Effect of Silimarin, Succinic Acid, and Their Combination on Bioenergetics of the Brain in Experimental Encephalopathy

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In rats with experimental encephalopathy caused by intoxication with 4-pentenoic acid inhibiting β-oxidation of medium- and long-chain fatty acids, hepatoprotector silimarin inhibited LPO, prevented deenergization and maintained high respiratory activity of brain mitochondria, and increased the rate and coupling of oxidation and phosphorylation. Succinic acid improved oxidation of substrates in motochondria and promoted activation of succinate-dependent ATP generation. Silimarin and succinic acid used together produced a synergistic protective effect on brain mitochondria surpassing the protective effects of individual preparations and prevented LPO activation.

Key Words: *silimarin; succinate; bioenergetics; experimental encephalopathy; cerebroprotective effect*

Silimarin and succinic acid (SA) exhibit antioxidant properties, improve bioenergetics of hepatocytes, stimulate neutralization of ammonium in the ornithine cycle of urea synthesis, phenol sulfation, and bilirubin glucuronidation under conditions of liver pathologies caused by 4-pentenoic acid (4-PA), an inhibitor of β-oxidation of medium- and long-chain fatty acids (MLFA) [1]. It was hypothesized that silimarin and SA in individual and combined administration restore metabolic processes in the brain disturbed due to insufficiency of the antitoxic function of the liver and release of toxic substances into circulation.

Here we studied the effect of silimarin, SA, and their combination on metabolic status of mitochondria in the brain of rats with experimental encephalopathy developed under conditions of inhibition of MLFA β-oxidation against the background of 4-PA intoxication.

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MATERIALS AND METHODS

Experiments were carried out on 70 mongrel male rats weighing 200-220 g obtained from laboratory animal clinic, Institute of Pharmacology, Tomsk Research Center, Siberian Division of Russian Academy of Medical Sciences. The animals were kept under standard vivarium conditions with natural illumination and free access to food and water. The experiments were performed in accordance with Manual on Experimental (Preclinical) Testing of New Pharmacological Agents [3].

The animals received daily intraperitoneal injections of 4-PA (ISN) in a dose of 20 mg/kg for 7 days [10]. The test preparations in effective therapeutic doses [6,7] were administered through a gastric tube for 14 days starting from day 8 of the experiment: 100 mg/kg silimarin (Madaus), 50 mg/kg SA, or their combination in the same doses. Controls received the solvent for silimarin and SA (1%

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starch suspension). The rats were decapitated under ether narcosis 12 h after the last dose of the preparations.

Functional state of mitochondria (MC) in brain homogenate was studied polarographically (RA-2 polarograph) by the rate of O_2 consumption in different metabolic states using a closed Clark electrode made in our laboratory [2,7]. We measured the rate of O_2 consumption before (V_{4P}), during (V_3), and after (V_{4O}) the cycle of phosphorylation of added ADP (0.1 mM) during oxidation of endogenous substrates, flavin adenine-dinucleotide (FAD)-dependent substrate succinate (1 mM), and NAD-dependent substrates malate and glutamate (3 mM each) and the time of phosphorylation of added ADP (T_P). For evaluation of the energetic status of mitochondria, we calculated the coefficient of oxidation-phosphorylation coupling (ADP/O). The contribution of oxidation of endogenous succinate during oxidation of NAD-dependent substrates by mitochondria was determined after addition of SDH inhibitor malonate (2 mM) to the incubation medium and aminotransferase inhibitor aminooxyacetate (0.5 mM). LPO activity was evaluated by the rate of MDA formation in the presence of oxidation initiator ascorbate by the content of conjugated dienes and Schiff bases.

The data were processed statistically using nonparametric Mann—Whitney test, error probability did not exceed 5% ($p<0.05$).

