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The mechanism of neurogenic pulmonary edema in epilepsy

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Abstract Neurogenic pulmonary edema (NPE) is found in many epilepsy patients at autopsy. It is a life-threatening complication, known for almost 100 years, but its etio-pathogenesis is still not completely understood. In this study, we used the tremor rat (TRM: tm/tm) as an animal model of epilepsy to investigate the potential mechanisms of NPE under epileptic conditions. We performed reverse-phase high-pressure liquid chromatography assay, H&E and Masson staining, TUNEL assay, and Western blot experiments to determine the role of seizures in NPE. We found the level of catecholamine was higher in TRM rats. Also the occurrence of alveolar cell apoptosis was increased.

Moreover, pulmonary vascular remodeling including the deposition of collagen and medial thickening was also found in TRM rats. Further study showed that cell apoptosis was mediated by increasing Bax, decreasing Bcl-2, and activating caspase-3. In addition, the protein level of phosphory-lated ERK (p-ERK) was found to be decreased while phosphorylated JNK and phosphorylated p38 were upregu-lated in TRM rats. Thus, these findings suggest that pul-monary vascular remodeling and alveolar cell apoptosis might be involved in epilepsy-induced NPE and that the mitogen-activated protein kinase signal pathway was involved.

Keywords Epilepsy · Tremor rat · Neurogenic pulmonary edema · Apoptosis

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Introduction

Epilepsy comprises a variety of neurological disorders characterized by recurrent seizures, and it affects 1–2 % of the population worldwide [1]. There are 50 million people with epilepsy worldwide; 40 million live in developing countries [2]. The mortality of patients with epilepsy is two to three times that of the general population [3, 4]. Although most epilepsies are considered multifactorial diseases, some specific types are considered single-gene disorders [1]. Nervous system infections are the most important reason for seizures. Sudden unexpected death in epilepsy (SUDEP) is defined as a sudden, unexpected, nontraumatic, witnessed or unwitnessed death and is a major clinical problem in epilepsy patients, especially those with chronic, uncontrolled epilepsy [5]. There are several pathophysiological events contributing to SUDEP, including central apnea, autonomic dysregulation of cerebral and cardiac blood flow, decreased

heart rate variability (HRV), and neurogenic pulmonary edema (NPE). The pulmonary conditions central apnea and NPE are among the most frequently implicated mechanisms in SUDEP cases [6].

NPE is a life-threatening complication following central nervous system injury. It has been well known for almost 100 years, but its etiopathogenesis is still not completely understood. The roles of central nervous system trigger zones, systemic sympathetic discharge, inflammation and anesthesia, and intracranial pressure in the etiopathogenesis of NPE have been considered in detail [7]. NPE develops rapidly following injury and significantly complicates the overall clinical status of the patient. It is characterized by marked pulmonary vascular congestion with perivascular edema, extravasation, and intra-alveolar accumulation of protein- rich edema fluid and intraalveolar hemorrhage [8–10]. Although several pathophysiological mechanisms have been proposed, the exact cascade leading to the development of NPE is unclear [9, 10]. Both a rapid, transient, and severe sympathetic discharge and the release of vasoactive substances are thought to be involved in this process [11]. NPE is found in many cases of epilepsy at autopsy [12]. A proven mechanism of seizure-induced NPE is the intense generalized vasoconstriction from the massive seizure-related outpouring of central sympathetic activity, which leads to an increase in pulmonary vascular resistance [13, 14]. The aim of this study is to explore the exact mechanism of epilepsy-induced NPE.

