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EXPERIMENTAL STUDY

Expression of Lung IL-1β and IL-6 in Pentylenetetrazol-Kindling Model Epilepsy

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Abstract

Objectives: Cytokines are thought to be involved in the pathogenesis of many neurological diseases such as epilepsy. It is predicted that ınterleukin (IL)-1β and IL-6 levels in brain tissue are effective in the initiation and maintenance of epileptic seizures. However, there is no study addressing the inflammatory process in the lung in epileptic conditions. It was aimed to contribute to the mechanism of respiratory pathologies accompanying epilepsy in this study.

Methods: The chemical pentylenetetrazol (PTZ) has been used to develop epileptic seizures in rats. To examine the effect of seizures on the formation of inflammation in the lung, the expression levels of IL-1β and IL-6 were measured by real-time polymerase chain reaction.

Results: We found that the expression levels of IL-6 and IL-1β in the lung tissue of rats in the animal model of epilepsy induced by PTZ increased significantly. In addition, we found that both IL-6 and IL-1β mRNA expression increased at a higher rate in epileptic females than males.

Conclusion: This is the first study evaluating the IL-1β and IL-6 expression levels in the lung. Our results support the existence of an inflammatory state in epileptic patients. The significance of our findings will be supported by future studies on pro-inflammatory and anti-inflammatory responses in the lung.

Keywords: Animal model; epilepsy; interleukin-1β; interleukin-6; lung.

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Introduction

Epilepsy is a neurological disease that affects more than 70 million people. It is caused by the uncontrolled increase in the excitability of the neurons. It is characterized by recurrent seizures that occur due to disruption of the excitatory and inhibitory balance of the brain.[1] Seizures accompanying epilepsy can cause death by disrupting the autonomic control of cardiac and respiratory function.[2] Partial and generalized seizures affect the parasympathetic, sympathetic, and adrenal medullary system, altering autonomic function in ictal, postictal, and interictal periods.

Seizures can cause apnea or suffocation.[3] Furthermore, pulmonary edema may lead to sudden unexpected death in epilepsy (SUDEP) with suppression of autonomic respiratory reflexes after seizures.[4] For this reason, it is crucial to clarify the molecular mechanisms of epilepsy accompanying respiratory disorders. Focal or systemic irregular inflammatory processes are thought to trigger epilepsy by causing abnormal neural connections.[5] Current preclinical and clinical data show that there is a relationship between epileptiform activity formation and brain inflammation. Epileptic seizures trigger the release of essential inflammatory mediators, including cytokines. Thus, secondary damage occurs in the brain structure and recurrent seizures develop.[6] Cytokines are important signaling molecules that play a role in immunity, inflammation, and functional change of cells.[7] They modulate inflammatory processes are mainly produced by glial cells and neurons during brain inflammation. However, an increase occurs in the expression of cytokine receptors during the inflammation process.[8] Clinical studies reveal


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Pentilentetrazol-Tutuşma Modeli Epilepside Akciğer IL-1 β ve IL-6 Ekspresyonu

Öz

Amaç: Sitokinlerin epilepsi gibi birçok nörolojik hastalığın patogenezinde rol oynadığı düşünülmektedir. Beyin dokusundaki IL-1β ve IL-6 seviyelerinin epileptik nöbetlerin başlamasında ve sürdürülmesinde etkili olduğu tahmin edilmektedir. Bununla birlikte, epileptik durumlarda akciğerdeki iltihaplanma sürecini ele alan bir çalışma yoktur. Bu çalışmada epilepsiye eşlik eden solunum patolojilerinin mekanizmasına katkı sağlanması amaçlanmıştır.

Gereç ve Yöntem: Kimyasal pentylenetetrazol, sıçanlarda epileptik nöbetler geliştirmek için kullanılmıştır. Nöbetlerin akciğerde inflamasyon oluşumu üzerindeki etkisini incelemek için IL-1β ve IL-6 ekspresyon seviyeleri RT-PCR ile ölçüldü.

Bulgular: Pentilentetrazol ile indüklenen epilepsi hayvan modelinde sıçanların akciğer dokusunda IL-1β ve IL-6 ekspresyon seviyelerinin önemli ölçüde arttığını bulduk. Ek olarak, hem IL-1β hem de IL-6 mRNA ekspresyonunun epileptik kadınlarda erkeklerden daha yüksek bir oranda arttığını bulduk.

