DOI 10.1007/s11062-019-09816-6
Neurophysiology, Vol. 51, No. 4, July, 2019

Effects of a Combined Mitochondria-Targeted Treatment on the State of Mitochondria and Synaptic Membranes from the Brains of Diabetic Rats

T. M. Kuchmerovska,[1] K.O. Dyakun,[1] M. M. Guzyk,[1] L. V. Yanytska,[2] and I. B. Pryvrotska[3]

Received January 12, 2019

On samples of the mitochondria and synaptic membranes isolated from rat brains using differential centrifugation, we tried to evaluate the neuroprotective efficacy of a combination of mitochondria-specific antioxidants, acetyl-L-carnitine (ALC) and α-lipoic acid (LA), with nicotinamide (NAm), against diabetes-induced disorders in the CNS. Three groups of adult male Wistar rats were examined; these were control intact rats (group C), animals with experimental streptozotocin (STZ)-induced diabetes (group D; 6 weeks after STZ injections), and diabetic rats treated during the two final weeks of the above period by a combination of ALC, LA, and NAm (separate daily injections; doses 100, 50, and 100 mg/kg body mass, respectively; group D+T). At the day of preparation of the organelle samples, the mean blood glucose levels in groups C, D, and D+T were 4.8, 20.3, and 15.4 mM, respectively. The intensity of reactive oxygen species (ROS) production in the brain mitochondria from rats of group D measured by fluorescent analyses using 2',7'-dichlorofluorescein diacetate was, on average, 37.2% greater than that in group C. Co-treatment provided a significant decrease in the above index in group D+T (27.8% in comparison with group D). Diabetes led to dramatic intensification of the CYP2E1 protein level in the liver of group D animals (242% *vs*. group C). In group D+T, this index was 33.1% lower than that in group D.

Keywords: diabetes, oxidative stress, acetyl-L-carnitine, alpha-lipoic acid, nicotinamide, CYP2E1, mitochondria, membrane potential, synaptic membranes, ATP, NAD^+.

INTRODUCTION

Type 1 diabetes (T1D) is at present qualified as a disease related (at least, to a great extent) to the T cell-mediated autoimmune disorder resulting from destruction and dysfunction of insulin-producing pancreatic islet β cells, which is induced by a complex interplay of genetic and environmental factors [1, 2]. This disease is accompanied by a number of complications. Among the latter, neuropathies associated with functional alterations in the nervous system are of great importance [3, 4]. Diabetes-associated neurological disabilities in T1D patients are manifested as cognitive impairment, depression, and memory loss, especially in a long-lasting disease related to chronic hyperglycemia [5]. Disorders in the peripheral nervous system are most frequent complications of *diabetes mellitus*, but this disease also dramatically affects the CNS [3, 6]. Diabetic encephalopathy is associated with disorders of a complex interplay between cerebral neurons, astroglia, and vascular components. Diabetes induces significant alterations of neurotransmission in the brain [7], but molecular mechanisms underlying diabetes-induced brain dysfunctions have not been completely elucidated. There is important evidence supporting the concept that correct functioning of the mitochondria is essential for the maintenance of an adequate state of central neurotransmission, and mitochondrial dysfunction is a crucial factor determining the neuronal damage in diabetes. Accumulation of long-chain fatty acids and impaired beta-oxidation due to deficiencies of carnitine and/or its esterified derivatives, such as acetyl-L-carnitine, may exert deleterious effects on the function of nerve cells in this disease;

[1] Palladin Institute of Biochemistry, NAS of Ukraine, Kyiv, Ukraine.
[2] Bogomolets National Medical University, Ministry of Public Health of Ukraine, Kyiv, Ukraine.
[3] Gorbachevsky Ternopil' State Medical University, Ministry of Public Health of Ukraine, Ternopil', Ukraine.
Correspondence should be addressed to T. M. Kuchmerovska (e-mail: tkuchmerovska@gmail.com)

this results in alterations in the plasma, loss of mitochondrial membrane integrity, and perturbations of intracellular metabolism and energy production.

It is known that some endogenous nutrients can considerably influence metabolism of other nutrients and of some non-nutrient substances. The importance that may be attributed to these interrelationships depends on the ability of such agents to maintain acceptable levels of all above-mentioned substances in different tissues for adequate functioning of the organism. The interaction of these substances may occur in different metabolic pathways.

There is strong evidence that a metabolic imbalance resulting from insulin deficiency and, especially, from the effects of this factor on CNS neurons might be a common denominator of metabolic disorders and cognitive and somatosensory dysfunctions [8]. The underlying pathogenic mechanisms are multiple; those include, in particular, abnormal signaling by advanced glycation end-products on neuronal and glial receptors, altered levels of neurotrophic factors, impairments of glucose utilization, activation of poly(ADP-ribose) polymerase in neurons, etc. [9, 10]. Cerebral dysfunction induced by diabetes can result from neuronal alterations including abnormal signaling provided by sodium and calcium channels, changes in electrochemical gradients on the plasma membranes, failures in the central neurotransmitter systems, and astrogliosis [11].

Impairments of the serotoninergic neuromediator system are significantly implicated in the pathophysiology of cognitive dysfunctions; depression and painful somatic symptoms also commonly occur in diabetes [12]. It should be recognized that the relative contribution of various biochemical and metabolic alterations of the synaptic functions associated with hyperglycemia has not been fully defined. In fact, the pathogenesis of brain dysfunctions induced by diabetes is rather complex; it is still highly important to prevent the irreversible development of diabetic neuropathy.

Oxidative and nitrosative stress induced by diabetes leads to dysfunctions of the mitochondria, which dramatically affect the cell functions and result in the loss of ATP-generating capacity and development of cognitive impairments [13]. It is believed that diabetes also can induce oxidative stress dependent on cytochrome P450 family enzymes (CYPs). Among the latter, the effect of CYP2E1 (EC 1.14.13.n7) seems to be one of the main causes of hepatotoxicity, because its expression in the liver is the highest [14]. In the brain, the CYP2E1 level is much lower than that in the liver, but this index was found to be noticeably shifted in the mitochondria of different brain regions (cortex, hippocampus, and pons) [15]. This is why the improvement of mitochondrial functions in the brain is important for more effective treatment of diabetes. Among the nutrients chosen for this purpose, there is a mitochondrial cofactor and antioxidant, alpha-lipoic acid (LA), a compound that increases the amount of endogenous antioxidants and improves mitochondrial bioenergetics [16], and acetyl-L-carnitine (ALC), a mitochondrial metabolite that improves the process of mitochondrial fatty acid beta-oxidation in tissues and helps to increase ATP production [17]. Furthermore, ALC is absorbed better and crosses the blood-brain barrier (BBB) more successfully than carnitine; it also provides acetyl equivalents for the production of acetylcholine [18]. The third nutrient is nicotinamide (NAm), an intermediate product of metabolism of pyridine nucleotides, which plays a variety of significant regulatory roles in the fundamental cellular processes [19, 20]. It could be expected that these compounds used in combination may more effectively reverse negative brain alterations induced by diabetes. Earlier, we revealed that NAm and its catabolic product 1-methylnicotinamide (MNA) could exert a strong neuroprotective action and could be efficacious with respect to brain abnormalities associated with T1D [21].

Our present study was performed to evaluate the neuroprotective efficacy of combined supplementation by mitochondria-targeted compounds, acetyl-L-carnitine, alpha-lipoic acid, and nicotinamide (ALC, LA, and NAm), as a multiple-action mean affecting several functional, biochemical, and metabolic aspects of diabetes-related brain dysfunction. We estimated the respective effects in samples of the mitochondria and synaptic membranes obtained from the brains of different (control and diabetic) experimental rats.

