Intravenous levetiracetam in the rat pilocarpine-induced status epilepticus model: Behavioral, physiological and histological studies

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1. Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent spontaneous seizures. Status epilepticus (SE), defined as continuous seizure activity lasting over 30 min, or repeated seizures between which patients do not recover to their baseline level of consciousness, is a medical emergency with high morbidity and mortality if not treated promptly (DeLorenzo et al., 1995, 1996). Recurrent seizures or status epilepticus may cause injury to hippocampus and other limbic structures in humans and in animal seizure models.

Status epilepticus is typically treated in a protocol-driven fashion with intravenous anticonvulsants. Up to 30% of SE patients do not respond to first or second line anti-epileptic drug (AED) treatment and require administration of anesthetic agents, but in this group morbidity and mortality increase significantly (Mayer et al., 2002; Walker, 1998). Because no new intravenous treatments have been developed and licensed for the treatment of SE, in spite of the fact that existing treatments frequently fail, it is important to discover new drugs with utility in the initial (pre-anesthetic) treatment of status epilepticus. Whereas traditional AEDs target Na+ channels, T-type Ca++ channels, GABA receptors or glutamate receptors (Urbanska et al., 1999; Fraser, 1999; Zona, 2001), it is likely that efficacious alternative agents will aim at new molecular targets to achieve specific therapeutic effects.

Levetiracetam (LEV), a novel anti-epileptic drug that has a unique profile in animal models of partial and generalized seizures, was initially identified as an anti-convulsant in a mouse audiogenic seizure model (Gower et al., 1992). It does not have anti-convulsant activity in either the maximal electroshock seizure (MES) or the subcutaneous pentylenetetrazole (PTZ) seizure rodent (Klitgaard et al., 1998; Loscher, 1999; Zona, 2001), but is a potent anti-convulsant in genetic rodent...
models (Klitgaard et al., 1998; Gower et al., 1995; Yan et al., 2005), the amygdala kindling model (Mazarati et al., 2004; Glien et al., 2002; De Smedt et al., 2005; Loscher et al., 1998), and spontaneous recurrent seizure models (Glien et al., 2002). LEV has been approved for clinical use in the U.S. since 1999 to treat partial epilepsy (Hovinga, 2001; Shorvon et al., 2000; Shorvon et al., 2000), and more recently to treat tonic-clonic and myoclonic seizures. LEV inhibits neuronal hyper-synchronization in cultured hippocampal slices (Margineanu and Klitgaard, 2000), and inhibits N-type Ca2+ channels (Niespodziany et al., 2001). It prevents the upregulation of BDNF and neuropeptide Y mRNAs, and downregulation of NPY1- and NPY5-like receptors in the kindling rat model (Husum et al., 2004). LEV binds to the synaptic vesicle protein SV2A (Lynch et al., 2004), where it has been hypothesized to interfere with neurotransmitter release. In animal studies it shows few adverse effects (Klitgaard et al., 1998; Lamberty et al., 2000), suggesting a high safety margin. The unique characteristics of LEV, including its anti-epileptic profile in animal models as well as its molecular target suggest that LEV represents a new class of AED.

The effect of LEV on prevention and treatment of status epilepticus has not been widely studied. In a previous report (Klitgaard et al., 1998), LEV administered intraperitoneally appeared to attenuate status epilepticus induced by systemic pilocarpine or kainate, but intravenous administration of LEV has not been studied.

