Effect of Inhibitors of Inducible and Neuronal NO Synthases on the Development of Audiogenic Stress-Induced Damage in Krushinskii–Molodkina Rats

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Experiments on the models of epileptiform seizure and hemorrhagic stroke (Krushinskii–Molodkina rats) showed that selective inhibitors of inducible and neuronal NO synthases (aminoguanidine and 7-nitroindazole) significantly decrease the mortality rate, reduce the severity of motor disorders, and prevent the development of intracranial hemorrhages under conditions of audiogenic stress.

Key Words: *nitric oxide; sodium nitrite; nitric oxide cycle; aminoguanidine; 7-nitroindazole*

Studying the role of different NO synthesis pathways in brain disorders is an urgent problem of modern physiology and medicine. NO is synthesized enzymatically with the following three isoforms of NO synthase: endothelial, inducible, and neuronal NO synthases [3,5,10,11]. Previous studies revealed the existence of enzymatic and nonenzymatic synthesis of NO from NO_2^- [10]. The NO synthase system and nitrite reductase system constitute the NO cycle and can be activated during hypoxia/ischemia [10]. Apart from the regulatory and neuroprotective properties of NO [3-5,7,11], this agent possesses cytotoxic activity and serves as a factor of neuronal damage and death [8,13]. The ambiguous role of NO in cerebral ischemia and epileptiform seizures is determined by various factors. They include activity of NO synthases, concentration of NO and products of NO metabolism, degree of oxygen starvation, and other factors [4,5,14].

Our previous studies on the model of epileptiform seizures (Krushinskii–Molodkina rats; KM) showed that NO-generating compounds have an opposite and dose-dependent effect on stress injuries [6,9]. By contrast, nonselective NO synthase inhibitor L-NNA in various doses increases the severity of stress injury under conditions of acoustic stimulation [9]. These data indicate that the role of various forms of NO synthases should be evaluated in experiments with selective inhibitors. Here we studied the effects of aminoguanidine and 7-nitroindazole (selective inhibitors of inducible and neuronal NO synthases, respectively) on stress injuries in KM rats under conditions of acoustic stimulation.

MATERIALS AND METHODS

Experiments were performed on male KM rats (n=90) aging 4-4.5 months and weighing 250-300 g. The study was conducted on adult KM rats due to the following two reasons. First, hemorrhagic strokes usually occur in the second half of life and therefore they should be modeled on adult rats, but not on young animals. And second, adult animals are tested for the severity of seizures and exhibit the highest seizure activity (according to the standard 4-point scale). Experiments should be conducted on animals with the highest severity of seizures, but not on young or adult specimens (older than 8 months). Moreover, experiments on animals of

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the same age group allow us to compare the effect of various factors.

Two experimental series were performed. In series I, inhibitor of inducible NO synthase aminoguanidine (Sigma) or inhibitor of neuronal NO synthase 7-nitroindazole (Sigma) in a dose of 2.5 mg/100 g was injected intraperitoneally to animals of the treatment groups (n=42). Control animals (n=20) received an intraperitoneal injection of physiological saline in an equivalent volume 60 min before the study.

In series II, inhibitor of inducible NO synthase aminoguanidine (2.5 mg/100 g) and inhibitor of neuronal NO synthase 7-nitroindazole (2.5 mg/100 g) were simultaneously intraperitoneally injected to animals of the treatment groups (n=14) 60 min before the study. Controls (n=14) received an intraperitoneal injection of physiological saline in an equivalent volume 60 min before the study.

Acoustic stimulation was delivered as described elsewhere [6]. The exposure to a loud electric bell (110-115 dB, 1.5 min) was followed by a series of weak and strong (80 dB) acoustic signals (10-sec length, 10-sec intersignal intervals). The duration of this treatment was 15 min. A strong sound (1-min length) was repeatedly delivered after 3 min. The excitability of CNS (latency, severity, and type of seizures) in treated and control rats was evaluated during acoustic stimulation. The mortality rate and degree of motor dysfunction were estimated under conditions of acoustic stress. There were the following three types of disorders: mild disorders (slight impairment of muscle tone with no limitation of movements); moderate disorders (paresis of the limbs, particularly of the hindlimbs, impairing animals locomotion); and severe disorders (the animals practically cannot move). The animals were decapitated immediately after the study. The brain was fixed in 10% formalin. The area of subdural and visible subarachnoid hemorrhages was measured with a binocular micrometer. The presence or absence of intraventricular hemorrhages was estimated on cross-sections of the brain (1-2 mm in width).

The results were analyzed by Wilcoxon test and Fisher test.

RESULTS

Aminoguanidine had a protective effect and prevented the development of stress injuries in LM rats (Fig. 1). It was manifested in a significant decrease in the percentage of treated animals with severe motor dysfunction (52.4 *vs.* 100% in the control group, p<0.001) and increase in the number of rats with moderate disorders (47.6 *vs*. 0% in the control group, p<0.001). No differences were found in other indexes for stress injuries in animals of the treatment and control groups. The severity of seizures in animals of the treatment group did not differ from the control. We revealed a tendency to a decrease in the mortality rate (Fig. 1) and incidence of intracranial hemorrhages (Fig. 2, *a*) in treated rats. The latency of seizures in treated animals was greater than in control specimens (5.0±0.3 and 4.0±0.3 sec, respectively; p<0.05).

