PHARMACOLOGY AND TOXICOLOGY

Mechanism Underlying the Protective Effect of Glycine in Energetic Disturbances in Brain Tissues under Hypoxic Conditions

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Glycine stabilizes energetics of brain mitochondria under conditions of brain hypoxia *in vivo* modeled by ligation of the common carotid artery in rats. Hypoxia reduced respiratory control in brain cortex mitochondria from 7.7±0.5 to 4.5±0.3. Preliminary oral administration of glycine almost completely prevented this decrease. In both *in vitro* models of hypoxia, similar phosphorylation disturbances were detected in both cortical slices and isolated brain mitochondria; they were effectively prevented by glycine. Hypoxia activates H_2O_2 generation in mitochondrial suspension. The process is significantly reduced in the presence of 5 mM glycine. It is concluded that both in the model of hypoxia *in vivo* and during *in vitro* modeling of hypoxia in cortical slices and mitochondria, glycine acts as a protector inhibiting generation of reactive oxygen species in mitochondria and preventing energetics disturbances in brain mitochondria.

Key Words: *glycine; mitochondria; hypoxia; phosphorylation*

Glycine known as the inhibitory neurotransmitter is effectively used in modern medicine for the therapy of ischemic stroke [2]. However, the mechanism underlying its anti-ischemic action is poorly understood. Glycine protects tissues against intoxication during hypoxia and reperfusion [10,12]. Glycine increases lifespan of cortical neurons under hypoxic conditions [11] and significantly reduces the content of oxidative stress products in the ischemic area as was shown in experiments with focal ischemia [5]. Antiischemic effect of glycine can be related to improvement of microcirculation, since it is known that application of glycine to rat *pia mater* leads to significant (by ~1.5-2 times) dilatation of arterioles [4]. It should be noted that glycine prevents ATP drop and protect cells against necrotic death in the model of chemical hypoxia of hepatic sinusoidal endothelial cells [3]. Authors suggest that glycine blocks opening of unspecific anion channel, activation of which leads to swelling and subsequent rupture of cell plasma membrane. It was demonstrated that glycine protects cardiomyocyte mitochondria against cyclosporin A-sensitive swelling developing after ischemia. This shows that glycine can prevent opening of non-specific mitochondrial pore [6].

Here we studied the mechanisms underlying the anti-ischemic effect of glycine *in vivo* on the model of stroke in rats and *in vitro* on the models of hypoxia in cortical slices and isolated brain mitochondria.

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MATERIALS AND METHODS

Ischemia *in vivo* was simulated by ligation of the left common carotid artery. Before surgery, the animals were intraperitoneally narcotized with chloral hydrate in a dose of 350 mg/kg. The skin on the neck was cut and the muscles were moved apart. The left common carotid artery was found, separated from the connective tissue and nerve bundles, and then ligated. The skin was sutured and powdered with streptocid. Glycine was administered orally in a dose of 40 mg/kg 4 times with 1-h intervals.

Hypoxia of *in vitro* cultured slices of brain cortex. The method of preparing and incubating slices was described elsewhere [9]. Slice thickness was ~500 μ. Incubation medium was artificial cerebrospinal fluid containing (in mM): 125 NaCl, 3.5 KCl, 1 $MgSO_4$, 25 $NaHCO_3$, 1.25 NaH_2PO_4 , 0.25 EGTA, and 5 D-glucose (pH 7.4). Incubation was carried out for 30 min at 37°C.

Preparation of homogenates for measurement of respiratory activity and mitochondrial membrane potential. After 24-h ischemia *in vivo,* the animals were decapitated, the brain was removed and cooled in the incubation medium at 0°C containing (in mM): 125 NaCl, 3.5 KCl, 1 $MgSO_4$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 0.25 EGTA, and 5 D-glucose (pH 7.4). Brain cortex was separated and homogenized at 0°C in a medium containing (in mM): 210 mannitol, 70 sucrose, 1 EDTA, 10 HEPES-Tris, and 1% BSA (pH 7.4).

Homogenates of brain cortex slices were prepared as described elsewhere [9]. Mitochondria were isolated by the standard method of differential centrifugation [9] with minor modifications. Isolation medium contained (in mM): 225 mannitol, 75 sucrose, 20 HEPES-Tris, 1 EDTA, 0.1% BSA.

Mitochondrial respiration was recorded using Clark-type electrode. Standard measurement medium contained (in mM): 120 KCl, 10 HEPES-Tris, 1 K_2HPO_4 (pH 7.4).

Generation of reactive oxygen species (ROS) in mitochondria was assessed using Amplex Red fluorescent labeling. Mitochondria were added to the standard measurement medium containing 10 μM of Amplex Red and 5 U/ml horseradish peroxidase. Fluorescence was recorded at 530 nm excitation and 600 nm emission wavelengths. Calibration was carried out by adding 10 μM hydrogen peroxide.

