4-PBA and metformin decrease sensitivity to PTZ-induced seizures in a malin knockout model of Lafora disease

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Abstract

Lafora disease (LD) is a rare adolescent-onset progressive myoclonic epilepsy caused by loss-of-function mutations either in the *EPM2A* gene encoding laforin or in the *EPM2B* gene encoding malin. Mouse models with deletion in the *Epm2a* or *Epm2b* gene display intracellular aggregates of polyglucosans (Lafora bodies) and neurological complications that resemble those seen in patients with LD. In the absence of laforin or malin expression, mice also exhibit different degrees of hyperexcitability, as reflected by an enhanced response to the convulsant drug pentylenetetrazol (PTZ). Malin knockout mice treated with 4-phenylbutyric acid (4-PBA) and metformin showed decreased amounts of Lafora bodies and polyubiquitin protein aggregates in the brain, diminished neurodegeneration, and amelioration of some neurological conditions. In this study, we analyzed the action of 4-PBA and metformin treatments on response to PTZ in a malin knockout model of LD. Both treatments decreased seizure susceptibility, bringing about a reduction in both seizure number and length, and eliminated the mortality induced by PTZ. These results show a neuroprotective role for 4-PBA and metformin and extend the beneficial effects reported in the malin knockout model of LD.

Key Words: Lafora disease; epilepsy; oxidative stress; autophagy; PTZ; malin knockout mouse

Introduction

Lafora progressive myoclonus epilepsy (OMIM 254780; ORPHA501) is a rare autosomal recessive disease that presents in adolescence with generalized seizures, myoclonic, absence, and visual seizures or cognitive decline. Rapid neurologic deterioration with progressive ataxia, dementia, dysarthria, amaurosis, and respiratory failure leads to death within 5 to 10 years of disease onset[1]. Patients with LD present Lafora bodies, which are intracellular inclusions of polyglucosan, a long, linear and poorly branched glycogen. Lafora bodies accumulate in brain, skin, heart, and other tissues. At present, there is no effective treatment for this disease. LD is caused by recessive mutations either in the *EPM2A* gene, which encodes the dual-specificity phosphatase laforin (OMIM 607566)[2-4], or in the *EPM2B* gene, encoding the E3 ubiquitin ligase malin (OMIM 608072)[5,6].

A number of mouse models of LD with targeted mutations in either the *Epm2a*[7] or the *Epm2b* gene[8-10] have been generated. In the absence of laforin or malin expression, *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mice develop Lafora bodies and neurological complications that resemble those seen in patients with LD. Thus, both *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mice, manifest with dyskinesia, impaired motor coordination and activity, deficits in episodic memory, and distinct extents of spontaneous epileptic activity[11]. Additionally, they also exhibit different degrees of hyperexcitability, as reflected by an enhanced response to the convulsant agent pentylenetetrazol (PTZ)[12], an antagonist of the $GABA_A$ receptor. Laforin and malin knockout mice phenotypically express with impaired macroautophagy and altered ubiquitin-proteasome system, resulting in defects in protein clearance mechanisms[10,13]. Both LD mouse models also display increased oxidative

stress and impaired antioxidant response in the brain[14]. In addition to polyglucosan aggregates, Lafora bodies also present ubiquitinated proteins, advanced glycation-end products, chaperones, autophagy components, and proteasome subunits.

A previous report from Sanz's and our group analyzed the effects of treatments with 4-PBA and with metformin in malin mutant mice[15]. 4-PBA is a chemical chaperone that sequesters misfolded and aggregated proteins associated with several human neurodegenerative diseases while metformin promotes autophagy through the activation of the AMP-activated protein kinase (AMPK) and acts as a neuroprotective agent in different neurodegenerative diseases[16]. Both treatments decreased the number of Lafora bodies and polyubiquitin protein aggregates in the brain, diminished neurodegeneration, and ameliorated neurological tests in mice lacking the malin protein[15].

In order to explore the effects of 4-PBA and metformin on the epileptic activity of these mice, we analyzed the susceptibility to PTZ-induced seizures in malin-deficient mice following treatment with these two drugs.

Materials and Methods

Animals and treatments

Malin-deficient mice were used for our study. Generation of malin mutant mice was performed by targeted deletion of the single exon encoding malin, as described in Criado et al[10]. 4-PBA at 20 mM and metformin at 12 mM (Sigma Chemicals, MO, USA) were dissolved in drinking water and administered *ad libitum* in malin knockout male mice at 3 months of age. 4-PBA and metformin treatments were administered for 2 months and animals were then tested for their sensitivity to PTZ. Four groups of 16 homozygous adult male mice were analyzed per condition: wild type mice; malin

knockout mice; malin knockout mice with 4-PBA treatment and; malin knockout mice with metformin treatment.

