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Title: Altered insulin relaxation of aortic rings in a rodent model of polycystic ovary syndrome

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

Design: A controlled experimental animal study.

Setting: An animal laboratory at a university research institute.

Sample: Aorta rings from Wistar rats and observed in an organ bath.

Methods: The PCOS model was induced by DHT treatment. After ten weeks, aorta rings of control, DHT and DHT plus vitamin D3-treated animals (n=10-10) were isolated. Insulin-dependent vasodilation of isolated aorta rings in normal Krebs-Ringer solution was compared to the response of rings treated with NO-synthase (by nitro-L-arginine methyl ester) or cyclooxygenase (by indomethacin) blockade.

Results: Insulin-dependent vasorelaxation decreased in both DHT-treated groups independent of vitamin D treatment. Impaired NO-relaxation and enhanced prostanoid contraction were observed.

Conclusion: DHT treatment caused deterioration of insulin-induced vasodilation on aorta rings. Vitamin D3 treatment prevented systemic insulin resistance; however, it did not influence vascular insulin resistance of the aorta. Although chronic, systemic insulin resistance causes endothelial dysfunction and atherosclerosis. Controlling insulin resistance with vitamin D3 alone did not resolve the endothelial dysfunction of the aorta caused by the hyperandrogenic state.

Suggested Reviewers:

Opposed Reviewers:
Dear Editor,

Attached to this letter You will find our manuscript entitled „Altered insulin relaxation of aortic rings in a rodent model of polycystic ovary syndrome”. In our experiments we used a simple rodent model of polycystic ovary syndrome and detected essential alterations.

Our primary aim was to determine the exact mechanism of action of DHT induced vascular damage, which we detected and published earlier in Your Journal (Sara et al., Fertil Steril 2012). Parallel to that we found a regional difference of vascular damage.

As one of the first alterations, we prove decreased insulin-dependent vasorelaxation of the aorta as a consequence of dihydrotestosterone treatment. Despite of the protective effects of vitamin-D administration on systemic and arteriolar insulin resistance in PCOS, the adjuvant vitamin D treatment does not influence the decreased aortic vasorelaxation induced by insulin. This regional difference can be explained by a direct deteriorating effect of hyperandrogenic state. This basic science study may give the key to the connections and first steps of diabetological and hyperandrogenic alterations and cardiovascular damage on the level of large vessels, often seen parallel in polycystic ovary syndrome. As our best knowledge, this is the first study to point out the possibility of a different dominance of the same mechanisms and different levels of vascular damage for similar stimuli in micro- and macro-vessels, suggesting possible mechanisms of timing differences in targeted-organ damage.

I hope, You will find our manuscript acceptable for publication in Your highly esteemed Journal.

Sincerely Yours,

Szabolcs Varbiro MD, PhD

Gabriella Masszi MD
Altered insulin relaxation of aortic rings in a rodent model of polycystic ovary syndrome

Running title: Effects of insulin on the aorta in PCOS

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Abstract
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Word count: 194.

Keywords: rat, vascular insulin resistance, dihydrotestosterone, vitamin D, PCOS.
It has been demonstrated that certain metabolic disturbances, such as hyperinsulinemia, insulin resistance, metabolic syndrome, diabetes mellitus and atherosclerosis, develop earlier in the majority of women with polycystic ovary syndrome (PCOS) than in the general population\textsuperscript{1,2}. In the present study we examined early functional changes (the earliest detectable lesions) of large vessels. Manneras et al. developed an adequate experimental model to study PCOS. Chronic DHT treatment of adolescent female rats induces a PCOS-like condition including early deterioration of carbohydrate metabolism\textsuperscript{3,4}. Insulin resistance that can be reversed by insulin sensitizers such as metformin develops in PCOS\textsuperscript{5}. The mode of action of metformin on the cardiovascular system in PCOS is well known\textsuperscript{6,7,8}. At the same time, vitamin D use has emerged as an adjuvant therapy in PCOS\textsuperscript{9}. Vitamin D therapy has positive effects on carbohydrate metabolism\textsuperscript{10,11} and has been suggested to prevent cardiovascular complications as well\textsuperscript{12,13}. Therefore, we investigated the effects of protective doses vitamin D in hyperandrogenic female (HAF) rats. Similar chronic vitamin D\textsubscript{3} therapy prevented heart failure and left ventricular hypertrophy in adolescent heart failure-prone SHR rats\textsuperscript{13}. Previously we reported that decreasing insulin induced vasorelaxation in small arteries after 70-day DHT administration\textsuperscript{14}. This effect was prevented by a parallel weekly dosage of vitamin D. Because NO relaxation, which deteriorates with chronic DHT treatment, was not influenced by vitamin D, other mechanisms of compensation may be involved. In the present study we aimed to clarify the effects of dihydrotestosterone on insulin-dependent vasodilatation on HAF rats’ aortic rings and the possible modulatory role of a protective dose of vitamin D. To confirm the reproducibility of these effects, we examined the NO-dependent relaxation in the aorta rings as well as the possible role of prostanoids in the compensatory mechanism. In this study we tested the two most important mechanisms regulating vascular tone. Earlier, we had demonstrated the positive effect of vitamin D on systemic insulin resistance\textsuperscript{14}. This normalizing action of systemic insulin levels by vitamin D was also suspected to be the key mechanism in compensation of resistance-arteriole relaxation induced
by insulin directly. We investigated the relevance of this theory as well.

