Free Radical–Mediated Cell Damage After Experimental Status Epileptics in Hippocampal Slice Cultures

RICHARD KOVÁCS,1 SEBASTIAN SCHUCHMANN,2 SIEGRUN GABRIEL,2 OLIVER KANN,2 JULIANNA KARDOS,1 AND UWE HEINEMANN2
1Department of Neurochemistry, Chemical Institute, Chemical Research Center, Hungarian Academy of Sciences, Budapest 1025, Hungary; and 2Johannes Müller Institute of Physiology, Humboldt University, D-10117 Berlin, Germany

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Kovács, Richard, Sebastian Schuchmann, Siegrun Gabriel, Oliver Kann, Julianna Kardos, and Uwe Heinemann. Free radical–mediated cell damage after experimental status epilepticus in hippocampal slice cultures. J Neurophysiol 88: 2909–2918, 2002; 10.1152/jn.00149.2002. Generation of free radicals may have a key role in the nerve cell damage induced by prolonged or frequently recurring convulsions (status epilepticus). Mitochondrial function may also be altered due to production of free radicals during seizures. We therefore studied changes in field potentials (fp) together with measurements of extracellular, intracellular, and intramitochondrial calcium concentration ([Ca2+]e, [Ca2+]i, and [Ca2+]m, respectively), mitochondrial membrane potential (ΔΨ), NAD(P)H auto-fluorescence, and dihydroethidium (HEt) fluorescence in hippocampal slice cultures by means of simultaneous electrophysiological and microfluorimetric measurements. As reported previously, each seizure-like event (SLE) resulted in mitochondrial depolarization associated with a delayed rise in oxidation of HEt to ethidium, presumably indicating ROS production. We show here that repeated SLEs led to a decline in intracellular and intramitochondrial Ca2+ signals despite unaltered Ca2+ influx. Also, mitochondrial depolarization and the NAD(P)H signal became smaller during recurring SLEs. By contrast, the ethidium fluorescence rises remained constant or even increased from SLE to SLE. After about 15 SLEs, activity changed to continuous afterdischarges with steady depolarization of mitochondrial membranes. Staining with a cell death marker, propidium iodide, indicated widespread cell damage after 2 h of recurring SLEs. The free radical scavenger, α-tocopherol, protected the slice cultures against this damage and also reduced the ongoing impairment of NAD(P)H production. These findings suggest involvement of reactive oxygen species (ROS) of mitochondrial origin in the epileptic cell damage and that free radical scavenging may prevent status epilepticus–induced cell loss.

INTRODUCTION

Single limbic seizures can lead to cell loss in the dentate gyrus (Benzzon et al. 1997), while recurrent seizures and convulsive status epilepticus results in widespread cell death in the hippocampal formation (e.g., Magloczky and Freund 1995). This is followed by severe gliosis (Niquet et al. 1994; Schmidt-Kastner and Ingvar 1994) and a functional and anatomical reorganization of the hippocampus, resulting eventually in appearance of spontaneous limbic seizures (Cavazos and Sutula 1990; Turski et al. 1989). The majority of patients with drug-resistant mesial temporal lobe epilepsy display similar pathological alterations in their hippocampi, known as Ammon’s horn sclerosis (Fisher et al. 1998; Mathern et al. 1997; Sommer 1880). In the past, about two-thirds of the patients had complicated febrile convulsions or a period of status epilepticus (Mathern et al. 1997). Preventing seizure and status epilepticus–induced cell death may therefore influence epileptogenesis.

A large number of studies linked seizure-induced cell damage to excitotoxic mechanisms (for a review see Meldrum 1993). Convolusions can result in augmented glutamate release (Sherwin 1999), leading to Ca2+ uptake through N-methyl-D-aspartate (NMDA) and voltage-gated Ca2+ channels. In fact, during convulsions induced by different means and in different models, [Ca2+]i decreases (for a review see Heinemann et al. 1986) while cytosolic Ca2+ concentration increases (e.g., Gloveli et al. 1999; Kovács et al. 2000). Mitochondria were reported to accumulate Ca2+ provided cytosolic Ca2+ rises exceed 400 nM or provided mitochondrial uptake dominates mitochondrial Ca2+ extrusion (Babcock et al. 1997; Colegrove et al. 2000; David 1999; Gunter et al. 1998; Pivovarova et al. 1999; Sparagna et al. 1995), thereby leading to depolarization of mitochondrial membranes (Duchen 1992; Schuchmann et al. 1998, 2000). Uptake of Ca2+ into mitochondria stimulates the tricarboxylate cycle resulting in augmented reduction of pyridine nucleotides, which may be one of the mechanisms of the coupling of neuronal and metabolic activity (Duchen 1999, 2000; Rutter et al. 1996). On the other hand, exposure of mitochondria to high [Ca2+] was shown to increase formation of ROS (Dykens 1994). Sustained depolarization of mitochondrial membranes and enhanced reactive oxygen species (ROS) formation could impair production of NADH and ATP. Indeed, rises in NAD(P)H auto-fluorescence associated with single seizure-like events (SLEs) in slices decline with time during status epilepticus (Schuchmann et al. 1999). Moreover, in vivo studies suggested a failure of ATP production after prolonged status epilepticus (Folbergrova et al. 1981; Gupta et al. 2001).

We have previously shown that recurrent SLEs are readily induced in organotypic slice cultures (Gutierrez et al. 1999) and that they led to considerable cell loss (Kovács et al. 1999). In a previous paper we described that a SLE induced by...
lowering of extracellular Mg\(^{2+}\) concentration is accompanied by intracellular and intramitochondrial Ca\(^{2+}\) accumulation, followed by rises in NAD(P)H auto-fluorescence, depolarization of mitochondria, and likely, increased production of ROS (Kovács et al. 2001). Here we report on changes of these signals during recurring SLEs, as a model of experimental status epilepticus. To demonstrate the involvement of ROS in seizure-induced cell damage we also examined the effects of the free radical scavenger α-tocopherol on SLE-associated alterations of the mitochondrial function. In subsequent experiments, we tested for possible neuroprotective effects of α-tocopherol.

