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MELATONIN AND SUCCINATE REDUCE RAT LIVER MITOCHONDRIAL DYSFUNCTION IN DIABETES

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Mitochondrial dysfunction and an increase in mitochondrial reactive oxygen species in response to hyperglycemia during diabetes lead to pathological consequences of hyperglycemia. The aim of the present work was to investigate the role of a specific functional damage in rat liver mitochondria during diabetes as well as to evaluate the possibility of metabolic and antioxidative correction of mitochondrial disorders by pharmacological doses of succinate and melatonin. In rat liver mitochondria, streptozotocin-induced diabetes was accompanied by marked impairments of metabolism: we observed a significant activation of α -ketoglutarate dehydrogenase (by 60%, $p < 0.05$) and a damage of the respiratory function. In diabetic animals, melatonin (10 mg/kg b.w., 30 days) or succinate (50 mg/kg b.w., 30 days) reversed the oxygen consumption rate V_3 and the acceptor control ratio to those in nondiabetic animals. Melatonin enhanced the inhibited activity of catalase in the cytoplasm of liver cells and prevented mitochondrial glutathione-S-transferase inhibition while succinate administration prevented α -ketoglutarate dehydrogenase activation. The mitochondria dysfunction associated with diabetes was partially remedied by succinate or melatonin administration. Thus, these molecules may have benefits for the treatment of diabetes. The protective mechanism may be related to improvements in mitochondrial physiology and the antioxidative status of cells.

Key words: *antioxidants, bioenergetics, experimental diabetes, liver, mitochondria, melatonin, succinate*

INTRODUCTION

It is important to distinguish the pathogenic mechanisms involved in the onset and progression of diabetes mellitus, one of the most common metabolic diseases in humans. Hyperglycemia causes many of the pathological consequences of both type 1 and type 2 diabetes. The main consequences of hyperglycemia of particular pathological relevance are 1) formation, auto-oxidation, and interaction with cell receptors of advanced glycation end products; 2) activation of various isoforms of protein kinase C; 3) induction of the polyol (sorbitol) pathway; 4) and increased hexosamine pathway flux (1). Enhanced oxidative stress and declines in antioxidant capacity are considered to play important roles in the pathogenesis of chronic diabetes mellitus and its complications (2, 3). Reactive oxygen species (ROS) may serve as second messengers in the insulin action cascade (redox paradox) (4). Glucose and lipid metabolism are largely dependent on the mitochondrial functional state and physiology. ROS formation by mitochondria, excessive mitochondrial oxidative damage and reduced mitochondria biogenesis contribute to mitochondria disruption, and, subsequently, to insulin resistance and

associated diabetic complications (5-7). The realization that mitochondria are at the intersection of aerobic cellular life and death has made them a promising target for drug discovery and therapeutic interventions (8). As was recently discussed, hyperglycemia induced mitochondrial dysfunction in liver holds importance in the context of non-alcoholic fatty liver disease in diabetic subjects (9).

The administration of succinic acid (50 mg/kg) as a bioenergetic regulator corrects oxidative phosphorylation disturbances in hepatic mitochondria under diabetes in rats and reduces blood glucose and cholesterol (10). Succinate protected isolated rat liver mitochondria from peroxidative damage, proteins from cross-links, mitochondrial membranes from permeability changes (11). The known antioxidant, pineal gland hormone, melatonin, has both receptor-dependent and independent mechanisms (12). Many of the beneficial effects of melatonin administration may depend on its action on the mitochondria (13, 14). Earlier we documented some beneficial effects of melatonin under experimental diabetes in rats: correction of impaired antioxidative status in liver tissue and regulation of nitric oxide bioavailability in the aorta (15). Long-term melatonin administration to diabetic rats reduced their

hyperlipidemia and hyperinsulinemia and enhanced insulin-receptor kinase and insulin receptor substrate-1 phosphorylation; this suggests the existence of a signaling pathway cross-talk between melatonin and insulin (16).

The aim of the present work was to investigate the role of a specific functional damage in rat liver mitochondria during diabetes as well as to evaluate the possibility of mitochondrial impairment corrections by the energetic substrate, succinate, and by the antioxidant, melatonin.

