Neuroprotective Effect of N-nitro-L-arginine Methylester Pretreatment on the Early Stage of Kainic Acid Induced Neuronal Degeneration in the Rat Brain

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Objective : Kainic acid(KA) enhances the expression of nitric oxide synthase, increases nitric oxide(NO), and thus evokes epileptic convulsion, which results in neuronal damage in the rat brain. NO may stimulate cyclooxygenase type-2 (COX-2) activity, thus producing seizure and neuronal injury, but it has also been reported that KA-induced seizure and neurodegeneration are aggravated on decreasing the COX-2 level. This study was undertaken to investigate whether the suppression of NO using the NOS inhibitor, N-nitro-L-arginine methyl ester(L-NAME), suppresses or enhances the activity of COX-2.

Methods : Silver impregnation and COX-2 immunohistochemical staining were used to localize related pathophysiological processes in the rat forebrain following KA-induced epileptic convulsion and L-NAME pretreatment. Post-injection survival of the rat was 1, 2, 3days and 2months, respectively.

Results : After the systemic administration of KA in rats, neurodegeneration increased with time in the cornu ammonis (CA) 3, CA 1 and amygdala, as confirmed by silver impregnation. On pretreating L-NAME, KA-induced neuronal degeneration decreased. COX-2 enzyme activities increased after KA injection in the dentate gyrus, CA 3, CA 1, amygdala and pyriform cortex, as determined by COX-2 staining. L-NAME pretreatment prior to KA-injection, caused COX-2 activities to increase compared with KA- injection only group by 1day and 2days survival time point.

Conclusion : These results suggest that L-NAME has a neuroprotective effect on KA-induced neuronal damage, especially during the early stage of neurodegeneration.

KEY WORDS : Cyclooxygenase-2(COX-2) · Kainic acid · Neurodegeneration · Nitric oxide(NO) · N-nitro-L-arginine methyl ester (L-NAME).

Introduction

The systemic administration of kainic acid(KA) enhances the expression of nitric oxide synthase(NOS), increases nitric oxide(NO), thus evokes status epilepticus and results in neuronal injury in the rat brain, especially in the hippocampus[1,12,16,25]. Recently, KA-induced seizure has been reported to enhance cyclooxygenase type-2(COX-2) expression in the rat forebrain[5,6,22]. COX is the rate-limiting enzyme in the synthesis of various prostanoids from arachidonic acid. Two types of COX are known, the first is the constitutive (COX-1) type and the other is the inducible one. Experimental evidence suggest that the enhanced expression of COX-2 and the accumulation of products of the arachidonic acid metabolism are implicated in the pathophysiology of epilepsy and cerebral neuronal degeneration[3,17]. Recent data suggests that NO may stimulate COX-2 activity, thus producing seizure and neuronal damage[6,22], but other studies have reported that certain COX-2 inhibitors aggravate KA-induced seizure and neuronal damage in the rat brain[2,7,23].

The present study was performed to investigate whether the suppression of NO by the NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), would suppress or enhance the activity of COX-2. We also examined the temporal activities

of COX-2 according to the specific cerebral regions. Finally, we investigated the possibility of relations between the degree of neuronal degeneration in each region and COX-2 activity. Accordingly, two unique histological methods (silver impregnation and COX-2 immunohistochemistry) were used to directly localize and characterize the pathophysiological changes occurring in selected regions of the rat forebrain, including the motor cortex, somatosensory cortex, dentate gyrus, CA 3, CA 1, amygdala and pyriform cortex.

Materials and Methods

Animals and processing

10-week-old male Fischer 344rats were used in this study. The animals were housed individually under a constant light-dark cycle (light on from 06:00 to 19:00h) with access to food and water supplied ad libitum. L-NAME (30mg/kg, i.p.) was administered 2h prior to KA, which was injected systematically (8mg/kg, s.c.). Seizure activity was monitored behaviorally, and 2h after convulsive status epilepticus. Seizures were terminated with diazepam (5mg/kg, i.p.) to prevent death. Rats that displayed continuous, generalized convulsive seizure activity after KA treatment were used in this study. Generalized convulsions were defined as repeated bouts of head nodding and forelimb clonus, with intermittent episodes of whole body clonus. Animals were sacrificed at 1, 2, 3days and 2months after KA administration (n=6per time point). Rats were anesthetized with pentobarbital sodium (50mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Brains were removed and postfixed in the same fixative overnight and subsequently cryoprotected with 20% sucrose in 0.0M phosphate-buffered saline(PBS), pH 7.4 for 48h. Frozen 40μm sections were prepared in the coronal plane using a cryostat.