Pilocarpine is a muscarinic cholinergic agonist. The pathophysiology and neuropathology of pilocarpine-induced SE has been thoroughly studied (Turski et al., 1989). Intraperitoneal administration of high doses of pilocarpine (300–400 mg/kg), or lower doses of pilocarpine (30–40 mg/kg) after pretreatment with lithium, results in the stereotyped appearance of behavioral and electrographic seizures lasting many hours (Treiman et al., 1990). Surviving animals typically go on to develop spontaneous recurrent seizures weeks to months after SE. Neuronal injury in rat brain after seizures is highly reminiscent of the injury seen in humans after prolonged SE, affecting mainly hippocampus and limbic structures (Cavalleiro et al., 1991). The effect of LEV on the onset of pilocarpine-induced SE in mice (Oliveira et al., 2005), and on pilocarpine-induced spontaneous recurrent seizures in rats has been studied (Glien et al., 2002). Here we sought to determine whether acute treatment with intravenous LEV could stop or attenuate the behavioral and electrographic manifestations of pilocarpine-induced SE and the subsequent development of neuronal injury in rats.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (125–175 g) (Charles River Laboratories; Wilmington, MA.) were used for all experiments. All experiments were conducted under an approved IACUC protocol in accordance with the regulations of the Center for Comparative Medicine and Laboratory Animal Services of Massachusetts General Hospital.

2.2. Induction of seizures by lithium-pilocarpine and administration of LEV

18–24 h prior to pilocarpine, animals were treated with lithium hydrochloride (127 mg/kg (3 meg/kg) i.p.). Thirty minutes prior to pilocarpine administration, animals were treated with scopolamine methyl bromide (1 mg/kg i.p.) to reduce peripheral cholinergic agonist-induced side effects. Animals were then treated with pilocarpine hydrochloride (30 mg/kg i.p.). Using this treatment paradigm, behavioral seizures typically begin within 20–40 min. The tail vein was cannulated and LEV was administered as a single bolus through the intravenous catheter at designated timepoints after onset of seizure activity.

2.3. Seizure behavior quantification

After pilocarpine injection, animals were closely observed and behavioral changes were recorded. A seizure severity grade was assigned according to a modified Racine scale previously used in our laboratory (Weiss et al., 1996; Hu et al., 1998) with regard to the animals’ maximal behavioral response (Grade 0, no response; Grade 1, wet dog shake (WDS) and/or behavioral arrest; Grade 2, WDS, staring, pawing, and clonic jerks; Grade 3, WDS, staring, pawing, clonic jerks, rearing and falling; Grade 4, continuous grade 3 seizures for more than 30 min (status epilepticus)). Injection of LEV or normal saline (PBS) was undertaken at specific timepoints after rats initially achieved a grade 3 seizure severity (rearing and falling). Approximately 90% of experimental animals achieved Grade 4 seizure severity, and only these animals were utilized for subsequent analyses. Ongoing behavior was observed and recorded. After 2 h of continuous or intermittent Grade 4 seizure activity, all animals were treated with diazepam (15 mg/kg i.p.) to minimize stress and discomfort and increase survival. Animals were given supplemental dextrose (3% solution) and allowed to survive 24 h prior to sacrifice.

2.4. Rat EEG recording

For EEG recordings adult male Sprague–Dawley rats weighing 200–250 g at the time of surgery were utilized. Rats were anaesthetized with ketamine (70 mg/kg i.p.) (McBride Laboratories; Bedford, OH) and pentobarbital (25 mg/kg i.p.) (Abbott Laboratories; N. Chicago, IL). Supplemental doses of 15–20 mg/kg ketamine were given as needed. Rats were placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA) and surface recording electrodes (Plastics One; Roanoke, VA) (1/8" stainless steel jewelers screws) were placed in right frontal, left frontal and left parietal locations. A bipolar twist electrode (Plastics One) was placed in the right hippocampus (AP ~3.5 mm from bregma, ML ± 2.0 mm from bregma, DV ~3.7 mm from dura, incisor bar 3.1 mm) using coordinates determined from the atlas of Paxinos and Watson (1986). Electrodes were inserted into a plastic pedestal (Plastics One), which was then stabilized on the skull with dental acrylic (Benco Dental; Wilkes Barre, PA).

Postoperatively, the animals were housed singly in clear plastic cages and were allowed to recover for 6–8 days during which time they were handled daily before recording was performed. Seizures were induced by pretreatment with lithium and then administration of scopolamine and pilocarpine as described above.

