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ELIMINATION OF NITROXIDE RADICAL DIALYZED IN THE HIPPOCAMPUS DURING KAINATE-INDUCED SEIZURE IN RATS

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Abstract

Time-dependent changes of the electron spin resonance (ESR) signal intensity of the nitroxide radical applied to the ventral hippocampus by *in vivo* brain dialysis were evaluated using ESR spectrometry in rats during convulsions induced by kainic acid [20mg/kg i.p., 0.2 ml; G(Ka)], and compared with the intensity changes in control rats (0.2 ml saline i.p., G(sl)). After the perfusion of the nitroxide radicals for 120 min by dialysis at the rate of 2 μl/min, the rats were administered kainic acid or saline intraperitoneally and the perfused liquor was switched to Ringer's solution at the same time. The dialysates of Ringer's solution were then collected at 20-min intervals in test tubes. Spectra were observed from the area perfused with the nitroxide radical in both groups. The half-life, which was estimated on the basis of the exponential decay of amplitude of the nitroxide radical perfused into hippocampal extracellular space, was used as a parameter of elimination activity in the brain of rats during convulsion status. The half-life in the rats during convulsion was significantly longer than that in the control rats ($p < 0.01$). This finding shows that the elimination activity in the extracellular space of the brain during convulsion was decreased compared with that in the control. These results suggest that hippocampal cellular vulnerability to free radical attack is present during convulsions and explains one of the mechanisms for selective cellular damage in the hippocampus.

Introduction

The production of free radicals has been suggested to participate in the neuronal

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pathology resulting from excitoxic insults, as well as in age-related neuronal disorders and other forms of neurodegeneration (Carney *et al.*, 1991; Coyle *et al.*, 1993; Lebel *et al.*, 1992; Monyer *et al.*, 1990). However, the extreme reactivity of free radicals has made their direct detection and quantification difficult, and the free radical involvement in pathologic conditions has therefore generally been inferred from the measurement of indirect markers of oxidative stress *in vitro*. Based on these measurements, free radicals have been suggested to be implicated in neuronal injuries, including the epileptogenesis of the kainic acid (KA)-induced seizures (Cohen *et al.*, 1987).

Seizure activity induced by KA results in the selective degeneration of vulnerable neuronal populations in limbic structures, including the hippocampus and the piriform cortex(Annadora *et al.*, 1995). The molecular and cellular events that are responsible for the selective vulnerability of these neurons to KA-induced seizure activity are not yet understood, although the excessive production of free radicals has been hypothesized to participate. It is generally acknowledged that KA administration results in the activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors in vulnerable populations, an event that has been shown to cause the formation of free radicals(Lafon-Cazal *et al.*, 1993).

Among the protective reductant systems against the generation of these free radicals, an important role is played by free radical scavengers in the brain, including ascorbic acid and α-tocopherol. It is possible that an imbalance between the free radical formation and the reductant system is among the important triggers of the cellular vulnerability of the hippocampal neurons to KA-induced seizure activity.

We hypothesize that the analysis of balance between free radical production and the reductant system during convulsions in the freely moving state and unanesthetized condition would provide important evidence in the clarification of the mechanism of cellular vulnerability to free radicals *in vivo*. To overcome the difficult technical problems of simultaneous detection of these free radicals and reductant, we measured the intracerebral elimination of nitroxide radical exogenously applied to the hippocampal extracellular space using an *in vivo* microdialysis technique.

Materials and Methods

Chemicals

The nitroxide, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL, from Sigma) was used. Carbamoyl-PROXYL was dissolved at a concentration of 300 mM in Ringer's solution (147 mM NaCl, 3.5 mM KCl, 2.3 mM $CaCl_2$; pH 6.8).

Kainic acid at 1% (w/v; from Sigma) dissolved in saline solution was used for injection to induce seizures.

