely high concentration of 500,000 – 3,000,000 ng/mL (Wang et al., 1982; Wang et al., 1998). There, a large portion of PSA is in the clipped form (Zang et al., 1995) and only trace amounts of PSA are complexed to A2M (Otto et al., 1998).

In contrast to male serum, where PSA levels can be readily detected by standard immunoassays, female serum levels of PSA can only be detected using ultrasensitive immunoassays because female PSA levels are normally less than 0.1 ng/mL (Yu & Diamandis, 1995a; Zarghami et al., 1997a). The main source of PSA in the circulation is believed to be the female breast as suggested by the finding of elevated levels of PSA in sera of women suffering from fibrocystic changes of the mammary tissue (Borchert et al., 1997). Even so, one cannot exclude the possibility that the Skene's gland can also contribute to serum PSA levels as noted in a case of Skene's gland adenocarcinoma (Dodson et al., 1994).

1.1.3 Urinary PSA

In male urine, only clipped form of PSA (<5%) could originate from serum since serum PSA-ACT complexes are too large to be filtered into the renal
effluent. In male urine, the main source of PSA is the periurethral glands (Iwakiri et al., 1993); these glands themselves do not contribute to serum PSA levels (Oesterling et al., 1996). Normal urinary PSA levels are of the magnitude 100-300 ng/mL. While elevated levels may indicate prostatic malignancy, urinary PSA is not a good marker of this due to periurethral PSA secretion (Takayama et al., 1997).

In females, urinary PSA levels are around 2 ng/mL (Breul et al., 1997) and its putative source is the periurethral glands (Skene's gland), as suggested by positive staining of PSA in this glands in immunohistochemical studies (Tepper et al., 1984; Wernert et al., 1992;).

1.2 The Human Glandular Kallikrein (hK2) Protein

Human glandular kallikrein (hK2) is also a 33 kDa single-chain glycoprotein. Because of its 80% sequence similarity to PSA on the protein level, it is not surprising that hK2 share many of the characteristics of PSA (Dube & Tremblay, 1997) including the same amino acid length, and the steps involved in the post-translational processing to its mature enzymatic form (McCormack, 1995). In fact, it was on the account of its high degree of similarity to PSA, that the initial efforts to isolate and purify this protein from prostate tissue were hampered. Expression of hK2 mRNA was demonstrated in the prostate (Chapdelaine, 1988) soon after the isolation of its DNA sequence using genomic hybridization technique with a mouse kallikrein cDNA serving as probe (Schedlich et al., 1987). However, successful isolation of hK2 from seminal plasma became possible only after
monoclonal antibodies were raised against synthetic peptides representing regions of least homology to PSA (Deperthes et al., 1995).

Despite its marked similarity to PSA, extensive studies of expressed and purified recombinant hK2 (Kumar et al., 1996) revealed the existence of some important differences between hK2 and PSA. hK2 is predicted to be glycosylated on Asn78 (Eerola et al., 1997; Mikolajczyk et al., 1998) and its secreted zymogen form is very rapidly converted to the mature, enzymatically active form (Kumar et al., 1996). In marked contrast to PSA, hK2 has a trypsin-like enzymatic specificity (Deperthes et al., 1996; Frenette et al., 1997a) with some 20,000-fold higher proteolytic activity than that of PSA (Mikolajczyk et al., 1997, 1998). hK2 is unique among kallikreins in its ability to activate itself (Mikolajczyk et al., 1997) which accounts for its transient existence as a proenzyme. In addition to autoactivation, the possible biological roles of hK2 include the activation of proPSA into enzymatically active PSA (Kumar et al., 1997; Lovgren et al., 1997; Takayama et al., 1997), and the cleavage of seminogelin I and II in seminal plasma to enhance sperm motility. However, a high biological importance of hK2 in cleaving the seminal clot is doubtful due to its presence at much lower concentration in seminal fluid than PSA (Deperthes et al., 1996). While hK2 cannot release Lys-bradikin the same way as hK1 can (Deperthes et al., 1997), hK2 has recently been demonstrated to be able to release bradykinin from high molecular weight kininogen; this ability may permit hK2 to play a role in enhancing sperm motility via increasing vascular permeability through bradykinin’s action (Charlesworth, 1999). Additional enzymatic activities include
the cleavage of IGFBP (Dube et.al., 1997) and the activation of uPA which converts plasminogen into plasmin (Frenette et.al., 1997b). Enzymatic activity of hK2 is now known to be inhibited by zinc ions (Lovgren et.al., 1999).

1.2.1 hK2 in Seminal Plasma, in Serum and Urine

Unlike PSA, very little information is known about the forms and levels of hK2 outside the prostate. In general, seminal plasma levels of hK2 are between 2000-12,000 ng/mL in healthy males, a much lower concentration that of PSA (Dube et.al., 1997; Finlay et.al., 1998). While in the prostate almost all of hK2 is in its uncomplexed form with a significant fraction of this being prohK2 (Rittenhouse et.al., 1998), in seminal plasma, around 50% of hK2 is found complexed to protein C inhibitor (PCI) (Deperthes et.al., 1995). In contrast, in serum hK2 exists mainly as a complex with ACT; hK2 complexed with PCI or A2M has not yet been found. Virtually nothing is known yet about hK2 levels in female serum and urine though its levels in nipple aspirate fluid and breast cyst fluid are under investigation for possible clinical usefulness in breast cancer.
2. THE ANDROGEN SYSTEM: FUNCTION AND DISFUNCTION

2.1 Androgens

Androgens are lipid soluble, steroidal hormones whose production is under the direct control of the hypothalamo-pituitary axis via follicle stimulating hormone (FSH) and luteinizing hormone (LH) secretion. The two most prominent androgens are testosterone, and its even more potent form 5α-dihydrotestosterone (DHT) (Fig II-2.1). In target tissues, testosterone is converted to DHT by the NADPH-dependent enzymes type 1 and type 2 5α-reductase. The former resides in the endoplasmic reticulum (ER) and nuclear membrane of epithelial cells in the scalp, skin and liver, while the latter form of the enzyme is mainly expressed in ER and nuclear membrane of the epididymis, seminal vesicle and the prostate gland (Rittmaster, 1995).

2.1.1 Androgens in Males

In males, most of the testosterone arises from the conversion of cholesterol in the testes (Fig. II-2.1.1a); however, a small amount of circulating testosterone originates from the conversion of dehydroepiandrosterone (DHEA) and androsterone in the adrenal glands; these two androgens are byproducts of andrenocorticotropic (ACTH)-induced cortisol synthesis (Fig. II-2.1.1b) (Gill, 1976; Simpson & Waterman, 1983). In the circulation, only 1-2% of testosterone is available for uptake by androgen sensitive tissues at any given time because
Androgens

- Testosterone
- 5-alpha-dihydrotestosterone (DHT)
- Androstenedione

Antiandrogens

- Cyproterone Acetate (CA)
- Hydroxyflutamide

Figure II-2.1: Structures of some Androgens and Antiadrogens
Figure II-2.1.1a: Androgen Production and Action from the Gonads
Figure II-2.1.1b: Androgen Production from the Adrenal Cortex
the rest is bound by sex hormone binding globulin (SHBG) (Petra, 1991). Being
the chief hormonal messengers in males, androgens are vital to many key
processes including muscle build, cell growth, brain activity, sex drive, secondary
male characteristics and for the growth and differentiation of the prostate in
addition to regulating its secretory activity.