The tremor rat (TRM) (tm/tm), a single-gene mutant, is the parent strain of the spontaneously epileptic rat (SER: zi/zi, tm/tm) found in the Kyoto:Wistar colony that exhibits both absence-like and convulsive seizures without any external stimuli [15]. Previous works have demonstrated that the absence-like seizures in TRM rats are characterized by paroxysmal occurrence of 5–7-Hz spike-wave-like complexes in hippocampal electroencephalograms (EEGs) at 8 weeks, similar to 3 Hz in human epilepsy [16–18]. Thus, this TRM rat is most likely to be a suitable animal model for epilepsy. In the current study, the TRM rat was selected as an animal model of epilepsy to determine the NPE of seizures, and significant lung injury was observed in the TRM rat. Therefore, the present experiments were designed to investigate the potential mechanisms of NPE in TRM rats. We suspected that pulmonary vascular remodeling and alveolar cell apoptosis might be involved in epilepsy-induced NPE.

Materials and methods

Materials

Antibodies against Bax, Bcl-2, caspase-3, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (CA, USA).

Rabbit polyclonal antibodies to ERK, phospho-ERK, JNK, phosphor-JNK, p38, phosphor-p38, collagen I, and collagen III were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit was from Roche (Mannheim, Germany). Enhanced chemiluminescence (ECL) reagent was from Amersham International (Amersham, UK). All other reagents were from common commercial sources.

Animal and lung tissue preparation

The animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Adult female/male Wistar rats and TRM rats at the age of 6 months were used in this study. Rats with a mean weight of 200 g were conditioned at a controlled ambient temperature of 22 ± 2 °C with 50 ± 10 % relative humidity and at a 12-h light–dark cycle (lights on at 8:00 a.m.). Standard rat chow and water were provided ad libitum to all rats. We anesthetized each rat with pentobarbital injection (120 mg/kg, i.p.), opened the thorax, and removed the heart and lungs to the flat plate. In some experiments, the lungs were quickly removed and further processed for immunostaining as described below.

Morphometric analysis

Rats were anesthetized, and tissue blocks were sliced and perfused with 4 % paraformaldehyde for overnight fixation. Fixed tissues were then dehydrated, cleared, and embedded in paraffin wax. The tissues were cut into 5-μm-thick sections and stained with H&E and Masson staining.

TUNEL assay

Alveolar cell apoptosis was measured by TUNEL assay according to the manufacturer's protocol. Under the light microscope, the normal nucleolus of alveolar cells was stained blue and the apoptotic nucleolus brown. Apoptotic cells in each group were carefully evaluated under double-blind conditions.

Western blot analysis

Rat lung tissues containing only alveolus were homogenized in lysis buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, Triton X-100 1 %, EDTA 1 mM, and PMSF 2 mM) containing protease inhibitors on ice. The homogenate was sonicated for 1 min and then centrifuged at 14,000 rpm for 15 min at 4 °C. The protein concentrations in the supernatant were confirmed using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). The

protocol for Western blot analysis was similar to previous descriptions [19, 20].

Reverse-phase high-pressure liquid chromatography (RP-HPLC) assay

The contents of catecholamine levels in rat blood samples were analyzed by RP-HPLC according to the published method [21]. Each sample was centrifuged at 4,000 rpm for 15 min at 4 °C. $HClO_4$ (200 μl, 0.1 M, Sigma), DHBA (50 μl, 2 μg/ml), and supernatants (250 μl) were added to a test tube. The mixture was shaken for 5 min and centrifuged at $14,000 \times g$ for 10 min at 4 °C. The supernatants were filtered with a 0.5-μm filter and then used for catecholamine detection. The levels of adrenaline (AD) and noradrenaline (NA) in rat plasma were measured by HPLC. DHBA (3,4-dihydroxybenzylamine) was used as an internal standard in each sample. The mobile phase contained SDS (sodium dodecyl sulfate, Sigma) 0.144 g, TEA (triethylamine, Merck) 0.5 ml, CA (citric acid, Merck) 10.5 g, SA (sodium acetate, Sigma) 4.1 g, ME (methanol, Merck) 120 ml, EDTA–2Na (ethylenediaminetetraacetic acid disodium salt, Sigma) 0.186 g, and tri-distilled water 880 ml. The flow rate was 0.8 ml/min. The fluorescence detector (RF-10Axl, Shimadzu) was excited at 280 nm while collecting emitted wavelengths at 330 nm.