METHODS

Experimental Design. All experiments were performed on adult male Wistar rats (body mass 180–250 g) fed a standard diet and having free access to water and chow *ad libitum*. After one week of acclimation, diabetes was induced by a single i.p. injection of freshly prepared solution of

streptozotocin (STZ, Sigma, USA) in citrate buffer (0.1 M, pH 4.5) at dose 60 mg/kg body mass after an overnight fasting. The animals were maintained at room temperature (20 ± 5°C) and a 12/12-h light/dark cycle; they were randomly divided into the following groups: control (C), diabetic (D, with the 6-week duration of experimental diabetes), and the diabetic group treated by separate i.p. injections of ALC, LA, and NAm at doses 100, 50, and 100 mg/kg body mass, respectively, daily, for 2 weeks starting 4 weeks after induction of diabetes (group D+T). The blood glucose level was measured by the Precision Xtra *Plus* Ketone meter (MediSense UK Ltd., Great Britain). Diabetic rats with mean blood glucose level 20.3 ± 1.7 mM were taken into experiments. After 6 weeks, experimental rats were fasted overnight with free access to water and were sacrificed in the morning via cervical dislocation under mild diethyl ether narcosis.

Samples of the Mitochondria and Synaptic Membranes. The brains of experimental rats were rapidly removed, weighed, and kept on ice. For obtaining brain homogenates, we used 0.32 M sucrose and 5 mM Tris-HCl buffer containing 0.2 μg/ml PMSF, pH 7.4. Briefly, brain homogenates were centrifuged at 1000 g for 10 min to eliminate the nuclei and cell debris; the resultant supernatant was centrifuged at 15,000 g for 20 min to obtain the mitochondria fraction. A part of the latter was suspended in Tris-HCl buffer after purification; another part of this fraction was used for isolation of the synaptic membranes from purified synaptosomes. The latter were subjected to osmotic shock by cold water at 4°C with continuous shaking, as described by Abita et al. [22] with a slight modification. Synaptic membranes after 30-min-long centrifugation at 105,000 g in a step-wise discontinuous sucrose gradient (0.8, 1.0, and 1.2 M layers) were separated between the 0.8 and 1.0 M above layers. Then this membrane fraction was collected and diluted with three volumes of 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at 105,000 g for 30 min. A final precipitate of the synaptic membranes was gently re-suspended in ice-cold homogenization medium. The obtained samples of purified mitochondria and synaptic membranes were used for experimental investigations. All described procedures were carried out at 0–4°C within 3 h.

Estimation of Oxidative Stress in the Mitochondria. Oxidative stress in the brain mitochondria was estimated according to the production of reactive oxygen species (ROSs), which were assessed using 2',7'-dichlorofluorescein diacetate (Sigma–Aldrich, USA) at a final concentration of 25 mM. This reagent is, after oxidation, transformed into fluorescent 2',7'-dichlorofluorescein (according to [23] with some our modifications). The fluorescence intensity of 2',7'-dichlorofluorescein, being directly proportional to the content of ROSs in the investigated samples, was recorded on an FL1 channel (515–535 nm) using the Coulter Epics XL flow cytometer (Beckman Coulter, USA).

Expression of CYP2E1 Protein by Western Blot Analysis. Liver samples (100 mg) were immediately frozen in liquid nitrogen; proteins were extracted by RIPA buffer (20 mM Tris-HCl, pH 7.6, 1% Triton-X100, 150 mM NaCl, 50 mM NaF, 0.2% SDS) with a mixture of protease inhibitors (Thermo Fisher Scientific, USA) added and stirred at 4°C for 5 min. The immunoblot analysis and quantification were performed as described previously [34]. For Western blot analysis, equal amounts of target proteins (80 μg) were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the nitrocellulose membrane (GE Healthcare, Great Britain). After staining, nonspecific binding sites were blocked in 5% skim milk powder (Apex Research, USA) in 0.1% Tween 20 in phosphate-buffered saline (PBST) for 1 h at room temperature. The membranes were washed three times in PBST and incubated with anti-CYP2E1 (1:500 to 1:1000 in 5% skim milk in PBST, Sigma–Aldrich, USA), and anti-β-actin antibodies (1:3000 in 5% skim milk in PBST, Sigma–Aldrich, USA) overnight at 4°C. After three washes in PBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Unspecific binding of the secondary antibody was controlled by excluding primary antibodies. Immunoreactive bands were visualized by measuring the chemiluminescence intensity. Densitometric analysis was performed using TotalLab TL120 (Nonlinear Inc., USA) software. The protein contents were expressed in arbitrary units (a.u.). All experiments were performed three times, and relative protein expressions were compared to β-actin expression set as the control.

Measurement of the Membrane Potential of Brain Mitochondria. Rhodamine 123 (R123) is widely used as a marker for the mitochondria in living cells; uptake of this dye is strictly dependent on the plasma potential and mitochondrial membrane potential [24]. We used this fluorescent dye for the measurement of the membrane potential in the rat

brain mitochondria. Briefly, freshly purified samples of these mitochondria (0.25 mg protein/ml) were placed in an incubation medium containing (in mM): NaCl, 132; KCl, 5.0; $MgCl_2$, 1.3; $CaCl_2$, 1.8; glucose, 10; NaH_2PO_4, 1.2; Tris-HCl buffer, 5.0 (pH 7.4). After a 5-min-long preincubation of the samples at 37°C, the assays were then incubated for 15 min in the presence of the fluorescent dye rhodamine-123 (final concentration 10 μM). After incubation was completed, the extracellular dye was removed by sedimentation (three times in ice-cold incubation medium). To measure the membrane potential, 200 μl of the mitochondria loaded with the dye was added to a cuvette with the incubation medium. The fluorescence intensity was measured on the Coulter Epics XL flow cytometer (Beckman Coulter, USA) equipped with an argon laser (λ_{excit} = 488 nm). The fluorescence intensity of the samples was recorded by the FL1 channel (515–535 nm).

Na^+,K^+-ATPase in Synaptic Membranes. The Na^+,K^+-ATPase (E.C. 3.6.1.3.) activity in the brain synaptic membranes was determined using a modification of the spectrophotometric method [25], which we used previously [21]. This method is based on measurement of the amount of inorganic phosphate (P_i) released from ATP with or without the Na^+,K^+-ATPase inhibitor ouabain (2.0 mM) during incubation of the synaptic membranes. The enzyme activity was expressed in μmol P_i released after ATP cleavage during 1.0 min per 1 mg of synaptic membrane proteins.

Measurement of Other Metabolites in the Rat Brain. The contents of ATP, NAD^+, and sorbitol were determined in protein- and ion-free perchloric acid extracts of the whole brain tissue by enzymatic assays [26]. The protein content was measured according to Bradford [28] and by the Lowry method [27] using bovine serum albumin as a standard.

Numerical data are expressed below as means ± ± s.d. Intergroup differences of the means were tested using the Mann–Whitney *U* test and considered to be statistically significant at $P < 0.05$.

RESULTS

Mortality. Fifty-six animals were used in the experiments. During one week after STZ injection, eight animals died spontaneously and were, therefore, ruled out from the examined groups. So, the final number of rats in groups C, D, and D+T was 14, 17 and 17, respectively.

Blood Glucose. After 6 weeks of the development of diabetes, the mean body mass of STZ-diabetic rats (group D) were 19.4% lower than that in group C. The mean blood glucose level in diabetic animals was much higher (20.3 mM), as compared to that in control rats (4.8 mM, $P < 0.05$; Fig. 1). Hyperglycemia began to be observed one week after STZ-provided induction of diabetes and was continued throughout the studied period. Following administration of the above-mentioned compounds to diabetic rats, the blood glucose content in group D+T (15.4 mM, on average) was moderately lower ($P < 0.05$) than in group D.

