ALTERATIONS OF ARTERIOLAR REACTIVITY IN A RAT POLYCYSTIC OVARY SYNDROME MODEL - EFFECTS OF PARALLEL VITAMIN D_3 ADMINISTRATION

PhD thesis

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Budapest, 2012

BACKGROUND

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disease and the most frequent disorder in women of reproductive age. Nowadays, PCOS is in the focus of research because of its increasing prevalence. PCOS affects approximately 4-10% of fertile women around the world, but some researchers estimate the overall rate to be between 4-25%. The aetiology of this complex and heterogenous disorder is still uncertain. Environment factors such as physical inactivity, malnutrition and obesity have crucial role in development of the disorder. The most common features of PCOS, called the “Rotterdam criteria”, are menstrual disorders (amenorrhoea) such as oligo- or anovulatory menstrual cycles; polycystic, large ovaries as detected by ultrasound; and clinical or laboratory signs of excess androgen. It often associated with insulin resistance (IR) obesity, acne, hirsutism, cardiovascular disorders (CVD). There is a wide variability in phenotypes; symptoms and severity vary among affected patients. PCOS also alters cardiovascular function through various mechanisms. The mechanisms underlying this increased risk and its possible therapeutic approaches are still under investigation. IR and obesity were proposed to have a central role in the development of CVDs. Although there is a higher incidence of cardiovascular risk factors among PCOS patients, the existence of a significantly higher CVD incidence compared to control patients is debated. In research yet little is known about cardiovascular alterations of PCOS. In the background of PCOS circle vicious of IR and hyperandrogenism can increase the risk of CVDs.

Vascular alterations in PCOS. In PCOS, increased arterial stiffness and pulse wave velocity were demonstrated by ultrasound assessments. The mechanism of these alterations is unclear, but endothelial dysfunction and altered collagen metabolism of the vessel walls may be involved. This is similar to the patterns seen in IR and metabolic syndrome. Human studies have shown both macro- and microvascular dysfunction measured by ultrasound in PCOS, as indicated by the reduction in acetylcholine(ACh)-dependent vasodilation. This impaired response to ACh is similar to that observed in non-insulin-dependent diabetes mellitus. It is proposed to be related to metabolic alterations, especially insulin resistance in PCOS. It was showed that those women who have a decreased internal carotid artery pulsatility index in PCOS, have a

higher cardiovascular risk as well. Women with PCOS have a high prevalence of IR, metabolic syndrome, early-onset atherosclerosis, and they might develop hypertension during their reproductive period. These abnormalities are partly explained by the pharmacological and biomechanical remodelling of resistance arteries. The intima-media thickness of the carotid arteries was confirmed to be greater in PCOS than in age- and BMI-matched controls. Similarly, coronary artery calcification was found to be greater in PCOS patients. An endothelial dysfunction is the first step in the formation of atherosclerosis. The exact molecular mechanisms of the vascular alterations in PCOS are still unclear. It was hypothesised that the accumulation of reactive oxygen species, toxic oxygen-derived products and nitrogen-derived products might contribute to an uncontrolled lipid peroxidation of the cell membranes. The stimulation of NADPH oxidase and the uncoupling of mitochondrial oxidative phosphorylation can impair NO synthesis. These might explain the cardiovascular disorders often seen in PCOS. Hyperinsulinemia and IR often associates with PCOS. The independent role of insulin in human vessel relaxation has been demonstrated. Insulin induced dilation on efferent arterioles in kidney was reported to be independent of EDRF/NO and prostaglandins, however the mechanism of insulin induced direct vascular effect remained unclear. Insulin was suggested to affect IP3 release of Ca^{2+} by a cGMP-dependent mechanism that would contribute to its vasodilatory effect. Insulin may also act through NO and Na^{+}-K^{+} ATP-ase in human muscle arterioles.

