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Corrections by melatonin of liver mitochondrial disorders under diabetes and acute intoxication in rats

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The aim of the present work was to investigate the mechanisms of oxidative damage of the liver mitochondria under diabetes and intoxication in rats as well as to evaluate the possibility of corrections of mitochondrial disorders by pharmacological doses of melatonin. The experimental (30 days) streptozotocin-induced diabetes mellitus caused a significant damage of the respiratory activity in rat liver mitochondria. In the case of succinate as a respiratory substrate, the ADP-stimulated respiration rate V_3 considerably decreased (by 25%, $p<0{\cdot}05$) as well as the acceptor control ratio (ACR) V_3/V_2 markedly diminished (by 25%, $p<0{\cdot}01$). We observed a decrease of the ADP-stimulated respiration rate V_3 by 35% ($p<0{\cdot}05$), with glutamate as substrate. In this case, ACR also decreased (by 20%, $p<0{\cdot}05$). Surprisingly, the phosphorylation coefficient ADP/O did not change under diabetic liver damage. Acute rat carbon tetrachloride-induced intoxication resulted in considerable decrease of the phosphorylation coefficient because of uncoupling of the oxidation and phosphorylation processes in the liver mitochondria. The melatonin administration during diabetes ($10 mg \cdot kg^{-1}$ body weight, 30 days, daily) showed a considerable protective effect on the liver mitochondrial function, reversing the decreased respiration rate V_3 and the diminished ACR to the control values both for succinate-dependent respiration and for glutamate-dependent respiration. The melatonin administration to intoxicated animals ($10 mg \cdot kg^{-1}$ body weight, three times) partially increased the rate of succinate-dependent respiration coupled with phosphorylation. The impairment of mitochondrial respiratory plays a key role in the development of liver injury under diabetes and intoxication. Melatonin might be considered as an effector that regulates the mitochondrial function under diabetes. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—melatonin; rat liver mitochondria; respiration; diabetes; oxidative stress

INTRODUCTION

Mitochondria play a key role in coordination of the main cellular functions. They provide energy required for all cellular processes as well as participate in cell signalling and in necrotic and apoptotic cell death. There is evidence that defective mitochondrial oxidative phosphorylation plays an important role in the pathogenesis of many diseases: Alzheimer's disease, diabetes, and ageing.[10] Mitochondria are major producers of free radical species in the cell (possibly also nitric oxide) and are major targets of oxidative damage.[8] The mitochondrial function is particularly susceptible to oxidative stress, leading to decreased mitochondrial adenosine-5'-triphosphate (ATP) synthesis, cellular calcium dyshomeostasis and induction of mitochondrial permeability transition, all of which predispose cells to necrosis or apoptosis.[11,13]

The main consequences of hyperglycemia of particularly pathological relevance in diabetes are as follows: formation, auto-oxidation and interaction with cell receptors of advanced glycation end products, protein glycation, protein kinase C activation and induction of the polyol pathway, as well as increased hexosamine pathway flux.[30] Many of these pathways have long been associated with oxidative stress.[30] Much of the hyperglycemia-related damages under both types 1 and 2 diabetes are suggested to be a consequence of elevated production of reactive oxygen species by the mitochondrial respiratory chain and mitochondrial oxidative damage.[30] Similarly, mitochondrial dysfunction is an early manifestation of hepatotoxic agent effects. The metabolic capacity of liver mitochondria is considerably impaired in rats with carbon tetrachloride-induced cirrhosis caused by both reduced mitochondria volume per liver and damaged metabolism of the remaining mitochondria.[15] Reduced oxygen consumption and mitochondrial enzyme activities as well as a diminished rate of ATP production were observed under intoxication.[15]

Melatonin (N-acetyl-5-methoxy-tryptamine) has a number of membrane-bound receptor-mediated actions, including regulation of circadian rhythms, reproduction, immunoregulation and retinal function.[3,5] Melatonin has

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been shown to be an effective antioxidant in a number of experimental models both *in vitro* and *in vivo*.[3,35,38] The antioxidative effects of melatonin range from direct radical scavenging to modulation of a number of processes, which may trigger a redox balance in the cell.[3,19,23,29] At the molecular level, melatonin has effects that optimize cellular functions (mitochondrial respiration, ATP production, gene transcription and translation, and protein synthesis).[5] The mitochondria-modulating effect of melatonin may account for some of the protective properties of indolamine and may be of physiological significance because it seems that the indolamine is concentrated into the mitochondria.[1,22] Pharmacological corrections of mitochondrial activity and cell bioenergetics and prevention of the mitochondrial oxidative damage are a beneficial therapeutic approach in many pathological states, such as diabetes, which are connected with mitochondrial dysfunctions. Mitochondria-targeted antioxidants are to have a therapeutic potential in diabetes.[11] At the same time, despite the prevalence of mitochondrial dysfunction in many diseases, mitochondria-specific therapies have not been well developed.[34] Correction of the mitochondrial functions is the basis of 'mitochondrial medicine'.[10]