Parts of the results were previously presented in abstract form.

**METHODS**

**Slice cultures**

Organotypic hippocampal slice cultures were prepared and maintained as described earlier by Stoppini et al. (1991). Briefly, 7- to 8-day-old rat pups were decapitated and the hippocampi were cut in 400-µm slices under sterile conditions in gassed (95% O\(_2\), 5% CO\(_2\)) ice-cold minimal essential medium (MEM) at pH 7.5. The slices were maintained on a millipore membrane (Millicell-CM, 0.4 µm, Millipore, Eschborn, Germany) between the culture medium (50% MEM, 25% Hank’s balanced salt solution, 25% Horse serum, 2 mM l-glutamine, 10,000 IU/ml penicillin, and 10,000 µg/ml streptomycin, pH 7.4, all from Gibco, Egggenstein, Germany), and the humidified, 5% CO\(_2\)-containing atmosphere of the incubator at 36.5°C. The culture medium was completely replaced on the first 2 days and thereafter twice a week. All experiments were done 9-10 days after preparation of cultures at a time when slice cultures had a thickness of 200–250 µm. Slice cultures with incomplete structure were excluded from the experiments. For studies on effects of α-tocopherol, 50 µg/ml ± α-tocopherol (106 µM, Sigma-Aldrich, Taufkirchen, Germany) was dissolved in ethyl alcohol (final concentration 0.1%) and added to the culture medium for 2 days prior to the experiment and also to the artificial cerebrospinal fluid (ACSF) immediately before experimentation. Control experiments were performed in the presence of the solvent, and no differences in the appearance and pattern of activity to the respective groups were found.

**Simultaneous electrophysiological and microfluorimetric measurements**

The slice cultures were preloaded with only one of the following dyes in culture medium in the incubator for 20–40 min: rhodamine-123 (Rhod-123, 26 µM, excitation 490 nm, emission 530 nm), rhod-2 AM (Rhod-2, 5 µM, excitation 530 nm, emission 590 nm), and dihydroethidium (HEt, 63 µM, excitation 515 nm, emission 610 nm). CalciumGreen-1 AM (CaGreen-1, 5 µM, excitation 490 nm, emission 530 nm) was added to the gassed ACSF at room temperature for ≥50 min. All dyes were from Molecular Probes (Leiden, Netherlands). The production of NAD(P)H was monitored by its auto-fluorescence at 460 nm on excitation at 360 nm (Aubin 1979; Schuchmann et al. 1998, 1999). To achieve rapid equilibration and staining of the relatively compact slice cultures, preliminary experiments were done to determine the appropriate dye concentrations. Since in the case of HEt, a considerable part of the dye can be oxidized during the staining procedure, we doubled the concentration previously used in dissociated cell cultures to shorten the incubation time to 20–25 min (Schuchmann and Heinemann 2000a). To control the availability of Rhod-123 a mitochondrial uncoupler, carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone (FCCP; 1 µM, Sigma-Aldrich, Taufkirchen, Germany) was applied after 2 h of epileptiform activity in two slice cultures.

After staining, a patch of membrane carrying one slice culture was excised and placed into the recording chamber. The chamber was perfused with gassed (95% O\(_2\), 5% CO\(_2\)) and warmed (36 ± 0.5°C) ACSF. The ACSF contained the following (in mM): 129 NaCl, 5 KCl, 1.25 NaHPO\(_4\), 1.8 or 0 MgSO\(_4\), 1.6 CaCl\(_2\), 21 NaHCO\(_3\), and 10 glucose, pH 7.4. In the present study, we used a model of experimental status epilepticus with repeated occurrence of SLEs induced by omitting Mg\(^{2+}\) from the ACSF and by elevating K\(^+\) to 5 mM, thereby facilitating activation of NMDA receptors, enhancing transmitter release, and increasing neuronal excitability (Mody et al. 1987). SLE did occur spontaneously, but their appearance could not be predicted and therefore would have required continuous monitoring of fluorescence increasing the risk of phototoxic damage particularly with UV illumination. Therefore each SLE was induced by a short stimulus train of 10 pulses (0.1 ms, 10–12 V) at 100 Hz applied to the mossy fibers with a glass pipette (tip diameter 10 µm) filled with ACSF. Inducing SLEs every 10 min had no effect on their appearance, but inhibited the occurrence of spontaneous SLEs, as revealed by the continuous monitoring with the ion-sensitive microelectrode. Imaging was usually started 50 s before initiation of a SLE. Control for phototoxic effects included continuous illumination with wavelength above 480 nm, which affected neither evoked field potential transients in normal medium nor the properties of low Mg\(^{2+}\)-induced SLEs.

Field potentials and changes in [Ca\(^{2+}\)]\(c\) were measured with a double-barreled ion-sensitive microelectrode in the stratum pyramidale of the CA3 region in the hippocampus. The electrodes were prepared and tested as previously described (Heinemann et al. 1977). They responded with 26–30 mV to a concentration change from 0.3 to 3 mM Ca\(^{2+}\). Changes in [Ca\(^{2+}\)]\(c\) were calculated according to the Nerst equation.

