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EFFECT OF 7-NITROINDAZOLE ON SUPEROXIDE PRODUCTION AND MnSOD ACTIVITY IN THE RAT BRAIN FOLLOWING KAINATE-INDUCED NEUROTOXICITY

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Abstract — We investigated the effect of 7-nitroindazole (7-NI), a selective neuronal nitric oxide synthase inhibitor in vivo, on superoxide concentration as well its influence on mitochondrial MnSOD activity since this activity is associated with the production of reactive oxygen species after kainate-induced neurotoxicity. The time course of *in vivo* oxidative damage in different brain regions was investigated. Measurements were performed at different times (5 min, 15 min, 2 h, 48 h, and 7 days) in the ipsi- and contralateral hippocampus, forebrain cortex, striatum, and cerebellum homogenates. Our results indicated that 7-NI had no statistically significant influence on superoxide concentrations in the tested brain structures compared to the control values. However, superoxide concentrations after kainate-induced neurotoxicity returned to the control values after pretreatment with 7-NI in all tested brain structures. Regarding the activity of MnSOD, our results demonstrated statistically significant increase 7 days after intrahippocampal KA treatment in all tested brain structures after pretreatment with 7-NI. The obtained results suggest that neuronal NO synthase inhibitors may be useful in the treatment of neurological diseases in which excitotoxic mechanisms play a role.

Key words: Kainate, glutamate neurotoxicity, 7-nitroindazole, brain, mitochondria, MnSOD, oxidative stress, superoxide

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INTRODUCTION

Oxidative stress and excessive activation of glutamate receptors are converging processes and represent sequential as well as interacting factors that provide a final common pathway for cell vulnerability in the brain. While excitotoxic and oxidative injury may occur independently, growing evidence indicates that reactive oxygen species (ROS) formation may also be a specific consequence of glutamate receptor-mediated neurotoxicity (D u g a n et al., 1995).

Kainic acid (KA) is an endogenous excitotoxin acting on glutamate receptors that leads to neurotoxic damage resembling the alterations observed in some neurological disorders. Stimulation of glutamate receptors induces superoxide production, which may be one of the mediators of excitotoxic neuronal injury in CNS. Free radical reactions are implicated in a variety of physiological and pathological processes and abnormalities associated with superoxide dismutase (SOD) have been documented in several neurodegenerative processes (C o y l e et al. 1993). Glutamate neurotoxicity is partly mediated by ROS formed as a consequence of several processes, including nitric oxide (A l a b a d i et al., 1999; N a k a k i et al., 2000; R a d e n o v i c et al., 2003, 2005b) and superoxide (L i et al., 2001; R a d e n o v i c et al., 2004) production. Superoxide radicals react rapidly with nitric oxide to form highly cytotoxic peroxynitrite, which acts through lipid peroxidation (L e e et al. 2001). Although there are a number of intracellular sources of free radicals, the mitochondria are thought to be the most important (C i r i o l o et al., 2001).

In view of the above, the present study was undertaken to examine whether the superoxide anion production and activity of mitochondrial MnSOD within the rat hippocampus, forebrain cortex, striatum and cerebellum after intrahippocampal

kainate injection can be modulated by pretreatment with 7-nitroindazole (7-NI), a selective neuronal nitric oxide synthase inhibitor *in vivo*.

MATERIALS AND METHODS

Animals

Adult rats of the Wistar strain *(Rattus norvegicus)* of both sexes with body weight of 200 ± 30 g were used for experiments. Groups of two or three rats per cage (Erath, FRG) were housed in an air-conditioned room at room 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 food and tap water *ad libitum*. These animals were anesthetized by giving them intraperitoneal injections of sodium pentobarbital (0.0405 g/kg b.w.) and then placed in a stereotaxic frame.

