

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Evaluation of the role of Protein Kinase G signaling pathway in cardiac dysfunction caused by diabetes mellitus and atherosclerosis

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2019

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1. Introduction and Aims

1.1. Cardiac dysfunction and Heart Failure

Although cardiovascular mortality slowly declines in recent decades, there is a continuing need for a deeper understanding of the pathomechanism of related diseases and for the development of novel drugs and therapeutic procedures.

Heart failure (HF) is a clinical syndrome typically characterized by subjective symptoms such as shortness of breath, tiredness, as well as diagnostic signs such as increased jugular venous pressure, peripheral- and pulmonary edema, pulmonary crackles. Cardiac dysfunction is a precursor of heart failure, referring to the underlying functional disorder that exists before manifest symptoms appear. The estimated prevalence of heart failure in the adult population is around 1-2% (~ 38 million patients), with a high mortality rate, and a 10-year survival rate of only 10%. Many disorders result in heart failure, with particular importance of diabetes mellitus and atherosclerosis, that are growing public health burden.

Diabetes mellitus (DM) is a major risk factor for heart failure, regardless of age, existing hypertension or coronary disease. Although proper glucose control reduces cardiovascular risk, the incidence of cardiovascular disease is significantly higher in the diabetic population, with a 2-5-fold increase at the risk of heart failure.

By definition, diabetic cardiomyopathy (DCM) is a left ventricular dysfunction that develops in diabetic subjects independently of other risk factors, and is most commonly associated with diastolic dysfunction and myocardial hypertrophy, and may be accompanied by systolic dysfunction in advanced stages.

A number of factors contribute to the pathophysiology of cardiomyopathy, as metabolic abnormalities, autonomic nervous system damage, inflammation, and oxidative stress. According to the recent studies, mitochondrial damage,

endoplasmic reticulum stress (ER stress) and calcium homeostasis disorders are particularly important. Currently, specific drugs are not indicated in diabetic cardiomyopathy or diastolic dysfunction.

Coronary artery disease (CAD) and ischemic heart diseases, as results of atherosclerosis, are the most common causes and major risk factors for the development of heart failure, in addition to hypertension. It is estimated that 70% of patients with heart failure have coronary artery disease, and in at least 50% of cases CAD is the primary cause of HF. Dyslipidemia is considered as a risk factor for heart failure alone, without induction of myocardial infarction. Elevated serum cholesterol levels can be directly associated with hypertension, increased vascular stiffness, left ventricular mass and wall thickness. Based on experimental evidence from animal models maintained an atherogenic diet, hypercholesterolemia may also result in systolic and diastolic dysfunction.

Based on most recent data, endothelial dysfunction and diminished activity of the cGMP-PKG signaling cascade may play a crucial role in the pathomechanism of cardiac dysfunction. The cGMP-PKG signaling pathway enhances the activity of cardioprotective pathways, offering therapeutic options. Activation of the cGMP-PKG system via the nitrogen monoxide (NO) or natriuretic peptide (NP) pathways mediates vasodilatation and vascular protection, which in itself is beneficial to the myocardium in both ischemic and under pressure- or volume-overload conditions. In addition, recent studies have confirmed the role of the cGMP-PKG system in directly influencing myocardial remodeling and diastolic function. In addition to anti-hypertrophic effects, phosphorylation targets of PKG enzyme are involved in other processes that primarily determine myocardial relaxation. PKG affects the elasticity of the giant protein titin, by phosphorylation of specific amino acids in the N2-bus region, thereby reducing the passive tension and rigidity of myofibrils.

It has also been proposed that activation of the cGMP-PKG system plays a role in stimulating the function of the SERCA pump, via the phosphorylation of

the phospholamban regulatory protein, thereby contributing to the relaxation of the cardiomyocytes.

The level of intracellular cAMP and cGMP are regulated by phosphodiesterase enzymes (PDEs). Inhibition of PDEs result in elevation of cGMP levels, and this mechanism is therapeutically exploitable in heart failure and pulmonary hypertension. The most recent finding related to PDE function is the confirmation of the role of the PDE9A enzyme. PDE9A is primarily responsible for the degradation of cGMP generated by the natriuretic peptide pathway, is up-regulated in human myocardium when hypertrophy and heart failure occur. Inhibition of PDE9A reversed the experimentally developed cardiac disease (pressure-overload) independent of NO-pathway. However, the expression of the enzyme in atherosclerosis and diabetes has not been studied.

The small molecule BGP-15 drug candidate (O- (3-piperidino-2-hydroxy-1-propyl)nicotinic acid-amidoxime) is an original molecule of Hungarian origin. Currently, BGP-15 is of scientific interest due to its effects on skeletal and cardiac muscles, with related publications recently have been published in high-impact journals. Gehrig et al. (Nature, 2012) showed that in mdx (muscular dystrophic) mice, BGP-15 treatment improved muscle strength and contractility of striated muscles, reduced kyphosis, and increased the life span of animals by inducing hsp72 and preserving the function of the SERCA2a pump. Sapra et al. (Nature Communications, 2014) developed a transgenic, heart-failure-atrial fibrillation mouse model, in which BGP-15-treatment (15 mg / kg / day) significantly reduced atrial enlargement, improved systolic function, reduced collagen-1 synthesis and prevented the development of arrhythmias. Based on the above, Kennedy et al. showed that BGP-15 has protective effects on both skeletal and cardiac muscles, but different and only partially detected or controversial mechanisms.

The effect of BGP-15 on myocardium and the underlying mechanisms are still questionable, and may differ in various disease models, thus investigating the cardiovascular actions of the drug candidate is a subject of ongoing research.

1.2. Aims

Based on the above, we aimed to investigate the followings:

1. Evaluation of cardiac dysfunction caused by type 2 diabetes mellitus in Goto-Kakizaki (GotoK) rat model, and comparison of the cardiovascular effects of the drug candidate BGP-15 drug with known pleiotropic antidiabetic agents.

Wistar control, GotoK diabetic, and GotoK animals treated with BGP-15, metformin and pioglitazone were used to the experiments. We attempted to investigate systolic and diastolic parameters by echocardiography, evaluated endothelial-dependent vasorelaxation, and evaluated the activity of the PKG pathway by assessing the phosphorylation of VASP protein, and determined the expression of SERCA2a, phospholamban, and PDE9A proteins.

2. Secondly, we attempted to provide a detailed echocardiographic description of atherosclerosis-induced cardiac dysfunction in the hypercholesterolemic CAL-NZW rabbit model, with the evaluation of endothelial-dependent vasorelaxation and particular interest regarding the cGMP-PKG-PDE9A signaling pathway.