RESULTS

4-PA acylates carnitine and prevents its participation in MLFA transport into mitochondria for β-oxidation [10]. Acute period of experimental encephalopathy after the end of 4-PA injections was characterized by a 1.3-1.7-fold decrease in the rates of respiration in all metabolic states during oxidation of endogenous substrates, exogenous succinate, and malate-glutamate mixture; T_P increased by 1.6-2.0 times and oxidation—phosphorylation coupling decreased by 25-55%. During utilization of endogenous substrates V_{4O} surpassed V_{4P}. These changes attested to weakened energetic respiration control. It is indicative that energization of mitochondria with the substrate added to the incubation medium partially restored the energetic control of respiration ($V_{4O}=V_{4P}$) due to different effects on oxidation—phosphorylation coupling: ADP/O coefficient decreased in case of utilization of NAD-dependent substrates and decreased in case of utilization of succinate. These data suggest that the disturbances in oxidative phosphorylation in brain mitochondria during 4-PA intoxication develop against the background of endogenous substrate deficiency. The use of inhibitors malonate and aminooxyacetate together with malate and glutamate led to changes in mitochondrial oxidation similar for NAD- and FAD-dependent substrates: decrease in the respiration rate in all metabolic states and ADP/O coefficient (Table 1). The rate of MDA production increased by 2.3 times, the content of conjugated dienes and Schiff bases increased by 2.1 and 1.3 times, respectively, compared to LPO parameters under normal conditions (Table 2).

In delayed period of inhibition of β-oxidation of fatty acids (day 14 after the end of 4-PA injections), metabolic disturbances in the brain tissue progressed. Inhibition of respiratory activity of mitochondria during oxidation of both types of substrates and signs of oxidation—phosphorylation uncoupling increased, the energizing effects of substrates persisted (V_{4O} decreased to V_{4P}), the rate of MDA production increased by 1.7 times, the content of conjugated dienes and Schiff bases 2.3 and 1.2 fold surpassed the levels observed immediately after the end of 4-PA treatment (Tables 1 and 2). Renewal of mitochondria takes about a week; therefore, new mitochondria immediately after formation exist under conditions of oxidation—phosphorylation uncoupling caused by MLFA. Moreover, these fatty acids disturb electron transport in the respiratory chain and stimulate generation of free radicals [9].

Thus, inhibition of MLFA β-oxidation drastically disturbs energy metabolism in the brain (signs of substrate deficiency inhibition and uncoupling of oxidative phosphorylation) against the background of LPO activation.

Therapy of experimental encephalopathy with hepatoprotector silimarin for 14 days improved metabolic processes in the brain. Respiratory activity of mitochondria in all metabolic states increased, V_{4O} decreased and became equal to V_{4P}, oxidation—phosphorylation coupling during oxidation of endogenous substrates, NAD-dependent substrates, and succinate increased. The majority of parameters of oxidative phosphorylation in brain mitochondria from rats receiving silimarin did not differ from normal (Table 1). Silimarin produced a pronounced antioxidant effect in experimental encephalopathy. The content of conjugated dienes and Schiff bases in brain homogenate did not differ from that in intact animals, the intensity of MDA production was 2.2-fold lower than in rats not protected with silimarin (Table 2). The antioxidant effect of silimarin flavonoids [4,5] play an important role in prevention of oxidative phosphorylation disturbances in brain tissue during inhibition of MLFA β-oxidation.

TABLE 1. Effect of Silimarin, SA, and Their Combination on Oxidative Phosphorylation in Brain Homogenate against the Background of Experimental Inhibition of MLFA β-Oxidation Caused by 4-PA (*M*±*m*; *n*=10)

