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Effect of K_{ATP} openers and blockers on AKT and mTOR mRNA levels in the hippocampus and cortex of rats with penicillin ınduced epilepsy

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Opening or closing of K_{ATP} channels affects some signaling pathways in the brain. These signaling pathways are thought to be associated with epilepsy. Here, we investigated the effect of K_{ATP} channel opener and blocker on the AKT mTOR mRNA expressions in the hippocampus and cortex regions in the penicillin model of epilepsy in rats. Four groups were created viz. Control (C), Epilepsy (E), Epilepsy-Opener (E-O) and Epilepsy-Blocker (E-B). Epileptic focus was created by administering penicillin into the brain, and the seizure-related AKT, mTOR mRNA levels were determined by qPCR on days 1st, 4th, and 8th after the seizure. The AKT mRNA expression levels in the hippocampus were statistically significant (P <0.05) in the E 1st day group. mTOR mRNA expression levels were observed to be significantly higher in the E 1st day and 8th day groups than in control and all opener groups. (P <0.05). mTOR mRNA expression levels were significantly higher in the E-B 1st day and 8th day group compared to the control and opener groups (P <0.05). AKT mRNA expression levels in the cortex were found to be statistically significantly higher in the E 1st day group than in the other groups. mTOR mRNA expression was significantly higher in the E 4th day group than in the control group. The results suggest that activation of the AKT/mTOR signaling pathway is effective in the development of epilepsy and that the effect of KATP channels on epilepsy may be via the AKT/mTOR signaling pathway.

Keywords: Antidiabetic, Cellular stress, Epileptogenesis, Glibenclamide, Pinacidil

Epilepsy is one of the most prevalent neurological illnesses, affecting about 50 million individuals globally[1]. Sudden, abnormal discharge of neurons, which leads to behavioural abnormalities, including short-term nervous dysfunction, sensory and movement disorders, can be considered among the clinical features of epilepsy[2,3].

ATP-sensitive K+ (K_{ATP}) channels are inwardly rectifying potassium channels that are widely expressed in the body. K_{ATP} is regulated by adenine nucleotides, which are activated by decreasing ATP and increasing ADP levels, and plays an important physiological role by combining cellular metabolism with membrane excitability[4]. K_{ATP} channels have important roles and are found in many cell types, including myocytes, pancreatic beta (β) cells, and neurons[5,6]. The K_{ATP} channels in the hippocampus have neuroprotective roles. The cellular stress causes a temporary membrane hyperpolarization by activating these channels, and as a result leads to a reduction in energy demand of cells ensuring adequate protection for the metabolically degraded cells[7]. The K_{ATP} channels functionally bind to A1 receptors located at the cholinergic terminals of the hippocampus, and glibenclamide and glipizide interact with the K_{ATP} channels, relieving this inhibitory neuromodulation[8].

Pinacidil, a K_{ATP} channel opener, acts on the K_{ATP} channels in smooth muscle and the sarcolemma and mitochondria[9] of cardiac muscle cells. Genetic, physiological, and pharmacological evidence suggests that certain K+ channels have roles in epileptogenesis and neuronal excitability modulation. As a result, various investigations on K+ channel openers have been undertaken[10–12]. K+ channel openers, such as diazoxide, have been demonstrated to have antiepileptic effects in both *in vitro* and *in vivo* models. As a result of this insight, the K_{ATP} channel may be a viable target for novel medications[13,14].

Glibenclamide is an antidiabetic drug that acts by blocking ATP-sensitive potassium channels[15,16]. However, it is also well known that glibenclamide binds to SUR1 subunits of ATP-sensitive potassium channels[17]. According to certain research,

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glibenclamide may have a possible function in the prevention of convulsions due to its capacity to control potassium currents[14]. According to research, the anticonvulsant effect may be related to regulation of GABA release caused by glibenclamide's interaction with potassium channels in the CNS[18,19].