The recording chamber was mounted on an epifluorescence microscope (Axiioskop, Carl Zeiss, Jena, Germany), and the fluorescent signal was detected using a 10× water immersion objective (numerical aperture 0.3) and a photomultiplier (SMT, Seefeld, Germany). Fluorescence signals were collected from area CA3, the hilus and part of area CA1, and the dentate gyrus (Fig. 1D). Fluorescence was excited using a xenon arc lamp, and the appropriate exciting wavelengths were set with a monochromator system (Deltascan, PTI, Wedel, Germany). All signals were captured on computer disk at 10 Hz. Data were analyzed using the program IgorPro 3.14 (Waveometrics). The fluorescent signal from the photomultiplier tube was given in percent as Δf/fo × 100, where fo was the average fluorescence from a 20-s period, 20–50 s before each stimulus. In the present study, we analyzed the time course of changes during subsequent recurring SLEs.

**Measurement of seizure-induced cell damage**

In a different set of experiments cell death measurements were made, as described previously (Kovács et al. 1999). In brief, slice cultures were first put in an interface chamber and perfused with normal ACSF for 40 min and with normal or Mg\(^{2+}\)-free ACSF for another 20 min. The evoked field responses and the epileptiform activity were recorded on chart writer and on a hard disk. Thereafter the slice cultures were stained with propidium iodide (excitation 530 nm) for 30 min in the incubator 1–3 h after the experimental period. Fluorescence pictures were obtained above 590 nm, using the same microscope configuration with a low light CCD camera (Hamamatsu, Herrsching, Germany) and constant settings of light intensity and camera gain: 512 × 480 pixel pictures were taken from the three subregions of the hippocampus, i.e., the DG-hilus, the area CA3, and the area CA1. The images were stored on hard disk using the Image Master for Windows software (PTI, Wedel, Germany). After background subtraction (a picture obtained from the same membrane without culture), six defined (20 × 20 pixels) regions of interest were analyzed in each.
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of the following subfields: CA1, CA3, hilus, and the stratum granulare of the DG. The fluorescence signal was measured in each region of interest and expressed in arbitrary units (0–255) on a gray scale. Three different experimental conditions were compared using propidium iodide staining: 1) slice cultures stained in the incubator without any additional treatment (medium group); 2) slice cultures treated for 3 h in normal, gassed ACSF in the interface recording chamber (control group); and 3) slice cultures treated for 1 h with ACSF and for a further 2 h with low Mg²⁺ ACSF in the recording chamber with continuous monitoring of the epileptiform activity (low Mg²⁺ group). Measurements in the medium group indicated the basal level of cell death in the slice cultures, which occurs during cultivation. Preliminary experiments revealed that the propidium iodide signals correlated well with LDH release after exposure to hypoxia with and without glucose deprivation (Graulich et al. 2002). For statistical evaluation of differences within and between groups, the Wilcoxon matched-pairs signed rank test and the Mann Whitney U test were used, respectively (SPSS Software package). Data are given as mean ± SD.

RESULTS

On elevation of K⁺ and omission of Mg²⁺ from the perfusate, stimulus trains induced a SLE lasting for about 1 min (see also Gutierrez et al. 1999; Kovács et al. 1999, 2000, 2001). In brief, the SLEs were characterized by field potential (fp) transients superimposed on a slow negative fp shift and by decreases in [Ca²⁺]e (Fig. 1, A and B). Following a SLE, interictal discharges were suppressed for 10 s. Spontaneous SLEs were similar in appearance and duration (Fig. 2C). SLEs induced by subsequent stimuli were similar in duration and amplitude (cf. Figs. 1–4). After about 15 SLEs, the clonic-like discharge mitochondria stayed depolarized (see also Gutierrez et al. 1999, 2000, 2001). In brief, the SLEs were characterized by field potential (fp) transients superimposed on a slow negative fp shift and by decreases in [Ca²⁺]e. The rise of the Rhod-123 signal indicates mitochondrial depolarization. Note that amplitudes of the Rhod-123 signal became smaller during the course of recurrent SLEs. Displayed signals represent 1st and last SLEs in a sequence of recurring SLEs, as well as two SLEs in between. Calibrations on the right apply to all records. B: sample recordings of Rhod-123 fluorescence signals indicating SLE-associated changes in [Ca²⁺]e, [Ca²⁺]i, and fp during subsequent SLEs. C: composite picture of a slice culture representing the field of view of the photomultiplier tube, containing the area CA3, the hilus and part of area CA1, and the dentate gyrus. The picture seen by the photomultiplier is added on a picture of a Nissl stained slice culture for better location of the field of view (bar represents 400 μm).

FIG. 2. Rhod-123 signals during subsequent SLEs. A: changes in Rhod-123 signals (ΔIF) recorded simultaneously with fp and changes in [Ca²⁺]i. The rise of the Rhod-123 signals indicates mitochondrial depolarization. Note that amplitudes of the Rhod-123 signal became smaller during the course of recurrent SLEs. Displayed signals represent 1st and last SLEs in a sequence of recurring SLEs, as well as two SLEs in between. Calibrations on the right apply to all records. B: amplitudes of Rhod-123 fluorescence rises as a function of recurring SLEs in the presence and absence of α-tocopherol (vertical bars present SD). Note that initially mitochondrial depolarization is somewhat smaller in presence of α-tocopherol. C: after transition to late recurrent discharges mitochondria stayed depolarized as indicated by the sustained increase in Rhod-123 fluorescence. D: carbonyl cyanide-m-(trifluoromethoxy)-phenylhydrazone (FCCP) application during late recurrent discharges caused a large increase of Rhod-123 fluorescence.