2.1.2 Androgens in Females

While the principal hormonal messengers are estrogens and progestins,
endogenous androgens, although at much lower levels than in males, are
produced in the female ovaries and adrenals. They act as precursors of estrogen
formation (Fig. II-2.1.1a) in the ovaries, play a role in muscle development,
enhance libido (Rittmaster, 1995) and influence normal follicle development
(Daniel et al., 1986). The most abundantly secreted androgens from the ovaries
are androstenedione (1.3 – 1.6 ng/mL) and testosterone (0.4 ng/mL), the latter
being the far more potent of the two; this accounts for 50% - 70% of total
testosterone in women (Rittmaster, 1995). Additional sources of circulating
testosterone in females include the peripheral conversion of androgen precursors
in the skin, liver and adipose tissue, accounting for the remaining 30%-50% of
testosterone in the circulation (Longcope et al., 1986).

As in males, the active form of testosterone is DHT in androgen sensitive
organs in females; subsequently, DHT is rapidly metabolized to androstenediol/
androstenedione glucuronide or sulfate conjugates. These compounds are used
as markers of androgen metabolism in skin to confirm hirsutism in females.
(Rittenmaster, 1993). In contrast to androgens in males which are under the direct regulation of FSH and LH action, in females FSH and LH regulates estrogen and cortisol synthesis to which androgens are only precursors or byproducts. Therefore in women, the actual levels of circulating androgens are indirectly determined by factors that influence ovarian and adrenal steroid synthesis (Rittmaster, 1995).

2.1.3 Androgenic dysfunction in females

In women, the most common form of androgenic dysfunction is hyperandrogenism, which affects almost 10%-25% of the female population in the United States (Derman, 1995). Hyperandrogenism may be the result of increased LH secretion, most often seen in patients suffering from polycystic ovary syndrome (PCOS), or it may be due to adrenal overstimulation caused by ACTH as is the case in women suffering from congenital adrenal hyperplasia (CAH). In the former case, predominantly ovarian overproduction of testosterone (1.5-2.0 ng/mL) is often associated with obesity, menstrual irregularity or anovulation and severe hirsutism (Redmond, 1998). In contrast, patients, whose hyperandrogenism is due to adrenal overstimulation, have elevated androstenedione levels (>4.0 ng/mL), remain slender, and have normal menstrual cycles and fertility (Redmond, 1998). In both cases, allopata and hirsutism (due to either elevated circulatory testosterone or to DHT hypersensitivity caused by increased type 2 5α-reductase activity in the skin), may or may not be present.
2.2 The Androgen Receptor: Structure and Function

The androgen receptor (AR) belongs to the superfamily of steroid/thyroid hormone receptors. The other members of this family are the estrogen receptor, progesterone receptor, mineralocorticoid receptor, glucocorticoid receptor, vitamin D, thyroid hormone receptor, retinoic acid receptor and the so called orphan receptors whose ligands are not yet known (Evans et al., 1988; Wahli & Martinez, 1991). All steroid hormone receptors are ligand activated transcription factors; in case of the AR receptor, its activating ligand is DHT (Kaufman et al., 1981). AR, in addition to inducing gene expression, may influence the stability of mRNA (Wolfe et al., 1992).

AR is encoded by the q11-12 region of the X chromosome, its codons spanning a 90 kb region (Brown et al., 1989). Its eight coding exons give rise to a 10.6 kb mRNA, of which 2.8 kb codes for its open reading frame; this mRNA is then translated into a 918 aa-long protein with varying stretches of polyglycine (17-23) and polyglutamine (16-27) residues (Kuiper et al., 1989). The N terminus of the AR is phosphorylated and has a DNA binding domain (DBD) highly conserved among steroid hormone receptors, while its C-terminal portion houses the ligand binding domain (Brinkman et al., 1989; Jenster et al., 1991). Just like all steroid hormone receptors, the AR protein is found in the cytoplasm in association with the HSP 70 and HSP 90; once ligand binding takes place, it is believed that the dissociation of HSP 90 allows a conformational change to take place, subsequently permitting the interaction of DBD with the chromatin (Smith & Toft, 1993). However, experimental evidence for the exact steps in the
mechanism of activation of the androgen receptor has not yet been obtained. So far, it has been shown experimentally that upon stimulation by androgens, the C-terminal portion of the androgen receptor forms a complex with its ligand; the complex is then translocated through the nuclear membrane into the nucleus where its DBD can interact with promoters of androgen responsive genes, resulting in transcriptional activation (Horwitz et al., 1996).

2.3 Antiandrogens

Steroidal antiandrogens, due to the similarity of their molecular shape to androgens (Fig 2.1), can bind the AR without eliciting a physiological response, thus competing with androgens for the occupation of the AR (Neri, 1976). In the clinical setting, antiandrogens are used to treat illnesses where the underlying cause is androgenic overexpression, such as in alopecia, hirsutism and androgen-dependent prostate cancer (PC) (Namer, 1988). Non-steroidal antiandrogens, such as flutamide and hydroxyflutamide, are pure antiandrogens since they have no steroidal effects (Habernicht et al., 1988). However, because of the fact that pure antiandrogens are known to cause an increased secretion of androgens from the testes due to the negative feedback of androgens on the hypothalamo-pituitary axis, treatment of PC is more effective using antiandrogens like cyproterone acetate (CA) which has both antiandrogenic and mild progestational effects (Wakeling et al., 1981). CA, like other antiandrogens, not only inhibit binding of DHT to its receptor, but also influence serum testosterone levels via suppression of pituitary gonadotrophins; for this reason its
side effects include breast enlargement, loss of sexual drive and impotence (Fishman & Geller, 1970). Another clinically useful antiandrogen is spironolactone; this drug is a commonly used aldosterone antagonist often administered in the treatment of hirsutism when adrenal androgen overproduction is the underlying cause. (Redmond, 1998).
3. REGULATION OF PSA AND hK2 GENE EXPRESSION

The regulation of the expression of a gene can occur either at the transcription initiation step, or post-transcriptionally via regulating the stability of the messenger RNA (mRNA), or at the post-translational stage through feedback mechanisms. By the far, the most common and best known mechanism of regulation occurs at the transcriptional initiation level; androgens exert their control at this step. Some of the best known examples for this comes from the study of genes expressed by the prostate and testis such as KGF, a gene that is responsible for the modulation of stromal cells' control on epithelial cell differentiation in the prostate (Yan et.al., 1992), the testicular 5α-reductase (Horton et.al., 1993) which is responsible for the conversion of testosterone to DHT, and the expression of the AR gene which acts as a transcriptional repressor of its own transcription (Quamby et.al., 1990) while it acts as a transcription activator for other genes like PSA and hK2. (Sun et.al., 1997).

