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NITRIC OXIDE PRODUCTION IN THE RAT BRAIN AFTER KAINATE-INDUCED SEIZURE

RADENOVIĆ LIDIJA *, JOVANOVIĆ MARINA **, VASILJEVIĆ IVANA**, NINKOVIĆ MILICA**, SELAKOVIĆ VESNA** and MALIČEVIĆ Ž**

*Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Serbia
*Institute for Medical Research, Military Medical Academy, Belgrade, Serbia

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We investigated the role of nitric oxide (NO) as a new neurotransmitter in the control of excitability and neurotoxicity of the hippocampus, forebrain cortex, striatum and cerebellum of the rat, as well as the possible functional interaction between NO and the glutamate system. Kainic acid is an endogenous excitotoxin acting on glutamate non-N-methyl-D-aspartate (non-NMDA) receptors, that leads to neurotoxic damage resembling the alterations observed in some neurological disorders. Stimulation of glutamate receptors induces neuronal NO release, which in turn modulates glutamate transmission.

We also investigated the effects of 7-nitroindazole (7-NI), a selective neuronal nitric oxide synthase inhibitor in vivo, on nitrite concentration after kainic acid injection (0.5 mg/ml, pH 7.2) unilaterally into the CA3 region of the rat hippocampus. The accumulation of nitrite, the stable metabolite of NO, was measured by the Griess reaction at different times (5 min, 15 min, 2 h, 48 h and 7 days) following kainate injection in the ipsi- and contralateral hippocampus, forebrain cortex, striatum and cerebellum homogenates.

7-NI (100 microM) can effectively inhibit NO synthesis in rat brain after kainate-induced intrahippocampal neurotoxicity, suppressed nitrite accumulation and attenuated neuronal damage induced by NMDA overactivity. All data showed that reduction of nitrite levels in the nervous system causes overactivity resulting from the absence of the NO-mediated modulatory action. The relatively transient nitric oxide synthase inhibitory effect of 7-NI following intracerebral injection should be taken into account when using this drug to evaluate the central effects of NO. The present results implicate neuronal NO generation in the pathogenesis of both direct and secondary excitotoxic neuronal injuries in vivo. As such they suggest that neuronal NO synthase inhibitors may be useful in the treatment of neurological diseases in which excitotoxic mechanisms play a role.

Keywords: brain, nitric oxide, nitrite, kainate, 7-nitroindazole

INTRODUCTION

Excitotoxicity, a process by which overactivation of glutamate receptors induces cell death, is responsible for many neurological disorders ranging from acute insults, such as stroke, hypoglycemia, ischemia, epileptiform seizures, to chronic neurodegenerative diseases, such as Huntington's disease, Alzheimer's disease or amyotrophic lateral sclerosis (Albin *et al.* 1992; Lipton et al. 1994). Excitatory amino acids act on the CNS through various receptors, which are classified into two groups: ionotropic and metabotropic. Ionotropic receptors act on cationic-specific ion channels and can be divided into three subtypes: NMDA, AMPA and kainate (KA) receptors (Hollmann *et al.* 1994; Gratacos *et al.* 2001). Glutamate receptors are the primary excitatory neurotransmitter receptors in vertebrate brain and are of critical importance to a wide variety of neurological processes. Recent reports suggest that ionotropic glutamate receptors may have a unique transmembrane topology not shared by other ligand-gated ion channels (Wo *et al.* 1994).

Stimulation of glutamate KA receptors induces neuronal nitric oxide (NO) release, which in turn modulates glutamate transmission (Alabadi *et al.* 1999; Nakaki *et al.* 2000). NO is a highly reactive signal molecule in the CNS. The agent is a gaseous chemical messenger that acts on interneuronal communications, synaptic plasticity, memory formation, receptor function, intracellular signal transmission and mediator release (Brown 1999; Heales *et al.* 1999; Lei *et al.* 1999). However, pathological conditions may occur when higher fluxes of these mediators are generated, such as during the process referred to as excitotoxicity, i.e. the excessive activation of glutamate KA receptors. This is a condition common to both acute and chronic neurological diseases (Sengpiel *et al.* 1998; Brorson et al. 1999; Cirili *et al.* 2001).

