Summary of Doctoral (Ph.D.) Thesis

Effects of type 2 diabetes mellitus on microvascular function

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1.INTRODUCTION

1.1. Cardiovascular diseases and microvascular dysfunction in type 2 diabetes mellitus

Type 2 diabetes mellitus (T2-DM) has reached epidemic level in the Western countries, including Hungary, and it is associated with a markedly increased incidence of cardiovascular diseases, accounting for ≈70% of deaths in the diabetic population. The chance of having myocardial infarction in a type 2 diabetic patient is the same to have a second myocardial infarction. Development of macrovascular diseases, such as atherosclerotic plaque formation and atherothrombosis, are common among patients with diabetes mellitus contributing to the increased cardiovascular risk. Previous studies revealed that even before the atherosclerosis of the large vessels, the microvascular function is altered, although the pathomechanism is not known. In type 2 diabetes microvascular dysfunction causes nephropathy, neuropathy, retinopathy, cerebral and myocardial microcircular disturbances. These alterations increase the morbidity and mortality of type 2 diabetic patients. Alterations in local vasoregulatory mechanisms intrinsic to the vascular wall, such as enhanced pressure-induced arteriolar tone and reduced endothelium-dependent dilation, have been reported previously as characteristics of T2-DM. Changes in the local vasoregulatory mechanisms of peripheral microvessels may have significant influence on tissue perfusion, vascular resistance and systemic blood pressure in T2-DM; however, the possible underlying mechanisms are still open to question.

1.2. Endothelial dysfunction in type 2 diabetes mellitus

In the recent decades, it has become evident that the endothelium is not only a passive inner lining of blood vessels. This 'organ' with a large surface (approximately 350 m^2) and a comparatively small total mass (approximately 110 g) is actively involved in vital functions of the cardiovascular system,

including regulation of perfusion, fluid and solute exchange, haemostasis and coagulation, inflammatory responses, vasculogenesis and angiogenesis. The endothelial cells synthesize different vasodilator and vasoconstrictor factors in response to physiological stimuli to maintain tissue perfusion. The most important vasodilators are the NOS-derived nitrogen-monoxide (NO), the cyclooxygenase (COX)-1 and 2-derived prostacyclin (PGI_2), and the endothelium-derived hyperpolarizing factor (EDHF). The relative contribution of the different vasodilators varies in the different vascular beds. The endothelial cells also produce vasoconstrictor factors. These are endothelin, angiotensin II and the COX-1 and COX-2-derived constrictor prostaglandins, prostaglandin H_2/tromboxane A_2 (PGH_2/TXA_2). When the physiological function of the endothelium is damaged, endothelial dysfunction develops. In endothelial dysfunction the delicate balance between vasodilator (NO, PGI_2, EDHF) and vasoconstrictor (endothelin-1, angiotensin II, and vasoconstrictor prostanoids) is disturbed. Previous studies have demonstrated that endothelial dysfunction of microvessels is an early manifestation of vascular complications, which may lead to disturbed regulation of tissue perfusion, predisposing diabetic patients to tissue ischemia, as well as early development of hypertension. A reduced endothelium-dependent arteriolar vasodilation and/or an enhanced smooth muscle–dependent vasoconstriction in microvessels has been demonstrated in T2-DM, alterations that could influence total peripheral resistance and increase myogenic tone.

1.3. Alterations in the myogenic tone in type 2 diabetes

In the microvessels another important mechanism regulating vascular resistance is the myogenic response. Elevation of the systemic blood pressure leads to smooth muscle mediated vasoconstriction of the microvessels. This is called myogenic response. If vasoconstrictors are synthesized in the vascular wall, the myogenic tone increases, whereas in response to endothelium-derived

vasodilators it decreases. A key role for altered regulation of microvascular resistance has been suggested by several earlier investigations that found specific impairment of microvascular vasoregulatory mechanisms in subjects with diabetes, mostly type 1 diabetes. Previous studies reported decreased myogenic tone of the microvessels in type 2 diabetes. On the other hand in the advanced stage of the disease the agonist-induced vasoconstriction and the pressure-induced myogenic tone were reported to be increased. It seems that the stage of diabetes affects the alterations of the myogenic tone, although the underlying mechanisms have not yet been fully elucidated.

1.4. Vasomotor dysfunction and oxidative stress in type 2 diabetes

It is now well established that moderate amounts of reactive oxygen species (ROS) play an important role in signal transduction processes, such as cell growth and posttranslational modification of proteins. An excessive and/or sustained increase in ROS production damages different macromolecules (DNA, proteins, lipids and carbohydrates), and causes morphological and functional damage of the cells. This phenomenon is called oxidative stress. Increased vascular ROS production has been implicated in the pathogenesis of certain diseases. Vascular ROS are typically generated by tightly regulated enzymes such as NO synthase (NOS) and NAD(P)H oxidase and xanthine oxidase. Vascular ROS may impair endothelial and smooth muscle function of the microvessels, leading to endothelial dysfunction. Excessive amounts of ROS inactivate NO, leading to decreased bioavailability of NO. ROS may also play an important role in regulating vascular smooth muscle function, by activating smooth muscle migration, apoptosis leading to vascular remodeling. Previous studies revealed that type 2 diabetes is associated with oxidative stress. Oxidative stress alters the endothelial and smooth muscle function of the microvessels, however the possible underlying mechanisms behind it are still not known.

