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Phosphoglycerate kinase (PGK) 1 succinylation modulates epileptic seizures and the blood-brain barrier

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Abstract: Epilepsy is the most common chronic disorder in the nervous system, mainly characterized by recurrent, periodic, unpredictable seizures. Post-translational modifications (PTMs) are important protein functional regulators that regulate various physiological and pathological processes. It is significant for cell activity, stability, protein folding, and localization. Phosphoglycerate kinase (PGK) 1 has traditionally been studied as an important adenosine triphosphate (ATP)-generating enzyme of the glycolytic pathway. PGK1 catalyzes the reversible transfer of a phosphoryl group from 1, 3-bisphosphoglycerate (1, 3-BPG) to ADP, producing 3-phosphoglycerate (3-PG) and ATP. In addition to cell metabolism regulation, PGK1 is involved in multiple biological activities, including angiogenesis, autophagy, and DNA repair. However, the exact role of PGK1 succinylation in epilepsy has not been thoroughly investigated. The expression of PGK1 succinylation was analyzed by Immunoprecipitation. Western blots were used to assess the expression of PGK1, angiostatin, and vascular endothelial growth factor (VEGF) in a rat model of lithium-pilocarpine-induced acute epilepsy. Behavioral experiments were performed in a rat model of lithium-pilocarpine-induced acute epilepsy. ELISA method was used to measure the level of S100β in serum brain biomarkers' integrity of the blood-brain barrier. The expression of the succinylation of PGK1 was decreased in a rat model of lithium-pilocarpine-induced acute epilepsy compared with the normal rats in the hippocampus. Interestingly, the lysine 15 (K15), and the arginine (R) variants of lentivirus increased the susceptibility in a rat model of lithium-pilocarpine-induced acute epilepsy, and the K15 the glutamate (E) variants, had the opposite effect. In addition, the succinylation of PGK1 at K15 affected the expression of PGK1 succinylation but not the expression of PGK1total protein. Furthermore, the study found that the succinylation of PGK1 at K15 may affect the level of angiostatin and VEGF in the hippocampus, which also affects the level of S100β in serum. In conclusion, the mutation of the K15 site of PGK1 may alter the expression of the succinylation of PGK1 and then affect the integrity of the blood-brain barrier through the angiostatin / VEGF pathway altering the activity of epilepsy, which may be one of the new mechanisms of treatment strategies.

Key words: angiostatin, epilepsy, phosphoglycerate kinase (PGK) 1, succinylation, vascular endothelial growth factor (VEGF)

Introduction

Epilepsy is the most common chronic disorder in the nervous system, mainly characterized by recurrent, periodic, and unpredictable seizures [1]. Epilepsy is a global public health problem affecting people worldwide [2, 3]. According to statistics, the global prevalence rate is about 0.7% (there are about 70 million people with epilepsy worldwide, with nearly 90% residing in low- and middle-income countries with limited medical re-

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sources [4]) in the world, and thereby accounts for about 0.75% of the global burden of disease [5]. It is estimated that 70% of patients have been effectively controlled, but about 30% of patients with epilepsy still suffer from intractable seizures, despite treatment with antiepileptic drugs [6]. Then it is of great significance to explore the mechanism of epilepsy to provide new therapeutic targets.

Post-translational modifications (PTMs) are functional modifications to regulate protein enzymatic activity in the specific amino acid residues, such as phosphorylation, acetylation, ubiquitination, succinylation, and glycosylation, allowing cells to respond rapidly to changes in the environment [7–9]. Over 450 PTMs have been identified to date [10]. Protein succinylation is a biochemical reaction in which a succinyl group (-CO-CH2-CH2-CO-) is attached to a protein molecule's lysine (K) residue. K succinylation plays an important regulatory role in living cells [11]. The succinyl group changed the charge on the modified residues from +1 to −1, and the charge changes were greater than acetylation, including charge changes (+1 to 0) [12, 13], which, in turn, leads to greater changes in the structure and function of succinylated proteins. Thus, K succinylation may regulate other novel and complex cellular activities [14]. Moreover, succinylated proteins are abundant in the mitochondrial metabolism [15]. Komine-Abe *et al.* reported that the protein acylation at K replaced this residue with arginine (non-acylation mimic, R) glutamate (succinylation mimic, E) [16] (Fig. 1).

As well-known, phosphoglycerate kinase (PGK) 1 is the first adenosine triphosphate (ATP)-generating glycolytic enzyme in the aerobic glycolysis pathway. PGK1 catalyzes the conversion of 1, 3-diphosphoglycerate to 3-phosphoglycerate [17]. In addition to cell metabolism regulation, PGK1 is involved in multiple biological activities, including angiogenesis, autophagy, and DNA repair. Because of its multi-faceted functions, high intracellular expression of PGK1 leads to cell proliferation. However, the high extracellular expression of PGK1 suppresses angiogenesis [18]. In addition, PGK1 is found to participate in the angiogenic process as a disulfide reductase [19].

Moreover, Boyd *et al.* reported that when the expression of PGK1 is insufficient, its participation in the glycolysis pathway is not effective, resulting in a decreased production of ATP and impaired neurological function, and they speculated that the abnormal secretion of PGK1 may affect neuronal development [20, 21]. Phosphorylation and acetylation are two aspects of PGK1 that have been studied deeply. In recent years, more and more PTMs have been found on PGK1, and succinylation is one of them [12, 22]. PGK1 has two functional domains: a catalytic domain; and a nucleotide-binding domain [23]. PGK1 is a multifunctional protein. In addition to cell metabolism regulation, it is involved in multiple biological activities, including angiogenesis, autophagy, and DNA repair [18]. The PGK1 is found to participate in the angiogenic process and exhibits disulfide reductase activity by reducing the disulfide bond of plasmin [24], which the over-expression causes an increase of angiostatin [21].

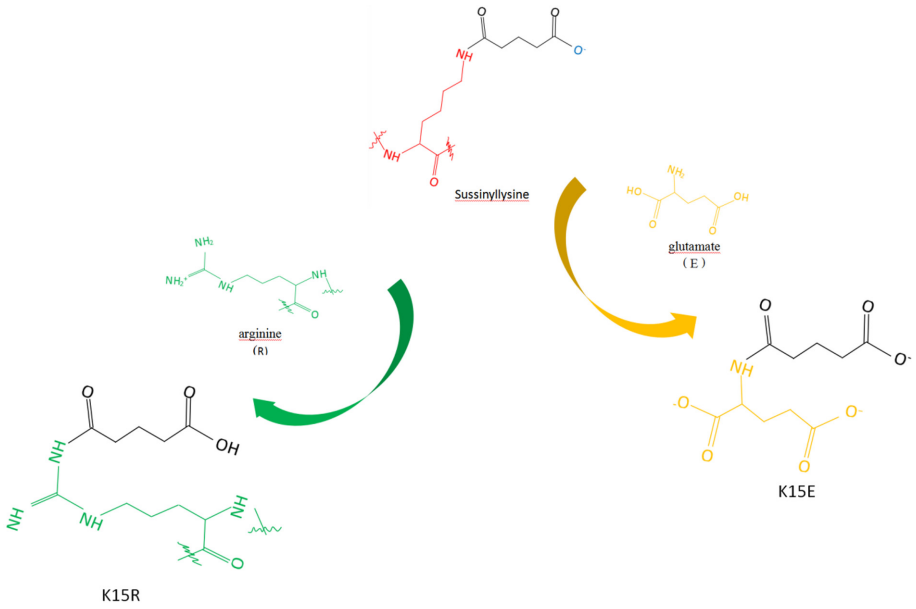

Fig. 1. The protein acylation at K replaced this residue with arginine (non-acylation mimic, R) glutamate (succinylation mimic, E).