Statistical analysis

Experimental data are presented as mean ± SEM. Statistical analysis was performed with one-way ANOVA (analysis of variance), followed by Dunnett's test where appropriate. Differences were considered to be significant at $P < 0.05$.

Results

Measurement of catecholamine levels in rat plasma by HPLC

Epilepsy has been found to be associated with higher levels of catecholamines, which could cause lung injury and pulmonary vasoconstriction. Pulmonary edema caused by the intravenous administration of epinephrine was similar to NPE [22, 23]. To determine whether the contents of catecholamine in plasma were increased in TRM rats, the levels of AD and NA were measured by HPLC. Our results showed that the products of AD and NA in plasma were both higher in TRM rats than in normal rats (Fig. 1a, b).

Morphometric analysis of pulmonary vascular remodeling in TRM rats

Previous studies have suggested that seizure-induced NPE leads to an increase in pulmonary vascular resistance [13, 14]. Remodeling of the pulmonary vessel wall is a major cause for the elevated pulmonary vascular resistance. The pulmonary vascular remodeling includes the medial thickening and deposition of collagen. Therefore, the morphology of pulmonary vessels was examined with hematoxylin-eosin and Masson staining. We found that pulmonary vascular walls in TRM rats were thicker than the vessels from normal rats observed with H&E (Fig. 2a). In addition, Masson staining showed that the deposition of collagen was significantly increased in TRM rats (Fig. 2b). The expression of collagen I and collagen III protein in rat alveolar homogenates from normoxic and TRM rats had similar results (Fig. 2c).