1.5. Altered prostanoid synthesis in type 2 diabetes

In previous studies it has been suggested that a low-grade vascular inflammation contributes to vascular diseases in certain pathological conditions. Among other factors, prostaglandins (PGs) are important mediators of several inflammatory mechanisms; however, it is also known that many PG derivatives have specific vasoactive properties, thereby contributing to the local regulation of arteriolar diameter. Two isoforms of the COX enzyme, encoded by distinct genes, have been isolated in mammalian cells. COX-1 is constitutively expressed in most tissues, such as vascular endothelial cells, and is involved in the maintenance of cellular homeostasis. In contrast, under normal conditions, COX-2 is expressed only at low or undetectable levels but is readily upregulated by inflammatory, mitogenic, and physical stimuli. Only a limited number of biochemical studies have investigated alterations in COX-2–dependent mechanisms related to DM, and the changes in microvascular function. Recent studies suggested that oxidative stress plays an important role in the induction of chronic inflammation within the vascular wall in type 2 diabetes although the underlying mechanisms remained obscure.

1.6. The vasoactive effects of levosimendan and OR-1896

Levosimendan is a Ca^{2+}-sensitizing cardiotonic agent. Besides its direct positive effects on the myocardial contractility, part of the hemodynamic benefit following levosimendan administration has been ascribed to vasodilation in the peripheral vessels. Levosimendan causes vasodilation both in the arteries and in the veins. It has an elimination half-life of only 1 h, whereas that of OR-1896 is about 75–80 h. Moreover, 40% of OR-1896 is bound to plasma proteins, as compared with 98% for levosimendan, and thus the active metabolite has a considerably larger free fraction than that of the parent drug. These pharmacokinetic features provide plausible explanations for the prolonged

hemodynamic effects of levosimendan metabolites, which last for up to 7–9 days after discontinuation of a 24-h infusion of levosimendan. Levosimendan activates various potassium channel types leading to smooth muscle cell hyperpolarization and vasodilation.

Potassium channels play an important role in the regulation of microvascular diameter. Although levosimendan has been suggested to exert vasodilation in the large conductance vessels, no experimental studies have as yet addressed the effects of levosimendan and OR-1896 on the microvascular dynamics.

2. AIMS

Based on the previous research described in the *Introduction* the following aims were defined:

1. To investigate the alterations in the endothelium and smooth muscle vasomotor function of the resistance vessels in different animal models of type 2 diabetes mellitus.

2. To reveal the underlying mechanisms responsible for microvascular vasomotor dysfunction and to investigate the specific role of oxidative stress and altered vascular prostanoid synthesis.

3. To characterize the direct microvascular effects of OR-1896, the levosimendan metabolite, which compound may have a potential role in the therapy of microvascular dysfunction in certain pathological conditions, such as type 2 diabetes.

3. METHODS

3.1. Animal models of type 2 diabetes

Diet-induced type 2 diabetes

Male Wistar rats were maintained on standard rat chow or on a high-fat diet (HFD) for 10 weeks. Wistar rats on normal laboratory diet served as controls.

Genetic model of type 2 diabetes

In the second series of experiments, a well characterized mouse model of type 2 diabetes mellitus was used. In the absence of a functional leptin receptor gene, the duration of food intake increases, and the homozygous db/db mice develop metabolic alterations similar as seen in type 2 diabetes mellitus.

3.2. Determination of blood pressure and calculation of total peripheral resistance

Determination of blood pressure

The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium, and the left common carotid artery was cannulated for continous monitoring of mean blood pressure with a physiological pressure transducer.

In conscious mice, systolic and diastolic blood pressures and heart rate were measured by the tail-cuff method and mean arterial pressure was calculated.

Echocardiography

Left venticular wall thickness, left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular diastolic area (LVDA), and left ventricular end systolic area (LVSA) was obtained in conscious control and db/db mice using echocardiography. Cardiac output data, and total peripheral resistance was also calculated.

3.3. Analytical procedures

The plasma level of total cholesterol, glucose and insulin was measured.

3.4. Isolation of gracilis muscle arterioles

With the use of microsurgical instruments and an operating microscope we isolated the gracilis muscle arteriole running intramusculary, which were then canulated and transferred into an organ chamber containing oxygenated physiological salt solution (T=37^{o}C, pH=7.4; O_2: 10%, CO_2: 5%, N_2: 85%). During an incubation period of 1 hour, a spontaneus myogenic tone developed in the isolated arterioles in response to the intraluminal pressure of 80 mmHg. Images of vessels were contiuously collected by a digital camera connected to a microscope, and the internal diameter at the midpoint of the isolated arteriole was measured offline.

3.5. Experimental protocols on isolated microvessels

Measurement of vasodilation using vasoactive agents in isolated rat garcilis arterioles

In the first series of experiments, cumulative concentrations of the endothelium-dependent dilator ACh, histamine, and the endothelium-independent dilator sodium nitorprusside were administered to the skeletal muscle arterioles and changes in the diameter were measured. The ACh- and histamine-induced arteriolar responses were observed in the presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine-methyl ester. Arterioles of control and HFD rats were also incubated in the presence of superoxide scavenger Tiron, and arteriolar responses were obtained again. The source of superoxide was examined using xanthine oxidase inhibitor allopurinol, or NAD(P)H oxidase inhibitor apocynin, agonist-induced arteriolar responses were obtained again in the presence of these inhibitors.

