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Pregabalin Suppresses Calcium-Mediated Proteolysis and Improves Stroke Outcome

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Abstract

Pregabalin, a Ca^{2+} channel $\alpha_2\delta$ -subunit antagonist with analgesic and antiepileptic activity, reduced neuronal loss and improved functional outcome in a mouse model of focal ischemic stroke. Pregabalin administration (5 – 10 mg/kg, i.p.) 30-90 min after transient middle cerebral artery occlusion/reperfusion reduced infarct volume, neuronal death in the ischemic penumbra and neurological deficits at 24 h post-stroke. Pregabalin significantly decreased the amount of Ca^{2+} /calpain-mediated α -spectrin proteolysis in the cerebral cortex measured at 6 h post-stroke. Together with the extensive clinical experience with pregabalin for other neurological indications, our findings suggest the potential for a therapeutic benefit of pregabalin in stroke patients.

Keywords

calcium; calpain; cerebral ischemia; neuronal death; pregabalin; presynaptic calcium channels

Introduction

Increasing the survival of neurons in the ischemic penumbra is a major goal for the development of drugs for treating stroke patients in the first few hours to days after the ischemic event (Hossmann, 2006; Tuttolomondo et al., 2009). Cell membrane depolarization, excessive release of the excitatory neurotransmitter glutamate, and Ca^{2+} influx through N-methyl-D-aspartate (NMDA) receptor channels and voltage-gated Ca^{2+} channels endanger neurons in the ischemic penumbra (Choi, 1988; Barone et al., 1997). Although NMDA receptor antagonists were effective in reducing brain damage in animal models of stroke, clinical trials in human stroke patients proved unsuccessful, in part because of severe side effects (Muir, 2006).

Voltage-gated Ca^{2+} channels (VGCC) are multi-subunit integral membrane protein complexes that include a Ca^{2+} -permeable α_1 subunit (of which there are at least 10 encoded by separate genes) and modulatory γ , β and $\alpha_2\delta$ subunits. Pregabalin (*S*-3-(aminomethyl)-5-methylhexanoic acid) is used to control neurogenic pain in various clinical conditions including fibromyalgia, diabetic neuropathy and complex regional pain syndrome (Lyseng-Williamson and Siddiqui, 2008). Although pregabalin is a structural analogue of the

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neurotransmitter γ -aminobutyric acid its pharmacological actions are mediated by binding to the $\alpha_2\text{-}\delta$ subunit of VGCC (Taylor et al., 2007). Its potent binding at this site reduces Ca^{2+} influx at presynaptic nerve endings, and therefore, reduces the release of several neurotransmitters including glutamate (Joshi and Taylor, 2006). Thus, in contrast to dihydropyridine inhibitors of L-type VGCC which block postsynaptic channels (Hanson and Smith et al., 2002), pregabalin acts mainly on presynaptic VGCC (Bauer et al., 2009), a mode of action expected to reduce excessive release of glutamate and protect neurons against Ca^{2+} overload in ischemic stroke. Because pregabalin targets a specific VGCC subunit not previously targeted for stroke treatment, we performed preclinical studies to evaluate the potential clinical efficacy and neuroprotective mechanism of action in a mouse model of focal ischemic stroke.

Methods

Animals

Male C57BL/6 mice (Jackson Laboratories), 4-6 months of age and weighing 25 – 29 g at the time of surgery, were used for all experiments. The mice were group-housed and maintained on a 12 h light/12 h dark cycle, with ad libitum access to water and rodent chow. All procedures were in accordance with the NIH guidelines for the care and use of animals in research, and were performed with approval from the National Institute on Aging Animal Care and Use Committee.

Drug treatment

In an initial set of studies, pregabalin (*S*-[+]-3-isobutyl- γ -aminobutyric acid, >99.9% purity) or 0.5% carboxymethyl cellulose (CMC; vehicle) was administered intraperitoneally (i.p.; 10 mg/kg) a pre-treatment (at 60 min before reperfusion, $n = 7$) and a post-treatment (just after, and 30 min after middle cerebral artery occlusion and reperfusion (MCAO/R; $n = 7$ at each time point). For dose-response studies, pregabalin was administered in doses of 1 mg/kg, 5 mg/kg, 10 mg/kg, and 30 mg/kg ($n = 7$ in each group) at 30 min after MCAO/R. For the evaluation of time-response, pregabalin groups were administered with pregabalin (10 mg/kg, i.p.) at 30, 60 or 90 min after MCAO/R ($n = 7$ at each time point).