Despite the fact that hepatic lesions under intoxication and diabetes are well characterized by biochemical, morphological and histochemical studies, the early events critical to the onset of toxic and diabetic hepatocyte impairments remain unclear. The aim of this study was to examine the liver mitochondria respiratory parameters under diabetes in rats. The corrections of mitochondrial disorders by pharmacological doses of melatonin were simultaneously evaluated. The effects of diabetes on rat liver mitochondrial functional activity were compared with those of rat intoxication, and the beneficial effect of melatonin under diabetes was compared with that under acute intoxication.

MATERIALS AND METHODS

Chemicals

Melatonin, carbon tetrachloride (CCl_4), 5, 5'-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), reduced glutathione (GSH), safranin O and trichloroacetic acid were from Sigma-Aldrich, St. Louis, MO. 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 solutions were made with water purified in the Milli-Q system.

Animal models

The investigations were performed using 80 male albino Wistar rats (150–180 g). A standard balanced diet and tap water were provided *ad libitum*. The animals were adapted to an intermittent 12-h light and dark phases cycle for 1 week and were divided into two groups for CCl_4 intoxication and experimental model of diabetes. Lights were on daily from 08:00 to 20:00 h.

Diabetes. Ten animals received physiological saline containing 5% ethanol intraperitoneally (i.p.) and were kept as controls. Thirty animals were injected with a single dose of STZ (45 mg·kg^{-1}, 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^{-1} (Blood Glucose Sensor Electrodes, MediSense; Abbot Laboratories, Bedford, UK). All the animals diagnosed as diabetic were further divided into two subgroups: the first subgroup was injected daily with physiological saline containing 5% ethanol (i.p.) (the group 'diabetes'), and the second subgroup received daily 10 mg·kg^{-1} body weight of melatonin (i.p.) ('diabetes+10 mg melatonin'). Melatonin was prepared as a 0·3% solution in the physiological saline, containing 5% ethanol and injected at 08:00 h. Melatonin was injected in the morning (not before nocturnal increase of the physiological melatonin level) because we evaluated nonspecific antioxidative effect of melatonin. The dose of melatonin was chosen on the bases of previous studies.[22,27] The experiment (melatonin treatment) began 7 days after the STZ injection, and the rats were sacrificed after 30 days of melatonin (or saline) administration.

CCl_4 intoxication. The animals were subdivided into four subgroups: (1) control animals treated only with olive oil (5 ml·kg^{-1}, intragastrically, i.g.) and ethanol (5%, i.p.) in the same volume as the melatonin solution; (2) treated with i.p. melatonin and i.g. olive oil; (3) treated with the 5% ethanol solution and CCl_4; and (4) treated with melatonin and CCl_4. CCl_4 was administered i.g. at a dose of 4 g·kg^{-1} body weight (2·5 ml·kg^{-1}) in a 50% olive oil solution at 9 h, and the rats were decapitated on the following day at 9 h. Melatonin (10 mg·kg^{-1} body weight) was injected three times: at 30 min before and then 2 and 6 h after the CCl_4 administration. The rats fasted for 24 h before CCl_4 treatment, but they received water *ad libitum*. Hepatic toxicity of CCl_4 was estimated by means of biochemical measurements and analysis of mitochondrial respiration activity.

The animals were killed by decapitation according to the rules defined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Blood samples were drawn by an abdominal aorta puncture into tubes containing hirudin (50 μg·ml^{-1}). After the removal of plasma by centrifugation, the erythrocytes were washed three times with cold PBS, pH 7·4, and haemolysed.

Isolation of rat liver mitochondria

Mitochondria were isolated by differential centrifugation from the liver.[14] The liver was quickly removed and placed into the ice-cold isolation medium containing 250 mmol·l^{-1} of sucrose, 20 mmol·l^{-1} of Tris-HCl and 1 mmol·l^{-1} of EDTA, pH 7·2. The tissue was cut into small pieces and homogenized in a glass-Teflon homogenizer with isolation

medium (at 2°C). The homogenate was centrifuged at 600 *g* for 10 min, and the supernatant was centrifuged at 8500 *g* for 10 min. The obtained pellet was washed in buffer containing 250 mmol·l^{-1} of sucrose and 5 mmol·l^{-1} of Tris-HCl, pH 7·2 (at 2°C). The final centrifugation was carried out at 8500 *g* for 10 min. The mitochondrial pellet was resuspended in the buffer to an approximate protein concentration of 35–40 mg·ml^{-1}. The protein concentration was determined by the method of Lowry *et al.*.[18]