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\([\text{Ca}^{2+}]\) decreased during each SLE during the course of recurring activity by \(0.48 \pm 0.2 \text{ mM} (n = 142)\). The maximal decrease in \([\text{Ca}^{2+}]\) associated with the first SLE was not significantly different from the decrease at the last SLE observed immediately before late recurrent discharges developed (\(P = 0.182; n = 11\)), suggesting that the \(\text{Ca}^{2+}\) influx from the extracellular space was rather constant during subsequent SLEs. Late recurrent discharges were accompanied by small \([\text{Ca}^{2+}]\) fluctuations (<0.1 mM) around a somewhat lowered baseline \([\text{Ca}^{2+}]\).

Baseline CaGreen-1 fluorescence signals between each evoked SLE remained rather constant over the course of the whole experiment with \(0.4 \pm 0.5\%\) loss of fluorescence signal over 100 s. Interictal activity caused only small fluctuations in the CaGreen-1 signal, thereby slightly increasing baseline fluorescence. Marked increases of the \([\text{Ca}^{2+}]\) were observed during each SLE. Despite unchanged \(\text{Ca}^{2+}\) influx from the extracellular space, the amplitude of the CaGreen-1 signal became smaller by \(33.3 \pm 16.5\% (n = 6)\) within the first six SLEs. Thereafter it remained quite stable as the CaGreen-1 signal decrease was \(33.5 \pm 8.6\%\) at the 15th SLE (\(n = 2\); Fig. 1A). The largest reduction of the signals was noted from the first to second SLE (\(-17.5 \pm 19.0\%; n = 7; P = 0.075\)). This difference was significant between the CaGreen-1 fluorescence rises associated to the first and to the last SLEs in a sequence (\(n = 6; P = 0.018\); Fig. 1C).

\([\text{Ca}^{2+}]\)m rose rapidly during the SLE as revealed by the Rhod-2 fluorescence signal. Recovery of \([\text{Ca}^{2+}]\)m was slower than that of the \([\text{Ca}^{2+}]\) signal and fast fluctuations were not present, suggesting that the \(\text{Rhod}-2\) signals arose predominantly from a different compartment than that of CaGreen-1 (Kovács et al. 2001). As was the case with CaGreen-1 signals, the amplitude of rises in \([\text{Ca}^{2+}]\)m declined during subsequent SLEs (Fig. 1, B and C), despite the relatively constant baseline Rhod-2 fluorescence (\(-0.6 \pm 0.7\%/100 \text{ s}\)). After eight SLEs, the amplitudes of rises in \([\text{Ca}^{2+}]\)m had declined by about \(53.6 \pm 11.8\%\) (Fig. 1C). Similarly to the CaGreen-1 fluorescence, the largest decrease (\(-20.2 \pm 9.0\%; n = 5; P = 0.043\)) could be observed from the first to the second SLE. After 14 SLEs—shortly before the late recurrent discharges started—[\([\text{Ca}^{2+}]\)m] rises were still present but their amplitudes were only \(40.9 \pm 12.2\% (n = 3)\) of those during the first SLE.

Changes in the mitochondrial membrane potential (\(\Delta \Psi\))

Changes in \(\Delta \Psi\) were measured using the dye Rhod-123. Rhod-123 accumulates in energized mitochondria, where its fluorescence is quenched on binding to matrix proteins. With depolarization of mitochondrial membrane, it is released from the mitochondrial, thereby increasing fluorescence (Bindokas et al. 1998; Duchen et al. 1992; Schuchmann et al. 2000). As reported previously (Kovács et al. 2001), a slight decrease of the baseline fluorescence was noted initially during a SLE. Subsequently, the Rhod-123 signal increased, indicating depolarization of mitochondrial membranes. The maximum fluorescence increase by \(12.3 \pm 5.7\%\) was reached at the end of the SLE at \(45.9 \pm 27.7\%\) after onset of the SLE (\(n = 15\)). The Rhod-123 fluorescence signal recovered within some 2–3 min to baseline and thus more slowly than [\([\text{Ca}^{2+}]\)].

Similar to \([\text{Ca}^{2+}]\)i and [\([\text{Ca}^{2+}]\)m signals, the SLE-associated Rhod-123 rises also declined from seizure to seizure (Fig. 2, A and B). After 10 SLEs, the Rhod-123 fluorescence signal change had significantly declined by \(46.2 \pm 15.8\% (P = 0.012, n = 8)\). At the 15th SLE, the signal had declined by \(70.0 \pm 13.8\% (n = 5)\) from its initial value. When SLEs changed to late recurrent discharges, a sustained increase of the Rhod-123 fluorescence could be observed, which was similar in amplitude to those, accompanied to the last segregated SLEs (Fig. 2C). To exclude the possibility that successive decline of the SLE associated Rhod-123 fluorescence changes is simply due to loss of the dye from the slice culture, the mitochondrial uncoupler FCCP (1 \(\mu\)M) was added to the perfusion after 2 h of continuous epileptiform activity. Rhod-123 fluorescence increased immediately, and this increase was about five times larger than that associated with previous SLEs, thereby indicating that Rhod-123 is not depleted from the slice culture at the end of the measurement (Fig. 2D).

Treatment of the slice cultures with \(\alpha\)-tocopherol caused a slight decrease of the Rhod-123 fluorescence rises associated with single SLEs. In \(\alpha\)-tocopherol–treated slice cultures, the rate of decline during subsequent SLEs was smaller than in control slice cultures and in some cases even a transient increase of the maximum amplitude could be noted. The Rhod-123 fluorescence rises at the 10th SLE in cultures treated with \(\alpha\)-tocopherol were not significantly different from the rises associated with the first SLE (3.52 \pm 51.3\%; \(n = 6; P = 0.6\)).