Fig. 1 Measurement of catecholamine level in rat plasma by the HPLC method. Adrenaline (AD) and noradrenaline (NA) are the two main types of catecholamine in plasma. **a**, **b** The levels of AD and NA in rat plasma were measured by HPLC method. Data are expressed as mean ± SEM ($n = 5$). **$p < 0.05$ vs. control
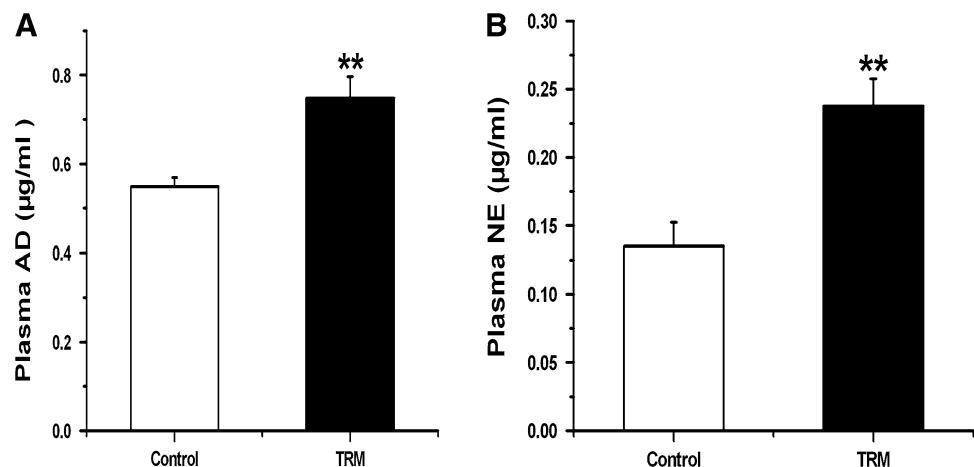
Fig. 2 H&E and Masson staining in pulmonary vessels from control and TRM rats. **a** H&E staining