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KAINATE-INDUCED OXIDATIVE STRESS AND NEUROTOXICITY IN THE RAT BRAIN

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Abstract - We investigated superoxide production and MnSOD activity after kainate injection into the CA3 region of the rat hippocampus. The measurements took place at different times in hippocampus, forebrain cortex, striatum, and cerebellum homogenates. Free radicals including superoxide are responsible for post-lesional cytotoxicity. Neuronal cells responded to oxidative stress in kainate-induced neurotoxicity and caused the protective mechanism to increase MnSOD levels. The increase of MnSOD in distinct brain regions functionally connected via afferents and efferents suggests that these regions are affected by the injury. It implies that MnSOD protects the cells in these regions from superoxide-induced damage and therefore may limit the retrograde and anterograde spread of neurotoxicity.

Key words: Brain, glutamate neurotoxicity, kainic acid, mitochondria, MnSOD, oxidative stress, superoxide

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INTRODUCTION

Glutamate neurotoxicity has been hypothesized to underlie several types of acute brain injury. 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 recently documented in several neurodegenerative processes (Coyle *et al.* 1993). However, the precise cellular mechanisms that lead to neurotoxicity under these conditions still remain unclear. Among the neurobiological conditions in which oxidative stress is believed to be involved are adult neurodegenerative diseases (Parkinson's disease, Huntington's disease, ALS, stroke, trauma, and seizures).

We were interested in changes induced by intrahippocampal kainate treatment. Kainic acid (KA) is an endogenous excitotoxin acting on glutamate receptors that leads to neurotoxic damage resembling the alterations observed in some neurological disorders. While excitotoxic and oxidative injury may occur independently, growing evidence indicates that reactive oxygen species formation may also be a specific consequence of glutamate receptor-mediated neurotoxicity (Dugan *et al.* 1995). Glutamate neurotoxicity is partly mediated by reactive oxygen species formed as a consequence of several processes, including nitric oxide (Alabadi *et al.* 1999; Nakaki *et al.* 2000; Radenović *et al.* 2003) and superoxide production (Li *et al.* 2001) production. Superoxide radicals react rapidly with nitric oxide to form highly cytotoxic peroxynitrite, which acts through lipid peroxidation (Lee *et al.* 2001). Although there are a number of intracellular sources of free radicals, the mitochondria are thought to be the most important (Ciriolo *et al.* 2001).

Superoxide dismutases (SOD) are the most effective endogenous scavengers of superoxide radicals, and changes in both CuZnSOD and MnSOD affect processes of aging and learning (Nicholls *et al.* 1999). Furthermore, mutations within the CuZnSOD gene leading to alternations in SOD activity contribute to several neuropathological conditions (Pinteaux *et al.* 1998), whereas SODs coupled to polyethylen glycol, lecithinized SOD, and SOD entrapped in liposomes act neuroprotectively (Cassarino *et al.* 1999).

Superoxide dismutases are considered to be a major factor in protection of nervous tissue against excitotoxic

and ischemic/hypoxic lesion. Reports about the localization of SOD after such an insult are controversial: CuZn-SOD, but mitochondrial MnSOD seems to be a key enzyme in oxygen metabolism in the brain. In contrast to the constitutive CuZnSOD, MnSOD is inducible and has the potential to protect neurons by its superoxide dismutating activity. Moreover MnSOD represents a reactive oxygen species (ROS) inducible enzyme which should allow the adaptation of brain cells to variation in ROS concentrations resulting from their oxidative metabolism. It has been recently shown that mice deficient in mitochondrial

