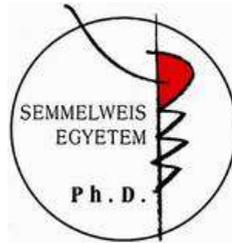


Endothelial dysfunction in experimental modells of atherosclerosis and polycystic ovary syndrome

Ph.D. Thesis

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INTRODUCTION

Endothelial dysfunction plays a key role in the pathogenesis of hypertension, atherosclerosis and vascular complications of diabetes. Endothelial dysfunction is often caused by oxidative stress, the acute and chronic overproduction of reactive oxygen species (ROS). Nitric oxide (NO) first described as endothelium-derived relaxing factor (EDRF) mediates relaxation of the vascular wall and is highly vulnerable to ROS as ROS reduce the bioavailability of NO leading to endothelial dysfunction. Besides NO scavenging, ROS induce inflammatory phenotype changes of the endothelium leading to the expression of inflammatory mediators and adhesion molecules. Oxidative stress-induced endothelial dysfunction is a predominant factor in the pathomechanism of atherosclerosis and hypertension and dramatically increases the risk of cardiovascular disease.

Cytokines released during these phenotypic changes of the endothelium are well known to stimulate cell-mediated inflammatory processes which result in structural changes characteristic to atherosclerosis. The role of Transforming Growth Factor β (TGF- β) in the development of atherosclerosis is controversial. TGF- β is assumed to be an atheroprotective cytokine due to its anti-inflammatory and lipid accumulation inhibitory effects. On the other hand, inhibiting endothelial regeneration, stimulating vascular oxidative stress and fibrotic process, TGF- β is shown to have atherogenic effects. Thus, the role of TGF- β in the initiation and progression of atherosclerosis is still unclear.

Oxidative stress-induced NO-deficient endothelial dysfunction is also a typical phenomenon in Polycystic Ovary Syndrome (PCOS) accompanying insulin resistance. PCOS is characterized by enlarged, cystic ovaries, driven by androgen overproduction. Hyperinsulinaemia and insulin resistance, appearing already in the very early stage of PCOS, substantially influence later disease progression. Cardiovascular complications and T2DM develop significantly earlier in patients with PCOS than in the normal female population.

The prevalence of vitamin D deficiency in PCOS is 72%. After vitamin D and parallel calcium administration menstrual disturbances recovered in majority of patients. Furthermore vitamin D deficiency correlated with elevation of several metabolic risk factors (BMI, fasting glucose, CRP, insulin, triglyceride) in PCOS and vitamin D supplementation normalized these parameters. Vitamin D deficiency is also a pathogenic factor in the development of insulin resistance in PCOS, but the eventual beneficial effects of vitamin D have not been demonstrated before.

AIMS

1. Examination of endothelial dysfunction in the presence and absence of specific vasoactive reagents in aorta rings of an experimental mouse model with elevated serum TGF- β .
2. Examination of ROS production in aorta of an experimental mouse model with elevated serum TGF- β .
3. Examination of long-term effects of elevated serum TGF- β on blood pressure, aortic and myocardial morphology.
4. Examination of insulin-induced relaxation in aortas of a rat PCOS model in the presence and absence of specific inhibitors.
5. Examination of possible modulatory role of vitamin D in a PCOS rat model.

METHODS

Elevated systemic TGF- β mouse model:

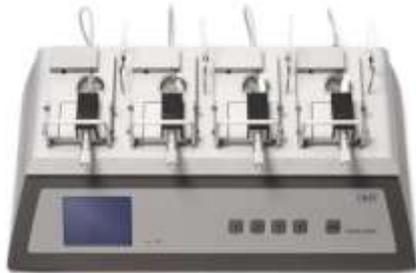
We examined the effect of high TGF- β blood level on endothelial dysfunction and atherosclerosis on a crossbred, TGF- β transgenic x apolipoprotein knockout (TGF- β -TGxAPO-E-KO) mice with elevated TGF- β levels and atherosclerotic background. APO-E-KO and TGF- β -TG mice and the background C57BL/6 mice were used as controls.

PCOS rat model:

We induced PCOS on twenty 21-28 days old female Wistar rats by 70 days long dihydrotestosterone (DHT) treatment. Half of the DHT treated animals received parallel vitamin D treatment as well. Other ten animals were sham operated.