Healthy control and hypercholesterolemic (HC) rabbits were used during the experiments. Cardiac function was evaluated by conventional, Doppler and STE echocardiography, endothelial-dependent vasorelaxation was determined, and the activity of the cGMP-PKG signaling pathway was investigated, along with the expression of VASP and PDE9A proteins.

2. Material and methods

2.1. The Goto-Kakizaki T2DM rat model

Diabetic animals from the Goto-Kakizaki substrain, and healthy rats from the Wistar strain were used to the experiments. Rats were obtained from AnimaLab Ltd, a distributor of Charles River Laboratories Inc. (Vác, Hungary). In accordance with European Union Directive 2010/63 / EU, all animals have received humane treatment, treatments and experimental procedures were carried out by appropriately qualified personnel, the study was registered by the Ethics Committee of the University of Debrecen (DEMÁB 25/2013)

2.2. Experimental protocol I (Goto-Kakizaki rat studies)

12 week-old GotoK and Wistar rats were randomly divided into 5 subgroups ($n = 6$ / group) as the followings: (I) healthy Wistar (Control) group, vehicle treatment; (II) GotoK diabetic group, vehicle treatment (“untreated group”); (III), GotoK + BGP-15 group, 10 mg / kg oral daily BGP-15 treatment; (IV) GotoK + MET group, 100 mg / kg / day oral metformin treatment; (V) GotoK + PIO group, 10 mg / kg oral pioglitazone treatment, daily. Prior to treatments, blood was collected from the tail vein of the animals to determine serum glucose and insulin levels. Animals were weighed weekly to calculate the exact dosage regimens. At the endpoint (week 12), blood was collected from the tail vein, followed by echocardiography. Thoracotomy was performed during deep (100/10 mg/kg) ketamine-xylazine anesthesia, the heart was excised, placed into a Ca^{2+} - free Krebs solution, the atria and chambers were immediately frozen in liquid nitrogen and stored at -80°C . The excised thoracic aorta was placed into ice-cold Krebs solution and was subjected immediately for ex vivo vasorelaxation studies.

2.3. The hypercholesterolemic rabbit model

For the second phase of the experiments, 20 week old male rabbits were obtained from Juráskó Ltd. (Debrecen). The rabbits were derived from crosses of

New Zealand White (NZW) and California (CAL) strain, having a higher body fat percentage compared to the commonly used New Zealand variety. Since our primary goal was to increase cholesterol and triglyceride values, we assumed the higher value of this rabbit strain compared to the traditional New Zealand animals. The control group received a normal rodent chow, while the hypercholesterolemic (HC) group was set on an "atherogenic" diet. Atherogenic chow contained 1% added cholesterol and 1% saturated fat compared to normal chow. Laboratory animals have been treated in a humane way, and all experiments were conducted in accordance with the Principles of Laboratory Animal Care Directive 2010/63 / EU and approved by the Ethics Committee of the University of Debrecen (DEM 25/2013)

2.4. Experimental design II (Rabbit model studies)

The rabbits were randomized to 2 subgroups: Control (I) ($n = 9$) received normal rodent chow; the HC (II) group ($n = 9$) was set on an atherogenic chow for 4 months. Bodyweights were recorded at the start and end points (weeks 0 and 16). At the endpoint, blood was collected from the marginal ear vein of the animals, followed by an echocardiographic examination. Subsequently, thoracotomy was performed under "deep" anesthesia (ketamine/xylazine, 100/10 mg/kg), and organ samples were isolated in Ca^{2+} -free Krebs solution (heart, left ventricle, aorta thoracica, kidney, lung), for morphometric assays. The thoracic aorta was excised, the upper section was stored in 4% formalin solution and the lower section was placed in ice-cold Krebs solution and immediately used for *ex vivo* vascular studies. The left ventricle was immediately frozen in liquid nitrogen for subsequent molecular biological analyses.

2.5. Chemicals

All reagents, antibodies, salts and buffer solutions used in Western blot, histological staining and assays were received from Sigma-Aldrich-Merck (Darmstadt, Germany) or Abcam (Cambridge, UK) suppliers. For *ex vivo*

vascular studies, norepinephrine hydrochloride (Arterenol®), acetylcholine chloride, adenosine 5'-triphosphate hydrate and modified Krebs buffer were used. The composition of the Krebs solution was the following: 118 mmol / L NaCl, 4.7 mmol / L KCl, 2.5 mmol / L CaCl₂, 1 mmol / L NaH₂PO₄, 1.2 mmol / L MgCl₂, 24.9 mmol / L NaHCO₃, 11.5 mmol / L glucose and 0.1 mmol / L ascorbic acid; dissolved in distilled water and vented with carbogen gas. The substances used for vascular assays were dissolved or diluted in this buffer. Organ samples (aorta, myocardium, kidney, liver) were isolated in CaCl₂-free Krebs buffer for further use. Metformin hydrochloride, pioglitazone hydrochloride and BGP-15 used for the treatment of Goto-Kakizaki animals (O- (3-piperidino-2-hydroxy-1-propyl) nicotinic acid amidoxime) were obtained from Sigma-Aldrich-Merck (Darmstadt, Germany). For oral gavage, the active ingredients were dissolved in a 1: 5 mixture of hydroxyethylcellulose and distilled water.

2.6. Morphometry, blood collection, metabolic parameters

Rats were weighed weekly to calculate the exact dosage regimens (weeks 0-12). After an overnight fasting, blood was collected from the tail vein and serum glucose was determined using an Accu-Chek blood glucose meter (Roche Diagnostics, Risch, Switzerland). The remaining sample was centrifuged at 4 ° C for 2 minutes at 10000 G, the plasma frozen and stored at -80 ° C. Plasma insulin levels were determined by radio-immunoassay (RIA) kit (RK-400CT, Hungarian Academy of Sciences, Isotope Research Institute). The HOMA-IR index (Homeostasis Model Assessment of Insulin Resistance) was determined using the following formula: HOMA-IR = fasting insulin (μ IU / mL) × fasting glucose (mmol / mL) / 22.5; while beta cell function (HOMA-B index) was assessed with the following formula: HOMA-B = 20 × fasting insulin (μ IU / mL) / fasting glucose (mmol / mL) - 3.5.