Arteriolar tone as a function of pressure in db/db mice

After 1 hour incubation period, spontaneous basal arteriolar tone developed in response to 80 mmHg intraluminal pressure. Changes in the diameter of arterioles were then measured in response to stepwise increases in intraluminal pressure from 20 to 120 mmHg in calcium-free (passive diameter), and calcium-containing (active diameter) PSS. Normalized arteriolar diameter (in calcium-containing PSS) was expressed as a percentage of corresponding passive diameters (in calcium-free PSS). Arterioles were incubated with catalase, PGH_2/TXA_2 antagonist SQ-29548, selective COX-1 inhibitor SC-560 or selective COX-2 inhibitor NS-398, and the pressure induced diameter changes were reassessed.

Arteriolar responses to exogenous H_2O_2

H_2O_2 was applied to the organ chamber and the diameter changes were recorded. H_2O_2-induced responses were also obtained after endothelium-denudation or in the presence of the PGH_2/TXA_2 receptor antagonsit SQ-29548.

Arteriolar responses to levosimendan and OR-1896

Cumulative concentrations of levosimendan or OR-1896 were administered to the rat gracilis muscle arterioles and the changes in diameter were measured. To assess the contribution of K^+ channels to the OR-1896-mediated vascular responses arterioles were incubated in the presence of the nonselective K^+ channel blocker tetraethylammonium (TEA), the selective K_{ATP} channel blocker glibenclamide, the selective BK_{Ca} channel blocker iberiotoxin (IBTX), or the selective K_V channel blocker 4-aminopiridine, and the arteriolar responses to cumulative concentrations of OR-1896 were then obtained again.

3.6. Quantification of reactive oxigen species

Quantification of vascular superoxide production by lucigenin-enhanced chemiluminescence assay

Vascular superoxide porduction was assessed in carotid arteries isolated from control and HFD rats by lucigenin-enhanced chemiluminescence. Scintillation counts were also obtained after the addition of sueproxide scavenger Tiron. In separate portocols carotid arteries were incubated with NADH or xanthine, allowing an estimation of the stimulated amount of superoxide porduced by the NAD(P)H oxidase or xanthine oxidase.

Detection of H_2O_2 by fluorescence

Dichlorodihydroflourescein (DCHF) was used to assess the vascular production of H_2O_2 in control and db/db carotid arteries.

3.7. Immunohistochemistry

Aceton fixed consecutive sections (10 μm thick) were made from pieces of embedded garcilis muscles from control and HFD rats. These were immunolabeled with monoclonal antibody against xanthine oxidase.

3.8. Western immunoblot

COX-1 and COX-2 protein expression was determined from control and db/db mice aortae. Anti- β-actin IgG was used for loading control.

3.9. Statistics

Statistical analyses were performed by a one way ANOVA, followed by the Tukey post hoc test. $P<0,05$ was considered statistically significant.

4. RESULTS

4.1. Diet-induced type 2 diabetes mellitus

Metabolic and other parameters

The body weight of HFD rats became significantly greater than those of controls fed the standard diet. Serum glucose, insulin, total cholesterol levels, and arterial blood pressure were significantly elevated in HFD rats compared with controls.

Agonist-induced arteriolar responses

In gracilis muscle arterioles isolated from control and HFD rats, there were no significant differences between the active and passive diameters at 80 mmHg intraluminal pressure. In arterioles of HFD rats, dilations in response to cumulative doses of ACh and histamine were significantly decreased compared with those of control vessels. Arteriolar responses to the NO donor SNP were not different between vessels of control and HFD rats. Inhibition of NO synthesis by L-NAME decreased ACh- and histamine-induced dilations in control arterioles, whereas it had no effect on ACh- and histamine-induced responses in arterioles of HFD rats. Administration of Tiron significantly enhanced histamine-induced dilation in arterioles isolated from HFD rats but did not affect ACh-evoked responses in HFD or agonist-induced dilations in control vessels. In arterioles of control rats, neither apocynin nor allopurinol affected agonist-induced dilations. On the other hand, in arterioles of HFD rats, allopurinol, but not apocynin, significantly enhanced ACh- and histamine-induced dilation.

Quantification of superoxide production by lucigenin-enhanced chemiluminescence

Vascular superoxide production was assessed in carotid arteries of control and HFD rats by the lucigenin-enhanced chemiluminescence method. An enhanced lucigenin chemiluminescence was measured in carotid arteries of HFD rats that

was inhibited by preincubation with superoxide scavenger Tiron. Also, lucigenin-enhanced chemiluminescence assay was performed in the presence of NADH or xanthine to measure the NAD(P)H oxidase- and xanthine oxidase-derived superoxide production, thereby assessing the enzyme activity of the NAD(P)H oxidase and xanthine oxidase in the vessels. In the presence of NADH, there was no significant difference in the stimulated superoxide production between the two groups of vessels, whereas the presence of xanthine resulted in a significantly enhanced superoxide production in carotid arteries from HFD rats compared with control vessels.