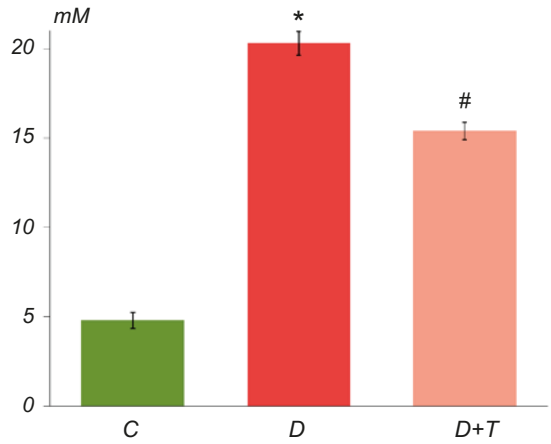
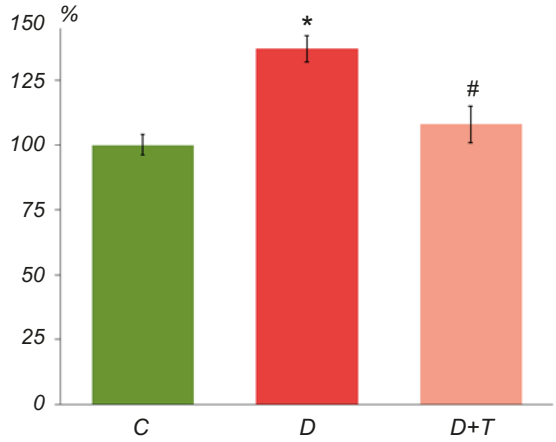


Fig. 1. Levels of glucose in the blood, mM, in rats of the control group (C), animals with experimental type 1 diabetes (D), and diabetic rats co-treated with acetyl-L-carnitine, alpha-lipoic acid, and nicotinamide (D+T). Means ± s.d. (n = 5) are shown. *$P < 0.05$ in comparison with the control group; # $P < 0.05$ in comparison with the diabetic group.

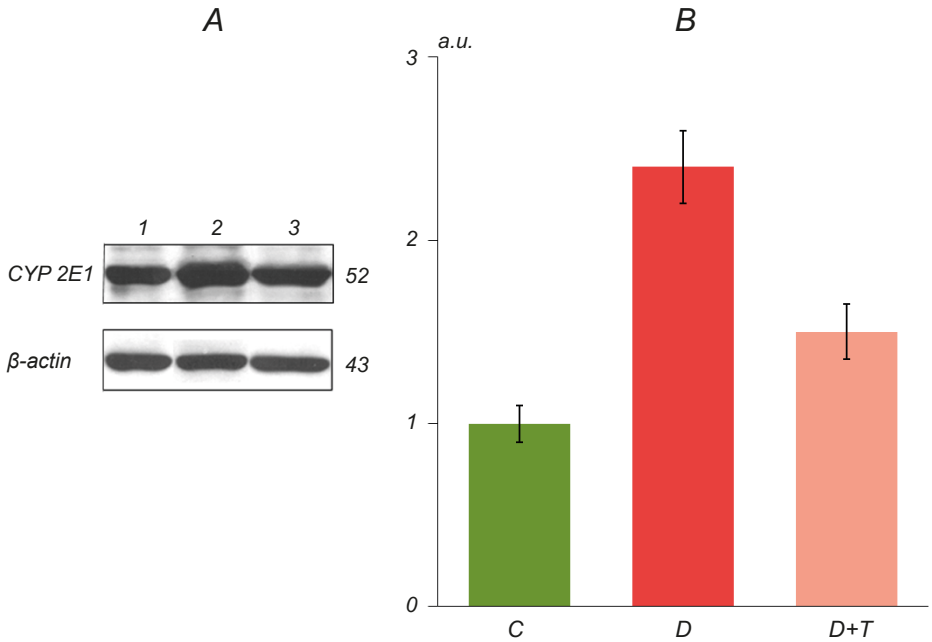
ROS Production in the Brain Mitochondria. It is known that hyperglycemia can induce oxidative cell damage in different organs through increasing the ROS production and alterations of the antioxidant defense system [13]. This, in turn, can result in the development of ROS-induced inflammatory processes in the brain as one of the most sensitive tissues in this respect. Previously, we showed that T1D in rats leads to strong oxidative stress in the brain and to activation of poly-ADP-ribosylation of nuclear proteins (a response to DNA damage) [29]. In the present study, we assessed whether the tested combined therapy is effective against the development of diabetes-related oxidative stress in the CNS. As is shown in Fig. 2, hyperglycemia-induced oxidative stress in animals of group D led to considerably more intense (on average, by 37.2%) ROS generation in the brain mitochondria compa-

red to the respective index in the control ($P < 0.05$). This fact is indicative of a strong imbalance between pro- and antioxidant processes in the diabetic brain. The tested co-treatment led to a significant decrease in the ROS production in the brain mitochondria of rats of group D+T (by 27.8% *vs*. group D; $P < 0.05$). These results again indicate that diabetes can lead to dramatic alterations in the brain neuronal terminals, thereby confirming the crucial role of such changes in the brain functioning.



F i g 2. ROS production in the brain mitochondria, %, according to the intensity of DCF fluorescence (control values are taken as 100%). Designations are similar to those in Fig. 1.

Assessment of the CYP2E1 Protein Level. Cytochrome P4502E1 is mainly responsible for the metabolism of many endogenous and exogenous compounds in the liver. However, the assessment of brain CYP2E1 functions *in vivo* is challenging due to the presence of significant metabolism of this enzyme in the liver. Because of this, different metabolites (including toxic ones capable of penetrating into the brain) are formed. This is why it was necessary to determine the CYP2E1 expression in the liver to elucidate whether the co-treatment used can influence the respective index. According to the data of Western blot analysis, the level of CYP2E1 protein in the liver of diabetic animals was much higher (242%; $P < 0.05$) as compared to that in control rats (Fig. 3). The elevated level of this enzyme in the liver may be indicative of cellular leakage and loss of functional integrity of the cell membranes, which accompanied intensification of pathological processes in the body of diabetic animals. Moreover, significantly increased expression of cytochrome P450 2E1 in diabetes may be associated with elevated oxidative stress and additional production of ROSs, because CYP 2E1 metabolizes such endogenous compounds, as fatty acids, lipid hydroperoxides, and ketone bodies, into aldehydes; many xenobiotics are metabolized



F i g. 3. Results of Western blot analysis: visualization of the immunoreactivity of a specific marker, CYP2E1 protein, in the liver tissues of rats of different experimental groups. Equal loading was confirmed by reprobing for β-actin. A) Representative Western blot of CYP2E1; B) results of densitometric analysis of immunoblots showing the CYP2E1 contents; vertical scale, arbitrary units (a.u.). Other designations are similar to those in Figs. 1 and 2.

into nucleophilic reactive species [30]. The tested co-treatment led to the noticeable reduction of CYP2E1 expression in the liver of rats of group D+T (by 33.1%, on average, *vs.* group D; $P < 0.05$). This can be one of the reasons providing protection against CYP 2E1-related oxidative stress in different body tissues.

Assessment of the Membrane Potential of the Brain Mitochondria. In the mitochondria isolated from the brain of diabetic rats, the mean level of Rh123 fluorescence under self-quenching conditions was by 32.4% more intense than that in the control. This significant ($P < 0.05$) shift resulted from reduction of the mitochondrial membrane potential compared to that in the non-diabetic group C, which may be indicative of noticeable depolarization of the membranes of these organelles. In the mitochondria from the brain of group D+T rats, the Rh 123 fluorescence was nearly similar to that in group C (Fig. 4).

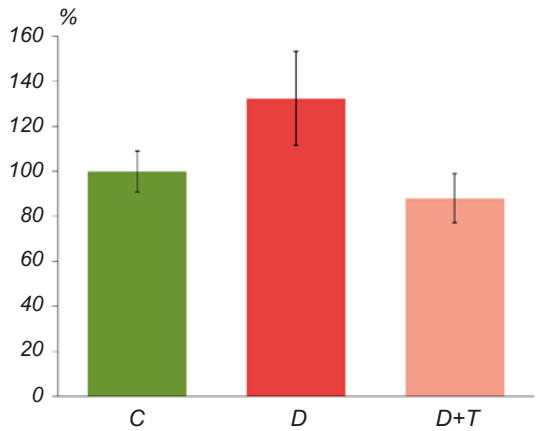


Fig. 4. Effects of diabetes and combined treatment (ALC+ LA + + NAm) on rhodamine 123 (R123) accumulation, % (control values are taken as 100%), which negatively correlates with the membrane potential of the brain mitochondria. Designations are similar to those in Fig. 1–3.

