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Blockade of endothelin B receptor improves the efficacy of levetiracetam in chronic epileptic rats

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ABSTRACT

Purpose: To elucidate the mechanisms that regulate p-glycoprotein (PGP) expression and function in pharmacoresistant epilepsy, we investigated the effect of an ET_B receptor antagonist (BQ788) and a p38 mitogen-activated protein kinase (p38MAPK) inhibitor (SB202190) on intractable seizures in chronic epileptic rats.
Methods: Lithium-pilocarpine-induced chronic epileptic rats were used in the present study. Animals were given levetiracetam (LEV), LEV + SB202190, LEV + BQ788, SB202190 or BQ788 over a 3-day period using an osmotic pump. Seizure activity was recorded by video-EEG monitoring with 2 h of recording per day at the same time of day. We also performed western blot after EEG analysis.
Results: Compared to control animals, PGP, ET_B receptor and p38MAPK expression was increased in the hippocampus of epileptic animals. Neither SB202190 nor BQ788 affected the spontaneous seizure activity in epileptic rats. Three of ten rats were responders and achieved complete seizure control or significant reduction in seizure activity by LEV. In four of ten rats, seizure frequency was unaltered by LEV (non-responders). LEV + SB202190 reduced seizure duration, but not seizure frequency, in both responders and non-responders. LEV + BQ788 alleviated seizure frequency and seizure duration in both responders and non-responders. Compared to responders, PGP and ET_B receptor expression was enhanced in the hippocampus of non-responders.
Conclusion: To the best of our knowledge, these findings are the first indications of the role of ET_B receptor in pharmacoresistant epilepsy. Therefore, the present data suggest that the regulation of the ET_B receptor-mediated signaling pathway may be important for identification of new therapeutic strategies for improving antiepileptic drug efficacy.

1. Introduction

The ATP-cassette-binding protein (ABC) family contains drug efflux transporters that exclude antiepileptic drugs (AED) from the brain to the blood. Among them, p-glycoprotein (PGP) is a well-characterized drug efflux transporter in the brain–blood barrier (BBB). Inhibitions of PGP expression and activity show substantially increased brain levels of anticonvulsant agents and improvement of anticonvulsant responses [1,2]. Therefore, drug efflux transporter expression/activity is one of the limiting factors in epilepsy pharmacotherapy [3].

Recently, we have reported that endothelin-1 (ET-1) expression is up-regulated in vessels within the rat piriform cortex following status epilepticus (SE), which is one of the epileptogenic insults [4,5]. ET-1 is a potent vasoactive 21-amino acid peptide that binds to the ET_A and ET_B receptors. ET_A receptor activation induces vasoconstriction [6], while ET_B receptor activation results in vasodilation via nitric oxide (NO) production [7–10]. In addition, ET-1 regulates PGP expression and transport activity in isolated, intact rat brain capillaries, but not in rat brain capillary endothelial cell lines [11–15]. In addition, ET-1 activates the p38 mitogen-activated protein kinase (p38MAPK) signaling pathway, which is involved in the regulation of PGP and ET-1 expression [16–22]. Interestingly, ET_B receptor activation potentiates the production and secretion of more ET-1 in an autocrine positive feedback loop [23–26]. With respect to these properties of ET-1, it is likely that increases in ET-1 concentration or ET_B receptor activation in the epileptic hippocampus would have the undesired effect of reducing AED efficacy via up-regulation of PGP expression. However, little is known about whether ET-1 is involved in the pharmacoresistance in chronic epilepsy. Therefore, we investigated the effect of an ET_B receptor

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antagonist (BQ788) and a p38MAPK inhibitor (SB202190) on intractable seizures in chronic epileptic rats.

2. Materials and methods

2.1. Experimental animals and chemicals

This study utilized the progeny of male Sprague-Dawley (SD) rats (7 weeks old) obtained from the Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were provided with a commercial diet and water ad libitum under controlled temperature, humidity and lighting conditions (22 ± 2 °C, 55 ± 5% and a 12:12 light/dark cycle with lights). Animal protocols were approved by the Institutional Animal Care and Use Committee of Hallym University (Chunchon, Republic of Korea). The number of animals used and their suffering were minimized in all cases. All reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA), except as noted.

2.2. SE induction

Animals were given LiCl (127 mg/kg i.p) 24 h before the pilocarpine treatment. Animals were intraperitoneally (i.p) treated with pilocarpine (30 mg/kg) 20 min after atropine methylbromide (5 mg/kg i.p.). Diazepam (Valium; Hoffman la Roche, Neuilly sur-Seine, France; 10 mg/kg, i.p.) was administered 2 h after onset of SE and repeated, as needed. Control animals received saline in place of pilocarpine. Animals were video-monitored 8 h a day for general behavior and occurrence of spontaneous seizures by 4 weeks after SE (Fig. 1). Rats showing spontaneous recurrent seizures were used as chronic epileptic animals.

