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Effect of Pioglitazone on Excitotoxic Neuronal Damage in the Mouse Hippocampus

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Abstract

Pioglitazone (PGZ), a synthetic peroxisome proliferator-activated receptor γ agonist, is known to regulate inflammatory process and to have neuroprotective effects against neurological disorders. In the present study, we examined the effects of 30 mg/kg PGZ on excitotoxic neuronal damage and glial activation in the mouse hippocampus following intracerebroventricular injection of kainic acid (KA). PGZ treatment significantly reduced seizure-like behavior. PGZ had the neuroprotective effect against KA-induced neuronal damage and attenuated the activations of astrocytes and microglia in the hippocampal CA3 region. In addition, MPO and NFκB immunoreactivities in the glial cells were also decreased in the PGZ-treated group. These results indicate that PGZ had anticonvulsant and neuroprotective effects against KA-induced excitotocix injury, and that neuroprotective effect of PGZ might be due to the attenuation of KA-induced activation in astrocytes and microglia as well as KA-induced increases in MPO and NFκB.

Key Words: Pioglitazone, Kainic acid, Neuroprotection, Hippocampus, Astrocyte, Microglia

INTRODUCTION

Epilepsy leads to the functional and neuropathological alteration in some brain regions. Especially, among the brain regions, the hippocampus is known as one of the most vulnerable brain region in epilepsy. Pyramidal cell loss and glial activation in the hippocampus are distinctive neuropathological changes in various animal models of epilepsy (Matsuoka *et al*., 1999; Sun *et al*., 2008; Yu *et al*., 2008; Hong *et al*., 2012; Hong *et al*., 2013). In addition, it has been widely accepted that neuroinflammatory reaction occurs in epilepsy. Pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, are increased in rodent brain in epilepsy, and the increase of pro-inflammatory cytokines is closely associated with the neuropathological changes and epileptogenesis (Ravizza *et al*., 2005; Vezzani and Granata, 2005; Sun *et al*., 2008; Vezzani *et al*., 2013).

Peroxisome proliferator-activated receptor (PPAR), which is a member of nuclear hormone receptor superfamily, has been well known to be ligand-activated transcriptional factors regulating lipid metabolism and glucose homeostasis (Vamecq and Latruffe, 1999). Among PPAR subtypes, PPARγ has been found to have multiple actions, including the promotion of lipogenesis, increase of insulin sensitivity and down-regulation of inflammatory processes (Chinetti *et al*., 2000; Berger *et al*., 2005). Pioglitazone (PGZ), a synthetic agonist of PPARγ, has been widely used as an anti-diabetic drug for treatment of type 2 diabetes mellitus. Recently, it has been suggested that PPARγ agonists, including PGZ and rosiglitazone, have an anti-inflammatory and anti-oxidant effects and have neuroprotective effects against neurological and neurodegenerative disorders (Breidert *et al*., 2002; Inestrosa *et al*., 2005; Zhao *et al*., 2006; Lee *et al*., 2011).

Although some studies showed the neuroprotective effect of rosiglitazone in animal models of epilepsy (Sun *et al*., 2008; Yu *et al*., 2008; Hong *et al*., 2013), the underlying mechanisms of the neuroprotective effect of PPARγ agonists are not fully elucidated yet. In addition, there is no study on the neuroprotective effect of PGZ in epilepsy. In the present study, therefore, we examined the effects of PGZ on excitotoxic neuronal damage and glial activation in the mouse hippocampus following intracerebroventricular injection of kainic acid (KA), which is an excitatory glutamate receptor agonist and causes severe status epilepticus with neuronal death and glial activation in

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the hippocampus, especially in the hippocampal CA3 region.

MATERIALS AND METHODS

Experimental animals

Male ICR mice (B.W., 23-25 g) were purchased from Samtako Bio Korea Co. (Osan, South Korea). The animals were housed in a conventional state under adequate temperature (23 ± 3°C) and relative humidity (55 ± 5%) control with a 12-h light/12-h dark cycle, and provided with free access to food and water. The procedures for animal handling and care were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chungnam National University (CNU-00151). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Pioglitazone treatment and KA injection

To elucidate the effects of PGZ (Actos, Takeda Pharmaceuticals, Osaka, Japan) against excitotoxic neuronal damage, the animals were divided into 3 groups (n=8 at each groups); 1) vehicle (0.1% (*w/v*) methyl cellulose)-treated control group (control-group), 2) vehicle-treated KA-treated group (KA-group), 3) 30 mg/kg PGZ-treated KA-treated group (PGZ-KA-group). Vehicle and PGZ were administered orally using feeding needle for 3 days before KA injection: The last administration was performed at 1 h before KA injection. Dose of PGZ was selected based on the previous studies (Okada *et al*., 2006; Abdallah, 2010).

