Published in final edited form as:
Neuroreport. 2011 January 5; 22(1): 15–18. doi:10.1097/WNR.0b013e3283413231.

Acetaminophen inhibits status epilepticus in cultured hippocampal neurons

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Abstract

Status epilepticus (SE) is a major neurological disorder and patients surviving SE often develop acquired epilepsy and cognitive deficits. Thus, it is important to stop SE and limit brain damage. However, rapid pharmacoresistance develops to anticonvulsants as seizure duration lengthens. Cannabinoids stop seizures in experimental models. Here we investigated if acetaminophen via its conversion to AM404 and subsequent increases in endocannabinoid levels would block SE-like activity. Exposure of cultured hippocampal neurons to a low Mg^{2+} medium elicits high-frequency epileptiform discharges that exceed 3 Hz (in-vitro SE). Acetaminophen (500 μM) blocked SE-like activity. Co-application of CB1 receptor antagonist SR 141716A (1 μM) blocked this inhibitory effect of acetaminophen on SE, indicating that acetaminophen was mediating its anticonvulsant effects through CB1 receptors.

Keywords

status epilepticus; endocannabinoids; acetaminophen; AM 404; hippocampal cultures

Introduction

Status epilepticus (SE) is a major neurological disorder associated with significant morbidity and mortality. SE causes significant damage to limbic system and patients surviving SE have a very high probability for developing acquired epilepsy and cognitive deficits [1]. It is therefore important to stop SE and limit brain damage as early as possible. Unfortunately, SE has been found to be refractory to many treatments and there is rapid development of tolerance to standard anticonvulsant drugs as seizure duration lengthens [2]. Thus, there is a need to develop newer drugs and treatment strategies for prompt treatment of SE.

Exposure of cultured hippocampal neurons to a low Mg^{2+} medium elicits high frequency epileptiform discharges in neurons that exceed 3 Hz in spike frequency. This model has been widely used to study SE mechanisms in an in vitro setting [3–6]. It is considered as a model of refractory SE, since standard anticonvulsant drugs such as phenytoin and phenobarbital do not stop the high frequency epileptiform discharges [7]. In addition, similar to humans [8], benzodiazepines such as lorazepam stop SE-like activity only initially and then display pharmacoresistance resulting in its loss of efficacy as SE continues [7]. Molecular changes such as loss of $GABA_A$ receptors following SE in animals are also observed in this preparation [4]. This in vitro model allows for precise control of extracellular milieu while

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mimicking electrographic properties, neuronal population synchronicity, and anticonvulsant sensitivity identical to SE observed in vivo and is therefore ideally suited to evaluate the effects of investigational compounds on SE-like activity.

The endocannabinoid system has come to forefront as novel target for treatment of seizures. This system comprises of at least two cannabinoid receptors (CB1 and CB2), the endogenous ligands (anandamide and 2-arachidonyl glycerol) and the protein machinery for the synthesis, degradation and reuptake of endocannabinoids. CB1 receptors are abundant in hippocampus and are located on both excitatory and inhibitory nerve terminals. Activation of inhibitory G-protein coupled presynaptic CB1 receptors results in decreased neurotransmitter release (Reviewed in: [9]). This action is thought to underlie the potent anticonvulsant effects of CB1 agonists in models of acquired epilepsy [10–12]. Plasticity changes in the endocannabinoid system have also been found following febrile seizure [13]. Cannabinoids have also been found to stop SE-like activity in hippocampal neuronal cultures [7]. It has also been reported that a CB1 receptor-dependent endocannabinoid tone plays an important role in modulating seizure frequency and duration and preventing the development of SE-like activity in epileptic neurons [14,15]. Thus, the endocannabinoid system plays a critical role in modulating neuronal excitability.

The development of medicinal cannabinoids has been greatly tempered by psychoactivity and abuse potential associated with CB1 agonists. Altering endocannabinoid levels by targeting enzymes that synthesize and degrade the endogenous ligands is thought to be an attractive strategy for therapeutic modulation of endocannabinoid system [9]. In this regard, the endocannabinoid reuptake inhibitors and fatty acid amide hydrolase (FAAH) inhibitors have been found to exert effects similar to CB1 receptor agonists but are not associated with abuse liability [16]. Acetaminophen or paracetamol has recently been suggested as a prodrug for activating the endocannabinoid system [17]. This commonly used analgesic/ antipyretic drug was reported to undergo deacetylation and then conjugation with arachidonic acid to yield N-arachidonoyl-phenolamine or AM 404- a potent endocannabinoid reuptake inhibitor [18]. AM 404 has been reported to increase levels on anandamide [19]. This conversion of acetaminophen to AM 404 is thought to underlie its pain alleviating effect since CB1 receptor antagonist blocked its analgesic effect in various pain models [20–22]. In addition, acetaminophen has also been reported to exhibit anxiolytic properties that are thought to be mediated by endocannabinoids and CB1 receptors [23]. We have previously demonstrated that endocannabinoids block SE-like activity in vitro [3]. Further, AM 404 has been shown to prevent naloxone induced seizures [24]. In light of these studies, we investigated if acetaminophen via its conversion to AM 404 and subsequent increases in endocannabinoid levels would block low Mg^{2+} induced SE-like activity in cultured hippocampal neurons.