Na^+,K^+-ATPase Activity in the Synaptic Membranes. We tried to answer the question: Is there a relationship between the mitochondrial membrane potential and impaired ion transportation via the synaptic membranes? For this purpose, we measured the activity of Na^+,K^+-ATPase in samples of the rat brain synaptic membranes. Maintenance of the Na^+/K^+ equilibrium on neuronal membranes by Na^+,K^+-ATPase of the plasma membrane-localized sodium pump is crucial for brain functioning [31]. An adequate Na^+,K^+-ATPase activity, maintenance of normal ion gradients across the cell membrane, and, thus, maintenance of the membrane potential and osmotic equilibrium in the cell are crucially necessary for proper cellular functioning. However, the impairment of the Na^+,K^+-ATPase functioning observed under pathological conditions leads to depolarization of neuronal terminals accompanied by excessive Ca^{2+} entry into the cell. This can lead to significant cell dysfunction as a result of abnormal neurotransmitter release and swelling of the neurons. That is why it was important to estimate the activity of the above enzyme in the synaptic membranes; the respective measurements could help us to understand its role in the regulation of the membrane potential on these membranes.

The effect of diabetes on the activity of Na^+,K^+-ATPase is illustrated in Fig. 5. It was found that Na^+,K^+-ATPase activity in the synaptic membranes of diabetic (group D) animals was significantly lower (66%, on average, $P < 0.05$) as compared to that in the respective membranes of control rats. This fact may reflect a lowered efficacy of the Na^+/K^+ pump. This means that Na^+,K^+-ATPase in the case of experimental diabetes cannot sufficiently maintain an adequate ion balance on the neuronal membranes. Moreover, accumulation of Ca^{2+} in synaptic endings induces considerable disorders in the secretion of neurotransmitters. This is why abnormal secretion of neurotransmitters may be predetermined by inhibition of Na^+,K^+-ATPase of presynaptic membranes under conditions of depolarization and

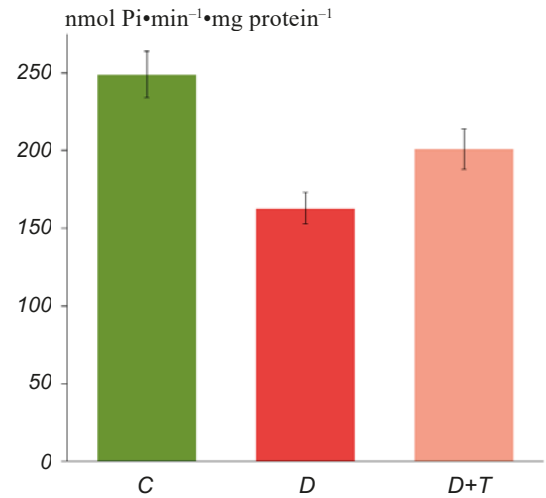


Fig. 5. Activity of Na^+, K^+-ATPase in the synaptic membranes isolated from the brains of rats of different experimental groups. The enzyme activity was determined by measuring the release of inorganic phosphate associated with the hydrolysis of ATP. The control value (subtracted) was determined in the presence of 2 mM ouabain. The data represent results from five independent experiments (each performed in quadruplicate) and are expressed in nmol Pi• min^{-1}•mg $protein^{-1}$ as means ± s.e.m. Other designations are similar to those in Figs. 1–4.

respective alterations of the passive and active transport of cations. So, it is important to emphasize that inhibition of Na^+,K^+-ATPase is one of the crucial reasons for the increased level of entry of Ca^{2+} ions into the nerve terminals upon their excitation. As was found, ouabain stimulates the transport of not only Na^+, but also of Ca^{2+} in the cells [32]. After the combined treatment by ALC, LA, and NAm, the activity of the above-mentioned enzyme in group D+T was 23.3% higher, on average, than the respective index in the synaptic membrane samples of diabetic animals of group D ($P < 0.05$).

Indices of Metabolic Alterations. There is accumulating evidence that dysregulation of intracellular sodium is an inherent consequence of a reduction in the cellular ATP amount. This triggers a secondary failure of extra- and intracellular homeostasis of other ions, in particular potassium, calcium, and protons; naturally, these shifts promote the development of excitotoxicity. Our data completely support this statement, since it was observed that the ATP content in the diabetic brain (group D) was 28.5% smaller, on average, than that in the control ($P < 0.05$; Table 1). Such alterations in the content of ATP induced by diabetes may be indicative of the impairment of functioning of the electron transport chain and coupling of the electron transfer process with ATP synthesis (oxidative phosphorylation of ADP). Moreover, for ATP biosynthesis, as well as for ATP-dependent processes (including Na^+,K^+-ATPase activity and generation of the membrane potential), it is important to maintain the proper level of nicotinamide adenine dinucleotides; this level should be considered a significant regulatory factor. This is why it was important to assess the effects of diabetes and co-treatment by the investigated compounds on the NAD^+ level in the rat brain. Experimental diabetes led to a 30.9% decrease in the NAD content in the rat brain, as compared to the control ($P < 0.05$; Table 1). Co-administration of ALC, LA, and NAm to diabetic rats (group D+T) provided nearly complete normalization of the NAD^+ level in the brain and partially restored the level of ATP. Under diabetic conditions, the glucose flux through the polyol pathway is considerably enhanced due to glucose overconsumption; this may be implicated as one of the important pathogenic mechanisms underlying the brain failures observed [3]. Indeed, as is shown in Table 1, diabetes was associated with a nearly twofold elevation (by 90%) of the sorbitol level in the brain compared to the control value ($P < 0.05$). Diabetic rats co-treated with the compounds used demonstrated practically full normalization of the brain sorbitol content.

Thus, our findings showed that there are reciprocal interrelations between hyperglycemia and CYP2E1-modulated ROS production and between oxidative stress-modulated hyperglycemia and brain dysfunctions associated with this disorder.

T a b l e 1. Diabetes-Induced Alterations of the Contents (mmol/g Tissue) of ATP, NAD^+, and Sorbitol in the Brain of Experimental Rats

Groups	Compounds		
	ATP	NAD^+	Sorbitol
Control (C)	2.14 ± 0.12	0.249 ± 0.016	0.076 ± 0.006
Diabetes (D)	1.53 ± 0.08*	0.172 ± 0.012*	0.144 ± 0.011*
Diabetes+combined treatment (D+T)	1.74 ± 0.07#	0.238 ± 0.014#	0.070 ± 0.005#

Footnotes: rats of group C were intact; in rats of group D, experimental diabetes was induced by streptozotocin injections; diabetic rats of group D+T were subjected to combined treatment by injections of acetyl-L-carmide (ALC), α-lipoic acid (LA), and nicotinamide (NAm). For more details, see Methods. *$P < 0.05$ in comparison with the C group, #$P < 0.05$ in comparison with the D group.

DISCUSSION

In our studies, both described earlier and the present one, we obtained convincing evidence that experimental diabetes induces strong dysfunctions of the CNS. The respective shifts include a spectrum of functional and biochemical alterations. We also found that the neuroprotective action of combined supplementation by ALC, LA, and NAm effectively attenuates deleterious effects of diabetes in the brain. One of the rationales for our study was to determine whether such co-treatment could improve metabolic indices and limit negative diabetes-induced shifts related to oxidative stress. It was found that diabetes-associated hyperglycemia leads to a significant increase in ROS production (Fig. 2). This can be a result of disorders in the mitochondrial electron transport chain. The mitochondria are the

major source of excessive ROS generation, which finally contributes significantly to the development of diabetic neuropathy.

