Endothelial Dysfunction in a Rat Model of PCOS: Evidence of Increased Vasoconstrictor Prostanoid Activity

Jennifer Keller, Maurizio Mandala, Peter Casson, and George Osol

Division of Reproductive Endocrinology and Infertility (J.K., P.C.) and Department of Obstetrics/Gynecology and Reproductive Sciences (M.M., G.O.), University of Vermont, Burlington, Vermont 05401; and Department of Cell Biology (M.M.), University of Calabria, 87036 Arcavacata di Rende (Cosenza), Italy

Clinical research demonstrates an association between polycystic ovary syndrome (PCOS) and endothelial dysfunction, a pathological state widely believed to be a hallmark of vascular disease; the underlying pathways, however, have not been defined. The purpose of this study was to characterize endothelial function in resistance arteries in a novel rat model of PCOS. Female rats were randomized at 3–4 wk to implantation of a 7.5-mg, 90-d dihydrotestosterone (DHT) pellet or a matched placebo. At 15–16 wk, experiments were performed on isolated mesenteric resistance arteries using a pressurized arteriograph. Endothelial function was assessed by the vasodilatory response of preconstricted arteries to acetylcholine (ACh) in the absence and presence of inhibitors for cyclooxygenase (indomethacin) and the thromboxane prostanoid receptor antagonist (SQ29,548). Distensibility was evaluated by measuring vessel diameter from 3–100 mm Hg, and elastin/collagen content was calculated on formalin-fixed vessels. Serum steroid levels were analyzed by sensitive RIA. DHT-induced PCOS rats were heavier, cycled irregularly, and had elevated blood pressure and smaller arterial lumens than controls. Furthermore, DHT vessels showed significantly reduced vasodilatory efficacy to ACh (with no change in sensitivity), reduced distensibility, and increased elastin content compared with controls. Within DHT animals, maximal dilation correlated negatively to DHT levels ($r = -0.72$) but not to body weight. Preincubation with either indomethacin or SC29,548 abrogated the dysfunction and restored full efficacy to ACh ($P < 0.05$). This is the first report to demonstrate the presence of endothelial dysfunction in a hyperandrogenic rat model of PCOS and to identify the role of vasoconstrictor prostanoids, allowing for more targeted research regarding the development of disease and potential therapeutic interventions. **(*Endocrinology* 152: 4927–4936, 2011)**

Polycystic ovary syndrome (PCOS) represents the most common endocrinopathy in women, affecting up to 7% of women of reproductive age (1). Although classically characterized as a reproductive disorder associated with irregular menses and hyperandrogenism, PCOS is now well recognized to also have a metabolic component. Patients with PCOS display a number of metabolic disturbances including insulin resistance, central obesity, dyslipidemia, hypertension, and low-grade chronic inflammation, often apparent at a young age (2–4). Although epidemiological data in patients with PCOS are not conclusive, this clustering of metabolic abnormalities may confer an increased risk of cardiovascular morbidity and mortality (3, 5) as it does in other patients with the metabolic syndrome (6).

The endothelium plays a crucial role in regulating vascular function, and dysfunction of endothelial cells is probably the earliest event in the process of lesion formation and atherosclerosis (7). Clinical assessment of endothelial dysfunction is noninvasive and allows for the de-

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Abbreviations: ACh, Acetylcholine; COX, cyclooxygenase; DHT, dihydrotestosterone; HOMA-IR, homeostasis model of insulin resistance; L-NAME, *N*-ω-nitro-L-arginine methyl ester; L-NNA, *N*-ω-nitro-L-arginine; NOS, nitric oxide synthase; PCOS, polycystic ovary syndrome; PSS, physiological saline solution; TP, thromboxane prostanoid.

tection of such preclinical vascular disease, which may be a useful prognostic and/or therapeutic monitoring tool for patients with cardiovascular disease risk factors (8).

Multiple cross-sectional studies demonstrate significantly impaired endothelial-dependent dysfunction, as measured by brachial artery flow-mediated dilation, in PCOS patients compared with controls (9–13). Although the association between insulin resistance and endothelial dysfunction has been clearly demonstrated in young obese subjects without PCOS (14), clinical research in PCOS patients suggests an association with endothelial dysfunction that is greater than that accounted for by insulin resistance alone (15–17). Because PCOS is a multifactorial disorder with considerable heterogeneity, and based on the small numbers of patients evaluated in clinical studies, the true extent of endothelial dysfunction and its relationship to hyperandrogenemia has not been defined. Furthermore, little is known about the pathway of impaired endothelial function in PCOS.

Endothelial cells actively regulate basal vascular tone and reactivity in both physiological and pathological conditions through the release of a variety of relaxing and contracting factors (18). Endothelial nitric oxide (NO) is widely recognized as a relaxing factor, released in response to acetylcholine (ACh), which plays an important role in maintaining normal vascular tone and blood pressure. Relaxing factors also include prostacyclin and the undefined endothelium-derived hyperpolarizing factor. Conversely, vasocontriction occurs when endothelial cyclooxygenases trigger the release of constrictor prostanoids, which activate thromboxane prostanoid (TP) receptors and cause contraction of the underlying vascular smooth muscle. The balance of relaxing and contracting factors most likely depends on a multitude of influences, including gender and sex-steroid environment (19–21) as well as the health or pathology (*e.g.* aging, hypertension, or diabetes) of the animal (7).

The primary aim of the present study was to use a recently developed rat model of PCOS to determine whether endothelial dysfunction was indeed present and, if so, to identify the molecular pathways responsible for the impairment. We chose a novel model described by Mannerås *et al.* (22) in which rats are exposed to the nonaromatizable androgen dihydrotestosterone (DHT) through a subcutaneous timed-release pellet implanted before puberty, resulting in the development of both the ovarian and metabolic characteristics seen in classic PCOS. Animals in this model demonstrate irregular menstrual cycles, polycystic ovaries characterized by cysts formed from atretic follicles, central adiposity, and insulin resistance.

We hypothesized that changes in both resistance artery structure and reactivity would be seen in the DHT-induced PCOS model and that the latter would manifest through alterations in endothelial vasodilatory or vasoconstricting factors known to affect vascular reactivity. We examined small mesenteric resistance arteries (150–300 μm), because the splanchnic vessels comprise a substantial portion of the total peripheral resistance seen by the heart and are therefore intimately involved in normal and pathological vascular tone.

