The Effects of Sex Steroids on Plasma Levels of Marker Proteins of Endothelial Cell Functioning

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Summary

We studied thirteen male-to-female (M→F) and ten female-to-male (F→M) transsexuals who, for four months, received cross-sex treatment with, respectively, ethinylestradiol and cyproterone acetate, and with testosterone enanthate. We assessed the effects of treatment on plasma levels of tissue-type plasminogen activator (tPA), von Willebrand factor (vWF), vWF-propeptide (vWF:AgII) and big-endothelin-1 (big-ET-1), four proteins that are markers of endothelial cell functioning. We also measured urokinase-type PA (uPA) and plasminogen activator inhibitor-type 1 (PAI-1), which may not be endothelium-derived but share major clearance pathways with tPA.

In M→F transsexuals, mean plasma levels of tPA (minus 4.4 ng/ml), big-ET-1 (minus 0.8 pg/ml), uPA (minus 0.5 ng/ml) and PAI-1 (minus 26 ng/ml) decreased (all Ps ≤0.02). The level of vWF increased (plus 24%; P = 0.005), while vWF:AgII did not change (P = 0.49).

In F→M transsexuals, levels of big-ET-1 increased (plus 0.4 pg/ml; P = 0.02), while tPA, uPA and PAI-1 did not change (all Ps >0.25). In this group vWF decreased (minus 14%; P = 0.06), but vWF:AgII did not change (P = 0.38).

Estrogens and androgens have clear effects on plasma levels of endothelial marker proteins. The mechanisms behind these effects are complex and appear to involve both altered secretion (big-ET-1) and processing and/or clearance (vWF and possibly tPA). Therefore, effects of hormones on the levels of endothelial marker proteins do not necessarily reflect changes in endothelial cell functioning, at least with regard to changes in vWF level associated with the oral administration of high doses of ethinylestradiol and cyproterone acetate to healthy men and the parenteral administration of testosterone to healthy women.

Introduction

Estrogen replacement therapy in postmenopausal women is associated with a decreased risk of cardiovascular disease. The nature of this effect is, however, incompletely understood. Apart from their effect on lipids, estrogens may affect the regulation of hemostasis, fibrinolysis, vasomotor tone and vascular smooth muscle proliferation (1), processes which play key roles in the pathogenesis of cardiovascular disease. These effects of estrogens may partly be the result of a direct action on the vascular endothelium. Evidence for this cannot easily be obtained, however, mainly because the endothelium is not directly accessible in humans. An increasingly used strategy to gain insight into vascular endothelial functioning is to measure plasma levels of endothelium-derived marker proteins, whereby changes in plasma levels are considered to reflect changes in endothelial functioning. Recent studies have focused on tissue-type plasminogen activator (tPA), von Willebrand factor (vWF) and endothelin-1 (ET-1), and, in general, have shown that increased levels of these proteins are predictive of an adverse cardiovascular outcome (2-6).

It is not known, however, to what extent changes in plasma levels of endothelium-derived marker proteins are caused by changes in their secretion rate, thus reflecting changes in endothelial functioning, and to what extent they reflect altered processing or clearance of the marker proteins.

To gain further insight in this issue, we investigated the effect of sex steroids on plasma levels of proteins marking endothelial cell functioning. We chose to do so in male-to-female (M→F) and female-to-male (F→M) transsexuals receiving cross-sex hormone treatment, because this uniquely allows the investigation of the effects of high doses of estrogens and of androgens in young subjects, in whom endothelial responses are relatively unaffected by subclinical atherosclerosis. Some of the data in this paper have been presented in abstract form elsewhere (7).

Patients, Materials and Methods

Patients

Thirteen M→F and ten F→M transsexuals, all whites, were studied at baseline and after four months of cross-sex hormone treatment. All subjects were eugonadal and healthy, had received no earlier hormone treatment, and did not use medications of any kind. M→F transsexuals were treated with ethinylestradiol (100 µg/day; Organon, Oss, the Netherlands) in combination with the anti-androgen cyproterone acetate (100 mg/day; Schering AG, Berlin, Germany), both orally. F→M transsexuals were treated with parenteral testosterone esters (250 mg/2 weeks intramuscularly; Sustanon 250, Organon, Oss, the Netherlands).