Irregular mTOR activity has been associated with promoting epileptogenesis in various types or models of epilepsy[20]. The primary role of the mTOR pathway in the pathophysiology of epilepsy is genetic disorders involving mutations of genes that directly regulate the mTOR pathway[21-26]. In animal models of epilepsy, secondary to brain damage, evidence was found that the mTOR pathway is activated abnormally and that the mTOR inhibitors can limit the development of spontaneous seizures[27,28] and potentially epileptogenic pathological changes[29], Still, the effects on epileptogenesis are not yet clear[30]. Although, the molecular events that cause mTOR activation are clearly defined in specific genetic models of epilepsy. Among the numerous signaling pathways that can regulate mTOR, the PI3K-AKT pathway is an important candidate for mediating the effects of seizures; this is because seizures cause intense glutamate release and glutamate can stimulate PI3K[31,32]. Earlier findings in the PTZ and kainate models support the mediator role of the AKT-PI3K pathway in seizure-induced mTOR activation[33]. On the contrary, the PI3K-AKT–mTOR cascade and mitochondrial K_{ATP} channels provide multiple cardioprotective effects, and this pathway is activated by ischemic preconditioning, post-conditioning, and pharmacological conditioning[34,35].

In this study, we investigated the impact of seizure on gene expression in the cortex and hippocampus, the areas which are known to get affected after a single seizure. We applied K_{ATP} channel opener pinasidil and its blocker glibenclamide on AKT and mTOR mRNA expression levels in the hippocampus and cortex of rats with penicillin model epilepsy on the 1st, 4th, and 8th days after seizure, and the effect of K_{ATP} channels was mediated by the AKT/mTOR signal pathway.

Materials and Methods

Experimental animals

Experimental animals to be used in the study were obtained from BAIBU Experimental Animals Application Research Center. All experimental animals have been treated based on the guiding principles approved by the animal ethical committee of Bolu Abant Izzet Baysal University as well as all the treatments comply with recommendations provided on the Declaration of Helsinki (Registration number:2018/36/A2). The animals were kept in the Experimental Animals Application Research Center in a relative humidity of 60-70% in a 12 h light/dark environment, and fed *ad libitum* until the study started and during the study period. Male rats of the Wistar albino breed aged 2-4 months were used. Figure 1 depicts the experimental design clearly. Four groups were created: Control (C), Epilepsy (E), Epilepsy-opener (E-O) and Epilepsy-Blocker (E-B). A total of 48 animals were used, with 12 animals in each group. Subgroups of each group were formed on 1st, 4th and 8th day. Rats were anesthetized with 1.2 g/kg (i.p.) urethane. The epileptic focus was created by intracortical (i.c.) administration of penicillin at a dose of 500 IU/2 µL. Drugs were applied 30 min after penicillin administration.[37] Hippocampus and cortex tissues were removed from animals 24 h (1st day), 4th and 8th day after penicillin administration. After that AKT, mTOR, mRNA expression levels were detected in the hippocampus and cortex.

Surgical operation

All rats were anesthetized with 1.2 g/kg urethane (i.p.) (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) and placed in a stereotaxic device. Left cerebral cortex 2 mm posterior to bregma and 3 mm lateral to sagittal skull bone was removed, and

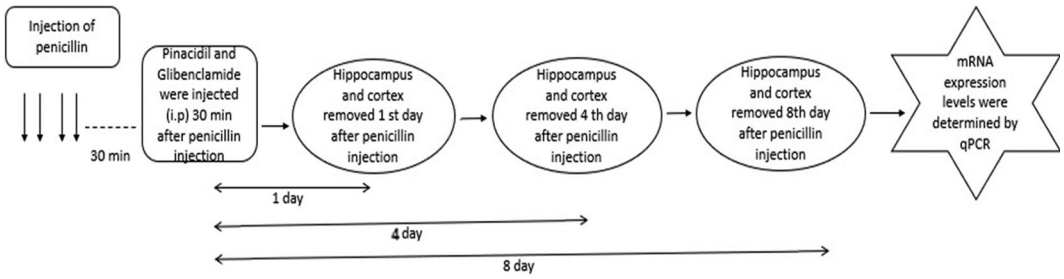

Fig. 1 — Experimental design

then dura matter was removed. To create epileptic focus 500IU with a Hamilton microinjector (701N, Hamilton Co., Reno, NV, USA) to a depth of 1.2 mm, 2 µL of penicillin G was injected[36].

