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Ischemic Preconditioning of the Rat Brain as a Method of Endothelial Protection from Ischemic/Repercussion Injury

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The studies reported here addressed the endothelium-protecting action of local and remote ischemic preconditioning of the brain in rats. Cerebral ischemia lasting 30 min was reproduced by thermocoagulation of the vertebral arteries with simultaneous clamping of the carotid arteries, the procedure being followed by reperfusion via the carotid arteries for 120 min (controls). The early and late phases of ischemic preconditioning and remote preconditioning were reproduced. Brain blood flow was recorded using high-frequency Doppler ultrasonography. The early and late phases of local ischemic preconditioning and the late phase of remote ischemic preconditioning were found to have endothelium-protecting actions apparent as improvements in the recovery of brain blood flow in the post-ischemic period in preconditioned rats, with lower levels of endothelial desquamation and cerebral edema. Blockade of nitric oxide synthesis eliminated the protective effects of both phases of preconditioning.

KEY WORDS: ischemia, reperfusion, ischemic preconditioning, brain, nitric oxide, endothelium.

Ischemic preconditioning is one of the most important endogenous mechanisms for protecting cells against ischemic and reperfusion injury [18]. Increases in the resistance of an organ's cells to ischemia arise after one or several transient episodes of ischemia/reperfusion. Early and late phases of ischemic preconditioning are identified [6]. The early ("classical") phase of ischemic preconditioning starts immediately after transient ischemia and lasts up to 180 min. If the time between transient occlusion and prolonged ischemia is 12–72 h, then the late phase of ischemic preconditioning becomes active (the second window of protection).

The literature contains only a few reports addressing the cytoprotective effects of local and remote ischemic preconditioning [3, 4, 6, 17–20]. Local ischemic preconditioning acts after transient occlusion of the major vessels supplying blood to the organ of interest before long-term ischemia. Remote (inter-organ) preconditioning occurs as a result of transient ischemia of another organ. There are some reports identifying the infarct-limiting action of remote ischemic preconditioning as a mechanism protecting the myocardium [18]. However, remote ischemic preconditioning of the brain has not been studied.

The aims of the present work were to demonstrate the possible endothelium-protecting actions of the early and late phases of local and remote ischemic preconditioning and to study the role of nitric oxide (NO) in this process.

METHODS

Studies were performed using mongrel male rats (from the Rappolovo supplier) weighing 250–300 g; animals were anesthetized with urethane (1200 mg/kg i.p.). Local ischemic preconditioning was reproduced by 5-min clipping of the carotid arteries. Remote ischemic preconditioning consisted of clipping the femoral arteries for 30 min. Prolonged four-vessel cerebral ischemia was reproduced by thermocoagulation of the vertebral arteries and simultaneous clamping of the carotid arteries [9]. Blood flow was reestablished via the carotid arteries after 30 min of four-vessel ischemia. Animals were divided into the following experimental groups (Fig. 1):

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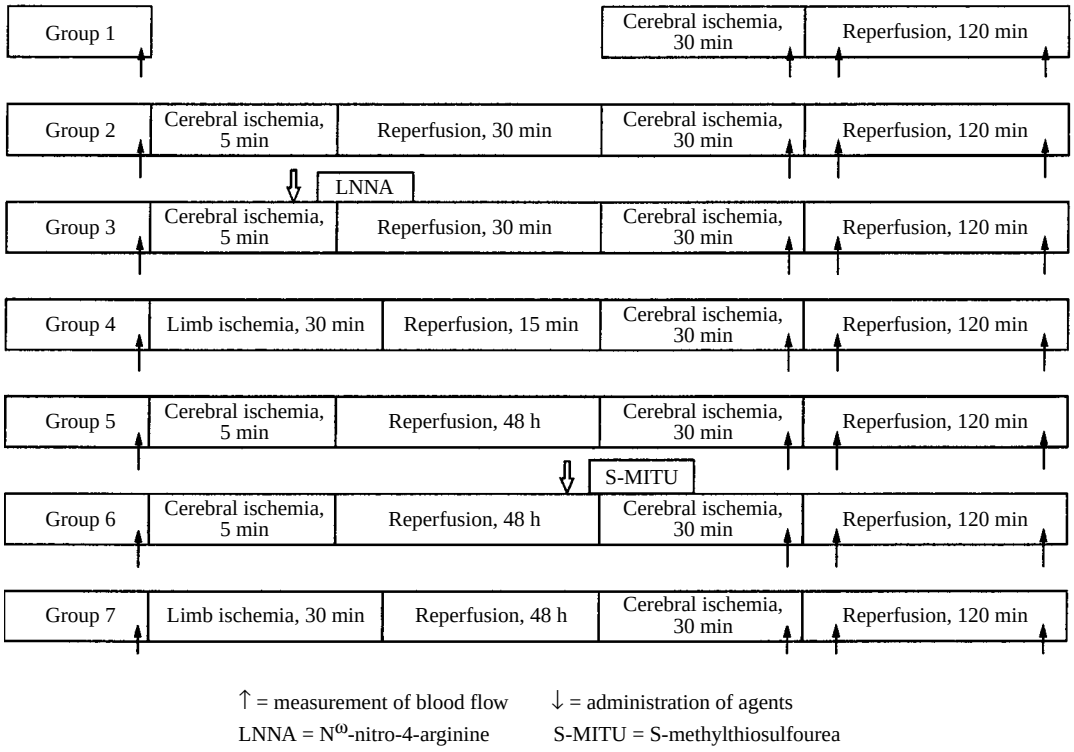