In the second series of experiments, the rabbits were weighed at the start- and endpoints (weeks 0, 16). At the end of week 16, after overnight fasting, blood

was collected from the marginal ear vein into Vacutainer Plast SSTII tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Serum lipid and insulin levels, liver enzymes, and other markers were determined by the Department of Laboratory Medicine, University of Debrecen. The insulin level was determined on the Liaison XL DiaSorin platform (DiaSorin Inc., Stillwater, MN, USA). The other 'routine' parameters were measured using the Roche Cobas Integrated Platform (Roche Diagnostics GmbH, Mannheim, Germany). The specified lipid parameters were: total cholesterol (TChol), low-density lipoprotein (LDLc), high-density lipoprotein (HDLc), apolipoprotein A and B (ApoA and ApoB), and triglyceride (TG). Liver enzymes: aspartate transaminase (AST) and alanine transaminase (ALT). Other markers include creatinine, troponin T, creatine kinase MB isoform (CK-MB) and C-reactive protein (CRP). Calculation of the atherogenic index (total cholesterol / HDLc) and ApoB/ApoA ratio was also performed. Serum NT-pro-BNP was determined by the "sandwich electrochemiluminescence immunoassay" (ECLIA) method using the Roche Cobas Integrated Platform. At the endpoint of the rabbit-studies, thoracotomy was performed under deep anaesthesia (ketamine/xylazine, 75/5 mg / kg), the heart was isolated, weighed, and cut at the height of the papillary muscles, then placed in a 4% formalin solution. Left ventricle samples (always from the same region) were immediately frozen in liquid nitrogen and stored at -80 ° C for subsequent molecular biological analyses. Kidney and lung samples were weighed (wet weight) and then completely dried at 60 ° C overnight and weighed again (dry weight). The upper third of the aorta thoracica was placed in 4% formalin for subsequent histological stains. The right tibia was excised, cleaned, and the length (cm) was measured, and weight of organs were normalized to bodyweight and tibial length as well.

2.7. *Echocardiography*

Transthoracic echocardiographic imaging (TTE) was performed with GE Vivid E9 ultrasound device (GE Healthcare, New York, USA) for both animal models, but with different transducers and settings. The rat model was tested by i13L linear probe at 14 MHz frequency. The rats were anaesthetized by ketamine-xylazine combination (i.m., 50/5 mg / kg), then the chest hair was removed with an electric hair trimmer, and the animals were positioned in a dorsal position. For the rabbit model, a 12S-D "sector" transducer was used at 12 MHz. The anesthesia was performed with a ketamine-xylazine combination (i.m., 35/3 mg / kg), the sternal hair was removed and the animals were placed laterally. From this point on, the two settings followed the same imaging protocol, in line with the American Society of Echocardiography's recommendations. First, from parasternal long-axis (PLAX) view, the aorta, the left ventricle and the atrium was visualized, using 2D and M-mode echocardiograph. At papillary muscle height, M-mode imaging was performed to determine wall thickness and left ventricle diameters in systole and diastole (left ventricular posterior wall thickness, interventricular septum thickness, left ventricel internal diameter, fractional shortening and ejection fraction were determined). Aortic diameter above the aorta and the maximum width of the atrium were measured. The same recordings were made from a short axis view (SAX) for a more accurate definition. Subsequently, the mitral anular plane systolic excursion (MAPSE) was measured from the apical 4-well section along with the mitral inflow velocity curves (E/A) in Doppler mode. Tissue Doppler imaging (TDI) was performed to determine the velocity of lateral and septal annuluses, for the precise evaluation of diastolic function. Velocity and pressure gradients of left ventricle outflow tract (LVOT) were also determined using the Doppler technique. In the rabbit model, 5 cycles of motion were recorded from apical views at the appropriate frame rate, and Global Longitudinal Strain (GLS) was determined using the STE technique, after manually tracing the

ventricular segments. Data was stored on a hard disk and analyzed using EchoPAC PC software (ver. 112, GE Healthcare, New York, USA). Some parameters were derived by post-calculation: Tei index = IVRT+IVCT/ET; indicative for myocardial performance. Left ventricle mass was calculated as LVmass = 0.8(1,04((LVIDd + IVSd + LVPWd)3 * LVIDd3))) + 0.6, where LVID: left ventricle internal diameter, IVS: interventricular septum thickness, LVPW: left ventricle posterior wall thickness. The left ventricular remodeling geometry was estimated by calculating the relative wall thickness (RWT%): RWT% = 2 (LVPWd) / LVIDd; and increased RWT referred to concentric remodeling. Echocardiographic results were evaluated using a statistical program after a tabular summary.

2.8. *Vascular studies*

Vascular functional measurements were performed following thoracotomy in both models. After sacrificing the rats, the proximal part of the abdominal aorta was isolated, and 2 mm wide rings were cut off (two rings from each animal). The rings were mounted horizontally at 10 mN resting tension, using wire instruments, in 10-mL vertical organ chambers (Experimetria TSZ-04, Experimetria Ltd, Budapest, Hungary). The chambers contained Krebs solution oxygenated with 95% O₂ and 5% CO₂ (36 °C; pH = 7.4). The isometric contractile force of the circulatory muscle layer of aortic rings was measured by a transducer (Experimetria SD-01, Experimetria Ltd, Budapest Hungary) and strain gauge (Experimetria SG-01D), and recorded by a polygraph (Medicor R-61 6CH Recorder, Medicor, Hungary). After a 60-min incubation period, 10 nmol/L norepinephrine was administered to the rings (a norepinephrine concentration near to the half maximal effective concentration (EC50)). After the stabilization of the contractile force (pre-contraction), a cumulative Ach E/c curve was constructed (from 10 nmol/L to 10 µmol/L). (Acetylcholine releases NO from endothelium by activating eNOS). Responses of aortic rings obtained from the

same rat were averaged. In rabbit samples, ATP concentration curve (0.1-100 $\mu\text{mol/l}$) was also generated. The effect of Ach (and ATP) was defined as a percentage decrease in the pre-contraction produced by norepinephrine (in addition to the resting tension). Normality of data sets was verified with Shapiro-Wilk test. Data sets (more than two) were compared using one-way ANOVA (with Geisser-Greenhouse correction) followed by Tukey post-testing. Difference of means was considered significant at $p < 0.05$.