Changes in \(\text{NAD(P)H}\) auto-fluorescence

Measurement of changes in \(\text{NAD(P)H}\) auto-fluorescence requires excitation with 360 nm. This carries the risk of phototoxic damage due to UV light exposure. Indeed, late recurrent discharges could start much earlier than during the other experiments, and \(\text{Fp}\) changes during SLEs declined in amplitude, presumably due to phototoxicity. This transition occurred on average after \(8 \pm 7\) SLEs. Measurements of changes in \(\text{NAD(P)H}\) signals had therefore to be restricted to the first 6 SLEs in control slice cultures. By contrast, in \(\alpha\)-tocopherol treated slice cultures transition to late recurrent discharges was noted significantly later, after \(17 \pm 4\) SLEs (\(n = 13, 12; P = 0.006\)).

In most cases the \(\text{NAD(P)H}\) fluorescence began to rise after an initial decrease and reached peak levels at \(48.1 \pm 15.7\% (n = 12)\) after onset of the first SLEs. \(\text{NAD(P)H}\) fluorescence then recovered slowly within 2–3 min. The individual rises in \(\text{NAD(P)H}\) auto-fluorescence declined in amplitude by \(35.5 \pm 23.9\%\) from the first to the sixth SLE (\(n = 5\); Fig. 3A). Also, recovery to baseline occurred faster. In two cases, the SLE associated \(\text{NAD(P)H}\) auto-fluorescence rises disappeared shortly before transition to late recurrent discharges. Instead, \(\text{NAD(P)H}\) auto-fluorescence was reduced during the whole course of a single SLE (Fig. 3A).

To test whether the decline of mitochondrial depolarization and \(\text{NAD(P)H}\) auto-fluorescence signals might depend on accumulating ROS-dependent damage of mitochondria, we repeated our experiments in the presence of \(\alpha\)-tocopherol. In the \(\alpha\)-tocopherol–treated slice cultures, the SLE-associated \(\text{NAD(P)H}\) rises were larger than in the control cultures (\(n = 112\) for control cultures, \(n = 212\) for \(\alpha\)-tocopherol–treated cultures; \(P < 0.001\)). Moreover, decline of the \(\text{NAD(P)H}\)
cultures than in the control ones. The displayed signals represent the smaller during recurrent SLEs and occasionally completely disappear. The cultures. Note that SLE-associated rises of NAD(P)H auto-fluorescence were larger during the whole course of the experimental status in the pooled maximal values of NAD(P)H auto-fluorescence vs. the number of SLEs (vertical bars present SD). Note, that the changes in ethidium fluorescence were obtained after the peak depolarization of mitochondria. In contrast to changes in Ca^{2+}, Rhod-123 fluorescence, and NAD(P)H auto-fluorescence, which declined in amplitude during recurring SLEs, the ethidium signals increased in amplitude in 5 of 12 cases. Moreover, the rise time became faster (Fig. 4A). Only two of the cultures showed a transient decline, while the remaining slice cultures had fairly constant ethidium fluorescence signal amplitudes. The amplitude of the ethidium fluorescence signal increased after 12 SLEs on average to 153 ± 73.2% (n = 9). The ethidium fluorescence signal showed a rather marked baseline decline between early SLEs and under control conditions. However, this was less steep during late recurrent discharges and in some cases, even an increasing baseline could be observed. CCD camera recordings revealed that at the beginning of a recording period, ethidium was signals during subsequent SLEs was delayed in presence of α-tocopherol (Fig. 3C). The NAD(P)H signal at the sixth SLE in α-tocopherol–treated cultures was practically unchanged (88.4 ± 50.3% of the amplitude of the first SLE, n = 12).

Changes in the ethidium fluorescence

To determine whether SLEs were associated with increased generation of free radicals we stained the slice cultures with HEt, which shows a fluorescence shift on oxidation to ethidium (Bindokas et al. 1996; Budd et al. 1997). At the chosen wavelength, this becomes apparent as an increase in fluorescence. HEt was shown to be particularly sensitive to oxidation by superoxide anion radicals and to co-localize with mitochondria (Bindokas et al. 1996, Robb et al. 1999). Similarly to Rhod-123 and NAD(P)H fluorescence, ethidium fluorescence decreased initially during a SLE but then rose to reach a maximum on average 67.7 ± 25.3 s (n = 12) after onset of a SLE. Thus peak levels in ethidium fluorescence were obtained after the peak depolarization of mitochondria. In contrast to changes in Ca^{2+}, Rhod-123 fluorescence, and NAD(P)H auto-fluorescence, which declined in amplitude during recurring SLEs, the ethidium signals increased in amplitude in 5 of 12 cases. Moreover, the rise time became faster (Fig. 4A). Only two of the cultures showed a transient decline, while the remaining slice cultures had fairly constant ethidium fluorescence signal amplitudes. The amplitude of the ethidium fluorescence signal increased after 12 SLEs on average to 153 ± 73.2% (n = 9). The ethidium fluorescence signal showed a rather marked baseline decline between early SLEs and under control conditions. However, this was less steep during late recurrent discharges and in some cases, even an increasing baseline could be observed. CCD camera recordings revealed that at the beginning of a recording period, ethidium was signals during subsequent SLEs was delayed in presence of α-tocopherol (Fig. 3C). The NAD(P)H signal at the sixth SLE in α-tocopherol–treated cultures was practically unchanged (88.4 ± 50.3% of the amplitude of the first SLE, n = 12).