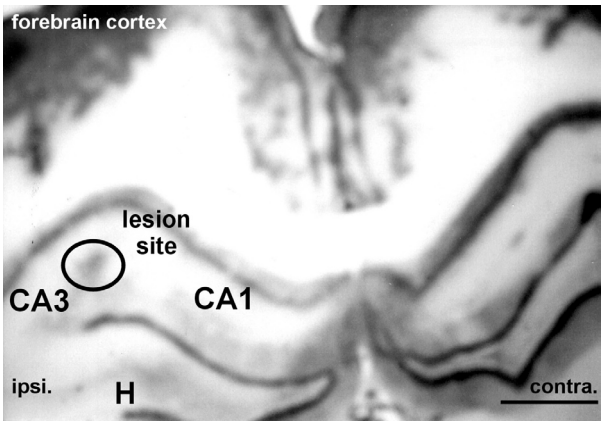

Fig. 1. Low-power light micrograph of both hippocampal hemispheres of a rat following unilateral kainic acid injection into the CA3 region (ipsi), stained with hematoxylin.The rat was allowed to survive for 2 h. The ipsilateral pyramidal cells of the CA3 region were slightly damaged around the injection site (white marks). Damage to CA1 pyramidal cells was not observed, nor was damage to dentate granule cells. There were no morphological signs of neuronal degeneration in the contralateral hippocampus. Scale bar = 500 μm.

SOD immunoreactivity was found to be located predominantly in astrocytes throughout the CNS. In contrast, the Mn-containing isoenzyme was predominantly confined to neurons and their processes throughout the brain and the spinal cord (L i n d e n a u *et al.* 2000). It seems reasonable to conclude that differences in the basal content

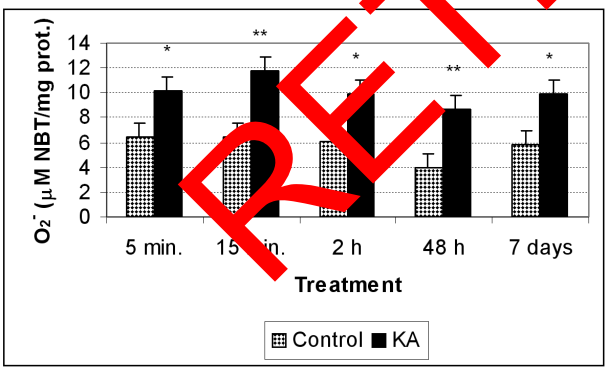

Figure 2. The effect of intrahippocampal kainate injection on superoxide production (O_2^-, μM NBT/mg prot.) in the rat hippocampus (ipsilateral) at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and control (sham-operated) animals (p<0.05). **Indicates a statistically very significant difference between kainate-treated and control (sham-operated) animals (p<0.01).

of SOD-isoenzymes may contribute to different cellular susceptibilities in neurodegenerative processes that are accompanied by oxidative stress.

Most of the studies cited above focused on CuZn-

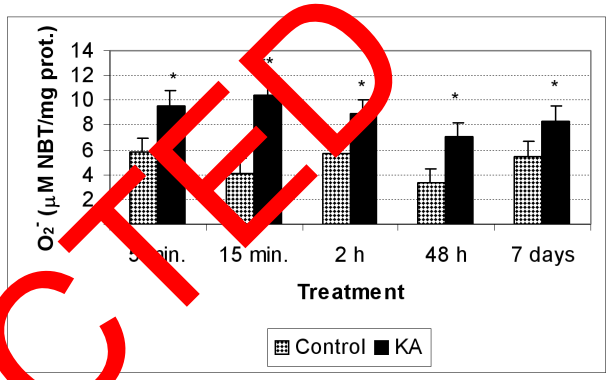

Figure 3. The effect of intrahippocampal kainate injection on superoxide production (O_2^-, μM NBT/mg prot.) in the rat forebrain cortex (ipsilateral) at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and sham-operated animals (p<0.05). **Indicates a statistically very significant difference between kainate treated and control (sham-operated) animals (p<0.01).

MnSOD suffer from early neurodegeneration (K i m *et al.* 2000). Also, L i *et al.* (1998) reported that cultured cortical neurons of heterozygous knockout mutant mice with reduced MnSOD activity are significantly more sensitive to glutamate-induced toxicity than the neurons of wild type mice. Kainate-induced increases of mitochondrial superoxide production and hippocampal neuronal loss were attenuated in transgenic mice overexpressing mitochondrial MnSOD (Liang *et al.* 2000).