**Methods**

**Drugs and Chemicals**

Rats were anesthetized with pentobarbital (Nembutal, Phylaxia-Sanofi, Budapest, Hungary) during surgical interventions or diethyl-ether during OGTT. Following chronic surgical interventions, 20 mg amoxicillin + 4 mg clavulanic acid (Augmentin GlaxoSmithKline (Memphis, USA)) dissolved in 0.2 ml saline was administered intramuscularly to prevent infections. Experimental polycystic ovary syndrome was achieved as described by Manneras et al.\(^3\), using 90-day continuous-release pellets containing 7.5 mg dihydrotestosterone (Innovative Research of America, Sarasota, Fl, USA, daily dose: 83 μg). For vitamin D supplementation, \(1,25 (OH)\_2 D\_3\) vitamin was used\(^{13}\) (Inj. Cacijex, 2μg/ml, Abbott Lab., Illinois, USA). Composition of the normal Krebs-Ringer (nKR) solution used in the in vitro studies was (in mM) 119 NaCl, 4.7 KCl, 2.5 CaCl\(_2\)-2H\(_2\)O, 1.17 MgSO\(_4\)-7H\(_2\)O, 20 NaHCO\(_3\), 1.18 KH\(_2\)PO\(_4\), 0.027 EDTA, and 11 glucose (Sigma Aldrich). The temperature of the solution was kept at 37 °C, and it was aerated with 5% CO\(_2\), 95% O\(_2\) which stabilized the pH at 7.4.

Norepinephrine, acetylcholine chloride, 17-β-estradiol, L-N\(^G\)-Nitroarginine methyl ester hydrochloride (L-NAME) and indomethacin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA and Budapest, Hungary). Human recombinant insulin (Actrapid Penfill 100 NE/ml) was obtained from Novo Nordisk (Copenhagen, Denmark). Drugs were freshly prepared in nKR solution on the day of the experiment.

**Animals**

Thirty adolescent, 21- to 28-day old, female Wistar rats, weighing 100-140 g were used
(Semmelweis University Animal Colony, Budapest, Hungary originated from Charles River Ltd). Twenty
animals received subcutaneous pellets of 7.5 mg dihydrotestosterone (DHT) beneath the back skin under
anesthesia (by Nembutal 45 mg/kg) and sterile conditions. Ten animals underwent sham operations
(Control group). Ten DHT-treated animals received 120 ng/100 g body weight/week $1,25\text{(OH)}_2 \text{D}_3$
vitamin (DHT+D3 group) vitamin D subcutaneously as previously described by Przybilski et al. We
administered weekly instead of daily dosage of vitamin D to reduce stress of the animals. The control
group and ten of DHT-treated animals received vitamin D vehicle (saline) sc. As described earlier, after
eight weeks of treatment, the Oral Glucose Tolerance Test (OGTT) was performed in short ether narcosis
to assess glucose homeostasis. Fasting glucose and insulin levels were similar in all groups.
Significantly higher insulin production was measured in DHT-treated rats. A significant difference
between the fasting and 120 min insulin levels was found in the DHT group only. Vitamin D treatment
prevented insulin elevation (Table). Serum fructose-amine was similar in all groups and was within the
reference range, indicating that the animals did not develop diabetes or blood glucose elevation
(Table). DHT treatment increased body weight in both DTH groups to a similar extent (Table). After ten
weeks there was no significant difference in blood pressures between groups, as measured directly by
carotid artery cannulation (Table). Animal ovaries were collected and freshly fixed for histological
examinations for controlling polycystic morphology as described earlier. No medical or surgical
complications were observed. Conventional rat chow and tap water were provided ad libitum. The study
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).