Parameter	Intact animals	4-PA for 7 days	4-PA after 14 days	Silimarin+ 4-PA	SA+ 4-PA	Silimarin+ SA+4-PA
Oxidation of endogenous substrates						
V_{4P}	38.0±1.7	22.8±1.8[1]	12.4±0.3[1,2]	29.3±1.2[1-3]	13.2±0.5[1,2,4]	39.3±1.5[2-5]
V_3	52.7±1.6	41.6±2.2[1]	31.6±1.5[1,2]	50.4±1.6[2,3]	24.7±1.3[1-4]	51.8±1.1[2,3,5]
V_{4O}	38.4±1.1	31.4±1.9[1]	13.8±0.8[1,2]	34.8±1.6[3]	20.7±1.4[1-4]	38.2±1.7[2,3,5]
ADP/O	1.65±0.2	0.90±0.04[1]	0.90±0.10[1]	1.60±0.20[2,3]	1.10±0.10[1,4]	1.70±0.10[2,3,5]
T_P	1.20±0.10	2.30±0.30[1]	2.80±0.10[1]	1.10±0.10[2,3]	1.70±0.08[1-4]	1.20±0.10[2,3,5]
SA oxidation						
V_{4P}	37.9±1.3	28.9±1.4[1]	16.5±1.4[1,2]	33.3±0.8[2,3]	24.5±1.2[1,3,4]	36.3±1.2[2,3,5]
V_3	91.7±3.7	57.3±2.5[1]	32.1±1.6[1,2]	73.5±1.7[1-3]	56.7±3.1[1,3,4]	79.4±1.1[1-5]
V_{4O}	36.3±1.3	25.5±1.9[1]	15.3±1.5[1,2]	36.8±1.6[2,3]	35.4±1.1[2,3]	36.5±0.7[2,3]
ADP/O	2.10±0.20	1.57±0.20	1.35±0.03[1]	2.12±0.10[2,3]	1.90±0.08[2,3]	2.00±0.10[3]
T_P	0.80±0.02	1.63±0.09[1]	1.60±0.02[1]	0.90±0.02[2,3]	1.10±0.03[2,3]	0.80±0.02[2,3]
Malate+glutamate oxidation						
V_{4P}	33.6±1.4	26.6±1.4[1]	16.3±0.4[1,2]	34.3±1.0[2,3]	42.4±2.4[1-4]	41.6±1.4[1-4]
V_3	85.1±3.1	71.4±3.5[1]	30.3±0.5[1,2]	83.6±1.8[2,3]	82.1±2.0[2,3]	85.4±1.3[2,3]
V_{4O}	29.8±1.2	20.1±0.7[1]	16.2±1.0[1,2]	29.8±1.3[2,3]	43.5±2.2[1-4]	37.4±1.0[1-5]
ADP/O	2.70±0.07	1.20±0.04[1]	0.90±0.06[1,2]	2.65±0.08[2,3]	1.70±0.02[1-4]	2.70±0.02[2,3,5]
T_P	0.80±0.08	1.30±0.03[1]	1.50±0.05[1,2]	0.90±0.07[3]	1.10±0.05[3]	0.80±0.02[2,3,5]
Malate+glutamate oxidation in the presence of malonate						
V_{4P}	29.2±1.9	22.8±1.3[1]	16.1±0.9[1,2]	32.5±1.2[2,3]	23.9±1.9[1,3,4]	31.6±1.3[2,3,5]
V_3	70.6±2.6	53.7±4.8[1]	28.1±2.5[1,2]	69.9±2.4[2,3]	42.4±1.2[1-4]	68.6±1.5[2,3,5]
V_{4O}	29.4±1.4	21.1±1.3[1]	13.9±0.1[1,2]	28.8±1.8[2,3]	26.6±1.9[2,3]	27.2±1.0[2,3]
ADP/O	2.02±0.07	1.42±0.04[1]	1.27±0.05[1,2]	1.90±0.08[2,3]	0.80±0.08[1-4]	2.10±0.08[2,3,5]
T_P	0.70±0.05	0.80±0.02	1.40±0.04[1,2]	0.80±0.06[3]	1.10±0.04[1-4]	0.80±0.03[3,5]
Malate+glutamate oxidation in the presence of aminooxyacetate						
V_{4P}	30.1±1.0	24.6±1.4[1]	24.1±0.9[1]	31.2±1.1[2,3]	23.9±1.9[1,4]	30.2±1.5[2,3,5]
V_3	41.8±2.9	32.9±1.6[1]	28.2±1.4[1]	42.5±1.2[2,3]	42.4±1.2[2,3]	41.4±0.9[2,3]
V_{4O}	28.4±1.3	23.0±1.0[1]	16.1±1.3[1,2]	29.3±1.7[2,3]	26.6±1.8[3]	27.4±1.1[2,3]
ADP/O	3.20±0.02	1.90±0.08[1]	1.40±0.02[1,2]	3.30±0.08[2,3]	2.80±0.08[1-4]	3.30±0.08[2,3,5]
T_P	1.00±0.09	1.30±0.06[1]	1.80±0.07[1,2]	0.90±0.08[2,3]	1.10±0.03[2,3]	0.80±0.04[2,3,5]

Note. Dimensions: respiration rate (V_{4P}, V_3, V_{4O}) in ng-atom O_2/min/mg mitochondrial protein, T_P in min/mg mitochondrial protein. Here and in Table 2: $p<0.05$ compared to [1]intact animals, [2]4-PA for 7 days, [3]14 days after termination of 4-PA injections, [4]silimarin, [5]SA.

