SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Functional and molecular biological study of the retinoprotective effect of BGP-15 in Goto-Kakizaki and Zucker Diabetic Fatty (ZDF) diabetic animal models

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DOCTORAL SCHOOL OF NUTRITION AND FOOD SCIENCES
Debrecen, 2023.

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The PhD defense takes place at the Lecture hall of „A" building of Internal Medicine, Faculty of Medicine, University of Debrecen
2023. july 14. 11 a.m.

Table of Contents

1. Introduction and Objectives

Diabetes mellitus is a worldwide problem in developing countries, affecting more and more people every year. The number of diabetes patients increased from 108 million in 1980 to 422 million in 2014, according to 2018 WHO statistics. Most of the chronic complications of diabetes arise from macro- and microangiopathy, such as coronary, cerebrovascular and peripheral vascular diseases, diabetic nephropathy, neuropathy and retinopathy.

Diabetic retinopathy, a common microvascular complication of diabetes mellitus, is one of the leading causes of blindness worldwide. High blood sugar and the consequent ischemia-reperfusion (I/R) damage destroy the function of retinal vessels. Deterioration of vision can be functionally measured using electroretinography (ERG) even in laboratory animals.

Although there are many genetic rat models of diabetes, such as Zucker diabetic fatty (ZDF), Otsuka Long-Evans Tokushima fatty (OLETF), biobreeding (BB), Wistar Bonn/Kobori (WBN/Kob) , Spontaneous Diabetic Torii (SDT) and Goto-Kakizaki (GK), there is no "best model" for 100 percent imitation of diabetes in humans. The ZDF rat is the result of a mutation in an originally obese/fatty rat strain, namely the Zucker rat, which results in the obese ZDF rats exhibiting glucose intolerance, hyperinsulinemia and eventually type 2 diabetes due to leptin resistance caused by the mutation. In addition to ZDF, OLETF and BB are also monogenic animal models, the former containing a G-protein mutation causing obesity and type II diabetes, and the latter a type I diabetes model due to a mutation causing an autoimmune response in lymphoid immune cells. GK, WBN/Kob and SDT rats belong to the group of animal models of polygenic diabetic retinopathy, each of which produces type II diabetes at different stages of life. During my work, we first performed experiments with Goto-Kakizaki (GK) rats; however, this model does not

develop obesity, which is common in the clinical environment, so in the second phase of my work, we worked on ZDF animals. In the case of the ZDF rat, the process of developing type II diabetes and many of its characteristics, such as the tendency to develop obesity, are the same as in humans. Type 2 diabetes develops in humans with similar symptoms, ranging from glucose intolerance to overt diabetes to serious consequences of diabetes, such as diabetic retinopathy. The pathological process of retinopathy can be clearly visualized using electroretinography (ERG), which method is also suitable for measuring the disease-reducing effects of certain experimental substances.

Our working group has already conducted several experiments with retinoprotective agents on diabetic animals to prevent damage to the retina. BGP-15 is a promising future target molecule for drug development, which, being chemically related to propranolol, was first developed for cardiovascular purposes, although it has since been tested in many different diseases related to diabetes and ischemia-reperfusion, such as cardiac ischemia-reperfusion , in kidney toxicity, neuropathy, myopathy, and especially against insulin resistance. Diabetes itself is also related to ischemia-reperfusion damage: for example, local ischemia increases the expression of vascular endothelial growth factor (VEGF), which then leads to proliferative retinopathy with neovascularization. Thus, retinal ischemia-reperfusion injuries are a proven part of the background events leading to the above-mentioned vision-damaging consequences of diabetes. At the same time, at the beginning of my research, no one had investigated BGP-15 in diabetic retinopathy, although it is a potential candidate for the treatment or prevention of such diseases due to its mechanism of action.

Based on these previous results, the aim of our first experiment was to assess the effects of systemic BGP-15 against the retina-damaging effect of glucose in an insulin-resistant animal model, the Goto-Kakizaki rat. Furthermore, using electroretinography, we wanted to compare the possible

retinoprotective effect of BGP-15 with standard anti-diabetic drugs: metformin, glibenclamide and pioglitazone, representatives of the main anti-diabetic groups. We also attempted to discover the mechanism of action of the retinoprotection observed in the case of treatments: we wanted to investigate the possible effect of BGP-15 on the expression of SIRT1 and MMP9.

As a basis for potential future drug development targets, our second study was conducted to assess the possible side effects of long-term BGP treatment: this trial aimed to find out whether BGP-15 causes early death over a long period of time, and if so, for what reasons . Here we used ZDF rats instead of GK in order to experiment with obesity-prone, diabetic animals in the long term: this has significant clinical implications, since most patients with type II diabetes are overweight. My work is based on the already mentioned electroretinography measurement, which is a relatively easy-to-implement and well-reproducible method that provides accurate information about the vision of animals and the function of the retina. In this second experiment, the animals suffered from completely uncontrolled diabetes during the one-year period and received the treatment substance, BGP-15, daily via feeding tube.

2. Materials and methods

2.1. Experimental animals

In our first experiment, male Goto-Kakizaki and Wistar rats (10 weeks old; 250–300 g) were obtained from Charles River Laboratories International Inc. (Wilmington, MA, USA) for our study. The Goto-Kakizaki (GK) model, according to Charles River, is a non-obese Wistar substrain that develops type 2 diabetes; control is the Wistar rat (Crl: WI). The animals were kept in cages and cared for in accordance with international standards for animal research (ARVO (Statement for the Use of Animals in Ophthalmic and Vision Research) and NIH (National Institute of Health) guidelines). All methods used during the

study were approved by the Institutional Animal Ethics Committee of the University of Debrecen (18/2013/DE MÁB). Animals had free access to water and were fed standard rodent chow ad libitum.

In our second experiment, eight-week-old male ZDF rats (fa/fa) and their controls, male so-called lean rats (-/-) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA). The housing and care of the animals in this case was also based on the rules adopted by the Workplace Animal Ethics Committee of the University of Debrecen, in accordance with international regulations (ARVO and NIH guidelines), and all methodological protocols were also approved by the same committee. The animals also had free access to water and rodent chow, which in this case was Purina 5008, recommended for ZDF rats.

2.2. Glucose-related measurements

In our first experiment, fasting blood glucose levels were analyzed at the beginning of the treatment period, then at the beginning of weeks 3, 8, 11 and immediately before the end of the study ("end point"). Furthermore, an Oral Glucose Tolerance Test (OGTT) was performed at all the above-mentioned time points, except at the end point. At the end of this experiment, all animals were subjected to a hyperinsulinemic euglycemic glucose clamp (HEGC) measurement to assess their insulin sensitivity or insulin resistance.

In our second experiment, we also performed an OGTT: after a 1-month acclimatization (baseline values), then at the beginning of the gastric probing period (initial values), and then after 1, 6, and 12 months of gavage.

All of the mentioned methods were performed after overnight fasting.

2.2.1 Fasting Plasma Glucose

For fasting blood glucose analysis, blood was taken from the tail vein, and then blood glucose was measured with an Accu-Check glucometer (Roche Diagnostics, Mannheim, Germany).