Drug administration

In this study, K_{ATP} channel-opener pinacidil (1 mg/kg), and K_{ATP} channel-blocker glibenclamide (5 mg/kg) were given intraperitoneally (i.p.). All drugs were applied 30 min after penicillin administration[37].

Q PCR method

To detect changes in gene expression levels, total RNA was isolated, cDNA synthesis was performed, and quantitative real-time PCR (qRT-PCR) experiments were performed.

RNA isolation

For RNA isolation from tissue samples, 1 mL of Trizole solution was added to a 50 mg tissue sample and homogenized. The tubes were incubated at room temperature (25°C) for 5 min, added 200 µL chloroform, and manually shaken quickly for 15 s. After 3 min, the tubes were centrifuged at 12,000×g, and 4°C for 15 min. The transparent coloured upper phase was taken into a new tube and 500 µL of 100% isopropanol was added. After incubation at room temperature for 10 min, the tubes were again centrifuged for 10 min at 12,000×g and 4°C. At this stage, the RNA in the sample formed a white precipitate at the bottom of the tube. The liquid in the tube was removed, taking care not to touch this precipitate, and the RNA precipitate was washed with 1 mL of 75% ethanol and centrifuged at 7500×g and 4°C for 5 min. The resulting RNA was dissolved with 20-50 µL of DEPC-ddH2O and its concentration was measured.

cDNA synthesis

For each sample, 1 µg of RNA, 2 µL of oligo dT, and DEPC-ddH2O were mixed with a final volume of 8µL and incubated for 5 min at 70°C. After 10 µL of 2X reaction buffer and 2 µL of reverse transcriptase enzyme were added, the samples were incubated for 1 h at 42°C and 5 min at 80°C. The cDNA samples were stored at –20°C.

Quantitative Real-Time PCR (*q RT-PCR*)

Primers that bind with high specificity to the target gene regions to be tested for RT-PCR experiments were designed. The oligo design was carried out using the Amplify program, and its properties such as melting temperatures (Tm) and primary-dimer formation were studied using the same program. To ensure that the selected primers do not bind to other unwanted regions (unspecific) in the genome, the primers were selected from the exon-intron junction regions. However, the specificity of the primers was confirmed by the *in silico* PCR method using the UC Genome Browser. To investigate the level of m RNA expression, 1 µL of cDNA, 1 µL of primer mixture (10 µM, forward+ reverse), 10 µL of 2X SYBR Green, and 8 µL of ddH2O were added to each q RT-PCR reaction. The following program was used for the reaction: 95°C for 5 min, [95°C for 15 s, 60°C for 30 s, 72°C for 30 s] × 40, 72°C for 5 min. Table 1 shows the details of primers used in this study.

Analysis of qRT-PCR results

Normalization with a housekeeping gene such as GAPDH was performed to prevent differences between samples and possible pipetting errors during the detection of mRNA expression levels. The analysis was performed using the duct method by the following equation.

ddCt = Ct (target gene) - Ct (house keeping gene)

Target gene expression = 2^(- ddCt)1 [38]

Statistical analysis

The difference between groups was analyzed with SPSS v.21 ANOVA and Post-Hoc LSD test. A *P* value of <0.05 was considered significant.

Results

In this work, an epileptic focal was produced by injecting penicillin into the brain, resulting in penicillin model epilepsy. The effects of K_{ATP} channel opener pinasidil and K_{ATP} channel blocker glibenclamide on AKT and mTOR mRNA expression levels in the hippocampus and cortex regions on Day 1, Day 4 and Day 8 of a single seizure were studied.

