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Effects of vitamin D_3 derivative – calcitriol on pharmacological reactivity of aortic rings in a rodent PCOS model

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Abstract:
Background: The aim of this study was to examine the effects of the hyperandrogenic state in dihydrotestosterone (DHT)-induced polycystic ovary syndrome (PCOS), the vascular responses to different vasoactive agents, and the modulatory role of vitamin D_3.
Methods: A PCOS model was induced by DHT application in 20 female Wistar rats. Ten of the DHT treated rats simultaneously received calcitriol treatment. After 10 weeks, myographs were used to test the reactivity of isolated thoracic aortic rings to norepinephrine and acetylcholine. Thereafter, the vascular rings were incubated with the NO-synthase blocker (nitro-L-arginine methyl ester) or the cyclooxygenase inhibitor (indomethacin) for 20 min, and the effects of norepinephrine and acetylcholine were re-evaluated.
Results: Norepinephrine-induced vasoconstriction was enhanced after DHT treatment, but this effect was attenuated by calcitriol administration. Vasorelaxation of DHT-treated thoracic aortic rings was impaired, but this could be partly reversed by calcitriol application. Impaired NO-dependent vasorelaxation in DHT-treated animals was mostly reversed by concomitant calcitriol administration, but this effect was diminished by prostanoid-dependent vasoconstriction.
Conclusions: These studies show that the enhanced sensitivity to vasoconstrictors and impaired NO-dependent vasorelaxation in hyperandrogenic PCOS rats could be partially reversed by calcitriol treatment.

Key words:
aorta, vascular reactivity, PCOS, calcitriol, vitamin D_3, DHT, NO

Introduction

It has been shown that certain metabolic disorders such as hyperinsulinemia, insulin resistance, metabolic syndrome, diabetes mellitus and atherosclerosis are characteristic defects in the majority of women with polycystic ovary syndrome (PCOS) [5]. In the present study, we examined early functional changes - the earliest detectable lesions – of large vessels. Manneras et al. recently developed an adequate experimental model to study PCOS [12]: chronic dihydrotestosterone (DHT) application to adolescent female rats induces a PCOS-like condition including early dysfunction of carbohydrate homeostasis [12, 21]. Following this regimen of DHT treatment in rats, the estrus cycle was not observed, but a threefold increase in serum androgen levels, polycystic ovaries and insulin resistance were detected [12, 16, 21].

The protective effects of insulin sensitizers on PCOS related vascular damage are well-known [1, 14], and vitamin D_3 treatment has also been suggested as an adjuvant therapy for PCOS [18]. Positive effects of vitamin D_3 therapy on carbohydrate metabolism [9] and prevention of cardiovascular complications have been reported [15, 20]. Therefore, we investigated the effects of protective doses of calcitriol in hyperandrogenic female (HAF) rats. Similar chronic calcitriol therapies prevented heart failure and left ventricular hypertrophy in adolescent heart failure-prone SHHF rats [15]. In the present study, we aimed to clarify the effects of DHT on norepinephrine-induced vasoconstriction, acetylcholine (ACh)-induced NO-dependent vasodilation and the possible protective effect of simultaneous calcitriol administration.

Materials and Methods

Drugs and Chemicals

Rats were anesthetized with pentobarbital for surgical interventions (Nembutal, Phylaxia-Sanofi, Budapest, Hungary). Our short preparation method for blood pressure measurement did not require deep anesthesia as a typical surgical preparation. We used anesthetics only just below the pain reflex, which was assessed through the cornea reflex. Following surgical interventions, 20 mg of amoxicillin and 4 mg of clavulanic acid (Augmentin GlaxoSmithKline (Memphis, USA)) were dissolved in 0.2 ml saline, and this solution was administered intramuscularly to prevent infections. Experimental polycystic ovary syndrome was achieved as described by Manneras et al. [12] by using 90-day continuous-release pellets containing 7.5 mg dihydrotestosterone (Innovative Research of America, Sarasota, FL, USA, daily dose: 83 μg). As an active treatment, 1,25-$(OH)_2$-D_3 vitamin was used [15] (calcitriol, Cacijex injections of 2 μg/ml, Abbott Labs., Illinois, USA). The normal Krebs-Ringer (nKR) solution used in the *in vitro* studies was composed of the following (in mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 1.17 $MgSO_4 \cdot 7H_2O$, 20 $NaHCO_3$, 1.18 KH_2PO_4, 0.027 EDTA, and 11 glucose (Sigma Aldrich Co., St. Louis, MO, USA and Budapest, Hungary). The solution was kept at 37°C, and it was aerated with 5% CO_2 and 95% O_2, which stabilized the pH at 7.4. Norepinephrine, acetylcholine chloride, L-N^G-nitro-arginine methyl ester hydrochloride (L-NAME) and indomethacin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA and Budapest, Hungary). Drugs were freshly prepared in nKR solution on the day of the experiment.

