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Research report

Cyclooxygenase-2 selective inhibitors aggravate kainic acid induced seizure and neuronal cell death in the hippocampus

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Abstract

Cyclooxygenase-2 (COX-2) in the brain is expressed constitutively and also increased in pathological conditions such as seizure, cerebral ischemia, and inflammation. This study examined the role of COX-2 in kainic acid-induced seizure and in the following neuronal death by using selective inhibitors. Systemic kainate injection (50 mg/kg; i.p.) in mice evoked seizure within 15 min and led to 29% mortality within 2 h. TUNEL-positive neuronal death peaked at 3 days after injection and was prominent in CA_{3a} regions of the hippocampus. NS-398 or celecoxib (10 mg/kg, COX-2 selective inhibitor) and indomethacin (5 mg/kg, nonselective inhibitor) exaggerated kainic acid-induced seizure activity and mortality. COX-2 selective inhibitors induced the seizure at earlier onset and more severe mortality within the first hour than indomethacin and aspirin. NS-398 also aggravated kainic acid-induced TUNEL positive neuronal death and decreased Cresyl violet stained viable neurons, and extended lesions to CA_1 and CA_{3b}. Kainic acid increased the levels of PGD_2, PGF_{2a} and PG E_2 in the hippocampus immediately after injection. Indomethacin attenuated the production of basal and kainic acid-induced prostaglandins. In contrast, NS-398 failed to reduce until the first 30 min after kainic acid injection, during which the animals were severely seizured. It has been challenged the endogenous PGs might have anticonvulsant properties. Thus, COX-2 selective inhibitor, including nonselective inhibitor such as indomethacin, aggravated kainic acid-induced seizure activity and the following hippocampal neuronal death even with variable prostaglandin levels.

Keywords: Cyclooxygenase-2 inhibitor; Kainic acid-induced seizure; Hippocampus; TUNEL-positive neuronal death; Prostaglandin

1. Introduction

Prolonged generalized seizure results in neuronal injury and death in the brain. In humans, this damage presumably involves the hippocampus and memory impairment as the major neurological sequela. Systemic administration of kainic acid evokes electrographic status epilepticus and neuronal injury, especially in the hippocampus, and has been proposed as a model of human seizures.

Cyclooxygenase-2 (COX-2) and their diffusible prostanoid products may be thought to play a role in postsynaptic signaling of excitatory neurons [16]. The COX-2 expression in the nervous system has been induced by pathological conditions such as cerebral ischemia, excitotoxic neuronal injury, and peripheral inflammation [1,12,27]. The COX-2 is implicated in the mechanism of neuronal death. Primarily, the neuronal cell death on ischemic injury model was prevented by COX-2 selective inhibitor [24]. However, although kainic acid increased the expression of COX-2 mRNA in the hippocampus [7], the role of COX-2 in kainic acid-induced seizure and the following neuronal death is still unclear. There are some reports kainic acid-induced neuronal death is reduced by treatment with COX inhibitors [4,6], but there is increasing evidence for a protective role of prostanoids in various tissues including nervous system [2,8,9,27]. Further exploration is needed to determine the specific roles of prostanoids in kainate-induced seizure and cell death, and the possible existence of factors associated with roles of COX isozymes and their prostanoids especially in brain damage.

Prostaglandins, COX metabolites of arachidonic acid, tend to increase in chemically or electrically induced seizure. In many experiments, it has been thought that endogenous prostaglandins may have anticonvulsant properties. Nonselective COX inhibitors such as indomethacin

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aggravated kainic acid-induced seizure [4,10]. Therefore, the exact role of COX isozymes such as COX-1 and COX-2 in kainic acid-induced seizure needs to be elucidated. In the present experiment, we investigated the effect of COX inhibitors, including COX-2 selective inhibitors, on kainic acid-induced seizure and the following hippocampal neuronal death.

2. Materials and methods

2.1. Injection of kainic acid and preadministration of cyclooxygenase inhibitors

Adult male mice weighing approximately 40 g were used for this experiment. Kainic acid (50 mg/kg, dissolved in saline, pH 7.0) or saline was injected intraperitoneally. The animals were pretreated with either one of test drugs or the corresponding vehicles before kainic acid. Indomethacin was given in a dose of 10 mg/kg or 5 mg/kg (i.p; dissolved in 100 mM sodium bicarbonate), or NS-398 and celecoxib, a selective COX-2 inhibitors, was given in a dose of 10 mg/kg (i.p.; dissolved in DMSO) 30 min before kainic acid. Aspirin, as a more COX-1 preferential inhibitor, was given in a dose of 10 mg/kg i.p. (dissolved in 5% ethanol).

2.2. Measurements of seizure activities

The behavior of the animals was evaluated for 2 h after injection of kainic acid according to the following rating scale [31]: 0 for normal, rare wet dog shakes (WDS), no convulsion; 1 for intermediate number of WDS, rare focal convulsions affecting the head and extremities; 2 for frequent WDS, frequent focal convulsions, appearance of generalized convulsions (no rearing, no salivation); 3 for frequent WDS, focal convulsions, frequent appearance of generalized convulsions with rearing (but not falling), salivation; 4 for frequent WDS, focal convulsions, frequent generalized convulsions with falling, salivation; 5 for continuous generalized limbic seizures, death within 2 h.

2.3. Tissue preparation and TUNEL staining

The animals were sampled by decapitation at 1, 3 and 6 days after kainic acid injection. The brains were removed