In preclinical and clinical studies, the altered blood-brain barrier has recently emerged as an important research direction for contributing to seizure genesis and resistance to antiseizure drugs in epilepsy [25, 26]. The blood-brain barrier is a dynamic, highly selective, semipermeable interface primarily formed by endothelial cells connected by tight junctions. The blood-brain barrier plays an important role in maintaining the internal environment's stability and regulating the central nervous system [26, 27]. The Blood-brain barrier dysfunction, a hallmark of brain injury, is a prominent manifestation of status epilepticus [28]. The dysfunction of the blood-brain barrier was shown to have a role in astroglial dysfunction, neuroinflammation, increasing neural excitability, reduction of seizure threshold, excitatory synaptogenesis, impaired plasticity, and epileptogenesis [28]. The destruction in the integrity of the blood-brain barrier (mainly located in the hippocampus, thalamus, or amygdala [29]) was first proposed by Cornford in 1986 [30].

Preliminary work of our team: succinylated proteins were identified by non-standard quantitative omics analysis in the hippocampus of rats. The study identified 3829 succinylation sites on 892 proteins, mainly in the glycolysis pathway. Subsequently, we performed a systematic bioinformatics analysis of the proteins. One is PGK1, one of the most important proteins in the glycolysis pathway. We discovered that the expression of PGK1 succinylation was down-regulated, but there was no change in the expression of PGK1 total protein in the hippocampus of pilocarpine-induced epileptic rats. 26 putative succinylation sites on PGK1 were identified by mass spectroscopy, and among these sites, only the K15 succinylation level of PGK1 is the most important in modal rats by absolute stoichiometry. Based on the above basis, we hypothesized that PGK1 succinylation might regulate the integrity of the blood-brain barrier to participate in epilepsy.

Materials and Methods

Construction of plasmids and lentivirus

To confirm whether PGK1 is succinylated, we mutated K15 to arginine (K15R) to non-acylation mimic, and glutamic acid (K15E) succinylation mimics PGK1 activity. The plasmid and lentivirus (Supplementary materials) packaging was constructed by Beijing Syngentech Co., Ltd. (Beijing, China). The PGK1 succinylated differential modification site of position K15 was mutated by CRISPR/CAS9, and the vector carried puro resistance and green fluorescent plasmid Flag-EGFP [31].

Total DNA was extracted from the *E. coli* colonies following the manufacturer's protocols (OMEGA, Beijing, China) and stored at −20°C to prevent degradation. The DNA concentration was measured at 280 nm (A280) with DNA/RNA purity and concentration measuring instruments. The A260/A280 ratio (1.7–1.9) was used to estimate the purity (85–95%) of the extracted DNA [32].

Cell culture and transfection

Cell line HEK293 was obtained from ZhongQiao (Shanghai, China). In a humidified cell incubator at 37°C containing an atmosphere of 5% CO_2, the cell line was conventionally incubated in Dulbecco's Modified Eagle Medium (ZhongQiao, Shanghai, China) contained 10% fetal calf serum and 1% penicillin and streptomycin. All experiments were performed on cells from passages 4–8 [33].

Initially, HEK293 cells were seeded into a 60 mm dish. Then the wild-type and mutant PGK1 plasmids DNA were transfected into HEK293 cells using lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to manufacturer instructions. The transfection steps were prepared according to the report of Liu *et al.* [34]. The GFP fluorescence was estimated as the transfection efficiency of plasmids under a fluorescence microscope. Every transfection was assayed in triplicate, and each transfection experiment was performed at least thrice. Western blot assays were performed 48 h later [35].

Rat and model

The care and experimentation of the rats were carried out according to an approved agreement by the Institutional Animal Care and Use Committee of Zunyi Medical University (KLLY (A)-2020-084), which follows to National Institutes of Health guide (NIH) guidelines. Male Sprague-Dawley rats (6–8 weeks old) were provided with a commercial diet and water *ad libitum* under controlled temperature (22 ± 2°C), humidity (55 ± 5%), and lighting (12:12 light/dark cycle with lights) conditions. Every effort was made to minimize the number of animals used [36].

Epilepsy was induced in rats with lithium-pilocarpine, as previously described [37, 38]. Sprague-Dawley rats (weighing approximately 220 g) were intraperitoneally injected with lithium (127 mg/kg, Sigma-Aldrich, St. Louis, MO, USA). After 18–20 h, pilocarpine hydrochloride (30 mg/kg, Sigma) was intraperitoneally injected, and seizure activity was scored using Racine's scale in 30 min. Only animals whose seizure activity reached scales IV and V were included in the analysis. Seizure activity was terminated by anesthetizing animals with diazepam [39]. Before 30 min pilocarpine injection,

atropine sulfate (1 mg/kg, Sigma) was applied to decrease peripheral cholinergic side effects. Normal animals received saline in place of pilocarpine [40].

Behavioral seizure severity was evaluated according to the Racine scale [40–42]:

- Phase 0: No response
- Phase I: Mouth and facial movement
- Phase II: Head nodding
- Phase III: Forelimb clonus
- Phase IV: Rearing with forelimb clonus
- Phase V: Rearing and falling with forelimb clonus

When phase V was identified, status epilepticus (SE) was considered [43]. Only animals that reached stage IV and presented recurrent seizures were used for further experiments. Different time points of the pilocarpine model were analyzed: 30 min after pilocarpine injection and then 5 min, 15 min, 30 min, and 45 min after the SE onset.

Rats were sacrificed at different times after SE. For Immunoprecipitation and Western blotting, the hippocampus was frozen in liquid nitrogen and then stored at −80°C for later use.

For ELISA and Western blotting, the serum and the hippocampus were immediately frozen in liquid nitrogen and then stored at −80°C for later use. For immunofluorescence analysis, the brain tissues were fixed with 4% paraformaldehyde and then incubated in 20% and 30% sucrose in PBS for 24 h each. The tissues were then sectioned at 10 μm and stored at −20°C for later experimentation.

