There is a need to investigate the role of nuclear factor kappa B in the regulation of cyclooxygenase-2 expression in the epileptic rat brain and cultured hippocampal neurons. Immunofluorescence and polymerase chain reaction was used to detect the expression of nuclear factor kappa B and cyclooxygenase-2. In cultured hippocampal neurons and rat brain: the control group compared with the normal group, nuclear factor kappa B expression in the hippocampal dentate gyrus, cerebral cortex, the piriform cortex brain regions were significantly increased (P < 0.01). This is accompanied by a significant increase in cyclooxygenase-2 protein and mRNA expressions in the hippocampus (P < 0.01). In the experimental group compared to the control group, the nuclear factor-kappa B expression in the hippocampal dentate gyrus, cerebral cortex, piriform cortex, and other brain regions was significantly lower (P < 0.01), with the accompanying decrease in cyclooxygenase-2 protein and mRNA expression (P < 0.01) in the hippocampus. In conclusion, κB-decoy can inhibit nuclear factor kappa B activation in epileptic rat brain and cyclooxygenase-2 overexpression.

Keywords
NF-κB; κB-decoy; cyclooxygenase-2; seizures

1. Introduction

Epilepsy is a serious disease of the nervous system. Repeated seizures cause brain damage in people with epilepsy. It has been revealed that the inflammatory reaction induced by seizures is one of the reasons for the pathological changes in the brain, especially hippocampal sclerosis, including neuronal loss, glial cells proliferation, abnormal mossy fiber sprouting, and so on (Samland et al., 2003).

The brain tissue of epileptic patients undergoing surgical resection shows strong inflammatory response, including up-regulation of IL-1β, TNF-α, and IL-6 and other pro-inflammatory factors (Deng et al., 2019). More and more evidence derived from in vitro experiments and animal models, proving that inhibition of inflammatory response has a significant neuroprotective effect and reduces the seizure degree and mortality (Serrano et al., 2011). However, recent reports showed that the inflammatory reaction is closely correlated with cyclooxygenase-2 (COX-2) overexpression and increased levels of prostaglandin. COX-2 is the rate-limiting enzyme of PGs (prostaglandins) that has a close relationship with inflammatory disease, which not only causes oxidative stress reaction but also can convert arachidonic acid to PGs when activated. Although COX-2 can be regulated by promoting inflammatory factors as well as some growth factors related to an inflammatory reaction, the mechanism is still unclear.

 Activated nuclear factor-kappa B (NF-κB) can efficiently induce the transcription of extensive target genes such as cell adhesion molecules and cytokines and plays a crucial role in the course of inflammation and immune reaction. It has been reported that NF-κB plays a decisive regulatory role in the macrophages of mice and human colon cancer cells (Kojima et al., 2000; Sanjewa et al., 2019). We observed that NF-κB regulates the expression of COX-2 in PC12 cells and hippocampal neurons. Activation of NF-κB and hippocampal reformation in kainate-induced epileptic animal models has been reported (Won et al., 1999). The neuronal death and astrocyte activation in lithium-pilocarpine-induced epileptic rats caused by NF-κB and COX-2 has also been detected. We, therefore, proposed that NF-κB could play an essential role in brain injury during epilepsy. Although it has been reported that NF-κB activation cause neuronal death and astrocyte activation leading to the pathological changes in the brain of epileptic animals (Kim et al., 2001; Prasad et al., 1994; Rong and Baudry, 1996; Voutsinos-Porche et al., 2004). There are no reports on whether activated NF-κB can regulate one of its downstream target genes COX-2 in epileptic conditions.