2.9. *Histology*

After the embedding of aorta samples fixed in 4% formalin solution into paraffin, sections of 7 μm thickness were prepared and placed on a slide for Hematoxyllin-eosin (HE) staining. After deparaffination (xylene, alcohol), the sections were placed in a hematoxylin solution for 15 seconds, and after differentiation (10 min tap water wash), slides were incubated with 0.1% eosin solution and one drop of 2% acetic acid for 4 min. After rinsing in 96% ethanol, the sections were dewatered in absolute alcohol and covered with a cover plate. After determining the intima and media thickness using a light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and morphometric software (Scion Corp., Torrance, CA, USA), the intima/media ratio was determined.

Masson's trichrome staining was used on myocardial samples to detect fibrotic tissues, according to the protocol of the manufacturer (Sigma-Aldrich). Briefly, after deparaffination (xylene, alcohol), Bouin's solution was used for fixation, followed by a 10-minute tap water wash, then a 10 min incubation with iron-hematoxylin solution. Subsequently, 10 minutes of incubation, washing, and differentiation in Bieblich (acid fushcine) dye were followed, in a special acid (phosphor-molybden/phosphor-tungsten acid). The blue color of collagen was then obtained by adding aniline blue dye and 1% acetic acid. Finally, the sections were dewatered in absolute alcohol and covered.

2.10. Western blot analyses

Western blot was performed on protein samples (300 mg) isolated from left ventricular myocardium in both animal models, and aortic thoracic samples (100 mg) of the rabbit model (only for PDE9A expression). Protein homogenate was prepared from the deep frozen tissues using a standard homogenization buffer (containing and a protease inhibitor) and a polytron homogenizer (T10 basic Ultra-Turrax, IKA, Germany). The total protein concentration of the samples was determined by a spectrophotometer (FLUOstar Optima, BMG Labtech, Ortenberg, Germany) using the BCA-method, then Laemmli buffer was added to the samples. Samples were separated by electrophoresis on a 12% SDS-polyacrylamide gel (120 V, 70-90 min) and transferred onto a nitrocellulose membrane (100 V, 90 min). The free binding sites of the membranes were blocked in 5% BSA dissolved in TBS-T buffer, for 120 min, followed by overnight incubation with primary antibodies corresponding to the protein of interest. Primary antibodies (produced in mice or rabbit) were purchased from Sigma-Aldrich-Merck (Darmstadt, Germany) and Abcam (Cambridge, UK). Primary antibodies were specific for the following proteins: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphodiesterase 9A (PDE9A), Protein Kinase G (PKG), vasodilator-stimulated phosphoprotein (VASP), ^{Ser239}phospho-vasodilator-stimulated phosphoprotein 16C2 clone (pVASP), sarco/endoplasmic reticulum ATPase 2a (SERCA2a), phospholamban (PLB), ^{Ser16}phospho-phospholamban (pPLB). From the primary antibodies detecting phosphorylated proteins, we chose those that the manufacturer guaranteed to recognize only the phosphorylated form, but not the complete, unphosphorylated protein. Antibodies were used at concentrations recommended by the supplier, typically at a dilution of 1: 1000 to 1: 5000. On the second day of the protocol, after TBS-T washing, the membranes were incubated with the corresponding (anti-mouse or anti-rabbit)

secondary HRP-conjugated (horse-raddish peroxidase) antibodies (1:10 000) for 90 min in TBS-T buffer containing 3% BSA. After washing, 3 ml of ECL substrate (Enhanced Chemiluminescent Substrate) was dropped onto the membranes (2 minutes), thus each labeled protein was detectable by C-Digit® blot scanner and Image Studio Digits ver. 5.2. software (LI-COR Inc., Lincoln, NE, USA). The expression level of each protein was indicated by pixel density calculated from the intensity of the detected light. After densitometric analysis, the pixel density was normalized to the corresponding housekeeping protein (GAPDH) of the same sample. Data were averaged from 3 repeated experiments, then statistically analyzed and plotted.

2.11. Determination of myocardial cGMP levels

The myocardial cGMP level was determined from left ventricular samples using direct competitive immunoassay (Abcam (Cambridge, UK)). A dilution series of standard cGMP provided by the manufacturer was prepared for this method, and myocardial samples were homogenized in 1 M hydrochloric acid. Measurements were carried out on a G-protein coated (antibody-binding), 96-well plate, and a spectrophotometer. The cGMP-content of the sample competed with the added standard HRP-conjugated cGMP for the binding sites. After washing and adding HRP substrate, the optical density at 450 nm was measured (FLUOstar Optima, BMG Labtech, Ortenberg, Germany), and absorbance was inversely proportional to the cGMP concentration of the sample. Samples were duplicated, then data was averaged and expressed as "pmol/mg tissue".

2.12. Statistical procedures

The results were reported as mean \pm SEM (standard error of the mean). Data were first tested for normality (D'Agostino-Pearson omnibus normality test). If only 2 groups were compared (rabbit model experiments), we used an unpaired t-test (Student's t-test), and if the data did not follow the normal distribution, the Mann-Whitney test was used to determine the differences. For the statistical

analysis of data obtained from rat model experiments, that requiring multiple group comparisons, the Kruskal-Wallis test was used (because of the small number of elements ($n = 6$)), with Dunn's post-hoc analysis. Differences were considered significant when the p value was less than 0.05, and the asterisks indicate the level of significance compared to the control, where: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; and ****: $p < 0.0001$. At some methods, the value of p is given precisely.

3. Results

3.1. Results of the Goto-Kakizaki experiments

3.1.1. Metabolic parameters

The initial body weight of GotoK animals was lower in the age-matched Wistar control animals, in line with the literature data. The body weight of metformin-treated animals increased to the smallest extent (week 12), while the weight of the pioglitazone-treated rats gained significant weight, thus the average bodyweight in this two group significantly differed at the endpoint (361.7 ± 12.27 vs. 411.3 ± 10.40 g). Fasting plasma glucose was elevated in all GotoK animals compared to the Wistar control, and although all antidiabetic agents reduced glucose levels, only pioglitazone-treated animals showed significantly lower values compared to the GotoK group (13.62 ± 0.6256 vs. 7.880 ± 0.3121 mmol/L). The HOMA-IR index of GotoK animals increased significantly compared to the Wistar control group, which indicates insulin resistance.

