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Possible Role of Nitric Oxide in Anxiety Following Transient Cerebral Ischemia in Mice

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Abstract. The possible role of nitric oxide (NO) in anxiety following transient cerebral ischemia by a 10-min bilateral carotid occlusion was examined in mice. Two days after the ischemia, mice showed a significant decrease in time spent on the open arms in the elevated plus-maze test; and likewise, they showed shortened social interaction time in the social interaction test, suggesting the induction of anxiety. Such anxiety behavior, however, was diminished 7 days after the treatment in both tests. A nonselective nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), and a selective inducible NOS (iNOS) inhibitor, *S*-ethylisothiourea (EIT), given twice after reperfusion, produced an anxiolytic effect in the elevated plus-maze test 2 days after the ischemia, while only the former produced antianxiety in the social interaction test. A relatively selective neuronal NOS (nNOS) inhibitor, 7-nitroindazole (7-NI), failed to decrease the level of anxiety in both tests. These results suggest that the production of NO participates in the anxiogenic behavior by the ischemia. Furthermore, NO generated by endothelial NOS (eNOS) or eNOS with iNOS, with no involvement of nNOS, plays an important role in the anxiety induced by the ischemia. Thus, we conclude that 10-min bilateral carotid occlusion provides a useful exploratory animal model for anxiety following transient cerebral ischemia.

Keywords: transient cerebral ischemia, nitric oxide, anxiety, elevated plus-maze test, social interaction test

Introduction

Brain, demanding high energy which is produced by the oxidation of D-glucose, is vulnerable to any deficiency in these elements, which subsequently leads to brain damage. A cerebral ischemia is a common but very crucial problem, and the most typical presentation is in the form of stroke. Stroke causes many physical and psychological disadvantages; for example, paralysis, apathy, irritability, learning difficulties, depression and anxiety. Numerous studies have reported that the post-stroke anxiety disorder is widely recognized, with a prevalence of 12 – 28% (1 – 3) in the clinical field. In an animal study using rats, it was described that global ischemia following cardiac arrest induced anxiety (4).

Nitric oxide (NO) is well known as a neural messenger molecule in the central nervous system. NO has been recognized to act as a neuroprotective or a neurodegenerative substance depending on the stage of the cerebral ischemic process and on its cellular source (5, 6). Moreover, nitric oxide synthase (NOS) inhibitors have been reported to show the anxiolytic-like effect in different exploratory animal models of anxiety, indicating that NO may be involved in the mechanism of anxiety (7, 8).

It is well established that three isoforms of NOS contribute to the NO formation, namely, neuronal NOS (nNOS) and endothelial NOS (eNOS), as constitutive isoforms, and inducible NOS (iNOS), as an inducible isoform. In these NOS isoforms, both nNOS and eNOS are localized to neurons and blood vessels in the periphery and in the brain. Among them, the iNOS is in-

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duced in response to the ischemic brain injury, infectious disease, or inflammation (9 – 11), leading to the inference that NO may participate in progression of neuronal damage after cerebral ischemia.

In the present study, we examined whether the anxiety in mice following the transient cerebral ischemia is produced, and if this is the case, how the NO is involved in the production of the transient cerebral ischemia induced anxiety.

Materials and Methods

Animals

Male ddY mice weighing 18 – 20 g (Otsubo Exp. Animals, Nagasaki) were purchased. They were housed in an air-conditioned room at 22 ± 1°C, humidity of 55 ± 5%, with a 12-h light-dark schedule. They were allowed free access to food and water. After reaching a weight of 22 – 28 g, they were used for the experiment. All procedures used in this study were approved by the University Animal Care and Use Committee.

Surgical procedures

Transient cerebral ischemia in mice was induced by the method of Kojima and Kaneto (12). Briefly, under pentobarbital sodium anesthesia, bilateral common carotid arteries were exposed through a surgical midline incision in the neck. Each artery was threaded through a small polyethylene tube, and both ends of the thread were ligated at the tip of the tube. Then the incision was sutured, leaving the tip of the tube and both ends of the thread out of the skin. Twenty-four hours after this surgery, both common carotid arteries were occluded for 10 min by pulling the artery into the tube. After the occlusion, the ligation was cut and the tubes were removed, allowing reperfusion of the blood. Sham operation was done in the same manner as the occlusion except the step of threading the arteries through a polyethylene tube.