Lentiviral vector injection

Two-three weeks before inducing SE, the rats were anesthetized and fixed in a stereotactic instrument rats (RWD, Shenzhen, China). Ten microliters of lentiviral particles were bilaterally injected into the dorsal blade of the CA1 area of adult rats through a Hamilton syringe (10 μl, RWD). According to "The Rat Brain" by George Paxinos and Charles Watson, determine the injection coordinate location. AP (3.72 mm), ML (2.0–2.2 mm), DV (2.8–3.0 mm). According to the report of Wang *et al.*, the dosages of viral solutions having optimal effects were decided [44]. The administration speed was 0.5 μl/min. Two-three weeks after virus administration, all surviving animals were used in the next experiment [45].

Western blotting

After the hippocampi were separated, they homogenized with ice-cold RIPA buffer (Solaibio, Beijing, China) containing PMSF (Solaibio) and Protease inhibitor (BOSTER, Wuhan, China) (100:1:1). After 20 min of ice lysis, the supernatant was collected by centrifugation at 4°C in a High-Speed Tabletop Refrigerated Centrifuge (Eppendorf, Germany). The protein concentration was measured using a BCA kit (Biyuntian, Shanghai, China).

Protein samples (40 μg each) were isolated by 10% SDS-PAGE (Beyotime, Jiangsu, China) and transferred to PVDF membranes (Beyotime). After blocking with skim milk (Sangong, Shanghai, China) in Tris-buffered saline containing Tween 20 (TBST) (Solarbio) at room temperature (RT, usually taken as being around 20°C) for 1 h in the shaker (Kylin-bell, Haimen, China). Then membranes were incubated with rabbit anti-PGK1 (1:1,000, ProteinTech Group, Chicago, IL, USA, GTX107614), mouse anti-PGK1 (2 μg per 100–500 μg of total protein, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-130335), rabbit anti-angiostatin (1:1,000, Beijing Biosynthesis Biotech Co., Ltd., Beijing, China; bs-1828R), rabbit anti-succinyllysine (1:1,000, Protein Post-translational Modification Biolab, Hangzhou, China, PTM-401), mouse anti-Flag (1:1,000, ZEN BIO, Chengdu, China, 390002), mouse anti-β-Tubulin (1:5,000, ZEN BIO, 200608), rabbit anti-VEGF (1:1,000, Wanleibio, Shenyang, China, WL000096) antibodies overnight at 4°C. After the membranes were incubated with HRP-conjugated secondary antibodies in TBST at RT or 1 h in the shaker, the membranes were dyed with ECL reagent (Thermo Fisher Scientific, Waltham, MA, USA) and developed using autoradiography film in the motored molecular imaging system (BIO-RAD, Hercules, CA, USA). Data quantification was done through Image J software.

Immunoprecipitation (IP)

Total lysates were prepared as described above. For Immunoprecipitation experiments, the cells were lysed with an immunoprecipitation lysis buffer containing protease inhibitors and phosphatase inhibitors cocktail, and then measured the protein concentration in a BCA kit (Beyotime). Protein A/G Magnetic beads (MCE, Shanghai, China) were incubated with anti-PGK1 antibody and rotated overnight at 4°C in a Four-dimensional rotary mixer. After being washed 3 times, Protein A/G beads were incubated with cell lysates and rotated at RT for 2 h in a Four-dimensional rotary mixer. After being washed with Immunoprecipitation lysis buffer, the beads with 1×SDS loading buffer (Solarbio) were boiled for 5–10 min. Eluted proteins were used for Western blotting analysis.

ELISA

The S100β level was detected using a kit from Jianglai (Shanghai, China). According to the merchant's in-

structions, check the level of S100β in the serum of rats.

Detection of ATP

The ATP level was detected using a kit from Solarbio. After being transfected, the cells were incubated in MEM containing 10% FBS for 48 h. After the cells were collected, the ATP level of the cell was detected with Ultraviolet spectrophotometry assays (Thermo Fisher Scientific). The calculation formula is ATP content (μmol / 10^6 cells) = $0.125 \times \Delta A_{Determination} \div \Delta A_{Standard}$.

Statistical analysis

IBM SPSS statistics Version 18.0 was used for statistical analysis of all. Data are presented as the means ± SEM. Statistical analysis was performed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Fisher's test. Differences were considered statistically significant at $P<0.05$.

Results

The expression level of the succinylation of PGK1 and the PGK1

Normal group rats received the same treatment with saline. Epilepsy seizures occurred in 95 rats (no occurred in 5 rats), and 13 of them died in the acute phase, and the survival rate of rats was up to 86%.

To investigate the possible role of the succinylation of PGK1 in epilepsy development and progression, we analyzed the succinylation of PGK1 protein expression levels in normal/epilepsy hippocampal tissue pairs at different point-in-time in rats. In Fig. 2a, rat brain homogenates seem to be immunoprecipitated withanti-PGK1 antibody, and the precipitates were subjected to Western blotting withanti-PGK1 antibody and anti-succinyllysine antibody. The succinylation of PGK1 protein expression levels gradually decreased, and there was a significant difference with the normal hippocampal tissues at 45 min (Fig. 2a). In addition, we compared the expression of PGK1 total protein. The expression of PGK1 total protein was not always significant compared with the normal tissues (Fig. 2b). Suggesting that epilepsy may contribute to the succinylation of PGK1 protein expression levels but not the expression of PGK1 total protein.

Transfected *in vivo* and *in vitro*

Previously, using a rat model of lithium-pilocarpine hydrochloride-induced epilepsy, hippocampus tissue was examined using LC-MS/MS and bioinformatics analysis.

