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The Degree of Oxidative Stress in the Rat Brain During Ischemia and Reperfusion in Conditions of Correction of the L-Arginine-No System

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The aim of the present work was to evaluate oxidative stress in the brains of rats during ischemia/reperfusion in conditions of correction of the L-arginine-NO system. Experiments on 128 rats with brain ischemia/perfusion in conditions of modulation of the L-arginine-NO system were used to study changes in the concentrations of (a) lipid peroxidation products, i.e., diene conjugates, malonic dialdehyde, and Schiff bases, and (b) antioxidant protection factors, i.e., retinol, α-tocopherol, and SH-groups. Administration of L-arginine and NO synthase inhibitors, i.e., the non-selective inhibitor N^{ω}-nitro-L-arginine methyl ester, the selective neuronal NO synthase inhibitor 7-nitroindasole, and the selective inhibitor of inducible NO synthase S-methylisothiourea, established that oxidative stress in rats with brain ischemia/perfusion is NO-dependent. NO formed by the various isoforms of NO synthase had different roles: hyperactivation of neuronal NO synthase was responsible for oxidative stress in both periods of brain ischemia/reperfusion, while increased inducible NO synthase activity was responsible in the late period.

KEY WORDS: brain, ischemia/reperfusion, oxidative stress, nitric oxide.

The lack of the desired success in using traditional treatment protocols for ischemic and reperfusional brain diseases leads to the need for the exact mechanisms of pathogenesis to be identified. The vast majority of post-ischemic brain conditions are accompanied by oxidative stress [1, 9]. Nitric oxide (NO), which has radical properties, plays an important role in supporting the homeostasis of brain tissue and in the pathogenesis of many brain diseases, including ischemic and reperfusional conditions [7]. Sources of active forms of oxygen and NO in ischemia/reperfusion may include neurocytes as well as extraneuronal structures (endothelial cells of brain vessels, glia, and leukocytes), these making up the nitrergic system [9, 16].

The roles of NO in mediating oxidative stress in ischemic and reperfusional lesions of various organs and especially the brain are multilateral and different in nature. The cytotoxic effects of NO are associated with its prooxidant properties, while NO can also be an antioxidant [22]. NO hyperproduction and the resulting increase in the activity of oxidative processes represent an important pathogenetic mechanism in brain lesions in this pathology [16]. At the same time, NO deficiency can also lead to activation of oxidative processes. The development of pathogenetic treatment aimed at the NO-dependent mechanisms supporting the homeostasis of brain tissue requires a detailed understanding of the role of NO in maintaining the prooxidant-antioxidant equilibrium in ischemic and reperfusional brain damage. The aim of the present work was to evaluate the level of oxidative stress in the rat brain during ischemia and reperfusion in conditions of correction of the L-arginine-NO system.

METHODS

Studies of measures characterizing oxidative processes were performed in 128 white mongrel male rats weighing 220–250 g; brain ischemia/reperfusion was modeled by 30-min bilateral occlusion of the common carotid arteries

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TABLE 1. Lipid Peroxidation Activity in Brain Tissue in Rats During the Early Period of Brain Ischemia/Reperfusion (I/R) after Administration of Modulators of the L-Arginine-NO System (n = 8)

Group	Diene conjugates, nmol/g	Malonic dialdehyde, μmol/g	Schiff bases, U/g
Control	23.8 ± 0.37	7.6 ± 0.16	315 ± 4.5
I/R	35.1 ± 0.30**	12.8 ± 0.45**	365 ± 3.7**
L-arginine + I/R	28.4 ± 0.42**##	11.6 ± 0.42**	330 ± 2.0*##
L-NAME + I/R	47.6 ± 0.75**##	17.0 ± 0.53**##	398 ± 5.3**##
7-NI + I/R	28.6 ± 0.73**##	11.3 ± 0.40**#	341 ± 1.8*##
S-MT + I/R	36.3 ± 0.49**	13.3 ± 0.40**	362 ± 3.4**
7-NI + S-MT + I/R	30.1 ± 1.22*#	11.4 ± 0.26**	343 ± 2.2**##
7-NI + S-MT + L-arginine + I/R	26.1 ± 0.48*##	9.4 ± 0.35*##	340 ± 1.5**##