Electroencephalographic activity was monitored in freely moving rats using a dedicated digital EEG machine (Cadwell Laboratories, Inc.; Kennewick, WA). Two control animals treated with intravenous saline were utilized to assess the effects of handling and injection on behavioral and EEG activity during SE. At the end of recording, animals were euthanized with 200 mg/kg i.p. pentobarbital sodium (Abbott). Brains were immediately fixed in 4% paraformaldehyde. Depth electrode placement in the hippocampus was confirmed two days later by gross inspection of coronal brain sections.

For each animal, 2 h of continuous EEG was visually inspected by an experienced electroencephalographer (AG) to determine the presence of electrographic seizure activity. Epileptiform activity was identified based on commonly used principles of EEG analysis, including the occurrence of epileptiform spikes. Spikes were recognized on the basis of morphology, electrical field and amplitude. Seizures were identified by a change in the baseline activity with an evolving pattern of higher amplitude rhythmic activity.

Power analysis was used to improve the sensitivity of detecting subtle EEG changes occurring after LEV administration. 300 s blocks of EEG were selected beginning 400 s before the first electrographic manifestation of ictal activity, 100 s after the first ictal electrographic change, 100 s after LEV administration (700 s after onset of Stage III ictal behavior), and 1000 s after LEV administration. Each block was analyzed to determine power in 8 Hz frequency bands from 0 to 40 Hz. Power measurements were normalized to the baseline block. Power was then compared between the samples obtained immediately prior to treatment, immediately after treatment, and 15 min after treatment.

2.5. TUNEL staining of neuronal injury loss

For examination of neuronal injury the in situ nick-translation method was used to detect DNA fragmentation. Following sacrifice by transcardiac perfusion with PBS and 4% paraformaldehyde, brains were harvested and post-fixed overnight. 50 µm coronal sections through the hippocampus were cut using a vibratome and collected into wells, each of which contained every sixth section. Sections were stained using the in situ nick-translation method (TUNEL) modified from Wijman et al. (1993). Briefly, floating sections were incubated in 2–SSC buffer (300 mM NaCl, 30 mM Na acetate) for 20 min at 80 °C to denature the DNA, and then treated with pronase (1 µg/ml) for 10 min. The reaction is stopped with 2% glycine and incubated in nucleotide complex (10 unit/ml DNA polymerase 1, 1 µM each of dCTP, dATP, dGTP, biotin-20UTP (Clonetech)) dissolved in buffer A (50 mM Tris–HCl, pH 7.5, 5 mM MgCl2, 0.005% BSA) for 1 h at room temperature. The sections were washed, treated with 0.1% H2O2 for 30 min, and then developed with avidin–biotin–peroxidase complex Vectastain ABC detection kit (Vector Lab) for 1 h. The colors were developed by microscopic examination of stained brain sections by two independent observers, both blinded to treatment. Inter-rater agreement was high. In cases where rater’s scores differed, an average of the two scores was used. There was never a difference of more than one grade between raters examining specific sections. Different brain regions were examined for the presence or absence of peroxidase reaction product. Only dense nuclear staining was interpreted as positive.
The abundance of positively stained neurons in specific structures was graded on a four-plus scale: "0": no neurons positive, "1": 0–25% positive, "2": 25–50% positive, "3": 50–75% positive, "4": 75–100% positive. Grading of injury in specific structures was performed on sections from a total of 8 saline- and 18 LEV-treated animals and then averaged. Quantification of TUNEL staining was compared using Student’s t test. Significance was defined as p < 0.05.