General Procedures

At baseline and follow-up, we assessed current smoking habits (yes or no), body mass index (kg/m²) and supine blood pressure (measured after 15 min of rest with an automatic device [Colin Sphygmomanometer, Colin Electronics, Komaki-City, Japan]). Blood samples were collected between 9 and 11 a.m. after an overnight fast for measurement of hormonal and metabolic variables and endothelium-derived marker proteins, using a carefully standardised procedure (8). In the F→M transsexuals, baseline blood samples were taken between days 4-6 of their menstrual cycle, i.e. presumably in the follicular phase, and blood samples at four months were taken at random between two consecutive testosterone injections.
Hormonal and Metabolic Variables

Serum LH, FSH, and estradiol were measured by immunoradiometric assays (interassay CVs [for low levels], 10%, 9%, and 8%; lower limits of detection, 0.3 U/l, 0.5 U/l and 90 pmol/l). Serum testosterone was measured by radioimmunoassay (interassay CV for low levels, 13%; lower limit of detection, 1.0 nmol/l). Serum glucose was measured by an hexokinase method (Boehringer Mannheim, Mannheim, Germany), serum insulin by an immunoradiometric assay (Medgenix Diagnostics, Fleurus, Belgium; interassay CV 12%), serum total cholesterol by an enzymatic colorimetric test (Boehringer Mannheim, Mannheim, Germany), serum HDL-cholesterol by enzymatic photometry (Quantolip assay; Immuno AG, Vienna, Austria), and serum triglycerides by enzymatic hydrolysis and subsequent colorimetry (Boehringer Mannheim, Mannheim, Germany). LDL-cholesterol was calculated by the Friedewald formula.

Marker Protein Assays

vWF:antigen was measured by an enzyme immunomassay adapted from Ingerslev (9), and expressed as percentage of vWF concentration in normal pooled plasma (NPP).

vWF:AgII (vWF-propeptide) antigen, which is stored and secreted in equinormal amounts with vWF (10), was determined by enzyme immunomassay as described (11). vWF-propeptide does not undergo multimerization and does not adhere to platelets or to vascular structures. Thus, it is thought that vWF and vWF:AgII are similar with respect to secretion from the endothelial cell, but differ with respect to further processing and clearance pathways (10, 11). Results are expressed as percentage of vWF:AgII in an NPP known to contain 0.72 μg vWF:AgII per ml (11).

tpA antigen was determined using a commercially available enzyme immunomassay kit (Thrombonostika t-PA; Organon Teknika, Turnhout, Belgium; ref 12).

Big-ET-1 antigen was measured with a two-site immunoenzymatic sandwich assay (Amersham, RPN 229, Little Chalfont, UK), after extraction of EDTA-plasma on C-18 columns. Samples and standards were incubated in the wells of a microtiter plate coated with anti-big-ET-122-38 antibodies; big-ET-1 bound was detected by HRP-labelled Fab’ fragments of anti-ET-1 antibodies. Sensitivity of the assay was 1.5 pg/ml. Cross-reactivity with ET-1 was 0.025%, with ET-3 <0.001% and with big-ET3 100%. We did not measure ET-1 because, in our experience, specific ET-1 assays are imprecise at physiological levels (unpublished).

PAI-1 antigen was determined using a commercially available enzyme immunomassay kit (Thrombonostika PAI-1; Organon Teknika, Turnhout, Belgium; ref 13).

Urokinase-type PA (uPA) antigen was determined by enzyme immunoassay (14), using Ukidan uPA (Serono, Aubonne, Switzerland) for calibration.

Although most circulating uPA and PAI-1 may not be endothelium-derived under normal circumstances (14, 15), they share a major clearance pathway with tPA through the low-density-lipoprotein receptor-related protein/macroglobulin receptor (e.g., 16, 17).

All 46 samples were assayed in a single run. The intra-assay variation coefficients for the marker protein ELISA assays ranged from 2.7 to 5.5%.

**Statistical Analysis**

All data are reported as mean ± SEM. If hormonal measurements were below the lower limit of detection, the value of that lower limit was used. Skewed data were logarithmically transformed. We compared baseline differences between the groups and differences between baseline and follow-up measurements in each group using standard paired or non-paired, parametric or non-parametric testing, as appropriate. All testing was two-sided, with P <0.05 as the level of significance. The power of this study to find a change in vWF:AgII of ≥15% in either group was >90% at α = 0.05 (see below).