2.2.2 Oral glucose tolerance test (OGTT)

For the OGTT measurements, the animals were given 2 g/kg glucose (Sigma-Aldrich-Merck KGaA, Darmstadt, Germany) through a stomach tube, and then the blood sugar level taken from the tail was measured after 15, 30, 60, 90 and 120 minutes with the above-mentioned glucometer. Fasting blood glucose level was used as a starting value (OGTT time 0 minutes). When evaluating the results of the OGTT, we used the following equation to calculate the area under the curve (AUC), where "n" is the number of measurement times:

$$AUC = (\left(\frac{c_1 + c_2}{2}\right) * (t_2 - t_1) + \cdots + \left(\frac{c_{n-1} + c_n}{2}\right) * (t_n - t_{n-1}))$$

2.2.3. Hyperinsulinemic euglycemic glucose clamp (HEGC)

At the end of the first experiment, after the fasting glucose measurement, the animals were anesthetized with a ketamine/xylazine mixture (100/10 mg/kg body weight; ketamine: Calypsol, Richter Gedeon Ltd., Budapest, Hungary; xylazine: Nerfasin, Le Vet BV, Oudewater, The Netherlands) and HEGC we performed a protocol on them according to a previously used method, as follows. First, we surgically opened the trachea and inserted a cannula. Two jugular veins were then cannulated for administration of glucose solution (20%) and insulin (6 mIU/min (milli international units per minute); Humulin R, Eli Lily, Indianapolis, IN, USA), and a carotid artery for blood collection. Blood sugar levels were measured with an Accu-Check glucometer from blood samples taken every 10 minutes. Euglycemia was maintained by adjusting the

rate of glucose infusion (GI). The steady-state GI was reached - and the experiment was considered complete - when the blood sugar level stabilized for at least 20 minutes. To measure plasma insulin, blood samples were taken from the carotid artery at the beginning and end of the HEGC protocol. Insulin was measured from the plasma of blood samples that were centrifuged for 2 min at 4 °C and 10,000 g (5415R Centrifuge, Eppendorf GmbH, Hamburg, Germany). After the HEGC protocol, all animals were euthanized with an overdose of the ketamine/xylazine combination anesthetic. After that, the eyes of the animals were removed for further microbiological analysis.

2.2.4 Calculated indices

From the results of direct measurements before and during the HEGC protocol - i.e. body weight (BW), fasting plasma glucose (FPG), fasting plasma insulin (FPI), glucose infusion rate (GI), insulin infusion rate (II), steady-state plasma glucose (SSPG), steady-state plasma insulin (SSPI) - various indices were calculated, the formulas of which are as follows:

GIR = glucose infusion rate (not to be confused with glucose infusion rate) (GI))

$$GIR = \frac{GI\ (\mu l/min)}{BW\ (g)} * 220$$

The correction factor used (220) is due to the concentration of the glucose infusion, which was 20% (220 g glucose in 1000 ml water; Glucose TEVA 20% Solution, Teva Ltd., Debrecen, Hungary), so the final unit of GIR will be: mg /minute/kg.

ISI = insulin sensitivity index; unit: (mg L)/(min kg mIU)

$$ISI = \frac{GIR\ (mg/min/kg)}{SSPI\ (mIU/l)}$$

MCRI = Metabolic Clearance Rate for Insulin; unit: mL/min

$$MCRI = \frac{II\ (mIU/min)}{SSPI\ (\mu IU/ml) - FPI(\mu IU/ml)} * 1000$$

The correction factor (1000) was used due to the conversion of the milli international unit (international unit, IU) into micro IU values.

QUICKI = Quantitative Insulin sensitivity Check Index (QUantitative Insulin sensitivity ChecK Index) (without measurement unit)

$$QUICKI = \frac{1}{log\ FPI(\mu IU/ml) + (logFPG(mg/dl) * 18)}$$

The correction factor (×18) was used due to the conversion from mmol/l to mg/dl.

To calculate insulin resistance (HOMA-IR), we used the homeostatic model of assessment (HOMA) Matthews et al. Based on.

$$HOMA - IR = \frac{FPI(\mu IU/ml) * FPG(mmol/l)}{22{,}5}$$

Since μIU/ml = mIU/l, the final unit of HOMA-IR will be (mIU × mmol)/l2.

To evaluate pancreatic β-cell function, we used the HOMA-B calculation method based on the same article.

$$HOMA - B = \frac{20 * FPI(\mu IU/ml)}{FPG(mmol/l) - 3{,}5}$$

Since μIU/ml = mIU/l, the final HOMA-IR unit will be: mIU/mmol

2.3. Calculation and evaluation of the survival curve

During the long course of our second study (52 weeks, i.e. 1 year), some animals died, and we recorded them. Data were then transferred to the statistical analysis program GraphPad Prism (version 7.0, GraphPad Software Inc., La Jolla, CA, USA) to generate Kaplan–Meier survival curves. The curves were then analyzed using the Mantel–Cox test and the Gehan–Breslow–Wilcoxon test.

2.4. Electroretinography (ERG)

At the end of both of our experiments, electroretinography measurements were performed: in our first experiment one day before HEGC, and in our second experiment immediately after the last OGTT. Animals were anesthetized with a combination of ketamine/xylazine (100/10 mg/kg) to perform electroretinography measurements as follows. The pupils of both eyes were dilated with one drop of cyclopentolate (Humapent, Teva Kft., Debrecen, Hungary), then a short fundus examination was performed with a hand-held ophthalmoscope (Heine Mini 2000 Ophthalmoscope, HEINE Optotechnik GmbH and Co. KG, Gilching, Germany) diabetic retinopathy for confirmation based on the literature. For ERG measurement, five needle electrodes were used to drive light-generated currents into an amplifier connected to an analog-to-digital converter (Bridge Amp and PowerLab, ADInstruments, Sydney, Australia), and waveforms were recorded using PowerLab Chart software (version 5.2.2, ADInstruments , Sydney, Australia) to make it visible on a computer. Two measuring electrodes were inserted lightly into the surface of the cornea (without perforating it), two reference electrodes were inserted into the earlobe of the animal, while the general grounding electrode was fixed in the midline of the animal, inserted into the skin of the bridge of the nose. Carbomer-based eye gel (Vidisic, BauschandLomb, Berlin, Germany) was used to prevent eye dryness and as a contact gel.

Based on the guidelines of the International Society for Clinical Electrophysiology of Vision (ISCEV), electroretinography measurements were performed after 20 minutes of dark adaptation. The scotopic retinogram was recorded in the dark by illuminating the animals' eyes with a stroboscope (20 cd/m2, 0.5 Hz) to elicit mixed rod and pin responses. The recorded electroretinograms show distinct, clearly identifiable electrical response peaks that stand out from the background noise, consistently occurring after and in

rhythm with the stroboscope light stimulus (ie, at the same 0.5 Hz frequency). As such, the strongly positive peaks of individual spikes are the maximum values of the b-waves, preceded by the characteristic negative peaks of the a-waves, as seen in other standard ERG systems. The amplitudes of the b-waves were measured from the preceding negative maximum to the positive maximum, while those of the a-waves were measured between the negative maximum and the preceding positive maximum. The electrical activity of both eyes of each animal was measured after 10 flashing light stimuli in order to create a common recording set (pool) for each group, from which statistical analyzes were then performed as detailed in the "Statistical analysis" section. Our previous results recorded with the same measurement system ensure that the applied experimental method provides reproducible and reliable data on retinal function and is related to retinal integrity.