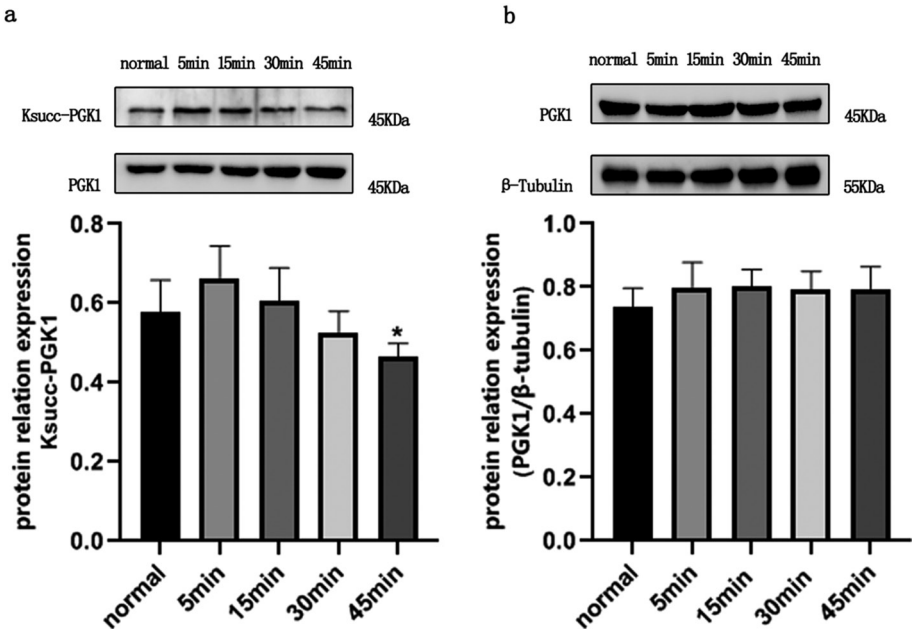

Fig. 2. Decreased succinylation of phosphoglycerate kinase (PGK) 1 protein expression levels but not PGK1 is decreased in a rat model of lithium-pilocarpine-induced acute epilepsy. (a) Representative Immunoprecipitation showed significantly decreased succinylation of PGK1 protein expression levels in the hippocampus of the rat model compared with normals (n=3 per group). (b) PGK1 expression levels were not significantly changed in the hippocampus of the rat model compared with normal rats (n=4 per group). All the succinylation of PGK1 protein expression levels were normalized by calculating the OD ratio of succinylation-PGK1 to PGK1 (Ksucc-PGK1/PGK1). All expression levels of PGK1 were normalized by calculating the OD ratio of PGK1 to β-tubulin (PGK1/β-tubulin) (*$P<0.05$).

The PGK1 of the glycolysis pathway demonstrated the succinylation of multiple sites. K15 of these is the most significant of the differential modification site. To investigate whether the succinylation of PGK1 is modified at the K15 site, we transfected HEK293 cells with GFP-fused the wild type, the scramble (scramble DNA of PGK1, a genome-scale tool to generate random structural variations (synthetic chromosome rearrangement and modification by loxPsym-mediated evolution)) [46], the K15R variants, and the K15E variants constructed to track the cellular distribution of this plasmid (Fig. 3a). The rats' brains were transfected *in vivo* via injection with GFP-fused scramble, K15R variants, and K15E variants to track the hippocampus tissues of PGK1 (Fig. 3b).

Cellular PTMs are highly dynamic in response to environmental stimuli [9]. Thus, we investigated whether the PGK1 activity was also dynamically regulated when the K15R non-acylation mimic mutation or the K15E

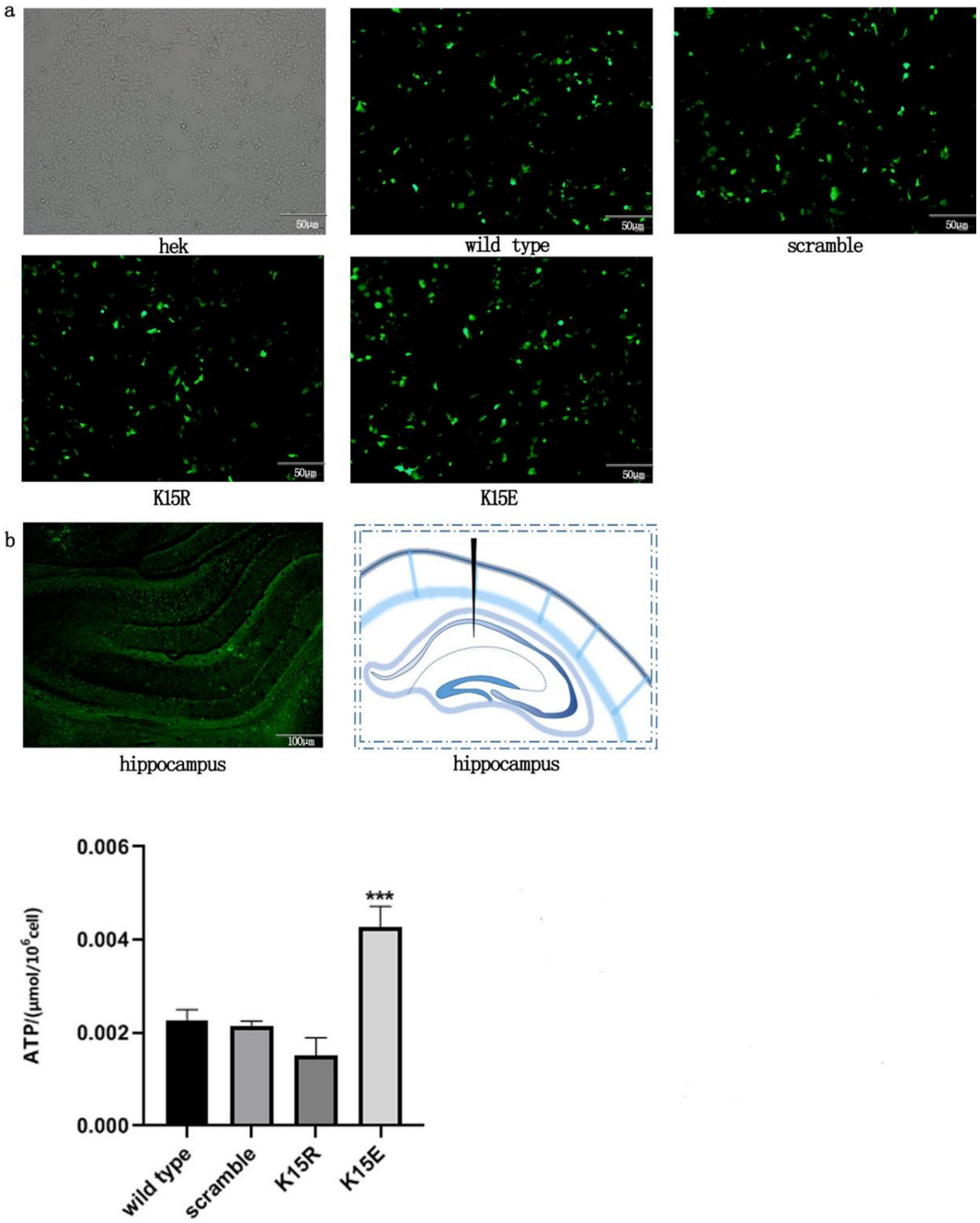

Fig. 3. *In vivo* and *in vitro* transfection. (a) Localization of the plasmid with GFP-fused the wild type, the scramble, the K15R variants, and the K15E variants in HEK293 cells. (b) Localization of the lentivirus with GFP-fused the scramble, the K15R variants, and the K15E variants in rats' hippocampus (CA1). (c) After being transfected with plasmids of the wild type, the scramble, the K15R variants, and the K15E variants in HEK293 cells, the ATP level of cells was detected with Ultraviolet spectrophotometry assays (**P*<0.05,****P*<0.01).

succinylation-mimic mutation (Fig. 3c). PGK1, the first ATP-generating enzyme in glycolysis, catalyzes the conversion of 1,3-diphosphoglycerate (1,3-BPG) to 3- phosphoglycerate (3-PG) and produces one molecule of ATP. The catalytic process is accompanied by the conversion between the open and closed conformation of PGK1. Liu *et al.* demonstrated that PGK1 in the closed conformation first releases the product ATP to reach a semi-open conformation and releases the product 3PG to achieve the fully open conformation, which could accept new substrates ADP and 1,3BPG for the next cycle. The PTMs could also regulate the ligands binding/releasing with different effects. For example, the phosphorylation of PGK1 at T243 modifications caused the exposure of the ADP/ATP binding site, which was beneficial for the substrates/products binding/releasing of PGK1 [47]. In addition, PGK1 activity is also mediated by different post-translational modifications (acetylation, O-GlcNAcylation *et al.*) [33]. However, the dynamic collaboration mechanism between the PGK1 conformation transition and the products releasing process remains poorly understood. The ATP level increased in K15E variants compared to the condition of the normal and scrambled in our experiment. Together these data show that the functional activity of PGK1 may be dynamically regulated in the succinylation of PGK1 at K15.