Notes (here and Table 2). Differences between values in the control and other groups: *$p < 0.05$, **$p < 0.001$; differences between values in the I/R group and other groups: #$p < 0.05$; ##$p < 0.001$.

with subsequent 30-min (the early period) or 24-h (the late period) reperfusion [13], after which brain tissue was collected in liquid nitrogen until studies were performed. These time points were selected on the basis of the time differences in the activation of different isoforms of NO synthase. Thus, the activity of constitutional NO synthase isoforms (endothelial NO synthase and neuronal NO synthase) increases quickly in ischemia, while the activity of inducible NO synthase increases at 6–12 h, reaching a peak by 24 h [12].

Modulation of the L-arginine-NO system was achieved by treatment with the NO substrate L-arginine and inhibitors of various NO synthases: the non-selective NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma), the selective neuronal NO synthase inhibitor 7-nitroindasole (7-NI, Sigma), and the selective inhibitor of inducible NO synthase S-methylisothiourea (S-MT, Sigma).

Animals were divided into eight groups (n = 16), each of which was divided into two subgroups (n = 8 in each). Rats of group 1 (sham-operated control 1) received i.v. isotonic NaCl (0.5 ml). Rats of groups 2–8 underwent modeling of brain ischemia/reperfusion. Rats of group 2 received i.v. isotonic saline (0.5 ml). Rats of groups 3–8 received i.v. doses of modulators of the L-arginine-NO system: group 3 received L-arginine (150 mg/kg), group 4 received L-NAME (5 mg/kg), group 5 received 7-NI (10 mg/kg), group 6 received S-MT (1 mg/kg), group 7 received 7-NI and S-MT, and group 8 received 7-NI, S-MT, and L-arginine at the same doses. Agents were given immediately after occlusion of the common carotid arteries. Experiments were performed in conditions of i.v. thiopental anesthesia (40–60 μg/kg).

The extent of oxidative stress in brain homogenates was assessed by measuring the concentrations of lipid peroxidation products: diene conjugates, malonic dialdehyde, and Schiff bases; the concentrations of antioxidant protection factors were also measured, i.e., retinol, α-tocopherol, and SH groups. The concentrations of lipid peroxidation products were measured by classical methods: diene conjugates using an SF-46 spectrophotometer (originated in Russia) [4], malonic dialdehyde on a Specord (Germany) spectrophotometer [15], and Schiff bases using an F-4010 (Hitachi, Japan) spectrophotometer [19]. The concentrations of antioxidant protection factors were measured using a Specord (Germany) spectrophotometer for SH groups [21] and an F-4010 (Hitachi, Japan) spectrophotometer for retinol and α-tocopherol [8]. Data were analyzed by variation statistics using Student's t test.

RESULTS

Measurements of lipid peroxidation products in brain tissue from rats subjected to brain ischemia/reperfusion (I/R) in conditions of correction of the L-arginine-NO system in rats of group 2 (I/R, control 2) in the early period showed significant increases in diene conjugates, malonic dialdehyde, and Schiff bases (Table 1), with decreases in retinol, α-tocopherol, and SH groups (Fig. 1). In the late period of brain ischemia/reperfusion, there was a more significant increase in Schiff bases (to 576 ± 6.2 U/g, $p < 0.001$) than in the early period ($p < 0.001$) (Table 2), and a decrease in retinol compared with control group 1 to 1.7 ± 0.04 nmol/g (control: 2.5 ± 0.05 nmol/g, $p < 0.001$), a decrease in α-tocopherol to 14.1 ± 0.48 nmol/g (20.1 ± 0.35 nmol/g, $p < 0.001$), and a decrease in SH groups to 3.0 ± 0.06 nmol/g (3.7 ± 0.05 nmol/g, $p < 0.001$). It should be noted that the decreases in retinol and α-tocopherol concentrations in the late period of brain ischemia-reperfusion were less significant than those in the early period ($p < 0.05$). Subsequent comparative analysis was performed between measures from groups 3–8 and group 2 (control 2), between measures from multiple groups, and between measures from individual groups in the early and late periods. Administration of L-arginine to rats of group 3 led in the early period to significant decreases in malonic dialdehyde and Schiff bases and increases in retinol (to 2.2 ± 0.05 nmol/g, $p < 0.001$), α-tocopherol (to 17.4 ± 0.35 nmol/g, $p < 0.05$), and SH groups (to 3.3 ± 0.04 nmol/g, $p < 0.05$). In the late period,

