

Altered insulin-induced relaxation of aortic rings in a dihydrotestosterone-induced rodent model of polycystic ovary syndrome

Gabriella Masszi, M.D.,^a Anna Buday, M.D.,^b Agnes Novak, M.D.,^{c,d} Eszter Maria Horvath, M.D., Ph.D.,^d Robert Tarszabo,^b Levente Sara, M.D.,^c Csaba Revesz, M.D.,^b Rita Benko, Ph.D.,^d Gyorgy L. Nadasy, M.D., Ph.D.,^d Zoltán Benyó, M.D., Ph.D., D.M.Sc.,^d Peter Hamar, M.D., Ph.D., D.M.Sc.,^b and Szabolcs Varbiro, M.D., Ph.D.^c

^a Department of Cardiology, Bajcsy Zs. Hospital; and ^b Department of Pathophysiology, ^c Second Department of Obstetrics and Gynecology, and ^d Institute of Human Physiology and Clinical Experimental Research, Faculty of Medicine, Semmelweis University, Budapest, Hungary

Objective: To clarify the effects of dihydrotestosterone (DHT)-induced polycystic ovary syndrome (PCOS) on the insulin-dependent vasodilatation of the thoracic aorta and the role of vitamin D in a rat model.

Design: Controlled experimental animal study.

Setting: Laboratory.

Animal(s): Thirty adolescent female Wistar rats.

Intervention(s): The PCOS model was induced by 10 weeks of DHT treatment (83 µg/d). One-half of the DHT-treated animals also received vitamin D (120 ng/kg/wk).

Main Outcome Measure(s): The aortic rings of the control, DHT, and DHT plus vitamin D-treated animals were isolated. The insulin-dependent vasodilation of the isolated aortic rings was compared in Krebs-Ringer solution and under blockade of nitric oxide (NO) synthase or cyclooxygenase.

Result(s): The insulin-dependent vasorelaxation decreased in both DHT-treated groups independently from the vitamin D treatment; NO-dependent and -independent relaxations were both impaired. In response to prostanoid, vasoconstriction was increased after DHT treatment. The NO-independent relaxation was partially improved by vitamin D treatment, which was neutralized by increased prostanoid-dependent vasoconstriction.

Conclusion(s): Previously, we found that vitamin D treatment prevented systemic insulin resistance; however, in this study, we did not detect any influence on the vascular insulin resistance of the aorta that was induced by DHT treatment. Consequently, controlling insulin resistance with vitamin D alone did not resolve the aortic endothelial dysfunction caused by the hyperandrogenic state. (Fertil Steril® 2013;99:573–8. ©2013 by American Society for Reproductive Medicine.)

Key Words: Rat, vascular insulin resistance, dihydrotestosterone, vitamin D, PCOS

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Reprint requests: Szabolcs Varbiro, M.D., Ph.D., Second Department of Obstetrics and Gynecology, Faculty of Medicine, Semmelweis University, Ullói ut 78/A H-1083, Budapest, Hungary (E-mail: varbiroszabolcs@gmail.com).

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Certain metabolic changes, such as hyperinsulinemia, insulin resistance, and metabolic syndrome, may already be present before the onset of polycystic ovary syndrome (PCOS) and may in fact mediate the onset of PCOS (1, 2). Type 2 diabetes mellitus and the risk for cardiovascular diseases develop earlier in the majority of women with PCOS than in the general population (1, 2). In the present study, we examined early functional changes in large blood vessels, most

likely the earliest detectable difference, Manneras et al. developed a reliable experimental model to study PCOS (3). Chronic treatment of adolescent female rats with dihydrotestosterone (DHT) induces a PCOS-like condition, including impaired insulin sensitivity (3, 4).