2.5. Western blot

Both experiments were concluded with molecular biological analysis of the eye. The animals were euthanized with an overdose of anesthetic mixture, then their eyes were excised and frozen. Whole grain samples were homogenized with a knife homogenizer (IKA-WERKE ULTRA-TURRAX dispersant, Staufen, Germany) in homogenizing buffer on ice. The homogenization buffer contained 25 mM Tris, 25 mM NaCl, 0.5 mM EDTA, protease inhibitor cocktail, and distilled water (all components were purchased from Sigma-Aldrich-Merck KGaA, Darmstadt, Germany). After centrifugation at 10,000 rpm for 20 min at 4°C, the cytosolic proteins were aspirated together with the supernatant to separate the supernatant cytosolic fraction from the pellet. The pellet was further incubated for 1 hour with homogenization buffer containing the surfactant Triton X-100 (also from Sigma-Aldrich-Merck KGaA, Darmstadt, Germany), and the supernatant nuclear fraction was separated by centrifugation (14,000 rpm, 10 min, 4°C), and the nuclear proteins were aspirated together with the supernatant.

The total protein concentration was measured from a small part of the supernatants of each fraction with a spectrophotometer (FLUOstar Optima, BMG Labtech, Ortenberg, Germany), after which the rest of the supernatants were added to the rest of the supernatants by adding Laemmli sample buffer (Sigma-Aldrich-Merck KGaA, Darmstadt, Germany) and boiling for 5 minutes prepared for SDS-polyacrylamide gel electrophoresis. Proteins were separated on a 12% gel (Hoefer miniVe PAGE SE300 vertical electrophoretic and electrotransfer unit, Hoefer Inc., Holliston, MA, USA) running at 4 mA for 100–120 min. The proteins were then electrophoretically transferred to a nitrocellulose membrane (GE Healthcare, Darmstadt, Germany) using the blotting module of the aforementioned device (Hoefer miniVE SE300). The exposed binding surface of the nitrocellulose membrane was blocked with a 3% BSA solution (Sigma-Aldrich-Merck KGaA, Darmstadt, Germany), and then the proteins were incubated overnight with primary antibodies. In our first experiment, we incubated with the following primary antibodies: anti-SIRT1 mouse monoclonal antibody (which recognizes SIRT1 (~110 kDa), Cat#ab110304, Abcam, Cambridge, UK); anti-MMP9 rabbit polyclonal antibody (which recognizes MMP9 (~92 kDa), Cat#ab38898, Abcam, Cambridge, UK); with anti-HistoneH3 recombinant monoclonal rabbit antibody (which recognizes histone H3 (~17 kDa), Cat#701517 ThermoFisher Scientific, Waltham, MA, USA); and anti-beta-actin mouse monoclonal antibody (which recognizes beta-actin (~42 kDa), Cat#A5316, Sigma-Aldrich-Merck KGaA, Darmstadt, Germany). In our second experiment, we used the following primary antibodies: anti-β-actin (Cat#A5316, Sigma-Aldrich-Merck KGaA, Darmstadt, Germany); anti-Histone H3 (Cat. No. 701517, Thermo Fisher Scientific, Waltham, MA, USA); anti-HSP70 (SAB4200714, Sigma-Aldrich-Merck KGaA, Darmstadt, Germany); and anti-nuclear factor κB (ab 16502, Abcam, Cambridge, UK). The next day, after washing the membranes with distilled water, horseradish peroxidase-conjugated secondary antibodies were used to visualize the desired proteins: anti-mouse

antibody (Cat#A4416) and anti-rabbit antibody (Cat#A0545; both from Sigma-Aldrich-Merck KGaA, Darmstadt , Germany). WesternBright™ enhanced sensitivity chemiluminescence substrate (Advansta Inc., Menlo Park, CA, USA) and LI-COR C-DiGit® blot scanner (LI-COR Inc., Lincoln, NE, USA) were used for detection. Three blots per group were analyzed using ImageJ software (version 1.51, National Institutes of Health, Bethesda, MD, United States). Results were obtained by normalizing blots to background and standardizing to a housekeeping protein (beta-actin or histone H3).

2.7. Experimental protocols and groups

2.7.1. First experiment

The aim of our first experiment was to compare the putative retinoprotective effect of BGP-15 with anti-diabetics traditionally used in human medicine in the Goto-Kakizaki rat model.

After two weeks of acclimatization (at 12 weeks of age), the animals were randomly divided into the following groups (n = 6 in each group): Goto-Kakizaki control group (untreated, diseased), Wistar control group (untreated, healthy) and four treated disease groups, in which Goto- Kakizaki rats were: BGP-15, metformin, glibenclamide and pioglitazone. The treatments were given daily by gavage through a stainless steel stomach tube in the following doses: BGP-15 10 mg/kg, metformin 100 mg/kg, glibenclamide 5 mg/kg and pioglitazone 10 mg/kg. All treatment materials were obtained from Sigma-Aldrich-Merck KGaA (Darmstadt, Germany). The two control groups received solvent by gavage. The doses were selected based on previous research. The weight of the animals was measured weekly during the 12 weeks of the study.

The following experiments were performed on these animals (in addition to the daily gavage) in chronological order: fasting blood glucose measurement, OGTT, ERG, HEGC, WB.

2.7.2. Second experiment

The purpose of our second experiment is to test the long-term use of BGP-15, to monitor possible side effects, and to test the experimental substance in an obese, II. in an animal model of type 2 diabetes, ZDF rats.

ZDF animals were randomly divided into two groups: ZDF control group (ZDF) and BGP-15-treated group (BGP). We created a third group of lean animals (lean). N=10 in each group. Starting at 16 weeks of age, all animals were gavaged orally (via orogastric feeding tube) daily for the duration of the study, which lasted 52 weeks (1 year). The treated group received 10 mg/kg of BGP-15 in methyl cellulose mucus, while the animals in the lean and ZDF groups were treated only with vehicle. The dose was based on our first experiment. BGP-15 was obtained from Sigma-Aldrich-Merck KGaA (Darmstadt, Germany).

The following experiments were performed on these animals in chronological order: survival curve analysis, fasting blood sugar measurement, OGTT, ERG, WB.

2.8. Statistics

GraphPad Prism software (version 7.0, GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. The Gaussian distribution was evaluated with the Shapiro-Wilk normality test. The normally distributed data were analyzed with one-way analysis of variance (ANOVA), while the non-normally distributed data were analyzed with the non-parametric Kruskal–Wallis test. In case of analysis of group values at different times, two-way analysis of variance was used. The comparison was considered significant if the probability values were less than 0.05 ($p<0.05$). The level of significance was indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$. All data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Results of the first experiment

3.1.1. Weightgain

During the 12 weeks of the experiment, the weight of the animals was measured weekly. A more or less uniform increase in body weight was observed in each group. The initial and final weights of the groups ± standard error of the means (±SEM) were as follows: Glibenclamide 313.3 ± 4.074 –376.0 ± 5.671 g, Metformin 312.8 ± 5.655 – 383.6 ± 12.268 g, Goto control 315.6 ± 4.070 – 397.4 ± 6.266 g, BGP-15 319.6 ± 5.566 – 405.6 ± 9.024 g, Pioglitazone 315.6 ± 3.658 – 433.2 ± 10.398 g, Wistar control 356.5 ± 3.989 – 568.2 ± 18.552 g; in each group, the first week (initial) and 12th week (final) weights were significantly different from each other ($p<0.0001$). The body weight increase ±SEM (136.3 ± 2.207%) of the pioglitazone group was significantly higher than the other patient groups (120.3 ± 0.788%, 121.9 ± 2.228%, 125.5 ± 0.940% and 126.8 ± 0.769%, Glibeclamide, Metformin, Goto control and BGP-15 groups in the order of mention). The data of healthy Wistar rats (156.9 ± 4.667%) was higher than that of all other groups ($p<0.0001$).

