Analysis of the vascular responses in a murine model of polycystic ovary syndrome

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Abstract
Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of the reproductive age, but the exact pathophysiological mechanisms involved remain unclear. Cardiovascular disease risk is increased in PCOS patients and endothelial damage has been observed. We recently developed a mouse model of PCOS with reproductive and metabolic characteristics resembling those observed in women with PCOS. In this model we studied vascular function with particular emphasis on markers of vascular endothelial function. Animals were treated for 90 days with dihydrotestosterone (DHT; 27.5 μg/day) or placebo using subcutaneous continuous-release pellets. Aortas were isolated for isometric force recordings in organ baths to investigate endothelial and vascular smooth muscle characteristics. Lungs were used to analyze endothelial nitric oxide synthase (eNOS) expression and phosphorylation. Asymmetric dimethylarginine (ADMA) levels were investigated in serum to assess endothelial damage. Expression of androgen receptor (Ar) mRNA was studied in aortas. DHT treatment (compared with placebo) induced i) a significant decrease in acetylcholine-induced aortic relaxations, with no change in calcitonin gene-related peptide- or sodium nitroprusside-induced relaxations, as well as 5-hydroxytryptamine-induced contractions; ii) no change in eNOS expression/phosphorylation in lungs or in plasma ADMA levels; and iii) a twofold increase in aortic AR expression. Our results suggest that, in DHT-exposed mice, hyperandrogenemia specifically decreases endothelial-dependent vasorelaxation without deterioration of smooth muscle function. This study may initiate further investigations to elucidate underlying mechanism for the phenotype that is present in these animals, as well as in PCOS patients.

Key Words
- PCOS
- dihydrotestosterone
- aorta
- non-genomic mechanisms
- vasorelaxation

Introduction
Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age and the most common form of hyperandrogenism (Carmina et al. 2006). It has a worldwide prevalence of 5–10% (Diamanti-Kandarakis et al. 1999, Asuncion et al. 2000, Azziz et al. 2004), but this can be higher (13–52%) in some
subpopulations (Rodin et al. 1998, Davis et al. 2002, Goodarzi et al. 2005). The exact mechanisms involved in the pathophysiology of PCOS are still unclear, but high levels of androgens are considered to play a crucial role in the etiology. PCOS patients suffer from dyslipidemia, obesity and metabolic syndrome (Wild 2002, Glueck et al. 2009), resulting in an increased risk for type 2 diabetes mellitus and cardiovascular diseases including hypertension and atherosclerosis (Charitidou et al. 2008, Wild et al. 2010, Goodarzi et al. 2011, de Groot et al. 2011). This predisposition is further aggravated by the associated endothelial dysfunction (Lakhani et al. 2005, Hudecova et al. 2010). Studies in PCOS women showed increased circulating levels of asymmetric dimethylarginine (ADMA), a marker of endothelial dysfunction. ADMA is a competitive inhibitor of nitric oxide synthase (NOS), thus increased ADMA levels can lead to decreased vascular relaxation. ADMA levels are often increased in cardiovascular diseases (Charitidou et al. 2008, Bayrak et al. 2012). Endothelial dysfunction in women with PCOS is often assessed by measuring flow-mediated dilation (FMD) of the brachial artery and intima media thickness of the carotid artery (CIMT). A decreased FMD and an increased CIMT have been observed in women with PCOS compared with normo-ovulatory woman (Kravariti et al. 2005, Meyer et al. 2009). Also women with PCOS often display increased insulin resistance. Insulin resistance is thought to be correlated with endothelial dysfunction and thus might play an important role in the development of endothelial dysfunction (Suzuki et al. 2007, Stepto et al. 2013). In addition, serum paraoxonase 1 (PON1) activity is decreased in women with PCOS (Soyman et al. 2011, Bayrak et al. 2012). PON1 is a HDL-associated enzyme that prevents LDL oxidation and thereby prevents the negative effects of LDL. Furthermore, hypertensive, but not normotensive, PCOS women displayed increased arterial stiffness (Sasaki et al. 2011). Taken together, these studies suggest that women with PCOS have impaired vascular function, which may be assigned at least partly to impaired endothelial function. However, the relationship between hyperandrogenemia and endothelial dysfunction remains unclear. The results of studies on the effects of androgens on vascular reactivity in women are controversial and probably depend on the study period and endogenous estradiol levels in women (Vitale et al. 2010). In females taking high doses of androgens decreased vascular function was found, which was assigned to a negative effect of testosterone on endothelial NOS (eNOS)-mediated responses (McCredie et al. 1998). It is unknown whether the effects of testosterone on endothelial function are direct effects mediated through the androgen receptor (AR) or indirect effects mediated via the conversion of testosterone into estrogens. The direct effect of androgens can be experimentally investigated by studying the effects of the nonaromatizable androgen dihydrotestosterone (DHT) on endothelial function.