Animals

Thirty adolescent, 21–28 day-old female Wistar rats (Semmelweis University Animal Colony, Budapest, Hungary originated from Charles River Ltd.) weighing 100–140 g were randomized into 3 treatment groups. Twenty animals received subcutaneous pellets of 7.5 mg dihydrotestosterone (DHT) underneath the back skin under anesthesia (induced with Nembutal at 45 mg/kg) and under sterile conditions. Ten animals underwent sham operations (referred to as the sham group). Ten DHT animals received 120 ng/100 g body weight/week of 1,25-$(OH)_2$-D_3 vitamin subcutaneously(DHT + D_3 group), as previously described by Przybylski et al. [15]. We applied one weekly dosage instead of daily administration [15], to reduce stress to the animals. The sham group and 10 DHT animals received the calcitriol vehicle (DHT + saline group). No medical or surgical complications were observed. Conventional rat chow and tap water were provided *ad libitum*. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care Commission (IRB approval: 22.1/2960/003/2009).

In vivo blood pressure measurement

Ten weeks after DHT pellet application, blood pressure was measured directly through the carotid artery in anesthetized rats (Nembutal 45 mg/kg *im*) with a Statham transducer connected to a Cardiosys CO-104 system (Experimetria, Budapest, Hungary).

Ex vivo pharmacological reactivity of thoracic aortic rings

After opening of the chest, the anesthetized animals were perfused transcardially with 10 ml of heparinized (10 IU/ml) nKR solution. After perfusion, each animal's aorta was removed. The distal part of the thoracic aorta (TA) was isolated, and four rings were prepared and placed into a vessel chamber filled with nKR solution aerated with carbogen (95% O_2 balanced with 5% CO_2, Lindegas, Répcelak, Hungary).

Thoracic aorta (TA) segments of 3 mm length from each experimental group were mounted on stainless steel vessel holders (200 µm in diameter) of a conventional myograph setup (610-M Multi Myograph System; Danish Myo Technology, Aarhus, Denmark). The organ chambers of the myographs were filled with 8 ml of nKR solution. The bath was warmed to 37°C, and the resting tension of the TA rings was adjusted to 15 mN, according to previous studies [3, 7].

Segments were exposed to 124 mM K^+ to elicit a reference contraction. After recovery, norepinephrine (10^{-9} – 10^{-6} M) and acetylcholine (10^{-8} – 10^{-5} M) dose-response curves were recorded. Thereafter, the vascular rings were incubated with either 10^{-4} M indomethacin or 10^{-4} M L-NAME for 20 min, and norepinephrine and ACh dose-response measurements were repeated to test different potential pathways of relaxation. Between measurements, the vessels were rinsed and allowed to recover for 20 min. Norepinephrine contraction was expressed as a percentage of K^+-precontraction. Aortic relaxations were tested after a stable plateau of contraction had been reached. Relaxant responses were expressed as a percentage of the precontraction induced by norepinephrine.

The isometric tension recording of the thoracic aorta segments was made with the MP100 system, and recorded data were analyzed with Acknowledge 3.7.3 software (BIOPAC Systems, Goleta, CA). Vasoactive substances were dissolved in physiological saline solution (0.9% w/v NaCl). All concentrations are expressed as the final concentration in the organ bath.

Histology

Ovaries and aortic rings of the animals were collected and freshly fixed for histological examinations. Tissue samples were immersion-fixed in 4% buffered formaldehyde and examined by light microscopy [hematoxylin and eosin staining; for evaluation Panoramic viewer software was used (3DHISTECH Ltd., Budapest, Hungary)]. Ovaries were examined for evidence of polycystic morphology, as described previously [16]. The results are shown in Figure 1 A (sham) and B (DHT treated).