3.1.2. Results of fasting plasma glucose measurements

During the experiment, the fasting blood sugar level of all patients in the Goto-Kakizaki group hovered around an average of 8-9 mmol/L - without any significant difference comparing any two groups - while the Wistar values were at a significantly lower level, on average 5-6 mmol/L. were around L (9.2 ± 0.589 mmol/L, 9.4 ± 0.526 mmol/L, 8.2 ± 0.171 mmol/L, 9.2 ± 1.059 mmol/L and 9.4 ± 0.692 mmol/L vs. 5.2 ± 0.178 mmol/L Glibenclamide, Metformin respectively , Pioglitazone, Goto control and BGP-15 vs Wistar control; $p < 0.05$). The fasting blood sugar values of the BGP-15, Pioglitazone and Wistar

groups did not change, the endpoint values were respectively: 98.87 ± 4.532%; 97.35 ± 6.116% and 108.6 ± 10.550%, of which the first two groups were statistically significantly different from the Goto control group (137.4 ± 5.219%). The values of the Glibenclamide and Metformin groups were as follows: 132.6 ± 10.15% and 117.8 ± 8.421%.

3.1.3. Results of OGTT measurements

Neither the areas under the curve (AUC) of the blood glucose levels during the oral glucose tolerance test (OGTT) nor the 120-minute OGTT values showed differences when comparing the treated groups and the untreated Goto control group. On the other hand, as expected, even the 120-minute OGTT values did not show signs of diabetes in the case of the healthy Wistar rats, as did the fasting blood sugar levels: during the entire experiment, the 120-minute OGTT values of the healthy Wistar group were 7.8 mmol/ remained below L (5.92 ± 0.073, 5.82 ± 0.183, 6.40 ± 0.148 and 6.63 ± 0.551 mmol/L ± SEM at the beginning of the experiment and in the 3rd, 8th and 11th weeks, respectively).

3.1.4. Results od HEGC measurements

As our results show, there were significant differences between the plasma insulin levels at the end of the hyperinsulinemic euglycemic clamp (HEGC) method: while the healthy Wistar rats presented a mean ± SEM value of 366.6 ± 141.3 mU/L, the BGP-15-treated Goto-Kakizaki rats showed 178.3 It turned out to be ± 54.71 mU/L, which was significantly different from the value of 513.0 ± 135.9 mU/L of the Goto control group (* = $p<0.05$). Glibenclamide could not alleviate insulin resistance, which was also evident from the relatively high insulin levels (626.3 ± 140.7 mU/L) at the end of the HEGC protocol, whereas both Metformin and Pioglitazone showed an insulin-sensitizing effect (their values were 266.6 ± 266.6 ± 72.93 and 291.5 ± 65.46 mU/L). If we look

at the direct fasting plasma glucose values, it is striking that not only Metformin (11.28 ± 0.822; $p < 0.05$ vs. Goto control (13.15 ± 0.650 mmol/L)) and pioglitazone (8.27 ± 0.463 mmol/L; $p < 0.0001$ vs. Goto control), but also BGP-15 (10.97 ± 0.460 mmol/L) was able to significantly reduce blood sugar ($p<0.05$ vs. Goto control), although this reduction was not as pronounced as in the case of pioglitazone. However, even pioglitazone was not able to reach the level of healthy animals ($p<0.05$ in the pioglitazone vs Wistar control (5.86 ± 0.506 mmol/L) comparison). Glibenclamide-treated fasting plasma glucose level was ±SEM 11.5 ± 1.016. There were no significant differences between groups in fasting plasma insulin and steady state blood glucose levels.

Further examination of the direct HEGC data also provided us with various derived quantities, such as the glucose infusion rate (GIR), insulin sensitivity index (ISI), metabolic clearance rate for insulin (MCRI), quantitative insulin sensitivity check index (QUICKI)), calculated for insulin resistance, so-called HOMA index (homeostasis model assessment of insulin resistance (HOMA-IR)) and HOMA calculated for B-cell function (index homeostasis model assessment of B-cell function (HOMA-B)). As can be seen from the ISI and MCRI graphs, the BGP-15 values ±SEM (3.596 ± 1.656 and 57.57 ± 18.500 respectively) were significantly different from the Goto control values ±SEM (respectively 0.956 ± 0.432 and 16.57 ± 5.095), however, compared to the healthy control values, they were not there was a difference (2.22 ± 0.776 and 25.67 ± 5.650, respectively), and even slightly (but statistically non-significant) even higher values were visible. In the case of the other diagrams, the differences did not reach the level of statistical significance (regarding reasonable comparisons).

3.1.5. Results of ERG measurements

Based on the ERG measurements, the group trends are very similar for both a- and b-waves. BGP produced higher a- and b-wave amplitudes (respectively 30.25 ± 0.342 µV and 97.39 ± 0.708 µV) than those observed in the Goto control group (respectively 19.7 ± 0.315 µV and 61.11 ± 0.672 µV). Moreover, BGP values were statistically significantly higher than healthy Wistar values (a- and b-wave amplitudes 26.54 ± 0.267 µV and 76.83 ± 0.767 µV, respectively). The effect of BGP treatment on these a- and b-wave amplitudes proved to be similar or even better than metformin and pioglitazone treatment (mean a- and b-waves of metformin: 24.66 ± 0.316 µV and 68.09 ± 0.628 µV, respectively µV; average a- and b-waves of pioglitazone: 29.25 ± 0.426 µV and 89.68 ± 0.862 µV, respectively). The aforementioned three treatments produced similar or higher amplitudes than healthy Wistar animals.

3.1.6. Western Blot Results

Primary antibodies against Sirtuin 1 and matrix metalloproteinase 9 were used on isolated proteins after separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transfer to a nitrocellulose membrane. Significant differences were observed for the aforementioned two proteins in the BGP-treated group.

SIRT1 expression was stimulated by BGP treatment: the standardized-normalized pixel density of the BGP-15-treated group was significantly higher than that of both the Goto control and Wistar control groups (1.539 ± 0.301 vs 0.6463 ± 0.094 and 0.2346 ± 0.083, $p < 0.01$ and $p < 0.0001$, in order of mention). There was no statistically significant difference between the Goto control and Wistar control groups. The BGP-15 value was also higher than all other groups (1.020 ± 0.205, 0.6371 ± 0.040 and 0.8204 ± 0.1015 respectively: glibenclamide, metformin and pioglitazone groups; $p<0.05$, $p<0.01$ and $p<0.01$

vs BGP) . In the glibenclamide and pioglitazone groups, the SIRT1 level was significantly higher than in the Wistar control group ($p<0.05$ in both comparisons).

MMP9 expression was significantly higher in the diabetic Goto control group compared to the healthy Wistar control group (2.716 ± 0.402 vs 1.167 ± 0.229 $p < 0.01$). This increase was reduced by all treatments (BGP-15, metformin, pioglitazone; 1.1210 ± 0.225, 1.466 ± 0.257, 0.7875 ± 0.093 and 0.4484 ± 0.047, respectively). The decrease of MMP9 as a result of BGP-15 treatment proved to be significant compared to the Goto control ($p<0.01$), so much so that the mean value of BGP was similar to that of the Wistar control group: there was no significant difference between the latter two groups. Glibenclamide was able to do what the BGP: value was significantly different from the Goto control ($p<0.05$), but not from the Wistar group. Metformin and pioglitazone were even more effective ($p < 0.001$ in metformin vs Goto control and $p < 0.001$ in pioglitazone vs Goto control comparisons).