2.3. Surgery

Control and epileptic rats were anesthetized (Zolretil, 50 mg/kg, I.M. Virbac Laboratories, France) and placed in a stereotaxic frame. Thereafter, animals were implanted with depth electrodes in the right hippocampus. Monopolar stainless steel electrodes (Plastics One, Roanoke, VA, USA) were lowered stereotaxically into the left dorsal hippocampus using the following coordinates: −3.8 mm posterior; 2.0 mm lateral; −2.6 mm depth. Connecting wire and electrode socket were then inserted in an electrode pedestal (Plastics One, Roanoke, VA, USA), and secured to the exposed skull with dental acrylic.

2.4. Drug trials, EEG analysis and Quantification of behavioral seizure activity

Fig. 1 illustrates the design of the drug trial methodology, which was a modified protocol based on Glien et al. [27]. After baseline seizure activity (vehicle treatment) was determined over 3 days, each drug or mixture of compounds was administered over a 3-day period using an osmotic pump (1003D, Alzet, Cupertino, CA, USA). The pump was placed in a subcutaneous pocket in the dorsal region under isoflurane anesthesia (3% induction, 1.5–2% for surgery and 1.5% maintenance in a 65:35 mixture of N_2O:O_2). Throughout surgery, the animals were positioned over a heated pad, and core temperature was monitored and maintained between 37 and 38 °C. Between trials, the minipump was changed out for another minipump filled with another mixture under isoflurane anesthesia. The concentration of each drug or mixture of compounds was LEV (UCB, Belgium, 500 mg/ml), LEV (500 mg/ml) + SB202190 (a p38MAPK inhibitor, 0.3 mg/ml), and LEV (500 mg/ml) + BQ788 (an ET_B receptor antagonist, 10 mg/ml). To identify the effect of the ET_B receptor or p38MAPK activity on seizure activity, some animals were given BQ788 (10 mg/ml) or SB202190 (0.3 mg/ml) alone (n = 5, respectively). Every day during the experiment, seizure activity was recorded by video-EEG monitoring with 2 h of recording per day at the same time. EEG signals were recorded with a DAM 80 differential amplifier (0.1–3000 Hz bandpass; World Precision Instruments, Sarasota, FL, USA) and the data were digitized (1000 Hz) and analyzed using LabChart Pro v7 (AD Instruments, NSW, Australia). EEG analysis was performed by uploading the data to an automated program (LabChart Pro v7, AD Instruments, NSW, Australia). Spectrograms were automatically calculated using a Hanning sliding window with 50% overlap. Behavioral seizure severity was also evaluated according to Racine's scale [28]: 1, immobility, eye closure, twitching of vibrissae, sniffing, facial clonus; 2, head nodding associated with more severe facial clonus; 3, clonus of one forelimb; 4, rearing, often accompanied by bilateral forelimb clonus; and 5, rearing with loss of balance and falling accompanied by generalized clonic seizures.

2.5. Western blot

Under urethane anesthesia (1.5 g/kg, I.P.), the left hippocampus was removed and homogenized in 50 mM Tris containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4), ethylene glycol tetraacetic acid (EGTA, pH 8.0), 0.2% Tergitol type NP-40, 10 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT). Tissue lysate proteins were then loaded onto a 10% polyacrylamide gel. After electrophoresis, gels were transferred to nitrocellulose transfer membranes (Schleicher and Schuell BioScience Inc.). To reduce background staining, the filters were incubated with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 45 min, followed by incubation with PGP (GeneTex, USA; diluted 1:1000), ET_B receptor (Millipore, USA;

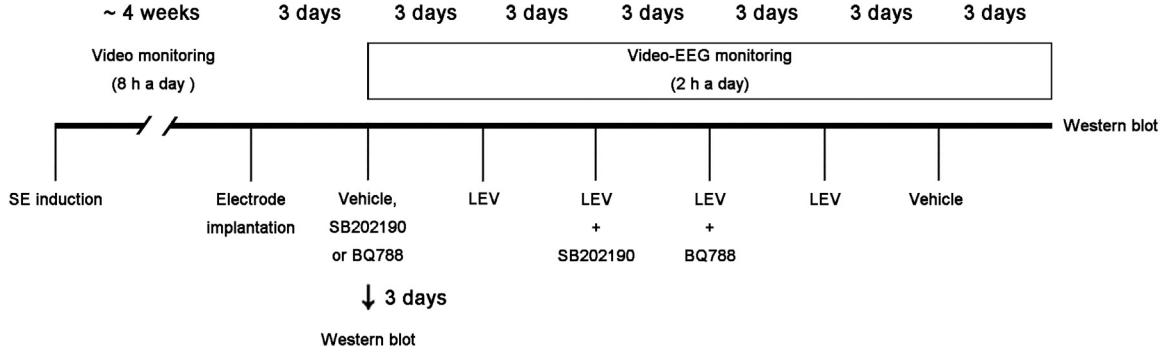

Fig. 1. Scheme of the experimental design. After baseline seizure activity (saline treatment) was determined during 3 days, each drug or a mixture of compounds was sequentially administered over a 3-day period using an osmotic pump.

