Regulation of the apoptosis/autophagy switch by propionic acid in ventromedial hypothalamus of rats with type 2 diabetes mellitus

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Abstract

The balance between autophagy and apoptosis in hypothalamus may be disrupted upon type 2 diabetes mellitus (T2DM). Since propionic acid (PA) exerts neuroprotective effects, the aim was to investigate its effects on apoptosis/autophagy switch in the ventromedial hypothalamus (VMH) in a T2DM rat model. Therefore male Wistar rats were divided into untreated control rats and rats in which T2DM was induced. The untreated T2DM rats were compared to a group that received metformin, a group that received sodium salt of PA and a group that got PA+metformin. Western blotting, RT-PCR, transmission electron microscopy and immunohistochemical staining were from VMH tissue were performed.

T2DM-induced apoptosis and mitoptosis, enlarged ER tubules/cisterns were observed in VMH, and accompanied by imbalance of pro- and anti-apoptotic factors. Elevation of pro-apoptotic Bax and caspase-3, decrease in autophagy marker LC3 and anti-apoptotic Bcl-xl were observed in VMH of T2DM rats. Metformin and PA partially improved VMH ultrastructural changes by reducing mitochondrial swelling and diminishing the number of apoptotic neurons. Metformin inhibited neuronal apoptosis, however caused reactive astrogliosis and accumulation of lipofuscin. Elevated number of autophagosomes was associated with LC3, Beclin-1 and Bcl-xl increase and decrease in Bax and caspase-3 in VMH of T2DM rats. PA switched cell fate from apoptosis to autophagy by elevating LC3 and Beclin-1 levels and increasing Bcl-xl that altogether may represent adaptive response to T2DM-induced apoptosis. PA+metformin administration lowered relative area of ER membranes/cisterns in VMH compared to untreated T2DM rats and metformin alone, and was balancing the ratio of pro-apoptotic, anti-apoptotic and autophagy markers.

In conclusion, T2DM was associated with apoptosis activation leading to impairments in VMH. PA in combination with metformin may be effective against diabetes-induced cell death by switching apoptosis to autophagy in VMH.

Introduction

Type 2 diabetes mellitus (T2DM) is one of the most common metabolic disorders worldwide [1]. Among the complications of diabetes, damage to the peripheral and autonomic nervous systems is classified as different forms of neuropathy, that are of high prevalence, and occur in up to half of all individuals with T2DM [2]. Besides peripheral neuropathy, diabetes is also associated with gradually developing injury of the central nervous system (CNS), including such important area as hypothalamus. This rarely considered complication is referred as “diabetic encephalopathy” and characterized by the strong impairments in molecular regulatory pathways and cognitive functions [3]. Among major risk factors for the T2DM-associated neuropathy are obesity, lifestyle factors, genetic predispositions, gut dysbiosis, and mitochondrial deregulation [4]. The main structures that regulate the body energy balance in accordance with the needs of the organism are the neurons of the ventromedial (VMH), lateral (LHA) and arcuate (ARK) nuclei of the hypothalamus. Local dysregulation of molecular pathways and cellular processes in these hypothalamic zones can be one of the fundamental reasons caused the disturbance in glucose

metabolism and/or the development of T2DM, and may be accompanied by the neuroinflammation in the hypothalamus and impaired neurogenesis in general [5, 6, 7]. Thus, a bidirectional link may be suggested between T2DM development and hypothalamic dysfunction and both may be considered as reasons and consequences for each other.

T2DM-induced cell damage is associated with both hyperglycemia or hyperlipidemia states, so-called glucolipotoxicity [8]. Excess of glucose and fatty acids overloads the cell and disrupt endoplasmic reticulum (ER) functioning. Chronically stressed ER may lead to disrupted proinsulin synthesis, folding, and protein processing, generate pro-apoptotic signals, induce oxidative stress, and initiate mitochondrial apoptosis [9]. Thus, the cell fate upon ER stress is determined by the balance of cell survival and death triggers mainly regulated by the unfolded protein response (UPR) signaling. ER stress-induced autophagy is required for cell survival, likely through compensatory removal of expanded and disorganized ER (which results from the UPR), along with the misfolded proteins, that probably form the ERAD (ER-associated degradation) mechanism [10]. ER stress-associated mechanisms may influence the autophagy process via the PERK/eIF2 α /ATF4 signaling pathway [11]. It was shown that ATG5 and ATG7 connect autophagy with ER stress through PERK signaling [12]. However, there is no data showing the link between PERK and autophagic/apoptotic regulatory molecules. Thus, particular attention should be paid to elucidating the process of switch between apoptosis and autophagy as an important factor in the development of T2DM-associated hypothalamic dysfunction.

Autophagy is an important mechanism ensuring the delivery of damaged organelles, abnormal and misfolded proteins to the lysosomes for degradation [13, 14]. This complex mechanism is responsible for maintaining cellular homeostasis under normal conditions, and is rapidly activated by different stimuli such as nutrient starvation, hypoxia, oxidative stress, pathogen infection and ER stress [15]. In pathological contexts, including diabetes, autophagy plays a pivotal role. It has been observed that autophagy regulates normal functioning of pancreatic β -cells and insulin-target tissues, such as skeletal muscle, liver, and adipose tissue [16]. Moreover, autophagic activity is important for maintenance of neuronal functioning since these cells are unable to dispose the aggregated proteins [17, 18]. ER stress, associated with chronic neurodegenerative disorders often stimulates autophagic activity, however, failure in clearing the aggregated proteins and the impairment of UPR leads to the accumulation of misfolded proteins and the progression of neurodegeneration [19, 20]. The association between ER stress, UPR and neuropathology is partially established, however, the role of this interplay upon T2DM-associated hypothalamic dysfunction should be further studied.

The mostly used autophagy markers are Beclin-1 and microtubule-associated protein 1 light chain 3 (LC3). Moreover, Beclin-1 also plays an important role in T2DM progression. It was shown that Beclin-1 may be involved in the pathogenesis of diabetic nephropathy with the mechanism related to the process of programmed cell death [21]. Related to brain, it was shown that Beclin-1-mediated autophagy in hippocampus probably played an important role in cognitive and affective disorders of streptozotocin-induced aged diabetic mice [22]. In order to measure autophagic flux, LC3 is frequently used as additional autophagy marker protein. LC3 is an ubiquitin-like protein that is associated with the autophagosomes

during the autophagic process and is generated by the conjugation of cytosolic LC3-I to phosphatidylethanolamine (PE) on the surface of nascent autophagosomes [23]. The ubiquitin-like conjugation of phosphatidylethanolamine (PE) to LC3 couples with the translocation of LC3 from the soluble fraction to autophagic membranes. Therefore, the localization of LC3-PE in autophagic membranes is a reliable marker of autophagy and considered a gold standard for autophagy process characterization [24]. Thus, it is important to study the involvement of LC3 and Beclin-1 in the hypothalamic dysfunction under T2DM conditions, because it was shown that autophagy may mediate the protective effect of metformin on hyperglycemia-induced apoptosis of cardiomyocytes [25].

Pharmacological UPR modulation and the regulation of the interplay between apoptosis and autophagy in disease-affected tissues may contribute to the prevention and/or treatment of neurodegeneration. It is of great significance to explore the new possible effective treatment options with the focus on apoptosis/autophagy modulation. First of all, in this study we used metformin, a well-known oral glucose-lowering agent, and a “gold” standard for the anti-diabetic monotherapy, due to its ability to decrease plasma glucose levels, and to exert anti-inflammatory, anti-apoptotic and anti-oxidative properties [26, 27]. Moreover, it is interesting to study the effect of metformin on apoptosis and autophagy in the light of the available data regarding its influence on these processes on different cell and animal models. For example, it was demonstrated that the ability of metformin to trigger autophagy by AMPK activation and subsequent inhibition of mTOR, which is one of major inhibitor of the autophagic flux [28], as shown for human gastric adenocarcinoma cells [29].