According to the specifications of the manufacturer of the MMP9 antibody, the antibody can also detect other forms of MMP9, since "the processing of the [MMP9] precursor results in different active forms of 64, 67 and 82 kDa" (Abcam). Other, lower molecular weight markings were indeed detectable on the blot and these were also evaluated; however, there were no statistically significant differences between the signals of each group (these data are not shown).

3.2. Results of the second experiment

3.2.1. Results of survival curve analysis

During the 12 months of treatment, 100% of the animals in the BGP-treated group survived the harmful effects of diabetes, compared to the untreated ZDF group, where 60% of the animals died, which proved to be a significant

difference in the Kaplan–Meier survival curves of these groups. between (**p < 0.01). In the lean group, 90% of the healthy control animals reached the end of the study alive, which was significantly different from the 40% value of the untreated ZDF group (*p < 0.05). Based on the Mantel–Cox and Gehan–Breslow–Wilcoxon tests, there was no significant difference between the BGP and lean groups.

Weight did not change differently between the treated and untreated ZDF groups, although both were significantly different from lean values throughout the study.

3.2.2. OGTT Results

Based on the measurements of the OGTTs, the values of the area under the curve (AUC) were similar in all groups. However, at the beginning of the treatment period, the diseased animal models were already significantly different from the healthy control (lean) animals (1338.873 ± 53.008 and 1511.077 ± 56.820 vs. 190.211 ± 5.892 for the ZDF and ZDF + BGP-15 vs. lean groups, $p < 0.0001$ for both comparisons). This difference remained throughout the study period. However, there was no significant difference between the two patient groups either at this time point or at the 1-month or 6-month time points. Not so at the 12-month time point, where the AUC value of the BGP-treated group was significantly different compared to the untreated ZDF group (1976 027 ± 264 024 vs. 3040 019 ± 308 145, $p < 0.0001$).

Looking at the 120-minute values of the OGTT, the two-way ANOVA statistical analysis showed a significant difference directly at baseline (7.630 ± 0.142 vs. 5.330 ± 0.078, in the ZDF vs. lean comparison, $p < 0.05$). , which - similarly to the AUC - remained throughout the study: the mean values of the lean group were below 7.5 mmol/l at all times (5.330 ± 0.078, 5.050 ± 0.110, 5.200 ± 0.116, 7.244 ± 0.116, 7.244 ± 3, 2 and 7.244 ± 6.07 mmol/L at baseline,

initial, 1 month, 6 months and 12 months, respectively). The sick groups showed higher values throughout the study; however, there was a significant difference between the treated and untreated patient groups at the 6-month and 12-month time points (30,275 ± 0.689 vs. 27.8 ± 1,548, ZDF vs. ZDF + BGP15, $p < 0.05$ at the 6-month time point , and 23.7 ± 1.522 vs. 16.84 ± 1.264, for ZDF vs. ZDF + BGP15, $p < 0.0001$ at 12 months).

Fasting blood glucose values (baseline OGGT values) showed the same trend as the 120-minute values.

3.2.3. ERG Results

According to the ERG measurements, the average amplitude of both a- and b-waves was found to be significantly different in all groups. The trends were the same for a-waves and b-waves: untreated ZDF animals produced a significantly lower amplitude than healthy (lean) animals (for a-waves it was 23.32 ± 0.4277 vs. 62.09 ± 0.4621 for ZDF and lean groups, for b-waves it was 68.07 ± 0.9519 vs. 198.4 ± 0.7796 for ZDF and lean groups, $p < 0.0001$ for both comparisons), while BGP-treated ZDF animals produced a significantly higher amplitude than the untreated ZDF group (40.88 ± 0.5149 for a-waves and 131.3 ± 1.408 for b-waves in BGP-treated ZDF animals; in both comparisons with the corresponding untreated ZDF values $p < 0.0001$).

3.2.4. Western Blot Results

According to Western blot measurements, significant differences were observed between the expression levels of heat shock protein 70 (HSP70) and nuclear factor kappa B (NFkB) in the different animal groups.

In the case of HSP70 values, an increased expression of this protein can be observed in both untreated and BGP-treated ZDF animals compared to

healthy (lean) animals (0.7402 ± 0.0087 and 0.9059 ± 0.0621 vs. 0.3250 ± 0.0184 for the ZDF and BGP vs lean groups, respectively $p < 0.0001$ in both comparisons). BGP-15 treatment further increased the expression of HSP70, which was also found to be significant compared to the mean value of the untreated ZDF group ($p < 0.05$).

Similarly, NFκB expression was increased in both groups of diabetic animals; however, this difference is only between ZDF vs. was significant in lean comparison (0.318 ± 0.0211 vs. 0.0933 ± 0.0210, $p < 0.001$), BGP vs. not in lean comparison (0.1735 ± 0.0435 vs. 0.0.33 vs. 0.0.9). BGP treatment significantly decreased NFκB expression compared to the untreated ZDF group ($p < 0.05$).

4. Discussion

Ocular complications of diabetes often result in the most serious ophthalmic consequences, such as deterioration or even loss of vision. As the incidence of diabetes mellitus increases, so does the prevalence of diabetic retinopathy. The importance of diabetic retinopathy is further emphasized by the fact that it is one of the most common causes of blindness in the world. Therefore, the discovery of new pharmacological agents that can counteract or at least alleviate or delay the harmful effects of diabetes is of primary importance. Although strict glycemic control can reduce and delay complications, unfortunately even current antidiabetic drugs are not able to effectively prevent the development of retinal damage: despite adequate treatment, diabetic retinopathy develops after a long time in diabetes, which is further aggravated by age and co-morbidities, such as hyperlipidemia.

The research of our working group is directed at the complex pharmacodynamic screening of different active substances in diseased animal models, the aim of which is to find new, emerging drug candidates for

subsequent human drug development. One aspect of our research work is the investigation of eye-related complications of diabetes with electroretinography measurements, keeping in mind the possible human translation possibilities. That is why, in our first experiment, we wanted to assess the possible retinoprotective effects of BGP-15, a hydroxamic acid derivative, in diabetic conditions and compare it with well-known antidiabetics such as glibenclamide, metformin and pioglitazone. However, it was also crucial to design a long-term diabetes trial in order to best model the long time spent in diabetes. In our second experiment, we wanted to observe the effects of BGP in the long term.

The Goto-Kakizaki (Goto) rat is a spontaneously diabetic, non-obese animal model for the study of type II diabetes and its sequelae, such as retinopathy. Thus, in our first experiment, the Goto rats produced a much less pronounced increase in body weight compared to their healthy control Wistar counterparts, the weight of the latter increased significantly during the experiment. However, there were significant differences between the individual Goto groups: pioglitazone, although a widely used antidiabetic, appeared to predispose the animals to obesity. According to the literature, this is quite controversial, since pioglitazone is increasingly accepted in the treatment of non-alcoholic fatty liver disease in both diabetic and non-diabetic patients, although other sources also mention weight gain as a side effect of the drug. In our experiment, the BGP group did not differ significantly in terms of body weight gain from the other Goto groups.

In our first experiment, the measurement of blood sugar levels - which is a standard part of any diabetes test - did not provide new information about the active ingredients used. Some fluctuation was observed, but according to the literature and the data of the distributor of the animal model (Charles River), this was normal. Although the changes in blood glucose levels of BGP-treated animals were significantly low throughout the experiment, BGP was unable to achieve as low end-point fasting blood glucose levels as, for example,

pioglitazone, but it was comparable to that of metformin, which is the treatment of choice for type II diabetes.

