Lower-limb veins are thicker and vascular reactivity is decreased in a rat PCOS model: concomitant vitamin D₃ treatment partially prevents these changes

Várbiró S, Sára L, Antal P, Monori-Kiss A, Tőkés A, Monos E, Benkő R, Csibi N, Szekeres M, Tarszabo R, Novak A, Paragi P, Nádasy GL. Lower-limb veins are thicker and vascular reactivity is decreased in a rat pcos model: concomitant vitamin D₃ treatment partially prevents these changes. Am J Physiol Heart Circ Physiol 307: H848–H857, 2014. First published July 11, 2014; doi:10.1152/ajpheart.01024.2013.—Polycystic ovary syndrome (PCOS) causes vascular damage to arteries; however, there are no data for its effect on veins. Our aim was to clarify the effects of dihydrotestosterone (DHT)-induced PCOS both on venous biomechanics and on pharmacological reactivity in a rat model and to test the possible modulatory role of vitamin D₃ (vitD). PCOS was induced in female Wistar rats by DHT treatment (83 µg/d, subcutaneous pellet). After 10 wk, the venous biomechanics, norepinephrine (NE)-induced contractility, and acetylcholine-induced relaxation were tested in saphenous veins from control animals and from animals treated with DHT or DHT with vitD using pressure angiography. Additionally, the expression levels of endothelial nitric oxide synthase (eNOS) and cyclooxygenase (COX-2) were measured using immunohistochemistry. Increased diameter, wall thickness, and distensibility as well as decreased vasoconstriction were detected after the DHT treatment. Concomitant vitD treatment lowered the mechanical load on the veins, reduced distensibility, and resulted in vessels that were more relaxed. Although there was no difference in the endothelial dilation tested using acetylcholine (ACh), the blocking effect of N-containing-arginine methyl ester (L-NAME) was lower and was accompanied by lower COX-2 expression in the endothelium after the DHT treatment. Supplementation with vitD prevented these alterations. eNOS expression did not differ among the three groups. We conclude that the hyperandrogenic state resulted in thicker vein walls. These veins showed early remodeling and altered vasorelaxant mechanisms similar to those of varicose veins. Alterations caused by the chronic DHT treatment were prevented partially by concomitant vitD administration.

Polycystic ovary syndrome (PCOS) is one of the most frequently occurring endocrine syndromes in reproductive-age women and is accompanied by a high risk for cardiovascular diseases. Females with PCOS have a prevalence of early-onset atherosclerosis, metabolic syndrome, and insulin resistance (6). It was shown previously that vascular biomechanical functions are defective in PCOS. Lakhani et al. (16) showed that women who have a low internal carotid artery pulsatility index in PCOS also have a high cardiovascular risk. These changes correlate with a hyperandrogenic state rather than with obesity (18). In PCOS, increased arterial stiffness and pulse wave velocity were demonstrated by ultrasound assessments (1, 16, 19). The mechanism of these alterations is unclear, but endothelial dysfunction and altered collagen metabolism of the vessel wall may be involved. This is similar to the patterns observed in insulin resistance and metabolic syndrome (4, 26). Cussons et al. (4) stated that vascular damage developed gradually in PCOS. A decrease in flow-mediated vasodilation in PCOS could be detected as the earliest sign of abnormality even with normal arterial stiffness. Recently, our group detected a similar early-onset alteration of vascular reactivity in the small resistance arteries, which might be the first step in the pathogenesis of hypertension (32, 33). These vascular alterations of hyperandrogenic PCOS were accompanied by insulin resistance (31). Clinically, both macro- and microvascular dysfunctions were observed by ultrasound in PCOS, as indicated by the reduction in acetylcholine (ACh)-dependent vasodilation. This impaired response to ACh is similar to that observed in non-insulin-dependent diabetes mellitus, and it may be related to metabolic alterations, especially the insulin resistance present in clinical PCOS (5, 14, 17). We also have experimental data on the arterial damage of large vessels (21). However, there are currently no experimental or clinical data on the potential alterations of the venous system accompanying PCOS.

The venous system has a decisive role in the regulation of circulation, determining capacitance function; this is especially true of the small veins. We hypothesized that in addition to arterial effects, changes in the hormonal environment during PCOS may affect the vascular reactivity of small veins. In addition to metformin, the use of vitamin D (vitD) as a possible adjuvant therapy to treat metabolic symptoms in females with PCOS was reported to improve vessel reactivity in skeletal muscle resistance arteries (32, 33). Data on the direct effects of vitD on venous contractility and biomechanics are still lacking. Continuing this line of research, in the present...
study, we aimed to examine the possible effects of vitD treatment on the venous walls in a pharmacologically induced PCOS model.