The succinylation of PGK1 protein expression levels is regulated at K15 *in vivo* and *in vitro*

Firstly, we analyzed HEK293 cells stably expressing the wild type, the scramble, K15R variants, and the K15E variants rescue construct. Immunoprecipitating showed the results (Fig. 4a). Wang *et al.* found that substitution at K by arginine substantially reduced PGK1 enzyme activity [48]. Leila *et al.* found that the lysine residues were replaced by glutamic acid to lead to increased thermostability [49]. As expected, the succinylation of PGK1 protein expression levels was decreased in K15R variants and increased in K15E variants by transfecting plasmid in HEK293 cells, compared with the normal and the scramble. Meantime, we compared the expression of PGK1 total protein (Fig. 4b). The expression of PGK1 total protein was not significant in the groups of K15R variants or K15E variants compared with both the wild-type group and the scramble group. Secondly, we analyzed the succinylation of PGK1 protein expression in the rats with lentivirus of the scramble (the scramble vectors express GFP under the control of the same promoter that was used for the K15R variants and the K15E variants), the K15R variants, and the K15E variants and a rat model of lithium-pilocarpine-induced acute epilepsy (lasted 45 min after successful modeling) (Fig. 4c). The results were keeping with the *in vitro* data, and the succinylation of PGK1 protein expression levels significantly decreased in the K15R variants, and increased in the K15E variants, compared to the rats with the scramble. Meantime, the rats transfected with the lentivirus and a rat model of lithium-pilocarpine-induced acute epilepsy were analyzed for the expression of PGK1 total protein in every group. It was observed to be no significance (Fig. 4d).

The succinylation of PGK1 is important for epilepsy

To better understand the impact of the succinylation of PGK1 on epilepsy. Two-three weeks after virus administration. We observed the changes of behavior (which lasted 60 min after successful modeling) in the rats with lentivirus of the scramble (the scramble vectors expressed GFP under the control of the same promoter that was used for the K15R variants and the K15E variants), the K15R variants and the K15E variants and a rat model of lithium-pilocarpine-induced acute epilepsy (Fig. 5). We found that: on the one hand, the K15R variants showed a shorter latency period, and the K15E variants displayed a longer latency period in rats compared to their corresponding the wild type and the scramble (Fig. 5a); on the other hand, the K15R variants showed more seizures, and the K15E variants displayed fewer seizures in rats compared to their corresponding the wild type and the scramble (Fig. 5b), suggesting that succinylation of PGK1 at K15 is functionally important in epilepsy.

The succinylation of PGK1 at K15 is involved in the expression of angiostatin

Next, the downstream effect of PGK1 was analyzed. In addition to cell metabolism regulation, PGK1 is involved in multiple biological activities, including angiogenesis (through reducing the disulfide bond of plasmin [24]), autophagy, and DNA repair [18]. Therefore, we investigated whether the succinylation of PGK1 at K15 also mediates angiostatin in epilepsy. In our study, we markedly showed that angiostatin expression decreased in epilepsy rats (Fig. 6a). In the presence of the K15R variants, the expression of angiostatin protein showed a lower level than in the wild type. Therefore suggests that the succinylation of PGK1 at K15 up-expression might render PGK1 activity to facilitate the cleavage of plasminogen to generate the vascular inhibitor angiostatin [19]. On the contrary, in the presence of the K15E variants, the expression of angiostatin protein showed a higher level than the wild type (Fig. 6b). In this study, we verified the effects of the K15R variants and the K15E variants in angiostatin and combined with the previous