3.1 PSA and hK2 Gene Regulation by the AR

3.1.1 Regulation by the androgen-AR complex

PSA and hK2 share their similarities when it comes to genetic organization as well. The PSA gene (hKLK3) is 5.9 kb while hK2 gene (hKLK2) is 5.2 kb, both having 5 exons and 4 introns with the conserved intron-exon junction (Lundwall, 1989; Riegean et.al., 1989; Schedlich et.al., 1989). On the molecular level, the regulation of PSA gene by the androgen-AR complex has been extensively studied in LNCaP cell lines; a marked increase of PSA mRNA levels
was noted as early as 2 hours post-androgenic induction (Wolf et al., 1992). Transactivation by the AR is achieved through the interaction of the DBD domain of the AR with the hKLK3 promoter and enhancer regions that contain three distinct androgen response elements (AREs) (Schuur et al., 1996; Cleutjens et al., 1996, 1997a, 1997b). These imperfect pallindromic sequences are often found, as is the case for PSA, in the 5' flanking regions of genes; however, they are known to be effective as well in introns or in 3' flanking sequences (Beato, 1991). Multiple AREs are known to have synergistic effects and both their spacing and topology relative to one another affect the degree of transcriptional activation by the AR (Beato, 1991). As shown in Figure III-3.1, a partially pallindromic, 15 bp-long ARE, very similar to the sequence of nuclear receptor binding sites, spans the region corresponding to 155 - 170 bp upstream from the start codon of hKLK3 (Beato et al., 1989, Riegrnan et al., 1991). Because the hKLK3 and hKLK2 promoters, except for a single base difference in this ARE, are identical in their sequence (Murtha et al., 1993), hK2 gene activation by AR is believed to occur through the same mechanism. The fact that hKLK2 proved to be even more responsive than hKLK3 to androgenic stimulation in vitro, is attributed to this single base difference in this ARE (Hsieh et al., 1997).

In addition to this ARE, 2 additional AREs in the hKLK3 promoter are found further upstream (Fig. III-3.1) demonstrating an even greater contribution than ARE1 alone to the androgen sensitivity of this gene (Zhang et al., 1997; Cleutjens et al., 1997). Taking advantage of this knowledge, androgenic upregulation has been demonstrated in LNCaP cells as well as in the breast
Figure III-3.1: Schematic Representation of the ARE Organization in the hKLK2 and hKLK3 Promoter
carcinoma derived T47D cells for both PSA (Zarghami et al., 1997) and hK2 (Hsieh et al., 1997) proteins. From time course experiments using various androgens as inducers in LNCaP cells, it is known that synthetic androgens such as mibolerone upregulate PSA and hK2 mRNA levels within 4-72 hours post-induction. However, nuclear run-on analysis demonstrated that the actual PSA and hK2 mRNA transcription returned to pre-hormonal treatment levels within 24 hours most likely due to the autologous downregulation of the AR (Wolf et al., 1992). Because the actual mRNA levels are maximal at time points when transcription is already back to its baseline levels, the persistence of high PSA mRNA levels observed in LNCaP cells can only be attributed to high stability of the PSA mRNA and possibly to other post-transcriptional events (Wolf et al., 1992). Therefore, androgenic regulation of PSA and hK2 gene expression via the AR occurs at the transcription initiation as well as post-transcriptional step.

3.1.2 Regulation via Non-Androgenic Activation of the AR/Bypass of AR Activation

While androgens, particularly DHT, and other, synthetic androgens have the highest affinity for the AR, they are not the only compounds that can be involved in PSA and hK2 gene regulation. Growth factors like insulin-like growth factor 1 (IGF-1) is able to activate the AR without the presence of androgens or able to further augment androgen-induced transcription (Culig et al., 1994). More recent discovery in the androgen-independent induction of PSA via the AR is the elucidation of the effects of protein kinase A signal transduction pathway (Sadar
et al., 1999) Surprisingly, the antiandrogen CA was shown to have an agonistic effect in vitro on PSA and hK2 expression most probably due to its progestational activity. (Wolf et al., 1992). The upregulating effects of CA in vivo was demonstrated in castrated rats by monitoring the extent of prostate growth in response to this antiandrogen (Wakeling et al., 1981).

Because the DBD of AR, PR, mineralocorticoid and glucocorticoid receptors (but not of the estrogen receptor) bind the same steroid response element such as the ARE of PSA (Beato, 1989), other steroids, namely glucocorticoids, mineralocorticoids and progestins were successfully demonstrated to be able to upregulate PSA and hK2 expression in T47D cell lines through the interaction of their respective receptors (Cleutjens et al., 1997; Skan et al., 1997; Zarghami et al., 1997; Hsieh et al., 1997).

On the other hand, downregulation of PSA and hK2 gene expression can be achieved using compounds other than pure antiandrogens. 5α-reductase inhibitors, such as finasteride, can regulate PSA expression through, or in conjunction with, the AR (Wang et al., 1997 Hsieh et al., 1997); similarly, N-4-hydroxyphenylretinamide (a retinoic acid homolog), and retinoic acid itself at high concentrations, can downregulate PSA expression (Lindzey et al., 1994). Most recently, the ability of cyclin D1 and D3 to complex with the AR, and inhibit PSA expression in a cell cycle independent manner was demonstrated in vitro (Knudsen et al., 1999).
3.2 Androgen, AR or other Steroid Receptor Independent Regulation

Besides the AR mediated mechanism, several other transcription factors and growth factors has been shown to possess the ability to influence hKLK3 transcription. Among them TGFα was demonstrated to downregulate PSA mRNA levels in LNCaP cells (Henttu & Vinko, 1993), while TGFβ was able to upregulate PSA mRNA expression under similar conditions (Gleave et al., 1992). Other transcription factors, namely AP1, c-fos and c-jun protooncogenes can also regulate PSA and hK2 transcription (Murtha et al., 1997; Sun et al., 1997). In addition, the PSA promoter has been shown to contain the CACCC motif, which is a known SP1 binding site and allows this transcription factor to cooperate with the known AREs of PSA in transcription regulation (Srahle et al., 1988).
4. PSA AND hK2 AS MARKERS OF DISEASE AND ANDROGEN IRREGULARITY

4.1 PSA and hK2 in Prostatic Diseases

In healthy males, PSA and hK2 are very tightly confined to the prostate gland where the uncomplexed PSA and hK2 are the most abundant forms of these two proteins. Since PSA levels can only be markedly elevated if the physical integrity of this glandular tissue is compromised, allowing PSA to leak into the circulatory system, PSA proved to be the most useful marker in detecting possible presence of cancerous tissue in the prostate. However, in practice, its usefulness as a specific marker of malignancy is limited by the fact that serum PSA levels of 4-10 ng/mL can be due not only to cancerous tissue, but also to the benign prostatic hyperplasia (BPH) (Oesterling et al., 1988; Catalona et al., 1994). Indeed, serum PSA becomes a truly definitive prostate cancer (PC) marker when its levels are above 10 ng/mL. In addition to BPH and PC, elevated serum PSA levels can be attributed to clinically insignificant states such as prostatitis, prostatic infarction, prior prostatic manipulation and even to physical activity (Rana & Chisolm, 1994; Oremek & Seiffert, 1996; Ornstein et al., 1997). Even with these existing limitations as a disease marker on its own, when serum PSA levels are tested in combination with digital rectal examination (DRE) as recommended for males above 50 years of age, it is still the best tool to assess the risk of prostate cancer (Catalona et al., 1991, 1993). Also, the measurement of other than total serum PSA was noted to be useful. Measuring
the ratio of free to total PSA (% free PSA) in serum was shown to be significantly higher in BPH than in PC; thus, % free PSA reduces the number of unnecessary biopsies (Marley et al., 1996).