showed that the wall thickness in TRM rats was higher compared to normal rats. **b** The deposition of collagen in pulmonary artery vessels was increased in TRM rats. **c** Collagen I and collagen III protein expression in rat alveolar homogenates from normoxic and TRM rats. All values are denoted as mean ± SEM from at least three separate experiments. **$p < 0.05$ vs. control
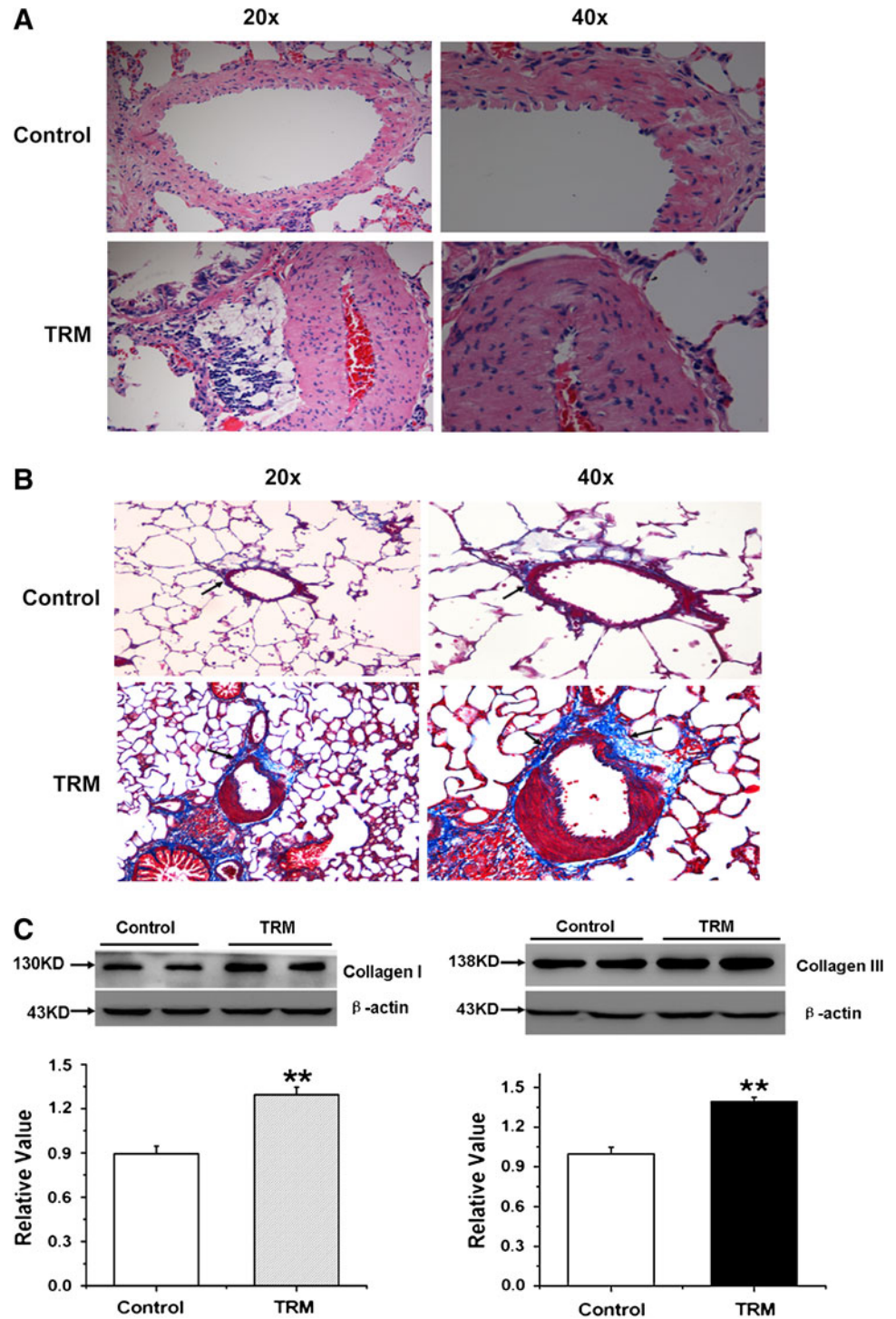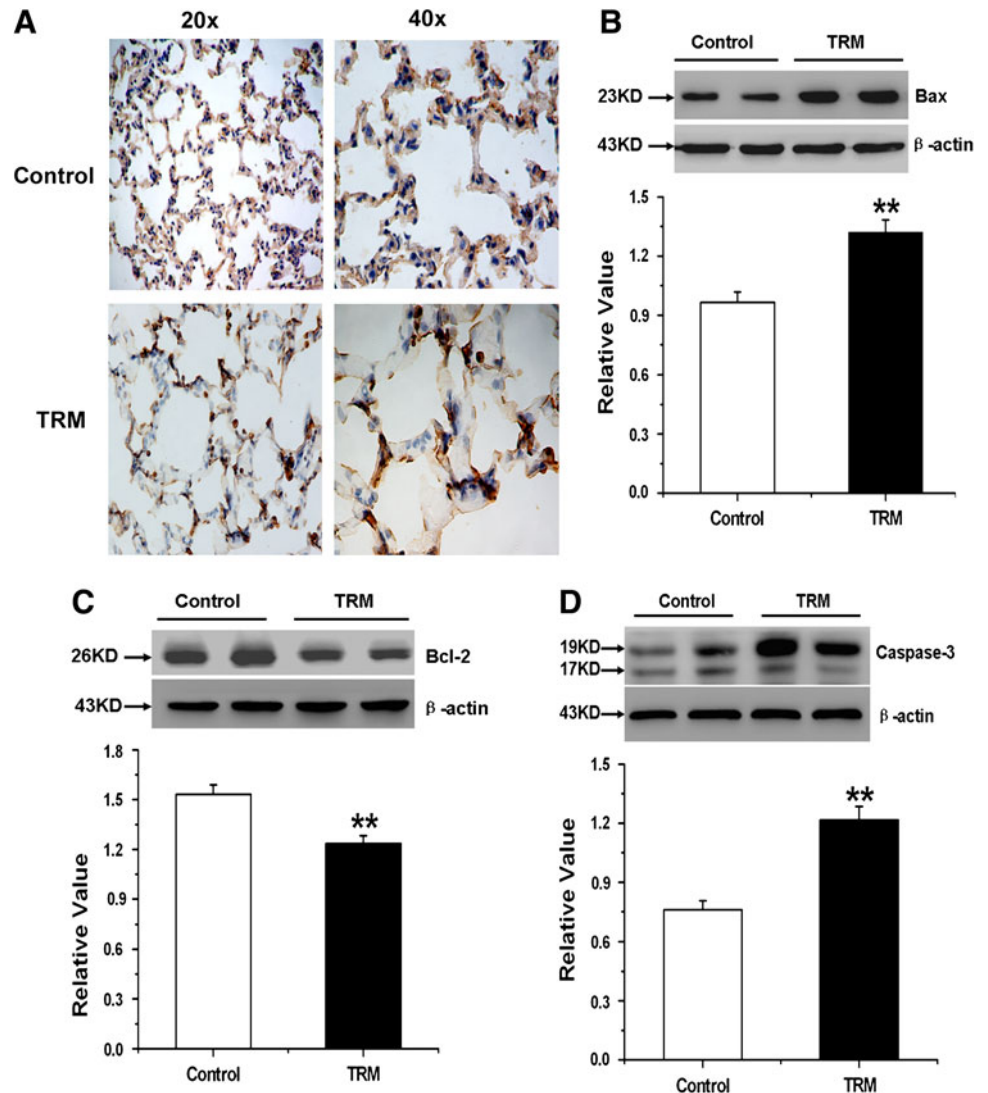