Immunohistochemistry

Compared with control arterioles, an enhanced xanthine oxidase immunostaining was detected in the gracilis arterioles of HFD rats, which was mainly localized in the endothelial layer of arterioles.

4.2. Genetic model of type 2 diabetes

Metabolic and systemic hemodinamic parameters

Previously, we had found that at 12 weeks of age, body weight, serum glucose, and serum insulin values of db/db mice were significantly elevated compared with age-matched wild-type animals, resembling data obtained from patients with obesity and T2-DM. Systolic and mean arterial pressures were significantly elevated in conscious db/db mice compared with wild-type mice. Calculated peripheral vascular resistance was significantly elevated in db/db mice compared with wild-type animals.

Pressure-induced arteriolar responses in control and db/db mouse gracilis muscle arterioles

At 80 mmHg, the diameter of arterioles of db/db mice was significantly reduced compared with that of arterioles from wild-type mice. There were no significant differences between passive arteriolar diameters in the 2 groups of animals obtained in calcium-free PSS at 80 mmHg. Stepwise increases in intraluminal pressure from 20 to 120 mm Hg elicited significantly greater reductions in the diameter of arterioles from db/db mice compared with control vessels at each pressure step. Endothelium denudation did not affect significantly the pressure-induced changes in the diameter of arterioles in either group. Incubation and presence of catalase did not affect the pressure-diameter curves of arterioles of control mice, whereas it shifted this curve significantly upward in arterioles of db/db mice, back to control levels. The presence of the PGH_2/TxA_2 receptor antagonist SQ-29548 did not affect the pressure-induced responses of arterioles of control mice, but it reduced the tone of arterioles of db/db mice back to control levels. The presence of the selective inhibitor of COX-1, SC-560, did not affect the basal tone of arterioles in either control or db/db mice. On the other hand, the presence of NS-398, a selective inhibitor of COX-2, caused a significant upward shift in the arteriolar pressure-diameter curve of vessels from db/db mice, but did not significantly affect that of arterioles isolated from control animals.

Western immunoblot

Western blot analysis was performed on aortae from both control and db/db mice. There were no significant differences in total COX-1 protein levels in the 2 groups, whereas COX-2 expression was significantly greater in aortae from db/db mice.

Detection of H_2O_2 by fluorescence

In carotid arteries of db/db mice, an enhanced DCFH fluorescence was detected, indicating an increased level of H_2O_2. Presence of catalase reduced DCFH fluorescence in arteries of db/db mice, whereas it did not affect fluorescence intensity in control vessels.

Arteriolar responses to exogenous H_2O_2

H_2O_2, in a dose-dependent manner, elicited substantial dilations in control arterioles, but it caused constrictions in db/db arterioles. Endothelium-denudation did not affect significantly H_2O_2-induced differences in arteriolar responses in the two groups of animals. Presence of the TP receptor antagonist SQ-29548 did not significantly affect H_2O_2-mediated dilations of control arterioles. However, SQ-29548 converted H_2O_2-induced constriction to dilation in arterioles of db/db mice. The TP receptor agonist U-46619-induced constrictions were not significantly different in the two groups of vessels, whereas the U-46619-induced constrictions were completely inhibited with SQ-29548.

4.3. Vasoactive effects of OR-1896 and levosimendan

OR-1896 elicited substantial dilations in the gracilis muscle arterioles in a concentration-dependent manner. The extent of the dilation induced by OR-1896 was similar to that induced by levosimendan. In the presence of TEA, the magnitude of the dilation in response to OR-1896 was attenuated markedly in the gracilis muscle arterioles. Glibenclamide, a selective blocker of K_{ATP} channels, gave rise to a great reduction in the magnitude of the OR-1896-induced dilations in the gracilis muscle arterioles. The selective BK_{Ca} channel inhibitor IBTX had no effect on the OR-1896-induced dilation but the K_V channel inhibitor 4-aminopiridin significantly decreased it.

5. DISCUSSION

Type 2 diabetes mellitus is associated with a markedly increased cardiovascular morbidity and mortality. Even before the onset of atherosclerosis and the morphological changes of the microvessels, the endothelial and smooth muscle function of the resistance arterioles can be altered, which is belived to contriburte to the increased cardiovascular risk. Several studies have demonstrated that vasomotor dysfunction of microvessels is an early manifestation of the vascular complications in T2-DM. Alterations in local vasoregulatory mechanisms intrinsic to the vascular wall, such as enhanced pressure-induced arteriolar tone and reduced endothelium-dependent dilation, have been reported previously as characteristic of T2-DM. Changes in the local vasoregulatory mechanisms of peripheral microvessels may significantly influence vascular resistance in T2-DM; however, the possible underlying mechanisms are not completely understood. The aforementioned prompted us to investigate the alterations of the vasomotor function in resistance artreioles in T2-DM. We used two different animal models of type 2 diabetes to reveal the possible alterations in the endothelium- and smooth muscle-dependent function of the microvessels. We aimed to investigate the underlying mechanisms, especially the role of oxidative stress and altered vascular prostanoid synthesis.