METHODS

Chemicals

Pentobarbital sodium (Nembutal; Phylaxia-Sanofi, Budapest, Hungary) was used for anesthesia (50 mg/kg ip). Following the surgical intervention, 20 mg of amoxicillin and 4 mg of clavulanic acid (Augmentin; GlaxoSmiithKline, Memphis, TN) in 0.2 ml of saline was administered intramuscularly to prevent infection. The protocol described by Mannerås et al. (20) was followed to induce experimental PCOS. Dihydrotestosterone (DHT), in 7.5-mg subcutaneous pellets (releasing 83 μg/day for 90 days according to the manufacturer’s instructions; Innovative Research of America, Sarasota, FL), was applied for 70 days. We purchased 1.25(0H)2 D3 vitamin (Inj. Cacijex, 2 μg/ml) from Abbott Laboratories.

The composition of the normal Krebs-Ringer solution used in the in vitro vascular studies was as follows (in mmol/l): 119 NaCl, 4.7 KCl, 1.2 NaH2PO4, 1.17 MgSO4, 24 NaHCO3, 5.5 glucose, and 0.034 EDTA. The Ca2+-free Krebs solution that was used to relax the vascular smooth muscle contained the following (in mmol/l): 92 NaCl, 4.7 KCl, 1.18 NaH2PO4, 20 MgCl2, 1.17 MgSO4, 24 NaHCO3, 5.5 glucose, 2.0 EGTA, and 0.025 EDTA. The temperature of the solution was maintained at 37°C, and the solution was bubbled with 5% CO2, 20% O2, and 75% N2, which stabilized the pH at 7.4.

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Norepinephrine (NE), acetylcholine, and NOS-nitro-l-arginine methyl ester (l-NAME) were obtained from Sigma-Aldrich (St. Louis, MO, and Budapest, Hungary); dilutions were freshly prepared on the day of the experiment. The cyclooxygenase-2 (COX-2) and endothelial nitric oxide (NO) synthase (eNOS) antibodies were obtained from Abcam (Cambridge, UK); the secondary antibody, normal goat serum, avidin-biotinylated enzyme complex kit, and 3,3'-diaminobenzidine (DAB) were purchased from Vector Laboratories (Burlingame, CA). The manufacturers’ protocols were used for the immunohistochemistry.

Animals

Thirty adolescent (21- to 28-day-old) female Wistar rats (provided by the Animal Facility of Semmelweis University in agreement with Charles River Laboratories that weighed 100–140 g when the study received 120 ng·100 g body wt

Anesthesia (DHT

Table 1. Results of vital parameters and carbohydrate metabolism

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls</th>
<th>DHT Treated</th>
<th>DHT + D3 Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>298 ± 8</td>
<td>354 ± 16*</td>
<td>353 ± 9†</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>122 ± 3</td>
<td>123 ± 6</td>
<td>123 ± 4</td>
</tr>
<tr>
<td>Blood sugar 0 min, mM/I</td>
<td>5.31 ± 0.15</td>
<td>5.35 ± 0.24</td>
<td>5.18 ± 0.27</td>
</tr>
<tr>
<td>Blood sugar 120 min, mM/I</td>
<td>6.11 ± 0.11</td>
<td>6.36 ± 0.22</td>
<td>7.09 ± 0.13</td>
</tr>
<tr>
<td>Insulin 0 min, ng/ml</td>
<td>0.42 ± 0.02</td>
<td>0.42 ± 0.03</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td>Insulin 120 min, ng/ml</td>
<td>0.71 ± 0.14</td>
<td>1.42 ± 0.33*</td>
<td>0.48 ± 0.07†</td>
</tr>
<tr>
<td>Glycated protein (fructosamine), mM/I</td>
<td>157 ± 3</td>
<td>151 ± 4</td>
<td>156 ± 4</td>
</tr>
<tr>
<td>DHT plasma levels, pg/ml</td>
<td>267.3 ± 14.1</td>
<td>370.9 ± 35.0*</td>
<td>438.4 ± 24.1†</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. DHT, dihydrotestosterone; D3, vitamin D3. The body weights of control animals were significantly lower than in DHT-treated animals (P < 0.01). The 120-min postload insulin level in the DHT group was significantly higher than in controls (P < 0.01). Vitamin D3 treatment normalized the insulin response during oral glucose tolerance test (P < 0.001). The control values of DHT level were significantly different from those of the DHT and DHT + D3 groups (P < 0.05). There were no significant differences in other parameters: blood pressure, blood sugar 0 min, blood sugar 120 min, insulin 0 min, and glycated protein levels. *DHT was significantly different from the control group; †DHT + D3 group was significantly different from the control; ‡DHT + D3 group was significantly different from the DHT group (n = 10 in each group).