Silver impregnation staining and analysis

Silver impregnation for the detection of degenerating cells was performed using Gallyas method[8]. This method reliably stains injured neurons and does not produce false-positive staining. The sections were immersed in a solution of 4.5% sodium hydroxide and 0.6% ammonium nitrate in distilled water. Subsequently, the sections were incubated in a solution containing 5.4% sodium hydroxide, 6.4% ammonium nitrate and 0.2% silver nitrate. After washing in a solution containing 0.012% ammonium nitrate, 0.5% sodium carbonate and 30% ethanol, the sections were incubated in a solution containing 0.012% ammonium nitrate, 0.05% citric acid, 0.55% formalin and 10% ethanol. All incubations were performed three times for 5min. After incubation, the sections were mounted directly on slide glasses from the last mentioned

Table 1. Summary of silver impregnated cells after KA administration and NAME pretreatment in the rat forebrain

		MC	SC	DG	CA3	CA1	AMG	PC
Control	1d	−	−	−	−	−	−	−
KA	1d	−	−	−	−	−	+	−
Group	2d	−	−	−	++	++	++	−
	3d	−	−	−	+	+++	+	−
	2m	−	−	−	++	++++	+	−
KA+NAME	1d	−	−	−	−	−	−	−
Group	2d	−	−	−	+	++	+	−
	3d	−	−	−	+	++	−	−
	2m	−	−	−	+	++	−	−

+ = 10~50cells/mm², ++ = 50~100cells/mm², +++ = 100~150cells/mm², ++++ = > 150cells/mm², MC : motor cortex, SC : sensory cortex, DG : dentate gyrus, CA : cornu ammonis, AMG : amygdala, PC : pyriform cortex, KA : kainic acid, NAME : N−nitro−L−arginine methyl ester

solution, dried, dipped in xylene and cover-slipped with Histomount media. Impregnated cells were counted under a microscope ($\times$200) in each cerebral region.

COX-2 immunohistochemical staining and analysis

Sections were incubated for 24h in PBS at 4°C containing COX-2 antiserum, 0.3% Triton X-100, 0.5mg/ml bovine serum albumin and 1.5% normal horse serum. Sections were then incubated with biotinylated secondary antibody (Vector, Burlingame, U.S.A.) diluted 1:200 for 90min and with avidin-biotin-peroxidase complex (1:100, Vector) for 1h at room temperature. The peroxidase was reacted with 0.02% 3, 3'-diaminobenzidine and 0.01% H_2O_2 for about 3min. After each incubation step, sections were washed three times with PBS for a total of 15min. Rat brain analysis was carried out using the atlas of Paxinos and Watson[19]. Under an image analyzer (Multiscan, Fullerton, CA), the average optical densities of immunohistochemically stained cells (N=15 to 20) in each cerebral region were measured.

Statistics

Mean ± S.E.M. of optical density was calculated and statistical analysis was performed using the paired sample test. Differences were considered statistically significant when P value was scored less than 0.05.