5.1. Diet-induced type 2 diabetes

In the first series of experiments type 2 diabetes was induced in male Wistar rats by 10 weeks of high fat feeding. After 10 weeks of HFD, body weight was significantly elevated, and this weight gain was accompanied by greater retroperitoneal fat pad mass. HFD rats exhibited higher mean arterial blood pressure. HFD for 10 weeks was also associated with elevated serum insulin, and total cholesterol levels. The serum glucose level was only slightly but significantly elevated, thus this model can be considered to represent an early manifestation of type 2 diabetes. In the present study, we investigated the

vasomotor function of isolated skeletal muscle arterioles (~160 μm internal diameter). We have found that gracilis muscle arterioles isolated from HFD rats did not show significant changes in the active and passive arteriolar diameters at intraluminal pressure of 80 mmHg compared with control vessels. We concluded that the smooth muscle function of the microvessels isolated from HFD rats is essentially preserved. In arterioles of HFD rats, endothelium-dependent dilations to ACh and histamine were significantly reduced compared to those of control responses, whereas dilations to the NO donor SNP were not different between the two groups. These findings indicated a selective impairment of endothelium-dependent dilations and endothelial dysfunction in skeletal muscle arterioles of HFD rats. These observations are in line with previous studies, which demonstrated that high-fat feeding in animals impairs endothelium-dependent relaxation of the large conductance arteries and extend those findings to microvessels. Several studies suggested that decreased NO synthesis and/or decreased NO bioavailability is the primary cause of endothelial dysfunction in certain pathological conditions. To test this hypothesis, agonist-induced arteriolar dilations were observed after the inhibition of NO synthesis. The NO synthase inhibitor L-NAME significantly reduced ACh- and histamine-induced dilations in control arterioles; however, it had no effect on responses of HFD vessels, suggesting a lack of NO mediation of agonist-induced dilations in arterioles of HFD rats. Previous studies suggested that increased ROS generation is the primary cause of the NO inactivation in diet-induced obesity. The results of our study support this hypothesis because, in skeletal muscle arterioles of HFD rats, the superoxide scavenger Tiron significantly enhanced histamine-induced dilations, suggesting a role for increased superoxide production interfering with NO signaling. To further substantiate the primary role for ROS, superoxide production was measured in carotid arteries by the lucigenin-enhanced chemiluminescence method. This study revealed an increased superoxide anion production in the carotid artery of HFD rats compared to those

of vessels obtained from control animals. It has been earlier suggested that excess production of vascular superoxide anion may be derived from different ROS-producing systems, including NAD(P)H oxidase and xanthine oxidase. In the present study, we have found that the xanthine oxidase inhibitor, allopurinol enhanced agonist-induced dilations in skeletal muscle arterioles of HFD rats, whereas the NAD(P)H oxidase inhibitor apocynin had no significant affect on these responses. In addition, a marked xanthine oxidase immunostaining was detected in the endothelial layer of HFD, but not in control gracilis arterioles, further substantiating a primary role for xanthine oxidase in mediation of enhanced arteriolar superoxide production. Taken together, our study demonstrates a key role for endothelial xanthine oxidase-derived superoxide production, which is responsible for the reduced NO-mediated dilations of skeletal muscle arterioles of type 2 diabetic rats.

5.2. Genetic model of type 2 diabetes

At 12 weeks of age, body weight, serum glucose, and serum insulin values of db/db mice were significantly elevated compared with age-matched wild-type animals, resembling data obtained from patients with obesity and T2-DM. Serum glucose is ~4 times higher than in control mice, and we propose that this model represent an advanced stage of type 2 diabetes mellitus with more severe microvascular dysfunction and systemic alterations. We have found that db/db mice exhibit an enhanced peripheral vascular resistance and elevated systemic systolic and diastolic blood pressure. In the next series of experiments, we aimed to elucidate the possible underlying mechanisms responsible for the enhanced peripheral vascular resistance using isolated gracilis muscle arterioles (~90 µm internal diameter). In response to stepwise increases in intraluminal pressure (from 20 to 120 mm Hg), the diameter of isolated skeletal muscle arterioles was significantly reduced in db/db compared with control mice, whereas the passive diameter of arterioles was not significantly different between the two groups.

These findings indicate that the active tone is greater in the arterioles of db/db mice. Removal of endothelium did not affect significantly the tone of arterioles in either group of vessels, suggesting that smooth muscle-dependent mechanisms are primarily responsible for the enhanced tone in arterioles of db/db mice. Based on these experimental data and previous studies we propose that the myogenic tone of resistance arterioles is increased in type 2 diabetes and these alterations are associated with increased peripheral vascular resistance and elevated systolic blood pressure. Arteriolar diameter is continuously modulated by dilator and constrictor factors, many of them intrinsic to the vascular wall. Early investigations reported enhanced release of a constrictor prostanoid from diabetic vessels. One of the most important constrictor prostanoids is PGH_2/TXA_2, which enhances myogenic tone in certain pathological conditions. We found that a PGH_2/TXA_2 receptor antagonist increased the diameter of arterioles of T2-DM mice back to control levels, whereas it did not affect the diameter of vessels from control animals. These findings indicate that endogenous release of constrictor PGs, PGH_2/TxA_2, may be responsible for the reduced diameter of arterioles of type 2 diabetic mice. Prostanoids are generated by the two COX isoforms from arachidonic acid. COX-1 is constitutively expressed in most tissues, whereas COX-2 is expressed only at low or undetectable levels but is readily upregulated by inflammatory, mitogenic, and physical stimuli. Recently, a role for prostanoid-mediated vascular inflammation has been shown to be associated with the development of vascular complications in T2-DM. Next we aimed to elucidate the possible role of the two COX isoforms in the development of the myogenic tone of the microvessel. Selective inhibition of COX-1 did not affect the myogenic tone of control or db/db arterioles, whereas in arterioles of T2-DM mice basal tone was reduced by the selective inhibitor of COX-2 to the control level. COX-2 protein levels in the aortas of T2-DM mice were markedly increased compared with those of control animals. We interpret these findings to mean that in arterioles of db/db mice,