diluted 1:1000) or p38MAPK (Cell Signaling, USA; diluted 1:1000) antibodies, and subsequently with HRP-conjugated secondary antibody. The PGP antibody (C219) used in the present study recognizes two PGP isoforms: multiple drug resistance protein 1 (MDR1) and MDR3 (Abcam, UK; diluted 1:50). Western blotting was conducted with an ECL Western Blotting Detection Kit (Amersham, USA) [29]. Optical density values were corrected by subtracting the average value for background noise. The optical density was then standardized by setting the threshold levels.

2.6. Data analysis

All data obtained from the quantitative measurements were analyzed using paired Student's *t*-tests or one way ANOVA to determine statistical significance. Bonferroni's test was used for post-hoc comparisons. A *p*-value below 0.05 was considered statistically significant [30].

3. Results

3.1. PGP, ET_B receptor and p38MAPK expression in the epileptic hippocampus

Compared to control animals, MDR1, MDR3, ET_B receptor and p38MAPK expression was increased 1.27-, 1.74-, 1.42- and 1.53-fold in the hippocampus of epileptic animals, respectively ($p < 0.05$, Fig. 2A and B). Linear regression analysis showed a directly proportional relationship between the intensity of ET_B receptor expression and that of MDR1 and MDR3 with linear correlation coefficients of 0.5638 and 0.6652, respectively ($p < 0.05$, Fig. 2C). The intensity of p38MAPK and that of MDR1 and MDR3 expressions also showed a direct proportional relationship with linear correlation coefficients of 0.9176 and 0.8736, respectively ($p < 0.05$, Fig. 2C). BQ788 did not affect MDR1, MDR3, ET_B receptor and p38MAPK expression in the