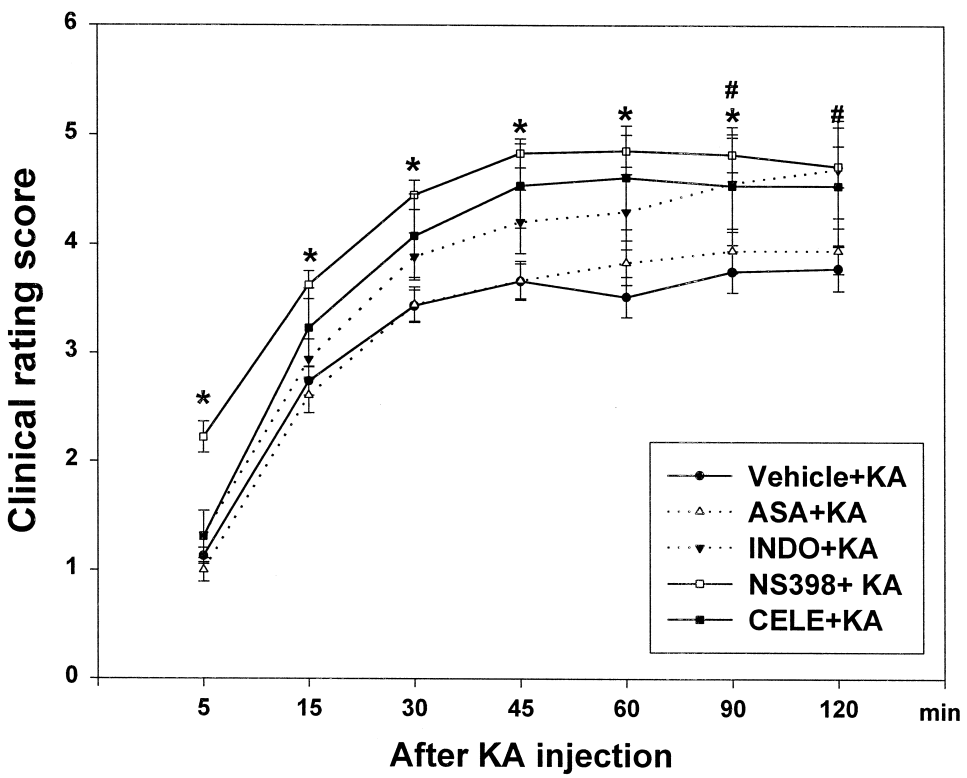

Fig. 1. Effect of COX inhibitors on kainic acid (50 mg/kg, i.p.)-induced seizure activity. Each of drugs was administered 30 min before kainic acid (KA) injection. Values are mean ± S.E.M. KA-treated group (50 mg/kg), $N = 21$; KA + INDO (indomethacin 5 mg/kg) group, $N = 20$; KA + ASA (aspirin 10 mg/kg) group, $N = 18$; KA + NS-398 (10 mg/kg) group, $N = 21$; KA + celecoxib (10 mg/kg), $N = 13$. Significance of the difference between drug KA alone treated group and corresponding group pretreated with drug ($P < 0.05$): * NS-389 treated group, # indomethacin treated group (ANOVA and Dunnett's test).

immediately and rapidly frozen in isopentane (−70°C). Coronal sections of the brain (20 μm thick) were cut on a cryostat, and every third section was mounted on the slides for TUNEL staining. Every fourth section was stained with Cresyl violet to assess neuronal injury using standard histological criteria.

DNA fragmentation was assessed using TUNEL technique. Terminal deoxynucleotidyl transferase was used to label the 3′-OH ends of fragmented DNA as previously described [11]. Frozen brain sections were fixed for 30 min in 4% paraformaldehyde, quenched with H_2O_2, washed with PBS three times, and incubated in a mixture of TdT and Digoxygenin-dUTP in TDT buffer (Oncor) for 120 min at 37°C. After washing with PBS, the sections were incubated in peroxidase-anti-digoxygenin antibody Fab fragment (Oncor) for 30 min at 37°C. To count the number

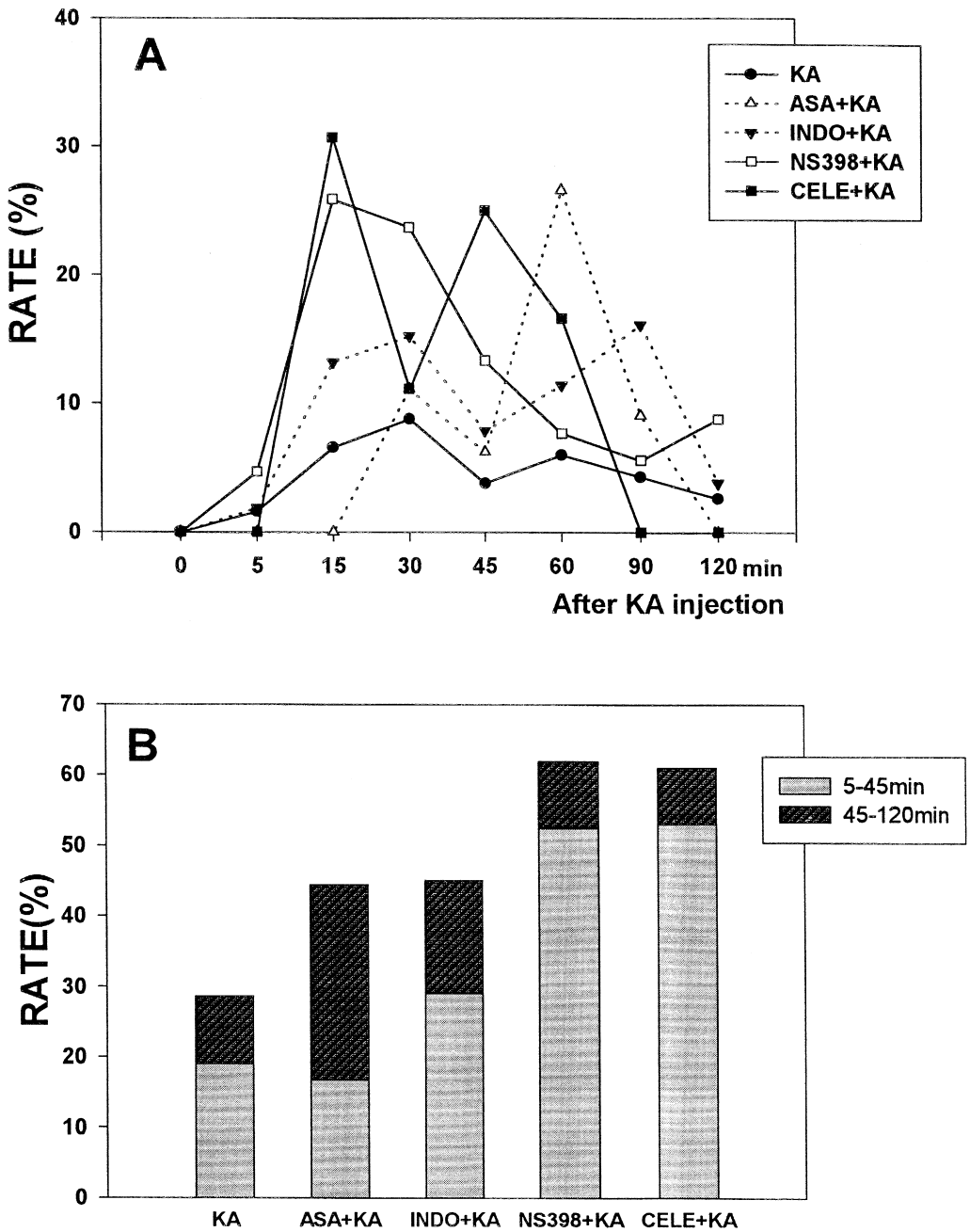

Fig. 2. Effect of COX inhibitors on kainic acid-induced mortality. Each of drugs was administered 30 min before kainic acid (KA) injection. KA-treated group (50 mg/kg); KA + INDO (indomethacin 5 mg/kg) group; KA + ASA (aspirin 10 mg/kg) group; KA + NS-398 (10 mg/kg) group; KA + celecoxib (10 mg/kg), $N = 13$. (A) The changes of mortality during various time periods after kainic acid injection. (B) Total mortality within 45 and 120 min after kainic acid injection.