We also analysed whether the baseline levels or the changes in vWF, tPA, PAI-1 and big-ET-1 were related to baseline levels of, or changes in conventional risk factors for cardiovascular disease (age, smoking habits, body mass index, blood pressure, and levels of LDL-cholesterol, HDL-cholesterol, triglycerides, glucose and insulin) using Pearson’s or Spearman’s correlation coefficient, as appropriate. For the correlation analyses, P values less than 0.005 were considered statistically significant. All calculations were performed using the Statistical Package for the Social Sciences.

**Results**

Tables 1 and 2 show the patients’ baseline characteristics and the response of metabolic variables and blood pressure to hormonal

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**Table 1** Baseline characteristics of the male-to-female (M→F) and female-to-male (F→M) transsexuals

<table>
<thead>
<tr>
<th></th>
<th>M→F (n=13)</th>
<th>F→M (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>27.5 (18-38)</td>
<td>21.3 (17-35)</td>
</tr>
<tr>
<td><strong>Body mass index (kg/m²)</strong></td>
<td>21 (17-25)</td>
<td>20 (17-25)</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF (%)</td>
<td>62 ± 4</td>
<td>81 ± 6</td>
</tr>
<tr>
<td>vWF:AgII (%)</td>
<td>98 ± 3</td>
<td>122 ± 7</td>
</tr>
<tr>
<td>tPA (ng/ml)</td>
<td>8 ± 1.1</td>
<td>9 ± 1.5</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>58 ± 9</td>
<td>94 ± 22</td>
</tr>
<tr>
<td>uPA (ng/ml)</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>big-ET-1 (pg/ml)</td>
<td>7.4 ± 0.3</td>
<td>7.7 ± 0.3</td>
</tr>
</tbody>
</table>

Data are mean (range), mean ± SEM, or number.

* P < 0.05 vs the M→F group

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**Table 2** Changes in metabolic variables and blood pressure in male-to-female and female-to-male transsexuals after four months of cross-sex hormone treatment

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>At 4 months</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male-to-female transsexuals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>49 ± 4</td>
<td>***</td>
<td>0.002</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>23.1 ± 2.0</td>
<td>1.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>LH (U/l)</td>
<td>3.4 ± 0.7</td>
<td>0.3 ± 0.0</td>
<td>0.002</td>
</tr>
<tr>
<td>FSH (U/l)</td>
<td>3.1 ± 0.9</td>
<td>0.5 ± 0.0</td>
<td>0.002</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.7 ± 0.4</td>
<td>4.7 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.19 ± 0.99</td>
<td>1.38 ± 0.16</td>
<td>0.03</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.8 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>0.002</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.2 ± 0.3</td>
<td>2.7 ± 0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>41 ± 3</td>
<td>59 ± 3</td>
<td>0.02</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.8 ± 0.2</td>
<td>4.6 ± 0.2</td>
<td>0.24</td>
</tr>
<tr>
<td>Glucose/insulin (x 10⁵)</td>
<td>0.13 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>122/67</td>
<td>122/68</td>
<td>0.8/0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>At 4 months</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female-to-male transsexuals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>34 ± 14</td>
<td>***</td>
<td>0.3</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>1.5 ± 0.2</td>
<td>26.4 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LH (U/l)</td>
<td>4.4 ± 0.7</td>
<td>2.2 ± 0.9</td>
<td>0.02</td>
</tr>
<tr>
<td>FSH (U/l)</td>
<td>4.3 ± 0.3</td>
<td>3.0 ± 0.6</td>
<td>0.09</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.2 ± 0.3</td>
<td>4.2 ± 0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.3 ± 0.10</td>
<td>0.97 ± 0.06</td>
<td>0.002</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>2.6 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>0.09</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>50 ± 7</td>
<td>43 ± 5</td>
<td>0.09</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.0 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Glucose/insulin (x 10⁵)</td>
<td>0.12 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>0.6</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>112/58</td>
<td>114/62</td>
<td>0.5/0.5</td>
</tr>
</tbody>
</table>

* All data shown are mean ± SEM, except blood pressure (mean and range)

*** Male-to-female transsexuals were treated with ethinylestradiol, which is not detected by the assay used
Changes in endothelial marker proteins in male-to-female transsexuals after four months of cross-sex hormone treatment. Data (mean ± SEM) are shown for tPA, uPA, PAI-1 and big-ET-1. ** P = 0.002; * P < 0.02 (Wilcoxon’s signed rank test).