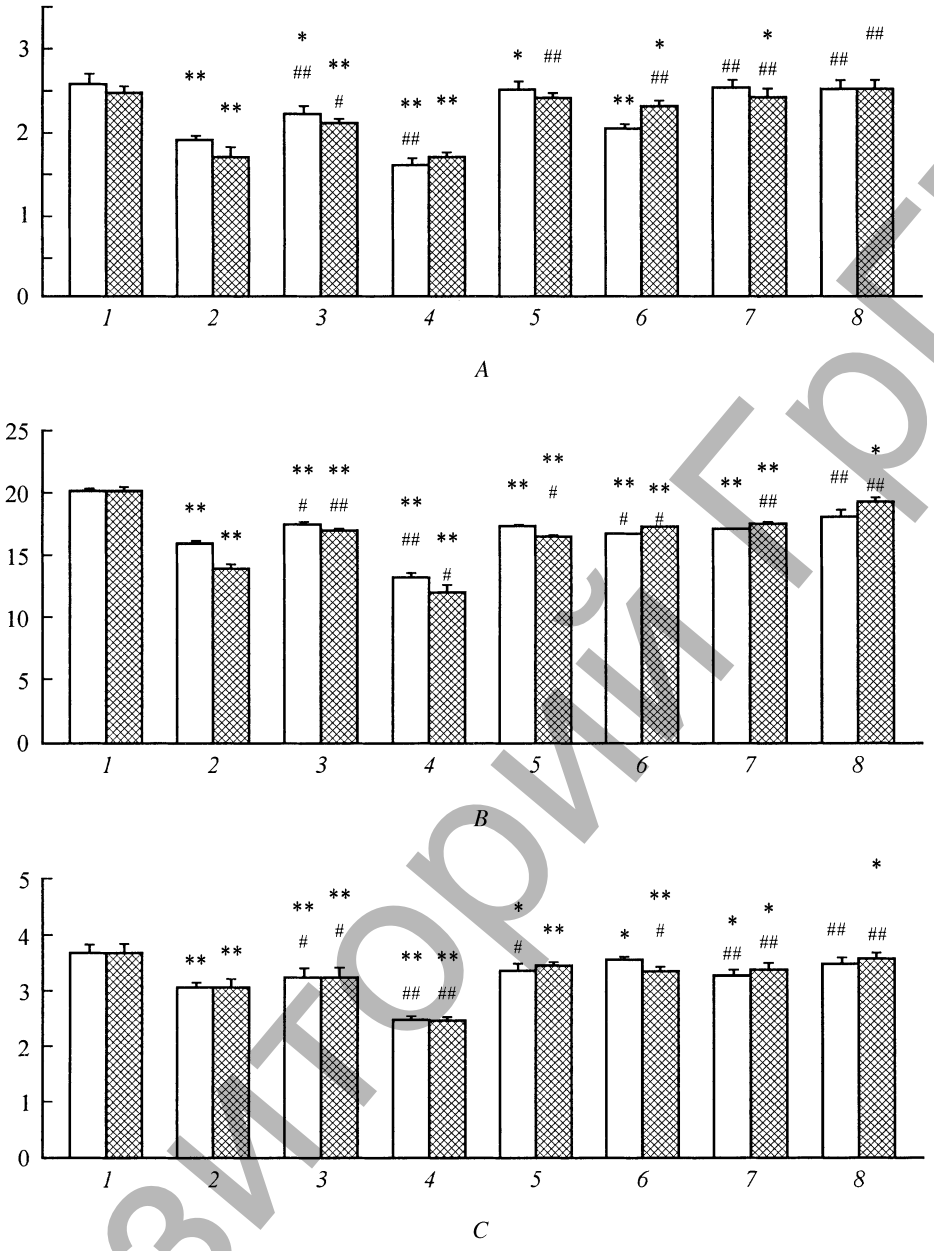

Fig. 1. Retinol (*A*), α-tocopherol (*B*), and SH group (*C*) levels in brain tissue from rats with brain ischemia/reperfusion (I/R) during modulation of the L-arginine-NO system. The vertical axes show the concentrations of retinol, nmol/g (*A*), α-tocopherol, nmol/g (*B*), and SH groups, μmol/g (*C*). White columns show the early period of I/R; dark columns show the late period of I/R. *1*) Control 1; *2*) I/R; *3*) I/R + L-arginine; *4*) I/R + L-NAME; *5*) I/R + 7-NI; *6*) I/R + S-MT; *7*) I/R + 7-NI + S-MT; *8*) I/R + 7-NI + S-MT + L-arginine. Differences between values in the control and other groups: $^{*}p < 0.05$; $^{**}p < 0.001$; differences between values in the I/R and other groups: $^{\#}p < 0.05$; $^{\#\#}p < 0.001$.

there were decreases in the levels of all measures of lipid peroxidation, i.e., diene conjugates, malonic dialdehyde, and Schiff bases, along with increases in retinol (to 2.1 ± 0.05 nmol/g, $p < 0.05$), α-tocopherol (to 16.9 ± 0.32 nmol/g, $p < 0.001$), and SH groups (to 3.3 ± 0.04 nmol/g, $p < 0.05$). However, in the late period of brain ischemia-reperfusion, there were significantly higher levels of diene conjugates and Schiff bases and lower levels of retinol than in the early period. Analysis of the results from this group provided evidence for the antioxidant effects of L-arginine in both the early and late periods of brain ischemia/reperfusion.

In rats of group 4, administration of L-NAME showed increases in lipid peroxidation products at both periods of brain ischemia/reperfusion, the increases in Schiff bases in