Table 1
Mortality in kainic acid alone group and indomethacin or NS-398 pretreated group according time course

	Mortality (within 45 min)	Mortality (within 120 min)
Vehicle + KA	19.0 (4/21)	28.6 (6/21)
INDO (5 mg/kg) + KA	30.0 (7/20)	45.0 (9/20)
INDO (10 mg/kg) + KA	76.9 (10/13)*	84.6 (12/13)*
NS-398 (10 mg/kg) + KA	52.4 (11/21)*	61.9 (13/21)*
NS-398 (20 mg/kg) + KA	64.3 (9/14)*	71.4 (10/14)*

* $P < 0.05$ vs. KA alone group (χ^2 test).

of TUNEL-positive cells, staining was visualized using 0.05% diaminobenzidine (DAB) and 0.002% H_2O_2 in PBS for 10 min, and then rinsed with water and counterstained with methylene blue.

For evaluation of density of Cresyl violet stained viable neurons and TUNEL positively stained neuronal death with DNA fragmentation, the blind semi-quantitative estimation using the following score was applied. If the 0%–25% population was stained positively, the score was 0; 26%–50% was 1; 51%–75% was 2; and more than 76% was 3, compared with vehicle treated group (Figs. 4 and 8).

2.4. Assay of prostaglandin E_2, prostaglandin D_2 and prostaglandin $F_{2\alpha}$

To ascertain COX activity in the hippocampus, the concentrations of prostaglandin (PG) E_2, D_2 and $F_{2\alpha}$ were measured according to the time course. After the head was decapitated, the hippocampus was dissected in the cold chamber and immediately frozen in liquid nitrogen. The tissue was homogenized in PBS containing indomethacin (100 μM) and extracted with 100% ethanol. After centrifugation, the supernatant was applied to Sep-Pak columns (Waters associate). Prostaglandin was eluted with 100% methanol, and methanol fraction was evaporated and resuspended in 1 ml PBS gel (0.1% gelatin in PBS buffer pH 7.4). To determine concentration of PG E_2, the 96 well plates were coated with mouse monoclonal PG E_2 antibody in 0.01 M pH 7.4 PBS. By incubation with 50 μl of PG E_2 acetylcholinesterase EIA tracer (Cayman) and prostaglandin antiserum antibody, PG E_2 in the tissue and the tracer was combined competitively for 1 h. After washing, 200 μl of Ellman's reagent was added for coloring PG E_2 acetylcholinesterase EIA tracer combined with antibody. At 405 nm spectrum the absorbance was measured and was calculated. The amount of PG $F_{2\alpha}$ was also measured using the same method above. PG D_2 was measured by radioimmunoassay. The 300 μl of extracted

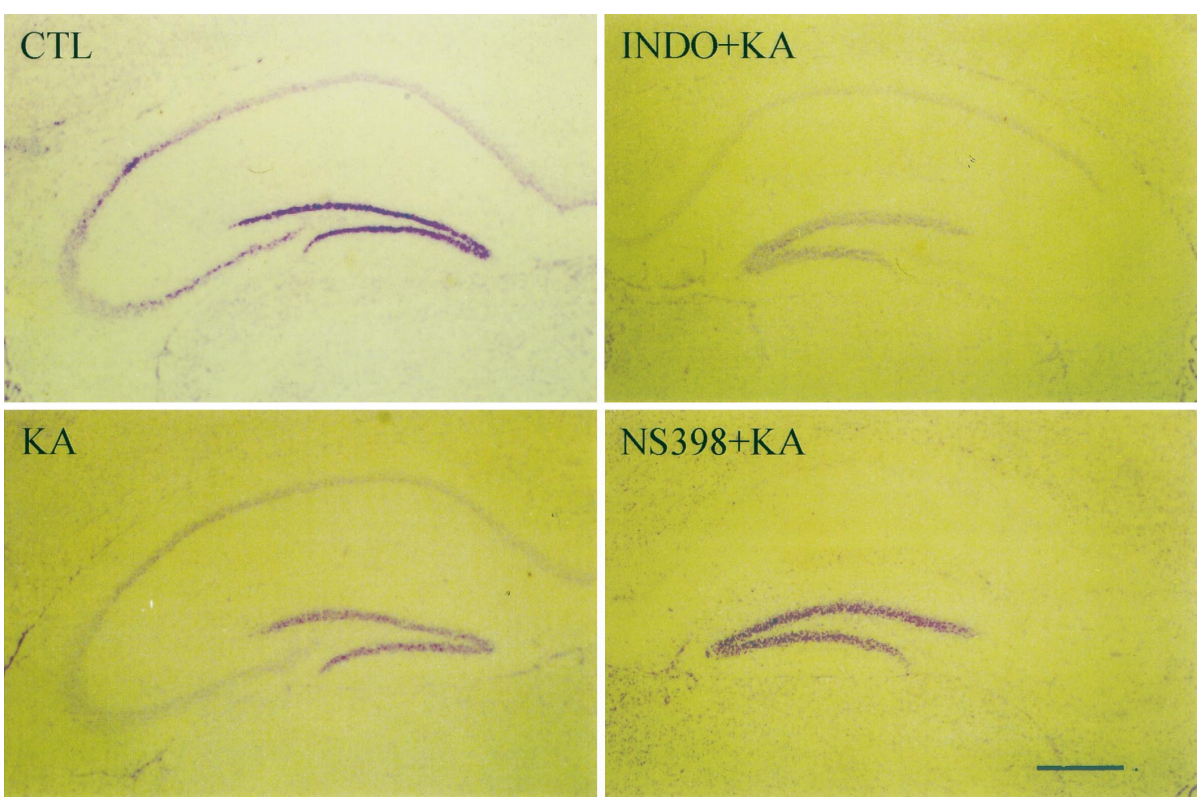

Fig. 3. Light microscopy pictures showing Cresyl violet stained viable neurons in the hippocampal regions 3 days after kainic acid injection. The CA_1 and CA_{3a} regions of the hippocampus were vulnerable to kainic acid-induced excitotoxic injury. (A) Vehicle-treated animal, (B) Kainic acid-treated animal, (C) Indomethacin-pretreated animal, (D) NS-398-pretreated animal. Scale bar = 500 μm.