Another strategy for the treatment of neuropathy in diabetes mellitus includes the pharmacological agents correcting the functional state of the elements of the "intestinal microflora – central nervous system" axis (gut-brain axis, GBA), with a special emphasis on the mitochondrial apparatus and energy metabolism in neurons. In animal models, enteric-soluble short-chain fatty acids (SCFAs) such as propionic acid (PA) and other intestinal metabolites have been shown to normalize fasting blood glucose, body weight and insulin tolerance, and generally have therapeutic potential in obesity [30]. It was also found that acetic and PA therapy reduces ER stress in bovine mammary epithelial cell culture [31].

However, some controversial effects of PA have been reported in the neurotoxic context [32]. A recent study indicated that autophagic flux is impaired in PA-treated hippocampal neurons. At the molecular level, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway was activated and autophagic activity was impaired [33]. In addition, SCFAs act as energy substances to protect intestinal barrier and inhibit autophagy. However, there is no data related to the influence of PA on apoptosis induced by T2DM condition. Thus, the direct effect of the use of PA as additional therapy on the neurons of the VMH in type 2 diabetes mellitus should be studied in detail, with the focus on regulation of apoptosis/autophagy interplay.

Considering abovementioned, the aim of this study was to explore the regulatory effect of PA and metformin administration, and their combination on the molecular mechanisms mediating the switch between autophagy and apoptosis as a system for maintaining intracellular homeostasis in the ventromedial nucleus of the hypothalamus in rats with the experimentally induced T2DM. Consequently,

T2DM rats were used to determine the levels of autophagy markers LC3 and Beclin-1 on transcriptional and translational level. The pro-apoptotic (Bax, caspase-3) and anti-apoptotic (Bcl-xl) markers were also measured on mRNA and Protein level as well as immunohistochemically to characterize the process of apoptosis in the VMH of rats with T2DM treated with metformin, PA and its combination. Moreover, the ultrathin sections of VMH after metformin and PA administration were examined with a transmission electron microscopy to visualize the possible morphological changes, including assessment of apoptotic signs after metformin and PA treatment, and their combined action.

Materials And Methods

Animals

The present study was performed on male Wistar rats (176.8 ± 8.3 g) of 2-6 months of age. All rats were kept in a humidity-controlled house ($24^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $65\% \pm 5\%$ humidity with 12-h dark/light cycling, and were allowed to drink and eat and freely the standard, balanced rodent diet (for control group). All experimental procedures with animals were carried out in an accordance with national guidelines and international laws concerning animal welfare: "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 1986), "Bioethical expertise of preclinical and other scientific research conducted on animals" No. 3447-IV (Kyiv, 2006). All procedures were approved by the Bioethics Committee of the Bogomolets National Medical University (Protocol No 123 from 23/12/2019).

Experimental design

Each experimental group consisted of 12 animals, previously acclimated for one week: 1) the untreated control group; 2) the group with experimentally induced T2DM by high-fat diet (HFD) treatment for 3 months and additionally by a single injection of streptozotocin (STZ, 25 mg/kg of b.w., Sigma, USA) followed by a placebo treatment with neutral saline *solution orally administered for 14 days*; 3) the group that received anti-hyperglycemic agent metformin dissolved in water for injection (GLUKOFAGE, Merck Sante, France) at a dose 60 mg/kg of b.w., for 14 days, orally on the background of T2DM; 4) the group that received sodium salt of propionic acid (PROPICUM[®], Flexopharm Brain GmbH & Co, Germany) dissolved in water for injection at a dose 60 mg/kg of b.w., for 14 days, orally on the background of T2DM; 5) the T2DM group that received concurrently metformin (60 mg/kg of b.w., for 14 days, orally) and sodium salt of PA (60 mg/kg of b.w., for 14 days, orally). Sodium salt of PA was kindly provided by Prof., Dr. Nina Babel (Center for Translational Medicine and Immune Diagnostics Laboratory, Medical Department I, Marien Hospital Herne, University Hospital of the Ruhr University Bochum, Herne, Germany).

The experimental design is shown in the Fig. 1a. To induce T2DM, rats were fed a homogenous HFD mixture: standard rodent feed (34%), pre-melted fat from lard (45%), medical bile acids (1%, for natural emulsification of fat in the intestine of rats and improvement of its enteral absorption), and dry fructose (20%). Additionally, STZ administration was performed once intraperitoneal after 3 months of HFD, and

after an injection, the standard rodent diet was returned for the next 2 weeks. After this period, all experimental animals with confirmed T2DM were randomly divided into 4 groups to treat them orally with placebo, metformin, PA and metformin+PA. In the T2DM placebo group rats were treated with the neutral saline *solution* at a similar volume as the volume of other compounds. After two-week treatment, rats were sacrificed by a decapitation under deep ether anesthesia. Blood samples were collected for serum separation to determine glucose and glycosylated hemoglobin (HbA1c) levels. For western blot, RT-PCR and histological analysis, VMH samples were collected. Sections for immunohistochemistry were between 2.04- and 3.0-mm posterior to Bregma.

Assessment of glycosylated hemoglobin level

The level of glycosylated hemoglobin (HbA1c) was determined by the ion-exchange chromatography-spectrophotometry method using a standard kit «HEMOGLOBIN A1C-DIRECT» (HbA1C-DIR, #31047, Bio Systems, Spain). 50 µl of whole blood collected previously in tubes with EDTA and 5 ml of distilled water were mixed, erythrocytes were lysed and then the mixture was applied on a microcolumn from the HbA1C-DIR kit. Filling buffer was used to perform the elution according to the manufacturer's protocol. The eluates containing the HbA1c fraction were collected and their optical density was measured spectrophotometrically (on 415 nm). The optical density of the sample with total hemoglobin was also determined and the results were expressed as a percentage (%) of the HbA1c fraction to the total hemoglobin.

Slice preparation and electron microscopy

Rat VMH samples were fixed in 2.5% glutaraldehyde for 4 hours and Millonig's phosphate buffer (pH 7.4) followed with fixation by 1% osmium tetroxide for 1 hour. After that brief washing of slices in distilled water, dehydration in a graded series of ethanol solution (70%, 80%, 90%, 100%) and acetone was done. Then slices were poured into a mixture of epon-araldite (Epoxy embedding medium, #45345-250ML-F, Epoxy embedding medium, hardener DDSA, #45346-250ML-F, Araldite, #10951-250ML, Sigma, USA). Semi-thin and ultrathin sections were cut with the ultramicrotome "LKB III" (Sweden). To confirm that we work with the required brain area semi-thin sections (2-3 µm) were stained with methylene blue according to the Hayat method [34]; ultrathin sections (600-900 Å) were contrasted with 2% uranyl acetate solution and lead citrate. Transmission electron microscope "PEM-125" (Ukraine) with a magnification of 6000-20000 times was used to examine the sections. By ImageJ software we calculated the areas of the ER membranes and the ER cisterns in each cell.

RNA isolation and quantitative real-time PCR analysis

Total RNAs from the ventromedial hypothalamus samples (30 mg) were isolated using GeneJET RNA Purification Kit (Thermo Fisher Scientific Inc., USA). mRNA concentrations and purity were determined by measuring the OD260/280 and OD260/230 ratios on DeNovix DS-11 FX+ (DeNovix Inc., USA). Purified RNA samples were reverse transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA). Quantification of mRNA levels was performed on 7500 Real-time

PCR System (Life Technologies Corporation, USA). Target genes were amplified for 40 cycles using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific Inc., USA). A two-stage RT-PCR amplification reaction was performed under the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and at 60°C for 50 s. The primer sequences are listed in the Table 1 (designed using Primer BLAST software). Gene expression relative to the housekeeping gene *β-actin* was quantified as the fold change using the $\Delta\Delta C_t$ method.