TABLE 2. Effect of Silimarin, SA, and Their Combination on LPO in Brain Homogenate against the Background of Experimental Inhibition of MLFA β-Oxidation Caused by 4-PA (*M*±*m*; *n*=10)

Parameter	Intact animals	4-PA for 7 days	4-PA after 14 days	Silimarin+ 4-PA	SA+ 4-PA	Silimarin+ SA+4-PA
MDA, nmol/mg protein/min	0.25±0.03	0.58±0.01[1]	0.98±0.05[1,2]	0.44±0.02[1-3]	0.62±0.0[1,3,4]	0.41±0.01[1-5]
Conjugated dienes, U/mg lipids	0.24±0.03	0.51±0.07[1]	1.15±0.09[1,2]	0.28±0.06[2,3]	0.48±0.07[1,3,4]	0.26±0.07[2,3,5]
Schiff bases, U/mg lipids	1.85±0.100	2.38±0.12[1]	2.85±0.10[1,2]	1.82±0.03[2,3]	2.19±0.09[1,3,4]	1.80±0.05[2,3,5]

In rats with experimental encephalopathy treated with SA, oxidation of endogenous substrates in brain mitochondria was characterized by low respiration rate in metabolic states V_3 and V_{4P}. V_{4O} surpassed V_{4P} by 57%, which together with the absence of significant growth of ADP/O coefficient attested to deenergization of mitochondria, suppression of energetic respiration control, and substrate deficiency, despite almost normal ADP phosphorylation rate. The use of succinate as the oxidation substrate increased the rate of mitochondrial respiration and practically normalized the rate and coupling of oxidative phosphorylation. Malate and glutamate used as the substrates increased the rate of phosphorylating respiration in mitochondria from rats receiving SA. The rate of respiration in controlled states (V_{4P} and V_{4O}) became similar and attained maximum values in all groups. The rate of ADP phosphorylation returned to values observed in intact animals, ADP/O coefficient was lower by 37%. We observed considerable normalizing effect of substrates on mitochondria from the brain of rats with experimental encephalopathy receiving SA. Malonate and aminooxyacetate abolished the normalizing effect of NAD-dependent substrates on parameters of oxidative phosphorylation. When aminooxyacetate and malonate were used, ADP/O coefficient was below the normal by only 12% and by 60%, respectively. These findings attest to important role of high SDH activity in the regulation of energy homeostasis in mitochondria and integrity of reactions of rapid substrate oxidation pathway bypassing slow sites of the Krebs cycle (citrate synthase and isocitrate dehydrogenase reactions) with the increase in the contribution of oxidation of endogenous succinate into ATP production (Table 1). Under conditions of SA treatment, the rate of MDA production, the content of conjugated dienes and Schiff bases in brain homogenate little differed from the corresponding levels after termination of 4-PA injections (Table 2).

Under conditions of combined treatment with silimarin and SA (compared to silimarine alone) we observed not only normalization of the majority of parameters of respiratory and phosphorylating activities, but also predominant oxidation of substrates by succinate oxidase system in mitochondria, which is of particular importance for the maintenance of the resistance of the energy production system [8]. Pronounced protective effect of the silimarin+SA combination under conditions of inhibition of MLFA β-oxidation manifested in considerable suppression of LPO.

Thus, silimarin and SA administered together against the background of experimental encephalopathy produce synergistic protective effect on brain mitochondria. For the realization of the antioxidant and energizing effects of SA in low doses affecting cytoreceptors [8] on energy metabolism, flavonolignans of silimarin should maintain the integrity of mitochondrial membranes and thiol enzymes under conditions of oxidation—phosphorylation uncoupling by MLFA excess. Preservation of the structure and function of mitochondria provides conditions for the maintenance of metabolic homeostasis of the brain and eliminates toxic effects of MLFA excess.

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