Unfolded protein response in gastric glandulocytes of rats with the pharmacological correction of type 2 diabetes

Abstract. Background. The cellular and molecular mechanisms underlying gastrointestinal complications caused by type 2 diabetes mellitus (T2DM) may involve accumulation of misfolded proteins in the endoplasmic reticulum that disrupts protein homeostasis and activates a signaling pathway termed the unfolded protein response (UPR). The goal of the present study was to assess the state of the UPR system in gastric glandulocytes of untreated and metformin- and propionate-treated T2DM rats. 

Materials and methods. Rats with induced T2DM received metformin, propionate, and their combination. Analysis of the levels of 78-kDa glucose-regulated protein (GRP78), activating transcription factor 6 (ATF6), protein kinase R-like endoplasmic reticulum kinase (PERK), and inositol-requiring enzyme 1 (IRE1) was performed by Western blotting and immunohistochemical assessment of slices.

Results. In T2DM rats, an increase in GRP78 vs. control (normal) group was found. Metformin and propionate treatment led to an increase in GRP78; under combination therapy, its content was registered at the level in untreated T2DM group. An increase in the ATF6 in T2DM rats was found, and all treatment regimens contributed to its growth. The PERK level in T2DM rats exceeded that in controls, and propionate treatment caused its decrease to the level observed in control group. An immunohistochemical assessment revealed a tendency to increase the intensity of immunoreaction for GRP78 in T2DM rats. With metformin treatment, an intensive immunoreaction for GRP78 was revealed. The general trend in T2DM rats was a significant increase in ATF6 expression.

Conclusions. Combination treatment with metformin and propionate led to a significant decrease in GRP78, which may indicate a positive effect of such therapy. New data on propionic acid effect on UPR in the stomach have been obtained that may be beneficial for developing possible treatment strategies in complications of gastropathy caused by diabetes.

Keywords: metformin; propionate; endoplasmic reticulum stress; gastropathy; experimental diabetes mellitus model

Introduction

Among diabetic complications, there are those associated with changes in the enteric nervous system often known as diabetic enteropathy [1]. Diabetes-induced neuronal damage causes impaired motility and changes in secretion along the gastrointestinal (GI) tract [2]. At present, there are practically no available data on the secretory function of gastric glandulocytes affected by type 2 diabetes mellitus (T2DM). Taking into account the significant T2DM prevalence and long-term oral therapies of diabetic patients, it is considered appropriate to study the features of structural and functional changes in the main glandular cells of the stomach. This is relevant because the secretory function of the stomach is directly associated not only with digestive functions and the chyme formation, but also with the drug absorption in the stomach [3]. The cellular and molecular pathogenic mechanisms underlying GI complications in T2DM have not been fully elucidated. It is assumed that these mechanisms may include cellular disorders, namely mitochondrial dysfunction and accumulation of misfolded proteins in the endoplasmic reticulum (ER) that disrupts protein homeostasis and activates a signaling pathway termed the unfolded protein response (UPR).
Numerous current studies identify propionic acid (PA) as an important part in maintaining tissue homeostasis. Treatment with propionate is reasonably considered to be a promising pharmacological strategy. Firstly, short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate are released during the breakdown of dietary fiber. They are responsible for affecting the intestinal epithelial barrier, immune system, the gut microbiota and are natural substances sometimes acting as bacterial inhibitors [4]. Secondly, there is a significant amount of experimental evidence on successful correction of pathological conditions by using propionate as a dietary supplement [5–8]. However, despite the beneficial effect of SCFA on host gastrointestinal activity, excessive PA concentrations were accompanied by propionic acidemia which confirms the need for reasonable administration of SCFA [9]. SCFAs cause a number of direct effects on GI physiology [10]. It was likely that the effect of SCFA on the host microbiota suggested a significant functional load on GI cells and resulted in ER stress. Current preventive and/or therapeutic measures for the T2DM complications include diet and nutritional supplement administration. Therefore, there is a need to test our hypothesis on the response system of unstructured proteins in glandulocytes as baseline indicators of ER stress in the background of an experimental T2DM rat model and with various pharmacological strategies.

So, the goal of the research was to assess the state of UPR signaling system in gastric glandulocytes of diabetic rats and to study the effects of metformin and PA to compare treatment strategies. To achieve the principal goal, we determined the levels of 78-kDa glucose-regulated protein (GRP78), activating transcription factor 6 (ATF6), protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1) by Western blotting in the stomach fundus of T2DM rats after treatment with metformin and PA and their combination. In addition, gastric sections after metformin and PA administration were examined using immunohistochemical markers GRP78, ATF6 and IRE1 to assess their distribution.

