Vascular reactivity changes in a polycystic ovary syndrome rat model and the effect of vitamin D3

PhD Theses

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INTRUDUCTION

The first name of policystyc ovary syndrome was given Stein-Leventhal after the two gynecologysts from Chicago, who firstly reported cases with infertility and hirsutism, irreglular menstrual cycles, and enlarged ovaries, in 1935. Polycystic ovary syndrome is an elusive disease and due to its varied symptomatology, spanning multiple specialities, women often contact medical professionals only when problems presented by infertility arise. PCOS, there is much more frequently prevalent as diagnosed, but the known cases of women are affected at least 5-8%. It is a systemic disease which accompanies patients their whole lives and at different ages raises different problems. The relevance of the problem shows that 50-60% of women responsible for this disease of infertility as a consequence of anovulation. The most characteristic symptoms of the Rotterdam consensus was identified; dysmenorrhoea, oligo or anovulation, an ultrasound confirmed enlarged policystyc ovaries, and clinical or laboratory signs of androgen excess. The presence of these two out of three enough for the diagnosis raising. It is often associated with insulin resistance, abdominal obesity, acne, hirsutism, and increased cardiovascular risk. It is important to know, that every tenth PCOS patients forty years of age will have diabetes, and 30-40%-of women with PCOS impaired glucose tolerance can be diagnosed. The increased risk of cardiovascular diseases and their therapeutic potential are both in the forefront of interest in PCOS. Eliciting both environmental and genetic factors can be included. Since the etiological origins are still not fully understood, therefore we undertook this series of experiments. First the PCOS was modelled in Wistar rats then pharmacological responsiveness of aortic rings was tested to vasoconstrictor and vasodilator effects. We examined the relaxing effects of insulin and estrogen, and the measure of different cell damages. We looked for these effects of vitamin D, knowing that it is used as an adjuvant therapy in PCOS.

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Vascular changes in PCOS

Several studies have documented endothelial dysfunction in PCOS. The flow mediated vasodilatation, the increase of the pulse wave velocity, and the thickening of the vessel wall was demonstrated. These changes are known to be early signs of atherosclerosis. The fertile age women with PCOS, insulin resistance, metabolic syndrome, premature atherosclerosis and hypertension frequently develops and persists even after menopause. These phenomena are unfavorable for long term cardiovascular risk. The relaxing capacity is reduced in PCOS. It is similar to that experienced in diabetes mellitus. Insulin induced dilation mediated through endothelial NO production, via endothelial NO-synthase involving the contribution of the insulin receptor. Endothelium independent part of vasorelaxation works mainly via reducing Ca^{2+} concentration in smooth muscle cells. But the role of EDHF, endothelium derived hyperpolarising factor was proved with the contribution of Ca^{2+} activated potassium channel in vasodilatation. Life style changes may play an improving role not only in favour clinical benefits but in enhancing the vasodilator capacity as well. Rats on walking belt showed vasodilator effect of physical activity.

Vitamin D

Vitamin D has become to known as a hormone in the last two decades, which can have regulatory roles at several major point the organism. It is formed in the skin to sunlight, which is completed in the liver and kidney, or it enters with the food intake. The interrelation between vitamin D deficiency and polycystic ovary syndrome has been studied by many researchers. More than 40% of the European inhabitants are suffering from vitamin D deficiency, north along it reaches the 60%. Vitamin D deficiency is accompanied PCOS nearly 80% of the cases. It has been demonstrated that vitamin D deficiency is connected with the pathophysiology of metabolic syndrome. This presumption is certificated by the fact, that the gene vitamin D regulates more than 3% of human genome inter alia that controls the lipid and glucose homeostasis and blood

pressure. The insuline resistance of obese women with PCOS could be corrected by giving vitamin D. The therapeutic use of vitamin D caused lipid profile changes in better direction, even the menstrual cycle could have been corrected and infertility has been solved in some cases. In experimental conditions other diseases were tested and showed beneficial results by giving vitamin D to the animals; e.g. heart failure of spontaneous hypertensive rats was cured. In another experiment the reduction of endothelium dependent contractions developed and hypertension was diminished by giving vitamin D to spontaneous hypertensive rats. In PCOS, as the insuline resistance is one of the basic alterations vitamin D therapy is suggested by the literature and Hungarian consensus statement as an adjuvant therapy.