Several experimental animal models for PCOS have been developed through prenatal or postnatal exposure to androgens, but these animal models were mainly used to investigate ovarian and metabolic function and the vascular phenotype was not studied (Manneras et al. 2007, Roland et al. 2010). In pregnant rats treated with testosterone, increased blood pressure and decreased endothelial function were observed (Chinnathambi et al. 2013). Recently, we have developed a mouse model of PCOS, facilitating future use of transgenic animals, in which prepubertal female mice were exposed to DHT for 90 days. These DHT-exposed mice display both reproductive and metabolic characteristics resembling those observed in women with PCOS, such as acyclicity, cyst-like follicles, increased adiposity, increased leptin and decreased adiponectin levels, and impaired glucose tolerance (van Houten et al. 2012). We used this mouse PCOS model to study the effect of hyperandrogenemia on vascular function. The effect of DHT on in vitro vascular responses to several vasoactive agents was analyzed, with particular emphasis on markers of vascular endothelial function (i.e. lung expression/phosphorylation of eNOS and serum levels of ADMA).

Materials and methods

Animals

C57BL/6J mice at postnatal day 19 were s.c. implanted with a 90-day continuous-release pellet containing either DHT (2.5 mg, 27.5 μg/day; n = 14) or placebo (n = 16; Innovative Research of America, Sarasota, FL, USA) as described previously (van Houten et al. 2012). A separate group of mice was implanted with a 60-day release DHT pellet (1.5 mg, 25 μg/day; n = 9) or placebo (n = 9; Innovative Research of America). Mice were killed at the end of the treatment period (60 or 90 days). Blood samples were collected by orbital puncture after the mice were anesthetized with isoflurane. Mice were killed by decapitation and tissues were isolated. The same animals as reported in the study by van Houten et al. (2012) were used in this study.

Mice were kept under standard animal housing conditions in accordance with the National Institutes of
Health Guidelines for the Care and Use of Experimental Animals. The experiments were performed with permission of the Local Ethics Committee.

**Tissues**

Thoracic aortas from the 60- (n=18) and 90-day-treated animals (n=18) were placed in cold Krebs buffer (4°C), aerated with 5% CO2 and 95% O2 and stored overnight for organ bath experiments (composition of Krebs buffer in mmol/l: NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25 and glucose 8.3; pH 7.4). In addition, aorta segments of the 90-day-treated mice (n=12) were directly snap frozen in liquid nitrogen and stored at −80°C until RNA isolation. Furthermore, from the 90-day-treated animals, the lungs were collected (n=6 for both DHT- and placebo-treated animals) and cut into pieces. Pieces were divided into two groups and incubated for 1 min with either vehicle or 100 μmol/l acetylcholine in Krebs buffer. Samples were snap frozen in liquid nitrogen and stored at −80°C for protein isolation. Lungs were used for this experiment, because lungs are very densely vascularized with small resistance vessels. A small amount of blood serum was available from all the treatment groups and was used for the measurement of ADMA as a measure of endothelial damage.