The vitamin D3 treatment prevented these negative effects of the hyperandrogenic status (22, 31).

Measuring the In Vitro Biomechanics and Pharmacological Reactivity of the Saphenous Vein

After 10 wk of treatment, the animals were reanesthetized (Nembutal, 50 mg/kg ip). The blood pressure was measured directly through the cannulation of the carotid artery. Then, the animals were bled under anesthesia, and the adductor muscle group and ovaries were removed from the cadavers. After the iliofemoral region was opened, the saphenous vein, which had an in vivo diameter of ~500–600 μm, was removed and placed in a vessel chamber filled with normal Krebs-Ringer (nKR) solution. The saphenous vein was cannulated at both ends with plastic microcannulas and carefully extended to its in vivo length. Both cannulas were connected to pressure-servo systems (Living Systems, Burlington, VT), and the veins were incubated and pressurized under a no-flow condition at 5 mmHg of intraluminal pressure.

The outer and the inner diameters of the veins were measured using pressure microangiometry with video microscopy. In this setup, the glass-bottomed tissue bath was positioned in the light path of an inverted Leica microscope. A magnified picture of the vessel was formed using a video camera (Leica DFC320) and Leica QWin software. The digitized pictures were saved, and the inner and outer diameters were measured off-line using Leica QWin image analysis software.

The saphenous veins were allowed to equilibrate for 30 min at 5-mmHg intraluminal pressure in an oxygenized nKR solution. After incubation, the pressure was first decreased to 2 mmHg and then increased to 20 mmHg in steps (2, 4, 6, 8, 10, 15, and 20 mmHg). The steady-state diameter at each step was measured. During NE contraction (after 10-min incubation with 10−6 M NE), the pressure-diameter curve was repeated as described above. After the NE curve was recorded, 10−6 M ACh was added to the organ bath at 5-mmHg intraluminal pressure. Following a 20-min equilibration period, the outer and inner diameters were measured. Next, 10−5 M l-NAME was administered, and the diameters were measured again after reaching equilibrium (25–30 min at 5 mmHg). Finally, the passive diameter was measured in Ca2+-free Krebs solution, as described above. We used an Etalon micrometer (Wild) for calibrations.
Biomechanical Calculations

From the original calibrated pressure diameter plots, the following geometrical and biomechanical parameters were computed for each intraluminal pressure level (22). The tangential stress was computed according to the Laplace equation \( \sigma_r = \frac{p \times r_i}{h} \), where \( \sigma_r \) is the tangential (circularfemoral) wall stress, \( p \) is the intraluminal pressure, \( r_i \) is the inner radius, and \( h \) is the wall thickness (\( h = r_o - r_i \), where \( r_o \) is the outer radius). The incremental distensibility was computed as \( \text{Dinc} = \Delta V/\Delta P \), where \( \text{Dinc} \) is the incremental distensibility and \( \Delta V \) the change in vessel lumen volume relative to the initial volume \( V \) in response to a pressure change of \( \Delta P \). The circumferential incremental elastic modulus was computed using the equation \( E_{\text{inc}} = \frac{\Delta \sigma_r / \Delta r_o}{r_o} \times \frac{r_o^2 - r_i^2}{r_o^2 - r_i^2} \), where \( E_{\text{inc}} \) is the incremental elastic modulus, \( r_i \) is the inner radius, \( r_o \) is the outer radius, and \( \Delta r_o \) is the change in the outer radius in response to the intraluminal pressure change of \( \Delta P \). The incremental distensibility was computed as \( \text{Dinc} = \Delta V/\Delta P \), where \( \text{Dinc} \) is the incremental distensibility and \( \Delta V \) the change in vessel lumen volume relative to the initial volume \( V \) in response to a pressure change of \( \Delta P \).