Other publication in the topic of dissertation theses (accepted, under publication)

Masszi G, Horvath EM, Tarszabo R, Benko R, Novak A, Buday A, Tokes AM, Nadasy LGY, Hamar P, Benyó Z, Varbiro S. (2012) Reduced Estradiol-Induced Vasodilation and Poly-(ADP-Ribose) Polymerase (PARP) Activity in the Aortas of Rats with Experimental Polycystic Ovary Syndrome (PCOS)
PLOS ONE DOI.10.1371/journal.pone.0055589 **IF: 4,092**

Other relevant publications of the author which support the thesis

Articles

1. Masszi G. (1997) A nők és az idősek hypertóniája. Orvostovábbképző Szemle IV(6):46-49
2. Masszi G. (1998)A hormonpótló kezelés és a hypertónia. Cardiologia Hungarica. 27(4):9-64.
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1. Masszi G. Cardiovascularis rizikó és védelem a menopausában. In: Cseh I, Dancsó J, Tóth K S. (szerk.), A menopausa időszerű kérdései. B + V Kiadó, Budapest, 2000:68-82.
2. Masszi G. A szív és érrendszeri megbetegedések prevenciója a változás kora után. In: Császár A, Masszi G (szerk.), Nők a változó korban. Harc a Női Szívekért Alapítvány, Budapest, 2011:121-139.

LIST OF PUBLICATIONS

The thesis is based on the following papers of the author

1. Masszi G, Buday A, Novak A, Horvath EM, Tarszabo R, Sara L, Revesz Cs, Benkő R, Nádasy Gy, Benyó Z, Hamar P, Varbiro Sz. (2012) Altered insulin induced relaxation of aortic rings in a dihydrotestosterone induced rodent model of polycystic ovary syndrome Fertil Steril. DOI.10.1016/j.fertnstert.2012.09.024 2./ **IF:3.564**

2. Masszi G, Novak A, Tarszabo R, Horvath EM, Buday A, Ruisanchez E, Tokes AM, Sara L, Benko R, Nadasy LGY, Revesz Cs, Hamar P, Benyó Z, Varbiro Sz. (2012) Effects of vitamin D3 on pharmacological reactivity of aorta rings in a rodent PCOS model Pharmacological Reports **IF:2,445**

Related publications

1. Sara L, Antal P, Masszi G, Buday A, Horvath EM, Hamar P, Monos E, Nadasy GL, Varbiro S. (2012) Arteriolar insulin resistance in a rat model of polycystic ovary syndrome. Fertil Steril. 97:462-468. **IF:3.564**

2. Sara L, Nadasy G, Antal P, Monori-Kiss A, Szekeres M, Masszi G, Monos E, Varbiro S. (2012b). Pharmacological reactivity of resistance vessels in a rat PCOS model - vascular effects of parallel vitamin D3 treatment. Gynecol Endocrinol. (doi:10.3109/09513590.2012.683079) **IF:1,461**

3. Sára L, Nádasy GyL, Antal P, Szekeres M, Monori-Kiss A, Horváth EM, Tőkés AM, Masszi G, Monos E, Várbíró Sz. (2010) Arteriolar biomechanics in a rat polycystic ovary syndrome model - Effects of parallel vitamin D3 treatment Acta Physiol Hung 99:(3) pp. 279-288. **IF:0.821**

AIMS

Our research aimed to analyze the significantly early changes in vascular reactivity on large conduit vessels, on the part of thoracic aortas in a proven PCOS rat model using 70 days of continuous dihydrotestosterone treatment. In that stage, when hypertension has not been developed yet, but insulin resistance, the characteristic metabolic alteration had been already occurred. We examined, whether vitamin D has positive effect on these? Our presumption was that already in the early stages of PCOS the vascular reactions change unfavorably, and vitamin D or estrogen itself locally applied could enhance it. We were curious about PARP activity to detect the early vascular injuries, and whether these could be influenced by an endogenous PARP inhibitor, vitamin D?