Materials and Methods

Animals

Female Wistar Hannover rats (n = 44) were purchased from Taconic Laboratories (Hudson, NY). At 3–4 wk of age, 16 pups received placebo pellets, and 28 received DHT pellets, which were surgically implanted at Taconic. After surgery, animals were allowed to fully recover, and then were shipped to the University of Vermont at 4–5 wk of age. All rats were housed individually in the Animal Care facility until the conclusion of the experiment at 15–16 wk of age. Rats were maintained under controlled conditions on a 12-h light, 12-h dark photoperiod and provided commercial chow and tap water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Rats were implanted with 90-d continuous-release pellets (Innovative Research of America, Sarasota, FL) containing 7.5 mg DHT, equivalent to a daily dose of 83 μg, as described in the original publication (22). The dose was chosen by the original authors to mimic the hyperandrogenic state in women with PCOS, whose plasma DHT levels vary widely, but on average are approximately 2-fold higher than those of healthy controls (23, 24). Rats in the control arm received matched placebo pellets also developed by Innovative Research of America. The pellet activity continued for 11–12 wk of DHT or placebo administration, *i.e.* until the animals were 15–16 wk of age.

Experimental data

Rats were weighed weekly from 5 wk of age. Daily feed weights were measured in young adult rats (wk 10–11) and again before experimentation (wk 14–15) in a subset of six DHT and six control rats. Blood pressures were measured noninvasively by determining the tail blood volume with a volume-pressure recording sensor and an occlusion tail cuff (CODA System; Kent Scientific, Torrington, CT). Blood pressure was measured at the same time of day (late morning) three times during the week before study conclusion to allow for rat acclimatization to the device.

The estrous cycle was monitored by microscopic analysis of the predominant cell type in vaginal smears obtained during the final week of the experiment. In an effort to control for the confounding effects of steroid hormones on vasodilation in the cycling controls compared with the irregularly cycling DHT animals, all control animals were studied during the diestrous phase, and DHT animals were studied in the pseudo-diestrous phase when vaginal smears demonstrated leukocytes were the dominant cell type.

At 15–16 wk of age, animals were weighed and then euthanized with an ip injection of Nembutal (pentobarbital sodium,

50 mg/kg; Ovation Pharmaceuticals, Deerfield, IL), followed by decapitation in a small-animal guillotine. The abdomen was opened, and a section of the mesentery 2 cm from the pylorus was removed and pinned in a Sylgard-lined petri dish filled with cold (4 C), oxygenated (aerated with a mixture of 5% CO_2, 10% O_2, and 85% N_2) physiological saline solution (PSS).

Hormonal assays

After decapitation, trunk blood from animals was collected and centrifuged (n = 8 DHT and 8 control), and serum samples were stored at −80 C. Samples were then shipped to the University of Southern California Reproductive Endocrinology Laboratory, where DHT, testosterone, and estradiol were measured using highly specific, sensitive, and validated extraction RIA as described previously (25–28). The assay sensitivities for DHT, testosterone, and estradiol were 4, 15, and 3 pg/ml, respectively. The interassay coefficients of variation ranged from 8–12%.

Fasting insulin and glucose were monitored after a 16-h fast (n = 6 DHT and 6 control). Fasting commenced at 1700 h at the beginning of the dark cycle, and the next morning, blood glucose and insulin levels were monitored at 0900 h. Glucose values were assessed from blood obtained through a small nick in the tail vein, measured with a standard glucometer (Life Scan One Touch Ultra, Milpitas, CA). Plasma was collected from decapitated animals, and plasma insulin was determined by rat-specific direct immunoassay (Alpco Diagnostics, Salem, NH). OD readings were converted to nanograms per milliliter using kit-provided standards and Prism software (GraphPad, San Diego, CA). Homeostasis model of insulin resistance (HOMA-IR), validated previously in rats (29), was calculated from fasting plasma glucose (FPG) and fasting plasma insulin (FPI) according to the report by Matthews *et al.* (30), with the formula HOMA-IR = FPI (milliunits per liter) × FPG (milligrams per deciliter)/405.

Vessel dissection and equilibration

Third-order mesenteric vessels were dissected free of connective tissue, and a 1- to 2-mm arterial segment was cannulated on a buffer-filled, pulled-glass pipette at room temperature in a custom-built arteriograph (Instrumentation and Model Facility, University of Vermont, Burlington, VT). Luminal contents were flushed at 10 mm Hg before securing the distal end onto a second cannula.

Intralumenal pressure was measured and maintained using a pressure-servo system (Living Systems Instrumentation, Burlington, VT) with an in-line transducer that was calibrated before each experiment. Mounted pressurized vessels were positioned on the stage of an inverted microscope (Nikon TMS; MVI, Avon, MA) equipped with a video dimension analyzer as described previously (31) and connected to data-acquisition software (WinDaq DI-720; DATAQ Instruments, Inc., Akron, OH). Vessels were continuously superfused with PSS and maintained at 37 C and pH 7.4, monitored in the bath near the vessel with a micro pH probe (Microelectrodes Inc., Bedford, NH). Vessels were equilibrated at 10 mm Hg for 10 min, followed by a 40-min equilibration at 50 mm Hg. This pressure was chosen for experimental procedures because it is a reasonable approximation of physiological pressures experienced *in vivo* and to avoid possible confounding effects of myogenic tone that appears at higher transmural pressures.

Vascular reactivity experiments

After equilibration, vessels from DHT animals (n = 8) and control animals (n = 8) were preconstricted with phenylephrine (0.5–2.5 μM) to produce a 45–55% reduction in lumen diameter. Once constriction was achieved and stable for 15 min, reactivity experiments were performed by exposing the vessel to increasing concentrations of the endothelium-dependent vasodilator ACh (10^{-8} to 10^{-5} M; n = 7) and recording the dilation at each concentration until the maximal response was obtained. After experiments were completed, vessels were superfused with PSS solution containing diltiazem (10^{-5} M) and papaverine (10^{-4} M) (relaxing solution) to assure maximal vasodilation.

Pharmacological sensitivity was determined by best-fit curve analysis (Sigma Plot), which allowed calculation of the concentration of ACh required to produce 50% of the maximal response (EC_{50}). Vasodilation efficacy for ACh was defined as the percentage of maximal vessel dilatation obtained during the reactivity experiments relative to the fully relaxed diameter obtained in relaxing solution at the end of each experiment.

Additional experiments were then performed in DHT-treated animals using pharmacological blockade with various agents in an effort to isolate individual relaxing and constricting factors. The first of these experiments involved the blockade of nitric oxide synthase (NOS) and cyclooxygenase (COX) enzymes using several inhibitors, including *N*-ω-nitro-L-arginine (L-NNA, 10^{-4} M) plus *N*-ω-nitro-L-arginine methyl ester L-NAME (10^{-4} M) for NOS and indomethacin (10^{-5} M) for COX (n = 6). Next, vessels were preincubated with indomethacin alone to isolate the effects of COX inhibition (n = 7). Finally, to better understand the involvement of constricting TP in the treated group, vessels from DHT animals were preincubated with a selective TP receptor antagonist, SQ29,548 (10^{-5} M; n = 6). All agents above were added to the superfusate after the first 20 min of equilibration at 50 mm Hg and thus were present for the final 20 min of equilibration before beginning reactivity experiments.