Surgical Procedure and In Vivo Microdialysis

Male Wistar rats (n=12) weighing 200-250 g at the time of surgery were anesthetized with sodium pentobarbital (37.5 mg/kg, i.p.). In this experiment, stereotaxic coordinates were determined according to the rat brain atlas of Pellegrino *et al.* (1986). The incisor bar was set on the intraaural line. Each rat was also stereotaxically implanted with a 22-G guide cannula, coordinated 5.6 mm posterior, 5.0 mm to the right of the bregma, and 3.0 mm below the surface of the skull. The 22-G guide cannula was firmly anchored to the skull with miniature screws and dental cement. The microdialysis probe filled with carbamoyl-PROXYL solution was inserted into the guide cannula. The probe's tip was covered with a 5.0 mm length of permeable hollow fiber (11 mm thick, 0.2 mm outside diameter, molecular weight cut-off 7000-8000, Cuprophan, Nikkiso, Japan). The dialysis cannula was connected to a microinfusion pump (BRC, Japan) for continuous perfusion with 300mM carbamoyl-PROXYL solution at the rate of 2.0 μl/min. The microdialysis study was carried out using a method that is basically the same as that described by Nakahara *et al.*(Nakahara *et al.*, 1989).

After the perfusion of the nitroxide radical for 120 min by *in vivo* brain microdialysis into the ventral hippocampus, each rat was administered 0.2 ml of saline solution containing kainic acid (15mg/kg) intraperitoneally (G(Ka), n=6). The control rats were instead administered 0.2 ml of physiological saline intraperitoneally (G(sl) n=6). All rats were fully recovered from pentobarbital anesthesia at the time of injection. At the same time as the injection, the perfused liquid (nitroxide radical solution) was replaced with Ringer's solution. The dialysates of Ringer's solution were collected at 20-min intervals in t-tubes for the first 120 min after injection in each group. The sampling of

dialysate was performed in rats in the free moving state. All of the samples were subjected to X-band electron spin resonance (ESR) spectrometry as soon as possible. None was frozen.

Ten mM 4-hydroxy-2,2,6,6,-tetramethyl-1-piperidine-1-oxyl (TEMPOL) in Ringer's solution was used as a standard sample to estimate the amplitude of nitroxide radical in the perfused sample.

To examine the direct interaction between kainic acid and carbamoyl-PROXYL, the following experiment was done. Twenty μl of 1 % kainic acid solution and 20 μl Ringer's solution containing 300mM carbamoyl-PROXYL were mixed together. The mixture was subjected to X-band ESR spectrometry. The ESR measurements were repeated every 20 min for 120 min after mixing the kainic acid solution and Ringer's solution containing 300 mM carbamoyl-PROXYL.

Condition of ESR

All samples were subjected to ESR analysis at room temperature in a capillary cell. The X-band ESR spectrometer (RE-1X, JEOL) settings for samples were as follows: magnetic field, 335.3 ± 5 mT; microwave frequency, 9.43 GHz; microwave power, 8 mW; modulation width, 0.05mT; receiver gain, 400 x; and time constant, 0.1 sec.

Results

Following 2-hr perfusion with Ringer's solution containing carbamoyl-PROXYL, we obtained the successive ESR spectra of the carbamoyl-PROXYL in the dialysate of G(sl) and G(Ka). Time-dependent changes of the amplitude were observed, characterized by a decrease without change in line-shape compared with the spectrum at 20 min after injection in each group. The change of the ESR spectrum amplitude reflects the change of the amount of paramagnetic species. Therefore, the ratio of the amplitudes derived from the dialysate and the standard sample was defined as the signal intensity.

Figure 1 shows typical plots of signal intensities against time after injection of carbamoyl-PROXYL in each group. We obtained a good linearity on the semilogarithmic scale with high reproducibility (correlation coefficient in G(Ka)= 0.923+/-0.005; in G(sl)= 0.914+/-0.007), which indicated that signal decayed exponentially. Thus, the half-

life of decay was used as a parameter reflecting radical eliminating-ability. As shown in Table I, the half-life of the ESR signal of carbamoyl-PROXYL obtained from the perfused area in the ventral hippocampus in G(Ka) was 63.47 min, and that in G(sl) was 51.64 min. The value of G(Ka) was significantly longer than that in G(sl) (Mann-Whitney U-test, $p < 0.01$).