METHODS

Thirty adolescent, 21-28 day-old, female Wistar rats, weighing 100-140 g were purchased from Semmelweis University Animal Colony (Budapest, Hungary, originated from Charles River Ltd). The animals were randomized into 3 treatment groups: control, DHT and DHT+vitamin D_3. No medical or surgical complications were observed throughout the whole period. Conventional rat chow and tap water were provided ad libitum. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Ex vivo pharmacological reactivity of thoracic aortic rings

After opening the chest, the 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. The distal part of the thoracic aorta (TA) was isolated, and eight rings were prepared and placed into a vessel chamber filled with nKR solution aerated with carbogen. Segments of the thoracic aorta of 3 mm length from each experimental group were mounted on the 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 normal Krebs Ringer solution. The bath was warmed to 37°C, and the resting tension of the thoracic aorta segments was 15 mN.

Insulin induced vasorelaxation

After giving 5 x 10^{-8}M norepinephrine to generate precontraction the insulin-mediated vasorelaxation was tested by the administration of increasing concentrations of insulin (50-150-300-600 mU/ml) in pre-contracted (5x10^{-8}M norepinephrine) vessels. Afterwards, 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 different potential pathways of relaxation. Indomethacin blocks the synthesis of all

Estrogen

DHT treatment changed the relaxation capacity of aortic rings. The relaxation capacity of E_2 was significantly reduced in a hyperandrogenic environment and this was not affected by vitamin D_3 treatment. The above mentioned results made it possible to reveal the main mechanism of cardiac and vascular alterations in PCOS.

PARP

The damaging effects of PCOS brought on by DHT pretreatment revealed themselves partly in the intensification of PARP activity (ovaries and leukocytes) and this unfavourable state was completely reversed by vitamin D_3 treatment in these tissues. In contrast, PARP activity was reduced in the aortic walls, both in the endothelium and smooth muscle cells after DHT treatment and vitamin D_3 administration did not change this result.

In conclusion, we can state that in an adequate rat model for PCOS the vascular reaction of large blood vessels changed in an altogether negative direction even at the earliest functional changes and vitamin D_3 treatment may have a partial positive effect on these changes.

CONCLUSION

Insulin

Our research was the first to show reduced insulin-dependent vasorelaxation capacity on aortas under hyperandrogenic conditions and the lack of the effect of vitamin D on this. The androgen effect-caused decrease in relaxation, as well as the relaxation brought on by insulin, proved to be partly NO-dependent. Vitamin D_3 had an altogether neutral effect on the aorta. This can be explained in relation to insulin caused vasorelaxation, by local constrictor prostanoid effects that counteracted the moderate improvement of NO-independent relaxation caused by vitamin D treatment.

Variances in vasoreactivity

Our research was the first to show that NA caused increase in the contraction of the aorta in a rat PCOS model is reduced and partly counteracted by a concurrent vitamin D_3 treatment. In a clinical sense, vitamin D_3 can have a preventive effect on prehypertensive changes. We showed that relaxation caused by ACh is also damaged in PCOS and this is partly corrected by vitamin D_3 treatment. We think that the correction is only partial because of local prostanoid effects (L-NAME and indomethacin incubation), which almost fully neutralize the NO-dependent relaxation capacity intensified by vitamin D_3.

Our results allow us to conclude that hyperandrogenic states cause prehypertensive blood vessel states and this can lead to a decrease in vasorelaxation capacity. That might have a role in the clinical manifestation of PCOS, which spurs us to consider an early intervention to reduce cardiovascular risk. Vitamin D_3 treatment can regenerate the raised levels of vasoconstrictor activity brought on by a hyperandrogenic environment. An important conclusion of our research is that adjuvant vitamin D_3 treatment in PCOS can block prehypertensive damage in large blood vessels.

prostanoids (including prostacyclin and thromboxane), L-NAME is a general inhibitor of NO synthases (i-NOS, e-NOS, c-NOS), so we could analyze more precisely the mechanisms.