Likewise, the OGTT results of our first experiment confirmed the animal model we used - since according to the World Health Organization (WHO), any 120-minute OGTT result above 11.1 mmol/liter blood sugar means diabetes mellitus. At the same time, we demonstrated for the first time that the effect of BGP-15 on the OGTT is comparable to well-known antidiabetic drugs. The results of the latter can be compared with the work of others: similar measurements can be found in the literature for metformin, pioglitazone and gliclazide, another sulphonylurea, only in human patients with type II diabetes.

During the HEGC protocol, in the case of plasma insulin levels, BGP-15 was able to reach a significantly low value compared to the Goto control: the less insulin needed to maintain a constant plasma glucose level of around 5.5 mmol/L, the less insulin-resistant an animal is. This is also completely consistent with derived indexes, such as the insulin sensitivity index. Our results demonstrate for the first time that a 12-week treatment with BGP-15 can improve insulin sensitivity in spontaneously diabetic rats to an extent comparable to known antidiabetic agents such as glibenclamide, metformin and pioglitazone. The results of the latter active substances are similar to those found in the scientific literature.

An important novelty in our first experiment is the demonstration that BGP-15 can counteract the damaging effects of type II diabetes on retinal function in spontaneously diabetic Goto-Kakizaki rats, which effect is comparable to pioglitazone and metformin treatment. As a comparison with the scientific literature: electroretinography experiments were previously performed on a diabetic mouse model induced by a high-fat diet, although those researchers could not prove the retinoprotective effect induced by metformin treatment. The situation was different with pioglitazone, which was confirmed to prevent glial cell apoptosis in a glaucomatous rat model, in which ischemia-

reperfusion (I/R) damage was induced by high intraocular pressure: here, pioglitazone alleviated I/R-induced ERG and VEP (visual evoked potential) waves decrease. Glibenclamide was tested in conjunction with ischemic preconditioning, a method known to reduce ischemic damage to the retina: in that trial, glibenclamide was significantly more effective when administered intraocularly before preconditioning. Similarly, in our first experiment, all treatments were applied before the development of diabetic ischemia - the damaging effect of diabetes on the microcirculation, which is responsible for almost all diagnostic symptoms of diabetic retinopathy. Such preventive measures may counteract the development of diabetic visual disturbances due to diabetic retinopathy with a future treatment with BGP-15 – the administration of this retinoprotective drug to diabetes-prone patients.

In our first experiment, BGP-15 and pioglitazone were able to generate higher electroretinographic waves than healthy Wistar rats, which is a surprising result, but not impossible. A possible reason for the smaller ERG waves is the deeper general anesthesia that may have occurred in healthy Wistars: due to their larger body weight, the Wistars received a larger amount of general anesthetic (ketamine/xylazine 100/10mg/kg body weight), and we observed that - presumably the due to their higher amount of fat – these animals woke up from anesthesia more slowly than the GK rats. In addition, there is a small chance that BGP-15 and pioglitazone may sensitize the retina to light by an unknown mechanism. This raises further, future experimental possibilities.

The mechanism of the retinoprotective effect of BGP-15 began to be outlined based on the western blot results of our first experiment: BGP can change the expression of the enzymes sirtuin 1 and matrix metalloproteinase 9, thereby alleviating the symptoms of diabetic retinopathy. Based on previous scientific research, these two proteins are connected to a common pathway: SIRT1 deacetylates and thereby inhibits protein complexes - which e.g. they include poly-ADP-ribose polymerase 1 (PARP1), nuclear factor kappa B (nfkB)

and activator protein 1 (AP1) - which, among other things, would initiate the transcription of MMP9 in the retina. However, in diabetes, the expression and activity of SIRT1 is also inhibited, so the aforementioned transcription factors stimulate the synthesis of MMP9 due to the inhibition of the inhibition. This leads to excessive transactivation and the consequent accumulation of MMP9 even in the mitochondria, damaging the mitochondrial membrane, as a result of which cytochrome c is released into the cytosol and apoptosis is activated.

In our first experiment, the SIRT1 level of the Wistar animals was unusually low, apparently even lower than that of the Goto control group, although the difference did not reach the level of statistical significance. According to other scientific articles, caloric restriction increases SIRT1 concentration, while, on the contrary, overweight and obesity are associated with SIRT1 down-regulation, which could be the reason for the lower-than-usual SIRT1 expression in our experiment in Wistar rats, which produced a significant weight gain during the experiment under.

According to the results of our first experiment, BGP-15 can counteract the events leading to mitochondrial damage mentioned above, as it can significantly increase SIRT1 expression, even more than anti-diabetics such as glibenclamide, pioglitazone or metformin. For an easier comparison with the scientific literature: according to previous results, metformin can increase the level of SIRT1 in the retina. In the case of glibenclamide, however, to the best of our knowledge, this is the first time that a SIRT1-enhancing effect has been demonstrated in the eye, although this could be inferred from other studies conducted on non-retinal tissues. In our first experiment, pioglitazone - consistent with the kidney and liver results - also produced an increase in SIRT1 expression in diabetic eyes, which is also a new result.

Along with this, we proved for the first time that BGP-15 can reduce the expression of MMP9 to the level of the healthy control. Glibenclamide has previously been shown to reduce MMP9 levels, but only in metastatic breast

cancer and the brain. Our results regarding the MMP9-reducing effect of glibenclamide in diabetics are novel. Metformin has been shown to reduce MMP9 synthesis in many different diseases, such as insulin-resistant diabetes, breast cancer, spinal cord injury, etc., but so far no scientific paper has demonstrated this effect in the eyes of diabetic animals, as in our first experiment. The same applies to pioglitazone: pioglitazone has been shown to decrease MMP9 levels in mouse peritoneal macrophages, lung and breast cancer cells, and in the serum of an atherosclerotic rabbit model. However, there is no mention in the scientific literature that the drug reduces the expression of MMP9 in the eye. Thus, in addition to proving the effectiveness of BGP-15, we established new facts about three well-known anti-diabetic agents, which could be deduced from previous results, but which no one has yet described for diabetics.

A recent paper on BGP-15 by another research group concluded that BGP protects mitochondria, which is fully consistent with our findings presented here. Although BGP was used in acetaminophen-induced hepatotoxicity in the above-mentioned article and the pathway of the mechanism of action (prevention of JNK activation and reduction of autophagy markers) is also different from ours, it is possible that there is a correlation between our results as follows.

In the case of diabetes, subclinical ischemia presumably plays an important role in the neurodegeneration of the retina, moreover, this neurodegeneration is present even without fully developed diabetic retinopathy, and even in well-maintained patients with good metabolic control. Oxidative stress alone can activate the c-Jun amino-terminal kinase (JNK) mitogen-activated protein (MAP) kinase pathway, which - by increasing mitochondrial outer membrane permeabilization (MOMP) and the formation of mitochondrial permeability transition pores (MPTP) – leads to mitochondrial membrane damage, which releases proapoptotic mediators and perhaps MMP9. The latter

enzyme is of great importance in the development of diabetic retinopathy, and damage to retinal mitochondria and oxidative stress have both been proven to be present in the diabetic retina, so the two paths must cross each other.