3.1.2. Endothelium-dependent vasorelaxation

In aortic samples of Wistar rats, $10 \mu\text{mol/L}$ Ach reduced the aortic tension to approximately 50% of the pre-contraction elicited by 10 nmol/L norepinephrine. In the group of GotoK rats without antidiabetic treatment, response to Ach showed a decrease in comparison with that of the Wistar rats, thus, the GotoK rats showed impaired endothelial function as compared to the Wistar rats. Treatment with BGP-15 and metformin did not significantly improve the deteriorated susceptibility of Goto-Kakizaki rat aorta to Ach, although metformin appeared to enhance the endothelium-dependent arterial relaxation at higher doses. In contrast, pioglitazone increased the response to Ach, that was statistically significant at 100 nmol/L and $1 \mu\text{mol/L}$ Ach concentrations (when compared to the GotoK group). Moreover, GotoK rats treated with pioglitazone

exhibited a greater ($p = 0.1261$ at $0.1 \mu\text{mol/L}$ Ach) endothelium-dependent arterial relaxation compared to the Wistar controls.

3.1.3. Echocardiography

By the end of week 12, diastolic cardiac function evaluated by echocardiography significantly deteriorated in the GotoK group compared to Wistar control. Diastolic dysfunction is indicated by the reversal of the e' / a' ratio measured by TDI in the GotoK group ($p = 0.0386$ vs. Wistar), indicating the reduced passive relaxation capability of the ventricular tissue. This is confirmed by the significant increase in the E wave and the E / e' ratio ($p = 0.002$ and $p = 0.0045$ vs. Wistar), indicative for elevated ventricular filling pressure. The duration of the ejection (ET) was significantly reduced in the GotoK group ($p = 0.05$ vs. Wistar), and the velocity of the outflow tract also increased (LVOT Vmax). All of the parameters described above changed significantly in the BGP-15 treated group. The e' / a' ratio increased ($p = 0.0023$ vs. GotoK), the velocity of the E and A waves decreased (to a value similar to the Wistar animals), the E/e' ratio, so the filling pressure decreased ($p = 0.0019$ vs. GotoK). The duration of ejection has increased, resulting in a significant improvement in the Tei index ($p = 0.0147$ vs. GotoK), which indicates an improvement in global cardiac function. The systolic parameters did not change significantly during the treatments. In the metformin-treated group, the rate of e'/a' and the outflow velocities were preserved, but no further parameters were significantly different from those of the GotoK group. Treatment with pioglitazone had no effect on the cardiac function determined by echocardiography.

3.1.4. Western blot results

The results of Western blot analyses on protein isolates from left ventricular samples can be summarized as the follows: significant differences were found between the groups in the level and phosphorylation of phospholamban (PLB)

and VASP proteins. The ratio of ^{Ser¹⁶}phospho-phosolamban to the non-phosphorylated form (pPLB/PLB) was significantly reduced in the untreated GotoK group ($p = 0.0171$ vs. Wistar). The pPLB/PLB ratio increased significantly in the myocardium of GotoK animals treated with BGP-15 ($p = 0.0348$ vs. GotoK). Metformin treatment showed a similar trend ($p = 0.1432$ v. GotoK). Since 239-serine amino acid of the VASP protein is the target of Protein Kinase G, Ser239 phospho-VASP/total VASP (pVASP/VAsP) ratios were also investigated. The pVASP/VASP ratio was similar to that of the phospholamban protein, ie. significantly increased in the BGP-15-treated group compared to untreated animals ($p = 0.0094$ vs. GotoK). Metformin-treatment produced similar result ($p = 0.0877$ vs. GotoK). The expression of SERCA2a did not differ significantly between treatment groups. Although the expression of PDE9A, responsible for the degradation of NP-cGMP was the highest in the GotoK group, and decreased in the BGP-15 treated group, these differences did not reach the pre-defined significance level ($p < 0.05$).

3.2. Result of the rabbit-model experiments

3.2.1. Echocardiography

After 16 weeks of atherogenic diet, significant differences were found between the echocardiographic parameters of the Control and HC groups, primarily in diastolic function. In the HC group, the area of the left atrium ($p < 0.0001$), the weight of the left ventricle ($p = 0.0005$), the relative wall thickness ($p = 0.005$), the velocity of the outflow tract ($p < 0.0001$) and the pressure gradient ($p < 0.0001$) significantly increased. These parameters indicate increased filling pressure, ventricular hypertrophy and aortic stenosis in the HC group. The ejection fraction (EF) was significantly reduced compared to Control ($p = 0.0026$), but remained within the physiological range. Among the values determined by

Doppler echocardiography, a decrease in the E/A and TDI e'/ a' ratios ($p < 0.0001$ and $p = 0.002$), deceleration time ($p = 0.0002$) and prolongation of isovolumic contraction time ($p < 0.0001$) and an increase in the E/e' ratio ($p = 0.0002$) was found in the HC group, parameters indicative for diastolic dysfunction and elevated filling pressures. The global performance indicator Tei-index declined significantly compared to the Control group ($p < 0.0001$). During Speckle Tracking echocardiography, global longitudinal systolic function was characterized by GLS (global longitudinal strain). The value of GLS decreased from about 20% (Control) to about 16% in the HC group ($p = 0.0007$), which specifically indicates the systolic dysfunction.

3.2.2. Morphometry

The animals in the HC group gained significant weight compared to the Control (4153 ± 86 vs. 3064 ± 87 g). The weight of the left ventricle (LV) was also increased, when was normalized to body weight and, for a more standardized parameter, to the length of the tibia (0.828 ± 0.038 ** vs. 0.605 ± 0.046). There was no difference between the groups in wet/dry tissue ratiops, suggesting the absence of significant edema.

3.2.3. Serum parameters

Lipid values of HC animals (total cholesterol, LDL, ApoB: $p < 0.0001$), liver enzymes, C-reactive protein (CRP) ($p = 0.0359$) and troponin T values ($p = 0.0092$) were significantly elevated in comparison to the Control. These results suggest marked hypercholesterolemia and mild systemic inflammation (CRP) in the hypercholesterolemic group.

3.2.4. Endothelium-dependent vasorelaxation and aortic PDE9A expression

Based on the results obtained from the studies performed on the isolated vascular rings, the response to noradrenaline was significantly different between the groups, as there was a significantly weaker contraction response in the HC group at 10 and 100 nmol/L concentrations. In both groups, acetylcholine caused relaxation (compared to pre-contraction) to 1 μ mol/L, and above produced contraction. The samples of the animals kept on the atherogenic diet produced significantly less relaxation in all concentration ranges. After fitting the Hill equation, the determined E_{max} decreased (50.9 ± 9.48 vs. 93.67 ± 2.66 , HC vs. Control), while the \log_{EC50} increased slightly (-7.59 ± 0.16 vs. -8.62 ± 0.36 , HC vs. Control) in the HC group in comparison to the Control. ATP induced significantly higher relaxation response in the samples of the Control group compared to the HC, thus the atherogenic diet also impaired the ATP-induced relaxation, in addition to the "classical" acetylcholine-dependent relaxation. In the thoracic aortic samples of HC animals, expression of PDE9A enzyme was significantly increased compared to the Control ($p < 0.0001$).