Distensibility

Passive distensibility was determined by assessing the lumen diameter of fully relaxed vessels (n = 8 DHT and 8 control) across a broad range of transmural pressure (2–100 mm Hg). Distensibility was normalized to lumen diameter at the opening pressure (the minimum pressure at which the vessel did not collapse), and was calculated by the following formula: $[(Ø_p - Ø_a)/Ø_a] \times 100$, where $Ø_p$ is the inner diameter at each pressure step and $Ø_a$ is the diameter at the opening pressure of 2–3 mm Hg.

Drugs and solutions

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), including salts for physiological solution, L-NNA, L-NAME, indomethacin, phenylephrine, ACh, diltiazem, and papaverine. The TP receptor antagonist SQ29,548 was purchased from Cayman Chemicals (Ann Arbor, MI). PSS was composed of the following (in mM): 119 NaCl, 4.7 KCl, 24.0 $NaHCO_3$, 1.2 $MgSO_4$, 0.023 EDTA, 1.6 $CaCl_2$, 1.2 $KH2PO_4$, and 11.0 glucose (pH 7.4).

Histological staining

Third-order mesenteric vessels (n = 8 DHT and 8 control) were formalin-fixed in an unpressurized state and paraffin-

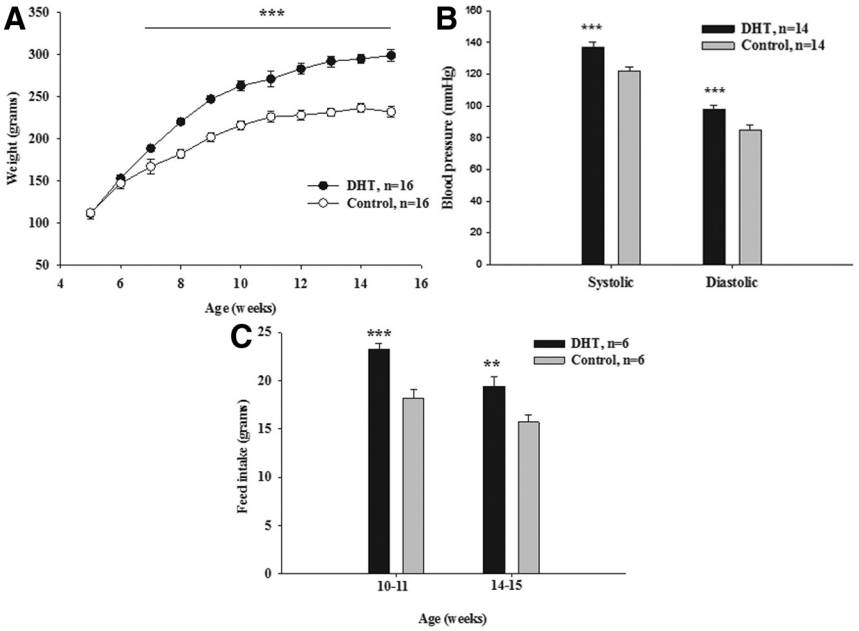

FIG. 1. Body weight and blood pressure. A, Growth curve of DHT-treated (PCOS) *vs.* control rats from 5–15 wk. DHT animals gained significantly more weight between 7 and 15 wk than control animals. B, Systolic and diastolic blood pressure in DHT and control animals, showing significantly elevated blood pressure in DHT animals. C, Mean weight of daily feed intake (grams) at 10–11 and 14–15 wk of age in DHT animals and controls. DHT-treated animals consumed significantly more during these time periods. Values are mean ± SE. ***, $P <$ 0.001 *vs.* controls; **, $P < 0.01$ *vs.* controls.

embedded, and 4- to 5-μm cross-sections were mounted on 3-in. glass slides. Elastic Van Gieson and Masson's trichrome stains (which stain elastic fibers and collagen, respectively) were applied to paraffin sections using standard histological procedures. Images were analyzed using an Olympus BX50 light microscope and a computer imaging program (Magnafare/Optronics, Goleta, CA) and optimized to contrast staining of elastin, collagen, and muscle fibers. Once established, the computer settings remained constant for all subsequent images. An imaging program (MetaMorph; Universal Imaging Corp., Downingtown, PA) was then used to delineate and quantify elastin and collagen content by color-threshold analysis; the total area of the arterial wall was measured and percentages of elastin and collagen were calculated accordingly.

Statistical analysis

Statistical differences between groups were determined from individual vessel data (SigmaPlot version 9.0; Systat Software Inc., San Jose, CA.) by ANOVA to evaluate inter- *vs.* intragroup variability, followed by multiple-comparisons tests to stratify differences between treatment means; P values <0.05 were considered significant.

All data are expressed as means ± SE, with n values representing the number of animals (one vessel used per animal). Differences between animal parameters (weight and blood pressure), vessel size, and structural/histological data were analyzed using the Student's t tests or Mann-Whitney Rank Sum tests, as appropriate. Relationships between variables (hormone levels, animal weight and blood pressure, and reactivity data) were determined using Pearson correlation coefficients.

Results

Animals

After 11–12 wk of DHT treatment, adult female rats weighed significantly more than control rats (299 ± 6.9 and 232 ± 6.0 g, respectively, $P < 0.001$) and demonstrated higher blood pressure (137/98 *vs.* 122/85 mm Hg, respectively, $P < 0.001$; Fig. 1, A and B, respectively). Weekly weight gain in DHT animals was significantly higher than in control animals beginning at 7 wk of age (Fig. 1A). Because of this evident difference, feed intake was assessed by measurement of daily feed weights in a subset of both DHT and controls to determine whether the difference in weight gain was due to hyperphagia *vs.* other factors (*e.g.* altered metabolism). DHT animals demonstrated significantly more daily feed intake than controls (Fig. 1C).

Vaginal smears revealed normal 4-d estrous cycles in control animals. Consistent with the original model, DHT animals cycled irregularly, and when acyclic, leukocytes were the dominant cell type on vaginal smear, representing a pseudo-diestrous state.

