Levetiracetam differentially alters CD95 expression of neuronal cells and the mitochondrial membrane potential of immune and neuronal cells in vitro

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INTRODUCTION

Epilepsy is a neurological seizure disorder that affects over 100 million people worldwide. Levetiracetam, either alone, as monotherapy, or as adjunctive treatment, is widely used to control certain types of seizures. Despite its increasing popularity as a relatively safe and effective anticonvulsive treatment option, its mechanism(s) of action are poorly understood. Studies have suggested neuronal, glial, and immune mechanisms of action. Understanding the precise mechanisms of action of levetiracetam would be extremely beneficial in helping to understand the processes involved in seizure generation and epilepsy. Moreover, a full understanding of these mechanisms would help to create more efficacious treatments while minimizing side-effects. The current study examined the effects of levetiracetam on the mitochondrial membrane potential of neuronal and non-neuronal cells, in vitro, in order to determine if levetiracetam influences metabolic processes in these cell types. In addition, this study sought to address possible immune-mediated mechanisms by determining if levetiracetam alters the expression of immune receptor–ligand pairs. The results show that levetiracetam induces expression of CD95 and CD178 on NGF-treated C172 neuronal cells. The results also show that levetiracetam increases mitochondrial membrane potential on C172 neuronal cells in the presence of nerve growth factor. In contrast, levetiracetam decreases the mitochondrial membrane potential of splenocytes and this effect was dependent on intact invariant chain, thus implicating immune cell interactions. These results suggest that both neuronal and non-neuronal anti-epileptic activities of levetiracetam involve control over energy metabolism, more specifically, mΔΨ. Future studies are needed to further investigate this potential mechanism of action.

Keywords: epilepsy, Keppra, splenocytes, C172, in vitro, Fas, Fasl
Kim et al. (10) showed that levetiracetam reduced gliosis in epileptic brains and inhibited IL-1β, and Stienen et al. (11) showed that the anti-inflammatory effects of levetiracetam on astrocytes may be mediated by TGFβ1.

In addition to effects in the CNS, levetiracetam could also be exerting its effects in the periphery. Supporting this notion are the results of a previous study demonstrating that levetiracetam inhibits the function of some CD8+ T lymphocytes (12). Such interactions with the peripheral immune system might explain the increased incidence of pharyngitis and rhinitis in levetiracetam-treated patients (13–18). However, studies are lacking that provide a thorough analysis of the effects of levetiracetam on peripheral immune cells.

Understanding the mechanism(s) of action of levetiracetam is important because this knowledge could lead to more efficacious treatments and better understanding of the epileptic condition. Due to the lack of a unified theory for the mechanism(s) of action, the current study was designed to determine if levetiracetam affects the m∆Ψ of peripheral immune cells and neuronal cells. Moreover, this study sought to address possible immunomodulated mechanisms by determining if levetiracetam alters the expression of immune receptor–ligand pairs.

MATERIALS AND METHODS

CELL LINES

C17.2

The C17.2 cell line is an immortalized mouse neural progenitor cell line capable of differentiation in vitro. The cell line was established by retroviral-mediated transduction of the avian myc oncogene into mitotic progenitor cells of neonatal mouse cerebellum from a CD1 × C57BL/6 mouse. The C17.2 line of neural stem cells responds to NGF by differentiating into more mature neuronal phenotypes and has been used extensively to monitor developmental regulation of mouse neurons (19). We employed this cell line as a model of mouse neuronal cells.

In vitro stimulations

C17.2 cells were either untreated, or treated with nerve growth factor (NGF) at 0.4 nM final concentration. All cells were treated with levetiracetam or vehicle for 48 h, at the following concentrations: 0.5 μM, 15 μM, 0.15 mM, or 1.5 mM.

Mice

Eight- to ten-week-old C57BL/6J male mice were purchased from Jackson Labs. Invariant chain (CD74)-deficient mice (Ii−/−) (C57BL/6 background) were purchased from Jackson Labs and bred at the Scott and White Healthcare animal facility to maintain homozygosity. Mice were housed in the Scott and White Healthcare animal facility according to IACUC regulations.

Spleen cell isolation

Mice were sacrificed and spleens were removed. Splenocytes were dissociated by passing splenens through 40-μm cell strainers. Red blood cells were lysed using GEY’S buffer (20). Cells were then cultured at 1.010⁶ cells/mL in 6 well plates. Cells were grown in RPMI 1640 (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen) in a humidified 5% CO₂ incubator at 37°C for the designated time period. Splenocytes were then treated with levetiracetam or vehicle for 48 h, at the following concentrations: 0.5 μM, 15 μM, 0.15 mM, or 1.5 mM.

Flow cytometry

For cell surface markers the cells were first blocked with FC Block (BD Bioscience) and then stained with the following antibodies: MHCII, CD3ε, CD80, CD86, Fas (CD95), and CD178 (BD Bioscience). Cells were analyzed using a BD FACs Canto II flow cytometer and the data was analyzed using FlowJo software (TreeStar Inc.).