Neurological deficits and the infarct volume were determined 24 h after MCAO/R.

Middle cerebral artery occlusion and reperfusion

The MCAO/R model was performed using the intraluminal suture technique described previously (Shimamura et al., 2006). Briefly, mice were anesthetized with isoflurane, a midline incision was made in the neck, and the right external carotid and pterygopalatine arteries were isolated and ligated with 6-0 silk thread. The internal carotid artery was occluded at the peripheral site of the bifurcation of the internal carotid artery (ICA) and the pterygopalatine artery with a small clip and the common carotid artery (CCA) was ligated with 6-0 silk thread. The external carotid artery (ECA) was cut, and 6-0 nylon monofilament coated with a mixture of silicone resin was advanced into the middle cerebral artery (MCA) until resistance was felt. The nylon thread and the CCA ligature were removed after 30 min of occlusion to initiate reperfusion. In the sham group, these arteries were visualized but not disturbed. Body temperature was maintained around 37°C by using heating pads and a heating ramp throughout the surgical procedure and afterward till the animal recovered from anesthesia. In a separate set of experiments anesthetized animals from all groups (4 mice per group) underwent cerebral blood flow (CBF) measurements using a laser Doppler perfusion monitor. All CBF measurements were conducted with the mouse fixed in a plastic frame with the probe placed in the region of cerebral cortex perfused by the MCA. There were no

significant differences in CBF between vehicle- and pregabalin-treated mice, before, during or after MCA occlusion.

Evaluation of neurological deficits

Neurological impairment was assessed by using a five-point neurological deficit score (0, no neurological deficit; 1, failure to extend left paw; 2, circling to the left; 3, falling to the left; and 4, unable to walk spontaneously) (Bederson et al., 1986) and were assessed in a blinded fashion.

Evaluation of infarct volume

The infarct area was evaluated by TTC staining. Briefly, at 24 h of reperfusion, the mice were killed with a lethal dose of isoflurane. The brains were immediately removed and placed into PBS (4°C) for 15 min, and 2-mm coronal sections were cut with a tissue cutter. The brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer at 37 °C for 20 min and then immediately fixed in 10% formalin overnight. The stained sections were photographed, and the digitized images were used for analysis. The borders of the infarct in each brain slice were outlined and the area quantified using a NIH image 6.1 software. For each brain section, the infarct area was determined by subtracting the area of the non-infarcted ipsilateral hemisphere from that of the intact contralateral hemisphere. The percentage of infarct volume was calculated by dividing the sum of the area of infarction by the total of that of contralateral hemisphere to avoid the influence of tissue edema (Swanson and Sharp 1994).

Histopathological analysis

Mice were deeply anesthetized with isoflurane and perfused transcardially with isotonic saline for 5 min followed by fixation with 10% formalin for overnight. The tissues were cut into 3 mm slabs which were then embedded in paraffin. Sections (4-5 µm thick) were cut in the coronal plane and stained with haematoxylin and eosin (HE) for evaluation of cells in the ischemic penumbra. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed according to the manufacturer's instructions (Roche Diagnostics). Briefly, after deparaffinization and rehydration, brain sections (adjacent to those used for HE staining) were incubated in sodium citrate buffer with 0.1% triton X-100 for 20 min at 4°C, followed by the TUNEL reaction mixture for 60 min at 37°C. The number of TUNEL-positive cells was counted in the penumbra of the cortex and the striatum (as the ischemic penumbra) at × 10 magnification.