The 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.[7] The electrode was calibrated by bubbling the polarographic cell with pure nitrogen (pO_2=0) and atmospheric air (atmospheric pO_2). The oxygen consumption by the electrode was negligible. The incubation medium contained 125 mmol·l^{-1} of sucrose, 20 mmol·l^{-1} of Tris-HCl, 50 mmol·l^{-1} of KCl, 20 mmol·l^{-1} of KH_2PO_4, 5 mmol·l^{-1} of $MgSO_4$ and 1 mmol·l^{-1} of EDTA, pH 7·5. The experiments were performed at 26·5°C using 5 mmol·l^{-1} of succinate or 4 mmol·l^{-1} of L-glutamate as substrates. Mitochondrial protein concentration in the probe was 1.0 mg·ml^{-1}. The functional state of the mitochondria was determined by the acceptor control ratio (ACR), equal to the ratio of the respiratory rates (V_3/V_2) of the mitochondria in States 3 and 2; by the respiratory control ratio (RCR), equal to the ratio of the respiratory rates (V_3/V_4) of the mitochondria in States 3 and 4; and by the coefficient of phosphorylation (ADP/O), according to the common terminology.[6] The rate of mitochondrial respiration corresponding to State 3 (V_3) was registered after addition of 180 μmol·l^{-1} of ADP (in the presence of 210 nm of ADP in the polarographic cell). State 1 corresponded to the respiration in the presence of endogenous substrates (V_1), State 2 corresponded to the respiration in the presence of substrate (glutamate or succinate) added (V_2) and State 4 corresponded to the respiration when the ADP added was exhausted (V_4).

Mitochondrial transmembrane potential

Mitochondrial transmembrane potential ($\Delta\psi$) was measured in the medium (200 mmol·l^{-1} of sucrose, 20 mmol·l^{-1} of Tris-HCl, 2·5 mmol·l^{-1} of $MgSO_4$, 2·5 mmol·l^{-1} of KH_2PO_4, 20 mmol·l^{-1} of KCl, and 1 mmol·l^{-1} of EDTA, pH 7·2) at 27°C using fluorescence of the cationic dye safranin O that is accumulated and quenched inside the energized mitochondria. The excitation wavelength was 495 nm (slit 3 nm), and the emission wavelength was 586 nm (slit 3 nm) [an SM 2203 spectrofluorimeter (Solar, Belarus)]; the dye concentration used was 8 μmol·l^{-1} ,[2] and the mitochondrial protein concentration was 0·3 mg·ml^{-1}. For mitochondrial membrane depolarization, we used the uncoupler, 2,4-dinitrophenol (36 μmol·l^{-1}), in the presence of NaN_3 (5 mmol·l^{-1}) as inhibitor of the respiratory chain. The membrane potential was determined using a calibration plot,[24] which represents the dependence of the fluorescence intensity of safranin O on the membrane potential value, according to the Nernst equation:

$$\Delta\Psi = 60\log[K^+]_{out}/[K^+]_{in}(mV),$$

where the $[K^+]_{in}$ is the intramitochondrial potassium concentration (120 mmol·l^{-1}), [2] and the $[K^+]_{out}$ is the extramitochondrial potassium concentration in the media that varies from 0 to 20 mmol·l^{-1} in the presence of ionophore valinomycine (0·28 μmol·l^{-1}). We used succinate and α-ketoglutarate as substrates for potential generation.

Biochemical measurements

A stable form of glycated haemoglobin containing 1-deoxy-1(N-valyl)fructose and blood plasma urea were assayed using a reagent set (Pliva-Lachema a.s., Brno, Czech Republic). The concentration of nonprotein thiols (predominantly, of GSH in mitochondria was determined spectrophotometrically by the method of Ellman [9] using the molar absorption coefficient $\varepsilon_{412}=1{\cdot}36{\cdot}10^4 M^{-1}{\cdot}cm^{-1}$. Mixed disulfides (GSSP) formed by glutathione and accessible sulfhydryl groups of mitochondrial proteins were determined by the method described by Rossi *et al.*.[32]

Statistical analysis

Data for 8–10 rats in each group are presented as a mean ± SD for the normally distributed parameters or as a median and interquartile range for the data showing departures from normality. We used the standard Student's *t*-test for the comparison of the raw and transformed data showing no departures from normality (according to Shapiro-Wilk's test) and the nonparametric Mann-Whitney *U*-test for the remaining variables. $p<0{\cdot}05$ was taken to indicate statistical significance.