Besides proving to be an invaluable tool in detection of PC, PSA is also a very powerful tool to be taken advantage of once the presence of PC is established. It has been noted that PSA concentrations in blood may be related to worsening histological differentiation (Gleason Grade). This is related to the fact that patients with poorly differentiated grade 4 tumours have higher serum PSA because these tumours occupy the interstitial space and can leak PSA into the extracellular fluid more readily (Oesterling et al., 1988, 1991; Partin et al., 1990). However, while serum PSA increases with advanced pathological stage, it does not predict staging well (Partin et al., 1990). Because of this, coupled to the fact that PSA levels per gram of tumour tissue decrease with Gleason Grade (Partin & Oesterling, 1994), the interpretation of serum PSA for prognostic purposes in prostate cancer is somewhat limited. Thus, with the exception of advanced metastatic disease characterized with exceptionally high levels of serum PSA, total serum PSA predicts the significance of PC only poorly (Partin & Oesterling, 1994).

More importantly, perhaps the greatest power of PSA lies in the fact that its biological half life is around 2-3 days, so that the time for serum PSA levels to return to baseline once PC tissue is surgically removed, occurs within 48 hours (Rittenhouse et al., 1998). Thus, PSA is an excellent marker of yet undetected
metastasis in cases when it does not return to baseline, as well as being useful in monitoring recurrence of tumorigenicity of the prostate.

Similarly, hK2 is proving to be very useful in the management of prostatic carcinoma, although in screening and early diagnosis the usefulness of hK2 is not probable owing to the fact that in serum it is present at much lower levels than PSA. hK2 levels alone or in combination with PSA may be very effective from a clinical standpoint. It is now known that hK2 levels are not directly proportional to PSA serum levels in PC and BPH; therefore, additional, potentially useful diagnostic information can be obtained by comparing the levels of these two proteins (Finlay et. al., 1998). The ratio of hK2 to free PSA was shown to be higher in PC patients than in non-cancer subjects (Kwiatkowski et. al., 1998) providing the field with a possible tool to distinguish between PC and BPH on serum measurements alone. In addition, hK2 may give a more specific information than PSA in the identification of PC since hK2 appears to be expressed highest in poorly differentiated PC cells, demonstrating a more tumour-associated profile than PSA (Corey et. al., 1997; Kawakami et. al., 1997). Histochemical staining for hK2 also shows that hK2 increases with Gleason grade in PC; thus, hK2 may prove to be a better staging and aggressiveness-evaluating tool than PSA (Darson et. al., 1997).

4.2 PSA and hK2 in Breast Tissue Tumorigenicity

Once its presence in extraprostatic tissues became apparent, the success of PSA as a tumorigenic marker in PC has prompted investigators to consider
whether PSA is of use in breast tissue malignancy. To-date it is known that PSA, besides being produced in normal and hyperplastic breast tissue, is also produced in 70% of breast tumours (Borchert et.al., 1997). PSA appears to be a favourable prognostic indicator for women with breast cancer (Yu et.al. 1995c; Levesque et.al., 1995). The usefulness of measuring PSA in nipple aspirate fluid to assess breast cancer risk has also been investigated (Sauter et.al., 1996). PSA seems to be in the uncomplexed form in breast milk and possibly in other breast secretions (Yu & Diamandis, 1995c). Just like PSA, hK2 has also been discovered to be present in breast carcinoma cell lines (Hsieh et.al., 1997); its usefulness in breast related disorders is currently under investigation.

4.3 PSA in Females with Elevated Androgens/Hyperandrogenism

Using ultrasensitive immunoassays, the serum level of PSA in healthy women was determined to be less than 0.1 ng/mL though some fluctuations were noted due to the menstrual cycle and individual differences (Yu & Diamandis, 1995a; Zarghami et.al., 1997b). Most probably because of responding to the high androgen levels, PSA is higher in women with idiopathic hirsutism in comparison to controls (Melegos et.al., 1997). Furthermore, Melegos and coworkers also noted a positive correlation of serum PSA with 3α-androstenediol glucuronide (3α-AG); induced PSA was almost as good a marker of hirsutism as 3α-AG. However, other investigations found that PSA did not respond to changing androgen levels from the ovaries or adrenals in hirsute women though serum PSA seemed to be clearly higher in these women than in
controls (Escobar-Morreale et al., 1998). The same study concluded that hirsute women, with the adrenals or the ovary as the underlying cause, had higher serum PSA than idiopathic hirsute women, suggesting that PSA may be an indicator of the degree of hyperandrogenism in females (Escobar-Morreale et al., 1998). Other examples of elevated PSA levels due to high circulating androgens, albeit the androgen is of an exogenous source, are in female-to-male transsexuals under testosterone treatment. In fact, it seems that in female-to-male transsexuals under androgenizing treatment, PSA levels are similar in magnitude to that found in women with PCOS (Pache et al., 1992) making PSA levels in female-to-male transsexuals a good model of serum PSA levels in hyperadrogenism.
II. RATIONALE

As presented in the introductory sections, the androgenic regulation of PSA and hK2 in vitro is already a well-established fact. However, apart from the evidence of urinary PSA upregulation in androgenized females (Breul et al., 1997), little else is known about PSA levels in response to androgenic stimulation in males or females. While there is some evidence for the existence of elevated levels of serum PSA in hyperandrogenic women (Melegos et al., 1997), a causative relationship between increased PSA levels in these women and the increased androgen levels could not be established (Escobar-Morreale et al., 1997). The demonstrated in-vitro upregulation of PSA and hK2 in response to the antiandrogen CA, a drug that is successfully used to suppress androgen availability for androgen-dependent tumours in vivo (Goldenberg et al., 1988) clearly points out the pitfalls of extrapolating in vitro results directly to the outcome of androgenic stimulation in vivo. The reason for conducting this study, then, is to establish whether androgenic regulation of PSA and hK2 determined in vitro also occurs in vivo. If the extent of PSA and hK2 changes in females due to androgenic stimulation regulation is markedly significant, an indirect but compelling evidence for hyperadrogenicity as the cause of higher PSA levels in hyperadrogenic females would be provided. Furthermore, either of these proteins may be a better marker of hyperandrogenism in females than the one currently in use. Lastly, determination of the existence of possible correlation between PSA
and hK2 levels in vivo under androgenic stimulation as well as androgenic suppression may further strengthen the role of hK2 in PSA activation.
III. HYPOTHESIS

Based on the following, previously validated facts that:

a) PSA and hK2 are upregulated by androgens and progestins in breast carcinoma cell lines and their upregulation is blocked by estrogens

b) cyproterone acetate is widely used as an effective antiandrogen in the management of prostatic carcinoma to starve tumor cells for androgens

c) the source of PSA and hK2 in male serum is the male prostate, while in urine the source of PSA appears to be the periurethral glands (Iwakiri, et.al., 1993) and both of these organs are androgen-driven

d) the source of PSA in female serum is not known but in urine it has been postulated to originate from the periurethral glands whose PSA secretion is upregulated in response to androgens (Breul et.al., 1994, 1997)

it is hypothesized that PSA and hK2 are upregulated in female plasma and urine in response to long term androgenic stimulation while PSA and hK2 is downregulated in male plasma and urine in response to a long-term antiandrogenic regime.
IV. OBJECTIVES

- to determine if androgenic regulation of PSA and hK2 established \textit{in vitro}
  applies to PSA and possibly hK2 \textit{in vivo}

- to determine the extend of PSA and hK2 changes in female plasma and urine
due to androgenic stimulation; if changes are markedly significant, either of
these proteins may be a better marker of hyperandrogenism in females than
the one currently in use

- to determine if a correlation exists between PSA and hK2 in vivo even under
androgenic stimulation; this may further strengthen the role of hK2 in PSA
activation
V. MATERIALS AND METHODS

1. Drug Treatment

32 female-to-male transsexuals (ages 16-37; mean age 25) were treated intramuscularly for 12 months with 250 mg of testosterone esters (SustamonR), administered every two weeks.