Immunoblotting

Tissue samples from the striatum and the cortex of ipsilateral hemisphere were homogenized in a buffer consisting of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, and protease inhibitors). The homogenates were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected and the total protein content determined using the BCA protein assay kit (Pierce, USA). Equal amounts of protein (20 µg) were separated by SDS-PAGE (8-12%) and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat milk for 1 h at room temperature, followed by an overnight incubation at 4°C with primary antibodies for anti-spectrin alpha chain (1:1000; Millipore) or GAPDH as a loading control for protein quantification. Membranes were then washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized using a chemiluminescence detection kit (Amersham Biosciences). Quantitative results were expressed as the ratio of the band intensity of the protein of interest to the band intensity of GAPDH.

Determination of pregabalin concentration in plasma

The analysis of pregabalin in plasma was performed using methods reported previously (Vermeij and Edelbroek, 2004). Briefly, each mouse was injected intraperitoneally with a single dose of 10 mg/kg of pregabalin dissolved in 0.5% CMC. Under anesthesia with isoflurane, blood samples were collected from each mouse by cardiac puncture according to the specific schedule (at times of 1 min, 5 min, 10 min, 15 min, 30 min, 60 min, and 120 min after dosing; $n = 4$ at each time point). Each collected blood sample was immediately centrifuged at 1500 g for 15 min at 4 °C to separate the plasma. Plasma samples were stored at -70°C until further processing. The plasma (200 μ l) was then mixed with 50 μ l of the internal standard (DL-2-aminopentanoic, 1 mg/ml) and 100 μ l of a 20% trichloroacetic acid. The mixture was vortexed for 15 s and then centrifuged for 10 min at 2500 $\times g$. The supernatant (10 μ l) was then mixed with 100 μ l of borate buffer (0.1 M, pH 10.0) and 10 μ l of the derivatizing reagent (*o*-phthalaldehyde, 2-mercaptopropionic acid, 0.1 M borate buffer). After two mixing cycles, performed by aspirating and dispensing 100 μ l of the mixture and after a reaction time of 1 min, 15 μ l of the reaction product was injected for analysis onto an HPLC (Agilent 1100 series attached to a zorbax xcb c-18 column, Agilent Technologies).

Statistical analysis

Data are presented as means $\pm$ SEM. Statistical analysis was performed by one-way ANOVA followed by Scheffe post-hoc test for pairwise comparisons. $P < 0.05$ was considered statistically significant.

Results

Twenty-four hours after MCAO/R, a well-defined infarct area was seen in the ipsilateral cerebral hemisphere (cerebral cortex and striatum) of vehicle-treated mice, whereas no brain damage was detected in sham-operated control mice (Supplementary Figure 1A). When mice were administered a single dose of pregabalin (10 mg/kg) 60 min prior to reperfusion, infarct size was not significantly different than that of vehicle-treated mice. However, infarct size was reduced by approximately 50% in mice treated with pregabalin immediately after the stroke (i.e., after the onset of reperfusion), and by approximately 70% in mice treated with pregabalin 30 minutes after the stroke (Fig. 1A). Neurological deficits, assessed at 24 h after ischemia, were significantly reduced in mice that had been treated with pregabalin at 0 or 30 min after the stroke compared to vehicle-treated mice (Fig. 1B).

To determine whether pregabalin treatment did, in fact, preserve neurons, we performed TUNEL staining to label cells with damaged DNA in brain tissue sections from mice in all 5 experimental groups. Large numbers of TUNEL-positive cells were present in the striatum and cerebral cortex of vehicle-treated mice 24 h after MCAO/R (Fig. 1C, D; Supplementary Figure 1B). In contrast, there were significantly fewer TUNEL-positive cells in the striatum and cortex of mice that had been treated with pregabalin at either 0 or 30 min after MCAO/R. Treatment with pregabalin 60 min prior to reperfusion did not significantly affect the numbers of TUNEL-positive neurons (Fig. 1C, D).

To determine the optimal treatment dose and the post-stroke therapeutic window, mice were subjected to MCAO/R and 30 min later were administered either 0, 1, 5, 10 or 30 mg/kg of pregabalin; neurological score and infarct volume were determined at 24 h post-stroke. Pregabalin doses of 5 and 10 mg/kg were the most effective in reducing infarct volume by more than 70% compared to pregabalin doses of either 1 or 30 mg/kg which reduced infarct volume by only 15-20% (Fig. 2A, B). Similar efficacies of the four different doses of pregabalin were observed in the neurological deficit scores, with 5 and 10 mg/kg being the

most effective (Fig. 2C). In the time-response study, pregabalin at 10 mg/kg, whether administered at 30 min, 60 min, or 90 min after reperfusion, significantly reduced both the infarct volume and the neurological deficits, with protection at 30 min being the greatest (Fig. 2D, E).