**Functional experiments**

Two-millimeter aortic rings (inner diameter 1–2 mm) were mounted in organ baths (Danish Myo Technology, Aarhus, Denmark) between two aluminum wires (wire diameter 40 μm), which were attached to a force displacement transducer and a computer on one side and a displacement device on the other side. The temperature-controlled organ baths (37°C) were filled with Krebs buffer and aerated with 5% CO2 and 95% O2. The tension of the aortic rings was normalized to 90% of the estimated diameter at 100 mmHg pressure and stabilized for 30 min (Mulvany & Halpern 1977).

The rings were exposed to 30 mmol/l KCl and after washout to 100 mmol/l KCl to compare reactivity and maximal contractile response of the different rings. For vasodilator studies, rings were precontracted with 10–100 mmol/l U46619, a thromboxane A2 analog, until a contraction of 50% of the contraction induced by 100 mmol/l KCl was reached. Smooth muscle cell-dependent vasorelaxation was determined in response to increasing concentrations of the vasodilating compounds calcitonin gene-related peptide (CGRP; rat α-CGRP, NeoMPS, Polypeptide Group; Strasbourg, France) and sodium nitroprusside (SNP; Sigma Chemical Co.). CGRP activates the CGRP receptor present in smooth muscle cells (Eftekhar & Edvinsson 2010). SNP stimulates the direct release of NO, which can enter the smooth muscle cells (Friederich & Butterworth 1995). Endothelium-dependent responses were examined using acetylcholine (Sigma Chemical Co.). To measure the maximal relaxation, 100 μmol/l SNP were added following the relaxation experiments to CGRP and acetylcholine. Contractile responses were investigated with increasing concentrations of the vasoconstrictor 5-hydroxytryptamine (5-HT; Sigma Chemical Co.). Half of the rings of the 90-day DHT-treatment group were first incubated for 30 min with 100 μmol/l (6R)-5,6,7,8-tetrahydro-L-biopterin-2HCl (BH4) (Calbiochem, Merck KGaA, Darmstadt, Germany) (an inhibitor of both reactive oxygen species (ROS) and eNOS uncoupling), 30 μmol/l N-acetyl-cysteine (NAC; Sigma Chemical Co.) or 100 μmol/l tempol (Sigma Chemical Co.; both ROS inhibitors) prior to precontraction with U46619. These concentrations have been determined previously (Durik et al. 2012). SNP, acetylcholine and 5-HT were dissolved in bidistilled water; NAC and tempol were both dissolved in DMSO.

**Quantitative real-time RT-PCR**

RNA of thoracic aortas was isolated using an RNA isolation kit for fibrous tissue (RNaseasy fibrous tissue; Qiagen). RNA was reverse transcribed using the Quantitect RT kit. A quantitative real-time RT-PCR was performed using TaqMan probes for the AR and β-actin as a housekeeping gene (Applied Biosystems). The expression of the target gene was normalized to the expression levels of β-actin using the 2−ΔΔCt method.

**Quantification of eNOS phosphorylation using western blotting**

Lung tissue was isolated, cut into small pieces and incubated with acetylcholine (100 μmol/l) or vehicle (Krebs buffer) for 1 min. Western blotting was performed with 15 μg of protein using total eNOS antibody (diluted 1:1000) or phosphorylated eNOS (Ser1177) antibody (diluted 1:1000). For visualization, a peroxidase-conjugated goat anti-rabbit antibody was used (all antibodies were obtained from Santa Cruz Biotechnology). The blot was scanned and analyzed with the use of ImageJ Software (NIH, Bethesda, MD, USA).
ADMA levels

Blood serum was collected from all treatment groups and serum levels of ADMA were measured using the ADMA ELISA kit (DLD Diagnostika GmbH, Hamburg, Germany).

Statistical analysis

The relaxant responses elicited by the vasorelaxant compounds were expressed as percentage of the maximum contraction induced by U46619 (10–100 nmol/l, 100%). The contractile responses to 5-HT were expressed as percentage of the previous response to 100 nmol/l KCl. All data are presented as mean ± S.E.M. Statistical analysis of the concentration response curves was accomplished with GraphPad Prism 5 Software (La Jolla, CA, USA), using unpaired t-tests and ANOVA. Moreover, differences in %AR expression (compared with the household gene β-actin using the 2^-ΔΔCt method) and the differences in eNOS expression and phosphorylation between the treatment groups were assessed using an unpaired t-test. Statistical significance was accepted at P<0.05 in all cases.