Previous studies suggested that mitochondrial Mn-SOD is important for the resistance to toxic cellular insults and plays a major protective role. It is known that MnSOD has a heterogeneous distribution in the brain (G o n z a l e z - Z u l u e t a *et al.* 1999). In the striatum, cholinergic neurons and somatostatin neurons are enriched with MnSOD. Cholinergic neurons of the basal forebrain are also highly MnSOD-enriched. In the hippocampus, enrichment with MnSOD mainly occurred in parvalbumin-containing neurons and MnSOD-positive interneurons were present in the stratum piramidale, with the highest packing densities being recorded in the subiculum and CA3. The highest packing density within

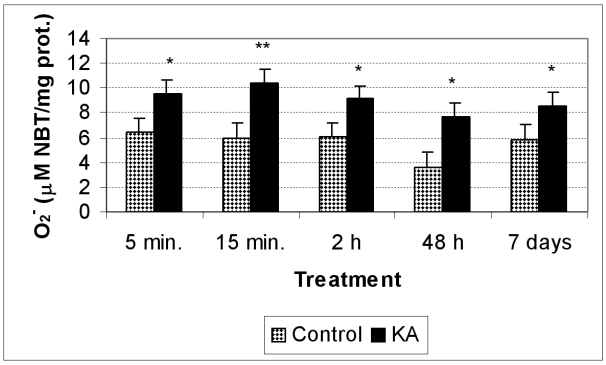

Figure 4. The effect of intrahippocampal kainate injection on superoxide production ($O_2^{\cdot}$, μM NBT/mg prot.) in the rat striatum (ipsilateral) at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and sham-operated animals ($p<0.05$). **Indicates a statistically very significant difference between kainate treated and control (sham-operated) animals ($p<0.01$).

the hippocampal formation occurred in the polymorphic cell layer of the dentate gyrus (B i d m o n *et al.* 1997).

In view of the above considerations, we studied superoxide anion production and activity of mitochondrial MnSOD within the rat hippocampus, forebrain cortex, striatum, and cerebellum after intrahippocampal kainate injection.

MATERIALS AND METHODS

Animals

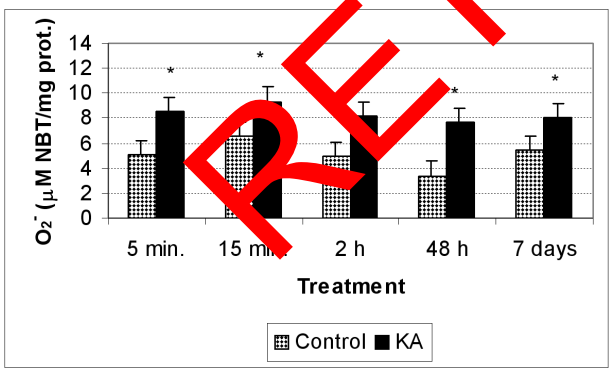

Figure 5. The effect of intrahippocampal kainate injection on superoxide production ($O_2^{\cdot}$, μM NBT/mg prot.) in the rat cerebellum (ipsilateral) at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and sham-operated animals ($p<0.05$). **Indicates a statistically very significant difference between kainate treated and control (sham-operated) animals ($p<0.01$).

Adult rats of the Wistar strain (*Rattus norvegicus*) of both sexes, with body weight 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 were placed in a stereotaxic frame.

Experimental procedure and intracerebral injection of drugs

The rats were divided into two 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. Animals of the first group received a 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) using a Hamilton microsyringe with a beveled tip. The second 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, decapitated, and the brains immediately removed. The ipsi- and contralateral hippocampus, forebrain cortex, striatum, and cerebellum from individual animals were quickly isolated 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 (crude mitochondrial fraction) obtained by this procedure was then frozen and stored at -70°C.