Methods

Acetaminophen was purchased from Sigma-Aldrich Co. (St. Louis, MO) and was dissolved in ethanol. CB1 receptor antagonist SR141716A was supplied through the NIDA Chemical Synthesis and Drug Supply Program and was made up in a vehicle stock solution of absolute ethanol, Emulphor-620 (Rhone-Poulenc Inc., Princeton, NJ) and 0.9% saline at a ratio of 1:1:18 that were then diluted at a minimum of 1:500 to a final working concentration in the physiological bath recording solution (pBRS) consisting of (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 0.002 glycine, pH 7.3, and osmolarity 325 ± 5 mOsm adjusted with sucrose. All the drugs were bath-applied using a multi valve perfusion assembly (Warner Instrument Corp., CT).

All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's

Institutional Animal Care and Use Committee. Studies were conducted on primary mixed hippocampal neuronal cultures prepared as described previously with slight modifications[10]. In brief, hippocampal cells were obtained from 2-day postnatal Sprague-Dawley rats (Harlan, Frederick, MD) and plated at a density of 2.5×10^4 cells/cm^2 onto a glial support layer previously plated onto poly-l-lysine coated (0.05 mg/ml) 35-mm Falcon cell culture dishes (B–D& Co., Franklin Lakes, NJ). Cultures were maintained at 37 °C in a 5% CO_2/95% air atmosphere and fed twice weekly with neuronal feed + condition medium. After 2 weeks, cultures were utilized for experimentation. Continuous epileptiform bursts (SE) were induced by exposing neuronal cultures to pBRS without added $MgCl_2$ (low Mg^{2+}). The SE continued until pBRS containing 1 mM $MgCl_2$ was added back to the cultures [3].

Whole-cell current clamp recordings were performed using previously established procedures [3]. Briefly, cell culture dish was mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo) continuously perfused with pBRS. Patch electrodes with a resistance of 2 to 4 MΩ were pulled on a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA), fire-polished and filled with a solution containing the following (in mM): 140 K^+ gluconate, 1 $MgCl_2$ and 10 Na-HEPES, pH 7.2, osmolarity 290 ± 10 mOsm adjusted with sucrose. Recordings were carried out using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA) in current-clamp mode. Data were digitized and transferred to videotape using a PCM device (Neurocorder, New York, NY) and then played back on a DC-500 Hz chart recorder (Dash II, Astro-Med, Warwick, RI).

Data is expressed as mean ± SEM and were analyzed by SigmaPlot 10 (SPSS Inc., Chicago, IL) using a one-way ANOVA with post-hoc Tukey's test. Experiments were performed on different cells and not in a repeat measures mode.

Results

Recordings from control neurons displayed occasional action potentials as shown in panel A (n= 10 neurons). As shown in panel B, removal of Mg^{2+} from the pBRS resulted in the development of continuous high-frequency epileptiform discharges in cultured hippocampal neurons (n= 20 neurons). This epileptiform activity consisted of repetitive burst discharges with each burst comprised of multiple spikes overlaying a paroxysmal depolarization shift. Spike frequency upon exposure to the low Mg^{2+} medium exceeded 3 Hz that meets the criteria of electrographic SE. The spiking was continuous and did not slow down throughout the 60-min of recording. On an average neurons were recorded for 15-min in low Mg^{2+} solution before the application of acetaminophen. Acetaminophen (500 μM) was bath applied after the induction of low Mg^{2+} induced SE. As shown in panel C, acetaminophen blocked low Mg^{2+} induced SE-like activity (n= 30 neurons). At 15-min following acetaminophen perfusion the spike frequency was not significantly different from low Mg^{2+} condition. However, by 30-min the SE-like activity was significantly blocked. Similarly, there was a time-lag in the inhibitory effect of acetaminophen to dissipate following washout of the drug. Twenty minutes following washout of acetaminophen, the spike frequency was still significantly depressed and the neurons reverted to low Mg^{2+} alone spike frequencies by 45-mins. Recent studies have shown that acetaminophen exerts analgesic and anxiolytic effect via CB1 receptors. We co-applied acetaminophen (500 μM) with the CB1 antagonist SR 141716A (1 μM). As shown in panel D, SR 141716A blocked the effect of acetaminophen on SE suggesting that acetaminophen was mediating its anti-SE effect via CB1 receptors (n= 20 neurons). The effect of acetaminophen on spike frequencies has been quantified in Table 1. SR 141716A alone did not have any effect on control or low Mg^{2+} treated neurons (data not shown and see: [14]).

