Direct and Continuous Monitoring of Intrahippocampal Nitric Oxide (NO) by an NO Sensor in Freely Moving Rat after *N*-Methyl-D-aspartic Acid Injection

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We directly and continuously monitored the endogenous nitric oxide (NO) in the rat hippocampus after intraperitoneal (i.p.) administration of *N*-methyl-D-aspartic acid (NMDA) in freely moving state. The sensor used here was Malinski-type porphyrinic probe modified so as to measure in the freely moving animals. When the rats received the subconvulsive dose of NMDA (20 mg/kg), a significant increase of the NO concentration in the hippocampus was continuously observed. The increase was up to 1 μ M at 25-30 min past administration. This elevation in NO concentration was totally blocked by pre-treatment with an NO synthase inhibitor, *N*^G-monomethyl-L-arginine (30 mg/kg, i.p.). These findings indicate that our sensor successfully measured the NMDA-induced NO in the hippocampus catalyzed by NO synthase.

1 INTRODUCTION

Hippocampus is rich in *N*-methyl-D-aspartic acid (NMDA) receptors¹⁾, which are thought to be involved in the important phenomena such as memory²⁾, neurotoxicity³⁾, and epilepsy⁴⁾. NMDA receptors have been shown to induce nitric oxide (NO) generation, via NO synthase, from arginine^{5,6)}. NO then activates soluble guanylate cyclase, leading to formation of cGMP. Recently, *in vivo* evidence for an NMDA-stimulated increase of cGMP concentration in rat hippocampus have been reported⁷⁾. Thus NO increase was indirectly shown but its concentration was unknown. In spite of increasing importance of measuring NO, to our knowledge, direct and quantitative measurements of NO in the hippocampus after activation of NMDA receptors has not been performed. NO is a free radical and has a short

half-life in the biological system. Therefore, it is difficult to measure *in vivo* by the standard chemical technique^{8,9)}.

Malinski *et al.*¹⁰⁾ have reported porphyrinic-based electrode which functioned as an NO sensor. The NO sensor detects the current of NO oxidation when a characteristic potential is applied. The NO sensor consists of polymerized Ni-porphyrin layer on the surface of carbon fiber electrode and Nafion membrane out-side of the porphyrin layer. With catalytic action for NO oxidation by the Ni-porphyrin, the sensor detects NO at high sensitivity. On the other hand, Nafion has a role of high selectivity for NO. Since NO and nitrous anion, metabolized production of NO, have almost same oxidative potentials around 0.65V vs. Ag/AgCl, high selectivity of NO sensor requires elimination of the nitrous anion on the measurement of NO. Nafion membrane does not allow to pass the nitrous anion.

In this study, we modified to reinforce the structure of the Malinski-type NO sensor so that we could measure the NO in the brain of a freely moving animal, and performed direct detection of the endogenous NO in the rat hippocampus after intraperitoneal (i.p.) administration of NMDA. We also tested the effect of an NO synthase inhibitor, *N*^G-monomethyl-L-arginine

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(NMNA), on the NMDA-induced NO elevation.

2 EXPERIMENTAL

2.1 Fabrication of NO sensor

The NO sensor was made according to the method of Malinski *et al.*⁽¹⁰⁾ with some modifications. Ten carbon fibers (10 μm in diameter) were connected to copper wire with Ag paste. The bundle of carbon fibers was insulated using a Teflon solution (Cytop, Asahi Glass Co. Ltd.), and then enclosed into a glass capillary tube (1 mm in outer diameter, 15 mm in length) by epoxy resin. The glass capillary and the copper wire were fixed by dental cement. The tip of the glass capillary tube was polished. A thin layer of polymeric porphyrin was deposited on the polished surface of carbon fibers by the cyclic potential scans (-200~1200 mV vs. Ag/AgCl, 50 mV/sec) in a solution of 0.1N NaOH, containing 1 mM nickel tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin, prepared with modifications of the procedure by Malinski *et al.*⁽¹¹⁾. Subsequently, the tip of this electrode was dipped in the Nafion solution (5% w/w in alcohol) for 15 sec. After being air dried for 5 min, the electrode was used as a working electrode of the NO sensor (Fig. 1). An Ag/AgCl electrode was employed as a reference electrode, and a platinum wire was used as a counter electrode.

Pure NO gas was bubbled through the O₂-purged phosphate buffer solution (PBS) (pH 7.4). Thus, saturated NO solution was obtained. The NO sensor was immersed in the O₂-purged PBS in the airtight electrolytic cell. NO solutions of various concentration were prepared by injecting the saturated NO solution

Fig. 1 Structure of the NO sensor.

into the cell using microsyringe. We have used differential double-pulse amperometry to obtain sensor responses for NO. Since differential voltammetry showed a peak for the catalytic oxidation of NO at 670 mV vs. Ag/AgCl, the potentials of the first and second pulses were set, respectively, at 650 mV and 690 mV vs. Ag/AgCl (Fig. 2) in order to improve the selectivity to NO. The response increased almost linearly with the concentration of NO in the range up to 5 μM . For each sensor, the responses were plotted against NO concentration to obtain a calibration curve. The electrochemical analysis was carried out by CS1090 (Cypress Co. Ltd.).