Elevated plus-maze test

The plus-maze was made of plexiglass and consisted of two opposite open arms (24 × 8 cm) and two enclosed arms (24 × 8 × 15 cm) with side and end walls. The arms extended from a central platform (8 × 8 cm) and were elevated to the height of 50 cm from the floor. The open arms, the central platform, and the floor of the enclosed arms were made of black plexiglass. At the beginning of the test, each mouse was placed at the center of the plus-maze facing one of the open arms. The cumulative time spent on the open arms and the number of open arm entries were measured during a 5-min observation period. A mouse was taken to have entered an arm when all four legs were on the arm. The time spent on the open arms was used as an index of anxiety (13, 14). Two days or 7 days after the transient cerebral ischemia, the mice were used for the one trial elevated plus-maze test.

Social interaction test

The test box was 28 × 18 × 13 cm and white light was at 32 cm above from the floor. At the beginning of the test, a pair of mice were placed at the opposite corner in this box. During a 10-min observation period, the following social interaction behaviors were observed: sniffing, grooming, genital investigation, following, facing and mounting. Data were expressed as cumulative time of the social interaction behavior (15, 16). Passive body contact was not regarded as a social interaction behavior. These paired mice were housed in different cages until using this test. Two days or 7 days after the transient cerebral ischemia, the mice were used for the one trial social interaction test.

Drugs and treatments

N^{ω}-Nitro-L-arginine methyl ester (L-NAME), *S*-ethylisothiourea (EIT), and 7-Nitroindazole (7-NI) were obtained from Sigma (St. Louis, MO, USA). L-NAME and EIT were dissolved in saline, and 7-NI was suspended in saline using a few drops of Tween-80. L-NAME (10 mg/kg), EIT (5 mg/kg) and 7-NI (120 mg/kg) were administered intraperitoneally twice at 10 min and 6 h after occlusion and reperfusion. Saline or vehicle was also administered by the same route as a control.

Statistical analyses

Data were expressed as the mean values ± S.E.M. Significance of the differences between the individual mean values was analyzed by Dunnett's test or Student's *t*-test. The difference was considered to be significant at $P<0.05$.

Results

Effect of bilateral carotid artery occlusion

Figure 1 showed the exploratory activity of mice assessed by the elevated plus-maze test, 2 or 7 days following the bilateral carotid artery occlusion. Time spent on the open arms of the occluded mice was significantly decreased at 2 days after the transient cerebral ischemia and the sham operation, compared with the control group; and the decreased time was recovered to the normal level at 7 days after transient cerebral ischemia as well as the sham group. Meanwhile, the number of open arm entries of occluded mice tended to decrease at 2 and 7 days after the transient cerebral