7-Nitroindazole had a strong protective effect on rats under conditions of acoustic stimulation (Fig. 1). The mortality rate of treated animals was signifiantly lower than in control specimens (0 and 35%, respectively; p<0.001). The percentage of treated rats with severe motor dysfunction was lower than in the control (14.3 and 100%, respectively; p<0.001). By contrast, the number of treated animals with mild disorders was higher than in the control (23.8 and 0%, respectively; p<0.01).

The average area of subdural and visible subarachnoid hemorrhages (Fig. 3, *a*) in treated animals was much lower than in the control (17.9±5.4 and 82.5±10.1 mm^2; p<0.01). The incidence of intraventricular hemorrhages was lower in the treatment group than in the control (48 and 95%, respectively; p<0.01). We revealed a significant increase in the latency of seizures in treated animals (6.7±0.5 *vs*. 4.0±0.3 sec in the control; p<0.01).

Combined treatment with inhibitors of neuronal and inducible NO synthases had a strong protective effect and prevented the development of stress injuries in rats (Fig. 3). The mortality rate of treated rats was lower than in the control (7.1 and 50%, respectively; p<0.05). The percentage of treated rats with severe motor dysfunction was much lower than in the control (7.1 and 92.9%, respectively; p<0.001).

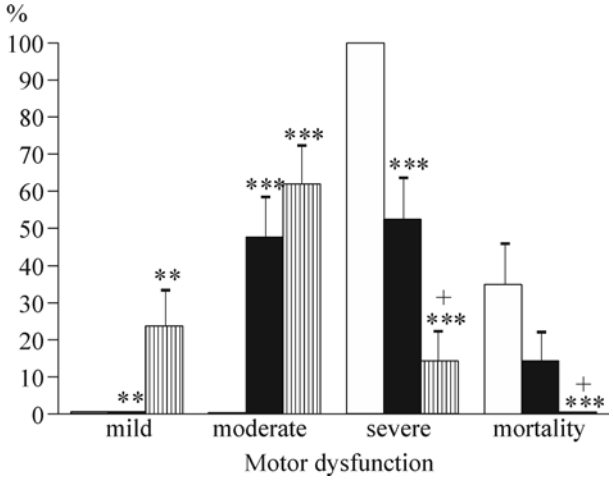

Fig. 1. Effect of an inducible NO synthase inhibitor aminoguanidine and neuronal NO synthase inhibitor 7-nitroindazole on stress injuries. Light bars, control; dark bars, aminoguanidine; shaded bars, 7-nitroindazole. **p<0.01 and ***p<0.001 compared to the control; ^+p<0.05 compared to aminoguanidine.

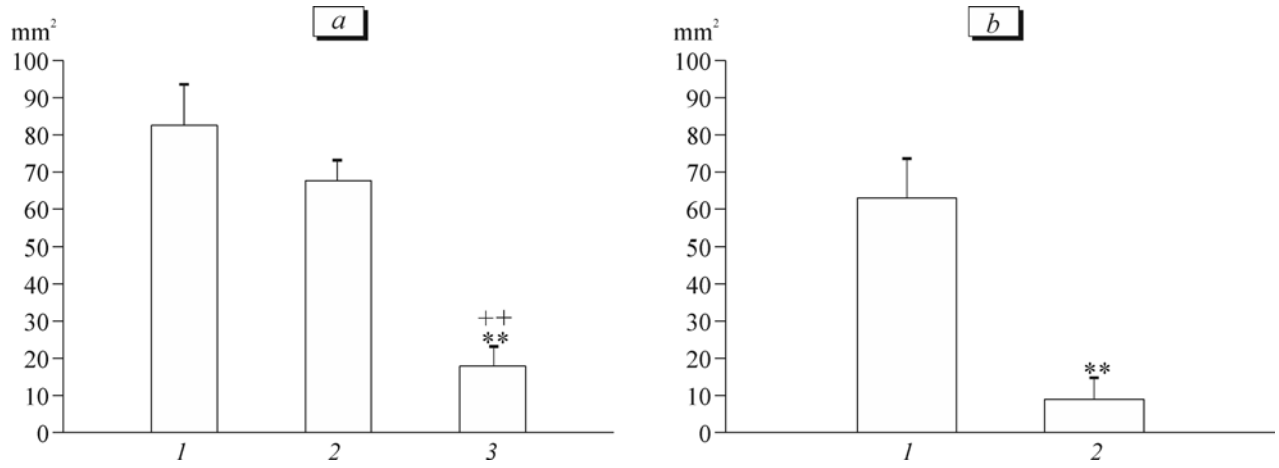

Fig. 2. Effect of selective NO synthase inhibitors on the formation of subdural and subarachnoid hemorrhages. (*a*) Control (*1*); aminoguanidine (*2*); 7-nitroindazole (*3*). (*b*) Control (*1*); aminoguanidine+7-nitroindazole (*2*). $^{**}p<0.01$ compared to the control; $^{++}p<0.01$ compared to aminoguanidine.