Statistical analysis

Following normality tests (F-tests), dose-tension curves were statistically analyzed by a repeated measures ANOVA test and discrete parameters (e.g., body weights) were analyzed by one-way ANOVA tests. The Newman-Keuls test was applied as a *post-hoc* test; $p < 0.05$ was uniformly accepted as a significant difference. Data are presented as the mean ± SEM.

Results

The mean arterial pressure was 122 ± 3 mmHg, 123 ± 6 mmHg and 123 ± 4 mmHg in the sham, DHT + saline and DHT + D_3 groups, respectively. Body weights at the end of the study were: 298 ± 8 g, 354 ± 16 g and 353 ± 9 g in the 3 groups, respectively.

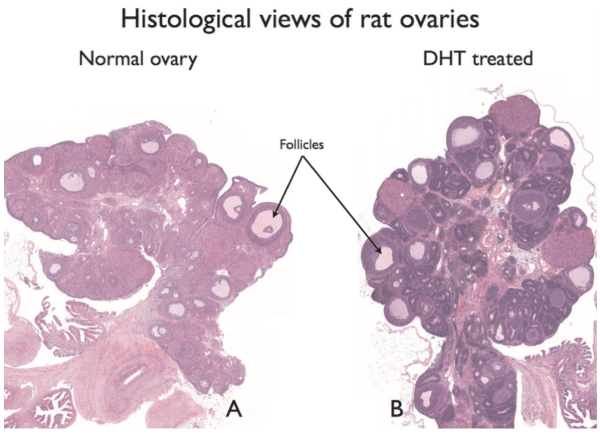

Fig. 1. Ovarian morphology following DHT treatment. Panel **A** shows the sham, and Panel **B** shows DHT-treated ovaries

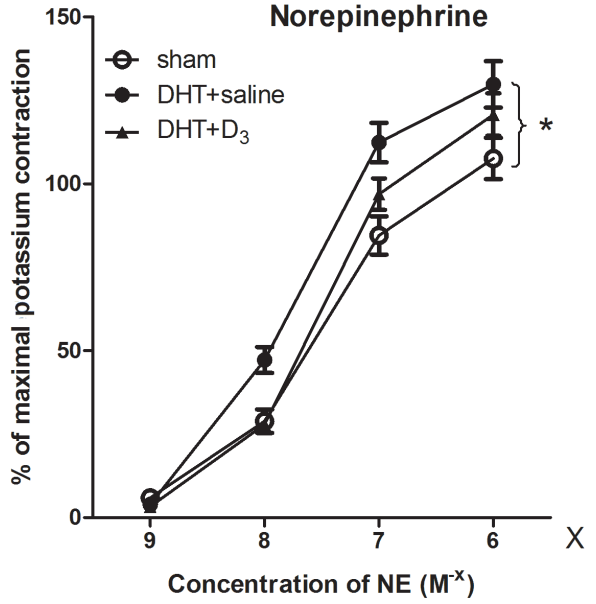

Fig. 2. Norepinephrine contraction of the vessels. The line graphs depict the contraction-response curves of aortic rings to increasing doses of 10^{-9} – 10^{-6} M norepinephrine for three groups: sham, dihydrotestosterone and dihydrotestosterone plus D_3 vitamin treated rats. The y axis shows contractions and the x axis shows norepinephrine concentrations. Each data point represents the mean ± SEM. Dihydrotestosterone-enhanced contractions (sham *vs.* DHT, * $p < 0.05$), and calcitriol reduced the responses to the levels of the sham group (non-significant for sham *vs.* DHT + D_3 and DHT *vs.* DHT + D_3)