Experimental procedure and intracerebral injection of drug

The rats were divided into three basic groups (according to drug treatment), each basic group consisting of five different subgroups (according to survival times) and each subgroup consisting of eight animals. The first group received an unilateral injection of KA (Sigma Chemical Co. U.S.A., 0.5 mg/ml, dissolved in 0.1 M saline, pH 7.2; 1 μL total volume) into the CA3 region of the hippocampus (coordinates 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 KA after pretreatment with 7-NI (Sigma Chemical Co. U.S.A., 0.5 mg/ml, dissolved in purified olive oil, pH 7.2; 1 μL). Finally, the third group received the same volume (1 μL) but only saline solution and served as a control (sham-operated). The animals were allowed to survive for 5 min up to seven days (5 min, 15 min, 2 h, 48 h, and 7 days). All animals were anesthetized and decapitated, after which the brains were immediately removed. The ipsi- and contralateral hippocampus, forebrain cortex, striatum, and cerebellum were quickly isolated from individual animals and homogenized in ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, and 50 mM K-Na phosphate buffer, pH 7.2. Homogenates were centrifuged twice at 1580g for 15 min at 4 °C. The supernatant obtained by this procedure was then frozen and stored at -70 °C.

Superoxide production and measurement

In these experiments, superoxide was measured from reduction of nitro blue tetrazolium (NBT) as previously described (S p i t z et al., 1989). Detection of this product was by spectrophotometric quantification of the colored formazan product formed from blue tetrazolium. Reduction of NBT was measured at 560 nm.

Superoxide dismutase assay

The assay of MnSOD activity by the adrenaline method (F r i d v i c h et al., 1995) was used. The method is based on measuring the degree of adrenaline autooxidation inhibition by MnSOD contained in the examined samples in 50 mM sodium carbonate buffer, pH 10.2, with 5 mM KCN. Enzymatic activity was expressed in units per milligram of protein.

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 L o w r y et al. (1951) using bovine serum albumin (Sigma) as 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 the experiment.

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

RESULTS

Behavioral changes after kainate injection

The purpose of this study was to investigate fine changes in superoxide and MnSOD levels during the process of excitotoxicity in various brain parts and modulations of these parameters after preatreatment with 7-NI. Our aim was to inject KA (appropriate dose) but to avoid any behavioral changes ('wet dog shake', focal seizure of the limbs and neck, hypersalivation, or generalized convulsion) and typical limbic seizures evolving into status epilepticus, since during status epilepticus hippocampal blood flow, oxygen supply, and body temperatures are modified. These effects are accompanied by severe damage to all subfields of the hippocampal formation. It is a condition of intense metabolic activation and could interfere with our results and measurements. We did not measure epileptic activity by electroencephalogram. Only normally behaving animals took part in the experiments.