Changes in vWF and vW F : AgII in male-to-female transsexuals after four months of cross-sex hormone treatment. Data shown are individual values (–––) for vWF (panel A; P = 0.005) and vW F : AgII (panel B; P = 0.49) and medians plus 95% confidence intervals (■).

Changes in vWF and vW F : AgII in female-to-male transsexuals after four months of cross-sex hormone treatment. Data shown are individual values (–––) for vWF (panel A; P = 0.06) and vW F : AgII (panel B; P = 0.38) and medians (■).

Baseline levels of tPA, big-ET-1, PAI-1 and uPA were similar in both groups. Baseline levels of vWF were lower in the M→F group as compared to the F→M transsexuals (62 ± 4% vs 81 ± 6%; P = 0.03 after correction for age), as were vW F : AgII levels (98 ± 3% vs 122 ± 7%; P = 0.005).

Testosterone treatment of F→M transsexuals was associated with a significant increase in big-ET-1 (from 7.7 ± 0.3 to 8.2 ± 0.3 pg/ml; P < 0.01).
change, plus 0.4 pg/ml; P = 0.02), and a decrease in vWF (minus 14%; P = 0.06; Fig. 3), whereas tPA (9.5 ± 1.5 vs 10.4 ± 1.4 ng/ml), uPA (1.0 ± 0.1 vs 1.0 ± 0.1 ng/ml) and Pai-1 (94 ± 22 vs 73 ± 13 ng/ml) levels did not change (all P > 0.25). vWF:AgII did not change either (122 ± 7% vs 118 ± 5%; P = 0.38) (Fig. 3).

There was no significant relationship between the baseline levels of conventional cardiovascular risk factors (age, smoking habits, body mass index, blood pressure, and levels of LDL-cholesterol, HDL-cholesterol, triglycerides, glucose and insulin) and those of the plasma marker proteins assayed, nor between percentage change in these risk factors and percentage change in levels of marker proteins (data not shown).

**Discussion**

Ethinylestradiol plus cyproterone acetate administration in M→F transsexuals was associated with decreases in tPA, big-ET-1, uPA and Pai-1, an increase in vWF, and no change in vWF:AgII. Only the increase in vWF is considered unfavourable from the point of view of cardiovascular risk (2-6, 18). Conversely, testosterone treatment of F→M transsexuals was associated with an increase in big-ET-1 and a decrease in vWF, i.e. effects that were in the opposite direction to those observed in the M→F transsexuals, whereas tPA, vWF:AgII, uPA and Pai-1 levels did not change. Whether the changes in the F→M transsexuals will affect cardiovascular risk cannot at present be assessed.

The decreases in tPA and Pai-1 observed in the M→F transsexuals are in agreement with earlier studies of estrogen administration in men (19, 20) and in pre- and postmenopausal women (21-23). The observation that treatment with high-dose testosterone does not affect tPA levels agrees with a study of hormonal contraception in men (24). Our finding of no change in Pai-1 levels, however, contrasts somewhat with the transient decrease observed in that study (24).

Can these changes be explained by effects of sex steroids on the vascular endothelium? Although changes in the plasma levels of the proteins studied here are often thought to be mediated by changes in their rate of synthesis and secretion from the endothelium, one could consider alternative explanations such as changed plasma clearance rates or changed circulatory metabolism.