KA injection was performed by the method of our previous studies (Yi *et al*., 2012; Yi *et al*., 2013). In brief, KA (Sigma, MO, USA) was prepared as a stock solution at 5 mg/ml in sterile 0.1 M PBS; aliquots were stored at -20°C until required. KA was injected at right lateral ventricle (anteroposterior, -0.4 mm; mediolateral, 1 mm; dorsoventral, -2.3 mm relative to bregma) using a 50-μl Hamilton microsyringe fitted with a 26-gauge needle inserted to a depth of 2.4 mm (0.1 μg/5 μl in PBS, i.c.v.). Control mice received an equal volume of sterile 0.1 M PBS.

KA-induced seizure activity

KA-induced seizure-like behaviors were examined according to the method and the criteria of the previous studies (McLin and Steward, 2006; Jeong *et al*., 2011). The motor and behavioral characteristics, as well as the severity of the seizures, were classified according to the Racine scale: stage 1, immobility; stage 2, forelimb and or tail extension, rigid posture; stage 3, repetitive movements, head bobbing; stage 4, rearing and falling; stage 5, continuous rearing and falling; and stage 6, severe tonic-clonic seizures. Mice of the KA-group and PGZ-KA-group were observed for 2 h after KA injection. The number of times reaching at least stage 4 was counted to examine whether PGZ had an effect on the KA-induced seizure-like behaviors.

Tissue processing for histology

At 3 days after KA injection, mice were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brains were removed and postfixed in the same fixative for 4 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections.

Cresyl violet staining

To examine neuronal damage in the CA3 at 3 days after KA injection, cresyl violet (CV) staining was done according to the method of the previous study (Lee *et al*., 2010). In brief, the sections were mounted on gelatin-coated microscopy slides. Cresyl violet acetate (Sigma, MO, USA) was dissolved at 1.0% (w/v) in distilled water, and glacial acetic acid was added to this solution. The sections were stained and dehydrated by immersing in serial ethanol baths, and they were then mounted with Poly-Mount (Polysciences, USA).

To evaluate the neuroprotective effect of PGZ, CV-positive cells in pyramidal layer of hippocampal CA3 region were counted using an image analyzing system (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ, USA). The studied tissue sections were selected with 240-μm interval, and cell counts were obtained by averaging the counts from each animal.

Immunohistochemistry

Immunohistochemical staining of the tissue sections were performed using the avidin-biotin peroxidase complex (ABC) method described previously (Yi *et al*., 2012; Yi *et al*., 2013). Briefly, Parallel free-floating sections were subjected to endogenous peroxidase blocking with 1% H_2O_2 in PBS, followed by treatment with blocking buffer (1% fetal bovine serum in PBS and 0.3% Triton X-100 for 30 min) and incubation with primary antibodies. Rabbit anti-glial fibrillary acidic protein (GFAP, 1:200, Chemicon International, Temecula, CA, USA), rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1, 1:200, Wako, Osaka, Japan), Myeloperoxidase (MPO, 1:500, Dako), NFκB (p65, Rel A, 1:200, Thermo Fischer Scientific Inc. IL, USA) were used as primary antibodies. After washing with PBS, tissues were exposed to biotinylated anti-rabbit or mouse IgG and streptavidin peroxidase complex. Immunostaining was visualized with diaminobenzidine, and tissues were mounted using Poly-Mount (Polysciences, USA).

To quantitatively analyze immunoreactivity, digital images of the hippocampal CA3 region were captured with an AxioM2 light microscope (Carl Zeiss, Germany) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. The density of immunoreactivity in CA3 region was evaluated on the basis of optical density (OD). The OD of background was taken from areas adjacent to the measured area. After the background density was subtracted, a ratio of the OD of image file was calibrated as % (relative optical density, ROD) using Adobe Photoshop version 8.0 and NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). A ratio of the ROD was calibrated as %, with the control-group designated as 100%.