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Effects of α-tocopherol on status epilepticus–induced cell death in slice cultures

In previous studies we showed that by using propidium iodide staining, long-lasting (over 2 h) epileptiform activity could lead to damage of slice cultures (Kovács et al. 1999). To test whether this damage might be related to the SLE-associated ROS production, we investigated the effects of α-tocopherol on cell survival. Three conditions were compared: 1) slice cultures being kept in the incubator (medium group); 2) slice cultures treated for 3 h in normal, gassed ACSF in an interface chamber (control group); and 3) slice cultures treated for 1 h with ACSF and for a further 2 h with low Mg²⁺ ACSF (low Mg²⁺ group). In the control and the low Mg²⁺ groups, the evoked fp responses were controlled at the end of the recording, while epileptiform activity was monitored throughout the whole experiment. As reported previously (Kovács et al. 1999), the basal level of spontaneous cell death after 9 days in vitro (DIV) was low. Exposure to normal ACSF with 95% O₂ increased the amount of stained cells throughout the different subfields of the hippocampus and decreased the amplitude of the evoked fps (Kovács et al. 1999; Pomper et al. 2001). These signs of cell damage were augmented after 2 h of recurrent seizure-like events (Gutierrez et al. 1999; Kovács et al. 1999). Neither treatment with α-tocopherol nor with the vehicle alone had an effect on the propidium iodide staining of the medium group (n = 17 slice cultures with, and n = 17 without α-tocopherol treatment; P > 0.5), but it significantly reduced cell death after exposure to 95% O₂ in normal ACSF (n = 26 slice cultures with, and n = 48 without α-tocopherol treatment; P < 0.05). It also significantly reduced propidium iodide fluorescence in slice cultures, which had experienced 2 h of recurrent SLEs (n = 30 slice cultures with, and n = 22 without, α-tocopherol treatment; P < 0.001). Figure 5 presents examples of the propidium iodide stained slice cultures, while Fig. 6 shows the average values of the propidium iodide fluorescence in the different subregions of the hippocampus. It is interesting to note that the differences between control and low Mg²⁺ groups were no longer significantly different in α-tocopherol–treated slice cultures (P = 0.066, 0.153, 0.393 for CA1, CA3, and DG.

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respective, \( n = 30 \) for low Mg\(^{2+}\) group, \( n = 26 \) for control group). According to the results of propidium iodide staining, the decrease of the evoked fi responses between the beginning and the end of the activity was smaller in the \( \alpha \)-TH treated low Mg\(^{2+}\) group, than in slice cultures of the untreated low Mg\(^{2+}\) group (75 ± 30% vs. 39 ± 10% for CA1, 71 ± 32% vs. 44 ± 18% for DG, and 56 ± 29% vs. 25 ± 15% for CA3).  

**DISCUSSION**

Here we present evidence that mitochondrial free radical production is involved in cell loss after frequently recurring SLEs in an in vitro model of status epilepticus. Omission of Mg\(^{2+}\) from the ACSF evoked recurring SLEs in hippocampal slice cultures. Every SLE resulted in intracellular as well as intramitochondrial Ca\(^{2+}\) accumulation. However, there was no evidence for accumulating shifts in the baseline [Ca\(^{2+}\)] and no signs of deregulation of intracellular Ca\(^{2+}\) homeostasis. Instead, SLE-associated intracellular and intramitochondrial Ca\(^{2+}\) transients decrease in amplitude during the course of recurring SLEs. Also, fluorescence signals indicating mitochondrial depolarization and enhancement of NAD(P)H production became smaller, while ethidium fluorescence signals—probably representing ROS production—increased in amplitude. These data suggest an accumulating mitochondrial impairment, which may be involved in the cell loss observed by propidium iodide staining after 2 h of continuous epileptiform activity. The successive changes in mitochondrial depolarization, NAD(P)H auto-fluorescence, and ethidium fluorescence were reduced by the free radical scavenger and lipid peroxidation chain-breaker, \( \alpha \)-tocopherol. Likewise, cell death induced by recurrent SLEs was also strongly reduced by application of \( \alpha \)-tocopherol, suggesting also ROS involvement in seizure-related cell damage.

**Mitochondrial Ca\(^{2+}\) uptake during recurrent SLEs**

During SLEs, there is a considerable intracellular accumulation of Na\(^+\), Cl\(^-\), and Ca\(^{2+}\) within cells, while K\(^+\) leaves the neurons (for a review see Heinemann et al. 1986; Lux et al. 1986). To restore ionic gradients, transport processes across the membrane have to be activated, which depend on the availability of ATP. Thus a SLE is followed by a long-lasting membrane hyperpolarization mediated by enhanced activity of the Na-K-ATPase (Ayala et al. 1973; Heinemann and Gutnick 1979; Schmitz et al. 1997). The increased energy demand requires strong coupling of neuronal and metabolic activity. Indeed, blockade of the Na-K-ATPase by ouabain decreased the respiration rate, while Na\(^+\) influx due to veratridine application led to a threefold increase of the mitochondrial respiration (Urenjak et al. 1991). It is well documented with respect to the vascular compartment that cerebral blood flow increases severalfold during a seizure, thereby supporting the tissue with more glucose and O\(_2\) (Horton et al. 1980; Ingvar 1986; Johnson et al. 1993; Kahane et al. 1999; Pinard et al. 1984). However, less is known on the mechanisms that couple neuronal with metabolic activity within neurons during seizures.

A possible link could be the uptake of Ca\(^{2+}\) into the mitochondria. The activity of three key dehydrogenases of the tricarboxylate cycle is stimulated by Ca\(^{2+}\) (McCormack and Denton 1993). There is experimental evidence that even physiological rises of [Ca\(^{2+}\)] can lead to mitochondrial Ca\(^{2+}\) accumulation with subsequent activation of pyruvate dehydrogenase (Rutter et al. 1996). Also, the activity of the electron transport chain can be enhanced on Ca\(^{2+}\)-dependent changes of the mitochondrial matrix volume (Halestrap 1989). Recently, Jouaville and co-workers have shown that histamine induced Ca\(^{2+}\) elevations readily increases the mitochondrial and cytosolic ATP concentration in HeLa cells (Jouaville et al. 1999).