Since 1996, the first transcription factor E2F "decoy" strategy approved by FDA (Food and Drug Administration), is used in treating endometrial hyperplasia after vessel bypass grafting and has been shown in several studies to authenticate its effect as gene therapy (Tomita et al., 2002). "Decoy" strategy has been applied in the treatment of NF-κB overactivation-related diseases like myocardial ischemic repercussion injury, glomerulonephritis, and so on in animal research (Morishita et al., 1998; Tomita et al., 2000). Herein, we designed and synthesized a double-stranded...
oligodeoxynucleotide (ODNs) -->B-decoy--which matches with the cis-element of NF-κB and a corresponding ODNs with irrelevant scrambled sequence--scrambled-decoy, up with vector ExGen500 form of complexes in vivo and in vitro experiments. These complexes were transfected into hippocampal neurons with the catalyzed NF-κB and then injected into the rat's cerebral ventricle with a micro-injection technique. The epilepsy model was then made. NF-κB activity and COX-2 expressions were determined in the hippocampal neurons and brain of rats to investigate the effects of NF-κB "decoy" on the expression of NF-κB and COX-2 in the neurons and the injured brain.

2. Methods

2.1 Design and synthesis of NF-κB-decoy

According to NF-κB enhancer specific sequence 5'-GGGACTTTCC-3', a double-stranded oligodeoxynucleotide (ODNs)-κB-decoy- was designed and synthesized -and this matches with the cis-element of NF-κB and a corresponding ODNs with irrelevant scrambled sequence -scrambled-decoy (Biological Engineering Company Limited, Dalian).

2.2 ExGen500 carrier parcels oligonucleotide

DNA stock solution (2 g/L) was diluted with 150 nM NaCl, and ExGen500 carrier (Fermentas) was diluted 18.3 times with sterile RNAse water. Then 13.2 μl diluted ExGen500 was added into DNA, slightly mixed, and cultured at room temperature for 10 min.

2.3 Culture method of hippocampus neurons

24 h-newborn SD rats were decapitated. The bilateral hippocampus was quickly stripped under a dissecting microscope, divided into three small pieces of size, and placed into a 0.125% glycine solution was diluted with 0.18% DEPC water was added to dissolve the RNA. Concentration and purity of extracted RNA were determined using Nanodrop.

2.4 Cell transfection and processing method

Hippocampal neurons were divided into 3 groups on the 9th day. The groupings were as follows: Experimental group: ExGen500 carrier and κB-decoy (1 μg DNA/plate), Control group: ExGen500 carrier and mismatch-decoy (1 μg oligonucleotide/plate) and, Normal group: ExGen500 carrier. 24 hours after transfection, each group was stained with LPS (100 ng/ml) for 12 h. The coverslips with attached cells were washed with 0.01 M PBS 3 × for 5 min each wash, fixed with cold acetone for 20 min at -20 °C and stored.

Hippocampal neurons were subdivided into 4 groups: experimental group (1-A) and control group (1-B) were transfected with 0.5 μg DNA/plate for 44 h, experimental group (2-A) and control group (2-B) were transfected with 1 μg DNA/plate for 44 h, experimental group (3-A) and control group (3-B) were transfected with 0.5 μg DNA/plate for 24 h, experimental group (4-A) and control group (4-B) were transfected with 1 μg DNA/plate for 24 h. Cells were collected after LPS (100 ng/ml) treatment for 12 h.

2.5 NF-κB and COX-2 immunocytochemical staining

The coverslips with attached cells were washed with 0.01 M PBS 3 × for 5 min each wash and washed with 1% H2O2 for 10 min, and blocked with 5% sheep serum (containing 0.3% triton-100 PBS) for 20 min. Primary antibody (NF-κB p65 1 : 100, CST #8242; COX-2 1 : 100, CST #12822) was applied, kept in a wet box and incubated at 4 ℃ overnight. Cells were rinsed in 0.01 M PBS 3 × for 5 min each wash, and then incubated with secondary antibody (1: 400) at room temperature for 1 h. After washing in 0.01 M PBS for 3 ×, and the avidin-biotin complex was applied and kept at room temperature for 2 h. DAB was applied and observed for color change under the light microscope. The reaction was terminated, dehydrated, and mounted with neutral gum on a glass slide.

2.6 Room temperature-polymerase chain reaction method for the detection of hippocampus neurons in COX-2 mRNA

The following primers were designed according to the rat COX-2 cDNA sequence: the forward: 5'-CTGTATCCCGCCTGCTGGTG-3', and reverse: 5'-ACTTGGCTTGATGGTGCGTCTT-3' (Dalian Bao biological Company).