Hormone concentrations

Table 1 shows the serum concentrations of estradiol, DHT, testosterone, and progesterone. Estradiol concentrations were not significantly different between the DHT and control animals during the diestrous phase. DHT animals had significantly higher concentrations of DHT and lower concentrations of testosterone than control animals. Progesterone levels were significantly different between the groups ($P = 0.03$); however, in both groups, the levels were below the peak levels typically seen in proestrus (32).

TABLE 1. Hormone concentrations

	Control (n = 8)	DHT (n = 8)
Estradiol (pg/ml)	4.6 ± 0.5	3.6 ± 0.7
Progesterone (ng/ml)	11.8 ± 2.5	4.9 ± 1.6[a]
DHT (pg/ml)	30.6 ± 3.0	253.2 ± 20.9[b]
Testosterone (pg/ml)	55.1 ± 7.0	33.0 ± 3.8[a]

All values were taken during the diestrous (control) or pseudo-diestrous (DHT) phase of the estrous cycle. Values are presented as mean ± SE.

[a] $P < 0.05$ *vs.* control.

[b] $P < 0.001$.

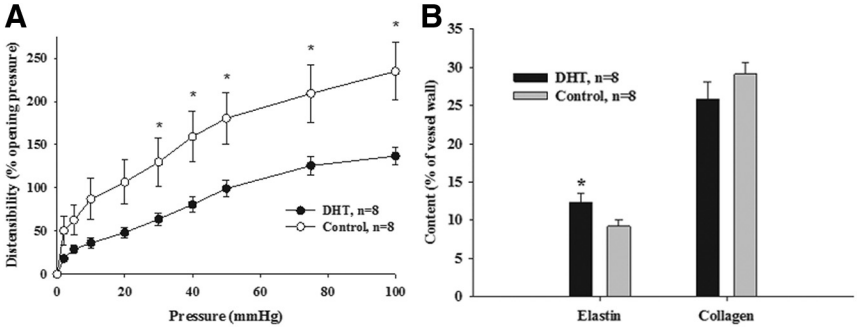

FIG. 2. Arterial distensibility and elastin/collagen content. A, Arterial distensibility, expressed as a percent increase in lumen diameter from an opening pressure of 2–3 mm Hg as a function of transmural pressure. B, Elastin and collagen content of uterine arteries from DHT and control rats showing a significant increase in elastin within the arterial wall. *, $P < 0.05$ *vs.* controls.

Fasting insulin levels were significantly higher in DHT animals (42.9 ± 12.3 μU/ml) compared with control animals (15.2 ± 3.4 μU/ml; $P = 0.03$; n = 6 in each group). HOMA-IR was higher in DHT rats than controls (index values are 7.5 ± 2.4 and 3.2 ± 1.1, respectively); however, this did not reach statistical significance ($P = 0.11$).

Vessel structure

Passive vessel lumen diameter at 50 mm Hg was smaller in the DHT group (204 ± 4.9 μm) compared with the control group (241 ± 10.5 μm; $P = 0.007$; n = 8 in each group), whereas wall thickness was not significantly different between the two groups (29.4 ± 2.7 μm DHT *vs.* 27.7 ± 3.2 μm control, $P = 0.49$).

Mesenteric arteries from DHT rats were 40–50% less distensible at higher pressures compared with the control rats (Fig. 2A; $P < 0.05$ for all values >30 mm Hg). Furthermore, quantification of elastin on stained histological slides demonstrated significantly increased content in DHT animals (12.4 ± 1.1 *vs.* 9.2 ± 0.9%; $P < 0.05$), but no difference was seen in collagen content (25.8 ± 2.3 *vs.* 29.1 ± 1.5%; $P = 0.24$) (Fig. 2B).

Vessel reactivity

Figure 3 graphically depicts sensitivity and efficacy dose-response curves to ACh in vessels from DHT *vs.* control animals. Sensitivity to ACh was not significantly different between the two groups, as demonstrated by the EC_{50} values of 0.25 ± 0.09 and 0.26 ± 0.07 μM/liter for control and DHT rats, respectively ($P = 0.80$). Although arteries from control animals showed essentially complete efficacy (99.9 ± 0.1%), DHT arteries dilated only 75.3 ± 5.6% on average ($P < 0.05$). Pearson correlation coefficients between maximal dilation, hormone concentrations, and animal weights in DHT animals revealed a significant negative correlation between serum DHT concentration and maximal dilation ($r = -0.72$; $P = 0.04$) but no correlation to other hormones or to weight (Table 2). The corresponding r^2 value of 0.52 indicates that serum DHT concentration accounts for approximately 52% of the variation in efficacy of vasodilation, with increasing hyperandrogenemia correlating with decreasing efficacy.

After the initial experiments demonstrating reduced efficacy of dilation in vessels from DHT animals, a second set of experiments was initiated to investigate the molecular basis for the dysfunction. Vessels were treated with L-NNA, L-NAME, and indomethacin to inhibit the vasodilatory factors nitric oxide and prostacyclin (n = 6). These vessels displayed full reactivity, showing no difference in efficacy compared with untreated controls (97.8 *vs.* 99.9%, respectively, no significant difference).

Based on these results, subsequent experiments aimed to evaluate the effect of constricting factors on reduced vasodilation in DHT animals. Figure 4 demonstrates the efficacy and sensitivity curves to ACh of vessels from DHT animals in the presence or absence of indomethacin to block the formation of constrictor prostanoids. Results showed restoration of full dilation (99.4 ± 3.6%; $P < 0.05$). Additionally, there was a significant difference in EC_{50} values in the presence or absence of indomethacin (0.05 *vs.* 0.26 μM, respectively, $P = 0.02$), indicating a leftward shift in the reactivity curve. In other words,

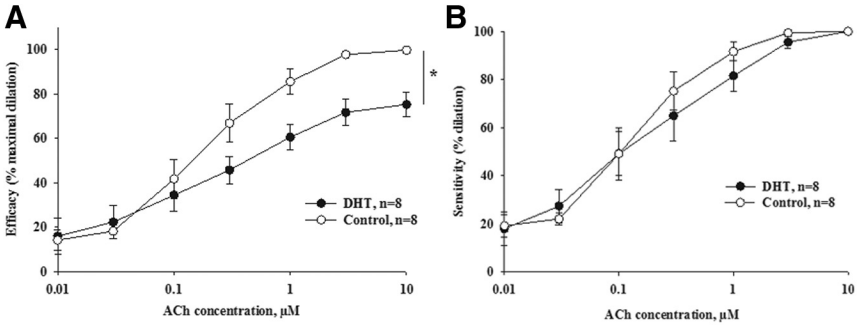

FIG. 3. Reactivity of vessels from DHT-treated *vs.* control animals to ACh. A, Efficacy: dose-response curve of preconstricted arteries to ACh relative to maximal dilation in relaxing solution shows a significant attenuation in DHT-treated animals. *, $P < 0.05$ *vs.* controls. B, Sensitivity: dose-response curve of preconstricted arteries relative to the maximal response to ACh. EC_{50} values (shown in text) were not significantly different.