Light microscopy

Brains were removed from the skull and fixed in 4% paraformaldehyde for at least 24 h before dehydration through a range of alcohols and embedding in paraffin wax. Frozen (-70°C) 50 μm-thick hippocampus brain sections were cut in a cryostat, thaw-mounted on gelatin-coated glass slides, and stained with hematoxylin and eosin.

Superoxide production and measurement

In these experiments superoxide was measured from reduction of nitro blue tetrazolium (NBT) as previously

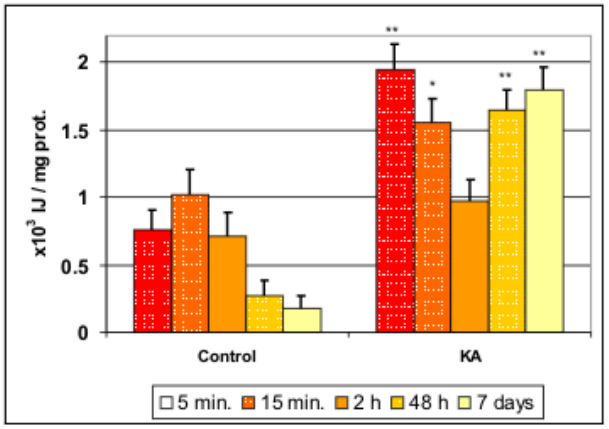

Figure 6. The effect of intrahippocampal kainate injection on MnSOD activity (x10³ IJ/mg prot.) in the ipsilateral hippocampus at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and control (sham-operated) animals (p<0.05). **Indicates a statistically very significant difference between kainate treated and control (sham-operated) animals (p<0.01).

described (S p i t z *et al.* 1989). Detection of this product was by spectrophotometric quantification of a colored formazan product formed from blue tetrazolium. Reduction of NBT was measured at 560 nm.

Superoxide dismutase assay

The assay of MnSOD activity by th[illegible] ad[illegible] method (F r i d o v i c h *et al.* 1995) wa[illegible] used. [illegible]he method is based on the measuruing a[illegible]eg[illegible]ee of adr[illegible]naline

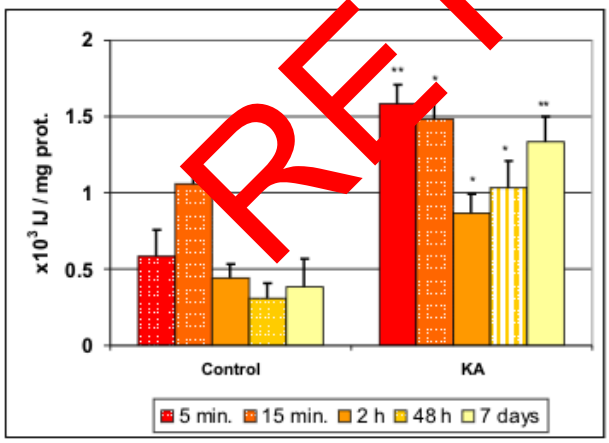

Figure 7. The effect of intrahippocampal kainate injection on MnSOD activity (x10³ IJ/mg prot.) in the ipsilateral forebrain cortex at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and sham-operated animals (p<0.05). **Indicates a statistically very significant difference between kainate treated and control (sham-operated) animals (p<0.01).

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 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 p[illegible]esen[illegible]tion an[illegible]analysis

All exper[illegible]ents [illegible]ere done with n = 8. Each assay was perfor[illegible]ed a[illegible]ast twi[illegible]e under identical conditions. Data ar[illegible]xpressed [illegible]eans ± SD. The statistical significance of [illegible]ifferences between groups was assessed b[illegible]tudent's t-t[illegible]t (paired and unpaired) for individual comparisons and regression analysis for overall significance (wit[illegible] $p < 0.05$ as significant and $p < 0.01$ as very s[illegible]nifica[illegible]).