Materials and methods

Male Wistar rats weighing (176.8 ± 8.3) g were kept on a standard balanced rodent diet and freely available water. T2DM was induced by a combination of a high-fat diet and with various pharmacological strategies. To achieve the principal goal, we determined the levels of 78-kDa glucose-regulated protein (GRP78), activating transcription factor 6 (ATF6), protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1) by Western blotting in the stomach fundus of T2DM rats after treatment with metformin and PA and their combination. In addition, gastric sections after metformin and PA administration were examined using immunohistochemical markers GRP78, ATF6 and IRE1 to assess their distribution.

All experimental procedures with the rats were carried out in accordance with national instructions and international laws on the humane treatment of laboratory animals: European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the law of Ukraine “On the protection of animals from cruelty” No. 3447-IV (Ukraine, 2006). The protocol of experiments on rats was approved by the Bioethics Commission of the Bogomolets National Medical University (Protocol No. 123 dated 26.09.2022).

The levels of GRP78, PERK, ATF6, and IRE1 proteins were detected by Western blotting. Protein lysates from the stomach fundus were prepared according to a standard protocol using a RIPA buffer as a homogenizer. 0.1 g of tissue was lysed for 20 min in RIPA (1 : 9) in the presence of a mixture of protease inhibitors (Sigma, USA). After being lysed, the samples were centrifuged (+4 °C, 16,000 g, 45 min), and then the supernatant was aliquoted. The aliquot of each lysate with an equal amount of protein (70 micrograms per lane) was separated using 10–15% SDS-PAGE. After the proteins being transferred to a nitrocellulose membrane (#HATF00010, pore size 0.45 microns, Merck Millipore, USA), protein-binding sites have been blocked with 5% non-fat milk in a phosphate-salt buffer with 0.05% Tween-20 (PBST) for 1 hour. The membranes were incubated overnight at +4 °C with primary GRP78 antibodies (1 : 2000, #PA5-34941, Invitrogen, USA), PERK (1 : 250, #PA5-79193, Invitrogen, USA), ATF6 (1 : 1000, #PA5-85935, Invitrogen, USA), IRE1 (1 : 250, #PA5-20190, Invitrogen, USA) and β-actin (1 : 5000, A3854, Sigma-Aldrich, USA) in a 3% bovine serum albumin with PBST. Membranes for target protein identifying were washed and incubated with antirabbit IgG, secondary antibody, HRP-conjugated (1 : 10000, #31460, Sigma-Aldrich, USA), and visualized immediately to actin determination. Enhanced chemiluminescence with p-coumaric acid (Sigma-Aldrich, USA) and luminol (Sigma-Aldrich, USA) was performed to visualize the protein bands. Relative levels of GRP78, PERK, ATF6, and IRE1 were normalized with β-actin and quantified using Gel-Pro Analyzer32, v3.1. The data were presented in conventional optical density units as a multiplicity of changes compared to the control values.

For immunohistochemical assessment, samples of the studied part of the stomach were fixed in a 10% formalin solution and processed by the paraffin technique (Leica Surgipath Paraplast Regular). Cross sections 4 μm thick were cut with a Thermo Microm HM 360 microtome and fixed on adhesive slides (HistoBond ++, Paul Marienfeld GmbH & Co. KG). GRP78 Polyclonal Antibody (#PA5-34941, Invitrogen, USA) and ATF6 (#PA5-85935, Invitrogen, USA) were used in a 1 : 200 dilution. The reaction product visualization was performed using a detection system based on diaminobenzidine (EnVision FLEX; Dako, Glostrup, Denmark). Incubation of the sections with primary and secondary antibodies was carried out at 24 °C for 20 and 10 min, respectively. The slides were examined with an Olympus BX51 microscope and photographed with an Olympus C–3040 Zoom digital camera using Olympus DP–Soft 3.2 software (Olympus, Tokyo, Japan). Quantification was performed on micrographs with a 400× magnification (2272 × 1704 RGB pixels, photo lumi-
nance mode, standardized exposure). ImageJ 1.46 was used as a system for image analysis (ver. 64-bit Java 1.8.0_172, Wayne Rasband, NIH, USA). Digital image processing was carried out according to the method [12]: the image deconvolution was performed, the integral intensity of the regions studied was obtained and then recalculated per the image unit area of 1 mm².