RESULTS

After 30 days of diabetes in rats, we observed the typical signs of hyperglycemia: a high glycosylated haemoglobin level and animal growth retardation (Table 1). The melatonin administration showed a slight hypoglycemic effect and some reduction of the glycosylated haemoglobin level. We observed a statistically significant increase of the rat blood urea under diabetes (by 40%, $p<0{\cdot}05$) as a result

Table 1. Blood glucose and urea, glycated haemoglobin and body weight in nontreated and melatonin-treated STZ-diabetic rats

Animal groups	Blood glucose, (mg·dl^{-1})	Body weight (final-initial), (g)	Glycated haemoglobin, (mol fructose/g Hb)	Blood urea, (mmol·l^{-1})
Control	105·3±5·9	35	2·7±0·6	7·7±2·2
Diabetes	522·7±179·4	8	6·1±0·6*	10·8±3·4*
Diabetes+ melatonin	405·3±147·8	24.2	4·8±1·7	10·8±2·8*

Data, presented as a mean±SD, represent values at the termination of the experiment (30 days of experimental diabetes).
*$p<0{\cdot}05$ versus control nontreated animals.

of an increase in protein catabolism, amino acid deamination and urea synthesis activation. The melatonin administration did not prevent this process. It was postulated that the mitochondrial ROS production and consequent mitochondrial oxidative damage might contribute to the onset, progression and pathological consequences of types 1 and 2 diabetes.[11] The experimental (30-days) STZ-induced diabetes mellitus resulted in a considerable damage of the respiratory activity in rat liver mitochondria (Tables 2 and 3). The basal (endogenous) respiration rate V_1 decreased by 15–20%. In the case of succinate as a respiratory substrate, the rate of oxygen consumption V_2 and the respiration rate V_4 after ADP consumption did not change, but the ADP-stimulated respiration rate V_3 considerably decreased (by 25%, $p<0{\cdot}05$) as well as the ACR V_3/V_2 and the RCR V_3/V_4 markedly diminished (by 25%, $p<0{\cdot}01$, and by 27%, $p<0{\cdot}01$, respectively). Similarly, we observed a decrease of the respiration rate V_2 by 20% and the ADP-stimulated respiration rate V_3 by 35% ($p<0{\cdot}05$) with glutamate as substrate, with no changes in the respiration rate V_4 being noticed. In this case, the ACR and the RCR also decreased (by 20%, $p<0{\cdot}05$, and 25%, $p<0{\cdot}01$, respectively). Surprisingly, the phosphorylation coefficient ADP/O did not change under diabetic liver damage. Therefore, the efficacy of oxygen consumption remained unchanged despite the respiration chain impairments. The melatonin administration during diabetes (10 mg·kg^{-1} body weight, 30 days daily) exerted a considerable protective effect on the liver mitochondria function, reversing the decreased respiration rate V_3 to the control values both for succinate-dependent respiration ($p<0{\cdot}05$ in comparison with diabetic animals) and for glutamate-dependent respiration ($p<0{\cdot}01$). Similarly, the melatonin treatment of diabetic rats reversed the effect of diabetes on the ACR and RCR values both for succinate-dependent respiration and for glutamate-dependent respiration (Tables 2 and 3).

Figure 1 represents the fluorescence intensity dependence of the potential-sensitive dye safranin O incorporated into the mitochondria on the mitochondrial potential (negative inside) that is used as a calibration curve. The mitochondrial Donnane potential calculated from this curve was equal to 45 mV. Using this dependence and the intensity of safranin O fluorescence in the liver mitochondria of diabetic and control animals, we calculated the values of the mitochondrial potential (Figure 2). When we used L-glutamate or α-ketoglutarate as substrates, the membrane potential was generated by electron flow through complexes I, III and IV of the respiratory chain. When mitochondria oxidized succinate in the presence of rotenone, the membrane potential was generated by electron flow through complexes II, III and IV. As seen from Figure 2, despite the changes in the rates of mitochondrial oxygen consumption, we did not observe any significant alterations in the mitochondrial membrane potential under diabetes and melatonin treatment. The treatment of diabetic animals, not control animals, with melatonin slightly increased the membrane potential (by 15% in comparison with control animals) in the mitochondria oxidizing succinate rather than α-ketoglutarate (Figure 2).

We did not observe marked changes in the mitochondrial GSH level and GSSP level under diabetes (Figure 3). In our experiments, the level of mixed protein-glutathione disulfides in rat liver mitochondria was $0{\cdot}12\pm0{\cdot}025$ nmol·mg^{-1} protein; it was about 1% from the mitochondrial GSH content. The melatonin administration had no effect on the GSH content in the liver mitochondria.