Mitochondrial membrane potential (m∆Ψ)

To assess the possibility that levetiracetam has direct effects on mitochondrial function, mitochondrial activity was assessed using MitoTracker Red CM-H2XRos (Life Technologies), a mitochondrial dye that fluoresces as a function of m∆Ψ. Tightly regulated m∆Ψ is essential for maintaining physiological function(s), including appropriate mitochondrial substrate selection for generating ATP and for maintaining cell viability. Cells were treated with MitoTracker Red and allowed to incubate in the dye for 20 min prior to analysis using a BD FACs Canto II flow cytometer. The flow cytometer measures mean fluorescence intensity per cell. Cells were untreated, treated with NGF, treated with levetiracetam or NGF + levetiracetam, as described above in the in vitro stimulation. For each treatment group, a minimum of four separate assays were performed in triplicate.

Lysosomal acidity

To assess the effects of levetiracetam on lysosomal pH, we used the fluorescent dye Lysosensor Green (Life Technologies). Lysosensor Green produces increased fluorescence intensity at lower pH. Cells were untreated, treated with NGF, treated with levetiracetam or NGF + levetiracetam, as described above in the in vitro stimulation. For each treatment group, a minimum of four separate assays were performed in triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc.). For comparisons between splenocytes from C57BL/6J and Ii−/−, a paired t-test was used with a significance cut-off of P < 0.05. For all other analysis, repeated measures ANOVA was used with post hoc planned comparisons using Dunnett’s correction factor.

RESULTS

Previous studies have indicated mitochondrial differences in the presence of levetiracetam (6, 7). Therefore, we determined if these differences were specific for neuronal or immune cells. Analysis of m∆Ψ in C17.2 cells revealed no significant differences in the absence of NGF (Figure 1A). In the presence of NGF, levetiracetam resulted in a significant increase (Figure 1A) in m∆Ψ at all concentrations tested (1.5 μM, p < 0.03; 15 μM, p < 0.05; 0.15 mM, p < 0.04; 1.5 mM, NS). It is pertinent to note that treatment with levetiracetam did not cause any observable alterations to the morphology of the C17.2 cells, either with or without NGF (data not shown). In contrast to the increased m∆Ψ in the presence of NGF and levetiracetam, the impact of levetiracetam on spleen cells (Figure 1B) was a significant reduction in m∆Ψ (p < 0.007). This reduction appeared to be invariant.
FIGURE 1 | Changes in mitochondrial membrane potential following treatment with levetiracetam. (A) Mean fluorescence intensity (MFI) of Mitotracker Red as a measure of relative mitochondrial membrane potential in C17.2 cells at 48 h post treatment with NGF with or without levetiracetam (Lev). (B) MFI Mitotracker Red in C57BL/6 splenocytes 48 h after treatment with or without 0.15 mM Lev. (C) MFI Mitotracker Red in II^D^ splenocytes 48 h after treatment with or without 0.15 mM Lev. (D) Table depicting percent change from NGF treatment alone, compared to NGF treatment in the presence of doses of levetiracetam. *Denotes a p-value < 0.05.

chain dependent, as splenocytes from mice deficient in invariant chain showed no significant changes in m\(\Delta\Psi\) in response to levetiracetam (Figures 1C,D).

Elevated m\(\Delta\Psi\) can be associated with elevated CD95 (21, 22). Therefore, to address the possibility that levetiracetam alters receptor–ligand pairs on neurons, we used the mouse neuronal stem cell line, C17.2, which can be differentiated in the presence of NGF. We assessed CD95, a member of the BGF superfamily and its ligand, FasL (CD178) to determine if levetiracetam can influence cell proliferation, differentiation, and survival. We also examined alterations in the co-stimulatory molecules B7.1 (CD80), or B7.2 (CD86) to assess the potential of levetiracetam to alter co-stimulation of T cell activation. The results from analysis of C17.2 cells revealed that levetiracetam treatment alone had no significant effects on CD95 (Figure 2A), CD178 (Figure 2B), CD80 (Figure 2C), or CD86 (Figure 2D). In the presence of NGF, no significant differences were observed for CD95, CD178, CD80, or CD86 at the 1.5 or 15\(\mu\)M concentrations. However, at 0.15 and 1.5\(\mu\)M, a significant increase in CD95 (p < 0.02 and p < 0.001, respectively) was observed. At these latter two concentrations, no significant differences were observed for CD80 or CD86. For CD178, no significant differences were observed for the three lowest concentrations of levetiracetam, but at the 1.5\(\mu\)M concentration, a significant increase was observed for CD178 (p < 0.05).