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

The injection site

Kainate was injected unilaterally into the CA3 subfield of the hippocampus of rats. The ipsilateral pyramidal cells of the CA3 region were slightly damaged 2 h after KA injection (Fig. 1). Pyramidal cells in the CA1 and CA3 subfields, granule cells in the dentate gyrus and hilar neurons which are known to be vulnerable in this model, show a pattern of damage around the injection site. That was found to be similar at any of the survival times tested, only the size of the lesion varied. Damage to CA1 pyramidal cells was not observed, non was damage to dentate granule cells. There were no morphological signs of neuronal degeneration in the contralateral hippocam-

pus at this early stage or at any other survival time tested. Under conditions of normal behavior in the rat, the damage was localized mainly in the CA3 region of hippocampus, where neuronal loss occurred. The purpose of these micrographs was to verify the injection site.

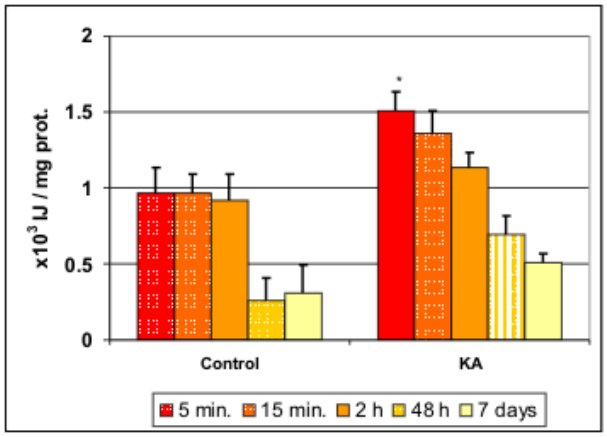

Figure 8. The effect of intrahippocampal kainate injection on MnSOD activity (x10³ IJ/mg prot.) in the ipsilateral striatum at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between kainate treated and sham-operated animals (p<0.05).

Superoxide production in the rat brain after intrahippocampal kainate injection

The results presented in Fig. 2-5 show the superoxide levels (O_2^-, µM NBT/mg proteins) in ipsilateral hippocampal, cortical, striatal, and cerebellar homogenates, respectively. Superoxide levels in the brains of control rats showed no significant differences between the left and right hemispheres in any of the tested structures (data not shown). Also, there was no statistically significant difference between mean superoxide levels obtained from each hemisphere after KA treatment in any of the tested brain structures, although the injection site was in the ipsilateral hippocampus (not presented).

Kainate injection resulted in generally higher superoxide levels (p<0.05) in all tested brain structures. The obtained superoxide levels were highest in the hippocampus (Fig. 1). Rapid increase in superoxide production at 5 min after KA injection and production levels continued to be above normal at all subsequent times (at 7 days finally) in all tested brain structures. Measurement at 15 min after KA injection in the hippocampus, in the forebrain cortex, and in the striatum showed statistically very significant differences (p<0.01) compared with the equivalent control group (Figs. 2, 3, 4). The results obtained for the contralateral hippocampus, forebrain cortex and striatum were similar.

Activity of MnSOD in the rat brain after intrahippocampal kainate injection

The results presented in Figs. 6-9 show the MnSOD levels (x10³ IJ/mg protein) in ipsilateral hippocampal, cortical, striatal and cerebellar homogenates respectively. There was no statistically significant difference between mean MnSOD levels obtained from each hemisphere after KA treatment, although the injection site was in the ipsilateral hippocampus. Activity of MnSOD in the brains of control rats showed no significant differences between the left and right hemispheres in any of the tested structures (not shown).