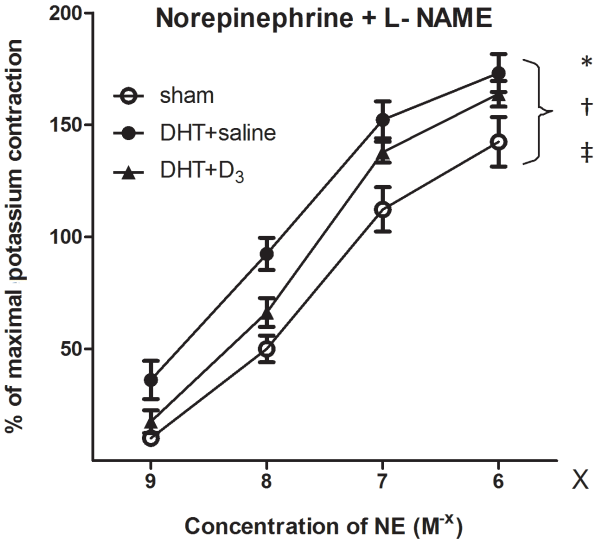

Fig. 3. Norepinephrine contractions under L-NAME incubation. Line graphs show the contraction response curves of aortic rings to increasing doses of norepinephrine (10^{-9} – 10^{-6} M) following L-NAME incubation. Each data point represents the mean values ± SEM. DHT enhanced contraction capacity (sham *vs.* DHT + saline, * $p < 0.0001$), but simultaneous calcitriol treatment partly reduced the contractility-enhancement due to DHT († $p < 0.05$ sham *vs.* DHT + D_3, ‡ $p < 0.05$ DHT *vs.* DHT + D_3)

Body weights of the DHT-treated animals differed significantly ($p < 0.05$) from those of the sham treated group, but not from each other (DHT + saline *vs.* DHT + D_3, not significant)

The histology results showed a similar morphology in the aorta for each of the 3 groups. Aorta wall thickness values were not significantly different: 113 ± 4 μm (sham), 122 ± 5 μm (DHT + saline) and 113 ± 6 μm (DHT + D_3)

Vasoconstrictor responses

The reference contractions of aortic rings exposed to 124 mM K^+ were similar and not significantly different in all 3 groups: 26.1 ± 0.4 mN (sham), 26.6 ± 0.5 mN (DHT + saline) and 25.7 ± 0.5 mN (DHT + D_3).

Norepinephrine contractions applied in a cumulative dose response manner were augmented significantly in the DHT-treated aortas compared to sham ($p < 0.05$). 1,25-$(OH)_2$-D_3 vitamin (calcitriol) treatment diminished the difference compared to sham. The DHT + D_3 group did not significantly differ from the DHT + saline or the sham group (Fig. 2).

Contractions in response to norepinephrine under L-NAME pre-incubation increased significantly ($p < 0.0001$) in aortas of DHT animals. Calcitriol treatment (DHT + D_3 group) partially reduced this contractility enhancement, with the results significantly differing from both the DHT + saline and the sham results ($p < 0.05$) (Fig. 3).

Following indomethacin incubation, there was a significant difference between the aortas of the sham and the DHT animals throughout the entire dose range ($p < 0.05$), with stronger contractions in the DHT animals. Calcitriol treatment significantly inhibited contractility-enhancement at small norepinephrine doses (10^{-9} – 10^{-8} M), but had no effect at higher norepinephrine doses. At low norepinephrine concentrations the DHT + D_3 curve approached the sham curve and differed significantly from the DHT + saline ($p < 0.05$) data, whereas at high norepinephrine concentrations the DHT + D_3 curve was similar to the DHT + saline curve, with both groups differing significantly ($p < 0.05$) from the sham (Fig. 4).

Vasorelaxation

Following the pre-contraction of the aortic rings induced by 10^{-6} M norepinephrine, acetylcholine (ACh)-induced relaxation was determined. Cumula-