Results

Silver impregnation staining

In the motor cortex, somatosensory cortex, dentate gyrus, and pyriform cortex, silver-impregnated cells were nearly not detected throughout the time course in both groups (Table 1), (Fig. 1). In CA 3, silver-impregnated cells were apparent at 2 and 3days, and at 2months after KA injection. After pretreatment with NAME, silver impregnated cell numbers decreased after 2days versus KA injection group (Table 1).
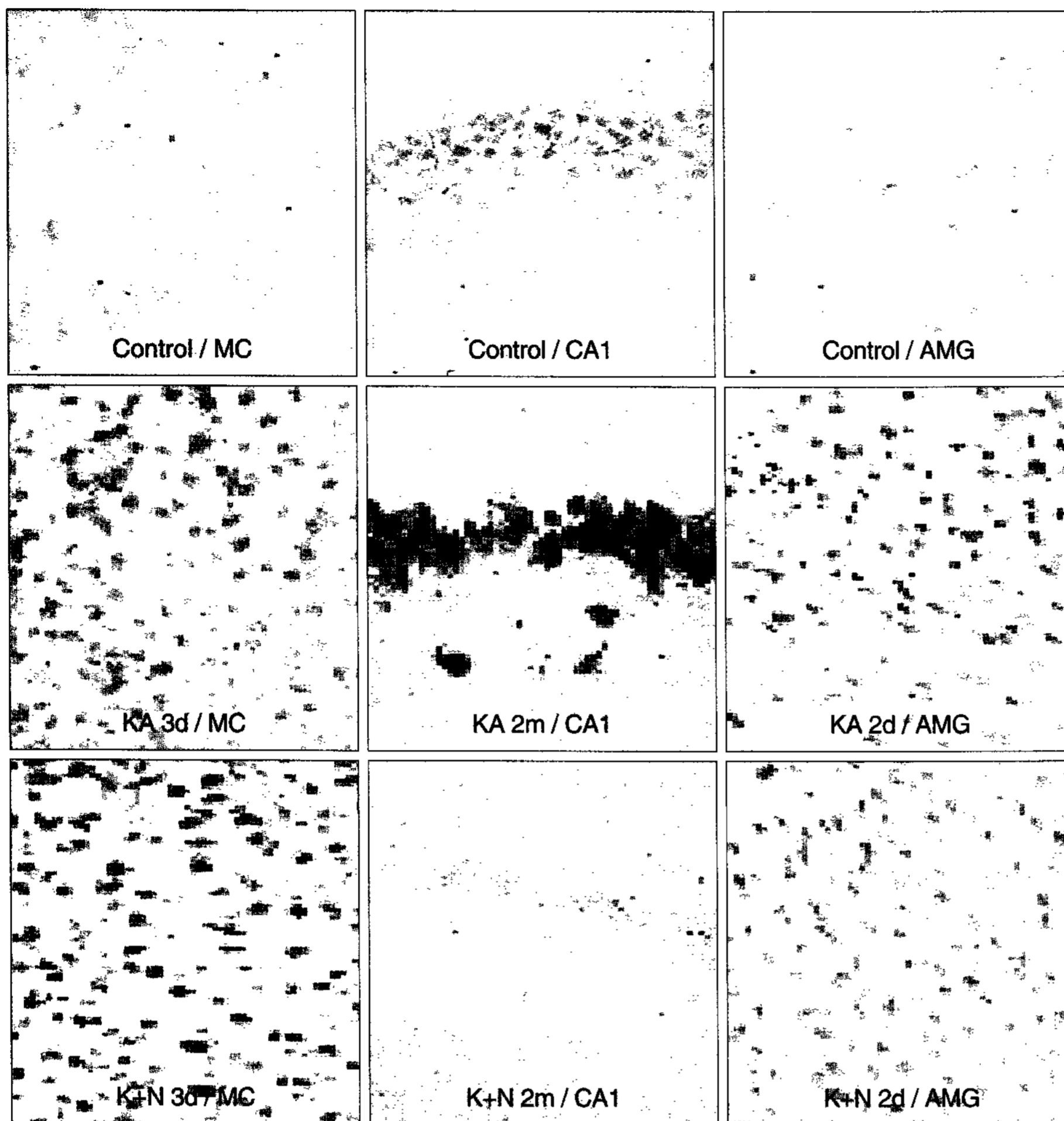
Fig. 1. View of regional changes in kainic acid(KA)−induced silver impregnation staining. Following KA injection and KA+N−nitro−L−arginine methyl ester(NAME) pretreatment, in the motor cortex, positively stained cells are little seen at 3days of time period. The silver−impregnated cells appear prominently in the cornu ammonis(CA) 1 at 2months and in the amygdala(AMG) at 2days after KA administration. With the NAME pretreatment, in the CA 1 and AMG, positively stained cells are much reduced in comparing of those of KA injection group. Silver−impregnated cells are dark brown to black in color (x100). MC : motor cortex, CA 1 : cornu ammonis 1, AMG : amygdala, KA : kainic acid injection, K+N : kainic acid injection+NAME pretreatment.

In CA 1, silver impregnated cells most prominently showed evidence of staining, and this was apparent 2days after KA injection and increased with time. Positively stained cell numbers reduced compared with the KA group 3days and 2months after NAME pretreatment followed by KA administration (Table 1), (Fig. 1). Silver impregnated cells were detected 1day and were visible throughout the time course of experiment in the amygdala. After NAME pretreatment and KA administration, silver impregnated cells were not only reduced compared with the KA administered group, but were seen only 2days after KA injection (Table 1), (Fig. 1).