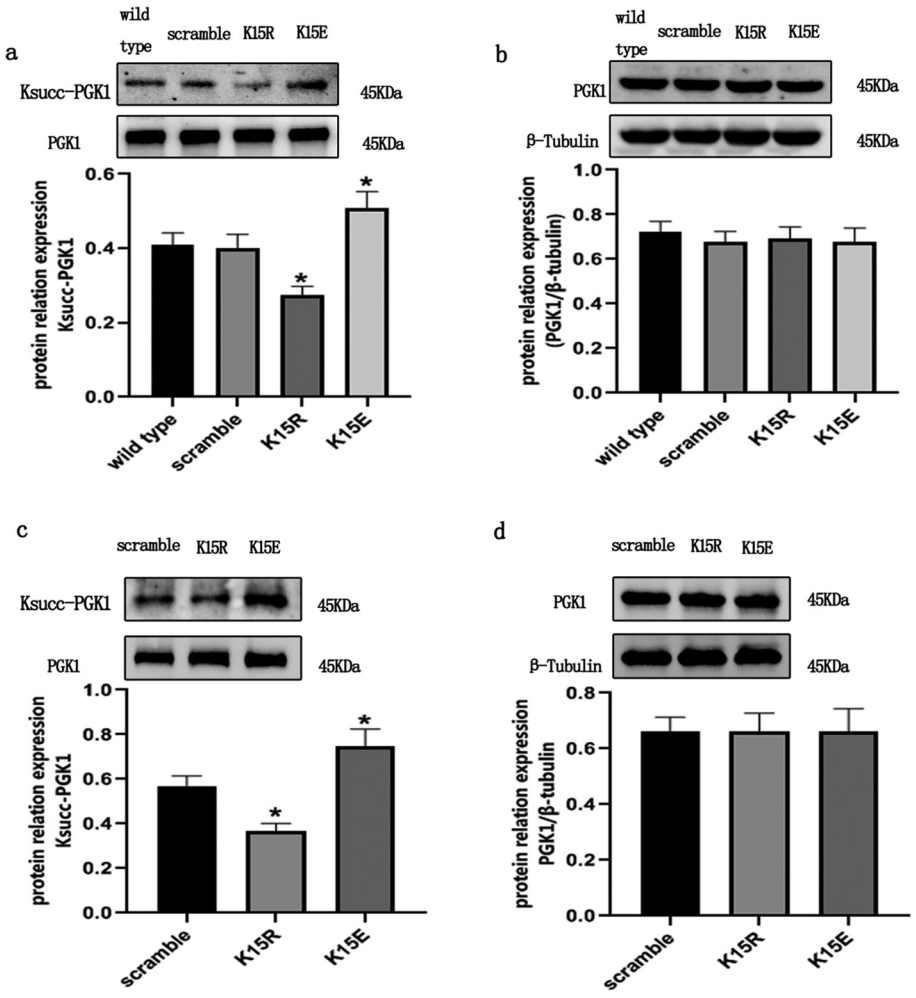

Fig. 4. The succinylation of phosphoglycerate kinase (PGK) 1 protein expression levels is regulated at K15 *in vivo* and *in vitro*. (A) Representative Immunoprecipitation shows that the succinylation of PGK1 protein expression levels was decreased significantly in the K15R variants and increased significantly in the K15E variants compared with the wild-type group by transfecting plasmid in HEK293 cells (n=3 per group). (B) Representative Western blots show that the expression of PGK1 total protein was insignificant in the groups of the K15R variants and the K15E variants compared with the wild-type group by transfecting plasmid in HEK293 cells (n=4 per group). (C) Representative Immunoprecipitation shows that the succinylation of PGK1 protein expression levels decreased significantly in the K15R variants and increased significantly in the K15E variants compared with the scramble group by transfecting with the lentivirus and a rat model of lithium-pilocarpine-induced acute epilepsy (n=3 per group). (D) Representative Western blots show that the expression of PGK1 total protein was insignificant in the groups of the K15R variants and the K15E variants compared with the scramble group by transfecting with the lentivirus and a rat model of lithium-pilocarpine-induced acute epilepsy (n=4 per group). All expression levels of the succinylation of PGK1 protein expression levels were normalized by calculating the OD ratio of the succinylation of PGK1 to PGK1 (Ksucc-PGK1/PGK1). All expression levels of PGK1 were normalized by calculating the OD ratio of PGK1 to β-tubulin (PGK1/β-tubulin)(*P<0.05).

verification of the effect of the K15R variants and the K15E variants on the succinylation of PGK1. Therefore, the results indicated that E and R modified point K's point mutations may affect angiostatin's expression.

The succinylation of PGK1 at K15 mediates VEGF and the blood-brain barrier integrity in epilepsy

Then, we explored whether the site of K15 could affect the expression of VEGF protein. Cheng *et al.* also reported that Shenmai injection promoted the normalization of tumor microvessels by increasing the antiangiogenic factor angiostatin and decreasing the pro-angiogenic factor VEGF [50]. Therefore, we investigated whether the succinylation of PGK1 at K15 also mediated the VEGF in epilepsy (Fig. 7). In our study, we found the VEGF level markedly in previous studies

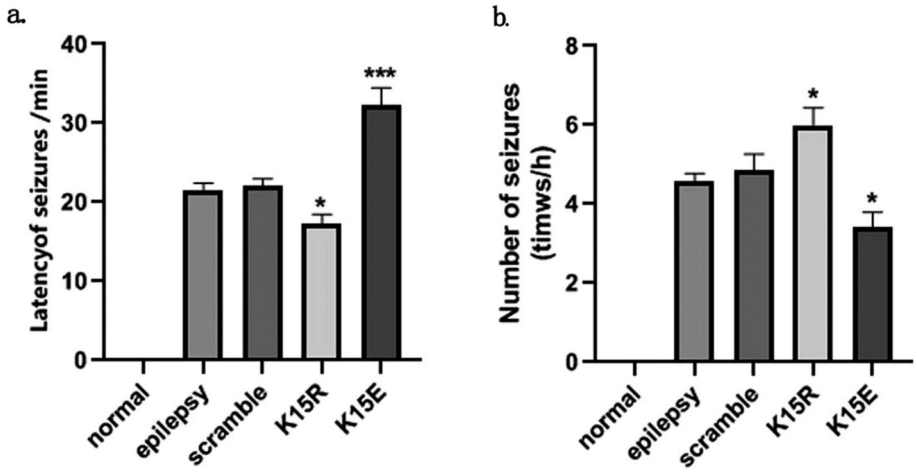

Fig. 5. The succinylation of phosphoglycerate kinase (PGK) 1 is important for epilepsy. (a) The latency in the group with the lentivirus of the K15R variants and a rat model of lithium-pilocarpine-induced acute epilepsy was significantly shorter than in the scramble, while the latency in the group with the lentivirus of the K15E variants and a rat model of lithium-pilocarpine-induced acute epilepsy was significantly longer than in the scramble (n=7 in each group). (b) Rats injected with the lentivirus of the K15R variants had more seizures than the scramble group. However, rats injected with the lentivirus of the K15E variants had fewer seizures than the scramble (n=7 in each group) (**P*<0.05,****P*<0.01).

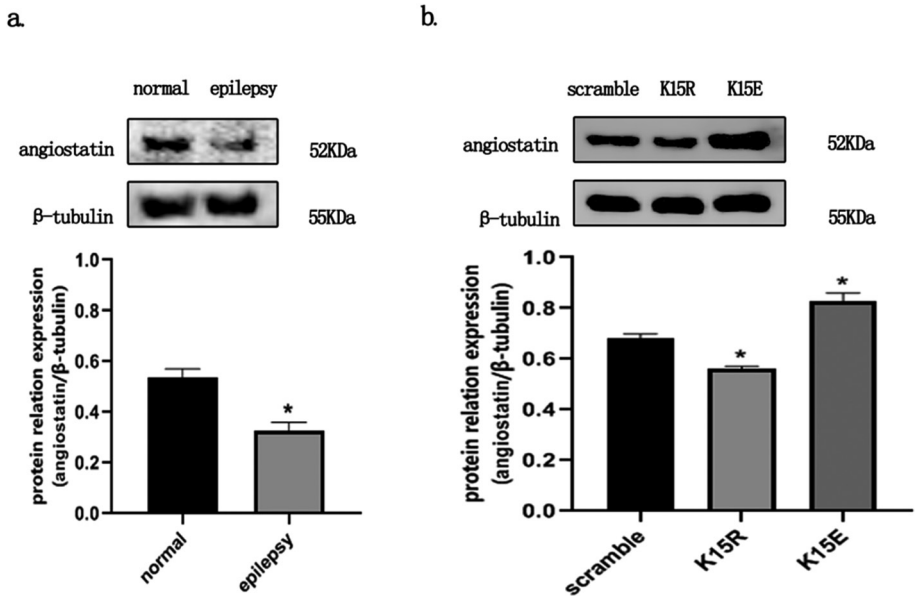

Fig. 6. The succinylation of phosphoglycerate kinase (PGK) 1 at K15 is involved in the expression of angiostatin. (a) Representative Western blots show that the expression of angiostatin total protein was decreased significantly in a rat model of lithium-pilocarpine-induced acute epilepsy compared with the normal group (n=4 per group; **P*<0.05). (b) Representative Western blots show that the expression of angiostatin total protein was decreased significantly in the K15R variants and increased significantly in the K15E variants compared with the scramble group by transfecting with the lentivirus and a rat model of lithium-pilocarpine-induced acute epilepsy (n=3 per group; **P*<0.05). All expression levels of angiostatin were normalized by calculating the OD ratio of angiostatin to β-tubulin (PGK1/β-tubulin).

increased in epilepsy (Fig. 7a). It is the same as van Lanen's research report [51]. We also found that in the presence of the K15R variants, the VEGF was significantly down-regulated compared to the wild type, but the VEGF level was significantly upregulated in the presence of the K15E variants (Fig. 7b). Thus, these data indicate that the succinylation of PGK1 at K15 maybe affects the angiostatin/VEGF passway.