In previous studies we have already shown that SLEs in hippocampal slice cultures are associated with Ca\(^{2+}\) uptake from the extracellular space to the cytosol, and hence into mitochondria (Kovacs et al. 2000). This is followed by an increase in the NAD(P)H fluorescence, suggesting that Ca\(^{2+}\) accumulation within mitochondria, apart from metabolites, is important for adaptation of mitochondrial activity to the needs of the cell (Kovacs et al. 2001).

During recurring SLEs, the increases in [Ca\(^{2+}\)] and [Ca\(^{2+}\)]m decline with time. Long periods of epileptiform activity were associated with considerable cell loss, without any sign of ongoing intracellular Ca\(^{2+}\) accumulation, which is often thought to be critically involved in causing cell death (Frantseva et al. 2000b; Nicholls and Budd 1998, 2000). The decline of [Ca\(^{2+}\)] and [Ca\(^{2+}\)]m rises is not a consequence of cell loss or leaking of the dye, because the largest decrease can be observed between the first and the second SLE, while the fluorescence rises remained stable at later SLEs. Studies with propidium iodide staining at different times of recurrent SLEs in comparison to control suggested that significant increase of the propidium iodide fluorescence appears only after more than 1 h of continuous activity. We also can exclude bleaching of the dye, because the slope of the baseline fluorescence before each SLE is shallow, and it did not significantly change over time. Since the values in this study are given as fluorescence changes relative to the baseline fluorescence, the signals should be rather insensitive to small changes in dye availability. There may be two possible explanations for the deterioration of Ca\(^{2+}\) signals. First, the contribution of intracellular Ca\(^{2+}\) stores to the rises in [Ca\(^{2+}\)]m might be decreased, perhaps due to depletion of the endoplasmatic reticulum Ca\(^{2+}\) stores during the first SLE as a consequence of activation of metabotropic receptors and Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Second, intracellular Ca\(^{2+}\) buffer capacity might be increased resulting in a decrease of free cytosolic [Ca\(^{2+}\)].

**NAD(P)H production during recurrent SLEs**

The decreased uptake of Ca\(^{2+}\) into mitochondria may led to reduced activation of the tricarboxylate cycle and thereby to a decrease of NADH synthesis. Indeed, both mitochondrial depolarization and NAD(P)H auto-fluorescence rises decreased from SLE to SLE. However, the decline of [Ca\(^{2+}\)]m cannot account for the ultimate failure of the NAD(P)H production as intramitochondrial Ca\(^{2+}\) accumulation is still observed at a time, when NAD(P)H auto-fluorescence increases have disappeared. Since investigations of the NAD(P)H auto-fluorescence require UV illumination, the decline in NAD(P)H signals could be partly due to oxidative photo-damage of the slice cultures. However, similar results were previously obtained in freshly isolated brain slices in pulsed nitrogen laser evoked auto-fluorescence measurements where phototoxic effects are expected to be minimal (Schuchmann et al. 2001).
Deterioration of NADH production could result in reduced ATP synthesis and a mismatch between cellular and metabolic activity. Indeed, reduced ATP levels were observed after prolonged status epilepticus (Folbergerova et al. 1981; Wasterlain et al. 1993). ATP depletion would result in impairment of normal neuronal functioning. Thus AHPs due to enhanced Na⁺-K⁺-ATPase activity following a SLE decline in amplitude and duration, when seizures recur frequently (Schmitz et al. 1997). This process might be involved in the transition to late recurrent discharges. Late recurrent discharges are particularly dangerous, as they cannot easily be interrupted by conventional anticonvulsant drugs, and therefore represent a pharmacoresistant phase of status epilepticus (Zhang et al. 1995). A comparable decline of the SLE associated NAD(P)H rises was recently described in slice preparations when SLEs were induced by Mg²⁺-free ACSF, while they did not readily occur in the 4-aminopyridine model. In this latter case, transition to late recurrent discharges is also missing or delayed (Schuchmann et al. 1999). A decline in AHPs was also noted when glutamate was repeatedly applied (Fukuda and Prince 1992). It was assumed that this decline is mediated by a direct effect of Ca²⁺ on the Na⁺-K⁺-ATPase because it could be prevented by lowering [Ca²⁺]. Alternatively, we suggest that accumulating impairment of NAD(P)H production might result in decreased ATP levels and subsequent reduction of the Na⁺-K⁺-ATPase activity.

**Effects of α-tocopherol on SLE-induced mitochondrial function changes**

It was recently reported that in human tissue from patients with mesial temporal lobe epilepsy and with Ammon’s horn sclerosis, the complex I of the respiratory chain is impaired (Kunz et al. 2000). Such alterations might result from ROS-dependent damage and they can also lead to further enhancement of production of superoxide anion radicals in a vicious cycle. As a consequence, synthesis of NADH will be decreased, which may lead to depletion of the ATP pool and in addition to exhaustion of glutathione (GSH) pools (e.g., Schuchmann and Heinemann 2000b). There is already some experimental evidence for involvement of ROS in the seizure-induced cell loss (Folbergerova et al. 1999; Frantseva et al. 2000a; Gupta et al. 2001). Moreover, seizure-associated generation of ROS was suggested by in vitro microfluorimetric methods (Frantseva et al. 2000b; Kovács et al. 2001).