**Ex vivo pharmacological reactivity of thoracic aorta rings**

After opening the chest, deeply anesthetized animals were transcardially perfused with 10 ml of
heparinized (10 IU/ml) nKR solution. After perfusion, the heart and aorta of each animal were removed and hearts 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, 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 TA rings was adjusted to 15 mN, as described in previous studies. Segments were exposed to 124 mM K⁺ to elicit a reference contraction. Twenty minutes later, pre-contraction was induced by norepinephrine (5x10⁻⁸ M) and endothelial relaxation was tested using 10⁻⁸ - 10⁻⁴ M acetylcholine. After recovery, insulin-mediated vasorelaxation was tested by administration of increasing concentrations of insulin (50-150-300-600 mU/ml) in pre-contracted (5x10⁻⁸ M norepinephrine) vessels. Thereafter the vascular rings were incubated with either 10⁻⁴ M indomethacin or 10⁻⁴ M L-NAME for 20 minutes, and the insulin dose-response measurement was repeated to test for different potential pathways of relaxation. Between measurements, vessels were rinsed and allowed a 20-minute recovery period. Aortic relaxations were tested after a stable plateau of contraction had been reached. Relaxant responses were expressed as a percentage of the pre-contraction produced by norepinephrine.

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

Dose-tension curves and discrete parameters (e.g., body weights) were analyzed statistically by one-way ANOVA. Blood glucose and insulin level changes in time were analyzed by two-way ANOVA. As a post hoc test, Newman-Keuls test was applied. P<0.05 was uniformly accepted as significantly different. Data were presented as the mean ± SEM.

Results

Insulin-induced relaxation of aorta rings

Insulin-induced relaxation was significantly lower in DHT-treated groups compared to control (Figure 1.), p<0.05 for C vs DHT and DHT+D₃. D₃ treatment had no significant effect on insulin-induced aorta relaxation.

L-NAME incubation significantly reduced insulin-induced relaxation in control rats. In the whole dose-relaxation curve, insulin-induced relaxation after L-NAME incubation was also reduced in DHT-treated rats compared to controls (Figure 2. A), p<0.01 for C vs DHT and p<0.05 for C vs DHT+D₃. However, vitamin D₃ did restore the loss of insulin-induced vasorelaxation between 150-600 mIU/ml, p<0.05 DHT vs DHT+D₃ for doses > 50 mIU/ml insulin (Figure 2. B, C and D).

Insulin-induced relaxation was also reduced by indomethacin. Following indomethacin incubation, insulin-induced relaxation curves were also shifted down in DHT-treated rats (Figure 3. A), p<0.05 for C vs DHT and C vs DHT+D₃. Again, vitamin D₃ treatment was able to improve the insulin-induced vasorelaxation in DHT-treated animals or doses > 50 mIU/ml (p<0.05 Figure 3. B, C and D).

Discussion
In our previous study, we demonstrated that vitamin D therapy reversed systemic insulin resistance in our early PCOS model caused by dihydrotestosterone treatment. The two-hour insulin value of the oral glucose tolerance test in the DHT-treated animals was nearly threefold higher than in controls. Vitamin D supplementation completely corrected insulin resistance. Similar results were observed following insulin-dependent vasorelaxation on small arteries.