Alveolar cell apoptosis in TRM rats was analyzed by TUNEL staining and the effects of NPE in epilepsy on apoptotic protein expression

NPE was thought to be a potential mechanism of epilepsy-induced lung injury. To elucidate the cause of NPE in TRM rats, alveolar cell apoptosis was analyzed using TUNEL staining. As shown in Fig. 3a, the apoptotic numbers of alveolar cells in TRM rats increased significantly compared to the control group. To further examine the lung injury induced by spontaneous epilepsy in TRM rats, we analyzed the expression of apoptotic proteins in alveolar homogenates. We found that the expression of Bcl-2 was downregulated and the expression of caspase-3 and Bax was increased in alveolar homogenates in TRM rats (Fig. 3b–d).

Fig. 3 Alveolar cell apoptosis in TRM rats was analyzed by TUNEL staining and detection of Bax, Bcl-2, and caspase-3 protein expression in rat alveolar homogenate by Western blot. **a** Nuclei with brown staining were increased in TRM rats, which were defined as apoptotic cells. **b–d** Western blot assay was examined for Bax, Bcl-2, and caspase-3 expression. Average data are represented by mean ± SEM ($n = 6$). **p < 0.05$ vs. control


Seizure-induced NPE influenced MAPK cascades in TRM rats

To examine whether epilepsy-induced lung injury led to alveolar cell apoptosis through the mitogen-activated protein kinase (MAPK) signal pathway, extracellular signal-regulated protein kinase (ERK), c-Jun NH$_2$-terminal protein kinase (JNK), and p38 protein expression were analyzed by Western blot. JNK and p38 have been reported to regulate apoptotic or death signals, whereas ERK1/2 mediates the cell growth and differentiation signal in the cell. We found that the total ERK1/2, JNK, and p38 were equivalent in the two groups, while p-ERK1/2 expression was decreased (Fig. 4a). In addition, the expression levels of phosphorylated JNK (p-JNK) and phosphorylated p38 (p-p38) were upregulated in TRM rats (Fig. 4b, c).

Discussion

Epilepsy is one of the most common neurologic diseases worldwide, affecting 3 % of the world's population. NPE is a complication following several central nervous system injuries, including severe head injury, subarachnoid hemorrhage, and epileptic seizures. NPE is found in many epilepsy patients at autopsy [12]. However, the exact pathogenic mechanism leading to its development is still unclear. In the present study, we used the spontaneously epileptic rat-TRM rat as an animal model of epilepsy to determine the NPE of seizures. This study reported for the first time that epilepsy-induced lung injury led to alveolar cell apoptosis through the mitogen-activated protein kinase (MAPK) signal pathway in TRM rats. In addition, the levels of AD and NA in plasma were both higher in TRM