The effects of diabetes and melatonin administration on mitochondrial functional activity were compared with those of CCl_4-induced rat intoxication and melatonin treatment. Tables 4 and 5 represent the parameters of liver mitochondria respiration for control, intoxicated and melatonin-treated animals. After 24 h of acute CCl_4 intoxication, we observed considerable mitochondrial dysfunction. The endogenous respiration rate (V_1) decreased by 30%, the succinate-dependent respiration rate and the glutamate-dependent respiration rate coupling with phosphorylation (V_3) markedly reduced (by 65%, $p<0{\cdot}0001$, and by 50%, $p<0{\cdot}01$, respectively (Tables 4 and 5). Under intoxication, the ACR and the RCR approached 1, reflecting the loss of respiration control. The coefficient of phosphorylation significantly decreased, demonstrating a considerable reduction of the efficacy of oxygen consumption by the mitochondria under intoxication. Under intoxication, the membrane potential slightly decreased (by 12%) for glutamate-dependent,

Table 2. Parameters of oxidative phosphorylation processes in rat liver mitochondria oxidizing succinate under STZ-induced diabetes (30 days). Effect of melatonin administration (10 mg·kg^{-1} body weight, 30 days daily).

	Basal respiration rate V_1, (ng atom O/min)	Substrate-dependent respiration rate V_2, (ng atom O/min)	ADP-stimulated respiration rate V_3, (ng atom O/min)	Respiration rate after ADP-consumption V_4, (ng atom O/min)	ACR (V_3/V_2)	RCR (V_3/V_4)	Coefficient of phosphorylation (ADP/O)
Control	12·2±1·8	35·7±4·3	140·9±10·7	32·0±3·6	4·05±0·19	4·55±0·31	1·86±0·08
Diabetes	9·1±1·4	35·0±2·5	107·4±7·6*	33·5±4·0	3·09±0·13†	3·32±0·2†	1·8±0·1
Diabetes+melatonin	11·2±1·5	31·6±3·4	134·8±4·9‡	28·14±2·0§	4·46±0·39§	4·91±0·36§	1·86±0·07

Data, presented as a mean±SEM, represent values at the termination of the experiment (30 days of experimental diabetes).
*$p<0{\cdot}05$ versus the control nontreated animals;
†$p<0{\cdot}01$ versus the control nontreated animals;
‡$p<0{\cdot}05$ versus the diabetic group;
§$p<0{\cdot}01$ versus the diabetic group.

Table 3. Parameters of oxidative phosphorylation processes in rat liver mitochondria oxidizing glutamate under STZ-induced diabetes (30days). Effect of melatonin administration (10mg·kg^{-1} body weight, 30days daily).

	Basal respiration rate V_1, (ng atom O/min)	Substrate-dependent respiration rate V_2, (ng atom O/min)	ADP-stimulated respiration rate V_3, (ng atom O/min)	Respiration rate after ADP-consumption V_4, (ng atom O/min)	ACR (V_3/V_2)	RCR (V_3/V_4)	Coefficient of phosphorylation (ADP/O)
Control	14·2±2·1	25·1±2·2	104·1±12·2	17·3±3·4	4·14±0·32	6·65±0·76	2·33±0·08
Diabetes	12·0±1·4	20·5±1·1*	66·0±4·9*	18·8±3·1	3·25±0·25*	4·01±0·53*	2·24±0·09
Diabetes+melatonin	11·7±1·5	18·6±2·4*	90·6±4·9‡	16·1±1·9	5·26±0·65†	5·84±0·54†	2·6±0·1†

Data, presented as a mean±SEM, represent values at the termination of the experiment (30days of experimental diabetes).
*$p<0·05$ versus the control nontreated animals;
†$p<0·05$ versus the diabetic group;
‡$p<0·01$ versus the diabetic group.

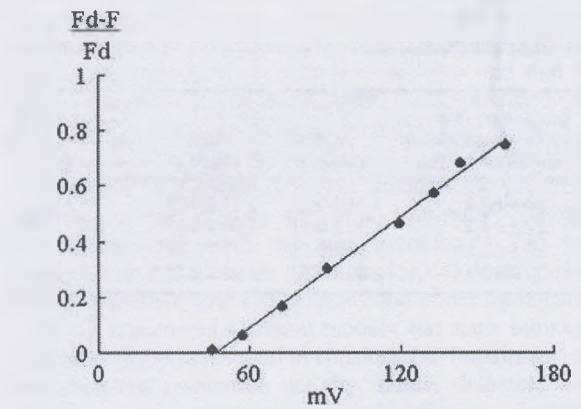

Figure 1. Dependence of the intensity of safranin O incorporated into the rat liver mitochondria on the mitochondrial membrane diffusion K^+ potential measured in the following medium: 200mmol·l^{-1} of sucrose, 20 mmol·l^{-1} of Tris-HCl, 1mmol·l^{-1} of EDTA, pH 7·2, supplemented with 8μ mol·l^{-1} of safranin O, 5mmol·l^{-1} of succinate, 0.28μmol·l^{-1} of valinomycine. F, dye fluorescence intensity in the mitochondrial suspension; F_d, dye fluorescence intensity in the mitochondrial suspension in the presence of 2,4-dinitrophenol (36μmol·l^{-1}). [K^+_{out}] was changed from 0 to 20mmol·l^{-1}. Protein concentration—0·3mg·ml^{-1}