Levels of AKT and mTOR mRNA expression in hippocampus

The level of AKT mRNA expression in the hippocampus was statistically significantly greater in the E Day 1 group (*P* <0.05) (Fig. 2A). The levels of mTOR mRNA expression in the hippocampal region were observed to be significantly greater in the E Day 1 and Day 8 groups compared to the control and

Table 1 — Details of Primers used in the study

Primers name	Primers	Temp (°C)
M TOR-F	TCTGCACTTGTTGTTGCCTC	57
MTOR-R	ACAATCGGGTGAATGATGCG	57
AKT-F	CAAGGAGATCATGCAGCACC	59
AKT-R	CATCTTGATCAGGCGGTGTG	59
GAPDH-F	ACCACCATGGAGAAGGCTGG	61
GAPDH-R	CTCAGTGATGCCCAGGATGC	61

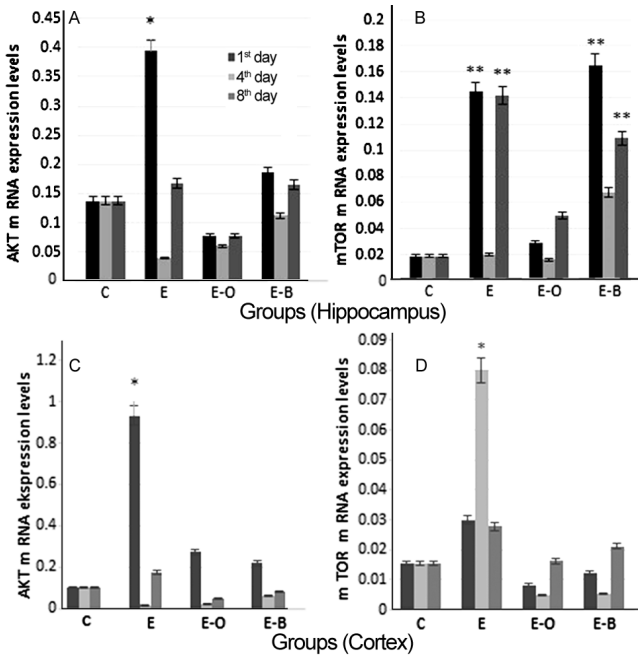

Fig. 2 — (A and C) AKT mRNA expression levels; and (B and D) mTOR mRNA expression levels in the hippocampus and cortex, respectively. **P* <0.05 compared with all groups and ***P*<0.05 compared with control and E-O groups.

opener groups (*P* <0.05). On Day 1 and Day 8, mTOR mRNA expression was considerably higher in the E-B group than in the control and opener groups (*P* <0.05) (Fig. 2B).

Levels of AKT and mTOR mRNA expression in cortex

The amount of AKT mRNA expression in the cortex was found to be statistically significantly greater in the E Day 1 group than in the other groups (*P* <0.05). (Fig. 2C) The E Day 4 group's mTOR mRNA expression levels in the cortical region were found to be statistically significantly greater than the other groups (*P* <0.05). (Fig. 2D)

Discussion

The mTOR (mammalian target of rapamycin) signaling system is involved in a variety of multicellular processes, including protein synthesis, cell growth and proliferation, and synaptic plasticity, which can impact neuronal excitability and may be involved in epileptogenesis. In epilepsy, frequent hyperactivation of mTOR signaling makes it a potential mechanism in pathogenesis, as well as an attractive target for therapeutic intervention[39]. It has been claimed that the mTOR signal transduction pathway, in particular, plays a crucial role in this respect. These pathways are involved in numerous pathological conditions, as well as in major physiological processes[40].

mTOR plays a role in promoting mechanisms of epileptogenesis in various animal models of epilepsy. mTOR inhibitors reduce the development of seizures as well as accompanying cellular and molecular abnormalities that promote epileptogeneses, such as glial proliferation, neuronal hypertrophy, and defective glutamate transporters[21,22,26]. Similarly, in the kainate and pilocarpine models of acquired temporal lobe epilepsy, the first stage of status epilepticus induces abnormal mTOR activation, and mTOR inhibitors reduce mossy fiber sprouting[29] and chronic epilepsy[27]. Therefore, there is intense interest in the overall role of the mTOR pathway in epileptogenesis and the potential use of mTOR inhibitors as treatments for multiple types of epilepsy[20].