Possible role of vitamin D in PCOS. Vitamin D is a secosteroid hormone that differs from other steroid hormones and can be obtained mainly from de novo synthesis in the skin. The accompanying metabolic alterations and obesity in PCOS might increase the risk of vitamin D deficiency. A recent study showed that vitamin D_3 deficiency occurred in approximately 72% of PCOS women. The level of vitamin D_3 was significantly lower in metabolic syndrome, and supplementation during infancy was observed to protect against T1DM. Vitamin D deficiency is strongly determined by the degree of obesity, and genetic variants of vitamin D_3 receptors can enhance the risk of deficiency. A rat vitamin D deficiency model was reported in which altered vascular endothelial and smooth muscle function and elevated blood pressure were observed. In accordance, increased arterial stiffness and endothelial dysfunction were demonstrated to be associated with vitamin D insufficiency in humans. Several researchers suggest that vitamin D deficiency may contribute to elevated blood pressure and that it has a crucial

role in the development of hypertension. Direct effect of vitamin D_3 on resistance vessels was demonstrated earlier by researchers. However, vitamin D_3 supplementation was assigned as an adjuvant therapy in PCOS, its effectiveness and possible role on metabolic symptoms need to be further characterised. However, even though several studies have confirmed the relationship between IR and vitamin D deficiency, the role of vitamin D_3 in glucose metabolism is still under debate.

AIMS

The aim of our study was to induce PCOS by a 70-day-DHT treatment in a modified animal model for examining early metabolic and functional changes - the earliest detectable lesions. We tried to determine the early effects of DHT treatment on carbohydrate metabolism. We examined the potentially beneficial effects of vitamin D_3. To identify early mechanical and pharmacological alterations in a morphologically stable skeletal muscle resistance vessel such as the gracilis arteriole in rat in PCOS. We also investigated the effect of low-dose vitamin D_3 treatment on the vascular biomechanical adaptation and the pharmacological responsiveness of the gracilis resistance arterioles. We aimed to clarify the effects of DHT on insulin-dependent vasodilatation on resistance arterioles to determine associations of the possible modulatory role of protective doses of vitamin D_3.

METHODS

Animals. Thirty adolescent (21- to 28-day-old) female Wistar rats that weighed 100-140 g when the study began were used. Twenty of the animals received subcutaneous pellets containing 7.5 mg DHT implanted under the back skin under anaesthesia (Nembutal 45 mg/kg i.p.) in sterile conditions (DHT-treated female groups), which induces polycystic ovary syndrome.Ten animals underwent sham operations. Ten DHT-treated animals received weekly doses of 120 ng /100 g 1,25 $(OH)_2$ D_3 vitamin (DHT+D3 group) subcutaneously. A vehicle was given to the remaining 20 animals. The investigation conforms to the Principles of Laboratory Animal Care NIH publication No. 85-23, revised 1985) as well as the Euroconform Hungarian Law on Animal Care (XXVIII/ 1998), and the study protocol was accepted by the institutional Animal Care Commission (IRB approval: 22.1/2960/003/2009).

Oral Glucose Tolerance Test (OGTT). After eight weeks of treatment, an oral glucose tolerance test was performed under short ether narcosis. Blood glucose and plasma insulin were measured following overnight fasting and 120 minutes after an oral glucose load of 0.3g / 100 g body weight given through a gauge.

In vivo blood pressure measurement. After ten weeks of DHT treatment blood pressure was measured directly through the carotid artery in anaesthetised rats (Nembutal 45 mg/ kg i.m.) with a Statham transducer connected to a Cardiosys CO-104 system (Experimetria, Budapest, Hungary).