In the present study we aimed to clarify detailed the mechanism of deterioration of insulin-dependent vasorelaxation on aorta rings. We demonstrated that dihydrotestosterone treatment reduced insulin-dependent relaxation of rat aortic rings. This loss of insulin-dependent dilation is the vascular form of insulin resistance. In contrast with systemic insulin resistance and local insulin-dependent relaxation in small vessels / arterioles, the net effect of vitamin D treatment on vascular insulin resistance caused by a hyperandrogenic state was neutral in aortic rings. Similar dissociation of metabolic and vascular insulin resistance has been demonstrated in aging. The difference of vitamin D effect between micro- and macro-vessels proposes the possibility of direct impairment of vessel wall by chronic DHT treatment. Based on vitamin D effects, we speculate that this impairment had no significant impact on insulin-induced vasorelaxation in the aorta, unlike its protective effect on insulin-induced relaxation in arterioles and elimination of systemic insulin resistance. The explanation for this could be the different dominance of the same mechanisms of insulin-dependent vasorelaxation in different vessel types.

As suggested by a recent publication, a key mechanism of insulin-dependent vasorelaxation is activation of the nitric oxide (NO) pathway. In accordance with this finding, our study showed that blocking the NO pathway by L-NAME decreased vasorelaxation in control aortas. Our results suggest that DHT treatment caused a decline of the insulin-dependent relaxation principally through deterioration of NO-dependent relaxation. The remaining relaxation after the NO pathway was blocked demonstrated that other relaxing pathways participate in this process. Following vitamin D treatment, further deterioration of NO-
dependent vasorelaxation was found, as the remaining tone following NO-blockade was lower than in the DHT group.

In agreement with previous studies demonstrating mild vasorelaxant effects of the cyclooxygenase inhibitor indomethacin on the aorta\textsuperscript{19}, we observed a moderate enhancement of vasorelaxation in aortas of all groups. Interestingly, after indomethacin pretreatment, loss of insulin-dependent vasorelaxation by DHT was partially reversed by vitamin D. Thus, indomethacin diminished the constrictor tone of aortas, which was strongest in the DHT + D\textsubscript{3} group (Figure 3.).

Taken together, we suggest that the local effect of vitamin D treatment is a partial decrease of NO-dependent relaxation and increase in constrictor prostanoid effects. However, the lack of vitamin D effects on insulin vasorelaxation without pretreatment suggests the involvement of other, unknown relaxation effects, such as EDHF or other mechanisms. However, in parallel with improving glucose metabolism\textsuperscript{20,21}, plasma NO-responsiveness might be elevated after vitamin D treatment\textsuperscript{20}, which may balance this effect.

Although long term systemic insulin resistance is accompanied by vessel damage and atherosclerosis, controlling insulin resistance alone did not resolve vascular damage of 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\textsuperscript{3,4} after 8-12 weeks of DHT treatment. Direct effects of the hyperandrogenic state on blood vessels should be taken into account.

Effects of vitamin D on vessel function have been studied. Acute application of vitamin D decreased prostanoid-dependent vasoconstriction in spontaneously hypertensive (SHR) rats to the level of control WKY rats by weakening endothelium-dependent 6-keto-PGF\textsubscript{1α}-mediated vasoconstriction in SHR\textsuperscript{12}.

Furthermore, it has been demonstrated that a 6-week vitamin D treatment normalized the enhanced ACh-dependent relaxation in SHR; however, this was not related to the intracellular Ca-balance, but it
correlated with the decrease of reactive oxidative free-radicals and COX-1 expression\textsuperscript{12}. At this time, there is no other information available relating the effect of vitamin D on vascular prostanoid metabolism. However, Wong described a prostanoid-dependent relaxation that contrast with the vasoconstrictor effect we found. The variation in the effect of vitamin D may be explained by the differences in the examined vessels and species. Considering the vascular effects of vitamin D in hyperandrogenic females, we have to consider the direct vascular effects of vitamin D, interactions with dihydrotestosterone and indirect vascular effects on improving systemic insulin resistance. However, the latter effect was not dominant in the rat aorta in regard to insulin dependent relaxation.

**Conclusion**

This study is the first to show a decreased insulin-dependent relaxation effect in the aorta following DHT treatment. Vitamin D supplementation helped avoid the elevation of the serum insulin level, but did not influence the decrease of insulin-dependent relaxation in the rat aorta. The diminished relaxation caused by the androgenic effect was partly NO-dependent. The net neutral effect of vitamin D on the rat aorta could be explained by the counterbalance of the local constrictor prostanoids and a moderate alteration of the NO-dependent relaxation against relaxing effects that were not studied here. 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 point out the possibility of a different dominance of the same mechanisms and different levels of vascular damage for similar stimuli in micro- and macro-vessels, suggesting possible mechanisms of timing differences in targeted-organ damage.