31 male-to-female transsexuals (ages 18-47; mean age 25) were treated orally with 100 μg of ethinyl estradiol per day in conjunction with 100 mg/day of the antiandrogen cyproterone acetate (CA) for a period of 12 months. Another group of 15 males were given transdermal 17-β-estradiol administered twice weekly in combination with 100mg/day of CA for 12 months. The remaining 10 males of the third group were treated orally with only 100 mg/day of CA for 12 months. Male-to-female transsexuals were of the age 18-47 (mean age 31).

2. Blood and Urine Sample Collection

From female-to-male transsexuals, venous blood samples were collected in the morning between 9 and 11 am after an overnight fast, at baseline and again after 4 months and 12 months of hormonal administration. Urine samples were collected during the 24-hour period prior to blood sampling, at baseline and at 4 months and 12 months of hormonal treatment.

Similarly, from male-to-female transsexuals, venous blood samples were collected between 9 and 11 am after an overnight fast at baseline, and again after 4 months of hormonal administration. From patients undergoing ethinyl
estradiol + CA treatment, venous blood samples were collected also after 12 months of hormonal administration. From all male subjects, fasting 2-h morning urine samples were collected at baseline and at 4 months, and where applicable, at 12 months of hormonal treatment.

Blood samples were separated within a few hours of sample collection; plasma and urine samples were stored at −20°C until immunological analyses were performed. At that time, the frozen plasma and urine samples were allowed to thaw to room temperature and were mixed using a vortexer prior to use.

3. PSA Immunoassays

PSA was measured in undiluted female plasma and in female urine initially diluted 1:10 in a 60 g/L bovine serum albumin (BSA) solution, pH 7.40. For samples obtained from males, PSA was measured in undiluted plasma and in urine initially diluted 1:100 in a 60 g/L bovine serum albumin (BSA) solution, pH 7.40. In both cases, measurements were carried out using a one-step time resolved fluorometric immunoassay (Ferguson et al., 1996). In this procedure, 96-well microtitre plates (Dynatech Laboratories, San Diego, CA) were used precoated with 500 ng of monoclonal anti-PSA antibody 8301 (Diagnostic Systems Laboratories, Webster, TX) per well. Standard PSA solution prepared from purified seminal plasma (gift from T. Stamey, Standford University, Palo Alto, CA) was diluted in 50 mM Tris buffer (pH 7.80) to final concentrations of 0, 5, 20, 100, 500, 2000, 10,000 ng/L. 100 μL of the standards and samples were added in duplicates to the wells. Then, to each well 50 μL of assay buffer (50mM
Tris, 500 mM KCl, 100 mL/L goat serum, 50 mL/L mouse serum, 60 g/L BSA, 10 g/L bovine immunoglobulin, 5 g/L Tween 20) was added, with the assay buffer containing the biotinylated monoclonal anti-PSA detection antibody 8311 (Diagnostic Systems Laboratories, Webster, TX) diluted to a final concentration of 250 ng per 100 µL well. Microtitre plates were then incubated for 1 hour with shaking. After incubation, plates were washed with wash solution (150 mM NaCl, 0.5 g/L Tween 20, 1 mM NaN₃ in 50 mM tris, pH 7.8) six times on a microtitre plate washer. To each well was added 100 µL of streptavidin-alkaline phosphatase (SA-ALP) conjugate, diluted from stock solution (Jackson Immunoresearch) in 6% BSA to a final concentration of 5 ng per well. Microtitre plates were incubated with shaking for 15 min. After this, wells were washed six times as before. At this point the 10 mM substrate diflunisal phosphate (DFP) (Cyberfluor, Toronto, ONT) was diluted in substrate buffer (100 mM Tris pH 9.1, 150 mM NaCl, 1 mM MgCl₂, 7.5 mM NaN₃) to a final concentration of 1 mM. Then 100 µL of this diluted DFP was added to each well, and the plates were incubated for 10 min with shaking. Immediately, 100 µL of the developing solution (400 mM NaOH, 3 mM EDTA, 2 mM Tb³⁺) was added to each well. The plates were then shaken briefly on the microtitre plate shaker, and the resulting fluorescence was measured on a Cyberfluor 615 Immunoanalyzer (Nordion International, Kanata, ONT). For the urine samples for which results were under the detection limit (i.e. below 1 ng/L), the measurement was repeated without prior dilution of the sample. PSA and hK2 concentrations are reported in ng/L for
both plasma and urine samples after adjustments were made to account for the dilutions, where applicable.

4. hK2 Immunoassay

In samples obtained from females, hK2 was measured in undiluted plasma and urine; in samples obtained from males, hK2 was measured in undiluted plasma and in urine diluted 1:5 in 6% BSA solution. hK2 was measured using a two-step time resolved immunofluorometric assay with a detection limit of 6 ng/L (Black et al., 1999). The procedures to perform this assay differ from the PSA assay described above only at the initial steps as follows:

a) anti-hK2 monoclonal capture antibody raised against recombinant hK2 (Hybritech Inc., San Diego, CA) had to be immobilized on polystyrene microtiter wells, by applying 300 ng of antibody per well diluted in coating buffer (50 mM Tris, pH 7.8) and incubating it over night;

b) standard solutions were prepared at 0, 5, 20, 100, 500, 2000 ng/L by diluting recombinant hK2 (gift from Dr. Wolfert, Hybritech Inc., San Diego, CA) in 6% BSA (50 mM Tris buffer solution, pH 7.8);

c) 100 μL of the samples and standard solutions was applied to the wells as described for PSA and incubated with the assay buffer for 1 hour with shaking. Wells were washed 6 times as described above, then 100 μL of the same biotinylated detection antibody, used for the PSA assay, was added to each well and the wells were incubated again for 1 hour with shaking. hK2
concentrations are reported in ng/L for both plasma and urine samples after adjustments were made to account for the dilutions, where applicable.

5. Immunoassay for Other Serum and Urine Parameters

To determine other parameters in serum and urine, commercially available immunoassay kits were utilized. The additional parameters and the type of immunoassay used are as follows:

Competitive chemiluminescence immunoassay was utilized to determine serum levels of free thyroxine (freeT4) (ACS:180 System, Chiron Diagnostics, Emeryville, CA, USA; in pmol/L). Radioimmunoassays were used to determine serum testosterone levels (Coat-A-Count, DPC, Los Angeles, CA, USA), and serum 5-alpha-dihydrotestosterone (DHT) levels after oxidation and extraction (Intertech, Strassen, Luxembourg; in nmol/L), serum 17-beta-estradiol levels, (double antibody, Sorin Biomedica, Saluggia, Italy; in pmol/L), serum androstenedione after extraction (Coat-A-Count, DPC; in nmol/L), serum dehydroepiandrosterone sulfate (DHEA-S) (Coat-A-Count, DPC; μmol/L), serum thyrotropin, (TSH) (ACS:180; in mU/L) and urine free-cortisol levels after extraction (Coat-A-Count, DPC, in nmol/24 hours).