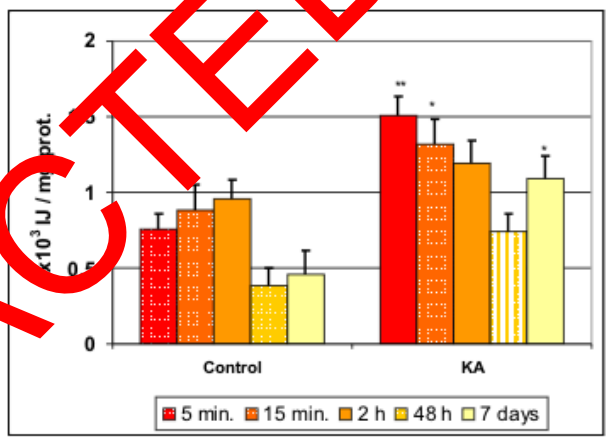

Figure 9. The effect of intrahippocampal kainate injection on MnSOD activity (x10³ IJ/mg prot.) in the ipsilateral cerebellum at different survival times. Data are means ± S.D. *Indicates a statistically significant difference between kainate-treated and sham-operated animals (p<0.05). **Indicates a statistically very significant difference between kainate treated and control (sham-operated) animals (p<0.01).

Levels of MnSOD at the injection site (control or KA treated) were highest in the hippocampus (Fig. 6). Intrahippocampal KA-induced neurotoxicity caused significant increase of MnSOD activity at 5 and 15 min, followed by further significant increase at 48 h and 7 days, in the hippocampus, in the forebrain cortex, and in the cerebellum (Figs. 6 , 7, 9). In the striatum, a significant increase of MnSOD activity was found only at the earliest tested time, at 5 min (Fig. 8). Neuronal cells responded to oxidative stress in KA-induced neurotoxicity by increasing MnSOD levels.

DISCUSSION

Mitochondria are the main cellular source of super-

oxide production, both during normal cell respiration and in association with oxidative stress, such as ischemia, trauma, etc., when uncontrolled release of glutamate occurs. Under normal conditions, a dynamic equilibrium exists *in vivo* between the oxidative damage potential and the antioxidant defense capacity. However, during episodes of oxidative stress, increased free radical production or reduced antioxidant reservoirs might upset this balance. Accumulation of free radicals may lead to the generation of a 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 cells. The superoxide radical is much less reactive and can cross cell membranes and act at a distance. As the first protective mechanism, SOD reacts with superoxide to produce hydrogen peroxide and molecular oxygen. The induction of mitochondrial MnSOD under pathological conditions is variable and related mainly to the type of injury (B i d m o n *et al.* 1997).

Mitochondria from different tissue sources display differential susceptibility to oxidizing species (H a e a l e s *et al.* 1999). However, it is now becoming apparent that there is within the brain a differential susceptibility of various brain cell types to oxidizing species. In contrast to astrocytes, neurons appear to be particularly vulnerable to the action of free radicals. Such vulnerability may arise from an inability to sustain cellular energy demands by glycolysis and an inferior capacity to handle oxidizing species (S e n g p i e l *et al.* 1998).

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 granule cell layer, and the caudate putamen (C a r o l l *et al.* 1998; B a i l e y *et al.* 2001), which is why we tested these particular brain regions: hippocampus, forebrain cortex, striatum, and cerebellum.

Neuronal superoxide production varies with metabolic activity and age. Developmental increase in mitochondrial superoxide production and oxidative DNA damage following KA seizures suggests that mitochondrial oxidative stress may be a key factor that renders the developing brain resistant to seizure-induced brain damage (P a t e l *et al.* 2003).

In the present study, an appropriate dose of KA (0.5 mg/ml) was used to cause small brain damage in the ipsilateral, but not contralateral, hippocampus; there were no behavioral or epileptic effects. We have shown that superoxide levels in the rat brain increased immediately after KA injection and continued to increase gradually throughout the experiments for a period of 7 days. This significant increase in superoxide production was correlated with MnSOD levels and presumably with the degree of neuronal injury.

Superoxide radical-induced brain injury has been described to be the main mechanism of injury caused by brain reperfusion after transient ischemia. It is belived that CuZnSOD and possibly also MnSOD to represent the main endogenous protective systems against such reperfusion injury. Moreover MnSOD may also be involved in limiting the damage in remote brain areas that were not ischemic by scavenging radicals formed in response to deafferentation (B i d m o n *et al.* 1997).