TABLE 2. Lipid Peroxidation Activity in Brain Tissue in Rats During the Late Period of Brain Ischemia/Reperfusion (I/R) after Administration of Modulators of the L-Arginine-NO System (n = 8)

Group	Diene conjugates, nmol/g	Malonic dialdehyde, μmol/g	Schiff bases, U/g
Control	24.0 ± 0.27	7.6 ± 0.27	318 ± 4.9
I/R	33.6 ± 0.63**	15.0 ± 0.76**	576 ± 6.2**
L-arginine + I/R	29.8 ± 0.49**#	13.1 ± 0.40**#	492 ± 9.6**##
L-NAME + I/R	45.4 ± 0.91**##	17.9 ± 0.51**	641 ± 6.5**##
7-NI + I/R	31.3 ± 0.73*##	12.4 ± 0.46**#	479 ± 12.1**##
S-MT + I/R	33.9 ± 0.61**#	11.3 ± 0.62**#	362 ± 3.4**##
7-NI + S-MT + I/R	27.8 ± 1.10*#	10.5 ± 0.6*#	343 ± 2.2*##
7-NI + S-MT + L-arginine + I/R	26.0 ± 0.38*##	9.3 ± 0.28*##	339 ± 1.5*##

the late period being more significant ($p < 0.001$). Rats in this group also showed decreases in the concentrations of antioxidant protection factors. During the early period, retinol decreased to 1.6 ± 0.04 nmol/g ($p < 0.001$), α-tocopherol to 13.2 ± 0.29 nmol/g ($p < 0.05$), and SH groups to 2.5 ± 0.03 nmol/g ($p < 0.001$); in the late periods, the α-tocopherol level decreased more significantly than in the early period, i.e., to 12.1 ± 0.28 nmol/g ($p < 0.05$). Thus, in contrast to the situation with L-arginine, rats with brain ischemia/reperfusion given the non-selective NO synthase inhibitor L-NAME showed increases in the level of oxidative processes in brain tissue at both periods, these being more marked in the late period of ischemia/reperfusion.