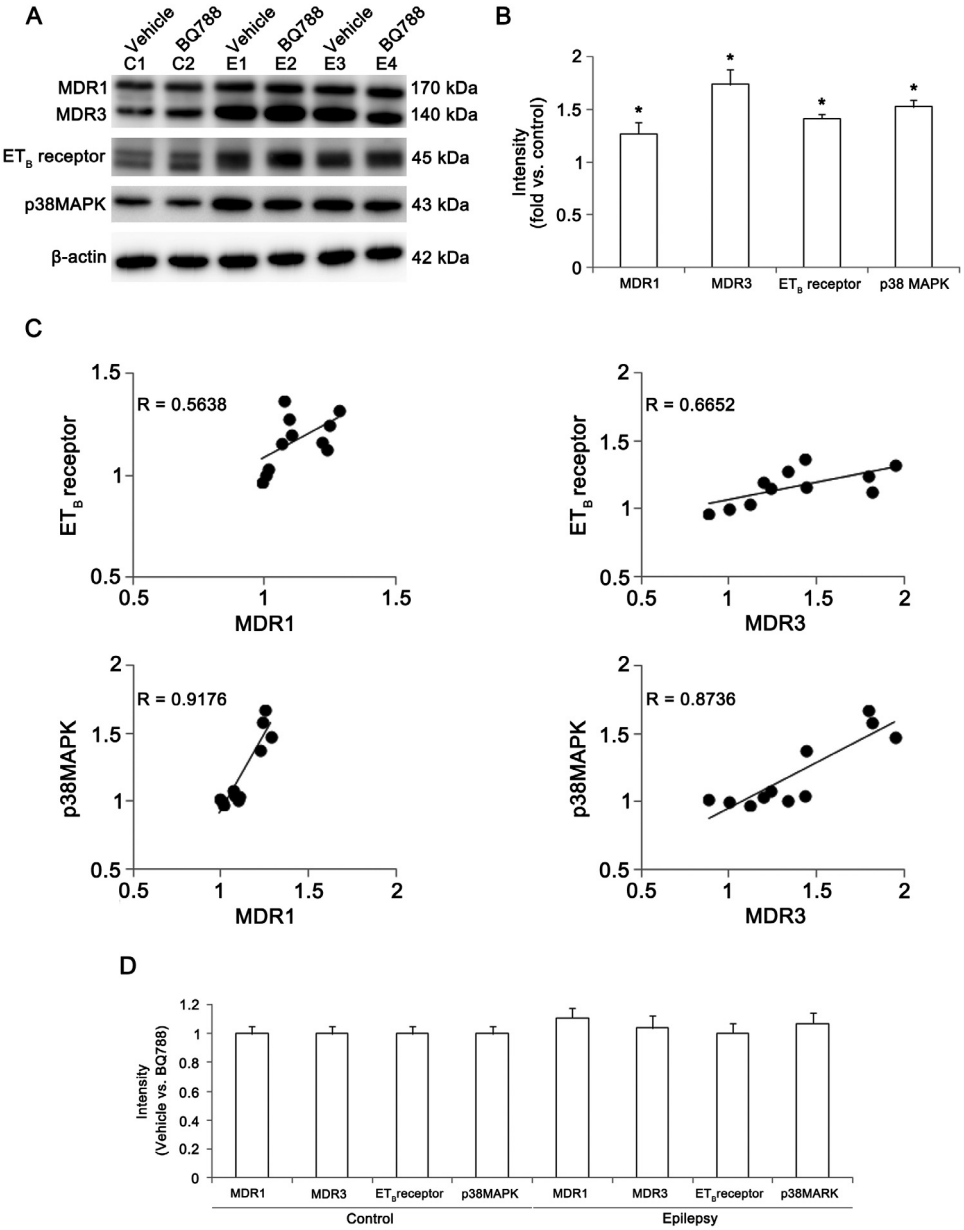

Fig. 2. PGP, ET_B receptor and p38MAPK expression in control and epileptic animals. (A) Western blot shows the up-regulation of PGP, ET_B receptor and p38MAPK expression in epileptic rats (E) compared to control animals (C). Arabic number indicates an individual animal number. (B) Quantitative values (mean ± S.E.M.) of PGP, ET_B receptor and p38MAPK expression in epileptic animals. $^*p < 0.05$ vs. control rats ($n = 5$, respectively). (C) Linear regression analysis of PGP, ET_B receptor and p38MAPK expression in the hippocampus. (D) Quantitative analysis of effect of PGP, ET_B receptor and p38MAPK expression (mean ± S.E.M.) in control and epileptic animals. $^*p < 0.05$ vs. vehicle in control rats ($n = 5$, respectively).

hippocampus of control and epileptic animals, compared to vehicle (Fig. 2A and D).

3.2. The effect of SB202190 and BQ788 on spontaneous seizure activity in epileptic animals

In the present study, control animals did not show behavioral or EEG seizure activity ($n = 10$). SB202190 and BQ788 infusion could not induce seizure activity in control animals ($n = 5$, respectively). The mean seizure frequency in epileptic animals was 8 ± 4.2/recording session (2 h) and the total seizure duration in epileptic animals was 684 ± 695 s. Vehicle, SB202190 and BQ788 infusion did not affected the mean seizure frequency and the total seizure duration in epileptic rats (Fig. 3).

3.3. The effects of LEV and co-treatment with SB202190 or BQ788 on spontaneous seizure activity in epileptic animals

LEV and co-treatment with SB202190 or BQ788 did not affect behavior and EEG activity in control animals ($n = 10$, data not shown). Similar to a previous study [27], three of ten rats were responders with complete seizure control ($n = 1$) or the significant reduction in seizure activity ($n = 2$) by LEV. Four of ten rats showed that seizure frequency was unaltered by LEV (non-responders). Rest animals (three of ten rats) showed a reduction of seizure frequency by LEV, but it was not statistically significant compared to vehicle due to large variation (from 0 to 12) among the daily recording. In responders, the mean seizure frequency was 2.2 ± 2.1/recording session and the total seizure duration was 68.5 ± 66 s during LEV treatment, while in non-responders the mean seizure frequency was 4.6 ± 1.4/recording session and the total seizure duration was 539.3 ± 559.7 s (Figs. 4 and 5). During LEV + SB202190 treatment, the mean seizure frequency was 3.7 ± 3.4/recording session and the total seizure duration was 55.8 ± 47.9 s in responders, while in non-responders, these values were 2.9 ± 3.2/recording session and total seizure duration of 131.2 ± 142.9 s. These findings indicate that LEV + SB202190 reduced seizure duration, but not seizure frequency, in both responders and non-responders compared to vehicle ($p < 0.05$, Figs. 4 and 5). During LEV + BQ788 treatment, the mean seizure frequency was 2.2 ± 3.3/recording session and the total seizure duration was 5.3 ± 6.7 s in responders, and in non-responders seizure frequency was 0.7 ± 1.5/recording session and seizure duration was 5 ± 11.2 s. These findings indicate that LEV + BQ788 reduced seizure frequency and seizure