After removing the culture medium, cells were rinsed with PBS 2 × for 3 min. 200 μl/well Trizol was added, oscillated in -20 ℃ freezer for 1 h, and kept at room temperature for 5 min. The cell suspension was removed into the EP tube after Percussion. Chloroform (40 μl/tube) was added and invered gently and kept at room temperature for 2-3 min, centrifuged at 12000 rpm for 15 mins at 4 ℃. The supernatant was collected into another tube and added 100 μl isopropanol into each tube and kept at room temperature (RT) to precipitate RNA for 10 min. It was then centrifuged at 12000 rpm for 10 min at 4 ℃ and supernatant discarded. The RNA pellet was washed with 75% ethanol (200 μl/tube). After air-drying, DEPC water was added to dissolve the RNA. Concentration and purity of extracted RNA were determined using Nanodrop.

Polymerase chain reaction (PCR) amplification was done with the following conditions: 94 ℃ 2 min, 1 Cycle; 94 ℃ 0.5 min, 50 ℃ 0.5 min, 72 ℃ 1.5 min, 28 cycles (reaction system following the kit provides steps).

2.7 Image analysis

The HPIAS series of color pathological image analysis system software was used for image analysis.

2.8 κB-decoy and mismatch -decoy-ExGen500 preparation

To prepare the κB-decoy or mismatched -decoy 5 μl of sterile 5% glucose solution was diluted with 1 μg κB-decoy or mismatched-decoy, and gently vortexed; another 5 μl of sterile 5% glucose solution was diluted with 0.18 μl ExGen500 and diluted in 5 μl ExGen500 (this order cannot be reversed). After vortexing, the mixture is placed at RT for 10 min.

2.9 Animal group and treatment

46 healthy adult male SD rats weighing 250-300 g were divided into three groups. The rats were housed in polypropylene
brains were transcardially perfused, removed, and post-fixed in 4% PFA overnight at 4 °C. Brains were dissected, and the hippocampus removed and promptly placed in -80 °C cryopreservation. The hippocampus was placed in an EP tube and 1 ml of Trizol and quickly homogenized (10,000 rpm) thoroughly. The mixture is kept for 5 min. 200 μl chloroform was added, inverted some times, and kept for 15 minutes. The mixture was centrifuged at 12,100 rpm at 4 °C for 15 min. The supernatant was carefully collected into a new EP tube; 500 μl of isopropanol was added, mixed by inverting 10 times, kept for 10 min and centrifuged at 12,000 rpm at 4 °C for 10 min. Discarded the supernatant, the pellet in the tube was washed with 75% alcohol, mixed by inversion and centrifuged at 12,000 rpm for 5 min. After removing the supernatant, the pellet was air-dried for 1 min and dissolved with DEPC water. RNA concentrations were determined using Nanodrop. Extracted RNA was converted to cDNA using the following conditions: 94 °C, 2 min; 1 Cycle. 94 °C, 0.5 min; 55 °C, 0.5 min; 72 °C, 1.5 min; 28 Cycles.

2.14 Statistical analysis
All statistical analyses were performed using SPSS18.0. Data are presented as mean ± SEM. Data between multiple groups were analyzed by 1-or 2-way analysis of variance followed by Fischer protected least significant difference post hoc tests. An unpaired t-test was used to analyze the differences between the 2 groups. P-Value < 0.05 was considered as the significance level for all analyses (*P < 0.05, **P < 0.01; # P < 0.05, ## P < 0.01).

3. Results
3.1 κB-decoy inhibit the expression and activation of NF-κB in hippocampal neurons
In normal cultured hippocampal neurons, the cytoplasm was pale yellow, and the nucleus was brown-yellow stained, indicating the low basal level of NF-κB expression and depict NF-κB was not activated. In the control group transfected with mismatch-decoy (1 μg oligonucleotide/well) and treated with LPS (100 ng/ml) for 12 h, NF-κB was activated significantly (P < 0.01). Hippocampal neurons in the experimental group transfected with κB-decoy (1 μg DNA/well) and treated with LPS (100 ng/ml) for 12 h, showed that NF-κB was not activated compared with the control group indicating that κB-decoy significantly inhibited or diminished NF-κB activation upon LPS treatment (P < 0.01, Fig. 1).