Sonuç: Bu, akciğerde IL-1β ve IL-6 ekspresyon düzeylerini değerlendiren ilk çalışmadır. Sonuçlarımız epileptik hastalarda inflamatuar bir durumun varlığını desteklemektedir. Bulgularımızın önemi, akciğerde proinflamatuar ve antiinflamatuar yanıtlar üzerine yapılacak gelecekteki çalışmalarla desteklenecektir.

Anahtar sözcükler: Akciğer; epilepsi; hayvan modeli; IL-1β; IL-6.

that the expression of interleukin-1β (IL-1β) and IL-6 pro-inflammatory cytokines increases after seizures.[9] Expressed in active microglia and astrocytes, IL-1β causes glutamate release from astrocytes and suppresses glutamate reuptake. Thus, the level of glutamate increases in neuronal synapses.[8] It has been reported that the level of IL-1β in the cerebrospinal fluid (CSF) significantly increases in patients with generalized tonic-clonic seizures.[10] However, it was determined that intrahippocampal IL-1β injection worsened and prolonged both electrographic and behavioral seizure activity in the epilepsy model induced by kainic acid in rats.[11] IL-6 is a pro-inflammatory cytokine found in low amounts in the central nervous system. IL-6 production increases with the stimulation of astrocytes and microglia.[12] Clinical data reveal that IL-6 level increases in both blood and CSF in correlation with the severity of epileptic seizures.[13] It was found that IL-6 mRNA expression was induced in the hippocampus, cortex, amygdala, and meninges after seizures in the rat model of status epilepticus induced by kainic acid. Supporting it, it has been found that the IL-6 receptor is upregulated in the hippocampus.[14] In this context, IL-1β and IL-6 inflammatory cytokines are predicted to be effective in the initiation and maintenance of epileptic seizures.

IL-1β and IL-6 mediated inflammation has been extensively examined in brain tissues.[15] However, there is no study addressing the inflammatory process in the lungs in epileptic conditions. In this study, a kindling epilepsy model was created in rats with pentylenetetrazol (PTZ). The gene expression levels of IL-1β and IL-6 inflammatory cytokines in the lung tissue of the experimental animal were measured by real-time polymerase chain reaction (RT-PCR). It was aimed to contribute to the mechanism of respiratory pathologies accompanying epilepsy.

Materials and Methods

In this study, Wistar Albino female and male rats (n=34, 280–380 g) were obtained from Kayseri Erciyes University Research Center. Animals were housed in a controlled environment (24±2°C and 60% humidity) under a 12 h light-dark cycle. They were provided with free access to tap water and standard food. All procedures were fully followed by the recommendations in the Guidelines for the Care and Use of Laboratory Animals, adopted by the National Institutes of Health (USA) and the Declaration of Helsinki. Rats were anesthetized intraperitoneally (i.p.) with ketamine and xylazine (90/10 mg/kg). All necessary efforts have been made to minimize the suffering of animals. The experimental protocol of this study was approved by Kayseri Erciyes University Animal Experiments Local Ethics Committee (HADYEK) (Ethics committee decision no: 19/027).

Experimental epilepsy model– Experimental animals were randomly divided into control and PTZ-kindling groups. The chemical PTZ has been used to develop epileptic seizures in animals. Repeated doses of PTZ (P6500, Sigma-Aldrich, St. Louis, MO, USA) were given in an epilepsy kindling model.

Male and female control groups– Male and female control groups (n=7/7) were formed with seven animals per experimental setup. The experimental animals were given equal injection of 0.5 cc intraperitoneal (i.p.) saline 3 times a week (Monday, Wednesday, and Friday) for 1 month. At the end of 1 month, animals were anesthetized using ketamine and xylazine agents.

Male and female PTZ-kindling groups– Male and female PTZ-kindling groups (n=10/10) were determined to be ten

Table 1. Behavioral categorization of the severity of epileptic seizures

Racine skorlama sistemi	
Stage 0	No answer
Stage 1	Ear and face twitching
Stage 2	Myoclonic jerks in the body
Stage 3	Rise on hind legs
Stage 4	Tonic-clonic seizures with the animal falling to the ground
Stage 5	Recurrent (generalized) severe tonic-clonic seizures
Stage 6	Death

Table 2. Primer sequences used to amplify the gene region

	Forward primer (5′–3′)	Reverse primer (3′–5′)
IL-6	TGA TGG ATG CTT CCA AAC TG	GAG CAT TGG AAG TTG GGG TA
IL-1β	CAC CTT CTT TTC CTT CAT CTT TG	GTC GTT GCT TGT CTC TCC TTG TA

animals per experimental setup. To create the chronic epilepsy model, the epileptic agent PTZ was administered according to the protocol given in the study.[16] PTZ adjusted at a dose of 35 mg/kg 3 times a week (Monday, Wednesday, and Friday) for 1 month was dissolved in saline (%0.9 NaCl solution) and administered i.p. as injected. The animals were placed in a cage alone and their behavior was observed within 30 min after PTZ treatment. Epileptic seizures were graded according to the previously developed Racine Scoring protocol[17] (Table 1).