Statistical analysis

The data shown here represent the means ± SEM. Differences among the means were statistically analyzed by two-tailed Student *t*-test in order to elucidate the neuroprotective effects of PGZ after KA injection. In addition, differences of the mean ROD among the groups were statistically analyzed by analysis of variance (ANOVA) followed by Tukey's multiple range method. Statistical significance was considered at $p<0.05$.

RESULTS

KA-induced seizure activity

To evaluate whether PGZ had an anti-convulsive effect in KA-induced seizures, we observed behavioral seizure activity after KA injection. KA- and PGZ-KA-treated mice demonstrated the increased repetitive head bobbing movements within 14 and 18 min (p=0.013), respectively. In addition to retarding seizure latency, the frequency of behavioral seizure activity corresponding to seizure stage 4 above was significantly attenuated in PGZ-KA-treated mice compared to KA-treated mice (Fig. 1).

Neuronal damage

Neuronal death in the CA3 region at 3 days after KA injection was examined using CV staining. In the control-groups, CV-positive cells were observed abundantly (Fig. 2A and 2D). In the KA-group, marked neuronal damage was detected in the stratum pyramidale (SP) of the CA3 region at 3 days after KA injection; in this group, CV-positive cells were significantly decreased, compared to that in the control-group (Fig. 2B and 2D). However, in the PGZ-KA-group, the numbers of CV-positive cells in the SP of the CA3 region were much higher than those of the KA-group; the numbers of CV-positive cells in the PGZ-KA-group were lower than those in the control-group (Fig. 2C and 2D).

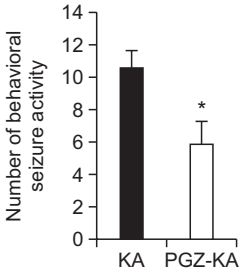

Fig. 1. Effect of PGZ on the number of seizure activity in KA- and PGZ-KA-groups. A. The frequency of seizure activity corresponding to seizure scale 4 above was significantly reduced in the PGZ-KA-group compare to the KA-group (n=8 per group; *p<0.05, significantly different from the KA-group). The bars indicate the means ± SEM.

Glial activation

In the control-group, GFAP-immunoreactive astrocytes with thread-like processes and small cytoplasm, and Iba-1-immunoreactive microglia with small cytoplasm were observed through the CA3 region (Fig. 3A and 3D). In the CA3 region of the KA-group, GFAP immunoreactivity was significantly increased and GFAP-immunoreactive astrocytes were observed as activated form with hypertrophied cytoplasm and thickened processes (Fig. 3B and 3G). Iba-1-immunoreactive microglia in the KA-group were increased and hypertrophied; in addition, Iba-1-immunoreactive microglia with dense cytoplasm were aggregated in the SP, where neuronal damage had occurred (Fig. 3E and 3G). In the PGZ-KA-group, activated astrocytes and microglia were also found in the CA3 region at 3 days after KA injection; however, their activation was much lower than that in the KA-group (Fig. 3C, 3F and 3G).

Immunoreactivities for MPO and NFκB

In the control-group, MPO-immunoreactive cells were hardly detected in the CA3 region (Fig. 4A). MPO immunoreactivity was increased markedly in the CA3 region, especially in the strata oriens and radiatum, of the KA group at 3 days after KA injection; in addition, MPO immunoreactivity was primarily detected in the glial cells of the CA3 region (Fig. 4B and 4G).