TABLE 2. Hormone correlations in DHT animals

	Estradiol	Testosterone	DHT	Weight
Maximal dilation	−0.148	−0.171	−0.723[a]	−0.345
Estradiol		−0.164	0.118	0.885[a]
Testosterone			−0.147	0.488
DHT				0.641

For all groups, n = 8.
[a] $P < 0.05$.

COX blockade increased sensitivity to ACh more than ten-fold.

In the final set of experiments, a TP receptor antagonist, SQ29,548, was used to specifically block the vasoconstricting actions of thromboxane through its primary receptor. The results of this blockade in vessels from DHT animals are seen in Fig. 5. Compared with DHT vessels in the absence of SQ29,548, complete (100%) vasodilation was restored in treated vessels ($P < 0.05$) without a significant change in sensitivity.

Group-wide comparisons between maximal efficacy and sensitivity (EC_{50}) are shown in Fig. 6. Figure 6A summarizes the reduced efficacy of dilation in DHT animals compared with control animals, which was reversed through the use of indomethacin (blockade of prostanoid production via COX inhibition) and also with SC29,548 (blockade of TP receptor). Sensitivity analysis is summarized in Fig. 6B. Blockade of COX via indomethacin resulted in a significantly lower EC_{50} value, indicating an increased sensitivity to ACh.

Discussion

This study presents the first evidence for resistance-artery endothelial dysfunction in a hyperandrogenic rat model of PCOS. Our data demonstrate that DHT-induced PCOS rats are heavier and more hyperinsulinemic, experience borderline hypertension, and exhibit alterations in both vessel structure and reactivity. Structurally, vessels from DHT animals were less distensible to passive dilation, a finding consistent with a significant increase in elastin content. Vascular reactivity was also significantly altered, as demonstrated by the reduced vasodilatory efficacy to ACh. Furthermore, we identified a role of vasoconstrictor prostanoids in the reduction of vasodilation efficacy and have shown that blockade of their production (via COX inhibition), or action (by TP receptor antagonism), abrogated the dysfunction and restored full dilation. These data provide an understanding of the molecular basis for the development of endothelial dysfunction in patients with PCOS.

Characterizing the nature of endothelial function in PCOS in animal models is clinically relevant as dysfunction of endothelial cells is probably the earliest event in the development of atherosclerotic lesions and cardiovascular disease (8), which remains the leading cause of morbidity and mortality among adults in developed countries (33). Numerous clinical trials have identified endothelial dysfunction in young patients with PCOS (9–13), undoubtedly influenced by the presence of factors related to the metabolic syndrome in these patients (14, 34, 35). Although our data do not resolve whether endothelial dysfunction results primarily from hyperandrogenemia, or from secondary effects arising from adiposity and insulin resistance, these findings provide a good foundation for understanding endothelial dysfunction in this complex syndrome.

The protective role of NO on the vascular wall has long been recognized (36), and disruption of the NO pathway has been demonstrated extensively to alter vascular reactivity and lead to the development of atherosclerosis (7). In the current investigation, however, vessels from DHT-induced PCOS rats demonstrated reduced vasodilation to ACh that appeared unrelated to NO and instead was primarily due to augmented vasoconstrictor release. This became apparent when the dysfunction was reversed after preincubation with a combination of inhibitors of NOS and COX, indicating that NO did not contribute to reduced vessel reactivity and that a constricting factor was likely responsible for causing the impairment in vascular relaxation. Subsequent experiments with indomethacin and SC29,548 confirmed this hypothesis and demonstrated the presence of a vasoconstric-

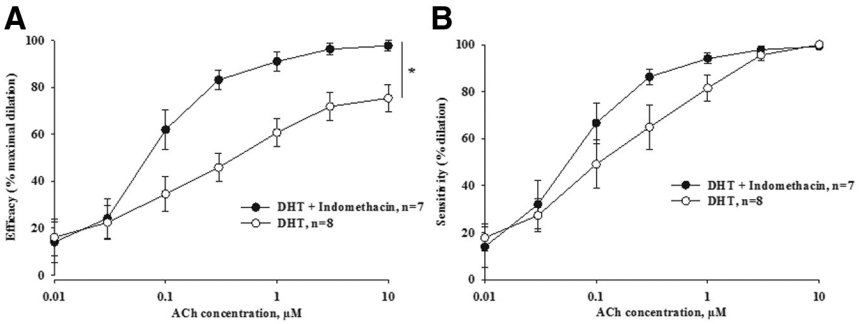

FIG. 4. Effects of COX inhibition with indomethacin on arterial vasodilator reactivity to ACh. A, Efficacy: dose-response curve of preconstricted arteries to ACh relative to maximal dilation in relaxing solution. *, $P < 0.05$ *vs.* untreated DHT vessels. B, Sensitivity: dose-response curve of preconstricted arteries relative to the maximal response to ACh. Note the left-shift in the curve of treated vessels; EC_{50} values (shown in text) demonstrate this significantly greater sensitivity.

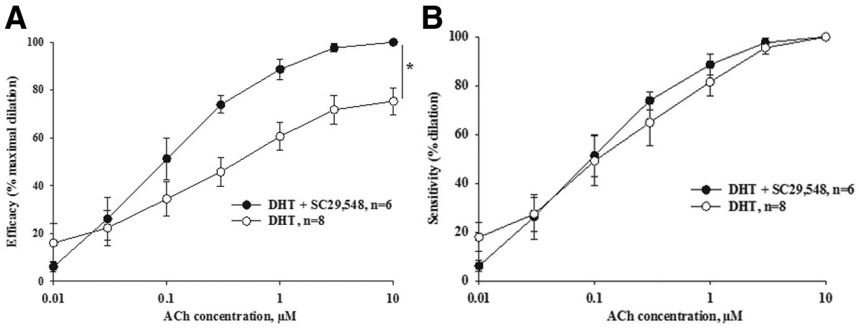

FIG. 5. Effects of TP receptor inhibition with SC29,548 on arterial vasodilator reactivity to ACh. A, Efficacy: dose-response curve of preconstricted arteries to ACh relative to maximal dilation in relaxing solution. *, $P < 0.05$ *vs.* untreated DHT vessels. B, Sensitivity: dose-response curve of preconstricted arteries relative to the maximal response to ACh. EC_{50} values (shown in text) were not significantly different.

tor prostanoid produced through the arachidonic acid pathway and acting through the TP receptor.