Results

Effect of 90 days DHT exposure on acetylcholine-induced relaxation in thoracic aortas

Acetylcholine-induced maximal relaxations were significantly smaller in aortas from 90-day DHT-treated mice than in those from placebo-treated mice (P=0.04; Fig. 1A). The pEC50 values did not differ between DHT- and placebo-treated animals (Table 1). Furthermore, no difference was observed in response to 100 µmol/l SNP, which was added after the concentration response curve to acetylcholine was finished (Fig. 1B).

Effect of 90 days DHT exposure on SNP- and CGRP-induced relaxations and 5-HT-induced contractions

In contrast to the relaxation response to acetylcholine described above, the maximal relaxations and pEC50 values to CGRP (Fig. 2A) and SNP (Fig. 2C) were not different between the 90-day DHT- and placebo-treated groups. Furthermore, no difference was observed in the subsequent response induced by 100 µmol/l SNP after the CGRP curve (Fig. 2B). Likewise, no differences were observed in the concentration response curves to 5-HT (Fig. 2D). E_max and pEC50 values are shown in Table 1.

Effect of BH4, NAC or tempol on the relaxations to acetylcholine in 90 days DHT-exposed vessels

Since impaired endothelial function might be caused by a decreased NO bioavailability or decreased NO production by eNOS, the relaxations to acetylcholine were also measured in the presence of 100 µmol/l BH4, 30 µmol/l NAC or 100 µmol/l tempol in thoracic aortas of mice treated for 90 days with placebo and DHT to investigate the role of ROS and eNOS uncoupling in the acetylcholine response. Both NAC and tempol are ROS inhibitors and BH4 is needed for eNOS functioning. Thirty minutes of incubation with NAC or tempol did not cause any difference in acetylcholine-induced relaxation in DHT- or placebo-treated mice (Fig. 3A and B). Interestingly, whereas incubation with BH4 had no effect on the acetylcholine-induced maximal vasorelaxation in placebo-treated mice, it induced a significant increase in vasorelaxation in DHT-treated animals (E_max DHT: 64 ± 4% vs E_max DHT+BH4: 81 ± 3%, P = 0.02). The pEC50 values did not differ (pEC50 DHT: 7.1 ± 0.1 vs pEC50 DHT+BH4: 6.8 ± 0.1, P = 0.4; Fig. 3B).

Table 1  E_max and pEC50 values for acetylcholine, CGRP, SNP and 5-HT in aortas of mice treated for 90 days with DHT or placebo

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<th>Placebo 90 days</th>
<th>DHT 90 days</th>
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<tbody>
<tr>
<td>Acetylcholine</td>
<td>E_max (%)</td>
<td>pEC50</td>
</tr>
<tr>
<td></td>
<td>75 ± 3</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>CGRP</td>
<td>89 ± 13</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>SNP</td>
<td>96 ± 7</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>5-HT</td>
<td>96 ± 7</td>
<td>6.9 ± 0.1</td>
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</table>

*Significantly different from placebo, P<0.05.
Endothelial damage as a primary or secondary phenomenon

To investigate whether the endothelial damage that we observed was a consequence of the metabolic changes in the DHT-treated mice, we studied relaxations to acetylcholine and SNP in mice that were treated with DHT for 60 days. DHT treatment for 60 days induced neither a reproductive nor a metabolic phenotype (results not shown), but also resulted in significantly lower plasma DHT levels at the end of the treatment period compared with the animals that were treated with the 90-day release pellet (0.8 ± 0.1 vs 2.8 ± 0.3 nmol/l, P < 0.05). Comparable with the 90-day treatment group, acetylcholine-induced maximal relaxations were significantly smaller in aortas from 60-day DHT-treated mice than in those from placebo-treated mice (P = 0.04; Fig. 4A). The pEC_{50} values did not differ between DHT- and placebo-treated animals (Table 2). Furthermore, no difference was observed in response to 100 μmol/l SNP, which was added after the concentration response curve to acetylcholine had been finished (Fig. 4B).