Fig. 1. Experimental protocols.

1) Cerebral ischemia (CI) for 30 min followed by reperfusion via the carotid arteries for 120 min (controls), $n = 8$ (n is the number of animals in the group);

2) cerebral ischemia for 5 min followed by reperfusion via the carotid arteries for 30 min (to model the early phase of local ischemic preconditioning), $n = 7$;

3) cerebral ischemia for 5 min followed by reperfusion via the carotid arteries for 30 after administration of N^{ω}-nitro-L-arginine (LNNA) at a dose of 10 mg/kg, given i.v. (to model the early phase of local ischemic preconditioning on the background of non-selective blockade of NO synthase), $n = 6$;

4) hindlimb ischemia for 30 min followed by reperfusion for 15 min (to model the early phase of remote ischemic preconditioning), $n = 6$;

5) cerebral ischemia for 5 min followed by reperfusion via the carotid arteries for 48 h (to model the late phase of local ischemic preconditioning), $n = 8$;

6) cerebral ischemia for 5 min followed by reperfusion for 48 h after administration of S-methylthiosulfourea (S-MITU) at a dose of 3 mg/kg, given i.p. (to model the late phase of local ischemic preconditioning in conditions of blockade of induced NO synthase), $n = 6$;

7) hindlimb ischemia for 30 min by clipping the femoral arteries followed by reperfusion for 48 h (to model the late phase of remote ischemic preconditioning), $n = 7$.

The preconditioning stimuli were followed in groups 2–7 by cerebral ischemia for 30 min followed by reperfusion for 120 min (using the protocol for control group 1) (Fig. 1).

In experiments addressing the late phase of local and remote ischemic preconditioning (groups 5–7), the preconditioning procedures (48 h before prolonged ischemia) were performed under Nembutal anesthesia (6 mg/100 g body weight, given i.m.).

Cerebral microhemodynamics were recorded by high-frequency Doppler ultrasonography (Minimax-Doppler-K apparatus from MiniMaks, St. Petersburg) with a probe working zone diameter of 0.65 mm and an operating frequency of 20 MHz [5]. Blood flow measurements were made transcranially with the probe placed on the parietal bone. Blood flow was measured at each stage of the experiment (initially, after ischemia, and during the reperfusion period) (Fig. 1).

The extent of post-ischemic cerebral edema was assessed in terms of the water content of brain tissue. Brains were removed 120 min after reperfusion and the dry weight:wet weight ratio was measured as a percentage [14].

The extent of desquamation of endotheliocytes was assessed in terms of the number of circulating endothelial cells in the blood [8]. These measurements were also made in healthy, sham-operated animals to provide controls for comparison with the normal levels of endotheliocytes in the circulating blood ($n = 7$).