Calculations of Pharmacological Reactivity of the Vessels

From the original calibrated pressure diameter plots, the following pharmacological parameters were computed for each intraluminal pressure level: full contraction of the segments, \( T_{\text{Full}} = 100 \times \left( \frac{R_{\text{Cafree}} - R_{\text{NE}}}{R_{\text{Cafree}}} \right) \); myogenic (spontaneous) tone, \( T_{\text{ACR}} = 100 \times \left( \frac{R_{\text{Cafree}} - R_{\text{KK}}} {R_{\text{Cafree}}} \right) \); and NE-induced tone, \( T_{\text{NE}} = 100 \times \left( \frac{R_{\text{NE}} - R_{\text{Cafree}}} {R_{\text{Cafree}}} \right) \). The ACh-induced relaxation (\( T_{\text{ACH}} \)) was computed relative to the NE-induced tone as follows: \( \frac{(R_{\text{ACR}} - R_{\text{NE}})}{R_{\text{NE}}} \). The ACh-induced tone alteration (\( T_{\text{ACH}} \)) was computed as a function of the NE-induced tone and as a percentage of the Ca\(^{2+}\)-free vessel radius (compared with the morphological diameter as an absolute reference point): 100 \times \left( \frac{R_{\text{ACR}} - R_{\text{NE}}}{R_{\text{NE}}} \right) \). The \( \text{L}-\text{NAME} \)-induced tone \( (T_{\text{L-name}}) \) was evaluated compared with the ACh-relaxation: \( \frac{(R_{\text{ACR}} - R_{\text{L-name}})}{R_{\text{ACR}}} \). In addition to the comparisons mentioned above, the \( \text{L}-\text{NAME} \)-induced tone \( (T_{\text{L-name}}) \) was given as a function of the ACh-induced tone: 1) \( 100 \times \left( \frac{R_{\text{L-name}} - R_{\text{NE}}}{R_{\text{NE}}} \right) \), 2) \( 100 \times \left( \frac{R_{\text{Cafree}} - R_{\text{L-name}}}{R_{\text{Cafree}}} \right) \), or 3) \( 100 \times \left( \frac{R_{\text{ACR}} - R_{\text{L-name}}}{R_{\text{ACR}}} \right) \).

Histology

Neighboring segments of the saphenous vein were isolated and used for histological examination. Ovaries of the animals were also collected and freshly fixed for histological study. All tissue samples were immersion fixed in 4% buffered formaldehyde, stained with hematoxylin and eosin, and examined using light microscopy. The ovaries were examined for polycystic morphology. The vessel samples were stained with resorcin-fuchsin to examine the content of the elastic fibers in the tunica media. For the measurements and photographs, the slides were scanned with a Pannoramic 250 Scanner (3DHistech) and analyzed with Pannoramic viewer software (3DHistech). ImageJ software was used to evaluate the elastic density. The digitized photographs of the vessels were converted to an 8-bit format. The tunica media was marked as the experimental area of the vessel walls for digital analysis. Grayscale comparisons were made.

eNOS and COX-2 Immunohistochemistry

To evaluate the alterations in the expression of eNOS and COX2 in the vessel endothelium and smooth muscle, we performed immunohistochemistry. Saphenous veins were collected, freshly fixed in 4% buffered formaldehyde, and embedded in paraffin for histological examinations. The sections were deparaffinized with xylene and rehydrated using an ethanol-to-water gradient. After being hydrated with PBS, the sections were cyclically heated and cooled in a sodium citrate-buffered solution. The endogenous peroxidases were quenched with 3% H\(_2\)O\(_2\) in methanol for 15 min at room temperature. Serum proteins were blocked using normal goat serum (NGS; Vector Laboratories, Burlingame, CA) in Tx-PBS for 60 min at room temperature. The sections were incubated for 60 min at 37°C with either 1:50 eNOS or 1:200 COX-2 monoclonal antibody (both dissolved in 150 µl of NGS; Abcam, Cambridge, UK). After being rehydrated with Tx-PBS, the sections were incubated with secondary antibody for 30 min (Vector Laboratories) and with a DAB kit for 7 min. Sections
were then analyzed using a Zeiss AxioImager.A1 microscope coupled with a Zeiss AxioCam MRc5 charge-coupled device camera (AxioVision Software).

**Statistical Analysis**

For statistical analysis, repeated-measures ANOVA was used. The in vitro parameters were plotted as a function of intraluminal pressure, and the curves were analyzed using paired comparisons of the treatment groups. One-way ANOVA was applied to compare the discrete parameters (e.g., body weights and parameters measured at discrete intraluminal pressures, including T_max and T_min). As a post hoc test, Tukey’s test was used. P < 0.05 was uniformly accepted as a significant difference. The data are presented as means ± SE. GraphPad Prism (version 5.0d; GraphPad Software) was used as the statistical software.

**RESULTS**

Basic Physiological Parameters and Ovarian Pathology

The mean arterial pressures were 122 ± 3, 123 ± 6, and 123 ± 4 mmHg for the control, DHT, and DHT + D3 groups, respectively. The mean body weight in the two DHT-treated groups was significantly higher than in the control group (P < 0.01; Table 1). However, the two treatment groups did not differ from each other at the end of the experiment (control: 298 ± 8 g; DHT: 354 ± 16 g; DHT + D3: 353 ± 9 g).