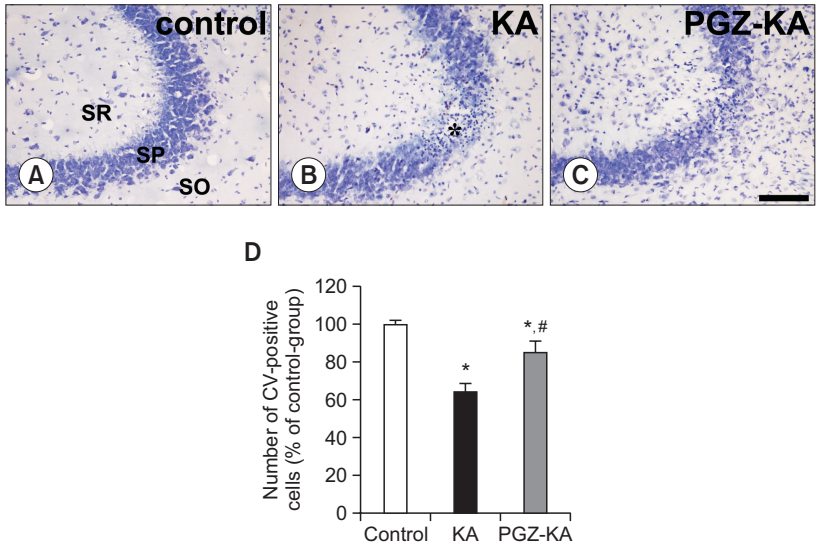

Fig. 2. CV staining in the hippocampal CA3 region of the control- (A), KA- (B) and PGZ-KA-(C) groups at 3 days after KA injection. In the KA-group, CV-positive cells are decreased in the stratum pyramidale (SP) of the hippocampal CA3 region (asterisk). However, in the PGZ-KA-group, many CV-positive cells are found compared to those in the KA-group. SO; stratum oriens, SP; stratum pyramidale, SR; stratum radiatum. Scale bar=100 μm. (D) Relative analysis in the number of CV-positive cells in the SP of hippocampal CA3 region (*p<0.05, significantly different from the control-group, $^{\#}p$<0.05, significantly different from the KA-group). The bars indicate the means ± SEM.

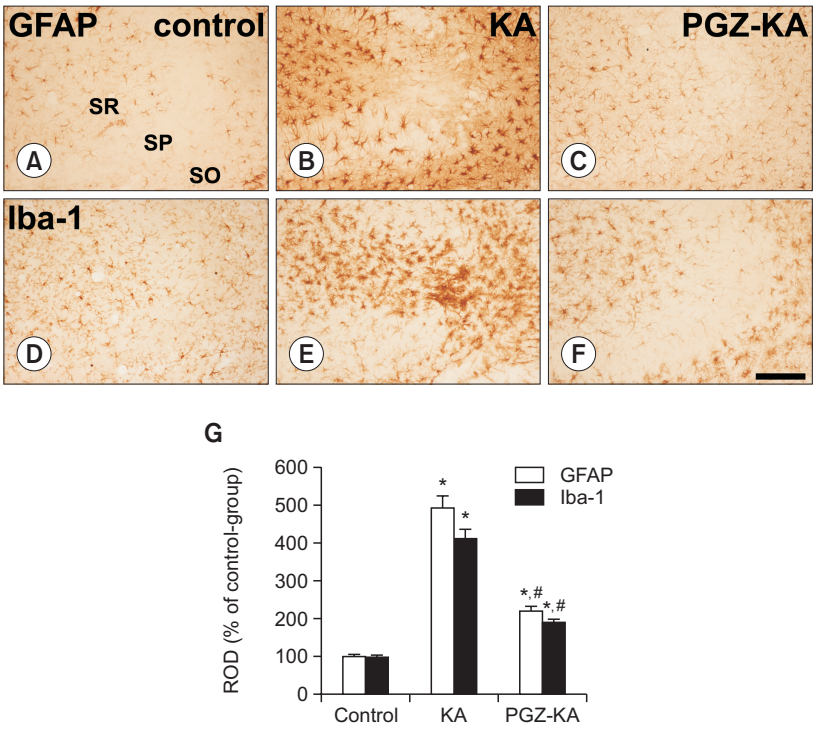

Fig. 3. GFAP (A-C) and Iba-1 (D-F) immunohistochemistry in the hippocampal CA3 region of the control- (A, D), KA- (B, E) and PGZ-KA- (C, F) groups at 3 days after KA injection. GFAP and Iba-1 immunoreactivity is markedly increased in the KA-group. However, in the PGZ-KA-group, GFAP and Iba-1 immunoreactivity are lower than those in the KA-group. SO; stratum oriens, SP; stratum pyramidale, SR; stratum radiatum. Scale bar=100 μm. (G) Relative optical density as % of GFAP- and Iba-1-immunoreactive structures in the CA3 region of the control-, KA- and PGZ-KA-groups (*p<0.05, significantly different from the control-group, #p<0.05, significantly different from the KA-group). The bars indicate the means ± SEM.

In the PGZ-KA-group, MPO immunoreactivity was much lower than that in the KA-group (Fig. 4C and 4G).