Immunochemiluminescent assays were used to assess serum LH and serum FSH levels (Amerlite, Amersham, UK; in U/L). In addition, commercially available, immunoradiometric assays were performed to measure serum levels of sex hormone binding globulin (SHBG) (Orion Diagnostica, Espoo, Finland; in nmol/L), serum prolactin and serum insulin levels (Biosource Diagnostics,
Fleurus, Belgium; in U/L) and serum levels of growth hormone (GH) (GH colour, Sorin Biomedica, Saluggia, Italy; in μg/L).

6. Statistical Analysis of Immunoassay Results

Because the distributions of all measured variables were non-Gaussian, statistical analysis was performed using non-parametric tests. If values were below the lower limit of detection, the value of that lower limit was used for statistical analysis (for LH 0.3 IU/L, and for FSH 0.5 IU/L). Wilcoxon's signed-ranks test was used to analyze the effects of hormonal administration at baseline versus 4 months' values, and, where applicable, versus 12 months' values. Association of PSA and hK2 in plasma and in urine with the other measured parameters was examined at pre-treatment, 4 month post-treatment, and where applicable, at 12 months post-treatment using Spearman's correlation coefficients.

7. Immunohistochemical Localization of PSA, hK2 and AR

Breast tissue sample, obtained from an androgenized female-to-male transsexual during cross-sex surgery, was analyzed for PSA and hK2. This tissue sample was also stained immunochemically for androgen receptor. Localization was carried out in our Histochemistry department as follows: Tissue sections were deparaffinized in xylol and hydrated through a graduated series of alcohols. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. The tissues were immersed in a pepsin digestive enzyme at 37°C for
10 minutes. A 5% universal tissue conditioner (Biomeda, Foster City, CA) was applied at room temperature for 10 minutes to block any non-specific binding. At 37°C, a breast tissue section was incubated with polyclonal rabbit anti-human PSA antibody (DAKO Corp. Denmark) diluted 1:200. Another Sample of this section was incubated the same way with monoclonal mouse anti-hK2 antibody Hybritech Inc., San Diego, CA) diluted 1:200. For AR staining, a section of this breast tissue was incubated with rabbit polyclonal antibody raised against AR (Biogenex, San Ramon, CA). In each case, incubation was carried out for 1 hour, then for 20 minutes with universal secondary antibody (Biomeda), followed by an autoprobe III peroxidase reagent (Biomeda) for another 20 minutes. After each step, a brief wash in buffer solution was performed. Aminoethyl carbazole with hydrogen peroxide served as the chromogenic substrate. Slides were counterstained with haematoxylin. Tissue from prostate was used as the positive control, and the primary antibodies were omitted and replaced with non-immune serum to serve as the negative control.
VI. RESULTS

1. RESULTS OF SAMPLES FROM FEMALE-TO-MALE TRANSSEXUALS

1.1 Immunoassay Results

1.1.1 PSA Results in Plasma and Urine

The data obtained in this study demonstrate a significant testosterone-induced PSA concentration increase in plasma and urine as estimated by the mean values of PSA before and after treatment. As seen in Fig. VI-1.1.1a, mean PSA in plasma increased from almost undetectable levels to 11 ng/L by the 4th month of steroidal treatment, and to 22 ng/L by the 12th month of treatment. The differences between pre-treatment and 4 months post-treatment plasma PSA levels were highly significant (P<0.001). Similarly, urinary PSA levels increased dramatically in response to testosterone treatment from 17 ng/L to 1,420 ng/L by the end of the 4th month of therapy, and further increased to 18,130 ng/L by the end of the treatment period (Fig. VI-1.1.1b). The differences between all three mean PSA values in urine were highly significant (P<0.001).

1.1.2 hK2 in Plasma and Urine

Baseline or post-stimulation hK2 concentration remained under the detection limit in female plasma; however, hK2 was readily detectable in urine where it demonstrated inducibility by testosterone as shown by the increase
Figure VI.1.1.1a: Mean Plasma PSA Concentrations at 0, 4 and 12 Months of Testosterone Treatment

Figure VI.1.1.1b: Mean Urinary PSA at 0, 4 and 12 Months of Testosterone Treatment
of mean values over time. Here, hK2 has increased from a mean of <6 ng/L prior treatment to 18 ng/L by the 4th month of treatment. By the end of the 12 month-long therapy, urinary hK2 has dramatically risen to 179 ng/L (Figure VI-1.1.2). Just as for urinary PSA, the differences detected between mean values of urinary hK2 were highly significant at 4 months versus prior to treatment (P<0.001) as well as at 4 versus 12 months (P=0.035).

1.2 Correlation of PSA and hK2 with Other Tested Parameters

Testosterone levels did not change beyond the 4th month while LH and FSH levels decreased during testosterone therapy (Table VI-1.2a). As indicated in Table VI-1.2b, after 4 months of therapy with testosterone, plasma PSA was found to correlate with serum insulin, whereas urinary PSA correlated with urinary hK2 and to a lesser extent, with LH and FSH. Before treatment, correlation of FSH or LH with either PSA or hK2 was not apparent. By the end of the 12 month treatment, plasma PSA had no noteworthy correlations with any of the variables tested. However, as seen in Table VI-1.2c, at the end of the treatment period there was a better correlation between urinary hK2 and PSA (r=0.723, P=0.003) compared to the correlation at 4 months post treatment, for the same set of subjects (Table VI-1.2b). In addition, urinary hK2 concentration correlated negatively with body mass index (BMI) and with serum insulin (Table V-1.2c). Other associations observed was the correlation of serum FSH with LH (r=0.639, P<0.0001) at 4 months of treatment, and at 12 months post treatment (r=0.858 P=0.001). Other associations relevant to this study include the expected
Figure VI-1.1.2: Mean Urinary hK2 Concentrations at 0, 4, and 12 Months of Testosterone Treatment
Table VI-1.2a: Mean testosterone, LH and FSH values at baseline, 4 and 12 months of hormonal treatment in serum

<table>
<thead>
<tr>
<th></th>
<th>Time of Measurement</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>at baseline</td>
<td>at 4 months</td>
<td>at 12 months</td>
<td></td>
</tr>
<tr>
<td><strong>Testosterone</strong>a</td>
<td>2.8</td>
<td>31.2</td>
<td>33.1</td>
<td></td>
</tr>
<tr>
<td>(nmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LH</strong>b</td>
<td>5.9</td>
<td>3.5</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>(IU/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FSH</strong>c</td>
<td>5.3</td>
<td>4.2</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>(IU/L)</td>
<td></td>
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*a*normal value for serum testosterone in females is < 3 nmol/L

*b*normal range for LH in female serum is 2-20 IU/L, with peak production of 30-140 IU/L

*c*normal range for FSH in female serum is 2-15 IU/L, with peak production of 20-50 IU/L
Table VI-1.2b: Spearman correlation values for parameters measured in serum 4 months post treatment with plasma, urinary PSA as well as with urinary hK2\(^{(1)}\)

<table>
<thead>
<tr>
<th></th>
<th>plasma PSA</th>
<th>Urinary PSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH with..</td>
<td>n.s.(^{(2)})</td>
<td>0.356 (p=0.04)</td>
</tr>
<tr>
<td>FSH with..</td>
<td>n.s.</td>
<td>0.379 (p=0.03)</td>
</tr>
<tr>
<td>Urine hK2 with..</td>
<td>n.s.</td>
<td>0.477 (p=0.006)</td>
</tr>
<tr>
<td>Insulin with..</td>
<td>0.662 (p=0.01)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

\(^{(1)}\) N=32 in all cases except with insulin where N=14. Only statistically significant correlations are presented.