3.2 κB-decoy inhibits COX-2 expression in hippocampal neurons
In normal cultured hippocampal neurons, COX-2 expression was not evident. However, when transfected with mismatch-decoy oligonucleotides (1 μg/well 24 h) followed by LPS (100 ng/ml) treatment for 12 h (control group), COX-2 showed significantly higher expression (P < 0.01). Compared to the control group, COX-2 expression was not evident or had lower expression levels in the experimental group. An indication that κB-decoy significantly down-regulate COX-2 expression in hippocampal neurons after treatment with LPS (P < 0.01, Fig. 2).
3.3 \( \kappa \)-B-decoy inhibits COX-2 mRNA expression in hippocampal neurons

Hippocampal neurons transfected with mismatch-decoy (1-A, 2-A) or \( \kappa \)-B-decoy for 24 h (1-B, 2-B) as showed in our previous study (Sun et al., 2008). Portions of the neurons were stimulated with LPS (100 ng/ml) for 12 h (control group: 3-B, 4-B; Experimental group: 3-A, 4-A). The results showed higher levels of COX-2 mRNA expression in the control group compared with the experimental group (3-A, 4-A) \((P < 0.01, \text{Fig. } 3)\).

3.4 \( \kappa \)-B-decoy inhibits the expression and activation of NF-\( \kappa \)-B p65 in the seizure rat brain

In normal rat hippocampus, amygdala, piriform cortex, entorhinal cortex, cerebral cortex, and other seizure-related brain areas, weak NF-\( \kappa \)-B p65 expression was observed as neurons with pale yellow staining. Comparatively, the control group showed deeper staining. The pattern of staining depicts that neurons in the experimental group showed the pale-yellow staining or brown staining in a small number of neurons in brain regions mentioned above. Most nuclei did not show any staining. NF-\( \kappa \)-B p65 expression was significantly reduced \((P < 0.01)\), indicating \( \kappa \)-B-decoy could inhibit or weaken NF-\( \kappa \)-B activation upon seizure induction in rats’ brains (Fig. 4).

3.5 \( \kappa \)-B-decoy inhibits COX-2 expression upon seizure induction in rat brain

Positive cells without COX-2 immunoreactivity in the normal rat hippocampal hilus, the piriform cortex, the cerebral cortex, and other seizure-related brain regions were observed. Compared with the normal group, cells were significantly increased, and COX-2 immunoreactivity was deeper in the control group \((P < 0.01)\). COX-2 immunoreactivity was significantly reduced in the experimental group \((P < 0.01)\), indicating that \( \kappa \)-B-decoy could inhibit or weaken COX-2 expression in seizure-induced rats’ brains (Fig. 5).

3.6 \( \kappa \)-B-decoy inhibits COX-2 mRNA expression in the seizure rat brain

The mRNA expression level of COX-2 in the hippocampus of epileptic rats transfected with mismatch-decoy was significantly increased than that of the normal group \((P < 0.01)\). Meanwhile, the expression of COX-2 in the hippocampus of epileptic rats transfected with \( \kappa \)-B-decoy was significantly lower than that of the control group 4 hours after the seizure \((P < 0.01)\). There was no significant difference in these time points (2 h, 8 h, 12 h) (Fig. 6).
4. Discussion

NF-κB, which was first found by (Sen and Baltimore, 1986), was initially found in the extract of B cell nucleus is a protein that can specially bind with the enhancer κB sequence (GC-GACTTTC) from the light chain immune κ gene. It can combine specific enhancer genes at the B locus to initiate and promote gene transcription.

NF-κB is a nuclear transcription factor that widely exists in various cells. It is the convergence point of many signal transduction pathways and plays a crucial role in the regulation of inflammatory response. It consists of two subunits of the Rel family, the most common heterodimer composed of p65 (RelA) and p50 (of NF-κB). The N-terminus of the P65 has about 300 amino acid residues. The Rel protein oncogene real encoded highly homologous domains known as the Rel homology domain (RHD). The RHD contains a DNA binding sequence, nuclear localization sequence (NLS), and the formation of a dimer structure required. It is closely related to the biological effects of NF-κB. In a resting state, NF-κB forms a dimer with its inhibitor protein IκB, which results in the inhibition of NLS by RHD being covered. Therefore, there is no trimer activity in the cytoplasm by NF-κB. Therefore, there is no active NF-κB in the cytoplasm. When the cells are stimulated by a variety of external signals, IκB is phosphorylated and degraded. When NF-κB is activated, and it promotes the expression of its related genes (Foo and Nolan, 1999; Ghosh, 1998; Liu et al., 1999).