Table 1: The sequences of the primers

Primer name	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
<i>Bax</i>	<i>CACGTCTGCGGGGAGTC</i>	<i>CATCCTCTCTGCTCGATCCT</i>
<i>Bcl-xl</i>	<i>GGTCTCTTCAGGGGAAACTG</i>	<i>TCCAAAACACCTGCTCACTC</i>
<i>Beclin-1</i>	<i>GAGGAATGGAGGGGTCTAAGG</i>	<i>TGGCTGTGGTAAGTAATGGAGC</i>
<i>LC3</i>	<i>GTGCATTTGGCTTGGAAACTC</i>	<i>CCTTTTAGAGAAGGCAGCAGG</i>
<i>Caspase-3</i>	<i>ACAACAACGAAACCTCCGTG</i>	<i>CACAAGCCCATTTCAGGGTA</i>
<i>β-actin</i>	<i>TGCAGAAGGAGATTACTGCCCTGG</i>	<i>GCTGATCCACATCTGCTGGAAGG</i>

Western blot analysis

Target protein levels (LC3, Beclin-1, Bcl-xL, Bax, caspase-3) were measured by western blotting. Protein extracts from frozen hypothalamus samples were prepared using a standard protocol with RIPA buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 1 mM EGTA; 0.1% SDS; 1% sodium deoxycholate; 10 mM sodium pyrophosphate). VMH samples (30 mg) were lysed for 20 min in RIPA buffer in the presence of protease inhibitor cocktails (PIC, Sigma, USA), then sonicated and centrifuged for 20 min (14000g) at +4°C. Protein concentration in supernatants was determined by Stoscheck method [35] and equal amounts of protein (30 µg per track) from each sample were loaded onto 15% PAAG for electrophoresis. Then protein samples were blotted on a nitrocellulose membrane (#HATF00010, Immobilon-NC transfer membrane, 0.45. µm pore size, Merck Millipore, USA). Membranes were blocked with 5% non-fat milk in PBS with 0.05% Tween-20 (PBST) for 1h followed by an incubation overnight at +4°C with primary antibodies against LC3 (1:1000, #Sc-134226, Santa Cruz Biotechnology, USA), Beclin-1 (1:1500, #PA5-20171, Invitrogen, USA), Bcl-xl (1:1000, #PA5-21676, Invitrogen, USA), Bax (1:500, #MA5-14003, Invitrogen, USA), caspase-3 (1:2500, #ab208161, Abcam, UK) and tubulin (1:1000, T5168, Sigma-Aldrich, USA) in PBST. Membranes were then washed and incubated with HRP-conjugated secondary antibodies: anti-rabbit IgG (1:8000, #A0545, Sigma-Aldrich, USA) or anti-mouse IgG (1:5000, #ab97057, Abcam, UK). Thereafter, chemiluminescence development was used to visualize bands with chemiluminescent agents: p-coumaric acid (Sigma-Aldrich, USA) and luminol (Sigma-Aldrich, USA). The relative levels of LC3, Beclin-1, Bcl-xL, Bax, and caspase-3 were normalized tubulin content. Quantification

of western blots was performed by measuring the optical densities of the respective bands on the films using [Gel-Pro Analyzer32](#), v3.1.

Immunohistochemical staining and quantification

Immunohistochemical studies were performed using polyclonal antibodies against Bax (#MA5-14003, Invitrogen, USA) and Bcl-xl (#PA5-21676, Invitrogen, USA) in dilution of 1:500. DAB (3,3'-diaminobenzidine) and the EnVision FLEX detection system (Dako, Denmark) were used for visualization.

After rat sacrificing, a transcardial perfusion was performed with 0.1M PBS (pH 7.4) and 4% paraformaldehyde (PFA) in PBS. The brain was postfixed in 4% PFA overnight, and after that, brain samples were dehydrated in the elevated concentrations of ethanol and embedded in paraplast (Leica-Paraplast Regular, 39601006, Leica Biosystems Inc., USA). Ventromedial hypothalamus sections were obtained using a microtome Microm HM360 (Microm International GmbH, Germany) to cut slices of 5 μ m thick and they were mounted on HistoBond[®]+ adhesive microscope slides (Marienfeld GmbH & Co. KG, Germany). All brain sections were within stereotaxic coordinates – 2.04...3.0 mm relative to Bregma point. To remove paraffin, slices were incubated in xylene and rehydrated in decreased concentration of ethanol solution (100%, 95%, 80% and 70%; for 2 min) and 2 steps of distilled water (for 2 min). Antigen unmasking was performed using 10 mM sodium citrate buffer (pH 6.0) at the temperature of 98°C for 30 min. When temperature reached 65°C, the slides were washed for 1 min in 3 volumes of EnVision[™] FLEX wash buffer. After that endogenous peroxidase activity was blocked with the EnVision FLEX peroxidase-blocking reagent solution for 2 min. Sections were incubated for 30 min in Bax or Bcl-xl antibodies (1:500) at room temperature, then, after washing, incubated with secondary HRP-conjugated antibody from the kit (EnVision FLEX/HRP) for 20 min. For visualization a mix of DAB (EnVision DAB+ Chromogen) with EnVision FLEX substrate buffer was used for 10 min at room temperature. Nuclei were stained with Gill III haematoxylin.

Brain sections were investigated using an Olympus microscope BX51 and documented by a digital camera Olympus C4040ZOOM with the software Olympus DP-Soft 3.2. For semi-quantitative evaluation, the intensity of staining was calculated according to the next scale: grade 0 for the absence or focal weak reaction; grade 1 for intense focal or diffuse weak reaction; grade 2 for moderate diffuse reaction; grade 3 for intense diffuse reaction; and grade 4 for the high intensity. Standard statistical methods were used to quantify obtained data. Significance of differences in mean values between comparing groups were assessed by Student's t-test. $p \leq 0.05$ was considered statistically significant.

Statistical analysis

The data distribution was analyzed using the Shapiro-Wilk test. Statistical differences between the groups were analyzed by the one-way ANOVA test with the following Tukey post-hoc test. Results with $p < 0.05$ were considered statistically significant. The Pearson's correlation coefficient (R) was calculated with a p-value 0.05 (95% confidence interval). All data were obtained based on two or three independent

experiments and provided as mean $\pm$ SEM. Statistical analysis was performed using IBM SPSS Statistics, version 23.0 (SPSS Inc., USA).

Results

3.1. Effects of metformin and PA on animal body parameters, peripheral glucose homeostasis and insulin sensitivity

The first part of the study was the verification of the development of metabolic syndrome after HFD, therefore we measured animal body parameters (weight, g and waist, cm), the levels of glucose and glycosylated hemoglobin (HbA1c) in the serum of animals and also performed an intraperitoneal insulin tolerance test.

We observed that after T2DM induction, the body weight and the waist of diabetic rats was higher (1.65- and 1.35-fold respectively) compared with control animals (Fig. 1b, 1c). Administration of metformin and PA, as well as their combination had no significant effects on body weight compared with T2DM group, however the body weight after metformin, PA and their combined action was still higher compared with control animals by 67%, by 63% and by 51% respectively. The same effect of compounds was detected on waist parameter. Thus, animals in T2DM group showed a significant increase in weight and waist compared with the control group reflecting the development of obesity. In turn, PA and metformin treatment had no lowering effects on these parameters.

To further confirm the development of stable hyperglycemia, the levels of glucose and glycosylated hemoglobin (HbA1c) in the blood were measured (Fig. 1d, 1e). In addition, to determine the sensitivity of insulin-responsive tissues in the rodent we performed an intraperitoneal insulin tolerance test (ipITT). 4 weeks after the injection of streptozotocin, fasting blood glucose level exceeded the control value by 1.98 times (Fig. 1f) reflecting the development of hyperglycemia. After treatment with metformin and PA for 2 weeks the glucose level did not change compared with T2DM rats. Rats treated with metformin and PA group showed a slight reduction by about 14% of blood glucose level compared to rats treated only with PA.

The next step was to estimate the level of HbA1c as an important marker of hyperglycemia. We observed the correlation of HbA1c level with glucose content in the blood. HbA1c level was higher by 74% in T2DM rats and by 70% in metformin group vs. control (Fig. 1e). PA also had an increasing effect on HbA1c levels compared with control animals (2.10-fold), with T2DM (by 21%) and metformin group (by 23.9%). In addition, we confirmed a slight reduction in HbA1c level in combined PA- and metformin-treated rats by 13% vs. T2DM group and by 36.6% vs. PA group, however it still remained higher than in control rats by 54%.