The immunocytochemical distribution of MnSOD was determined in the rat hippocampus by A k a i *et al.* (1990), who reported that CA1 pyramidal cells were weakly immunostained, whereas CA3 pyramidal cells were strongly reactive. These differences in the intensity of the MnSOD immunostaining reactions may relate to variations in the sensitivity of subfields of the hippocampus to ischemia. The hippocampus is especially vulnerable to ischemic damage. Neurons in the CA3 region and dentate hilus demonstrate fast progressive damage, while CA1 pyramidal cells demonstrate delayed neuronal damage. The delayed CA1 pyramidal cell loss could be caused by post-ischemic neuronal hyperactivity if hippocampal interactions are lost after ischemia. Because CA3 neurons constitute the main input to CA1 pyramidal cells, decreased activity of CA3 neurons indicates less excitatory input to CA1 neurons. Also, MnSOD was localized in the cerebral cortex and hippocampus of patients with A l z h e i m e r-type senile dementia (M a e d a *et al.* 1997).

Our results show that there is a clear, transient increase of inducible MnSOD in all tested brain regions after intrahippocampal KA treatment. A rapid increase, within 5 min, was found in the ipsilateral and contralateral areas, which possibly receive a few direct connections from the lesioned area. The data indicate that the onset of MnSOD upregulation was better in the hippocampus, which may be intrinsically more protected by MnSOD from toxic effects than other areas. The first increase in MnSOD occurred between 5 min and 2 h. The delayed increases at 48 h and 7 days suggest a rapid *de novo* syn-

thesis involving transcription of the gene and translation of its mRNA. It seems that the mechanisms or time points of the induction may be different. Most probably, direct injury leads to an instant induction of MnSOD expression, whereas more time is needed to transfer the signal via afferents or efferents to the remote regions.

This finding indicates that protection against superoxide radicals not only takes place around the lesioned area, but also may exist in more remote brain areas-regions that are affected by the injury. It suggests that Mn-SOD protects the cells in these regions from superoxide-induced damage and therefore may limit the retrograde and anterograde spread of neurotoxicity.

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КАИНИЧНА КИСЕЛИНА ИЗАЗИВА ОКСИДАТИВНИ СТРЕС И НЕУРОТОКСИЧНОСТ У МОЗГУ ПАЦОВА

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Претпоставља се да је присутна количина MnSOD у организму довољна за неутралисање физиолошке брзине стварања супероксида радикала. Показали смо да излагање организма деловању виших концентрација супероксидног радикала доводи до повећане продукције MnSOD. Интерцеребрална апликација количине киселине, агонисте глутаматних рецептора, у селективно осетљиви CA3 регион хипокампуса пацова доводи до неуротоксичног оштећења неурона у овој структури, посредовано стварањем слободних радикала као медијатора оштећења. С обзиром да неуротоксичност доводи до оксидационог оштећења и повећаног стварања супероксидног радикала, пратили смо ниво активности MnSOD у различитим можданим структурама (ипси- и контралатерални хипокампус, кортекс, стријатум и церебелум), у различитим временским интервалима у односу на тренутак изазивања неуротоксичног ефекта (5 min, 15 min, 2 h, 48 h и 7 дана). Детектовали смо врло брзо повећање нивоа супероксид анјон радикала које се задржава током целог експеримента закључно са 7 даном. Статистички најзначајније повећање продукције забележено је у 15 min након апликације каиничне киселине у свим тестираним структурама. У хипокампусу пацова (место апликације) измерене су највише концентрације супероксидног анјон радикала, док се церебелум (физички најудаљенија структура) показао као најрезистентнија тестирана мождана структура на изазвану ексцитотоксичност. Такође, детектовали смо врло брзо повећање нивоа MnSOD већ после 5 min до 2 h у свим испитиваним можданим структурама. Накнадно повећање активности ензима 48 h и 7 дана по апликацији каината објашњавамо поновном *de novo* синтезом овог индуцибилног ензима који има протективни ефекат на иницирану неуротоксичност и оксидативни стрес.