Expression and phosphorylation of eNOS as well as serum levels of ADMA

To investigate whether there were differences in eNOS availability and functioning between the treatment groups, total eNOS and eNOS activation through Ser1177 phosphorylation were analyzed in lungs of 90-day placebo- and DHT-treated animals after 1 min incubation with acetylcholine or vehicle. Values were corrected for β-actin expression. No differences were seen in the total expression level of eNOS (Fig. 5A). Also DHT treatment had no effect on the eNOS Ser1177 phosphorylation level (Fig. 5B). Likewise, serum levels of ADMA, a competitive inhibitor of eNOS, did not significantly differ between 90-day DHT- and placebo-treated animals (Fig. 6).
Effect of DHT exposure on ArmRNA expression in thoracic aortas

To investigate if AR signaling might be involved in the difference in endothelial functioning between the DHT- and placebo-treated animals, Ar expression was examined. A 2.2-fold increase in Ar mRNA expression was observed in thoracic aortas of mice treated for 90 days with DHT when corrected for β-actin expression (P < 0.02; Fig. 7).

Discussion

General

Our results show that in aortic vessels chronic DHT exposure i) specifically decreased the vasodilatation to acetylcholine but not to vasodilators directly acting at the smooth muscle layer, such as SNP and ii) increased Ar mRNA expression. This implies a decreased endothelial function, unrelated to a deterioration of vascular smooth muscle function and/or an eNOS decreased expression/phosphorylation. These results suggest that this DHT-induced mouse model, besides having a PCOS-resembling metabolic and reproductive phenotype, also has a vascular phenotype. This mouse model for PCOS therefore may be an interesting model to investigate the cardiovascular alterations observed in PCOS patients.

Specific decrease in aortic endothelial function after DHT treatment in mice: resemblance with other PCOS experimental models

Our data show that the aortic vasodilatation to acetylcholine, which acts on the vascular endothelium to induce vascular smooth muscle relaxation (Furchgott & Zawadzki 1980), was significantly decreased after chronic treatment with DHT. In contrast, the vasodilatation to SNP, an NO donor acting directly on vascular smooth muscle, remained unaffected. These findings suggest that chronic DHT treatment results in a decreased aortic endothelial function. Consistent with this conclusion, the concentration response curves to the direct vasodilator CGRP and to the vasoconstrictor 5-HT remained unaffected upon chronic DHT exposure. Because CGRP and 5-HT receptors have been shown to be present on the membrane of vascular smooth muscle cells and, therefore, can produce direct vascular effects (Furchgott & Zawadzki 1980, Villalon & Centurion 2007, Villalon & Olesen 2009), our findings suggest that chronic DHT treatment does not cause deterioration of vascular smooth muscle function. In contrast, in a rat model of PCOS, contractions to noradrenaline, mediated via the PLC signaling pathway similarly as contractions to 5-HT in our model, were decreased (Sara et al. 2012). Thus, although contractions to 5-HT remained unaffected after chronic DHT treatment, we cannot exclude that the effect of other constrictors might be affected in our model. On the other hand, our results are in agreement with a recent study in a DHT-induced rat PCOS model, using a comparable DHT treatment schedule as in our mouse study, where DHT treatment resulted in specific decrease in acetylcholine-induced vasodilator responses (Keller et al. 2011). Endothelial dysfunction was also observed in a rat PCOS model developed by daily injections of the antiprogestin mifepristone (Lakhani et al. 2006). Clearly, endothelial dysfunction can be a predictor of cardiovascular disease or hypertension. This is also seen in women with PCOS (Wild et al. 2010, Goodarzi et al. 2011, de Groot et al. 2011); therefore it is important to elucidate the underlying mechanisms for the development of better treatment options.