Discussion

This study for the first time reports inhibitory effect of acetaminophen on low Mg^{2+} induced SE-like activity in cultured hippocampal neurons. The onset of acetaminophen action was delayed and was maximally effective by approximately 30-min at which time it inhibited the high frequency spiking activity characteristic of SE. The results suggest that there is a time-lag for conversion of acetaminophen to AM 404 and then for subsequent buildup of endocannabinoids to concentrations high enough to stop SE-like activity. This is in line with the observation by Hogestatt et al., who reported that AM 404 was detectable in rat brains 20-min after a 300 mg/kg i.p. injection of acetaminophen [18]. Following acetaminophen washout, the SE-like activity remained inhibited for almost 20-min despite the presence of low Mg^{2+} medium and then returned to pre-acetaminophen levels by 45-min. The results indicate that after the removal of acetaminophen endocannabinoid levels remain elevated at concentrations sufficient to inhibit high frequency spiking before the transporters reduce their levels. A 500 μM dose of acetaminophen was chosen, since Hogestatt et al also showed that significant accumulations of AM 404 in brain homogenates occurred only beyond 100 μM concentration [18]. While we did observe a reduction in SE-like activity at 300 μM dose of acetaminophen, to maximize the formation of AM 404 we chose the 500 μM concentration. A dose-response relationship would be performed in future studies to find the ED_{50} concentration of acetaminophen to stop SE-like activity. While co-application of SR 141716A produced a significant inhibition of acetaminophen's effect, it could not revert the neuron to low Mg^{2+} alone spike patterns suggesting involvement of mechanisms beyond CB1 receptor activation. Indeed, acetaminophen and in turn AM 404 are potent COX-2 and prostaglandin synthesis inhibitors [18]. These inflammatory molecules are up regulated during seizure events and their inhibition along with CB1 receptor activation could underlie the inhibitory effect of acetaminophen on SE-like activity. It will also be interesting to investigate interaction between diazepam and acetaminophen in our in vitro model of SE. The slow onset suggests that SE would remain inhibited by the time benzodiazepines start to lose their efficacy and therefore acetaminophen could be a novel adjuvant to benzodiazepines for treatment of SE. Indeed, acetaminophen has been reported to enhance the protective activity of valproate against maximal electroshock-induced seizures [25].

Conclusion

Acetaminophen blocked SE-like activity in cultured hippocampal neurons partly via CB1 receptor dependent mechanism. This clinically important observation needs to be investigated further; particularly research into mechanisms of action of acetaminophen and possible drug interactions in SE settings are warranted.

Acknowledgments

The CounterACT Program, National Institutes of Health Office of the Director and the National Institute of Neurological Disorders and Stroke [Grant# UO1NS058213] supported this work. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the federal government. This study is also supported by NINDS [Grant# RO1NS051505 and RO1NS052529] to RJD.

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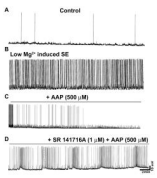

Figure.

Acetaminophen blocks low-Mg^{2+} induced SE-like activity in cultured hippocampal neurons. **A.** Current-clamp recording from a representative control neuron displaying baseline activity consisting of intermittent action potentials. **B.** Induction of continuous epileptiform activity (SE) in a neuron upon low Mg^{2+} treatment. **C.** Acetaminophen (AAP, 500μM) blocks low Mg^{2+} induced SE. **D.** Co-application of CB1 receptor antagonist SR 141716-A (1 μM) blocked the effects of acetaminophen (500 μM) against low Mg^{2+}-induced SE.

Table 1

Effect of acetaminophen on low Mg^{2+} induced spike frequency

Experimental group	Spike frequency (Hz) (mean ± SEM)
Control	0.089 ± 0.02
Low Mg^{2+} alone	3.545 ± 0.17
Low Mg^{2+} + acetaminophen 15-min	3.14 ± 0.27
Low Mg^{2+} + acetaminophen 30-min	0.263 ± 0.06*
Acetaminophen washout 20-min	1.12 ± 0.11*
Acetaminophen washout 45-min	3.18 ± 0.12
SR 141716A + acetaminophen	1.53 ± 0.15*

Data represented as mean ± S.E.M. of spike frequency during low-Mg^{2+} SE for various conditions (n= 10–30). Asterisk denotes the statistical differences between the spike frequencies of these conditions.