\(^{(2)}\) n.s.: not significant.
**Table VI-1.2c:** Spearman correlation values for parameters measured in serum 12 months post treatment with serum, urine PSA and with urinary hK2\(^{(1)}\)

<table>
<thead>
<tr>
<th></th>
<th>Urine PSA</th>
<th>Urine hK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine hK2 with..</td>
<td>0.723 (p=0.003)</td>
<td></td>
</tr>
<tr>
<td>BMI with..</td>
<td>n.s.(^{(2)})</td>
<td>-0.692 (p=0.006)</td>
</tr>
<tr>
<td>Insulin with..</td>
<td>n.s.</td>
<td>-0.681 (p=0.007)</td>
</tr>
</tbody>
</table>

\(^{(1)}\) N=14 for all cases.

\(^{(2)}\) n.s.; not significant.
correlation between testosterone and DHT at 4th months (r=0.719, P=0.004) and at 12 months of treatment (r=0.802, P=0.001). Testosterone at 4 months of hormonal therapy correlated well with serum GH levels (r=0.692, P=0.006).

Furthermore, we examined by Wilcoxon ranked sign test if any of the measured parameters, in addition to plasma PSA and urinary PSA and hK2, changed significantly with testosterone treatment. The results can be summarized as follows: body mass index, testosterone, and DHT showed statistically significant increase post-testosterone administration; estradiol, LH, FSH, SHBG and thyroxine decreased while androstenedione, DHEAS, GH, insulin, urinary free cortisol, prolactin, and TSH showed no significant change.

1.3 Immunohistochemical Staining Results

PSA, hK2 and AR were immunohistochemically localized in breast tissue of a patient who was on testosterone therapy and has undergone breast removal surgery. PSA, hK2 and AR positivity can be seen in several cells as cytoplasmic staining (Fig. VI-1.3a and VI-1.3b) while AR positivity can be seen as primarily nuclear staining with some cytoplasmic staining in some of the cells (Fig. VI-1.3c). True positivity these results was determined based on the staining characteristics of an AR negative ovarian carcinoma tissue (negative control) which showed no staining for AR, and prostatic tissue (positive control) which showed intense staining for PSA, hK2 and AR.
Figure VI-1.3a: Histochemical Staining for PSA in Breast Tissue
Figure VI-1.3b: Histochemical Staining for hK2 in Breast Tissue
Figure VI-1.3c: Histochemical Staining for the Androgen Receptor in Breast Tissue
2. RESULTS OF SAMPLES FROM MALE-TO-FEMALE TRANSSEXUALS

In general, there was a dramatic suppression of both plasma and urinary PSA and hK2 levels under all three types of drug regimes. At 4 months, the most effective suppression of PSA and hK2 in plasma was seen with ethinyl estradiol plus cyproterone acetate treatment, while in urine the treatments with either ethinyl estradiol or estradiol patch seemed more suppressive than cyproterone acetate alone. However, overall the differences between the various treatments were not statistically significant. The differences between pre-treatment and post-treatment values of PSA or hK2 concentrations in either plasma or urine were highly significant (P<0.001 by Wilcoxon ranked sign test). A statistically significant correlation was noted between plasma PSA and plasma hK2 (r=0.53, P=0.002, N=56) for pretreatment values, and for at the 4 months of treatment period (r=0.60, P<0.0001, N=56).

2.1 Immunoassay Results for Patients Under Ethinyl Estradiol + CA Treatment

2.1.1 PSA and hK2 Immunoassay Results

Figures VI-2.1.1a and VI-2.1.1b present graphically plasma PSA and plasma hK2 concentrations before, at 4 months and at 12 months of treatment for patients receiving ethinyl estradiol plus cyproterone acetate. The decrease in both plasma PSA and plasma hK2 was found to be statistically significant at 4 months of the therapy (P<0.001).
**Figure VI-2.1.1a:** Median Plasma PSA Under Ethinyl Estradiol + Cyproterone Acetate Treatment

**Figure VI-2.1.1b:** Median Plasma hK2 Under Ethinyl Estradiol + Cyproterone Acetate Treatment
2.1.2 Urinary PSA and hK2

Similarly to plasma levels, median urinary levels of PSA and hK2 also indicated suppression due to antiandrogenic treatment (Fig. VI-2.1.2a and VI-2.1.2b). Indeed, for both PSA and hK2, suppression seemed to be even more dramatic by the 4th month of treatment in urine than in plasma. However, just like for plasma values, statistically significant difference was only apparent for median values of samples obtained at baseline and at the 4th month (P<0.001).

2.2 CA Treatment with or without Transdermal Estradiol

2.2.1 Plasma and Urinary PSA

As demonstrated in figure VI-2.2.1a, median plasma PSA levels declined by the 4th month of treatment (P<0.001). Statistically significant difference between the two treatments could not be found, suggesting that transdermal estradiol does not augment the suppressive effect of CA in a significant manner. The same pattern holds true for urinary PSA as well under both treatments (Fig. V-2.2.1b).

2.2.2 Plasma and Urinary hK2

As indicated by figures V-2.2.2a and V-2.2.2b, both plasma and urinary hK2 declined in a pattern similar to that of PSA by the 4th month of treatment, and this decline proved to be statistically significant (P<0.001) in both cases. The addition of transdermal estradiol on top of CA did not make a significant difference in terms of the extent of decline in plasma or urinary hK2 levels.
**Figure VI-2.1.2a:** Median Urinary PSA under Ethinyl Estradiol + Cyproterone Acetate Regime

**Figure VI-2.1.2b:** Median Urinary hK2 under Ethinyl Estradiol + Cyproterone Acetate Regime
**Figure VI-2.2.1a:** Median PSA in Plasma under CA and Transdermal Estradiol + CA Treatments

**Figure VI-2.2.1b:** Median PSA in Urine under CA and Transdermal Estradiol + CA Treatments
Figure VI-2.2.2a: Median hK2 in Plasma under CA and Transdermal Estradiol + CA Treatments

Figure VI-2.2.2b: Median hK2 in Urine under CA and Transdermal Estradiol + CA Treatments
2.3 Correlation of PSA and hK2 with Other Tested Parameters

A good correlation was noted between plasma hK2 and BMI at 12 months post-treatment \((r=0.635, \ P=0.006, \ N=17)\). Apart from this result, no other significant correlation was found to exist between either PSA or hK2 and the other biochemical parameters measured.
VII. DISCUSSION

1. ANDROGEN-INDUCED PSA AND hK2 UPREGULATION IN VIVO

The presence of PSA in various female tissues is a relatively new concept but since its discovery in female breast tumours, it is gaining attention as a possible diagnostic/prognostic marker of breast cancer (Yu & Diamandis, 1995, 1997). While in-vitro and in-vivo studies provided good indication for androgenic regulation of PSA (Zarghami et al., 1997a), previous efforts could confirm neither presence nor upregulation of PSA in normal female serum after testosterone administration (Escobar-Morreale et al., 1998). However, this study has succeeded in demonstrating the presence of very low baseline amounts of PSA in female plasma, and quite significant plasma PSA increase in response to testosterone therapy. By the end of the 12 month-long treatment, the average increase of mean plasma PSA was of the order of 20-fold. In addition, the finding of dramatic upregulation of PSA in urine post-testosterone treatment agree with previous results (Breul et al., 1997). In urine, the increases at 4 and at 12 months post-testosterone treatment were approximately 80-fold and 1,000-fold, respectively, over baseline concentrations.