3.2.5. Histology

Hematoxylin-eosin staining on the aorta sections showed atherosclerotic plaques containing foam cells. The plaque-free, control vessels showed low intima/media ratios, whereas this ratio in the samples of the HC animals was dramatically increased (1.623 ± 0.079 vs. 0.043 ± 0.004). Masson's trichrome staining was conducted for only representative purposes, on a small number of samples. Fibrotic tissue traces of blue color can be detected in the left ventricular myocardium of HC animals, while these were absent in Control samples.

3.2.6. Western blot and myocardial cGMP-levels

In the left ventricle samples of HC animals, PDE9A expression significantly elevated in comparison to the Control samples ($p < 0.001$). Expression of both unphosphorylated VASP and Ser239 phospho-VASP (pVASP) increased in the HC group relative to Control, but the pVASP / VASP ratio did not differ among groups, suggesting unchanged Protein Kinase G activity. This was also confirmed by the level of myocardial cGMP-content: no significant difference was found between cGMP levels in Control and HC groups. In contrast, the PKG protein expression was significantly elevated in the HC group ($p < 0.001$), thus, increased expression was not followed by an increase in enzyme activity.

4. Discussion

4.1. Findings of the Goto-Kakizaki rat studies

As expected, GotoK animals showed elevated fasting glucose and insulin levels, and were insulin-resistant (HOMA-IR). In our experimental setup, only pioglitazone was able to exert significant reductions in serum glucose levels, although the glucose levels of BGP-15 and metformin-treated animals were also slightly lower. Pioglitazone significantly improved the response of isolated aortic rings to acetylcholine, ie. the endothelial-dependent vasorelaxation, while metformin-treatment only showed a non-significant effects, while treatment with BGP-15 had no effect on vasorelaxation. However, despite the beneficial effects of pioglitazone, weight gain was observed in treated animals, which is undesirable in T2DM. As regards cardiac function, we obtained significantly different results, as pioglitazone and metformin-treatments had only a negligible effect on diastolic dysfunction observed in GotoK animals, whereas cardiac function in BGP-15-treated animals were significantly enhanced, the dysfunction was delayed. Echocardiography revealed that the E wave, the ratio of e'/a' and the E/e' ratios, indicative for myocardial motility and loading pressure were preserved in the GotoK+BGP-15 group. The ejection time, the Tei index describing the performance of the myocardium, and the velocity of the outflow tract were similar to those of the Wistar control animals, and were significantly improved in comparison to the values of untreated GotoK animals.

During molecular biological analyses, the expression and activity of the SERCA2a pump, a significant determinant of diastolic function was assessed. Expression of the SERCA pump did not differ in samples of animals, despite, the phosphorylation level of the phospholamban protein, the determinant of the activity of the SERCA pump, showed significant differences among groups. In the untreated GotoK group, the phosphorylation of PLB decreased, while elevated

in the BGP-15-treated GotoK animals, indicating the preserved function of the SERCA pump. The phosphorylation of the Ser16 amino acid of PLB may be a result of increased Protein Kinase G activity. This was further confirmed by monitoring the phosphorylation of the unique PKG target, VASP protein, which showed similar pattern. Since BGP-15 did not affect endothelial-dependent relaxation, we assumed that elevated PKG activity may not related to the NO pathway (although it is possible that BGP-15 may activate different signaling pathways in the vascular wall and in the heart muscle). Based on the above, we examined the expression of the PDE9A enzyme, that is responsible for the degradation of NP-cGMP. Although PDE9A expression was the elevated in the untreated GotoK group, and decreased in the BGP-15 treated group, differences were below the significance threshold (small number of elements), therefore it may be rational to assess PDE9A expression in similar models with higher “n” values.

In summary, since BGP-15 treatment exerted non-significant effects on metabolic parameters of the GotoK rat, and BGP-15 did not affect endothelial-dependent vasorelaxation, the significant improvement of diastolic function may be the result of the preserved activity of the PKG enzyme, causing phosphorylation of the PLB, thus preserved SERCA function and more complete myocardial relaxation.

4.2. Findings of the HC rabbit studies

During the treatment, rabbits in the HC group developed pronounced diastolic dysfunction with all its echocardiographic signs. E/A and e'/a' ratio declined, DecT and IVRT elongated, E/e' increased. The left ventricular mass was significantly higher in the HC group, leading to an increase in wall thickness (IVS) and as well an increase in relative wall thickness (RWT%), suggesting concentric hypertrophic remodeling. Pathology was also confirmed by the

appearance of fibrotic tissue, experienced during the representative staining. Global cardiac function impairment was indicated by an increase in the Tei index. Regarding systolic functions, the ejection fraction only slightly decreased, but the GLS (longitudinal strain of the myofibers) measured by STE echocardiography was significantly deteriorated in the group on the atherogenic diet. The increased pressure and velocity gradients of the outflow tract indicate persistent aortic stenosis in the HC group. Left ventricular and cardiac enlargement were also confirmed by ex vivo measurements, but no signs of peripheral edema (wet/dry weight ratios) were observed. Based on the results of blood parameters, marked dyslipidemia (total cholesterol, LDL, ApoB), along with a mild but significant increase of inflammatory and cardiac markers (CRT, troponin T) was observed. This was accompanied by the histological appearance of atherosclerosis (foamy plaques on the aorta), and by a significant reduction in endothelial-dependent vasorelaxation, i.e., NO bioavailability. An important novel finding obtained by Western blot studies was the elevated expression of PDE9A on aortic samples that may contribute to the development of endothelial dysfunction, by the increased degradation of NP-cGMP. Based on molecular biological studies on myocardial tissue, atherosclerosis-induced cardiac dysfunction was associated with increased myocardial expression of PDE9A, which was first described by our team in atherosclerotic heart disease. Similarly, expression of the VASP protein (unphosphorylated and phosphorylated on Ser239) increased, which may contribute to the hypertrophic response in the HC group. The activity of Protein Kinase G (measured by pVASP/VASP ratio), a key enzyme initiating cardioprotective pathways, was shown to be unchanged, butPKG protein expression was significantly elevated in the HC group. Thus, the increased PKG expression was not accompanied by an increase in its activity, possibly due to the relative lack of cGMP. Myocardial level of cGMP was well correlated with the pVASP/VASP ratio and confirms the unchanged PKG enzymatic activity with increased expression. A limiting factor in cGMP-elevation may be an increase in

the expression of the PDE9A enzyme, that is responsible for cGMP degradation. Based on our findings, PDE9A upregulation can be detected in early-stage cardiac dysfunction associated with atherosclerosis, and may become a future drug target in atherosclerotic heart diseases.