COX-2–dependent release of constrictor PGs, most likely PGH_2/TxA_2, derived primarily from vascular smooth muscle cells, mediate the enhanced pressure-induced reductions of arteriolar diameter. In the next series of experiments we set out to characterize the vasoactive effects of H_2O_2. Cumulative doses of H_2O_2 resulted in substantial dilations in both endothelium-intact and -denuded arterioles of control animals. In contrast, in arterioles of db/db mice, H_2O_2 resulted in constrictions, which were similar in magnitude regardless of whether or not the endothelium was present. The results obtained in control vessels confirm previous observations showing that, depending on the vessel type, H_2O_2 elicits vasodilation via prostaglandins or directly activating potassium channels in vascular smooth muscle cells. Interestingly, in recent studies it was found that H_2O_2 caused TxA_2/PGH_2 receptor-mediated contraction in different vessel types. H_2O_2-induced constrictions were converted to dilations by the TxA_2/PGH_2 receptor antagonist SQ-29548 in arterioles of db/db mice. On the other hand, TxA_2/PGH_2 receptor agonist U-46619-induced constriction was similar in the two groups, showing that there is no difference in the sensitivity of TxA_2/PGH_2 receptors between arterioles of control and db/db mice. These data together suggest that, in diabetic arterioles, H_2O_2 induces TxA_2/PGH_2 release, which prevents the development of H_2O_2-mediated dilation. To elucidate the possible role of H_2O_2 in pressure-induced responses, arterioles were studied in the presence of catalase, aiming to reduce the level of H_2O_2. Catalase had no effect on the tone of control arterioles at any pressure step investigated, whereas, in arterioles of db/db mice, catalase elicited an increase in diameter; hence, it reduced the level of arteriolar tone to the level of control at each pressure step. Taken together, our findings suggest that, in arterioles isolated from the skeletal muscle of db/db mice, the production of H_2O_2 is markedly enhanced, which contributes to the increased TxA_2/PGH_2-mediated basal tone.

We propose that vascular oxidative stress plays a central role in the pathogenesis of type 2 diabetic vasomotor dysfunction. In the early stage of type 2 diabetes

vascular oxidative stress leads to endothelial dysfunction, whereas the smooth muscle function remains intact (results on animals treated with high fat diet). However, in the advanced stage of disease the smooth muscle cell related myogenic function is also altered. These could be one of the initial steps in the pathological pathway contributing to the impairment of dilator capacity of resistance arterioles and enhancement of arteriolar tone (results on db/db mice).

5.3. Vasoactive effect of levosimendan and OR-1896

The microcirculation and tissue perfusion is disturbed in type 2 diabetes, which may lead to increased cardiovascular morbidity and mortality. Therapeutic approaches aiming to restore microvascular function in type 2 diabetes are particularly important. Levosimendan, and its long-lived metabolite OR-1896 are hypothesized to have vasoactive effects. Previous studies have shown that levosimendan-induced vasodilation is associated with the activation of various potassium channels in large conductance vessels, and relatively little attention has been devoted to OR-1896. In this study, we set out to characterize the direct vascular effects of levosimendan and OR-1896 in microvessel with spontaneously developing vascular tone. Our study revealed a pronounced vasodilator potential for levosimendan and the long-lived levosimendan metabolite OR-1896 in the skeletal muscle microvessels of the rat, which were similar in the magnitude. In this set of experimetns an attempt was made to employ K^+ channel function modulators to allow the characterization of the mechanism of OR-1896-induced arteriolar vasodilation. More than 50% of the OR-1896-induced vasodilation could be prevented by nonselective blocker of K^+ channel, TEA at maximal OR-1896 concentration, implying that at least part of the OR-1896-induced dilation is mediated by potassium channel activation. The selective inhibitor of K_{ATP} channels significantly decreased OR-1896-induced vasodilation, indicating that the OR-1896-induced activation of these channels contributes to its vasodilator action. To assess the potential role of BK_{Ca}

channels and K_V channels in the OR-1896-induced vasodilation, in another series of experiments, the selective BK_{Ca} channel inhibitor IBTX or the selective K_V channel inhibitor 4-aminopiridin was used. 4-aminopiridin significantly reduced the magnitude of the OR-1896-induced dilations in the gracilis arterioles, but IBTX had no effect on the dilations of the skeletal muscle arterioles. Collectively, our present data suggest for the first time that, in addition to Ca^{2+} sensitization in cardiac cells, the levosimendan metabolite OR-1896 acts on a distinct molecular target in arterioles, that is, it activates K^+ channels leading to substantial vasodilation. It can be assumed that OR-1896 induces dilation in the skeletal muscle arterioles through the activation of K_{ATP} and K_V channels. Furthermore, our findgins suggest the involvement of the long-lived metabolite OR-1896 in mediating certain of the levosimendan-induced vasodilating effects. We propose that different potassium channel activating agents, for example levosimendan and OR-1986, can be beneficial in the treatment of microvascular dysfunction for instance in type 2 diabetes mellitus.