K^+ channels play several positive roles in the neurological and cardiovascular systems. One of these channels is the K_{ATP} channel. K_{ATP} channels are named according to their localization in the cell. The K_{ATP} channels located on the cytoplasmic membrane are called cytoplasmic or sarcoplasmic K_{ATP} channels. The K_{ATP} channels found in the mitochondrial inner membrane are referred to as mitochondrial K_{ATP} channels. There are tissue-specific types of these specific channels. There are also blockers and openers specific to these channels. K_{ATP} channels are triggered in response to a drop in the quantity of ATP in the cell, and the cell becomes hyperpolarized as a result of the influx of K ions into the cell. K_{ATP} channels are closed under normal conditions. K_{ATP} channel openers and blockers may be able to mimic similar effects in the treatment of a variety of diseases, including epilepsy. Cytoplasmic K_{ATP} channels are associated with the electrophysiological characteristics of the cell, while mitochondrial K_{ATP} channels are associated with cell death.

The PI3K-AKT-mTOR cascade of the Reperfusion injury salvage kinase pathway (RISK) signal, on the other hand, modulates cell survival and tissue protection from ischemia during ischemia. AKT and mTOR proteins become active during this process, and their target is mitochondrial K_{ATP} channels[41].

The effects associated with K_{ATP} blockers are controversial. There is evidence that K_{ATP} blockers treatment promotes seizures in the penicillin model of epilepsy. Yet, in the penicillin model of epilepsy

model, the K_{ATP} blockers 5HD and HMR1098 administered before and after the seizure was found to increase seizures[42].

In a cardiac study, it was discovered that the AKT-mTOR-K_{ATP} signaling pathway is responsible for the preconditioning used for ischemia reperfusion. In this work, AKT mTOR-K_{ATP} antagonists were used, and the favorable effect of preconditioning was reported to diminish when this signaling pathway was inhibited[43,44].

The role of the AKT-mTOR-K_{ATP} signaling pathway in the penicillin model of epilepsy was examined in this study. In this investigation, openers and closures that work on both mitochondrial and cytoplasmic channels were utilized. Although activation of the mTOR pathway in the heart is protective, it has been demonstrated in numerous epilepsy models that mTOR activation in the epiletic process initiates this process[45].

In electrophysiological tests conducted by our team, the effects of both the general opener and the blocker, as well as the specific opener and closers of the K_{ATP} channel, were demonstrated, and the openers' anticonvulsant effects were demonstrated[42]. According to the findings of this study, the action of K_{ATP} openers on epilepsy may be mediated by the AKT-mTOR signaling pathway.

In this study, however, after the seizure focus was established, the impacts of the seizure AKT-mTOR signaling pathway were explored until the eighth day, as well as how mRNA expression is impacted. The role of mTOR in post-traumatic epilepsy has been studied (PTE). Rapamycin suppression of mTOR improved epileptic brain damage in patients with post-traumatic epilepsy[43]. Although there was no post-traumatic epilepsy in this study, it was similar to the first and fourth stages of a seizure. The protective effect of K_{ATP} opener pinacidil on the 1st and 8th days can be explained by a decrease in AKT/mTOR mRNA levels in these groups.

As a result, in the penicillin model of epilepsy, it was determined that while K_{ATP} channel openers act on the AKT/mTOR signal pathway on the 1st, 4th and 8th days after seizure, AKT and mTOR mRNA expression levels drop, whereas blockers increase mRNA expression levels.

Conclusion

This study has demonstrated that activation of the AKT/mTOR signaling pathway is effective in the development of epilepsy and that the effect of K_{ATP} channels on epilepsy may be via the AKT/mTOR signaling pathway. However, due to budget crunch, we could not verify protein by immunohistochemical staining of AKT and mTOR in the tissue and also the TUNEL staining.

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Conflict of Interest

Authors declare no competing interests.

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