**Ovarian morphology.** From the histological analysis, we detected polycystic morphology in the DHT-treated groups and normal ovaries in the control group. Multiple premature cysts were detected peripherally without dominant follicles in the DHT-treated animals. The follicle diameters were significantly smaller in the DHT-treated ovaries than in the control group (1,609 ± 617 and 2,334 ± 451 pixels at ×40 magnification, P < 0.05). The follicles of the DHT + D3 ovaries were not significantly different from those of either of the other two groups (2,054 ± 442).

Saphenous Vein Geometry

The DHT treatment increased the relaxed outer (Fig. 1A) and inner radii (measured in a Ca²⁺-free medium), a response prevented by a parallel vitD treatment (P < 0.05 for DHT vs. DHT + D3 comparison; Fig. 1A). However, this difference between the DHT and DHT + D3 groups nearly disappeared at higher pressures. The DHT treatment increased the venous wall thickness (Fig. 1B), which was significantly smaller in the control group than in the DHT-treated groups (P < 0.05; Fig. 1B). Similar to the wall thickness, the cross-sectional areas of

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Fig. 2. A: tangential wall stress in a Ca²⁺-free solution. The tangential wall stress did not differ in the 3 groups. B: tangential wall stress in norepinephrine (NE)-contracted segments. The tangential wall stress was significantly greater in the control group than in the other 2 groups through the entire pressure range (P < 0.05). From 2 to 10 mmHg the stress was significantly different in the 3 groups, with the highest values in the control group and the lowest in the DHT group (P < 0.001). C: distensibility in a Ca²⁺-free solution. The relaxed distensibility was significantly greater in the DHT group than in the other 2 groups throughout the entire pressure range (P < 0.005). D: distensibility of NE-contracted segments. Each group was significantly different from the other 2 throughout the entire pressure range (P < 0.001). The distensibility was greatest in the DHT animals and lowest in the control animals. The values are expressed as means ± SE. *DHT was significantly different from the control group; †DHT + D3 was significantly different from the control group; ‡DHT + D3 group was significantly different from the DHT group (n = 10 in each group).

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the walls were different throughout the entire pressure range ($P < 0.001$).

**Saphenous Vein Elasticity**

The mechanical load on the vessel wall was measured. Under passive circumstances, the tangential wall stress was not affected by the treatment with either DHT or vitD in a Ca$^{2+}$-free solution at $P = 5$ mmHg, the mean tangential wall stresses were $8.02 \pm 1.44$, $8.57 \pm 0.83$, and $6.68 \pm 1.13$ kPa for the control, DHT, and DHT + D$_3$ groups, respectively (not significant; Fig. 2A). During the NE contraction, the tangential wall stress was significantly lower throughout the entire pressure range for the DHT-treated groups than in the control group ($P < 0.05$; Fig. 2B). In the physiological pressure range (between 2 and 10 mmHg), the vitD treatment generated a partial elevation of the wall stress ($P < 0.001$; Fig. 2B).

The isobaric distensibility was significantly higher throughout the entire pressure range for the DHT group than for the other two groups in the Ca$^{2+}$-free medium and during NE contraction ($P < 0.005$ and $P < 0.001$; Fig. 2, C and D). The parallel vitD treatment generated a partial recovery but not a full restoration of the control values.

The DHT treatment alone did not alter the vessels’ isobaric elastic moduli. The DHT plus vitD-treated vessels had significantly lower elastic moduli throughout the pressure range than the DHT alone or the control group in the Ca$^{2+}$-free medium ($P < 0.001$; Fig. 3A). A similar alteration of the vessel elasticity resulting from the vitD treatment was detected when the elastic moduli were characterized as a function of the tangential wall stress. The elastic moduli were the lowest in the DHT + D$_3$ group in the entire pressure range in the Ca$^{2+}$-free solution ($P < 0.05$ for the control compared with the DHT + D$_3$ group and for the DHT group compared with the DHT + D$_3$ group, whereas the DHT group did not differ from the control group; Fig. 3B).