In view of the above, the present study was undertaken to examine whether the production of NO after intracerebral injections of kainate can be modulated with pretreatment with 7-nitroindazole (7-NI), the selective neuronal nitric oxide synthase (nNOS) inhibitor.

MATERIALS AND METHODS

Animals

Adult rats of the Wistar strain (Rattus norvegicus) of both sexes, with body weight 200 ± 30 g, were used for the experiments. Groups of two or three rats per cage (Erath, FRG), were housed in an air-conditioned room at temperature of 23 ± 2 °C with 55 ± 10% humidity and with lights on 12 h/day (07.00-19.00). The animals were given a commercial rat diet and tap water *ad libitum*. These animals were anaesthetized by intraperitoneal injections of pentobarbital sodium (0.0405 g/kg b.w.) and were placed in a stereotaxic frame.

Experimental procedure and intracerebral injection of drugs

The rats were divided into three basic groups (according drug treatment), each basic group consisting of 5 different subgroups (according survival times) and each subgroup consisting of 8 animals. The first group received an unilateral injection of kainic acid (Sigma Chemical Co. U.S.A., 0.5 mg/ml, dissolved in 0.1 M saline, pH 7.2; 1μL total volume) into the CA3 region of the hippocampus (coordi-

nates from bregma: anteroposterior: -3.3 mm, dorsoventral: 3.2 mm, and lateral: 3.0 mm) by using a Hamilton microsyringe with a beveled tip. The second group received kainic acid and 7-nitroindazole (Sigma Chemical Co. U.S.A., 0.5 mg/ml, dissolved in purified olive oil, pH 7.2; 1μL). Finally, the third group received the same volume (1 μL) saline and served as a control (sham-operated). The animals were allowed to survive for 5 min up to seven days (5 min, 15 min, 2h, 48h and 7 days). All animals were anaesthetized, decapitated and the brains immediately removed. Ipsi- and contralateral hippocampus, forebrain cortex, striatum as well as cerebellum from individual animals were quickly isolated and homogenized in ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, 50 mM K-Na phosphate buffer, pH 7.2. Homogenates were centrifuged twice at 1580 g for 15 min at 4 °C. The supernatant obtained by this procedure was then frozen and stored at -70 °C.

Nitrite measurement

Nitrite and nitrate determinations in biological material are increasingly being used as markers of nitric oxide production. We detected nitrite in the rat brain homogenates (the ipsi- and contralateral hippocampus, forebrain cortex, striatum, and cerebellum) by Griess' method (Guevara *et al.* 1998). NO production was quantified by measuring nitrite, a stable oxidation end product of NO (Green *et al.* 1982). Briefly, nitrite production was determined by mixing 50 μL of the assay buffer with 50 μL of Griess reagent (1.5% sulfanilamide in 1M HCl plus 0.15% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water, v:v). After 10 min of incubation at room temperature, the absorbance at 540 nm was determined and nitrite concentrations were calculated from the sodium nitrite (Sigma) standard curve. All measurements were performed in triplicate.

Protein measurement

The content of protein in the rat brain homogenates (hippocampus, striatum, forebrain cortex and cerebellum, ipsilateral and contralateral) was measured by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as the standard. All measurements were performed in triplicate.

Data presentation and analysis

All experiments were done with $n = 8$. Each assay was performed at least twice under identical conditions. Data are expressed as means ± SD. The statistical significance of differences between groups was assessed by Student's *t*-test (paired and unpaired) for individual comparisons and regression analysis for overall significance (with $p < 0.05$ as significant and $p < 0.01$ as very significant).

Materials

Chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade. All drug solutions were prepared on the day of experiment.