Statistical processing of the research results was performed using the IBM SPSS Statistics software, version 23.0 (SPSS Inc., USA). Data distribution was analyzed using the Shapiro-Wilk test, and statistical intergroup differences were analyzed using one-way ANOVA with Tukey or Bonferroni correction. The intergroup differences were considered to be statistically significant at p < 0.05. All data were obtained on the basis of two or three independent experiments.

Results

To assess the state of the UPR system, a content of GRP78, PERK, ATF6, and IRE1 in homogenates of the stomach fundus was detected by Western blotting. In T2DM rats, GRP78 level was 1.5 times higher vs. control group (p < 0.05) (Fig. 1). Metformin and propionate treatment led to a 2.4- and 1.6-fold increase in GRP78, respectively, compared to the experimental untreated T2DM group (p < 0.05). When metformin was co-administered with propionate, GRP78 content was at the level of untreated T2DM group.

The ATF6 content in experimental untreated T2DM rats increased by 1.3 times vs. control group (p < 0.05) (Fig. 1). When metformin was administered to T2DM rats, an increase by 1.6 times in the level of ATF6 was found vs. control group (p < 0.05). In gastric homogenates of T2DM rats receiving PA and combination therapy, the ATF6 content increased by 1.5 and 1.4 times, respectively, vs. control group.

The PERK level increased, as well as of all components in the UPR system (1.8 times vs. control group, p < 0.05) (Fig. 1). Metformin treatment and combination therapy led to a 1.7- and 1.9-fold increase in PERK levels, respectively (p < 0.05), compared to those in non-treated T2DM rats. Propionate-treated rats showed a decrease in PERK levels in gastric tissue to the control level. No changes in the IRE1 content were detected in untreated T2DM and metformin-treated groups (Fig. 1). PA administration led to a nonsignificant increase in the IRE1 level; with co-administration of drugs, it exceeded the control group by 1.4 times (p < 0.05).

Notes: * — p < 0.05 vs. control; † — p < 0.05 vs. T2DM; ‡ — p < 0.05 vs. metformin treatment; †† — p < 0.05 vs. PA treatment.

Figure 1 — Effects of metformin and PA treatment on relative levels of GRP78, PERK, ATF6, and IRE1 (-fold) in stomach fundus of T2DM rats. Immunoblotting analysis: representative immunoblots (A), levels of GRP78 (B), ATF6 (C), IRE1 (D) and PERK (E) was quantified using beta-actin as a loading control.
The immunohistochemical detection of GRP78 in the gastric fundus wall showed a significant specificity in the localization of the expression of this protein in cells (Fig. 2). GRP78-positive cells were mainly found in the lamina propria of gastric mucosa, and only individual cells were identified in the tela submucosa. However, the cytoplasmic immunoreaction to GRP78 was intense in the epithelial cells of the gastric glands. The dependence of the localization of GRP78-positive cells in the glands was also found. In the control group, these cells were identified at the bottom of the glands and up to 1/2 of the height (depth or lumen) of the gland.

Expression was weak (compared to the experimental groups) in individual cells or groups of cells in each gland. In the diabetic group, GRP78-positive cells were found at the level of 1/3 to 1/2 of the depth of the gastric glands. A tendency to increase the intensity of immunoreaction to
GRP78 was found. In metformin-treated T2DM rats, intensive immunoreaction was detected for GRP78 (vs. control group, p < 0.001, ANOVA post-hoc Bonferroni test; it was higher compared to T2DM (p < 0.001) according to the Tukey-Kramer test), and a visually increased number of GRP78-positive cells was observed. In the group of propionate-treated T2DM rats, the immunohistochemical features of GRP78-positive cells were similar to those in the diabetic untreated group. There was no significant difference in the integral immunoreaction density for GRP78 between the compared groups. In T2DM group receiving combination therapy, some heterogeneity in the detection of GRP78-positive cells was found. A general trend was an increase in GRP78 expression in epitheliocytes compared to the control group, but no significant differences were found compared to the untreated diabetic group.

Note. * — p < 0.05 compared to the controls.