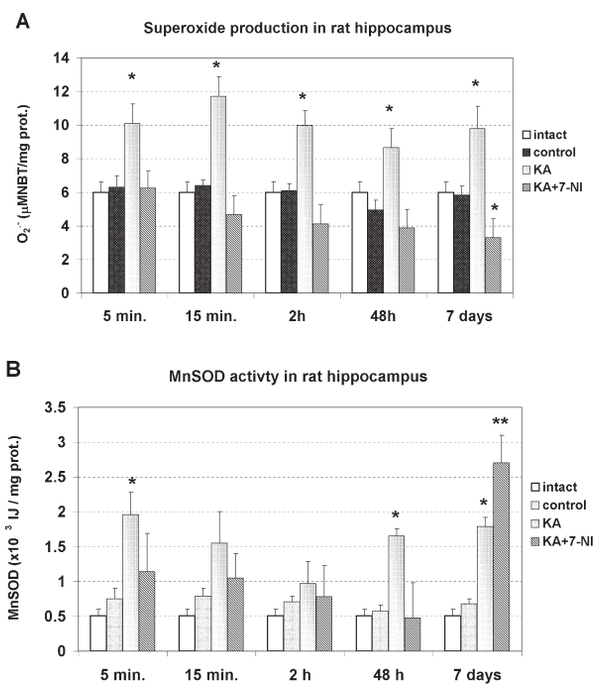

Fig. 1. A - Effect of pretreatment with 7-NI on superoxide production (O_2', μM NBT/mg prot.) in the ipsilateral rat hippocampus, at different survival times after intrahippocampal kainate injection. Data are means ±S.D. *Indicates a statistically significant difference between kainate treated and control (sham-operated) animals ($p<0.05$). B - Effect of pretreatment with 7-NI on MnSOD activity (x103 IJ/mg prot.) in the ipsilateral rat hippocampus at different survival times after intrahippocampal kainate injection. Data are means± S.D. *Indicates a statistically significant difference between kainate treated and control (sham-operated) animals ($p<0.05$). **Indicates a statistically very significant difference between animals treated with 7-NI plus kainate treated and control (sham-operated) animals ($p<0.01$).

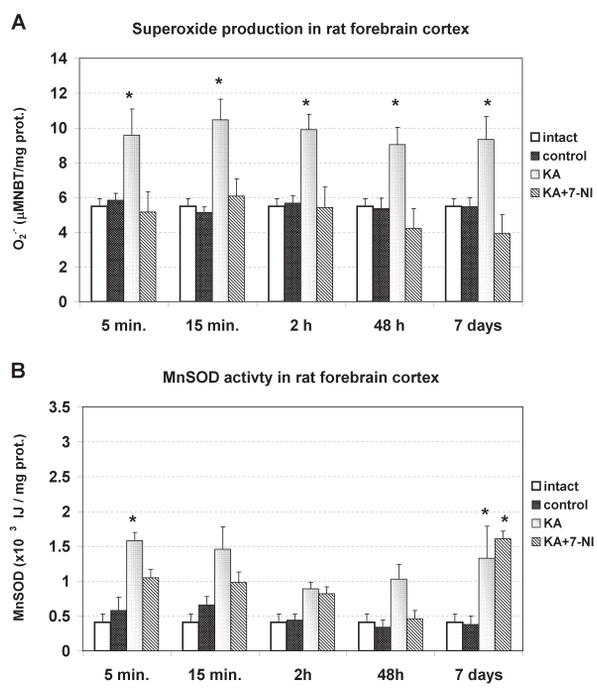

Fig. 2. A - Effect of pretreatment with 7-NI on superoxide production (O_2', μM NBT/mg prot.) in the ipsilateral rat forebrain cortex, at different survival times after intrahippocampal kainate injection. Data are means±S.D. *Indicates a statistically significant difference between kainate treated and control (sham-operated) animals ($p<0.05$). B - Effect of pretreatment with 7-NI on MnSOD activity (x103 IJ/mg prot.) in the ipsilateral rat forebrain cortex at different survival times after intrahippocampal kainate injection. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and between animals treated with 7-NI plus kainate and control (sham-operated) animals ($p<0.05$).