COX-2 immunohistochemical staining

In the motor and somatosensory cortex, COX-2 activities increased by 1 and 2days after KA injection. At 3days, COX-2 reached a peak level and then its activity reduced below

the control level. After NAME pretreatment and KA injection, COX-2 activity was enhanced significantly at 1day, 2days and 3days after KA injection and returned to the control level at 2months after injection (Table 2), (Fig. 2). In the dentate gyrus, CA 3, amygdala and pyriform cortex, COX-2 activity increased significantly over the same time course (peak at 3days) and then reduced below the control level by 2months after KA injection. On NAME pretreatment, COX-2 activity was higher than those in the KA group at 1day and 2days but at 3days it had significantly reduced below the activity of the KA-injected group (Fig. 2). In CA 1, COX-2 activity had increased but not significantly, 2 and 3days after KA injection and reduced below the control level, with statistical significance, 2months after KA injection. After NAME pretreatment and KA injection, the enzyme activity was higher than that in the KA-injected group throughout (Fig. 2). COX-2 enzyme activity in the KA-injected group peaked 1day after KA injection (except CA 3, which peaked at 3days) and then was somewhat reduced at 2days, but increased again to a peak level at 3days. In contrast, in NAME and KA injection group, COX-2 enzyme had a peak at 1day and then gradually decreased to the basal level with time.

Discussion

Kainic acid(KA) is known to be a type of glutamate analogue, and a potent neuroexcitant. It is widely used in animal models of epilepsy and neuronal degeneration. KA enhances the expression of NOS, increases NO, and thus evokes status epilepticus, which results in neuronal injury in the rat brain, especially in the hippocampus[1,12,13,16]. It has been reported that the pattern and extent of neurodegeneration may be dependent on the spread of seizure activity to regions interconnected by excitatory neural pathway[4] or receptor
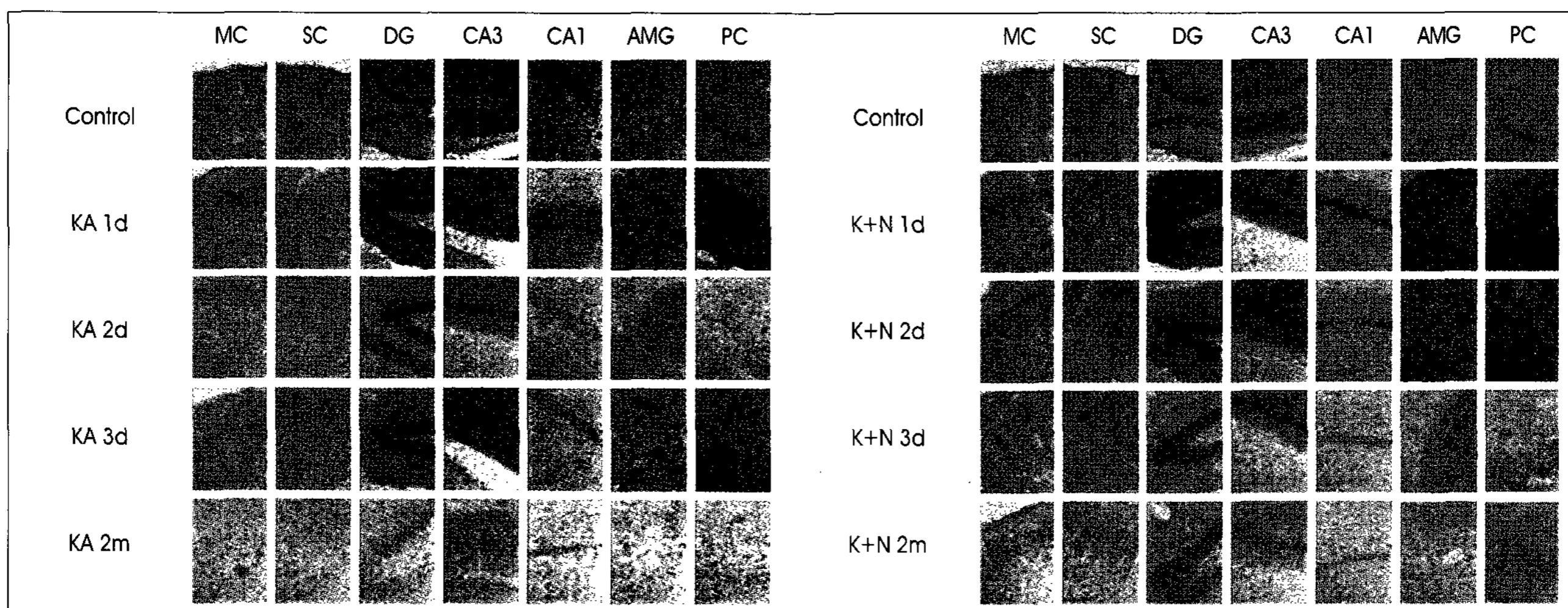
Fig. 2. Illustration of spatial distribution of kainic acid(KA)-induced cyclooxygenase type-2(COX-2). All of the brain regions show increased COX-2 activity over 3days versus the control group, and then the activity decreased below it at 2months. In the motor cortex(MC), there is an increased enzyme activity over 3days (peak at 3days) and then decreased below the control level. In the sensory cortex(SC), dentate gyrus (DG), cornu ammonis(CA 3), amygdala(AMG), and pyriform cortex(PC), increased activity is observed at 1day and then which ameliorated at 2days. At 3days, COX-2 enzyme re-increased to the peak level. In CA 1, COX-2 activity is prominent by 1day and tended to decrease to the control level but still increased by 2 and 3days. After N-nitro-L-arginine methyl ester(NAME) pretreatment, in the MC and SC, there is an increased COX-2 activity over a time period versus the control group and KA injection only group. In the DG, CA 3 and CA 1, COX-2 activity increasing at 1day, 2 and 3days and then decreasing below the control level. The enzyme activity increasing at 1day and 2days in the AMG and PC versus the KA group but decreasing at 3days. MC : motor cortex, SC : sensory cortex, DG : dentate gyrus, CA 3 : cornu ammonis 3, CA 1 : cornu ammonis 1, AMG : amygdala, PC : pyriform cortex, KA : kainic acid injection, K+N : kainic acid injection+NAME pretreatment.