3. Results

3.1. LEV attenuates seizure activity in established status epilepticus

In initial experiments we examined the effect of intravenous LEV administered 30 min after animals achieved Grade 3 seizure activity. We chose this behavioral stage as it represents the onset of rearing and falling behavior and is straightforward to ascertain. LEV doses from 100 to 1200 mg/kg were examined. Following administration of LEV, we observed a dose-dependent attenuation of seizure activity marked by disappearance of rearing and falling behavior. A behavioral effect of LEV was initially observed at a dose of 800 mg/kg where attenuation of Grade 3 seizure activity lasted an average of 6.8 (±4.1) min. At higher doses, duration of attenuation increased to a plateau of approximately 27 min at 1000–1200 mg/kg (Fig. 1). At these doses, animals appeared sedated within 1 min of injection and then appeared to recover over 2–5 min to walk around their cages with slight head bobbing and exploratory behavior. Gradually rats then developed stronger and stronger body and head jerking until the reappearance of rearing and falling behavior. At doses of 800 and 900 mg/kg, all animals survived. Higher doses were associated with an increasing mortality rate. With doses of 1000 mg/kg or greater, the survival rate dropped to 50–60% (6 out of 10) at 1000 mg/kg, 6 out of 11 at 1100 mg/kg, and 4 out of 8 at 1200 mg/kg.

Clinical studies suggest that earlier treatment of status epilepticus may be more effective. We therefore examined the effect of LEV when administered 10 min after the onset of pilocarpine-induced seizures. Control animals were treated with vehicle alone (n = 11) (Fig. 1). After doses as low as 200 mg/kg LEV injected 10 min after the onset of seizures, ictal behavior was briefly attenuated (21 min compared to 13.6 min in controls), at higher doses, longer periods of attenuation were observed, with an average duration of attenuation of 56.6 (±20) min after LEV 1100 mg/kg. The behavioral changes were similar to those observed when animals were given LEV 30 min after seizure onset, with longer period of slight head bobbing and walking-seeking behavior. Once severe seizure activity recurred, animals again showed strong jerking accompanied by rearing and falling.

We compared seizure latency between animals pre-treated with LEV (1100 mg/kg) immediately before administration of pilocarpine (n = 5) to animals treated with pilocarpine alone (n = 13). In untreated animals, the average time to seizure onset for rats after pilocarpine injection was 26 (±3.96) min. Following LEV pretreatment, one animal had no seizures. The average time to seizure onset in the remaining 4 animals was 97 (±38) min (p = 0.00003, t-test) confirming a result previously demonstrated by others (Klitgaard et al., 1998; Oliveira et al., 2005).

Next we examined the effect of intravenous LEV on electrographic ictal activity. We examined both scalp and depth recordings from the hippocampus. One week after the surgical placement of EEG recording electrodes, rats were pre-treated with LiCl and then seizures were induced with pilocarpine. We examined several doses of LEV, ranging from 50 to 1000 mg/kg, injected 10 min after the onset of seizures in twelve animals. In saline-treated animals both surface and depth electrodes displayed continuous spikes and spike and wave discharge that correlated with Grade 3 behavioral seizure activity. No dose of LEV resulted in a consistent change in the appearance of either the cortical or hippocampal EEG, although in some animals treated with 800 mg/kg LEV, the EEG became slightly discontinuous with slightly less frequent epileptic discharges of lower amplitude. Power analysis was used to improve the sensitivity of detecting subtle EEG changes occurring after LEV administration. There was no significant change in power in any frequency domain examined. While there was a slight decrease in the overall amplitude of the EEG after LEV administration, this decrease was not statistically significant (Fig. 2). In the terminal phase of the experiments, complete cessation of ictal electrographic discharge did not occur after diazepam administration (15 mg/kg) but was observed after pentobarbital administration.

We used histopathological assessment of DNA fragmentation to examine the effect of LEV treatment on neuronal injury in limbic structures after pilocarpine-induced status epilepticus. For these experiments animals were treated with intravenous diazepam 2 h after the onset of seizures to reduce convulsive behavior and promote survival. Animals were sacrificed 24 h later. In control animals (n = 8), after seizures neuronal injury was found in the CA1 region of the hippocampal pyramidal cell layer, amygdales, specific thalamic nuclei, and in cingulate and perirhinal cortex (Fig. 3). In rats treated with LEV (800–1200 mg/kg, N = 18) either 10 min (N = 9) or 30 min (N = 9) after pilocarpine-induced seizures, we found a statistically significant reduction in neuronal injury in CA1, thalamus, amygdala and cortex as compared to controls (Fig. 4). In our sample, the timing of LEV administration (10 or 30 min after behavioral onset) did not affect the magnitude of reduction in neuronal injury.