MATERIAL AND METHODS

Chemicals

Melatonin, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), succinic acid disodium salt hexahydrate, reduced glutathione (GSH), trichloroacetic acid (TCA), 2,6-dichlorophenol-indophenol, *tert*-butyl hydroperoxide (tBHP), 1-chloro-2,4-dinitrobenzene (CDNB) and safranin O were from Sigma-Aldrich (St. Louis, MO, USA/Steinheim, Germany). Streptozotocin (Streptozocin) (STZ) was from Fluka Chemie AG (Buchs, Switzerland). All other reagents were of analytical grade and were purchased from Reakhim (Moscow, Russia). All the water solutions were made with water purified in the Milli-Q system.

Animal model

The investigations were performed using 60 male albino Wistar rats (150-180 g). A standard balanced diet and tap water were provided *ad libitum*. Lights were on daily from 08.00 to 20.00 h. Ten animals received physiological saline containing 5% ethanol intraperitoneally (i.p.) and were kept as controls. Experimental animals were injected with a single dose of STZ (45 mg/kg, i.p.), dissolved in 0.01 M citrate buffer, pH 4.5, immediately before use. Seven days later, blood glucose levels were determined in whole blood samples. The rats injected with STZ were considered diabetic if their fasting blood glucose was >200 mg/dL (Blood Glucose Sensor Electrodes, MediSense, Abbot Laboratories, Bedford, UK). The first subgroup of the hyperglycemic animals diagnosed was injected daily with physiological saline containing 5% ethanol (i.p.) (the diabetes group); the second subgroup received daily 10 mg melatonin/kg b.w. (i.p.) (diabetes+10 mg melatonin); the third subgroup received daily 50 mg succinate/kg b.w. (i.p.) (diabetes+50 mg succinate); and the fourth subgroup received daily 10 mg melatonin/kg b.w.+50 mg succinate/kg b.w. (i.p.) (diabetes+10 mg melatonin+50 mg succinate). Melatonin was prepared as a 0.3% solution in physiological saline, containing 5% ethanol, and succinate was prepared as a 1.0% solution in physiological saline. Melatonin as well as succinate was injected in the morning at 08.00; thus, we evaluated the effect of exogenous melatonin administration that was used as an antioxidant. The rats were sacrificed after 30 days of melatonin and succinate (or saline) administration. Melatonin (10 mg/kg) (15, 17) and succinate (50 mg/kg) (10) were administered at doses that were applied earlier. The animals were killed by decapitation according to the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the study was approved by the Ethics Committee of the Institute for Pharmacology and Biochemistry of the National Academy of Sciences of Belarus.

Blood samples were drawn by an abdominal aorta puncture into tubes containing hirudin (50 µg/ml). After removing of plasma by centrifugation, the erythrocytes were washed three

times with cold phosphate buffered saline (150 mM NaCl, 10 mM Na₂HPO₄, pH 7.4).

Isolation of rat liver mitochondria

Mitochondria were isolated by differential centrifugation from the liver as follows (18). The liver was quickly removed and was homogenized in a glass-Teflon homogenizer with ice-cold isolation medium containing 250 mM sucrose, 20 mM Tris-HCl and 1 mM EDTA, pH 7.2, at 2°C. The homogenate was centrifuged at 600 g for 10 min, and the supernatant was centrifuged at 8,500 g for 10 min. The obtained pellet was washed in buffer containing 250 mM sucrose, 20 mM Tris-HCl, pH 7.2 (at 2°C). The protein concentration was determined by the method of Lowry *et al.* (19). Respiration of mitochondria was measured using a laboratory-made oxygen Clark-type electrode and a hermetic polarographic cell (volume 1.25 ml) with constant gentle stirring. The incubation medium contained 50 mM sucrose, 20 mM Tris-HCl, 125 mM KCl, 2.5 mM KH₂PO₄, 5 mM MgSO₄, 0.5 mM EDTA, pH 7.5. The experiments were performed at 25°C using 5 mM succinate or L-glutamate as substrates. The mitochondrial protein concentration in the probe was 1.0 mg/ml. The functional state of mitochondria was determined by the acceptor control ratio (ACR), equal to the ratio of the respiratory rates (V_3/V_2) of mitochondria in states 3 and 2, and the coefficient of phosphorylation (ADP/O). State 2 corresponded to the respiration in the presence of the substrate (glutamate, or succinate) added (V_2), and the rate of mitochondrial respiration corresponding to state 3 (V_3) was registered after addition of 180 µM ADP (in the presence of 210 nm ADP in the polarographic cell).