Table 2  $E_{\text{max}}$ and $pE_{50}$ values for acetylcholine in aortas of mice treated for 60 days with DHT or placebo

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<th>Placebo 60 days</th>
<th>DHT 60 days</th>
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<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$ (%)</td>
<td>$pE_{50}$</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>75 ± 4</td>
<td>7.3 ± 0.2</td>
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*Significantly different from placebo, P < 0.05.
ROS inhibition and eNOS uncoupling

Binding of acetylcholine to its receptor on endothelial cells leads to the activation of eNOS. We measured expression and phosphorylation of eNOS in lung tissue, which is densely vascularized, as an alternative for the aortic tissues, because aortic tissue was not available in sufficient quantities due to the functional studies. We did not observe any effect of DHT treatment, although we obviously cannot exclude that there may be differences in eNOS expression and phosphorylation between lung and aorta tissues. eNOS signaling can be disturbed by ROS, which decrease available NO, induce eNOS uncoupling and decrease the effectiveness of eNOS (Fleming 2010). Both tempol and NAC are ROS inhibitors but acting through different mechanisms (Zafarullah et al. 2003, Simonsen et al. 2009, Wilcox 2010). BH4 is a cofactor of eNOS and in the presence of reduced BH4 levels eNOS uncoupling occurs, leading to superoxide production instead of NO (Fleming 2010, Roe & Ren 2012). Since no differences in acetylcholine-induced aortic vasodilatation occurred after the treatment with NAC or tempol it is reasonable to suggest that ROS formation is not responsible for the decreased vasodilatation to acetylcholine in DHT-treated mice. In contrast, the increase in acetylcholine-induced aortic vasodilatation after treatment with BH4 implies that eNOS uncoupling was increased, leading to a disturbed eNOS function; however, we cannot explain why we did not observe increased ROS production as a consequence.

Increase in Ar mRNA expression after DHT treatment

We observed a strong increase in Ar mRNA expression in the chronically DHT-treated animals. This is in accordance with earlier studies reporting increased AR expression in endometrium of PCOS patients (Apparao et al. 2002, Villavicencio et al. 2006), as well as in ovaries in a rat PCOS model (Zurvarra et al. 2009). It is known that AR signaling can be both genomic and nongenomic and may, in turn, activate other signaling pathways (Bennett et al. 2010). Thus, it is tempting to suggest that an increased AR signaling may underlie the reduced endothelial function (i.e. a decreased vasorelaxation to acetylcholine) observed in the aortas after treatment with DHT. Admittedly, no experimental evidence is reported in the literature to support this view.

Endothelial dysfunction as a primary or secondary phenomenon

Chronic androgen exposure can increase blood pressure in women with PCOS and rats treated chronically with DHT or testosterone displayed elevated blood pressure (Keller et al. 2011, Chinnathambi et al. 2013). Increased blood pressure can lead to decreased endothelial function. However, our PCOS mice also display a metabolic phenotype. To decide whether hyperandrogenemia directly induces endothelial dysfunction or whether this is primarily mediated through metabolic alterations, we also studied endothelial function in mice implanted
with a 60-day DHT-release pellet, which resulted in lower serum DHT levels at the end of the experiment (60 days after implantation). These 60-day DHT-treated mice did not develop the metabolic or reproductive PCOS-resembling phenotype. Yet, these mice did show decreased vasorelaxation to acetylcholine to a similar extent as the 90-day DHT-treated mice. This suggests that the endothelial dysfunction observed upon DHT treatment most likely is a direct effect of androgens, not secondary, on metabolic changes.

In conclusion, our results in a DHT-induced mouse model for PCOS suggest that hyperandrogenemia specifically decreases the endothelium-dependent vasorelaxation, without deterioration of smooth muscle function. This study may initiate further investigations to resolve the mechanisms behind the vascular pathologies observed in PCOS patients.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**
J A Visser and A MaassenVanDenBrink contributed equally to this work.

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