One week after the last PTZ injection (13th injection), animals in both the female and male epilepsy groups were given a high dose of 50 mg/kg PTZ to show improved seizure sensitivity. It was thought that the pattern was formed effectively, and kindling occurred in all animals in the PTZ-kindling group with phase 4 or 5 type seizures.[18]

Dissection of the lung– After completing the formation of the PTZ-kindling model, animals injected with ketamine/xylazine (90/10 mg/kg, i.p.) were sacrificed by exsanguination while under anesthesia, and lung dissection was performed.

Genetic analysis

Total RNA isolation and cDNA recovery– IL-1β and IL-6 expression in the lungs was studied by RT-PCR. Total RNA was isolated using Accuzol™ Total RNA Extraction Reagent (K-3090, USA Bioneer, Inc.) according to the manufacturer's protocol. The RNAs obtained were converted into cDNA using the High Capacity cDNA Synthesis Kit with RNase Inhibitor (A.B.T.™) and stored at –20°C. The purity and quantitation of the samples were made with DS-11FX+Micro Volume Spectrophotometer (Denovix, USA).

RT-PCR– Primer sets (S-1001, USA Bioneer, Inc.) designed according to the PCR instrument and kit were used (Table 2). The prepared samples were placed in the Exicycler™ 96 (Ver.4) Real-time Quantitative Thermal Block (A-2060-1, USA Bioneer, Inc.) device. The detection of the signals and the evaluation of the results were made with the software of Exicycler™ 96 (Ver.4). The cycle of quantitation (Ct) of each sample was recorded, and the data were analyzed by normalization to β-actin values using the $2^{-\Delta\Delta Ct}$ method.[19]

Statistical analysis– SPSS program was used for statistical analysis. Results are presented as the mean standard error margin. Comparison between groups was made with one-way ANOVA test and post hoc Tukey test was used for paired comparisons. Values of $p<0.05$ were considered statistically.

Results

The PTZ-kindling epilepsy model has been successfully established in rats– Seizures were induced in all rats and the epileptic animal model was correctly constructed. No gender-related change was observed in the seizure scoring of male and female rats (Fig. 1a). The onset time of the epileptic seizure was measured in seconds for the male and female PTZ kindling epilepsy group. While in male rats seizures started at 266±66 s, the first seizure occurred after 308±95 s in females (Fig. 1b).

Increased levels of IL-6 and IL-1β in lung tissue of rats with epileptic seizures – To examine the effect of seizures on the formation of inflammation in the lung, the expression of pro-inflammatory cytokines IL-6 and IL-1β at the mRNA level was measured by RT-PCR. The mRNA level of IL-6 in epileptic female (n=7) and male rats (n=7) lung tissue samples compared to the saline-treated control group (n=10) was 12.07 times (**$p<0.01$) and 2.91 (**$p<0.01$), respectively, fold increase was observed. It was found that expression of IL-1β in the lung increased 35.07 times (***$p<0.001$) in female rats injected with PTZ and 3.52 times (*$p<0.05$) in male rats compared to control rats (Fig. 2). It was determined that both IL-6 and IL-1β mRNA expression increased at a higher rate in epileptic females than males.

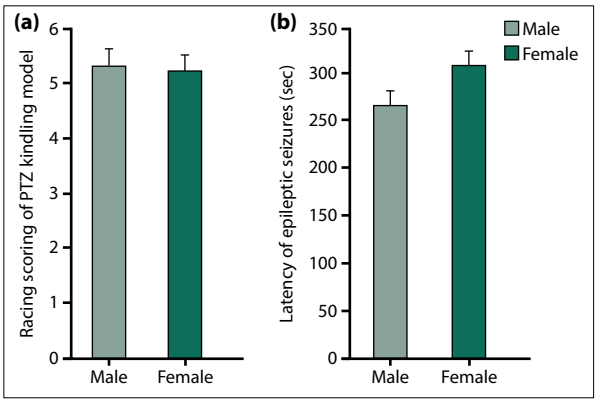

Fig. 1. Racine scoring and seizure latency in the kindling model created in rats. **(a)** Scoring of the seizure scores of female (5.31±0.58) and male (5.2±0.47) rats given 50 mg/kg PTZ after the 13th injection is shown. **(b)** The graph shows the time in seconds until the onset of seizures in both female (308±95) and male (266±66) rats. Bars representing the values of the seizure score and latency are expressed as ± SEM. There are seven animals in each of the female (purple) and male (orange) groups.