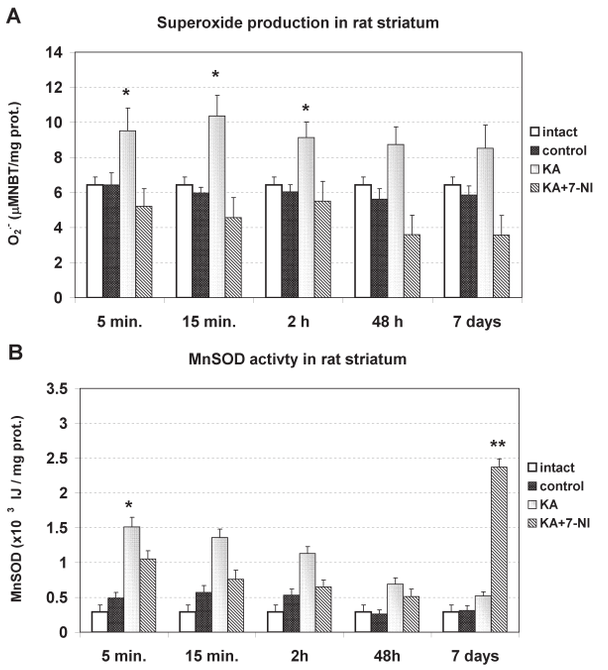

Fig. 3. A - Effect of pretreatment with 7-NI on superoxide production ($O_2^{\cdot}$, μM NBT/mg prot.) in the ipsilateral rat striatum, at different survival times after intrahippocampal kainate injection. Data are means ± S.D. *Indicates a statistically significant difference between kainate-treated and control (sham-operated) animals ($p<0.05$). B - Effect of pretreatment with 7-NI on MnSOD activity (x103 IJ/mg prot.) in the ipsilateral rat striatum at different survival times after intrahippocampal kainate injection. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and control (sham-operated) animals ($p<0.05$). **Indicates a statistically very significant difference between animals treated with 7-NI plus kainate and control (sham-operated) animals ($p<0.01$).

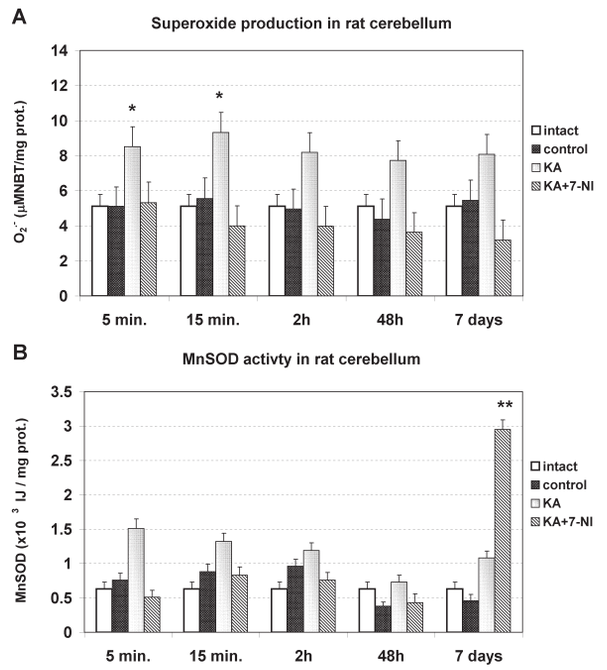

Fig. 1. A - Effect of pretreatment with 7-NI on superoxide production ($O_2^{\cdot}$, μM NBT/mg prot.) in the ipsilateral rat cerebellum, at different survival times after intrahippocampal kainate injection. Data are means ± S.D. *Indicates a statistically significant difference between kainate-treated and control (sham-operated) animals ($p<0.05$). B - Effect of pretreatment with 7-NI on MnSOD activity (x103 IJ/mg prot.) in the ipsilateral rat cerebellum at different survival times after intrahippocampal kainate injection. Data are means ±S.D. *Indicates a statistically significant difference between kainate treated and control (sham-operated) animals ($p<0.05$). **Indicates a statistically very significant difference between animals treated with 7-NI plus kainate and control (sham-operated) animals ($p<0.01$).

Superoxide production and MnSOD activity in the rat brain

The results presented in Figs. 1-4 show superoxide levels ($O_2^{\cdot-}$, μM NBT/mg protein) and MnSOD activity (MnSODx10^3 IU/mg protein) in ipsilateral hippocampal, cortical, striatal, and cerebellar homogenates, respectively. Results of comparing superoxide and MnSOD levels in the intact group of animals and sham-operated animals show the effect of mechanical injection. There was no significant difference between superoxide and MnSOD levels in these two groups, which means that mechanical injection alone is not sufficient to trigger oxidative stress and/or excitotoxicity. We therefore considered sham-operated animals as controls. In the control group, superoxide production and MnSOD levels showed no significant differences between the left and right hemispheres in any of the tested structures. Also, there was no significant difference between mean superoxide levels and MnSOD levels obtained from each hemisphere after 7-NI pretreatment in any of the tested brain structures, even when the injection site was in the ipsilateral hippocampus (results not presented). A selective neuronal nitric oxide synthase inhibitor *in vivo*, 7-NI showed the same pattern in all of the tested brain structures.