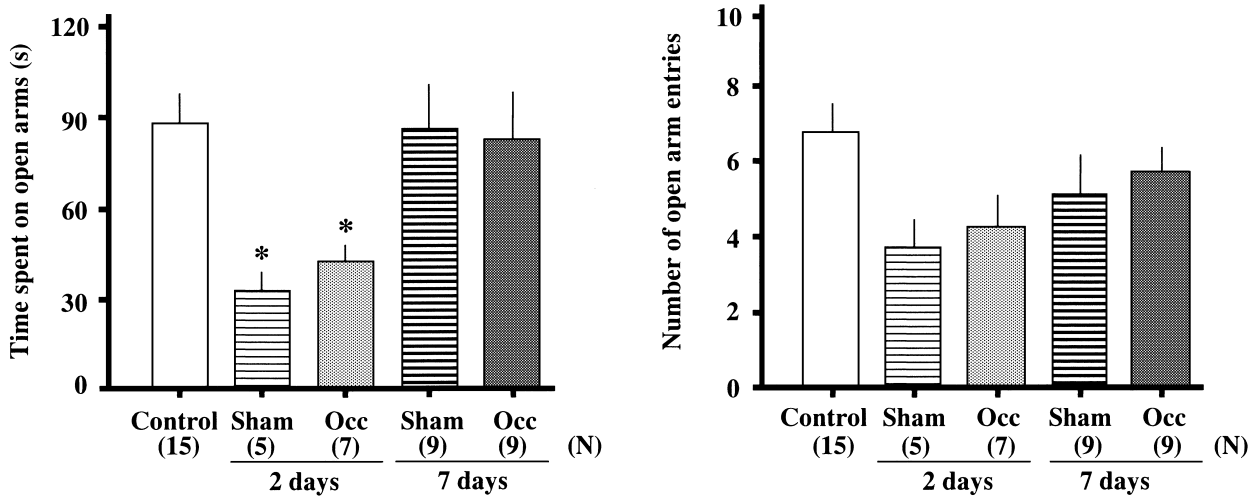

Fig. 1. Anxiogenic behavior of mice 2 and 7 days after the bilateral carotid artery occlusion in the elevated plus-maze test. Mice received bilateral carotid artery occlusion for 10 min and reperfusion, and they were tested in the elevated plus-maze test 2 and 7 days after the occlusion. The left panel shows time spent on the open arms and the right panel shows the number of open arm entries. Each value indicates the mean ± S.E.M. **P*<0.05, compared with the control group.

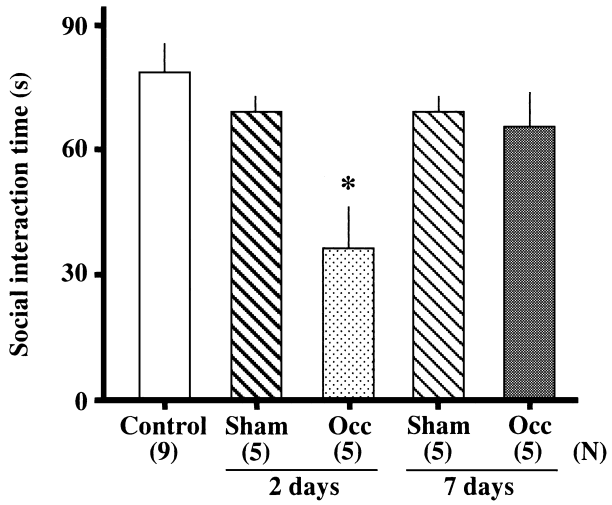

Fig. 2. Changes in social interaction time of mice 2 and 7 days after the bilateral carotid artery occlusion. Mice received bilateral carotid artery occlusion for 10 min and reperfusion, and they were tested in the social interaction test 2 and 7 days after the occlusion. Each value indicates the mean ± S.E.M. **P*<0.05, compared with the control group.

ischemia, but there was no significant difference compared with the control. No changes of locomotor activities were observed in all experimental animals (data not shown). Figure 2 showed the social interaction time of mice assessed by the social interaction test, 2 or 7 days following bilateral carotid artery occlusion. The social interaction time of occluded mice was significantly reduced at 2 days after transient cerebral ischemia, and then, the shortened time was recovered to the control level at 7 days after the transient cerebral ischemia. Differing from the results of the elevated plus-maze test 2 days after the occlusion, the sham-operation was substantially without effect on the social interaction time.

Effect of NOS inhibitors on the exploratory activity in the elevated plus-maze test

Table 1 showed the effect of NOS inhibitors on the exploratory activity of mice 2 days after the operation in the elevated plus-maze test. In the occluded mice, the treatment with L-NAME, a nonselective NOS inhibitor, significantly increased the time spent on the open arms. Moreover, this value was almost the same as that of the control as shown in Fig. 1. Treatment of the occluded mice with a selective iNOS inhibitor, EIT, likewise abolished the attenuation of time spent on the open arms in the elevated plus-maze test. In contrast, 7-NI, a relatively selective nNOS inhibitor, failed to counteract the decreased time spent on the open arms observed by the occluded mice. In the sham-operated mice, the attenuated time was actually unaffected by these NOS inhibitors. On the other hand, there were no significant differences in the number of open arm entries among all experimental groups. Additionally, no changes of locomotor activities were observed in all experimental animals (data not shown).

Effect of NOS inhibitors on the exploratory activity in the social interaction test

Effect of NOS inhibitors on the exploratory activity of mice 2 days after the operation in the social interaction

Table 1. Effect of NOS inhibitors on the anxiogenic behavior of mice 2 days after the bilateral carotid artery occlusion in the elevated plus-maze test

Parameter	Treatment			
	Saline or vehicle	L-NAME	EIT	7-NI
Time spent on open arms				
Sham	100 ± 19.6 (10)	148 ± 27.5 (7)		
	100 ± 9.8 (9)		135 ± 17.9 (8)	
	100 ± 14.6 (10)			100 ± 12.5 (11)
Occ	100 ± 9.0 (15)	154 ± 19.0* (14)		
	100 ± 10.7 (12)		168 ± 21.3* (12)	
	100 ± 14.2 (12)			100 ± 9.1 (14)
Number of open arm entries				
Sham	100 ± 17.1 (10)	99 ± 16.5 (7)		
	100 ± 19.4 (9)		123 ± 16.8 (8)	
	100 ± 19.3 (10)			104 ± 17.9 (11)
Occ	100 ± 9.7 (15)	99 ± 10.1 (14)		
	100 ± 14.3 (12)		126 ± 14.1 (12)	
	100 ± 11.0 (12)			101 ± 9.0 (14)