Biomechanics of a Musculocutanous Arteriole (Pressure Arteriography). After opening the iliofemoral region, the arteriole, which had an in vivo diameter of approximately 150 μm and is the blood supply for the gracilis muscle, was removed and placed into a vessel chamber filled with normal Krebs-Ringer (nKR) solution. It was cannulated at both ends with plastic microcannulas and extended to its in vivo length. Both cannulas were connected to pressure-servo pumps, and the arterioles were pressurised under a no-flow condition to 50 mmHg intraluminal pressure. The outer and the inner diameters of the arterioles were measured by video-microscopic pressure microangiometry. In this setup, the glass-bottomed tissue bath was positioned in the light path of an inverse Leica microscope. A magnified picture of the vessel was formed with the aid of a video camera (Leica DFC320) and Leica QWin software. The digitalised pictures were saved, and off-line measurements of the inner and outer diameter were made using Leica QWin image analysis software. The gracilis arterioles were allowed to equilibrate for 30 minutes at 50 mmHg intraluminal pressure in an oxygenised nKR solution. Following incubation, the pressure was decreased to 10 mmHg and then increased to 100 mmHg in 10-mmHg pressure steps. The steady-state diameter was measured. The diameter was also measured during norepinephrine contraction after a 10-minute incubation period with 10^{-6}M NE. After recording the NE-diameter (pre-contraction) at 50 mmHg, dose-diameter curves were recorded in the presence of 30, 60, 120, 240 and 600 mU/ml of insulin, leaving an equilibration period of 8-10 minutes for each dose, which allowed a stable diameter to be reached and measured. After rinsing, NE was added to the organ bath in the same dose as previously described. After a 10-minute incubation the pressure-diameter curve was repeated as described above. After recording the NE-curve, at P=50 mmHg, 10^{-6} M ACh was added to the organ bath. Following a 20-minute equilibration period, the outer and inner

diameters were measured. Next, 10^{-5} M L-NAME was administered, and the diameters were again measured after reaching equilibrium (25-30 minutes at P=50 mmHg). Finally, the passive diameter was measured in a Ca^{2+}-free Krebs solution. Arteriolar segments were incubated for 20 minutes, and the steady-state, fully relaxed diameter was then measured as described above. The effect of insulin + NE was compared to the NE-induced contraction without insulin and also was compared to full relaxation. We used a micrometre etalon (Wild) for the calibrations.

Histology. The ovaries were formaldehyde fixed, stained with hematoxylin-eosine and histologically examined for controlling PCOS morphology. The morphometry was measured using the Pannoramic Viewer software (3DHISTECH Ltd., Budapest, Hungary).

Statistical analysis. For statistical analysis, repeated-measures ANOVA was used. In vitro parameters were plotted as a function of intraluminal pressure, and the curves were analysed by paired comparisons of the treatment groups. A one-way ANOVA was applied for the discrete parameters (e.g., blood pressure) and two way ANOVA for analyzing glucose and insulin curves of OGTT. As a post hoc test, Tukey`s test was used. $P<0.05$ was uniformly accepted as a significant difference.

RESULTS

Physiological parameters. The mean arterial pressure was 122 ± 3 mmHg, 123 ± 6 mmHg and 123 ± 4 mmHg for the controls, the DHT-group and the DHT+vitamin D_3-treated group, respectively (non-significant). The body weights at the end of the experiment were 298 ± 8, 354 ± 16 and 353 ± 9 g for the same groups, respectively. The DHT-treated animals had significantly higher body weights than the controls ($p<0.01$), but there was no difference between the two DHT-treated groups.

Ovarian morphology. We detected a polycystic morphology in the DHT-treated groups and normal ovaries in the controls by histological analysis. Follicle diameters were significantly smaller in DHT-treated ovaries compared to control (1609 ± 617 and 2334 ± 451 pixels at 40x magnification, $p<0.05$). Follicles of DHT+D3 ovaries weren't significantly different from the other two groups (2054 ± 442).

Glucose metabolism. The blood glucose levels and the fasting insulin levels were not significantly different among the experimental groups. However, significant differences were found among the groups for the 120 min postload insulin values (0.71 ± 0.14, 1.42 ± 0.33, 0.48 ± 0.07 ng/ml for C, DHT and DHT+D3). DHT-treated animals had higher plasma insulin levels than the controls, and this difference was eliminated by the D_3 vitamin treatment ($p<0.001$). The fasting and 120 min postload insulin levels were significantly different only in the DHT group ($p<0.001$). Serum fructose amine was similar in all groups and within reference range indicating, that the animals did not develop diabetes or blood glucose elevation.

Arteriole geometry. The relaxed outer radii for the different groups were 167 ± 13 μm (C), 193 ± 6 μm (DHT) and 166 ± 10 μm (DHT + vitamin D_3). The relaxed inner radii of the gracilis arterioles were 96 ± 3 μm (DHT), 84 ± 6 μm (C), 83 ± 5 μm (DHT+D3). DHT treatment increased both the outer and inner radii, which were diminished by a parallel vitamin D_3 treatment ($p<0.05$ for both comparisons). The wall thicknesses for the different groups in the Ca^{2+}-free medium were as follows: 20 ± 2, 19 ± 1, and 20 ± 1 μm in the control, DHT- and DHT + vitamin D_3-treated groups, respectively. In a Ca^{2+}-free solution, the cross section areas for the vessel walls were 12080 ± 1627, 12542 ± 1090, and 11348 ± 660 μm^2 for these groups, which were not significant.