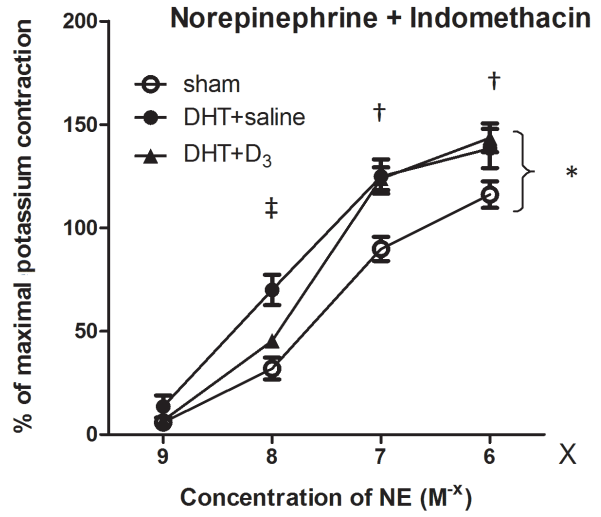

Fig. 4. Norepinephrine contractions under indomethacin incubation. Line graphs show the contraction response curves of aortic rings to increasing doses of norepinephrine (10^{-9} – 10^{-6} M) after indomethacin incubation. Contractions of aortas of DHT-treated rats exceeded sham rats within the entire dose range (sham *vs.* DHT + saline, * $p < 0.05$). Administration of calcitriol at low doses (10^{-8} M) of norepinephrine had a protective effect (‡ = $p < 0.05$ DHT *vs.* DHT + D_3, and not significant for sham *vs.* DHT + D_3). This protective effect of calcitriol was absent at higher (10^{-7} – 10^{-6} M) norepinephrine concentrations († $p < 0.05$ sham *vs.* DHT + D_3, and not significant for DHT *vs.* DHT + D_3)

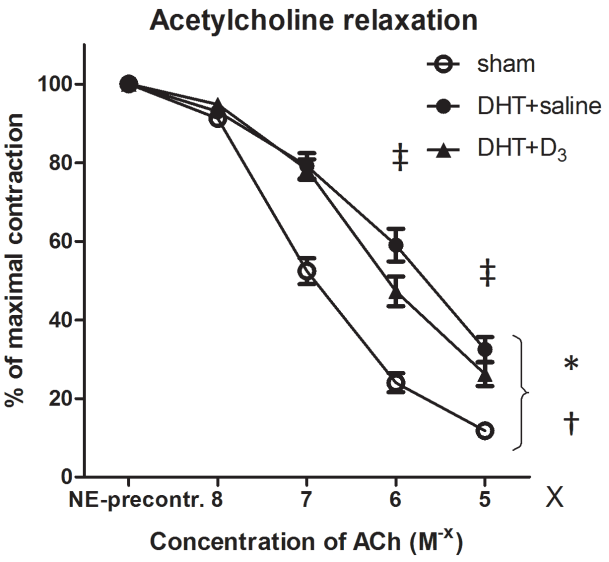

Fig. 5. Acetylcholine relaxation. Following norepinephrine (10^{-6} M) pre-contraction, cumulative doses of ACh (10^{-8} – 10^{-5} M) induced dose dependent relaxations. Within the entire dose range of ACh, only the sham group differed significantly from both the DHT + saline (* $p < 0.05$) and the DHT + D_3 († $p < 0.05$) treated groups. However, at higher doses of ACh, 10^{-6} – 10^{-5} M, calcitriol (DHT + D_3) enhanced relaxation significantly compared to DHT + saline (‡ $p < 0.05$)

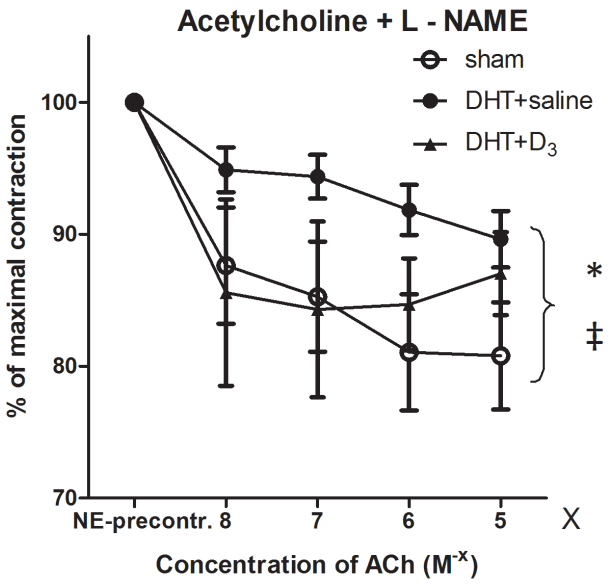

Fig. 6. Acetylcholine relaxation after L-NAME pre-incubation. Following L-NAME incubation, significant differences were measured in ACh-relaxation throughout the entire measurement range between the DHT + saline group and both the sham (* $p < 0.05$) and the DHT + D_3 (‡ $p < 0.05$) groups. There was no difference between the sham and the DHT + D_3 treated groups

tive dose-response curves of the sham group were significantly different from both the DHT-saline and DHT-D_3 groups throughout the entire dose range from 10^{-8} to 10^{-5} M.