Table 2. Summary of COX-2 immunoreactivity after KA administration and NAME pretreatment in the rat forebrain

		MC	SC	DG	CA3	CA1	AMG	PC
Control	1d	62.8±1.4	61±1.3	65.9±5.4	72.2±2.9	67.4±3.6	80.4±4.9	81.1±3.3
KA	1d	65.2±5.2	73.7±1.4ᵃ	92.9±1.6ᵃ	100.7±1.9ᵃ	80.6±1.4ᵃ	112.1±6.5ᵃ	122.1±4.0ᵃ
Group	2d	64.1±2.0	63.6±2.2	87.5±2.1ᵃ	77±0.6ᵃ	72.7±2.7	93.1±1.5ᵃ	92.2±1.3ᵃ
	3d	71.1±1.8ᵃ	73.5±2.8ᵃ	95.6±1.7ᵃ	128.3±1.2ᵃ	71±1.7	110.8±2.0ᵃ	123.1±2.9ᵃ
	2m	57.2±3.7	57.8±3.6	61.5±2.3	68.9±1.2	45.1±1.2ᵃ	57.5±3.0ᵃ	55.7±2.4ᵃ
KA+NAME	1d	82±1.2ᵃ,ᵇ	81.6±1.3ᵃ,ᵇ	125.6±5.1ᵃ,ᵇ	124.9±2.2ᵃ,ᵇ	100.2±6.4ᵃ,ᵇ	126±9.3ᵃ	146.5±2.6ᵃ
Group	2d	77.4±1.4ᵃ,ᵇ	84.8±2.5ᵃ,ᵇ	103.7±2.8ᵃ,ᵇ	123.1±3.9ᵃ,ᵇ	91.3±7.3ᵃ,ᵇ	111.1±6.5ᵃ,ᵇ	139.4±2.9ᵃ,ᵇ
	3d	78.1±2.4ᵃ,ᵇ	86.1±1.8ᵃ	84.6±3.0ᵃ,ᵇ	110.1±2.9ᵃ,ᵇ	80.5±1.9ᵃ,ᵇ	93.5±2.9ᵃ,ᵇ	113.7±5.3
	2m	69.4±4.4ᵇ	68.2±2.4ᵇ	63.8±2.4	67.5±0.8	48.7±2.9ᵃ	62.3±0.9ᵃ	67.4±2.0ᵃ,ᵇ

Data are mean S.E.M. of optical density. a ; P<0.05 compared with control group, b ; P<0.05 compared with KA group. Abbreviation : Refer to the table notes of Table 1

subtype distribution[15]. Several previous studies that have used KA-induced seizure models have shown that L-NAME, a NOS inhibitor, attenuates hippocampal neuronal damage by lowering the activity of NO[18,20,26]. The present study shows that KA-induced neuronal degeneration mainly occurs in CA 3, CA 1, and amygdala, known as the limbic system, and confirms the neuroprotective effects of L-NAME by silver impregnation staining.