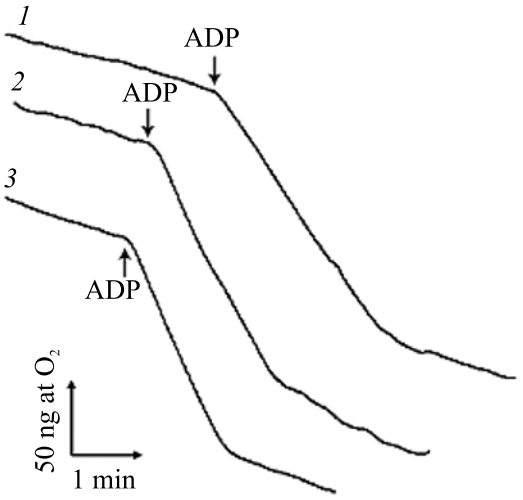

Fig. 1. Glycine prevents functional impairments in mitochondria of the brain cortex after 24-h ischemia (unilateral ligation of the left carotid artery). *1*) homogenate respiration after 24-h ischemia; *2*) homogenate respiration after 24-h ischemia in the presence of glycine; *3*) functional status of brain cortex homogenate without ischemia (control).

RESULTS

Anti-ischemic effect of glycine *in vivo* on rat model of ischemic stroke. Glycine was administered orally to animals before stroke modeling. In 24 h after ligation of the left common carotid artery, functional status of brain cortex mitochondria (respiratory control, RC) was impaired in the left and right hemisphere (Fig. 1). The effect of ligation of the left common carotid artery on RC was pronounced in the left hemisphere (Table 1).

In animals treated with glycine, phosphorylation activity of mitochondria in brain cortex homogenates

TABLE 1. Parameters of Mitochondrial Energetics in Homogenates of Rat Brain Cortex on *In Vivo* Ischemia Model

Ligation of the left common carotid artery in rats	RC after Chance (V3/V4)		ADP/O
	left hemisphere	right hemisphere	
Intact animal (control)	7.7±0.5	7.1±0.4	1.9±0.1
24-h ischemia	4.5±0.3*	5.8±0.3*	1.8±0.1
24-h ischemia+glycine	6.4±0.5+	7.0±0.4+	1.8±0.2

Note. $p<0.05$ in comparison with: *control, +24-h ischemia. V3 and V4: rates of respiration in states 3 and 4 after Chance, respectively.

was completely preserved (Fig. 1, curve *2*) and RC was close to the control (Table 1).

In *in vivo* ischemia model, glycine stabilized the phosphorylation system of brain cortex mitochondria. Significant effect of glycine was observed in both hemispheres (Table 1). For elucidation of the molecular mechanisms of this phenomenon, two models were used: brain cortex slices and isolated brain mitochondria. Both objects were subjected to hypoxia for 10-30 min.

Effect of glycine on the mitochondrial phosphorylating system on the model of brain cortex slices (under hypoxic conditions). Mitochondrial respiration was recorded in the homogenate from intact tissue (Fig. 2, curve *1*). Activation of oxidative phosphorylation by adding ADP stimulated respiration.

After incubation of brain cortex slices under hypoxic conditions for 30 minutes, phosphorylation function of mitochondrial in brain cortex homogenates was completely disturbed, because respiration was not stimulated in response to addition of ADP, a substrate of ATP synthase (Fig. 2, curve *2*).

Preliminary addition of 5 mM glycine to the incubation medium partially stabilized oxidative phosphorylation. Under these conditions, registration of mitochondrial respiration in homogenates demonstrated markedly enhanced respiration in response to addition of ADP. V3/V2 was 18±2% of the control (Fig. 2, curve *3*); this effect was observed in 5 independent experiments. It should be noted that functional activity of the brain under hypoxic conditions is retained for 3-5 minutes, whereas phosphorylation in mitochondria in the presence of glycine persists for 30 min.

Effect of glycine on phosphorylating system on the model of isolated brain mitochondria. After 10-min incubation of mitochondria, RC decreased to 67±5% in comparison with controls (Table 2). In

TABLE 2. Reduction of RC in Isolated Brain Mitochondria under Hypoxic Conditions

Incubation condition	RC (V3/V2 after Chance) in comparison with intact mitochondria during succinate oxidation, %
Intact mitochondria (without hypoxia)	100
10-min hypoxia	67±5+
10-min hypoxia in the presence of 5 mM glycine	85±5*

Note. $p<0.05$ in comparison with: +intact mitochondria, *10-min hypoxia. V3 and V2: rates of respiration in states 3 and 2 after Chance, respectively. Reduced RC in comparison with RC of intact mitochondria is demonstrated.