After pre-contraction by 10^{-6} M norepinephrine, the cumulative dose-response curves (10^{-8} – 10^{-5} M) of acetylcholine-induced relaxation demonstrated that the sham group differed from both DHT + saline and DHT + D_3 treated groups ($p < 0.05$ for both comparisons) in the entire dose range. However, at higher doses of ACh, the relaxation of the DHT + D_3 group was significantly larger than that of the DHT + saline group (Fig. 5).

After L-NAME incubation (Fig. 6), significant differences were detected in ACh-relaxation over the entire measurement range between the DHT + saline group and the other two groups (sham and DHT + D_3 treated rats) ($p < 0.05$, Fig. 6). Calcitriol normalized the ACh-induced relaxation because there was no difference between the sham- and DHT + D_3-treated groups.

A comparison of the ACh-induced relaxation of the experimental groups after indomethacin incubation showed that the three curves were practically identical (without any significant differences) throughout the entire dose range (Fig. 7).

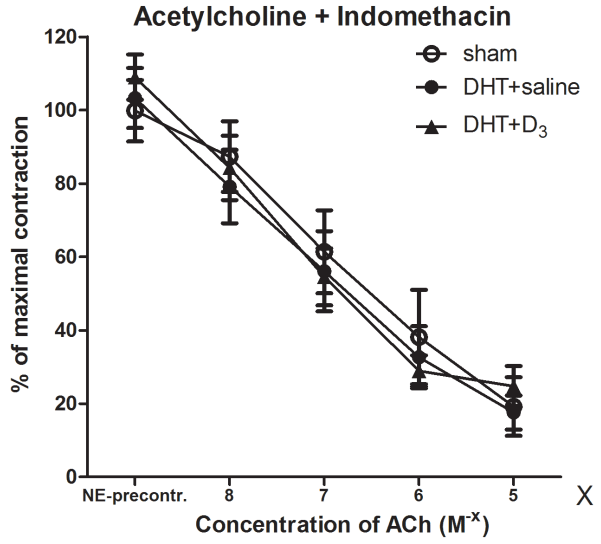

Fig. 7. Acetylcholine relaxation after indomethacin pre-incubation. ACh-induced relaxation was measured as a percentage of contraction induced by 10^{-6} M norepinephrine following indomethacin incubation. The groups did not significantly differ from each other throughout the entire dose range

Discussion

In this study, we have, for the first time, demonstrated early changes in conduit vessel function and quantified the effects of parallel 1,25-$(OH)_2$-D_3 vitamin (calcitriol) therapy in a rat PCOS model.

In a DHT-induced hyperandrogenic state, noradrenaline-induced contractions of rat aortas were enhanced. This effect was diminished by simultaneous calcitriol therapy (Fig. 2).

ACh-induced relaxation was more intense in the aortas of sham treated animals than in the two DHT groups (Fig. 5). ACh relaxation was decreased in DHT animals and calcitriol treatment did not return relaxation values to those of the sham group. However, following incubation with L-NAME, aortic rings of the DHT groups remained more contracted, and calcitriol-treatment enhanced relaxation significantly enough to return the DHT + D_3 group to sham levels (Fig. 6).

Indomethacin incubation diminished tone differences of the treatment groups. We assume that ACh-dependent relaxation differences contribute to prostanoid-dependent vascular effects, such as altered constrictor/dilator prostanoid balance (Fig. 7).

Increasing NO release is counterbalanced by constrictor prostanoids in the aortic rings of DHT + calcitriol treated animals during ACh-relaxation.

The rat model we used in our experiments is an approved model of PCOS [12, 21]. Eight to 12 weeks after similar DHT application, rat ovaries developed polycystic deformations, as well as significantly higher androgen levels [12]. In this model, during the course of 90 days of DHT application, Yanes detected numerous metabolic changes [21] such as insulin resistance [16, 21], higher cholesterol levels and greater oxidative stress [21]. These metabolic changes can influence the pharmacological responsiveness of blood vessels as well.

Longer term (90 days) DHT application, as in the aforementioned experiment, caused hypertension as well, while in our 70-day treatment blood pressure changes were not observed among the studied animals. The increased contractility in an already normotensive state, as well as the lower ACh-related relaxation capacity can be considered prehypertensive changes, similar to those found by Keller et al. on mesenteric arteries in hypertension following a 90-day regimen [8]. In the present study, we found a high degree of depletion of relaxation reserves, which can be a self-boosting local factor of the development of hypertension. The partial-compensation experienced in rats treated with vitamin D_3 has a local effect against the development of high blood pressure. However, in response to long term pressor effects, the antihypertensive impact of calcitriol treatment diminished.