Vasoconstrictor prostanoids, or endothelium-derived contracting factors, oppose the relaxing effect of NO and play a significant role in endothelial dysfunction in various pathological states such as aging, diabetes, and hypertension (37). Prostanoid release is evoked by stretch or by agonists (ACh) that elevate endothelial calcium, which triggers phospholipase A2, freeing arachidonic acid for further metabolism by COX. The primary products of COX metabolism include prostaglandin I_2 (prostacyclin), generally considered an endothelium-derived vasodilator, and thromboxane A_2, a potent vasoconstrictor (38). A considerable amount of data in animal models demonstrates the significance of endothelium-derived contracting factors in pathological states, and some human studies have also shown an improvement in endothelial dysfunction with the use of COX inhibitors (39, 40) and TP-receptor blockers (41–43).

In addition to the restoration of full vasodilatory function, vessels pretreated with indomethacin were more than 10 times more sensitive to ACh (lower EC_{50}), indicating that blockade of vasoconstrictor prostanoid formation augmented the vasodilatory potency of ACh. On the other hand, the fact that TP-receptor blockade with SC29,548 restored full vasodilator efficacy without any reduction in EC_{50} suggests that constrictor prostanoids may be activating additional receptors in this model of PCOS. Determining the identity and localization of these receptors, however, was beyond the scope of our study.

Strengths of this study include the novel rat model of PCOS used, which exhibits the multiple endocrine and metabolic characteristics present in patients with PCOS, including hyperandrogenemia, irregular cycles, polycystic ovarian morphology, increased adiposity, and decreased insulin sensitivity (22). Previous animal models have induced PCOS through a variety of ways including constant light, mifepristone, estradiol valerate, or letrozole (44–47). These models have succeeded in producing anovulatory or oligoovulatory rats, but without the concurrent metabolic features of insulin resistance and adiposity seen clinically in a large percentage of patients with PCOS (48, 49). For assessment of endothelial function, we focused on a model with a combination of hyperandrogenemic and hyperinsulinemic features, mimicking the most prevalent aspects of clinical PCOS and including metabolic disturbances relevant to cardiovascular risk (4, 50). Our principal findings both validate the original model as described by Mannerås *et al.* (22) by confirming irregular cycles, increased adiposity, and elevated fasting insulin levels, and also reveal alterations in vascular physiology including elevated blood pressures, structural remodeling, and endothelial dysfunction. Using this model, we were able to examine the underlying mechanisms of endothelial dysfunction in resistance arteries that play a primary role in the control of blood pressure and regional blood flow.

The original model aimed to create a hyperandrogenic state that mimics the levels seen in patients with PCOS, and the nonaromatizable androgen DHT was chosen to avoid confounding effects of estrogen. Based on available data, DHT levels in women are relatively stable across the menstrual cycle, averaging 7–8 mg/dl (70–80 pg/ml) in normal patients and 14–18 ng/dl (140–

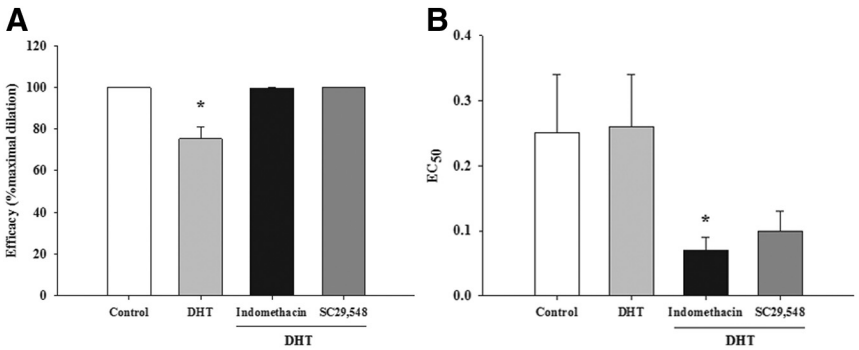

FIG. 6. A, Summary of maximal efficacy (response to 10 μM ACh) from each of the four groups. Note restoration of full dilation in DHT vessels in the presence of indomethacin or SC29,548 (ANOVA followed by *post hoc* Dunnet's test; *, $P < 0.05$ for DHT *vs.* all other groups). B, Summary of EC_{50} values (the concentration of ACh, in μM, required to produce 50% of the maximal response) from each of the four groups. Vessels from DHT animals treated with COX inhibitor indomethacin were significantly more sensitive to ACh dilation than untreated DHT vessels (*, $P < 0.05$, DHT *vs.* DHT plus indomethacin).

180 pg/ml) in women with PCOS (23, 24). However, because DHT in patients with PCOS is produced via conversion of testosterone in androgen-sensitive tissues, the use of serum values likely represents an underestimation of local DHT levels and androgen bioactivity (51). In our model, rats demonstrate a supraphysiological DHT level (253 ± 21 pg/ml), severalfold higher than the control animals (31 ± 3 pg/ml). This level exceeds the reported 2–3-fold increase in PCOS women; nonetheless, serum levels of hyperandrogenemia in patients with PCOS vary widely (23, 24) and may not reflect local tissue levels. Because the phenotype of this animal model most closely represents the syndrome of human PCOS, the findings here likely illustrate effects of hyperandrogenemia along a continuum of dysfunction.

DHT levels showed a strong inverse correlation to maximal dilation, suggesting a negative androgenic effect in female rats on endothelial function. This finding is consistent with clinical studies that suggest endothelial dysfunction in PCOS patients above that attributable to metabolic risk factors alone (15–17), and may also help describe the finding of endothelial dysfunction in patients with lean PCOS (52). Although a definitive conclusion cannot be drawn from our data regarding whether endothelial dysfunction is a primary or secondary effect of hyperandrogenemia, maximal dilation did not significantly correlate to body weight; therefore, the effects are not likely due to obesity alone.

Likewise, the role of insulin resistance cannot be resolved from the present data. Decreased insulin sensitivity was clearly demonstrated in the original DHT model as determined by a euglycemic-hyperinsulinemic clamp. Although not the focus of this study, we analyzed a subset of six DHT and six control rats to confirm insulin resistance. Fasting insulin levels were indeed elevated in DHT rats; however, this subgroup was underpowered to detect a difference in HOMA-IR. Future studies will analyze insulin sensitivity and the effects of dietary and pharmacological modifications on endothelial function, to determine whether effects are ascribable primarily to DHT or to secondary effects.