TABLE 1. Volume Blood Flow (ml/sec·cm^3) in the Brains of Rats Measured by Doppler Ultrasonography

Group of rats	Model	Baseline data		End of prolonged ischemia		Reperfusion, 5 min		Reperfusion, 120 min	
No. 1	Local ischemia without preconditioning	0.0136	0.0007	0.0013	0.0003	0.0025	0.0005	0.0108	0.0005
No. 2	Early phase of local preconditioning	0.0136	0.0007	0.0073	0.0007**	0.0087	0.0013**	0.0134	0.0004*
No. 3	Early phase of remote preconditioning + LNNA	0.0136	0.0007	0.0014	0.0003	0.0033	0.0005	0.0080	0.0008**
No. 4	Early phase of remote preconditioning	0.0136	0.0007	0.0020	0.0002	0.0028	0.0005	0.0109	0.0005
No. 5	Late phase of local preconditioning	0.0136	0.0007	0.0027	0.0003	0.0077	0.0005**	0.0133	0.005*
No. 6	Late phase of remote preconditioning + S-MITU	0.0136	0.0007	0.0018	0.0005	0.0027	0.0007	0.0101	0.0003
No. 7	Late phase of remote preconditioning	0.0136	0.0007	0.0021	0.0005	0.0050	0.005*	0.0126	0.0005*

Notes. Differences compared with group 1: *$p < 0.05$; **$p < 0.01$. LNNA = N$^\omega$-nitro-L-arginine; S-MITU = S-methylthiosulfourea.

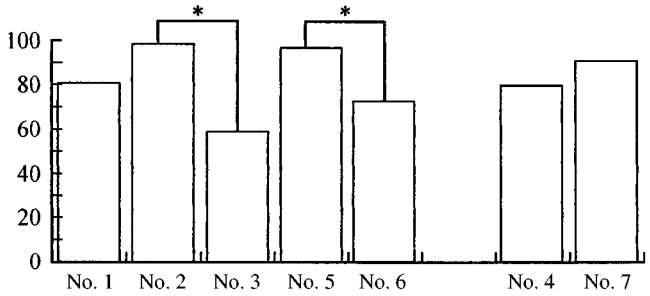

Fig. 2. Brain blood flow in a rat after 120 min of reperfusion. Significant differences with group 1 ($p < 0.05$) were seen in all groups apart from 4 and 6. *$p < 0.05$. *1–7*) Rat group identification numbers. The vertical axis shows blood flow 120 min after reperfusion, % of baseline.

Rats were decapitated at 120 min and the cerebral cortex was removed and placed in 2.5% glutaraldehyde in phosphate buffer, with post-fixation in 1% OsO_4 for preparation of electron microscope specimens. Specimens for light microscopy (stained with hematoxylin) were prepared using standard methods. Neocortical and hippocampal zones were studied.

The statistical significance of differences between series of values was evaluated using the Wilcoxon–Mann–Whitney test run on Statistica 97.

RESULTS

In the control group (group 1), occlusion of the vertebral arteries decreased blood flow to 11% compared with baseline. Subsequent clamping of the carotid arteries led to a decrease in blood flow by 89.8% of baseline. At the start of reperfusion (5 min), brain blood flow began to recover, reaching 79.4% of initial by 120 min (Table 1).

In rats with early local ischemic preconditioning (group 2), blood flow was significantly greater than in the control group both at the start and at 120 min of reperfusion ($p < 0.01$). Similar blood flow dynamics in the post-reperfusion period were seen in the group with late local ischemic preconditioning (group 5) (Table 1).

In conditions of blockade of nitric oxide synthesis with N$^\omega$-nitro-L-arginine (group 3), the protective effect of early local ischemic preconditioning was completely eliminated. Use of the inhibitor of inducible NO synthase, S-methylthiosulfourea (group 6), also showed loss of the protective effect of the late phase of local ischemic preconditioning; brain blood flow in these groups was no different from controls at 120 min of reperfusion.

The early phase of remote ischemic preconditioning (group 4) had no effect on the state of post-ischemic brain blood flow, values not being different from control, while the late phase of remote ischemic preconditioning (group 7) facilitated recovery of blood flow at 120 min of reperfusion as compared with the control group, i.e., to 91.6% ($p < 0.05$) (Table 1; Fig. 2).

Cerebral edema provides a measure of ischemic brain damage. Brain tissue water content in the control group was 78.1 ± 0.35%. The early phase of local and the late phase of

TABLE 2. Degree of Hydration of the Brains of Rats of the Various Groups

Measure	Group of rats													
	1		2		3		4		5		6		7	
Water content, %	78.10	0.35	77.46	0.32*	78.15	0.15	77.74	0.01	77.95	0.16	78.35	0.30	77.25	0.29*

Notes. Difference compared with group 1: *$p < 0.05$.