Because the blood pressures of the animals in our study did not differ significantly from each other, the detected changes in the pharmacological reactivity of aortas should be considered as consequences of either the hyperandrogenic state or of insulin resistance, as our research team has previously demonstrated using this model [16]. In women with PCOS, significant pharmacological reactivity changes develop, as well as mechanical damage to the large blood vessels [5, 11]. Changes in smooth muscle and endothelial-related relaxation, as well as vasoconstriction, were studied in PCOS and under hyperandrogenic circumstances: Kravariti measured a significant decrease in both endothelial-dependent and smooth muscle-related (nitrate-mediated) relaxation [10]. The degree of insulin resistance, the level of the hyperandrogenic state and cholesterol levels were all independent factors related to diminished flow. It has been suggested

that in women with PCOS, endothelial dysfunction is present from their twenties onward, independently of obesity levels [10].

Flow-dependent dilation has been studied by numerous groups, all of which found non-significant differences between PCOS patients and healthy individuals [2, 17]. The apparent contradiction is resolved by Cussons' data which points to gradual damage. Cussons described a decrease in flow-mediated vasodilation at a physiological arterial stiffness as the earliest detectable change [4]. In conclusion, early (possibly when the patient is in their 20s and 30s) and gradual development of initial changes may be the first steps to hypertension and metabolic syndrome [4].

The pharmacological responsiveness to PCOS therapy is a subject of intense research. Agarwal demonstrated that metformin treatment reduced arterial stiffness, aortic and brachial pulse wave velocities, and the aortic augmentation index, but it also improved endothelium-dependent and independent vascular responses and endothelial function [1]. During insulin-sensitizer therapy, either metformin or pioglitazone caused enhancement of flow-mediated vasodilation in PCOS women [14]. The therapies used to treat PCOS have been found to alter cardiovascular risk [1, 13]. Contraceptives containing high hormone levels may increase cardiovascular risk, while metformin treatment decreases cardiovascular risk [13]. However, small doses of contraceptives are not likely to influence cardiovascular risk substantially [1, 2]. Vitamin D is an accepted adjuvant therapy in PCOS [18] and it was described that in small doses it has a cardiovascular protective effect [15, 20]. However, there has been no information on the effects of vitamin D and its' active form, calcitriol on vascular adaptation and pharmacological responsiveness to constrictor and vasodilator stimuli on large blood vessels in PCOS. Earlier, Weishaar and Simpson described an enhanced vasoconstriction of aortic rings in vitamin D deficient male rats [19], which was reversible following vitamin D_3 replacement. This effect was time and dose dependent – occurred following nine weeks of treatment and was not measured following six weeks [19]. We measured similar increase in the contractility of aortic rings in hyperandrogenic female rats, which was balanced by a longer (ten weeks) calcitriol treatment. An important implication of our study is that vitamin D_3, applied as an adjuvant treatment in PCOS, can delay the development of prehypertensive-induced damage on large blood vessels and may potentiate the vascular protective effects of metformin. Other vitamins might also have positive vascular effects in glucose imbalance on androgen dependent vascular function as demonstrated by Fernandes et al. [6].

DHT treatment lowered norepinephrine-induced contraction and reduced acetylcholine- as well as insulin-induced dilation of resistance vessels. Calcitriol treatment restored insulin relaxation and norepinephrine-induced contractility; however, it failed to alter NO dependent relaxation [16]. On small and large arteries we found similarities and differences in vascular reactivity. This finding could explain the different effects of vasorelaxants on the aorta and on arterioles.

In the present study, we demonstrated for the first time that in a rat model of PCOS enhanced contractions in response to norepinephrine can be partly counter-balanced by simultaneous therapy with calcitriol. We also demonstrated that acetylcholine-dependent vasorelaxation was not affected by calcitriol, most likely due to a constrictor-prostanoid effect, which mainly neutralized the enhanced NO-dependent relaxation. According to our results, the hyperandrogenic state resulted in prehypertensive blood vessel changes, and the depletion of vasorelaxant reserves. Calcitriol treatment reversed the vasoconstrictor reactivity that was elevated by DHT administration, and partially counter-balanced the prehypertensive changes of the aorta.

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