The insulin resistance that occurs in PCOS can be reversed by insulin sensitizers such as metformin (5). Metformin decreases serum glucose and insulin levels. Furthermore, metformin has beneficial effects on the cardiovascular system. Agarwal et al. have proved that metformin reduces arterial stiffness and improves endothelial function in young women with PCOS (6). Palomba et al. and Naka et al. showed the enhancement of flow-mediated dilatation and of vascular endothelial function by metformin (7, 8). At the same time, vitamin D use has emerged as an adjuvant therapy in PCOS (9). Vitamin D therapy has positive effects on carbohydrate metabolism (10, 11) and has been suggested to prevent cardiovascular complications as well (12, 13). Therefore, we investigated the effects of protective doses of vitamin D in hyperandrogenic female (HAF) rats. A similar chronic vitamin D therapy prevented heart failure and left ventricular hypertrophy in adolescent heart failure-prone spontaneously hypertensive rats (SHR) (13). Previously, we reported that after 70 days of DHT administration, the insulin-induced vasorelaxation decreased in the small arteries of HAF rats (14). This effect was prevented by a concomitant weekly dosage of vitamin D. Because nitric oxide (NO)-dependent relaxation, which deteriorated with chronic DHT treatment, was not influenced by vitamin D, other mechanisms of compensation might be involved. In the present study, we aimed to clarify the effects of DHT on the insulin-dependent vasodilatation of the aortic rings of HAF rats and the possible modulatory role of a protective dose of vitamin D. To confirm these effects, we examined NO-dependent relaxation in the aortic rings as well as the possible role of prostanoids in the compensatory mechanism. In this study, we tested the two essential mechanisms regulating vascular tone: the relaxing capacity under pretreatment with L-N^G-nitroarginine methyl ester hydrochloride (L-NAME, i.e., NO synthase blocker) or indomethacin (i.e., cyclooxygenase blocker). Earlier, we demonstrated the positive effect of vitamin D on systemic insulin resistance (14). The DHT-dependent reduction of insulin-induced vasorelaxation found in small arteries was improved by parallel vitamin D administration (14). We sought to demonstrate a similar phenomenon in large vessels, such as the aorta, in a HAF rat model.

METHODS

Drugs and Chemicals

The composition of the normal Krebs-Ringer (nKR) solution used in the *in vitro* studies was (in mmol/L) 119 NaCl, 4.7 KCl, 2.5 CaCl₂·2H₂O, 1.17 MgSO₄·7H₂O, 20 NaHCO₃, 1.18 KH₂PO₄, 0.027 EDTA, and 11 glucose (Sigma Aldrich). The solution was maintained at 37°C and aerated with 5% CO₂ and 95% O₂, which stabilized the pH at 7.4.

Norepinephrine, acetylcholine chloride, 17β-estradiol, L-NAME, and indomethacin were obtained from Sigma-Aldrich. Human recombinant insulin (100 NE/mL Actrapid Penfill) was obtained from Novo Nordisk. The drugs were freshly prepared in nKR solution on the day of the experiment.