Arteriole elasticity. The mechanical load on the vessel wall was measured, and the tangential wall stress was significantly higher throughout the entire pressure range for the DHT-treated group compared with the other two groups in both a Ca^{2+}-free solution and during norepinephrine contraction ($p<0.05$). Vitamin D_3 treatment induced a partial reversal of wall stress in the NE-contracted segments and an overcompensation in the Ca^{2+}-free solution (both for DHT vs. DHT + D3 and the control vs. DHT + D3, $p<0.05$). Isobaric distensibility was significantly higher throughout the entire pressure range for the DHT group compared with the other two groups in both the Ca^{2+}-free medium and during norepinephrine contraction ($p<0.05$). In response to a parallel treatment with vitamin D_3, relaxed distensibility fully returned to the control in Ca^{2+}-free solution. After NE contraction only a partial recovery was detected in DHT+D3 group. Wall elastic moduli were not affected by DHT and vitamin D_3 treatment (in a Ca^{2+}-free solution, the log elastic moduli were 2.8 ± 0.14, 2.7 ± 0.16 and 2.4 ± 0.11 log(Pa) in the control, DHT- and DHT + vitamin D_3-treated groups, respectively, ns.). There was no difference in the elastic moduli as a function of tangential wall stress.

Arteriolar contractility DHT treatment significantly reduced NE tone, which was almost fully restored by vitamin D_3 treatment ($p<0.01$ between the control and DHT, $p<0.05$ for the other comparisons). These segments do maintain substantial myogenic (spontaneous) tone. The tone was also lower following DHT treatment, and interestingly, co-administration of D_3 vitamin further decreased myogenic tone (the control and DHT-treated rats, $p<0.01$, as well as the D_3 vitamin-treated and untreated DHT rats, $p<0.05$, were significantly different). The sum of the two contractions, which represent the ability of the segments to fully contract (range of total pharmacological adaptation), was significantly lower after DHT treatment and was partially restored by vitamin D treatment (the control and DHT-treated rats, $p<0.01$, as well as the D vitamin-treated and untreated DHT rats, $p<0.05$, were significantly different).

Endothelial dilation. Segments contracted by NE were relaxed in response to 1 μM acetylcholine, and this relaxation was significantly larger in the control compared with the DHT-treated group. ACh dilations were severely diminished in the testosterone-treated female animals. Vitamin D_3 treatment did not compensate this alteration but produced only a nonsignificant trend toward restoring relaxation. ($p<0.05$ for both treatments comparison with the controls). L-NAME induced substantial contraction in the control segments, which was missing in both DHT-treated groups.

Insulin-induced vascular relaxation of gracilis arterioles Insulin administered to NE-precontracted gracilis arteriole segments induced concentration-dependent relaxation in the control animals. This relaxing effect was much reduced in the DHT-treated group ($p<0.001$). The administration of vitamin D_3 partially restored the insulin-induced relaxation ($p<0.001$). Another significant effect of Vitamin D_3 treatment was revealed when we compared the NE-induced contractions of segments before and after in vitro treatment with elevating concentrations of insulin. Typically, NE-induced contractions were more profound after insulin treatment. This difference was reduced in vitamin D_3-treated animals ($p<0.05$).