Animals used for the procedures were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

RESULTS

Nitrite levels in the rat hippocampus

The results presented in Table 1. show the nitrite levels (mM/mg proteins) in ipsilateral and contralateral hippocampal homogenates respectively. KA injection

resulted in generally higher levels of nitrite production at all tested times. There was no statistically significant difference between mean nitrite levels obtained from each hemisphere, although the injection site was in the ipsilateral hippocampus. 7-NI treatment followed with KA, very clearly inhibitied nitrite production in this brain structure. The early tested times of 5 min (2.99 ± 1.18), 15 min (2.84 ± 0.56) and 2 h (2.89 ± 1.49) showed statistically significant difference (according to Students t-test; $p<0.05$) compared with the equivalent control groups, in the ipsilateral side of brain. The results obtained for the contralateral hippocampus were similar. Measurements at 15 min (2.46 ± 1.23, $p<0.05$) and 2 h (2.25 ± 0.68, $p<0.05$) showed statistically significant differences compared with the equivalent control groups.

Table 1.The effect of intrahippocampal drug injection on nitrite levels (mM NO^-_2/mg prot.) in the rat hippocampus (ipsilateral and contralateral), at different survival times. Data are means ± S.D.

Time	Hemi s	Control	KA	KA + 7-NI
5 min.	I	8.75 ± 1.51	9.76 ± 1.83	2.99 ± 1.18 *
	C	6.67 ± 1.72	6.85 ± 1.2	4.61 ± 1.44
	I	6.32 ± 1.45	7.97 ± 2.18	2.84 ± 0.56 *
	C	6.62 ± 1.24	6.86 ± 2.21	2.46 ± 1.23 *
2 h	I	8.23 ± 1.3	8.65 ± 1.9	2.89 ± 1.49 *
	C	8.81 ± 1.74	8.96 ± 1.85	2.25 ± 0.68 *
48 h	I	6.18 ± 1.52	7.94 ± 1.67	5.36 ± 1.8
	C	6.04 ± 1.15	6.81 ± 1.43	4.68 ± 1.68
7 days	I	5.32 ± 1.66	7.79 ± 1.56	4.62 ± 2.04
	C	6.11 ± 1.71	6.57 ± 1.67	5.20 ± 1.96

*Indicates a statistically significant difference between treated (KA- and KA+7-NI-treated) and control (sham-operated) animals ($p < 0.05$).

Nitrite levels in the rat forebrain cortex

There was a significant reduction in nitrite levels after KA + 7-NI treatment at all tested times, especially at 5 min (4.14 ± 1.66, $p<0.05$) and 2 h (2.25 ± 0.79, $p<0.05$) in the ipsilateral side, as well as 5 min (4.07 ± 0.77, $p<0.05$) and 2 h (2.68 ± 0.98, $p<0.05$) in the contralateral side of the brain (Table 2). There was no statistically significant difference between mean nitrite levels obtained from each hemisphere.

Nitrite levels in the rat striatum

The striatum, the main component of the basal ganglia, receives glutamatergic inputs from the cortex and thalamus and therefore considerable attention has been given to the role of excitotoxicity in striatal disorders. The effect of

Table 2. The effect of intrahippocampal drug injection on nitrite levels (mM NO^-_2/mg prot.) in the rat forebrain cortex (ipsilateral and contralateral), at different survival times. Data are means ± S.D.

Time	Hemi s	Control	KA	KA + 7-NI
5 min.	I	8.04 ± 1.29	7.45 ± 1.2	4.14 ± 1.66 *
	C	8.59 ± 1.78	6.14 ± 1.98	4.07 ± 0.77 *
15 min.	I	6.89 ± 2.07	5.26 ± 2.09	3.52 ± 0.87
	C	5.58 ± 1.38	4.68 ± 1.16	2.64 ± 1.09
2 h	I	6.67 ± 1.63	6.19 ± 1.96	2.25 ± 0.79 *
	C	5.76 ± 1.57	5.59 ± 1.06	2.68 ± 0.98 *
48 h	I	6.59 ± 1.5	6.27 ± 1.38	3.49 ± 0.99
	C	5.91 ± 0.73	5.39 ± 1.2	3.46 ± 1.99
7 days	I	4.74 ± 0.87	4.55 ± 1.59	3.14 ± 1.22
	C	4.98 ± 1.27	4.00 ± 1.45	3.44 ± 1.21

*Indicates a statistically significant difference between treated (KA- and KA+7-NI-treated) and sham-operated animals ($p < 0.05$).