Thus, based on the persistent hyperglycemia and elevated HbA1c level as stable and accurate biomarkers, we verified the development of experimentally induced T2DM in rats after 3-month HFD

followed by STZ injection. Administration of PA and metformin had almost no effect on these parameters during the T2DM course.

Additionally, to determine the sensitivity of insulin-responsive tissues, we performed intraperitoneal tolerance test (ipITT) based on the measurement of glucose remaining in the circulation after an intraperitoneal insulin injection (Fig. 1f). Animals were fasted for approximately 6 hours (morning fast), fasted blood glucose levels (basal) were determined before an intraperitoneal insulin injection. Within 15 min after injection of insulin, blood glucose levels in control animals increased by 1.3-fold (5.77 ± 0.29 mmol/l) compared with the basic control value (4.25 mmol/l) and went back to starting level after 30 min. At 60 min after the insulin injection, the glucose level was minimal (2.7 ± 0.08 mmol/l), and then after 15 minutes the level increased to 3.8 ± 0.14 mmol/l. In the T2DM group the basal glucose level (7.26 ± 0.45 mmol/l) was higher than in control rats. After 30 min it reached a maximum value – 11.73 ± 0.96 mmol/l, followed by a progressive decrease (to 10.17 ± 0.27 mmol/l on the 45th min and to 7.8 ± 0.21 mmol/l on the 60th min) reaching initial levels after 90 min post injection (6.88 ± 0.55 mmol/l).

Thus, as expected in the course of T2DM model verification, rats from T2DM group exhibited impaired glucose tolerance and insulin sensitivity, which confirmed the impairments in glucose homeostasis.

3.2. Ultrastructural changes of VMH in rats with T2DM after metformin and PA administration assessed by electronic microscopy

To detect the ultrastructural changes in the VMH of diabetic rats and to evaluate the possible influence of metformin and PA administration, electron microscopic study was performed.

According to electron microscopy examinations of VMH neurons of control rats (Fig. 2a, 2b), we observed well-defined nuclei, with a small perinuclear space, which passed into ER. The Golgi complex and mitochondria were clearly defined. In some neurons, single small autophagosomes and lysosomes were found.

In T2DM group we found that the electron density of the cytoplasm and nucleus was increased in most neurons (Fig. 2c, 2d) compared to the control rats. In VMH neurons of diabetic rats, partial fragmentation of elements of the Golgi complex and an enlargement of the tubules of the granular ER were observed. Most mitochondria in VMH neurons showed signs of diabetes-associated structural damage: in particular, defragmentation, matrix edema and destruction of cristae. In a small percentage of neurons, considerable mitochondrial destruction was detected with the formation of mitophagosomes. Autophagosomes and lysosomes were also detected in the higher frequency compared with control (Fig. 2c, black arrows – lysosomes; 2d, yellow arrows – autophagosomes). Most of the nuclei of VMH neurons in diabetes mellitus group underwent pyknotic changes with the appearance of deep invaginations of the nuclear membrane (Fig. 2c, 2d, white arrows) and in addition, fragmentation of nuclei was observed in single cells. Neurons with apoptotic changes, axon and dendritic loss, and alignment of the plasma membrane in VMH of T2DM rats were predominated. "Light" neurons were practically not detected. Swelling of synaptic terminals with loss of vesicles was detected in the neuropil of VMH in the T2DM

group. Astrocytes surrounding VMH neurons showed signs of cytoplasmic edema and a decrease in the total number of organelles.

Metformin administration slightly improved diabetic-induced changes in VMH. After metformin treatment neuronal Golgi complex was clearly visible, the ER cisterns in most cells did not differ from the untreated control rats (Fig. 2e, 2f). A clear outline of the tubules of the granular ER with the abundance of ribosomes on their surface was also noticed, however the structure of mitochondria was more preserved than in T2DM group. The volume of the ER cisterns was reduced compared with T2DM group. In addition, administration of metformin on the background of T2DM was accompanied by a clearly visible accumulation of lipofuscin granules in the cytoplasm of VMH neurons (Fig. 2f, green arrows), as well as the development of reactive astrogliosis with the excessive proliferation and hypertrophy of the surrounding astrocytes. Moreover, an accumulation of autophagosomes, an increase in the number of lysosomes, axoplasm swelling, and damage of the myelin sheath were still observed in VMH of rats treated with metformin.

Treatment of T2DM rats with propionic acid was accompanied by an improved visualization of the contours of intracellular membrane structures in VMH neurons, mostly similar to control group (Fig. 2g, 2h). Tubules of rough endoplasmic reticulum were well preserved with clearly visible ribosomes located on its surface without accumulating in the cytoplasm. We observed that the volume of ER cisterns was relative to the control (Fig. 2g, 2h). The interesting point that observed in T2DM + PA group was the identification of two types of autophagosomes in VMH neurons: primary with a double-layer membrane and secondary with a single-layer membrane containing cellular detritus, that might be a sign of the accumulation of abnormally folded proteins. Administration of PA to animals with T2DM visually reduced the number of pre-apoptotic and apoptotic dark neurons with pyknotically altered nuclei compared with T2DM. In the T2DM+PA group, “light” neurons with large light nuclei were predominated with an increased number of deep invaginations as compared with the control. In a small number of neurons, a partial enlargement of the perinuclear space was observed. Altogether, these observations may indicate the development of compensatory and adaptive processes aimed at increasing the area of contact between the nucleus and the cytoplasm. After PA administration astrocytes were with no signs of swelling. Thus, according to these observations, PA probably in more extent than metformin prevented diabetes-induced alterations of VMH cytoarchitectonics.

In the group with combined administration of metformin and propionic acid (Fig. 2i, 2j), we found that their concurrent administration led to an increase in the quantity of microglial cells in the VMH region of the hypothalamus. Additionally, combined administration of metformin and PA was accompanied by a slight decrease in pre-apoptotic and apoptotic neurons, in comparison with the T2DM + metformin and T2DM + PA groups. In “light” neurons, ER tubules were well-visualized, however with a small number of ribosomes (Fig. 2i, 2j, red arrows). In “dark” neurons, an enlargement of ER cisterns and vesicles was observed. In VMH neurons of T2DM rats treated with the combination of drugs, the number of swollen mitochondria, lysosomes (Fig. 2j, black arrows), and lipofuscin granules were increased compared with control, however their number was less than in the T2DM group and metformin group. The nuclei of

“light” neurons demonstrated significantly more invaginations of the nuclear membrane compared with dark neurons. Interestingly, astrocytes were less swollen near “dark” neurons when comparing with T2DM group.

Thus, the results of electron microscopy demonstrated diabetes-related changes in VMH neurons, manifested in the form of fragmentation of membrane organelles including mitochondria, an increase in the number of cells with apoptotic changes and neuropil edema. These changes were improved to a different extent by administration of drugs that partially prevented ER damage and mitochondrial swelling, reduced the number of apoptotic neurons and enhanced the glial response.

Additionally, since increasing evidence has shown a strong link between ER stress and the pathology of obesity and type 2 diabetes that may lead to leptin and insulin resistance, we performed an assessment of morphometric parameters of ER in the hypothalamus of rats with T2DM and after metformin and propionic acid administration (Fig. 2k, 2l). The sum of the relative areas of ER membranes and cisterns was considered an overall fraction of ER in the cell.