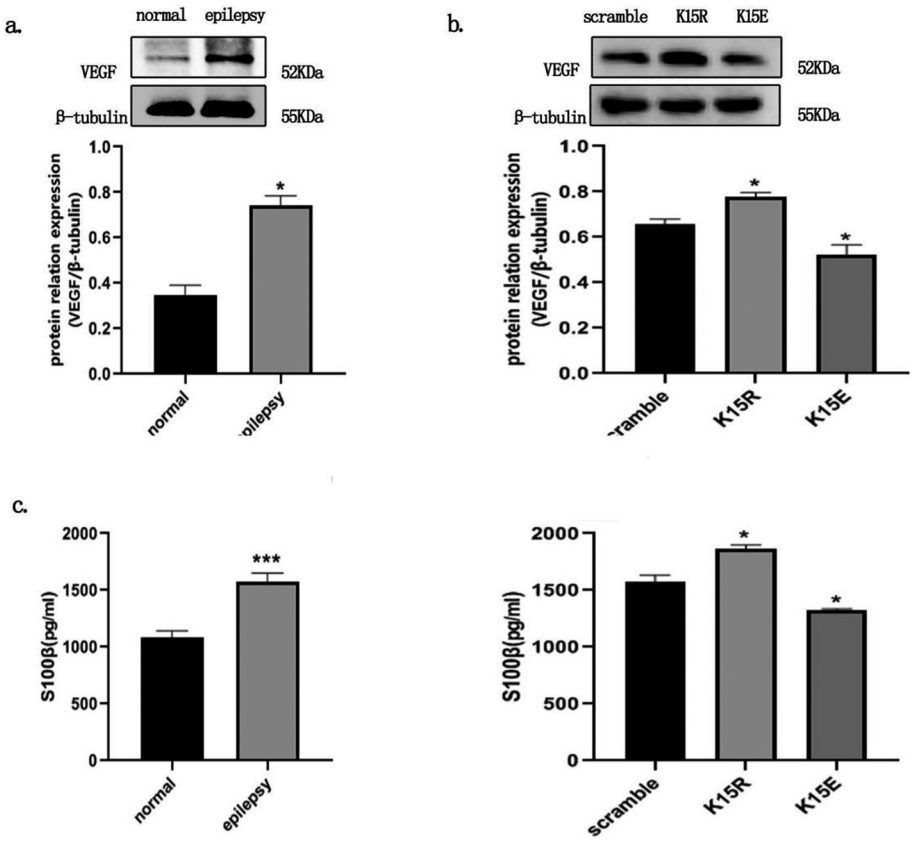

Fig. 7. The succinylation of phosphoglycerate kinase (PGK) 1 at K15 mediates vascular endothelial growth factor (VEGF) and the blood-brain barrier integrity in epilepsy. (a) Representative Western blots show that the expression of VEGF total protein was increased significantly in a rat model of lithium-pilocarpine-induced acute epilepsy compared with the normal group (n=4 per group; **P*<0.05). (b) Representative Western blots show that the expression of VEGF total protein was increased significantly in the K15R variants and decreased significantly in the K15E variants compared with the scramble group by transfecting with the lentivirus and a rat model of lithium-pilocarpine-induced acute epilepsy (n=4 per group; **P*<0.05). (c) ELISA data showed that the level of S100β in serum was increased significantly in a rat model of lithium-pilocarpine-induced acute epilepsy compared with the normal group. ELISA showed that the level of S100β in serum was increased significantly in the K15R variants and decreased significantly in the K15E variants compared with the scramble group by transfecting with the lentivirus and a rat model of lithium-pilocarpine-induced acute epilepsy (n=4 per group; **P*<0.05, ****P*<0.01). All expression levels of VEGF were normalized by calculating the OD ratio of VEGF to β-tubulin (VEGF/β-tubulin).

Finally, we explored whether the succinylation of PGK1 at K15 could alter the vessel permeability and leakage (Fig. 7c). In animal research, the level of S100β in serum of brain biomarkers following epilepsy is significantly increased as in previous studies [52]. Consistently, in the presence of the K15R variants, the S100β was significantly upregulated, and the S100β was significantly down-regulated in the presence of the K15E variants, compared to the wild type in rats. Altogether, our data suggest that the succinylation of PGK1 at K15 may be critical for the blood-brain barrier integrity in epilepsy.

Discussion

Epilepsy is a chronic and recurrent disease caused by the highly synchronized abnormal discharge of brain neurons, especially those closely related to the limbic system, such as the hippocampus and amygdala [53]. According to a meta-analysis, the annual incidence of epilepsy is approximately 67.77 per 100,000 persons; age and gender have less effect on the incidence; the incidence is quite high in low- and middle-income nations [54]. Up to 10% of people in the world may experience a seizure as a single event [55].

PTMs are protein function regulators which play an important role in the structure and function of metabolic enzymes [56]. Lysine succinylation is a naturally

occurring PTM that is ubiquitous in organisms. Lysine succinylation plays an important role in regulating protein structure, function, and cellular metabolism [57]. Emerging clinical evidence indicates that lysine succinylation dysregulation is associated with the progression of various diseases, including cancers and neurodegeneration disorders [58]. Morris-Blanco *et al.* reported that Sirtuin (SIRT) 5 is an important mitochondrial enzyme for protection against metabolic and ischemic stress following protein kinase C epsilon activation in the brain [59], suggesting that the disorder of succinylation modification is closely related to the progression of nervous system diseases. In addition, Some studies have shown that the PTMs of PGK1, including acetylation, phosphorylation, ubiquitination, and succinylation, are the key ways to regulate the activity of the PGK1 protease [7, 56, 60–64]. PGK1 catalyzes the reversible transfer of a phosphoryl group from 1, 3-bisphosphoglycerate (1, 3-BPG) to ADP, producing 3-phosphoglycerate (3-PG) and ATP. PGK1 is critical in coordinating glycolytic energy production with one-carbon metabolism, serine biosynthesis, and cellular redox regulation [65].

This study is the first to report the relationship between the succinylation of PGK1 and acute epilepsy in rats. In this paper, we found that the expression of the succinylation of PGK1 was decreased with the prolongation of seizure time gradually in a rat model of lithium-pilocarpine-induced acute epilepsy. In addition, we present the novel finding that lentivirus-mediated K15R variants and the K15E variants in the hippocampus decreased or increased the susceptibility of rats to seizures in the model. Is it possible that the decrease in succinylated PGK1 after 45 min is a consequence rather than a cause of the epileptic seizure? Anyway,the expression level of succinylation can affect seizures. We want to prove that the expression level of succinylation can modify seizures.