Animals

Thirty adolescent 21–28-day-old female Wistar rats weighing 100–140 g were used (Simmelweis University Animal Colony, Budapest, Hungary; originated from Charles River). Experimental polycystic ovary syndrome was achieved as described by Manneras et al. (3), with the use of 90-day continuous-release pellets containing 7.5 mg DHT (Innovative Research of America; daily dose 83 μg). Ten animals underwent sham operations (control group). The rats were anesthetized with pentobarbital (Nembutal; Phylaxia-Sanofi) during surgical interventions. Following chronic surgical interventions, 20 mg amoxicillin + 4 mg clavulanic acid (Augmentin; Glaxo Smith Kline) dissolved in 0.2 mL saline solution was administered intramuscularly to prevent infections. Ten DHT-treated animals received 120 ng/100 g body wt./wk of 1,25(OH)₂-vitamin D₃ (DHT+D₃ group). The vitamin D was given subcutaneously as previously described by Przybilski et al. We administered a weekly—for 10 weeks—instead of daily dosage (13, 14) of vitamin D to reduce the stress to the animals. 1,25(OH)₂-vitamin D₃ was chosen (injectable Calcijex, 2 μg/mL; Abbott Laboratories) because this is the active form of vitamin D and the action of other forms depends on liver and renal function (13, 14). The control group and ten of the DHT-treated animals received vitamin D vehicle (saline solution) subcutaneously. As described earlier, after 8 weeks of treatment, the oral glucose tolerance test (OGTT) was performed in short ether narcosis to assess glucose homeostasis (fasting and 120-minute blood glucose and plasma insulin levels were measured after the administration of 20 mg glucose/100 g body weight, in gauge) (14). The fasting glucose and insulin levels were similar in all groups. At the 120-minute time point of the OGTT, the insulin level measured in the DHT-treated rats was three times higher than in both the control and the DHT+D₃-treated animals (14). A significant difference between the fasting and the 120-minute insulin levels was found only in the DHT group (14), which developed insulin resistance after DHT treatment (14). Vitamin D treatment prevented insulin elevation (14). The serum fructoseamine level was similar in all groups and within the reference range, indicating that the animals did not have diabetes or elevated blood glucose (14). The ovaries of the animals were collected and immediately fixed for histologic examinations to determine whether polycystic morphology was present as described earlier (14). No medical or surgical complications were observed. Conventional rat chow (S8106-S011 SMR/M-Z+H, with physiologic vitamin D content; Spezialdiäten) and tap water were provided *ad libitum*. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and was approved by the Institutional Animal Care Commission (Institutional Review Board approval 22.1/2960/003/2009).

Ex Vivo Pharmacologic Reactivity of Thoracic Aortic Rings

After opening the chest, the deeply anesthetized animals were transcatheterially perfused with 10 mL heparinized (10 IU/mL) nKR solution. After perfusion, the heart and aorta of each animal were removed, and the hearts were weighed. 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% O₂ balanced with 5% CO₂; Lindegas).

Segments of the thoracic aorta of 3 mm length from each experimental group were mounted on the stainless steel vessel holders (200 μ m diameter) of a conventional myograph setup (610-M Multi Myograph System; Danish Myo Technology). The organ chambers of the myographs were filled with 8 mL nKR solution. The bath was warmed to 37°C, and the resting tension of the TA rings was adjusted to 15 mN, as described in previous studies (15, 16).

The segments were exposed to 124 mmol/L K⁺ to elicit a reference contraction. Twenty minutes later, precontraction was induced by norepinephrine (5 \times 10⁻⁸ mol/L), and endothelial relaxation was tested using 10⁻⁸–10⁻⁵ mol/L acetylcholine. After recovery, the insulin-mediated vasorelaxation was tested by the administration of increasing concentrations of insulin (50–150–300–600 mU/mL) in precontracted (5 \times 10⁻⁸ mol/L norepinephrine) vessels. Afterward, the vascular rings were incubated with either 10⁻⁴ mol/L indomethacin or 10⁻⁴ mol/L L-NAME for 20 minutes, and the insulin dose-response measurement was repeated to test different potential pathways of relaxation. Indomethacin blocks the synthesis of all prostanooids (including prostacyclin and thromboxane), and L-NAME is a general inhibitor of NO synthases (all three forms). Between measurements, the vessels were rinsed and allowed a 20-minute-long recovery period. Aortic relaxations were tested after a stable plateau of contraction had been reached. The relaxant responses were expressed as a percentage of the precontraction produced by norepinephrine.

The isometric tension of the TA segments was recorded with a MP100 system, and the recorded data were analyzed with Acq-Knowledge 3.7.3 software (Biopac Systems). Vasoactive substances were dissolved in physiologic saline solution (0.9 v/v% NaCl). All concentrations are expressed as the final concentration in the organ bath. NO-dependent vasorelaxation was counted as a relative difference in insulin-related direct and L-NAME-induced relaxations.