Estradiol levels were not significantly different between the control and DHT groups in the diestrous phase. Progesterone levels were slightly higher in control than DHT rats, yet the levels in both groups were well below those seen after ovulation (40–60 ng/ml) (32). In one study, progesterone antagonized the vasoprotective effects of estrogen (53), whereas in another, it stimulated endothelial NOS activity, NO production, and relaxation of vessels (20). Whether the reduced vasodilator efficacy in DHT rats is related to a reduction in progesterone levels, therefore, cannot be determined.

In summary, the results of this study demonstrate significant endothelial dysfunction in mesenteric resistance arteries in a new rat model for PCOS. This is evidenced by significant attenuation of the vasodilatory response to ACh and appears to be due to an increase in the release of vasoconstrictor prostanoids acting through the TP receptor. Structural changes in arteries from DHT-treated rats also occurred, as evidenced by a reduction in distensibility, increased elastin content, and smaller arterial lumen diameters. Because the splanchnic circulation contributes significantly to peripheral resistance, these findings are consistent with the observed elevation in blood pressure and may contribute to the morbidity and cardiovascular abnormalities associated with this clinically significant disease. Future studies will be directed at resolving whether the endothelial dysfunction is directly induced by hyperandrogenemia or by secondary metabolic alterations such as increased insulin resistance and obesity.

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Address all correspondence and requests for reprints to: Jennifer L. Keller, 111 Colchester Avenue, Smith 422, Burlington, Vermont 05401. E-mail: Jennifer.Keller@vtmednet.org.

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References

1. **Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO** 2004 The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab 89:2745–2749
2. **Hopkinson ZE, Sattar N, Fleming R, Greer IA** 1998 Polycystic ovarian syndrome: the metabolic syndrome comes to gynaecology. BMJ 317:329–332
3. **Talbott E, Clerici A, Berga SL, Kuller L, Guzick D, Detre K, Daniels T, Engberg RA** 1998 Adverse lipid and coronary heart disease risk profiles in young women with polycystic ovary syndrome: results of a case-control study. J Clin Epidemiol 51:415–422
4. **Wild RA, Carmina E, Diamanti-Kandarakis E, Dokras A, Escobar-Morreale HF, Futterweit W, Lobo R, Norman RJ, Talbott E, Dumesic DA** 2010 Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society. J Clin Endocrinol Metab 95:2038–2049
5. **Wild S, Pierpoint T, McKeigue P, Jacobs H** 2000 Cardiovascular disease in women with polycystic ovary syndrome at long-term fol-

low-up: a retrospective cohort study. Clin Endocrinol (Oxf) 52:595–600
6. **Grundy SM, Brewer H}B Jr, Cleeman JI, Smith Jr SC, Lenfant C** 2004 Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Arterioscler Thromb Vasc Biol 24:e13–e18
7. **Vanhoutte PM** 2009 Endothelial dysfunction: the first step toward coronary arteriosclerosis. Circ J 73:595–601
8. **Verma S, Buchanan MR, Anderson TJ** 2003 Endothelial function testing as a biomarker of vascular disease. Circulation 108:2054–2059
9. **Tarkun I, Arslan BC, Cantürk Z, Türemen E, Sahin T, Duman C** 2004 Endothelial dysfunction in young women with polycystic ovary syndrome: relationship with insulin resistance and low-grade chronic inflammation. J Clin Endocrinol Metab 89:5592–5596
10. **Meyer C, McGrath BP, Cameron J, Kotsopoulos D, Teede HJ** 2005 Vascular dysfunction and metabolic parameters in polycystic ovary syndrome. J Clin Endocrinol Metab 90:4630–4635
11. **Carmina E, Orio F, Palomba S, Longo RA, Cascella T, Colao A, Lombardi G, Rini GB, Lobo RA** 2006 Endothelial dysfunction in PCOS: role of obesity and adipose hormones. Am J Med 119:356 e351–e356
12. **Dokras A, Jagasia DH, Maifeld M, Sinkey CA, VanVoorhis BJ, Haynes WG** 2006 Obesity and insulin resistance but not hyperandrogenism mediates vascular dysfunction in women with polycystic ovary syndrome. Fertil Steril 86:1702–1709
13. **Hudecova M, Holte J, Olovsson M, Lind L, Poromaa IS** 2010 Endothelial function in patients with polycystic ovary syndrome: a long-term follow-up study. Fertil Steril 94:2654–2658
14. **Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD** 1996 Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. J Clin Invest 97:2601–2610
15. **Kravariti M, Naka KK, Kalantaridou SN, Kazakos N, Katsouras CS, Makrigiannakis A, Paraskevaidis EA, Chrousos GP, Tsatsoulis A, Michalis LK** 2005 Predictors of endothelial dysfunction in young women with polycystic ovary syndrome. J Clin Endocrinol Metab 90:5088–5095
16. **Paradisi G, Steinberg HO, Hempfling A, Cronin J, Hook G, Shepard MK, Baron AD** 2001 Polycystic ovary syndrome is associated with endothelial dysfunction. Circulation 103:1410–1415
17. **Sorensen MB, Franks S, Robertson C, Pennell DJ, Collins P** 2006 Severe endothelial dysfunction in young women with polycystic ovary syndrome is only partially explained by known cardiovascular risk factors. Clin Endocrinol (Oxf) 65:655–659
18. **Furchgott RF, Vanhoutte PM** 1989 Endothelium-derived relaxing and contracting factors. FASEB J 3:2007–2018
19. **Buys ES, Sips P, Vermeersch P, Raher MJ, Rogge E, Ichinose F, Dewerchin M, Bloch KD, Janssens S, Brouckaert P** 2008 Gender-specific hypertension and responsiveness to nitric oxide in sGCalpha1 knockout mice. Cardiovasc Res 79:179–186
20. **Qiao X, McConnell KR, Khalil RA** 2008 Sex steroids and vascular responses in hypertension and aging. Gend Med 5(Suppl A):S46–S64
21. **Steinberg HO, Paradisi G, Cronin J, Crowde K, Hempfling A, Hook G, Baron AD** 2000 Type II diabetes abrogates sex differences in endothelial function in premenopausal women. Circulation 101:2040–2046
22. **Manneråas L, Cajander S, Holmäng A, Seleskovic Z, Lystig T, Lönn M, Stener-Victorin E** 2007 A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. Endocrinology 148:3781–3791
23. **Fassnacht M, Schlenz N, Schneider SB, Wudy SA, Allolio B, Arlt W** 2003 Beyond adrenal and ovarian androgen generation: Increased peripheral 5 alpha-reductase activity in women with polycystic ovary syndrome. J Clin Endocrinol Metab 88:2760–2766
24. **Silfen ME, Denburg MR, Manibo AM, Lobo RA, Jaffe R, Ferin M, Levine LS, Oberfield SE** 2003 Early endocrine, metabolic, and sonographic characteristics of polycystic ovary syndrome (PCOS): comparison between nonobese and obese adolescents. J Clin Endocrinol Metab 88:4682–4688
25. **Goebelsmann U, Arce JJ, Thorneycroft IH, Mishell Jr DR** 1974 Serum testosterone concentrations in women throughout the menstrual cycle and following HCG administration. Am J Obstet Gynecol 119:445–452
26. **Probst-Hensch NM, Ingles SA, Diep AT, Haile RW, Stanczyk FZ, Kolonel LN, Henderson BE** 1999 Aromatase and breast cancer susceptibility. Endocr Relat Cancer 6:165–173
27. **Serafini P, Lobo RA** 1985 Increased 5α-reductase activity in idiopathic hirsutism. Fertil Steril 43:74–78
28. **Goebelsmann U, Bernstein G, Gale J, Kletzky O, Nakamura R, Coulson A, Korelitz J** 1979 Serum gonadotropin, testosterone, estradiol and estrone levels prior to and following bilateral vasectomy. New York: Academic Press
29. **Cacho J, Sevillano J, de Castro J, Herrera E, Ramos MP** 2008 Validation of simple indexes to assess insulin sensitivity during pregnancy in Wistar and Sprague-Dawley rats. Am J Physiol Endocrinol Metab 295:E1269–E1276
30. **Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC** 1985 Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28:412–419
31. **Halpern W, Osol G, Coy GS** 1984 Mechanical behavior of pressurized in vitro prearteriolar vessels determined with a video system. Ann Biomed Eng 12:463–479
32. **Freeman M, (Ed.).**1994 The neuroendocrine control of the ovarian cycle of the rat. 2nd ed. New York: Raven Press
33. **Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB** 1998 Prediction of coronary heart disease using risk factor categories. Circulation 97:1837–1847
34. **Arcaro G, Zamboni M, Rossi L, Turcato E, Covi G, Armellini F, Bosello O, Lechi A** 1999 Body fat distribution predicts the degree of endothelial dysfunction in uncomplicated obesity. Int J Obes Relat Metab Disord 23:936–942
35. **Brook RD, Bard RL, Rubenfire M, Ridker PM, Rajagopalan S** 2001 Usefulness of visceral obesity (waist/hip ratio) in predicting vascular endothelial function in healthy overweight adults. Am J Cardiol 88:1264–1269
36. **Furchgott RF, Zawadzki JV** 1980 The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373–376
37. **Félétou M, Huang Y, Vanhoutte PM** 2010 Vasoconstrictor prostanoids. Pflugers Arch 459:941–950
38. **Coleman RA, Smith WL, Narumiya S** 1994 International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 46:205–229
39. **Chenevard R, Hürlimann D, Béchir M, Enseleit F, Spieker L, Hermann M, Riesen W, Gay S, Gay RE, Neidhart M, Michel B, Lüscher TF, Noll G, Ruschitzka F** 2003 Selective COX-2 inhibition improves endothelial function in coronary artery disease. Circulation 107:405–409
40. **Widlansky ME, Price DT, Gokce N, Eberhardt RT, Duffy SJ, Holbrook M, Maxwell C, Palmisano J, Keaney Jr JF, Morrow JD, Vita JA** 2003 Short- and long-term COX-2 inhibition reverses endothelial dysfunction in patients with hypertension. Hypertension 42:310–315
41. **Belhassen L, Pelle G, Dubois-Rande JL, Adnot S** 2003 Improved endothelial function by the thromboxane A2 receptor antagonist S 18886 in patients with coronary artery disease treated with aspirin. J Am Coll Cardiol 41:1198–1204
42. **Lesault PF, Boyer L, Pelle G, Covali-Noroc A, Rideau D, Akakpo S, Teiger E, Dubois-Randé JL, Adnot S** 2011 Daily administration of