In fact, MMP9 may be the link: while MMP9 is overexpressed in diabetes due to SIRT1 deficiency, there are also results that confirm the activation of MMP9 in the diabetic retina by a so-called extracellular signal-regulated kinase (ERK) MAP kinase pathway. . Thus, accumulated and activated MMP9 damages the mitochondrial membrane, the same result as for the JNK MAP kinase pathway, which can be inhibited by BGP-15. However, we did not measure the activity of MMP9 in our experiments, which is one of the shortcomings of the studies. Further experiments are also needed to assess possible interactions between BGP and the ERK MAP kinase pathway, whether the agent can inhibit MMP9 activation.

Overall, if we want to evaluate all the results of our first trial, we can conclude that BGP-15, pioglitazone and metformin do not use the same mechanism of action. Although the effect of pioglitazone in increasing SIRT1 is only a non-significant trend, and metformin does not increase it at all, in contrast, both substances showed a much stronger MMP9-lowering effect than BGP-15. However, although metformin reduces MMP9 levels, it still performed worse functionally than pioglitazone and BGP-15. The fact that in our first study metformin was not able to strongly reduce the blood sugar level of Goto-Kakizaki animals may be related to its lower efficiency in function, although based on ERG measurements it still increased the function of the retina and proved to be good in reducing MMP9 as well. For BGP-15, hypoglycemia is unlikely to contribute to its functional effect, although its glucose-lowering effect was comparable to that of metformin. It is clear that these agents are involved in different mechanisms of action in different ways and thus may act through different pathways: glibenclamide was not outstanding in any of the above-mentioned comparisons; metformin was best at reducing MMP9 and

somewhat good at increasing function and reducing blood glucose, but did not change SIRT1 levels; pioglitazone was shown to be the best in reducing MMP9 and blood sugar levels and improving retinal function, but not in increasing SIRT1; in our first experiment, BGP-15 proved to be the best in increasing SIRT1 and improving retinal function, and it was also good in reducing blood sugar and MMP9.

One of the most important results of our second experiment was the survival of the animals treated with BGP during the 12 months. There are few studies that operated on ZDF rats for a long period of time, such as 9 months, in other articles on diabetic retinopathy, but they measured peripheral blood parameters or performed histological analyses. Cardiovascular function and central nervous system protein changes, as well as endothelial dysfunction, were analyzed in 12-month-old ZDF animals, respectively. kidney function has already been examined in 12-month-old ZDFxSHHF hybrid rats, but to our knowledge, there is no article that contains information about the retinal function of diabetic ZDF rats that were diabetic for a whole year, i.e. 52 weeks. Similarly, the longest BGP treatment published so far was 12 weeks (3 months) in the case of a muscular dystrophy model and in our first experiment on Goto-Kakizaki rats. To the best of our knowledge, such a long BGP treatment has not been published before our second experiment. It is not unprecedented that a drug treatment can prolong survival in a diseased animal model; although our second experiment resulted in the first publication containing the results of such an effective, long-term BGP treatment in an animal model with type II diabetes maintained for 52 weeks, in ZDF rats.

Based on our OGTT results, it is unlikely that BGP exerts the aforementioned prevention by regulating glucose homeostasis; although we showed some significant differences in our second experiment, the effect may instead be related to oxidative stress. Diabetes, even type II diabetes, is associated with a higher mortality risk, probably due to the accompanying

oxidative stress, which has been shown to be one of the causes of the macro- and microcirculatory complications of diabetes. Although we currently have little information on the exact mechanism of action of BGP-15, the fact that none of the animals treated with BGP died during the 12 months in diabetes is at least very telling.

The function of the retina produces electrical signals, which are weakened in diabetes due to microcirculation problems. Thus, the effect of BGP-15 seen in the electroretinographic results of our second experiment can be related to the reduction of such microcirculatory complications. A remarkable novelty of our experiment is that BGP-15 is able to counteract the detrimental effect of long-term diabetes on the function of the retina of ZDF rats. Such retinal protection in diabetes is usually experienced in the case of neuropeptides, trophic factors, antioxidants, or anti-inflammatory agents. Although BGP is neither a neuropeptide nor a trophic factor, it may exert certain antioxidant or anti-inflammatory properties in the retina, as previously described in non-retinal cell lines. Different effector molecules have already been reported behind the effects of BGP, including, for example, HSP70 in diaphragm muscle cells, histone deacetylases in mouse endothelial fibroblast cells, RAC-alpha serine/threonine protein kinase (AKT) in cardiac muscle cells, or Sirtuin 1 (SIRT1) in the eye cells of whole eye homogenate (our first experiment). On the other hand, BGP-15 inhibits poly-ADP-ribose polymerase 1 (PARP1) in the myocardium and the c-Jun N-terminal kinase (JNK) pathway, and reduces the expression of MMP9 (our first experiment). These molecular targets are part of inflammatory and ischemic cascades and also occur in diabetic eyes, based on which in our second experiment we investigated the role of HSP70 and NFkB in the functional retinoprotective effect of BGP.

Our experimental results demonstrate for the first time that BGP-15 can increase the expression of HSP70 in the eyes of ZDF rats. The 70-kDa heat shock proteins (HSP70) are ubiquitous chaperone molecules that support the

proper folding of many proteins, inhibit their aggregation, and, if necessary, assist in their removal. HSP70 proteins have been proven to play a protective role in many central nervous system diseases, in which the aggregation of aberrant or defectively folded proteins initiates inflammatory processes ending in neuronal death. Thanks to this neuroprotective effect, we thought about the possible protective role of HSP70 in eye diseases as well. Based on the previously demonstrated relationship with BGP-15, we analyzed HSP70 in our second experiment to see if we could find a significant increase in its expression, which we believe may contribute to the functional retinoprotective effect of the treatment.

Advanced glycation endproducts (AGEs) and the consequent increased oxidative stress and low-level inflammation also underlie the vascular complications of diabetes. And HSP70 is able to inhibit these inflammatory processes by binding NFkB, thereby reducing NFkB-induced iNOS expression and thus reducing the formation of reactive oxygen radicals (ROS) and peroxynitrites. In addition, HSP70 inhibits the activation and translocation of NFkB and tumor necrosis factor alpha (TNFα). This was the reason why this nuclear factor also became the target of our study. According to our Western blot results obtained in our second experiment, BGP-15 can reduce the expression of NFkB in the diabetic eyes of ZDF rats, which is a new result. This is consistent with our previous findings regarding SIRT1 (our first experiment): SIRT1 physiologically inactivates NFkB and PARP1; however, in diabetes, SIRT1 expression is decreased, leading to excessive transactivation of genes harmful to the diabetic eye, such as MMP9, an enzyme known to be involved in mitochondrial damage in diabetic retinopathy. NFkB-dependent inflammation has previously been shown to be an important driver of endothelial insulin resistance, and inhibition of this protein improves the insulin transduction cascade and extends lifespan in mice. Similar to our experiments, carotenoids have already been shown to have a beneficial effect on the

development of diabetic retinopathy by effectively reducing NFkB levels in the eyes of a streptozocin-induced diabetic rat model.