5. Summary

Both diabetes mellitus and atherosclerosis induce pathological changes in the vasculature and in the myocardial tissue, that may result in cardiac dysfunction. Recent investigations emphasize the diminished activity of the cGMP-PKG signaling pathway behind the pathophysiology. Our aim was to characterize cardiac dysfunction induced by diabetes in the Goto-Kakizaki rat, and to compare the cardiovascular effects of the drug candidate BGP-15 to such actions of well-known pleiotropic antidiabetic agents, with particular interest regarding the Protein Kinase G pathway. Secondly, we aimed to investigate cardiac and endothelial dysfunction as a result of atherogenic diet in a hypercholesterolemic rabbit model, concerning the role of the same, cGMP-PKG-PDE9A signaling pathway. According to our results, BGP-15 delays the onset of diabetic cardiomyopathy in the Goto-Kakizaki rat, independent from any beneficial metabolic or vascular effect. BGP-15 seems to increase phospholamban phosphorylation at Ser16, that may preserve SERCA2a pump function. This mechanism may be related to increased activity of PKG enzyme, as showed by elevated pVASP/VASP ratios in the myocardial tissues of rats. Furthermore, our findings demonstrate that atherosclerotic cardiovascular disease may be associated with the upregulation of PDE9A enzyme in aortic vessels and myocardial tissues of rabbits. This is accompanied by the increased expression but not activity of the PKG enzyme in the heart, thus the upregulation of PDE9A may contribute to diminished activity of cardioprotective pathways under atherosclerotic conditions.

6. Novel results of the doctoral thesis

In the light of our original aims, the following new findings were obtained on the basis of the above two studies:

- BGP-15 treatment improves diastolic cardiac function in type 2 diabetic Goto-Kakizaki rat, irrespective of metabolic and vascular effects; and the beneficial effects of BGP-15 may be partly a result of the increased phosphorylation of phospholamban on Ser16 amino acid, thereby preservation of the SERCA2a pump function through activation of the Protein Kinase G signaling pathway.
- Manifestation of cardiac dysfunction related to atherosclerosis on rabbit model results in increased expression of PDE9A in the vasculature and in the myocardial tissues, which is associated with an increase in PKG expression in cardiomyocytes, with unchanged enzymatic activity, thus upregulation of PDE9A may contribute to the suppression of cardioprotective signals.

7. List of publications



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Registry number: DEENK/47/2019.PL
Subject: PhD Publikációs Lista

Candidate: Dániel Priksz
Neptun ID: BW72WL
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. Bombicz, M., **Priksz, D.**, Gesztelyi, R., Kiss, R., Hollós, N., Varga, B., Németh, J., Tóth, A., Papp, Z., Szilvássy, Z., Juhász, B.: The Drug Candidate BGP-15 Delays the Onset of Diastolic Dysfunction in the Goto-Kakizaki Rat Model of Diabetic Cardiomyopathy. *Molecules*. 24 (3), 1-18, 2019.
DOI: <http://dx.doi.org/10.3390/molecules24030586>
IF: 3.098 (2017)
2. **Priksz, D.**, Bombicz, M., Varga, B., Kurucz, A., Gesztelyi, R., Balla, J., Tóth, A., Papp, Z., Szilvássy, Z., Juhász, B.: Upregulation of Myocardial and Vascular Phosphodiesterase 9A in A Model of Atherosclerotic Cardiovascular Disease. *Int. J. Mol. Sci.* 19 (10), 1-18, 2018.
DOI: <http://dx.doi.org/10.3390/ijms19102882>
IF: 3.687 (2017)

List of other publications

3. Szabó, K., Gesztelyi, R., Lampé, N., Kiss, R., Gáléné Remenyik, J., Pesti-Asbóth, G., **Priksz, D.**, Szilvássy, Z., Juhász, B.: Fenugreek (Trigonella Foenum-Graecum) Seed Flour and Diosgenin Preserve Endothelium-Dependent Arterial Relaxation in a Rat Model of Early-Stage Metabolic Syndrome. *Int. J. Mol. Sci.* 19 (3), 1-21, 2018.
DOI: <http://dx.doi.org/10.3390/ijms19030798>
IF: 3.687 (2017)
4. Kurucz, A., Bombicz, M., Kiss, R., **Priksz, D.**, Varga, B., Hortobágyi, T., Trencsényi, G., Szabó, R., Posa, A., Gesztelyi, R., Szilvássy, Z., Juhász, B.: Heme oxygenase-1 activity as a correlate to exercise-mediated amelioration of cognitive decline and neuropathological alterations in an aging rat model of dementia. *Biomed Res. Int.* 2018, 1-13, 2018.
IF: 2.583 (2017)