Figure 3 — Immunohistochemical detection of ATF6 in the stomach fundus affected by T2DM and in the background of pharmacocorrection: A) controls; B) diabetes; C) metformin; D) propionate; E) combination therapy. A micrograph, 400×. The result of quantitative assessment of the immunopositive response for ATF6 in the stomach fundus (F)
We investigated the features of the immunohistochemical response to ATF6. In the control group, a weak positive reaction to ATF6 was found in the epitheliolytic nuclei of the fundic glands (Fig. 3). Reaction products were typically not found in the cytoplasm. In the diabetic group, both nuclei with a significantly more intense positive response (vs. control group) and ATF6-negative epitheliolytic nuclei were identified. Signs of weak cytoplasmic immunoreaction were also noted.

In T2DM group, a general trend to a significant increase in ATF6 expression in epithelial cells vs. control group (p < 0.001) and an increase in the number of such cells in the tubular gland (along its length) were registered. In metformin-treated T2DM group, significantly more intense immunoreaction to ATF6 vs. control group (p < 0.001) was also found, and both a nuclear and weak cytoplasmic responses were also detected. In propionate-treated group, the morphological pattern was similar to that in untreated T2DM rats and metformin-only treated T2DM rats. The integral density of the immunopositive response to ATF6 has no differences from the other groups studied, but it was significantly higher than that in controls, by almost 48 % (p < 0.001). In T2DM rats receiving combination therapy, cytoplasmic immunoreaction to ATF6 exceeded this indicator in control group (p < 0.001). At the bottom of the glands, cytoplasmic and, somewhat less often, nuclear immunoreactions were observed. The overall trend of ATF6 expression was similar to that in other groups studied. It should be noted that diabetic mucocytes increased ATF6 expression compared to the mucocytes in control group.

Discussion

The endoplasmic reticulum is a specialized perinuclear organelle involved in the synthesis of secretary and membrane proteins and lipids. ER stress activates the UPR mechanism instigating three main ER signaling pathways: PERK, IRE1, and ATF6 [13].

The primary functional stress sensor is considered to be the GRP78 chaperone. It regulates folding in the ER, responds to fluctuations in transmembrane sensors ATF6, PERK, and IRE1, and also detach from the ER membrane, move to the nucleus, and activate the intranuclear transcription chain. As a result of transcriptional activation, the GRP78 level is established/restored, and the endoplasmic reticulum-associated protein degradation (ERAD) of misfolded structures is initiated. It seems that this mechanism is absolutely logical and quite simple. However, the study of these elements of the UPR system on experimental models showed that there is no clear understanding of the role of each regulator, no correlation between the content of these proteins in cells and the degree of ER stress, recovery after stress, cellular adaptation, processes of nuclear transcription of components, etc. These issues remain to be further investigated. It is considered that PERK signaling is activated immediately as a rapid response to the action of a damaging pattern; it inhibits the overall protein synthesis in the cell. However, this early protective process is unfavorable for long-term cellular function.

The cellular adaptation to long-term (chronic) stress is determined by the activity of the ATF pathway, which renews overall splicing in the cell including to restore the level of the major chaperone GRP78 and ERAD proteins. The role of IRE1 signaling in mammals has not yet been definitively determined, and the functional pleiotropy of IRE1 by which it can switch its activity from pro-survival to pro-apoptotic thereby defining the fate of cells [14] is still being discussed.

Our study on the functional state of gastric glandulocytes in the T2DM model showed an increase in the content of all UPR elements. This supports the hypothesis that ER stress is present in cells. However, an additional 2.4-fold increase in the GRP78 content in the stomach tissue of metformin-treated T2DM rats occurred unexpectedly. Especially clearly it was demonstrated by the results of the IGH study.

Metformin is widely accepted first-line antihyperglycemic agent for most T2DM patients. Despite practical data about metformin effect on the UPR system in various cells, it has been shown to reduce ER stress in kidney cells [15], angiotensin II-induced ER stress and hypertension in mice [16], and also protect human islet cells from the damaging effects of pro-inflammatory cytokines [17]. In our previous work, we also showed that metformin treatment can restore GRP78 levels to the control one and reduce ATF6 and IRE1 content in the hypothalamus compared to untreated diabetic rats [15]. Considered that metformin for T2DM should be taken orally for a long time, an increase in functional stress on glandulocytes was logical as an additional effect on the glandular epithelium in any treatment regimen.

Our null hypothesis was an increase in functional stress on the gastric glands with additional oral administration of another medicine, propionate. Accordingly, we expected an increase in ER stress in glandulocytes. The addition of propionate to therapy for T2DM is based on an attempt to provide neuroprotection and prevent diabetic encephalopathy, since propionate is actively used in the treatment of neuroinflammation in various conditions and diseases [18–21].