prostaglandin diluted in PBS gel (0.1% gelatin in PBS buffer, pH 7.4) was added in 100 μl of 165 Ci/mmol [^{3}H]PGD_2 (DuPont NEN, USA) solution, and 200 μl of PGD_2 antibody. The reaction lasted for 12 h at 4°C and the PGD_2 and [^{3}H]PGD_2 were combined competitively. The 600 μl of charcoal (1 mg/ml dextran, 6 mg/ml charcoal in PBS, Sigma, USA) was added to eliminate the remaining PGD_2 for 15 min at 4°C. The radioactivity of incorporated free PGD_2 was measured by liquid scintillation counter. The scintillation cocktail was composed of three xylene and one Triton X-100 containing 0.3% PPO. The amount of PG D_2 was calculated by PGM-2 program.

2.5. Statistics

Mean ± S.E.M. was calculated and statistical analysis was performed by analysis of variance and Dunnett's test in case of multiple comparisons. For evaluation of mortality in drug pretreated animals the χ^2-test was used. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Behavior

Kainic acid induced-seizure was observed within 2 h after application. The seizure rating scores began to increase at 5 min (1.10 ± 0.07), and peaked at about 45 min (3.66 ± 0.16). Pretreatment of the mice with the COX inhibitors 30 min before kainic acid injection further increased the seizure rating scores and mortality. Indomethacin (5 mg/kg) increased the seizure rating after 1 h (4.30 ± 0.21), while NS-398 (10 mg/kg) potentiated the seizure at all time points, even at 5 min (2.20 ± 0.15) after kainic acid injection (Fig. 1). The difference ($P < 0.05$) between kainic acid alone group and drug-pretreatment

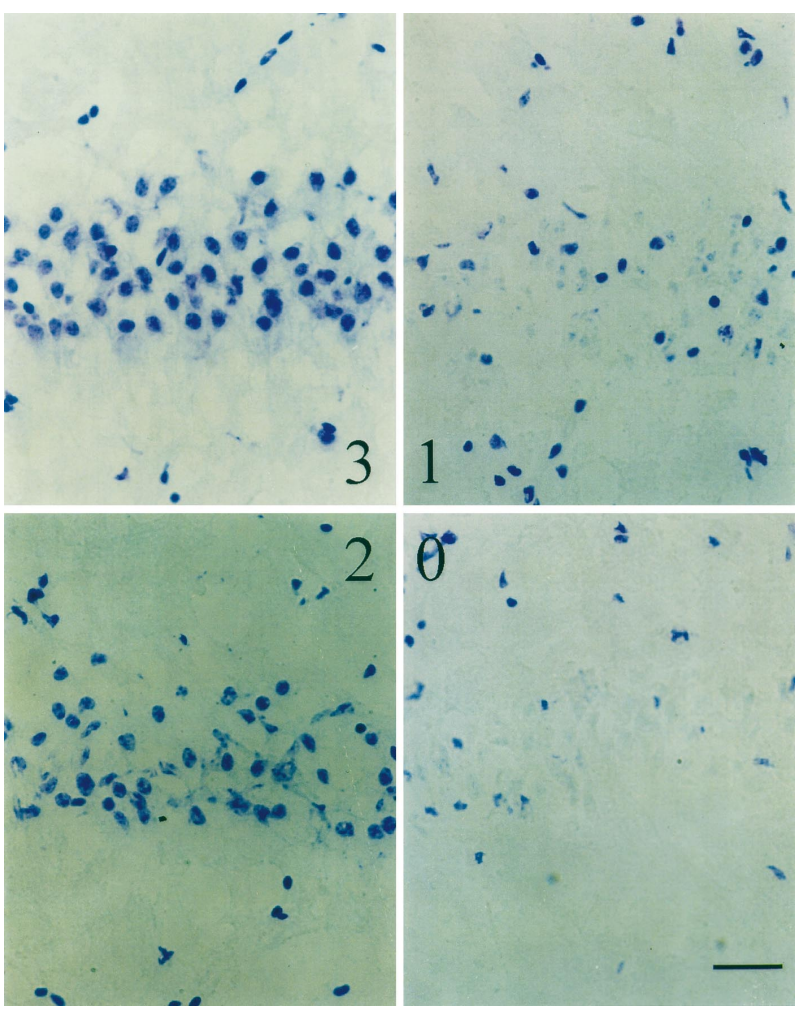

Fig. 4. High power pictures showing Cresyl violet stained viable neurons in hippocampal CA_1 region (grade 3–0). The pictures were obtained from vehicle-treated animal, kainic acid-treated animal, indomethacin-pretreated animal, and NS-398-pretreated animal. Scale bar = 50 μm.

group (indomethacin, NS-398 or celecoxib, but not aspirin) was significant.

The mortality induced by kainic acid had two phases, i.e., the first and second peaks; the first mortality peak (5–45 min) and the second peak (45–120 min). Indomethacin exaggerated the two peaks, while NS-398 and celecoxib augmented the first peak, and aspirin augmented the second peak (Fig. 2A).

The dose relationship of drugs was summarized in Table 1. Indomethacin (10 mg/kg) increased seizure activ-