4. Discussion

The main finding of change the electrographic appearance of ictal activity. Earlier treatment has a more prolonged behavioral effect than delayed treatment. Moreover, pretreatment with intravenous LEV significantly delayed the onset of convulsive activity after administration of systemic pilocarpine. In addition, treatment with high doses of LEV attenuated pilocarpine-status-induced neuronal injury in hippocampus as assayed by analysis of DNA fragmentation.

We chose to use a well-characterized chemically-induced model of status epilepticus to examine the effect of acute intravenous
administration of LEV on ongoing seizure activity, and used clinical observation of ictal behavior as our primary endpoint to assess efficacy. Because pilocarpine induces a highly stereotyped behavioral response that progresses through a series of well defined clinical stages of severity, we were able to detect partial responses to treatment, which we captured as “time in grade”. Based on the hypothesis that intravenous LEV would reduce seizure intensity, we chose to define a reduction in severity grade from Grade 3 to Grade 2 as a response. Moreover, we were able to quantify the duration of reduction to allow statistical analysis of the effect and dose comparison. Using this approach we established a clear dose–response relationship between LEV dose and duration of grade reduction. Our results complement Mazarati’s finding in the self-sustaining status epilepticus model that intravenous injection of LEV 10 min after perforant path stimulation shortened seizure duration at doses of 200 mg/kg or greater, and in combination with diazepam, LEV suppressed seizures immediately (Mazarati et al., 2004).

Importantly, we found that the dose–response curve is shifted to the left when LEV is administered 10 min, as compared to 30 min, after clinical seizure onset. A similar finding in a study of diazepam treatment of pilocarpine-induced seizures (Walton and Treiman, 1988) supports the clinical observation that earlier treatment is more likely to suppress status epilepticus in patients (Lowenstein, 2005).

We also examined the effect of pretreatment with intravenous LEV on the latency to onset of pilocarpine-induced seizures and confirmed that pretreatment significantly delayed the appearance of initial behavioral changes (Klitgaard et al., 1998; Oliveira et al., 2005). We therefore speculate that LEV raises the threshold for occurrence of status epilepticus and may have a role in preventing status in individuals who are predisposed to this serious clinical condition.

Although the behavioral effect of intravenous LEV treatment after the onset of seizures was incomplete, i.e. seizures were not fully terminated, we found clear evidence of a reduction in seizure-induced neuronal injury as assayed by staining for DNA fragmentation. Gibbs found that LEV administration early after seizure onset protected against mitochondrial dysfunction in the self-sustaining status epilepticus model, suggesting one possible mechanism of LEV-mediated neuroprotection in status epilepticus (Gibbs et al., 2006; Gibbs and Cock, 2007).

![Fig. 2. EEG recording from an animal prior (upper tracing) and 11 min after (lower tracing) treatment with LEV 800 mg/kg administered 10 min after the onset of status epilepticus. Recording is bipolar between left and right central skull screws. Vertical bar = 75 µV, horizontal bar = 1 s.](image)