According to our data, propionate treatment for T2DM does not lead to stomach problems in terms of the parameters studied. But metformin and PA combination showed a significant decrease in the GRP78 content, which may indicate a positive effect of this therapeutic strategy. It is possible that propionate has such a positive effect on glandulocytes due to its affinity for propionic acid as a product of intestinal microbiota metabolism. There were no available data on the effect of PA on the UPR in the stomach, so we can conclude that completely new data have been obtained on this issue, which may be beneficial in developing possible strategies for treating complications of diabetic gastropathy. Analysis of fluctuations in transmembrane sensors ATF6, PERK and IRE1 affected by the DM and various therapeutic effects proved the main fact: upon compensation of ER stress and the ERAD system, various pathways are activated that may complement, model, and regulate each other. Hence, we found that metformin treatment caused an increase in ATF6 and PERK and did not activate IRE1 regulation. On the contrary, propionate treatment activated IRE1 signaling and did not affect PERK. Combination therapy with metformin and propionate equally increased ATF6 and IRE1; PERK was increased to values similar to that in metformin use.
Therefore, the study of the UPR system in the gastric glandulocytes and the ER structural features found no increase in functional stress and no increase in ER stress in gastric cells during propionate treatment. On the contrary, we revealed an effective restoration of the ER state on the background of combination therapy with metformin and propionate, which indicates the possible prospects of this therapeutic strategy.

To analyze the effect of T2DM and various pharmacological strategies on the transcription processes, it is advisable to study the expression of the above-mentioned proteins by evaluating mRNA content under these conditions.

Conclusions

In general, T2DM-induced ER stress suggests impaired gastric homeostasis. Metformin treatment worsens the ER stress in gastric glandulocytes. However, the addition of propionate to metformin therapy did not worsen stress indicators, but, on the contrary, resulted in normalization of the state of the fundic glands.

This study allowed us to find out the safety of additional propionate therapy, which can be used for T2DM to improve the condition of various systems, primarily for neuroprotection. PA treatment was more effective than metformin therapy, and partially normalized the ratio of UPR sensors to regulators. However, the most pronounced effect was observed in the group of combination therapy (metformin and PA). Thus, combination therapy with propionic acid and metformin can be considered a promising candidate in T2DM.

To further elucidate the role of transmembrane sensors of the UPR system in transcription processes, it is necessary to study the expression of the above-mentioned proteins by evaluating mRNA content on the background of T2DM and various pharmacological strategies.

References

Відгук неструктурованих протеїнів у гландулоцитах шлунка щурів із цукровим діабетом 2 типу на тлі фармакологічного впливу

Ключові слова: метформін; пропіонат; стрес ендоплазматичного ретикулуму; гастропатія; експериментальна модель цукрового діабету

Резюме. Актуальність. Клітинні та молекулярні механізми, що лежать в основі шлунково-кишкових ускладнень при цукровому діабеті 2 типу (ЦД2), можуть включати накопичення неправильно згорнутих білків в ендоплазматичному ретикулумі, що порушує білковий гомеостаз і активує сигнальний шлях, який називається відповіддю на незгорнуті білки (ВНБ).

Мета дослідження: оцінити стан системи ВНБ у гландулоцитах шлунка щурів при цукровому діабеті 2 типу та при лікуванні метформіном і пропіонатом.

Матеріали та методи. Лікування щурів з індукованим ЦД2 проводили метформіном, пропіонатом та комбінацією цих препаратів. Рівні GRP78, ATF6, PERK, IRE1 оцінювали за допомогою вестерн-блоту та імуногістохімічного дослідження зрізів.

Результати. При ЦД2 встановлено збільшення вмісту GRP78 відносно контролю. Призначення метформіну й пропіонату призводило до зниження GRP78, при комбінованій терапії він був на рівні групи ЦД2. Виявлено збільшення концентрації ATF6 при діабеті, а використання пропіонату відновлювало його до рівня контрольної групи. Імуногістохімічне дослідження виявило тенденцію до збільшення інтенсивності імунореакції до GRP78 у щурів із ЦД2. При лікуванні метформіном спостерігали зниження умісту GRP78.

Висновки. Уведення комбінації метформіну і пропіонату призвело до зменшення вмісту GRP78, що може свідчити про позитивний ефект цих препаратів при ЦД2. Отримані дані можуть допомогти розробити ефективні стратегії лікування, що можуть бути корисними при розробці можливих стратегій лікування ускладнень гастропатій, що трапляється при цукровому діабеті.