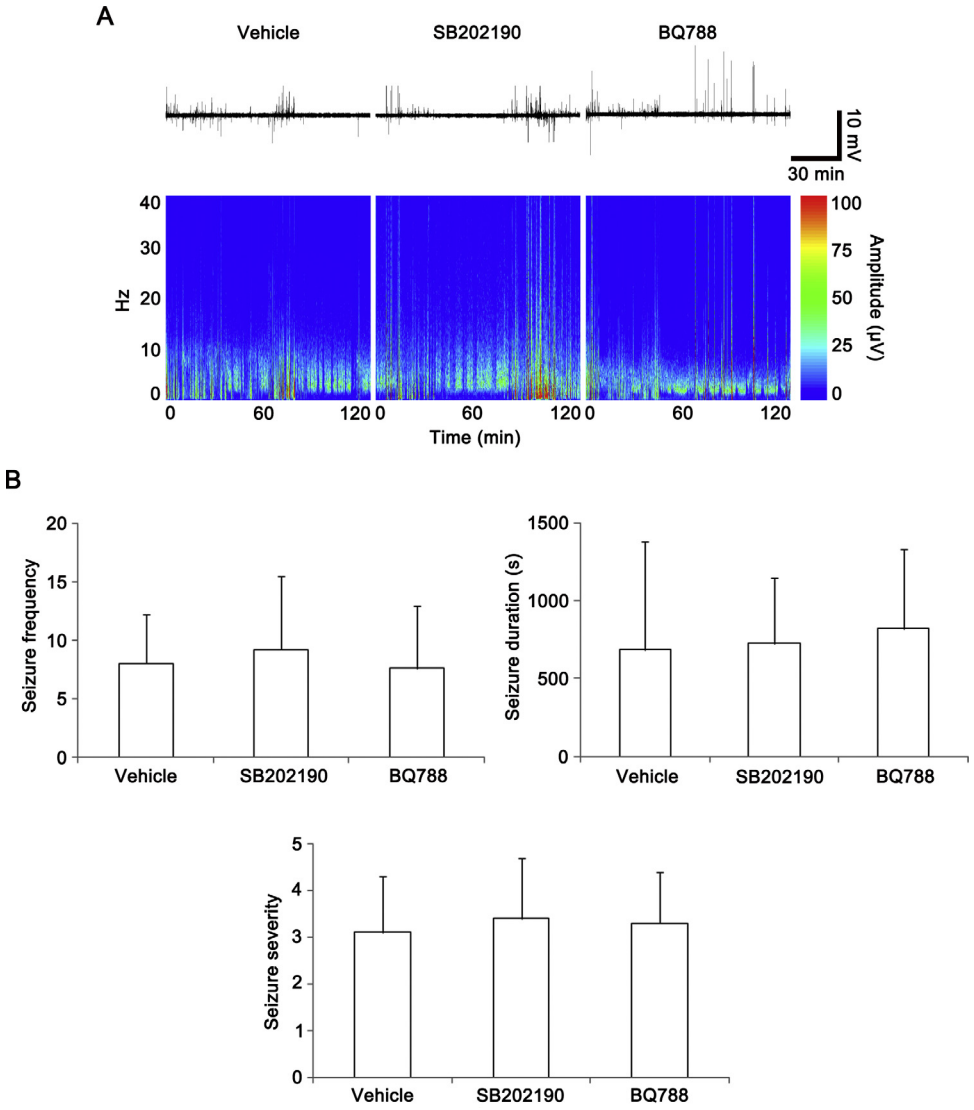

Fig. 3. The effect of SB202190 and BQ788 on spontaneous seizure activity in epileptic rats. (A) *Representative EEG traces and* frequency-power spectral temporal maps. (B) Quantitative values (mean ± S.D) of mean seizure frequency (left), total seizure duration (right) and seizure severity (low) during 2 h of recording a day. There is no difference in mean seizure frequency, total seizure duration and seizure severity among the three groups.