Activation of NF-κB can effectively induce the transcription of target genes and regulate the expression of many factors involved in immune and inflammatory responses, such as cell adhesion molecules, cytokines, and iNOS. Previous studies have shown that NF-κB plays a vital role in epileptic brain injury (Singh et al., 2018). However, whether the activation of NF-κB during epilepsy can induce brain inflammation and other pathological changes, especially in regulating the expression of its downstream target gene, cyclooxygenase-2, has not been reported.

Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme involved in the synthesis of prostaglandin (PG). It does cause not only oxidative stress reaction but also transforms arachidonic acid into PGs and other harmful substances. COX-2 is primarily present at the sites of inflammation. Barbalho et al. (2016) have shown that epilepsy can induce the up-regulation of COX-2 in clinical samples and experimental models of epilepsy. The epileptic inflammatory response is closely related to the high expression of
activated COX-2 in the brain and increased level of prostaglandins (PGs). The activation of COX-2 in the brain of epileptic animals can promote neuronal death caused by epileptic activity and can cause pathological changes such as abnormal nerve growth and glial proliferation (Keun et al., 2006; Kunz and Oliw, 2001).

It can shorten the time from seizure behavior to recovery state in epileptic animals and contribute to repeated seizures of epilepsy (Ciceri et al., 2002; Okada et al., 2001; Takemiya et al., 2003). The selective inhibitor of COX-2 can prolong the latency of epilepsy, reduced seizure behavior (Dhir and Kulkarni, 2006a; Dhir et al., 2006b; Gobbo and O’Mara, 2004) as well as reduced animal mortality. Besides, it can also inhibit the death of neurons and activation of glial cells in the hippocampus (Ciceri et al., 2002; Keun et al., 2006; Kunz and Oliw, 2001). It has also been found that hippocampal sclerosis and clinical astrocyte activation in patients with temporal lobe epilepsy are associated with COX-2 (Das et al., 2012; Desjardins et al., 2003).

Prostaglandin E2 (PGE2) can dynamically regulate the excitability, synaptic transmission, and plasticity of neuronal membranes, which is related to the reduced susceptibility of animals to epilepsy. Desjardins et al. (2003) have shown that high expression of activated COX-2 alters PGE2 levels in the brain, leading to pathological changes and hippocampal remodeling in epilepsy-sensitive animals. These studies indicate that COX-2 is closely related to epilepsy and is involved in brain-associated pathological changes caused by epilepsy. Although COX-2 may be a pro-inflammatory factor and participates in brain inflammation, the regulatory mechanism of COX-2 activation in the central nervous system remains unclear. Activation of NF-κB can effectively induce extensive gene expression and play a key role in inflammation and immune response. It is reported that NF-κB plays an active role in the expression of COX-2 in rat macrophages and human colon cancer cell lines (Dhir and Kulkarni, 2006a; Dhir et al., 2006b).

The results show that the expression of COX-2 can be changed either by activating or inhibiting the activity of NF-κB in cultured hippocampal neurons as well as epileptic rat brain. Therefore, we chose synthetic double-stranded NF-κB trapping oligodeoxynucleotide (κB-decoy) as the strategy for inhibiting NF-κB. κB-decoy is the double helix oligonucleotide (ODN) containing NF-κB binding site. When the NF-κB comes out from NF-κB-1:κB complexes, κB-decoy binds with the free NF-κB, which leads to the closure of NF-κB and affects the transcription of the target gene. This strategy has the following advantages compared with the other NF-κB inhibitor: (1) a potential drug targets (transcription factors); (2) synthesis of the trap ODN is relatively simple,
Figure 4. kB-decoy inhibits the expression of NF-κB in seizure-induced rat brain. ([A] Immunocytochemistry of NF-κB expression in seizure-induced rat’s brain (× 400). [B] The optical density of immunocytochemistry of NF-κB. N: Normal; S: scrambled-decoy + Pilocarpine; D: kB-decoy + Pilocarpine; * compared with N group; # compared with S group.