TABLE I

The half-life of the ESR signal of carbamoyl-PROXYL

	G(Ka)	G(sl)
Mean (min)	63.47*	51.64
Range (min)	48.75-98.33	39.34-60.53

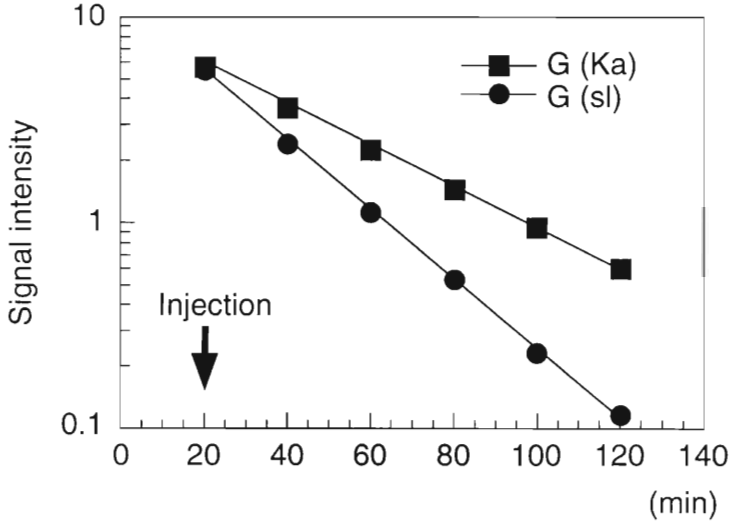

Figure 1. Typical plots of signal intensities vs the time after perfusion of carbamoyl-PROXYL into the hippocampal extracellular space in G(Ka) and G(sl).

When the ESR signals from the carbamoyl-PROXYL solution mixed with kainic acid solution were measured for 120 min after mixing, no changes of signal intensities were observed. The result indicates the absence of an interaction between kainic acid and carbamoyl-PROXYL.

Discussion

Estimation of the eliminating-activity within the biological system of the anesthetized animal have been developed which employ the half-life of nitroxide radicals as measured by a L-band ESR system (Bacic *et al.*, 1989; Berliner *et al.*, 1989; Ishida *et al.*, 1989; Yokoyama *et al.*, 1995). This is the first evaluation of the intracerebral eliminating activity using animals in the free moving and unanesthetized state.

In this study, as a measure of the balance between free radical generation and the intracerebral eliminating-ability, time-dependent changes of signal intensities of nitroxide radical exogenously applied by *in vivo* microdialysis were examined in rats in the free moving state. Subsequently, the dialysates following the perfusion of carbamoyl-PROXYL showed an exponential decay in the ESR signal intensity. The signal intensities of nitroxide radicals injected via peripheral vessels are well known to decay due to excretion via the kidneys and depletion of the paramagnetism of the liver (Bacic *et al.*, 1989; Couet *et al.*, 1984; Nakagawa *et al.*, 1984). In this experimental protocol, the decreasing signal intensities might have been due to two factors, as follows: the excretion of the applied nitroxide radical from the hippocampal extracellular space to the extra-brain space by successive dialysis of Ringer's solution following carbamoyl-PROXYL perfusion, and scavenging by reductant in the brain. The first factor, the excretion of nitroxide radical by perfusion of Ringer's solution, is common to both G(Ka) and G(sl). Therefore, the eliminating-ability of carbamoyl-PROXYL in terms of the half-life reflects an intracerebral depletion of paramagnetic substances, perhaps due mainly to reduction in the brain.

The significant difference in the half-life between G(Ka) and G(sl) is thought to have been caused by the intracerebral depletion of paramagnetism. In view of the absence of an interaction between kainic acid and carbamoyl-PROXYL, kainic acid itself apparently has no effect in depriving the paramagnetism of carbamoyl-PROXYL. This result suggests that the intracerebral depletion of paramagnetic substances during the seizure status is decreased compared with that of the control rat. It was found in this study that cellular vulnerability to free radicals would occur during seizure *in vivo*. We conclude that this cellular vulnerability plays an important role for neurodegeration in

KA-induced seizures. The present method is considered to be useful in an assessment of the estimation of the intracerebral eliminating-ability of free radicals in rats in the freely moving state.

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