5. Kiss, R., Szabó, K., Gesztelyi, R., Somodi, S., Kovács, P., Szabó, Z., Németh, J., **Priksz, D.**, Kurucz, A., Juhász, B., Szilvássy, Z.: Insulin-Sensitizer Effects of Fenugreek Seeds in Parallel with Changes in Plasma MCH Levels in Healthy Volunteers.
Int. J. Mol. Sci. 19 (3), 771-, 2018.
DOI: <http://dx.doi.org/10.3390/ijms19030771>
IF: 3.687 (2017)
6. Varga, C., Veszelka, M., Kupai, K., Börzsei, D., Deim, Z., Szabó, R., Török, S., **Priksz, D.**, Gesztelyi, R., Juhász, B., Radák, Z., Pósa, A.: The Effects of Exercise Training and High Triglyceride Diet in an Estrogen Depleted Rat Model: the Role of the Heme Oxygenase System and Inflammatory Processes in Cardiovascular Risk.
J. Sports Sci. Med. 17 (4), 580-588, 2018.
IF: 1.99 (2017)
7. Vecsernyés, M., Szokol, M., Bombicz, M., **Priksz, D.**, Gesztelyi, R., Fülöp, G. Á., Varga, B., Juhász, B., Haines, D. D., Tósaki, Á.: Alpha-MSH induces vasodilatation and exerts cardioprotection via the heme-oxygenase pathway in rat hearts.
J. Cardiovasc. Pharmacol. 69 (5), 286-297, 2017.
DOI: <http://dx.doi.org/10.1097/FJC.0000000000000472>
IF: 2.227
8. Bombicz, M., **Priksz, D.**, Varga, B., Kurucz, A., Kertész, A. B., Takács, Á., Pósa, A., Kiss, R., Szilvássy, Z., Juhász, B.: A Novel Therapeutic Approach in the Treatment of Pulmonary Arterial Hypertension: allium ursinum Liophylisate Alleviates Symptoms Comparably to Sildenafil.
Int. J. Mol. Sci. 18 (7), 1-19, 2017.
DOI: <http://dx.doi.org/10.3390/ijms18071436>
IF: 3.687
9. Szokol, M., **Priksz, D.**, Bombicz, M., Varga, B., Kovács, Á., Fülöp, G. Á., Csípő, T., Pósa, A., Tóth, A., Papp, Z., Szilvássy, Z., Juhász, B.: Long term osmotic mini pump treatment with alpha-MSH improves myocardial function in Zucker Diabetic Fatty rats.
Molecules. 22 (10), 1-18, 2017.
IF: 3.098
10. Varga, B., **Priksz, D.**, Lampé, N., Bombicz, M., Kurucz, A., Szabó, A. M., Pósa, A., Szabó, R., Kemény-Beke, Á., Gáléné Remenyik, J., Gesztelyi, R., Juhász, B.: Protective Effect of *Prunus Cerasus* (Sour Cherry) Seed Extract on the Recovery of Ischemia/Reperfusion-Induced Retinal Damage in Zucker Diabetic Fatty Rat.
Molecules. 22 (10), [1-12], 2017.
DOI: <http://dx.doi.org/10.3390/molecules22101782>
IF: 3.098





11. Bombicz, M., **Priksz, D.**, Varga, B., Gesztelyi, R., Kertész, A. B., Lengyel, P., Balogh, P., Csupor, D., Hohmann, J., Bhatto, H. P., Haines, D. D., Juhász, B.: Anti-Atherogenic Properties of Allium ursinum Liophylisate: impact on Lipoprotein Homeostasis and Cardiac Biomarkers in Hypercholesterolemic Rabbits.
Int. J. Mol. Sci. 17 (8), E1284, 2016.
IF: 3.226
12. Fehér, P., Ujhelyi, Z., Váradi, J., Fenyvesi, F., Róka, E., Juhász, B., Varga, B., Bombicz, M., **Priksz, D.**, Bácskay, I., Vecsernyés, M.: Efficacy of Pre- and Post-Treatment by Topical Formulations Containing Dissolved and Suspended Silybum marianum against UVB-Induced Oxidative Stress in Guinea Pig and on HaCaT Keratinocytes.
Molecules. 21 (10), 1269, 2016.
DOI: <http://dx.doi.org/10.3390/molecules21101269>
IF: 2.861
13. Kovács, Á., Fülöp, G. Á., Kovács, A., Csípő, T., Bódi, B., **Priksz, D.**, Juhász, B., Beke, L., Hendrik, Z., Méhes, G., Granzier, H. L., Édes, I., Fagyas, M., Papp, Z., Barta, J., Tóth, A.: Renin overexpression leads to increased titin-based stiffness contributing to diastolic dysfunction in hypertensive mRen2 rats.
Am. J. Physiol.-Heart Circul. Physiol. 310 (11), H1671-H1682, 2016.
DOI: <http://dx.doi.org/10.1152/ajpheart.00842.2015>
IF: 3.348
14. Kertész, A. B., Bombicz, M., **Priksz, D.**, Balla, J., Balla, G., Gesztelyi, R., Varga, B., Haines, D. D., Tósaki, Á., Juhász, B.: Adverse Impact of Diet-Induced Hypercholesterolemia on Cardiovascular Tissue Homeostasis in a Rabbit Model: time-Dependent Changes in Cardiac Parameters.
Int. J. Mol. Sci. 14 (9), 19086-19108, 2013.
DOI: <http://dx.doi.org/10.3390/ijms140919086>
IF: 2.339
15. Juhász, B., Kertész, A. B., Balla, J., Balla, G., Szabó, Z., Bombicz, M., **Priksz, D.**, Gesztelyi, R., Varga, B., Haines, D. D., Tósaki, Á.: Cardioprotective Effects of Sour Cherry Seed Extract (SCSE) on the Hypercholesterolemic Rabbit Heart.
Curr. Pharm. Design. 19 (39), 6896-6905, 2013.
IF: 3.288

Total IF of journals (all publications): 45,904

Total IF of journals (publications related to the dissertation): 6,785

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

01 March, 2019



8. Acknowledgement

I am grateful to Dr. Béla Juhász, my supervisor, to his given advices not only in my professional work, but also in other areas of life. I'm grateful for his attention and friendly attitude, by which he helps me and my colleagues day by day.

I would like to thank Dr. Zoltán Szilvássy, who provided me with the opportunity to do my work at the Institute of Pharmacology and Pharmacotherapy with all possible support. With his attitude and skills, he sets an example to me and my colleagues in our professional work and life.

I am sincerely grateful to my colleague and friend, Mariann Kozma, who, with her creativity, essentially contributes to our research work. With her friendship and humor, she supports me day by day. I would like to thank to my colleagues Dr. Balázs Varga and Okszána Kisził for their professional help and sincere friendship.

I am grateful to the current and former staff of the Institute of Pharmacology and Pharmacotherapy for the high level of professional work and support. I would like to thank Szegváriné Andrea Erdős and Krisztina Oláh for their help in performing all the experiments.

I am grateful to our collaborative partners, the Department of Clinical Physiology, to Dr. Attila Tóth and Dr. Zoltán Papp, and Dr. Árpád Kovács, Dr. Gábor Fülöp and Dr. Tamás Csípő for their help and ideas. I'm grateful to the staff of the UD Laboratory of Medical Medicine.

I would like to thank our TDK students and our former colleagues at the Faculty of Pharmacy, for their honest support. I thank my friends, Dr. Balázs Gaál and Dr. Ádám Pék for their advices and help.

I'm sincerely grateful for the persistent support and love for my wife, Dr. Zsuzsa Priksz, and all my family members.

I dedicate this work to my beloved Grandfather, Mihály Priksz.

The doctoral thesis was supported by the GINOP-2.3.4-15-2016-00002 project. The project is co-financed by the European Union and the European Regional Development Fund.