Table 3. The effect of intrahippocampal drug injection on nitrite levels (mM NO^-_2/mg prot.) in the rat striatum (ipsilateral and contralateral), at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between treated (KA- and KA+7-NI-treated) and sham-operated animals ($p<0.05$).

Time	Hemi s	Control	KA	KA + 7-NI
5 min.	I	10.33 ± 1.2	9.96 ± 1.74	3.03 ± 1.04 **
	C	8.25 ± 1.23	9.21 ± 1.84	3.08 ± 1.59 **
15 min.	I	5.97 ± 1.78	7.28 ± 1.45	3.34 ± 1.81 *
	C	6.59 ± 1.92	9.24 ± 1.24	4.14 ± 1.49
2 h	I	9.30 ± 1.92	4.66 ± 1.36 *	1.90 ± 0.45 **
	C	10.22 ± 1.25	5.05 ± 0.73 *	2.89 ± 0.82 **
48 h	I	6.60 ± 1.7	5.45 ± 1.3	3.41 ± 1.68
	C	5.69 ± 1.53	6.24 ± 1.2	3.26 ± 0.94
7 days	I	6.41 ± 1.62	5.12 ± 1.84	4.23 ± 1.54
	C	4.76 ± 1.78	5.01 ± 1.29	3.63 ± 1.48

**Indicates a statistically very significant difference between treated (KA- and KA+7-NI-treated) and control (sham-operated) animals ($p<0.01$).

intrahippocampal drug injection on nitrite production in striatum is shown in Table 3. The effect of KA injection on nitrite levels measured at 2 h (4.66 ± 1.36, $p<0.05$) for the ipsilateral and 2 h (5.05 ± 0.73, $p<0.05$) for the contralateral side was a significant decrease. KA + 7-NI treatment at all tested times showed a significant reduction in nitrite levels, especially at 5 min (3.03 ± 1.04, $p<0.01$), 15 min (3.34 ± 1.81, $p<0.05$) and 2 h (1.90 ± 0.45, $p<0.01$) and at 5 min (3.08 ± 1.59, $p<0.01$) and 2 h (2.89 ± 0.82, $p<0.01$), for the ipsi-and contralateral sides respectively again early tested times (Tab. 3). There was no statistically significant difference between mean nitrite levels obtained from each hemisphere.

Nitrite levels in the rat cerebellum

The results obtained for this brain structure were very similar to those for the striatum. The effect of KA injection on nitrite levels measured at 15 min for the ipsilateral (9.79 ± 1.53, $p<0.05$) and contralateral side (12.71 ± 1.41, $p<0.05$) showed a significant increase (Tab. 4). There was a significant reduction in nitrite levels after KA + 7-NI treatment at all tested times, especially at 5 min (2.45 ± 0.73, $p<0.01$), 15 min (2.76 ± 0.9, $p<0.05$) and 2 h (2.11 ± 0.83, $p<0.01$) ipsilaterally, and 5 min (3.75 ± 1.02, $p<0.01$), 15 min (3.09 ± 0.78, $p<0.05$) and 2 h (3.09 ± 1.69, $p<0.01$) contralaterally, again early tested times (Tab. 4). There was no statistically significant difference between mean nitrite levels obtained from each hemisphere.

Table 4. The effect of intrahippocampal drug injection on nitrite levels (mM NO^-_2/mg prot.) in the rat cerebellum (ipsilateral and contralateral), at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between treated (KA- and KA+7-NI-treated) and sham-operated animals ($p<0.05$).