6. SUMMARY

Type 2 diabetes is associated with the development of microvascular dysfunction, but the underlying mechanisms has not yet been fully elucidated. This prompted us to investigate the alterations of the endothelium- and smooth muscle-dependent vasomotor function of the microvessels in animal models of type 2 diabetes. We also set out to characterize the direct vascular effects of OR-1896, a drug that can be beneficial in restoring microvascular function in type 2 diabetes. The key, novel findgins of our studies are the followings: 1.) In high fat diet-induced type 2 diabetes mellitus nitric oxide-mediation of endothelium-dependent dilation of rat skeletal muscle arterioles is reduced due to an enhanced xanthine oxidase-derived superoxide anion production. 2.) In a genetic model of type 2 diabetes (db/db mice) arteriolar production of H_2O_2 is enhanced, which leads to increased synthesis of the cyclooxygenase-2 derived constrictor prostaglandins tromboxane A_2/prostaglandin H_2 in the smooth muscle cells, which in turn enhances basal arteriolar tone. 3.) OR-1896 elicits a substantial vasodilation in skeletal muscle arterioles of the rat by activating promarily K_{ATP} and K_V channels.

In summary, our present data suggest that vascular oxidative stress plays a key role in the pathogenesis of arteriolar vasomotor dysfunction and consequently enhanced peripheral resistance in the early and advanced stage of type 2 diabetes. We propose that agents activating potassium channels on microvessels, such as OR-1896 can be useful in the therapy of diabetes by improving microvascular function, decreasing peripheral resistance and arterial blood pressure.

LIST OF PUBLICATIONS:

***In extenso* publications related to the thesis**

Erdei N, Toth A, Pasztor ET, Papp Z, Edes I, Koller A, Bagi Z. High-fat diet-induced reduction in nitric oxide-dependent arteriolar dilation in rats: role of xanthine oxidase-derived superoxide anion. Am J Physiol Heart Circ Physiol. 2006;291(5):H2107-15. IF: 3,724

Erdei N, Bagi Z, Edes I, Kaley G, Koller A. H_2O_2 increases production of constrictor prostaglandins in smooth muscle leading to enhanced arteriolar tone in Type 2 diabetic mice. Am J Physiol Heart Circ Physiol. 292(1):H649-56. 2007. IF: 3,724

Bagi Z, **Erdei N**, Toth A, Li W, Hintze TH, Koller A, Kaley G. Type 2 diabetic mice have increased arteriolar tone and blood pressure. Enhanced release of COX-2-derived constrictor prostaglandins. Arterioscler Thromb Vasc Biol. 25(8):1590-5.2005. IF: 7,053

Erdei N, Papp Z, Pollesello P, Edes I, Bagi Z. The levosimendan metabolite OR-1896 elicits vasodilation by activating the K(ATP) and BK(Ca) channels in rat isolated arterioles. Br J Pharmacol. 2006;148(5):696-702.
IF: 3,825

Bagi Z, **Erdei N**, Papp, Z, Edes I, Koller A. Up-regulation of vascular cyclooxygenase-2 in diabetes mellitus. Pharmacol Rep. 2006. 58 Suppl:52-6.
IF: 0,764

Other *in extenso* publications:

Szerafin T*, **Erdei N***, Fulop T, Pasztor ET, Edes I, Koller A, Bagi Z. Increased cyclooxygenase-2 expression and prostaglandin-mediated dilation in coronary arterioles of patients with diabetes mellitus. Circ Res. 2006;99(5):e12-7. **társ elsőszerzős közlemény* IF: 9,854

Fulop T, Jebelovszki E, **Erdei N**, Szerafin T, Forster T, Edes I, Koller A, Bagi Z. Adaptation of Vasomotor Function of Human Coronary Arterioles to the Simultaneous Presence of Obesity and Hypertension. Arterioscler Thromb Vasc Biol. 2007. 27(11):2348-54. IF: 6,883

Published abstracts:

Erdei N, Bagi Z, Tóth A, Édes I, Kaley G, Koller A A szelektív COX-1 és COX-2 gátlás hatása a mikrovaszkuláris rezisztenciára 2-es típusú diabetes mellitusban. Cardiologia Hungarica, 35, A18, 2005.

Lizanecz E, **Erdei N**, Bagi Z, Édes I, Tóth A A TRPV1 szerepe az értónus szabályozásában. Cardiologia Hungarica, 35, A24, 2005.