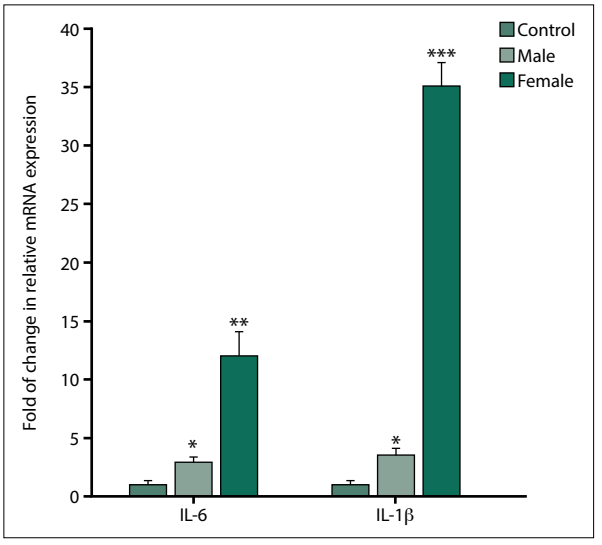

Fig. 2. Relative change of mRNA expression level of pro-inflammatory cytokines in the lung as a result of seizures. Relative variation of total IL-1β and IL-6 mRNA expression in lung tissue samples of PTZ-injected female (purple, n=10) and male (orange, n=10) rats compared to control group rats (black, n=7) is seen. Bars representing the relative change in expression level are expressed as ± SEM. (Values of *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant).

Discussion

One of the possible targets of epilepsy prevention strategies is the inflammatory response that occurs in the brain after initial epileptogenic injury. Evidence from experimental and clinical studies suggests that inflammatory mediators in the brain play a role in epileptogenesis and the neuropathology of epilepsy. Seizure formation may lead to an increase in gene expression of various pro-inflammatory proteins and may lead to neurological damage.[20] Epileptic seizures can induce cytokine production, which affects the pathogenesis and course of epilepsies.[15] We know that epileptic seizures cause significant changes in autonomic nervous system (ANS) function. Therefore, epileptic seizures may affect various organs and systems under the control of ANS. IL expressions examined in blood and serum were significantly higher in epilepsy patients.[13] The examination of IL-1 and IL-6 expression in the lung in epilepsy, where ANS may also be affected, is a preliminary study.

Changes in IL-1β and IL-6 levels in epilepsy are known, especially in brain tissue. A rapid increase in IL-1β was found in active microglia and astrocytes by immunohistochemical analysis in the forebrain of rats with epilepsy during acute seizures.[21,22] Increased IL-1β and IL-6 levels are associated with the duration and frequency of epileptic seizures. The fact that these ILs are among the most focused pro-inflammatory cytokines both in patients and in epilepsy model studies[23] reveals the importance of our study. We observed that the mRNA level of IL-6 increased 12.07 and 2.91 times, respectively, in lung tissue samples of epileptic female and epileptic male rats. We found that the expression of IL-1β in the lung increased 35.07 times in epileptic female rats and 3.52 times in epileptic male rats. Both IL-6 and IL-1β mRNA expression increased at a higher rate in epileptic females than males. This further increase may indicate that respiratory pathology in epilepsy may be more common in females. The American Academy of Neurology examined more than 20 other risk factors for SUDEP, including gender, in the 2017 SUDEP Practice Guidelines. These factors were not associated with increased or decreased risk for SUDEP.[24] In studies conducted on brain tissue, no relationship was found between gender and IL-1β and IL-6.[10,25,26] Although studies on lung tissue are limited, it is known that there are sex differences in innate immunity in mammals.[27] IL-1β and IL-6 expression is stimulated in the inflammatory response by activation of the TLR-NFκB pathway.[28] It has also been reported that TLR pathway gene expression is higher in females than in males.[27] Although we have found a difference in lung tissue by gender, more studies are needed on sex differences between tissues in epilepsy.

IL-6, which is an important group of cytokines in the regulation of the acute phase response to injury and infection, also functions as an activation signal for other cytokines

in the brain tissue.[29,30] An increase in IL-6 levels has been reported in patients with tonic-clonic seizures.[26] In addition, an increase in the level of IL-6 in the CSF was observed in the post-ictal state.[21] When applied externally, IL-6 has been observed to increase the severity of chemical-induced seizures in rats[31] and the increase in IL-6 expression is related to the spread and duration of the seizure.[32] Thus, the knowledge that IL-6 increases neuronal damage has been brought to the literature.[30] Increased IL-6 level in lung tissue correlates with the increase in the brain. This increase may suggest a more in-depth examination of inflammation in both the central nervous system and ANS.