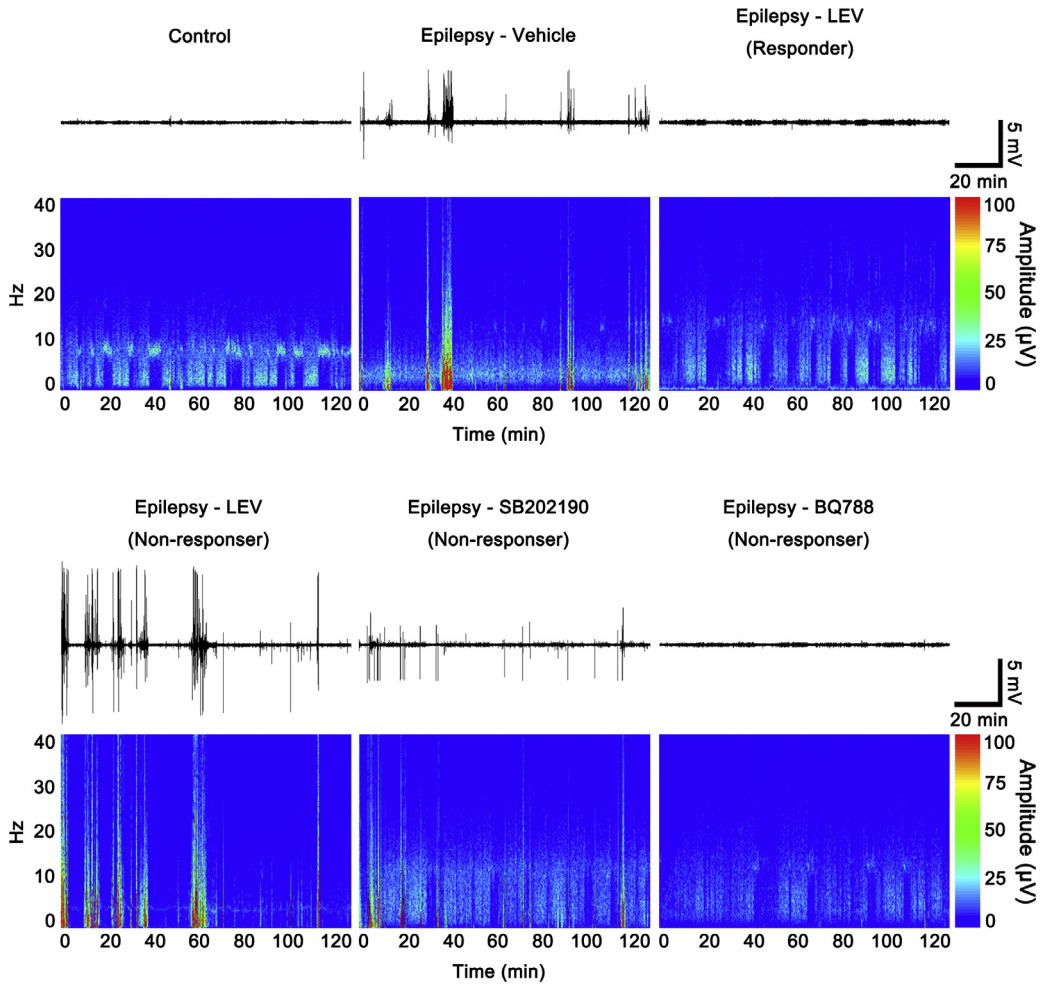

Fig. 4. *Representative EEG traces and* frequency-power spectral temporal maps demonstrating the effect of LEV and co-administration with SB202190 or BQ788 on spontaneous seizure activity in epileptic rats.

duration in both responders and non-responders compared to vehicle ($p < 0.05$, Figs. 4 and 5). The withdrawal of BQ788 rebounded seizure frequency and seizure duration in non-responders to vehicle-treated levels ($p < 0.05$, Figs. 4 and 5). Similarly, the withdrawal of LEV rebounded seizure frequency and seizure duration in responders to vehicle-treated levels ($p < 0.05$, Figs. 4 and 5).

3.4. Increases in MDR3 and ET_B receptor expression in the hippocampus of non-responders

To investigate the correlation of PGP, ET_B receptor and p38MAPK expression in the epileptic hippocampus with the efficacy of LEV, we performed western blot after EEG analysis. Compared to responders, MDR3 and ET_B receptor expression in the hippocampus was increased to 1.39- and 1.54-fold of responders ($p < 0.05$, Fig. 6A and B).

4. Discussion

Pilocarpine is an agonist at muscarinic acetylcholine receptors. Systemic administration of pilocarpine induces limbic seizures and SE, which provokes spontaneous, recurrent seizures after a seizure-free latent period. Pilocarpine in combination with lithium allows a reduction of the pilocarpine dose required to cause SE, but no increased mortality [31–33]. Therefore, the pilocarpine and lithium-pilocarpine model are useful to study epileptogenesis and AED efficacy for the control of seizures [27,34]. Indeed, Leite and Cavalheiro [34] used the pilocarpine model to investigate the anticonvulsant effect of conventional AEDs such as phenobarbital, carbamazepine and valproate, and reported the usefulness of the pilocarpine model to evaluate the efficacy of AEDs against complex partial seizures. In addition, Glien et al. [27] reported that spontaneous recurrent seizures in the pilocarpine model show an interindividual variability to LEV, which resembles that in patients with temporal lobe epilepsy. The present study showed that 30% of rats were responders to LEV, and that 40% of rats were non-responders to LEV. Similar to the observation in the present study, Glien et al. [27] found that 25% in this model were responders to LEV, 25% were non-responders, and 50% were variable responders. Therefore, the present data suggest that the pilocarpine model may be one of the most useful models to investigate pharmacoresistant epilepsy.

It has been well documented that overexpression or hyperactivity of PGP may involve the development of pharmacoresistent epilepsy or the efficacy of AEDs to control seizure activity [3,35,36]. Indeed, MDR1 expression is up-regulated in the human epileptic brain [37]. In the present study, both MDR1 and MDR3 expression was increased in the hippocampus of epileptic animals compared to controls. This discrepancy may be a consequence of the difference in species between humans and rodents, because MDR3 is the major form of PGP expression in the murine brain vessels [38]. Interestingly, the present data revealed that only MDR3 expression in the hippocampus of non-responders was higher than those of responders. Although the function or