Histology

The ovaries of the animals were collected and freshly fixed for histologic examinations. The tissue samples were immersion fixed in 4% buffered formaldehyde and examined with the use of light microscopy (hematoxylin-eosin staining; Panoramic viewer software was used for evaluation; 3D Histech). The ovaries were examined for controlling polycystic morphology as described earlier (12).

Statistical Analysis

The dose-tension curves were analyzed by repeated-measures analysis of variance (ANOVA). The discrete parameters were

analyzed statistically by one-way ANOVA. The Newman-Keuls test was applied for post hoc analysis. *P* < .05 was uniformly accepted as significantly different. The data are presented as mean \pm SEM.

RESULTS

Basic Parameters of the Animals

At the end of the experiments, the body weights of the animals were 298 \pm 8 g, 354 \pm 16 g, and 353 \pm 9 g for the control (C) and DHT- and DHT+D₃-treated groups, respectively (14). The body weight was higher after the DHT treatment, independently from vitamin D coadministration (*P* < .05 for both C vs. DHT and C vs. DHT+D₃, nonsignificant for DHT vs. DHT+D₃) (14). After 10 weeks, there was no significant difference in the blood pressures of the groups, as measured directly by carotid artery cannulation: 122 \pm 3 mm Hg, 123 \pm 6 mm Hg, and 123 \pm 4 mm Hg for the control group, the DHT-treated group, and the DHT+D₃-treated group, respectively (nonsignificant) (14). From histologic analysis, polycystic ovaries were found 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 (1,609 \pm 617 and 2,334 \pm 451 pixels at \times 40 magnification, respectively; *P* < .05). The diameters of the follicles of the DHT+D₃ ovaries were not significantly different from those of the other two groups (2,054 \pm 442 pixels).

Insulin-Induced Relaxation of Aortic Rings

The insulin-induced relaxation was significantly lower in the DHT-treated groups than in the control group (Fig. 1; *P* < .05 for C vs. DHT and C vs. DHT+D₃). The D₃ treatment had no significant effect on the insulin-induced aortic relaxation.

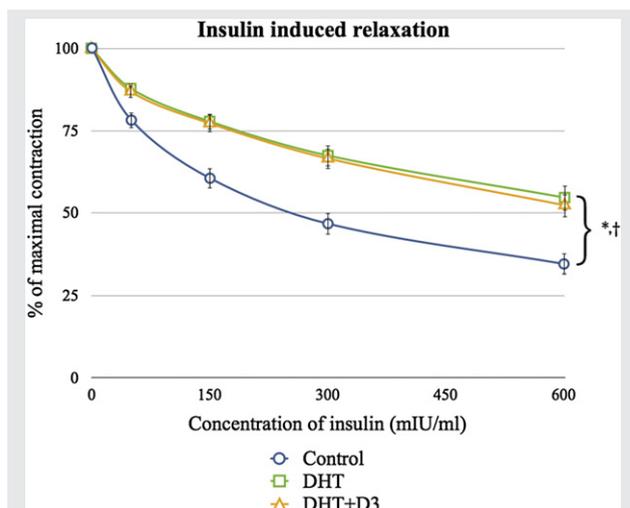
The L-NAME incubation significantly reduced the insulin-induced relaxation in the control rats. For the entire dose range, the insulin-induced relaxation after L-NAME preincubation was also reduced in the DHT-treated rats compared with the control rats (Fig. 2A; *P* < .01 for C vs. DHT; *P* < .05 for C vs. DHT+D₃). A repeated-measures ANOVA revealed that vitamin D treatment improved insulin-induced relaxation after L-NAME incubation overall (*P* < .05 DHT vs. DHT+D₃). Figure 2B shows the NO-dependent portion of the relaxation of the different groups upon insulin challenge. The DHT-treated groups were different from the control groups (for both comparisons: *P* < .001) but not from each other (DHT vs. DHT+D₃; ns).