The mouse colonies were bred at the IIS-Fundación Jiménez Díaz Animal Facility and were maintained on a 12:12-hour light/dark cycle under constant temperature (23ºC), with access to food and water *ad libitum.* The experiments were conducted in accordance with the Declaration of Helsinki principles and the guidelines of the Institutional Animal Care and Use Committee, and were approved by the IIS-Fundación Jiménez Díaz ethical review board.

PTZ treatment

PTZ (Sigma Chemicals, MO, USA) was administered intraperitoneally as a single injection at 50 mg/kg. After administration of a convulsive dose of PTZ, mice displayed intervals of hyperactivity, twitching, and hyperextension of the limbs that at times progressed to generalized tonic-clonic seizures, and occasionally to death, usually within the first 20 min after injection. The percentage of mice showing PTZ-induced generalized seizures, and the lethality were monitored over a period of 45 min. The time interval between drug administration and development of generalized tonic-clonic seizures and the length of the seizures were also analyzed. The appearance of additional PTZ-induced seizures for periods up to 2 h after PTZ administration was also evaluated in 8 animals per condition, although no concomitant episodes were observed.

Statistical analysis

Generalized seizures and lethality values are given as percentages of animals that respond to PTZ treatment. The chi-square test was used to perform the pairwise comparison. Latency time and seizure length values are given as means ± standard error of means (SEM), and differences between groups were analyzed by one-way ANOVA

followed-up by Student's t-test for pairwise comparison. Statistical significance was considered to be reached at *p<0.05; **p<0.01; ***p<0.001 (Graph-PadPrism2.0) (n=16 per condition).

Results

In recent years, research has focused on the search for therapies that cure or ameliorate the symptoms present in patients with LD. Here, we analyzed the action of 4-PBA and metformin on the susceptibility to PTZ-induced seizures in these malin knockout mice. After 2 months of treatment with 4-PBA or metformin, a convulsive dose of PTZ was injected intraperitoneally in control, malin knockout, and malin knockout mice after treatment with 4-PBA or metformin. The percentage of animals showing generalized seizures and mortality, seizure latency (i.e., time between PTZ injection and onset of tonic-clonic seizures) and seizure length was analyzed. Following injection of PTZ, the mice displayed freezing and convulsive activity, which later progressed to generalized tonic-clonic seizures, sometimes associated with death. PTZ treatment induced seizures in 50% of control mice, whereas in mice lacking malin, the percentage increased to 78% ($p<0.05$). Both 4-PBA and metformin treatments decreased the percentage of *Epm2b*$^{-/-}$ mice undergoing seizures, reaching the control values after 4-PBA treatment, and decreasing this percentage below control levels after metformin treatment (Fig. 1A). Although the differences were not statistically significant, PTZ-induced mortality tends to be less in malin knockout (17%) and 4-PBA-treated malin knockout mice (6.25%) when compared with controls (25%). After metformin treatment, the lethal effect of PTZ disappeared ($p<0.05$) (Fig. 1B). Seizure latency was lower for malin mutant mice when compared to control mice. Both 4-PBA and metformin treatments increased the latency for PTZ-induced seizure onset in malin

knockout mice, eliminating the statistical significance between control and malin knockout mice (Fig. 1C). The length of PTZ-induced seizures in the malin knockout model was significantly increased when compared to control mice ($p<0.05$). After 4-PBA treatment, no significant differences were observed between malin knockout and wild type, while seizure length was even shorter after metformin treatment ($p<0.001$) (Fig. 1D). Thus, 4-PBA and metformin decrease PTZ-induced seizures, mortality, and seizure length, ameliorating the hyperexcitability detected in mice lacking the malin protein.

Discussion

In this study, we have used the *Epm2b-/-* malin-deficient mouse model of LD to search for new treatments that could improve the neurologic symptoms and epilepsy present in patients suffering LD. To this end, we tested the effects of 4-PBA and metformin, two pharmacological agents involved in the compilation of misfolded and aggregated proteins and the activation of autophagy, on the increased sensitivity of this model to PTZ.