Multiple forms of cytochrome P450 (including CYP2E1) are present in the brain mitochondria [15]. Considering this, we can suppose that oxidative stress in the cerebral mitochondria under diabetes conditions (Fig. 3) is also associated with abnormal CYP2E1 activity in the brain. This index is obviously raised due to an increased permeability of the BBB for the products of intensified lipid peroxidation in the liver and also to intensified cell apoptosis and mitochondrial dysfunction. Such interpretation is strongly confirmed by a dramatic (more than twofold) elevation of the CYP2E1 protein expression induced in the liver by diabetes. So, highly elevated oxidative stress in the brain mitochondria is not only a consequence of diabetes itself; it is also a result of increased CYP2E1 expression in the liver and, indirectly, of the same process in the brain. It should be noted that increased CYP2E1 expression and the resultant exacerbated oxidative stress can lead to intensified apoptotic cell death not only in the liver, but also in other tissues, including those of the brain. It is logical to suggest that the enhanced effects of CYP2E1 enzyme, in addition to those of oxidative stress *per se*, are one of the key causes of diabetes-induced brain intoxication. It cannot be ruled out that co-treatment with the agents we have proposed can attenuate CYP2E1-mediated apoptosis-determined cell death as a result of considerable limitation of the CYP2E1 expression in the liver and the respective indirect suppression of neurotoxicity in the brain. Combined treatment by these compounds may probably be used as an effective therapeutic approach for the prevention of development of brain dysfunctions.

Earlier, we have revealed that an amide form of vitamin B_3, nicotinamide (NAm), and its biologically active derivatives are capable of exerting a pronounced neuroprotective action and ameliorating diabetes-induced neuropathy and retinopathy [21, 29, 33, 34]. It should not be ruled out that the NAm action is realized, at least partly, at the level of regulation of the processes of synaptic transmission and also through interaction of NAm with other biologically active compounds. A wide range of NAm effects, exerted both independently and via the actions of its biologically active derivatives, provides strong modulation and regulation of metabolism and considerably affects cell viability. Moreover, we have recently shown that diabetes also strongly influences the exocytotic machinery responsible for neurotransmitter release [7]. To confirm the statement that brain dysfunctions induced by diabetes significantly depend on the functional state of the mitochondria, we measured the mitochondrial membrane potential using rhodamine123. This allowed us to detect its rapid changes associated with fast changes in the synthesis of superoxide anions in the mitochondria, the phenomenon leading to additional intensification of oxidative stress in the brain. Rhodamine-123 is accumulated in the mitochondria with the subsequent formation of aggregates whose emission we have measured [35]. Our results obtained with the use of this fluorescent dye demonstrated that the membrane potential in the brain mitochondria is considerably reduced in diabetic rats (Fig. 4). Taken together, these findings provide strong evidence that the development of oxidative stress results, to a significant extent, in damaging the mitochondria (see [36]).

The data presented in Fig. 4 allow us to conclude that combined treatment with ALC, LA, and NAm partly but significantly normalizes the mitochondrial membrane potential in the brain. Such normalization of the above potential appears to be caused, to a noticeable extent, by the replenishment of carnitine and betaine, which intensifies shuttling of fatty acids into the mitochondria for beta-oxidation. We, in particular, included LA as a co-supplement because it is a naturally occurring cofactor for mitochondrial alpha-keto acid dioxygenases, which may participate in the cellular glucose-dependent ATP production. Moreover, this agent easily penetrates into a variety of tissues where it can be reduced to a more potent antioxidant, dihydrolipoic acid. The latter increases the intracellular ascorbate and glutathione concentrations [37]. Although dihydrolipoic acid is quickly removed from most cells, it also induces cystine-cysteine uptake and can, thereby, intensify glutathione synthesis. As is is known, NAm alone plays a significant role in physiologically important functions, but it could be expected that its combination with other biologically active compounds can provide stronger therapeutic effects in some widespread neurodegenerative disorders [29, 33, 34, 38]. Indeed, we revealed that treatment using a combination of NAm with such biologically active compounds, as ALC and LA, seems to be rather promising for the protection of the nervous system and for effective recovery without undesirable side effects.

It is generally known that normal glucose metabolism, one of the crucial determinants of the tissue ATP level, is also absolutely essential for brain function. In excitable tissues, Na^+,K^+-ATPase is involved in the maintenance of sodium and potassium gradients across the plasma membranes at the expense of ATP hydrolysis, which provides the maintenance and regulation of the transporter functions [39]. It is important that there is positive correlation between the ATP availability and the efficacy of the Na^+ pump, which has been detected in different tissues. Indeed, we elucidated that Na^+,K^+-ATPase of the synaptic membranes may be considerably involved in improving the brain ATP level; this fact confirms the positive effects on the energy utilization provided by combined treatment of diabetic rats with ALC, LA, and NAm (Fig. 5).

It cannot be ruled out that changes in Na^+,K^+-ATPase activity might be, in part, due to accumulation of sorbitol in the brain tissue. Such accumulation results from poor back penetration of this compound through the cell membranes and its slow metabolism. This leads to subsequent osmotic swelling of nerve cells and, consequently, augmentation of their dysfunctions. The disturbance of ion homeostasis, resulting not only from decreased Na^+,K^+-ATPase activity but also from hyperosmolarity associated with sorbitol accumulation, might cause excessive cell hydration and enhanced Na^+ inflow combined with concomitant K^+ loss. Moreover, it has been found that the electrogenic role of Na^+,K^+-ATPase in the synaptic membranes might contribute to the impact of membrane depolarization in diabetes and inadequate functioning of the sodium pump in synaptic endings. A marked decrease in the membrane potential and probable alterations of the electrogenic properties of Na^+,K^+-ATPase induced by diabetes might be expected to significantly disturb functions of the presynaptic structures and synaptic signaling. This assumption agrees with the fact that monoamine transporters belong to the Na^+/Cl^--dependent family [40].

Moreover, we tried to evaluate the relations between diabetes-associated brain dysfunctions and alterations in the intracellular NAD^+ and ATP levels. Drops in the NAD^+ and ATP contents in the brains of diabetic rats (Table 1) should lead to inhibition of glyceraldehyde-3-phosphate dehydrogenase, which is involved in ATP resynthesis during glycolysis [41]. Earlier, we found that the plasma and liver tryptophan concentrations were not significantly affected by diabetes. The NAD^+ synthesis *de novo* is also unlikely to be affected by the increased levels of branched-chain amino acids, all of which produce excessive amounts of NAD^+ in its reduced form in the cytosol and mitochondria, but can not increase the NAD^+ contents [42]. Chronic combined treatment with ALC, LA, and NAm exerts, as was observed, a profound normalizing effect on the NAD^+ and ATP contents in the brains of diabetic rats (Table 1). This fact is in line with our data published recently; we found that NAm counteracts multiple manifestations of diabetic encephalopathy and retinopathy in diabetic rats, mostly via pleiotropic mechanisms [33, 34]. Moreover, NAm through its complex effects (especially as a PARP-1 inhibitor) can suppress the ROS production, Ca^{2+} influx, apoptosis, and cell injury in neuronal populations [43]. It cannot be ruled out that the corrective effect of NAm on cerebral nerve cells under co-treatment conditions can be realized either by a direct effect of this compound on p53 expression (significant inhibition of the latter) or by preventing NAD^+-dependent deacetylation of p53 induced by sir2α and/or sir2 [44, 45]. Our previous findings and published data showed that NAm, being a multifunctional agent, also possesses the ability to regulate inflammatory processes in the cells (by providing a decrease in the level of proinflammatory cytokines, such as interleukin 1β, interleukin-6, interleukin-8, and tissue necrosis factor TNF-α), and this occurs in the blood, liver, and pancreas of rats [34, 46]. We do not reject the possibility that the antidiabetic efficacy of NAm, one of the components in the tested complex treatment, may also be associated with the formation of 1-methylnicotinamide, an endogenous methylated NAm derivative. This derivative considerably reduces the deleterious effects of diabetes on brain functions [19]. It should also be noted that the NAm antioxidant ability also should not be underestimated [47]. It is generally known that both acetyl-L-carnitine (ALC), a well-known cofactor in the process of mitochondrial fatty acid β-oxidation, and alpha-lipoic acid (LA), i.e., other components of the combined treatment, are effective mitochondria-specific antioxidants [48, 49]. ALC, due to its antioxidant properties is also involved in reparation of the membranes and their stabilization [48]. Under conditions of reduced activity of Na^+,K^+-ATPase in the synaptic membranes (the effect of diabetes) and a decreased content of ATP in the brain, we observed an about twofold increase (90%, on average) in the content of sorbitol (Table 1). This inevitably should lead to changes in the osmolarity and transport of