The reciprocal changes in vWF in the M→F and the F→M transsexuals contrast with the stable levels of vWF:AgII (vWF-propeptide) in both groups. Because vWF and vWF:AgII are released from endothelial cells in equimolar amounts but appear to have different degradative pathways (10, 11), our data indicate changes in vWF processing and/or clearance rather than changes in endothelial cell synthesis or release. Some of the secreted vWF, but not of the propeptide, remains trapped at the site of release (11, 25-27). This fraction may change after hormone treatment, but such an effect would have to be quite marked to result in changes in plasma vWF. Another possibility is that sex steroids influence vWF levels by altering intravascular proteolytic degradation (28). Finally, estrogen has been shown to increase vWF synthesis in cultured human umbilical vein endothelial cells (HUVECs; 29). We have not succeeded in reproducing these observations in HUVECs, nor in microvascular endothelial cells (unpublished data ML and TK) and cannot at present explain these discrepancies. Taken together, these data suggest that the effects of estrogens on vWF are complex and may depend on biological sex and on the type of estrogen under study. Nevertheless, our findings imply that changes in vWF plasma levels do not necessarily reflect changes in endothelial functioning, at least in response to the administration of high doses of sex steroids, but may well reflect changes in clearance and/or intravascular processing.

Similarly, the hypothesis that the decrease in tPA in the M→F transsexuals might be due to increased clearance rather than to decreased synthesis/secretion deserves serious consideration. First, incubation of cultured human endothelial cells with ethinylestradiol or progestagens does not affect the synthesis and secretion of tPA (30). Second, the parallel decrease of uPA, which shares a major hepatic clearance pathway with tPA (and Pai-1) through the low density lipoprotein receptor-related protein/α2-macroglobulin receptor (16, 17) but is not synthesized by endothelial cells under physiological conditions (14, 15), suggests that increased clearance may explain our findings for tPA (and possibly uPA) in the M→F transsexuals. This suggestion is strengthened by the observation (unpublished data JJE) that oral treatment of rats with ethinylestradiol increased the clearance of endogenous tPA while decreasing the steady-state tPA plasma level. We stress, however, that we cannot exclude other mechanisms as an explanation for the decrease in tPA. Specifically, Chandler et al. (31) have shown that the clearance of tPA:PAI complexes is slower than that of free tPA. The observed decrease in tPA might thus be secondary to a decrease in Pai-1. However, whether estrogens can decrease Pai-1 synthesis is unknown. The same applies to uPA synthesis. Also, it is unknown to what extent the endothelium, under physiological conditions, contributes to Pai-1 and uPA plasma levels (14, 15).

These data thus favour the hypothesis that the effects of estrogen on plasma levels of vWF and possibly of tPA, uPA and Pai-1 are due to changes in clearance and/or processing, and not to changes in endothelial cell functioning.

The observed changes in big-ET-1 support our earlier finding of a sex steroid-dependent gender difference in plasma endothelin-like immunoreactivity (32). The assay we used previously (32) could not distinguish between ET-1 and ET-3, whereas here we used an assay that is specific for big-ET-1. Taken together, these data suggest that the endothelin-1 pathway is sensitive to sex steroids, which may partly account for the gender difference in vasomotor regulation (33). Big-ET-1 is cleaved by an endothelin-converting enzyme to generate ET-1. Big-ET-1 is cleared from plasma more slowly than is ET-1, presumably through a different pathway (34). Therefore, our data are consistent with an effect of sex steroids on endothelial release of big-ET-1 and ET-1. Sex steroids may alter ET-1 synthesis directly or through changes in nitric oxide synthesis (35), an increase of which decreases ET-1. Production (33). A recent study did not find an effect of 17β-estradiol on ET-1 synthesis by HUVECs (36), but this does not exclude an effect on arterial or microvascular endothelium. Alternatively, sex steroids might affect ET-1 secretion through changes in insulin levels (37), but our data do not support this notion.

Our study was neither randomised nor placebo-controlled, because transsexual patients are generally unwilling to postpone hormonal treatment. We thus cannot fully exclude time-dependent effects. However, our study design uniquely allowed the investigation, in otherwise young and healthy subjects, of high doses of sex steroids, which implies that any time-dependent effects should have been quite marked to seriously confound our conclusions.

We conclude that estrogens and androgens may simultaneously have positive and negative effects on the risk of atherothrombotic disease. These hormones have clear but complex effects on plasma levels of marker proteins of endothelial cell functioning, in which both altered synthesis and secretion and changes in metabolism and clearance are likely involved. Therefore, the effects of estrogens and androgens on the plasma levels of endothelial marker proteins do not...
necessarily reflect changes in endothelial functioning, at least with regard to changes in vWF level associated with the oral administration of high doses of ethinylestradiol and cyproterone acetate to healthy men and the parenteral administration of testosterone to healthy women.

Acknowledgements

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