NOS inhibitors were administered twice at 10 min and 6 h after the occlusion and reperfusion. The time spent on the open arms and the number of open arm entries of sham-operated and occluded mice treated with NOS inhibitors were expressed as the percent of the sham-operated or occluded mice treated with saline or vehicle, respectively. Each value indicates the mean ± S.E.M. **P*<0.05, compared with the occlusion group treated with saline.

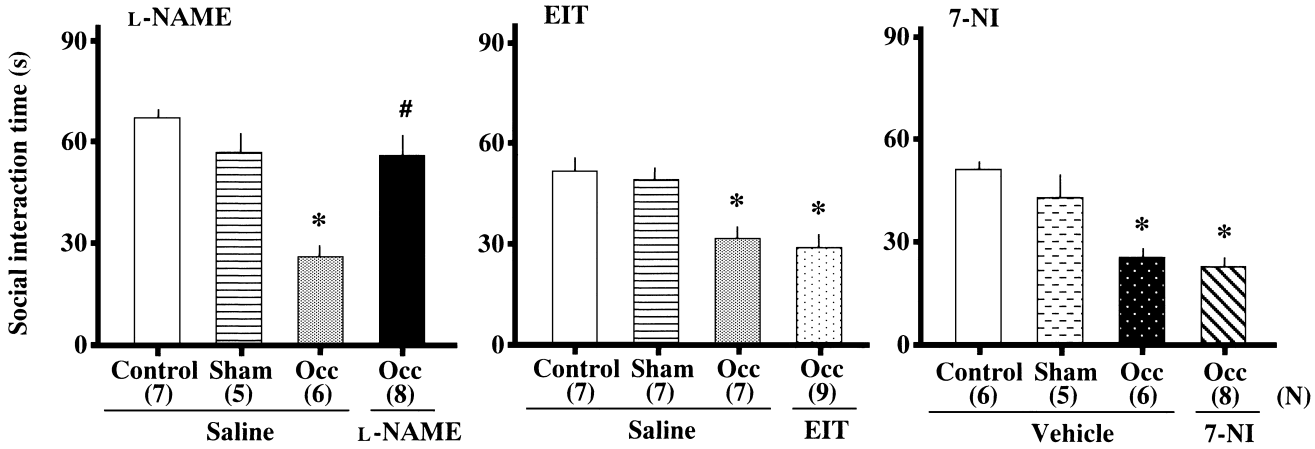

Fig. 3. Effect of NOS inhibitors on the anxiogenic behavior of mice 2 days after the bilateral carotid artery occlusion in the social interaction test. NOS inhibitors were administered twice at 10 min and 6 h after the occlusion and reperfusion. Each value indicates the mean ± S.E.M. **P*<0.05, compared with the sham group treated with saline or vehicle. $^{\#}P$<0.05, compared with the occlusion group treated with saline.

test is shown in Fig. 3. In all groups, the social interaction time of sham-operated mice was virtually equal to that of control mice. In the occluded mice, treatment with L-NAME alone significantly recovered the shortened interaction time. In contrast, treatment with EIT and 7-NI failed to counteract the shortened social interaction time observed by the occluded mice.

Discussion

The elevated plus-maze test that allows us to measure the natural aversion to the elevated open spaces of the apparatus is widely accepted as a method for determining the anxiety state in rodents (13, 14). In this study, mice with a 10-min transient cerebral ischemia showed

a significant decrease in time spent on the open arms compared with control mice when tested 2 days after the operation, indicating an increase of anxiety in the ischemic mice.