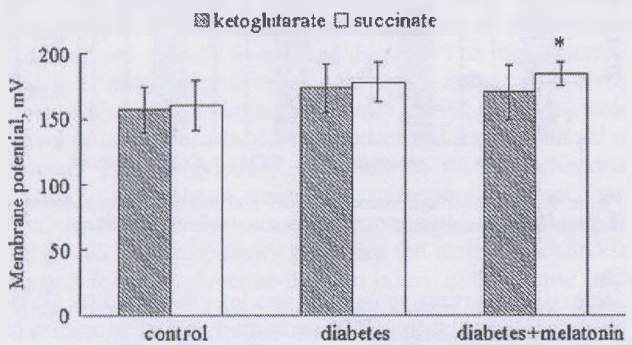

Figure 2. Rat liver mitochondrial membrane potential under diabetes. Effect of melatonin. Potential was measured by fluorescence of the cationic dye safranin O incorporated into the mitochondria

not for succinate-dependent respiration (Figure 4). For both substrates, we did not observe membrane potential changes after melatonin administration to the control animals. Rat

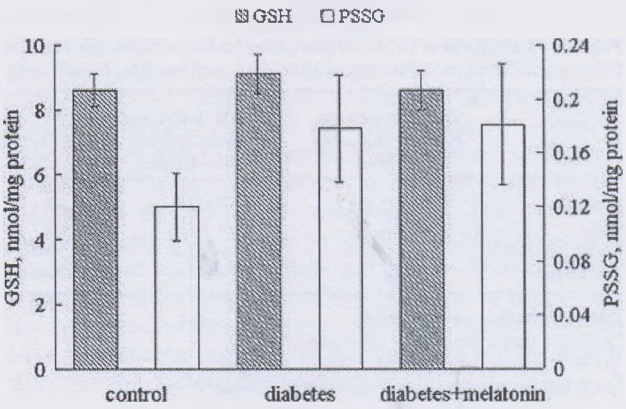

Figure 3. Rat liver mitochondrial GSH and mixed protein-glutathione disulfide levels under diabetes. Effect of melatonin

intoxication under melatonin administration diminished the membrane potential generated by glutamate-dependent respiration by 45% ($p<0·05$) and the membrane potential generated by succinate-dependent respiration by 20% (Figure 4). One can suggest interaction of melatonin with complex I of the mitochondrial respiration chain.

The fundamental impairments in the mitochondria under intoxication were associated with the oxidation of intramitochondrial GSH by 25% ($p<0·05$) and the increased level of mixed disulfides of glutathione with intramitochondrial proteins (Figure 5). The melatonin administration in a dose of 10mg·kg^{-1} to the control animals increased the rates of the glutamate-dependent respiration V_2 by 60% ($p<0·05$), V_3 by 15% and V_4 by 45% ($p<0·05$) (Table 5) but did not influence the parameters of succinate-dependent respiration. At the same time, the melatonin administration to intoxicated rats led to an increase in the rate of succinate-dependent respiration coupled with phosphorylation (V_3) by 30% ($p<0·05$) in comparison with that for mitochondria from intoxicated animals (Table 4).

DISCUSSION

Results of many studies suggest a role of oxidative stress (high levels of free radicals and a simultaneous decline of

Table 4. Parameters of oxidative phosphorylation processes in rat liver mitochondria oxidizing succinate after 24h of acute CCl_4 (4g·kg^{-1} body weight, i.g.). Effect of melatonin administration (10mg·kg^{-1} body weight, three times).

	Basal respiration rate V_1, (ng atom O/min))	Substrate-dependent respiration rate V_2, (ng atom O/min)	ADP-stimulated respiration rate V_3, (ng atom O/min)	Respiration rate after ADP-consumption V_4, (ng atom O/min)	ACR (V_3/V_2)	RCR (V_3/V_4)	Coefficient of phosphorylation (ADP/O)
Control	15·7±3·4	47·8±5·3	153·9±11·2	49·9±5·3	3·20±0·40	3·13±0·30	1·64±0·1
CCl_4	11·1±4·8	61·6±4·6	54·2±3·6‡	58·4±4·2	1·00±0·12‡	1·10±0·08‡	0·0
Melatonin+CCl_4	10·9±2·1*	60·2±4·8	71·9±5·0‡§	60·4±4·6	1·13±0·13‡	1·18±0·14‡	0·0
Melatonin	13·6±2·4	53·6±6·7	141·4±15·4	45·9±4·8	2·6±0·3	3·03±0·40	1·48±0·2

Data presented as mean±SEM.
*$p<0·05$ versus the control nontreated animals;
†$p<0·01$ versus the control nontreated animals;
‡$p<0·001$ versus the control nontreated animals;
§$p<0·05$ versus the CCl_4-intoxicated animals.