2.2 NO detection after NMDA simulation

Male Wistar rats (200-250g) were used. The animals were anesthetized with pentobarbital (50 mg/kg, i.p.), and the working electrode was stereotaxially implanted into the right hippocampus. The counter electrode and the reference electrode were placed on the dura over the contralateral parietal cortex. In a preliminary experiment, 20, 40, and 80 mg/kg of NMDA was i.p. injected to the rats. There was no behavioral change after administration of 20 mg/kg of NMDA, although NMDA doses more than 40 mg/kg produced myoclonic jerk, or running, or both. Therefore, in this study, 20 mg/kg (5 ml/kg) of NMDA (n=5) dissolved in saline was i.p. given. Controls (n=5) i.p. received an identical volume of saline alone. To test the effect of the NO synthase inhibitor, 4 animals received i.p. injection of 30 mg/kg (5 ml/kg) of NMMA dissolved saline. Thirty min later, 20 mg/kg (5 ml/kg) of NMDA was i.p. given. The NO measurements were conducted 2~4 days after implantation. The oxidation current of NO was measured by the differential double-pulse amperometry (650 mV/600 msec, 690 mV/200 msec)

Fig. 2 Schematic diagram of differential double-pulse amperometry for NO detection.

for freely moving rats for 30 min. The current was continuously recorded against time. The current was converted to NO concentration using the calibration curve.

3 RESULTS

Typical calibration curve of the NO sensor was presented in Fig. 3. The current values showed a linear elevation in association with NO concentration. The calibration curve indicates that our NO sensor can measure NO concentration on the order of 10^{-6} M. The detection limit was calculated as about $0.3 \mu\text{M}$ at an S/

Fig. 3 Calibration curve of the NO sensor which shows the relationship between current and NO concentration.

N ratio of 3. When the NO calibrations were repeated with the same sensor, similar current-responses were obtained. However, NO-response of the sensor was lost 1 week after the fabrication.

Saline, NMDA, or NMMA did not cause any behavioral change at the dose used here. Saline did not influence the NO concentration (Fig. 4, open circle). However, NMDA injection led to significant augmentation in NO concentration, which began to elevate at about 15 min and reached the maximal level at about 27 min (Fig. 4, closed circle). The maximal NO level was high by about $1 \mu\text{M}$ from the initial level. On the other hand, any animals given pre-treatment with NMMA did not exhibit significant increase of NO (Fig. 4, closed square).

4 DISCUSSION

The present study shows the first and direct measurements of NO in the hippocampus of rats. A significant elevation in NO concentration after i.p. administration of a subconvulsive dose of NMDA was observed. The parenteral NMDA, at a large dose, is known to produce severe epileptic seizures, accompanied by cerebral ischemia^{3,4}. Epileptic seizures and cerebral ischemia may be pathophysiological conditions associated with an elevation of NO concentration⁶. However, since no identifiable change

Fig. 4 Time course of NO concentration after injection of saline or NMDA in freely moving rats. ΔNO indicates degree of elevation (postinjection NO minus preinjection NO). Values are mean $\pm$ standard error (Student's t-test: $p < 0.01$, *)

in the behavior was caused by NMDA, it is unlikely that other mechanisms (such as epileptic seizure and cerebral ischemia) than the simple activation of NMDA receptor are major factors involved in the NO elevation observed here. The present result also includes that the NMDA-induced elevation of NO concentration is totally blocked by pre-treatment with the NO synthase inhibitor NMMA. This finding is consistent with the general concept that NO synthase which is activated by the excitation of NMDA receptors stimulates NO generation in the hippocampus⁶⁾.

Recently, Malinski *et al.*¹²⁾ have reported an increase in NO concentration in the rat cerebral cortex during cerebral ischemia using their sensors of cylinder electrodes of carbon fibers. Thus, the experiments were conducted with the rats under anesthesia. Rigidity of our sensor was reinforced using the glass capillary filled with epoxy resin for detection in the freely moving animals. The used glass capillary (o.d., 1 mm) is permissible size for implantation into the rat brain. So, using this sensor, simultaneous monitoring of NO concentration and behavior can be continuously made. *In vivo* measurement of NO in the brain is important for understanding the possible roles of NO directly associated with the behavioral changes, such as epilepsy.

For this reason, the NO sensor is considered to be useful tool.

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