In vitro and *in viv*o experiments, the mutation of PGK1 at K15 altered the expression of the succinylation of PGK1. *In vivo, t*he Immunoprecipitation and the Western blot data in a rat model of lithium-pilocarpine-induced acute epilepsy are consistent with the data in the HEK293 cells. The mutation at the K15 site of PGK1 did not affect the total protein level of PGK1. However, when the K15 site of PGK1 mutates R, the expression of the succinylation of PGK1 decreases. When the K15 site of PGK1 mutates E, expression of the succinylation of PGK1 increases. The decrease in succinylated PGK1 after 45 min probably due to seizures. Anyway, changing the expression level of succinylation can affect seizures. We want to prove that changing the expression level of succinylation can modify seizures.

Amino acids in the construction of biological organisms are basic materials which constitute the main elements of which proteins and peptides are made, and thus play an essential part in regulating the whole metabolism of living organisms [66]. L-lysine (low polarity and positive at neutral pH) and L-arginine (polar side group, positively charged at neutral pH) [67] are positively charged polar amino acids (basic amino acids), while glutamic acid (Glu) is polar negatively charged amino acids (acidic amino acids). The charge of the amino acid plays a major role in the interaction strength with the lipid bilayer membrane [68]. At the same time, it is also confirmed by literature that the mutation of K to R on the side chain by using a similar structure can simulate the non-acylation state, and the mutation of K to E can simulate the succinylation state on the side chain [16, 58]. *In vitro* experiments, the HEK293 cells were used as the tool cells. The HEK293 cell line with known neuronal developmental properties was one of the most widely used cell lines in research [69] and readily infected in a strain-dependent fashion [70].

Most notably, we found that the K15 site point of PGK1 may regulate the blood-brain barrier integrity through angiostatin/VEGF pathways to modulate seizure activity. In rodents, the blood-brain barrier integrity in seizures is impaired within hours and lasts for weeks [71]. Epilepsy has been associated with a dysfunction of the blood-brain barrier integrity. Unfortunately, whether the PGK1 succinylation is involved in seizure through the blood-brain barrier integrity has not been reported. Many studies suggest that PGK1 is also a protein kinase, which involves angiogenesis by reducing the disulfide bond of the plasmin, and the over-expression of plasmin leads to the increase of angiostatin [21, 24]. Some studies have also shown that angiostatin plays an important role in the proliferative pathological processes, such as tumors and cardiovascular diseases [72], and is considered to be an important regulator of the “angiogenesis switch” [19]. VEGF is the central medium of vascular permeability, called the “vascular permeability factor” [73]. Previous experimental and human studies show the up-regulation of VEGF following seizures in epilepsy [74]. Studies have shown that angiostatin (angio-3, derived from the plasminogen kringle 3) can inhibit VEGF and regulate the downstream pathway of VEGF [36]. Yang *et al.* found that the upregulation of VEGF resulted in downregulation and altered distribution of tight junction proteins such as ZO-1, Occludin, and Claudin-5 [75]. VEGF is a cytokine primarily involved in angiogenesis that can destroy blood-brain barrier integrity in acute stroke [76]. VEGF contributes to endothelial cells’ proliferation, survival, migration, and progenitors [77].

Additionally, the blood-brain barrier provides the

necessary neuronal microenvironment via efficient co-operation between endothelial cells and other specialized cell types, such as pericytes and astrocytes. Equally important the blood-brain barrier components are tight junctions, which limit the blood-brain barrier integrity by sealing the interendothelial space between neighboring endothelial cells. Tight junction consists of transmembrane proteins, such as the junctional adhesion molecules occludin, claudin-3, and claudin-5, and cytoplasmic accessory proteins, such as zona occludens 1. Endothelial cells also express a variety of transporter proteins to deliver essential nutrients that cannot penetrate the brain by diffusion. S100β belongs to a family of calcium-binding proteins, expressed and released mainly by astrocytes and oligodendrocytes in the brain Serum levels of S100β inform about the blood-brain barrier integrity and function. On the other hand, it is well known that increased peripheral inflammation may contribute to the blood-brain barrier integrity [78]. For example, serum S100B protein levels were also measured in serum using a S100B kit distributed by Diasorin, Charles House, Toutley Road, Wokingham, Berkshire, run on the Liaison XL chemiluminescence analyzer [79]. Trunk blood was collected during the decapitation procedure [78]. The serum (the antecubital vein) S100β levels were measured by the following commercially available enzyme-linked immunosorbent assay [80]. The serum concentrations of S100 are considered biomarkers of the blood-brain barrier integrity. S100 showed a significantly increased postictal concentration in epilepsy [26]. The calcium-binding protein S100B is widely expressed in the brain and predominantly in astrocytes and is a specific nervous system protein [81].

The expression profiles of PGK1 and PGK2 in the Human Protein Atlas and found that among the 69 human cell lines, there were three glioma cell lines, and the mRNA level of PGK1 was significantly higher than that of PGK2. Chen *et al.* research suggested that PGK1, but not PGK2, is the responsible driver gene in glioma [82]. Martı'nez *et al.* found that immunohistochemistry revealed the localization of PGK-1 mainly in astrocytes [83]. Yang *et al.* found it can protect neurons by enhancing PGK1 activity and promoting glycolysis [84]. However, how K15 coordinate between PGK1 between the neuron and the glial cell is largely unclear.

In conclusion, the mutation of the K15 site of PGK1 may alter the expression of the succinylation of PGK1 and then affect the blood-brain barrier integrity through the angiostatin / VEGF pathway altering the activity of epilepsy (Fig. 8), which may be one of the new mechanisms of treatment strategies.

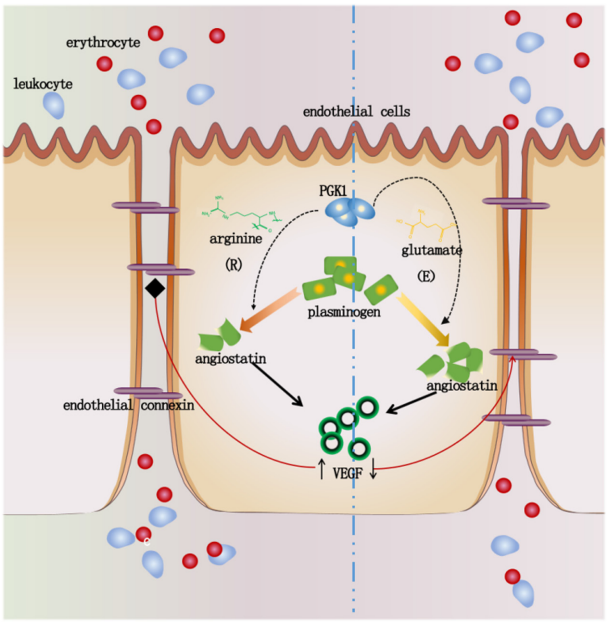

Fig. 8. Mechanism diagram of the phosphoglycerate kinase (PGK) 1 succinylation participating in the study of epilepsy and the blood-brain barrier integrity.

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