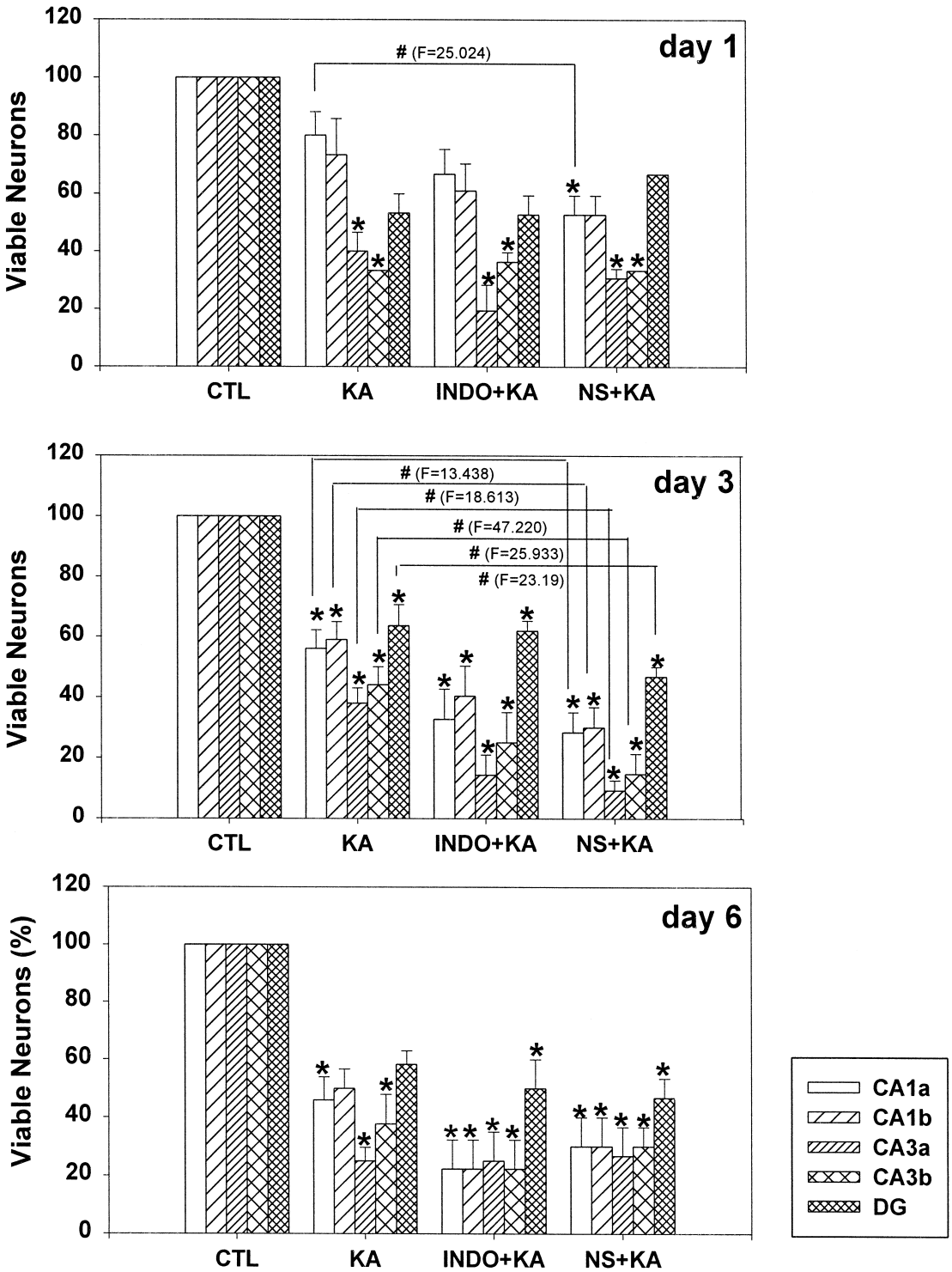

Fig. 5. Effects of COX inhibitors on kainic acid-induced neuronal death in the hippocampal region. The numbers of viable neurons represent the percent of Cresyl violet stained neurons compared with vehicle-treated control group. Values are mean ± S.E.M. of six experiments ($N = 8–12$). $^{*}P < 0.05$ vs. control vehicle group; or $\#P < 0.05$ vs. kainic acid alone group (ANOVA and Dunnett's test).

ity remarkably, and finally killed most of the animals (84.6%) within 2 h. Indomethacin (5 mg/kg) increased mortality within 2 h by 1.5 times compared to kainic acid alone. Mortality in NS-398 (10 mg/kg) treated group increased by more than 2 times the kainic acid treated group within 45 min, and NS-398 (20 mg/kg) group had more mortality. But in NS-398 and other COX inhibitors themselves without kainic acid, neither seizure nor increased mortality occurred. Chi-square test indicated that the mortality rate in NS-398 (10 and 20 mg/kg) and indomethacin (10 mg/kg) pretreated group was significantly higher than that in vehicle group ($P < 0.05$) within 45 min.

3.2. Delayed neuronal death

Cresyl violet stained neurons were reduced by kainic acid especially in the CA_3 region of the hippocampus (Figs. 3–5). TUNEL-positively-stained neurons were detected in CA_1 and CA_{3a} regions (Figs. 6–8). In NS-398 pretreated group numerous TUNEL-positive neurons were found at day 1 and viable neurons were reduced in the CA_1, and also in the CA_3 region. Indomethacin had similar effects as NS-398 group, but had slower progress. On day 3, TUNEL-positive neurons in indomethacin or NS-398 group extended to the CA_{3b} where TUNEL-positive neurons were rare in vehicle group (Fig. 6). The dentate nuclei were relatively resistant to TUNEL-positive neuronal death induced by kainic acid. On day 6, TUNEL-positive neurons in vehicle group disappeared, while the positive neurons remained in COX inhibitor pretreated groups (Fig. 8). NS-398 tended to injure neurons more rapidly than indomethacin, but the degree of neuronal loss seemed to be similar after 6 days (Fig. 5).

3.3. Prostaglandin E_2, D_2 and $F_{2\alpha}$

The concentrations of PG D_2, E_2 and $F_{2\alpha}$ in the hippocampus were measured at 15, 30, 60 min and 1 day after kainic acid injection. Endogenous PG D_2, a major PG of the nervous system, was known to have anticonvulsant properties, proven by treatment of its analogue or antagonist [3]. The PG $F_{2\alpha}$ increased remarkably by kainic acid [5]. The PGs began to increase by kainic acid within minutes. Indomethacin decreased not only to the basal levels of all PGs, but also kainic acid-induced production of the PGs at 15, 30, and 60 min after injection of kainic acid. In contrast, COX-2 selective inhibitors showed more complex effects on prostaglandin production. NS-398 significantly reduced the basal PG, but failed to inhibit the kainic acid-induced PG E_2 and D_2 production, instead they greatly augmenting kainic acid-induced PG E_2 pro-