**Saphenous Vein Smooth Muscle Tone**

NE had less of an effect on the DHT group than on the other two groups ($P < 0.001$). Each group was significantly different from the others for the entire pressure range. The DHT treatment reduced the NE tone significantly, a response that was partially avoided by the vitD treatment ($P < 0.001$; Fig. 4C). These segments do maintain a substantial myogenic (spontaneous) tone. The tone was higher after treatment with the DHT alone, but the coadministration of vitD decreased the myogenic tone at lower pressures ($P < 0.05$; between 2 and 6 mmHg; Fig. 4B). The control group was not different from the other two groups. This difference disappeared at higher pressures (Fig. 4B). The sum of the two contractions (Fig. 4A), which represents the full range of pharmacological vascular adaptation, was significantly lower after the DHT treatment. This alteration was not affected by the vitD treatment, but the low

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**Fig. 3.** A: elastic moduli in a Ca$^{2+}$-free solution. The elastic moduli did not differ between the control and DHT groups. The elastic moduli of the DHT + D$_3$ animals were significantly lower than those in the other 2 groups ($P < 0.001$). B: elastic moduli as a function of tangential wall stress in a Ca$^{2+}$-free solution. The values for the abscissa show the elastic moduli (log kPa) for the veins from the control rats as well as from the DHT- and DHT + D$_3$ groups, respectively (not significant; Fig. 2A). The DHT + D$_3$ group was significantly different from the DHT group. C: density after resorcin-fuchsin staining. The 3 bars show the relative grayscale density after resorcin-fuchsin staining. The density correlates with the elastin content of a certain area of the vessel wall. The densities did not differ between the control and DHT + D$_3$ groups. A significantly lower density was detected in the DHT group ($P < 0.005$). The values are expressed as means ± SE. *DHT was significantly different from the control; †DHT + D$_3$ was significantly different from the control; ‡DHT + D$_3$ group was significantly different from the DHT group. D–F: representative pictures of the veins after resorcin-fuchsin staining are shown. Optical measurements were made in the media ($n = 10$ in each group).
pressure values of the full contraction were moved to a more relaxed range in the DHT + D₃ group (P < 0.005; Fig. 4A).

Endothelial Reactivity

The segments that were contracted by NE were relaxed in response to 1 μM ACh, revealing the endothelial relaxation capacity. When the ACh-induced relaxation was measured relative to either the maximal relaxation in the Ca²⁺-free solution or the maximal contraction induced by NE, there was no significant difference among the groups (Fig. 5A). However, when 1-NAME was added, it induced a substantial contraction that was significantly lower in the DHT-treated group than in the control or the DHT + D₃ segments (Fig. 5, B and C). That is, vitD treatment fully reversed the alteration induced by the PCOS (P < 0.05; Fig. 5, B and C).

Histology and Immunohistochemistry

Saphenous vein wall histology. After the resorcin-fuchsin staining, a significant difference in the vessel wall intensity was detected. The DHT group showed a significantly lower intensity than the other two groups (P < 0.005; Fig. 3C). Figure 3, D–F, shows examples of the different groups.

Immunohistochemistry. The COX-2 and eNOS expression in the endothelium and in the media were measured using immunohistochemistry and calculated as described above. The DHT treatment decreased the COX-2 staining intensity in the endothelium significantly compared with that in the control. This effect of the DHT treatment was fully reversed by the vitamin D₃ coadministration (P < 0.05; Fig. 6A). Although the same trend was detected in the smooth muscle layer of the media, the difference was not statistically significant (Fig. 6B). There was no difference in the eNOS enzyme protein expression intensity between the groups in either the endothelium or the smooth muscle (Fig. 6, C and D). Demonstrative immunohistochemical staining samples of the different groups can be observed in Fig. 6, E–J.

DISCUSSION

Our study used a modified version of the PCOS model originally described by Mannerås et al. (20). Polycystic morphology of the ovaries, a hyperandrogenic state, and insulin resistance were detectable after 70 days of treatment in this model; however, the arterial blood pressures of the different animal groups did not differ. Because the hyperandrogenic state is the cornerstone of the human disease, we conclude that

Fig. 4. Vascular smooth muscle-dependent alterations of tone [outer radii (Rₒ)]. A: full contraction of the veins. The DHT-treated group was significantly different from the control group throughout the entire pressure range (P < 0.005). NE had the greatest effect on the veins from the control animals. The vitamin D₃ treatment did not improve the maximum effect from NE. B: the myogenic tone of the arterioles. The DHT + D₃ group was significantly different from the DHT group in the lower pressure range (between 2 and 4 mmHg, P < 0.05). The myogenic tone was greatest in the DHT group and lowest in the DHT + D₃ group. The control group did not differ from either of the other 2 groups. There was no difference in the 3 groups at higher pressures (between 6 and 20 mmHg; not significant). C: NE-induced tone compared with the spontaneous tone in an normal Krebs-Ringer (nKR) solution. NE had less of an effect on the DHT group than on the other 2 groups (P < 0.001). Each group was significantly different from the others for the entire pressure range. The values are expressed as means ± SE. *DHT was significantly different from the control; †DHT + D₃ was significantly different from the control; ‡DHT + D₃ group was significantly different from the DHT group (n = 10 in each group).
high DHT levels in female animals and polycystic morphology together are sufficient to define PCOS disease (see Ref. 31 as well as Ovarian morphology above).