**Acknowledgements**
This study was supported by a Servier Grant from the European Foundation for the Study of Diabetes as well as by grants from the Hungarian NIH (ALAP1-01298/2009), ETT (427/2009) and NFÜ (TÁMOP 4.2.1/B-09/1/KMR-2010-001). This study was sponsored by research grants from the Hungarian Society of Hypertension, National Grants of Hungarian Government Fund and Hungarian NIH (K81972, NF69278).

There is no conflict of interest.

Contribution to authorship:

Gabriella Masszi: performed chronic treatment and experiments, wrote manuscript.

Anna Buday: performed experiments, wrote manuscripts, assisted in OGTT.

Agnes Novak: performed experiments.

Eszter Maria Horvath: insulin and glucose measurements, assisted in OGTT, corrected the manuscript.

Robert Tarszabo: performed experiments.

Levente Sara: performed animal operations and chronic treatments.

Csaba Revesz: performed OGTT measurements.

Rita Benko: performed experiments.

Gyorgy L Nadasy: helped in study design, animal preparations.

Zoltán Benyó: professor of physiology, experiments were performed in his lab, directed vascular physiology measurements. Corrected study design and manuscript.

Peter Hamar: professor of pathophysiology. Directed OGTT and metabolic measurements. Corrected study design and manuscript.

Szabolcs Varbiro: designed experimental protocol, performed animal operations and chronic treatment, wrote manuscript.
Ethics approval: The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Semmelweis University Animal Care Commission (IRB approval: 22.1/2960/003/2009).

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Figure legends

**Figure 1. Insulin dependent relaxation of aorta rings**

Line graphs show concentration response curves of insulin-induced relaxation of aortic rings obtained from normal controls compared to dihydrotestosterone-treated, and dihydrotestosterone- and vitamin D₃-treated rats. The ordinate shows relaxation as a percentage of the contractions induced by norepinephrine (5x 10⁻⁸M). Each data point represents the mean±SEM from 40 rings, p<0.05 for C versus DHT and C versus DHT + D₃. There was no difference between DHT treated groups. The present data suggest that insulin induced vascular (aortic) relaxation is impaired in dihydrotestosterone treated rats and vitamin D₃ is not able to improve it.

**Figure 2. Insulin dependent relaxation of aorta rings after L-NAME pretreatment**

Panel A: Insulin-induced relaxation after L-NAME incubation was significantly larger in controls compared to DHT treated groups for the whole dose-relaxation curve (p<0.01 for C vs DHT and p<0.05 for C vs DHT+D₃). However, between 150-600 mIU/ml, relaxation of aorta rings were more pronounced in the DHT+D₃ animals than in the DHT group (p<0.05). Panel B, C and D show insulin relaxation curves before and after L-NAME incubation in the different groups, respectively (p<0.05 in all group).

**Figure 3. Insulin-dependent relaxation of aorta rings after indomethacin pretreatment**

Panel A: Insulin induced relaxation after indomethacin incubation was significantly larger in controls than in the DHT treated groups for the whole dose – relaxation curve (p<0.05 for C versus DHT and C versus DTH + D₃). For doses > 50 mIU/ml, vessels from DHT+D₃ animals relaxed more than those isolated from the DHT group (p<0.05). Panels B, C and D show insulin relaxation curves before indomethacin and
after indomethacin incubation in the different groups, respectively. Insulin-dependent relaxation increased in all groups after indomethacin pretreatment (p<0.05 in all group).

Word count: 2326.
Figure 1.
Figure 2. Insulin induced relaxation after L-NAME incubation.
Insulin induced relaxation after Indomethacin incubation

**Figure 3.**

**A**

- Control
- DHT
- DHT + D3

- % of maximal contractions
- Concentration of Insulin (mIU/ml)

**B**

- Control before Indomethacin
- Control after Indomethacin

- % of maximal contractions
- Concentration of Insulin (mIU/ml)

**C**

- DHT before Indomethacin
- DHT after Indomethacin

- % of maximal concentration
- Concentration of Insulin (mIU/ml)

**D**

- DHT + D3 before Indomethacin
- DHT + D3 after Indomethacin

- % of maximal contraction
- Concentration of Insulin (mIU/ml)