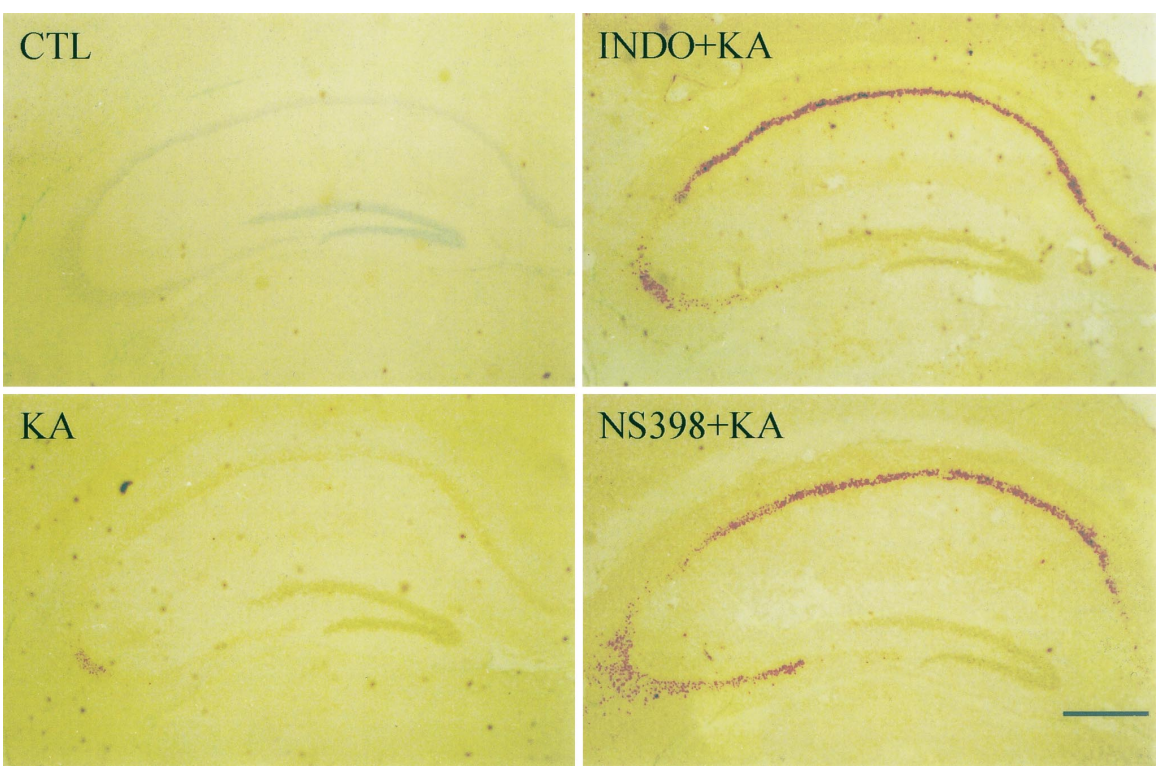

Fig. 6. Light microscopy pictures showing positively TUNEL stained neurons in the hippocampal regions 3 days after kainic acid injection. The TUNEL positively stained neuronal death was aggravated by pretreatment of indomethacin or NS-398. (A) Vehicle-treated animal, (B) Kainic acid-treated animal, (C) Indomethacin-pretreated animal, (D) NS-398-pretreated animal. Scale bar = 500 μm.

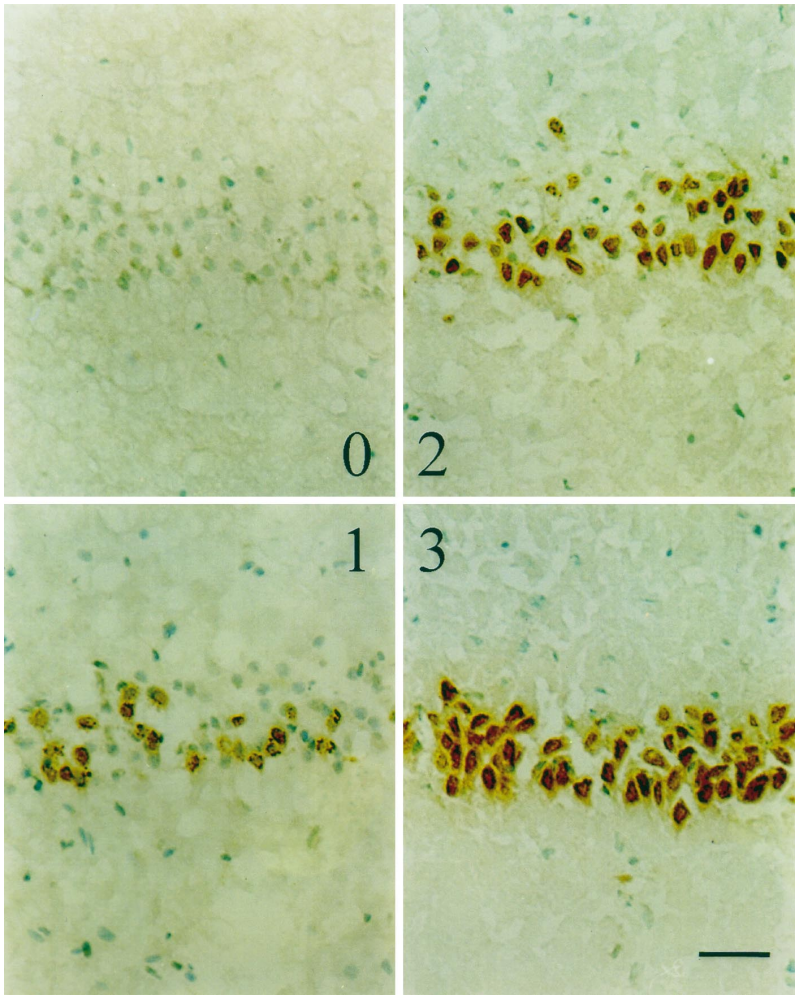

Fig. 7. High power pictures showing positively TUNEL stained neurons in hippocampal CA_1 region (grade 3–0). The pictures were obtained from vehicle-treated animal, kainic acid-treated animal, indomethacin-pretreated animal, and NS-398-pretreated animal. Scale bar = 50 μm.

duction during the first 15 min. Celecoxib showed the similar results (data was not shown), except it could not reduce the basal PGs levels. Indomethacin could inhibit completely all kinds of COX isoforms associated with kainic acid, while the selective inhibition of COX-2 could not inhibit another COX activity thought to be COX-1 in common. After 60 min, the levels of prostaglandin in the tissues tended to reduce in COX-2 selective inhibitor treated group (Fig. 9).

4. Discussion

Kainic acid injections in mice resulted in seizure followed by irreversible neuronal cell loss, especially in the hippocampal CA_{3a} region [17]. In the present study, the pretreatment of cyclooxygenase-2 (COX-2) inhibitors including COX-2 selective inhibitor aggravated kainic acid-induced seizure and mortality. Also, TUNEL-positive neuronal death was more extensive with expanding lesions of the hippocampal CA1 and CA_{3b} regions.