monovalent cations both inside and outside the cells. Taking into account such mechanisms of the ALC action, the increase in Na^+,K^+-ATPase activity in the synaptic membranes may occur because of partial restoration of the potential on the plasma membranes, the phenomenon clearly observed after co-treatment. Moreover, ALC, as a molecule produced by acetylation of carnitine in the mitochondria, can also be considerably involved in energy production in the case of repletion of the intracellular NAD^+ level and inhibition of the activated polyol pathway in diabetic rats. Significant normalization of the brain NAD^+ level after combined administration of the tested compounds is most probably related to increased pyruvate flux in the Krebs cycle rather than to inhibition of the glycolytic pathway. Taking into account all the above, we believe that the mechanism of action of the investigated compounds may be based, to a significant extent, on a combination of depolarization of the synaptic membranes with parallel activation of Na currents. It is also possible that the effects of these compounds, especially the effect of NAm on the Na^+, K^+-pump mediated by NAD^+, are related to enzymatic modification by a certain protein kinase. This is a result of deployment of a signaling cascade in which, as can be assumed, there is a functional connection between NAD^+ binding with the synaptic membranes [50] and activation of the signal transduction system.

Thus, the positive effects of chronic co-administration of the tested compounds are related to their ability to modify mostly those membrane-associated processes that are strictly dependent on the level of energy production in the cell**.** The positive action of these compounds may be also related to their involvement in the intracellular redox-sensitive and signaling pathways. Acceptance of this statement is based on the diabetes-induced decrease in the sorbitol level in the brain after the proposed combined treatment, an effect indicative of the possibility of the direct influence of the mentioned agents on aldose reductase (AR, EC 1.1.1.21), a key enzyme of the polyol pathway. Although a number of this pathway inhibitors have been synthesized at present (some of them have even begun to be used therapeutically), the effects of none of them appear to be completely satisfactory. We suppose that the investigated compounds, being "natural" molecules, possess some structural features making them capable of activating the respective center of aldose reductase because these molecules have a polar head group and a hydrophobic ring system, like other AR inhibitors [33, 34]. At the same time, another hypothesis can be proposed. It recognizes the pathogenic importance of oxidative stress and its strong linkage to the polyol pathway and emphasizes a greater importance of cofactor turnover rather than sorbitol accumulation [51]. Moreover, under conditions of diabetic neuropathy, activation of the polyol pathway of glucose metabolism accompanied by the increased consumption of NADPH in the AR-mediated reaction, suppresses, in turn, NADPH-dependent recycling of reduced glutathione. This inevitably leads to intensification of free radical and peroxidation processes in the nervous system [52].

In this study, we have demonstrated that a delineation and/or a similar-direction mode of action of investigated compounds would be of interest for a better understanding of the mechanisms by which diabetes-induced negative alterations in the brain may be compensated by the proposed combined treatment. However, there are a few aspects that should be emphasized within the existing context, although without any possible direct conclusions in terms of causality or direction of the interrelations. One of the probable mechanisms of neuroprotection by NAm (as a precursor of pyridine nucleotide biosynthesis) is inhibition of PARP-1 activity through NAD^+; this activity serves as a basis for the formation of ADP-ribosylated proteins, thus participating in the regulation of various cellular processes [33, 34]. This statement is consistent with our previous data related to diabetes-induced DNA damage followed by overconsumption of NAD^+ via stimulation of poly ADP-ribosylation [29]. It cannot be ruled that NAm and a few its derivatives can, under the respective pathological conditions, inhibit transfer of ADP-ribose from NAD^+ to proteins in different tissues and organs.

Among the components for combined treatment of diabetic animals, we used ALC. It is known that L-carnitine plays a significant role in the functioning of the mitochondria and as a shuttle for acetyl groups from inside to outside the mitochondrial membrane it also plays a key role in glucose metabolism. One of our possible explanations for this neuroprotective aspect of the combined therapy is the following: ALC participates in the transport of fatty acids (as acylcarnitine esters) across the inner mitochondrial membrane to the matrix, where they are oxidized, provide the production of energy, and maintain energy homeostasis [53]. In addition to the participation of ALC in the β-oxidation of

long-chain fatty acids, this compound also promotes cell detoxification, stimulates biosynthesis of acetylcholine, and promotes metabolic reactions involving coenzyme A and glucose [54]. Thus, the positive effect of ALC used in combined therapy may also be realized through its ability to affect cholinergic transmission in the brain, to ameliorate cognitive dysfunction, and to improve memory [55]. There are certain data that accumulation of long-chain fatty acids, as well as impairments of the process of β-oxidation due to a deficiency of ALC and/or its esterified derivatives and lipoic acid, may aggravate the dysfunction of nerve cells in diabetes, including negative alterations in the integrity of the cytoplasmic and mitochondrial membranes, in intracellular metabolism, and in energy production [56]. Lipoic acid (LA), a mitochondria-directed antioxidant, easily crosses the BBB and can be accumulated not only in the brain but also in other tissues; it can be converted to dihydrolipoic acid (DHLA) by lipoamide dehydrogenase. It was also demonstrated that LA and DHLA, after rapid gastrointestinal transport of LA to the blood plasma, are characterized by a similarly rapid clearance, due to both intense uptake by tissues (liver, brain, heart and skeletal muscles) and effective renal excretion [57].

Despite the differences between the metabolic effects of ALC, LA, and NAm, combined therapy with these compounds is noticeably more effective than with separate using of each of those. The positive action of these compounds on brain functions could be ascribed, at least partly, to their effects providing glucose level lowering and modifications of some extrabrain metabolic processes and those of cellular signaling. However, it seems unlikely that a slight improvement of hyperglycemia after the applied treatment could be a crucial effect sufficient to strongly affect diabetes-induced negative cerebral alterations and to shift the observed indices to the extent observed in our experiments. It should be emphasized that co-treatment with these compounds may protect the CNS both directly, by acting at the level of synaptic connections, and indirectly, by modifying brain metabolism impaired by diabetes.

The efficacy of combined treatment of metabolic disorders at the mitochondrial level is confirmed by the fact that long-term dietary supplementation of rats with a combination of N-acetylcysteine, α-LA, and α-tocopherol attenuated age-related alterations in amyloid beta metabolism [58].

Thus, it is reasonable to suggest that the combination of ALC, α-LA (as the main antioxidant), and NAm applied for the treatment of brain disorders caused by diabetes is one of the prospective options based on the use of the rational, natural, and non-toxic compounds. Therefore, the proposed combined treatment can be used as an effective addition to the main treatment of diabetes. The combination used may be supplemented by other components, most likely by micronutrients. Such combinations can serve a basis for the development of numerous new antidiabetic drugs, which probably can also be used for the treatment of other pathologies of the nervous system.