In addition to examining neuronal cells, we also examined peripheral immune cells from the spleen. We examined numbers of T cells and numbers of MHCII^+^ cells (which includes macrophages and B cells), as well as CD95 expression on these cells. The results showed that levetiracetam treatment resulted in no significant effect on the number of CD3^+^ T cells (Figure 3A), MHCII^+^ (Figure 3B) cells, nor on the levels of CD95 expression by T cells (Figure 3C), and non-T cells (Figure 3D). In addition, we examined overall levels of MHCII and CLIP on non-T cells (Figures 4A,B) to address the possibility that levetiracetam alters immunogenicity of peripheral immune cells. No changes were observed for either of these variables (Figures 4A,B). To further detect levetiracetam-induced changes in processing or presentation by immune cells, we assessed lysosomal acidity and found no significant changes (Figure 4C).

**DISCUSSION**

Levetiracetam is well established as a beneficial anti-seizure medication and as an adjunct to other anti-seizure medications. The molecular mechanisms accounting for the efficacy of levetiracetam for seizure activity are largely unknown. The results from the present study suggest that levetiracetam induces expression of CD95 and CD178 on NGF-treated C17.2 neuronal cells. The results also demonstrate that the increased m\(\Delta\Psi\) in response to levetiracetam on C17.2 neuronal stem cells requires the presence of NGF. This is likely due to the differentiating effect of NGF on neural stem cells. In contrast, the study shows that levetiracetam lowers the m\(\Delta\Psi\) of splenocytes and this effect is dependent on intact invariant chain. These results suggest that both neuronal and non-neuronal anti-epileptic activities of levetiracetam involve control over energy metabolism, more specifically, m\(\Delta\Psi\).

Epilepsy has traditionally been considered primarily a neuronal disease. Growing evidence also implicates astrocytes, microglia, peripheral leukocytes, and blood–brain barrier breakdown in the pathogenesis of epilepsy. Here, we show two novel observations.
that potentially link peripheral leukocytes to neurons. Our results suggest that levetiracetam affects mitochondrial energy metabolism as reflected by changes in m ΔΨ. Interestingly, these changes are inversely related when comparing splenocytes to NGF-treated neuronal stem cells. That is, levetiracetam causes a statistically significant increase in the membrane potential of NGF-treated C17.2 cells and a significant decrease in m ΔΨ of levetiracetam-treated spleen cells. It is pertinent to note that in mice deficient for CD74, the levetiracetam-induced change to splenocyte m ΔΨ was ameliorated. Thus, it is possible that levetiracetam-induced changes in mitochondrial activity result from cell–cell contact because CD74 can be expressed on the cell surface and mediate interactions with other cells through its cognate ligand, CD44. Alternatively, the requirement for CD74 to see the effects.

Previous studies have suggested that mitochondrial dysfunction contributes to the epileptic condition (23). The putative functional significance of levetiracetam-induced alterations to m ΔΨ in the epileptic brain is its known effects on proton transport. Previous studies have demonstrated that alterations to the m ΔΨ in neurons (24) and non-neuronal cells (25), directly influences ion concentrations in the cytosol, thereby influencing plasma membrane potential. Alterations to m ΔΨ have also been shown to influence oxidative stress (26), which may be another anti-epileptic mechanism. A third potential mechanism through which altered m ΔΨ could influence seizures is by altering the cytosolic pH. An acidification of cytosol as a result of protonation is known to hyperpolarize the plasma membrane (27), which may raise the seizure threshold. Support for this latter suggestion is observed in epileptic hippocampal slices where the pattern of epileptic activity corresponds to m ΔΨ and ion concentration (23).

Previous work from our lab demonstrated that Fas/FasL interactions can facilitate neurite outgrowth subsequent to nerve crush injury (28). CD95 and its ligand CD178, a member of the NGF/NGF receptor superfamily of death-inducing receptor–ligand pairs. Many members of this superfamily are involved in cell fate decisions including cell death, cell proliferation, and differentiation. We addressed the possibility that if levetiracetam altered mitochondrial activity, it might also affect CD95 expression because elevated m ΔΨ can be associated with elevated CD95. The results from the current study are consistent with this idea because we found that in neuronal cells, in the presence of NGF and >0.15 mM levetiracetam, m ΔΨ is increased as is Fas expression. Therefore, levetiracetam may be involved in stabilizing m ΔΨ in the presence of elevated levels of NGF.

Another potential effect of levetiracetam on leukocytes could be related to some of the side-effects associated with levetiracetam. In particular, an increased incidence of pharyngitis and rhinitis has been observed in levetiracetam-treated patients (13–18).
findings are consistent with a role for alterations to an effective immune response that may involve alterations in CD74 expression and function. A second possibility is related to the finding of reduced m∆Ψ in splenocytes, which may reflect altered levels of immune function, including increased inflammation accounting for pharyngitis and rhinitis.

Overall, the data from the current study indicate that levetiracetam differentially affects the m∆Ψ of neuronal C17.2 and non-neuronal splenocytes. The results also show that in the presence of elevated NGF, neuronal C17.2 cells express CD95 and CD178. The results from this study could help to explain some of the mechanisms of action of levetiracetam, including some of its side-effects. More studies are needed to better understand the implications of these findings so that more efficacious treatments with minimal side-effects can be developed.

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