Here we found that SLE-associated oxidation of HEt to ethidium increased in amplitude and its onset became earlier from seizure to seizure during recurrent ictaliform activity. Since HEt is preferentially oxidized by superoxide radicals (Bindokas et al. 1996), this indicates a cumulative increase of superoxide radical formation. We previously tested the specificity of the HEt fluorescence changes in dissociated cultures on exposure to glutamate, which also lead to changes in fluorescence signals of dihydrolrhodamine and dihydrocarboxyfluorescein, two other fluorescent indicators of ROS and found the HEt signal to correlate with radical formation (Schuchmann and Heinemann 2000a). Our data are also in agreement with the results of Frantseva and co-workers, who used dihydrorhodamine to indicate production of free radicals during bicuculline-induced epileptiform activity (Frantseva et al. 2000b). There are some experimental indications that ethidium fluorescence changes may originate from other sources than oxidation of HEt by superoxide anion radicals (Budd et al. 1997, Vergun et al. 2001). Under our particular conditions, we cannot completely exclude that ethidium release from depolarized mitochondria, at least partially, contributes to our signal. Three facts point against this possibility, which were already discussed to some extent in our previous paper (Kovács et al. 2001). First, the time course of the changes of Rhod-123 signals, which indicate depolarization of mitochondrial membranes, was much faster than ethidium fluorescence signals during a single SLE. Second, Rhod-123 changes declined from SLE to SLE, whereas SLE-associated ethidium fluorescence signals rather increased. This is not simply due to its accumulation in the mitochondria, as CCD camera recordings revealed that the oxidation end product, ethidium is mainly accumulated within the nucleus. Third, α-tocopherol caused only a slight, nonsignificant decrease of the amplitude of the Rhod-123 rises, whereas it was able to decrease or even completely inhibit the SLE-associated rises in ethidium fluorescence. The time course of the successive increase of ethidium fluorescence rises fit well with the regular decline in NAD(P)H auto-fluorescence rises as well as with the decline in subsequent mitochondrial depolarizations. All these facts point to an accumulating impairment of mitochondrial functions during experimental status epilepticus. The causality is hard to define since the decline in NAD(P)H auto-fluorescence signals could either be a result of direct oxidation of NAD(P)H by ROS (Schuchmann et al. 2001) but it might also represent damage of the mitochondrial function. Decreased levels of NAD(P)H may also lead to weakening of the antioxidative defense of the cell due to reduced glutathione regeneration by the NAPDH-dependent glutathione reductase, thereby enhancing ROS-induced damage. Indeed, we have previously reported that glutathione levels decline after repeated application of glutamate in dissociated mouse hippocampal cultures (Schuchmann and Heinemann 2000b). A further consequence of an NAD(P)H loss might be a decrease in the mitochondrial membrane potential. Such constant depolarization could be observed during late recurrent discharges.

The cumulative damage of the mitochondrial function was also inhibited by α-tocopherol, because the decline of the NAD(P)H auto-fluorescence signals was significantly delayed in the presence of α-tocopherol. Even the amplitude of every single rise was on average larger, which could be explained if we assume that part of the NAD(P)H is consumed by ROS under control conditions. It is noteworthy, that the decline of mitochondrial depolarizations was also delayed in the presence of α-tocopherol, which supports the idea, that α-tocopherol helps to maintain mitochondrial function.

α-Tocopherol accumulates in mitochondrial and plasma membranes and it is preferentially oxidized by lipid peroxyl radicals (Ham and Liebler 1995). Thus its protective effects are downstream from the oxidation of HEt to ethidium, which already occurs at the superoxide level. This implies that the inhibitory effect of α-tocopherol on HEt oxidation and NAD(P)H auto-fluorescence decline may only represent a small portion of the protective effects against cell damage. More likely, such a treatment interacts with the overall antioxidant capacity of the cells. Therefore we tested in subsequent experiments whether α-tocopherol rescued cells in organotypic cultures from status epilepticus–induced damage. We em-
ployed the widely used cell death marker, propidium iodide, for indication of cells with injured membranes (Noraberg et al. 1999). As reported previously, recurring SLEs caused damage of slice cultures and increased the propidium iodide fluorescence (Kovác et al. 1999). However, the propidium iodide fluorescence intensity remained low when slice cultures were treated with α-tocopherol, indicating that free radical production and lipid peroxidation are involved in cell loss after frequently recurring SLEs. This effect was already evident after exposure to normal ACSF, as carbogen bubbling of the ACSF represents an oxidative stress for slice cultures accommodated to the normal air oxygen tension in the incubator (Pomper et al. 2001). These results would be of particular interest if the increased local blood flow during seizures is able to deliver more O2 to the tissue than consumed by the enhanced metabolism (Johnson et al. 1993; Kahane et al. 1999). However, we are aware that the high oxygen levels, which are used to supply slices and slice cultures, are probably not reached in vivo. In unpublished subsequent control experiments with more physiological oxygen pressure we found, however, similar degrees of cell death as in the present experiments (I. Eyopoglu, T. N. Lehmann and U. Heinemann, unpublished data).

As we reported previously, the amplitude of the evoked field potential responses decreased significantly after experimental status epilepticus. Treatment with α-tocopherol prevented this decline to some extent, thus supporting our conclusion that it has a neuroprotective effect.

The suggested neuroprotective effect of α-tocopherol was independent of an anticonvulsant effect as the duration of SLEs and seizure-related decreases of [Ca2+] on were unaffected by the drug. In animals, α-tocopherol was found to be effective against ferrous chloride seizures, hyperbaric oxygen seizures, and penicillin-induced seizures, where ROS production may have a role in the development of the seizures itself (Levy et al. 1990, 1992). However, it has no direct anticonvulsant effects in the maximal electroshock and threshold pentylentetrazol models as well as in amygdala-kindled seizures and kainic-acid seizures (Levy et al. 1990, 1992). We therefore suggest that the neuroprotective role of α-tocopherol is based on its ability to prevent changes in mitochondrial function during SLE-associated enhancement of ROS production.

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