The insulin-induced relaxation was also reduced by indomethacin. After indomethacin incubation, the insulin-induced relaxation curves were also impaired in the DHT-treated rats (Fig. 3; *P* < .05 for C vs. DHT and C vs. DHT+D₃). Again, vitamin D treatment was able to improve the insulin-induced vasorelaxation in the DHT-treated animals at doses of 150–600 mU/mL.

DISCUSSION

In our previous study, we demonstrated that vitamin D therapy reversed systemic insulin resistance in our early

FIGURE 1



Insulin-induced vascular relaxation in the treatment and control groups. *Line graphs* show the concentration-response curves of the insulin-induced vascular relaxation of aortic rings obtained from the normal control rats compared with the DHT-treated and DHT and vitamin D₃-treated rats. The *ordinate* shows relaxation as a percentage of the contractions induced by norepinephrine (5×10^{-8} mol/L). Each data point represents the mean \pm SEM from 40 rings. $P < .05$ for *C vs. DHT and †C vs. DHT+D₃. There was no difference between the DHT-treated groups. These data suggest that insulin-induced vascular (aortic) relaxation is impaired in the DHT-treated rats and that vitamin D₃ is not able to improve that relaxation.

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DHT-induced PCOS model (14). The 2-hour insulin value in the OGTT in the DHT-treated animals was nearly threefold higher than in the control animals. The vitamin D supplementation completely reversed the insulin resistance. Similar results were observed after insulin-dependent vasorelaxation of small arteries (14).

In the present study, we aimed to clarify the detailed mechanism for the deterioration of the insulin-dependent vasorelaxation of aortic rings. We demonstrated that DHT treatment reduced the insulin-dependent relaxation of rat aortic rings. This loss of insulin-dependent dilation is the vascular manifestation of insulin resistance. In contrast to systemic insulin resistance and local insulin-dependent relaxation in small vessels, i.e., arterioles, the net effect of vitamin D treatment on vascular insulin resistance caused by a hyperandrogenic state was neutral in the aortic rings. A similar dissociation of the metabolic and vascular insulin resistance has been demonstrated for aging (17), which proves that such dissociation is not without precedence. Because the effects of vitamin D on micro- and macrovessels differed (14; this study), it is possible that the remaining direct effect of chronic DHT treatment impaired large vessel wall reactivity. Vitamin D had no significant impact on the insulin-induced vasorelaxation in the aorta of DHT-treated rats; however, vitamin D was protective to insulin-induced relaxation in arterioles, and this may be related to elimination of systemic insulin resistance in DHT-treated rats (14). The explanation of

this could be a change in vasodilation mechanisms associated with insulin in different vessel types.

As suggested by a recent publication (18), a key mechanism of insulin-dependent vasorelaxation is the activation of the NO pathway. In accordance with this finding, our study showed that blocking the NO pathway by L-NAME decreased vasorelaxation in the control aortas. Our results suggest that the DHT treatment caused a decline in the insulin-dependent relaxation principally through deterioration of the NO-dependent relaxation. The remaining relaxation after the NO pathway was blocked, demonstrating that other relaxation pathways participate in this process. The vitamin D treatment limited the deterioration of the NO-independent (in the presence of L-NAME) insulin relaxation induced by the DHT treatment; however, the vitamin D treatment did not alter the NO pathway significantly (Fig. 2).