The number of treated animals with mild disorders was higher than in the control (64.3 and 0%, respectively; $p<0.001$). The average area of subdural and visible subarachnoid hemorrhages (Fig. 2, *b*) in treated animals was much lower than in the control (9.0±5.8 and 63.0±9.9 mm^2; $p<0.01$). The incidence of intraventricular hemorrhages was lower in the treatment group than in the control (11.4 and 78.6%, respectively; $p<0.01$). We revealed an increase in the latency of seizures in treated animals (4.8±0.5 *vs*. 2.4±0.2 sec in the control; $p<0.01$).

Our results indicate that selective NO synthase inhibitors have a protective effect and prevent stress injuries in KM rats. The protective effect was most significant after administration of a neuronal NO synthase inhibitor or combined treatment with inhibitors of neuronal and inducible NO synthases. It can be suggested that acoustic exposure in these rats is accompanied by overproduction of NO. This process is catalyzed by neuronal and inducible NO synthases and results in the development of stress injuries. NO overproduction is reduced under the influence of selective enzyme inhibitors. Our results are consistent with published data that some brain disorders (ischemia, hypoxia, and strokes) can be accompanied by hyperproduction of NO by neuronal and inducible NO synthases [1,2,5,8,12,15].

Previous experiments showed that nonselective NO synthase inhibitor L-NNA has an opposite effect on the same model. This agent was shown to contribute to the development of stress injuries. It can be suggested that the adverse effect of L-NNA is related to blockade of endothelial NO synthase, which exhibits the protective properties. NO hyperproduction due to increased activity of endothelial NO synthase can reduce the severity of destructive processes in the brain under pathological conditions. This effect is associated with vasodilation, increase in the blood flow rate, and other changes [1,2,5,10]. We believe that activation of endothelial NO synthase or increased synthesis of NO in the blood plays a role in the development of hemorrhagic stroke. Our previous studies showed that administration of a NO-generating compound sodium nitrite in low dose (0.5 mg/100 g) 60 min before audiogenic stress improves the survival rate and decreases the severity of neurological disorders in these animals. By contrast, nonselective NO synthase inhibitor L-NNA increased the mortality rate and percentage of animals with severe neurological disturbances. Sodium nitrite in a dose of 0.5 mg/100 g was shown to abolish the adverse effect of nonselective NO synthase inhibitor L-NNA [9]. These data indicate that

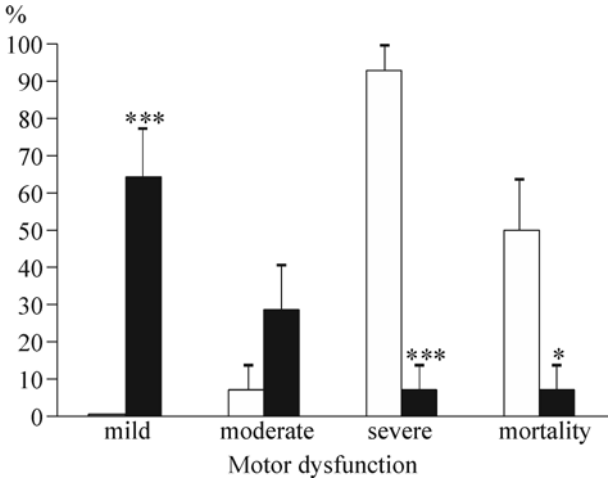

Fig. 3. Combined action of inducible NO synthase inhibitor aminoguanidine and neuronal NO synthase inhibitor 7-nitroindazole on stress injuries. Light bars, control; dark bars, aminoguanidine+7-nitroindazole. $^{*}p<0.05$ and $^{***}p<0.001$ compared to the control.

preactivation of NO synthesis in the blood relieves the symptoms of hemorrhagic stroke. The positive effect of NO-generating compound in low dose is probably related to hypotensive properties of this agent (*i.e.*, decrease in blood pressure). Vasodilating activity of NO contributes to improvement of blood circulation and decrease in the risk of hemorrhagic stroke. Hemorrhagic stroke is usually accompanied by glutamate neurotoxicity, persistent increase in Ca^{2+} concentration, and significant activation of neuronal NO synthase. Neuronal NO synthase should be inhibited under these conditions.

Our results confirm the notion that NO synthase isoforms (isoenzymes) play various roles in stress injuries. The increase in NO content in the vascular endothelium before hemorrhagic stroke has the vasodilating and protective effects. By contrast, activation of NO synthesis in the nervous tissue during hemorrhagic stroke may increase the degree of acute destructive processes. This state is characterized by a simultaneous increase in the generation of reactive oxygen and nitrogen species. Reactive nitrogen species cause the production of peroxynitrite anions. They undergo protonation and degradation with the formation of highly reactive NO_2 and OH radicals. The results of our experiments do not contradict the previously described concept of intracellular spatial confinement [10].

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