Biochemical measurements

A stable form of glycated haemoglobin (GHb) containing 1-deoxy-1(N-valyl)fructose and the activities of marker enzymes of hepatic cell membrane injury, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed in blood plasma using a reagent kits from Pliva-Lachema (Brno, Czech Republic). The concentration of total and protein thiols in mitochondria was determined spectrophotometrically by the method of Ellman (20) using the molar absorption coefficient $\epsilon_{412} = 1.36 \cdot 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Mixed disulfides (PSSG) formed by glutathione and accessible sulfhydryl groups of mitochondrial proteins were determined by the method described by Rossi *et al.* (21). The level of accumulated lipid peroxidation products (thiobarbituric acid-reactive substances, TBARS) was determined according to Stocks and Dormandy (22).

α -Ketoglutarate dehydrogenase (KGDH) activity was assayed as the rate of NAD reduction that was measured spectrophotometrically at 340 nm upon addition of fractured mitochondria (by rapid freezing-thawing of mitochondria, three times) to the medium containing 0.1 M potassium phosphate buffer, pH 7.4, 5.0 mM MgCl₂, 40.0 µM rotenone, 2.5 mM α -ketoglutarate, 0.1 mM CoA, 0.2 mM thiamine pyrophosphate, and 1.0 mM NAD at 25°C (23). The protein concentration in the reaction mixture was 50 µg/ml. The activity of mitochondrial succinate dehydrogenase (SDH) was spectrophotometrically determined by the rate of 2,6-dichlorophenol-indophenol reduction at 600 nm upon addition of fractured mitochondria (final protein concentration of 50 µg/ml) to the reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.4, 25 mM sodium succinate, 0.5 mM phenazinemetasulfate, 2.5 mM sodium azide, 0.05 mM 2,6-dichlorophenol-indophenol (23).

Mitochondrial glutathione peroxidase (GPx) activity was measured by the rate of GSH oxidation according to the method

of Martinez *et al.* (24). The reaction mixture contained 0.1 M Tris-HCl, pH 7.0, 1 mM EDTA, 12 mM sodium azide, 2 mM tBHP and 4.9 mM GSH (as cosubstrates of GPx). The reaction was started by addition of fractured mitochondria and was stopped by 0.2 ml 25% TCA after 10-min incubation at 37°C. The protein concentration in the reaction mixture was 100 µg/ml. The activity was measured as the amount of GSH oxidized in the GPx reaction using Ellman's reagent.

The activity of mitochondrial glutathione-S-transferase (GST) was determined employing the method of Habig *et al.* (25). The reactions were carried out in the presence of 10 µg mitochondrial protein, 1 mM CDNB (as substrate), 5 mM GSH and 100 mM potassium phosphate buffer, pH 6.5, at 25°C in the final volume of 2 ml. The conjugation of CDNB with glutathione was monitored at 340 nm, using the molar absorption coefficient of 9,600 M⁻¹cm⁻¹.

Catalase activity was measured in rat liver cell cytoplasm by the method of Aebi (26). The reaction mixture contained 4.5 µg/ml protein, 20 mM hydrogen peroxide (H₂O₂), 50 mM potassium phosphate buffer, pH 7.0. The H₂O₂ decomposition was monitored at 240 nm by the use of the molar absorption coefficient of 36 M⁻¹cm⁻¹ and at 25°C for 3 min.

Statistical analysis

Data for 8-10 rats in each group are presented as a mean ±S.E.M. We used the standard unpaired Student *t* test for the comparison of the data, *p*<0.05 was taken to indicate statistical significance.