It is noteworthy to mention that urinary PSA could not have originated by clearance from plasma alone, since around 95% of PSA in blood is the 100 kDa PSA-ACT which is too large to pass into the glomerular filtrate. This indicates that the source of PSA in urine may be different from that of PSA in the blood. Interestingly, in a previous case study of a female presented with Skene's gland carcinoma, the patient had a serum PSA level of 5,900ng/L and, in response to
surgery, this serum PSA level became undetectable (Dodson et al., 1994). While in this special case it seems that the source of excess serum PSA was indeed the gland of Skene, this finding also points out that under normal circumstances, the Skene's gland does not contribute significantly to serum PSA levels; this hypothesis had been confirmed in males (Oesterling et al., 1996). Relying on previous studies of PSA and hK2 levels in breast tumour tissues and nipple aspirate fluid (Yu et al., 1994; Sauter et al., 1996, 1998) as well as on the histochemical data of this study confirming presence of PSA and hK2 in the breast (Figures VI-1.3a and VI-1.3b), it is quite reasonable to suggest that the source of PSA in blood may be the female breast. Based on the results of this study, it can be proposed that PSA production is under androgenic control in breast tissue as well as in the gland of Skene. The latter is the most likely source of both PSA and hK2 in urine. Contrary to PSA, hK2 in plasma was not detectable with our method. However, in urine, the greatly increased levels due to administration of testosterone clearly indicate that just like PSA, hK2 is under androgenic control. There is a good correlation between urinary PSA and hK2 and this further supports the notion that hK2 arises from the same source as PSA. Furthermore, the fact that PSA itself is known to be activated by hK2 provides a physiological rationale for their coexpression. Serum LH levels are known to be lower in testosterone treated female-to-male transsexuals than in controls (Pache et al, 1992). According to our data, LH and FSH decreased and this decrease can be accounted for by testosterone
induced negative feedback on the hypothalamo-pituitary level (Gooren, 1989; Foresta et.al., 1997). Interestingly, hK2 in urine correlated negatively with BMI and with serum insulin. As expected, DHT increased in response to testosterone treatment and correlated with testosterone. Testosterone also had a good correlation with GH, which may be explained by the fact that, at least in males, androgen-stimulated inhibin secretion by the gonads can affect sensitivity of pituitary cells to GnRH, which in turn, affects GH release into the circulation (Burtis & Ashwood, 1994). Since the female ovary also secretes inhibin, (Pache et.al, 1992) the same principle may apply.

In addition to the above, we have observed negative correlation of FSH with urinary free cortisol, possibly reflecting negative feedback on the adrenals. A statistically significant decrease in LH and FSH throughout the treatment was noted as well as in SHBG and free thyroxine (free T4) without a significant TSH change. This may be explained by the fact that SHBG in serum is noted to change in response to androgen drug use (Plymate et.al., 1983). Decreased amount of SHBG may be attributed to the increased sequesterization by the increased amounts of DHT converted from testosterone.

It should be further emphasized that androgens are not the sole physiological upregulators of the PSA and hK2 genes. In-vitro studies (Hsieh et.al, 1997; Zarghami et.al., 1997) and in-vivo data (Katsaros et.al., 1998; Diamandis et.al., 1999) clearly demonstrate upregulation of these genes by progestins. However, this study demonstrates, for the first time in vivo, that testosterone can upregulate quite significantly the PSA and hK2 genes in target
tissues and mediate dramatic plasma and urinary PSA elevations post treatment. This finding gives credence to previous reports of increased serum PSA levels in patients with hyperandrogenic syndromes (Melegos et al., 1997; Escobar-Morreale et al., 1998). The target tissues have not yet been identified. Most likely, urinary PSA and hK2 are produced by the periurethral glands and secreted into the urine. Although this study does not present definite proof, it seems likely that urinary PSA and hK2 are very sensitive to androgen stimulation and therefore may serve as biochemical indicators of hyperandrogenism in women. This would especially be useful since the ability of such markers as 3α-AG to truly reflect the levels of circulating androgens and hyperandrogenism is under dispute (Rittmaster, 1995).
2. ANTIANDROGENIC PSA AND hK2 SUPRESSION IN VIVO

Androgen blocking therapy is a common choice for the treatment of androgen-dependent prostatic carcinomas which leads to histological changes of the prostate as well as to dramatic changes in PSA levels (de Voogt, 1987; Akakura et al., 1993; Stephanov et al., 1995). The administration of cyproterone acetate with or without estradiol to male-to-female transsexual subjects was to aid in assumption of female secondary sexual characteristics. Transsexuals are free of burden of any extra, circulatory sex steroid levels, and their estrogen feedback on LH does not differ from non-transsexual controls (Gooren, 1990). Because of this, any observed changes in PSA and hK2 levels due to antiandrogen treatment should be expected to be similar for non-transsexual males if they were placed under a similar drug regime. As our results suggest, there is an extensive downregulation of PSA and hK2 both in plasma and in urine under all three types of antiandrogenic treatments. Albeit slightly less effectively than the other two treatment modes, the therapy based solely on cyproterone acetate (Androcur) administration resulted in a very significant PSA and hK2 downregulation in vivo, as estimated by the corresponding median values, both in plasma and in urine. This attests to the fact that cyproterone acetate can successfully out-compete testosterone in vivo, even though it only has 10% of testosterone’s affinity for the androgen receptor (Pinsky et al., 1983). This clearly points out, then, that in the management of prostatic adenocarcinoma, where cyproterone acetate is used to suppress growth of the tumour for extended periods of time, a drop in plasma PSA or hK2 level may not be an accurate
indicator of tumour volume reduction; rather, a drop in plasma PSA and hK2 may reflect cyproterone acetate-induced PSA and hK2 suppression in normal and possibly in cancerous prostatic tissue.
3. CONCLUSIONS

This study successfully demonstrated that testosterone can dramatically upregulate plasma and urinary PSA as well as urinary hK2 in females in vivo. Therefore, especially PSA present both in serum and urine at detectable levels at baseline, appears to be a very good candidate as a marker of hyperandrogenism in females. This is now being followed up with additional studies. In addition, cyproterone acetate, alone or in combination with ethinyl estradiol, was able to dramatically suppress plasma and urinary PSA, as well as plasma and urinary hK2, in male-to-female transsexuals. The data obtained suggest that for prostate cancer patients treated with cyproterone acetate, PSA or hK2 suppression may not accurately reflect success of treatment; rather, it reflects the downregulation of PSA and hK2 genes in normal and likely in cancerous prostatic tissue.
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