Weak NFκB immunoreactivity was found in the CA3 region of the control group (Fig. 4D). In the KA-group, NFκB immunoreactivity was significantly increased compared to that in the control-group; in addition, NFκB-immunoreactive cells resembled astrocytes and microglia morphologically (Fig. 4E and 4G). NFκB immunoreactivity in the PGZ-KA-group was markedly decreased compared to that in the KA-group, and there is no significant difference in NFκB immunoreaction between the PGZ-KA-group and control-group (Fig. 4F and 4G).

DISCUSSION

In this study, we examined the anticonvulsant effect of PGZ against KA-induced seizure, and we found that PGZ treatment significantly reduced seizure-like behavior. Our present result is in a line with results of the previous studies, which showed the anticonvulsant effect of PPARγ agonists in animal models of epilepsy. It was reported that PGZ protected against pentylenetetrazole-induced seizure and delayed seizure latency onset (Abdallah, 2010). PGZ has been also known to delay the development of seizure responses and to shorten the duration of convulsion in the genetically epilepsy-susceptible EL mice (Okada *et al*., 2006).

It has been well known that pyramidal neurons in hippocampal CA3 region are very vulnerable to KA-induced acute excitotoxic neuronal death (Beal, 1992; Penkowa *et al*., 2005; Kim *et al*., 2010). Some studies reported the neuroprotective effect of rosiglitazone in animal models of epilepsy. It was reported that rosiglitazone could protect the hippocampal neurons against lithium-pilocarpine induced status epilepticus injury (Sun *et al*., 2008; Yu *et al*., 2008; Hong *et al*., 2013). In the present study, we found that PGZ had the neuroprotective effect against KA-induced neuronal damage in the hippocampal CA3 region. To the best of our knowledge, this is the first study to show the neuroprotective effect of PGZ in KA-induced animal model of epilepsy. In addition, it was suggested that the anticonvulsive effect of PGZ might be associated with attenuating neuroinflammation and preventing apoptosis in brain. Therefore, it can be postulated that the neuroprotective effect of PGZ may be related to the anticonvulsive effect of PGZ.

Astrocytes play important roles in the central nervous system, such as regulation of ion homeostasis and neuronal functions (Horner and Palmer, 2003). Astrocytes have been known to be associated with the increased neuronal excitability in epilepsy (Ricci *et al*., 2009). In addition, It was suggested that activation of astrocytes and reactive astrogliosis, which are induced by seizure, contributes to epileptogenesis, spread of seizure activity and cognitive impairment, and that rosiglitazone could attenuated the astrocyte activation after status epilepticus (Hong *et al*., 2012). Microglia are known as the principal immune cells and resident macrophages of the brain. In various brain insults including seizure, microglia undergo the reactive changes with altered morphology, pro-