RESULTS

Biochemical impairments and oxidative stress under diabetes

In our experiments, 30-day STZ-induced diabetes mellitus in rats resulted in elevated levels of blood glucose and glycosylated haemoglobin as well as in reduction of animal body weight (Table 1). Similarly, the activities of the markers of liver damage, ALT and AST, in rat blood plasma increased by 50% (*p*<0.01) and 10% (*p*<0.05), respectively (Table 1). Succinate, but not melatonin, administration to diabetic animals reduced hepatolysis, diminishing elevated blood plasma ALT and AST

activities. Diabetes resulted in significant impairments of rat tissues and organs: the kidney weight / body weight ratio increased in diabetic rats (Table 1). In our experiments, the melatonin injection to diabetic animals, but not succinate or melatonin plus succinate, recovered this parameter to the control value (*p*<0.01 in comparison with the diabetic animals) but did not affect body weight (Table 1). The level of lipid peroxidation products (TBARS) increased in the heart (by 20%, *p*<0.05) but not in the liver tissues of diabetic rats. The melatonin (but not succinate) administration prevented elevation of TBARS levels in heart tissue (Table 2). The melatonin or succinate (or both) administration to diabetic rats reduced the hepatic TBARS levels (*p*<0.05 in comparison with diabetes) (Table 2). The level of reduced glutathione decreased in red blood cells (by 25%, *p*<0.05) (Table 2). The liver mitochondrial protein thiol group content and mitochondrial total thiol group content did not change as a result of diabetes (Table 2). The level of PSSG increased in mitochondria of diabetic rats (by 50%, *p*<0.05). We evaluated the activities of antioxidative and glutathione metabolizing enzymes: liver mitochondrial GPx activity (did not change), liver mitochondrial GST activity (the main detoxifying enzyme) (decreased by 12%, *p*<0.05) and cytoplasmic catalase activity (decreased by 45%, *p*<0.001). Thus, the activities of the enzymatic antioxidative defense system diminished in the liver of diabetic rats (Table 2). Melatonin administration to diabetic animals enhanced the depressed activity of catalase (by 35%, *p*<0.05) in the cytoplasm of liver cells and mitochondrial GST (by 20%, *p*<0.05) in comparison with diabetes, respectively (Table 2).

Mitochondrial impairments under diabetes

30-day diabetes was accompanied by a considerable impairment of mitochondrial respiratory activity. In the case of succinate as a respiratory substrate, the ADP-stimulated respiration rate *V*₃ markedly decreased (by 25%, *p*<0.05) (Fig. 1A) and the acceptor control ratio (ACR) *V*₃/*V*₂ was also diminished (by 25%, *p*<0.01) (Fig. 1B). We observed a drop in the respiration rate *V*₂ (by 20%, *p*<0.05) and the ADP-stimulated respiration rate *V*₃ (by 35%, *p*<0.05) with glutamate as substrate (Fig. 1A). In this case, the ACR also decreased (by 20%, *p*<0.05). Surprisingly, the phosphorylation coefficient ADP/O did not change during diabetic liver damage (Fig. 1B).

Table 1. Blood glucose, glycated haemoglobin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and animal body and kidney weights in normal and STZ-treated diabetic rats. Effect of succinate and melatonin administration.

	Control (n=10)	Diabetes (n=8)	Diabetes+ succinate (n=8)	Diabetes+ melatonin (n=8)	Diabetes+ melatonin+ succinate (n=8)
Blood glucose, [mg/dl]	105.3±1.9	522.7±67.8 ^b	468.3±72.0 ^b	405.3±60.4 ^b	492.5±45.5 ^b
Glycated haemoglobin, [µmol fructose/g Hb]	2.7±0.3	6.1±0.3 ^a	5.6±0.6 ^a	4.8±0.7 ^a	6.1±0.8 ^a
ALT, µkat/l	0.95±0.08	1.43±0.09 ^b	0.99±0.05 ^d	1.51±0.15 ^b	1.32±0.19
AST, µkat/l	1.09±0.05	1.21±0.03 ^a	0.85±0.03 ^{b,e}	1.52±0.06 ^c	1.04±0.14
Weight, kidney, [g/100 g b.w.]	3.25±0.14	5.55±0.17 ^c	4.98±0.41 ^a	4.14±0.30 ^d	4.99±0.28 ^b
Body weight (initial), [g]	155±6.9	167.9±6.5	175±10.1	180.8±6.8	172.5±8.4
Body weight (final), [g]	190±8.9*	175±10.9	179.2±16.1	205±6.3	175.8±13.6

^a- statistically significant in comparison with control, *p*<0.05; ^b- statistically significant in comparison with control, *p*<0.01; ^c- statistically significant in comparison with control, *p*<0.001; ^d- statistically significant in comparison with diabetes, *p*<0.01; ^e- statistically significant in comparison with diabetes, *p*<0.001; * - statistically significant in comparison with initial body weight; n- the number of animals.