Examination of endothelial function on mouse aortas:

Endothelium-dependent relaxation was measured on isolated aortic rings of 4 month old mice by miograph before and after incubation with NOX inhibitor (apocynin) and ROS scavenger (SOD).



1. figure: 610-M Multi Myograph System

Superoxide detection with dihydroethidine staining in mice:

Superoxide production was detected on isolated aortic rings of 4 month old mice by dihydroethidine (DHE) and Hoechst staining. Images were obtained from each layer by confocal microscopy and compared by software.

In vivo measurement of blood pressure:

Carotis was cannulated and in vivo blood pressure was measured on 8 month old, anesthetized mice.

Aortic and myocardial morphology:

Wall/lumen ratio was measured on aortic sections isolated from 8 month old mice. Semi thin sections from myocardium of 8 month old mice were analyzed according to orientator method capillary volume density and fibrocyte volume density were compared.

Analysis of atherosclerotic lesions:

Atherosclerotic lesion severity was assessed using en face preparations of descending aortas isolated from 8 month old mice. Stained plaques were quantified by microscopy and computer-aided morphometry.

Oral Glucose Tolerance Test (OGTT), and insulin levels in PCOS rat model:

After 8 weeks of DHT treatment, OGTT was performed under ether narcosis. Blood glucose and plasma insulin were measured following overnight fasting and 120 minutes after oral glucose load of 0.3g/100 g body weight given by gavage.

Insulin-induced relaxation of aorta rings in PCOS rat model:

Insulin-induced relaxation was measured on isolated aortic rings of PCOS rat model by miograph. After incubation with NOS inhibitor (L-NAME) and prostanoid blocker (indomethacin) relaxations were measured again.

RESULTS

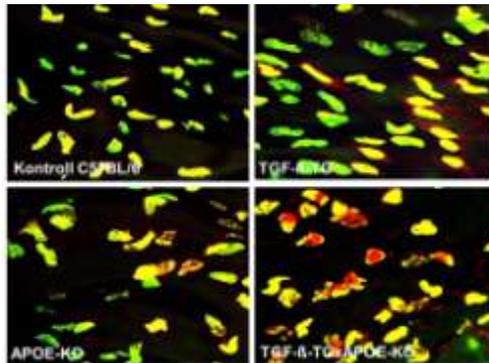
Effects of elevated systemic TGF- β levels:

Endothelium-dependent relaxation:

Endothelial relaxation was significantly impaired in TGF- β -TGxAPO-E-KO mice compared to the controls but significantly improved after incubation with apocynin or SOD.

Superoxide production:

Intense DHE staining was observed in the TGF- β -TGxAPO-E-KO mice compared to controls.



2. figure: DHE staining in mice aorta

In vivo blood pressure:

Blood pressure was significantly elevated in APO-E-KO compared to control (C57BL/6) mice. TGF- β -TGxAPO-E-KO animals had the highest blood pressure significantly higher compared to APO-E-KO.

Aortic and myocardial morphology:

Aortic wall/lumen ratio was significantly elevated and capillary length density was lower in TGF- β -TGxAPO-E-KO mice. Relative fibrocyte volume was higher in animals with TGF- β transgene compared to C57BL/6 and APO-KO mice.

Plaque formation:

Significant staining was observed in APO-E-KO mice. Plaque area and staining intensity demonstrated more prominent atherosclerotic lesions in TGF- β -TGxAPO-E-KO mice compared to APO-E-KO animals.

Endocrine and vascular effects of polycystic ovary syndrome in rats:

Glucose and insulin levels:

Blood glucose levels and fasting insulin levels did not significantly differ among the experimental groups. However, DHT-treated animals had higher plasma insulin levels than controls. Vitamin D treatment eliminated the insulin resistance of DHT-treated mice.

Insulin-induced relaxation after DHT treatment:

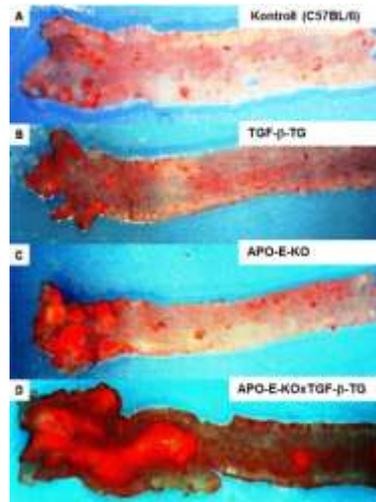
Insulin-induced relaxation was significantly lower in DHT-treated groups compared to controls. Vitamin-D treatment had no significant effect on insulin-induced aorta relaxation.