DISCUSSION

As a model of human polycystic ovary syndrome, ten weeks of dihydrotestosterone treatment starting during adolescence in female rats induced significant metabolic and vascular alterations in our studies. Systemic IR without altered glucose metabolism was

developed after 70-day DHT treatment. The two hours insulin value of oral glucose tolerance test in the DHT-treated animals was nearly two times higher than in controls. Chronic DHT treatment of female rats, which is an animal model for human PCOS, induced significant alterations in the biomechanical and pharmacological properties of a skeletal muscle arteriole. We observed an increase in arteriolar diameter. The relaxed lumen was dilated but was not accompanied by an elevation in wall mass (eutrophic remodelling). This elevated wall stress was accompanied by an increase in isobaric distensibility. The elastic moduli did not change, which demonstrates that passive elastic element remodelling was limited. There was a substantial alteration in the contractility and spontaneous (myogenic) tone of the segments. The additional contraction generated by 1 μM NE was reduced in the DHT-treated segments. Endothelial dilation was diminished in the DHT-treated groups. The present study demonstrated that insulin-induced vascular relaxation of arterioles was diminished. These loss of insulin dependent dilation of arterioles is the vascular form of IR. While increased IR in human PCOS disease and in DHT treated female animals has been described previously, the alteration of the well-known vasodilator activity of insulin was not studied.

Vitamin D_3 is an adjuvant therapy for PCOS, and the cardiovascular protective effect of low-dose vitamin D_3 therapy is widely accepted. In our study vitamin D_3 therapy was demonstrated to reverse systemic IR in our early PCOS model caused by DHT treatment. Vitamin D_3 supplementation entirely corrected IR. Chronic vitamin D_3 treatment restored vascular diameter. Our experiments demonstrate that vitamin D_3 can reverse morphological remodelling, elevation of wall stress and distensibility in a resistance vessel. It improved NE-induced contraction and myogenic tone was further reduced. However, loss of endothelial relaxation was unaffected. It was shown that changes after DHT treatment are partially counteracted by vitamin D_3. Investigation of these basic mechanisms revealed that the first effect from a hyperandrogenic state on musculocutaneous gracilis arterioles is that NO-dependent relaxation is impaired. However, vitamin D_3 treatment has no effect on the NO-dependent abnormalities; other mechanisms are responsible for compensation. In animals treated with vitamin D_3, the insulin-evoked relaxation returned to the control level in arterioles. In addition, the resistance artery effects of vitamin D_3 treatment, which improves both the alterations associated with PCOS and IR, had not been investigated yet.

Our results show basic changes in vascular biomechanics in response to DHT treatment; the arteriolar walls become more rigid with increased tangential wall stress compared with the controls. However, the tangential wall stress differences that were detected even in normotensive animals might be considered prehypertensive abnormalities. The enhanced mechanical load of the arteriolar wall is the local factor for the self-perpetuating, vicious cycle of emerging hypertension. For the decreased spontaneous tone observed in the vitamin D_3-treated group, tangential wall stress is not elevated. This has a local effect against hypertension development. Thus, the mechanical load of the vessels increases. Under passive circumstances, vitamin D_3 treatment fully restores the biomechanical balance of the vessel wall. However, in response to norepinephrine, mechanical load of the vessel wall increases compared with the control group, although it does not reach the level of the DHT-treated group. As a result of the smooth muscle pharmacological reactivity changes, the total adaptation range for skeletal muscle perfusion significantly decreases in response to DHT treatment. Although vitamin D_3 treatment significantly improves this range, it does not reach the level of the control group. In studying the basis for this phenomenon, an obvious, significant decrease in NE response is noted after DHT treatment. This is almost restored by vitamin D_3 treatment. A decrease of relaxation is detected in response to DHT treatment. This decrease is even more significant when DHT is combined with vitamin D_3. Another interpretation of these findings is that DHT treatment results in diminished adaptive reserve capacity in both directions. Vitamin D_3 applied with DHT significantly improves pharmacological reactivity but decreases the vessels' spontaneous tone even compared with the control.

In our experiments, vitamin D_3 treatment significantly decreased the vascular IR of arterioles caused by DHT. During NE-induced precontraction, DHT treatment decreased insulin-dependent relaxation, thus producing vascular IR. However, similar relaxation was observed in the control and vitamin D_3-treated groups. In response to insulin, the NE sensitivity of the vitamin D_3-treated group practically remained unchanged. In the control and DHT-treated groups, a 20% increase in contractility was observed after rinsing and during the second contraction compared with the first norepinephrine contraction. No differences were found between the first and second NE contractions in the vitamin D_3-treated group, which suggests that vitamin D_3 treatment improves NE sensitivity. However, the most prominent insulin-dependent relaxation was detected in

the vitamin D_3-treated group. Comparing the insulin-dependent relaxation to ACh relaxation, highly significant differences were noted. ACh caused stronger relaxation in the control group than in the two treated groups. Moreover, this difference remained the same or increased during L-NAME blockade. These results suggest that the relaxing effect of insulin is independent from NO in the gracilis arterioles and that vitamin D_3 restores normal vascular tone independently from the NO system.