Table 2. The total (TSH) and protein (PSH) thiol groups, mixed glutathione-protein disulfides (PSSG), lipid peroxidation product levels and antioxidative enzyme activities in rat liver cell mitochondria under diabetes. Effects of succinate and melatonin administration.

	Control (n=10)	Diabetes (n=8)	Diabetes+ succinate (n=8)	Diabetes+ melatonin (n=8)	Diabetes+ melatonin+ succinate (n=8)
TSH, [nmol/mg protein]	91.9±1.8	92.2±1.2	94.1±2.1	93.7±1.6	93.8±1.5
PSH, [nmol/mg protein]	83.4±1.8	83.1±1.1	85.3±2.1	85.1±1.4	85.0±1.5
PSSG, [nmol/mg protein]	0.12±0.03	0.18±0.03 ^a	0.13±0.03	0.18±0.02	0.24±0.03 ^a
TBARS, liver homogenate, [nmol/mg protein]	0.31±0.01	0.35±0.02	0.28±0.01 ^d	0.24±0.01 ^{c,c}	0.27±0.02 ^d
TBARS, heart homogenate, [nmol/mg protein]	0.32±0.02	0.39±0.02 ^a	0.42±0.05	0.34±0.02 ^d	0.48±0.03 ^{b,d}
GST, [nmol CDNB/min/mg protein]	35.2±1.7	30.8±0.6 ^a	32.2±2.5	36.7±2.4 ^d	29.4±2.2 ^a
GPx, [nmol GSH/min/mg protein]	466.5±6.4	478.0±38.9	466.3±35.8	480±21.9	430±55.8
Catalase, liver cell cytoplasm, [μmol H ₂ O ₂ /min/mg protein]	517.7±33.2	281.7±27.2 ^c	350.6±36.4 ^b	386.1±20.6 ^{b,d}	342.9±44.8 ^a
GSH, erythrocytes, [mM]	1.8±0.4	1.4±0.3 ^a	1.0±0.1 ^a	1.4±0.1	1.2±0.1 ^a

^a- statistically significant in comparison with control, $p < 0.05$; ^b- statistically significant in comparison with control, $p < 0.01$; ^c- statistically significant in comparison with control, $p < 0.001$; ^d- statistically significant in comparison with diabetes, $p < 0.05$; ^e- statistically significant in comparison with diabetes, $p < 0.01$; n- the number of animals.

The activity of the key enzyme of the Krebs cycle, KGDH, rose (by 60%, $p < 0.05$) (Fig. 2); probably, this reflected the activation of protein degradation and amino acid turnover during diabetes. Despite the substantial damage in respiratory activity, the activity of SDH, respiratory complex II, did not change (Fig. 2).

Succinate administration during diabetes in rats exerted a protective effect on the liver mitochondrial function. We observed an enhancement of the reduced ADP-stimulated oxygen consumption rate V_3 (by 25%, $p < 0.05$ in comparison with diabetic animals) (Fig. 1A), when we used succinate as a respiratory substrate. The ACR considerably increased to the control values ($p < 0.01$ in comparison with diabetic animals) (Fig. 1B). Similarly, succinate administration to diabetic animals reversed the ADP-stimulated oxygen consumption rate V_3 (increased by 30%, $p < 0.05$ in comparison with diabetic animals) and completely reversed the ARC value when mitochondria oxidized glutamate as a substrate (Fig. 1A, 1B). Melatonin administration during diabetes exerted a marked protective effect on the liver mitochondria function, reversing the decreased respiration rate V_3 to the control values both for succinate-dependent respiration ($p < 0.05$ in comparison with diabetic animals) and for glutamate-dependent respiration ($p < 0.01$). Similarly, the melatonin treatment of diabetic rats reversed the effect of diabetes on the ACR value both for succinate-dependent respiration and for glutamate-dependent respiration (Fig. 1A, 1B). The combined administration of melatonin and succinate to diabetic animals also reversed the V_3 rate value, and the ACR value for both oxidizing substrates (Fig. 1A, 1B) and only succinate administration prevented a significant KGDH activation during diabetes (Fig. 2).