In this study, the constant DHT treatment of female rats induced significant alterations in the vascular geometry, elasticity, and reactivity of the saphenous vein. The relaxed lumen was dilated, and this dilation was accompanied by an elevation in wall mass (“hypertrophic remodeling”). The wall stress did not change because of the thickening of the vessel wall, but the isobaric distensibility increased. The elastic moduli did not change either, which demonstrates that remodeling occurred without drastic alterations in the force-bearing connective tissue components. Previous ex vivo studies demonstrated that increases in shear stress and venous blood pressure can independently induce larger vessel diameter and early-phase remodeling in vein walls (7, 11). DHT increased smooth muscle cell (SMC) proliferation via pathways that are both dependent and independent of androgen receptors (25). Similar flow-induced alterations could be detected in varicosity (24). The association of varicosity with sex steroids was examined in previous human studies (3, 13); however, there is no published clinical report on the prevalence of venous varicosity in polycystic ovary syndrome. In our study, the increased venous diameter and wall mass that developed in response to DHT treatment may have an adaptive characteristic and may be caused by developing body weight (obesity) (10) induced by elevated blood flow, resulting in higher wall stress. In subjects with PCOS, only data on the large arteries have been published. These arteries become less elastic and more rigid; the stiffness and intima-media thickness of the common carotid artery increases, and its distensibility decreases (6, 19, 34).

The potential of low-dose vitD as an adjuvant therapy for PCOS and a cardiovascular protective agent is becoming widely accepted in clinical practice (35). Our experiments demonstrate that vitD can modify the morphological remodeling of the venous wall and oppose the varicose effect of the hyperandrogenic state. Previously, a supraphysiological concentration of vitD was shown to reduce the expression of tropoelastin in cultured SMCs (9, 28). Tukaj and Wrzolkowa (36) reported that the administration of low-dose calcitriol enhanced the proliferation of vascular SMCs in the aorta.

In the present work on veins, an important alteration of vessel tone was the NE-related decrease in contractility throughout the entire pressure range, whereas the myogenic tone of the DHT-treated segments increased slightly at lower physiological pressures. However, the capacity for ACh-stimulated endothelial dilation was not diminished, and the NO blockade by l-NAME had little effect on the DHT-treated segments. Despite the attenuated effect of l-NAME, the level of eNOS expression in the segments did not differ significantly from the eNOS expression in the control group. These observations can be explained if we suppose that the DHT-treated

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**Fig. 5. Alterations of nitric oxide (NO)-dependent dilation. A: acetylcholine (ACh)-induced relaxation. The abscissa shows the ratio of the ACh-induced relaxation (10^{-6} M) to the maximal relaxation in Ca^{2+}-free solution (R_{Cafree} – R_{ACh}/R_{Cafree}). There was no difference in the ACh relaxation among the three groups. B: NÖ-nitro-l-arginine methyl ester (l-NAME)-induced tone (10^{-5} M). The abscissa shows the ratio of the outer radii in l-NAME-induced tone to those in maximal relaxation in a Ca^{2+}-free solution for the veins from the control rats as well as the DHT- and DHT + D{3}-treated rats (R_{Cafree} – R_{-NAME}/R_{Cafree}). The l-NAME-induced vasoconstriction was significantly lower in the DHT group than in the other two groups (P < 0.05). C: radii in l-NAME-induced tone (10^{-5} M) are compared with those in ACh-induced relaxation (10^{-6} M). The abscissa shows the ratio of the outer radii for the veins from the 3 experimental groups after ACh relaxation and following NO-blocking by l-NAME (R_{ACh} – R_{-NAME}/R_{ACh}, %). This ratio was lower for the DHT group than for the other 2 groups (P < 0.05). Values are expressed as means ± SE. *DHT was significantly different from the control (n = 10 in each group).**
group had a smaller “NO relaxation component” of the tone without stimulation and that the DHT treatment did not affect the capacity for a similar relaxation upon ACh stimulation. A significantly lower expression of eNOS and nNOS has been shown in varicose veins as a result of chronic venous stasis, which suggested to the authors that mechanisms other than NO release should be involved in pathological dilation (8). Decreased bioavailability might contribute according to observations on mesenteric venules by Brookes et al. (2), who found that increased vascular oxidative stress limited the effect of NO. The insulin resistance previously observed in PCOS (21, 31) can also interfere with the NO pathway (38). In a recent study, the scaffolding domain of caveolin-1 was found to control basal NO release in mesenteric venules, and this mechanism might be involved here (37).