and it can be targeted in specific tissues; (3) do not have to clarify the precise molecular structure of the targeted transcription factor; (4) Trap-ODN can block the effects of various transcription factors connected to the same cis-acting element which means that it is better than antigens ODN. To avoid the shortcomings that the double chain ODN can be degraded by nuclease after entering the cell, we can use phosphorothioate, modify hexose, DNA methylation, modify three phosphoric acid ester, and borane for the ODN. Its main advantage is that it is similar to natural ODN with high toxicity and non-specificity. If the paper clip, annular or dumbbell-
Figure 5. B-decoy inhibits the expression of COX-2 in the seizure-induced rat brain. (A) Immunocytochemistry of COX-2 expression in the seizure-induced rat’s brain (× 400). (B) The optical density of immunocytochemistry of COX-2. N: Normal; S: scrambled-decoy + Pilocarpine; D: B-decoy + Pilocarpine; * compared with N group; # compared with S group.
shaped ODN is being designed, it does not only keep the advantages mentioned above but also avoid the disadvantages. Therefore, the sequence 5'-GGGACTTTCC-3’, which does not have a palindrome structure and has the highest affinity with NF-κB, is designed as a circular dumbbell decoy ODN. It contains two copies of the specific binding sequence of NF-κB to improve the effectiveness of the trap ODN.

NF-κB immunohistochemistry results show that in cultured hippocampal neurons, the expression of NF-κB was significantly increased in the control group (transfected with mismatch-decoy) compared with the normal group. While the expression of NF-κB was significantly decreased in the experimental group (transfected with κB-decoy) compared with the control group. In the rats’ hippocampus, piriform cortex, cerebral cortex, and other epilepsy-related brain regions, the expression of NF-κB was significantly increased in the control group (transfected with mismatch-decoy) compared with the normal group. While the expression of NF-κB was significantly decreased in the experimental group (transfected with κB-decoy) compared with the control group, these results indicate that κB-decoy is effective in inhibiting the activation of NF-κB in neurons of epilepsy-related brain areas.
COX-2 immunohistochemistry and RT-PCR results show that in cultured hippocampus neurons, the expression of COX-2 was significantly increased in the control group (transfected with mismatch-decoy) compared with the normal group. However, compared with the control group, the expression of COX-2 was significantly decreased in the experimental group (transfected with κB-decoy). These results show that κB-decoy can reduce the mRNA and protein expression levels of COX-2 in the cultured hippocampal neurons. In the rats’ hippocampus, piriform cortex, cerebral cortex, and other epilepsy-related brain areas, the expression of COX-2 are significantly increased in the control group (transfected with mismatch-decoy) compared with the normal group. While the expression of COX-2 is decreased substantially in the experimental group (transfected with κB-decoy) compared with the control group, all these results prove that κB-decoy can reduce the mRNA level and protein level of COX-2 in the cultured hippocampal neurons as well as epileptic rat brain.

5. Conclusions

In conclusion, the activation of NF-κB and high expression of COX-2 in the brain of epileptic rats can be inhibited by κB-decoy and, in turn, inhibit inflammation and oxidative stress during epilepsy.

Author contributions

Jie Zhao and Yiping Sun designed the research study. Jing Xu, Yongshun Zhao, and Qifa Li performed the research. Biying Ge, Shufang Dai, and Kemin Liu provided help and advice on the immunohistochemistry experiments and PCR. Hong Xu analyzed the data. LeiFu and Yiping Sun wrote the manuscript. All authors contributed to editorial changes in the manuscript.

Ethics approval and consent to participate

All procedures were subjected to the Institutional Animal Care and Use Committee guidelines and were approved by the Institutional Ethics Committee of Dalian Medical University.

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Conflict of Interest

The authors declare no conflict of interest.

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