Quantitative measurement of the parameters of the ER showed a 2.25-fold enlargement of the ER cistern areas in the T2DM group as compared with the control and the slight tendency to increased total ER fraction (by 19%), however, without statistically significant difference (Fig. 2k, 2l). Administration of drugs caused similar decreasing effects on the relative area of ER cisterns and total ER fraction. Metformin reduced the relative area of the ER cisterns in T2DM rats by 36% (vs. T2DM), that was confirmed on the electron microscopy images presented previously, however, this parameter was still by 65% higher than in the control. Total fraction of ER in the cell had no significant changes in metformin group compared with control, however it was lower than in T2DM rats (Fig. 2k). PA administration reduced the area of ER cisterns (by 40.5% vs. control, by 316% vs. T2DM, by 232% vs. metformin) and total fraction of ER in the cell (by 39% vs. control and by 66% vs. T2DM) in VMH neurons. Finally, the combined administration of PA and metformin exerted profound lowering effect on the relative area of ER cisterns (by 53% vs. control, by 344% vs. T2DM, by 253% vs. metformin) and on the total fraction of ER in the cell (by 50% vs. control, by 80% vs. T2DM, by 38% vs. metformin) (Fig. 2l). Thus, under the influence of compounds on the background T2DM interesting trend was observed: after T2DM-induced elevation, ER parameters (relative area of ER cisterns, and the total fraction of ER in the cell) were lowered to a different extent not only when comparing with T2DM but also vs. control values after the treatment with metformin, PA and their combined exposure.

3.3. The influence of metformin and PA on the apoptosis/autophagy markers in the VMH of rats with T2DM

Since we detected an elevated number of autophagosomes in T2DM rats and after treatment, we aimed to assess the autophagy process based on the measurement of two main markers – LC3 and Beclin-1. Thus, LC3 is considered an autophagosomal marker in mammals, and has been frequently used to study autophagy in neurodegenerative and neuromuscular diseases, tumorigenesis, bacterial, and viral infections [36]. In T2DM group LC3 protein level was reduced by 25% compared to the control (Fig. 3a,

3b), suggesting a slight inhibition of autophagy. Metformin, PA and their concurrent administration to T2DM rats significantly increased LC3 protein content by 1.82, 3.95 and 2.79 folds respectively. However, there was no differences in the LC3 mRNA level between groups, suggesting that all changes occurred on translational level (Fig. 3c).

Another important marker that play a central role in autophagy and membrane trafficking in physiological and pathological processes, Beclin-1, was measured to further confirm the activation of autophagy after compound administration. Moreover, since Beclin-1 is localized primarily within cytoplasmic structures, including the ER, mitochondria and the perinuclear membrane [37], and we observed T2DM-induced changes in the architectonics of these cell structures, it would be interesting to juxtapose it with the Beclin-1 fluctuations. Interestingly, no differences in Beclin-1 content were found between control and T2DM group. However, after metformin (by 2.33 folds) and PA (by 3.15 folds) administration and their combination (by 2.91 times) Beclin-1 level was increased, that was in agreement with LC3 protein level fluctuations (Fig. 3a, 3d). Beclin-1 mRNA levels remained unchanged in all 5 groups of animals (Fig. 3e), confirming the hypothesis, that all T2DM-induced changes occurred on the translational level. Thus, the similar increasing effects of PA and metformin on the autophagy markers was detected, suggesting the possibility of these compounds to activate the autophagy process in VMH to a different extent.

Since Beclin-1 can also be involved in autophagic suppression of apoptosis [38] and considering the strong link between Beclin-1 and Bcl2/Bcx-xl complex, the next step was to estimate the state of Bax/Bcl-xl apoptotic regulatory axis. It is known that Bcl-xl has apoptosis-inhibiting properties, therefore we examined its content by different methods. Compared to the control, we found that experimentally induced T2DM was accompanied by a strong 10-fold decrease in the expression of the apoptosis-inhibiting protein Bcl-xl in the VMH (Fig. 3f, 3g). Metformin administration led to a slight elevation of Bcl-xl protein content compared with T2DM group, however PA alone and PA together with metformin exerted more pronounced increasing effects on the expression of Bcl-xl protein, which was even higher than in the VMH of control animals (by 14% and 67% vs. respectively). Bcl-xl gene expression almost did not change in the groups compared with control level (Fig. 3h).

Immunohistochemical staining of VMH sections with Bcl-xl antibody and following semi-quantitative assessment of the obtained IHC images confirmed changes observed on the protein level (Fig. 3i). According to IHC studies, the VMH region of the control rats has demonstrated a concise Bcl-xl expression in the predominant number of neurons and glial cells (Fig. 3k). In VMH neurons, the Bcl-xl protein was visualized as moderately and/or intensively labeled macules (Fig. 3k-1) near the perinuclear space (Fig. 3k-2). A small number of VMH neurons of the control group with a low level of Bcl-xl expression showed its diffuse localization in the cytoplasm (Fig. 3k-3). In glial cells, Bcl-xl was detected as granules of different sizes in the perinuclear space (Fig. 3k-4). Interestingly, it should be noted that a moderate Bcl-xl staining was observed in the endothelium of the blood capillaries of the VMH region (Fig. 3k-5).

In T2DM group there was a significant decrease in Bcl-xl expression in neurons, glial cells, and endothelium of VMH (Fig. 3i). In most cells Bcl-xl expression was not detected, or a weak diffuse staining was determined in the cytoplasm of VMH neurons. Only a few neurons from all observed areas in T2DM animals had macules with a weak Bcl-xl expression that was in agreement with low Bcl-xl protein level in T2DM group.

Metformin administration led to a mild increase in Bcl-xl expression in VMH neurons and, to a lesser extent, in glial cells and vascular endothelium (Fig. 3j, 3i). Bcl-xl in VMH neurons appeared as stained macules in the perinuclear space, however in separate VMH neurons, a weak staining of the entire cytoplasm was observed. In turn, PA administration induced further increase in Bcl-xl expression in the VMH, almost to the control values (Fig. 3j, 3i). There was a significant elevation of the number of neurons with well-stained macules, which were larger than in the control. Glia cells also had enlarged immunoreactive granules with more intense staining than in control group. The expression of Bcl-xl in the vascular endothelium also increased in comparison with the VMH of the T2DM group, but was lower than in control samples. Finally, co-administration of metformin and PA induced the more pronounced elevation of Bcl-xl expression in VMH, even when comparing with control samples (Fig. 3j, 3i). Most neurons had intensively stained macules, the size of which was larger than in the control. A significant Bcl-xl increase was also detected in glial cells compared with all groups, however Bcl-xl expression in the vascular endothelium was similar to the control. Thus, after inducing T2DM the activation of anti-apoptotic protein Bcl-xl was decreased, probably contributing to the enhancement of apoptosis in VMH neurons. Treatment with PA and metformin led to an increase of Bcl-xl protein level in all VMH types of cells, and the combination of metformin and PA exerted the most effective anti-apoptotic influence.

Bax protein is well-known for apoptosis-promoting biologic functions. Moreover, since Bax is a key regulator of apoptosis that mediates the release of cytochrome c to the cytosol via oligomerization from a monomer to a dimer in the outer mitochondrial membrane before pore formation [39], it is important to determine its monomer and dimer forms. Western blot analysis revealed that the content of Bax-monomer in T2DM group was higher by 5.16 times than in the control animals (Fig. 4a, 4b).

The same pattern was observed after separate metformin and PA administration – Bax-monomer level was as in the VMH of T2DM animals (Fig. 4a, 4b). Only the concurrent combination of metformin + PA decreased the T2DM-induced monomeric Bax level by 1.5-fold vs. T2DM. At the same time after T2DM-associated elevation, Bax dimeric form was reduced after all types of treatments compared vs. T2DM (metformin – by 2.2-fold, PA – by 1.9-fold, combined treatment – by 2.7-fold), (Fig. 4a, 4c). Interestingly, in VMH of T2DM rats, the expression of Bax mRNA remained unchanged compared with the control, however, the administration of metformin contributed to a significant increase in its expression compared to the T2DM group, and even compared vs. control (by 1.45 times, Fig. 4d). These data are not unexpected, since it is known that metformin may significantly elevate p53 and Bax levels and induce apoptosis in human MCF breast cancer via targeting ERK signaling [40]. In contrast, PA administration reduced Bax mRNA level compared with T2DM rats by 1.81 times (Fig. 4d), therefore when we studied the

combined treatment, we observed the compensation of increasing effect of metformin on Bax and decreasing action of PA, resulted in the Bax level relative to that in the control and T2DM groups.