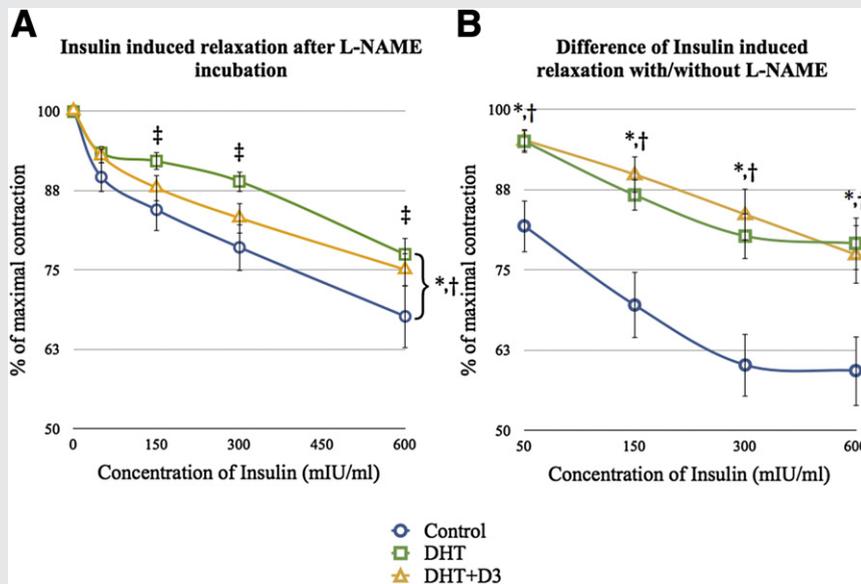
Earlier studies demonstrated mild vasorelaxant effects of the cyclooxygenase inhibitor indomethacin on the aorta (19); those findings are consistent with our observations of a moderate enhancement of vasorelaxation in the aortas of all groups. Interestingly, after indomethacin pretreatment the loss of insulin-dependent vasorelaxation by DHT was partially reversed by vitamin D. Thus, indomethacin diminished the constrictor tone of aortas, which was stronger in the DHT+D₃ group than in the DHT-alone group (Fig. 3). Previously, Keller et al. (20) described an increase in prostanoid-dependent vasoconstriction in resistance vessels in a similar 90-day DHT-induced rat model of PCOS. In our study, a further increase was found in prostanoid vasoconstriction after concomitant vitamin D application.

Considering these results, we suggest that the local effect of vitamin D treatment is a partial increase in NO-independent relaxation and an increase in constrictor prostanoid effects. However, the lack of effect of vitamin D on insulin vasorelaxation without pretreatment suggests the involvement of other, unknown, relaxation effects, such as endothelium-derived hyperpolarizing factor (21) or other mechanisms. Concomitantly with improving glucose metabolism (22, 23), plasma NO responsiveness might be elevated after vitamin D treatment (21), which may balance this effect.

Long-term systemic insulin resistance is accompanied by vessel damage and atherosclerosis (24, 25); however, controlling insulin resistance by vitamin D treatment alone did not resolve the vascular damage to the aorta caused by the hyperandrogenic state in our model. The DHT-induced model of PCOS in rats has been demonstrated to resemble human PCOS in that polycystic ovaries develop and androgen levels are elevated threefold (3, 4) after 8–12 weeks of DHT treatment. The direct effects of the hyperandrogenic state on blood vessels should be taken into account. Malkin et al. have shown that acute testosterone treatment induced vasodilation, whereas chronic treatment decreased endothelium-dependent and endothelium-independent vasorelaxation and increased norepinephrine-induced vasoconstriction (26). Gonzales et al. described an increase in thromboxane-induced tone after testosterone pretreatment (27).

The effects of vitamin D on vessel function have been studied. The acute application of vitamin D decreased