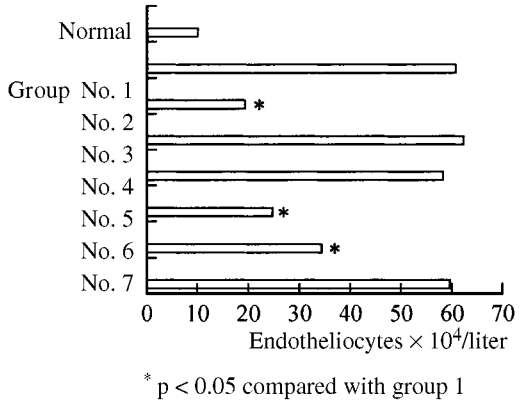

Fig. 3. Numbers of circulating endothelial cells (10^4/liter) in the blood of rats of the various groups.

remote ischemic preconditioning decreased the water content of the brain, while administration of N^{ω}-nitro-L-arginine to rats with ischemic preconditioning blocked their protective effects (Table 2).

The extent of endotheliocyte desquamation showed a partial correlation with brain blood flow, though this method was less sensitive than blood flow measurements (Fig. 3).

In healthy rats, the mean level of endotheliocytes circulating in the blood was $9.8 \pm 1.1 \cdot 10^4$/liter. Ischemia/reperfusion (group 1) was accompanied by a six-fold increase in this measure, which is evidence for endothelial damage (Fig. 3). As shown in Fig. 3, the most marked decreases in the level of circulating endotheliocytes in the blood (as compared with group 1) were seen in the early and late phases of local ischemic preconditioning (groups 2 and 5) ($p < 0.01$). Blockade of nitric oxide synthesis with N^{ω}-nitro-L-arginine completely eliminated the protective effect of the early phase of local ischemic preconditioning, though this did not occur after administration of S-methylthiosulfourea in experiments addressing the late phase of preconditioning. The level of circulating endothelial cells in experiments with remote ischemic preconditioning approached the level seen in the control group (Fig. 3).

Histological studies showed that there were no significant differences between groups of animals, which is probably associated with the short durations of the reperfusion periods. Morphological assessment of the cytoprotective action of ischemic preconditioning and the significance of nitric oxide in this mechanism is therefore not possible. Light and electron-microscopic studies of the brain revealed diffuse dystrophic changes (mainly shrinking of neurons without any glial reaction) in all preparations without marked focal characteristics. Signs of damage to the blood-brain and blood-CSF barriers (pericapillary microhemorrhages in the cerebral cortex and intraventricular hemorrhages) were seen, probably associated with damage to the villous microcirculatory system in the vascular plexus of the endbrain in conditions of post-ischemic reperfusion. Because of the relatively uniform damage, seen even within a group of animals, it was not possible to detect significant differences in the level of ischemic damage between groups of animals.

DISCUSSION

Control of regional blood flow, particularly the microcirculation, has several components, the most important of which is endothelium-dependent humoral regulation. Endothelial cells are known to have a part in controlling the tone of vessels, their permeability, and their thrombogenic, thrombus-resistance, adhesive, and some other properties. Ischemia and post-ischemic reperfusion lead to marked disturbances of blood flow in the vessels of organs, inducing the “no-reflow” phenomenon. Development of the “no-reflow” phenomenon is associated with increases in the permeability of microvessels and edema of the perivascular glia [11], adhesion of leukocytes to the endothelium [15], edema of endothelial cells [21], and increases in thrombogenicity and decreases in the thrombosis resistance of vessels [2, 16]. Disturbances to the functional properties of the endothelium of the vessels in the ischemic organ probably have major functional importance in the mechanisms of this state [7]. Ischemic preconditioning is one of the most powerful mechanisms protecting organs from ischemic and reperfusion damage, especially in the heart [4, 17]. The major effect of ischemic preconditioning is a decrease in the extent of ischemic damage to cells, i.e., a cytoprotective effect, which is seen in a variety of organs. Studies in recent years have drawn attention to a further effect of ischemic preconditioning, i.e., prevention of the development of post-ischemic endothelial dysfunction [19]. This action of

ischemic preconditioning on vessels decreases leukocyte and thrombocyte adhesion to the endothelium of the ischemic organ, preserves vascular tone, and prevents development of the "no-reflow" phenomenon. The endothelium-protecting action of ischemic preconditioning has been described in the small intestine [3, 20] and the heart [19].