In addition, the results of immunohistochemical semiquantitative analysis of Bax expression in VMH confirmed changes in Bax protein content after metformin and PA administration on the background of T2DM. VMH region of control animals showed different levels of Bax expression depending on the cell types. Endothelial Bax expression was observed at low and moderate levels, in neurons and glia – in small amounts both in the cytoplasm and in the nuclei (Fig. 4f). T2DM was associated with a significant increase in Bax expression in VMH cells (Fig. 4e, 4f, 4g), mostly in the endothelial cells (Fig. 4g-1). In VMH neurons, there was an elevation in cytoplasmic Bax expression (Fig. 4g-2), but not in the nucleus. Small diffusely stained areas in the perinuclear space were found in glial cells (Fig. 4g-3). Metformin treatment slightly decreased Bax expression in comparison with T2DM group (Fig. 4e). Diffuse Bax staining in the cytoplasm of VMH neurons was observed. Bax-positive glial cells showed moderate nuclear localization of Bax, however it was rarely observed in the cytoplasm and in the Golgi complex. Propionic acid did not affect the level of Bax expression in VMH compared with T2DM group (Fig. 4e, 4f). In the endothelium of blood vessels, expression of Bax protein was observed from moderate to high level. A significant part of VMH neurons after PA action showed moderate Bax expression in the cytoplasm. In glial cells Bax was localized in the perinuclear space in the form of granules. Unexpectedly, co-administration of metformin and PA led to a significant Bax decrease in VMH (Fig. 4e, 4f), however it was still 3-fold higher than in control animals. Interestingly, in endothelial cells, Bax expression was slightly decreased compared with the T2DM group. In turn, the number of intensively stained neurons was significantly higher than in control, but the intensity of labeling was lower than in the metformin and PA groups. In addition, after combined treatment with metformin and PA Bax was localized in the perinuclear space of the glial cells.

Thus, the development of T2DM was associated with an elevated Bax expression in VMH cells, especially in the cells of the endothelium of blood vessels that may indicate the activation of Bax oligomerization process. This triggers a pro-apoptotic effect that in turn may contribute to the development of diabetes-associated brain endothelial dysfunction. Combined treatment with metformin and PA was effective in inhibiting pro-apoptotic factor Bax and in activation of anti-apoptotic factor Bcl-xl that may mediate their neuroprotective effect on the background of T2DM.

The next part of the study was to estimate the occurrence of apoptosis based on the level of another trigger apoptotic marker – caspase-3, an executioner caspase whose activation by proteolytic cleavage often plays a role in apoptosis [41]. Neuron injury caused by an excessive stimulation by the excitatory neurotransmitter glutamate leads to widespread activation of caspase-3 [42], in part in neurons undergoing apoptosis, but primarily in the nuclei of astrocytes that are not undergoing apoptosis. We found that caspase-3 protein level in VMH of T2DM rats was dramatically increased by 4.4-fold compared with the control (Fig. 4h, 4i). The administration of the studied substances may have possible anti-apoptotic and neuroprotective effect expressed in diminishing caspase-3 level after metformin (by 8.8 times), after PA (by 1.94 times), and expectedly when their action was combined – by 2.93 folds vs. T2DM. However, caspase-3 level after all treatments was still 1.5-fold higher than in control.

To summarize, in animals with experimentally induced T2DM a critical increase in the level of caspase-3 was observed, that may reflect the high intensity of apoptotic events in VMH. The combination of metformin and PA was effective against apoptosis, but the caspase-3 content remained still 6 times higher compared with the control group.

Last, to further characterize autophagy/apoptosis process and find representative correlation links between all studied parameters, we calculated the Pearson's ratio (Table 2). Reasonably, we found a high positive correlation between autophagy markers Beclin-1 and LC3, Beclin-1 and anti-apoptotic factor Bcl-xl, and also between Bcl-xl and LC3. The level of pro-apoptotic factor Bax was linked with caspase-3 content by high positive correlation link, however caspase-3 was negatively correlated with Bcl-xl, that was in agreement with their main functions in the cell.

Table 2: Correlation (Pearson's ratio) matrix highlighting the link between apoptosis/autophagy markers in rat VMH

	Beclin-1	Caspase-3	Bcl-xl	Bax dimer	LC3
Beclin-1	1	-,042	,537**	,061	,781**
Caspase-3	-,042	1	-,417*	,779**	-,109
Bcl-xl	,537**	-,417*	1	-,344	,477*
Bax dimer	,061	,779**	-,344	1	-,214
LC3	,781**	-,109	,477*	-,214	1

Note: * - $p < 0.05$; ** - $p < 0.01$

Discussion

Local alterations of molecular pathways and cellular processes in hypothalamic area, especially in ventromedial hypothalamus, can be one of the fundamental reasons caused the disturbance in glucose metabolism and/or the development of T2DM, and may be accompanied by hypothalamic neuroinflammation. Animal studies may help to identify the mechanisms underlying diabetes-associated dysregulation of hypothalamic functions, confirm a bidirectional link with T2DM pathogenesis, and may also help to screen potential treatment options. In pathological contexts, including diabetes and neurodegenerative disorders, the particular attention has been paid to crosstalk between autophagy and apoptosis [43]. Since the balance between survival and loss of hypothalamic neurons may have an impact on the coordinated control of feeding and thermogenesis [44, 45], it was important to evaluate whether the HFD can induce apoptosis of cells in hypothalamus, and to investigate how possible treatment options may influence cell death processes.

Autophagy is a catabolic pathway activated by cellular stress, such as starvation or pathogen infection, followed by engulfment of proteins and organelles by double-membrane vesicles, called autophagosomes. Autophagy can result in cellular adaptation, as well as cell survival or cell death [46]. Despite the mechanisms of autophagy and apoptosis have differences, some proteins modulate both autophagy and apoptosis and these switch points may be considered as potential targets for therapeutic interventions. Thus, the interconnected molecular regulators between autophagy and apoptosis serve as switching points critical to the ultimate outcome of the cell [47]. For instance, Bcl-2 and Bcl-xl, the anti-apoptotic proteins, inhibit both autophagy and apoptosis through interacting respectively with Beclin-1 and Bax/Bak using their Bcl-2-homology (BH)-3-binding pockets [48, 49]. Bcl-2/Bcl-xl complex by binding to Beclin-1 prevents the interaction between Beclin-1 and the class III PI3K complex (PI3KC3) to inhibit autophagy [37]. Several pathways have been reported to interfere with autophagy, such as the Akt pathway and MAPK pathways including ERK, p38 MAPK, and JNK [50]. Previous studies showed that Bcl-2/Bcl-xl phosphorylation may be a regulatory switch between autophagy and apoptosis [51]. Since defects in autophagy may underlie the development of neurodegenerative diseases [52], particular attention should be paid to elucidating the process of switch between apoptosis and autophagy as an important factor in the development of T2DM-associated hypothalamic dysfunction. Our study was performed to investigate the crosstalk between autophagy and apoptosis in VMH after the treatment of metformin and PA by studying apoptosis-promoting (Bax, caspase-3), apoptosis-inhibiting (Bcl-xl) and autophagy-associated (LC3, Beclin-1) proteins.

As possible compounds that may have modulatory action on autophagy and apoptosis were considered metformin and sodium salt of propionic acid. As a comparison drug we chose metformin – a “gold” standard for the anti-diabetic monotherapy, due to its well-known lowering effects on glucose level, anti-inflammatory, anti-apoptotic and anti-oxidative properties [53], however there was no data about metformin neuroprotective action. In order to test another approach for the treatment of neuropathy in diabetes mellitus influencing gut-brain axis, we considered enteric-soluble SCFAs such as propionic acid as a supplementary therapy since it has been reported its normalizing effect on fasting blood glucose, body weight and insulin tolerance [54]. However, the direct effect of the PA usage as an additional therapy on the neurons of the VMH in type 2 diabetes mellitus should be studied in detail, therefore we used the complex approach in order to visualize cellular changes in VMH and to characterize the molecular impairments in the regulation of apoptosis/autophagy interplay.