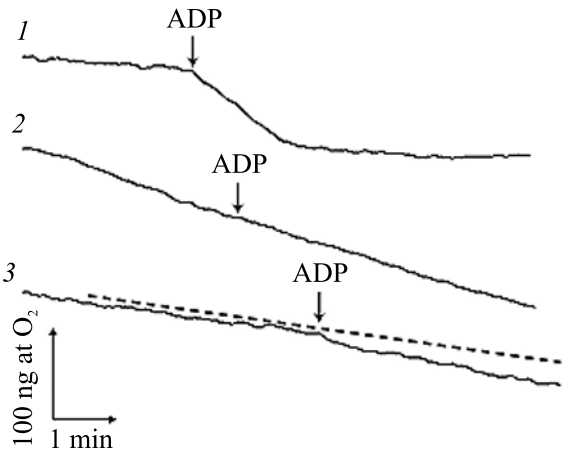

Fig. 2. Registration of homogenate respiration from brain cortex slides. Hypoxia (30 min) blocks the response to adding of ADP (curve *2*). Glycine contained in incubation medium contributes to pronounced stimulation of the respiration after adding of ADP (curve *3*). *1*) intact tissue; *2*) 30 min of hypoxia; *3*) 30 min of hypoxia+glycine (5 mM). The dashed line denotes the rate of respiration in the absence of ADP.

the presence of 5 mM glycine in the incubation medium, RC decreased to a lesser extent: to 85±5% of the control values. Hence, glycine protects mitochondrial phosphorylating function under hypoxic conditions.

These data suggest that mitochondria are the target of glycine in the brain cortex.

The biochemical mechanism underlying impairements of brain mitochondrial energetics under hypoxic conditions. Published data suggest that hypoxia is associated with activation of peroxidation processes in the brain tissue [1]. In light of this, we directly measured H_2O_2 accumulation in mitochondria after short-term exposure to hypoxia.

Oxidation of succinate was accompanied by rapid generation of H_2O_2 (Fig. 3, curve *3*). After 10-min incubation, the rate of H_2O_2 generation significantly increased (Fig. 3, curve *1*). However, after incubation in the presence of glycine, the rate of H_2O_2 formation was significantly lower (Fig. 3, curve *2*).

The results show that glycine protects energetics of brain mitochondria in all three models of hypoxia, most probably, due to attenuation of ROS generation.

The observed abnormal phenomenon of RC reduction against the background of constant ADP/O ratio (Table 1) is related to uncoupling caused by fatty acids, which occurs with participation of the nucleotide translocator [7]. In this type of uncoupling, the rate of O_2 consumption increases in respiratory state 2 (after Chance), but not in state 3 in the presence of added ADP. During phosphorylation, transfer of fatty acids by the translocator is blocked and uncoupling effect is suppressed. Hence, respiration rate, on the one

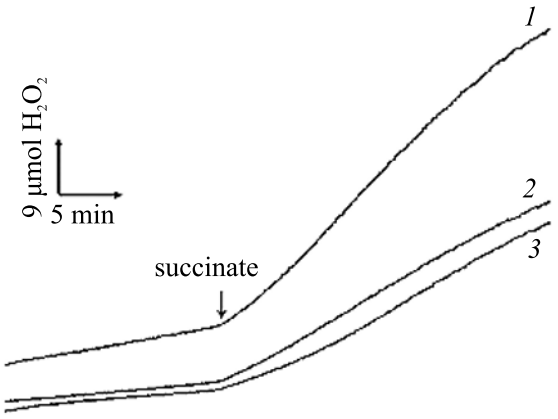

Fig. 3. Registration of H_2O_2 formation in the isolated brain mitochondria. Glycine reduces the rate of H_2O_2 formation in mitochondria. *1*) mitochondria after 10-min incubation under hypoxic conditions; *2)* 10-min hypoxia+glycine (5 mM); *3*) control mitochondria (without hypoxia). Additives: 5mM succinate.

hand, is inhibited due to suppression of uncoupling, and on the other hand, is accelerated by activation of phosphorylation. For this reason, RC (V3/V2) is low. At the same time, uncoupling effect of fatty acids remains suppressed until complete conversion of ADP into ATP and ADP/O remains at the maximum level.

Thus, two processes apparently mediate the effect of hypoxia on the mitochondrial phosphorylation system: first, activation of peroxidation reactions (Fig. 3) and second, activation of lipolysis by reactive oxygen species [8]. Decreased RC observed *in vivo* is probably due to accumulation of fatty acids and, consequently, activation of lipolysis (Table 1). Glycine (Fig. 3) prevents peroxidation processes and blocks the mitochondrial uncoupling.

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