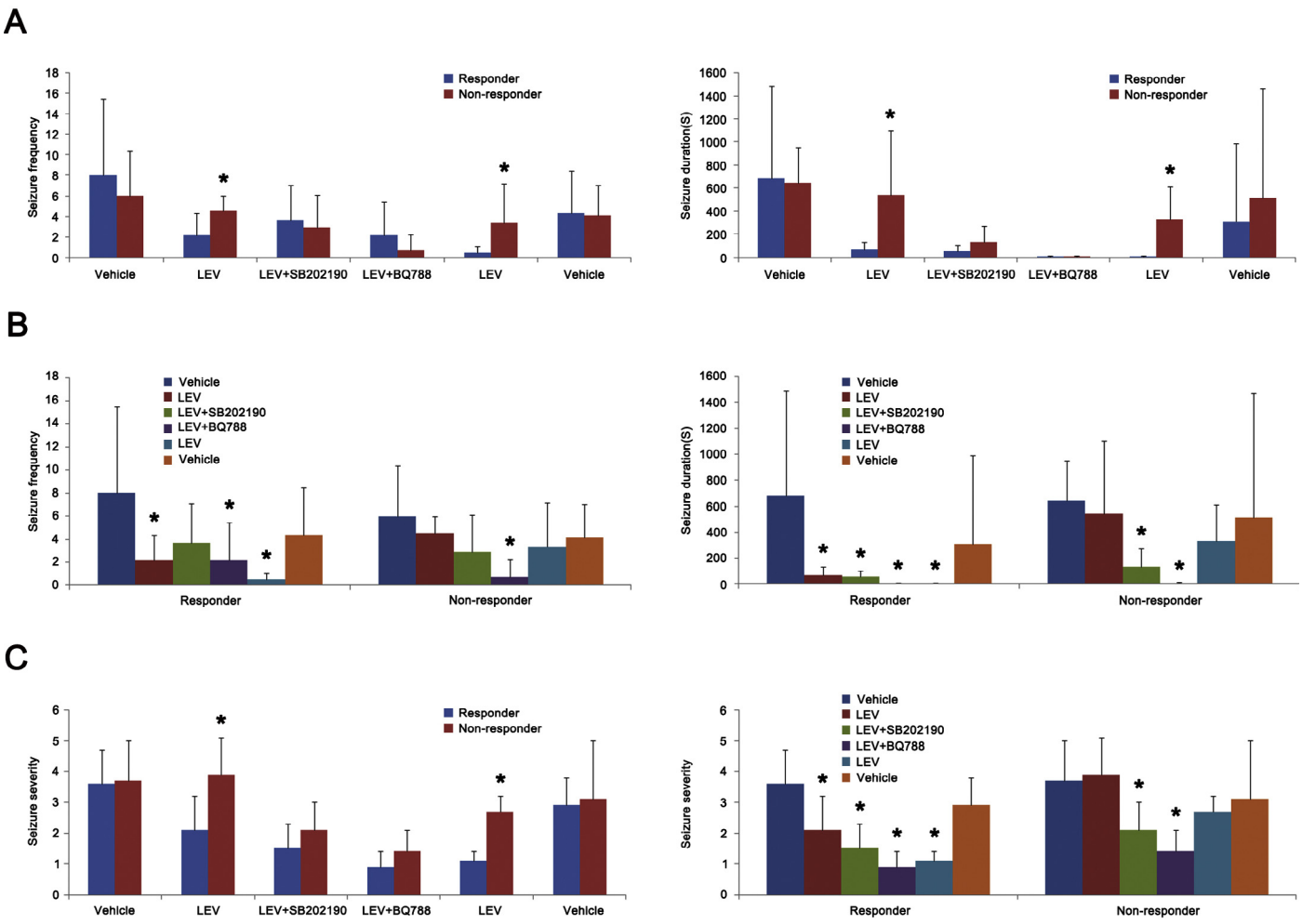

Fig. 5. The effect of LEV and co-administration with SB202190 or BQ788 on spontaneous seizure activity in epileptic rats. (A) Quantitative values (mean ± S.D.) of mean seizure frequency (left) and total seizure duration (right) during 2 h of recording a day in responders and non-responders. $^{*}p < 0.05$ vs. responders. (B) Quantitative values (mean ± S.D.) of effect of LEV and co-administration with SB202190 or BQ788 on mean seizure frequency (left) and total seizure duration (right) during 2 h of recording a day in responders and non-responders. $^{*}p < 0.05$ vs. vehicle. (C) Quantitative values (mean ± S.D.) of seizure severity in responders and non-responders (left) and quantitative values (mean ± S.D.) of effect of LEV and co-administration with SB202190 or BQ788 on seizure severity in responders and non-responders (right). $^{*}p < 0.05$ vs. responders (left) or $^{*}p < 0.05$ vs. vehicle (right).

substrates of MDR3 remain unclear, our findings indicate that up-regulation of MDR3 expression may play an important role in the pharmacoresistance to LEV in epileptic rats. The present data also point to a specific role of PGP over-expression and its hyperactivity as a limiting factor in epilepsy pharmacotherapy. Thus, inhibition of PGP expression or its activity may be one of the therapeutic targets for preventing the development of pharmacoresistent epilepsy and improving AED therapy.