CONCLUSIONS

In this study we used an experimental animal model of PCOS to examine early metabolic and vascular lesions. Our results show that the hyperandrogenic state resulted in more rigid, less flexible artery walls. These arteries show reduced endothelium- and smooth muscle-dependent vasorelaxation and constriction as well as a complete loss of NO-dependent relaxation compared with the controls. In the relaxed vessels, concurrent vitamin D_3 treatment corrected wall mechanics to the control level. Herein, we provide this first data for the effect of vitamin D_3 on the mechanical properties of arteries in PCOS. This is the first study investigating the vascular adaptation and insulin sensitivity of gracilis arterioles in a PCOS model. This is the first study demonstrating vascular insulin resistance of the gracilis arterioles and the counteracting effect of vitamin D_3 in an experimental PCOS model. The observed alterations of resistance arteries form an important prehypertensive pathway of vascular damage in PCOS and might explain the observed clinical effectiveness of vitamin D treatment.

ACKNOWLEDGEMENTS

This study was carried out at the Institute of Human Physiology and Clinical Experimental Research, Semmelweis University Budapest in 2011. I want to express my thanks to my project leader, colleauge and friend, **Szabolcs Várbíró**, lecturer of the 2nd Department of Obstetrics and Gynecology, who invented this novelty protocol, managed my study, supported my work strenuously, made a lot of sacrifices and expended most of his time on this project. I want to express my thanks to **György L. Nádasy**, associate professor of the Institute of Human Physiology and Clinical Experimental Research, who was always ready to help; taught and introduced me to special methods of microangiometric measurements and experimental processes. He gave an indispensable help during the study and evaluation of our results. I wish to thank to **Professor Zoltán**

Benyó director of the Institute of Human Physiology and Clinical Experimental Research for helping us to design and finish this study. **I** am very grateful to **Professor Emil Monos** for giving us possibility for carrying out this project in his laboratory. I want to convey my thanks to **Eszter M. Horváth** for her advices and help in technical problems. I am really thankful to **Professor Attila Pajor**, director of the 2nd Department of Obstetrics and Gynecology, Semmelweis University who has been helping me since I started my career. He gave me useful advices and permitted me of being for this study in the last year. I wish to express my gratitude to **Ildikó Murányi** for her devoted efforts in solving technical problems in the laboratory work. I am very grateful to **Péter Antal, Gabriella Masszi, Anna Buday, Péter Hamar, Mária Szekeres, Anna Monori-Kiss, Anna-Mária Tőkes, Ágnes Novák, Csaba Révész, Rita Benkő, Róbert Tarszabó** for their help and efforts**.** I wish to thank to **Peter Tóth**, associate professor of the 2nd Department of Obstetrics and Gynecology, who was my first master of my postgraduate course. I express my gratitude and thanks to my wife, **Katalin**, my daughter, **Boróka**, my **parents, brother** and **sister**, and my **colleauges** for their encouraging support during my work.

LIST OF PUBLICATIONS

The thesis is written on the basis of the following publications:

Sara, L., Antal, P., Masszi, G., Buday, A., Horvath, E.M., Hamar, P., Monos, E., Nadasy, G.L., Varbiro, S. *(2012)* Arteriolar insulin resistance in a rat model of polycystic ovary syndrome. *Fertility and Sterility. 97(2): 462-468. IF 3,958 (2010)*

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Valent S, Oláh O, Sára L, Pajor A, Langmár Z. *(2011)* Ultrasonography in the diagnosis of ovarian and endometrial carcinoma [Az ultrahangvizsgálat szerepe a méhtest és a petefészek rosszindulatú daganatainak diagnosztikájá ban] *Orvosi Hetilap. 152(47): 1887-1893.*

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