To modulate the development of metabolic syndrome, rats were fed with HFD for three months. Animals in T2DM group demonstrated a significant increase in weight and waist volume compared with the control rats that reflect the development of obesity. An elevation of blood glucose and HbA1c level in the serum of T2DM rats, and impaired glucose tolerance and insulin sensitivity (based on ipITT) were considered accurate biomarkers verified the development of experimentally induced T2DM. As expected, metformin and PA treatment did not influence nor body weight and waist of rats neither glucose and HbA1c level.

It is known that in rodents, the consumption of fat-rich diets blunted leptin and insulin anorexigenic signaling in the hypothalamus by a mechanism dependent on the *in situ* activation of inflammation [55]. Since inflammatory signal transduction may lead to the activation of apoptotic signaling pathways, it was important to investigate at the cellular level possible changes in the VMH cytoarchitectonics of rats with T2DM and the influence of the metformin and propionic acid administration. Therefore, we performed an examination of VMH ultrastructural changes by electronic microscopy, an evaluation of morphometric parameters of ER and determined the levels of apoptosis/autophagy representative markers on mRNA level by RT-PCR, on protein level – by western blotting, and, in addition, assessed their distribution immunohistochemically.

We observed HFD-induced profound changes in VMH: tubules and cisterns of *rough* ER in most neurons were enlarged; an accumulation of fragmented mitochondria with signs of cristae *destruction* and matrix swelling were detected, that may reflect the activation of mitoptosis, programmed destruction of mitochondria; nuclei were pyknotically altered. Thus, in T2DM group most neurons were with signs of apoptosis and dendritic loss. These changes were accompanied by an imbalance between pro- and anti-apoptotic molecular regulators: first, by a dramatic elevation of pro-apoptotic factors Bax, that may translocate to the outer mitochondrial membrane and undergo oligomerization, leading the release of apoptogenic factors such as cytochrome c, and caspase-3, that confirmed cellular changes observed by electron microscopy; second, by a slight decrease in autophagy marker LC3 and anti-apoptotic factor Bcl-xl, that may contribute to the inhibition of autophagy. Thus, the development of T2DM was associated with an activation of apoptosis and inhibition of autophagy that may underlie pathological processes in hypothalamus and, as a result, impaired glucose homeostasis and the development of neuropathy. Our results were in line with the study showing that the fat-rich diet induced neuronal apoptosis and that the presence of a functional TLR4 receptor protected hypothalamic cells from apoptotic damage [55]. Moreover, an induction of cell death in the hypothalamus and/or the whole brain can mimic metabolic inflammation that may mediate the development of central leptin and insulin resistance, resulting in a broad range of metabolic disorders including overeating, glucose intolerance and hypertension.

We found that the administration of drugs partially improved the morphological signs of damage in the hypothalamus by reducing mitochondrial swelling, diminishing the number of apoptotic neurons and also enhancing the glial response. It is not surprising, since it was shown that metformin demonstrated neuroprotective effects in primary cortical neurons [56]. However, to our knowledge there are no studies related to possible influence of metformin on apoptosis/autophagy switch in VMH on the background of T2DM. Metformin administration resulted in an increase in the number of lysosomes and autophagosomes, swelling of myelinated *fibers*, an accumulation of autophagosomes in axoplasm, the reduction of the volume and relative area of the ER cisterns compared to T2DM group. The structure of mitochondria was more preserved than in T2DM group. Elevated number of lysosomes and autophagosomes as signs of autophagy were associated with an elevation of the levels of anti-apoptotic protein Bcl-xl, autophagy markers LC3 and Beclin-1, and a decrease in pro-apoptotic proteins as Bax and caspase-3 compared with T2DM. However, it should be noted that administration of metformin on the

background of T2DM was accompanied by a clearly visible accumulation of lipofuscin granules in the cytoplasm of VMH neurons, as well as the development of reactive astrogliosis. Accumulation of lipofuscin is considered a sign of "cell aging", and also inhibits of the ubiquitin-proteasomal system, adequate stress response and increases oxidation of unsaturated fatty acids that altogether may lead to neuronal degeneration. Furthermore, experimental work on *C. elegans* and human primary cells demonstrated that treatment with metformin caused age-related disruptions in conservative metabolic pathways and led to the development of mitochondrial dysfunction [57]. Therefore, despite the effective influence on hyperglycemia, metformin monotherapy did not prevent the development of diabetes-induced hypothalamic impairments. Another interesting observation after metformin treatment that needed to be further studied was the presence of reactive astrogliosis, characterized by an abnormal increase in the number of astrocytes, their enhanced proliferation and hypertrophy. Therefore, our next step was to test PA as an additional therapy to correct neurodegenerative diabetes-induced changes and mitigate the potential negative effects of metformin treatment.

Administration of PA on the background of T2DM caused the similar effects on VMH architectonics as it was observed in metformin group, but in greater extent. Relative area of ER membranes and cisterns as a total fraction of ER in the cells were progressively decreased after PA action. The administration of PA was characterized by a significant number of autophagosomes and a decrease in the number of apoptotic cells compared with the T2DM group. It was in line with a significant elevation on LC3 and Beclin-1 levels, suggesting an activation of autophagy process, that was correlated with an increase in Bcl-xl content up to control values. However, despite that fact that PA administration led to a decrease in the number of apoptotic dark neurons with pyknotically altered nuclei compared with diabetes, unexpectedly pro-apoptotic protein Bax (both dimer and monomer forms) as well as caspase-3 were upregulated after PA administration, showing the tendency to the launch of apoptosis, however, it was not reflected in cellular level in the form of apoptotic signs. Bax may interact with Beclin-1 and inhibit autophagy [47] thus may represent a new crosstalk point. Moreover, it is known that Bax may oligomerize and trigger mitochondria-dependent apoptosis, however, we observed an elevation of the only Bax monomer, while Bax dimer was downregulated compared with T2DM group, that may explain the absence of the signs of apoptosis and mitoptosis during electron microscopy examinations. In addition, the positive point needed to be emphasized here is that the difference compared with metformin was that astrocytic glia demonstrated no signs of swelling. Generally, it can be assumed that PA activated anti-apoptotic mechanisms in neurons by stimulating an autophagy as an important protective way to prevent an accumulation of misfolded proteins. These statements require further research to understand the mechanisms of switch between apoptosis and autophagy in VMH under the influence of PA.

The last group we estimated was rats after combined administration of metformin and propionic acid on the background of T2DM. Based on the electron microscopy data, showing the effects of separate metformin and PA administration after HFD-induced diabetes, we expected that their combination would be more effective than the separate administration. Nevertheless, after combined treatment we found an increase in the quantity of microglial cells and also observed a significant reduction of the percentage of light neurons compared with the control group. Interestingly, it should be noted profound lowering effect

of combined administration of PA+metformin on the relative area of ER membranes and cisterns compared with all 3 groups (control, T2DM and metformin) reflecting the restoration of normal ER functioning. Additionally, combined administration of metformin and PA was accompanied by a slight decrease in pre-apoptotic and apoptotic neurons, in comparison with the T2DM + metformin and T2DM + PA groups. These observations correlated with a decrease in caspase-3 and Bax monomer and dimer levels, confirming diminished apoptotic changes compared with other treatment group. Considering the highest Bcl-xl level in the group of combined treatment compared with others, we may suggest that apoptosis was inhibited by the activation of Bcl-xl pathway. Moreover, it is known that Bcl-xl may inhibit both autophagy and apoptosis through interacting respectively with Beclin-1 and Bax. At the same time, we suggested that autophagy in this group was triggered by elevated levels of LC3 and Beclin-1. Thus, considering all the results, we can conclude that combined therapy with metformin and PA was optimal based on the measurement of the ratio of pro-apoptotic, anti-apoptotic and autophagy markers in the VMH of T2DM rats.