Insulin-induced relaxation after L-NAME incubation:

L-NAME incubation reduced insulin-induced relaxation in all experimental groups, with strongest reduction in the control group. Vitamin D partially restored the loss of insulin-induced vasorelaxation in the presence of L-NAME.

Insulin-induced relaxation after indomethacin incubation:

Insulin-induced relaxation was restored by indomethacin in all experimental groups but was still lower in the DHT-treated group compared to controls. Vitamin D partially restored the loss of insulin-induced vasorelaxation in the presence of indomethacin.



3. figure: Plaque formation in mice aorta

DISCUSSION

The role of elevated systemic TGF- β in the development of endothelial dysfunction and atherosclerosis:

Recent studies provided novel evidence for TGF- β - induced ROS production and cytoskeletal alterations in human endothelial cells and that oxidative stress can lead to vascular endothelial damage and dysfunction adds a new dimension to our knowledge regarding the role of TGF- β in atherosclerosis. Our study demonstrated that systemic overexpression of TGF- β was associated with increased vascular ROS production and endothelial dysfunction in the APO-E knockout mouse model of atherosclerosis. Further, we have also demonstrated that NADPH oxidase activation contributed to TGF- β -induced endothelial impairment associated with more severe atherosclerotic plaque formation, more severe hypertension and fibrosis of the heart.

In this study, isolated aortic rings of 4 months old APO-E-KO mice with elevated plasma TGF- β had impaired endothelial relaxation suggesting that endothelial dysfunction of APO-E-KO mice is exacerbated by elevated systemic TGF- β . Furthermore, NOX inhibition with apocynin and scavenging ROS by superoxide dismutase (SOD) partly improved endothelial function, suggesting that a stimulated NOX system is responsible for impaired endothelial relaxation in TGF- β -TGxAPO-E-KO mice.

In the background of significantly inhibited endothelium dependent vascular relaxation we observed enhanced superoxide formation. Our data provide evidence, that NADPH oxidase induced ROS production is responsible for endothelial dysfunction in case of high systemic TGF- β levels.

Vascular endothelial dysfunction plays a pivotal role in the pathogenesis of atherosclerosis and hypertension. In our study overproduction of circulating TGF β_1 elevated blood pressure, markedly increased plaque formation and induced NOX mediated myocardial hypertrophy and fibrosis in TGF- β -TGxAPO-E-KO mice.

Interestingly in our study high systemic TGF- β_1 levels alone were not sufficient to significantly inhibit endothelium-

dependent relaxation of the aorta in non-APO-E-KO, TGF- β -TG control mice. A possible explanation is that an ongoing vascular wall injury in APO-E-KO mice, such as oxidized LDL (oxLDL) and other lipid deposition and consequent inflammation may be necessary for the deleterious effects of TGF β to develop.

Our present study may explain some recent findings and may offer TGF- β as a new therapeutic target in human subjects, where atherosclerosis has been associated with elevated systemic TGF- β levels.

Vascular insulin resistance in DHT-induced PCOS model and potential modulatory role of vitamin D:

A validated rat model of polycystic ovary syndrome was used by induction of PCOS in rats with 10 weeks of dihydrotestosterone treatment. Similarly to human PCOS increased androgen levels and development of several metabolic, hormonal and ovarian abnormalities was observed in the rat model.

As a sign of metabolic disorders the two-hour insulin value of the oral glucose tolerance test was nearly threefold higher in the DHT-treated animals than in controls, demonstrating insulin resistance. Vitamin D supplementation completely reversed insulin resistance. Considering these facts, our experimental model was suitable for studying early initial abnormalities and vascular damage. DHT treatment reduced insulin-dependent relaxation of rat aortic rings. This loss of insulin-dependent dilatation is the vascular form of insulin resistance. In contrast to our previous study that demonstrated reversal of insulin-dependent vasorelaxation of small arteries, insulin resistance of aortic rings was not reversed by vitamin D.