Our findings support the idea that the combined influence of certain compounds on the diabetic brain is more successful than the separate effects of these compounds (due to the involvement of these components in reversal metabolic alterations, more efficient maintenance of the ion gradients on the plasma membranes, elevation of the membrane potential, modulation of the synaptic functions, etc.). The rather effective chronic application of the combined therapy during four weeks after onset of T1D allows us to believe that this opens an important line of research on the mechanisms of late cerebral complications in some diseases. These results form a basis for future investigations, with prospects for better understanding of not only the mechanisms of actions of the used compounds on brain dysfunctions related to diabetes, but also to a more precise insight into the latter widespread pathology.

In conclusion, the close relationships between the diabetes-caused development of oxidative stress, impaired energy metabolism, mitochondrial dysfunction, and changes in the osmolarity and transport of monovalent cations inside and outside cerebral cells were found. The results obtained point to the existence of significant neuroprotective effects of the combined treatment by the investigated compounds under conditions of diabetes and suggest the important role of CYP2E1 in diabetes-induced ROS production (directly in the liver and indirectly in the brain). Nevertheless, further studies are necessary to provide more profound understanding of not only diabetic neuropathies, but also of such neurological and psychiatric disorders, as Alzheimer's disease, Parkinson's disease, depression, dementia, etc. These pathologies have a multifactorial nature, and their treatment requires new approaches with the use of various agents

and their combinations for effective correction of pathological events.

The experimental procedures were in agreement with the protocols for the care and use of laboratory animals approved by the National (First National Congress of Ukraine on Bioethics, Kyiv, 2001) and international principles proclaimed by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, Strasbourg, 1986. All efforts were made to minimize the number of used animals and their suffering.

The authors, T. M. Kuchmerovska, K. O. Dyakun, M. M. Guzyk, L. V. Yanytska, and I. B. Pryvrotska, confirm the absence of any conflicts in commercial or financial relations, relationships with organizations and/or persons that in any way could be related to the study, and also in interrelations of the co-authors.

REFERENCES

1. S. T. Jerram and R. D. Leslie, "The genetic architecture of type 1 diabetes," *Genes*, **8**, No. 8, 209 (2017), doi: 10.3390/genes8080209.
2. M. Rewers, H. Hyöty, A. Lernmark, et al., "The environmental determinants of diabetes in the young (TEDDY) study: 2018 update," *Curr. Diab. Rep.*, **18**, No. 12, 136 (2018).
3. K. A. Adeshara, A. G. Diwan, and R. S. Tupe, "Diabetes and complications: Cellular signaling pathways, current understanding and targeted therapies," *Curr. Drug Targets*, **17,** No. 11, 1309–1328 (2016).
4. V.R. Drel, P. Pacher, R. Stavniichuk, at al., "Poly(ADP-ribose) polymerase inhibition counteracts renal hypertrophy and multiple manifestations of peripheral neuropathy in diabetic Akita mice," *Int. J. Mol. Med.*, **28**, No. 4, 629–635 (2011).
5. W. Li, E. Huang, and S. Gao, "Type 1 *diabetes mellitus* and cognitive impairments: A systematic review," *J. Alzheimers Dis.*, **57**, No. 1, 29–36 (2017).
6. S. A. Hamed, "Brain injury with *diabetes mellitus*: evidence, mechanisms and treatment implications," *Expert Rev. Clin. Pharmacol.*, **10**, No. 4, 409–428 (2017).
7. I. Trikash, V. Gumenyuk, and T. Kuchmerovska, "Diabetes-induced impairments of the exocytosis process and effect of gabapentin: the link with cholesterol level in neuronal plasma membranes," *Neurochem. Res.*, **40**, No. 4, 723–732 (2015).
8. E. Blázquez, E. Velázquez, V. Hurtado-Carneiro, and J.M. Ruiz-Albusac, "Insulin in the brain: its pathophysiological implications for states related with central insulin resistance, type 2 diabetes and Alzheimer's disease," *Front. Endocrinol.*, **5**, 161 (2014), doi: 10.3389/fendo.2014.00161.
9. J. M. Forbes and M. E. Cooper, "Mechanisms of diabetic complications," *Physiol. Rev.*, **93**, No. 1, 137–188 (2013).
10. S. Dewanjee, S. Das, A.K. Das, at al., "Molecular mechanism of diabetic neuropathy and its pharmacotherapeutic targets," *Eur. J. Pharmacol.*, **833**, 472–523 (2018).
11. A. Verkhratsky, M. Trebak, F. Perocchi, at al., "Crosslink between calcium and sodium signaling," *Exp. Physiol.*, **103**, No. 2, 157–169 (2018).
12. M. W. Jann and J. H. Slade, "Antidepressant agents for the treatment of chronic pain and depression," *Pharmacotherapy*, **27**, 1571–1587 (2007).
13. P. Kumar, G. N. Rao, B. B. Pal, and A. Pal, "Hyperglycemia-induced oxidative stress induces apoptosis by inhibiting PI3-kinase/Akt and ERK1/2 MAPK mediated signaling pathway causing downregulation of 8-oxoG-DNA glycosylase levels in glial cells," *Int. J. Biochem. Cell. Biol.*, **53**, 302–319 (2014).
14. F. J. Gonzalez, "Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1," *Mutat. Res.*, **569**, Nos. 1/2, 101–110 (2005).
15. S. V. Bhagwat, M. R. Boyd, and V. Ravindranath, "Multiple forms of cytochrome P450 and associated monooxygenase activities in human brain mitochondria," *Biochem. Pharmacol.*, **59**, No. 5, 573–582 (2000).
16. F. Seifar, M. Khalili, H. Khaledyan, at al., "α-Lipoic acid, functional fatty acid, as a novel therapeutic alternative for central nervous system diseases: A review," *Nutr. Neurosci.*, **22**, No. 5, 306–316 (2019), doi:10.1080/1028415X.2017.1386755. Epub 2017 Nov 29.
17. G. Sergi, S. Pizzato, F. Piovesan, et al., "Effects of acetyl-L-carnitine in diabetic neuropathy and other geriatric disorders," *Aging. Clin. Exp. Res.*, **30**, No. 2, 133–138 (2018).
18. B. Picconi, I. Barone, A. Pisani, et al., "Acetyl-L-carnitine protects striatal neurons against in vitro ischemia: the role of endogenous acetylcholine," *Neuropharmacology*, **50**, No. 8, 917–923 (2006).
19. N. Braidy, R. Grant, and P. S. Sachdev, "Nicotinamide adenine dinucleotide and its related precursors for the treatment of Alzheimer's disease," *Curr. Opin. Psychiat.*, **31**, No. 2, 160–166 (2018).
20. S. Nesci, F. Trombetti, V. Ventrella, et al., "The inhibition of the mitochondrial F1FO-ATPase activity when activated by Ca^{2+} opens new regulatory roles for NAD," *Biol. Chem.*, **399**, No. 2, 197–202 (2018).
21. T. Kuchmerovska, I. Shymanskyy, S. Chlopicki, and A. Klimenko, "1-Methylnicotinamide (MNA) in prevention of diabetes-associated brain disorders," *Neurochem. Int.*, **56**, 221–228 (2010).
22. I. P. Abita, R. Chicheportiche, and M. Schweitz, "Effect of neurotoxins (veratridine, sea anemone toxin, tetrodotoxin) on transmitter accumulation. Release by nerve terminals *in vitro*," *Biochemistry*, **16**, 1838–1864 (1977).
23. C. P. LeBel, H. Ischiropoulos, and S. C. Bondy, "Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress," *Chem. Res. Toxicol.*, **5**, 227–231 (1992).
24. A. Baracca, G. Sgarbi, G. Solaini, and G. Lenaz, "Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F_0 during