Apart from the "classical" ischemic preconditioning, the effects of remote, or inter-organ, preconditioning have been described in the myocardium – this is preconditioning of an organ to ischemia by creating transient ischemia/reperfusion of another organ. Infarct-limiting and antiarrhythmic actions have been seen with remote preconditioning [6, 18].

The data obtained in the study reported here provide evidence that stimulation of nitric oxide formation in the endothelium is one of the most important mechanisms maintaining brain blood flow in the post-ischemic period. The importance of nitric oxide in the pathogenesis of ischemic/reperfusion brain damage is unknown. It is now apparent that nitric oxide induces different effects depending on the duration of ischemia and reperfusion and may have a double action – both damaging and protective [1, 10, 12]. Which of these effects nitric oxide will have in any particular episode of ischemia/reperfusion depends on the quantity of NO formed and the duration of reperfusion. In the nervous system, nitric oxide can be formed by means of three NO synthases: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). In conditions of prolonged ischemia/reperfusion, the quantity of nitric oxide formed by iNOS can be excessive, and it starts to show its cytotoxic action [10]. On the other hand, at the early periods of ischemia/reperfusion damage, nitric oxide is formed mainly in endothelium by eNOS, and this is important as a mechanism decreasing the adhesive properties of the endothelium, decreasing vascular tone, and, at the early stages of post-ischemic reperfusion, nitric oxide has protective actions [12].

The positive effect of ischemic preconditioning reported here was associated with the formation of nitric oxide, which prevented the development of the "no-reflow" phenomenon, improving measures of brain blood flow in the post-ischemic period. Blockade of NO synthesis increased the severity of impairments to blood flow in ischemia and in the post-ischemic period. Both the early and late phases of local ischemic preconditioning had marked protective effects, improving all measures of the state of the microcirculation. Early remote ischemic preconditioning had no effect on blood flow measures, which is in general agreement with our previous data on ischemic preconditioning of the intestine but contradicts data on the efficacy of remote ischemic preconditioning of the myocardium. Thus, ischemia/reperfusion of the limb (the early phase), partially protecting the myocardium from subsequent ischemic damage [18], had no action in ischemia of the intestine [3], or in ischemia of the brain, as demonstrated by the present studies. This is probably associated with the different protocols for reproducing remote ischemia and different criteria for assessing ischemic preconditioning of the heart, intestine, and brain. However, the late phase of remote ischemic preconditioning significantly improved blood flow in the brain during the reperfusion period.

The involvement of nitric oxide in ischemic preconditioning is a very controversial question. Thus, blockade of nitric oxide synthesis has been shown not to affect local [15] or remote [18] ischemic preconditioning of the myocardium, while in the intestine [3, 20] and brain (present results), activation of NO synthesis is suggested as an important mechanism of ischemic preconditioning. This apparent contradiction should be resolved by selection of identical criteria for assessing the efficacy of ischemic preconditioning. Studies of the heart were performed to investigate the mainly cytoprotective effect of ischemic preconditioning, while the present study, as well as experiments on the intestine [3, 20], used its endothelium-protecting action as one of the major criteria for assessment. Stimulation of endothelial NO synthase and increases in the formation of nitric oxide play a leading role in the mechanisms of endothelial protection induced by ischemic preconditioning, which is supported by numerous studies of the endothelium-protecting effect of ischemic preconditioning in the myocardium [17], intestine, and brain.

The early phase of remote ischemic preconditioning was not effective in our experiments. This would appear to be associated with the fact that ischemia-reperfusion of different organs induces dysfunction of the endothelium at the system level. The main mechanism of this dysfunction is impairment of the formation and/or bioavailability of nitric oxide [2, 16]. Decreases in NO formation apparently also occur in the brain vessels. Early remote preconditioning did not therefore have any endothelium-protecting action.

Thus, the data obtained here demonstrate the protective effect of nitric oxide both at the early and late phases of post-ischemic reperfusion of the brain and its involvement in the endothelium-protecting actions of both phases of local and the late phase of remote ischemic preconditioning of the brain.

Thus, the early and late phases of local ischemic preconditioning had endothelium-protecting actions in ischemia/reperfusion of the brain. Nitric oxide (NO) is one factor protecting the endothelium in local ischemic preconditioning of the brain; during the time period of the second protective window, the positive action of preconditioning is associated with inducible NO synthase. Late but not early remote ischemic preconditioning had a significant endothelium-protecting effect.

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