![Fig. 3. Neuronal injury manifested by DNA fragmentation after Pilocarpine-Induced Status Epilepticus is reduced after treatment with intravenous levetiracetam. Rats were sacrificed 24 h after induction of SE. Only dense black nuclear staining was interpreted TUNEL staining. The number of positively TUNEL stained neurons in specific limbic structures were counted and compared to the total number of neurons (counter-stained with cresyl violet). Treatment with LEV (A, C) reduced number of neurons displaying DNA fragmentation compared to animals treated with PBS alone (B, D) in CA1 (A, B), entorhinal cortex (C, D). Note dense black nuclei in positively stained cells. Bar = 500 microns.](image)
The mechanism of behavioral attenuation in the absence of change in EEG remains unclear. Whereas many anti-epileptic drugs target Na⁺ channels, T-type Ca²⁺ channels, the GABAergic systems, or glutamate receptors, LEV has an atypical anti-convulsant profile in animal models where it is active against audiogenic seizures and seizures induced by kindling stimulation, but not against maximal electroconvulsive shock or pentylenetetrazole-induced seizures. The mechanism of action of LEV remains incompletely defined. LEV binds to the synaptic vesicle protein SV2A with high affinity (Lynch et al., 2004). SV2A is a glycoprotein that exists in all synaptic vesicles and membranes and plays an important role in synaptic vesicle cycling and neurotransmitters release into the synaptic cleft. An SV2A mouse knockout model manifests abnormal neurotransmission that results in early development failure, severe seizures and death (Crowder et al., 1999). It is possible that transient LEV-mediated modulation of SV2A function could underlie the transient behavioral response we observed even in the absence of a detectable effect on electrographic discharge.

Relatively high doses of LEV were required to attenuate ictal behavioral activity in our study in comparison to the doses used to block spontaneous seizures or kindling seizures, and relative to doses used clinically. Mazzarati et al., (2004) and Gibbs et al., (2006) also found a relatively high dose requirement in their studies of LEV activity against self-sustaining status epilepticus. We did not measure LEV serum levels in our study. It is possible that rodents metabolize LEV rapidly, especially in the setting of status, leading to reduced bioavailability. In rats the elimination half-life of LEV in serum is between 1.8 and 2.8 h (Doheny et al., 1999) whereas in humans the serum half-life is 6–8 h (Patalos, 2000), demonstrating a substantial species difference in pharmacokinetics. Additional pharmacokinetic studies will be required to address this issue. Interestingly, while LEV is rapidly absorbed and transported across the blood–brain barrier, there is a significant delay between $T_{\text{max}}$ (serum) and $T_{\text{max}}$ (CSF) (0.25–0.50 h vs. 1.33–1.92 h) (Doheny et al., 1999). Because the efficacy of treatment of SE is highly dependent on the duration of SE prior to treatment, it is possible that a high LEV dose is required to achieve adequate CSF concentrations in a timely manner. It is also possible that the systemic chemovconvulsant model we used dictates the need for unusually high doses of LEV. In particular, the chemovconvulsant remains in circulation throughout the treatment period, presenting an ongoing challenge to homeostatic mechanisms. Moreover, because LEV does not appear to act as a specific neurotransmitter antagonist, its unique mechanism of action may impose a higher dose requirement to achieve efficacy. This notion is supported indirectly by the relatively high dose requirement in clinical use, usually expressed in grams per day rather than milligrams per day as with many anti-convulsant drugs.

We have shown that intravenous LEV, administered at high doses, reduces the intensity of the behavioral response to pilocarpine in the lithium-pilocarpine model of status epilepticus. We have also demonstrated that intravenous LEV, again at high dose, reduces the severity of neuronal injury in hippocampus after Li-Pilo SE. It is therefore surprising that we were unable to demonstrate significant change in the ictal EEG pattern recorded from animals with Li-Pilo SE treated with LEV. This paradox focuses attention on the mechanism of the behavioral and protective effects of LEV. While a specific biochemical effect of LEV might mediate the behavioral and neuroprotective responses we observed, an alternative hypothesis is that LEV acts downstream of the cerebral cortex to modify the expression of epileptiform activity. If true, this hypothesis would raise the concern that treatment of status with intravenous LEV may convert convulsive SE into non-convulsive SE by acting to dissociate cortical structures from sub-cortical output pathways. Additional studies will be required to address these issues.

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Conflicts of Interest: Dr. Cole has received consulting fees and honoraria from UCB Pharma, Abbott Laboratories, GlaxoSmithKline, and Pfizer. No other authors have conflicts to disclose.

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