COX, a rate limiting enzyme for prostaglandin synthesis, has two isoforms, i.e., COX-1 and COX-2. While COX-1 is expressed constitutively in the peripheral tissue, COX-2 is induced by inflammation and other noxious conditions [12]. In the normal brain, COX-2 is expressed in neurons dynamically and may be associated with the NMDA receptor-related excitability [1]. In pathological conditions such as ischemic brain injury or Alzheimer's disease, the expression of COX-2 is thought to be associated with neuronal damage. Therefore, the regulation of this enzyme may offer neuroprotection [21,28,33]. Selective inhibition of COX-2 might be helpful to survive neurons in the inflammatory process of neuronal degeneration. The early induction of COX-2 may fuel tissue damage through prostanoids and free radicals, and delayed induction in remote area may be associated with inflammatory response. In many experiments [6,17,23–25,33] per-

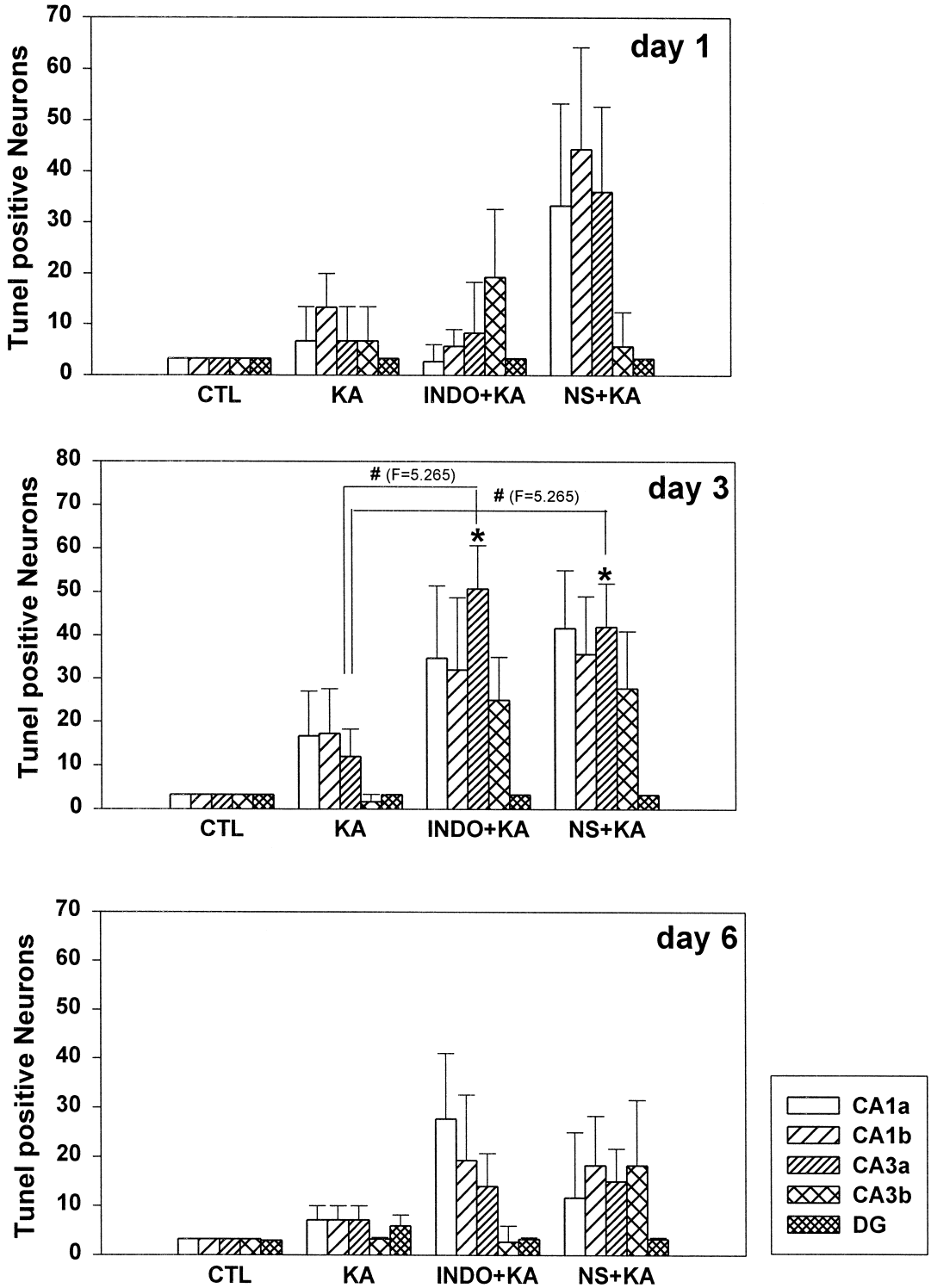

Fig. 8. Effects of COX inhibitors on kainic acid induced TUNEL positively stained neuronal death in the hippocampus. Values are mean ± S.E.M. of six experiments ($N = 8–12$). $^{*}P < 0.05$ vs. control group; or $\#P < 0.05$ vs. kainic acid alone group (ANOVA and Dunnett's test).

formed with ischemic and excitotoxic injury models, nonselective COX inhibitor and selective COX-2 inhibitors showed neuroprotective properties. In noxious conditions, the release of arachidonic acid and subsequent activation of COX with the formation of reactive oxygen species (ROS) would be implicated in cell damage. Although there are many experiments supporting for the roles of COX-2 in neuronal death as a damaging factor, it is also possible that COX-2 is involved in the rescue program of noxious conditions [2,8,9,34,35].