DISCUSSION

There is interest in determining whether antioxidants will reduce mitochondria oxidative damage and prevent diabetic complications (6). Excess ROS accumulation stimulates various serine/threonine kinases, including IKK β , JNK and PKCs, and inflammatory signaling, resulting in reduced insulin signaling cascade (insulin resistance) (7). The protective effect of antioxidants (α -lipoic acid, N-acetyl-cysteine, melatonin) on oxidative stress-induced insulin resistance relate to their ability to preserve the intracellular redox balance or to block the activation of stress-sensitive kinases.

Numerous studies have evaluated increased oxidative stress in mitochondria, as well as alterations in mitochondrial metabolism and function in different tissues under diabetes (9, 27). However, the results reported are controversial. Thus, it was reported that 9 weeks after STZ-induction of diabetes in rats, respiratory function of liver mitochondria declined as assessed by the mitochondrial membrane potential and respiratory ratios, succinate dehydrogenase and cytochrome C oxidase activities: all of them were significantly augmented (27). However, a decline in state 3 respiration in heart mitochondria was shown only in diabetic animals exhibiting a marked reduction in body weight (28). The data argue against hyperglycemia being a direct cause of the decline in state 3 oxygen consumption observed in cardiac mitochondria of type 1 diabetic rats (28). Moreira *et al.* (29) similarly concluded that 4-week and 9-week STZ-induced diabetes in rats was not accompanied by brain mitochondria dysfunction (4-week diabetic rats showed even higher mitochondrial respiratory chain enzymatic activities, compared to control rats), suggesting that oxidative stress associated with type 1 diabetes is not directly related to aberrant mitochondrial