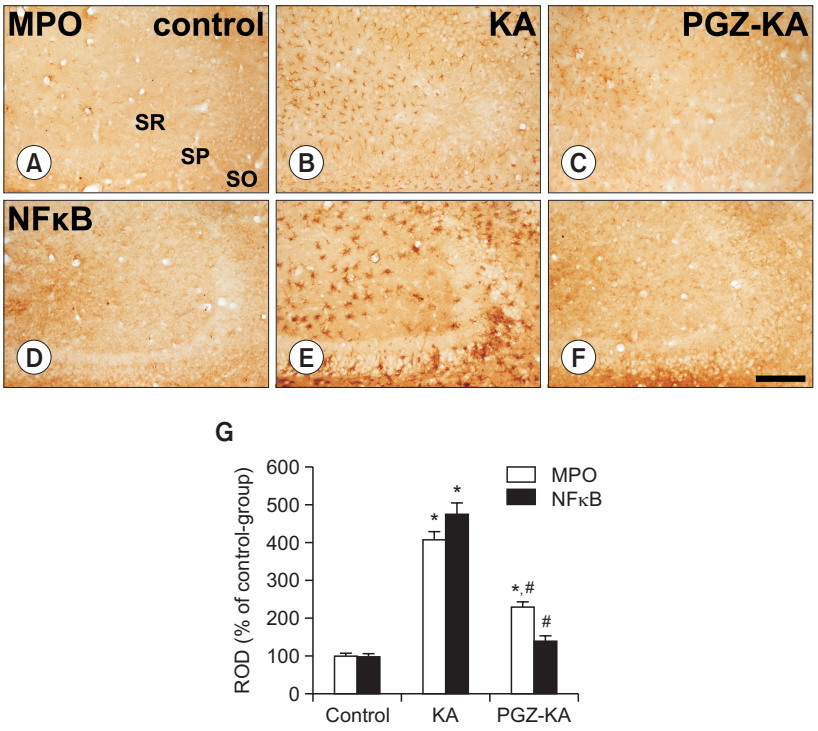

Fig. 4. MPO (A-C) and NFκB (D-F) immunohistochemistry in the hippocampal CA3 region of the control- (A, D), KA- (B, E) and PGZ-KA- (C, F) groups at 3 days after KA injection. MPO and NFκB immunoreactivity in the PGZ-KA-group is much than those in the KA-group. SO; stratum oriens, SP; stratum pyramidale, SR; stratum radiatum. Scale bar=100 μm. (G) Relative optical density as % of MPO- and NFκB-immunoreactive structures in the CA3 region of the control-, KA- and PGZ-KA-groups (*p<0.05, significantly different from the control-group, #p<0.05, significantly different from the KA-group). The bars indicate the means ± SEM.

liferation and production of pro-inflammatory cytokines and reactive oxygen species (Minghetti and Levi, 1998; Tooyama *et al*., 2002). It has been known that inhibition of microglia activation could decrease the status epilepticus-induced early brain injury and neuronal death (Chung and Han, 2003). It was also reported that microglia were notably increased and highly activated in the hippocampus after status epilepticus, and that neuroprotective effect of rosiglitazone against status epilepticus-induced hippocampal neuronal death was closely related to the suppression of microglia activation (Sun *et al*., 2008; Yu *et al*., 2008). In addition, PPARγ agonists have been known to decrease the induction of pro-inflammatory genes, such as TNF-α and IL-1, in glia (Bernardo *et al*., 2000; Luo *et al*., 2006). In the present study, we found that PGZ attenuated the KA-induced activations of astrocytes and microglia in the hippocampal CA3 region. Therefore, based on the previous studies, it is likely that decreases of astrocytes and microglia activation by PGZ may be a possible mechanism with the neuroprotective effect of PGZ against excitotoxic neuronal damage by KA.

MPO is known to be involved in the developments of Parkinson disease, and blockade of MPO activity can ameliorate decrease neuropathological conditions (Choi *et al*., 2005). It has been well known that MPO has properties of cytokine and regulates inflammatory signaling cascades, and that MPO triggers pro-inflammatory response in rotenone-exposed microglia (Kumar *et al*., 2005; Lau *et al*., 2005; Chang *et al*., 2011). Recently, it was reported that MPO could increase its own expression and activity in rat primary astrocytes and microglia (Chang *et al*., 2013). NFκB has been known to be expressed highly in many neuropathological conditions, and plays roles in inflammatory responses (Miyamoto and Verma, 1995). In addition, NFκB in glial cells, not in neuronal cells, is thought to be related to the induction of pro-inflammatory cytokines (Bales *et al*., 1998). It has been well known that NFκB is activated in glial cells of the hippocampus in a few days after KA treatment (Matsuoka *et al*., 1999; Lerner-Natoli *et al*., 2000). In this study, we observed that NFκB immunoreactivity in glia of the KA-group was significantly increased compared to that in the control-group. This result was consistent with a previous study which showed that NFκB immunoreactivity was strong in glial cells 3 and 7 days after KA injection (Matsuoka *et al*., 1999). In addition, it was reported that reduction of glial NFκB was related to the down-regulation of microglial and astroglial response and the drastic reduction in lesion volume against excitotoxic injury in postnatal brain (Acarin *et al*., 2001). In this study, we also found that both MPO and NFκB immunoreactivities in the glial cells of the PGZ-KA-group were significantly decreased compared to that in the KA-group. Therefore it can be postulated that decreases in MPO and NFκB immunoreactivities by PGZ treatment may be closely associated with the reduction in activations of astrocytes and microglia by PGZ.

In summary, PGZ had anticonvulsant and neuroprotective effects against KA-induced excitotoxic injury. In addition, neuroprotective effect of PGZ might be due to the attenuation of KA-induced activation in astrocytes and microglia as well as KA-induced increases in MPO and NFκB expressions.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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