Despite we were trying to comprehensively characterize the processes of apoptosis and autophagy in the VMH of T2DM rats in the course of experiments, there is a number of limitations in this study. Due to technical limitations, such as the small weight and size of rat VMH, we cannot perform the immunohistochemical studies of all proteins, therefore we decided to only visualize Bax/Bcl-xl axis. In addition, in this study we considered caspase-3 as apoptotic marker protein, however, it was shown that in some brain cell types caspase-3 activation plays non-apoptotic roles, including in cell differentiation, cell cycle regulation, and cell migration [58]. In addition, in the CNS, caspase-3 participates in cytoskeletal remodeling in neurons and astrocytes [59] and in the differentiation of astrocyte subpopulations [60]. The further work should be done to distinguish the non-apoptotic role of caspase-3 depending on brain cell types after the T2DM and under the influence of metformin and PA. It is also remaining undiscovered by which mechanisms PA exerts its effects on VMH, therefore additional investigations are required.

Thus, our findings demonstrated the presence of T2DM-induced activation of apoptosis and impairments in cell integrity of VMH. Diabetes-induced imbalance of pro- and anti-apoptotic proteins was more pronounced at the protein level than at the transcriptional, indicating a significance of transcriptional homeostasis. PA administration may switch apoptosis to autophagy that may mediate neuroprotective effect of PA on the background of T2DM development. A combination of PA and metformin may be beneficial in modulating cell death in hypothalamus upon T2DM.

Conclusions

T2DM-induced enhanced apoptosis and mitoptosis may trigger nerve cell dysfunction in VMH. Drug administration slightly improved ultrastructural changes in VMH. Metformin monotherapy led to a decrease in neuronal apoptosis, however caused enhanced cell aging and accumulation of lipofuscin granules and cell debris in VMH. PA administration partially normalized VMH architectonics and switched cell fate from apoptosis to autophagy that may be considered as adaptive and compensatory response to T2DM-induced apoptosis. Thus, PA and its combination with metformin may have beneficial effects

for counteracting diabetes-induced cell death by activating autophagy and inhibiting apoptosis in VMH. In our opinion PA may be considered as one of the promising substances in addition to metformin for correcting diabetic neuropathy and impairments in hypothalamus via modulating the interplay between apoptosis and autophagy.

Declarations

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Figures

Figure 1

Experimental design and timescale (**a**). T2DM was developed after 3 months of high-fat diet (HFD) with following single injection of streptozotocin (STZ, 25 mg/kg of b.w.). Each experimental group included 12 animals: 1) the untreated control group; 2) the group with experimentally induced T2DM; 3) the group that received anti-hyperglycemic agent metformin at a dose 60 mg/kg of b.w., for 14 days, orally on the background of T2DM; 4) the group that received sodium salt of propionic PA at a dose 60 mg/kg of b.w.,

for 14 days, orally on the background of T2DM; 5) the group that received metformin (60 mg/kg of b.w., for 14 days, orally) and PA (60 mg/kg of b.w., for 14 days, orally) on the background of T2DM. The body weight **(b)**, waist (c), blood glucose **(d)** and glycosylated hemoglobin (HbA1c) levels **(e)** of diabetic animals and after metformin and PA treatment. Intraperitoneal insulin tolerance test **(f)** on control rats (n=6, solid line) and rats with T2DM (n=24, dashed line) before administration of substances. Blood samples were collected from the tail at indicated time points and analyzed for glucose concentration (mmol/l). Values are given as mean $\pm$ SEM (n=6); * p<0.05 vs. control, § p<0.05 vs. T2DM, # p<0.05 vs. metformin administration, @ p<0.05 vs. PA administration.

Figure 2

Ultrastructural changes of VMH neurons in T2DM rats and after treatment (electron microscopy). Gallery of micrographs obtained by SEM ($n = 12$ neurons for each group): **a, b** – control rats (Magnification – x4800); **c, d** – rats from T2DM group (Magnification on C – x8000, D – x9600); **e, f** – T2DM rats treated with metformin (Magnification – x9600); **g, h** – T2DM rats treated with propionic acid (Magnification – x9600); **i, j** – T2DM rats treated with both metformin + propionic acid (Magnification on **a** – x6400, **b** – x8000). The red arrows indicate the rough endoplasmic reticulum, yellow arrows – autophagosomes, black arrows – lysosomes, white arrows – deep invaginations of the nuclear membrane and green arrows – lipofuscin granules. Scale bar: 200 nm. Structural parameters of the ER after metformin and propionate administration on the background of T2DM: ImageJ software was used to calculate the areas of the ER membranes and the ER cisterns (**k**) and its sum (total fraction of ER in cell, **l**) in each cell based on the electronic microscopic microphotographs. Values are given as mean $\pm$ SEM ($n=12$); * $p<0.05$ vs. control, § $p<0.05$ vs. T2DM, # $p<0.05$ vs. metformin administration, @ $p<0.05$ vs. PA administration.

Figure 3

Effects of metformin and PA treatment on LC3, Beclin-1 and Bcl-xl mRNA expression and protein content in VMH of diabetic rats. Immunoblotting analysis: representative immunoblots are shown (**a**, **f**) and level of proteins was quantified using tubulin as a loading control for hypothalamus lysates. The bar graphs of LC3 (**b**), Beclin-1 (**d**) and Bcl-xl (**g**) are presented as means $\pm$ SEM ($n = 6$ /group). Quantitative real-time PCR analysis of LC3 (**c**), Beclin-1 (**e**) and Bcl-xl (**h**) mRNAs expression in rat VMH: data were normalized to both β -actin/GAPDH and pooled from three independent experiments ($n = 6$ rats/group). Immunocytochemical analysis of Bcl-xl-positive VMH cells: representative histogram (**i**) and images (**j**)

are shown. DAB (3,3'-diaminobenzidine) staining was used to visualize Bcl-xl-positive cells, Hematoxylin Gill was used for nuclear staining. Scale bars indicate 150 μ m (magnification x400).

Immunocytochemical analysis of Bcl-xl-positive VMH cells of the control rats (**k**): 1 – moderately and/or intensively labeled macules; 2 – macules near the nucleus; 3 – neurons with diffuse Bcl-xl expression in the cytoplasm; 4 – glial cells with Bcl-xl expression; 5 – Bcl-xl staining in the endothelium of the blood capillaries of the VMH (magnification of fragment x3200). All data are shown as means $\pm$ SEM;

* $p < 0.05$ vs. control, § $p < 0.05$ vs. T2DM, # $p < 0.05$ vs. metformin administration, @ $p < 0.05$ vs. PA administration.

Figure 4

Effects of administration of metformin and PA on apoptotic and anti-apoptotic markers. Immunoblotting analysis of Bax in rat VMH: representative immunoblots show **(a)** the monomeric and dimeric forms **(b, c)** were quantified using tubulin as a loading control for hypothalamus lysates. The bar graphs of Bax protein content **(b, c)** are presented as means $\pm$ SEM (n = 6/group). RT-PCR of Bax in rat VMH **(d)**: data were normalized to both β -actin/GAPDH and pooled from three independent experiments (n = 6

rats/group). Immunocytochemical analysis of Bax-positive VMH cells: representative histogram (**e**) and images (**f**) are shown. DAB (3,3'-diaminobenzidine) staining was used to visualize GRP78-positive cells, Hematoxylin Gill was used for nuclear staining. Scale bars indicate 150 μ m (magnification x400). Immunocytochemical analysis of Bax-positive VMH cells of T2DM rats (**g**): 1 – blood capillaries of the VMH with high Bax expression; 2 – VMH neurons showed cytoplasmic expression of Bax; 3 – glial cells demonstrated small diffusely labeled areas of the cytoplasm located near the nucleus (magnification of fragment x3200). Caspase-3 protein level after metformin and PA administration on the background of T2DM: immunoblotting analysis of caspase-3 in rat VMH: representative immunoblots are shown (**h**) and quantified using tubulin as a loading control for hypothalamus lysates. The bar graph of caspase-3 (**i**) is presented as mean $\pm$ SEM (n = 6/group); * p<0.05 vs. control, § p<0.05 vs. T2DM, #p<0.05 vs. metformin administration, @ p<0.05 vs. PA administration.