It is a fairly general approach that the expression of HSP70 is induced in cellular stress, instead of which the most recent articles study extra- and intracellular HSP70 (eHSP72 and iHSP72) separately. The former is the pro-inflammatory and the latter anti-inflammatory inducible form of the 70kDa heat shock protein family. Thus, the overall picture is more complex, and since HSP70 and NFkB interact, as mentioned in the previous paragraphs, they need to be discussed together. In diabetes, although the level of iHSP72 decreases, the level of eHSP72 increases. Our hypothesis is that the summation of these changes appears in our second experiment as an increase in the total HSP70 level of diabetic ZDF animals. Others have shown that a longer time in diabetes increases eHSP72 more than a shorter time, and that iHSP72 levels decrease in diabetes - without treatment - and it is also known that the anti-inflammatory effect of iHSP72 is mainly mediated by NFkB inhibition. These are presumably the reasons why, in our second, long experiment, the total HSP70 level is also high (compared to the healthy group) and the NFkB level is also high in the untreated ZDF group. Our hypothesis is that we indirectly proved that this high level of HSP70 is due to the increased eHSP72, which is confirmed by our high NFkB result, showing a presumably high e/i ratio of the total HSP level. For this reason, some studies in a so-called He also recommended the introduction of the H-index (e/i ratio). The BGP-treated animals are also diabetic, ZDF rats, so they presumably have the same high levels of eHSP70, with one difference. Here we see an even higher level of total HSP70, in fact, a parallel decrease in NFkB level can be observed, and as already mentioned, the anti-inflammatory effect of iHSP72 has been proven to be primarily mediated by NFkB inhibition. Since NFkB levels were significantly decreased, while total HSP70 levels were increased in our BGP-treated group compared to untreated ZDF animals, we believe that this increase in total HSP70 is likely due to an increase in NFkB-

lowering, protective iHSP72 levels. So our second hypothesis is that we indirectly demonstrated that BGP-15 increases the level of the beneficial iHSP72, which then shifts the e/i ratio towards iHSP72, as it was able to decrease the level of NFkB, which is characteristic of iHSP72. In our second experiment, the establishment of these two mutually reinforcing hypotheses was made possible by the fact that the proteins were not isolated from isolated retinas, but from whole eyeball samples, as seen in other studies. On the one hand, the entire eyeball also contains blood vessels - which is the reasonable and main location of eHSP72, since blood is also extracellular - and on the other hand, the increased vascular permeability characteristic of diabetic retinopathy further increases the interstitial appearance of eHSP in the whole eye homogenate, contributing to the high total HSP70 level in our untreated experiment in the ZDF group. In addition, vascular permeability is also a consequence of NFkB activation due to hyperglycemia and inflammation. And in our second experiment, where BGP treatment reduced the level of NFkB, it presumably also reduced vascular permeability, so the contribution of eHSP72 to the measured total HSP70 level was presumably also reduced, further strengthening our hypothesis that the increase in total HSP70 in the treated animals is due to the beneficial iHSP72 the consequence of an increase in level. A limitation of our study is that we did not measure eHSP72 and iHSP72 separately, which means that their exact ratio cannot be determined. Thus, it cannot be ruled out that, in addition to increasing iHSP, BGP-15 could also decrease eHSP, in which case the e/i ratio could have become even more beneficial due to BGP. Therefore, in the future, to facilitate a more comprehensive understanding, we consider it necessary to isolate the retina for iHSP72 measurement and measure eHSP72 separately from blood to further strengthen our hypothesis that BGP-15 can shift the ratio of eHSP72/iHSP72 in a beneficial way in favor of iHSP72.

5. Summary

A purpose of the experimental work was to utilize electroretinography, a retinal function measurement. With the help of this method we successfully demonstrated that by increasing the expression of mitochondria-protecting SIRT1 and simultaneously reducing the expression of mitochondria-damaging MMP9, BGP-15 is able to counteract the damaging effect of diabetes on retinal function.

And as the final conclusion of our second experiment, we can conclude that BGP-15 is not only not harmful in the long term, but can also reduce the serious consequences of diabetes and the mortality related with it. By identifying more and more of its molecular targets, we are getting closer to understanding the mechanism of action of this special agent: the inhibition of NFkB expression and the increased level of HSP70 in the eye both contribute to its functional retinoprotective effect. In conclusion, BGP-15, this emerging hydroxy acid derivative, is an excellent candidate for future antidiabetic drug development as a potential drug against the adverse consequences of diabetes, such as diabetic retinopathy, due to its ability to counteract the functional damage of the retina due to prolonged exposure to diabetes.

6. Official publication list

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Subject: PhD Publication List

Candidate: Zita Wachal
Doctoral School: Doctoral School of Nutrition and Food Sciences

List of publications related to the dissertation

1. **Wachal, Z.**, Szilágyi, A. T., Takács, B., Szabó, A. M., Priksz, D., Bombicz, M., Szilvássy, J., Juhász, B., Szilvássy, Z., Varga, B.: Improved Survival and Retinal Function of Aging ZDF Rats in Long-Term, Uncontrolled Diabetes by BGP-15 Treatment.
   Front. Pharmacol. 12, 1-11, 2021.
   DOI: http://dx.doi.org/10.3389/fphar.2021.650207
   IF: 5.988

2. **Wachal, Z.**, Bombicz, M., Priksz, D., Hegedűs, C., Kovács, D. K., Szabó, A. M., Kiss, R., Németh, J., Juhász, B., Szilvássy, Z., Varga, B.: Retinoprotection by BGP-15, a Hydroximic Acid Derivative, in a Type II Diabetic Rat Model Compared to Glibenclamide, Metformin, and Pioglitazone.
   Int. J. Mol. Sci. 21 (6), 1-19, 2020.
   DOI: http://dx.doi.org/10.3390/ijms21062124
   IF: 5.924

List of other publications

3. Blaga, Z., Czine, P., Takács, B., Szilágyi, A. T., Szekeres, R., **Wachal, Z.**, Hegedűs, C., Buchholcz, G., Varga, B., Priksz, D., Bombicz, M., Szabó, A. M., Kiss, R., Gesztelyi, R., Romanescu, D. D., Szabó, Z., Szűcs, M., Balogh, P., Szilvássy, Z., Juhász, B.: Examination of Preferences for COVID-19 Vaccines in Hungary Based on Their Properties: Examining the Impact of Pandemic Awareness with a Hybrid Choice Approach.
   Int. J. Environ. Res. Public Health. 20 (2), 1-16, 2023.
   DOI: http://dx.doi.org/10.3390/ijerph20021270
   IF: 4.614 (2021)

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   DOI: http://dx.doi.org/10.3390/ijerph20021270
   IF: 4.614 (2021)

7. Acknowledgements

I express my gratitude to Prof. Dr. Zoltán Szilvássy (UD, Faculty of Medicine, Department of Pharmacology and Pharmacotherapy) for making it possible to carry out my work in the department he leads.

I would like to thank my supervisor, assistant professor Dr. Balázs Varga (UD, Faculty of Medicine, Department of Pharmacology and Pharmacotherapy), for guiding my experimental work, and for supporting me throughout my PhD training and always giving me guidance when I was writing my dissertation.

I express my gratitude to all the staff of the Department of Pharmacology and Pharmacotherapy, especially Andrea Szegváriné Erdős and Krisztina Oláh.

I thank my family, my husband, Béla, my daughters, Zizi, Jazi and Hanna for their loving support!

I thank my friends for the time I spent relaxing and recharging with them!

The research is supported by and was realized in the framework of tender GINOP-2.3.4-15-2020-00008 Development of a Complex Health Industry Multidisciplinary Competence Center at the University of Debrecen for the development of new innovative products and technologies. The research was also supported by tender projects with identification numbers of GINOP-2.3.4-15-2016-00002, GINOP-2.3.2-15-2016-00043, TKP2020-IKA-04, TKP2020-NKA-04, EFOP-3.6.3-VEKOP-16 -2017-00009, as well as NKFIH-1150-6/2019.