Several recent studies have reported that NO activates cyclooxygenase both in vivo and in vitro[21,22], and increases COX-2 mRNA and COX-2 enzyme protein expression after chemical seizure induction in the hippocampus of the rat[14,27]. The enhanced expression of COX-2 immunoreactivity and the accumulation of the brain prostaglandin E2 have been observed during the preconvulsive period, suggesting its role in the

triggering mechanism of seizure and neuronal degeneration[18]. The present data shows that COX-2 is mainly expressed in the allocortex and amygdala under normal conditions and this is similar to the pattern of COX-2 immunoreactive neurons reported previously[27]. After the systemic administration of KA, COX-2 enzyme activities were enhanced in the same area until 3days after KA injection. Pretreatment of L-NAME prior to KA enhanced COX-2 enzyme activity after seizure in most parts of the rat forebrain, especially in the allocortex and amygdala, at 1day and 2days after KA injection. However, at 3days after KA injection, COX-2 activity was attenuated, especially in the dentate gyrus, CA 3, amygdala and pyriform cortex. We could not conclude as to why there was a reciprocal COX-2 activity 3days after KA injection between the two group, but it might be related with peak enzyme activity after KA administration versus KA and NAME injection.

The physiological importance of COX-2 expression induced by glutamate receptor activation is poorly understood. Some authors have reported that COX-2 derived prostanoids appear to regulate the activity of glutamate receptors, and thus exaggerated KA-induced seizure activity and mortality in mice[25]. On the other hand, endogenous prostaglandins(PG) are

known to have anticonvulsive properties[23]. Thus COX-2 inhibitors, such as indomethacin, markedly suppress the basal production of PGs and KA-induced PGs. As a result of the suppression of endogenous prostanoids, KA-induced epileptic convulsions are aggravated, which enhances neuronal degeneration[2].

The present study was designed to determine whether KA-induced COX-2 would aggravate or ameliorate the neuronal degeneration after convulsive epilepsy because decreased COX-2 activities are known to exaggerate seizure activity[3,7]. The pathway(s) leading to COX-2 activation by NO are unknown but may be involved in an interaction at the iron-heme center of the enzyme. Moreover, it is known that COX contains an iron-heme center at its active site[11], and that NO interacts with iron containing enzyme, leading to the stimulation or inhibition of the enzymic activity[9,10]. The interaction between NO and the COX pathway may be a critical determinant in the optimal function of COX, thus the regulation of COX activity by NO may represent an important role, whereby the initial inflammatory response can be amplified or attenuated[23,25]. This study characterized the temporal progression of neuronal pathologies and the enhancement of COX-2 activity caused by the systemic administration of KA by using silver impregnation and COX-2 immunohistochemical staining. Our data show that neuronal degeneration by KA is markedly reduced in certain parts of the rat forebrain including the CA 3, CA 1 and amygdala, demonstrating that NAME, a NOS inhibitor, has a neuroprotective effect on neuronal cells. COX-2 expressions 1day and 2days after KA administration were significantly enhanced by NAME pretreatment on the present study. So during the early phase of neurodegeneration, increased COX-2 expression could play a protective role on neuronal cells because neuronal degeneration is significantly lower at this time. However, at 3days after the NAME pretreatment and KA injection, reciprocal COX-2 activity was observed and COX-2 remained elevated but without statistical significance compared with KA injection only group in most limbic areas until 2months after the NAME pretreatment and KA injection. To resolve as to whether COX-2 has a neuroprotective or a neurodegenerative action on neural tissue according to the time course after KA administration, additional studies are required.

Conclusion

We investigate the interactions between NO and COX-2 with using L-NAME in KA-induced rat epilepsy model. With these results, we can strongly suggest that L-NAME, a NOS inhibitor, has a neuroprotective effect on the early stage of KA-induced neuronal damages.

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