Because the ET1-ET_B receptor axis regulates PGP expression and transport activity [11–15], we hypothesized that over-expression of PGP may be one of the undesirable consequences from prolonged ET_B receptor activity through a positive feedback loop

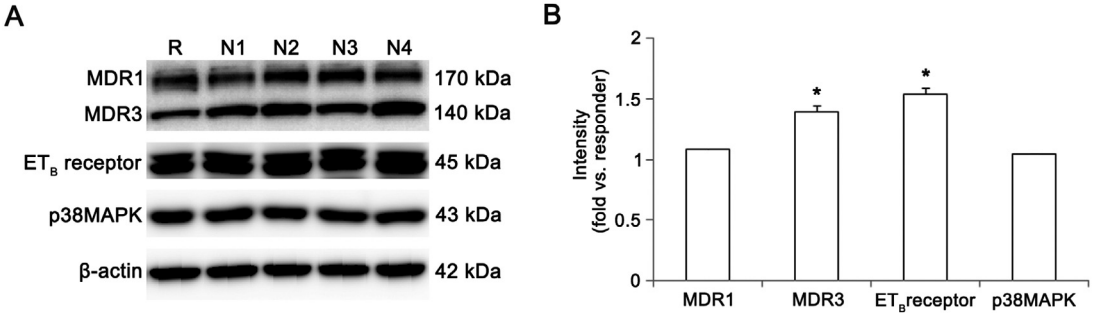

Fig. 6. PGP, ET_B receptor and p38MAPK expression in responders and non-responders. (A) Western blot shows the up-regulation of MDR3 and ET_B receptor expressions in non-responders (N) compared to responders (R). Arabic number indicates an individual animal number. (B) Quantitative values (mean ± S.E.M.) of PGP, ET_B receptor and p38MAPK expression in non-responders. $^{*}p < 0.05$ vs. responders.

between ET-1 and the ET_B receptor following SE [5,23–26]. In the present study, neither SB202190 nor BQ788 affected the spontaneous seizure activity in epileptic rats. The present data also showed that BQ788 treatment did not affect MDR1, MDR3, ET_B receptor or p38MAPK expression in the hippocampus of control and epileptic animals compared to vehicle. However, both LEV + SB202190 and LEV + BQ788 reduced seizure activity in non-responders to LEV alone. Therefore, our findings indicate that both BQ788 and SB202190 may enhance the efficacy of LEV by inhibiting PGP activity rather than its expression, although ET_B receptor or p38MAPK itself may not be involved in ictogenesis in chronic epilepsy rats.

Recently, we reported that LEV has no protective effect against SE-induced vasogenic edema formation [39], although LEV has anti-inflammatory properties [30]. Bauer et al. [40] reported that tumor necrosis factor-α (TNF-α) induces ET-1 release and action through ET_A and ET_B receptors, nitric oxide synthase, protein kinase C and nuclear factor-κB (NF-κB) and finally increased PGP expression and transport activity. Similarly, we have reported that pilocarpine-induced SE results in vasogenic edema via TNF-α/ET-1-mediated p65-Thr 485 NF-κB phosphorylation [5,41], which is one of the risk factors in pharmacoresistent epilepsy [42]. In addition, p38MAPK is one of the downstream signaling molecules for ET-1-mediated signal transduction [43,44], and p38MAPK activation increases PGP activity in various cancers [45–47]. Therefore, the inflammatory responses induced by seizure activity may raise the possibility of the development of refractory epilepsy via increased PGP expression and/or its activity. With respect to these previous reports, it is likely that add-on of BQ788 or SB202190 may enhance the anti-inflammatory properties and AED efficacy of LEV through the inhibition of ET-1/p38MAPK-mediated inflammatory signals in the epileptic hippocampus.

Unexpectedly, the present study shows that LEV + SB202190 did not affect seizure frequency in responders compared to vehicle. Because p38MAPK involves seizure tolerance (preconditioning), which is a phenomenon where brief seizures reduce the subsequent seizure severity [48], the fact that SB202190 had no effect on responders to LEV may result from the reduction in p38MAPK-mediated seizure tolerance. Further research would be needed to elucidate the role of p38MAPK in the regulation of seizure tolerance.

In conclusion, we demonstrated that co-treatment of BQ788 with LEV reduced seizure frequency and duration in chronic epileptic rats showing no response to LEV alone. In contrast, co-application of SB202190 decreased seizure duration only. These findings indicate that the ET_B receptor function may involve the development of pharmacoresistant epilepsy. To the best of our knowledge, the present study is the first indication of the role of the ET_B receptor in PGP related to refractory seizures. Therefore, the ET_B receptor will be an important therapeutic target for intractable epilepsy.

Conflict of interest

The authors declare that they have no conflict of interest.

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