Fig. 4 Seizure-induced neurogenic pulmonary edema influenced MAPK cascades in TRM rats. **a–c** Western blot analysis was performed for phosphorylated ERK1/2, JNK, and p38 as well as total ERK1/2, JNK, and p38 in rat alveolar homogenate. All data are expressed as mean ± SEM ($n = 6$). **$p < 0.01$ vs. control
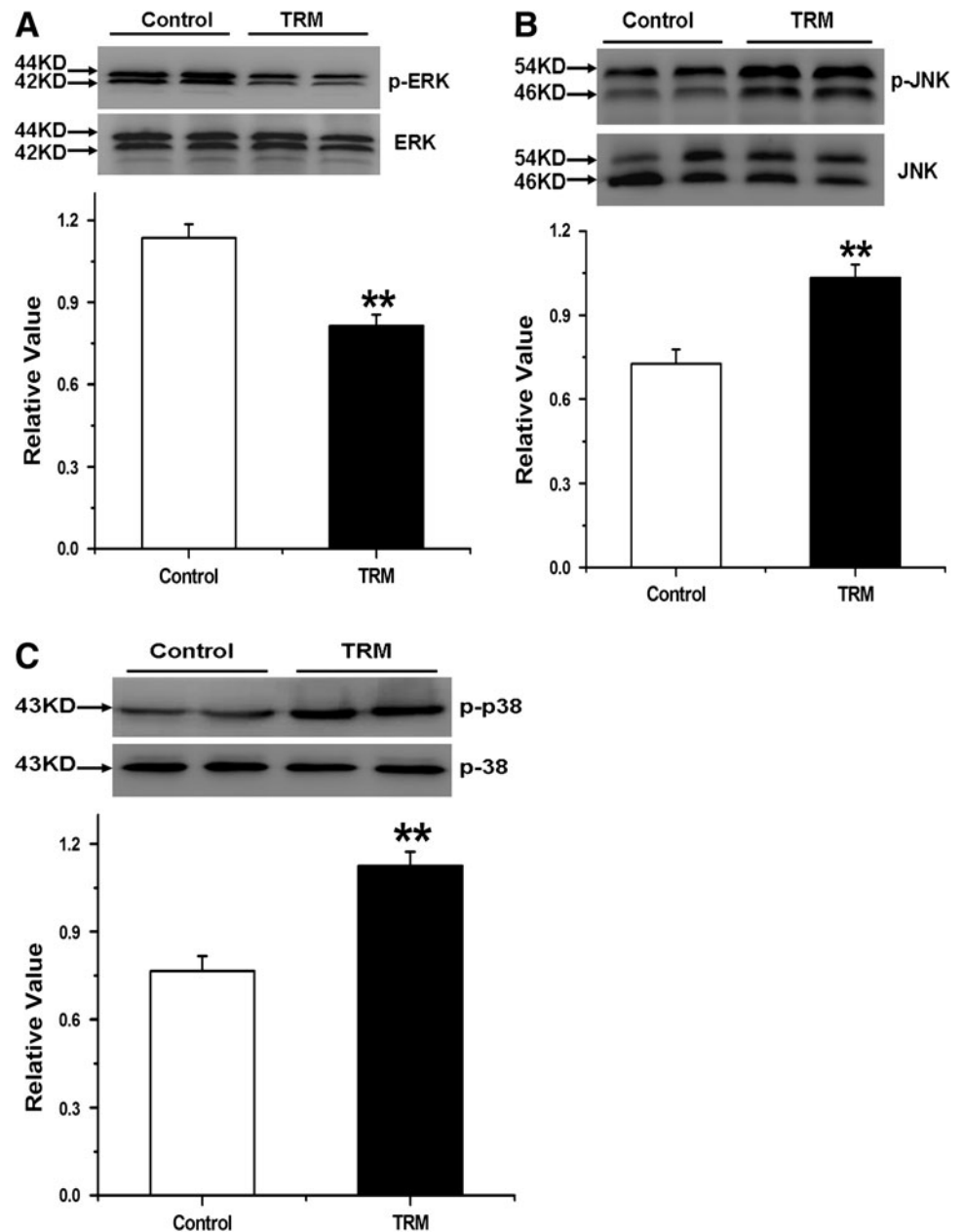
rats than in normal rats. This can cause pulmonary vascular vasoconstriction, leading to pulmonary vascular resistance. Another major discovery is that pulmonary vascular walls in TRM rats were thicker than the vessels from normal rats, and Masson staining showed that the deposition of collagen was significantly increased in TRM rats.

NPE in patients with epilepsy has been reported in previous studies [12, 24–27]. It creates an increase in pulmonary interstitial and alveolar fluid. In adults, it has been reported with prolonged seizure activity. NPE patients developed acute respiratory distress syndrome rapidly after the onset of status epilepticus. The etio-pathogenesis of NPE is still not completely understood.