Table 5. Parameters of oxidative phosphorylation processes in rat liver mitochondria oxidizing glutamate after 24h of acute CCl_4 (4g·kg body weight, i.g.). Effect of melatonin administration (10mg·kg^{-1} body weight, three times).

	Basal respiration rate V_1, (ng atom O/min))	Substrate-dependent respiration rate V_2, (ng atom O/min)	ADP-stimulated respiration rate V_3, (ng atom O/min)	Respiration rate after ADP-consumption V_4, (ng atom O/min)	ACR (V_3/V_2)	RCR (V_3/V_4)	Coefficient of phosphorylation (ADP/O)
Control	14·3±4·1	18·7±2·3	63·1±4·7	20·6±2·2	3·41±0·32	3·11±0·29	1·6±0·1
CCl_4	10·6±3·1	20·8±4·6	27·5±4·2†	27·5±4·2	1·36±0·51†	1·00±0·02‡	0·0
Melatonin+CCl_4	13·6±3·1	21·1±3·5	29·5±3·7†	26·5±2·8	1·39±0·53*	1·11±0·09‡	0·0
Melatonin	15·9±3·4	33·0±3·6*	73·4±7·7	30·4±3·3*	2·23±0·22	2·41±0·22	1·7±0·2

Data presented as mean±SEM.
*$p<0·05$ versus the control nontreated animals;
†$p<0·01$ versus the control nontreated animals;
‡$p<0·001$ versus the control nontreated animals.

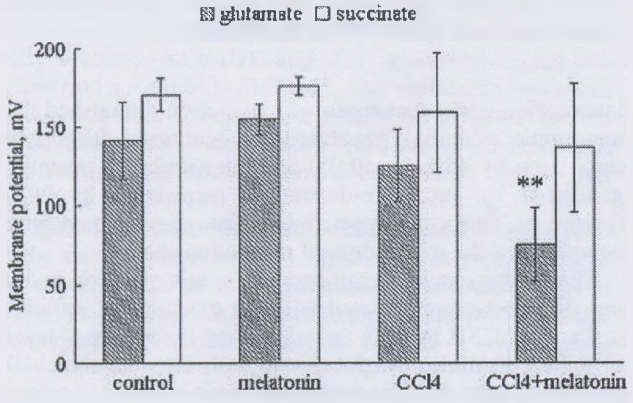

Figure 4. Rat liver mitochondrial membrane potential under acute CCl_4 intoxication. Effect of melatonin. Potential was measured by fluorescence of the cationic dye safranin O incorporated into the mitochondria

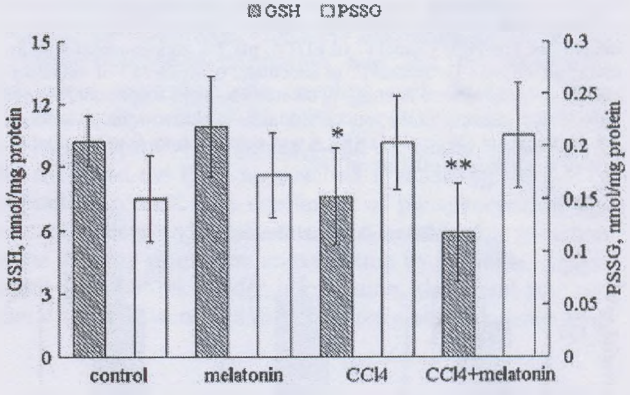

Figure 5. Rat liver mitochondrial GSH and mixed protein-glutathione disulfide levels under acute CCl_4 intoxication. Effect of melatonin

antioxidant defense mechanisms) in the development of insulin resistance, diabetes and diabetic complications, and evidence for a protective effect of antioxidants.[21,30] An elevated membrane electrochemical potential $\Delta\mu_{H+}$ favors mitochondrial free radical production, and limiting the magnitude of this potential under State 4 conditions should decrease free radical production.[33] Reactive oxygen species interfere with insulin signalling and inhibit the translocation of glucose transporter GLUT4 in the plasma membrane.[30] By the same way, intoxication by acetaminophen resulted in increasing the reactive oxygen species generation by GSH-depleted mitochondria, JNK activation and translocation of activated JNK to the mitochondria where JNK induces mitochondrial permeability transition and inhibits mitochondria bioenergetics.[12] It was shown that enhanced oxidative stress caused by diabetes is accepted to lead to endothelial dysfunction, and melatonin treatment (10mg·kg^{-1} i.p. for 8 weeks) protected the contractile functions of the aorta and the corpus cavernosum and prevented the oxidative stress under diabetes.[27] Similarly, melatonin administration for 15 days