FIGURE 2

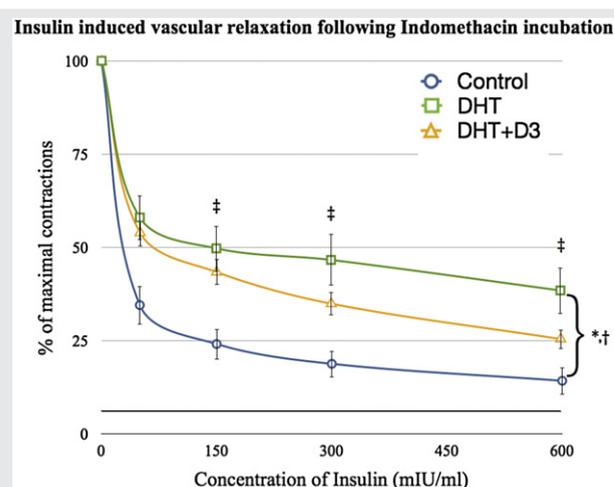


Insulin-induced vascular relaxation after incubation with L-NAME in the treatment and control groups. (A) Insulin-induced relaxation after L-NAME incubation was significantly larger in the control group than in the DHT-treated groups for the entire dose-relaxation curve ($*P < .01$ for C vs. DHT; $^{\dagger}P < .05$ for C vs. DHT+D₃). However, repeated-measures analysis of variance revealed that vitamin D₃ treatment improved overall insulin-induced relaxation after L-NAME incubation. The relaxation of aortic rings was more pronounced in the DHT+D₃ animals than in the DHT group ($^{\ddagger}P < .05$). (B) The NO-dependent portion of the relaxation on the insulin dose-response curve for the different groups. The DHT-treated groups were different from the control group (for both comparisons: $*^{\dagger}P < .001$) but not from each other (DHT vs. DHT+D₃: ns).

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prostanoid-dependent vasoconstriction in SHR to the level of the control Wistar Kyoto rats by weakening the endothelium-dependent 6-keto-prostaglandin F_{1 α} -mediated vasoconstriction in the SHR (12).

FIGURE 3



Insulin-induced vascular relaxation after incubation with indomethacin in the treatment and control groups. Insulin-induced relaxation following indomethacin incubation was significantly larger in the control than in the DHT-treated groups for the entire dose-relaxation curve ($P < .05$ for $*C$ vs. DHT and $^{\dagger}C$ vs. DHT+D₃). For doses from 150 mIU/mL to 600 mIU/mL, vessels from the DHT+D₃ animals relaxed more than those from the DHT-only animals ($^{\ddagger}P < .05$).

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Furthermore, it has been demonstrated that 6-week vitamin D treatment normalized the enhanced acetylcholine-dependent relaxation in SHR; this enhancement was not related to the intracellular Ca balance but correlated with the decreased amounts of reactive oxidative free radicals and cyclooxygenase-1 expression (12). Currently, there is no other information available relating the effect of vitamin D to vascular prostanoid metabolism. However, Wong et al. described a prostanoid-dependent relaxation (12) that is in contradiction to the vasoconstrictor effect we found. The variation in the effect of vitamin D may be explained by the differences in the examined vessels or species or may be the consequence of an interaction between the effect of vitamin D and increased prostanoid constriction caused by DHT treatment (20). When studying the vascular effects of vitamin D in hyperandrogenic females, we have to consider the direct vascular effects of vitamin D, interactions with DHT, and indirect vascular effects on improving systemic insulin resistance. However, the latter effect was not dominant in the rat aorta in terms of insulin-dependent relaxation.

Based on these studies, in clinical conditions we have to count the balancing effects of vitamin D in PCOS-related vascular damage. Drugs used in PCOS affect vascular function; they might deteriorate or improve it (28). It is important to know the net effects and potential interactions all of the drugs used to start optimal therapy for our patients.

CONCLUSION

This study is the first to show a decreased insulin-dependent relaxation effect in the aorta after DHT treatment. Vitamin D

supplementation helped to avoid elevation of the serum insulin levels (14) but did not change the decrease in the insulin-dependent relaxation in the rat aorta. The diminished relaxation caused by the androgenic effect was partially NO dependent. The net neutral effect of vitamin D on the rat aorta might be explained by the balance between the local constrictor prostanoids and a moderate alteration of the NO-independent relaxation; these effects have not been studied yet. In this study, we tested the two most important mechanisms that lead to normal (control) vascular tone; however, testing other mechanisms will be necessary in the future. This is the first study to note the possibility of a different dominance of the same mechanisms and different levels of vascular damage for similar stimuli in micro- and macrovessels; these differences suggest possible mechanisms with timing differences in target-organ damage.

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