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. Vitamin D treatment in the presence of L-NAME limited the deterioration of NO independent insulin relaxation induced by the DHT treatment, but did not alter NO-pathway significantly.

These results suggest that insulin relaxation is probably

NO independent in the gracilis arterioles but dominantly NO-dependent in the aorta. Thus, vitamin D could restore normal vascular tone independently from the NO system only in small vessels.

In agreement with previous studies we observed mild vasorelaxant effects of the cyclooxygenase inhibitor indomethacin in aortas of all groups. Interestingly, following indomethacin pretreatment loss of insulin-dependent vasorelaxation by DHT was partially reversed by vitamin D.

Taken together, we suggest that the local effect of vitamin D treatment in the aortic wall is a partial increase of NO-independent relaxation and increase in constrictor prostanoid effects. This simultaneous stimulation of both constrictory and relaxing effects may explain the lack of significant effect of vitamin D in aortas.

CONCLUSION

In conclusion, elevated circulating TGF- β in APO-E-KO mice but not in C57BL/6 normal mice induced endothelium mediated vasomotor dysfunction, through stimulation of NADPH-oxidase derived oxidative stress. Furthermore, high circulating TGF- β was associated with aortic wall thickening, acceleration of plaque formation, and consequent hypertension, with myocardial hypertrophy and fibrosis. Thus, TGF- β may exacerbate the ongoing inflammation in an atherosclerotic surrounding in APO-E-KO mice. This mechanism may provide a link between systemic overproduction of TGF- β and acceleration of atherosclerosis.

In dihydrotestosterone induced polycystic ovary syndrome in rats, systemic and vascular insulin resistance developed. Vitamin D supplementation reduced systemic but not vascular insulin resistance. The diminished aorta relaxation caused by the androgenic effect was partially NO-dependent. We propose, that the net neutral effect of vitamin D on PCOS rat aortas was due to the opposing effects of local constrictor prostanoids and a moderate alteration of the NO-independent relaxation.

PUBLICATIONS RELATED TO THE DISSERTATION

Buday A, Orsy P, Godo M, Mozes M, Kokeny G, Lacza Z, Koller A, Ungvari Z, Gross ML, Benyo Z, Hamar P **Elevated systemic TGF-beta impairs aortic vasomotor function through activation of NADPH oxidase-driven superoxide production and leads to hypertension, myocardial remodeling, and increased plaque formation in apoE(-/-) mice** AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY 299:(2) pp. H386-H395. (2010) **IF: 3.880**

Masszi G*, Buday A*, Novak A, Horvath EM, Tarszabo R, Sara L, Revesz C, Benko R, Nadasy GL, Benyo Z, Hamar P, Varbiro S (* megosztott elsőszerek) **Altered insulin-induced relaxation of aortic rings in a dihydrotestosterone-induced rodent model of polycystic ovary syndrome.** FERTILITY AND STERILITY 12: pp. S0015-0282. (2012) **IF: 3.775**

FURTHER PUBLICATIONS

Sara L, Antal P, Masszi G, Buday A, Horvath EM, Hamar P, Monos E, Nadasy GL, Varbiro S **Arteriolar insulin resistance in a rat model of polycystic ovary syndrome.** FERTILITY AND STERILITY 97:(2) pp. 462-468. (2012) **IF: 3.775**

Masszi G, Novak A, Tarszabo R, Horvath EM, Buday A, Ruisanchez E, Tokes AN, Sara L, Benko R, Nadasy GL, Revesz C, Hamar P, Benyo Z, Varbiro S **Effects of vitamin D+ derivate calcitriol on pharmacological reactivity of aortic ring sin a rodent PCOS model** PHARMACOLOGICAL REPORTS 2012 (*in press*)

Gabriella Masszi, M.D.; Robert Tarszabo; Eszter Maria Horvath; Rita Benko; Agnes Novak; Anna Buday; Anna Maria Tokes; Levente Sara; Gyorgy L Nadasy; Peter Hamar; Zoltán Benyó; Szabolcs Varbiro. **Reduced Estradiol-Induced Vasodilatation and Poly-(ADP-Ribose) Polymerase (PARP) Activity in the Aortas of Rats with Experimental Polycystic Ovary Syndrome (PCOS)** PLOS ONE 2012 (*in press*)

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