The innate immune system producing IL-1β plays a role in the initiation of seizures. The adaptive immune system can promote neurodegeneration. In addition, it can increase epileptogenesis by invading T lymphocytes and antibody complement activation. Disorders in abnormal cytokine production, potassium homeostasis, and cytotoxic glutamate buffering are thought to induce seizures.[22,33] This multifactorial induction of seizures and promotion of epileptogenesis makes it difficult to develop successful antiepileptogenic treatment strategies. For this reason, since the use of IL-1β signaling inhibitors is seen as a promising strategy,[29] our study will be useful in elucidating the pathology of epilepsy. Experimental studies have shown that mRNA expression of inflammatory cytokines is increased in rodent forebrain after epileptic seizures.[34] Increased IL-1β immunoreactivity in human epileptic tissue, and high cytokine levels have also been reported in serum and CSF from epileptic patients.[35] IL-1β increases the synthesis of other cytokines such as IL-6 and tumor necrosis factor (TNF)-α, which disrupt GABAergic neurotransmission in microglia.[36] In the experimental model created by inducing generalized tonic-clonic seizures by PTZ associated with high expression of TNF-α and IL-1β in the rat hippocampus, an increase in brain TNF-α and IL-1β was observed.[37] In the PTZ model epilepsy, the effects of methyl methionine sulfonium chloride called Vitamin U on lung tissue were investigated.[38] These findings in the lung tissue may indicate neurogenic pulmonary edema (NPE) in epilepsy. As it is known, NPE was defined by Shanahan in 1908 in patients with epilepsy.[39] NPE is found at autopsy in many epilepsy patients. At present, NPE is considered a pressure-dependent pulmonary edema. Inflammatory factors such as IL-1, IL-8, IL-6, and TNF, which are transported by circulating blood, participate in NPE pathology.[40] The studies have shown that subarachnoid hemorrhage (SAH), a brain injury, induced neutrophil flow and increased IL-1β in the lung.[41] In addition, active IL-1β increase was found in NPE animals after SAH.[42] It was emphasized that IL-1β causes disruption of the blood-lung barrier after SAH and therefore may be a therapeutic target for NPE therapy.[43] Systemic IL-6 concentration was shown to be an independent predictor for NPE in patients with non-traumatic intracerebral hemorrhage.[44] Based on this, the barrier damage effect of these ILs and the therapeutic target status gains importance in epilepsy. However, there are no studies on changes in IL-1β and IL-6 lung tissue in seizures caused by PTZ. Since there are not enough studies examining the role and effect of IL-1β and IL-6 for peripheral tissues in chronic epilepsy, our research will pave the way for other studies. The brain and lung interact strongly in complex ways. The treatment strategies should also be developed to prevent inflammation in the pulmonary system or lung damage in patients with epilepsy. Strict monitoring of respiratory and cerebral parameters is necessary to optimize the treatment of patients.

Conclusion– In summary, we found that the expression levels of IL-6 and IL-1β in the lung tissue of rats in the animal model of epilepsy induced by PTZ increased significantly. In addition, we found that both IL-6 and IL-1β mRNA expression increased at a higher rate in epileptic females than males. Although, there are studies in the literature showing that IL-1β and IL-6 mRNA and protein levels increase in epileptogenesis or seizure in the brain in both epilepsy patients and experimental rat model studies. As a result of this study, we observed that it was the first study in which the mRNA levels of IL-1R and IL-6 in the lung were evaluated. The further significance of our findings depends on future studies on pro-inflammatory and anti-inflammatory responses in the lung that will enable us to better understand the pathogenesis of epilepsy and will be a source study for them.

Ethics Committee Approval– This study approved by the Kayseri Erciyes University Animal Experiments Local Ethics Committee (Date: 13.02.2019, Decision No: 19/027).

Peer-review– Externally peer-reviewed.

Authorship Contributions– Concept: N.I., E.A.; Design: N.I., E.A.; Supervision: N.I., E.A.; Materials: N.I., E.A.; Data collection &/or processing: B.K., S.S., N.G.; Analysis and/or interpretation: B.K., S.S., N.G.; Literature search: B.K., S.S., N.G.; Writing: N.I., E.A.; Critical review: N.I., E.A.

Conflict of interest– The authors declare that they have no conflict of interest.

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