To provide insight into why pre-stroke treatment with pregabalin was less effective than post-stroke treatment, we measured plasma pregabalin concentrations at increasing time points after i.p. administration of pregabalin (10 mg/kg). Absorption was very rapid following the i.p. dose, being detectable within 1 min and increasing to a peak of 13.2 µg/mL at 15 min after dosing, then decreasing exponentially with a $t_{1/2}$ of 35.5 min; by 2 h plasma pregabalin levels had decreased to less than 3 µg/mL (Supplementary Figure 2).

Calpains are Ca^{2+} -activated proteases believed to play a role in the degeneration of neurons in ischemic stroke (Bartus et al., 1995). To elucidate the neuroprotective mechanism of action of pregabalin, we measured levels of the calpain-mediated cleavage product of the neuronal protein α -spectrin in the cerebral cortex and striatum. Levels of calpain-cleaved α -spectrin (145 kDa) increased progressively from 4 to 24 h after the onset of reperfusion (Fig. 3A). Pregabalin at 10 mg/kg, significantly decreased the amount of calpain-cleaved α -spectrin in the cerebral cortex measured at 6 h after reperfusion (Fig. 3B). However, pregabalin did not significantly reduce the amount of calpain-cleaved α -spectrin in the striatum, possibly because striatal neurons are more vulnerable to death in this stroke model.

Discussion

It was previously reported that the mechanism by which pregabalin inhibits pain is by suppressing Ca^{2+} influx through $\alpha_2\delta$ subunit-containing VGCC (Bian et al., 2006; Taylor et al., 2007), and this is a likely mechanism by which pregabalin protected neurons against ischemic brain injury in the present study. Excessive release of glutamate from presynaptic terminals occurs during and after cerebral ischemia, resulting sustained Ca^{2+} influx through postsynaptic NMDA receptor channels and VGCC. The high Ca^{2+} levels trigger the activation of proteases, including calpains and caspases, that may mediate death of neurons in the ischemic penumbra (Dutta et al., 2002; Glazner et al., 2000). Neuroprotection by pregabalin was associated with reduced Ca^{2+} -mediated proteolysis of spectrin in neurons in the ischemic cerebral cortex, consistent with suppression of excitotoxic Ca^{2+} overload. We chose to measure calcium-mediated spectrin cleavage as an indirect indicator of intracellular calcium levels in neurons, because such spectrin cleavage has been widely used as a marker of ischemic neuronal damage (Lee et al., 1991; Pike et al., 2004). Calpain substrates other than spectrin are involved in calcium-mediated neuronal damage, and there are also many calpain-independent cell death pathways including the classic mitochondria-mediated apoptotic pathway. While it may be possible to measure calcium levels in neurons using optogenetic probes and in vivo imaging, this method is problematic because it requires that the animals be anesthetized during the imaging; because the anesthetic affects intracellular calcium levels, the interpretation of data obtained using these methods is problematic.

An unexpected result was that post-stroke pregabalin treatment was more effective than pre-stroke treatment in reducing brain damage and improving functional outcome. Our pharmacokinetic data revealed that pregabalin was cleared from the blood relatively rapidly with a half-life of less than 40 minutes. Therefore, when given 1 hour prior to reperfusion, the pregabalin concentration was already considerably diminished compared to levels achieved when administered post-stroke. Interestingly, we found that when administered 30 min after the onset of reperfusion, pregabalin exhibited a U-shaped dose response curve with doses of 5 and 10 mg/kg being more effective in reducing brain damage and improving functional outcome compared to either 1 or 30 mg/kg. One possible explanation for the U-

shaped dose response is that the lowest dose was not inhibiting Ca^{2+} channels maximally, whereas the highest dose was causing non-specific adverse effects on neuronal viability.