In rats of group 5, administration of the selective inhibitor of neuronal NO synthase 7-NI led to decreases in the concentrations of lipid peroxidation products in the early and late periods; some measures of lipid peroxidation – malonic dialdehyde and Schiff bases – were significantly greater, while antioxidant protection factors – α-tocopherol (16.3 ± 0.36 nmol/g, $p < 0.001$) and SH groups (3.1 ± 0.03 nmol/g, $p < 0.05$) – were lower than in the early period. The nature of changes in lipid peroxidation products and antioxidant protection in rats of this group provide evidence that NO formed by neuronal NO synthase has a significant role in mediating the mechanisms of oxidative stress in the rat brain during both periods of ischemia/reperfusion. Comparison of lipid peroxidation and antioxidant factor levels revealed no overall differences in the extents of the antioxidant effects in rats given L-arginine and 7-NI.

Administration of the selective inhibitor of inducible NO synthase S-MT (group 6) produced no changes in brain tissue levels of lipid peroxidation products or antioxidant protection factors in the early period of brain ischemia/reperfusion. However, in the late period, there were significant decreases in diene conjugates, malonic dialdehyde, and Schiff bases, along with increases in retinol (to 2.3 ± ± 0.06 nmol/g, $p < 0.001$), α-tocopherol (to 16.9 ± 0.24 nmol/g, $p < 0.05$), and SH groups (to 3.2 ± 0.04 nmol/g, $p < 0.05$). Comparison of the effects of 7-NI and S-MT showed more significant decreases in Schiff bases in the brain tissues of rats given S-MT as compared with Schiff bases in rats given 7-NI ($p < 0.001$). Analysis of the results obtained from this group provided evidence for a defining role for NO formed by inducible NO synthase in the mechanisms of oxidative stress in the brain tissues of rats in the late period of brain ischemia/reperfusion.

Combined treatment of rats of group 7 with 7-NI and S-MT led, in the late period of brain ischemia/reperfusion, to more marked decreases in malonic dialdehyde and Schiff bases and a more marked increase in SH groups than seen in group 5 (7-NI); there were also more marked decreases in diene conjugates and Schiff bases than in group 6 (S-MT), though there were no differences in measures of lipid peroxidation and antioxidant protection in these groups in the early period.

Rats of group 8, given combined 7-NI, S-MT, and L-arginine, showed the most significant decreases in the activity of oxidative processes. As compared with group 7 (7-NI + S-MT), there were significant decreases in diene conjugates and malonic dialdehyde at both time points, such that values were no different from those recorded in control group 1. In terms of differences in antioxidant protection in the early and late periods of brain ischemia/reperfusion, it should be noted that differences persisted only in relation to α-retinol, at 19.1 ± 0.24 nmol/g ($p < 0.05$), and SH groups, at 3.5 ± 0.02 nmol/g ($p < 0.05$).

DISCUSSION

Analysis of the results shows that subtotal brain ischemia/reperfusion in rats led to increases in the activity of oxidative processes in brain tissues in both periods of brain ischemia/reperfusion, though the degree of oxidative stress in the late period was more significant. Administration of L-arginine led to decreases in the concentrations of lipid peroxidation products and increases in the concentrations of antioxidant protection factors in rat brain tissues, with a consequent decrease in the level of oxidative stress at both periods of brain ischemia/reperfusion. The positive effects of L-arginine may be associated with the antiradical and antioxidant effects of the NO formed from it, inhibi-

tion of the effects of inositol-1,4,5-triphosphate, and inhibition of leukocyte adhesion, and inhibition of the accumulation of leukocytes in the reperfused tissue [5, 6]. Deficiency of L-arginine in brain neurons and inadequate levels of NO formation within them lead to increases in superoxide anion formation [20]. It has been suggested that the antioxidant effect of L-arginine in brain ischemia/reperfusion may be due to activation of NO synthesis involving endothelial NO synthase, indirect inhibition of the activity of neuronal and inducible NO synthases as a result of improvements in brain circulation and decreases in the ischemic zone, as well as its direct antioxidant action.

Administration of the selective inhibitor of nNO synthase 7-NI decreased the level of oxidative stress in rat brain tissues in the early and late periods to the same extent as L-arginine. The mechanisms of glutamate excitotoxicity in ischemic brain damage are linked to the increased activity of neuronal NO synthase and the hyperproduction of NO mediated by this enzyme [9, 16]. It is evident that the involvement of NO formed by the neuronal isoform of NO synthase mediates the mechanisms of oxidative stress in reperfusional processes in the brain.