Intrahippocampal injection of KA resulted in generally higher levels ($p<0.05$) of superoxide pro-

duction in all of the tested brain structures. The obtained levels of superoxide production were the highest in the hippocampus (Fig. 1A). Rapid increase in superoxide production was found at 5 min after KA injection, and these higher levels continued to be above normal at all of the tested times (with 7 days as the final time point) in all of the tested brain structures (Figs.1A-4A). The results obtained for the contralateral hippocampus, forebrain cortex and striatum were similar (data not shown).

In the hippocampus, MnSOD levels were highest at the injection site (in both control and KA treated animals). Intrahippocampal injection of KA caused significant increase in MnSOD levels after 5 and 15 min, followed by further significant increase at 48 h and 7 days in the hippocampus and the forebrain cortex (Figs. 1B, 2B). In the striatum, a significant increase in MnSOD levels was found only at the earliest tested time, e. g., at 5 min (Fig. 3B).

Intrahippocampal injection of KA after pretreatment with 7-NI resulted in lower superoxide production around the control levels in all of the tested brain structures as compared with the equivalent group of KA-treated animals (Figs. 1A-4A). Such injection resulted in very significant (according to the Students *t*-test; $p<0.01$) increase of MnSOD activity only at the latest tested time (7 days) in virtually all of the tested brain structures as compared with the equivalent group of KA treated animals and the control group (Figs. 1B-4B). The effect of 7-NI was striking 7 days from injection in all of the tested brain structures, but MnSOD activity was the highest in the hippocampus (2.69 ± 0.29 MnSODx10^3 IU/mg protein, $p<0.01$; Fig. 1B).

DISCUSSION

Mitochondria are the main cellular site of superoxide production, both during normal cell respiration and in association with oxidative stress, such as that caused by ischemia, trauma etc. when uncontrolled release of glutamate occurs. Under physiological conditions, a dynamic equilibrium exists *in vivo* between potential oxidative damage and the antioxidant defense capacity. However, during episodes of oxidative stress, increased free radical production or reduced antioxidant reservoirs can upset this balance. Accumulation of free radicals may lead to generation of the more toxic and short-lived hydroxyl radical, which in turn attacks membrane phospholipids, proteins, and DNA, causing oxidative damage to these molecules and thereby destroying the cells.

The superoxide radical is much less reactive, and it can cross cell membranes and act at a distance. As the first protective mechanism, superoxide dismutase (SOD) reacts with superoxide to produce hydrogen peroxide and molecular oxygen (Heales et al.,1999). The induction of mitochondrial MnSOD under pathological conditions is variable and related mainly to the type of injury (Bidmon et al., 1999). The MnSOD isoenzyme is predominantly localized in neurons and their processes throughout the brain and the spinal cord (Lindenau et al., 2000). It seems reasonable to conclude that differences in the basal content of SOD-isoenzymes may contribute to different cellular susceptibilities in neurodegenerative processes that are accompanied by oxidative stress. Mitochondrial MnSOD seems to be a key enzyme in oxygen metabolism in the brain, and it is considered a major factor in protection of nervous tissue against excitotoxic and ischemic/hypoxic lesion (Budd et al., 1996; 1997). MnSOD represents an ROS-inducible enzyme that should allow the adaptation of brain cells to variation in ROS concentrations resulting from their oxidative metabolism (Gonzalez – Zulueta et al., 1999).

Regional distribution of KA receptors of the rat brain was found to be highest in deep layers (layer 5) of the forebrain cortex, the cerebellar granular cell layer, and the 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.