Time	Hemi s	Control	KA	KA + 7-NI
5 min.	i	10.66 ± 1.75	9.26 ± 1.4	2.45 ± 0.73 **
	c	10.42 ± 1.78	10.07 ± 1.9	3.75 ± 1.02 **
15 min.	i	5.94 ± 1.98	9.79 ± 1.53 *	2.76 ± 0.9 *
	c	7.33 ± 1.01	12.71 ± 1.41 *	3.09 ± 0.78 *
2 h	i	10.31 ± 1.38	6.54 ± 1.22	2.11 ± 0.83 **
	c	9.85 ± 1.38	6.48 ± 1.4	3.09 ± 1.69 **
48 h	i	4.74 ± 1.15	6.53 ± 1.22	4.02 ± 2.06
	c	5.60 ± 2.08	6.38 ± 1.58	2.75 ± 1.01
7 days	i	5.75 ± 1.21	5.98 ± 1.63	4.33 ± 1.78
	c	6.12 ± 1.05	6.14 ± 1.71	4.84 ± 1.31

**Indicates a statistically very significant difference between treated (KA- and KA+7-NI-treated) and control (sham-operated) animals ($p<0.01$).

DISCUSSION

The role of nitric oxide (NO) in cerebral insult remains controversial. While numerous studies have used models of ischemia and hypoxia, few have examined nitric oxide in the kainate model of excitotoxicity. Animals exposed to kainic acid (KA) induced status epilepticus display a striking pattern of selective neuronal vulnerability in the hippocampus. Neurons in the hilus/CA3 and CA1 subfields appear particularly sensitive whereas dendate gyrus granule cells are resistant (Becer *et al.* 1999; Lere *et al.* 2002), which is likely to be due to the high concentration of kainate receptors on their membranes. Regional distribution of kainate receptors of the rat brain was found to be highest in deep layers (layer 5) of the forebrain cortex, cerebellar granule cell layer and caudate putamen (Carroll *et al.* 1998; Bailey *et al.* 2001), which is why we tested these particular brain regions: hippocampus, forebrain cortex, striatum and cerebellum. Also, intraperitoneal injection of KA produced behavioral changes ("wet dog shake', focal seizure of the limbs and neck, hypersalivation, or generalized convulsion) (Bagetta *et al.* 1995).

Kainic acid enhances hippocampal NO generation (Kashikara *et al.* 1998) and injection of KA results in differential regulation of neuronal nitric oxide synthase (nNOS) mRNA and NO formation in the rat hippocampus (Kashikara *et al.* 2000). The literature results implicate neuronal nitric oxide generation in the pathogenesis of both direct and secondary excitotoxic neuronal injuries *in vivo.* As such they suggest that neuronal nitric oxide synthase inhibitors may be useful in the treatment of neurological diseases in which excitotoxic mechanisms play a role. Type nNOS has been detected in the cerebellum, the hypothalamus, the striatum, the hippocampus and the medula oblongata (Torreilles *et al.* 1999).

In the present study an appropriate dose of kainate (0.5 mg/ml) was used to cause small brain damage in the ipsilateral, but not contralateral, hippocampus; no behavioral or epileptic effects. It has been previously shown that nitric oxide formation was determined in different regions of the rat brain during kainate-induced seizures (Mulsch *et al.* 1994; Yasuda *et al.* 2001). In our experiments, at various times following intrahippocampal kainate injection nitrite levels were measured in the four rat brain structures. Cortical areas are known to contain the highest packing densities of nNOS-positive interneurones such as the piriform and entorhinal cortices (Bidmon *et al.* 1999), indicating that, in normal animals, neurotransmission and probably cognitive information processing would be affected by the pharmacological modulation of nitric oxide production.

We have shown that the nitric oxide end-product levels in the ipsilateral hippocampus increased immediately after kainate injection and continued to increase gradually throughout the experiments. Under conditions of normal behavior in the rat, the damage was localized mainly in the CA3 region of hippocampus, where neuronal loss occurred. No significant changes in nitrite concentration were noted in the contralateral hippocampus, forebrain cortex, striatum or cerebellum of rats receiving intracerebral injections of kainate. 7-Nitroindazole (7-NI), a selective neuronal nitric oxide synthase inhibitor *in vivo,* at any dose used did not affect basal nitrite levels before intracerebral injections of kainate.