ATP synthesis," *Biochim. Biophys. Acta.*, **1606**, Nos. 1/3, 137-146 (2003).
25. W. B. Rathbun and M. V. Betlach, "Estimation of enzymatically produced orthophosphate in the presence of cysteine and adenosine triphosphate," *Anal. Biochem.*, **28**, 436–445 (1969).
26. H. U. Bergmeyer, *Methods of Enzymatic Analysis*, Academic Press, New York, London, Vol. 4 (1974).
27. O. W. Lowry, N. J. Rosebrough, R. L. Farr, and R. J. Randall, "Protein measurement with the Folin reagent," *J. Biol. Chem.*, **193**, 265–275 (1951).
28. M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem.*, **72**, 248–254 (1976).
29. T. Kuchmerovska, I. Shymanskyy, G. Donchenko, et al., "Poly(ADP-ribosyl)ation enhancement in brain cell nuclei is associated with diabetic neuropathy," *J. Diabetes Complications*, **18**, No. 4, 198–204 (2004).
30. M. R. Lakshman, M. Garige, M. A. Gong, at al., "CYP2E1, oxidative stress, post-translational modifications and lipid metabolism," *Subcell. Biochem.*, **67**, 199–233 (2013).
31. A. S. Pivovarov, F. Calahorro, and R. J. Walker, "Na^+/K^+-pump and neurotransmitter membrane receptors," *Invert. Neurosci.*, **19**, No. 1, 1 (2019), doi: 10.1007/s10158-018-0221-7
32. T. Feldmann, M. Shahar, A. Baba, et al., "The Na(+)/Ca(2+)-exchanger: an essential component in the mechanism governing cardiac steroid-induced slow Ca(2+) oscillations," *Cell Calcium*, **50**, No. 5, 424–432 (2011).
33. M. M. Guzyk, A. A. Tykhomyrov, V. S. Nedzvetsky, et al., "Poly (ADP-ribose) polymerase-1 (PARP-1) inhibitors reduce reactive gliosis and improve angiostatin levels in retina of diabetic rats," *Neurochem. Res.*, **41**, No. 10, 2526–2537 (2016).
34. M. M. Guzyk, K. O. Dyakun, L.V. Yanytska, at al., "Inhibitors of poly(ADP-ribose) polymerase-1 as agents providing correction of brain dysfunctions induced by experimental diabetes," *Neurophysiology,* **49**, No. 3, 183–193 (2017).
35. R. C. Scaduto Jr. and L. W. Grotyohann, "Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives," *Biophys. J.*, **76**, No. 1, 469–477 (1999).
36. M. M. Guzyk1, K. O. Dyakun1, L. V. Yanitska, and T. M. Kuchmerovska, "Influence of poly(ADP-ribose) polymerase inhibitors on some parameters of oxidative stress in blood leukocytes of rats with experimental diabetes," *Ukr. Biochem. J.*, **85**, No. 1, 62–70 (2013).
37. R. Castañeda-Arriaga and J. R. Alvarez-Idaboy, "Lipoic acid and dihydrolipoic acid. A comprehensive theoretical study of their antioxidant activity supported by available experimental kinetic data," *J. Chem. Inform. Model.*, **54**, No. 6, 1642–1652 (2014).
38. I. C. Fernandez, M. Del Carmen Camberos, G. A. Passicot, et al., "Children at risk of diabetes type 1. Treatment with acetyl-L-carnitine plus nicotinamide – case reports," *J. Pediatr. Endocrinol. Metab.*, **26**, Nos. 3/4, 347–355 (2013).
39. M. J. Clausen and H. Poulsen, "Sodium/potassium homeostasis in the cell," *Met. Ions Life Sci.*, **12**, 41–67 (2013).
40. G. E. Torres and S. G. Amara, "Glutamate and monoamine transporters: new visions of form and function," *Curr. Opin. Neurobiol.*, **17**, No. 3, 304–312 (2007).
41. H. P. Hammes, X. Du, D. Edelstein, et al., "Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy," *Nat. Med.*, **9**, No. 3, 294–299 (2003).
42. T. Kuchmerovska, I. Shymanskyy, L. Bondarenko, and A. Klimenko, "Effects of nicotinamide supplementation on liver and serum contents of amino acids in diabetic rats," *Eur. J. Med. Res.*, **13**, No. 6, 275–280 (2008).
43. C. C. Shen, H. M. Huang, H. C. Ou, et al., "Protective effect of nicotinamide on neuronal cells under oxygen and glucose deprivation and hypoxia/reoxygenation," *J. Biomed. Sci.*, **11**, No. 4, 472–481 (2004).
44. J. Luo, A. Y. Nikolaev, S. Imai, et al., "Negative control of p53 by Sir2alpha promotes cell survival under stress," *Cell*, **107**, No. 2, 137–148 (2001).
45. M. M. Guzyk, T. M. Tykhonenko, K. O. Dyakun, et al., "Altered sirtuins 1 and 2 expression in the brain of rats induced by experimental diabetes and the ways of its correction," *Ukr. Biochem. J.*, **91**, No. 1, 21–29 (2019).
46. J. S. Ungerstedt, M. Blomback, T. Soderstrom, "Nicotinamide is a potent inhibitor of proinflammatory cytokines," *Clin. Exp. Immunol.*, **131**, No. 1, 48–52 (2003).
47. E. Turunc Bayrakdar, Y. Uyanikgil, L. Kanit, et al., "Nicotinamide treatment reduces the levels of oxidative stress, apoptosis, and PARP-1 activity in Aβ(1-42)-induced rat model of Alzheimer's disease," *Free Radic. Res.*, **48**, No. 2, 146–158 (2014).
48. G. Traina, "The neurobiology of acetyl-L-carnitine," *Front. Biosci. (Landmark Ed.)*, **21**, 1314–1329 (2016).
49. D. Ziegler, P. A. Low, and W. J. Litchy, "Efficacy and safety of antioxidant treatment with α-lipoic acid over 4 years in diabetic polyneuropathy: the NATHAN 1 trial," *Diabetes Care*, **34**, No. 9, 2054–2060 (2011).
50. T. M. Kuchmerovskaya, P. K. Parhomets, G. V. Chichkovskaya, et al., "Nature of the brain synaptic membranes that bind nicotinamide adenine dinucleotide," *Neurochemistry,* **4**, No. 4, 373–378 (1985).
51. L. J. Yan, "Redox imbalance stress in *diabetes mellitus*: Role of the polyol pathway," *Anim. Model Exp. Med.*, **1**, No. 1, 7–13 (2018).
52. W. H. Tang, K. A. Martin, and J. Hwa, "Aldose reductase, oxidative stress, and *diabetes mellitus*," *Front. Pharmacol.*, **3**, 87–94 (2012).
53. G. C. Ferreira and M. C. McKenna, "L-Carnitine and acetyl-L-carnitine roles and neuroprotection in developing brain," *Neurochem. Res.*, **46**, No. 6, 1661–1675 (2017).
54. T. Aureli, M. E. Di Cocco, C. Puccetti, et al., "Acetyl-L-carnitine modulates glucose metabolism and stimulates glycogen synthesis in rat brain," *Brain Res.*, **796**, Nos. 1/2, 75–81 (1998).
55. G. C. Ferreira and M. C. McKenna, "L-Carnitine and acetyl-L-carnitine roles and neuroprotection in developing brain," *Neurochem. Res.*, **42**, No. 6, 1661–1675 (2017).

56. T. M. Hagen, J. Liu, J. Lykkesfeldt, et al., "Feeding acetyl-L-carnitine and lipoic acid to old rats significantly improves metabolic function while decreasing oxidative stress," *Proc. Natl. Acad. Sci. USA*, **99**, No. 4, 1870–1875 (2002).
57. H. Schupke, R. Hempel, G. Peter, et al., "New metabolic pathways of alpha-lipoic acid," *Drug Metab. Dispos.*, **29**, No. 6, 855–862 (2001).
58. M. Sinha, A. Bir, A. Banerjee, et al., "Multiple mechanisms of age-dependent accumulation of amyloid beta protein in rat brain: Prevention by dietary supplementation with N-acetylcysteine, α-lipoic acid and α-tocopherol," *Neurochem. Int.*, **95**, 92–99 (2016).