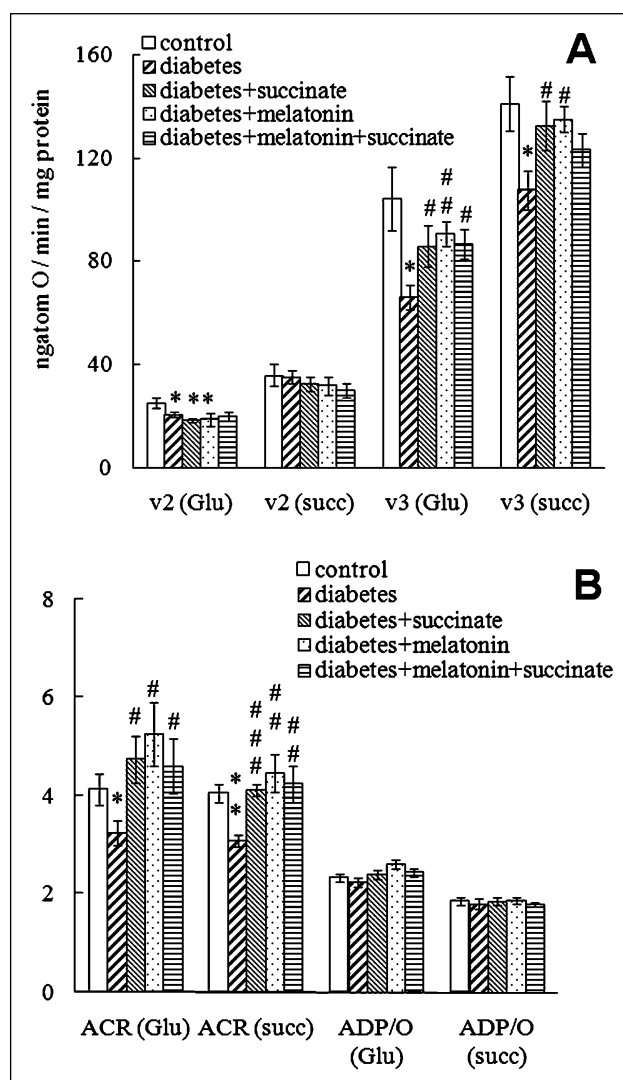


Fig. 1. Parameters of respiratory activity of liver mitochondria in normal and streptozotocin-treated diabetic rats. Effect of succinate and melatonin administration: substrate-dependent respiration rate V_2 and ADP-stimulated respiration rate V_3 (A) as well as acceptor control ratio (V_3/V_2) and coefficient of phosphorylation ADP/O (B). Glutamate (Glu) and succinate (succ) were used as respiratory substrates. Data for 8-10 rats in each group are presented as a mean \pm S.E.M. The standard unpaired Student *t* test was used for the comparison of the data. * - statistically significant in comparison with control, $p < 0.05$; ** - statistically significant in comparison with control, $p < 0.01$; # - statistically significant in comparison with diabetes, $p < 0.05$; ## - statistically significant in comparison with diabetes, $p < 0.01$; ### - statistically significant in comparison with diabetes, $p < 0.001$.

physiology. Recently it was shown that glycated LDL, which accumulates in patients with diabetes, increased ROS production by mitochondria and attenuated the activities of key enzymes in a mitochondrial electron-transport chain (30).

In the current experiments, we observed a considerable impairment of rat liver mitochondria function during diabetes, concomitant with liver damage. Significant diminishing of the ADP-dependent oxygen consumption rate V_3 and impairment of the coupling oxidation and phosphorylation processes in liver mitochondria (the ACR values decreased) without changes in the

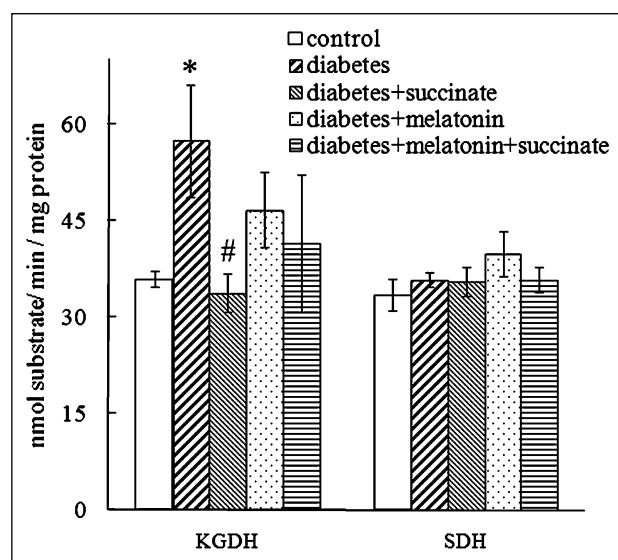


Fig. 2. Activities of α -ketoglutarate dehydrogenase (KGDH) and succinate dehydrogenase (SDH) of liver mitochondria in normal and streptozotocin-treated rats. Effect of succinate and melatonin administration. Data for 8-10 rats in each group are presented as a mean \pm S.E.M. The standard unpaired Student *t* test was used for the comparison of data. * - statistically significant in comparison with control, $p < 0.05$; ** - statistically significant in comparison with control, $p < 0.01$; # - statistically significant in comparison with diabetes, $p < 0.05$; ## - statistically significant in comparison with diabetes, $p < 0.01$; ### - statistically significant in comparison with diabetes, $p < 0.001$.

efficacy of oxygen consumption (the ADP/O did not change during diabetes) were shown. 30-day diabetes resulted in an increase of animal growth retardation, absolute and relative kidney weight (Table 1), and in typical signs of elevated oxidative stress. In rat liver mitochondria, diabetes was accompanied by marked impairments of metabolism: we observed a significant activation of KGDH, a key enzyme of the Krebs cycle, without changes in SDH activity. At the same time we did not observe alterations in the mitochondrial thiol group levels, or in the mitochondrial membrane potential (data not shown) as a result of diabetes. The reduction of the oxygen consumption rate V_3 with glutamate or succinate as substrates, with KGDH or SDH activity diminution being unobserved, could be explained either by impaired substrate oxidation in the electron-transport chain or by reduced H^+ translocation through the inner mitochondrial membrane.