($200\mu g \cdot kg^{-1}$ i.p.) effectively normalized the impaired antioxidant status in STZ-induced diabetes [4] and may be useful in delaying the complicated effects of diabetes. Strategies to decrease mitochondrial radical production and oxidative damage may have a therapeutic potential. It has recently been shown that melatonin ($10 mg \cdot kg^{-1}$) improves methanol intoxication-induced oxidative liver injury in rats, preventing reduction in all antioxidant enzyme activities, decreasing significantly the levels of malondialdehyde and protein carbonylation, as well as necrosis and portal inflammation.[16] In our earlier experiments, the melatonin administration prevented structural and functional liver damage under CCl_4 intoxication [38] but did not cause a pronounced recovery of the mitochondrial functional activity.[20] Recently, close relationships have been found between insulin, the endocrine system and melatonin.[28] Peschke showed that melatonin influenced insulin secretion both *in vivo* and *in vitro* and that these effects were MT(1)-receptor and MT(2)-receptor mediated.[28] Melatonin ($10 mg \cdot kg^{-1}$) can increase the activities of mitochondrial respiratory complexes I and IV after its administration in a time-dependent manner and prevented a decrease in the activity of these respiratory complexes induced by ruthenium red administration.[22] The results suggest that melatonin in pharmacological doses protected against oxidative stress in diabetes and under intoxication.

In our experimental animal models, the acute intoxication as well as diabetes resulted in considerable impairment of the mitochondrial respiration activity. Under diabetes, we observed a pronounced decrease of the ADP-stimulated oxygen consumption V_3, as well as the acceptor and respiration control ratios without an appreciable change in the ADP/O ratio. In comparison, under intoxication, we found that the respiration rate, V_3, and the phosphorylation coefficient considerably decreased, and the RCR and the ACR diminished, approaching 1, which indicates uncoupling of the oxidation and phosphorylation processes and loss of respiration control. At the same time, the mitochondrial membrane potential under intoxication and diabetes did not change significantly. Mitochondrial dysfunction may be the first sign of toxic and diabetic liver tissue damage. The impairment of rat liver mitochondrial respiration under intoxication correlated with the disturbed mitochondrial redox balance (reduced level of intramitochondrial GSH level and increased level of protein glutathionylation). It is known that mitochondrial redox (GSH) status modulates mitochondrial functional activity.[26] It was shown earlier that complexes I, III and IV of the electron respiratory chain are the main mitochondrial targets for hyperglycemia-induced injury in the mouse heart under diabetes.[37] Two months of diabetes were not shown to alter the renal cortical mitochondrial respiration. In contrast, 12months of diabetes caused a significant inhibition of State 3 respiratory activity and an increase of respiratory activity in State 4.[31] The investigation of a possible functional defect in cardiac mitochondria from diabetic rats indicates a decline in State 3 respiration only in animals having a marked decrease in body weight; heart mitochondria from hyperglycemic rats showed an increase in State 4 glutamate-dependent oxygen consumption.[17] Four-week and 9-week diabetes did not substantially affect the brain mitochondrial function.[25]

The direct inhibition of the respiration chain enzyme complexes and damage of mitochondrial membrane may be signs of the mitochondrial dysfunction in diabetes and under intoxication. Meanwhile, there are different molecular mechanism(s) and different targets of mitochondrial damage in diabetes and under intoxication. The melatonin administration in a pharmacological dose affected the mitochondrial function and prevented mitochondrial dysfunction, demonstrating mitochondria-specific activity. Under diabetes, melatonin reversed the decreased succinate-dependent and glutamate-dependent respiration V_3 rates and the ACR and RCR. Under intoxication, the pharmacological dose of melatonin ($10 mg \cdot kg^{-1}$) induced some increase of the succinate-dependent V_3 rate and further dissipation of the glutamate-dependent membrane potential of rat liver mitochondria. It has been shown that melatonin may recycle NADH by electron donation and, by this way, may improve the efficiency of NADH as an energy carrier and antioxidant. Interactions between melatonin and NADH may be implicated in mitochondrial metabolism.[36] The effects of melatonin might be caused by both its radical scavenging properties, its metabolic effects and specific interaction with complexes of the respiratory chain. Our results suggest that the melatonin, while regulating mitochondrial function, may have a therapeutic potential for the correction of diabetic liver damages and diabetic complications.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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