Bagi Z, **Erdei N**, Tóth A, Édes I, Kaley G, Koller A Increased level of hydrogen peroxide enhances myogenic tone in arterioles of type 2 diabetic mice. J. Muscle Res. Cell. Motil. 26(1), 69. 2005.

Erdei N, Tóth A, Kaley G, Bagi Z, Koller 2-es típusú diabetes mellitusban a vaszkuláris hydrogen peroxide növeli a COX-2 érfali expresszióját és fokozza az arteriolák konstriktor prosztaglandin termelését. Acta Physiol. Hung. 2005.

Bagi Z, **Erdei N**, Papp Z, Pollesello P, Édes I A levosimendan és metabolitja, az OR-1896 vazodilatációt okoz patkány izolált arteriolákban. Cardiologia Hungarica, 36, A13, 2006.

Erdei N, Tóth A, Édes I, Koller Á, Bagi Z A xantin-oxidáz eredetű szuperoxid-anion csökkenti az endothel-függő arterioláris vazodilatációt magas zsírtartalmú diétán tartott patkányokban. Cardiologia Hungarica, 36, A17, 2006.

Szerafin T, **Erdei N**, Pásztorné Tóth E, Édes I, Koller Á, Bagi Z Bradikinin fokozott ciklooxigenáz-2-függő prosztaglandin mediálta dilatációt okoz diabéteszes betegek koronária mikroereiben. Cardiologia Hungarica, 36, A26, 2006.

Erdei N, Fehér A, Tóth A, Papp Z, Édes I, Koller Á, Bagi Z Az oxidative stressz szerepe az elhízás következtében kialakuló mikrovaszkuláris endothel diszfunkció kialakulásában. Acta Physiol Hung. 2006.

Bagi Z, **Erdei N**, Tóth A, Édes I, Koller Á Interaction between hydrogen peroxide and prostaglandin metabolism leads to microvascular dysfunction in type 2 diabetes. J. Vasc. Res. 43 (Suppl 1), 2006.

Erdei N, Tóth A, Pásztorné Tóth E, Papp Z, Édes I, Koller Á, Bagi Z Obesity-induced reduction in nitric oxide-dependent arteriolar dilation in rats. Role of xanthine oxidase-derived superoxide. . J. Vasc. Res. 43 (Suppl 1), 2006.

Koller A, **Erdei N**, Tóth A, Édes I, Tóth J, Bagi Z High intraluminal pressure reduces tachyphylaxis to angiotensin II in isolated arterioles. FASEB Journal, 20(4), A 306, 2006.

Szerafin T, **Erdei N**, Édes I, Bagi Z Role of prostaglandin-mediated agonist-induced dilations of isolated human coronary arterioles obtained from patients with diabetes mellitus. Eur. Surg. 38 (Suppl 207), 2006.

Erdei N, Papp Z, Pollesello P, Édes I, Bagi Z The levosimendan metabolite OR-1896 elicits vasodilation by activating the KATP and BKCa channels in isolated arterioles of the rat. Eur. J. Heart Failure 5 (Suppl 1), 2006.

Erdei N, Fehér A, Beleznai T, Tóth A, Koller Á, Édes I, Bagi Z A koronária arteriolák megváltozott vasomotor működése a metabolikus szindróma állatkísérletes modelljében. Cardiologia Hungarica, 37, A15, 2007.

Bagi Z, **Erdei N**, Szerafin T, Edes I, Koller A. Multiple effects of diabetes mellitus on the vasomotor responses of human coronary arterioles. FASEB JOURNAL 21 (6): A1226-A1226 APR 2007

Koller A, **Erdei N**, Feher A, Edes I, Bagi Z. Increased soluble guanylate cyclase (sGC) activity may compensate for the high fat diet-induced reduction in NO bioavailability of rat coronary arterioles. FASEB JOURNAL 21 (6): A1226-A1226 APR 2007

Koller A, **Erdei N**, Edes I, Toth J, Bagi Z. High intraluminal pressure via increased release of hydrogen peroxide maintains arteriolar responsiveness to angiotensin II. FASEB JOURNAL 21 (6): A1248-A1248 APR 2007

Koller A., **Erdei N**, Feher A, Beleznai T, Edes I, Bagi Z. High fat diet leads to increased no sensitivity of rat coronary arterioles: role of soluble guanylate cyclase. MICROCIRCULATION 14: 4-5, 459. 2007

Koller A, **Erdei N**, Toth J, Bagi Z. High Intraluminal Pressure Reduces AT1 Receptor Internalization and Maintains Arteriolar Constrictions to Angiotensin II. Circulation, Oct 2007; 116: II_14.

Bagi Z, Fulop T, Jebelovszki E, **Erdei N**, Szerafin T, Forster T, Koller A, Edes I. Adaptation of Coronary Arteriolar Dilations to The Simultaneous Presence of Obesity and Hypertension: Role of Increased Nitric Oxide Sensitivity. Circulation, Oct 2007; 116: II_230.

Erdei N, Feher A, Beleznai T, Edes I, Koller A, Bagi Z. Abstract 244: Oxidative Stress via Activating Soluble Guanylate Cyclase Leads to Increased Nitric Oxide Sensitivity of Coronary Arterioles in High Fat Diet-induced Obesity. Circulation, Oct 2007; 116: II_28.