In addition, pretreatment with 7-NI was effective in preventing the effects of kainate. 7-NI inhibited the increased NO production 5 min after intracerebral injection. Under the conditions of this experiment, 7-NI produced a rapid (within 2 h) decrease in nitrite levels in all four brain regions. Previously, it was demonstrated

that 7-NI can produce designated changes in brain NO content, facilitating the use of 7-NI to probe the pharmacological implications of NO in the central nervous system (Bush *et al.* 2001). It was suggested that excessive production of NO is involved in the mechanisms triggering seizures and neurodegeneration produced by kainate (Bagetta *et al.* 1995).

The study presented here shows that after intrahippocampal injection 7-NI produced an approximately 50 % decrease in nitrite levels in all four brain regions, which was sustained. Correlation of the inhibitory effect was demonstrated in all tested brain structures whereby the striatum were shown to be the most sensitive and the forebrain cortex most resistant to 7-NI activity. The pathophysiological significance of decreased NO activity in selectied areas of the brain may indicate that treatment with 7-NI leads to protection of brain neurons against neuronal injuries by impairment of cellular energy metabolism and oxidative stress (Strorch *et al.* 2000). Our present results clearly show that NO synthase activity was significantly decreased in the group of pretreated animals. The combination of KA with nNOS inhibitor reduces the neurotoxic effects of KA in the rat brain. This study suggests that extremely fine levels in the different neural cell types can modulate excitotoxicity.

In conclusion, the present data indicate that the inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects neurons from seizure-induced toxicity in all tested brain regions. The relatively transient NOS inhibitory effect of 7-NI following intracerebral injection should be taken into account when using this drug to evaluate the central effects of nitric oxide.

Address for correspondence:
Lidija Radenovic, Ph.D. Assistant Professor
Department of General Physiology and Biophysics
Institute of Physiology and Biochemistry
Faculty of Biology, University of Belgrade
Studentski trg 3, 11000 Belgrade, Serbia, Yugoslavia
e-mail: lira@ibiss.bg.ac.yu

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STVARANJE AZOT OKSIDA U MOZGU PACOVA POSLE INTRACEREBRALNE APLIKACIJE KAINIČNE KISELINE

RADENOVIĆ LIDIJA, JOVANOVIĆ MARINA, VASILJEVIC IVANA NINKOVIC MILICA, SELAKOVIĆ VESNA i MALICEVIC Ž

SADRŽAJ

Intracerebralna aplikacija kainične kiseline (0.5 mg/ml, pH 7.2), agoniste kainatnih ne-NMDA receptora, u selektivno osetljiv CA3 region hipokampusa pacova dovodi do ekscitotoksičnog oštećenja neurona u ovoj strukturi, posredovano stvaranjem azot oksida (NO) kao medijatora oštećenja. S obzirom da ekscitotoksičnost dovodi do oksidacionog oštećenja i povećanog svaranja NO, praćenje dinamike stvaranja NO na nivou različitih moždanih struktura predstavlja senzitivni pokazatelj disfunkcije neurona u različitim vremenskim intervalima u odnosu na trenutak izazivanja ekscitotoksičnih efekata (5 min, 15 min, 2 h, 48 h i 7 dana). Stvaranje NO je praćeno preko akumulacije nitrita, stabilnih metabolita azot oksida, Griess-ovom metodom. Na stepen izazvane ekscitotoksičnosti u svim praćenim moždanim strukturama (ipsi- i kontralateralni hipokampus, korteks, striatum i cerebelum) neuroprotektivno je delovala primena 7-nitroindazola (100 μM), inhibitora NO-sintetaze u neuronima. Najočigledniji efekat 7-NI se postiže (već posle 2 h) u ranim praćenim terminima, što znači da veoma brzo i efikasno reaguje na povećanju akumulaciju nitrita u smislu smanjenja i eliminacije, pa samim tim i prevencije. Nije konstatovana razlika između pojedinih testiranih moždanih struktura, već je zaključeno da je primenjeni tretman podjenako efikasan i na nivou hipokampusa, korteksa, striatuma i cerebeluma. Na taj način je potvrđena naša predpostavka o izboru ove supstance u prevenciji intoksikacije kainatom.