We observed some beneficial effects of the melatonin and succinate administration on the complications of diabetes. In our experiments, the melatonin administration had no effect on diabetic growth retardation but partially prevented diabetic increase of kidney weight. Other authors demonstrated that melatonin supplementation to hamsters, rats, and mice reduced their body weight and the mass of white adipose tissue (31). Succinate-treated diabetic animals showed decreased ALT and AST blood plasma activities in comparison with diabetic group values. Melatonin treatment prevented GST inactivation and succinate treatment prevented KGDH activation under diabetes. The more important thing is that melatonin, as well as succinate administration to diabetic animals improved the mitochondrial physiology, *i.e.* increased the respiration rate of isolated rat liver mitochondria and prevented a loss of respiratory control, increasing the acceptor control ratio value. Generally, the

simultaneous administration of melatonin and succinate to diabetic rats did not show any additional beneficial effect in comparison with the melatonin or succinate injections.

Succinate and its mitochondrial metabolites may participate in triggering of insulin release by pancreatic islets (32). By acting as ligands for appropriate receptors, succinate and α -ketoglutarate have unexpected signaling functions beyond their traditional roles in the regulation of energy homeostasis and cellular metabolism (33). Mitochondrial oxidation of succinate in isolated diabetic rat hepatocytes and succinate carbon incorporation into proteins were markedly lowered, probably due to impairment in the Krebs cycle activity (34). At the same time we observed a significant activation of α -ketoglutarate dehydrogenase in hepatic mitochondria of diabetic rats.

Melatonin exhibits a variety of biological activities, including antioxidative protection of cells and anti-inflammatory functions (35, 36). The highest intracellular concentration of melatonin seems to be in mitochondria (37), which suggests its involvement in regulation of mitochondrial function. Based on the observations that melatonin treatment does not measurably influence food intake and only has a limited impact on the physical activity, the weight loss promoting effect of melatonin must be attributed to alterations in energy metabolism, *i.e.* increased energy expenditure induced by melatonin (31). Uncoupling action of melatonin on oxidative phosphorylation in isolated mitochondria has been observed by Lopez *et al.* (38). We demonstrated this mild uncoupling effect of melatonin in experimental rat model (39).

Melatonin influences insulin secretion, with this effect being mediated by specific high-affinity, pertussis-toxin-sensitive G-protein coupled receptors MT (1) as well MT (2) in pancreatic islets (40). At the same time the reduced insulin levels in type 1 diabetes (streptozotocin-induced rat model of diabetes) associated with higher melatonin concentrations in the blood and elevated insulin levels observed in type 2 diabetes are associated with reduced melatonin levels (40). The results suggest that a melatonin-insulin antagonism may exist (41). It was shown earlier that melatonin inhibits glucose-induced insulin secretion in isolated rat islets without interfering with glucose metabolism (42). Peschke *et al.* pointed out the fact that melatonin protects the β -cells against functional overcharge and, consequently, hinders the development of type 2 diabetes (43). The high concentrations of blood plasma melatonin found in type 1 diabetes are supposed to be beneficial for preventing diabetic lesions. At the same time the possible effect of melatonin on insulin secretion by pancreatic islets should be verified. Melatonin directly inhibits the mitochondrial permeability transition pore in a dose-dependent manner ($IC_{50}=0.8 \mu M$) in rat liver mitoplasts; this inhibition may contribute to melatonin's anti-apoptotic effects during transient brain ischemia (44). The combination of an antioxidant, melatonin, and a PARP inhibitor, nicotinamide, caused an essential reversal of biochemical alterations in diabetic neuropathy (45). The results of our study suggest that the antioxidant melatonin and the energetic substrate succinate, being signaling molecules, may be useful for the pharmacotherapy of diabetic complications.

In summary, the data support an important role of mitochondria dysfunction in the development of liver injury during diabetes as well as the possibility of corrections of mitochondrial disorders by melatonin and succinate. The mechanism of mitochondrial dysfunction might be impairment of cellular and mitochondrial redox-balance, changes of mitochondrial metabolism, damage of the mitochondrial membrane and components of the electron-transport chain. Succinate, an energetic mitochondrial substrate, and melatonin, a powerful antioxidant, effectively prevented the diabetic lesions of rat liver mitochondria and should be considered as effectors

regulating mitochondria function. The effects of melatonin might be due to both its radical scavenging properties, its signaling effects and its interaction with complexes of the respiratory chain.

Conflict of interests: None declared.

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Received: July 27, 2011

Accepted: August 17, 2011

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