the TP receptor antagonist terutroban improved endothelial function in high-cardiovascular-risk patients with atherosclerosis. Br J Clin Pharmacol 71:844–851

43. **Gelosa P, Sevin G, Pignieri A, Budelli S, Castiglioni L, Blanc-Guillemaud V, Lerond L, Tremoli E, Sironi L** 2011 Terutroban, a thromboxane/prostaglandin endoperoxide receptor antagonist, prevents hypertensive vascular hypertrophy and fibrosis. Am J Physiol Heart Circ Physiol 300:H762–h768
44. **Lakhani K, Yang W, Dooley A, El-Mahdi E, Sundaresan M, McLellan S, Bruckdorfer R, Leonard A, Seifalian A, Hardiman P** 2006 Aortic function is compromised in a rat model of polycystic ovary syndrome. Hum Reprod 21:651–656
45. **Mahajan DK** 1988 Polycystic ovarian disease: animal models. Endocrinol Metab Clin North Am 17:705–732
46. **Alexanderson C, Stener-Victorin E, Kullberg J, Nilsson S, Levin M, Cajander S, Lönn L, Lönn M, Holmäng A** 2010 A single early postnatal estradiol injection affects morphology and gene expression of the ovary and parametrial adipose tissue in adult female rats. J Steroid Biochem Mol Biol 122:82–90
47. **Kafali H, Iriadam M, Ozardali I, Demir N** 2004 Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. Arch Med Res 35:103–108
48. **Ovalle F, Azziz R** 2002 Insulin resistance, polycystic ovary syndrome, and type 2 diabetes mellitus. Fertil Steril 77:1095–1105
49. **Carmina E, Koyama T, Chang L, Stanczyk FZ, Lobo RA** 1992 Does ethnicity influence the prevalence of adrenal hyperandrogenism and insulin resistance in polycystic ovary syndrome? Am J Obstet Gynecol 167:1807–1812
50. **Talbott EO, Zborowskii JV, Boudraux MY** 2004 Do women with polycystic ovary syndrome have an increased risk of cardiovascular disease? Review of the evidence. Minerva Ginecol 56:27–39
51. **Fritz MA, Speroff L** 2011 Clinical gynecologic endocrinology and infertility. 8th ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams, Wilkins
52. **Orio Jr F, Palomba S, Cascella T, De Simone B, Di Biase S, Russo T, Labella D, Zullo F, Lombardi G, Colao A** 2004 Early impairment of endothelial structure and function in young normal-weight women with polycystic ovary syndrome. J Clin Endocrinol Metab 89:4588–4593
53. **Wassmann K, Wassmann S, Nickenig G** 2005 Progesterone antagonizes the vasoprotective effect of estrogen on antioxidant enzyme expression and function. Circ Res 97:1046–1054