In the present study, an appropriate dose of KA (0.5 mg/ml) was used to cause slight brain damage in the ipsilateral, but not contralateral, hippocampus with no behavioral or epileptic effects. We have shown that superoxide levels in the rat brain increased immediately after KA-injection and remained increased throughout the experiment (7

days was the longest survival time examined). This significant increase in superoxide production correlated with MnSOD levels and presumably with the degree of neuronal injury. Neuronal cells responded to oxidative stress in KA-induced neurotoxicity and activated a protective mechanism to increase MnSOD levels (R a d e n o v i c et al., 2004). After pretreatment with 7-NI, KA-induced increased superoxide levels returned to the control values in all of the tested brain structures. A neuroprotective effect of pretreatment with 7-NI was also found in measurement of MnSOD activity. Our results demonstrated statistically significant increase of MnSOD activity 7 days after intrahippocampal KA treatment in all of the tested brain structures. The delayed increase after 7 days suggests rapid *de novo* synthesis involving transcription of the gene and translation of its mRNA. It seems that the mechanisms or time points of induction may be different. Direct injury probably leads to an instant induction of MnSOD expression, whereas more time is needed to transfer the signal via afferents and efferents to remote regions.

It was previously shown (M o n t e c o t et al., 1997) that during status epilepticus 7-NI significantly reduced the increase of hippocampal blood flow and prevented an increase of tissue oxygen partial pressure. Also, seven days later, the hippocampal damage in the CA1 and CA3 layers was significantly less in 7-NI-treated rats than in vehicle-treated rats. The authors concluded that the inhibition of neuronal nitric oxide synthase by 7-NI protects neurons from seizure-induced toxicity despite reducing blood flow and oxygen supply to the hippocampus. In addition, 7-NI can effectively inhibit NO synthesis in the rat brain after kainate-induced neurotoxicity and suppressed nitrite accumulation (R a d e n o v i c et al., 2003, 2005a).

In conclusion, increase of superoxide production and MnSOD activity in distinct brain regions that are functionally connected via afferents and efferents suggests that these regions are affected by the injury. Pretreatment with 7-NI protects the cells in these regions from KA-induced damage and therefore may limit the retrograde and anterograde spread of neurotoxicity. The obtained results suggest that neuronal NO synthase inhibitors may be useful in the treatment of neurological diseases in which excitotoxic mechanisms play a role.

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ЕФЕКАТ 7-НИТРОИНДАЗОЛА НА ПРОДУКЦИЈУ СУПЕРОКСИДА И АКТИВНОСТ MnSOD У МОЗГУ ПАЦОВА НАКОН КАИНАТОМ ИЗАЗВАНЕ НЕУРОТОКСИЧНОСТИ

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Интрацеребрална апликација каиничне киселине доводи до неуротоксичног оштећења неурона посредовано стварањем слободних радикала као медијатора оштећења. С обзиром да неуротоксичност доводи до оксидационог оштећења и повећаног стварања супероксидног радикала, пратили смо и ниво активности MnSOD у различитим можданим структурама (ипси- и контралатерални хипокампус, кортекс, стриатум и церебелум), у различитим временским интервалима у односу на тренутак изазивања неуротоксичног ефекта (5 мин, 15 мин, 2 часа, 48 часа и 7 дана). Претретманом са 7-нитроиндазолом (7-NI) покушали смо да модулишемо мерене параметре оксидативног стреса и делујемо неуропротективно на изазвану неуротоксичност. Детектовали смо врло брзо зна-

чајно смањење нивоа супероксид анјон радикала после изазване неуротоксичности које се задржава током целог експеримента закључно са 7 даном у свим тестираним можданим структурама. Претретман са 7-NI утицао је на активност MnSOD која је показала статистички најзначајнији пораст 7 дана после апликације каината, што објашњавамо поновном *de novo* синтезом овог индуцибилног ензима који има протективни ефекат на иницирану неуротоксичност и оксидативни стрес. Добијени резултати указују да примена инхибитора неурон специфичне NO-синтазе (7-NI) може бити потенцијално корисна у третману неуролошких обољења у којима је заступљена неуротоксичност.