Endogenous prostanoids are important components of the vascular tone control. Racz et al. (30) showed that the COX-2-thromboxane A2 pathway has a pivotal role in venous vasoconstriction. In PCOS, an increased vasoconstrictor prostanoid activity was found (12). In our study, the decreased COX-2 expression in the venous endothelium of the DHT group was accompanied by unaltered endothelial vasodilation. The reduction of COX-2 expression in the smooth muscle component of the venous wall in this study did not reach the level of statistical significance. DHT was reported to increase COX-2 expression in an androgen receptor-dependent manner and decrease it in an adrenoceptor-independent manner in cultured human coronary vascular SMCs, depending on the physiological or pathological state of the cells (27). Further experiments are required to determine the importance of additional adaptive mechanisms for veins such as the alteration of COX-1 expression and the shift of NOS isoforms (15).

VitD did not affect the reduction of the adaptation range (the difference between the maximum contraction and the maximum relaxation) of the segments, which was a characteristic effect of DHT. However, the vitD cotreatment improved the NE reactivity and decreased the myogenic tone at physiological pressures, results that are similar to those obtained in arterioles (33). The vitD treatment resulted in vessels that were more relaxed with improved mechanisms of vascular adaptation. The reversal of the decline of L-NAME-induced tone and of endothelial COX-2 expression can also be explained as improved adaptation mechanisms of these vessels. A concentration of vitD similar to a low physiological concentration (1 nM) was reported to enhance NO production and eNOS activity through a vitamin D receptor-mediated mechanism in isolated endothelial cells (human umbilical vein endothelial cell culture) (23). In our study, the blocking of the NO-dependent vasodilation in the DHT + D3 group resulted in a tone similar to that of the control group, reversing the effect of PCOS. These findings can be explained if we suppose that the basal venous tone depends on basal NO release, which is reduced by PCOS and increased by VitD treatment. According to our observations, such alterations of basal NO release do not seem to be induced by an altered expression of eNOS. This fact can explain the similar ACh dilations of the three groups. In veins, vitD should stimulate the alternative relaxant mechanisms mentioned above, which may relieve the NO pathway. We are

Fig. 6. A: cyclooxygenase-2 (COX-2) expression in the venous endothelium. The abscissa shows the relative intensity of COX-2 expression as measured using quantitative immunohistochemistry. There was a significantly lower level of COX-2 expression in the endothelium of the DHT animals than in that of the other 2 groups (P < 0.05). B: COX-2 expression in the smooth muscle. Although the expression of COX-2 was lower in the DHT group, the difference was not significant. C and D: endothelial nitric oxide synthase (eNOS) expression in the endothelium and smooth muscle. The abscissa shows the relative intensity of eNOS expression. There was no significant difference among the 3 groups. Values are expressed as means ± SE. *DHT was significantly different from the control group; ‡DHT + D3 group was significantly different from the DHT group. E–G: immunohistochemical staining of samples of the DHT and DHT + D3 groups for eNOS. There was no difference in the eNOS enzyme expression among the groups in either the endothelium or the smooth muscle. H–J: immunohistochemical staining of samples of the DHT and DHT + D3 groups for COX-2. The DHT treatment decreased the COX-2 expression intensity in the endothelium significantly compared with that in the controls (n = 10 in each group).
aware of the fact that there are certain limitations of our study. The number of chronic preparations did not allow us to extend our observations toward prostanoid pathways in addition to NO, and the background of altered contractility also remains mostly obscure. Further studies are needed to clarify detailed molecular mechanisms in PCOS.

Conclusions

In this PCOS animal model, we aimed to test the most important mechanisms, components, and therapeutic effects that arose upon arterial studies made earlier. Ours is the first study to explore the details of the vascular biomechanical and pharmacological adaptation of veins in an experimental PCOS model. The hyperandrogenic state resulted in thicker but less flexible vein walls, which are also observed in varicosity. These veins showed wall remodeling that can be interpreted as an early sign of a varicose pathology and altered vasorelaxant mechanisms. These alterations of the veins differed to some degree from those observed earlier in resistance arteries. The varicose alterations caused by the chronic DHT treatment were partially avoided by concomitant vitD administration.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