Administration of the selective inducible NO synthase inhibitor S-MT was shown to be followed by decreases in lipid peroxidation activity and increases in the levels of antioxidant protection factors, predominantly in the late period of brain ischemia/reperfusion. This provides grounds for suggesting that NO formed by the inducible isoform of NO synthase mediates the mechanisms of oxidative stress in the late reperfusion period. Sources of inducible NO synthase in brain tissue may include not only macrophages of leukocyte and glial origin, but also endothelial cells [14] and thrombocytes and neurocytes [11]. The prooxidant action of the nitric oxide formed by neuronal and inducible NO synthases is associated with its ability to form the strong oxidant peroxynitrite which, like NO, can make a significant contribution to ongoing physiological processes in the brain. Neurodegenerative changes are facilitated by increases in the concentrations of peroxynitrite and its products (hydroxyl radicals and nitrites) [10, 17].

The involvement of neuronal and inducible NO synthase in the pathogenesis of oxidative stress in brain ischemia/reperfusion is supported by experimental data from the simultaneous use of two selective inhibitors of neuronal NO synthase, 7-NI, and inducible NO synthase, S-MT. During the early period of brain ischemia/reperfusion, the activity of oxidative stress in the presence of 7-NI and S-MT was no different from the activity of oxidative stress in the group given 7-MI only, while activity in the later period was lower than in groups given 7-NI and S-MT only. The non-selective NO synthase inhibitor L-NAME, contrary to our expectations, had no protective effects, which included the absence of any effects on the extent of lipid peroxidation and decreases in the concentrations of antioxidant protective factors in brain tissue at both periods of brain ischemia/reperfusion. L-NAME, unlike 7-NI, irreversibly inhibits the constitutional (neuronal and endothelial) isoforms of NO synthase and reversibly inhibits inducible NO synthase. Analysis of measures of the prooxidant-antioxidant state in groups given 7-NI and L-NAME provided grounds for suggesting that NO formed by endothelial NO synthase has antioxidant effects.

The mechanism of the antioxidant action of NO involves its ability to operate as a radical trap, facilitating the removal of radicals and reductions in their toxicity [21]. The antioxidant action of NO in vessel walls is mediated by inhibition of lipoxygenase-dependent lipid and lipoprotein oxidation [9, 21]. In neurons, this results from suppression of NMDA receptor activity of neurons, which in conditions of L-arginine deficiency may also result in the increased formation of oxygen radicals [9, 19]. In addition, NO can limit oxidative damage by modulating cellular and physiological processes [2, 22]. Correction of the L-arginine-NO system induces a shift in the prooxidant-antioxidant equilibrium, apparently associated not only with potentially high levels able to react with the multitude of target molecules responsible for the development of oxidative stress, but also with decreases in the contributions of other factors to the antioxidant potential of the body, particularly changes in the oxygen affinity of hemoglobin [3].

In addition, the antioxidant effects of NO formed by endothelial NO synthase may be associated with its ability to enhance the perfusion of brain tissues via NO-dependent dilation of vessels [10]. NO decreases the activity of oxidative processes by leukocytes sequestered in the vessels and tissues of the reperfused organ, which is associated with its antiadhesive properties [20]. The greatest antioxidant effect was seen in the group given both selective NO synthase inhibitors and L-arginine, which can be regarded as a result of suppression of the prooxidant effects of NO formed by neuronal and inducible NO synthase and increases in the antioxidant effects of NO of endothelial origin.

Thus, complex analysis of the separate and combined use of selective and non-selective inhibitors along with L-arginine provides grounds for considering that NO is involved in the activation of oxidative processes in brain tissue at both periods of brain ischemia/reperfusion. However, the roles of NO formed by the various isoforms of NO synthases are different: activation of oxidative processes at both periods of brain ischemia/reperfusion is associated with hyperactivation of the neuronal isoform of NO synthase and in the later period with increased activity of the inducible NO synthase isoform.

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