As mentioned above, mutations in either the *Epm2a* or the *Epmd2b* genes in mice give raise to many of the neurological and behavioral abnormalities found in LD patients, including certain neurologic deficits, different degrees of epilepsy and increased sensitivity to PTZ. LD mouse models also display Lafora bodies, increased oxidative stress and impaired antioxidant response in the brain. It is known that laforin and malin proteins form a functional complex, and that different mutations in the genes encoding both proteins lead to the development of LD. Unfortunately, the underlying molecular mechanisms of this pathology are not fully understood and the development of novel therapies has been arduous, and somehow hopeless. Although laforin and

malin have been involved in the regulation of glycogen metabolism, recent reports indicate that they are also implicated in alternative physiological pathways, such as endoplasmic reticulum stress response and protein clearance. Lafora bodies also contain certain proteins in addition to polyglucosan aggregates. Thus, ubiquitinated proteins, advanced glycation-end products, chaperones, autophagy components, and proteasome subunits, all form part of Lafora bodies. LD can be therefore considered as another example of a pathology in which the processes of protein clearance and endoplasmic reticulum stress are defective[17]. We have previously described the effects of treatments with 4-PBA and metformin in *Epm2b*$^{-/-}$ mice on decreasing the number of Lafora bodies, reducing neurodegeneration and improving motor behavior and memory[15]. We also described that 4-PBA increased the levels of the chaperone BIP/Grp78, involved in the amelioration of proteostasis dysfunction, and that metformin induced the activation of the AMPK complex. Here, we report the effects of treatments with 4-PBA and metformin on the response of *Epm2b-/-* mice to PTZ administration, and show that both treatments ameliorate the sensibility of malin-deficient mice to this epileptogenic agent. Thus, treatments with 4-PBA and metformin show further beneficial results in our malin knockout LD mouse model, probably acting as neuroprotective agents. As these compounds are already approved for clinical practices in different pathologies, we argue that they could be tested in clinical trials.

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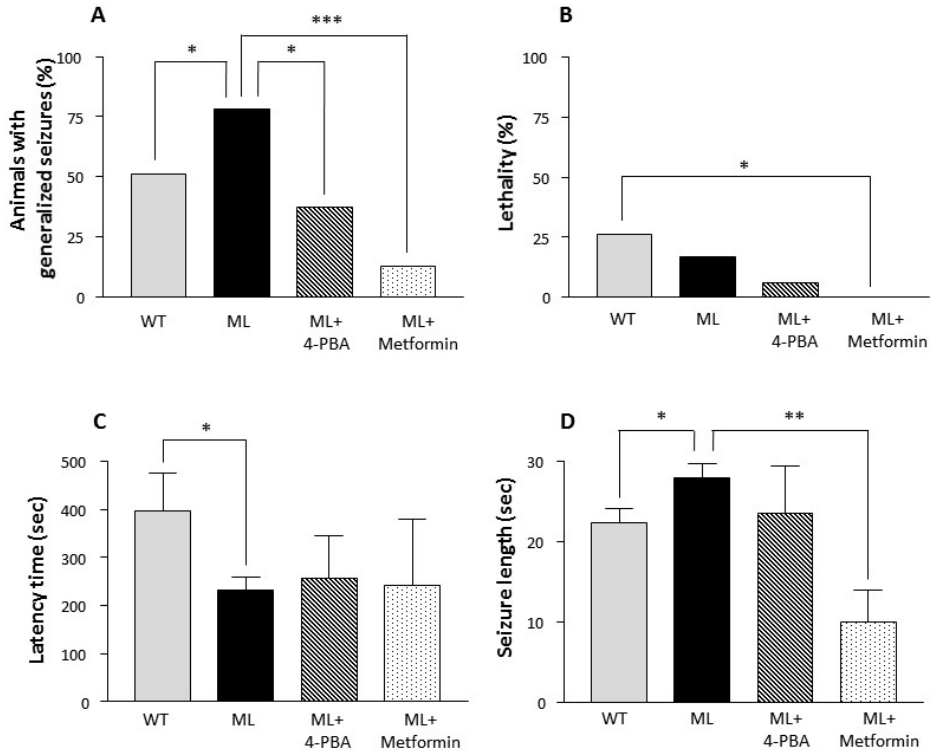

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255 Figure legend

256 **Figure 1. Sensitivity of *Epm2b*$^{-/-}$ mice to PTZ after treatment with 4-PBA and**
257 **metformin. (A)** 4-PBA and metformin decreased the percentage of mice with
258 generalized seizures after i.p. PTZ injection. The chi-square test yields $\chi^2 = 15.38$ on 3
259 degrees of freedom with a p-value of 0.0015. **(B)** Reduced mortality induced by PTZ
260 after 4-PBA administration, and lack of PTZ lethal effects after metformin treatment.
261 The chi-square test yields $\chi^2 = 7.823$ on 3 degrees of freedom with a p-value of 0.0498.

(C) After 4-PBA and metformin treatments the differences between control and *Epm2b*$^{-/-}$ in latency time (with a p-value of 0.0431 in Student´s t-test) are no longer present. **(D)** Seizure length was lowered by 4-PBA, and more notoriously by metformin. The one-way ANOVA showed a p-value of 0.0294 and an F-ratio of 3.483 ML: malin knockout mice; WT: wild type mice.