Measurements of the vasoreactivity

Segments were exposed to 124 mM K^+ to elicit a reference contraction. Twenty minutes later, precontraction was induced by norepinephrine ($5x10^{-8}$M) and endothelial relaxation was tested using 10^{-8}-10^{-5}M acetylcholine. 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 Indometacin, or 10^{-4}M L-NAME for 20 minutes, and norepinephrine and acetylcholine dose-response measurements were repeated in order to test different potential pathways of relaxation.

Relaxation caused by estradiol

The vasorelaxing effect of estradiol was measured on the precontracted aortic rings, the precontraction was caused by ($5x10^{-8}$M) norepinephrine. A 20-minute time interval was set for the effects of each concentration administered to set in. Dose effect curves were measured, using percentages of the rate of relaxation in relation to the relaxation of precontracted blood vessels.

Histological examination

The ovaries and other tissues were fixated in a fresh state for the histological examination on the day of extraction. We looked for histological signs of PCOS using a light microscope and haematoxylin-eosin staining after immersion fixation, the samples were stored in a 4% formaldehyde solution. The thickness of the aortic walls was measured using histological examinations as well. ‘Pannoramic viewer’ (3DHISTECH Ltd. Budapest, Hungary) software was used to evaluate the results.

Immunohistochemistry

Leukocytes were collected from the earlier drawn venal blood using Histopaque-1083 (Sigma Aldrich, St. Louis, USA). Methanol fixation smears were prepared from the cell suspension. Ovary and aortic samples were formalin fixated and embedded in paraffin then 5 micrometer thick slices were made of the samples. After deparaffination and antigen exploration monoclonal mouse anti-PAR antibodies were used to show poly(AdenosinDiPhosphate-Ribose) (PAR). Secondary labeling was achieved using biotinylated anti-mouse horse antibody. Nickel-enhanced diaminobenzidine (DAB) was used to visualize the labeling. A semiquantitive scoring scale was used for the evaluation of poly(ADP-ribose) stained smears, for the description of PAR activity (score from 1-10).

Statistics

Dose-response curves were evaluated using repeated measures ANOVA. Discrete parameters were analyzed by one way ANOVA. Newman Keul's analysis was used as a post hoc test. $P<0.05$ was the cutoff for significance.

RESULTS

Reactions to insulin

Our research led us to find that insulin-dependent relaxation in the PCOS model was significantly reduced in spite of vitamin D administration. Both the NO-dependent and the NO-independent routes were damaged. The vasorelaxation improving effect of vitamin D was altogether neutralized by the increase of prostanoid-dependent vasoconstriction.

Vasoreactivity (NA, ACh) responses

Our analysis of vasoreactivity led us to find that blood vessels exposed to DHT had an increase in vasoconstrictor capacity in response to noradrenaline – this effect was partly reduced by vitamin D, and at the same time the vasorelaxation was reduced, which was somewhat improved by vitamin D. The decrease in NO-independent vasorelaxation was almost fully compensated by vitamin D, but this effect was reduced by prostanoid-dependent vasoconstrictive effects.

Blood vessel responses to estradiol administration

The vasodilator effect of estradiol in PCOS on DHT-affected aortic rings was significantly reduced, and although NA-caused vasoconstriction was reduced and vasorelaxation improved by vitamin D; the vasorelaxation effect on DHT-affected blood vessels was not corrected.

Measurement of PARP activity

PARP activity was shown using poly-(ADP-ribose) immunostaining. PARP activity increased in PCOS-affected ovaries and circulating leukocytes; and this was significantly reduced by vitamin D. At the same time the PAR-staining was reduced in the aortic wall endhotelium and smooth muscle cells and this effect was not further affected by vitamin D administration.