Up to now, there are hemodynamic assays, the pulmonary capillary permeability doctrine and blast injury theory. The first two are more recognized. In order to obtain a concept of lung damage, hemodynamic measurements were examined, including left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP), and the maximum rates of the left ventricular pressure rise and fall ($+dp/dt_{max}$ and $-dp/dt_{max}$) were measured at the same time by a pressure transducer interfaced with a BL-420E organism function experiment system [28]. A number of studies have found that epilepsy produces activation of the sympathetic nervous system in both patient and animal models [29]. Therefore,

we measured the catecholamine product. Our results showed that the levels of AD and NA were significantly increased in TRM rat plasma. The level of catecholamines could cause lung injury and pulmonary vasoconstriction [22, 23]. Vasoconstriction, remodeling of the pulmonary vessel wall, and deposition of collagen are major causes of elevated pulmonary vascular resistance and pulmonary arterial pressure (PAP) [30, 31]. Consistent with this notion, we found that pulmonary vascular walls in TRM rats were thicker than vessels from normal rats. Meanwhile, Masson staining showed that the deposition of collagen was significantly increased in TRM rats.

Apoptosis, also called programmed cell death, has attracted much attention in some studies [32, 33]; it plays a fundamental role in the genesis of various diseases. The mitochondrial membrane potential is greatly influenced by proteins that belong to the Bcl-2 family [34]. The proapoptotic protein BAX and the antiapoptotic protein Bcl-2 are often studied together as indicators of apoptosis. In the present study, we found that the amount of apoptosis in alveolar cells increased significantly in TRM rats and was consistent with the downregulation of Bcl-2, the upregulation of Bax, and the activation of caspase-3.

MAPKs are phosphorylated in response to a series of extracellular stimuli. At least three subfamilies of the MAPK superfamily have been identified: ERK, JNK, and p38-MAPK [35]. An original study has proved that ERKs are important for cell survival, whereas JNKs and p38-MAPKs were deemed stress responsive and thus involved in apoptosis. JNK and p38-MAPK are potently activated by various forms of inflammatory signals or stress [36]. Activation of p38 MAPK has been shown to be involved in acute lung injury [37]. The differential contribution of different MAPK family members to cell death has been examined after withdrawal of nerve growth factor (NGF) from rat PC-12 pheochromocytoma cells. NGF withdrawal led to sustained activation of the JNK and p38 enzymes and inhibition of ERKs [38]. However, the ERK signal cascade is also known to be central to the activation of cellular processes even in the lung microenvironment, including lung inflammatory fibrosis, cytokine-induced ALI and ARDS, etc. [39–41]. In this study, we found that levels of P-JNK and P-p38 were increased in TRM rats, while P-ERK1/2 decreased. The results demonstrated that the epilepsy induced alveolar cell apoptosis may partly through the activation of MAPKs pathway in TRM rats. Distinct MAPK pathways control and determine cell fate in response to a variety of stimuli. MAPK signaling may either protect or enhance sensitivity to apoptosis depending on the cell type, stimuli, and latency of the MAPK activation. Therefore, the conflicting role of the p-ERK signal may be dependent on the various animal models, cell type, or a variety of stimuli.

The whole body develops tremor in TRM rat at 2 weeks of age, and the tremor gradually disappears at 6–8 weeks of age [18]. The 5–7 spike-and-wave complex associated with behavioral absence-like seizures starts at about 14 weeks of age in *tm/tm* rats and at about 26 weeks of age in *tm/+* rats [16, 42]. TRM shows vacuole formation in the CNS and aplastic gonads in both sexes [18]. By positional cloning, a genomic deletion was found within the critical region of more than 200 kb in which the aspartoacylase gene (*Aspa*) is located [43]. Abnormal accumulation of *N*-acetyl-L-aspartate (NAA) is observed especially in the pons and thalamus, and leads to epilepsy. Since aspartoacylase is not expressed in the lung, the cause of lung damage may be seizures.

In conclusion, our results imply that pulmonary vascular remodeling and alveolar cell apoptosis were involved in neurogenic pulmonary edema in TRM rats. The mechanisms were likely to include, at least in part, the activation of a mitochondria-initiated pathway as well as the mitogen-activated protein kinase pathway. These findings might provide us with good therapeutic targets for prompt intervention to treat this disorder in genetic epilepsy. Prompt respiratory support and treatment of the acute neurological insult can prevent further cerebral hypoxic injury.

Conflict of interest The authors declare no conflict of interest.

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