However, sham-operated mice have also shown similar behavior to that observed in occluded mice in this elevated plus-maze test. Likewise, Dhooper et al. (4) have reported the decrease in the time spent on the open arms of sham-operated rats, and they concluded that this phenomenon may result from the general malaise effect by anesthetizing. Accordingly, we attempted to differentiate the nature of anxiety attributed to the decrease of time spent on the open-arms observed in both occluded mice and sham-operated mice in the elevated plus-maze test. Here, we have chosen the social interaction test, an alternative method, for the assessment of anxiety. It is a test based on spontaneous social interaction behaviors of rodents. The social interaction behavior indicates active interaction elicited as a result of the formation of a relation between the mice placed in a novel test box; a decrease in the social interaction behavior was demonstrated to correlate with an increase of anxiety (15, 16). In our experiments, a significant decrease in the value of the social interaction time was observed in only the occluded mice 2 days after the operation. The value of the social interaction time of sham-operated mice was almost equal to that of the control mice. These results suggest that the elevated plus-maze test would have detected an apparent anxiogenic behavior in sham-operated mice. However, the transient cerebral ischemia is likely to produce an anxiogenic state in the occluded mice. These findings from the present animal studies provide evidence for the presence of post-stroke anxiety disorders in the clinical field. On the other hand, the level of anxiety 7 days after occlusion in both occluded and sham-operated mice was almost the same as that in the control mice. This result may suggest that certain functions such as feedback mechanisms compensate for the anxiogenic state 7 days after transient cerebral ischemia in mice.

Numerous reports have demonstrated that NO is involved in the mechanisms of cerebral ischemia. There is accumulating evidence indicating that NO acts as a protective or a destructive substance depending on the stage of evolution of the cerebral ischemic process and on its cellular source. There is appreciable evidence to suggest that NO originating from eNOS may improve microvascular flow by relaxation of cerebrovascular smooth muscles and may inhibit platelet aggregation and leukocyte adhesion (17 – 19). Thus, NO production at the vascular level seems only protective during the early stages of cerebral ischemia. On the other hand, it has been reported that nNOS activity increases 10 min after focal ischemia and it returns to the normal level 60 min later (20, 21). In the late stages of cerebral ischemia (>6 h), iNOS is expressed and large amounts of NO produced by this enzyme contribute to the progression of tissue damage (22 – 24). These reports have suggested that the role of NO in ischemic brain injury depends on the stage of evolution of the cerebral ischemic process and on the NO producing systems of NOS isoforms. Therefore, we examined the effects of different types of NOS inhibitors on the level of anxiety assessed by the elevated plus-maze test and the social interaction test following transient cerebral ischemia in mice, to estimate the participation of NO in the anxiogenic behavior induced by the cerebral ischemia.

Administration of L-NAME, a nonselective NOS inhibitor, produced an anxiolytic effect after transient cerebral ischemia in occluded mice in both tests. This result implies that the anxiogenic behavior induced by transient cerebral ischemia participates in the production of NO. Moreover, in the elevated plus-maze test, the administration of EIT, a selective iNOS inhibitor, also had an anxiolytic effect, similar to that of L-NAME; however, administration of 7-NI, a relatively selective nNOS inhibitor, failed to improve the level of anxiety in occluded mice. These results obtained from the elevated plus-maze test suggest that iNOS-induced NO plays an important role in the formation of anxiety following the transient cerebral ischemia in mice. Differing from the results of the elevated plus-maze test, the administration of EIT had no anxiolytic effect in occluded mice in the social interaction test. The administration of 7-NI also failed to retrieve the decreased social interaction time in occluded mice. These findings obtained from the social interaction test indicate that neither nNOS- nor iNOS-induced NO is concerned with the formation of anxiety in occluded mice. This discrepancy between the results from the two paradigms might reflect the different types of anxiety produced by these two models. The elevated plus-maze model appears to measure fear of exposure to an open space, whereas the social interaction model tests fear of novelty. Additionally, although an apparent anxiogenic effect induced by sham operation in the elevated-plus maze test could not be practically neglected, the effect is unlikely to occur by a mechanism related to the NO formation, because NOS inhibitors have no influence on the anxiety induced by sham operation, and the operation does not cause the appearance of anxiogenic behavior itself in the social interaction test.

Taken together, we have demonstrated that NO systems participate in the production of anxiogenic behavior by transient cerebral ischemia; and furthermore, we showed that NO generated by eNOS or eNOS with iNOS, but not nNOS, plays an important role in the

anxiety induced by ischemia.

In conclusion, NOS inhibitors would be of great therapeutic value because of their effectiveness for ischemia-induced anxiety. Additionally, mice with a 10-min bilateral carotid occlusion was suggested as a useful exploratory animal model of anxiety induced by transient cerebral ischemia.

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