The approach of targeting VGCC for stroke has a strong scientific rationale. The mostly widely studied drug that targets VGCC is nimodipine, which blocks L-type Ca^{2+} channels. While providing significant neuroprotection in rodent ischemic stroke models, nimodipine did not demonstrate efficacy in stroke patients (Tomassoni et al., 2008). However, L-type Ca^{2+} channels are concentrated primarily in cell bodies and proximal dendrites of neurons (Mize et al., 2002) and so, in contrast to pregabalin, nimodipine may not suppress the influx of Ca^{2+} into presynaptic terminals which is critical for glutamate release. Pregabalin has been used in millions of patients with pain syndromes and anxiety disorders, and exhibits an excellent safety profile (Shneker and McAuley, 2005). In light of its selective action on presynaptic Ca^{2+} influx and the established efficacy of pregabalin for other neurological disorders, our findings suggest that clinical trials of pregabalin in stroke patients are warranted.

Research Highlights

- Pregabalin, an antagonist of $\alpha_2\delta$ Ca^{2+} channels, improves outcome in a mouse model of stroke.
- Pregabalin is effective in reducing neuronal damage when administered after a stroke.
- A reduction in calpain-mediated proteolysis contributes to the neuroprotective action of pregabalin.
- Widely used to treat chronic pain, our findings suggest that pregabalin may benefit stroke patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Pregabalin reduces cell degeneration in the cerebral cortex and striatum, and improves functional outcome, when administered after focal ischemic stroke. Mice were administered pregabalin (10 mg/kg) either 60 min prior to, or 0 or 30 min after the onset of reperfusion; control mice were administered vehicle 30 min after the stroke. Neurological scoring was performed 24 h after the stroke and brains were then removed for histological analysis. **A.** Infarct volumes determined from TTC-stained brain sections. **B.** Neurological deficit scores. **C.** Images showing TUNEL-stained cells in the cerebral cortex of mice in the indicated experimental groups. **D.** Relative numbers of TUNEL-stained cells in the striatum and cortex of mice in the indicated treatment groups. For all three graphs the values are the mean and SEM (n = 7 mice per group). *p<0.05, **p<0.01, ***p<0.001 compared to the value for vehicle-treated mice.

Figure 2.

Determination of the therapeutic dose range and post-stroke treatment window of efficacy for pregabalin. **A-C.** Mice were treated with the indicated doses of pregabalin, administered 30 min after the stroke; clinical evaluation and euthanasia were performed 24 h after the stroke. **A.** Examples of TTC-stained serial brain sections from mice in the different groups. **B.** Infarct volumes. **C.** Neurological deficit scores. **D, E.** Mice were treated with 10 mg/kg at the indicated post-stroke time points, or with vehicle at 30 min post-stroke. Clinical evaluation and euthanasia were performed 24 h after the stroke. **D.** Infarct volumes. **E.** Neurological deficit scores. Values are the mean and SEM (n = 6-8 mice per group). *p<0.05, **p<0.01, ***p<0.001 compared to the value for vehicle-treated mice.

Figure 3.

Pregabalin treatment ameliorates Ca^{2+} -mediated proteolysis in cerebrocortical neurons in a mouse model of ischemic stroke. **A.** Immunoblot (upper) showing examples of relative levels of calpain-cleaved α -spectrin (a neuron-specific cytoskeletal protein) in tissue samples from the striatum and cortex of mice killed at the indicated post-stroke time points. Results of densitometric quantification of levels of calpain-cleaved α -spectrin (lower). Values are the mean and SEM ($n = 4$ mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the value for sham mice. **B.** Immunoblot (upper) showing examples of relative levels of calpain-cleaved α -spectrin in tissue samples from the striatum and cortex of mice in the indicated treatment groups (10 mg/kg pregabalin administered 30 min post-stroke) killed 6 h after a stroke. Results of densitometric quantification of levels of calpain-cleaved α -spectrin (lower). Values are the mean and SEM ($n = 4$ mice). ** $p < 0.01$ compared to the value for sham mice. # $p < 0.05$ compared to the value for mice not treated with pregabalin.