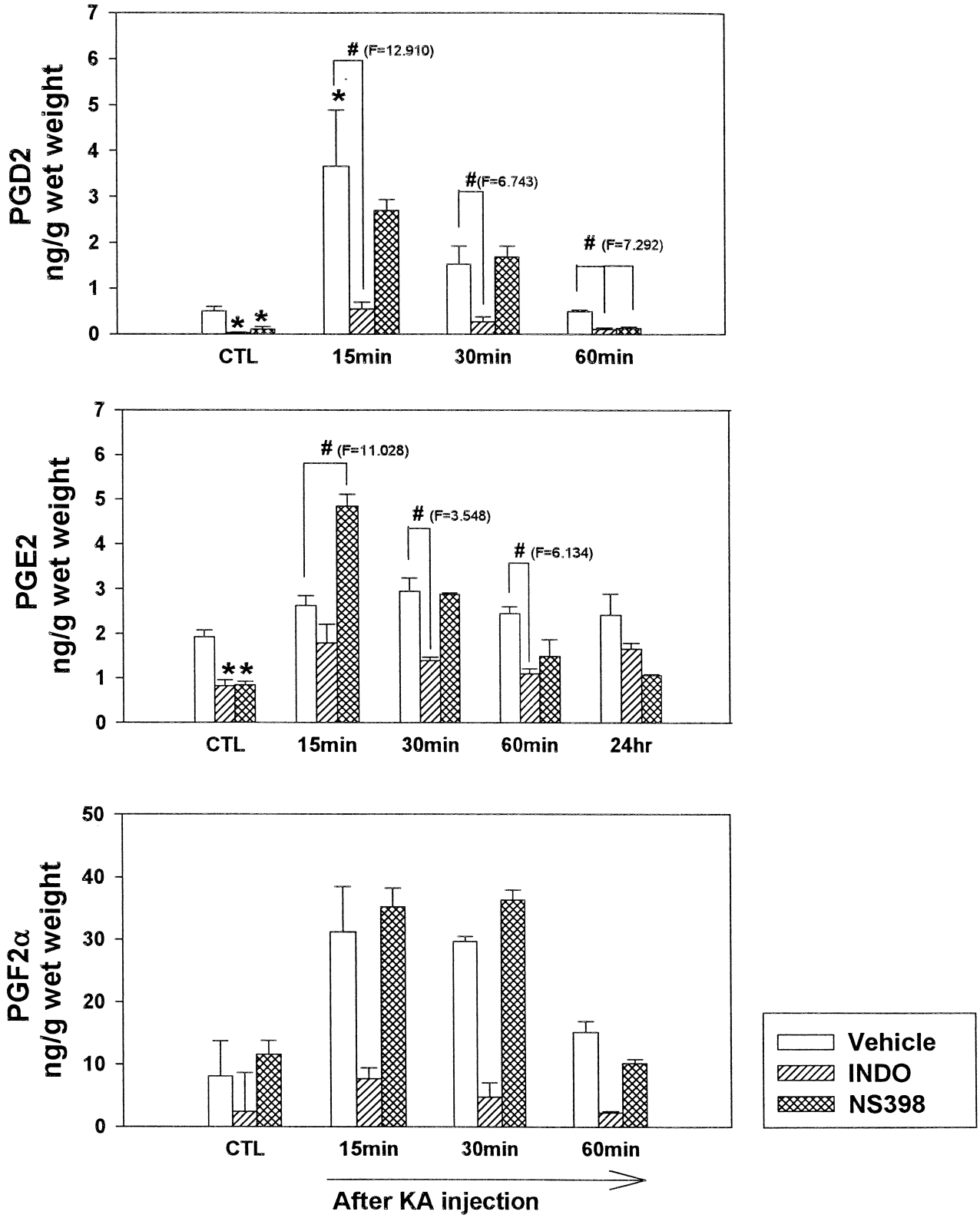

Fig. 9. Effects of COX inhibitors on the concentration of prostaglandin D_2, $F_{2\alpha}$ and E_2, in the hippocampus following kainic acid injection. Values are mean ± S.E.M., PGE_2, $N = 5$; PGD_2, $N = 5$; $PGF_{2\alpha}$, $N = 2$. $^*P < 0.05$ vs. control group; or $\#P < 0.05$ vs. kainic acid alone group (ANOVA and Dunnett's test).

The kainic acid-induced neuronal damage has been explained as the result of glutamate excitotoxicity and related zinc toxicity [30]. The neuronal lesion by kainic acid was associated with localization of kainic acid recep-

tors, and increased glutamate release modulated by COX metabolite [26,32]. The COX-2 expression may be prevented by NMDA blockers or glucocorticoid [1], and kainic acid-induced neuronal death may be due to glutamate-related calcium influx and following activation of a number of enzymes that generate free radicals. The kainic acid-induced hippocampal damage could be explained as free radical production by kainic acid [13,18].

Along with PG increase, induction of COX-2 by kainic acid or glutamate in the hippocampus were intimately associated with the following neuronal damage [20]. These results implied the possibility that COX-2 inhibitors could prevent kainic acid-induced neuronal death. Therefore, we addressed carefully whether or not the neuroprotective effect of COX-2 selective inhibitors in ischemic injury model also existed in kainic acid model. The present experiment also considered whether the COX-2 selective inhibitor could aggravate kainic acid-induced seizure because indomethacin and other nonselective COX inhibitors were known to exaggerate the seizure activity [4,10].

The increases in all kinds of prostanoids such as prostaglandin D_2, $F_{2\alpha}$, E_2, and TXB_2 in the hippocampus by kainic acid were reported [5]. Antiinflammatory drugs that inhibit PG production could aggravate the kainic acid-induced seizure [4,10]. Also, the level of prostanoids decreased in convulsion prone gerbils [29]. The above results suggested that the endogenous prostaglandin had anticonvulsant properties. In this study, we measured the changes of prostaglandin E_2, D_2 and $F_{2\alpha}$ in the hippocampal tissue by kainic acid injection. Prostaglandin level increased within a few minutes after kainic acid injection and indomethacin markedly suppressed basal production and kainic acid-induced prostaglandins. COX-2 selective inhibitor such as NS-398 was able to reduce the basal PG level, raising doubt as to whether NS-398 with the dose of 10 mg/kg could accomplish the selective inhibition of COX-2. However, in many other experiments, NS-398 (10 mg/kg) selectively and sufficiently inhibited COX-2 activity, and completely suppressed inflammatory responses without conventional COX-1 dependent side effects, such as gastric hemorrhage [14,22]. But in this experiment, NS-398 could not suppress kainic acid-induced PG production. Moreover, NS-398 increased PG E_2 by about 2 times more than kainic acid alone. This data created some confusion. We thought that perhaps the free arachidonic acid released by kainic acid and the increased substrates might activate both COX-1 and COX-2 activity. Because COX-1 or COX-2 activity might be changed according to the amount of arachidonic acid, selective inhibition of COX-2 could change the activity of another COX isoform [30]. Another explanation for the increased release of PGs by COX-2 selective inhibitors could be associated with change of local cerebral blood flow. COX-2 selective inhibitors could change the blood flow via vasoconstriction [15,19], and accumulate the PGs instantly. The effect of COX and metabolites such as PGs on vascular system might explain the differential effect of COX-2 on neuronal death such as ischemia or kainic acid model. This study suggested that COX isozymes act differentially according to types of neuronal injury. We showed that COX-2 inhibitors aggravated kainic acid-induced neuronal damage in vivo model through exaggeration of kainic acid-induced seizure activity.

Because COX-2 selective inhibitors aggravated the kainic acid-induced seizure with the maintenance of a high level of PG at early time points, it could be challenged that endogenous prostaglandin might have anticonvulsant activity. However, PG E_2 and PG D_2 in the basal state were decreased by pretreatment of COX-2 inhibitors, and also after 60 min, the levels of PG tended to decrease by COX-2 selective inhibitors. It is necessary to further explore the exact roles of each PG and COX isozymes on seizure activity and on protection or damage in neurodegenerative process.

Acknowledgements

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